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Mind the Gap

A new molecular model for the intracellular trafficking of Connexin43

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Abbreviations

Abbreviations

7-keto	7-Ketocholesterol
25-OH	25-Hydroxycholesterol
AMP	Adenosine Monophosphate
AMPK	AMP-Activated Protein Kinase
AMSH	Associated Molecule with the SH3 Domain of STAM
AP2	Adaptor Protein 2
APC	Anaphase-Promoting Complex
Arg	Arginine
ARH	Autosomal Recessive Hypercholesterolemia Protein
Asp	Aspartic Acid
ATP	Adenosine Triphosphate
BRCA1	Breast Cancer Type 1 Susceptibility Protein
BSA	Bovine Serum Albumin
c-Src	Cellular-Sarcoma
CaM	Calmodulin
cAMP	Cyclic Adenosine Monophosphate
Cav	Caveolin
Cbl3	Casitas B Lymphoma Protein 3
CCP	Clathrin Coated Pit
CCV	Clathrin Coated Vesicles
CD4	Cluster of Differentiation Antigen 4 Protein
cDNA	Complementary DNA
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
cGMP	Cyclic Guanosine Monophosphate
CHIP	Carboxyl-Terminus of Hsc70-Interacting Protein
CHOL	Cholesterol
CHX	Cyclohexamide
CIE	Clathrin-Independent Endocytosis
CK1	Casein Kinase 1
CLASPs	Clathrin-Associated Sorting Proteins
CLIC/GEEC	Clathrin-Independent Carrier/GPI-AP-enriched Early Endosomal Compartment
CME	Clathrin-Mediated Endocytosis
CONT	Control
CP	Core Particle
CT	Carboxyl-Terminal

Cx	Connexin
Cx43	Connexin43
Cys	Cysteine
Dab2	Disabled 2
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
Doa4	Degradation of Alpha 4
Drebrin	Developmentally Regulated Brain Protein
DTX	Deltex
DUB	Deubiquitinating Enzyme
E1	Ubiquitin-Activating Enzyme
E2	Ubiquitin-Conjugating Enzyme
E3	Ubiquitin-Protein Ligase
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EHD1	Eps15 Homology Domain-containing 1
ENaC	Epithelium Na^+ Channel
Eps15	Epidermal Growth Factor Receptor Substrate 15
ER	Endoplasmic Reticulum
ERAD	Endoplasmic Reticulum Associated Degradation
ERC	Endocytic Recycling Compartment
ERK	Extracellular Signal-Regulated Kinase
ERp29	Endoplasmic Reticulum Protein 29
ESCRT	Endosomal Sorting Complex Required for Transport
FGF	Fibroblast growth Factor
FIL	Filipin III
$GABA_A$	Gamma-Aminobutyric Acid Type A
GAP	GTPase-Activating Protein
GDP	Guanosine Diphosphate
GEF	Guanine Nucleotide Exchange Factor
GFP	Green Fluorescent Protein
GJ	Gap Junction
GJIC	Gap Junction Intercellular Communication
GLUE	Gram-Like Ubiquitin-Binding in Eap45
GLUT4	Glucose Transporter 4

Gly	Glycine
GTP	Guanosine Triphosphate
HBSS	Hank's Balanced Salt Solution
HCH	Hexachlorocyclohexane
HEK293	Human Embryonic Kidney 293 Cells
HECT	Homologous to the E6-AP Carboxyl-Terminus
His	Histidine
HMW	High Molecular Weight
Hrs	Hepatocyte Growth Factor-Regulated Tyrosine Kinase Substrate
Hsc70	Heat Shock Cognate Protein 70
Hsp90	Heat Shock Protein 90
IκBα	Inhibitor of NFκB Kinase Alpha
IKK	IκB Kinase
IL-1β	Interleukin-1β
Ile	Isoleucine
ILV	Intralumenal Vesicle
IP	Immunoprecipitation
IP3	Inositol 1,4,5-Triphosphate
Itch	Itchy E3 Ubiquitin Protein Ligase Homolog
JAMM	JAB1/MPN/Mov34 Metalloenzyme
LAMP1	Lysosome-Associated Membrane Protein 1
LAPTM5	Lysosomal Protein Transmembrane 5
LC3	Light Chain 3
LEC	Lens Epithelial Cells
Leu	Leucine
LMW	Low Molecular Weight
Lys	Lysine
MAPK	Mitogen-Activated Protein Kinase
MHC	Major Histocompatibility Complex
MJB	Josephin Domain
MVB	Multivesicular Body
NBD-TMA	[2-(4-nitro-2,1,3-benzoxadiazol-7-yl)aminoethyl]trimethylammonium
Nedd4	Neuronal Precursor Cell-Expressed Developmentally Downregulated 4
NFκB	Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells
NP	Non-Phosphorylated
NRK	Normal Rat Kidney Cells
NT	Amino-Terminal

OCP1	Organ of Corti Protein 1
ODDD	Oculodentodigital Dysplasia
OxLDL	Oxidized Low-Density Lipoprotein
OTU	Ovarian Tumour Domain
PA28	Proteasome Activator 28$\alpha\beta$
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PDGFR	Platelet-Derived Growth Factor Receptor
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-Kinase
pKa	Logarithmic Scale Acid Dissociation Constant
PKA	Protein Kinase A
PKC	Protein Kinase C
PKG	Protein Kinase G
PM	Plasma Membrane
PMA	Phorbol 12-Myristate 13-Acetate
PMSF	Phenylmethylsulfonyl Fluoride
PPXY	Proline Rich Motif
Pro	Proline
PTB	Phosphotyrosine-Binding Domain
PtdIns(4,5)P_2	Phosphatidylinositol-4,5-Biphosphate
PtdIns(3)P	Phosphatydilinositol-3-Phosphate
PVDF	Polyvinylidene Fluoride
PY-motif	Proline Rich Motif
RFP	Red Fluorescent Protein
RING	Really Interesting New Gene
RIP1	Receptor-Interacting Protein 1
RIPA	Radioimmunoprecipitation Buffer
RITC	Rhodamine-B-Isothiocyanate
RNA	Ribonucleic Acid
RP	Regulatory Particle
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SCF	Skp, Cullin, F-box Containing Complex
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Ser	Serine

shRNA	Short Hairpin RNA
siRNA	Small Interfering RNA
SNARE	Soluble N-ethylmaleimide-sensitive Factor Attachment Protein Receptor
STAM	Signal Transducing Adaptor Molecule
SUC	Sucrose
TDG	Thymine-DNA Glycosylase
TFR	Transferrin Receptor
TGN	Trans-Golgi Network
Thr	Threonine
TNF-α	Tumour Necrosis Factor-alpha
Tsg101	Tumour Susceptibility Gene 101
Tyr	Tyrosine
Ub	Ubiquitin
UBC	Ubiquitin-Conjugating Domain
UBD	Ubiquitin-Binding Domain
UBL	Ubiquitin-Like Domain
UBPY	Ubiquitin-Specific Processing Protease Y9
UCH	Ubiquitin Carboxyl-Terminal Hydrolase
UEV	Ubiquitin E2 Variant
UIM	Ubiquitin-Interacting Motif
USP	Ubiquitin-Specific Protease
v-Src	Viral-Sarcoma
Val	Valine
V_j	Tranjunctional Voltage
V_m	Membrane Voltage
Vps	Vacuolar Protein Sorting
WB	Western Blot
WT	Wild Type
YFP	Yellow Fluorescent Protein
YXXØ	Tyrosine Sorting Signal
ZO-1	Zonula Occludens 1

Resumo

Resumo

As gap junctions (GJ) são estruturas intercelulares multiméricas que permitem a comunicação directa entre células eucariotas, nomeadamente, a transferência de pequenos metabolitos, iões e mensageiros secundários. Para formar uma GJ, duas células adjacentes precisam de contribuir com um hemicanal, ou conexão, sendo este, por sua vez, composto por seis subunidades de uma proteína denominada conexina (Cx). A Cx43, um membro da família alfa das conexinas, pode ser encontrada em tecidos tão diversos como o coração, cristalino, retina, epiderme, cérebro, rim e medula óssea.

A extensão da comunicação intercelular através de gap junctions (CIGJ) está directamente relacionada com o número e estado funcional dos canais presentes na membrana plasmática. Por isso, processos que influenciam a estabilidade de conexinas na membrana plasmática são críticos para regular a comunicação intercelular. Apesar da CIGJ poder ser regulada através da abertura e do fecho do poro do canal, a degradação de conexinas, particularmente da Cx43, desempenha um papel fundamental na regulação da CIGJ. De facto, e quando comparada com outras proteínas da membrana plasmática, a Cx43 tem um tempo de vida invulgarmente curto, de menos de 5 horas. Assim, é previsível que a desregulação da CIGJ tenha impacto em vários órgãos e tecidos. Por exemplo, no olho, o cristalino e o epitélio pigmentado da retina dependem da CIGJ para a manutenção da sua homeostase, e eventos que interfiram com a comunicação através de gap junctions poderão comprometer irreversivelmente a viabilidade destes tecidos ou ter importantes implicações funcionais.

Nesta tese começamos por investigar os eventos moleculares que levam à disrupção da CIGJ em células epiteliais do cristalino. Procedemos depois com a investigação dos eventos moleculares que levam à internalização e degradação da conexina43, e que contribuem assim para a regulação da comunicação intercelular através de gap junctions.

A comunicação intercelular no cristalino depende de uma extensa rede de gap junctions que são essenciais para a manutenção da transparência do cristalino. Como o cristalino está continuamente exposto a insultos oxidativos e as membranas plasmáticas do cristalino contêm a maior proporção de colesterol de qualquer membrana biológica, investigámos se a acumulação de produtos de oxidação do colesterol poderia interferir com a comunicação intercelular. Os resultados apresentados neste trabalho demonstram, pela primeira vez, que o oxisterol 7-ketocolesterol induz aumentos na CIGJ, que são muito provavelmente devidos a um aumento da estabilidade da proteína na membrana plasmática e a um consequente aumento na formação de placas de gap junctions. A sobreregulação da CIGJ no cristalino poderá afectar uma variedade de eventos altamente regulados, incluindo a diferenciação das células epiteliais do cristalino em fibras, o que, em último caso, poderá comprometer a transparência do cristalino levando ao desenvolvimento da catarata.

A degradação da Cx43 como forma de regular a CIGJ, tem sido motivo de debate há mais de duas décadas, e tanto o proteassoma como o lisossoma já foram implicados neste mecanismo. Enquanto que a degradação da Cx43 pelo lisossoma está bem estabelecido, o papel do proteassoma no "turnover" da Cx43 é ainda pouco compreendido. No entanto, foi sugerido recentemente que o efeito da inibição do proteassoma na internalização de GJ está relacionado com a deplecção de ubiquitina livre, uma vez que está descrito que a conjugação da ubiquitina a uma variedade de proteínas membranares funciona como um sinal de endocitose. Alternativamente, o proteassoma poderá mediar a degradação de uma proteína ainda desconhecida, envolvida na regulação da estabilidade da Cx43 ao nível da membrana plasmática. Apesar de já ter sido demonstrado que a Cx43 é ubiquitinada, e que a fosforilação poderá servir como um sinal para a ubiquitinação da proteína, os mecanismos moleculares envolvidos na regulação da internalização e degradação da Cx43 continuam por esclarecer. Dados apresentados neste trabalho demonstram, pela primeira vez, que a ubiquitinação da Cx43 é mediada pela E3 ligase Nedd4 e que a conjugação de ubiquitina à Cx43 é necessária para a subsequente interacção com a proteína endocítica Eps15. De forma consistente, demonstramos ainda que a interacção entre os motivos de interacção com a ubiquitina da Eps15 e a forma ubiquitinada da Cx43 é necessário para que ocorra a internalização da Cx43.

Demonstramos também que a ubiquitinação da Cx43 induz a internalização da proteína através de um mecanismo que é independente do motivo de endereçamento YXXØ, que medeia a internalização constitutiva da proteína por uma via dependente de clatrina. É provável que este mecanismo necessite da ubiquitinação de algumas, mas não de todas, as subunidades de um hemicanal. Ao passo que o papel da clatrina na internalização da Cx43 está bem estabelecido, os resultados apresentados neste estudo sugerem que, alternativamente, a Cx43 ubiquitinada poderá ser internalizada através de um mecanismo independente de clatrina.

Está descrito que a ubiquitinação de algumas proteínas membranares também funciona como um sinal de endereçamento intracelular, que direcciona proteínas membranares para degradação no lisossoma. Neste estudo demonstramos que a ubiquitinação da Cx43 também pode sinalizar o seu tráfego intracelular para o lisossoma, através de um mecanismo que envolve a Hrs e a Tsg101, que são subunidades dos complexos ESCRT. Também foi demonstrado que a enzima de desubiquitinação UBPY poderá estar envolvida na modulação do tráfego intracelular da Cx43, promovendo o seu endereçamento para o lisossoma, onde é degradado. Por último, fornecemos provas de que a Cx43 internalizada pode ser reciclada de volta à membrana plasmática através de vias de reciclagem rápida e lenta, dependentes de Rab4 e Rab11 respectivamente, constituindo assim um novo nível de regulação para controlar a CIGJ.

Apesar de caracterizarmos parcialmente as vias de internalização e de tráfego intracelular seguidas pela Cx43, muitas questões continuam por resolver, incluindo os mecanismos moleculares que determinam a via de internalização seguida pela Cx43 ubiquitinada bem como os

mecanismos de interacção entre enzimas desubiquitinantes e proteínas Rab envolvidas no redireccionamento de Cx43 internalizada de volta à membrana plasmática. No entanto, este trabalho providencia uma base sólida para estudos futuros que tenham como objectivo uma melhor caracterização dos determinantes moleculares e mecanismos envolvidos na internalização e degradação da Cx43.

Abstract

Abstract

Gap junctions (GJ) are specialized cell–cell contacts that provide direct intercellular communication (IC) between eukaryotic cells, allowing for the transfer of small metabolites, ions and second messengers. Each of two adjacent cells contributes with one hemichannel to form a complete gap junction channel. Each hemichannel, or connexon is, in turn, composed by six subunits of a protein called connexin (Cx). Cx43, a member of the alpha connexin family, is the most widely expressed connexin protein, being found in tissues as diverse as the heart, lens, retina, skin, brain, kidney and bone marrow.

The extent of gap junction intercellular communication (GJIC) is directly related to the number and functional state of gap junction channels present at the plasma membrane. Therefore, processes that influence the stability of connexins at the plasma membrane are critical for regulating intercellular communication. Although GJIC can be regulated through the gating of the channel pore, degradation of connexins, particularly Cx43, appears to play an unexpected and critical role in regulating GJIC. Indeed, Cx43 has a remarkably short half-life, of less than 5 hours, when compared to other plasma membrane proteins. Disruption of GJIC is likely to have an impact upon a variety of organs and tissues. For example, the eye lens and retinal pigmented epithelium rely on GJIC and disruption of communication through gap junctions is likely to have important functional implications.

In this thesis we start by investigating the molecular events that lead to disruption of GJIC in lens epithelial cells. We further proceed by investigating the molecular events that lead to internalization and putative degradation of connexin43, and thus contribute to regulating intercellular communication through gap junctions.

Intercellular communication in the eye lens relies on an extensive network of gap junctions essential for maintenance of lens transparency. Since the lens is continuously exposed to oxidative insult and lens plasma membranes contain the highest cholesterol content of any biological membrane we investigated whether accumulation of cholesterol oxidation products might interfere with intercellular communication. Results presented in this work show, for the first time, that the oxysterol 7-ketocholesterol induces an increase in GJIC, which is most likely due to an increased stability of the protein at the plasma membrane and to an increased abundance of Cx43 assembly into gap junction plaques. The upregulation of GJIC in the lens may disrupt a variety of highly regulated events, including differentiation of lens epithelial cells into fibres, which ultimately compromises lens transparency leading to the formation of cataract.

Degradation of Cx43 as a means to regulate GJIC, has been a matter of debate for over the last two decades and both the proteasome and the lysosome have been implicated. Whereas degradation of Cx43 by the lysosome has long been established, the role of the proteasome in Cx43 turnover is poorly understood. However, recently it has been suggested that the proteasome

may act by regulating the availability of free ubiquitin, as the conjugation of ubiquitin to a variety of plasma membrane proteins is known to function as an endocytic signal. Alternatively, the proteasome could mediate the degradation of a putative and still unknown protein involved in the regulation of Cx43 stability at the plasma membrane. Indeed, it has been shown that Cx43 is a substrate for ubiquitination and that treatments that promote Cx43 internalization, such as phosphorylation, also induce the ubiquitination of the protein. However, the molecular players and regulatory mechanisms involved in the internalization and degradation of Cx43 have remained elusive. Data presented in this work show, for the first time, that Cx43 ubiquitination is mediated by the E3 ligase Nedd4 and that the conjugation of ubiquitin to Cx43 is required for subsequent interaction with the endocytic protein Eps15. Consistently, we show that the interaction between the ubiquitin-interacting motif of Eps15 and the ubiquitinated form of Cx43 is required for Cx43 internalization.

We have further shown that ubiquitination of Cx43 induces the internalization of the protein through a mechanism that is independent on the YXXØ sorting motif of Cx43, that mediates the constitutive internalization of the protein. This mechanism is likely to require the ubiquitination of some, but not all, subunits of one hemichannel. While the role of clathrin in Cx43 internalization has been well established, the results presented in this study suggest that ubiquitinated Cx43 can also be internalized through a clathrin-independent mechanism.

Ubiquitination of some integral membrane proteins is also known to function as an intracellular sorting signal, which directs membrane proteins for degradation in the lysosome. In this study we show that ubiquitination of Cx43 can also act as a signal for its intracellular sorting to the endolysosomal compartment, through a mechanism involving the ESCRT machinery components Hrs and Tsg101. The deubiquitinating enzyme UBPY was also shown to modulate the intracellular trafficking of Cx43, by promoting its intracellular sorting to the lysosome for degradation. Finally, we provide evidence that internalized Cx43 can be recycled back to the plasma membrane through both a rapid and a slow recycling pathway, thus adding a new level of regulation to control GJIC.

Although we partially characterize the internalization and intracellular trafficking routes followed by Cx43, many questions remain unresolved, these include the molecular mechanisms that determine the internalization pathway followed by ubiquitinated Cx43 as well as the interplay between deubiquitinating enzymes and Rab proteins in redirecting internalized Cx43 back to the plasma membrane. Nevertheless, this study provides a solid basis for future research aiming at further characterizing the molecular players and mechanisms involved in Cx43 internalization and degradation.

Chapter 1

Chapter 1: Introduction

Intercellular communication is essential for the survival of all multicellular organisms. The evolution of increasingly fine tuned communication mechanisms between cells allowed for the development of more complex organisms, with specialized cells, tissues and organs.

Cells can communicate with each other through the release of signalling molecules into the extracellular environment. These signalling molecules can then travel through the extracellular medium until reaching their target cells, which can be located either in the immediate vicinity of the originating cell, such as in the case of neurotransmitters, or over long distances, such as in the case of hormones. Upon reaching their target cell, signalling molecules are recognized by specific receptors on the cell surface, initiating signalling cascades that ultimately lead to specific cellular responses by inducing the expression (or repression) of genes. These signalling cascades normally involve the modulation of small molecules or ions (such as ATP, AMP, Ca^{2+}, etc) often referred to as secondary messengers. However, not all intercellular communication relies on the release of signalling molecules into the extracellular environment. In fact, cells can communicate directly with each other through gap junctions, channel structures that establish direct communications between the cytoplasm of adjacent cells.

Gap Junctions

Gap junctions are specialized cell-cell contacts that provide direct intercellular communication between eukaryotic cells, allowing for the direct passage of small molecules inferior to 1 kDa (such as ATP) and ions between adjacent cells. Gap junctions are composed from a few to many thousands of gap junction channels. Each channel, in turn, is formed by the docking of two hemichannels from adjacent cells. Hemichannels are hexameric protein structures. In vertebrates, the most well documented protein family of hemichannel monomers are the connexins; other hemichannel forming proteins include pannexins and their invertebrate homologues, the innexin protein family. While connexins and pannexins/innexins are structurally and functionally similar, they share little sequence homology and are evolutionarily distinct. Gap junction structures formed by connexins and innexins have been widely reported, however, reports concerning the ability of pannexins to form functional gap junctions are scarce.

The function of gap junctions in cell and tissue biology is of the utmost importance and, unsurprisingly, gap junction intercellular communication exists in nearly every mammalian cell type (Goodenough et al, 1996; Saez et al, 2003). In vertebrates, connexin gap junctions play critical roles in embryonic development, coordinated contraction of excitable cells, tissue homeostasis, normal cell growth and differentiation. Connexin gene mutations have been linked to several diseases (Laird, 2006). The first discovered connexin-linked human disease was chromosome-X-

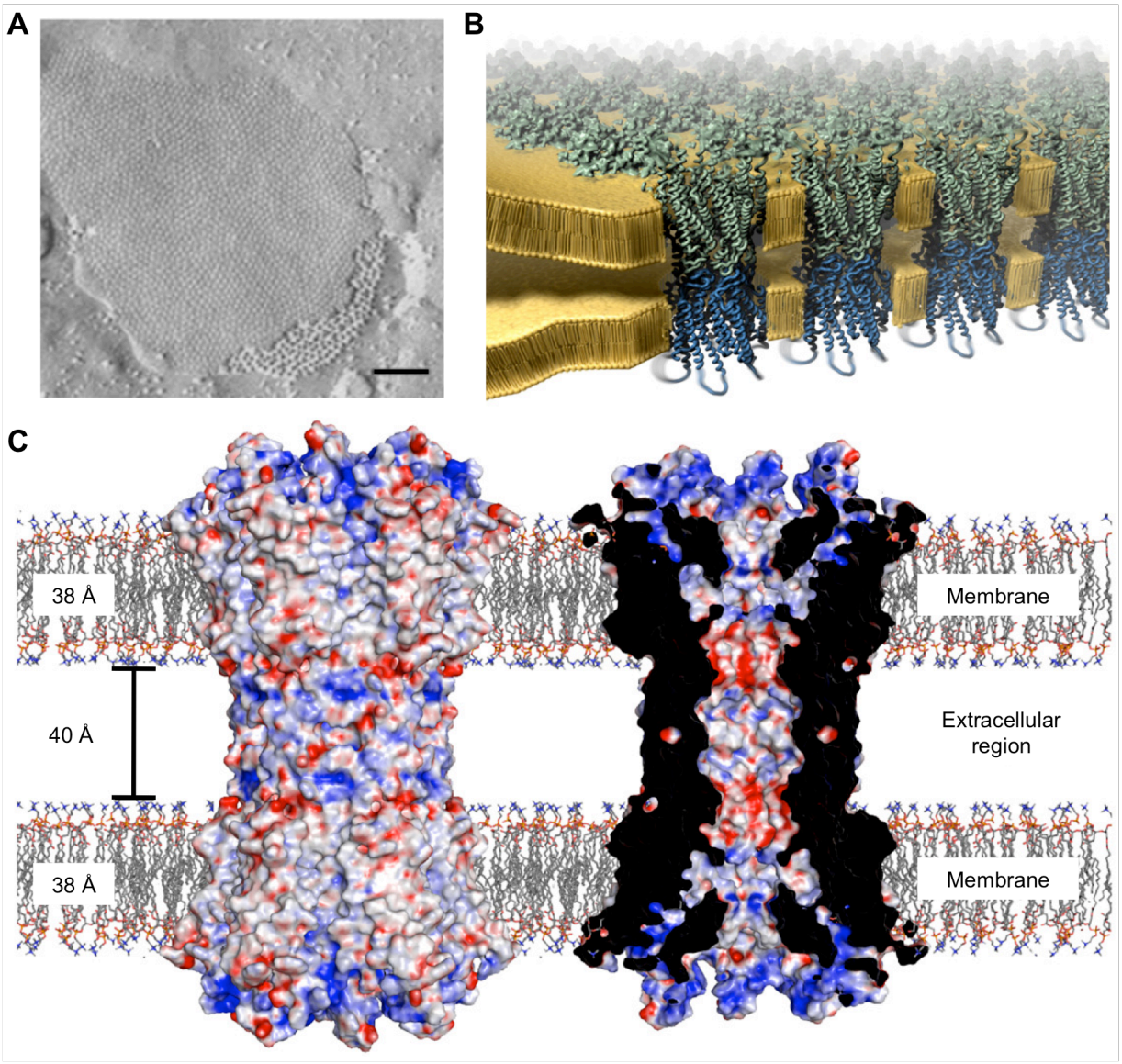

Figure 1 - Structure of gap junction channels. (A) Freeze-fracture replica of gap junctions of cultured B-16 melanoma cells. Scale bar, 0.1 μm (Larsen et al, 1979). (B) Schematic drawing of a gap junction plaque. The gap junction channels are drawn as a Cα trace spanning two adjoining membranes with an inter-membrane space of 40 A° (Nakagawa et al, 2010). (C) Surface charge representation of the Cx26 gap junction channel. The outer surface is represented on the left while the channel interior sectioned along the pore axis is represented on the right. The displayed potentials range from 20kT/e (blue) to -20kT/e (red) (Nakagawa et al, 2010).

linked Charcot-Marie-Tooth disease (Bergoffen et al, 1993) which is caused by mutations in Cx32 that result in aberrant Cx32 trafficking, misassembly of gap junction channels or abnormal gating properties (Krutovskikh & Yamasaki, 2000; Zhou & Griffin, 2003). Since then, several other human conditions have been linked to mutations in connexin family members. Mutations in Cx26, Cx30, Cx30.3 and Cx31 can give rise to sensorineural hearing loss and hyperproliferative skin disorders (Richard et al, 2003), while oculodentodigital dysplasia (ODDD) has been linked to mutations in the Cx43 gene (Paznekas et al, 2003).

Connexins

All members of the connexin family contain four transmembrane domains, two extracellular loops and one cytosolic loop. Both the amino- and carboxyl-terminal of connexins are located in the cytosolic face of the plasma membrane. At present, 21 different connexin genes have been reported in humans, and 20 in mice. The transmembrane domains and extracellular loops are highly conserved throughout the connexin family; however, many key functional differences between connexins stem from amino acid sequence differences in these domains. Differences between the primary amino acid sequences of connexin family members are mostly found in the cytoplasmic loop and the carboxyl-terminal of the proteins.

There are two nomenclature systems for connexin proteins. The first system identifies connexins according to their predicted molecular weight in kDa, for example, Cx32, Cx40, Cx43, etc (Cx = Connexin). The second nomenclature system identifies connexins according to their sequence homology, length of their cytoplasmic domains and by order of discovery. As such, Cx43, the first connexin of the alpha family to be discovered, is named gja1 (gj = gap junction). In this nomenclature two more protein families have been described, beta (b) and gamma (c). Connexins unrelated to either the α, β or γ families are classified as divergent connexins and identified as being a part of the delta family (d).

Many tissues and cell types express two or more members of the connexin family. Cardiomyocytes express Cx40, Cx43 and Cx45 (Beyer et al, 1995; Gros & Jongsma, 1996; Moreno, 2004), while the lens expresses Cx43, Cx46 and Cx50 (Paul et al, 1991; White et al, 1992). Expression of multiple members of the connexin family within the same cell type may allow for compensatory mechanisms to take place when a specific connexin protein is either mutated or lost. This has been demonstrated in several studies where the endogenous expression of several connexins was able to compensate for the deletion of a specific connexin gene (Houghton et al, 1999; Simon et al, 1997). However, this does not mean that all connexins are redundant when present in cells expressing more than one type of connexin protein; in some cell types the presence of a specific connexin protein is sometimes essential for the normal function of the cell, regardless of the presence of more members of the connexin family (White, 2003). In the developing lens, knockout of Cx46 results in the formation of cataracts while knockout of the Cx50 gene results in the formation of cataracts and a lens growth defect. Interestingly, knockin of the Cx46 gene into the loci of the Cx50 gene is capable of preventing the formation of opacities in the lens, but does not rescue the growth defect, demonstrating the requirement of Cx50 for normal development of the lens (Martinez-Wittinghan et al, 2004; Sellitto et al, 2004).

Of all the connexin family members, Cx43 is the most ubiquitously expressed and is endogenously expressed in at least 34 distinct tissues and 46 cell types that include cardiomyocytes,

Human		Mouse	Major expressed organ or cell types
Molecular mass nomenclature	HUGO nomenclature	Molecular mass nomenclature	
hCx23		mCx23	-
hCx25	-	-	-
hCx26	GJB2	mCx26	Breast, cochlea, placenta, hepatocytes, skin, pancreas, kidney, intestine
hCx30	GJB6	mCx30	Brain, cochlea, skin
hCx30.2	GJE1	mCx29	Brain, spinal cord, Schwann cells
hCx30.3	GJB4	mCx30.3	Skin, kidney
hCx31	GJB3	mCx31	Cochlea, placenta, skin
hCx31.1	GJB5	mCx31.1	Skin
hCx31.9	GJC1 (GJA11)	mCx30.2	-
hCx32	GJB1	mCx32	Hepatocytes, secretory acinar cells, Schwann cells
-	-	mCx33	Sertoli cells
hCx36	GJA9	mCx36	Neurons, pancreatic β-cells
hCx37	GJA4	mCx37	Endothelium, granulosa cells, lung, skin
hCx40	GJA5	mCx40	Cardiac conduction system, endothelium, lung
hCx40.1	-	mCx39	-
hCx43	GJA1	mCx43	Many cell types
hCx45	GJA7	mCx45	Cardiac conduction system, smooth muscle cells, neurons
hCx46	GJA3	mCx46	Lens
hCx47	GJA12	mCx47	Brain, spinal cord
hCx50	GJA8	mCx50	Lens
hCx59	GJA10	-	-
hCx62	-	mCx57	Retinal horizontal cells

Table 1 - Connexin genes and their expression. Adapted from (Oyamada et al, 2005).

keratinocytes, astrocytes, endothelial cells and smooth muscle cells (Solan & Lampe, 2009). Unsurprisingly, Cx43 is also the most widely studied connexin protein.

Connexons

Hemichannels formed by the oligomerization of 6 monomeric connexin units are called connexons. Connexons can be composed of a single type of connexin (homomeric) or multiple types of different connexins (heteromeric). Similarly, gap junction channels can be formed by the docking of two identical connexon hemichannels (homotypic) or through the docking of connexons composed of different connexin proteins (heterotypic). The ability of a specific connexin to participate in the assembly of a heteromeric connexon varies from connexin to connexin. As a general rule, connexin proteins of the alpha family cannot form heteromeric connexons with connexin proteins of the beta family. Examples of heteromeric channels formed by endogenously expressed connexins include the α family members Cx46-Cx50 in the lens (Jiang & Goodenough, 1996) and Cx43-Cx46 in the lung (Das Sarma et al, 2001) and the β family members Cx32-Cx26 in the liver (Ahmad et al, 1999; Harris, 2001). Connexins that do not belong to either family (i.e. γ and δ connexins) can form

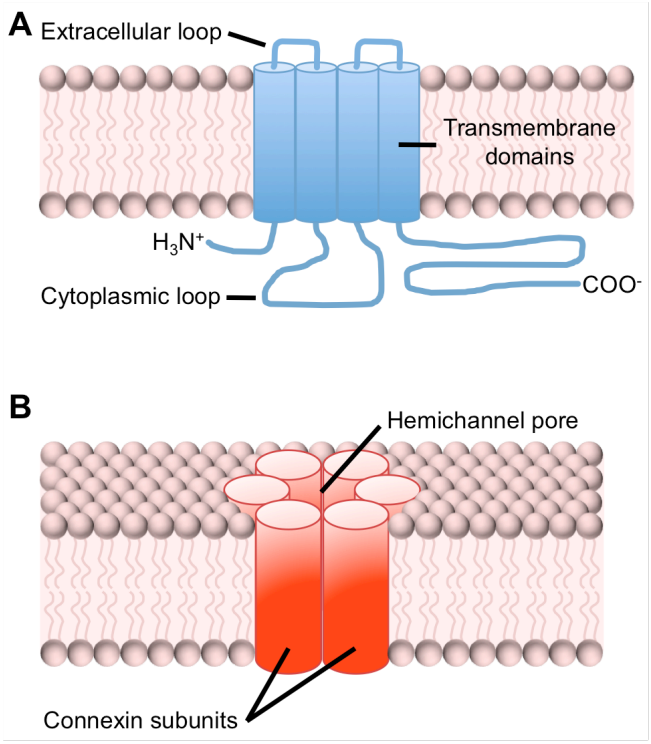

Figure 2 - Basic structure of connexins and connexons. (A) Representation of a single connexin subunit inserted at the plasma membrane. Connexin protein subunits are tetra-spanning membrane proteins with two extracellular loops and one cytoplasmic loop. Both the amino- and carboxyl-terminals of the protein face the cytoplasm. (B) Schematic drawing of a connexon. Six connexin subunits oligomerize to form a connexon or hemichannel. Each of the subunits contributes to the formation of the channel pore.

heteromeric connexins with members of either the α or β family, but not with both. Cx45, a member of the γ family has been shown to form heteromeric connexons with Cx43 (Koval et al, 1995), while mCx29, a member of the δ family has been shown to form heteromeric connexons with Cx32 (Altevogt et al, 2002).

However, the ability of different connexins to form heteromeric connexons is not only dependent on direct compatibility between connexins, but is also regulated by the cellular environment. For example, endogenous Cx43 and Cx46 can form heteromeric connexons in type I alveolar epithelial cells, but they do not hetero-oligomerize in type II alveolar epithelial cells or osteoblastic cells (Abraham et al, 1999; Das Sarma et al, 2001; Koval et al, 1997). Selective connexin hetero-oligomerization can also take place within a cell. Vascular endothelium and smooth muscle cells express Cx37, Cx40 and Cx43, which can all hetero-oligomerize with each other. However, it was found that Cx37 was specifically excluded from myoendothelial junctions between endothelial cells and smooth muscle cells, while still being incorporated in gap junction channels connecting adjacent endothelial cells or adjacent smooth muscle cells (Isakson & Duling, 2005).

Likewise, the ability of different connexons to assemble a heterotypic gap junction channel also varies with the connexin composition of each connexon. Although, and contrary to what happens with hetero-oligomerization, heterotypic gap junction channel assembly is not entirely restricted by connexin family subtypes. For example, Cx32 and Cx43 cannot form heterotypic channels,

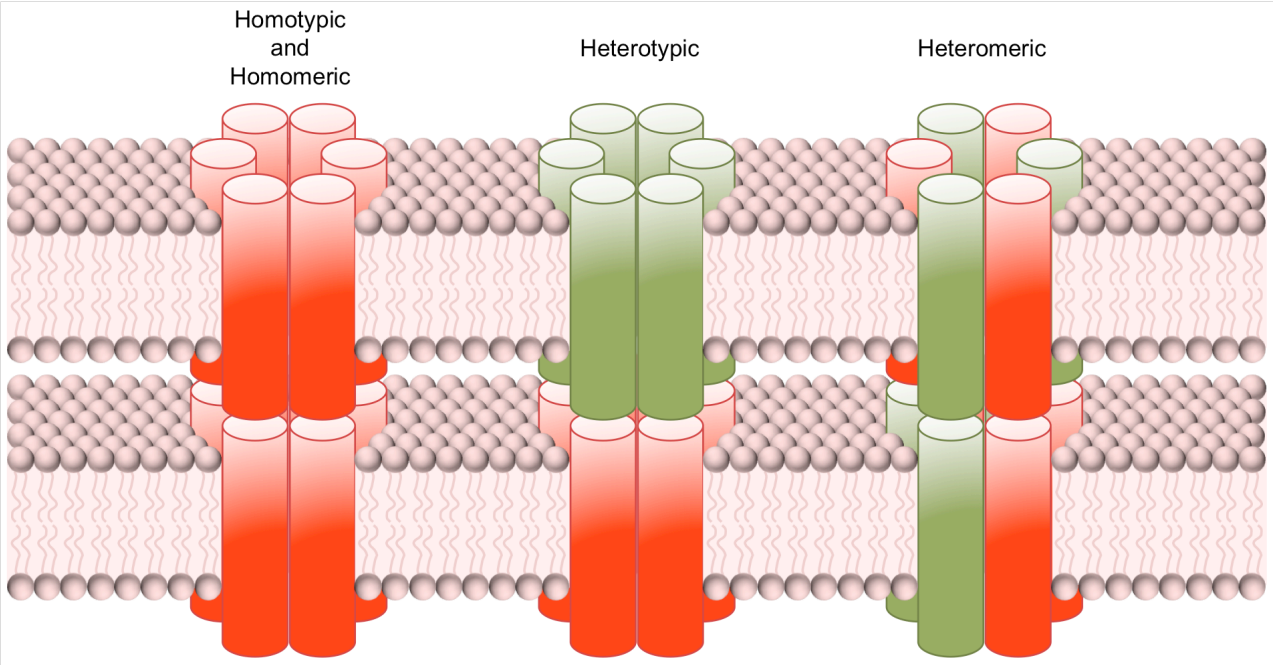

Figure 3 - Types of gap junction channel. Each cylinder represents an individual connexin protein. From left to right: Homotypic and homomeric channels composed of a single connexin type; Heterotypic channels composed of two types of connexin expressed in two different cells; Heteromeric channels composed of two different connexins expressed within a single cell.

however, the α connexins Cx46 and Cx50 can form heterotypic channels with either one (White et al, 1995). Heterotypic compatibility between connexins appears to be determined by the extracellular loops and the cytoplasmic loop of connexin proteins (Bruzzone et al, 1994; White et al, 1994). The selectivity in heterotypic gap junction assembly is an important property of connexons, allowing cells expressing different connexins to remain structurally and functionally independent, which in turn allows for the presence of independent communication pathways within tissues or organs such as the retina or the brain.

The permeability properties of gap junction channels are also directly affected by their connexin composition (Goldberg et al, 2004). While several studies have shown that the relative selectivity of gap junction channels towards monovalent cations is similar across a number of connexins: Cx43, Cx40, Cx37 and Cx45 (Beblo & Veenstra, 1997; Veenstra et al, 1995; Veenstra et al, 1994; Wang & Veenstra, 1997), the same does not remain true when examining the selectivity of channels towards cyclic nucleotides. Homomeric hemichannels formed by Cx32 display no preference towards cAMP or cGMP, however, heteromeric channels composed of Cx32 and Cx26 are less restrictive to the passage of cGMP compared to cAMP, indicating that Cx26 is selective against cAMP (Bevans et al, 1998). Another study shows that Cx32 gap junction channels are more restrictive towards the passage of AMP and ATP compared to gap junction channels formed by Cx43 (Goldberg et al, 2002b). Interestingly, while the passage of ATP through Cx43 channels is 100 fold better than the passage of the same negatively charged molecule through Cx32 channels, this same study also demonstrated that adenosine is transferred several fold more easily through

Cx32 gap junctions when compared to Cx43 gap junctions, indicating that Cx32 gap junction selectivity for this particular molecule may be dependent on charge, with the passage of negatively charged species of adenosine being restricted. However, the selectivity of Cx32 gap junction channels does not appear to be dictated by charge alone, since other studies have shown that the anionic metabolite IP3 passes more easily through Cx32 channels than through channels formed by either Cx43 or Cx26 (Niessen et al, 2000). This diversity in connexon composition and permeability allows for the functional diversity of gap junctions in different tissues and organs. Thus, gap junction channels are capable of modulating the passage of different molecular effectors in different cell types, according to the specific needs of each tissue and organ.

Assembly of oligomeric integral membrane proteins usually occurs in the organelle in which they are synthesized, however, connexon assembly can occur either in the endoplasmic reticulum (ER) or in the Golgi network, although recent reports suggest that for some connexin species, ER connexon oligomerization may be a product of connexin overexpression, rather than a physiologically relevant event (Vanslyke et al, 2009). Nevertheless, connexins are only transported to the plasma membrane after being oligomerized into a connexon unit. Upon reaching the plasma membrane, connexons may remain as a hemichannel or assemble into a gap junction channel by docking to a hemichannel of an adjacent cell. Docking of two hemichannels to form a gap junction channel is done through non-covalent interactions between the two extracellular loops of connexins from adjacent cells. Newly formed gap junction channels are then added to the periphery of gap junction plaques, while older gap junction channels are internalized from the centre of the gap junction plaque.

Connexon hemichannels were originally thought to be permanently closed, remaining inactive until they docked to a connexon from an adjacent cell to form a functional gap junction channel. However, it has been demonstrated that hemichannels are capable of opening to establish communication between the cytoplasm and the extracellular environment. In fact, important physiological functions have already been described for connexon hemichannels, such as calcium signalling, regulation of cell proliferation and death as well as the normal function and development of several cell types (Evans et al, 2006). Cx43 hemichannels have been shown to be important in bone remodelling through the release of Prostaglandin E(2) following mechanical stimuli (Burra & Jiang, 2009). Connexin hemichannels have also been shown to participate in inositol 1,4,5-trisphosphate (IP3) release in the cochlea (Gossman & Zhao, 2008) while Cx26 and Cx30 hemichannels are also important for the propagation of Ca^{2+} in the inner ear through the release of ATP into the extracellular medium (Anselmi et al, 2008), these roles of hemichannels in the inner ear may indicate that some of the hearing loss associated to mutations in connexin proteins may result not only from defects in gap junctional communication but also from defects in hemichannel function, similarly to what has recently been suggested for Cx26 mutations that cause skin diseases (Lee et al, 2009). In the heart, connexin hemichannels have an osmoregulatory role, with

both negative and positive effects in myocardial infarcts and cardiac physiology (John et al, 2003; John et al, 1999). Hemichannels composed of the same connexin species can have opposite functions in promoting cell survival depending on the cell type where it is being expressed and the stimuli it receives. In a rat C6 glioma cell model, Cx43 hemichannels were shown to promote apoptosis in response to treatments with cytochrome C (Decrock et al, 2009), while in osteoblastic cells, Cx43 hemichannels were shown to mediate the anti-apoptotic effects of bisphophonates (Plotkin et al, 2002).

However, despite all the important functions that have been ascribed to connexin hemichannels, recent reports have suggested that some of the functions attributed to connexin hemichannels may in reality be due to the presence of pannexin hemichannels (Shestopalov & Panchin, 2008).

Post-translational modification of Connexins

Phosphorylation

Protein phosphorylation consists in the addition of a phosphate group (PO_4) to a protein residue. Phosphorylation (and dephosphorylation) of proteins is a very common post-translational modification, functioning as a molecular switch that modulates the activity of most proteins. The most common protein phosphorylation residues are serine and threonine, followed by tyrosine.

Many connexin species have been demonstrated to be phosphoproteins: Cx31, Cx32, Cx36, Cx37, Cx40, Cx43, Cx45, Cx46, Cx50 and Cx56 (Lampe & Lau, 2004; Solan & Lampe, 2009). Consistent with its importance in modulating most aspects of connexin function, phosphorylation of connexins occurs mostly in the carboxyl-terminal (CT), however, phosphorylation in the cytoplasmic loop has been described for at least Cx36, Cx50 and Cx56 (Berthoud et al, 1997; Urschel et al, 2006; Wang & Schey, 2009). Currently, there are no reports of connexins being phosphorylated on the amino-terminal (NT).

Phosphorylation has been implicated in many important metabolic events of connexin life. Phosphorylation of Cx36 by protein kinase A (PKA) has been linked to decreased gap junctional communication between amacrine cells in the retina (Urschel et al, 2006). In contrast, activation of PKA has been linked to increased gap junctional communication in T84 cells expressing Cx32 (Chanson et al, 1996) and in a human hepatoma cell line expressing Cx40 (van Rijen et al, 2000). Additionally, dephosphorylation of Cx40 has been linked to a decrease in gap junctional communication in microvascular endothelial cells (Bolon et al, 2008), further reinforcing the importance of phosphorylation in Cx40 gap junctional communication. Cx45 gap junctions have also been demonstrated to be modulated by phosphorylation in HeLa cells (van Veen et al, 2000), while protein kinase C-gamma ($PKC\gamma$) has been implicated in regulating Cx46 and Cx50 gap junctional communication in the lens (Zampighi et al, 2005).

Due to its widespread expression and importance in many tissues and cell types, more is known about Cx43 phosphorylation than for all other connexin species. For example, phosphorylation of Cx43 has been implicated in Cx43 transport to the plasma membrane, as activation of PKA enhances transport of the protein to the plasma membrane and increases gap junctional communication (Atkinson et al, 1995; Paulson et al, 2000). Assembly of Cx43 hemichannels into gap junction channels has been shown to involve casein kinase 1 (CK1) activity, as mutation of the serines targeted by CK1 impairs the ability of Cx43 to form gap junctional channels (Cooper & Lampe, 2002). Activation of PKC by phorbol 12-myristate 13-acetate (PMA) induces Cx43 phosphorylation and internalization (Lampe, 1994; Pitts & Burk, 1987). PKC induced phosphorylation is also important for Cx43 channel gating (Lampe et al, 2000). Phosphorylation of Cx43 by mitogen-activated protein kinase (MAPK) has been implicated in channel gating by reducing the time Cx43 channels remain in the open state (Cottrell et al, 2003). Furthermore, phosphorylation was shown to act as a stimuli for Cx43 proteasome-dependent degradation in lens epithelial cells (Girao & Pereira, 2003), while both PMA and EGF (epidermal growth factor) induced phosphorylation of Cx43 has been shown to induce Cx43 ubiquitination and subsequent internalization (Leithe & Rivedal, 2004a; Leithe & Rivedal, 2004b). Lastly, phosphorylation of Cx43 is also involved in regulating the interaction of Cx43 with other proteins. For example, Cx43 phosphorylation has been shown to be important for its interaction with the E3 ligase Nedd4 (Leykauf et al, 2006).

Ubiquitination

Ubiquitination is another common post-translational modification of proteins that is characterized by the covalent attachment of the 76 amino acid protein ubiquitin to lysine residues on the target protein. The most commonly described function of ubiquitination is the targeting of proteins for degradation by the proteasome (Hershko & Ciechanover, 1998). However, unlike phosphorylation, to date only two connexin family members have been shown to be ubiquitinated: Cx43 (Laing & Beyer, 1995) and more recently the Cx50 chicken lens orthologue Cx45.6 (Yin et al, 2008).
Cx26 has been described to interact with organ of Corti protein 1 (OCP1) a subunit of the SCF E3 ubiquitin ligase complex (Henzl et al, 2004), however ubiquitination of Cx26 was not demonstrated. The importance of this interaction for Cx26 function is also still unknown.
In primary lens cell cultures, Cx45.6 turnover was shown to be decreased in the presence of proteasome inhibitors, suggesting that Cx45.6 degradation is proteasome dependent. Cx45.6 phosphorylation was also shown to be involved in regulation of Cx45.6 ubiquitination in N2A cells (Yin et al, 2008). In another study using primary embryonic lens epithelial cells, both Cx45.6 and Cx56 (the chicken lens ortologue of Cx46) were shown to accumulate at the plasma membrane in the presence of proteasome inhibitors, suggesting that the ubiquitin proteasome pathway may be

involved in regulating the stability of these two proteins at the plasma membrane (Boswell et al, 2009).

Due to the numerous studies focusing on Cx43, more is known about the importance of ubiquitination in regulating Cx43 function than for any other connexin protein. It has been demonstrated that Cx43 is a substrate for ER associated degradation (ERAD) (VanSlyke & Musil, 2002), a process by which newly synthesized misfolded proteins are ubiquitinated and targeted for proteasomal degradation. In response to mild oxidative or thermal stress, ERAD degradation of Cx43 was reduced, leading to increased gap junction assembly and GJIC. Ubiquitination of Cx43 at the plasma membrane has been suggested to target the protein for internalization (Leithe & Rivedal, 2004a; Leithe & Rivedal, 2004b), while more recent studies have shown that ubiquitination of Cx43 is necessary for the late endossomal sorting of Cx43 to the lysosome for degradation (Auth et al, 2009; Leithe et al, 2009). Thus, ubiquitination of Cx43 is a major post-translational modification, regulating gap junction intercellular communication (GJIC) both at the biosynthesis level, by regulating Cx43 exit from the ER and subsequent transport of the protein to the plasma membrane, as well as regulating Cx43 degradation by targeting Cx43 present at the plasma membrane for internalization and lysosomal degradation.

Connexin43

Cx43, a member of the alpha connexin family, was the second connexin family member to be identified (Beyer et al, 1987), following the characterization of Cx32 (Kumar & Gilula, 1986; Paul, 1986), and is also the most widely expressed connexin protein, being found in at least 35 tissues and 46 cell types (Solan & Lampe, 2009). Cx43 has been found to be expressed in tissues as diverse as the heart, lens, retina, skin, brain, kidney and bone marrow (Laird, 2006).

Knockout of Cx43 in mice was shown to be lethal, with mouse embryos dying shortly after birth due to defects in the right ventricular outflow tract of the heart (Reaume et al, 1995), which was later shown to be caused by defects in the neural crest cells required for the normal development of that region of the heart (Lo et al, 1997). However, knockout of the Cx43 gene did not appear to cause any major defects in other organs.

Mutations in the Cx43 gene have also been linked to oculodentodigital dysplasia (Paznekas et al, 2003; Paznekas et al, 2009). Abnormalities observed in ODDD affect the eye, dentition, and digits of the hands and feet. ODDD is also characterized by neurological problems, conductive hearing loss, cardiac defects, and anomalies of the skin, hair, and nails. Considering that Cx43 is the most universal connexin found in the human body, it is somewhat surprising that patients carrying this autossomal dominant mutation in the Cx43 gene aren't considerably more ill than they appear to be. In fact, patients suffering from this disease appear to live long lives in relatively good health (Laird, 2006). It is likely that more severe defects in Cx43 knockout mice and humans suffering

from ODDD are prevented due to functional compensation by the other connexin family members.

Cx43 oligomerization and delivery to the plasma membrane

Unlike most multimeric ion channels that oligomerize in the ER (Deutsch, 2003), Cx43 oligomerization occurs in the trans-Golgi network (TGN) after exiting the ER (Musil & Goodenough, 1993). A recent study demonstrated that the site of connexon formation was not dictated by connexin species, cell type or whether the connexin is native to the cell type, but rather by the level of connexin synthesis (Vanslyke et al, 2009). One hypothesis would be that a molecular chaperone could associate with newly synthesized connexins in the ER, preventing them from oligomerizing until they reached the TGN. Overexpression of Cx43 would then saturate the limited amount of the chaperone protein leading to Cx43 oligomerization in the ER instead. However, no evidence for such a chaperone protein was found in this study, leading to the proposition of an alternative hypothesis in which Cx43 oligomerization in the TGN was largely a concentration-driven self-assembly process, similar to what is suggested for later stages of gap junction formation (Castro et al, 1999; Valiunas et al, 1997). Thus, in physiologically relevant conditions, levels of Cx43 monomers would only reach the required concentration for oligomerization in the TGN (Vanslyke et al, 2009). Nonetheless, another recent study reported that the ER-associated chaperone ERp29 helps stabilize monomeric Cx43 facilitating its oligomerization in the TGN (Das et al, 2009). Interference with normal ERp29 function, either by depletion of ERp29 through RNAi or through the overexpression of a dominant negative mutant of the protein, was shown to decrease Cx43 gap junction formation and lead to the oligomerization of Cx43 in the ER, suggesting that premature oligomerization of Cx43 impedes efficient trafficking along the secretory pathway. Furthermore, ERp29 chaperone activity was shown to be able to distinguish between different classes of connexins since interference with ERp29 activity had little effect on the beta connexin family member Cx32. This result is in agreement with results obtained by Vanslyke et al, showing that overexpression of Cx32 did not affect the normal oligomerization of endogenous Cx43 in LA25 NRK cells (Vanslyke et al, 2009).

Upon reaching the plasma membrane Cx43 connexons may either remain as an uncoupled hemichannel, connecting the cytoplasm directly with the extracellular medium or, as more commonly described, connect with a connexon from an adjacent cell to form a functional gap junction. Incorporation of Cx43 into gap junction plaques has been shown to confer resistance to solubilization with mild detergents such as Triton X-100 (Musil & Goodenough, 1991), thus, forms of Cx43 that are insoluble in Triton X-100 are considered to be forming gap junction plaques. Incorporation of Cx43 hemichannels into gap junction plaques was shown to require previous phosphorylation of specific serine residues by CK1 (Cooper & Lampe, 2002). Inhibition of CK1 was shown to increase the amount of non-junctional Cx43 present at the plasma membrane as well as

reduce the levels of Triton X-100 insoluble Cx43. Furthermore, expression of mutant forms of Cx43 where the serine residues targeted by CK1 were mutated decreases the ability of cells to form gap junctions, indicating that conformational changes that occur after phosphorylation by CK1 were important for gap junction assembly (Cooper & Lampe, 2002).

Caveolins (Cav) are a structural component of caveolae and vesicles derived from the TGN, that serve as scaffolding proteins to cluster lipids and signalling molecules (Williams & Lisanti, 2004). Cav-1 and Cav-2 have been shown to interact with Cx43 and are involved with trafficking of the protein to the plasma membrane and to cholesterol and sphingolipid rich plasma membrane domains, commonly termed lipid rafts (Langlois et al, 2008; Schubert et al, 2002). Disruption of lipid rafts with methyl-β-cyclodextrin or depletion of Cav-1 and Cav-2 with siRNA, was shown to reduce GJIC, while overexpression of Cav-1 in HEK293FT cells (which express Cx43 but are caveolin deficient) enhanced GJIC. Furthermore, Cx43 and caveolins were shown to colocalize mainly in the TGN with little colocalization found at the plasma membrane (Langlois et al, 2008). Studies using Cav-1 knockout mice also showed that the absence of Cav-1 lead to reduced expression of Cx43 in the aorta. Gap junction formation and GJIC were also decreased in arteries of Cav-1 knockout mice (Saliez et al, 2008). In accordance with what is suggested for other connexins (Locke et al, 2005), Cx43 and caveolins may traffic together to the plasma membrane in lipid rafts, where they then disassociate as Cx43 is inserted into gap junctional plaques (Langlois et al, 2008). Activation of PKCγ by PMA or insulin growth factor 1 was shown to translocate PKCγ to lipid rafts where it colocalized with Cav-1 and phosphorylated Cx43, leading to the disassembly of Cx43 gap junction plaques and reduced GJIC. The disassembly of Cx43 gap junctions plaques following PKCγ activation was suggested to be due to relocalization of Cx43 to Cav-1 containing lipid rafts and not due to the internalization of Cx43 (Lin et al, 2003). A later study demonstrated that PKCγ mediated reduction of Cx43 GJIC could be induced by oxidative stress, suggesting that the rapid disassembly of gap junction plaques triggered by PKCγ phosphorylation of Cx43 could function as a protective mechanism against the spread of oxidative damage (Lin & Takemoto, 2005). A recent study demonstrated that Cx43 also interacts and colocalizes with Cav-3, suggesting that it may also play a role in regulating Cx43 GJIC (Liu et al, 2009).

Connexin gap junction plaques isolated from various cell types and organisms have shown that gap junctions are localized in membrane microdomains rich in cholesterol (Malewicz et al, 1990). Supplementation of exogenous cholesterol to cultured hepatoma cells was shown to increase gap junction assembly and permeability in a dose-dependent manner (Meyer et al, 1990). In studies using cardiac cells, Heptanol was shown to block Cx43 GJIC by reducing the fluidity of cholesterol-rich membrane domains, which in turn decreased the open probability of Cx43 gap junction channels (Bastiaanse et al, 1993). Oxidized phospholipids have also been suggested to take part in the pathogenesis of atherosclerosis by inducing modifications in Cx43 expression and

phosphorylation (Isakson et al, 2006; Johnstone et al, 2009).

Cx43 has been reported to interact with several components of the cytoskeleton (Dbouk et al, 2009; Olk et al, 2009). Sedimentation experiments were used to show that Cx43 interacts directly with microtubules (Giepmans et al, 2001a). Using several Cx43 protein constructs Giepmans et al demonstrated that amino acids 228 to 263 in the juxtamembrane region of the CT domain were essential for the interaction between Cx43 and microtubules. This region was also shown to contain a putative microtubule binding motif. Cx43 was also shown to interact directly with both α-tubulin and β-tubulin. Immunofluorescence and immunoelectronic microscopy experiments also showed that the distal ends of microtubules colocalized with Cx43 gap junctions (Giepmans et al, 2001a; Giepmans et al, 2001b). A recent study demonstrated that microtubules have an important function in the delivery of Cx43 to the plasma membrane. Using live-imaging to follow the intracellular transport of Cx43-YFP (yellow fluorescent protein), Shaw et al. demonstrated that treatments that impaired normal microtubule formation also delayed the delivery of Cx43 to the plasma membrane. Additionally, microtubules appeared to deliver Cx43 directly to plasma membrane domains containing N-cadherin, a major component of adherens junctions, thus suggesting that microtubules may be involved in the direct delivery of Cx43 hemichannels to points of cell-cell contact (Shaw et al, 2007).

Actin cytoskeleton filaments have long been known to associate with connexins. Annular gap junctions have been shown to be surrounded by actin-containing filaments (Larsen et al, 1979). Furthermore, invaginating gap junctions were also shown to be surrounded by a network of actin-containing microfilaments, suggesting that the microfilament network and sheath of actin act in concert to provide the mechanical force necessary for internalization of gap junctions and formation of annular gap junctions (Watanabe et al, 1988). Actin filament bundles have also been suggested to help stabilize Cx43 gap junctions at the plasma membrane (Wall et al, 2007). Although actin has been shown to colocalize or co-immunoprecipitate with Cx43, no direct interaction between the two proteins has been shown, suggesting that Cx43 interacts with actin through linker proteins. Drebrin (Developmentally Regulated Brain Protein) is one of these linker proteins. Butkevich et al. demonstrated that Drebrin interacts directly with Cx43. Furthermore, depletion of Drebrin with siRNA was shown to impair GJIC due to Cx43 gap junction internalization and degradation, while Drebrin overexpression stabilized Cx43 gap junctions at the plasma membrane (Butkevich et al, 2004). Drebrin was later shown to colocalize with Cx43 at gap junction plaques (Xu et al, 2006). As such, Drebrin is a prime candidate for linking Cx43 to the actin cytoskeleton, stabilizing Cx43 gap junction plaques at the plasma membrane. Recently, RhoA and F-actin were shown to have a role in modulating the permeability of the Cx43 channel pore. RhoA is a member of the Rho GTPases family, which are major regulators of the actin cytoskeleton (Etienne-Manneville & Hall, 2002). Derangeon et al. reported that activation of RhoA by cytotoxic necrotizing factor 1 enhanced GJIC while inhibition of RhoA by *Clostridium botulinum* C3

exoenzyme reduced GJIC. Both treatments had little effect on the distribution of gap junction plaques and Cx43 phosphorylation, indicating that the observed changes in GJIC following RhoA stimulation were due to changes in the permeaselectivity of the Cx43 channel pore and not by variations in the number of gap junction channels at the plasma membrane or cell-cell contacts. Additionally, RhoA was shown to exert its effect upon Cx43 gap junctions through the actin cytoskeleton as agents that stabilized actin filaments, such as phalloidin, markedly reduced the effects of RhoA activation or inhibition (Derangeon et al, 2008). As such, the actin cytoskeleton is not only important for stabilizing Cx43 at the plasma membrane, but also plays a role in modulating the channel pore.

Adherens junctions are membrane structures responsible for the mechanical coupling of cells. Considering how gap junction formation requires the close proximity of cells, it comes as no surprise that adherens junctions and gap junctions are closely linked. Cx43 GJIC was first shown to be modulated by the calcium-dependent cell adhesion molecule N-cadherin (Jongen et al, 1991). Blockage of the extracellular domains of Cx43 with antibodies prevented adherens junction formation, likewise, blockage of the extracellular domains of N-cadherin also prevented gap junction formation (Meyer et al, 1992). Furthermore, Cx43 was shown to co-assemble with N-cadherin and several other N-cadherin associated proteins in a process that was important for the trafficking of Cx43 to the plasma membrane. Depletion of either Cx43 or N-cadherin through siRNA was shown to decrease levels of N-cadherin and Cx43 at the plasma membrane and reduce GJIC (Wei et al, 2005). Although cadherins and adherens junctions were originally thought to only be important for establishing points of close cell-cell contact, thus enabling gap junction formation, the discovery that several protein members of the adherens junction complex are signal transduction molecules has led to the suggestion that adherens junctions may be involved in the transduction of signals relating to the status of cell-cell coupling, however, such signalling has yet to be formally demonstrated (Herve et al, 2007).

Cx43 has been shown to interact with several elements of tight junctions. Tight junctions are transmembrane protein structures that connect adjacent cells providing both barrier and fence functions. Tight junction proteins include occludins and claudins as well as proteins of the zonula occludens (ZO) family that function as scaffolds, connecting occludins and claudins to the actin cytoskeleton and signal transduction molecules (Koval, 2006). Cx43 was shown to interact and colocalize with the tight junction proteins occludin and claudin-5 in porcine blood-brain barrier endothelial cells (Nagasawa et al, 2006). Cx43 was the first connexin protein to be found to interact with ZO-1 (Giepmans & Moolenaar, 1998; Toyofuku et al, 1998), subsequently many other connexin members of the alpha family have been shown to interact with ZO-1 (Herve et al, 2007). However, to date no member of the other connexin families have been shown to interact with ZO-1, suggesting specificity of the alpha connexins for this interaction. Cx43-ZO-1 interactions are often found only at the periphery of gap junction plaques (Hunter et al, 2005). Disruption of the

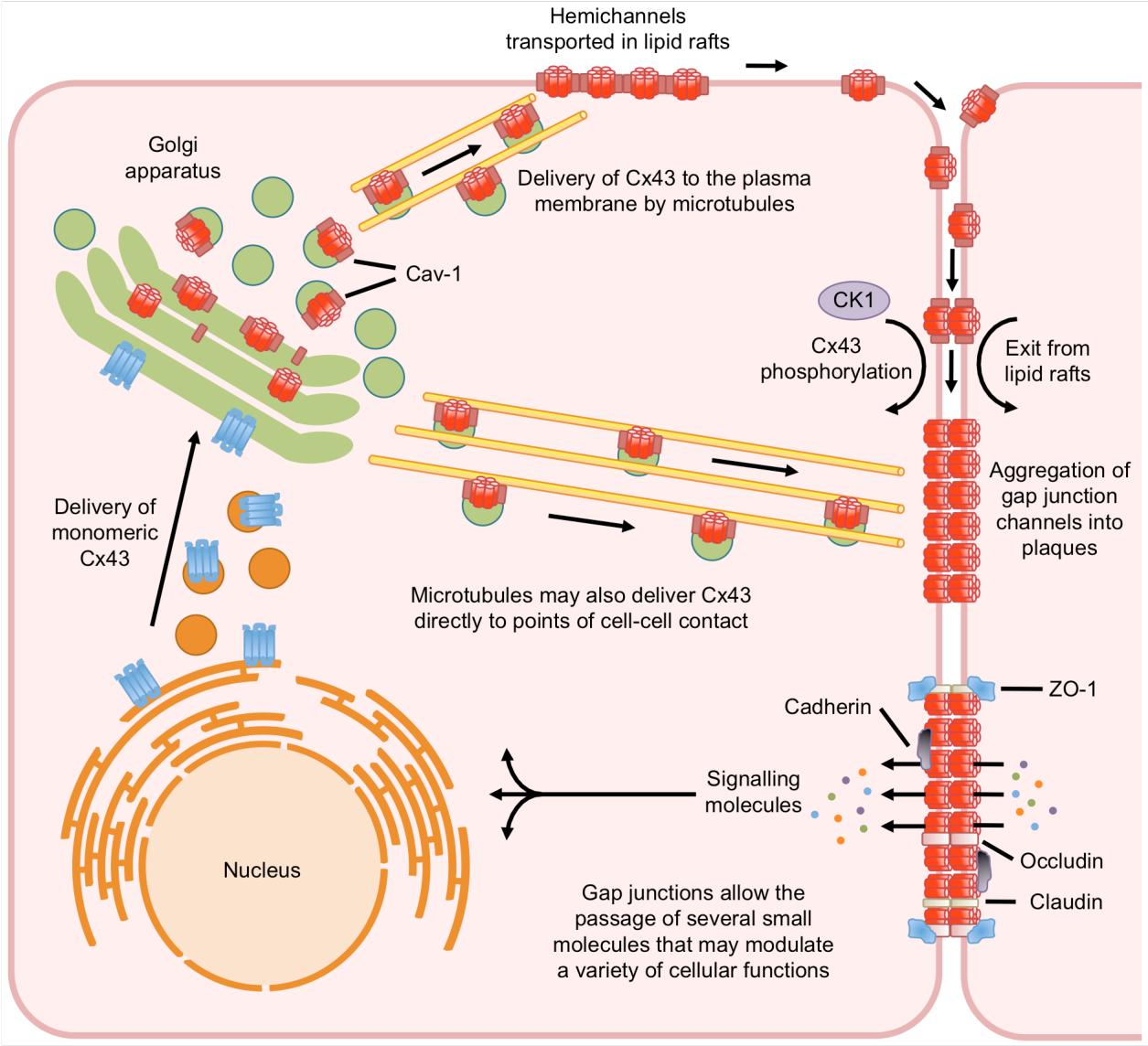

Figure 4 - Cx43 delivery and incorporation into gap junction plaques. A simplified model detailing part of the life cycle of Cx43. After being correctly folded and inserted into the membrane of the endoplasmic reticulum, monomeric Cx43 is trafficked to the trans-Golgi network where it oligomerizes into hexameric connexons and associates with Cav-1. Connexons are then transported through microtubules to the plasma membrane where they localize to lipid raft domains. Connexons can then dock with connexons from adjacent cells to form gap junction channels that diffuse laterally through the plasma membrane until aggregating at gap junction plaque sites. During this process, Cx43 is phosphorylated by CK1. Several proteins are known to interact with Cx43 at gap junction plaques, possibly modulating Cx43 function and signal transduction.

interaction between ZO-1 and Cx43 was shown to lead to the formation of abnormally large Cx43 gap junction plaques due to increased accretion of non-junctional Cx43 gap junctions, and not by increased Cx43 expression or decreased Cx43 turnover, thus suggesting that ZO-1 may be important in regulating the size of Cx43 gap junction plaques. A more recent study demonstrated that the interaction between Cx43 and ZO-1 was modulated by the proteasome (Girao & Pereira, 2007). Inhibition of the proteasome was shown to decrease the interaction of Cx43 with ZO-1 and lead to the formation of abnormally large gap junction plaques. This effect of the proteasome on gap junction plaque size was shown to specifically involve the modulation of the Cx43-ZO-1

interaction since inhibition of the proteasome had no effect on the size of gap junctions plaques formed by a mutant Cx43 in which the ZO-1 interacting domain was blocked with a tag. This study suggested that the proteasome modulated this interaction by degrading a putative Cx43-binding protein, which in turn regulated Cx43 and ZO-1 interaction (Girao & Pereira, 2007). The non-receptor tyrosine kinase c-Src was also shown to modulate the interaction between Cx43 and ZO-1. Binding of c-Src to Cx43 through its SH2 domain and subsequent Cx43 phosphorylation on Tyr265, reduced the interaction of Cx43 with ZO-1, impairing Cx43 mediated GJIC and leading to its internalization (Toyofuku et al, 2001). A more recent study by Gilleron et al further elucidated the role of c-Src in Cx43-ZO-1 dissociation and Cx43 internalization (Gilleron et al, 2008). γ-hexachlorocyclohexane (HCH) is a non-genomic carcinogen that is known to be a potent inducer of Cx43 internalization (Defamie et al, 2001). Treatment of 42GPA9 Sertoli cells with HCH was shown to recruit c-Src to the plasma membrane and induce Cx43 internalization. Furthermore, while ZO-1 was found on both sides of gap junction plaques in untreated cells, following treatment with HCH, ZO-1 would only be found on one side of the gap junction plaque, opposite to the side where c-Src is recruited. Additionally, during internalization of Cx43, ZO-1 would only be found in the inner side of the invaginating gap junction plaque and inside annular gap junction vesicles, while c-Src associated to Cx43 would be found in the outer side of annular gap junctions and was absent from the interior of the annular gap junction (Gilleron et al, 2008). These observations suggest a role for c-Src in initiating Cx43 gap junction internalization by disrupting the interaction between Cx43 and ZO-1. These findings are in agreement with earlier studies demonstrating that Cx43 phosphorylation induced its internalization and that proteasome inhibitors stabilized the phosphorylated form of Cx43, preventing its internalization and subsequent degradation (Fernandes et al, 2004; Girao & Pereira, 2003).

Cx43 has also been shown to interact with another zonula occludens protein, ZO-2 (Singh et al, 2005). ZO-1 and ZO-2 were shown to preferentially interact with Cx43 in different phases of the cell cycle, suggesting that both of these scaffold proteins may compete to influence gap junction patterning during cell cycle.

Cx43 channel gating

Similar to other transmembrane channels, gap junction channels are not permanently open and feature a gating mechanism that can be regulated through several factors, such as voltage, pH, calcium or phosphorylation (Gonzalez et al, 2007; Moreno & Lau, 2007; Peracchia, 2004).

To detect changes in voltage, the gating mechanisms of connexins must possess a voltage sensor. Typically, charged amino acids can be considered sensors since their electric charge position or electrical dipole orientation are sensitive to changes in the electric field. Modifications in the position of the charged group would then induce conformational changes in the protein leading

to the opening or closure of the channel pore. Connexin voltage gating can be modulated both by transjunctional voltage (V_j), which refers to the voltage difference between the two cells involved in forming the gap junction, and membrane voltage (V_m), which refers to the voltage difference between the interior of the cell and the extracellular medium. Cx43 is sensitive to both voltage differences (Gonzalez et al, 2007).

The V_j sensor of connexins appears to be located in the cytoplasmic amino-terminal domain (Gonzalez et al, 2007). Cx43 gap junctions appear to have two V_j sensitive gating mechanisms which allow for 3 functional states of the gap junctional channel, a main open state, a residual state (with lower conductance) and a closed state. Transitions from the main open state to the residual state are usually fast (<1-2 ms), while transitions from the residual state to the closed state are much slower, in the range of tens of milliseconds (Banach & Weingart, 2000). As such, these two gating processes have been termed as "fast V_j-gating" and "slow V_j-gating" respectively. These kinetically distinct gating states of Cx43 appear to be the result of two different gating mechanisms. The first evidence pointing towards this conclusion appeared in studies using a mutant form of Cx43 in which the CT domain had been truncated. CT truncated Cx43 mutants were shown to lose their fast gating component while the resulting differences in gating properties were largely attributed to slow V_j-gating (Revilla et al, 1999), indicating that not only were the gating mechanisms of fast V_j-gating and slow V_j-gating distinct, but also that the CT domain of Cx43 was important for the fast V_j-gating mechanism. Additionally, the differences found in slow V_j-gating kinetics in the Cx43 CT truncated mutants also indicate that the two gating mechanisms interact electrically in series. The action of the CT domain of Cx43 in fast V_j-gating was also shown to be blocked by the fusion of relatively large proteins to the CT domain, such as aequorin (Martin et al, 1998) or GFP (Bukauskas et al, 2000), possibly by limiting CT domain mobility or by interfering with some of its intramolecular interactions. These findings led to the suggestion that the fast V_j-gating mechanism of connexin proteins might follow the "ball and chain" model initially proposed to mediate fast inactivation in other unrelated ion channel families (Armstrong & Hille, 1998). According to this model, following stimulation, the CT domain of Cx43 ("chain") would fold into a "ball" (acting as a "gating particle") and move towards the entrance of the pore, physically blocking the passage of metabolites. Further support for this model appeared when it was shown that expression of a separate fragment containing the CT domain could restore fast V_j-gating in CT truncated Cx43 mutants (Anumonwo et al, 2001; Moreno et al, 2002). Therefore, even as a separate fragment, the CT domain of Cx43 can bind to the channel pore blocking its function. In Cx43, it has been suggested that the receptor site for the CT gating particle resides within the cytoplasmic loop of the protein. Fragments of Cx43 containing the second half of the cytoplasmic loop (termed the "L2" region) have been shown to interact with fragments containing the CT domain in a pH-dependent manner in vitro (Duffy et al, 2002). Expression of the fragment containing the cytoplasmic loop of Cx43 was also shown to block fast V_j-gating, suggesting that the

L2 peptide could interact with the CT domain of Cx43, therefore functioning as a competitive inhibitor that blocks its interaction with the native L2 region of Cx43 (Seki et al, 2004).

In Cx43, the transmembranar voltage gaiting (V_m-gating) mechanism seems to be independent of both V_j-gating mechanisms. Additionally, and unlike V_j-gating, V_m-gating induces transitions from an open state directly to a fully closed state of the channel pore (Gonzalez et al, 2007). Structural components of the V_m-gating sensor have been shown to reside in the CT domain of Cx43 near the forth transmembranar domain. Truncation of the CT domain of Cx43 at amino acid 257 fully abolished fast V_j-gating having little effect on V_m-gating (Revilla et al, 2000). On the other hand, truncation of the Cx43 CT domain at amino acid 242, which is the minimum length to retain channel-forming ability (Dunham et al, 1992), abolished sensitivity to V_m (Revilla et al, 2000). In this same study amino acids Arg243 and Asp245 were shown to be essential for V_m gating, indicating that, as opposed to the case of the V_j sensor, the V_m sensor appears to be located within the CT domain of Cx43.

pH-dependent gating of Cx43 was first described in a study detailing the inhibition of ionic communication by carbon dioxide (Turin & Warner, 1977), in which acidification of the intracellular medium would lead to the closure of Cx43 gap junction channels. Similar to the case of voltage dependent gating, the “ball and chain” model is also the proposed model for pH-dependent gating of Cx43 (Ek et al, 1994; Liu et al, 1993; Morley et al, 1996). A study where a fragment containing the CT domain of Cx43 was overexpressed, was shown to lead to the inhibition of pH-dependent gating of Cx43, supporting the “ball and chain” model for pH-gating (Morley et al, 1996). In later studies it was shown that this fragment interacted in vitro with the L2 region of the cytoplasmic loop in a pH-dependent manner (Duffy et al, 2002). As with voltage-gating, pH-gating would require a pH sensor in the protein. Histidine residues were proposed as the pH sensors of connexins due to the fact that histidine is the only amino acid whose sidechain dissociation constant from protons (pKa) falls within physiological range (Creighton, 1993). pH gating of Cx43 would then result from conformational changes induced by intracellular protons (Spray & Burt, 1990). Accordingly, His95 in the cytoplasmic loop of Cx43 was shown to be important for pH-dependent gating (Ek et al, 1994). Later studies also showed that acidification lead to conformational alterations and protonation of the histidines present in the L2 region (second half of the cytoplasmic domain) (Duffy et al, 2002), indicating that histidines may indeed function as a pH sensor for Cx43 gating. Additionally, studies have also shown that non-histidine residues located in the CT domain are also essential for pH-dependent gating (Ek-Vitorin et al, 1996), perhaps due to their importance in the function of the CT domain as a gating particle.

Although it was initially proposed that acidification of the intracellular medium with the concomitant rise in H^+ would directly modulate pH-gating of connexins, others have proposed that pH-gating of connexins is mediated by an intermediate molecule that binds to the connexin protein, given that in some cellular models connexins are insensitive to intracellular acidification as in the case of Cx43

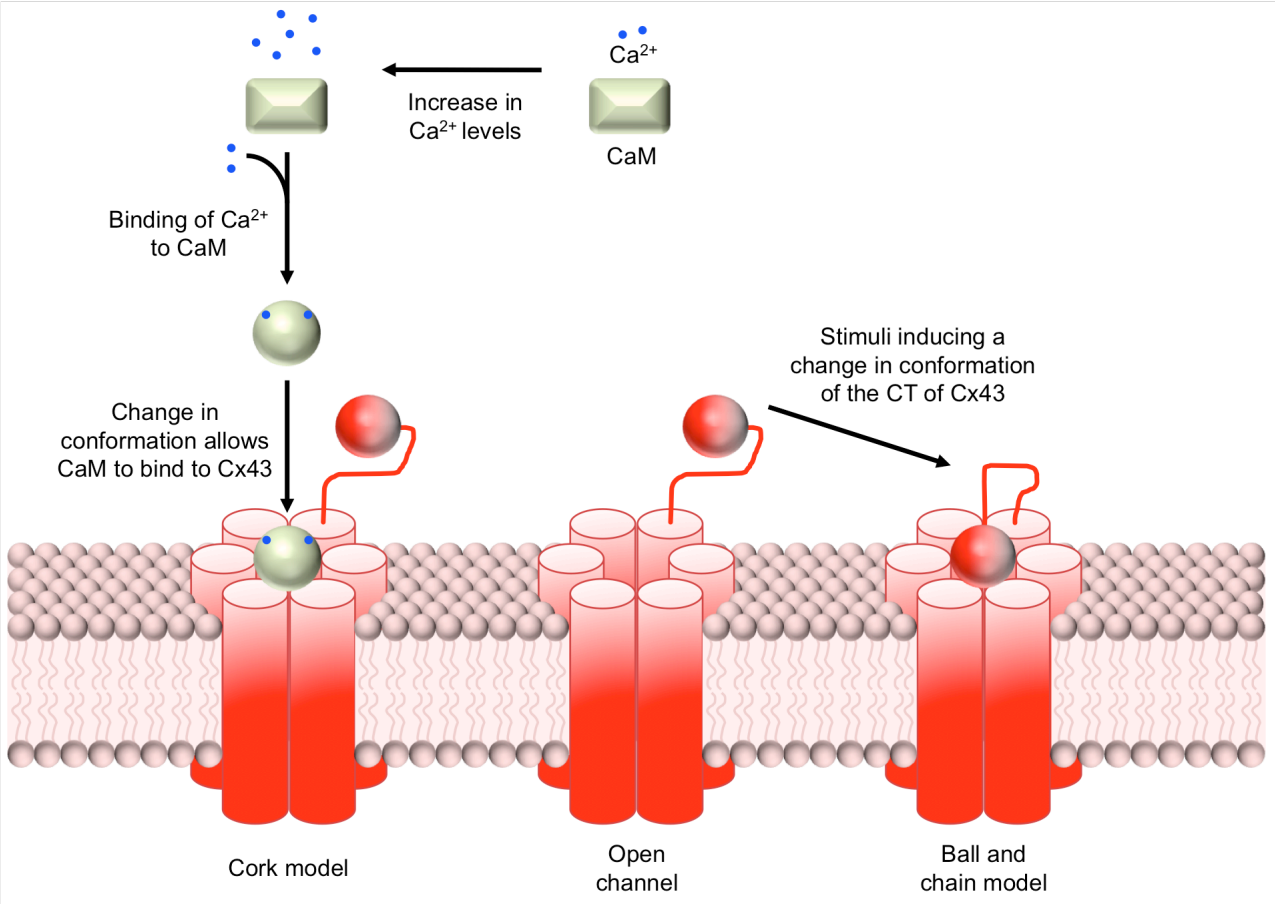

Figure 5 - Gating models for connexin gap junction channels. The centre connexon represents an open channel. Following an increase in intracellular calcium levels, Ca^{2+} binds to calmodulin inducing a conformational change in the protein, allowing it to bind to the cytosolic loop of Cx43 and physically obstruct the channel pore. Alternatively, stimuli that do not involve an increase in intracellular calcium levels may induce a change in the conformation of the carboxyl-terminal of Cx43, enabling it to physically obstruct the channel pore. For simplicity purposes only one of the six carboxyl-terminals of each connexon is represented.

gap junctions expressed in oocytes (Johnston & Ramon, 1981). Calcium and the Ca^{2+} binding protein calmodulin (CaM) were proposed to be this mediator (Peracchia et al, 1983), with later studies suggesting that acidification of the intracellular medium can induce the release of Ca^{2+} (Peracchia, 1990a; Peracchia, 1990b). CaM is a soluble acidic protein with high affinity for Ca^{2+}. Following Ca^{2+} attachment, CaM undergoes a conformational change that exposes two hydrophobic pockets, allowing it to bind to its molecular targets (Peracchia, 2004). CaM was first shown to bind to a synthetic peptide containing the first 16 amino acids of the NT domain of Cx43 (Torok et al, 1997). However, a later study could not detect any interaction between CaM and a similar peptide containing the first 21 amino acids of the NT domain (Duffy et al, 2002). Later studies showed that CaM inhibitors could block the Ca^{2+} induced gating of Cx43 in lens epithelial cells (Churchill et al, 2001; Lurtz & Louis, 2003). Using a search programme for CaM binding sites (Yap et al, 2000), Zhou et al recently identified a putative CaM binding site in Cx43 corresponding to amino acids 136 to 158 in the cytoplasmic loop of the protein (Zhou et al, 2007). The same study also demonstrated that activated CaM was capable of interacting directly with peptides

encompassing this region of Cx43. Mutation of the CaM binding site on Cx43 was shown to impair gap junction assembly and GJIC, indicating that this region of the cytoplasmic loop was important for the structure of the channel pore. GJIC in cells expressing these Cx43 mutants were also insensitive to increases in intracellular Ca^{2+}, further ascribing an important role for CaM in mediating the response of gap junctions to Ca^{2+}. Lastly, it was shown that the attachment of yellow fluorescent protein to the carboxyl-terminal of Cx43 did not affect CaM mediated gating (Zhou et al, 2007). Another recent study demonstrated that CaM mediated gating of Cx43 did not require the CT domain of Cx43 (Lurtz & Louis, 2007). Since it was previously shown that both the truncation of the CT domain and the attachment of GFP to the CT of Cx43 blocked fast V_j-gating (Bukauskas et al, 2000; Revilla et al, 1999), these results suggest that the mechanism by which CaM mediates Cx43 gating must differ from the "ball and chain" model suggested for fast V_j-gating and pH-gating. This is in accordance with the "cork" mechanism previously suggested by Peracchia et al, in which increases in intracellular Ca^{2+}, would lead to CaM association with Ca^{2+}, with subsequent activation of the protein. Activated CaM would then translocate to gap junction plaques, where it bound to connexin, physically obstructing the channel pore, similar to a "cork" (Peracchia et al, 2000).

CaM is not the only molecular intermediate involved in Cx43-gating modulation. Connexin phosphorylation is also an important modulator of gap junction channel gating (Moreno & Lau, 2007). Activation of protein kinase C (PKC) has been shown to induce changes in Cx43 gap junction channel conductance, favouring an intermediate conductance level (Moreno et al, 1992). This effect of PKC on Cx43 conductance levels was later reported to depend on Ser368 (Lampe et al, 2000). Protein kinase G (PKG) was shown to induce a similar shift in conductance levels in rat Cx43 but not in human Cx43 (Kwak et al, 1995; Takens-Kwak & Jongsma, 1992). This species specific response in Cx43 GJIC was shown to depend on Ser257 of rat Cx43, an amino acid that is not present in human Cx43 (Kwak et al, 1995). Cx43 gap junction channel gating has also been shown to be modulated through mitogen-activated protein kinase (MAPK) phosphorylation. MAPK was first shown to phosphorylate Cx43 in vitro (Kanemitsu & Lau, 1993), while a later study mapped the phosphorylation sites of MAPK to serines 255, 279 and 282 in the carboxyl-terminal of Cx43 (Warn-Cramer et al, 1998). Treatment of Cx43 channels reconstituted in liposomes with MAPK was also shown to reduce permeability of the channels to sucrose and Lucifer yellow (Kim et al, 1999). Another study that used a variety of Cx43 mutants, suggested that MAPK mediates the gating effect of v-Src upon Cx43 (Zhou et al, 1999). Cx43 mutants in which the serines targeted by MAPK were replaced by alanines were shown to be more insensitive to v-Src induced gating than wild type Cx43. Silencing or chemical inhibition of components of the MAPK pathway were also shown to inhibit the effects of v-Src upon Cx43 channel gating. The involvement of v-Src in Cx43 gating was first documented in works showing that mammalian cells expressing a constitutively active form of the protein presented lower gap junctional communication as measured by the transfer of a fluorescent dye (Crow et al, 1990; Zhou et al, 1999). It was later

shown that v-Src phosphorylated Cx43 on tyrosines 247 and 265 of the carboxyl-terminal (Lin et al, 2001). Subsequent studies using Cx43 mutants showed that v-Src gating of Cx43 not only blocked the transfer of fluorescent dyes but also reduced gap junction channel conductance. Co-expression of v-Src with wild type Cx43 was shown to induce a reduction in channel conduction, while co-expression of v-Src with the Cx43Y247F/Y265F double mutant showed no significant difference in channel conductance when compared to control cells expressing only wild type Cx43 (Cottrell et al, 2003). This is in stark contrast with results previously reported by Zhou et al, who showed that the replacement of Tyr247 and Tyr265 had no effect on v-Src induced gating of Cx43 (Zhou et al, 1999). These differences may be explained by the way v-Src was activated in each study. In the study by Zhou et al, v-Src was acutely activated with a temperature shift, while in the study by Cottrell et al, a constitutively activated v-Src was used. In the latter case it is possible that a prolonged exposure of cells to active v-Src might induce a negative feedback mechanism that inactivates the MAPK pathway, thus making these cells more insensitive to v-Src induced Cx43 channel gating (Moreno & Lau, 2007). Cottrell et al also showed that v-Src phosphorylation of Cx43 could affect the selectivity of the channel pore. Permeability to Lucifer yellow, a 457 Da protein with a net charge of 2^-, was shown to be reduced further than permeability to NBD-TMA, a 280 Da protein with a net charge of 1^+, when Cx43 was co-expressed with v-Src. Additionally, the reduction in NBD-TMA permeability was greater than expected when considering only the predicted changes in electrical conductances. These results suggest that phosphorylation of Cx43 by v-Src may alter the permeability of the channel pore by a process that restricts the passage of larger molecules (Cottrell et al, 2003).

Several mechanisms have been suggested to explain the process by which Cx43 phosphorylation may induce channel gating. Similar to fast V_j-gating or pH-gating, and given the many phosphorylation events that occur in the CT of Cx43, phosphorylation may induce conformational changes in the protein that favour or restrict interaction of the CT with the cytoplasmic loop of Cx43, thus phosphorylation may have a role in the “ball and chain” mechanism of Cx43 gating. Alternatively, phosphorylation of Cx43 may permit or restrict interactions with other molecules directly involved in occluding the entrance to the channel pore, thus phosphorylation of Cx43 may also have a role in the “cork” mechanism suggested for CaM mediated gating of Cx43.

Cx43 internalization

Connexins have a markedly short half-life, of less than 5 hours, when compared to other plasma membrane proteins (Girao & Pereira, 2003; Laird, 2006; Leithe & Rivedal, 2007). Considering that connexin mediated GJIC can be regulated through the gating of the gap junction channel, it is intriguing that cells would opt to regulate GJIC in such an energetically costly manner by constantly synthesizing and degrading Cx43. This might be partially explained by the observation that gap

junctional channels have a higher probability of being in an open state than closed (Christ & Brink, 1999), suggesting that gap channel gating is a rather limited regulatory mechanism. Thus, connexins may retain a short half-life in order to better respond to physiological changes in cells or tissues that require finer and more efficient changes in gap junction coupling (Bukauskas & Verselis, 2004; Harris, 2001).

The first studies involving internalization of gap junctions were observations made through electron microscopy depicting large double-membrane vesicular structures termed “annular junctions” that were suggested to be the result of the internalization of gap junction plaques (Archard & Denys, 1979; Kitson et al, 1978; Marquart, 1977). Later studies using specific antibodies confirmed that these structures were formed in large part by connexin proteins (Murray et al, 1997; Nagy et al, 1997; Naus et al, 1993). Annular junctions were later shown to indeed result from the internalization of connexin gap junctions (Jordan et al, 2001). Co-culture experiments using green fluorescent protein tagged Cx43 (Cx43-GFP) expressed in normal rat kidney (NRK) cells (that endogenously express Cx43) showed that large areas of gap junction plaques containing Cx43-GFP could be internalized into adjacent non transfected cells as vesicular like structures. Microinjection of anti-Cx43 antibodies also revealed that antibody-tagged gap junctions could be found in adjacent cells that were in contact with the microinjected cell (Jordan et al, 2001).

A study using Cx43 proteins containing tetracysteine tags marked with biarsenical fluorophores, has shown that newly synthesized Cx43 proteins are added to the periphery of existing gap junction plaques while older Cx43 proteins were mostly localized in the inner regions of the plaque from where they were internalized (Gaietta et al, 2002). This study demonstrates that gap junction plaques are not a static entity, but rather one that exists in a constant state of degradation and renewal.

Endocytosis of Cx43 has been reported to follow a clathrin-dependent pathway (Gumpert et al, 2008; Leithe & Rivedal, 2004a; Piehl et al, 2007). Clathrin was first suggested to interact with annular gap junction vesicles based on electron microscopy data showing structures similar to clathrin coats surrounding both invaginating sections of gap junctions as well as gap junction containing vesicles near the plasma membrane (Larsen et al, 1979). Years later, another study detected colocalization of Cx43 and clathrin in intracellular vesicles, through confocal microscopy and immunolabeling with antibodies directed against Cx43 and clathrin (Huang et al, 1996). A later study detailing the internalization of Cx43 in response to treatment with EGF, reported that EGF-induced internalization of Cx43 could be blocked by subjecting cells to hypertonic conditions (Leithe & Rivedal, 2004a), a method previously shown to inhibit clathrin-mediated endocytosis (Hansen et al, 1993; Heuser & Anderson, 1989; Wu et al, 2001). Recent studies have further elucidated the clathrin-mediated endocytosis of Cx43, showing that knockdown of several proteins critical for clathrin-mediated endocytosis inhibited Cx43 internalization. Piehl et al. reported that knockdown of clathrin in HeLa cells reduced the number of internalized annular gap junctions.

Furthermore, the clathrin adaptor disabled 2 (Dab2) and the GTPase dynamin were both found to colocalize with gap junctions and annular gap junctions (Piehl et al, 2007). Gumpert et al. further demonstrated that the siRNA depletion of Dab2, dynamin and the clathrin adaptor AP2 (adaptor protein 2) also significantly decreased gap junction internalization (Gumpert et al, 2008).

Although the role of clathrin-mediated endocytosis in Cx43 internalization has been extensively characterized, it is conceivable that Cx43 gap junction plaques may also be internalized through clathrin-independent endocytic pathways. In studies where clathrin-dependent endocytosis was inhibited either by siRNA depletion of clathrin or by incubation of cells in hypertonic conditions, the presence of internalized annular gap junction plaques was only reduced by roughly 55%, in comparison, internalization of transferrin, a protein previously shown to be internalized exclusively by clathrin coated pits (Mellman, 1996), was nearly abolished (Gumpert et al, 2008; Piehl et al, 2007). This suggests that a great part of Cx43 gap junctions are internalized through clathrin-independent mechanisms. A possible alternative mechanism may involve caveolar endocytosis. The interaction of Cx43 with Cav-1 (Langlois et al, 2008; Schubert et al, 2002), suggests that Cx43 may also be internalized through caveolae, a specialized invagination of the plasma membrane rich in Cav-1 that can bud from the plasma membrane (Kiss & Botos, 2009). However, more work is needed to ascertain a possible role for Cav-1 and caveolae in Cx43 internalization.

Both the lysosome and the proteasome have been implicated in regulating Cx43 turnover (Laing et al, 1997). This same study also showed that inhibition of the proteasome lead to the accumulation of Cx43 at the plasma membrane, suggesting that proteasomal activity was required for regulating the extent of Cx43 at the plasma membrane. Ubiquitinated Cx43 has been identified in gap junctional plaques in conditions where delivery of newly synthesized proteins to the plasma membrane was inhibited, indicating that Cx43 ubiquitination can occur at the plasma membrane (Rutz & Hulser, 2001). High glucose conditions were shown to induce Cx43 phosphorylation and subsequent internalization in retinal endothelial cells. In these conditions, the increased internalization of Cx43 was shown to require an active proteasome (Fernandes et al, 2004). Furthermore, PMA and EGF-induced phosphorylation and subsequent ubiquitination of Cx43 also lead to proteasome-dependent internalization of the protein in a rat liver epithelial cell line, further establishing ubiquitination as an internalization signal for Cx43 (Leithe & Rivedal, 2004a; Leithe & Rivedal, 2004b). In a subsequent study, Leithe et al demonstrated that Cx43 gap junctions are redistributed from the plasma membrane to intracellular vesicles positive for markers of early and late endossomes and for the endolysosomal protease cathepsin D. They further showed that annular gap junctions were broken down from double membrane structures to connexin-enriched multivesicular endosomes with a single limiting membrane prior to their arrival at the lysosome (Leithe et al, 2006). These results further reinforced the notion that while proteasome activity is required for Cx43 internalization, degradation of the protein occurs in the endolysosomal compartment. More recently it was shown that treatment of cells with PMA or EGF decreased the

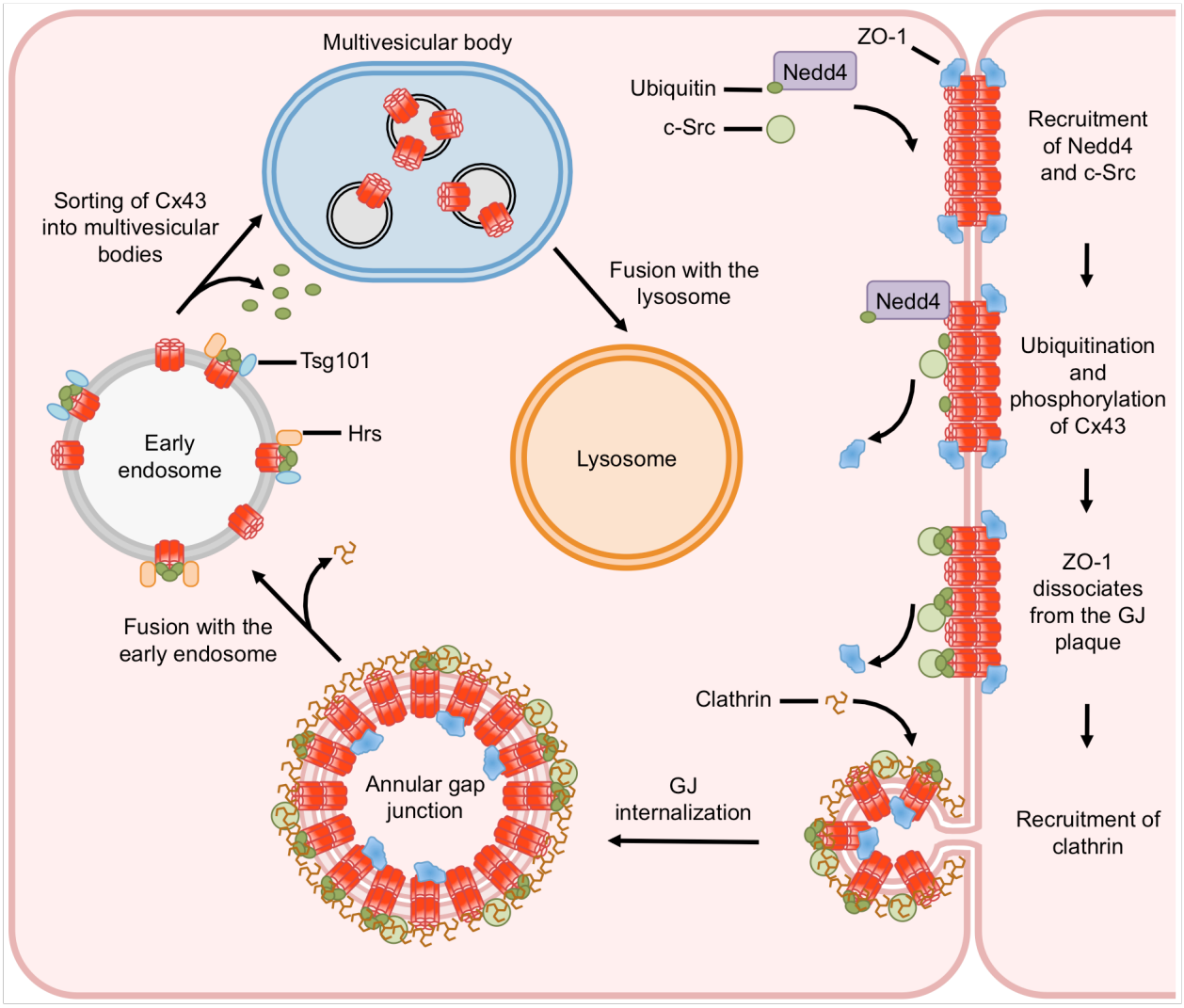

Figure 6 - Cx43 internalization and degradation. A simplified model detailing part of the life cycle of Cx43. Nedd4 is recruited to Cx43 gap junction plaques where it presumably mediates the ubiquitination of the protein. c-Src is recruited to the gap junction plaque on the side that will be internalized, phosphorylates Cx43, and induces the dissociation of ZO-1. Clathrin is then recruited to the nascent endocytic vesicle and both sides of the gap junction plaque are internalized into one of the adjacent cells as a double membrane structured termed annular gap junction. The annular gap junction is broken down into a single membrane vesicle and fuses with the early endosome. Following the maturation of endosomes, ubiquitinated Cx43 is sorted into multivesicular bodies in a process mediated by the ESCRT components Hrs and Tsg101. Lastly, the multivesicular bodies fuse with the lysosome where Cx43 is degraded.

Triton X-100 resistance of Cx43 in a process mediated by MAPK. This loss of detergent resistance of Cx43 was shown to occur at the plasma membrane and was suggested to be one of the first steps in Cx43 internalization (Sirnes et al, 2008).

The CT domain of Cx43 contains a protein-protein interaction PY-motif (XPPXY, with P = Proline and X = any amino acid) corresponding to amino acids 282-286, and an overlapping putative tyrosine-based sorting signal (YXXØ, with Ø = hydrophobic amino acid) corresponding to amino acids 286-289 (Thomas et al, 2003). Using SKHep1 cells, Thomas et al showed that mutation of essential residues in either domain, P283L for the PY-motif and V289D for the tyrosine-based sorting signal, lead to the accumulation of total Cx43 levels in cells, however, this effect was more pronounced for the V289D mutant. Mutation of the overlapping amino acid Y286 to alanine showed

no cumulative effects, which lead the authors to suggest that the putative PY-motif had a limited role in Cx43 turnover. The Y286A mutant was also shown to have a longer half-life when compared to wild type Cx43. Furthermore, treatment of cells with the lysosome inhibitor NH_4Cl had little effect on total levels, subcellular localization and GJIC of the Y286A mutant when compared to the wild type form of the protein. These results suggest that the tyrosine-based sorting motif is important for regulating Cx43 stability by targeting the protein for internalization and degradation by the endocytic/lysosomal compartment (Thomas et al, 2003).

Fragments of the CT of Cx43 were shown to interact with all three WW domains of the E3 ligase Nedd4 (Neuronal precursor cell-expressed developmentally downregulated 4) in a process modulated by Cx43 phosphorylation. Surface plasmon resonance was used to show that the PY-motif of Cx43 binds only to the WW2 domain of Nedd4. The interaction of Cx43 with Nedd4 was confirmed in vivo through immunofluorescence and immunoprecipitation. Silencing of Nedd4 was also shown to lead to the accumulation of Cx43 at the plasma membrane, indicating that Nedd4 may have a role in regulating Cx43 internalization, however the role of Nedd4 in Cx43 ubiquitination was not investigated (Leykauf et al, 2006).

The ubiquitin binding protein Tsg101 (tumour susceptibility protein 101) is involved with late endossomal sorting of ubiquitinated proteins to the lysosome (Raiborg & Stenmark, 2009). Tsg101 has been shown to interact with the carboxyl-terminals of several connexins (Cx30.2, Cx36 and Cx43) as well as co-precipitate with several other full-length connexins (Cx31, Cx43 and Cx45). Silencing of Tsg101 with siRNA also led to the accumulation of total levels of Cx43 and Cx45 as well as increased gap junction intercellular communication. On the other hand, silencing of Tsg101 failed to affect the levels of Cx31 (Auth et al, 2009). Thus, Tsg101 appears to be involved in the internalization of several connexins, however, the importance of previous ubiquitination of the connexin protein for Tsg101 function was only demonstrated in another study showing that Tsg101 and the ubiquitin binding protein Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) are necessary for Cx43 sorting into the endolysosomal compartment (Leithe et al, 2009). Leithe et al. also suggested that the observed stabilization of Cx43 at the plasma membrane following proteasome inhibition is due to a reduction in the levels of free ubiquitin. As such, depletion of free ubiquitin would prevent Cx43 ubiquitination, internalization and sorting to the endolisosomal compartment where it would be degraded.

The ubiquitin-proteasome pathway

The ubiquitin-proteasome pathway is the main cytosolic proteolytic system in eukaryotes, being implicated in a wide variety of cell functions with relevance to health and disease. In the history of life sciences, protein degradation was a neglected area for years with most researchers dedicating their attention to the study of nucleic acids and how the information contained within was translated

into protein synthesis. Protein degradation was at the time considered to be a mostly non-specific dead-end process. The discovery of the lysosome did little to change this view since, at the time, it appeared to only be involved in the non-specific degradation of extracellular proteins. However, the discovery of the ubiquitin-proteasome pathway of protein degradation revolutionized the field, demonstrating that the degradation of cellular proteins is a highly complex, temporally controlled and tightly regulated process (Glickman & Ciechanover, 2002).

Degradation of a protein by the ubiquitin-proteasome pathway involves two distinct and successive steps: the first step entails the covalent attachment of a polyubiquitin chain to the target protein; the second step involves recognition and degradation of the targeted protein by the 26S proteasome with the release of free and reusable ubiquitin (Glickman & Ciechanover, 2002; Hershko & Ciechanover, 1998).

Ubiquitin is a highly evolutionarily conserved 76 amino acid protein that is found in all eukaryotes, but not in members of the Eubacteria and Archaea superkingdoms. Conjugation of ubiquitin to the protein substrate proceeds through a three step process. The first step involves the activation of ubiquitin by the ubiquitin-activating enzyme E1. In this reaction, ATP-dependent energy is used to form a high-energy thiol ester intermediate, E1-S~ubiquitin. In the second step, the activated ubiquitin moiety is transferred to an E2 (ubiquitin-carrier protein or ubiquitin-conjugating enzyme) where another thiol ester intermediate is formed, E2-S~ubiquitin. In the last step, the activated ubiquitin is transferred from the E2 enzyme to the target protein with the help of an E3 ubiquitin-protein ligase enzyme that specifically binds to the target substrate. The ubiquitin molecule is normally transferred to an $–NH_2$ group of an internal lysine in the substrate, though in a few cases ubiquitin can also be conjugated to the NH_2-terminal amino group of the substrate. The covalent attachment of several ubiquitin moieties in series to a substrate, with the formation of a polyubiquitin chain, generally targets the protein for degradation by the 26S proteasome (Glickman & Ciechanover, 2002; Hershko & Ciechanover, 1998).

The ubiquitin-conjugation machinery has a pyramidal structure, with the number of components increasing the closer one is to the substrates. The human genome is estimated to encode two potential E1s, approximately 30 E2s and over 600 E3s (Li et al, 2008).

Ubiquitin-activating enzyme E1

Similar to ubiquitin, the E1 enzyme is an essential protein. Inactivation of the *UBA1* gene in yeast, which encodes the only E1 enzyme present in its genome, has been shown to be lethal (McGrath et al, 1991). This single E1 enzyme is responsible for the activation of all ubiquitin molecules, which it can then transfer to any type of E2 enzyme in the cell. Thus, the E1 enzyme does not confer any specificity to the ubiquitin-conjugation system.

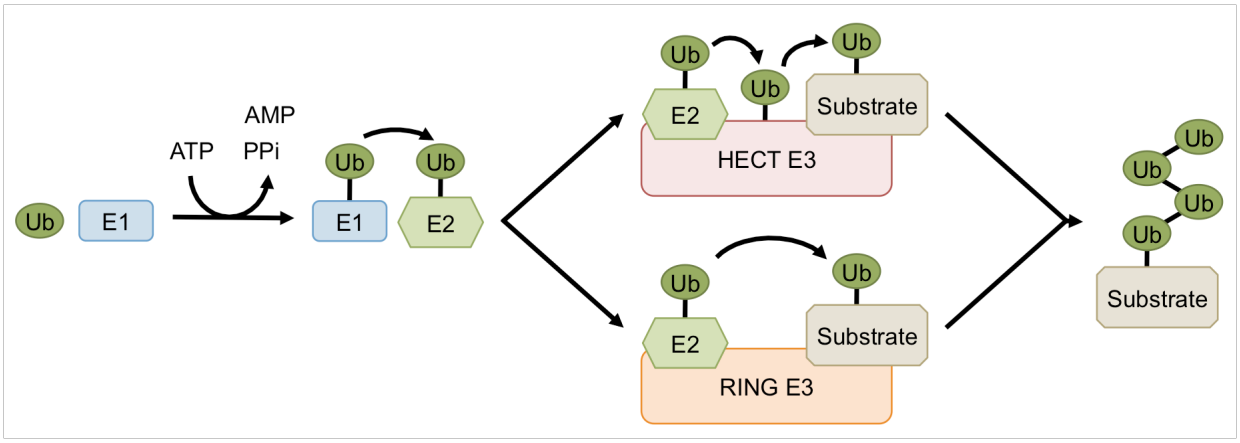

Figure 7 - The ubiquitination cascade. The conjugation of ubiquitin to target substrates is a sequential process involving several enzymes. First, the ubiquitin-activating enzyme E1 forms a high-energy thiol ester intermediate with ubiquitin, in a process that requires ATP. The activated ubiquitin moiety is then transferred to an E2 ubiquitin-conjugating enzyme. In the final step, the ubiquitin moiety is transferred to the substrate with the assistance of an E3 ubiquitin-protein ligase enzyme that specifically binds to the target protein. E3 ligase enzymes can be subdivided into two major groups, the RING domain containing E3s and the HECT domain containing E3s, as well as a few other minor groups. RING domain containing E3s facilitate the transfer of the activated ubiquitin moiety directly from the E2 to the target substrate (bottom). HECT domain containing E3s, however, first transfer the ubiquitin moiety to a conserved Cys residue in the HECT domain before transferring the ubiquitin moiety to the target substrate (top). A polyubiquitin chain is formed on the target substrate after several rounds of ubiquitin conjugation.

Ubiquitin-conjugating enzymes E2

E2s, or ubiquitin-conjugating enzymes, confer the first level of specificity to the ubiquitin system. E2s catalyze the covalent attachment of ubiquitin to target proteins with the aid of E3s. All E2 enzymes share an active-site ubiquitin-binding Cys residue and a UBC domain required for binding to distinct E3s. Although a single type of E2 can bind to several different E3s, some members of the E2 family appear to be involved with specific cellular mechanisms. For example, yeast Ubc6 and Ubc7 appear to be involved with degradation of proteins from within the endoplasmic reticulum (ER) (Bays et al, 2001; Plemper et al, 1997), while yeast Ubc11 has been shown to act along with the anaphase promotor complex (APC), which is the E3 enzyme that targets cell cycle regulators (Page & Hieter, 1999).

Ubiquitin-protein ligases E3

E3s, or ubiquitin-protein ligases, are responsible for catalyzing the transfer of activated ubiquitin from E2 enzymes to the target substrate. They are also the major determinants of specificity in the ubiquitin-conjugation system since E3s are responsible for recognizing all substrates that are ubiquitinated in the cell. As such, they constitute the largest group of enzymes in the ubiquitin system. E3s can operate as a single molecule or as a protein complex with ancillary proteins that aid in substrate recognition. In either case the E3 enzyme or complex binds to both E2 and the substrate to catalyze the transfer of ubiquitin. In most cases, such as in the case of RING domain

containing E3s, the E3 enzyme catalyzes this reaction by acting as a scaffold that brings the E2 close to the substrate, allowing for the efficient transfer of activated ubiquitin from E2 to the substrate. In the case of HECT domain containing E3s, the activated ubiquitin is first transferred to an internal Cys residue on the E3 before being conjugated to the substrate.
Even though E3 enzymes are highly heterogenous, due to all the different substrates that they must recognize and bind to, they can be classified into two major groups, the RING domain containing E3s and the HECT domain containing E3s, as well as a few other minor groups.

RING E3s

RING (Really Interesting New Gene) domains are typically characterized by a pattern of conserved Cys and His residues that form a cross-brace structure that binds two atoms of Zinc. The basic sequence expression of the canonical RING is Cys-X_2-Cys-$X_{(9-39)}$-Cys-$X_{(1-3)}$-His-$X_{(2-3)}$-Cys-X_2-Cys-$X_{(4-48)}$-Cys-X_2-Cys (where X is any amino acid). However, numerous RING variants have already been described, including some in which the Cys and His residues are swapped or where a Cys residue is replaced by another amino acid capable of coordinating zinc (Deshaies & Joazeiro, 2009). The RING domain of E3s is responsible for recruiting activated E2 enzymes. However, how RING domain E3s catalyze the transfer of activated ubiquitin from the E2 to the substrate is still poorly understood. Conventionally, bisubstrate enzymes catalyze reactions by positioning both substrates in close apposition to facilitate the chemical reaction, but in the case of all RING E3s that have been studied to date by X-crystallography, the substrate-docking site is located 50-60 Å from the location where the activated ubiquitin is expected to be. Two models have been suggested to resolve this problem. Deffenbaugh et al. suggested that the activated E2 must dissociate from the RING domain and encounter the substrate by diffusion (Deffenbaugh et al, 2003). However in cullin-RING E3s, it has been suggested that the gap between the substrate and E2~Ub could be bridged by a major conformational change in the RING domain (Petroski et al, 2006). Additionally, it has been suggested that RING E3s also catalyze the transfer of ubiquitin by inducing a conformational change in the activated E2 that facilitates ubiquitin discharge from the active site (Seol et al, 1999; Skowyra et al, 1999). RING domain E3s can exist as a single unit or as a multisubunit protein complex. While the single unit E3s must contain both the E2 binding domain (the RING domain) and the substrate binding domain, in the case of RING E3s that are part of a multisubunit complex, the substrate binding domain can sometimes be found in different subunits. Some well described multisubunit RING E3s include the SCF and the APC complexes.
SCF complexes (Skp1-Cullin-Fbox) usually include 4 proteins, Hrt1/Rbx1/Roc1, Skp1, a member of the Cdc53/Cullin-1 family and an F-box protein. In this complex, the Hrt1/Rbx1/Roc1 protein is the RING domain containing protein responsible for binding to the E2, while the F-box protein is involved in substrate recognition. Hrt1/Rbx1/Roc1, Skp1 and members of the Cdc53/Cullin-1 family

components are most likely common to all SCF complexes, while the specific substrate binding F-box protein is the most variable component, therefore the different SCF complexes are designated according to the variable F-box component (Glickman & Ciechanover, 2002). SCF complexes have a large variety of substrates, such as IκBα (Yaron et al, 1998) or β-catenin (Kitagawa et al, 1999), but are probably involved in targeting phosphorylated substrates (Glickman & Ciechanover, 2002).
The Anaphase-Promoting Complex or Cyclosome (APC/C) contains 11 subunits in yeast and 12 subunits in humans (Page & Hieter, 1999; van Leuken et al, 2008). Similar to SCF complexes, APC/C has a large cullin-like subunit, APC2 (Yu et al, 1998), while the subunit containing the RING domain is APC11 (Leverson et al, 2000). Together, these two subunits of APC/C are sufficient to drive E2-dependent ubiquitination *in vitro* (Tang et al, 2001). Substrate recognition by the APC/C is dependent on the additional binding of one of two activating subunits containing WD40 domains, Cdc20 or Cdh1 (Visintin et al, 1997), although the activity of APC/C can also be modulated by the phosphorylation status of its various subunits (Glickman & Ciechanover, 2002). The APC/C complex is involved in targeting cyclins for degradation, important regulators of the cell cycle, as well as securin, which regulates the separation of sister chromatids (Acquaviva & Pines, 2006).

HECT E3s

The second largest group of E3 ligases are characterized by the presence of a 350 amino acid residue HECT domain (Homologous to the E6-AP Carboxyl-Terminus) that is found at the carboxyl-terminal of proteins. Most HECT ligases also contain protein-protein or protein-lipid interaction domains located towards the amino-terminal of the protein. The HECT domain is a bilobal domain where the active Cys residue is located towards the carboxyl-terminal while the amino-terminal region binds the E2 enzyme. Substrate recognition occurs in regions outside of the HECT domain. Unlike RING domain containing ubiquitin ligases, that catalyze the transfer of activated ubiquitin from an E2 enzyme directly to the substrate, HECT domain E3s first directly bind the activated ubiquitin through the conserved Cys residue present within the HECT domain before transferring the ubiquitin moiety to the substrate. The three dimensional structure of the HECT domain can vary from protein to protein (Rotin & Kumar, 2009). For example, the HECT domain of E6AP has an L shaped structure (Huang et al, 1999), while the HECT domain of WWP1 has an inverted T structure (Verdecia et al, 2003). To catalyze the transfer of activated ubiquitin from the E2 to the conserved Cys residue on the E3 protein, both the E2 and E3 thiol groups must be positioned closely. However, while for WWP1 the active Cys residues in both enzymes are predicted to be within 6 Å of each other (Verdecia et al, 2003), for E6AP the active Cys residues are separated by 41 Å, which suggests that a large conformational change has to occur within the HECT domain during catalysis (Huang et al, 1999).
Based on the structure homology of the amino-terminal, HECT E3 ligases can be divided into three

groups: the Nedd4 family, the HERC family and other HECTs. Nedd4 family members are characterized by the presence of a C2 domain at the amino-terminal and two to four WW domains. The C2 domain binds to phospholipids, targeting the protein to the plasma membrane, endosomes and multivesicular bodies. The WW domains bind to PY-motifs in substrate proteins. The HERC family members are characterized by the presence of one or more RLD domains which interact with chromatin. The other HECT family members contain a varied assortment of protein domains including WWE domains, ankyrin repeats, UBA domains, zinc fingers, PABC domains, RING domains, IQ motifs and other domains (Rotin & Kumar, 2009).

A well characterized member of the HECT E3 family is Nedd4 and its *Saccharomyces cerevisae* orthologue Rsp5. In yeast, Rsp5 is an essential protein and has been shown to participate in several cellular functions, including mitochondrial inheritance, chromatin remodelling, transcription regulation, endocytosis regulation, and sorting of cargo into MVBs from endocytic vesicles or from the Golgi apparatus to endosomes (Rotin & Kumar, 2009). In mammals, Nedd4 has been suggested to be a positive regulator of cell proliferation and animal growth. Similar to what was shown for Rsp5, Nedd4 has also been shown to control the sorting of some transmembrane proteins such as LAPTM5 (Pak et al, 2006). In addition to Nedd4L, Nedd4 can also bind to PY-motifs of the epithelial Na^+ channel (ENaC), ubiquitinating the protein and driving its endocytosis from the plasma membrane (Staub et al, 1996). More recently, Nedd4 has been shown to be involved in regulating the internalization of Cx43. Nedd4 was shown to interact with the carboxyl-terminal of Cx43 and depletion of Nedd4 by siRNA led to the accumulation of Cx43 at the plasma membrane (Leykauf et al, 2006). However, the importance of Nedd4 for Cx43 ubiquitination was not investigated.

E4 and U box E3s

E4s are a small family of E3 ligases that are normally associated with the elongation of ubiquitin chains. All E4s contain a modified version of the RING domain, denominated U box, that lacks the metal-chelating residues of the RING domain. Most of the conserved Cys residues of the RING domain are absent from the U box, and the structure is likely stabilized by hydrogen bonds and salt bridges (Glickman & Ciechanover, 2002). Several U box proteins have been shown to elongate ubiquitin chains in a process dependent of both E1 and E2s, but independent of the presence of any E3 enzymes (Hatakeyama et al, 2001). Thus, U box proteins are suggested to target ubiquitinated proteins, catalyzing the elongation of a polyubiquitin chain that would make the target more susceptible to be recognized by the proteasome and degraded (Koegl et al, 1999). This model requires the presence of an E3 enzyme to catalyze the addition of the first ubiquitin moiety to the substrate. However, some U box-containing E3s have been shown to target their substrates directly, without requiring their previous ubiquitination (Hatakeyama et al, 2001). A well described

member of this family of E3s is CHIP (Carboxyl-terminus of Hsc70-Interacting Protein). CHIP interacts with the chaperon proteins Hsc70 and Hsp90 through TPR domains located towards its amino-terminal, while the E2 is recruited through the U box domain located at the carboxyl-terminal. Misfolded proteins bound to the chaperon proteins are ubiquitinated by CHIP and directed for proteasomal degradation, as is the case for the degradation of CFTR (Cystic Fibrosis Transmembrane conductance Regulator) (Meacham et al, 2001). Thus, CHIP functions as a bridge connecting refolding/chaperon machinery to proteasomal degradation (McDonough & Patterson, 2003).

Functions of Ubiquitin Conjugation

Similar to how the phosphorylation of a protein may elicit different cellular responses depending on the type of phosphorylation, i.e., which position or residue type (Tyr, Thr or Ser) is phosphorylated on the protein, ubiquitin conjugation may also elicit different responses depending on if the protein is monoubiquitinated on a single Lys residue or multimonoubiquitinated on several Lys residues. The monoubiquitin moiety itself may be subjected to further ubiquitin conjugation on one of its seven Lys residues (polyubiquitination). Additionally, the polyubiquitin chains themselves can also vary depending on which Lys on the ubiquitin moiety is used to produce the chain links, which furthers adds to the variability of the ubiquitin signal. Lastly, the carboxyl-terminal group of ubiquitin can also be linked to the amino-terminal group of another ubiquitin to form a linear ubiquitin chain. Canonically, ubiquitin conjugation is usually linked to proteasomal degradation of the targeted protein, however, along the years and with the characterization of the various types of ubiquitin signals, several new functions of ubiquitin conjugation, that are independent of proteasomal degradation, have been described (Glickman & Ciechanover, 2002; Li & Ye, 2008; Mukhopadhyay & Riezman, 2007; Pickart & Eddins, 2004).

One of the first non-proteasomal functions of ubiquitin conjugation to be described was the signalling of plasma membrane proteins for internalization. Monoubiquitination, multimonoubiquitination and polyubiquitination through Lys63-linked chains have all been suggested to function as signals for internalization (Mukhopadhyay & Riezman, 2007). Ubiquitination signals are thought to mediate interactions with several endocytic proteins that contain ubiquitin-binding domains (UBD), such as Epsin, Eps15 and Eps15R. Monoubiquitination and multimonoubiquitination also function as a sorting signal at endosomes, targeting its substrates to the interior of the multivesicular body (MVB), and leading to their subsequent degradation in the lysosome (Komada & Kitamura, 2005). Most proteins that are not ubiquitinated are recycled back from endosomes to other cellular compartments. Aside from its role in triggering endocytosis and endosomal sorting, monoubiquitination may also directly regulate the function of proteins. Many UBD-containing proteins are themselves monoubiquitinated, such as the endocytic

adaptor proteins (Hicke et al, 2005). Sts1 and Sts2 are UBD-containing proteins that bind to ubiquitinated epithelial growth factor receptor (EGFR) complexes, inhibiting their endocytosis. Once monoubiquitinated, Sts2 was shown to form an intramolecular interaction between its UBD and the ubiquitin moiety, preventing its interaction with ubiquitinated EGFR, thus allowing the endocytosis of the receptor. Similarly, other endocytic adaptors containing UBDs, such as Eps15 and Hrs, were also shown to form these intramolecular interactions once monubiquitinated (Hoeller et al, 2006).

The addition of a Lys48-linked polyubiquitin chain of at least four ubiquitin moieties is the canonical signal for directing substrates for proteasomal degradation. However, Lys48-linked polyubiquitin chains have also been implicated in other mechanisms non related to proteasomal degradation (Li & Ye, 2008). An example of one of these non-proteolytic functions is the regulation of the yeast transcription factor Met4. Following polyubiquitination, Met4 transcriptional activity decreases, however polyubiquitination of Met4 has little effect on its half-life (Kaiser et al, 2000; Kuras et al, 2002). Met4 contains an UBD that binds to the nascent polyubiquitin chain, preventing the elongation of the chain to levels that would make it a suitable target for proteasome recognition. Thus, Lys48-linked chains can inactivate the function of a substrate protein without leading to its subsequent proteasomal degradation (Flick et al, 2006). Lys48-linked polyubiquitin chains are also involved in the activation of the chaperone p97. p97 is capable of recognizing Lys48-linked polyubiquitinated substrates and subsequently extract them from immobilized cellular compartments or from a large protein complex. This “segregase” function of p97 has been described for the extraction of ERAD substrates from the ER, for the activation of the yeast membrane bound transcription factor Spt23 and also for the extraction of Aurora B kinase from chromatin during mitosis (Li & Ye, 2008).

Besides its importance in targeting plasma membrane proteins for internalization, Lys63-linked polyubiquitin chains have also been shown to be a major regulator of the NFκB signalling pathway. In basal conditions the transcription factor NFκB is retained in the cytoplasm by the inhibitor IκB. However, following activation by pro-inflammatory cytokines, such as TNF-α (tumour necrosis factor-α) and IL-1β (interleukin-1β), an upstream regulator of IκB, the IκB kinase (IKK), is polyubiquitinated, leading to the phosphorylation of IκB which subsequently targets it for proteasomal degradation. Other activators and repressors in this signalling pathway also bind to Lys63-linked polyubiquitin modified proteins, eventually leading to the activation of NFκB and its translocation to the nucleus (Mukhopadhyay & Riezman, 2007). Lys63-linked polyubiquitin chains are also involved in targeting substrates for degradation through a proteasome-independent mechanism termed autophagy. Autophagy is a molecular process by which organelles or part of the cytoplasm is engulfed in a double membrane structure termed autophagosome that subsequently fuses with lysosomes, enabling the degradation of large structures that are

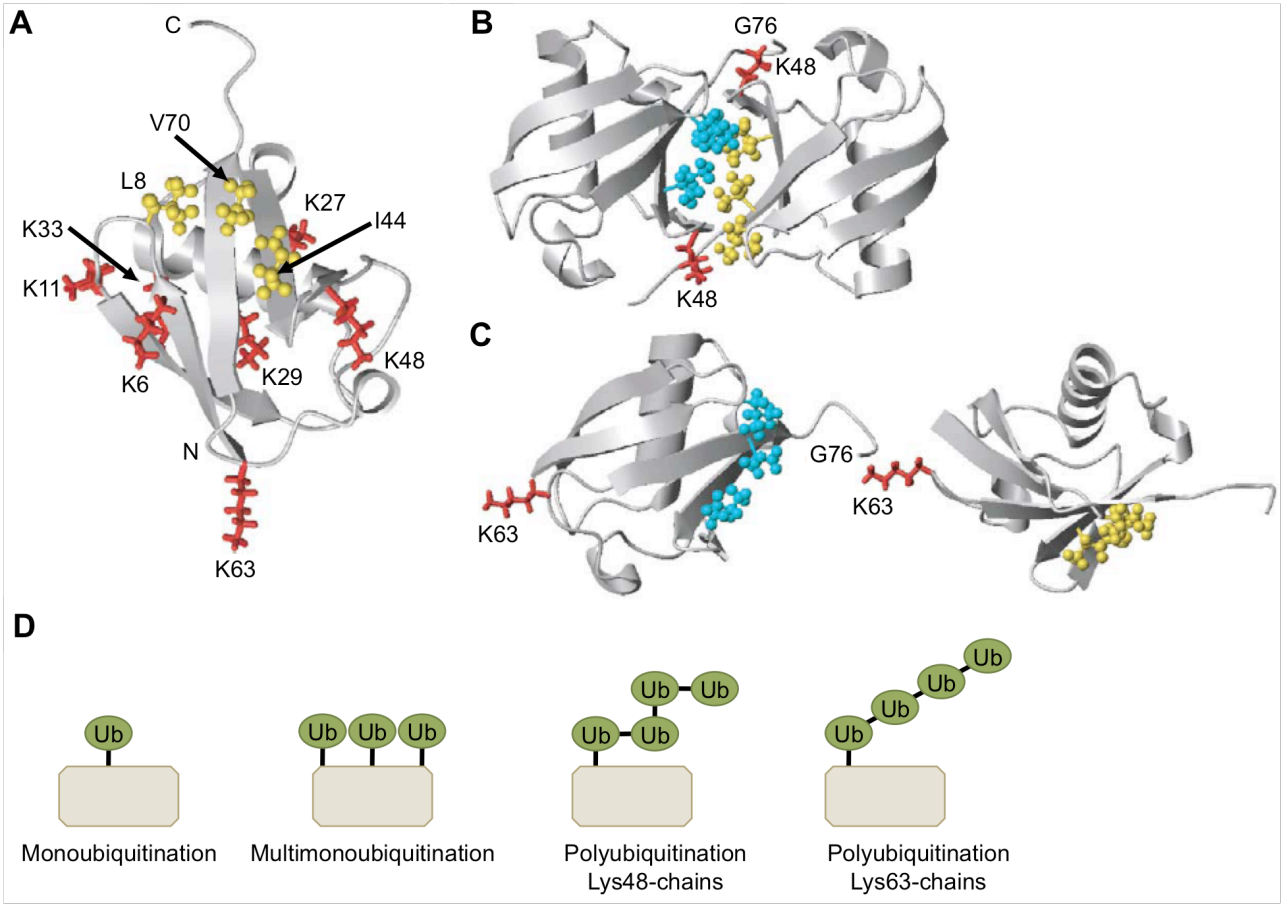

Figure 8 - Types of ubiquitin conjugation. (A) Ribbon diagram of ubiquitin. The seven lysine residues are shown in red and labeled, while the Leu8–Ile44–Val70 hydrophobic patch is shown in gold ball-and-stick. (B and C) Solution conformation of two ubiquitins linked through Lys48 (B) or Lys63 (C). The hydrophobic patches on the ubiquitin units are shown in ball-and-stick (colored gold or cyan) along with the side chains of Lys48 and Lys63 in stick representation (red). The location of Gly76 forming an isopeptide bond with the corresponding lysine is indicated. As shown in the figure, Lys48-linked ubiquitin chains have a more closed structure when compared to the linear structure of Lys63-linked chains (Pickart & Fushman, 2004). (D) Schematic representation of several different types of ubiquitin conjugation. From left to right: Monoubiquitination, the addition of a single ubiquitin moiety to a target protein; Multimonoubiquitination, the addition of several individual ubiquitin moieties to different lysine residues of the target protein; Polyubiquitination, the addition of an ubiquitin chain to a target protein. Different types of polyubiquitin chains can be formed depending on the lysine residue on ubiquitin that is used to extend the chain. Although it has been shown that all of the seven lysine residues on ubiquitin form polyubiquitin chains in vivo (as well as linear ubiquitin chains formed by conjugation to the NH_2-terminal amino group of ubiquitin), Lys48- and Lys63-linked polyubiquitin chains remain the most well described.

inaccessible to the proteasome (Reggiori & Klionsky, 2005). p62 is a protein that belongs to the Ubl/UBA protein family that contains both an ubiquitin-like domain (UBL) and an UBD, thus enabling it to recognize ubiquitinated proteins. p62 also interacts directly with LC3, which is an essential component of the autophagic machinery. p62 can also oligomerize to form protein bodies that contain ubiquitinated misfolded proteins, which in turn display partial colocalization with autophagosomes. These observations have led to the suggestion that misfolded proteins bearing Lys63-linked chains are first recognized by p62. Polymerization of p62 would then sequester these misfolded proteins in large protein bodies, that are recognized by LC3 who then signals their degradation by the autophagy pathway (Li & Ye, 2008).

Although the formation of polyubiquitin chains linked through each of the seven Lys residues of

ubiquitin has been detected in yeast (Peng et al, 2003), aside from Lys48- and Lys63-linked chains, little is known about the physiological functions of polyubiquitin chains linked through the other five Lys residues of ubiquitin. Lys29-linked chains have been shown to target the Notch signalling modulator DTX for lysosomal degradation (Chastagner et al, 2006). Another study has shown that the conjugation of Lys29- or Lys33-linked chains to two AMPK-related kinases may regulate the enzymatic activity of these enzymes (Al-Hakim et al, 2008). Lastly, the tumour suppressor BRCA1 E3 complex was shown to assemble Lys6- or Lys29-linked chains on itself, which in turn may regulate the stability of the E3 enzyme (Nishikawa et al, 2004).

The Proteasome

The proteasome is a 2.5 megadaltons complex made up of two copies each of at least 33 different subunits that are highly conserved among eukaryotes (Finley, 2009; Glickman & Ciechanover, 2002), and functions primarily to degrade proteins that have been modified by the covalent attachment of ubiquitin moieties. It works through a multi-step process in which ubiquitinated substrates are first recognized by the proteasome complex, followed by substrate unfolding, translocation into the catalytic pore and deubiquitination, finally ending with the cleavage of peptide bonds. The proteasome exists in various forms, the most commonly described being the 26S proteasome. The 26S proteasome can be subdivided into two subunits, the 28-subunit core particle (CP, also known as the 20S particle) and a regulatory particle (RP, also known as the 19S particle or PA700) consisting of 19 subunits in yeast and probably the same number in other eukaryotes. The RP can be further subdivided into two subunits: the base and the lid. The proteolytic active sites of the proteasome are sequestered in the interior of the CP. Access to the interior of the CP is restricted, and few substrates are capable of entering the CP without the aid of the RP. Recognition of ubiquitinated substrates is an intrinsic propriety of the RP, and it is also the RP that is responsible for substrate unfolding, deubiquitination and translocation into the CP (Finley, 2009).

The Core Particle

The CP has 28 subunits arranged into four heptaheteromeric rings, exhibiting a two fold symmetry and a hollow cylindrical shape (Groll et al, 1997). Each inner ring is formed by β-type subunits, three of which contain proteolytic active sites (β1, β2 and β5), making a total of 6 catalytic sites for each proteasome. Each active site favours the cleavage of specific peptide sequences. The β1 subunit exhibits caspase like activity, preferring to cleave on the carboxyl-terminal side of acidic residues. β2 exhibits trypsin like activity preferring to cleave after tryptic residues. Finally, β5 exhibits chymotrypsin like activity, preferring to cleave after hydrophobic residues. Because the

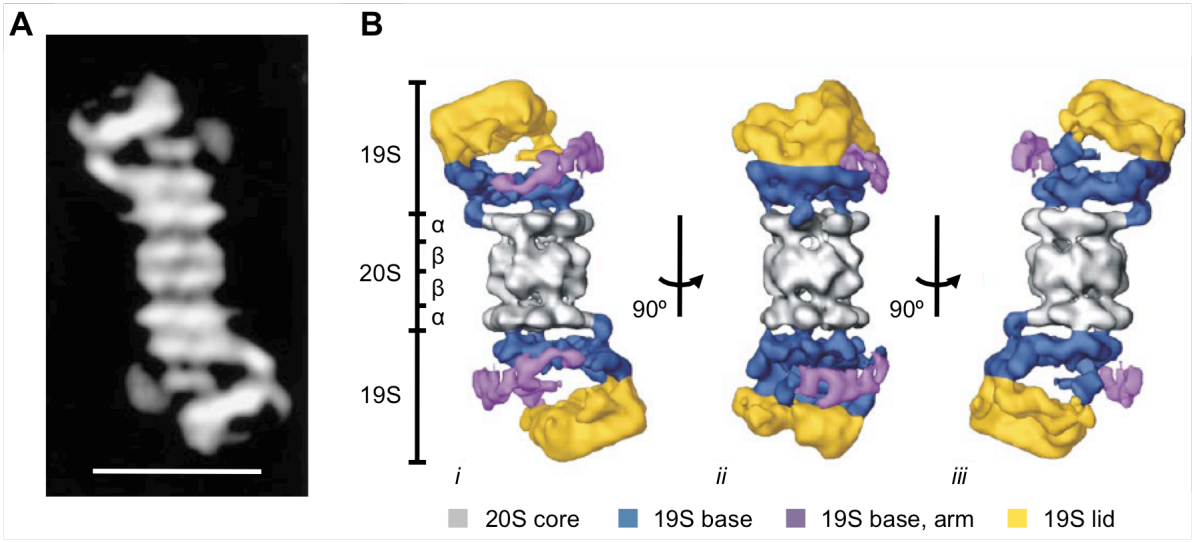

Figure 9 - Structure of the 26S proteasome. (A) 26S proteasome from *Xenopus laevis oocytes.* The image was generated by averaging of electron micrographs of negatively stained proteasomes. Scale bar, 20 nm (Walz et al, 1998). (B) Three-dimensional reconstructions of the 26S proteasome. *i–iii,* side views, with the approximate location of proteasome components indicated (left) with viewing directions parallel to the C2 axis (*i*), normal to the C2 axis (*ii*), and parallel to the C2 axis (*iii*), in opposite direction to *i* (da Fonseca & Morris, 2008).

active sites are enclosed in the interior space of the CP, substrates must first gain access to this space. In the closed form of the CP, the amino-termini of the α-subunits converge axially to close the entrance to the channel (Groll et al, 2000; Groll et al, 1997). Although purified CP can hydrolyze small peptides and some unfolded proteins, it cannot degrade multiubiquitinated proteins (Glickman & Ciechanover, 2002). Binding of the RP to the outer side of the α-subunit ring, activates the CP, allowing the access of substrates to the catalytic sites.

Aside from its major importance in clearing ubiquitin tagged proteins from cells, proteasome based proteolysis also has an important immunological function in the processing of antigens to be presented at the plasma membrane by Major Histocompatibiliy Complex (MHC) class I molecules. In mammals, following stimulation by γ-interferon, the three catalycally active β-subunits of the proteassome are replaced by inducible variants with different cleavage specificities (β1i, β2i and β5i), forming a proteasome variant known as the immunoproteasome (Borissenko & Groll, 2007; Goldberg et al, 2002a). β1i is thought to enhance the production of peptides with a hydrophobic carboxyl-terminal instead of an acidic one, while β2i enhances the production of peptides with a basic termini (Unno et al, 2002). These product peptides bind more favourably to MHC class I molecules, accounting for the enhanced antigen presentation conferred by immunoproteasomes (Goldberg et al, 2002a). More recently, another variant of the CP has been described in thymic cortical epithelial cells in which, besides the replacement of β1 and β2 for their inducible variants, β5 is replaced by a novel variant, β5t, to form a proteasome known as thymoproteasome (Murata et al, 2007). Similar to β1i and β2i, β5t also presents cleavage specificities that differ from both β5 and β5i subunits. β5 and β5i both produce peptides with a hydrophobic carboxyl-terminal, while β5t

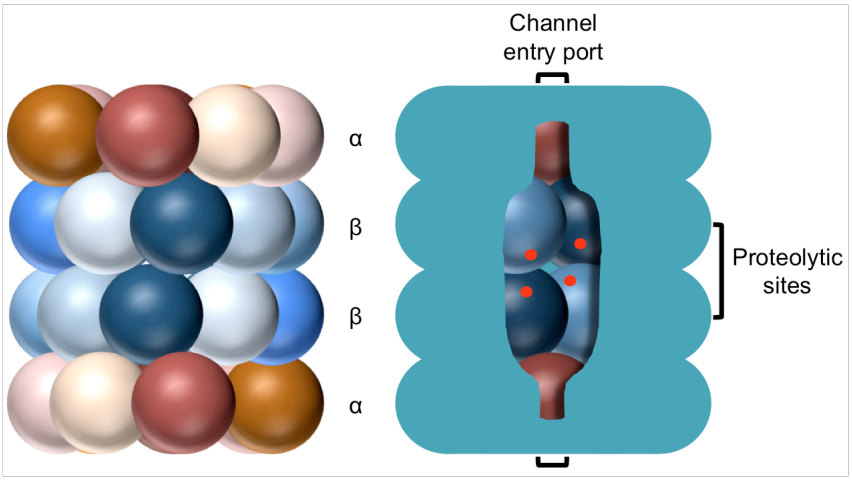

Figure 10 - Schematic representation of the 20S core particle. The 20S core particle is formed by four hollow heptaheteromeric rings, exhibiting a two fold symmetry. The inner ring is formed by β-type subunits, three of which contain proteolytic active sites. Left: surface representation of the CP. Right: Medial section of the CP displaying the channel in its closed state. Red dots represent the proteolytic active sites. Adapted from (Finley, 2009).

favours the production of peptides with a more hydrophilic termini, that weakens, but does not eliminate, the interaction of the peptides with MHC class I molecules. The thymoproteasome is thought to be critically important for thymic education and the generation of mature CD8+ T cells (Murata et al, 2007).

The Regulating Particle

Besides functioning as an activator of the CP, by opening the gate formed by the α-ring in the CP, the RP also regulates the activity of the CP through several other mechanisms: selecting substrates, unfolding and deubiquitinating substrates, as well as translocating them into the CP. The RP contains at least 19 subunits and can be subdivided into two functional elements, the base assembly, which binds directly to the CP, and the lid assembly, which binds to the base (Finley, 2009; Glickman & Ciechanover, 2002). The RP can assemble at either end of the CP to form the 26S proteasome. Isolated proteasomes usually consist of a mixture of free CP, singly capped CP or double capped CP (Glickman, 2000). While in yeast most proteasomes are present as double capped forms (Russell et al, 1999), in mammals the ratio of RP to CP is lower, and therefore free 20S and single capped forms are often present (Brooks et al, 2000).

The base of the RP is composed of 10 subunits. Six of the subunits are ATPases of the AAA family that are found in many multicellular machines such as translocators, transporters, membrane fusion complexes and proteases, and are designated as Rpt1-6 following their nomenclature in yeast (Glickman & Ciechanover, 2002). The 6 Rpt subunits form a ring that binds directly to the outer α-subunit ring of the CP, and as such are critical components for the formation of the 26S proteasome. Additionally, degradation of typical proteasome substrates is ATP-dependent, indicating a central role for this ATPase ring in the reaction mechanism of proteasomal

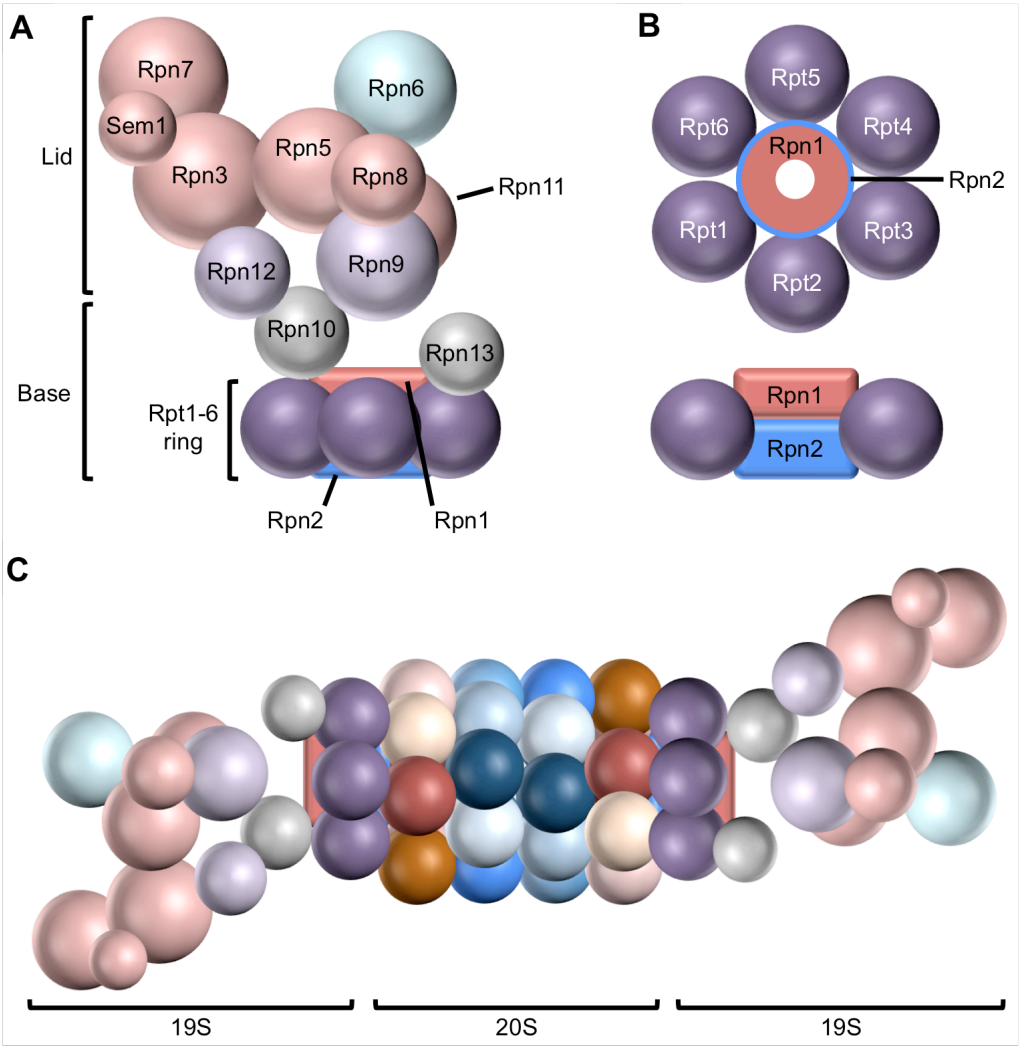

Figure 11 - Schematic representation of the 19S regulating particle. The 19S regulating particle contains at least 19 subunits and can be divided into two functional elements, the base assembly, which binds directly to the outer α-subunit ring of the core particle, and the lid assembly which binds to the base. (A) Schematic representation of the 19S regulating particle, displaying the base and lid assemblies. Adapted from (Rosenzweig et al, 2008; Sharon et al, 2006). (B) The Rpt1-6 ATPase ring of the base assembly surrounding a channel formed by the Rpn1-2 subunits. The upper image displays a top side view of the base assembly while the lower image displays a side view of the base assembly where one of the Rpt subunits was removed to better visualize the position of the Rpn1-2 subunits. Adapted from (Rosenzweig et al, 2008). (C) The 26S proteasome displaying two 19S regulating particles bound to the ends of a single 20S core particle.

degradation. This probably occurs by mediating the translocation of the unfolded substrate through the translocation channel formed at the center of the Rpt ring. Other components of the base include the scaffolding proteins Rpn1 and Rpn2 as well as the ubiquitin receptors Rpn10 and Rpn13 (Finley, 2009).

Rpn1 and Rpn2 form a channel that can attach directly to the CP, indicating that the Rpn1/Rpn2 channel might be a part of the substrate translocation channel of the base, however the role and mechanism of action of this channel is currently unknown. Another function of these proteins is to serve as scaffolds mediating the binding of other proteins to the proteasome. Currently, all known binding partners of Rpn1 and Rpn2 act directly on ubiquitin conjugates, either serving as ubiquitin receptors, such as in the case of the integral proteasome subunit Rpn13, or as enzymes that

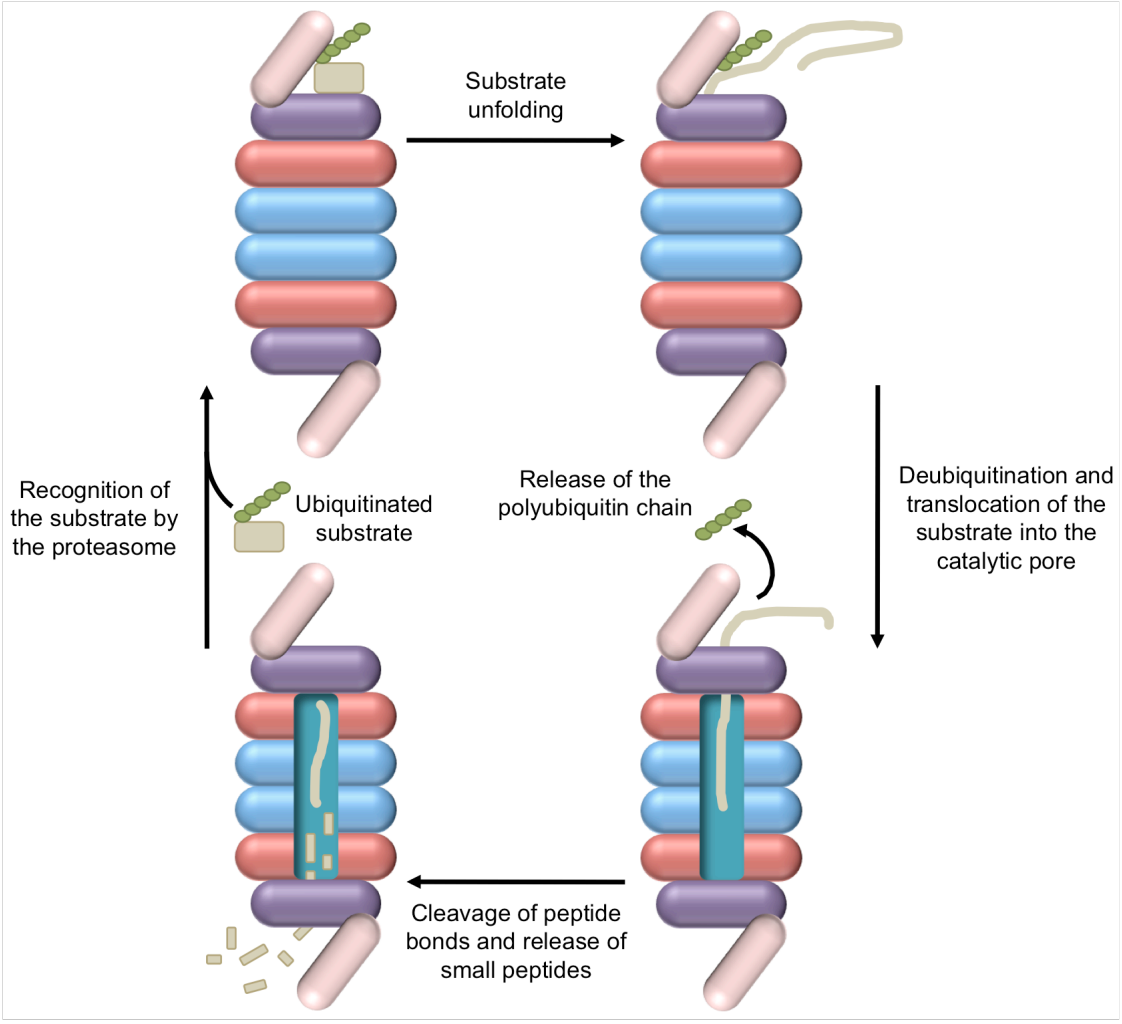

Figure 12 - Degradation of ubiquitinated substrates by the proteasome. The proteasome first recognizes the polyubiquitin chain attached to the substrate most likely through the Rpn10 or Rpn13 subunits of the base assembly. The next step involves the unfolding of the substrate. The unfolded substrate is then translocated into the catalytic pore, in an ATP-dependent process mediated by the Rpt1-6 ring of ATPases of the base assembly. Once a substrate is committed to degradation, the Rpn11 subunit of the lid assembly deubiquitinates the substrate, with the release of a free polyubiquitin chain. The substrate is then broken down into smaller peptides that subsequently exit the proteasome.

disassemble or extend ubiquitin chains, such as the ubiquitin ligase Hul5 and the deubiquitinating enzyme (DUB) Ubp6. Another DUB, Uch37, is associated to Rpn2 by directly binding to Rpn13. Because the factors that recognize, disassemble or extend ubiquitin chains are collected at Rpn1 and Rpn2, these subunits represent a major site in the proteasome for the regulation of chain dynamics and proteasome activity (Finley, 2009).

Rpn10 and Rpn13 both function as ubiquitin receptors for the proteasome, being capable of binding to ubiquitin chains as well as to proteins containing ubiquitin-like domains. Rpn10 is important for the structural integrity of the RP, since deletion of its von Willebrand A domain leads to the dissociation of the lid and base (Glickman et al, 1998). In humans, Rpn13 contains a receptor site for the DUB Uch37, suggesting that ubiquitin chains bound by Rpn13 may be disassembled by Uch37.

The lid consists of nine subunits arranged in a disklike shape that can detach from the RP base as

a discreet complex and reattach to it. None of the lid subunits are ATPases, and as of yet, only the Rpn11 subunit has a known function (Finley, 2009; Glickman & Ciechanover, 2002). Degradation of ubiquitinated substrates by the proteasome requires the lid component of the RP, suggesting an important function of the lid in substrate recognition or initial removal of the ubiquitin chain from the target (Glickman et al, 1998). Curiously, certain non-ubiquitinated proteins can be efficiently processed by proteasomes in which the lid has been detached, as well as by archaeal or prokaryotic proteasomes that do not natively possess a lid. This suggests that the lid may have appeared alongside the development of the ubiquitin targeting system in order to direct ubiquitinated substrates towards the proteasome for degradation (Glickman & Ciechanover, 2002). Rpn11 is a DUB and its activity is critical for the normal function of the proteasome. Before being degraded, the ubiquitin chain present in substrates must be removed, in order to facilitate the entrance of the substrate into the CP. This may be necessary due to the fact that the ubiquitin molecule is physically very stable, and as such, the presence of ubiquitin on the target protein may slow down the unfolding step of proteasomal degradation. The deubiquitinating activity of Rpn11 is only exerted upon proteolytic substrates. Furthermore, deubiquitination by Rpn11 is not observed in the absence of ATP. Since Rpn11 is unlikely to be an ATPase, this suggests that Rpn11 may only exert its DUB activity upon substrates that are proceeding along the pathway of degradation. Although no function has as yet been described for the remaining 8 subunits of the lid, they may serve to activate, position or regulate the specificity of Rpn11 (Finley, 2009).

The Proteasome Activator 28αβ

The proteasome activator 28αβ or PA28, is a heteroheptamer or hexamer of 28 kDA subunits that binds to the CP opening the CP channel. In contrast to the RP, PA28 lacks ATPase activity and does not bind to ubiquitinated proteins. Expression of the PA28 subunits is induced by γ-interferon, and is required for efficient presentation of antigens at the cell surface by MHC class I molecules (Finley, 2009; Glickman & Ciechanover, 2002). In most cases, proteasomes cleave proteins into small peptides varying between 3 and 23 amino acids in length (Kisselev et al, 1999; Nussbaum et al, 1998). However, only a fraction of the peptides produced by the proteasome are of the correct length to be presented by MHC class I molecules. PA28 appears to be involved in altering the cleavage specificity of the proteasome, despite not being in direct contact with the catalytically active β-subunits. The mechanism through which this happens is still unclear, however it has been suggested that PA28 might facilitate the exit of substrates from chimeric RP-CP-PA28 proteasomes. Rapid peptide exit could prevent overdigestion of substrates, allowing for the exit of peptides with an adequate size for MHC class I presentation (Finley, 2009; Glickman & Ciechanover, 2002).

Deubiquitinating enzymes

Similar to how protein phosphorylation is a reversible process, protein ubiquitination can also be reversed through the action of deubiquitinating enzymes, also known as DUBs. DUBs are proteases that process ubiquitin or ubiquitin-like gene products, reverse the modification of proteins by ubiquitin or ubiquitin-like proteins, and remodel polyubiquitin chains on target proteins. DUBs have been implicated in the regulation of numerous cellular functions including cell cycle regulation, proteasome and lysosome dependent protein degradation, gene expression, DNA repair, kinase activation and microbial pathogenesis (Reyes-Turcu et al, 2009). DUBs play several roles in the ubiquitin pathway. First, DUBs are essential for activating newly expressed ubiquitin. Ubiquitin is always synthesized either as a proprotein fused to one of two ribosomal proteins or as a linear polyubiquitin chain that must be processed to yield the free ubiquitin monomer. Furthermore, the carboxyl-terminal of the polyubiquitin gene product also contains an additional amino acid that must be removed to activate ubiquitin (Baker & Board, 1987; Ozkaynak et al, 1987; Wiborg et al, 1985). DUBs are also involved in the recycling of ubiquitin that may have been trapped by the reaction of small cellular nucleophiles with the thiol ester intermediates that are formed during the ubiquitination process (Pickart & Rose, 1985). DUBS also play a major role in reversing the modification of proteins by ubiquitin or ubiquitin-like conjugation (Nijman et al, 2005; Wilkinson, 1997). Lastly, DUBs are also responsible for the recycling of monomeric ubiquitin from free polyubiquitin chains that are synthesized de novo by the conjugating machinery or that have been released from target proteins by other DUBs (Piotrowski et al, 1997; Wilkinson et al, 1995).

Like most proteases, DUB activity is tightly controlled in order to avoid the cleavage of inappropriate substrates. DUB activity can be regulated through phosphorylation, ubiquitination or sumoylation (the conjugation of proteins with the ubiquitin-like protein SUMO), modifications that are likely to affect activity, localization or half-life of DUBs (Reyes-Turcu et al, 2009). Similar to other enzymes, besides their catalytic domain, DUBs also contain additional ubiquitin-binding domains as well as several protein-protein interaction domains. These domains enable the binding and recognition of different ubiquitin chain linkages and can also direct the assembly of multiprotein complexes that localize DUBs and assist in substrate selection (Reyes-Turcu et al, 2009; Reyes-Turcu & Wilkinson, 2009).

The human genome encodes over 100 putative DUBs that can be subdivided into at least five protein families. These 5 families can be grouped into two major groups, papain-like cysteine proteases which include the ubiquitin carboxyl-terminal hydrolases (UCH), the ubiquitin-specific proteases (USP/UBP), the ovarian tumour domain (OTU) DUBs and the Josephin domain (MJD) DUBs. The other group includes the JAB1/MPN/Mov34 metalloenzyme (JAMM) domain zinc-dependent metalloprotease family (Reyes-Turcu et al, 2009).

Ubiquitin carboxyl-terminal hydrolase domain DUBs are characterized by the presence of a 230

amino acid UCH catalytic core domain. Examples of these DUBs are Uch-L1, Uch-L3, BAP1 and the proteasome binding DUB Uch37.

Ovarian tumour domain DUBs are characterized by the presence of an OTU core domain composed of five β-strands, sandwiched between helical domains that vary in size among OTU family members. An example of an OTU DUB is otubain 1 (Komander & Barford, 2008; Lin et al, 2008b; Messick et al, 2008; Nanao et al, 2004).

Josephin domain DUBs contain a catalytic domain that is similar to UCH domains. Examples of these DUBs include Ataxin-3, a protein implicated in the neurodegenerative disorder spinocerebellar ataxia type 3 (Mao et al, 2005).

Ubiquitin-specific protease domain DUBs are characterized by the presence of an USP domain fold and are the largest DUB family. The USP domain fold is highly conserved among USPs, despite low sequence similarity (Avvakumov et al, 2006; Hu et al, 2002; Hu et al, 2005; Komander et al, 2008; Renatus et al, 2006). The USP fold consists of three subdomains, the finger, palm and thumb, which together form a structure that is often compared to a right hand. Examples of these DUBs include yeast Ubp1-16. Another member of this DUB family, UBPY (ubiquitin-specific processing protease Y9 also known as USP8), is a deubiquitinating enzyme that associates with endossomes in response to EGF and is implicated in receptor downregulation in mammalian cells (Clague & Urbe, 2006). UBPY shows little discrimination between Lys48 and Lys63-linked chains and appears to be important for the endossomal sorting of internalized plasma membrane proteins such as EGFR (Mizuno et al, 2005; Row et al, 2006). It can also protect proteins, such as STAM2, from proteasomal degradation (Row et al, 2006).

Lastly, JAB1/MPN/Mov34 Metalloenzyme domain DUBs differ from all other DUB families in that their catalytic site does not depend on a cysteine residue to form a thiol group with ubiquitin, instead, their catalytic activity involves Zinc ions present in the JAMM domain (Reyes-Turcu et al, 2009). Another important feature of JAMM DUBs is that they are not inhibited by ubiquitin aldehyde, a potent inhibitor of papain-like DUBs. Ubiquitin aldehyde inhibition results from its reaction with the cysteine active site of papain-like DUBs, forming a stable covalent bond. JAMM DUBs do not employ a covalent enzyme-ubiquitin reaction intermediate, and as such, are not inhibited by ubiquitin aldehyde. Instead, JAMM DUBs can be inhibited by zinc ion chelators such as TPEN (Finley, 2009; Yao & Cohen, 2002). An example of a member of this family is AMSH (Associated Molecule with the SH3 domain of STAM).

Ubiquitin-like proteins

The post-translational modification of proteins by conjugation to another protein is not exclusive to ubiquitin, a number of other small proteins are also capable of being conjugated to amino residues of proteins in processes similar to ubiquitin conjugation. Although these proteins sometimes have a

UBL*	Identity with ubiquitin (%)	Comments on UBL
Known UBLs		
Ubiquitin	100	Precursors encoded by multiple genes
Rub1 (NEDD8)	55	Substrates are cullins and p53
FUBI (also known as MNSF-β or FAU)	38	Derived from a ribosomal-protein precursor
FAT10	32 and 40**	Contains a β-grasp fold Substrates unknown
ISG15	32 and 37**	Production induced by type I interferons
Smt3 (SUMO1, SUMO2, SUMO3)	18	SUMO encoded by 3-4 genes in vertebrates, depending on the species
Atg8	ND	Three known isoforms in humans Contains a β-grasp fold
Atg12	ND	~20% identical to Atg8
Urm1	ND	Related to small sulphur-carrying proteins MoaD and ThiS Contains a β-grasp fold
UFM1	ND	Contains a β-grasp fold
Putative UBLs		
BUBL1, BUBL2	Variable (up to 80%)	Putative autoprocessed proteins in ciliates
UBL-1	40	A precursor to ribosomal proteins in nematodes
SF3A120	30	UBL domain at the carboxyl-terminal No data about conjugation
Oligoadenylate synthetase	30 and 42**	UBL domain at the carboxyl-terminal No data about conjugation

Table 2 - Known and putative UBLs. ND, not detectable by standard BLAST searches. *UBLs are listed as the yeast (*Saccharomyces cerevisae*) symbol if the UBL is present in yeast, otherwise vertebrate symbols are listed. Known vertebrate orthologues with symbols that differ from yeast proteins are listed in parentheses. **The identities listed are for each of two ubiquitin-related domains. Adapted from (Hochstrasser, 2009).

primary sequence that can be very different from that of ubiquitin, they all share the same distinct three-dimensional core structure characteristic of ubiquitin, the β-grasp fold. The conjugation of these proteins is catalyzed by distinct but evolutionarily related enzyme cascades to those used for ubiquitin conjugation. As such, these proteins have been termed ubiquitin-like proteins or UBLs (Glickman & Ciechanover, 2002; Hochstrasser, 2009).

UBLs are divided into two groups, those that are free-standing moieties that can be conjugated to other proteins and those that are synthesized as fusion proteins. Although the first group of proteins are capable of forming isopeptide bonds with internal lysines of target proteins, just as ubiquitin does, no modification by poly-UBL chains is known. The second group is heterogeneous, with the only common denominator being a region with sequence homology to ubiquitin. UBLs in this group do not end with Gly-Gly sequences, the UBL is permanently fused and they are not processed by DUB-like proteases (Glickman & Ciechanover, 2002).

Although the covalent attachment of UBLs to proteins does not target substrates for proteasomal degradation, UBLs appear to be intimately linked to the ubiquitin-proteasome pathway, regulating the interaction of ubiquitin and proteasome machinery with their target substrates. Conjugation of

UBLs to proteins can have several general functions (Hochstrasser, 2009). UBL conjugation may facilitate protein association by providing an additional binding site, such as in the case of the binding of SUMO-conjugated RanGAP1 with the nuclear-pore complex (Mahajan et al, 1997). UBL conjugation may also cause a conformational change in the target protein that enhances or decreases its affinity for interaction with other proteins. An example of this is the sumoylation of thymine-DNA glycosylase (TDG) which induces a conformational change in TDG that lowers its affinity for DNA (Steinacher & Schar, 2005). Modification of a protein by different UBLs may also recruit different binding factors. These modifications may be mutually exclusive and involve the same attachment site. For example, the modification of proliferating cell nuclear antigen (PCNA) by SUMO, ubiquitin or ubiquitin polymers causes PCNA to bind different factors (Ulrich, 2004). Another example is the binding of SUMO to IκBα, which precludes its ubiquitination and degradation as it occurs on the same residues that serve as polyubiquitin anchors as well (Desterro et al, 1998). Lastly, and given the bulk of UBLs, they may also exert their function through steric hindrance by simply blocking the interaction of two proteins. A potential example of this is the sumoylation of the vaccinia A40R that prevents association and aggregation between A40R monomers (Palacios et al, 2005).

UBLs that are expressed as permanent fusions with other proteins appear to act as interaction modules for binding to ubiquitin-binding domains. Binding of specific UBL domain-containing proteins can link the proteasome to potential substrates. For instance, Bag-1 can help target misfolded proteins to the proteasome (Luders et al, 2000), while Rad23 targets the proteasome to sites of DNA damage and repair (Russell et al, 1999a). Additional UBL domain-containing proteins are also linked to the ubiquitin system. The DUB Ubp6 and the E3 enzymes UIP28 and Parkin all contain UBL domains (Glickman & Ciechanover, 2002).

Internalization of membrane proteins

The plasma membrane of eukaryotic cells is composed of a mixture of different lipids as well as a complex assortment of different transmembrane proteins used for recognition, adhesion, nutrient uptake and signalling. The composition of the plasma membrane is not static, proteins present at the plasma membrane are constantly being renewed and the surface expression of different proteins is modulated in response to both extracellular and intracellular stimuli. Intracellular trafficking mechanisms control the lipid and protein composition of the plasma membrane and are also critical for the function of endosomes and lysosomes (Bonifacino & Traub, 2003; Doherty & McMahon, 2009; Traub, 2009).

Endocytosis of plasma membrane lipids and proteins as well as extracellular components can proceed through a diverse assortment of vesicular mechanisms. In general, vesicle-mediated transport between the plasma membrane and intracellular organelles (or between the organelles

themselves) follows similar steps. First, during biogenesis there is a restricted assembly of vesicle coat components at a designated site on the donor membrane. This is followed by membrane deformation linked to cargo concentration in order to form a coated bud. Upon membrane scission the intracellular vesicle is uncoated and transported to the proximity of an acceptor compartment. Lastly, the vesicle fuses with the acceptor membrane delivering its contents (Traub, 2009). The most well studied mechanism of internalization is clathrin-mediated endocytosis, however, several other clathrin-independent endocytosis mechanisms are known, including Caveolae-mediated endocytosis; CLIC/GEEC pathway (clathrin-independent carrier/GPI-AP-enriched early endosomal compartment); IL2Rβ pathway; Arf6 dependent endocytosis; Flotillin dependent endocytosis; phagocytosis; macropinocytosis, circular dorsal ruffles and entosis (Doherty & McMahon, 2009).

Clathrin-mediated endocytosis

Clathrin-mediated endocytosis (CME) is the most widely studied mechanism of internalization in eukaryotic cells. A wide variety of integral membrane proteins are substrates for this internalization pathway, that is characterized by the packaging of cargo into clathrin coated vesicles (CCVs). CCVs are not restricted to endocytosis events and can also be used for the intracellular trafficking of membranes and proteins between organelles, with the use of different adaptor proteins (Doherty & McMahon, 2009).

The assembly of a clathrin coated vesicle occurs through several sequential steps. The first step involves the nucleation de novo of the vesicle during which clathrin adaptor proteins form a transient complex with lipids of the plasma membrane that is stabilized by accessory proteins. The most well described clathrin adaptor protein (AP) is AP2, which is capable of interacting with phosphatidylinositol-4,5-biphosphate (PtdIns(4,5)P_2) at the plasma membrane. Following this first step the nascent clathrin coated vesicle can either fail to form a CCV and its components can dissolve, or further lateral extension can occur with the addition of more adaptor and accessory proteins that recruit clathrin to the nascent pit. This in turn promotes the polymerization of clathrin into curved polyhedral lattices, that stabilize the deformation of the attached membrane. Clathrin polymerization, coupled with the action of other proteins, aids in the formation and constriction of the vesicle neck, helping to bring the membranes surrounding the neck into close apposition. Concurrently with this process, co-assembled clathrin-associated sorting proteins (CLASPs), such as disabled 2 (Dab2), autosomal recessive hypercholesterolemia protein (ARH), Stonin 2, Epsin 1 and epidermal growth factor receptor substrate 15 (Eps15), can also cluster cargo molecules within the forming sorting scaffold. Lastly, the GTPase membrane scission protein dynamin is recruited to the neck of the vesicle, forming an oligomeric dynamin spiral to enwrap the vesicle neck. Upon GTP hydrolysis, the dynamin spiral is constricted, and with the aid of tension generated by actin linkage, the vesicle is severed from the plasma membrane. After vesicle

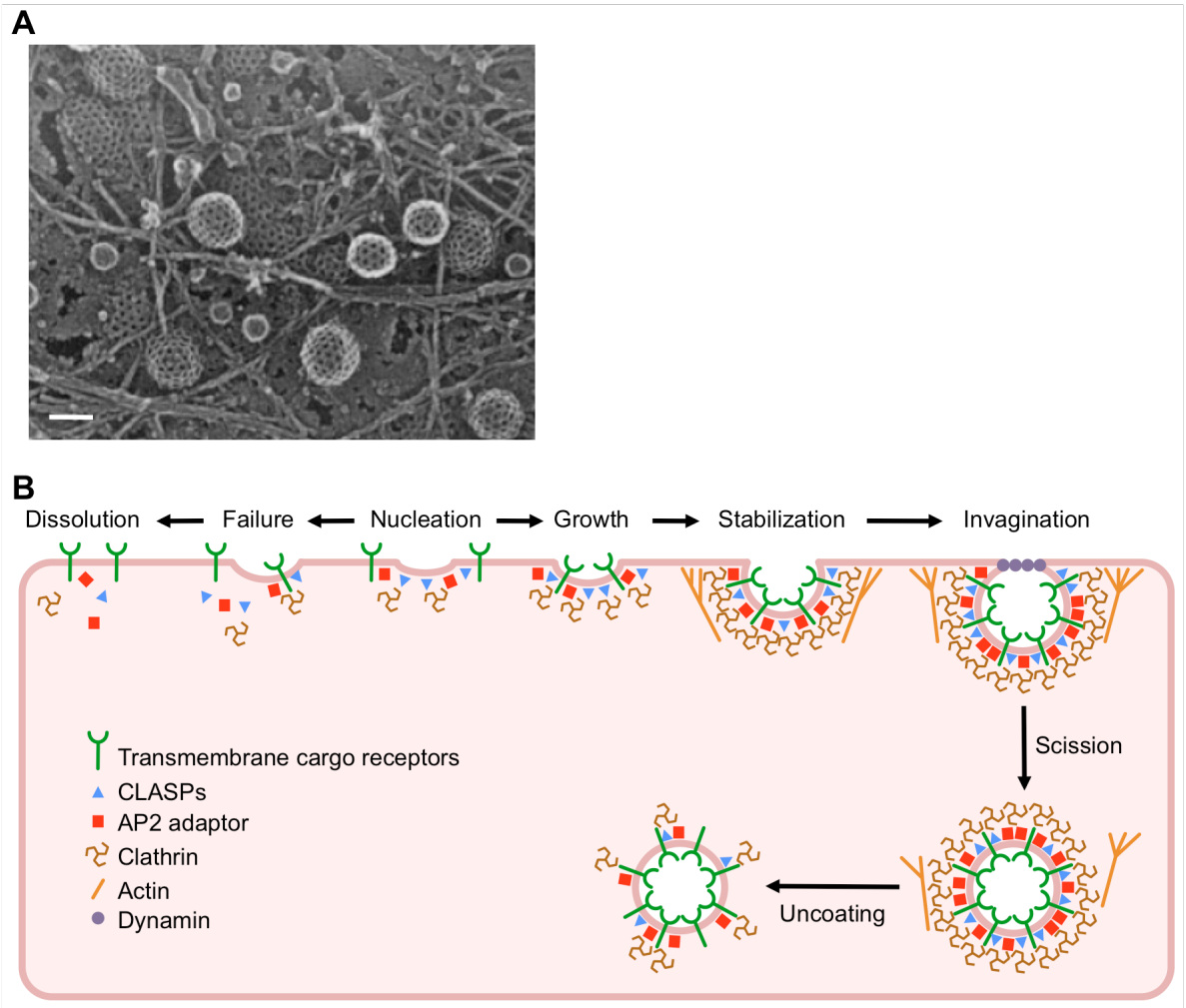

Figure 13 - Clathrin-mediated endocytosis. (A) Visualization of clathrin coat structures at the plasma membrane of HeLa SS6 cells. Cells were grown on glass coverslips and "unroofed" using sonication. The exposed plasma membranes were fixed, critical point dried and rotary shadowed with PT/C from an angle of 27º. Scale bar, 125 nm (Meyerholz et al, 2005). (B) A model illustrating the assembly and internalization of a clathrin coated vesicle. Assembly zones are probably nucleated de novo at sites of the plasma membrane where AP2 and CLASPs accumulate. From this point, the nascent pit may either fail to assemble, leading to the dissolution of the accessory components, or it may grow, recruiting additional accessory components, clathrin, actin and cargo to be internalized. These help to stabilize the membrane deformation. Once the coated pit is fully formed, dynamin is recruited to the connection point between the plasma membrane and the bulbous coated bud. Following GTP hydrolysis, and with the help of the tension force exerted by actin microfilaments, the vesicle detachs from the plasma membrane. The resulting vesicle moves quickly away from the plasma membrane and is rapidly uncoated. The uncoated vesicle can then fuse with other intracellular compartments, delivering its cargo. Adapted from (Traub, 2009).

release, the clathrin coat is removed from the vesicle by auxilin and Hsc70. The naked vesicle is then trafficked through the cell until reaching an intracellular compartment where it releases its cargo (Doherty & McMahon, 2009; Traub, 2009).

Caveolae-mediated endocytosis

Caveolae are specialized forms of lipid rafts that are generated by the oligomerization of caveolin proteins. Caveolae are detergent resistant, highly hydrophobic and are composed mainly of cholesterol and sphingolipids. They appear as flask or omega shaped plasma membrane

invaginations, which are present in many, but not all, eukaryotic plasma membranes (Doherty & McMahon, 2009; Kiss & Botos, 2009; Mosesson et al, 2008). There are three mammalian caveolin proteins, caveolin1 and 2 are found widely in non muscle cells while caveolin3 is muscle specific. Cells that do not express Cav-1 (or Cav-3 in muscle cells) are devoid of morphologically evident caveolae. Overexpression of Cav-1 in caveolae-deficient cells is sufficient to produce the flask-shaped plasma membrane invaginations characteristic of caveolae, implying that Cav-1 is necessary for the formation of these structures (Lipardi et al, 1998). Cav-2, however, does not seem to be required for caveolae formation, but may play a role in the formation of deep plasma membrane attached caveolae (Sowa et al, 2003). Caveolae appear to form in the Golgi complex, where they acquire their characteristic detergent insolubility. Interaction of oligomerized Cav-1 with Cav-2 renders the latter detergent insoluble, and induces the delocalization of Cav-2 from the Golgi complex to the plasma membrane. Exit of precaveolae from the Golgi complex is accelerated by cholesterol (Pol et al, 2005) and inhibited by glycosphingolipid depletion (Cheng et al, 2006). Cholesterol depletion flattens caveolae and increases the mobility of Cav-1 at the plasma membrane (Rothberg et al, 1992). Once the precaveolae reaches the plasma membrane they become relatively immobile structures (Kiss & Botos, 2009). Similar to how clathrin acts as a coat protein for CCVs, Cav-1 and PTRF-cavin (p-cavin) appear to function as coat proteins for caveolae. The recruitment of p-cavin to membrane domains containing oligomerized caveolin, cholesterol and phosphatidylserine stabilizes the membrane curvature of caveolae, producing the classical flask shaped structure of caveolae. p-cavin also appears to only interact with mature caveolae at the plasma membrane and not with non-caveolar caveolin present in the Golgi complex (Hill et al, 2008; Liu & Pilch, 2008).

While the abundance of caveolae in many cell types is consistent with a role of these structures in the trafficking of membrane proteins, the effective role of caveolae in the endocytosis of plasma membrane proteins remains controversial. Studies detailing the dynamic properties of GFP-tagged Cav-1 revealed that the internalization rate of Cav-1 is surprisingly slow (van Deurs et al, 2003). Moreover, overexpression of Cav-1 was also shown to inhibit the endocytosis of cargo localized in caveolae, suggesting that Cav-1 itself can be responsible for stabilizing caveolae on the plasma membrane (Minshall et al, 2000). Additionally, although caveolar-type structures can be observed in regions of the cell distant form the plasma membrane, it has been shown that many of these structures are still directly connected to the plasma membrane (Parton et al, 2002; Sandvig et al, 2008). Another problem with assessing the real function of caveolae in endocytosis is that Cav-1-associated cargos can also be found within other clathrin-independent vesicles such as those formed by the CLIC-GEEC internalization pathway, which raises the possibility that previous manipulations thought to affect caveolar endocytosis may in fact be acting on other endocytic pathways (Doherty & McMahon, 2009). Nevertheless, although caveolae are not normally involved in endocytosis, interaction of caveolae or caveolin with specific ligands can trigger the rapid

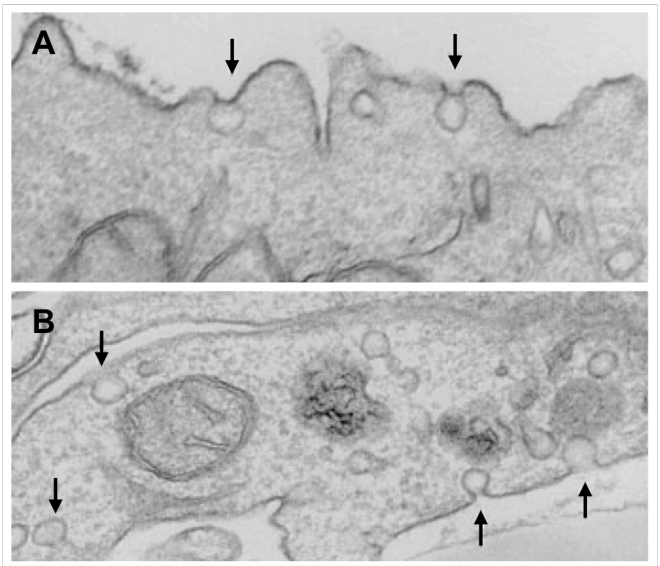

Figure 14 - Caveolae-mediated endocytosis. Electron micrographs of caveolin-expressing FRT cells. Cav-1 transfected FRT cells were grown on filters for five days and then fixed and treated for electron microscopy. (A) Apical side. (B) Basal side. Arrows indicate caveolae, which are characterized as coat-free flask-shaped plasmalemmal invaginations with the diaphragm at the neck (Lipardi et al, 1998).

internalization of caveolae and several substrates of this pathway have already been described (Kiss & Botos, 2009). Caveolae internalization has been shown to be regulated by kinases and phosphotases. Phosphorylation of tyrosine residues on Cav-1 and Cav-2 has been shown to induce caveolae internalization (Kiss et al, 2004; Lee et al, 2002; Tiruppathi et al, 1997), while treatment with vanadate, a tyrosine phosphatase inhibitor, stimulates internalization by causing hyperphosphorylation of Cav-1 (Botos et al, 2007). Besides its role in the pinching off of CCVs, dynamin also appears to be important for the scission of caveolae from the plasma membrane (Henley et al, 1998; Oh et al, 1998). Lastly, internalized caveolae have been shown to form large grape-like structures containing Cav-1, termed caveosomes. The fate of caveosomes appears to be regulated by its cargo. Caveosomes containing SV40 virus do not fuse with lysosomes, allowing the virus to escape lysosomal degradation (Pelkmans et al, 2001). However, other studies show that cargo internalized through caveolae can be translocated to early endosomes, a component of the classical endosomal degradation pathway (Kiss & Botos, 2009). Although caveolae are often considered a major pathway for the internalization of plasma membrane proteins, many questions concerning this pathway remain unanswered, including the molecular effectors responsible for accumulating cargo in caveolae.

Endocytic adaptor and accessory proteins

As mentioned above, the endocytosis of plasma membrane proteins can occur through several different mechanisms, which in turn can internalize a wide assortment of different substrates. Thus, in order to internalize a specific protein within the wide variety of possible substrates, endocytosis

machinery requires components capable of recognizing specific substrates or endocytic signals. These components are usually referred to as endocytic adaptors and accessory proteins. Considering how clathrin-mediated endocytosis is the best characterized internalization mechanism, more is known about the process of clathrin coated pit (CCP) formation and cargo recruitment than for any other internalization mechanism. As such, this segment will focus on describing the function of endocytic adaptors and accessory proteins for clathrin-mediated endocytosis.

Adaptor proteins are thought to initiate CCP formation through two ways. First, they are responsible for selecting and accumulating specific cargo at the plasma membrane into the nascent CCP. Second, they are also responsible for recruiting structural and regulatory components (clathrin and scaffold proteins) that help stabilize early endocytic protein complexes that are essential for the formation of CCPs (Maldonado-Baez & Wendland, 2006).

The term endocytic adaptor is generally reserved for proteins that bind to the lipid PtdIns(4,5)P_2, and also to clathrin and to sorting signals within the cytosolic regions of transmembrane cargo. Two major classes of endocytic adaptors participate in CCV formation: multimeric adaptor proteins, such as AP2, and monomeric adaptor proteins, such as CLASPs (Maldonado-Baez & Wendland, 2006).

Adaptor proteins share a common basic structure with a region containing folded domains that binds to plasma membrane components and unstructured regions that contain multiple peptide-binding motifs, that provide recruiting areas for clathrin and accessory proteins. This modular organization of endocytic adaptors allows them to interact simultaneously with several partners. These interactions can be classified into three groups according to the binding partner: lipid-adaptor interactions (Owen, 2004), cargo-sorting-signal-adaptor interactions (Robinson, 2004), and accessory-protein-adaptor interactions (Maldonado-Baez & Wendland, 2006).

AP2 is the main non-clathrin constituent of purified endocytic CCVs, and also the best characterized endocytic adaptor. AP2 is a stable complex comprised of four non-identical subunits termed α-subunit, β2-subunit, μ2-subunit and σ2-subunit (Collins et al, 2002; Matsui & Kirchhausen, 1990). Besides providing a bridge between clathrin and the plasma membrane, some subunits of AP2 also possess domains capable of recognizing protein sorting signals (described further below) present in the substrate. Although AP2 possesses lipid binding domains that allow its interaction with PtdIns(4,5)P_2, the association of AP2 with the plasma membrane is more stable when the adaptor is simultaneously binding a cargo molecule (Maldonado-Baez & Wendland, 2006). Deletion of genes that encode components of AP2 are lethal in several metazoan models, however depletion of AP2 subunits in *Saccharomyces cerevisiae* is non-lethal and has a minimal effect on clathrin function, which suggests that although AP2 is a major component of CCVs, some organisms have developed other proteins that overlap with AP2 function (Huang et al, 1999a; Traub, 2009; Yeung et al, 1999), CLASPs are such proteins. Examples of CLASP proteins include

Epsin, Dab2, ARH and Eps15. Most CLASPs bind both to AP2 and clathrin through short peptide interaction motifs located in the structurally disordered carboxyl-terminal region (Owen, 2004). This might explain why AP2 can associate with the plasma membrane in the absence of cargo. Furthermore, due to the considerable connectivity between CLASPs and because they also possess lipid interaction domains, they are capable of nucleating the assembly of fewer and diminutive but still operational clathrin coated structures when AP2 levels are depleted (Traub, 2009).

Besides their function in stabilizing the interaction between AP2 and the plasma membrane, CLASPs also have a role in inducing membrane curvature to form the CCP. Biophysical considerations suggest that membranes prefer a more flat structure, thus elements external to the plasma membrane are needed to induce the high membrane curvature present in the nascent endocytic vesicles. Studies have shown that clathrin polymerization alone is insufficient for membrane curvature generation (Nossal, 2001). CLASPs such as those belonging to the Epsin family of proteins, can help drive membrane deformation by the insertion of an amphipathic helix into the plasma membrane. This initial deformation is then stabilized by the polymerization of the clathrin coat (Ford et al, 2002).

CLASP proteins also participate in the recognition of endocytic cargo. For example, both Epsin and Eps15 are involved in the recruitment of ubiquitinated cargo into endocytic vesicles (Sorkina et al, 2006; Wendland, 2002).

Protein sorting signals

The composition of the plasma membrane is constantly being renewed in response to extra and intracellular stimuli. To ensure the proper response of the cell, plasma membrane proteins must be specifically and selectively internalized. Considering that each endocytic pathway can internalize multiple substrates, cells require a system to target plasma membrane proteins for degradation. The endocytosis machinery can recognize protein substrates through the direct binding to short polypeptide motifs present in the primary sequence of proteins, which are normally constitutively active, but also to post-translational modifications such as phosphorylation and ubiquitination (Bonifacino & Traub, 2003; Doherty & McMahon, 2009; Maldonado-Baez & Wendland, 2006; Traub, 2009).

YXXØ sorting signals

YXXØ sorting signals (in which X is any amino acid and Ø is a bulky hydrophobic amino acid) are found in a wide variety of integral membrane proteins, such as LAMP-1 or the transferrin receptor. Although YXXØ motifs have been shown to be essential for the rapid internalization of a variety of

plasma membrane proteins, their function is not limited to endocytosis. YXXØ motifs have been implicated in the intracellular sorting of transmembrane proteins to the lysosome and lysosome-related organelles and also in the sorting of proteins to the basolateral membrane of polarized epithelial cells (Bonifacino & Traub, 2003). Although the YXXØ tetrapeptide is the minimal motif to confer sorting information to proteins, the X residues and other residues flanking the motif may also confer sorting specificity to the signal. For instance, lysossomal-sorting YXXØ motifs tend to have acidic residues at the X positions and a glycine preceding the critical tyrosine residue (Harter & Mellman, 1992; Rous et al, 2002). YXXØ signals interact with the endocytic machinery through the μ2-subunit of AP2 (Owen & Evans, 1998). For some proteins this interaction can be modulated through phosphorylation. Phosphorylation of $GABA_A$ (γ-aminobutyric acid type A) receptor γ2-subunit on either tyrosine of its YGYECKL signal impedes its interaction with AP2 (Kittler et al, 2008). YXXØ signals can also be recognized by the α-subunit of AP2, as is the case for the CD317 protein (Masuyama et al, 2009).

Dileucine sorting signals

Dileucine sorting signals are characterized by the consensus sequence [DE]XXXL[LIM] and are involved in mediating the internalization of proteins such as the cluster of differentiation antigen 4 protein (CD4) and Tyrosinase (Bonifacino & Traub, 2003; Traub, 2009). Similar to YXXØ signals, Dileucine signals have also been shown to be important in signalling the trafficking of integral membrane proteins between intracellular compartments (Bonifacino & Traub, 2003). Dileucine sorting signals are structurally different from YXXØ signals and do not compete with it for entry into CCVs (Marks et al, 1996). Dileucine sorting signals also interact with the endocytic machinery through the AP2 complex, however they bind to a different subunit, the σ-subunit, through a domain that is adjacent to the $PtdIns(4,5)P_2$-binding site on the α-subunit (Kelly et al, 2008). The interaction between the [DE]XXXL[LIM] signal and the σ-subunit of AP2 can also be modulated by phosphorylation. In the case of CD4, its dileucine motif SQIKRLL must be phosphorylated on the serine residue to enable the binding of the protein to the AP2 complex (Pitcher et al, 1999).

[FY]XNPX[YF] sorting signals

Unlike YXXØ sorting signals, [FY]XNPX[YF] signals are not normally recognized by the μ2-subunit of AP2. Instead, [FY]XNPX[YF] signals are decoded by a subfamily of CLASPs containing the phosphoTyr-binding domain (PTB), which includes Dab2 and ARH. PTB domains are capable of binding both [FY]XNPX[YF]-containing cargo and $PtdIns(4,5)P_2$ simultaneously (Stolt & Bock, 2006). Even though YXXØ and [FY]XNPX[YF] signals both feature critical Tyr residues in their

sequence the PTB domain is selective towards [FY]XNPX[YF] signals. For example, the expression of Dab2 has been shown to enhance the uptake of [FY]XNPX[YF]-containing cargo, but not YXXØ-containing cargo (Chetrit et al, 2009).

Ubiquitin as a sorting signal

All the short peptide sequences mentioned above are normally constitutively active, with proteins bearing those motifs being rapidly and efficiently internalized through clathrin-mediated endocytosis. However, certain membrane proteins, such as signalling receptors, ion channels and transporters, persist at the cell surface and are internalized in a temporally defined manner. This defined regulation in the internalization of plasma membrane proteins usually requires a post-translational modification of the target protein to signal it for internalization. In *Saccharomyces cerevisiae*, the predominant internalization signal is the conjugation of ubiquitin to lysine residues of the targeted protein. These signals are recognized through a subfamily of CLASPs that include Epsins and Eps15. Epsin and Eps15 recognize ubiquitinated cargo through their ubiquitin-interacting motifs (UIM), and in turn, both of these CLASPs bind strongly to AP2. Additionally, Epsin can also associate with Eps15 and recruit clathrin, which enables it to assemble clathrin coats even in the absence of AP2 (Traub, 2009).

Monoubiquitination, multimonoubiquitination and Lys63-linked polyubiquitin chains have all been reported to function as endocytosis signals, however, several studies report that monoubiquitination appears to be a weak internalization signal (Barriere et al, 2006; Hawryluk et al, 2006). This is explained by observations showing that the UIM domains present in endocytic adaptors such as Eps15 have a relatively weak affinity for monoubiquitin. Instead, UIM domains have been shown to bind more strongly to polyubiquitin signals, especially Lys63-linked chains. Additionally, multimonoubiquitination of a single protein or monoubiquitination of several subunits of a protein complex was also suggested to be a strong endocytic signal since the close proximity of several monoubiquitin moieties would resemble a polyubiquitin signal, which is the preferred binding partner for UIM domains.

Ubiquitination of plasma membrane proteins is itself a regulated process. The main E3 ligase responsible for targeting proteins for internalization in *Saccharomyces cerevisiae* is Rsp5. Although Rsp5 contains several WW motifs that interact with PPXY motifs on target substrates, no plasma membrane cargo seems to interact directly with Rsp5 through these domains (Lin et al, 2008a; Nikko et al, 2008). Instead, prior phosphorylation of Rsp5 substrates is required for substrate internalization (Belgareh-Touze et al, 2008; Lundh et al, 2009; Paiva et al, 2009).

Curiously, besides its role in targeting plasma membrane proteins for internalization, ubiquitin also regulates the activity of UIM-containing endocytic adaptor proteins (Hoeller et al, 2006; Woelk et al, 2006). Eps15 was shown to be monoubiquitinated by the Nedd4 ubiquitin ligase. The proposed

model first involves the self-ubiquitination of Nedd4. This provides a binding site for the UIM domains of Eps15, which would then enable Nedd4 to ubiquitinate Eps15 (Woelk et al, 2006). The monoubiquitination of Eps15 (and other sorting proteins containing UIM domains) impairs its ability to regulate the trafficking of ubiquitinated transmembrane proteins. Monoubiquitination of Eps15 was suggested to induce the formation of an intramolecular interaction between the UIM domains of Eps15 and the monoubiquitin moiety. According to this model, following monoubiquitination, the UIM domains of Eps15 would be occupied in this intramolecular interaction, preventing the endocytic adaptor from interacting with ubiquitinated cargo. Accordingly, fusion of an ubiquitin molecule to the carboxyl-terminal of Eps15 prevented its recruitment to the plasma membrane and inhibited its association and colocalization with EGFR-containing endocytic vesicles (Hoeller et al, 2006).

Endosomal sorting of ubiquitinated membrane proteins

Following internalization, endocytosed cargos have several distinct molecular fates. One possibility that awaits internalized cargo is their transport to the lysosome for degradation. Endosomes can be defined as being either early or late, based on the relative time it takes for endocytosed material to reach either stage. During the maturation process of endosomes their structure and composition is altered, with late endosomes showing characteristics of typical multivesicular bodies (MVBs). The transition between the two stages is thought to proceed through the progressive involution of the limiting membrane of endosomes to form intralumenal vesicles (ILVs) and with the concomitant removal of recycling material via the fission of tubular elements that give rise to recycling endosomes. When the resulting MVBs fuse with lysosomes (or with the vacuole in yeast), the ILV membrane is degraded by lipases and its transmembrane content processed by lysosomal proteases (Raiborg & Stenmark, 2009; Williams & Urbe, 2007).

The best characterized signal directing membrane proteins to the degradative MVB pathway is ubiquitination. Ubiquitinated cargo arriving at endosomes are sorted into ILVs and are not recycled back to the plasma membrane, transported retrogradely to the secretory pathway or retained in the limiting endosome membrane. At the endosomal membrane, ubiquitinated cargo is recognized by the endosomal sorting complex required for transport (ESCRT) machinery, a series of protein complexes that are highly conserved in eukaryotes. The ESCRT machinery performs three distinct but connected functions: it recognizes ubiquitinated cargo and prevents their recycling or retrograde transport; it deforms the endosomal membrane to initiate ILV formation and accumulation of cargo; and it also catalyzes the abcission of the endosomal invaginations, forming ILVs containing the sorted cargo. The ESCRT machinery consists of the four complexes, ESCRT-0, -I, -II and -III, plus several accessory components. ESCRT-0, -I and -II all possess ubiquitin-binding domains that are used to recognize cargo to be sorted into ILVs. Although there is no clear

model that explains how ubiquitinated cargo can be transferred from one ESCRT complex to the next, the directional flow of cargo from ESCRT-0 to -I and finally to -II is plausible when one considers the sequential recruitment of the complexes (Raiborg & Stenmark, 2009; Williams & Urbe, 2007).

ESCRT-0

In mammals, the ESCRT-0 complex consists of the subunits Hrs and STAM (signal transducing adaptor molecule); in yeast the ESCRT-0 complex is formed by Vps27 and Hse1. ESCRT-0 can be considered as a filter that retains ubiquitinated membrane proteins in the endosomal membrane (Raiborg & Stenmark, 2009). Both Hrs and STAM are capable of binding ubiquitin, which allows the ESCRT-0 complex to recognize cargo destined for lysosomal degradation. Both subunits of ESCRT-0 contain an amino-terminal VHS (Vps27, Hrs and STAM) domain of unknown function, in addition to ubiquitin and clathrin binding domains (Hofmann & Falquet, 2001; McCullough et al, 2006; Raiborg et al, 2001). The Hrs subunit in particular contains a FYVE zinc-finger domain which it uses to bind to phosphatydilinositol-3-phosphate (PtdIns(3)P), this interaction is responsible for the recruitment of the ESCRT-0 complex to endosomes (Raiborg et al, 2001). Hrs also has an important role in recruiting the downstream ESCRTs by direct interaction with the ESCRT-I component Tsg101 (Clague & Urbe, 2003).
A third ubiquitin-binding protein, Eps15b (an isoform of Eps15), has been found to be associated with ESCRT-0 in human cells. Although the exact function of this protein is not known, it was shown to be important in sorting epidermal growth factor receptors for degradation (Roxrud et al, 2008).

ESCRT-I

ESCRT-I was the first ESCRT complex to be identified. The ESCRT-0 complex initially wasn't classified as a member of the ESCRTs, until later evidence showed that it functions to recruit ESCRT-I (Bache et al, 2003; Katzmann et al, 2003; Lu et al, 2003). The ESCRT-I complex has a 1:1:1:1 ratio of four subunits: Tsg101 or Vps23 in yeast, Vps28, Vps37 and Mvb12 (Chu et al, 2006; Morita et al, 2007). The structure of the ESCRT-I complex contains a headpiece (that binds to ESCRT-II), a rigid stalk and an endpiece that contains an UEV domain. The UEV domains of Tsg101 and Vps23 are capable of binding ubiquitin (which they use to recruit ubiquitinated substrates) and also to PSAP-like motifs in Hrs/Vps27, and together with additional interactions contribute to the recruitment of ESCRT-I to endosomes. As such, in the absence of ESCRT-0, recruitment of ESCRT-I to endosomal membranes in inhibited (Bache et al, 2003; Katzmann et al, 2003; Lu et al, 2003).

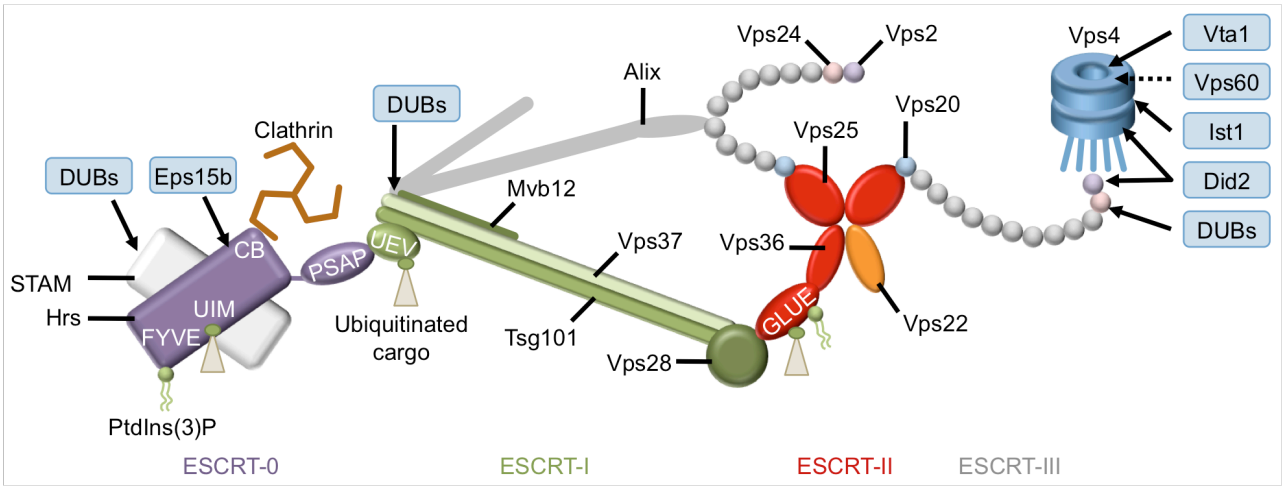

Figure 15 - Composition and molecular interactions of the ESCRT machinery. Interactions between the four ESCRT complexes are indicated, as are interactions with ubiquitinated cargo and accessory molecules such as PtdIns(3)P, deubiquitinating enzymes (DUBs), Alix and the ATPase Vps4. Protein domains are labelled in white. Dashed arrow indicates interaction predicted by genetic studies but not yet confirmed biochemically. Adapted from (Raiborg & Stenmark, 2009).

ESCRT-II

ESCRT-II is composed of one Vps36, one Vps22 and two Vps25 subunits. The core of the ESCRT-II complex is a flat y-shaped heterotetramer, with Vps22 and Vps36 forming the base of the y and the two copies of Vps25 forming the arms (Hierro et al, 2004; Teo et al, 2004). All of the subunits of ESCRT-II have two tandem repeats of a winged-helix domain, a common type of protein-protein and protein-DNA interaction module (Hierro et al, 2004; Teo et al, 2004; Wernimont & Weissenhorn, 2004). Additionally, Vps36 has a GLUE (Gram-like ubiquitin-binding in Eap45) domain that can bind both to ubiquitin and to PtdIns(3)P (Hirano et al, 2006; Slagsvold et al, 2005). ESCRT-II interacts with the Vps28 subunit of ESCRT-I through its Vps36 subunit. In addition to the GLUE domain of Vps36, the first helix of Vps22 also has lipid-binding properties, though less specific, and is likely to participate in the membrane recruitment of ESCRT-II (Teo et al, 2006).

ESCRT-III

ESCRT-III consists of small, highly charged subunits that assemble into higher-order multimers on membranes (Raiborg & Stenmark, 2009). The core complex of ESCRT-III contains the subunits Vps20, Vps32, Vps24 and Vps2, which are assembled in a highly ordered manner. Vps20 is the subunit responsible for initiating the assembly of ESCRT-III by first binding to the Vps25 subunit of ESCRT-II. Vps20 then interacts with Vps32, which triggers the assembly of Vps32 into filamentous oligomers that are capped by Vps24. Finally, Vps2 associates with Vps24, recruiting the ATPase Vps4 to the ESCRT-III complex (Saksena et al, 2009; Teis et al, 2008; Teo et al, 2004).

Complex	Metazoan protein	Ubiquitin-binding domain	Selected interacting proteins
ESCRT-0	Hrs	UIM	Clathrin, Eps15b, Tsg101
	STAM1, 2	UIM (VHS)	AMSH, UBPY
ESCRT-I	Tsg101	UEV	Hrs
	Vps28	NA	Vps36
	Vps37A, B, C, D	NA	NA
	Mvb12A, B	NA	NA
ESCRT-II	Vps22	NA	NA
	Vps25	NA	Vps20
	Vps36	GLUE	Vps28
ESCRT-III	Vps2A, B	NA	Vps4
	Vps20	NA	Vps25
	Vps24	NA	AMSH, UBPY
	Vps32A, B, C	NA	NA
Vps4	Vps4A, B	NA	Vps2
	Ist1	NA	NA
	Vps46A, B	NA	NA
	Vta1	NA	NA
	Vps60	NA	NA
Other	Alix, HD-PTP	NA	AMSH, UBPY

Table 3 - ESCRTs subunits. NA, not applicable. Note that the assignment of accessory proteins to Vps4 rather than to ESCRT-III is arbitrary in light of our incomplete understanding of their functions. Adapted from (Raiborg & Stenmark, 2009).

The ESCRT-III complex appears to be involved in the deforming of the endosomal membrane and in the abscission of ILVs into endosomes. Overexpression of Vps32, but not of other ESCRT-III subunits, in mammalian cells was shown to cause the protrusion of buds and tubules from the plasma membrane, triggered by spiralled filaments formed by Vps32 multimers (Hanson et al, 2008). Furthermore, *in vitro* assembly experiments show that Vps24 and Vps2 are capable of forming hollow tubules. Vps4 is recruited into these tubules, depolymerizing them from the inside following ATP hydrolysis (Ghazi-Tabatabai et al, 2008; Lata et al, 2008).

The opposite topology of ILV budding requires that the abscission machinery be recruited from inside of the membrane stalk, unlike how dynamin is recruited to the outside of the membrane stalk to induce the budding of CCPs. Equivalent membrane stalks are also severed during cytokinesis and viral budding from the plasma membrane, and accumulating evidence indicates that ESCRT-III and Vps4 represent a highly conserved machinery for this type of membrane fission. In fact, phylogenetic analyses indicate that ESCRT-III is the most ancient and conserved of the ESCRTs

(Leung et al, 2008; Raiborg & Stenmark, 2009). Thus, it is likely that the ESCRTs originated from a device for cell division in unicellular organisms that later evolved to recognize ubiquitinated membrane proteins in endosomes to mediate their translocation into ILVs. Although there is no definite evidence as to how ESCRT-III mediates membrane abscission, the ability of ESCRT-III subunits to assemble into circular arrays suggests a model where Vps4-mediated removal of individual Vps32 subunits from one end of the spiral polymer at the neck of the invagination may lead to the constriction of these circular arrays culminating in membrane scission (Saksena et al, 2009). Alternatively, ESCRT-III mediated clustering of cargo with bulky intraluminal domains could also contribute to both membrane deformation and scission (Raiborg & Stenmark, 2009).

Deubiquitination and endosomal sorting

Although ubiquitination is important to signal membrane cargo for vesicular sorting, proper deubiquitination of cargo is also necessary to ensure the normal function of the sorting system. For instance, it has been shown that the DUB Doa4 (degradation of alpha 4) is essential for MVB sorting (Amerik et al, 2000). Doa4 is recruited to endosomes through ESCRT-III and the accessory protein Bro1, where it is responsible for the deubiquitination of endosomal cargo before their entry into ILVs. The importance of Doa4 for proper endosomal sorting was suggested to be related to the recycling of ubiquitin, since if ubiquitin were allowed to degrade alongside endocytosed cargo, intracellular levels of free ubiquitin would be compromised (Swaminathan et al, 1999). Additionally, the DUB activity of Doa4 may also be important for reactivating components of the ESCRT machinery that have been inactivated by monoubiquitination (Nikko & Andre, 2007).
Two other DUBs have also been described to localize to endosomes in mammalian cells, AMSH and UBPY. Both DUBs can be recruited at two points of the ESCRT system: at the ESCRT-0 subunit STAM, or also at ESCRT-III and the accessory protein Alix (McCullough et al, 2006; Mizuno et al, 2006; Row et al, 2006). Given their association with the ESCRT-0 complex, these DUBs presumably serve to deubiquitinate cargo that is not destined for endosomal sorting to the lysosome. Some authors also suggest that they can activate Hrs and STAM by reversing the autoinhibitory monoubiquitination of these proteins (Raiborg & Stenmark, 2009). Curiously, UBPY and AMSH have different substrate specificity, with AMSH showing a preference for Lys63-linked polyubiquitin chains, while UBPY has no preference between Lys48 and Lys63-linked chains (Row et al, 2006). Given their different substrate specificity, its possible that these DUBs may be involved in the remodelling of ubiquitin chains attached to endosomal cargo. This different substrate specificity may also explain the antagonistic functions these two DUBs show in relation to some membrane proteins. For instance, while AMSH seems to promote the recycling of EGFR, UBPY promotes its endosomal sorting (Clague & Urbe, 2006).

Endocytic recycling

Endocytosis is one of the major mechanisms that regulate the lipid and protein composition of the plasma membrane. Additionally, it is estimated that cells internalize the equivalent of their cell surface one to five times per hour (Steinman et al, 1983). As such, pathways that deliver materials to the plasma membrane must be robust and coordinately regulated. Lipids and proteins can reach the plasma membrane through two broad mechanisms, the biosynthetic pathway and endocytic recycling, the latter of which being responsible for most of the material being delivered to the plasma membrane. The recycling of receptors back to the plasma membrane was one of the first characterized examples of recycling of endocytosed proteins, and since then, the recycling of many other types of integral membrane proteins has been described (Maxfield & McGraw, 2004). Endocytic recycling is also essential for maintaining the difference between apical and basolateral membranes in polarized cells (Wang et al, 2000). As an example, in hepatocytes, most basolateral membrane proteins are first delivered to the apical membrane from where they are subsequently delivered to the basolateral membrane following an intracellular route (Kipp & Arias, 2000; Schell et al, 1992; Zegers & Hoekstra, 1998).
Due to their nature and function, organelles involved in intracellular sorting pathways are not stable structures, with their membrane composition constantly shifting according to arriving and departing cargo. In fact, the characteristics of entire organelles may shift over time, leading to their transformation into a different organelle with a different function, such as in the case of early endosomes maturing into late endosomes.

Early endosomes

The early endosome is defined as the organelle that receives material from primary vesicles that have been generated by endocytosis. Markers of the early endosome include the small GTPase Rab5, phosphoinositide 3-kinase (PI3K) and its product phosphatydilinositol-3-phosphate (PtdIns(3)P). Regardless of the mode of entry, endocytosed cargo is usually delivered to the early endosome where sorting occurs. From there cargo can be routed to late endosomes and lysosomes for degradation, to the trans-Golgi network or to recycling endosomal carriers that bring the cargo back to the plasma membrane (Grant & Donaldson, 2009; Maxfield & McGraw, 2004).
Although clathrin-mediated internalization of proteins relies on the recognition of sorting signals (that can either be short polypeptide sequences or post-translational modifications such as ubiquitination), the predominant sorting mechanism of early endosomes is based more on organelle geometry than on the recognition of a specific sorting motif in the cargo proteins (Maxfield & McGraw, 2004). Most of the membrane of early endosomes is removed by the pinching off of narrow-diameter tubules (Dunn et al, 1989; Mayor et al, 1993). The surface-area-to-

volume ratio of these tubules is greater than that of the vesicular portion of the sorting endosome, and therefore, the pinching off of tubules preferentially sorts recycled membrane from the soluble molecules. As such, membrane proteins that arrive to the early endosome without any specific targeting information will be transported from the sorting endosome with the bulk of the membrane. On the other hand, the transport of membrane proteins to the late endosome requires specific targeting signals to separate them from the bulk of the plasma membrane.

The lumen of the early endosome is mildly acidic (Johnson et al, 1993; Presley et al, 1997); which can induce conformational changes in proteins that lead to the release of ligands from receptors. Thus, the now free receptor protein can be trafficked back to the plasma membrane for further rounds of signalling, while the ligand is retained in the lumen of the early endosome, eventually being degraded in the lysosome. This functions as one of the protein sorting mechanisms in early endosomes, separating the bulk of receptor proteins from their ligands (Maxfield & McGraw, 2004). The ESCRT system mentioned in the previous section also functions as a sorting mechanism in early endosomes that diverts ubiquitin tagged membrane proteins to ILVs, eventually being degraded in the lysosome alongside the luminal content of the early endosome.

Although recycling back to the plasma membrane seems to be the default pathway for cargo internalized through clathrin-dependent endocytosis, the recycling of cargoes internalized through clathrin-independent pathways might involve a positive selection process (Grant & Donaldson, 2009).

Recycling cargo exiting early endosomes have two possible destinations: the rapid recycling route, that delivers them directly to the plasma membrane; or the slow recycling route, that first delivers cargo to the endocytic recycling compartment (ERC) before they arrive at the plasma membrane.

Rapid recycling route

The rapid recycling route is characterized by the recycling of proteins directly from early endosomes, or from earlier stages in the endocytic pathway, back to the plasma membrane. The GTPase Rab4 is a commonly used marker for rapid recycling endosomes. Rab4 has been shown to be important for the rapid recycling of the transferrin receptor (TFR) and glycosphingolipids from early endosomes (Choudhury et al, 2004; Maxfield & McGraw, 2004; van der Sluijs et al, 1992), however the exact role that Rab4 plays in this recycling route remains unknown. Indeed, while the overexpression of a dominant negative form of Rab4 was shown to inhibit fast recycling, siRNA-mediated depletion of Rab4 was shown to increase rapid recycling, perhaps by blocking trafficking between the early endosome and ERCs (Deneka et al, 2003; Yudowski et al, 2009).

Another Rab GTPase involved in regulating rapid recycling is Rab35. Rab35 localizes to the plasma membrane and early endosomes and has been shown to be required for the rapid recycling of TFR (Kouranti et al, 2006).

Slow recycling route

The slow recycling route involves the transport of cargo proteins from the early endosome to the ERC and from the ERC to the plasma membrane. The above-mentioned model of geometry based sorting of proteins by the early endosome postulates that during maturation narrow-diameter tubules pinch off from the early endosome. These are thought to give rise to the ERC, while the main body of the early endosome becomes the multivesicular body (Grant & Donaldson, 2009; Maxfield & McGraw, 2004). In fact, live imaging studies have shown that over time early endosomes lose Rab5 and acquire Rab11, one of the molecular markers of the ERC (Sonnichsen et al, 2000). The ERC is morphologically defined as a tubular compartment that is largely devoid of fluid. Besides Rab11, EHD1 (Eps15 homology domain-containing 1) is also considered a molecular marker of the ERC (Grant & Donaldson, 2009).

While it is still mainly unknown why proteins sometimes recycle back to the plasma membrane through fast recycling endosomes and other times through the ERC, the delivery of cargo to the ERC might function to prevent their entry into degradative compartments (Grant & Donaldson, 2009).

Transport from the ERC to the plasma membrane has been suggested to proceed through several distinct pathways according to cargo. In HeLa cells distinct recycling pathways have been described for TFR and for cargo that was internalized through clathrin-independent pathways, as the recycling endosomes that carry TFR are separate from the tubular recycling endosomes that carry cargo internalized through clathrin-independent endocytosis (Naslavsky et al, 2003; Naslavsky et al, 2004; Radhakrishna & Donaldson, 1997). Although both types of recycling endosomes are Rab11-dependent, the trafficking of tubular recycling endosomes is much more regulated (Grant & Donaldson, 2009).

As a molecular marker of the ERC, the GTPase Rab11 is an important regulator of recycling. Interfering with Rab11, or with the many proteins that interact with it, inhibits recycling and often alters the positioning of the ERC in the cell. Rab11 has been shown to be important for the recycling of TFR, a canon substrate for clathrin-dependent endocytosis, but also for MHC class I and β-integrin, that internalize through clathrin-independent pathways (Grant & Donaldson, 2009).

The Rab GTPase proteins

Rab GTPases constitute the largest family of small GTPases, proteins that catalyze the dephosphorylation of GTP into GDP. They function as docking sites for other molecules and through their indirect interactions with coat components, motor proteins and SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) the Rab GTPases serve as multifaceted organizers of almost all membrane trafficking processes in eukaryotic cells

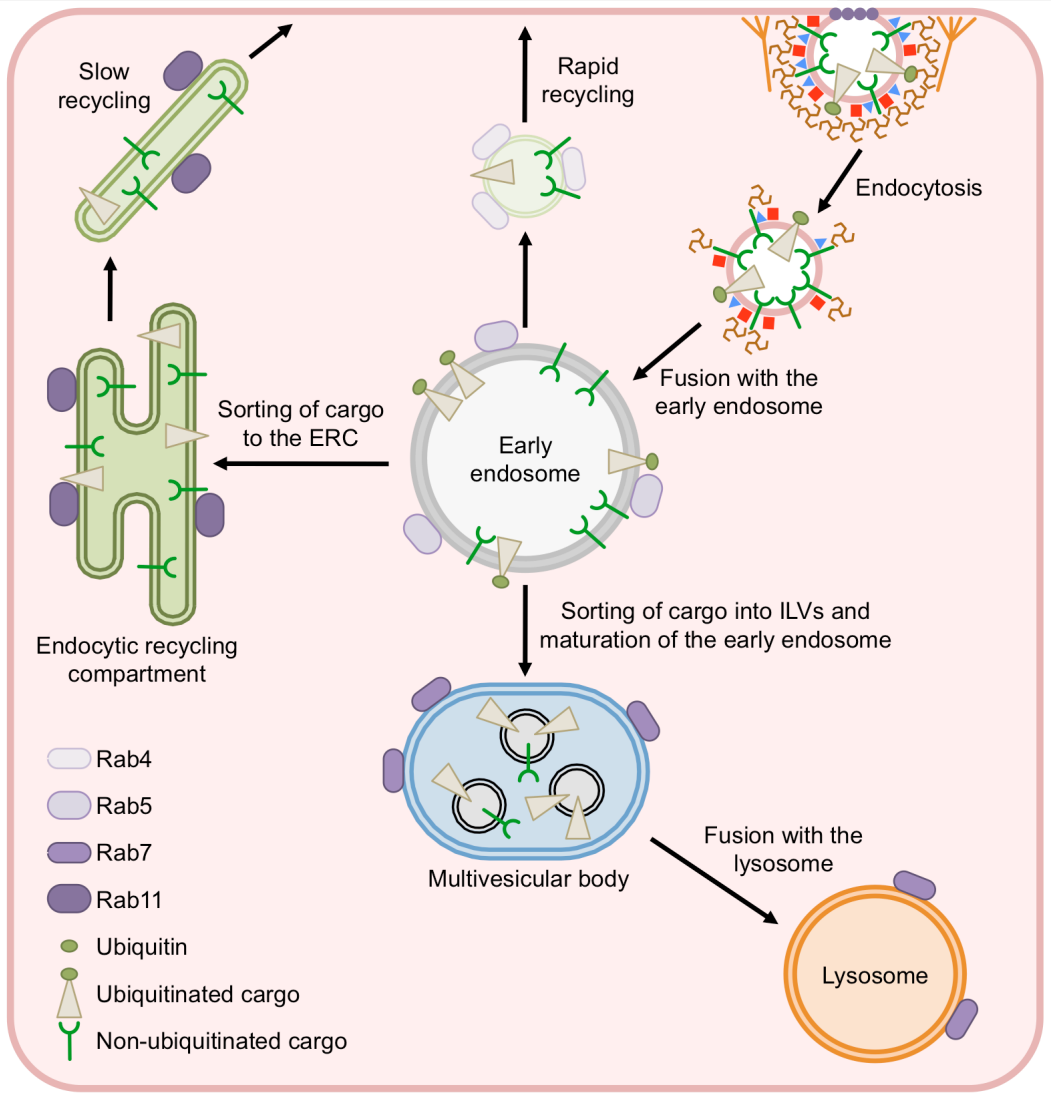

Figure 16 - Pathways of endocytic recycling. Regardless of the internalization pathway used, most cargo is delivered to the early endosome. Rab5 is a marker of this compartment. During the maturation process of the early endosome, cargo to be delivered to the lysosome is internalized into intralumenal vesicles. For this process to occur, cargo must contain specific sorting motifs, such as an ubiquitin moiety, though other sorting motifs exist. The mature multivesicular body, or late endosome, then fuses with the lysosome with the aid of Rab7. Alternatively, cargo present at the early endosome can be recycled back to the plasma membrane through two major pathways. Rapid recycling traffics cargo directly from the early endosome back to the plasma membrane through vesicles positive for Rab4. Slow recycling first traffics cargo to the endocytic recycling compartment before delivering the cargo to the plasma membrane through vesicles positive for Rab11. Endocytic recycling is considered to be the default route followed by plasma membrane proteins that reach the early endosome. Additionally, ubiquitinated cargo can also be recycled back to the plasma membrane as long as they are deubiquitinated before being sorted into ILVs by the ESCRT machinery present at early endosomes (Grant & Donaldson, 2009; Raiborg & Stenmark, 2009; Stenmark, 2009).

(Stenmark, 2009). For example, Rab5 has been shown to be involved in mediating cargo sequestration into and budding of endocytic vesicles, uncoating of CCVs, vesicle motility along microtubules and the tethering of vesicles to acceptor membranes. In humans there are more than sixty members of the Rab family that are localized to distinct intracellular membranes (Stenmark, 2009).

Rab proteins alternate between two conformational states: a GTP bound “on” form and a GDP bound “off” form, which allows Rab proteins to function as molecular switches. The conversion of

Rab proteins between the GDP- and GTP-bound forms involves major conformational changes in two variable regions: switch I and switch II (Pfeffer, 2005). The exchange of GDP for GTP is catalyzed by GEFs (guanine nucleotide exchange factors) that facilitate the release of GDP from the Rab protein (Delprato et al, 2004). The high cytosolic concentration of GTP ensures that GTP binds as soon as GDP has been released. In their GTP-bound form, Rab proteins are capable of interacting with several types of effector molecules, including sorting adaptors, tethering factors, kinases, phosphatases and motor proteins. Rab proteins are inactivated following GTP hydrolysis, which is driven both by the intrinsic GTPase activity of the Rab protein and by GTPase-activating proteins (GAPs) (Stenmark, 2009). Although there are some examples of Rab effectors that prefer the GDP-bound form (Shirane & Nakayama, 2006), this form is considered to be the inactive state of Rab GTPases.

Due to their crucial role in intracellular membrane trafficking, functional impairment of Rab pathways are associated with diseases, such as immunodeficiency, cancer and neurological disorders (Stenmark, 2009).

Chapter 2

Chapter 2: Objectives

Gap junction intercellular communication (GJIC) mediated by connexin proteins is essential for the survival of all vertebrate organisms, being critical for processes such as embryonic development, coordinated contraction of excitable cells, tissue homeostasis, normal cell growth and differentiation. In fact, connexin gene mutations have already been linked to several human diseases. The extent of GJIC is directly related to the number and functional state of gap junction channels present at the plasma membrane, therefore, processes that influence the stability of connexins at the plasma membrane are critical for regulating intercellular communication. Although GJIC can be regulated through the gating of the channel pore, degradation of connexins, particularly Cx43, appears to play an unexpected and critical role in regulating GJIC. The underlying goal of this thesis is the characterization of the molecular mechanisms that modulate Cx43 internalization, intracellular trafficking and degradation.

The physiological importance of GJIC is well illustrated in the eye lens where inner fibre cells fully depend on a complex network of gap junctions for nutrition and signalling. The lens plasma membrane is also unique among eukaryotic cell membranes due to its extremely high content of cholesterol and deficit of polyunsaturated fatty acids. As the main unsaturated lipid present in lens membranes, cholesterol is prone to oxidation yielding a variety of oxidation products. Some of these cholesterol oxides were shown to be increased in human cataractous lenses, 7-ketocholesterol being the predominant oxysterol present in human cataracts. The accumulation of oxysterols at the plasma membrane may alter intercellular communication by a variety of mechanisms, most of which unclear. Therefore, one of the purposes of this work was to investigate if oxysterols alter intercellular communication through Cx43 gap junctions in lens epithelial cells.

Both the proteasome and the lysosome have been implicated in the degradation of Cx43. While degradation of Cx43 by the lysosome has long been established, the role of the proteasome in Cx43 turnover is still poorly understood. Initial studies suggested that the proteasome could be directly involved in connexin degradation, however, more recently, it has been hypothesized that the proteasome acts by regulating the stability of Cx43 at the plasma membrane. Although several reports have suggested that ubiquitin may be implicated in gap junction internalization, direct evidence of ubiquitin-dependent internalization/degradation of Cx43 has not been clearly demonstrated. Recently it was demonstrated that Cx43 interacts with the ubiquitin ligase Nedd4, suggesting that Nedd4 also plays a role in GJ internalization and/or degradation. Although it was suggested that Nedd4 binds to and ubiquitinates Cx43 molecules at the cell surface, leading to their internalization and subsequent degradation probably via the interaction with accessory proteins, Nedd4-mediated ubiquitination of Cx43 has not been demonstrated. In mammalian cells, the monoubiquitination of plasma membrane proteins is associated with the internalization and sorting of cargo receptors. Although the exact mechanism and molecular players involved in

ubiquitin-dependent internalization of cell surface proteins is still unknown, several studies have consistently demonstrated the existence of ubiquitin receptors playing a key role in the internalization of membrane proteins. Indeed, a number of endocytic proteins, including Eps15, Epsin and Hrs, contain ubiquitin-binding domains that recognize ubiquitinated proteins and sort them along the endocytic pathway, thus acting as a link between cargo and components of the endocytic and sorting machinery. Thus, a major objective of this study is to evaluate the importance of ubiquitination as a signal for the internalization of Cx43, as well as to identify the molecular players involved in the ubiquitination of Cx43 and in the recognition and internalization of ubiquitinated Cx43.

Clathrin has long been suggested to mediate the internalization of annular gap junctions, with several recent studies showing that impairment of clathrin-mediated endocytosis (CME) leads to the accumulation of Cx43 at the plasma membrane. However, other recent studies suggest that large portions of Cx43 gap junctions are internalized through clathrin-independent mechanisms. As such, another aim of this study is to identify and characterize this clathrin-independent endocytic pathway for Cx43.

Although several reports have suggested that Cx43 is ubiquitinated, it is currently unknown whether ubiquitinated Cx43 is a substrate for deubiquitination, and the physiological relevance of such modification for the intracellular sorting of Cx43. Another aim of this study is to identify putative deubiquitinating enzymes (DUBs) that may be involved in the intracellular sorting of Cx43.

Following internalization and their arrival at the early endosome, endocytosed cargos may have a variety of different fates. Proteins may either be delivered to the trans-Golgi network, be recycled back to the plasma membrane or remain in association with the early endosome while it matures into late endosomes, which eventually fuse with lysosomes. Although Cx43 internalization and subsequent degradation is well established, little (or nothing) is known about the intracellular sorting mechanisms of Cx43 or about any putative endocytic recycling mechanisms directing Cx43 back to the plasma membrane. This work also aims to characterize the intracellular trafficking of Cx43.

Altogether, this study is aimed at characterizing the molecular mechanisms that modulate the internalization and intracellular trafficking of Cx43.

Chapter 3

Chapter 3: Material and Methods

Antibodies and reagents

The rabbit anti-Cx43 polyclonal antibody, obtained from Zymed (CA, USA) was raised against a peptide corresponding to a segment of the third cytoplasmic domain (carboxyl-terminal portion) of rat Cx43 (Cat No 71-0700). The mouse anti-Cx43 monoclonal antibody obtained from Zymed (CA, USA) was raised against a cytoplasmic sequence located near the carboxyl-terminus of rat Cx43 (Cat No 13-8300). The mouse monoclonal anti-c-myc antibody, obtained from Zymed (CA, USA) was raised against a 32 amino acid synthetic peptide (residues 408-439) derived from the carboxyl-terminus of the human c-myc protein (Cat No 13-2500). The mouse anti-GFP monoclonal antibody (Cat No 33-2600) was obtained from Zymed (CA, USA). The mouse anti-Cx43 monoclonal antibody obtained from BD Transduction Laboratories (BD Biosciences, CA, USA) was raised against a peptide sequence that represents amino acid residues 252-270 of rat Cx43. The rabbit polyclonal antibodies against Eps15 (H-896) were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). The rabbit polyclonal antibodies against Nedd4 (ab14592), GFP (ab290) and Tsg101 (ab30871) were obtained from Abcam (Cambridge, UK). The mouse monoclonal P4D1 antibodies against ubiquitin were obtained from Covance (CA, USA). The mouse monoclonal FK1 antibodies against ubiquitin were obtained from Biomol (Exeter, UK). The rabbit polyclonal antibodies against ubiquitin were kindly provided by Dr. Fu Shang (Tufts University, Boston, MA, USA). The mouse monoclonal antibodies against V5 were obtained from Invitrogen (Paisley, UK). The rabbit polyclonal antibodies against FLAG were obtained from Alexis Biochemicals.
Unless otherwise noted, all other reagents were obtained from Sigma. Oxysterols were dissolved in ethanol.

Cell culture

For primary cultures of lens epithelial cells, eyeballs were removed from adult bovines and the anterior capsule of the lens, with the attached epithelium, was cut along the equator and cultured in a 24 well-plate containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco BRL Life Technologies, Inchinnan, UK), 100 U/ml penicillin, 100 µg/ml streptomycin, at 37°C with 5% CO_2. The epithelial cells were then allowed to spread out from the capsule into the plate. The cells were kept in culture for 48 hours before treatments. At this stage they reached 90% confluence. $1x10^6$ cells were plated onto 60 mm Petri dishes.
The kidney derived cell lines NRK, Cos-7 and HEK293FT were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). The

retinal pigment epithelium cell line ARPE-19 (LGC Promochem, Teddington, UK) was cultured in Ham's F12/DMEM (1:1) supplemented with 10% fetal bovine serum, antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) and GlutaMax (1x). Transient transfections of cells were performed with Lipofectamine 2000 (Invitrogen), according to manufacturer's recommendations.

Plasmid constructions

Cx43 cDNA was cloned into a modified pENTR GFP C2 vector (Hume et al, 2006; Lopes et al, 2007). Site-directed mutagenesis was performed to generate the Cx43P283L and Cx43Y286A mutants from Cx43 cDNA. Site-directed mutagenesis was performed to generate the UbK48R(AA) mutant from ubiquitin cDNA. myc and UbK48R(AA) were then subcloned into a pCMV7 vector containing Cx43 to generate the myc-Cx43-Ub mutant. Correct mutagenesis was verified by sequencing the constructs. Plasmids expressing GFP-Cx43 were generated by cloning Cx43 cDNA into a pENTR GFP C2 vector. Plasmids expressing GFP-Cx43Y286A were generated by subcloning Cx43Y286A cDNA into a pEGFP vector. Plasmids expressing V5-Cx43 were generated by cloning Cx43 cDNA into a pENTR vector containing V5. Plasmids expressing RFP-Cx43 were generated by cloning Cx43 cDNA into a pENTR vector containing RFP. Plasmids expressing GFP-Rab11 were generated by cloning Rab11 cDNA into a pENTR vector containing GFP. Mouse Rab5a and Rab7a cDNA were amplified from AtT20 cells by RT-PCR and cloned into a pENTR GFP C2 vector to obtain plasmids expressing GFP-Rab5 and GFP-Rab7 (Hume et al, 2006). The UBPY cDNA was kindly provided by Dr. F Fiore (European Institute of Oncology, Milan, Italy). The V5-UBPY plasmids were generated by subcloning UBPY cDNA into a pENTR vector containing V5. Plasmids expressing Nedd4 and the Nedd4(C/A) mutant were kindly provided by Dr. Dale S Haines (Temple University School of Medicine, Philadelphia, PA, USA). Plasmids expressing Eps15 and the Eps15(ΔUIM) mutant were kindly provided by Dr. Simona Polo (University of Milan, Italy). Plasmids expressing Hrs-FLAG were kindly provided by Dr. Sylvie Urbé (University of Liverpool, Liverpool, UK). Plasmids expressing YFP-Rab4 were kindly provided by Dr. P van der Sluijs (Erasmus Medical Center, Rotterdam, The Netherlands).

siRNA and shRNA-mediated knockdown

siRNA targeting Eps15 (s4773 (CAGCAUUCUUGUAAACGGAtt) or s4774 (CUACCUUACUAGCCCAUAUtt)) or Nedd4 (s9416 (GGAAGAUCCAAGAUUGAAAtt) or s9417 (GGCGAUUUGUAAACCGAAUtt)) and a non-targeting control sequence were obtained from Ambion (Silencer Select Pre-designed siRNA). shRNA targeting UBPY was generated by inserting the sequence GGTCTCCATGAAGATCTAA into a plasmid under the U6 promotor. Prior to transfection, cells were grown until they reached 40-50% confluency. siRNA or shRNA was

complexed with Lipofectamine 2000 (Invitrogen), according to manufacturer's recommendations, and added to the cells medium to a final concentration of 20 nM. Eps15, Nedd4 or UBPY knockdown was achieved by transfecting twice at intervals of 24 hours, and experiments were performed 24 hours after the second transfection.

Immunoprecipitation and Western blot

Cells were rinsed with PBS at 4°C, resuspended in lysis buffer (190 mM NaCl, 50 mM Tris-HCl, 6 mM EDTA, 1% Triton X-100, pH 8.3) supplemented with protease inhibitor cocktail (Roche), 2 mM PMSF, 10 mM iodacetamide (that alkylates cysteine residues at the active site of deubiquitinating enzymes (DUB), preventing protein deubiquitination), and incubated on ice during 10 minutes. The samples were then centrifuged at 10.000 g, for 10 minutes, and the supernatants used for immunoprecipitation. Briefly, protein A was incubated with polyclonal antibodies directed against Cx43 (Zymed, Cat No 71-0700), Eps15, Nedd4 or GFP (Abcam, ab290), while protein G was incubated with monoclonal antibodies directed against the V5 or myc tags. Non-specific antibodies or non-immune rabbit serum were used as controls. Incubations proceeded for 1 hour, at 4 °C, followed by incubation with supernatants, for 3 hours at 4 °C. The samples were then centrifuged and the protein A/G-sepharose sediments washed 3 times in an appropriate washing buffer (500 mM NaCl, 50 mM Tris-HCl, 6 mM EDTA, 1% Triton X-100, pH 8.3), resuspended in Laemmli buffer and denatured at 37°C for 30 minutes or at 100ºC for 5 minutes. Samples for the connexon immunoprecipitation experiments were resuspended in Laemmli buffer without β-mercaptoethanol in order to preserve the integrity of connexons, before being denatured at 100 ºC for 5 minutes.
For Western blot analysis of the immunoprecipitated proteins, samples were separated by SDS-PAGE, transferred to a PVDF or nitrocellulose membrane, and probed with appropriate antibodies. Inputs represent about 5% of the total amount of protein in the lysates before immunoprecipitation.

Isolation of Triton X-100 soluble fractions

To isolate the Triton X-100 soluble fraction, cells were rinsed with PBS at 4°C, scraped and resuspended in lysis buffer (190 mM NaCl, 50 mM Tris-HCl, 6 mM EDTA, 1% Triton X-100, pH 8.3) supplemented with protease inhibitor cocktail (Roche), 2 mM PMSF, 10 mM iodacetamide, 10 mM NaF and 500 mM Na_3VO_4. Following incubation on ice for 30 minutes, samples were ultra-centrifuged for 50 minutes at 100.000 g at 4°C and the supernatant collected (Triton X-100 soluble fraction), while the pellet was resuspended in lysis buffer containing 1% SDS and solubilized by sonication (Triton X-100 insoluble fraction). After denaturation with Laemmli buffer for 30 minutes at 37°C, the proteins were separated by SDS-PAGE, transferred to PVDF membranes and probed with appropriate antibodies.

Immunofluorescence

Lens epithelial cells grown on glass coverslips were fixed with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS), containing Ca^{2+} and Mg^{2+}. The samples were then washed with PBS, permeabilized with 1% v/v Triton X-100 in PBS, and blocked with goat serum (1:10) for 20 minutes. Incubation with primary mouse anti-Cx43 monoclonal antibodies (Zymed) proceeded for 1 hour at room temperature. The samples were then washed three times with PBS before incubation with secondary antibodies for 1 hour at room temperature. The specimens were rinsed in PBS and mounted with Glycergel (Dako, Glostrup, Denmark). All solutions were made up in 0.2% w/v BSA (Sigma) containing 0.02% sodium azide (Sigma, St Louis, MO, USA) in PBS. For controls, primary antibodies were omitted.
Preparations using cell lines grown on glass coverslips were fixed with 4% PFA in PBS. The samples were then washed with PBS, permeabilized with 0.2% v/v Triton X-100 in PBS, and blocked with goat serum (1:10) for 20 minutes prior to incubation with primary antibodies. Incubation with primary antibodies against Cx43 (Transduction Lab), Eps15 (Santa Cruz Biotechnology), ubiquitin, myc (Zymed), FLAG (Alexis Biochemical) and Tsg101 (Abcam) proceeded for 1 hour at room temperature. The samples were then washed three times with PBS before incubation with secondary antibodies for 1 hour at room temperature. The specimens were rinsed in PBS and mounted with Glycergel (Dako) or MOWIOL 4-88 Reagent (Calbiochem). All solutions were made up in 0.2% w/v BSA (Sigma) containing 0.02% sodium azide (Sigma) in PBS. For controls, primary antibodies were omitted. For DAPI staining, cells were incubated with the DAPI reagent for 10 minutes.
The images were collected by confocal microscopy using a Bio-Rad MRC-600 or a Zeiss LSM 710 or by fluorescence microscopy using a Leica CTRMIC.

Dextran uptake

Lens epithelial cells grown on glass coverslips were incubated with 10 mg/ml RITC-dextran (Mr 10200 Da) in DMEM for 30 minutes, at 37°C. Cells were then rinsed with PBS and incubated with fresh DMEM without dextran and returned to the incubator for an additional 30 minutes. The cells were subsequently rinsed with PBS and fixed with 4% PFA and imaged by fluorescence confocal microscopy.

Dye transfer assay for gap junctional intercellular communication

Lens epithelial cells grown on glass coverslips were assayed for gap junction-mediated intercellular coupling as described by Le and Musil (Le & Musil, 1998). Briefly, the culture medium

from a confluent monolayer of LEC was removed and saved. The cells were rinsed three times with Hank's balanced salt solution containing 1% bovine serum albumin (HBSS), after which a 27-gauge needle was used to create multiple scrapes through the cell monolayer in the presence of Dulbecco's phosphate buffered saline containing 0.5% rhodamine-dextran and 0.5% lucifer yellow. After exactly 1 minute, the culture was rinsed three times with HBSS and then incubated for an additional 8 minutes in the saved culture medium to allow the loaded dye to transfer to adjoining cells. The cells were then rinsed, fixed with 4% paraformaldehyde and imaged on a fluorescence microscope under UV light.

Biotinylation of cell surface proteins

Cells grown on 60 mm culture dishes were rinsed twice with 5 ml of ice-cold PBS containing 0.5 mM $MgCl_2$ and 1 mM $CaCl_2$, followed by the addition of 3 ml of the same ice-cold solution containing 1 mg/ml of freshly added SULFO-NHS-SS-biotin (Pierce, Rockford, IL, USA). After 30 minutes at 4º C, to stop subcellular trafficking, the medium was discarded and the plates were washed 3 times with PBS containing 0.5 mM $MgCl_2$, 1 mM $CaCl_2$ and 100 mM glycine. The cells were scraped in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EGTA, containing 1% Triton, 0.5% DOC, 0.1% SDS and supplemented with protease inhibitor cocktail (Roche), 2 mM PMSF, 10 mM iodoacetamide, 10 mM NaF and 500 μM Na_3VO_4; pH 7.5). After 15 minutes on ice the cells were sonicated and the homogenates were centrifuged at 14000 rpm, for 10 minutes. The protein content of the supernatants was determined and the same quantity of protein was transferred to 1.5 ml Eppendorf microfuge tubes containing 200 μl of Neutravidin beads (Pierce, Rockford, IL, USA). After 2 hours of incubation at 4º C under agitation, the beads were washed four times with RIPA buffer. The final pellets were resuspended in 150 μl 2x Laemmli buffer and incubated 1 hour at 37º C. The beads were pelleted and the solubilized proteins were separated by SDS-PAGE, transferred to PVDF or nitrocellulose membranes and probed with antibodies directed against Cx43.

To determine the stability of Cx43 at the plasma membrane, biotinylated cells were incubated for an additional 2 hours in DMEM, at 37°C, in the absence or presence of cholesterol or oxysterols. The culture medium was then rejected and the cells extracted in situ by incubation in DPBS containing 0.5% Triton X-100, for 10 minutes, at room temperature. The samples were then processed as described above.

Statistical analysis

Data present in this manuscript is representative of at least three independent experiments. Data are expressed as means ± S.D. Comparison between groups was performed using a Student's t test.

Chapter 4

Chapter 4: 7-Ketocholesterol modulates intercellular communication through gap junctions in bovine lens epithelial cells

Abstract

Connexin43 is an integral membrane protein that forms intercellular channels called gap junctions. Intercellular communication in the eye lens relies on an extensive network of gap junctions essential for the maintenance of lens transparency. The association of Cx43 with cholesterol enriched lipid raft domains has been previously demonstrated. The objective of this chapter is to assess if products of cholesterol oxidation (oxysterols) affect gap junction intercellular communication.

Primary cultures of lens epithelial cells (LEC) were incubated with 7-ketocholesterol (7-keto), 25-hydroxycholesterol (25-OH) or cholesterol and the subcellular distribution of Cx43 was evaluated by immunofluorescence confocal microscopy. The levels of Cx43 present in gap junction plaques was assessed by its insolubility in Triton X-100 and quantified by Western blot. The stability of Cx43 at the plasma membrane following incubation with oxysterols was evaluated by biotinylation of cell surface proteins. Gap junction intercellular communication was evaluated by transfer of the dye Lucifer yellow. The results obtained showed that 7-keto induces an accumulation of Cx43 at the plasma membrane and an increase in intercellular communication through gap junctions. However, incubation with cholesterol or 25-OH did not lead to significant alterations in the subcellular distribution of Cx43 nor in intercellular communication. Data further suggests that increased intercellular communication results from increased stability of Cx43 at the plasma membrane, presumably forming functional gap junctions, as suggested by decreased solubility of Cx43 in 1% Triton X-100. The increased stability of Cx43 at the plasma membrane seems to be specific and not related to disruption of the endocytic pathway, as demonstrated by dextran uptake.

Results demonstrate, for the first time, that 7-keto induces an increase in gap junction intercellular communication, that is most likely due to an increased stability of the protein at the plasma membrane and to increased abundance of Cx43 assembled in gap junction plaques.

Introduction

Gap junction channels consist of two connexons that are located at the plasma membrane of two adjacent cells. Each connexon is composed of six subunits termed connexins. These channels allow the passage of molecules with a molecular mass below 1 kDa, such as small metabolites, ions, and second messengers (Kumar & Gilula, 1996). The physiological importance of intercellular communication through gap junctions is well illustrated in the eye lens where inner fibre cells fully

depend on a complex network of gap junctions for nutrition and signalling (Mathias et al, 1997; Rae et al, 1996). The lens is an avascular organ containing a central mass of fibre cells covered by an anterior monolayer of epithelial cells. In the lens, gap junctions allow the passage of small molecules between metabolically active epithelial cells, which produce most of the ATP used by the lens, and the fully differentiated fibre cells that present low metabolic activity. At the equatorial region of the lens, epithelial cells exit the cell cycle and undergo significant morphological and biochemical changes that result in the formation of fully differentiated fibre cells, where virtually all organelles, including the nuclei, are absent (Sue Menko, 2002). Three connexin genes are expressed in the vertebrate lens; α1 (Cx43) connexin that is expressed mostly in epithelial cells (Beyer et al, 1989); α3 (Cx46) and α8 (Cx50) connexins which are expressed in fibre cells (Paul et al, 1991; White et al, 1992).

The lens plasma membrane is unique among eukaryotic cell membranes due to its extremely high content of cholesterol and deficit of polyunsaturated fatty acids. In fact, lens membranes contain the highest cholesterol content of any known biological membrane (Li et al, 1985; Zelenka, 1984). As the main unsaturated lipid present in lens membranes, cholesterol is prone to oxidation yielding a variety of oxidation products. Some of these cholesterol oxides (or oxysterols) were shown to be increased in human cataractous lenses (Girao et al, 1998), 7-ketocholesterol being the predominant oxysterol present in human cataracts.

Accumulation of oxysterols at the plasma membrane may alter intercellular communication by a variety of mechanisms, most of which unclear. For example, oxysterols may alter lipid bilayer order and, therefore, affect intercellular communication (Verhagen et al, 1996). It has been shown that Cx43 at the plasma membrane is localized in specialized domains called caveolae (Schubert et al, 2002). The presence of products of cholesterol oxidation on caveolae disturbs the function of such domains (Liu et al, 2000; Myers & Stanley, 1999; Pike & Miller, 1998). Moreover, Cx43 trafficking, assembly, and turnover are regulated by multiple mechanisms, including those mediated by the cytoskeleton (Lauf et al, 2002; Theiss & Meller, 2002; Thomas et al, 2001). Cholesterol oxides were shown to disrupt the cytoskeleton network organisation, through a mechanism that involves activation of Rho GTPases (Girao et al, 2003a).

The objective of this study is to evaluate if oxysterols alter intercellular communication through Cx43 gap junctions in lens epithelial cells.

Results

7-keto stabilizes Cx43 at the plasma membrane

Cholesterol has been shown to increase gap junction assembly and cell-cell communication. In this study we evaluated the effect of products of cholesterol oxidation on the subcellular distribution of

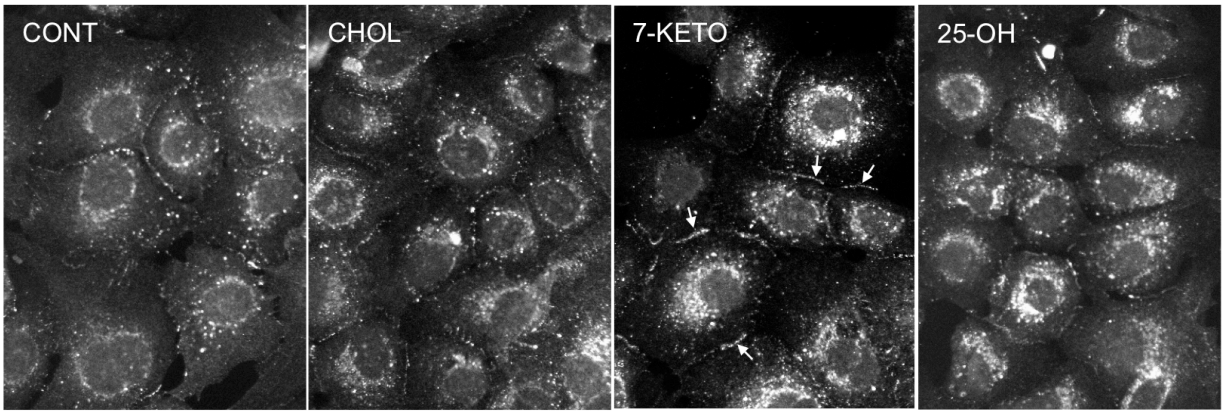

Figure 17 - Effect of oxysterols on the subcellular distribution of Cx43. LEC were treated with 20 µg/ml cholesterol, 7-keto or 25-OH for 3 hours. Cells incubated in 0.2% ethanol were used as controls. The cells were fixed and stained with antibodies directed against Cx43 and imaged by confocal microscopy.

Cx43 and GJIC in LEC.

To investigate the effects of cholesterol oxides on the subcellular distribution of Cx43, primary cultures of lens epithelial cells were incubated with 20 µg/ml cholesterol, 7-keto or 25-OH, for 3 hours. Cells incubated with 0.2% ethanol were used as controls. The cells were fixed and stained with antibodies directed against Cx43 and imaged by immunofluorescence confocal microscopy (Figure 17). In control cells Cx43 staining appeared as small punctate spots at the plasma membrane and cell-cell interfaces. After 3 hours of incubation with 7-keto the abundance of Cx43 at the plasma membrane (as evaluated by the number of punctate staining) was higher while incubation with cholesterol or 25-OH did not result in major alterations on Cx43 staining at the plasma membrane. A perinuclear staining was also observed following incubation with both 7-keto or 25-OH.

There are a variety of factors reported to affect the amount of Cx43 at the plasma membrane. These are often cell specific and lead to either recruitment of Cx43 to the plasma membrane or to stabilization of the protein at the membrane. The observation that the total amount of Cx43 does not change following treatment with 7-keto (Figure 18A) strongly suggests that Cx43 is not upregulated in response to cholesterol oxides.

In order to determine if accumulation of Cx43 at the plasma membrane following incubation with 7-keto was associated with an increased stabilization of the protein at the plasma membrane, cell surface proteins were labelled with biotin. The cells were incubated in the absence or presence of 20 µg/ml cholesterol, 7-keto or 25-OH, for 30 minutes, prior to biotinylation. The stabilization of Cx43 at the plasma membrane was evaluated as the amount of biotinylated Cx43 remaining at the cell surface 2 hours after biotinylation (Figure 18B). The internalized Cx43 was washed out from the cells following permeabilization with 0.5% Triton X-100 (Govindarajan et al, 2002). In controls, the levels of Cx43 at the plasma membrane decreased about 70%, while in cells incubated with 7-keto the decrease was only of about 37% (Figure 18C). Cells incubated with cholesterol or 25-OH

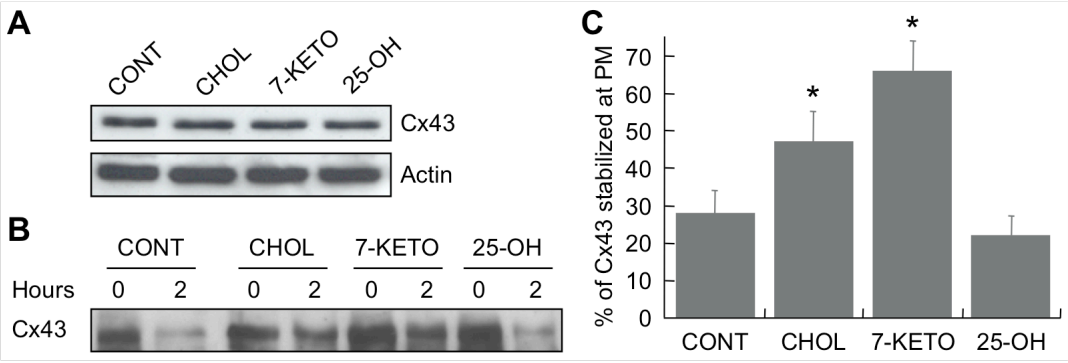

Figure 18 - Effect of oxysterols on Cx43 stability at the plasma membrane. (A) The total amount of Cx43 following treatment with 20 µg/ml cholesterol, 7-keto or 25-OH, for 3 hours, was determined by Western blot using antibodies directed against Cx43. Actin on the same samples is included to demonstrate comparable loading of the lanes. (B) LEC were treated with 20 µg/ml cholesterol (lane 3), 7-keto (lane 5) or 25-OH (lane 7) for 30 min prior biotinylation. Cells incubated in 0.2% ethanol were used as controls (lane 1). To determine the stability of Cx43 at the plasma membrane, the biotinylated cells were returned to the incubator for an additional 2 hours in DMEM, in the absence (lane 2) or presence of cholesterol (lane 4), 7-keto (lane 6) or 25-OH (lane 8). After extraction in situ by incubation in DPBS containing 0.5% Triton X-100, the biotinylated surface Cx43 was detected with antibodies directed against Cx43 following isolation with Neutravidin beads. (C) The results obtained in B are depicted in a graph and correspond to the Cx43 remaining at the plasma membrane 2 hours after biotinylation. The values are the average of three individual experiments ± SD. * indicates statistically significant differences from controls ($p < 0.01$).

showed a decrease in the amount of Cx43 at the plasma membrane of about 53% and 80%, respectively (Figure 18C).

Stabilization of Cx43 at the plasma membrane is not related to a decrease in endocytosis

Endocytosis of dextran is often used as a functional test for the endocytic pathway (Govindarajan et al, 2002; Kauppi et al, 2002; Song et al, 2002). To demonstrate that the stabilization of Cx43 induced by 7-keto results from a specific effect on the protein, rather than a general effect on endocytic pathways, the uptake of RITC-dextran was evaluated in LEC in culture (Figure 19). The internalized fluorescently labelled dextran was assessed by confocal microscopy. For negative controls, cells were incubated at 4°C to inhibit endocytosis. Results show that endocytosis is slightly enhanced in cells treated with 7-keto or cholesterol, as revealed by the increased fluorescence inside cells. Conversely, endocytosis was not affected following treatment with 25-OH. Despite the increase in endocytosis following treatment with 7-keto, Cx43 accumulates at the plasma membrane, indicating that the effect of 7-keto is most likely associated with stabilization of Cx43 at the plasma membrane.

7-keto increases intercellular communication through gap junctions

The functional implications of Cx43 redistribution following incubation with 7-keto was investigated in primary cultures of lens epithelial cells by scrape loading/dye transfer assays. Intercellular communication was quantified as the distance travelled by the dye Lucifer yellow 8 min after

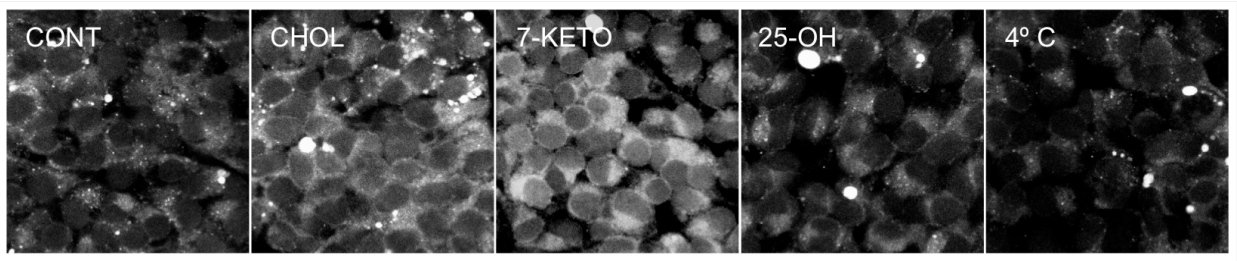

Figure 19 - Effect of oxysterols on the endocytic pathway. Lens epithelial cells were incubated with RITC-dextran for 30 min, at 37°C, following incubation with 20 µg/ml cholesterol, 7-keto or 25-OH for 2 hours. Cells incubated with 0.2% ethanol were used as controls. The cells were incubated for an additional 30 min in the absence of the dye, and subsequently fixed with 4% PFA and imaged by fluorescence confocal microscopy. For negative controls, cells were incubated at 4°C to inhibit endocytosis.

loading of the dye. The results obtained are presented in a histogram (Figure 20). Incubation of LEC with 7-keto resulted in a 57% increase in GJIC, as compared to controls, while incubation with cholesterol did not significantly affect intercellular communication. On the other hand, when cells were incubated with 25-OH there was a slight decrease in intercellular communication through gap junctions.

7-keto induces partition of Cx43 into Triton X-100 insoluble fractions and accumulation of phosphorylated forms of Cx43

Formation of functional gap junctions requires incorporation of Cx43 into gap junction plaques. Thus, by increasing intercellular communication 7-keto should consistently stabilize Cx43 in GJ. To confirm this hypothesis primary cultures of lens epithelial cells were incubated with 20 µg/ml cholesterol, 25-OH or 7-keto, for 3 hours. Cells incubated with 0.2% ethanol were used as controls. Phosphorylation of Cx43 and its assembly into GJ plaques results in an increased insolubility in 1% Triton X-100 (Musil & Goodenough, 1991). The results presented in Figure 21A and 21B show that incubation with 7-keto led to a 2 fold increase in the amount of Triton X-100 insoluble Cx43 (Figure 21A, lane 3, and 21B). Incubation of cells with cholesterol resulted in a 53% increase in the amount of Cx43 partitioned into the Triton insoluble fraction (Figure 21A, lane 2, and 21B) while cells incubated with 25-OH presented a decrease of about 20% (Figure 21A, lane 4, and 21B).
Incorporation of Cx43 into GJ is associated with phosphorylation of the protein (Cooper & Lampe, 2002; Lampe & Lau, 2000; Musil & Goodenough, 1991). The presence of phosphorylated Cx43 in the Triton insoluble fraction was determined by Western blot using polyclonal antibodies that recognize the phosphorylated forms of Cx43 (Figure 21A, middle panel). Three major forms of Cx43 are often recognized by Western blot: the non-phosphorylated (NP) form and two slower migrating bands corresponding to the phosphorylated species (P1 and P2). However, in Figure 21A, due to overexposure of the film, the non-phosphorylated form could not be quantified. Thus, only the phosphorylated forms P1 and P2 were considered and quantified (Figure 21A and 21C).

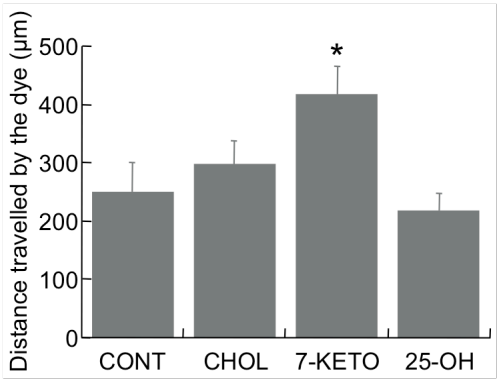

Figure 20 - Effect of oxysterols upon gap junction intercellular communication. LEC were treated with 20 µg/ml cholesterol, 7-keto or 25-OH for 3 hours. Cells were assayed for intercellular communication by Lucifer yellow dye transfer, following scrape loading. Intercellular communication was evaluated as the average distance travelled by the dye Lucifer yellow along the monolayer and is represented as a histogram. The values are the average of three individual experiments ± SD. * indicates statistically significant differences from controls ($p < 0.05$).

The results show that the amount of phosphorylated Cx43 increased in cells incubated with 7-keto (Figure 21A, lane 3) suggesting its assembly into gap junction plaques.

Discussion

In this chapter we demonstrate, for the first time, that 7-keto can modulate gap junction intercellular communication, probably through a mechanism that involves stabilization of Cx43 at the plasma membrane. Data presented in this study shows that incubation of LEC with 7-keto leads to an accumulation of the protein at the plasma membrane and to an increase in cell-cell communication. Additionally, an increased partition of Cx43 into Triton X-100 insoluble fractions after treatment with 7-keto suggests that Cx43 is incorporated into gap junctions plaques. This hypothesis is further supported by the observation that the amount of total Cx43 is not altered following incubation with 7-keto. Such changes in subcellular distribution may thus account for the increased intercellular communication. On the other hand, the biotinylation experiments showed that the rate of endocytosis of Cx43 is significantly reduced following incubation with 7-keto. Biotin labels mainly non-junctional Cx43, therefore and taken together, the biotinylation and solubility in Triton X-100 experiments suggest a role for 7-keto in the stabilization of Cx43 at the plasma membrane. Moreover, at least part of the Cx43 that is stabilized at the plasma membrane is likely to be incorporated into functional gap junctions, as revealed by the increased intercellular communication. Thus, it is conceivable that the accumulation of Cx43 into gap junctions induced by 7-keto is a consequence of an increase in the amount Cx43 at the plasma membrane available to form gap junction plaques.

It is not likely that perinuclear accumulation of Cx43 following treatment with 7-keto or 25-OH can account for the accumulation of Cx43 at the plasma membrane. Indeed, although both 7-keto and 25-OH lead to an accumulation of Cx43 in the perinuclear region it is only 7-keto that stabilizes

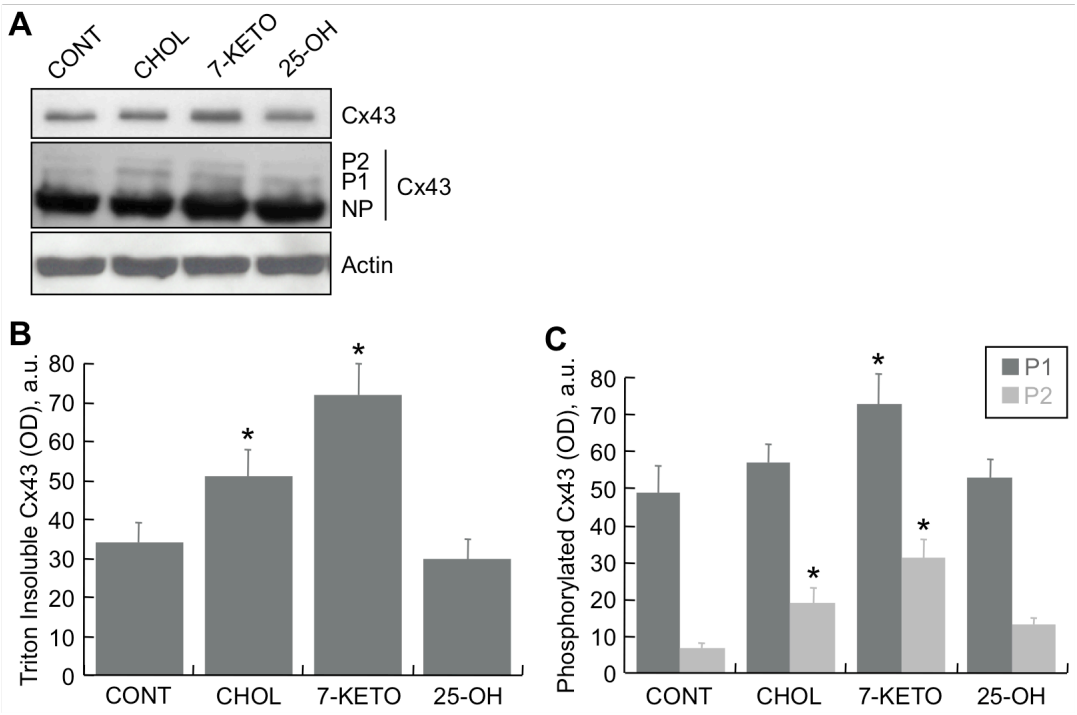

Figure 21 - Effect of oxysterols on Cx43 phosphorylation and solubility in Triton X-100. (A) LEC were incubated with 20 µg/ml cholesterol, 7-keto or 25-OH for 3 hours. Cells incubated with 0.2% ethanol were used as controls. The Triton X-100 insoluble fractions of cell lysates were separated by SDS-PAGE, transferred to PVDF membranes and probed with antibodies directed against Cx43 (upper panel). To allow quantification of the phosphorylated forms of Cx43 the film was overexposed (middle panel). Actin on the same samples is included to demonstrate comparable loading of the lanes (lower panel). (B and C) The intensity of the bands was determined by laser scanning of the films followed by quantitative densitometric analysis and the results obtained are depicted in a graph. The values are the average of three individual experiments ± SD. * indicates statistically significant differences from controls ($p < 0.05$).

Cx43 at the plasma membrane.

The findings in this study further suggest that the effects of 7-keto on Cx43 endocytosis are rather specific and most likely involve activation of specific signalling pathways. This hypothesis is supported by two main observations: (1) the effects of 7-keto cannot be mimicked by other cholesterol oxides such as 25-OH and (2) the effects of 7-keto are exerted specifically on Cx43 as the endocytic pathway is not inhibited (internalization of dextran is not diminished). Taken together these observations suggest that the effects of 7-keto reported in this study are not the result of unspecific cholesterol oxidation at the plasma membrane but rather reflect a physiological role for 7-keto in LEC intercellular communication. Presumably, a similar effect may also be present in other cells and tissues, where changes in intercellular communication are likely to compromise cell or tissue function.

The effects of cholesterol oxidation on endocytosis are controversial. In fact, Chow et al. have shown that OxLDL (rich in 7-keto) promotes endocytosis in endothelial cells (Chow et al, 1998), while Borsum et al. showed that OxLDL induces a decrease in the activity of endocytic pathways (Borsum et al, 1985). The results presented in this study indicate that 7-keto slightly enhances endocytosis in LEC. This emphasizes that Cx43 stabilization is a specific phenomena unrelated to alterations in the endocytic pathway.

Two main pathways for Cx43 internalization have been suggested: via clathrin coated pits (Larsen et al, 1979; Naus et al, 1993) and via caveolae (Schubert et al, 2002). Caveolae are membrane lipid rafts rich in cholesterol and sphingolipids that are involved in signal transduction (Galbiati et al, 2001; Razani et al, 2000; Shaul & Anderson, 1998), endocytosis (Schnitzer et al, 1996) and cholesterol transport (Fielding & Fielding, 1995; Smart et al, 1996). Indeed, connexins, including Cx43, were shown to specifically target to lipid raft/caveolae and directly interact with Cav-1, a membrane protein that acts as a scaffolding protein to cluster lipids and signalling molecules within caveolae (Schubert et al, 2002). Lin et al. showed that phosphorylation of Cx43 regulates the distribution of the protein within caveolae in lens epithelial cells (Lin et al, 2003b). Disruption of caveolae following incubation with cholesterol oxidase was reported to affect the activity of various membrane receptors sequestered in these membrane domains, including the endothelin receptor type A (Okamoto et al, 2000), GLUT4 (Shigematsu et al, 2003) and the platelet-derived growth factor receptor (PDGFR) (Liu et al, 2000). The presence of oxysterols, namely 7-keto, on caveolae has been shown to interfere with enzymes that concentrate in such membrane domains (Blair et al, 1999; Myers & Stanley, 1999). Additionally, *in vitro* studies demonstrated that 7-keto interacts with caveolin (Sleer et al, 2001). Therefore, by disturbing caveolae structure and/or function, 7-keto may affect proteins involved in regulating the stability of Cx43 at the plasma membrane. The identity of such proteins is largely unknown as are the mechanisms whereby Cx43 is extracted from the plasma membrane, endocytosed and eventually degraded. Protein kinases are likely candidates to assist in the regulation of Cx43 stability and endocytosis. Indeed, the carboxyl-terminal domain of Cx43 was shown to be phosphorylated by a variety of kinases including PKC (Solan et al, 2003), v-Src (Lin et al, 2001), MAPK (Cottrell et al, 2003; Warn-Cramer et al, 1998) or casein kinase I (Cooper & Lampe, 2002). Hyperphosphorylation of Cx43 was also shown to be associated with decreased GJIC and with increased protein degradation (Girao & Pereira, 2003; Lampe & Lau, 2000; Qin et al, 2003). However, recent studies reported increased GJIC following phosphorylation of Cx43. For example, it was shown that casein kinase 1 directly phosphorylated Cx43, promoting gap junction assembly (Cooper & Lampe, 2002) while FGF upregulated intercellular communication between lens epithelial cells through the activation of extracellular signal-regulated kinase (ERK) (Le & Musil, 2001). Initial phosphorylation of Cx43 is normally involved in the assembly of the protein into gap junction plaques (Cooper & Lampe, 2002; Lampe & Lau, 2000; Musil & Goodenough, 1991). Significantly, 7-keto was shown to activate a variety of protein kinases (Deckert et al, 2002; Myers & Stanley, 1999). It is thus conceivable that the presence of 7-keto at the plasma membrane may enhance Cx43 assembly and formation of gap junction plaques by a mechanism that probably involves phosphorylation of Cx43. The importance of cholesterol oxidation in the lens is not without significance. Indeed, cholesterol is the most abundant lipid in lens membranes and it has been shown before that cholesterol oxides are present at detectable levels in the lens and more so in human cataracts (Girao et al, 1998).

Although the ratio of cholesterol/phospholipid is higher in the inner regions of the lens (Li et al, 1985), where oxysterols are more likely to accumulate, this does not exclude that cholesterol oxides in fibre cells may still affect the function of lens epithelial cells nor that cholesterol oxides may interfere with intercellular communication through gap junctions formed by Cx46/Cx50 in inner regions of the lens. It has been previously shown that cholesterol oxides affect the organization of the cytoskeletal network in LEC, through a mechanism mediated by Rho GTPases (Girao et al, 2003a). Since the cytoskeleton plays a major role in the trafficking and recruitment of connexins into gap junction plaques (Lauf et al, 2002; Theiss & Meller, 2002; Thomas et al, 2001) it is conceivable that the effect of 7-keto on GJIC may be caused by a mechanism involving cytoskeleton disruption. Although the data presented in this study does not allow the demonstration of a mechanism whereby 7-keto increases Cx43 stability at the plasma membrane, it certainly provides a new insight into the effects of cholesterol oxides in LEC and into its physiological implications in intercellular communication. The inner layers of the lens fully depend on a complex network of gap junctions through which nutrients and signal molecules produced in the outer layers reach the inner fibres. Through a model similar to that described for Cx43 in lens epithelial cells, upregulation of intercellular communication through gap junctions formed by Cx46/Cx50 in deeper cortical regions of the lens may compromise lens transparency leading to the formation of cataract. Additionally, the increase in intercellular communication induced by 7-keto may also disrupt a variety of highly regulated events, including differentiation of LEC into fibres. Several studies have shown that connexins play an important role in lens differentiation and development (Gong et al, 1997; Gu et al, 2003; Qin et al, 2003; White, 2002). For example, it has been shown that 7-keto induces differentiation of LEC, by an unknown mechanism (Girao et al, 2003b). Thus, by interfering with GJIC, cholesterol oxides may affect highly regulated differentiation programs and compromise normal lens growth and transparency, contributing to the cataractogenic process.

Conclusions

The results obtained in this study show for the first time that 7-keto induces an increase in GJIC, that is most likely due to an increased stability of the protein at the plasma membrane and to an increased abundance of Cx43 assembled into gap junction plaques. The upregulation of GJIC in the lens may disrupt a variety of highly regulated events, including differentiation of LEC into fibres that, ultimately, compromises lens transparency leading to the formation of cataract.

Chapter 5

Chapter 5: Eps15 interacts with ubiquitinated Cx43 and mediates its internalization

Abstract

Gap junctions are specialized cell–cell contacts that provide direct intercellular communication between eukaryotic cells. Regulation of GJIC by degradation of Cx43 has been a matter of debate over the last two decades and both the proteasome and the lysosome have been implicated. However, the underlying mechanism and molecular players involved remain elusive. In this study we demonstrate, for the first time, that the ubiquitin ligase Nedd4 is involved in Cx43 ubiquitination. Indeed, depletion of Nedd4 with siRNA resulted in a decrease of the amount of ubiquitin attached to Cx43. Ubiquitinated membrane proteins are often recognized and targeted by endocytic adaptors containing ubiquitin-binding domains, such as Eps15. By co-immunoprecipitation and immunofluorescence we show interaction of Cx43 with Eps15 and colocalization of these proteins mainly at the plasma membrane. Moreover, depletion of Eps15 results in an accumulation of Cx43 at the plasma membrane. Furthermore, the interaction of Eps15 with Cx43 requires the ubiquitin-interacting motifs of Eps15 suggesting that the interaction occurs through ubiquitin attached to Cx43. Data presented in this chapter is consistent with a new molecular model in which Nedd4-mediated ubiquitination of Cx43 is required to recruit Eps15, through its ubiquitin-interacting motifs, and targets ubiquitinated Cx43 to the endocytic pathway. This provides the basis for future studies aiming at identifying the molecular players and mechanisms involved in Cx43 internalization and degradation.

Introduction

Gap junctions are specialized cell–cell contacts that provide direct intercellular communication between eukaryotic cells. Each channel consists of two hemichannels termed connexons, each composed of six connexin subunit proteins. Connexins have four membrane-spanning domains, two extracellular loops and three cytoplasmic domains. GJ can be composed of a few to many thousands of individual channels.

The extent of gap junction intercellular communication is a direct result of the number and functionality of connexin-based pores. Therefore, processes that influence the stability of connexins at the plasma membrane are critical for regulating intercellular communication. It is now well established that rapid degradation of connexins is one of the mechanisms involved in regulation of GJIC. Indeed, one of the most remarkable aspects of gap junction turnover is the exceptional metabolic lability of connexins (Girao & Pereira, 2003; Laird, 2006; Leithe & Rivedal, 2007). While the half-life of the vast majority of plasma membrane proteins exceed 24 hours, connexins turnover with a half-life of only 1.5-5 hours.

Recent studies have shown that docked connexons cannot be separated under physiological conditions. As a consequence, entire GJ plaques are internalized by one of two adjacent cells, as large double-membrane vesicular structures termed “annular GJ” (Leithe et al, 2006). These “annular GJ” are then fragmented into smaller vesicles and subsequently degraded by endo-lysosomal pathways. Clathrin itself and proteins associated to clathrin-mediated endocytosis (CME) play a critical role in “annular GJ” internalization, translocation and degradation (Gumpert et al, 2008; Piehl et al, 2007). The internalization of double-membrane structures by one of two adjacent cells is clearly distinct from the majority of other established mechanisms for the turnover of junctional complexes.

Both the proteasome (Beardslee et al, 2000; Fernandes et al, 2004; Girao & Pereira, 2003; Laing & Beyer, 1995; Laing et al, 1997; Musil et al, 2000) and the lysosome (Laing et al, 1997; Musil et al, 2000) have been implicated in the degradation of Cx43. Whereas degradation of Cx43 by the lysosome has long been established, the role of the proteasome in Cx43 turnover is still poorly understood. Initial studies suggested that the proteasome could be directly involved in connexin degradation (Laing & Beyer, 1995). However, more recently, it has been hypothesized that the proteasome acts by regulating the stability of Cx43 at the plasma membrane, most likely by degrading a connexin-interacting protein (Girao & Pereira, 2007; Musil et al, 2000). For example, a model has been proposed in which proteasome-dependent degradation of a putative Cx43 binding protein regulates Cx43-ZO-1 interaction which in turn regulates Cx43 internalization (Barker et al, 2002; Girao & Pereira, 2007). Cx43 is known to interact with several proteins that may regulate channel assembly and/or the stability of Cx43 at the plasma membrane. These include cytoskeletal proteins and anchoring proteins, such as E-cadherin (Fujimoto et al, 1997), Cav-1 (Schubert et al, 2002), and ZO-1 (Giepmans et al, 2001b; Toyofuku et al, 1998). More recently, it was demonstrated that Cx43 interacts with the ubiquitin ligase Nedd4 through the binding of its carboxyl-terminal PY-motif (XPPXY) with the WW2 domain of Nedd4, suggesting that Nedd4 also plays a role in GJ internalization and/or degradation. Although it was suggested that Nedd4 binds to and ubiquitinates Cx43 molecules at the cell surface, leading to their internalization and subsequent degradation probably via the interaction with accessory proteins (Leithe & Rivedal, 2007; Leykauf et al, 2006), Nedd4-mediated ubiquitination of Cx43 has not been demonstrated.

Binding of ubiquitin moieties can direct substrates to different pathways. For example, binding of polyubiquitin chains is associated with protein degradation (Lys48-linked chains) and with endocytosis (Lys63-linked chains). In many instances a single ubiquitin binds to one (monoubiquitination) or multiple (multimonoubiquitination) lysine residues on the substrate. In mammalian cells, the monoubiquitination of plasma membrane proteins is associated with the internalization and sorting of cargo receptors. Although the exact mechanism and molecular players involved in ubiquitin-dependent internalization of cell surface proteins is still unknown, several studies have consistently demonstrated that ubiquitin receptors play a key role in the

internalization of membrane proteins (Hicke & Dunn, 2003; Miranda & Sorkin, 2007; Mukhopadhyay & Riezman, 2007; Traub & Lukacs, 2007). Indeed, a number of endocytic proteins, including Eps15, Epsin and Hrs, contain ubiquitin-binding domains that recognize ubiquitinated proteins and sort them along the endocytic pathway, thus acting as a link between cargo and components of the endocytic and sorting machinery (Kirkin & Dikic, 2007; Traub & Lukacs, 2007). Moreover, some of these ubiquitin receptors are often themselves monoubiquitinated (Di Fiore et al, 2003; Hicke et al, 2005; Hoeller et al, 2006; Woelk et al, 2006).

Although the involvement of clathrin in Cx43 endocytosis is widely accepted, the role of ubiquitin in this process is still a matter of debate. The results presented in this chapter show, for the first time, that Cx43 ubiquitination is mediated by Nedd4 and that the conjugation of ubiquitin to Cx43 is required for subsequent interaction with the endocytic protein Eps15. Therefore, the interaction between the ubiquitin-interacting motifs (UIM) of Eps15 and the ubiquitinated form of Cx43 is shown to be required for Cx43 internalization.

Results

Cx43 is multimonoubiquitinated

Several studies carried out in different cell types have suggested that both the lysosome and the proteasome are involved in Cx43 degradation. Although Cx43 ubiquitination has previously been demonstrated (Laing & Beyer, 1995; Leithe & Rivedal, 2004a; Leithe & Rivedal, 2004b), its role in Cx43 internalization and/or degradation remains elusive. We first assessed whether the NRK cell line, that endogenously expresses high amounts of Cx43, presents detectable levels of ubiquitin conjugated to Cx43. For this purpose, endogenous Cx43 was selectively immunoprecipitated using polyclonal antibodies, followed by Western blot and probing with antibodies against ubiquitin. To distinguish between mono and polyubiquitinated Cx43 we used two different antibodies directed against ubiquitin: the P4D1 monoclonal antibody, that recognizes polyubiquitinated and monoubiquitinated proteins, and the FK1 monoclonal antibody that only recognizes polyubiquitinated proteins (Haglund et al, 2003). The results presented in Figure 22 show that a significant fraction of immunoprecipitated Cx43 reacts with the P4D1 antibody, that can recognize monoubiquitinated proteins. On the other hand, when the immunoprecipitated material was probed with FK1 antibodies, it was not possible to detect any band, suggesting that, as described before for rat liver epithelial cells (Leithe & Rivedal, 2004a; Leithe & Rivedal, 2004b), Cx43 is multimonoubiquitinated. The presence of a band with 50 kDa (asterisk), that reacts both with Cx43 and P4D1 antibodies, most likely corresponds to the single monoubiquitinated form of Cx43.

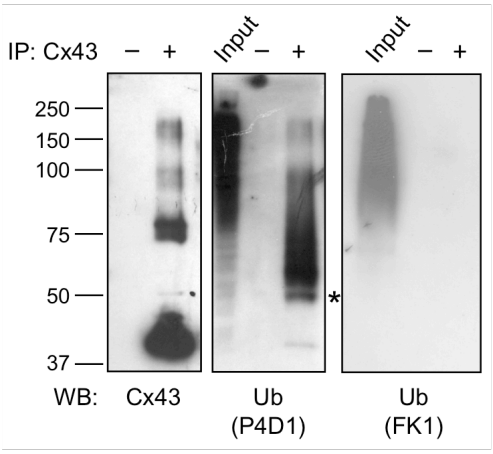

Figure 22 - Cx43 is multimonoubiquitinated. Lysates from NRK cells were immunoprecipitated with polyclonal antibodies against Cx43. Immunoprecipitates were then analyzed by Western blot using monoclonal antibodies against Cx43 and two different antibodies against ubiquitin; P4D1 (that recognizes both monoubiquitinated and polyubiquitinated proteins) and FK1 (only recognizes polyubiquitinated proteins). A significant fraction of immunoprecipitated Cx43 cross-reacts with the P4D1 antibody. Conversely, when the immunoprecipitated material was probed with FK1 antibodies, it was not possible to detect any band, suggesting that Cx43 is multimonoubiquitinated. The presence of a band with 50 kDa (*), that cross-reacts both with Cx43 and P4D1 antibodies, most likely corresponds to the single monoubiquitinated form of Cx43.

Cx43 ubiquitination depends on Nedd4

A recent study by Leykauf et al. demonstrated that the WW2 domain of Nedd4 binds to a sequence in the carboxyl-terminal of Cx43 that contains a PY motif (Leykauf et al, 2006). However, in that study the authors did not show that Nedd4 is involved in Cx43 ubiquitination. Thus, in the present study we assessed whether Cx43 ubiquitination is indeed mediated by Nedd4.

To first evaluate if the two proteins interact in our experimental model, Cos-7 cells were co-transfected with DNA encoding Cx43 and Nedd4. Cx43 or Nedd4 were then selectively immunoprecipitated using polyclonal antibodies against each of these proteins, followed by Western blot and probing with antibodies against Nedd4 or Cx43, respectively. As shown in Figure 23A, and consistent with previous reports (Leykauf et al, 2006), Nedd4 co-precipitates with Cx43.

To address the putative role of Nedd4 in Cx43 ubiquitination, we evaluated the levels of ubiquitinated Cx43 in cells depleted of endogenous Nedd4, following gene silencing with siRNA against Nedd4. As shown in Figure 23B, transfection of Cos-7 cells with two different siRNAs for Nedd4 resulted in almost total depletion of endogenous Nedd4 (upper panel). To analyze the effect of Nedd4 depletion upon the conjugation of ubiquitin to Cx43, Cx43 was selectively immunoprecipitated using polyclonal antibodies, followed by Western blot and probing with antibodies against ubiquitin (P4D1). The results obtained show that silencing of Nedd4 results in a decrease in ubiquitination levels of Cx43 (Figure 23B, compare lane 2 with lane 3 in the upper right panel probed with ubiquitin). However, as shown in the lower panels of Figure 23B, the total amount of Cx43 does not vary in cells transfected with siRNA for Nedd4.

Previous studies have reported that multiple ubiquitin ligases can be part of an ubiquitination

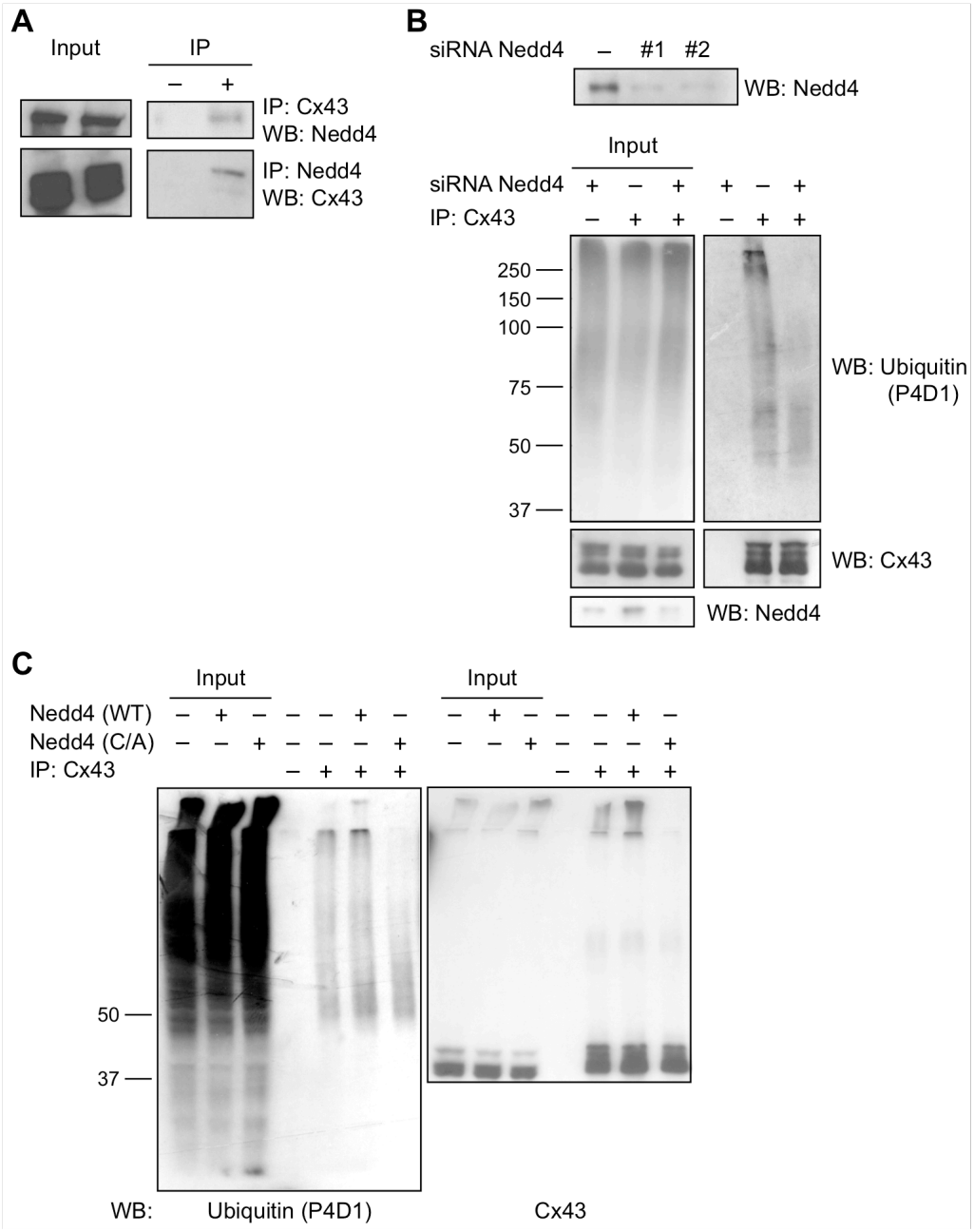

Figure 23 - Cx43 ubiquitination depends on Nedd4. (A) Lysates from Cos-7 cells overexpressing both Nedd4 and Cx43 were immunoprecipitated with polyclonal antibodies against Cx43 (upper panel) or Nedd4 (lower panel). Immunoprecipitates were then analyzed by Western blot and probed with monoclonal antibodies against Cx43 (lower panel) or polyclonal antibodies against Nedd4 (upper panel). (B) Lysates from Cos-7 cells transfected with Cx43 and two different siRNA for Nedd4 were analyzed by Western blot using antibodies against Nedd4 (upper panel) or immunoprecipitated with polyclonal antibodies against Cx43 (lower panels), Western blotted and probed for ubiquitin (P4D1) or Cx43. Depletion of endogenous Nedd4 by transfecting cells with siRNAs for Nedd4 (#1) resulted in a dramatic decrease in the ubiquitination levels of Cx43 (lower right panel), although the total amount of Cx43 does not vary. (C) Lysates from Cos-7 cells transfected with Cx43 and either the wild type or the catalytically inactive form of Nedd4 (Nedd4(C/A)) were immunoprecipitated with polyclonal antibodies against Cx43 and then analyzed by Western blot using monoclonal antibodies against ubiquitin (P4D1) or Cx43. Overexpression of the Nedd4(C/A) mutant induced a robust decrease in Cx43 ubiquitination levels (compare lane 7 with lane 5 and 6, left panel), while the total amount of Cx43 does not vary.

complex, such as in the case of Itch and A20 regulated ubiquitination of RIP1 (Shembade et al, 2008) or Cbl3 and Itch regulated ubiquitination of EGFR (Courbard et al, 2002), as such we hypothesized that silencing of Nedd4 by siRNA could disrupt a putative E3 ligase complex

responsible for ubiquitinating Cx43. To address this possibility, we tested the effect of overexpressing a catalytically inactive mutant of Nedd4, in which the active cysteine in the HECT domain was replaced by an Alanine. Cos-7 cells were co-transfected with plasmids encoding Cx43 and either the wild type or an inactive form of Nedd4. Cx43 was then selectively immunoprecipitated using polyclonal antibodies, followed by Western blot and probing with antibodies against ubiquitin (P4D1). Levels of ubiquitinated Cx43 significantly decreased in cells overexpressing an inactive form of Nedd4 (Figure 23C, compare lane 7 with lanes 5 and 6 in the left panel), while the total amount of Cx43 does not vary (Figure 23C, right panel).
Taken together, these data show, for the first time, that the ligase activity of Nedd4 is required for Cx43 ubiquitination.

Cx43-Nedd4 interaction is required for Nedd4-dependent ubiquitination of Cx43

To further establish whether ubiquitination of Cx43 requires binding to Nedd4, we used a mutated form of Cx43, in which Pro283 of the PY-motif was replaced by a leucine (Cx43P283L). To first investigate the role of the PY-domain in binding to Nedd4, Cos-7 cells were co-transfected with plasmids encoding Nedd4 and either the wild type or the mutated form of Cx43 (Cx43P283L). The results presented in Figure 24A show that the amount of Nedd4 that co-immunoprecipitates with the mutated form of Cx43 is significantly lower when compared to the wild type (compare lane 2 with lane 4). These results demonstrate that the PY-motif in Cx43 is involved in the interaction with Nedd4. Consistently, data presented in Figure 24B shows that the Cx43P283L mutant that weakly binds to Nedd4 is less ubiquitinated when compared to wild type Cx43, as demonstrated by the lower levels of both low and high molecular weight proteins that are immunoprecipated with Cx43 and cross react with ubiquitin (compare lane 4 and lane 6 in the rightmost panels). Moreover, a band with a molecular weight of about 50 kDa, that reacts both with antibodies against Cx43 and ubiquitin (asterisk), which presumably corresponds to the single monoubiquitinated form of Cx43, is present in much lower levels in cells overexpressing the mutated form Cx43P283L. Although the amount of Cx43P283L that interacts with Nedd4 is much lower than wild type Cx43, it is still possible to detect ubiquitinated Cx43 in cells overexpressing Cx43P283L. This may be explained by the presence of endogenous Cx43 in the cells overexpressing Cx43P283L. Moreover, although the interaction of Nedd4 with Cx43P283L is greatly impaired, some degree of interaction is still detectable, which may lead to the ubiquitination of this mutant of Cx43. Furthermore, it is possible that another E3 protein might be involved in Cx43 ubiquitination, through a mechanism independent of the PY-motif of Cx43.
Taken together these data show that an intact PY-domain of Cx43 is important for the interaction with Nedd4 and is, at least in part, required for Cx43 ubiquitination.

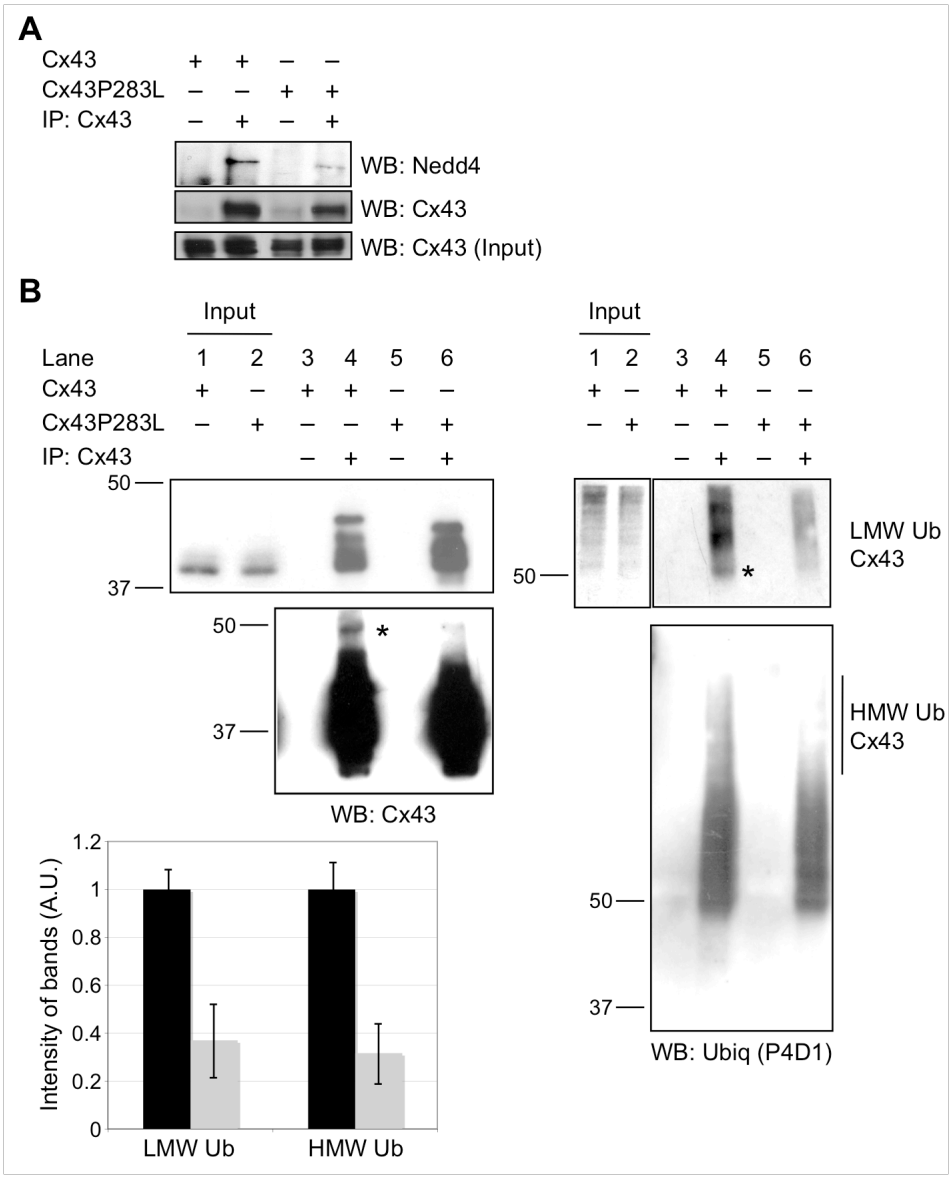

Figure 24 - The interaction between Cx43 and Nedd4 depends on the Proline-rich motif of Cx43. (A) Lysates from Cos-7 cells transfected with Nedd4 and either the wild type or mutated form of Cx43 (Cx43P283L) were immunoprecipitated using polyclonal antibodies against Cx43 and then analyzed by Western blot using monoclonal antibodies against Cx43 or polyclonal antibodies against Nedd4. The amount of Nedd4 co-immunoprecipitated with Cx43P283L is significantly lower when compared to wild type (compare lane 2 with lane 4). (B) Lysates from Cos-7 cells following transfection with Nedd4 and either the wild type or mutated form of Cx43 (Cx43P283L) were immunoprecipitated with polyclonal antibodies against Cx43 and then analyzed by Western blot using monoclonal antibodies against Cx43 or ubiquitin (P4D1). The lower panels correspond to longer exposures of the same membranes shown in the upper panels (LMW Ub Cx43 = Low molecular weight forms of ubiquitinated Cx43; HMW Ub Cx43 = High molecular weight forms of ubiquitinated Cx43). The intensity of the bands was measured and plotted in a graph (Black bars = Cx43wt; Grey bars = Cx43P283L). Cx43P283L is substantially less ubiquitinated (roughly two to three times) than wild type Cx43. Moreover, a band with a molecular weight of about 50 kDa (*), that reacts both with antibodies against Cx43 (lower left panel, lane 4) and ubiquitin (upper right panel, lane 4), and presumably corresponds to the single monoubiquitinated form of Cx43, is much weaker in cells overexpressing the mutant Cx43P283L.

Eps15 interacts with Cx43

The binding of ubiquitin to membrane proteins is known to promote the internalization and sorting of cargo. Several endocytic adaptors, including Eps15, have been traditionally linked to clathrin-

dependent endocytosis, namely through interaction with AP2. However, it is currently accepted that some of these adaptor proteins can also direct ubiquitinated cargos to clathrin-dependent or clathrin-independent endocytosis. Indeed, studies on EGFR showed that Eps15, through its UIMs, acts as an ubiquitin receptor that recognizes ubiquitinated proteins and sorts them along the endocytic pathway.

Considering that the binding of ubiquitin to Cx43 localized at the plasma membrane may act as an endocytic signal, this suggested a role for UIM-containing endocytic proteins, such as Eps15, in mediating gap junction endocytosis. To address this possibility, we investigated the interaction of Cx43 with Eps15 in NRK cells that express high levels of both Cx43 and Eps15. The interaction of Cx43 with Eps15 was evaluated by selectively immunoprecipitating Cx43 using polyclonal antibodies, followed by Western blot and probing with antibodies against Eps15. The results obtained and presented in Figure 25A show that a significant fraction of Eps15 co-precipitates with Cx43.

Cx43 colocalizes with Eps15 at the plasma membrane

To further confirm the interaction of Cx43 with Eps15, NRK cells were fixed and stained with monoclonal antibodies directed against Cx43 and polyclonal antibodies directed against Eps15. Cells were subsequently imaged by immunofluorescence confocal microscopy. The results show that a significant amount of Cx43 colocalizes with Eps15 (Figure 25B). Although most of the colocalization observed occurs at the plasma membrane (arrows), the two proteins can also be detected in the same cytoplasmic vesicles (arrowheads). These results demonstrate that Eps15 interacts with Cx43, most likely at the plasma membrane, thus suggesting a role for Eps15 in gap junction internalization.

The UIM of Eps15 is required for its interaction with Cx43

The carboxyl-terminus of Eps15 contains two UIMs, thus, we investigated if these domains are required for the interaction with Cx43. To address this question, we overexpressed a mutant of Eps15 lacking the UIMs, and its ability to bind Cx43 was compared with full-length Eps15. Consistent with what is observed for the two endogenous proteins in NRK, Cx43 and wild type Eps15 still interact when overexpressed in Cos-7 cells. However, when the wild type Eps15 was replaced by a mutant Eps15 lacking the UIMs, the interaction was dramatically reduced (Figure 26A, compare lane 10 with lane 12), indicating that interaction of Cx43 with Eps15 occurs through the UIM of Eps15. To investigate whether Cx43 ubiquitination is required for the interaction with Eps15, we performed several experiments where Cx43 ubiquitination was impaired. In the first experiment we overexpressed the Cx43 PY-mutant (Cx43P283L), which was previously shown to

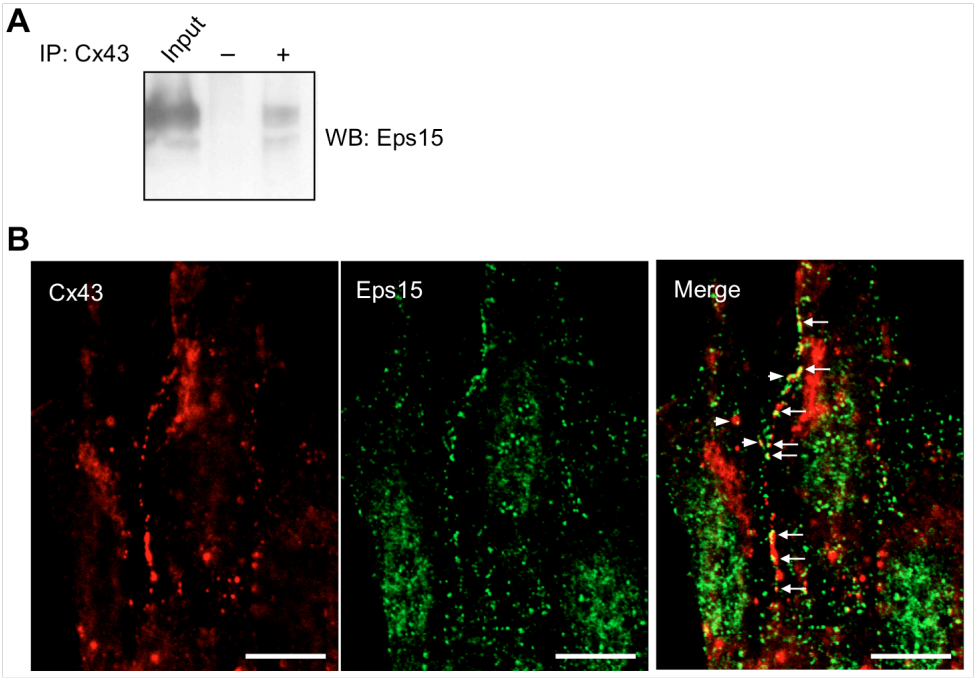

Figure 25 - Cx43 interacts with Eps15 mainly at the plasma membrane. (A) Lysates from NRK cells were immunoprecipitated with polyclonal antibodies against Cx43. Immunoprecipitates were then analyzed by Western blot and probed with antibodies against Eps15. Eps15 co-immunoprecipitates with Cx43. (B) NRK cells were fixed and stained with monoclonal antibodies directed against Cx43 (BD Transduction) and polyclonal antibodies against Eps15. A significant amount of Cx43 colocalizes with Eps15. Although most of the colocalization observed occurs at the plasma membrane (arrows), the two proteins can also be detected in the same cytoplasmic vesicles (arrowheads). Scale bars, 10 μm.

poorly interact with Nedd4 and be less ubiquitinated (Figure 24). Data presented in Figure 26A shows that the PY-mutant also interacts poorly with Eps15 as compared to the wild type (compare lane 6 with lane 10). We then impaired Cx43 ubiquitination by either overexpressing an inactive form of Nedd4 (Nedd4 (C/A)) or by siRNA silencing Nedd4. Cos-7 cells were co-transfected with plasmids encoding Cx43, Eps15 and either the wild type or an inactive form of Nedd4 (Figure 26B), and we then evaluated the amount of Eps15 that co-immunoprecipitates with Cx43 in each condition. The results presented in Figure 26B (right panels) show that the interaction between Eps15 and Cx43 was significantly impaired in cells overexpressing the inactive form of Nedd4 when compared to cells overexpressing the wild type form of Nedd4. The importance of Nedd4 for interaction of Cx43 with Eps15 was further confirmed by gene silencing with siRNA against Nedd4. Indeed, in cells depleted of Nedd4 the amount of Eps15 that co-immunoprecipitates with Cx43 is significantly lower (Figure 26C, upper panel). Taken together, this data indicates that the interaction of Eps15 with Cx43 requires its prior ubiquitination, most likely by Nedd4. To further confirm that Eps15 preferentially interacts with endogenous ubiquitinated Cx43, Eps15 was selectively immunoprecipitated using polyclonal antibodies, blotted and probed for Cx43. As shown in Figure 26D, a 50 kDa band, which presumably corresponds to single monoubiquitinated Cx43, can be observed. It is noteworthy that unmodified Cx43 does not co-precipitate with Eps15, indicating specificity for the ubiquitinated form. These results further confirmed the hypothesis that

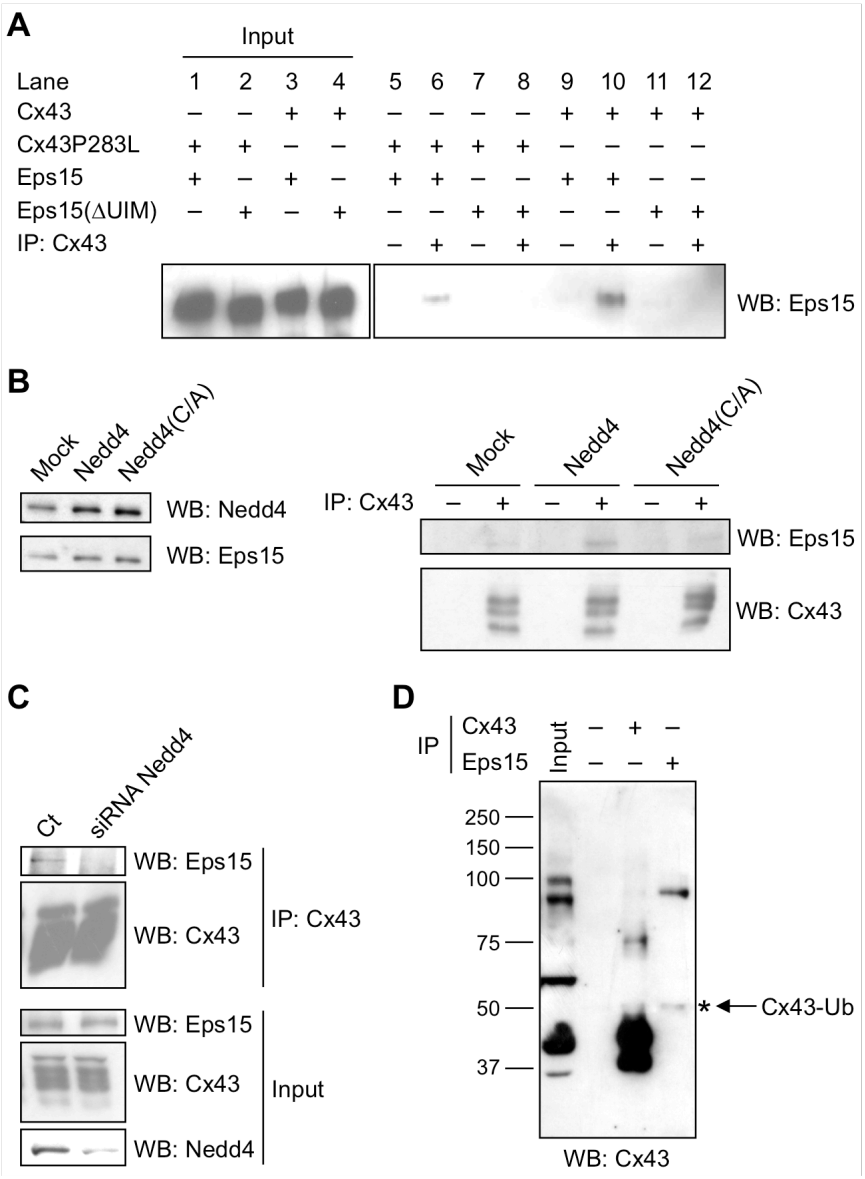

Figure 26 - The interaction of Cx43 with Eps15 depends on the UIMs of Eps15 and requires the PY-motif of Cx43. (A) Cos-7 cells were transfected with either wild type or the mutant Cx43 (Cx43P283L) together with full-length Eps15 or a mutant of Eps15 lacking the domain containing the two UIMs (Eps15(ΔUIM)). Lysates were immunoprecipitated with polyclonal antibodies against Cx43 and immunoprecipitates were then analyzed by Western blot and probed with antibodies against Eps15. Data shows a clear interaction between Cx43 and Eps15, however, the interaction of Cx43 with the mutant Eps15 lacking its UIMs is lost to a significant extent (compare lane 10 with lane 12). Full-length Eps15 also shows a lower interaction with Cx43P283L, the mutant Cx43 which is also less ubiquitinated than the wild type form of Cx43 (compare lane 6 with lane 10). (B) Lysates from Cos-7 cells transfected with Cx43, Eps15 and either the wild type or mutant form of Nedd4 (Nedd4(C/A)) were immunoprecipitated with polyclonal antibodies against Cx43 and then analyzed by Western blot and probed with antibodies against Eps15 or monoclonal antibodies against Cx43 (right panels). Left panels correspond to the input levels of Nedd4 and Eps15. Eps15 co-precipitates substantially less with Cx43 in cells overexpressing the catalytically inactive mutant Nedd4(C/A) when compared to cells overexpressing the endogenous form of the protein. (C) HEK293FT cells were transfected with Cx43 and siRNA directed against Nedd4 (#1). Lysates were then immunoprecipitated with polyclonal antibodies against Cx43 and analyzed by Western blot using antibodies against Eps15. Eps15 co-precipitates significantly less with Cx43 in cells depleted of Nedd4 when compared to control cells. (D) NRK cell lysates were immunoprecipitated with polyclonal antibodies against Cx43 or Eps15 and immunoprecipitates were then analyzed by Western blot and probed with monoclonal antibodies against Cx43. A 50 kDa band, that is attributed to monoubiquitinated Cx43, can be observed following immunoprecipitation with Eps15 antibodies (lane 4).

Eps15 interacts and targets mainly ubiquitinated Cx43.

Eps15 is required for Cx43 internalization

To further examine the functional significance of the interaction between Cx43 and Eps15, endogenous Eps15 was depleted using siRNA and the effect of Eps15 depletion on Cx43 degradation was evaluated by Western blot. NRK cells were transiently transfected with Eps15 siRNA and the expression levels of endogenous Eps15 were evaluated 48 hours after transfection. Transfection with two different siRNAs for Eps15 resulted in a significant depletion of endogenous Eps15 (data not shown). Results presented in Figure 27A show a ~70% decrease in the amount of Eps15, in cells transfected with siRNAs for Eps15 compared with non-targeting siRNA. However, this reduction in Eps15 levels has no effect on Cx43 protein levels, comparing with non-targeting siRNA transfected control cells (Figure 27A). To analyze whether Eps15 depletion leads to altered subcellular distribution of Cx43, transfected cells were immunolabelled with antibodies against Cx43 and subsequently imaged by immunofluorescence confocal microscopy. The effect of Eps15 depletion was further evaluated by measuring the size of gap junction plaques. Results presented in Figure 27B show that depletion of Eps15 by siRNA results in an accumulation of Cx43 at the plasma membrane, with the formation of larger plaques, as compared to cells transfected with non-targeting siRNA, strongly suggesting that Eps15 is required for Cx43 internalization. To further confirm the accumulation of Cx43 at the plasma membrane following knockdown of Eps15, cells were subjected to cell surface protein biotinylation. Biotinylated proteins from cell lysates were then precipitated with Neutravidin beads, followed by Western blot analysis with antibodies directed against Cx43. Data present in Figure 27C shows that Eps15 silencing results in a 2 fold increase of Cx43 at the plasma membrane.

Discussion

The results presented in this chapter demonstrate for the first time that Nedd4 is involved in Cx43 ubiquitination and that the binding of ubiquitin to Cx43 is required and sufficient for further recognition by the endocytic adaptor Eps15. Indeed, cells depleted of Nedd4 presented significantly lower levels of ubiquitinated Cx43. Moreover, we show that the interaction between Eps15 and Cx43 occurs through the ubiquitin-interacting motifs (UIM) of Eps15 and the ubiquitin moieties attached to Cx43, and that this interaction is required for Cx43 internalization.

The reduced levels of ubiquitination of a mutant form of Cx43 that has lower affinity for binding to Nedd4 (Cx43P283L) confirms that ubiquitination of Cx43 is, at least in part, dependent upon binding to Nedd4. Overexpression of a catalytically inactive mutant of Nedd4 impaired Cx43 ubiquitination, indicating a role for the ligase activity of Nedd4 in Cx43 ubiquitination. Although it was previously shown that Nedd4 binds to Cx43, to our knowledge, this is the first study demonstrating its role in Cx43 ubiquitination.

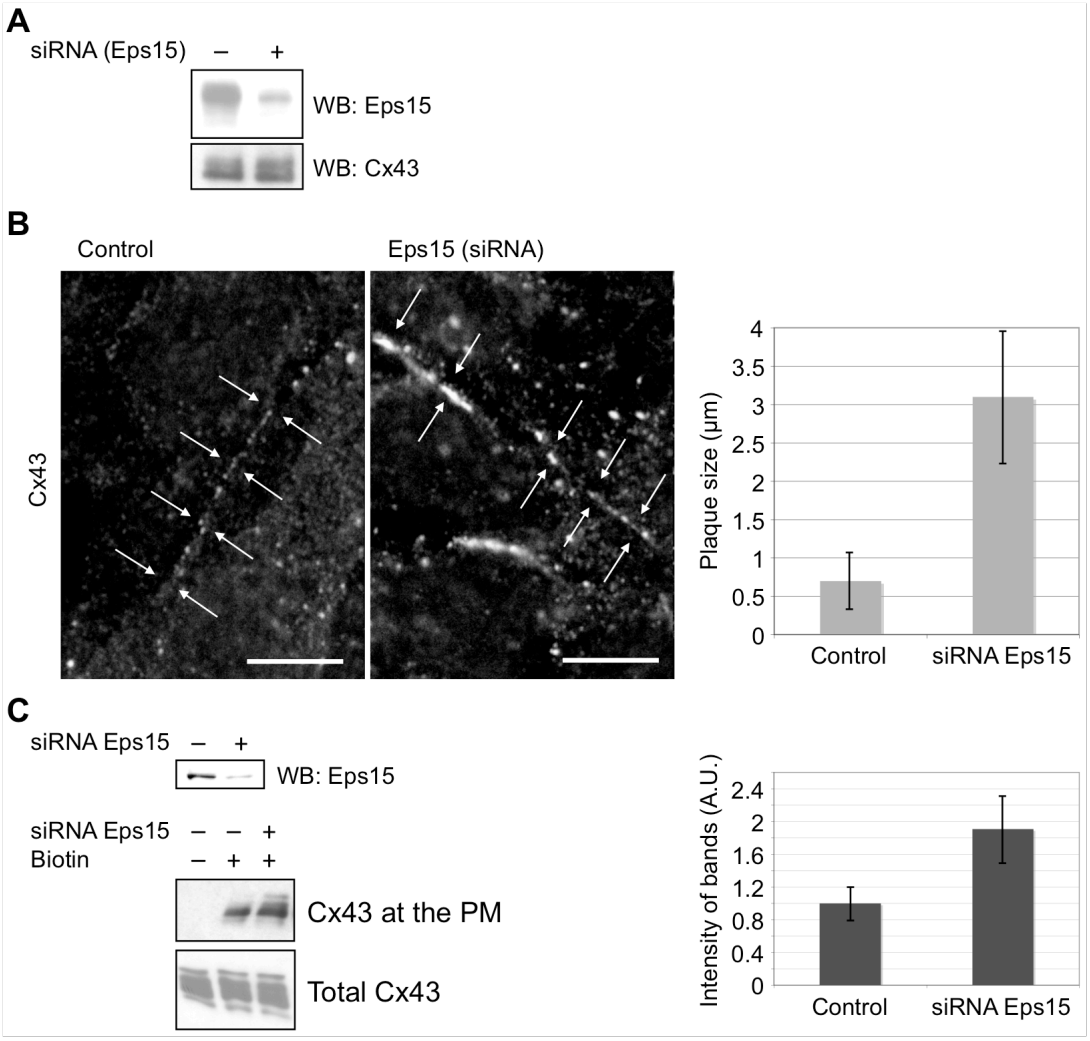

Figure 27 - Gap junction internalization depends on Eps15. (A) Lysates of NRK cells transfected with siRNA for Eps15, were analysed by Western blot using monoclonal antibodies against Cx43 or polyclonal antibodies against Eps15. The reduction in the levels of Eps15 has no effect on the levels of Cx43. (B) NRK cells were transfected with siRNA for Eps15 or a non-targeting siRNA. Cells were then stained with monoclonal antibodies directed against Cx43 (BD Transduction). Depletion of Eps15 by siRNA results in an accumulation of Cx43 at the plasma membrane, with the formation of larger plaques, as compared to cells transfected with non-targeting siRNA. Scale bars, 5 µm. (C) HEK293FT cells were transfected with Cx43, and siRNA directed against Eps15. Cells were then subjected to cell surface protein biotinylation and the biotinylated fraction of the cell lysates was precipitated with Neutravidin beads. Precipitates were then analyzed by Western blot using monoclonal antibodies against Cx43. Cx43 accumulates at the plasma membrane (PM) in cells depleted of Eps15 when compared to control cells. Data represents the average with standard deviation of three independent experiments.

Although the exact mechanism through which ubiquitin directs cell surface protein internalization is still unknown, several studies have shown that ubiquitin receptors play a key role in internalization. Indeed, it is well established that a number of endocytic proteins, including Eps15, Epsin and Hrs, contain ubiquitin-binding domains that are able to recognize ubiquitinated proteins and sort them along the endocytic pathway. For example, it was previously shown for EGFR that non-clathrin mediated internalization of ubiquitinated EGFR depends on its interaction with proteins harbouring UIMs, thus ascribing an important function to these proteins in coupling ubiquitinated cargo to clathrin-independent internalization (Sigismund et al, 2005).

Having established the role of Nedd4 in Cx43 ubiquitination, we further attempted to ascribe a

biological role to such modification, as well as unveil some of the molecular players involved in GJ internalization. By analogy with EGFR, it was hypothesized that ubiquitination of Cx43 may act as an internalization signal through a mechanism that involves Eps15 as a recognition adaptor. In this model, the UIM of Eps15 would interact with ubiquitinated Cx43 and target the protein for endocytosis. Indeed, we showed that interaction of Cx43 with Eps15 is dependent upon the UIM of Eps15 as well as Cx43 ubiquitination. The role of Eps15 on Cx43 internalization was confirmed by Eps15 gene silencing. Depletion of Eps15 led to the accumulation of Cx43 at the plasma membrane forming large plaques, which most likely reflect defects in gap junction internalization.

Over the last years, several studies have demonstrated the involvement of clathrin in gap junction internalization (Gumpert et al, 2008; Larsen et al, 1979; Piehl et al, 2007). In addition to the tyrosine-based motif, it was also demonstrated that the PY-motif may have a role in Cx43 turnover (Thomas et al, 2003). Indeed, a mutation in the PY-motif of Cx43, that interacts with Nedd4, also stabilizes Cx43. On the other hand, depletion of clathrin induces only a partial inhibition of Cx43 internalization. Together, these results strongly suggest that CME is the main endocytic pathway involved in Cx43 internalization, although a non-clathrin alternative pathway may also be involved in Cx43 turnover and regulation of GJIC. Although Eps15 has been traditionally linked to clathrin-dependent endocytosis, due to its interaction with clathrin, the involvement of Eps15 in targeting Ub-conjugated cargo to clathrin-independent endocytosis has also been recently demonstrated. Thus, Eps15 may participate in different endocytic pathways, depending on which signal the cargo protein presents and on the endocytic route used by the cargo. Therefore, in addition to its canonical role in CME it is conceivable that, as suggested for EGFR, Eps15 may direct ubiquitinated Cx43 to non-clathrin mediated internalization (Sigismund et al, 2005).

Although previous reports show that Cx43 accumulates at the plasma membrane in conditions where the proteasome is inhibited, little is known on the role of the proteasome in Cx43 internalization or degradation. It is conceivable that the proteasome may be responsible for degradation of a putative protein involved in Cx43 stabilization at the plasma membrane (either by directly interacting with Cx43 or by inhibiting the action of Nedd4 or Eps15). Alternatively, inhibition of the proteasome may lead to the depletion of free ubiquitin levels in the cell, required for signalling Cx43 internalization. Results presented in this chapter show that ubiquitination of Cx43 can serve as an internalization signal for the protein, however, downregulation of Cx43 internalization through siRNA silencing of Nedd4 (Leykauf et al, 2006) or Eps15 does not significantly increase total levels of Cx43. Rutz et al. demonstrated that ubiquitinated Cx43 can be found within gap junction plaques (Rutz & Hulser, 2001). Based on this observation and on the data we describe here, we suggest that the interaction of Eps15 with ubiquitinated Cx43 regulates the internalization of Cx43 involved in gap junctions. This hypothesis is based, in part, on immunofluorescence data showing an accumulation of Cx43 at the plasma membrane, with the formation of larger plaques, following knockdown of Eps15. Indeed, depletion of Eps15 results in a

4 fold increase in the size of gap junction plaques, as compared to cells transfected with non-targeting siRNA. However, biotinylation experiments show less than a 2 fold increase in the amount of Cx43 at the plasma membrane. Taking into account that biotin seems to have less access to connexins found within gap junction plaques in comparison to the extrajunctional surface pool, this result is consistent with our hypothesis, that Eps15 regulates internalization of Cx43 preferentially localized at gap junction plaques.

Conclusions

Altogether, these observations and the data presented in this chapter led us to propose a model in which Nedd4-mediated ubiquitination of Cx43 acts as a triggering signal for gap junction internalization, through a mechanism that involves recognition of ubiquitinated Cx43 by Eps15. While the role of clathrin in Cx43 internalization has been well established, the results presented in this study envision an alternative mechanism for regulation of gap junction internalization, that involves Cx43 ubiquitination and Eps15-mediated endocytosis. Although Eps15 may mediate clathrin dependent or independent endocytosis, the role of this endocytic adaptor in Cx43 internalization is unclear and should be a matter of future research.

Chapter 6

Chapter 6: Internalization and intracellular trafficking of Cx43

Abstract

Gap junctions (GJ) are specialized cell-cell contacts that provide direct intercellular communication (IC) between eukaryotic cells. In recent years ubiquitination of Cx43 has been proposed as a regulatory mechanism of GJIC, however, the underlying mechanism and molecular players involved remain elusive. In this chapter we demonstrate that ubiquitinated Cx43 is internalized through a mechanism that is independent on the YXXØ sorting signal. Indeed, expression of a Cx43-Ub chimera was shown to drive the internalization of a mutant Cx43 in which the YXXØ-motif was surpressed. Cyclohexamide-chase and cell surface protein biotinylation experiments demonstrate that oligomerization of Cx43-Ub into Cx43 hemichannels decreases protein half-life and reduces the stability of the protein at the plasma membrane. Furthermore, the destabilization of Cx43 at the cell surface by Cx43-Ub was shown to depend on the interaction with Eps15. Ubiquitinated Cx43 was shown to undergo internalization through clathrin-independent endocytosis. Immunofluorescence and immunoprecipitation experiments also show that Cx43 colocalizes with the ESCRT components Hrs and Tsg101, and that Cx43 intracellular trafficking is modulated by the deubiquitinating enzyme UBPY. Moreover, data shows that Cx43 can be recycled back to the plasma membrane, presumably by two independent pathways, the rapid and slow endocytic recycling pathways. The results presented in this chapter further elucidate the molecular mechanisms underlying ubiquitin-mediated internalization of Cx43 and provide an initial characterization of the intracellular trafficking pathways of the protein.

Introduction

Gap junctions are specialized areas of the plasma membrane that directly connect adjacent cells, allowing for the transfer of small metabolites, ions and second messengers. Two adjacent cells must supply one hemichannel each to form a functional gap junction channel. Each hemichannel, or connexon, is, in turn, composed of six connexin subunits. Connexons can result from the oligomerization of a single or multiple types of connexins. The function of gap junctions in cell and tissue biology is of the utmost importance, and unsurprisingly, gap junction intercellular communication is present in nearly every mammalian cell type (Goodenough et al, 1996; Saez et al, 2003). In vertebrates, connexins play critical roles in embryonic development, coordinated contraction of excitable cells, tissue homeostasis, normal cell growth and differentiation. Cx43, a member of the alpha connexin family, is the most widely expressed connexin, being found in tissues as diverse as the heart, lens, retina, skin, brain, kidney and bone marrow (Laird, 2006; Laird, 2009).

The extent of gap junction intercellular communication is directly related to the number and function of gap junction channels present at the plasma membrane. Although GJIC can be regulated by the gating of the channel pore, degradation of connexins, particularly Cx43, also plays a somewhat unexpected role in regulating GJIC. Indeed, Cx43 has a markedly short half-life, of less than 5 hours, when compared to other plasma membrane proteins (Girao & Pereira, 2003; Laird, 2006; Leithe & Rivedal, 2007). Gap junctions are not static entities, but rather exist in a constant state of degradation and renewal. As such, newly synthesized connexin hemichannels are constantly being added to the periphery of existing gap junction plaques while older hemichannels are internalized from the inner regions of the plaque (Gaietta et al, 2002). In contrast to most plasma membrane proteins, which are internalized through single membrane vesicles, both membrane sides of a gap junction plaque are internalized by one of two adjacent cells, as large double membrane vesicular structures termed annular gap junctions (Leithe et al, 2006).

Both the lysosome and the proteasome have been implicated in regulating Cx43 turnover (Beardslee et al, 2000; Fernandes et al, 2004; Girao & Pereira, 2003; Laing et al, 1997; Musil et al, 2000). While degradation of Cx43 by the lysosome has long been established, the role of the proteasome in Cx43 turnover is still poorly understood. Initial studies suggested that the proteasome could be directly involved in connexin degradation (Laing & Beyer, 1995), however, more recently it was suggested that the proteasome acts by regulating the stability of Cx43 at the plasma membrane, most likely by degrading a still unknown connexin-interacting protein (Girao & Pereira, 2007; Musil et al, 2000). For example, a model has been proposed in which proteasome-dependent degradation of a putative Cx43 binding protein regulates Cx43-ZO-1 interaction, which in turn regulates Cx43 internalization (Girao & Pereira, 2007).

It is well established that in mammalian cells, ubiquitin acts as a triggering signal for the internalization and sorting of a variety of cargo receptors, thus acting as a link between cargo and components of the endocytic and sorting machinery (Hicke & Dunn, 2003; Miranda & Sorkin, 2007; Mukhopadhyay & Riezman, 2007; Traub & Lukacs, 2007). However, to promote the internalization of membrane proteins, ubiquitin needs to be recognized by ubiquitin receptors, such as Eps15, Epsin and Hrs, that contain ubiquitin-binding domains which recognize ubiquitinated proteins and sort them along the endocytic pathway (Hicke & Dunn, 2003; Kirkin & Dikic, 2007; Miranda & Sorkin, 2007; Mukhopadhyay & Riezman, 2007; Traub & Lukacs, 2007). Moreover, some of these ubiquitin receptors are often themselves monoubiquitinated (Di Fiore et al, 2003; Hicke et al, 2005; Hoeller et al, 2006; Woelk et al, 2006).

Several studies have also shown that Cx43 is a substrate for ubiquitination (Laing et al, 1997). For example, data obtained by immunofluorescence and immunogold labelling, suggests that at least part of the Cx43 found at gap junction plaques is ubiquitinated (Rutz & Hulser, 2001). Moreover, treatments that induce Cx43 internalization also induce ubiquitination of the protein (Leithe & Rivedal, 2004a; Leithe & Rivedal, 2004b). Furthermore, the E3 ligase Nedd4 was shown to interact

with Cx43 (Leykauf et al, 2006), and we have consistently shown that Nedd4 mediates the ubiquitination of Cx43, through a mechanism that requires the Proline-rich motif (PY) of Cx43 (Chapter 5). We also demonstrated that Eps15 binds to ubiquitinated Cx43 through its ubiquitin-interacting motifs (UIMs) and that this interaction is, at least in part, required for the internalization of Cx43. Although several reports have suggested that ubiquitin may be implicated in gap junction internalization, direct evidence of ubiquitin-dependent internalization/degradation of Cx43 has not been clearly demonstrated.

Monoubiquitination, multimonoubiquitination and Lys63-linked polyubiquitin chains have all been reported to function as endocytosis signals, however, several studies report that monoubiquitination appears to be a weak internalization signal (Barriere et al, 2006; Hawryluk et al, 2006). This is explained by observations showing that the UIM domains present in endocytic adaptors such as Eps15 have a relatively weak affinity for monoubiquitin. However, UIM domains have been shown to bind with more avidity to polyubiquitin signals, especially Lys63-linked chains. Additionally, multimonoubiquitination of a single protein or monoubiquitination of several subunits of a protein complex was also suggested to be a strong endocytic signal since the close proximity of several monoubiquitin moieties would mimic a polyubiquitin signal, which binds strongly to UIM domains (Barriere et al, 2006).

Clathrin has long been suggested to mediate the internalization of annular gap junctions (Larsen et al, 1979), with several recent studies showing that impairment of clathrin-mediated endocytosis (CME) leads to the accumulation of Cx43 at the plasma membrane (Gumpert et al, 2008; Leithe & Rivedal, 2004a; Piehl et al, 2007). However, it is conceivable that Cx43 gap junction plaques may also be internalized through clathrin-independent endocytic (CIE) pathways. In studies where CME was inhibited, the presence of internalized annular gap junction plaques was only reduced by roughly 55%, whereas internalization of transferrin, a protein previously shown to be internalized exclusively by clathrin coated pits (Mellman, 1996), was nearly abolished (Gumpert et al, 2008; Piehl et al, 2007). This suggests that large portions of Cx43 gap junctions are internalized through clathrin-independent mechanisms. An alternative mechanism may involve caveolar endocytosis. The interaction of Cx43 with Cav-1 (Langlois et al, 2008; Schubert et al, 2002), suggests that Cx43 may also be internalized through caveolae, a specialized invagination of the plasma membrane rich in Cav-1 that can bud from the plasma membrane (Kiss & Botos, 2009).

Following internalization, endocytosed cargos may have a variety of different fates, although, as a general rule, all endocytosed cargo is delivered to the early endosome. From there proteins can be delivered to the trans-Golgi network, be recycled back to the plasma membrane or remain in association with the early endosome while it matures into late endosomes, which eventually fuse with lysosomes. During the maturation process of endosomes their structure and composition are altered, with late endosomes showing characteristics of typical multivesicular bodies (MVBs). The transition between the two stages is thought to proceed through the progressive involution of the

limiting membrane of endosomes to form intralumenal vesicles (Raiborg & Stenmark, 2009; Williams & Urbe, 2007). The best characterized signal directing membrane proteins to the degradative MVB pathway is ubiquitination. At the endosomal membrane, ubiquitinated cargo is recognized by the endosomal sorting complex for transport (ESCRT) machinery, a series of protein complexes that are highly conserved in eukaryotes. The ESCRT machinery consists of four complexes, ESCRT-0, -I, -II and –III, plus several ancillary components. Several subunits of the ESCRT complexes, such as Hrs of ESCRT-0 and Tsg101 of ESCRT-I, possess ubiquitin-binding domains that are thought to be responsible for substrate recognition. Furthermore, several deubiquitinating enzymes (DUBs), such as Doa4, AMSH or UBPY, have been shown to bind to elements of the ESCRT machinery. DUBs presumably serve a regulatory function in the ESCRT complexes, being responsible for recycling free ubiquitin, rescuing substrates from endolysosomal sorting and activating components of the ESCRT machinery (Clague & Urbe, 2006; Raiborg & Stenmark, 2009). However, little is known about the molecular players that direct internalized gap junction plaques to the endolysosomal compartment or whether the Cx43 protein is a substrate for deubiquitinating enzymes.

Cargo that escapes sorting to late endosomes is generally recycled back to the plasma membrane. Endocytic recycling can proceed via two major pathways, the rapid recycling pathway, which traffics proteins directly from the early endosome back to the plasma membrane in Rab4 positive vesicles, or the slow recycling pathway, which first directs cargo to the endocytic recycling compartment, which is positive for Rab11, before being delivered back to the plasma membrane (Grant & Donaldson, 2009; Maxfield & McGraw, 2004). Although Cx43 internalization and subsequent degradation in the lysosome is well established, little (or nothing) is known about any putative endocytic recycling mechanisms directing Cx43 back to the plasma membrane (VanSlyke & Musil, 2005).

Results

Cx43 colocalizes with ubiquitin at the plasma membrane

Ubiquitination of plasma membrane proteins is a well described internalization signal. Indeed, in the previous chapter we show that ubiquitination increases the interaction/association between Cx43 and Eps15, and that Eps15 is required to mediate the internalization of Cx43. Nevertheless, many questions still remain unanswered on the role of ubiquitin in Cx43 internalization. Thus, we first sought to characterize the subcellular distribution of Cx43 and ubiquitin. For this purpose NRK cells (that endogenously express high levels of Cx43) were fixed and stained with monoclonal antibodies directed against Cx43 and polyclonal antibodies directed against ubiquitin. Cells were subsequently imaged by immunofluorescence confocal microscopy. As shown in Figure 28, a

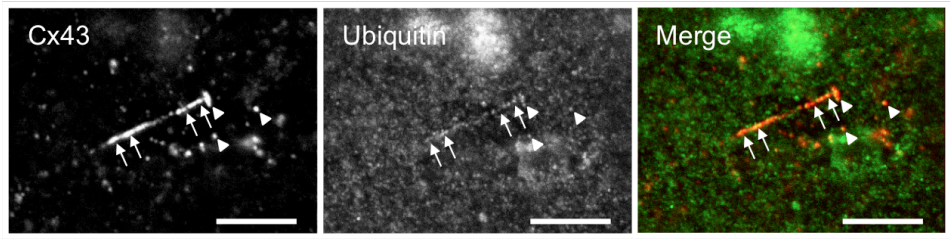

Figure 28 - Cx43 and ubiquitin colocalize at the plasma membrane and intracellular vesicles. NRK cells were fixed and stained with monoclonal antibodies directed against Cx43 (BD Transduction) and polyclonal antibodies against ubiquitin. A significant amount of Cx43 colocalizes with ubiquitin. Although most of the colocalization observed occurs at the plasma membrane (arrows), the two proteins can also be detected in the same cytoplasmic vesicles (arrowheads). Scale bars, 5 μm.

significant amount of Cx43 colocalizes with ubiquitin, at the plasma membrane, suggesting that much of the Cx43 present at the plasma membrane is ubiquitinated. However, based on this data the possibility that ubiquitin is bound to proteins located in close proximity to Cx43 cannot be ruled out. Although most of the colocalization occurs at the plasma membrane (arrows), most likely at gap junction plaques, some colocalization can also be detected in cytoplasmic vesicles localized in the close vicinity of the plasma membrane, that presumably correspond to early steps of annular gap junction internalization (arrowheads).

This immunofluorescence data strongly suggests that Cx43 and ubiquitin colocalize mostly at the plasma membrane and/or at internalized vesicles, thus suggesting a role for ubiquitin in gap junction internalization.

A Cx43-Ub chimera oligomerizes into the same connexons as Cx43

Following the observation that ubiquitin colocalizes with Cx43 at gap junction plaques, we hypothesized that the incorporation of ubiquitinated Cx43 into connexin hemichannels would be sufficient to trigger the internalization of non-ubiquitinated Cx43 present in the same hemichannels. Several studies have demonstrated that ubiquitination of various plasma membrane proteins is both necessary and sufficient to target them for internalization, in a process mediated by endocytic adaptors containing ubiquitin-binding domains (UBDs). A common strategy to evaluate the role of ubiquitin in the internalization of membrane proteins involves the overexpression of a chimeric protein formed by the membrane protein fused to an ubiquitin molecule. We constructed a chimeric Cx43 protein in which an ubiquitin molecule was fused in frame to the carboxyl-terminal of Cx43 (Cx43-Ub). To prevent this fusion protein from being conjugated to other proteins by the ubiquitination machinery, the two terminal glycines were mutated to alanine. Furthermore, to prevent the chimeric protein from being recognized by the ERAD machinery and degraded by the proteasome, Lys48 was also mutated to Arg, to prevent the extension of a Lys48 polyubiquitin chain on the fused ubiquitin. Lastly, to allow the specific detection of this chimeric protein through immunofluorescence, a myc tag was fused in frame to the amino-terminal of Cx43, completing the

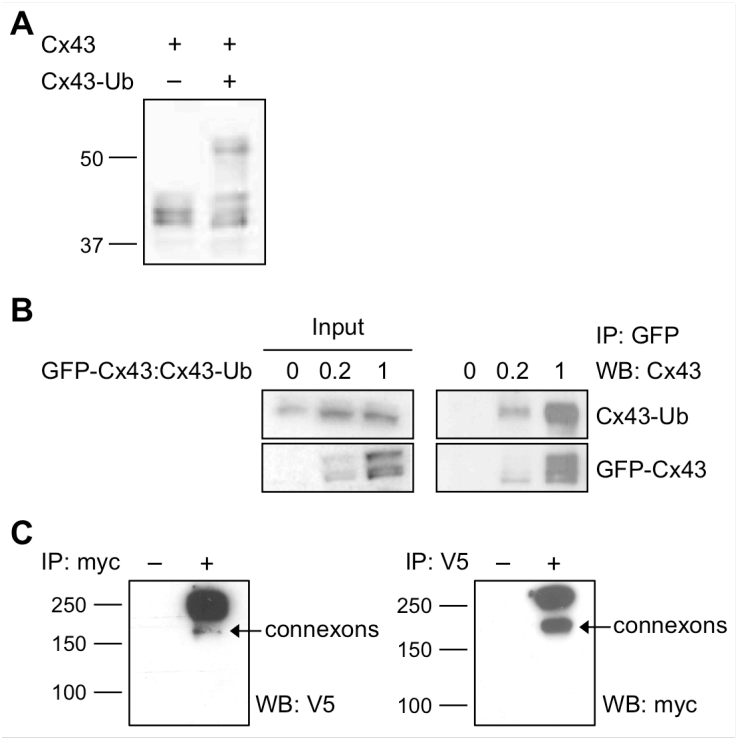

Figure 29 - Cx43 and Cx43-Ub oligomerize into the same connexons. (A) Lysates of Cos-7 cells co-transfected with Cx43 and Cx43-Ub were analyzed by Western blot using polyclonal antibodies directed against Cx43. (B) Lysates of Cos-7 cells, co-transfected with varying proportions of GFP-Cx43 and Cx43-Ub, were immunoprecipitated with polyclonal antibodies directed against GFP. Precipitates were then analyzed by Western blot using monoclonal antibodies directed against Cx43. Monomeric Cx43-Ub is selectively co-precipitated with GFP-Cx43. (C) Lysates of Cos-7 cells, co-transfected with V5-Cx43 and myc-Cx43-Ub, were immunoprecipitated with monoclonal antibodies directed against V5 or myc. Samples were denatured with Laemmli buffer without β-mercaptoethanol, which preserves the integrity of oligomeric connexons, and the precipitates were then analyzed by Western blot using monoclonal antibodies directed against V5 or myc. Connexons containing Cx43-Ub selectively co-precipitate with Cx43 and vice-versa.

Cx43-Ub chimera. To first evaluate the expression levels of Cx43 and Cx43-Ub, cDNA encoding these proteins was transfected into Cos-7 cells and the presence of each of the two proteins determined by Western blot, using polyclonal antibodies against Cx43. As shown in Figure 29A, the presence of a slower migrating band, with a molecular weight of about 50 KDa, that is recognized by Cx43 antibodies, is compatible with the expression of a chimeric protein.

Transport of Cx43 to the plasma membrane requires its prior oligomerization into connexons, a process that occurs in the trans-Golgi network. To investigate whether Cx43-Ub oligomerizes into connexons with wild-type Cx43, thus forming heteromeric hemichannels, Cos-7 cells were co-transfected with varying quantities of plasmids encoding Cx43-Ub and a construct in which GFP was fused in frame to the amino-terminal of Cx43 (GFP-Cx43). Polyclonal antibodies directed against GFP were then used to selectively immunoprecipitate the GFP-Cx43 chimera. As depicted in Figure 29B, monomeric Cx43-Ub precipitates only with the GFP antibodies when co-expressed with GFP-Cx43, suggesting that Cx43-Ub oligomerizes into the same connexons as GFP-Cx43. Moreover, as the amount of expressed Cx43 increases, the levels of Cx43-Ub that co-immunoprecipitate with the protein also increase. To further confirm that Cx43-Ub can oligomerize with wild type Cx43 to form heteromeric connexons, experiments were performed in

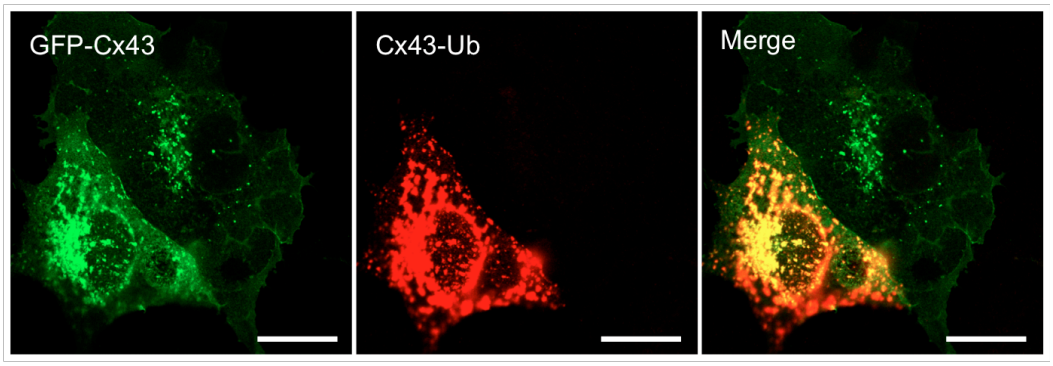

Figure 30 - Cx43 and Cx43-Ub colocalize in the same intracellular vesicles. Cos-7 cells were transfected with GFP-Cx43 together with myc-Cx43-Ub. Cells were then fixed and stained with monoclonal antibodies directed against myc. Cx43 and Cx43-Ub display extensive colocalization, further reinforcing that they oligomerize into the same connexons. Scale bars, 50 μm.

conditions where the structural integrity of connexons was maintained. Cos-7 cells were transfected with plasmids encoding myc-tagged Cx43-Ub and V5-tagged wild type Cx43. Following immunoprecipitation using antibodies directed against either tag, samples were denatured with Laemmli buffer without β-mercaptoethanol, in order to maintain the integrity of connexons. As shown in Figure 29C, connexons containing myc-Cx43-Ub were readily detected when wild type Cx43 was selectively immunoprecipitated using antibodies directed against V5. The opposite was also true, with V5-Cx43 being detected when Cx43-Ub was selectively immunoprecipitated using antibodies directed against the myc tag. Taken together, these results demonstrate that Cx43-Ub is capable of forming heteromeric connexons with Cx43.

To investigate the subcellular distribution of hemichannels containing Cx43-Ub, Cos-7 cells were co-transfected with cDNA encoding GFP-Cx43 and myc-tagged Cx43-Ub. Cells were subsequently fixed and stained with monoclonal antibodies directed against the myc tag, and imaged by immunofluorescence microscopy. Results depicted in Figure 30 show an extensive colocalization of the two proteins, thus reinforcing that they are being incorporated into the same hemichannels.

Expression of the Cx43-Ub chimera induces the internalization and degradation of Cx43

Once we established that Cx43-Ub incorporates into Cx43-containing hemichannels, we attempted to evaluate the effect of the presence of ubiquitin moieties upon Cx43 channels. First, we investigated the effect of Cx43-Ub overexpression upon the stability of endogenous Cx43. For this purpose, we used a retinal epithelial cell line (ARPE-19) that endogenously expresses detectable amounts of Cx43. The half-life of endogenous Cx43, either in the presence or absence of Cx43-Ub, was determined by a cyclohexamide (CHX)-chase assay. Following transfection with plasmids encoding Cx43-Ub, protein synthesis was inhibited with 50 μg/ml of CHX and cells were collected at several time points. Samples were then analysed by Western blot using polyclonal antibodies directed against Cx43 and the density of the detected bands was measured and plotted in a graph.

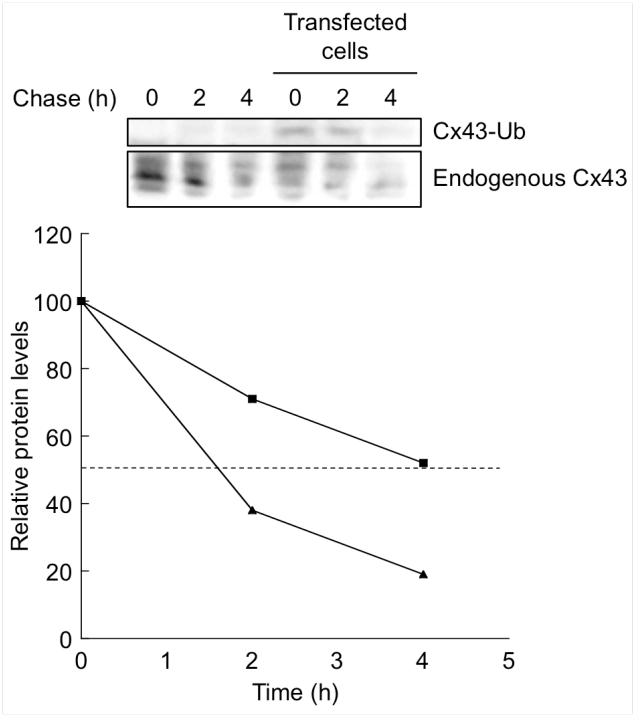

Figure 31 - Expression of the Cx43-Ub chimera reduces the half-life of endogenous Cx43. ARPE-19 cells transfected with Cx43-Ub, were incubated with 50 μg/ml cyclohexamide for 0, 2 and 4 hours. Lysates were then analyzed by Western blot using polyclonal antibodies directed against Cx43. The intensity of the endogenous Cx43 bands was measured and plotted in a graph (Squares = non-transfected cells; Triangles = transfected cells). Expression of Cx43-Ub reduced the half-life of endogenous Cx43 from 4 to 1.5 hours.

The results presented in Figure 31 show that the overexpression of Cx43-Ub results in a decrease of endogenous Cx43 half-life from around 4 hours to 1.5 hours. Similar results were obtained when Cx43 was co-expressed with Cx43-Ub in Cos-7 cells. As shown in Figure 32A, the presence of Cx43-Ub results in a decrease in Cx43 half-life from 2.5 hours to 1 hour.

It was previously shown for various membrane proteins that the conjugation of ubiquitin may act as a triggering signal for their internalization. Therefore, we decided to evaluate the effect of the presence of ubiquitin moieties on the stability of Cx43 at the plasma membrane surface, by co-expressing Cx43 with the Cx43-Ub chimera. To determine the half-life of Cx43 at the plasma membrane, transfected Cos-7 cells were first treated with 50μg/ml of CHX, during different time frames, before being subjected to cell surface protein biotinylation. Biotinylated proteins from cell lysates were then precipitated with Neutravidin beads, followed by Western blot analysis with antibodies directed against Cx43. Results presented in Figure 32B show that the co-expression of Cx43-Ub induces a reduction in the surface stability of wild type Cx43 from 1 hour to roughly 40 minutes.

To investigate the effect of the Cx43-Ub chimera on the subcellular localization of Cx43, Cos-7 cells were transfected with GFP-Cx43, either in the presence or absence of Cx43-Ub, after which the cells were fixed and stained with antibodies directed against ubiquitin or the myc tag, and imaged by immunofluorescence microscopy. When GFP-Cx43 is expressed alone (Figure 33,

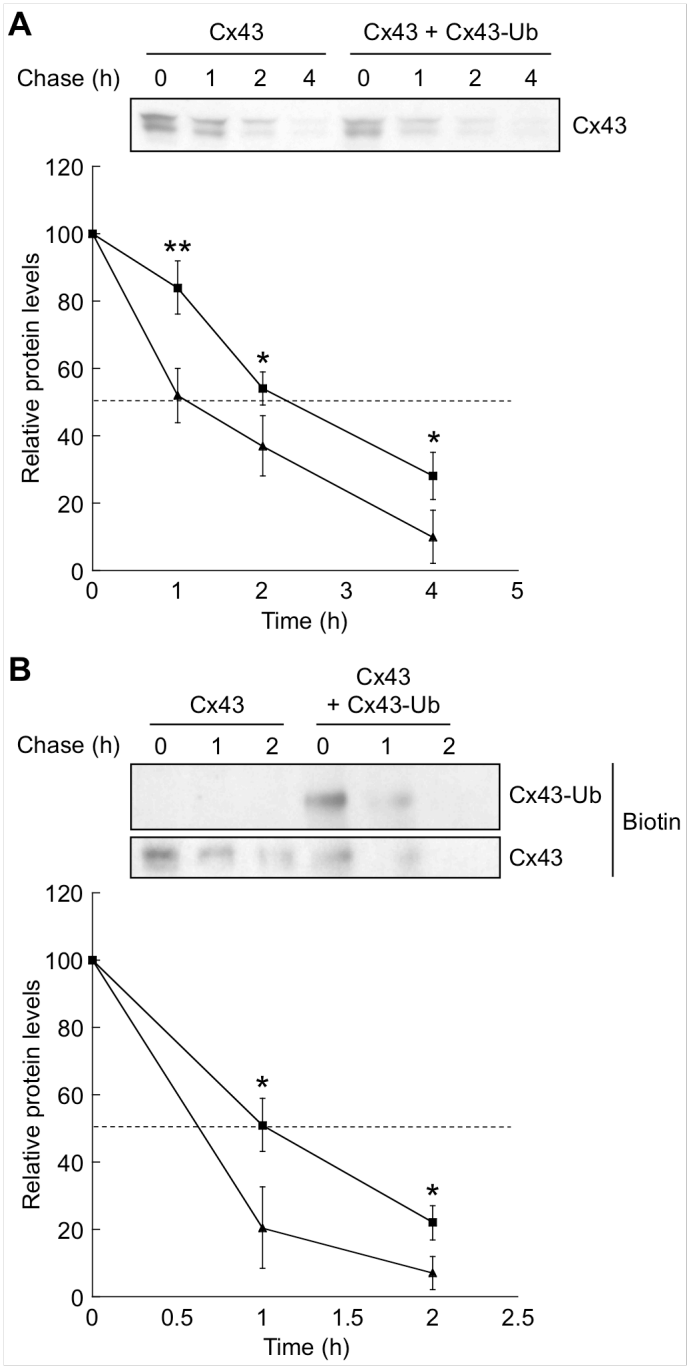

Figure 32 - Ubiquitination of Cx43 reduces its half-life and cell surface stability. (A) Cos-7 cells transfected with Cx43 either alone or together with Cx43-Ub, were incubated with 50 μg/ml cyclohexamide for 0, 1, 2 or 4 hours. Lysates were then analyzed by Western blot using polyclonal antibodies directed against Cx43. The intensity of the bands was measured and plotted in a graph (Squares = Cx43; Triangles = Cx43 + Cx43-Ub). Co-expression of Cx43 with Cx43-Ub reduced its half-life from 2.5 hours to 1 hour. (B) Cos-7 cells transfected with Cx43 either alone or together with Cx43-Ub, were incubated with 50 μg/ml cyclohexamide for 0, 1 or 2 hours. Cells were then subjected to cell surface protein biotinylation and the biotinylated fraction of the cell lysates was precipitated with Neutravidin beads. Precipitates were then analyzed by Western blot using polyclonal antibodies against Cx43. The intensity of the bands was measured and plotted in a graph (Squares = Cx43; Triangles = Cx43 + Cx43-Ub). Co-expression of Cx43 with Cx43-Ub reduced its surface half-life from 1 hour to 40 minutes. The values are the average of three individual experiments ± SD. Asterisks indicate statistically significant differences from controls (* $p < 0.05$; ** $p < 0.01$).

upper panels), extensive gap junction plaques can be found along cell-cell interfaces. In these conditions, GFP-Cx43 displays a weak colocalization pattern with ubiquitin. However, when Cx43-

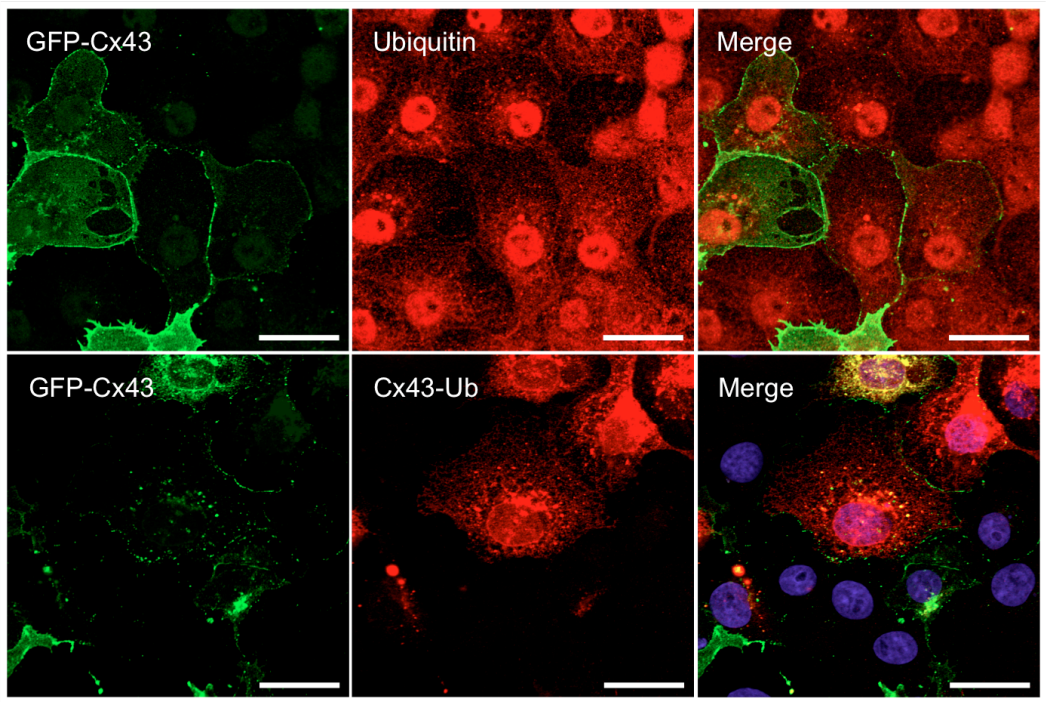

Figure 33 - Ubiquitination induces the internalization of gap junction plaques. Cos-7 cells were transfected with GFP-Cx43 either alone (upper panels) or together with myc-Cx43-Ub (lower panels) and then fixed and stained with polyclonal antibodies against ubiquitin (upper panels) or monoclonal antibodies against myc (lower panels). Nuclei were stained with DAPI. Co-expression of Cx43-Ub induces the internalization of gap junction plaques. Scale bars, 100 μm.

Ub is co-expressed with the GFP-Cx43 chimera (Figure 33, lower panels) there is a substantial decrease in GFP-Cx43 gap junction plaque size. Taken together, the immunofluorescence data and the biotinylation data strongly suggest that the presence of ubiquitin in Cx43-containing channels induces the internalization and subsequent degradation of gap junctions.

It is well established that the attachment of ubiquitin to membrane proteins, either by enhancing protein internalization or by modulating intracellular sorting, results in their degradation in the lysosome. To further confirm that incorporation of Cx43-Ub into hemichannels induces their lysosomal degradation, we evaluated the amount of Cx43 present in cells overexpressing the Cx43-Ub chimera either in the presence or absence of the lysosome inhibitor NH_4Cl. The results presented in Figure 34 show that lysosomal inhibition results in the accumulation of both Cx43 and Cx43-Ub, following 4 hours of chase in the presence of CHX (compare lane 6 with lane 8). Although we show that the co-expression of Cx43-Ub induces the internalization and degradation of Cx43, it cannot be excluded that a functional ubiquitin conjugation system is still required for internalization of Cx43. This is likely to explain the accumulation of Cx43 and Cx43-Ub in the presence of proteasome inhibitors as these treatments lead to the depletion of free ubiquitin (compare lane 6 with lane 7).

The small Rab GTPases are known to regulate the intracellular trafficking of membrane proteins along the endocytic pathway. For example, vesicle traffic from the plasma membrane to early endosomes is regulated by the small GTPase Rab5 (Bucci et al, 1992), while Rab7 is involved with

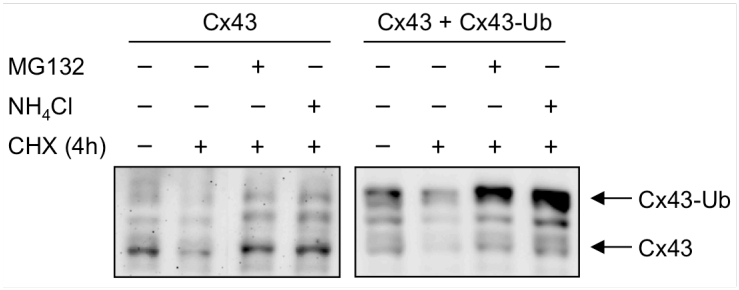

Figure 34 - Inhibition of the proteasome or the lysosome stabilizes Cx43-Ub. Cos-7 cells transfected with Cx43 either alone or together with Cx43-Ub, were incubated with 50 μg/ml cyclohexamide for 4 hours, either in the presence or absence of the proteasome inhibitor MG132 (40 μM) or the lysosome inhibitor NH_4Cl (10 mM). Lysates were then analyzed by Western blot using polyclonal antibodies against Cx43. Inhibition of either the proteasome or the lysosome stabilizes both wild type Cx43 and the Cx43-Ub chimera.

trafficking from early to late endosomes (Feng et al, 1995) and from late endosomes to lysosomes (Meresse et al, 1995). To investigate whether Cx43-Ub localizes to endocytic vesicles, cells co-expressing GFP-tagged Rab5 or Rab7 and Cx43-Ub were analyzed by confocal microscopy. Results presented in Figure 35 clearly show the accumulation of Cx43-Ub in intracellular vesicles containing Rab5 or Rab7, thus demonstrating that at least part of the detected vesicles containing Cx43-Ub are being transported to lysosomes.

Ubiquitin triggers the internalization of an endocytic-impaired mutant of Cx43

Cx43 contains within its carboxyl-terminal a putative tyrosine-based sorting signal domain corresponding to the consensus sequence YXXØ (where X is any amino acid and Ø is a hydrophobic amino acid). It was previously shown that mutation of Tyr286, which is critical for the sorting function of the YXXØ motif on Cx43, increases the half-life of the protein, induces its accumulation at the plasma membrane and increases intercellular communication (Thomas et al, 2003), suggesting that the YXXØ motif is the prime determinant of the stability of Cx43 at the cell surface. Thus, we decided to investigate the role of ubiquitin as an internalization signal in channels containing an endocytic defective mutant of Cx43. Besides its critical role as a part of the YXXØ domain on Cx43, Tyr286 is also an integral part of the PY-motif on Cx43, which is important for the interaction and ubiquitination of Cx43 by Nedd4. Therefore, we first generated a Cx43 mutant in which the tyrosine at position 286 was replaced with an alanine (Cx43Y286A), and investigated whether this mutation impaired Cx43 ubiquitination. Cos-7 cells were transfected either with the wild type or the mutated form of Cx43, after which the proteins were selectively immunoprecipitated using polyclonal antibodies against Cx43. The levels of ubiquitinated protein were then determined by Western blot using antibodies against Cx43. The results presented in Figure 36 consistently show that the Cx43Y286A mutant is less ubiquitinated as compared to wild type Cx43. Taken together with the previous data showing that ubiquitin induces the internalization and degradation of Cx43, it is possible to suggest that the increased stability of the Cx43Y286A

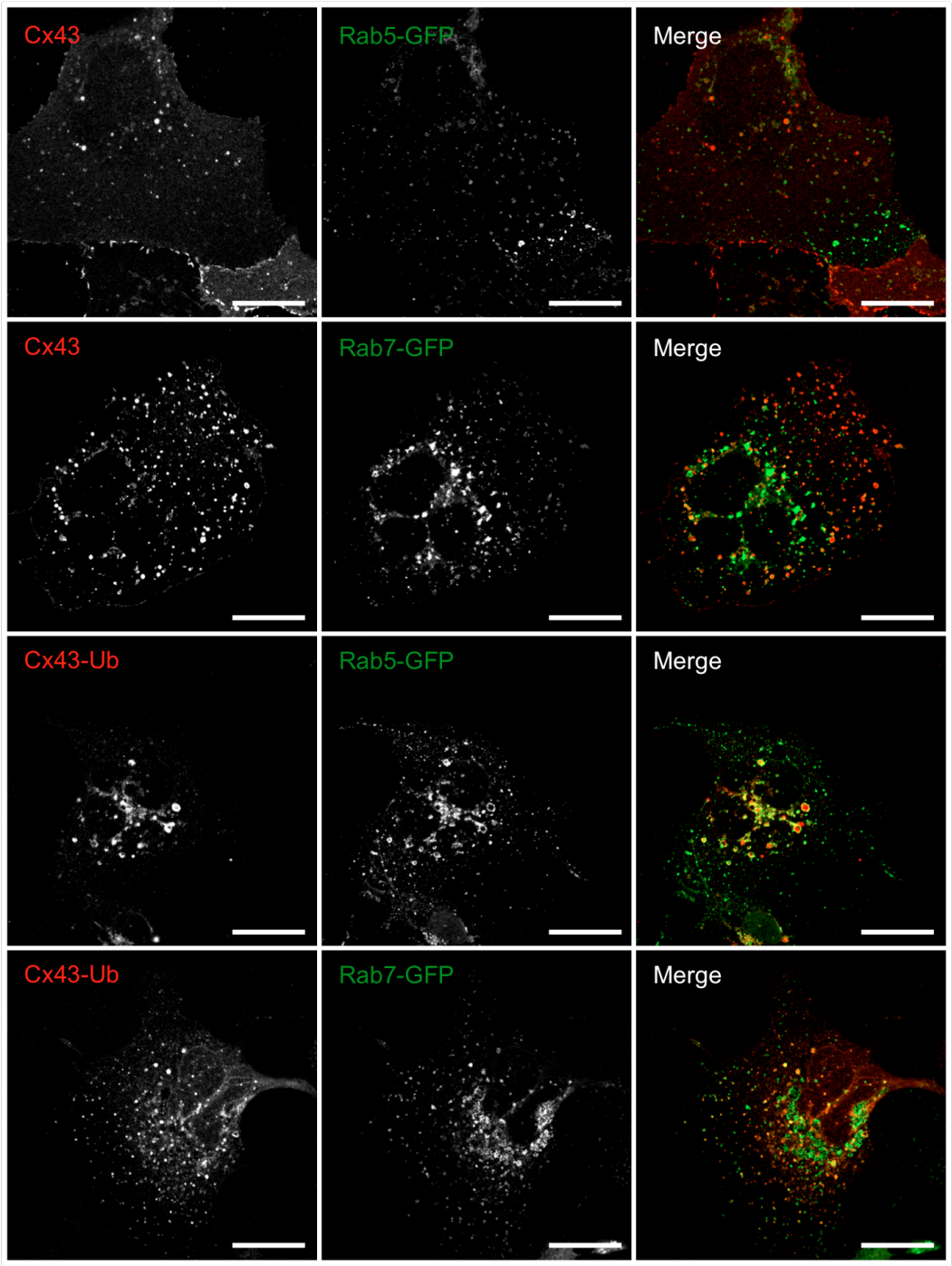

Figure 35 - Cx43 and the Cx43-Ub chimera colocalize with markers of endosomal vesicles. Cos-7 cells were co-transfected with V5-Cx43 or myc-Cx43-Ub and either Rab5-GFP or Rab7-GFP. Cells were then fixed and stained with monoclonal antibodies directed against V5 (upper panels) or myc (lower panels) before being imaged by confocal microscopy. Cx43-Ub displays increased colocalization with the endosomal markers Rab5 and Rab7 when compared to wild type Cx43. Scale bars, 50 μm.

mutant may not only be due to a defect in the tyrosine-based sorting signal, but also due to an ubiquitination defect.

Since internalization of Cx43 mainly occurs through a mechanism that depends on the tyrosine-based sorting signal, and mutations on this motif are known to dramatically stabilize Cx43 at the plasma membrane, we hypothesized that when YXXØ-driven internalization of Cx43 is impaired,

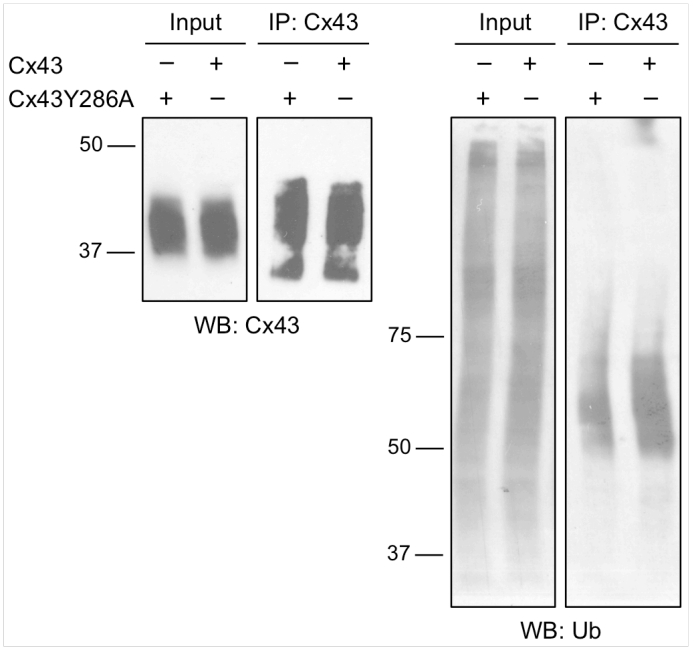

Figure 36 - Mutation of Tyr286 on Cx43 impairs its ubiquitination. Lysates from Cos-7 cells, transfected with either wild type or mutant Cx43 (Cx43Y286A), were immunoprecipitated with polyclonal antibodies against Cx43. Immunoprecipitates were then analyzed by Western blot and probed with monoclonal antibodies against Cx43 or ubiquitin (P4D1). Cx43Y286A is substantially less ubiquitinated than wild type Cx43.

ubiquitin could act as an internalization signal, triggering the endocytosis of this Cx43Y286A mutant. As with the wild type form of Cx43, we first evaluated the effect of Cx43-Ub on Cx43Y286A half-life. Consistent with a previous report (Thomas et al, 2003), mutation of Tyr286 on Cx43 increased its half-life from roughly 2.5 hours to 5.5 hours, thus reinforcing the importance of the YXXØ sorting motif for Cx43 internalization (compare Figure 32A with Figure 37A). However, co-expression of Cx43-Ub with Cx43Y286A markedly reduced its half-life from 5.5 to only 3 hours (Figure 37A).

To further investigate the effect of incorporating Cx43-Ub into Cx43Y286A-containing hemichannels, the cell surface half-life of Cx43Y286A was determined when expressed alone or together with Cx43-Ub, by cell surface protein biotinylation. Results presented in Figure 37B show that mutation of Tyr286 on Cx43 induces a two fold increase in the half-life of Cx43 at the cell surface, further reinforcing the importance of this residue for Cx43 internalization (compare Figure 32B with Figure 37B). Similarly to what was observed for Cx43 surface half-life, co-expression of Cx43-Ub induces a significant reduction in the surface stability of the Y286A mutant from 2 hours to less than 1 hour. Consistent with the results obtained with the biotinylation assays, the subcellular distribution of the Cx43Y286A mutant is still affect by the presence of Cx43-Ub. Indeed, using a construct in which GFP was fused to the amino-terminal of Cx43Y286A (GFP-Cx43Y286A), we show that this construct localizes to the plasma membrane where it appears to form large gap junction plaques, when compared to cells expressing GFP-Cx43 (compare the upper panels of Figure 33 with the upper panels of Figure 38). Furthermore, and as demonstrated

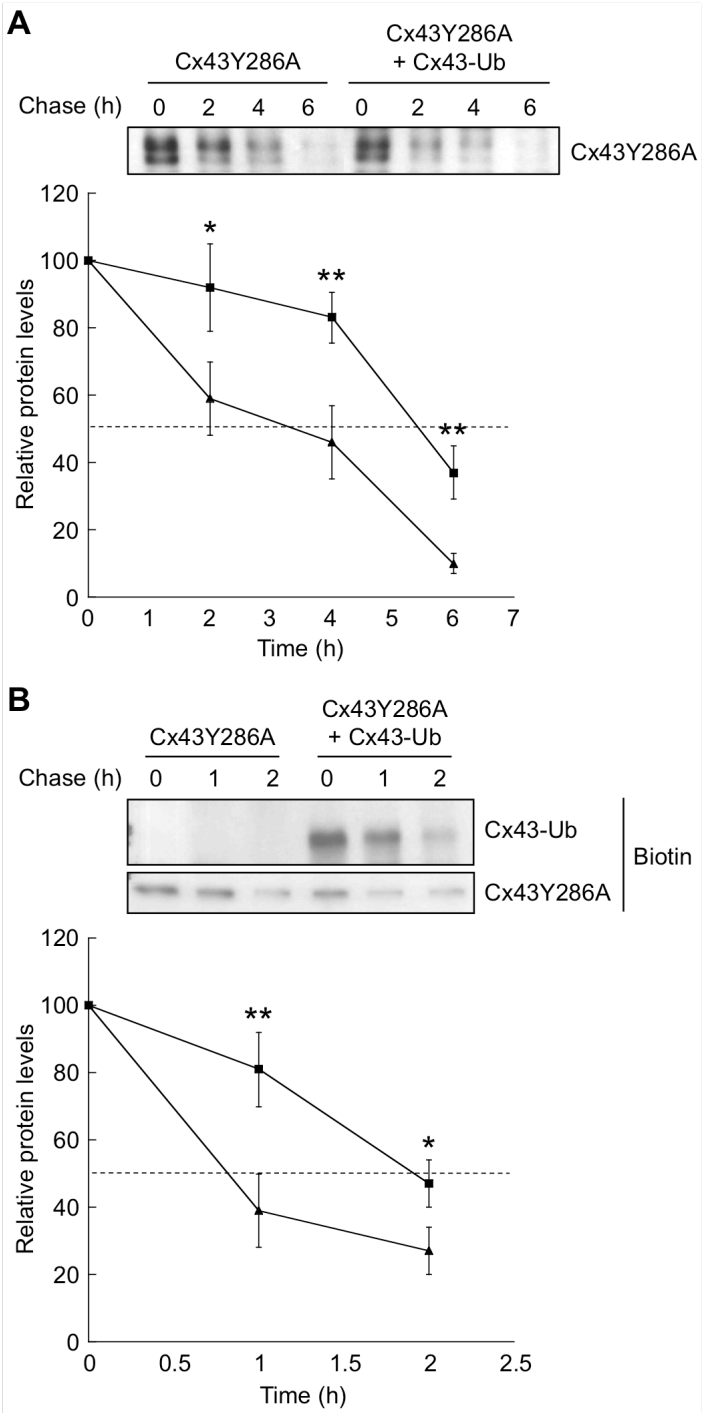

Figure 37 - Ubiquitination reduces the half-life and cell surface stability of the endocytic-impaired mutant Cx43Y286A. (A) Cos-7 cells transfected with Cx43Y286A either alone or together with Cx43-Ub, were incubated with 50 μg/ml cyclohexamide for 0, 2, 4 and 6 hours. Lysates were then analyzed by Western blot using polyclonal antibodies directed against Cx43. The intensity of the bands was measured and plotted in a graph (Squares = Cx43Y286A; Triangles = Cx43Y286A + Cx43-Ub). Co-expression of Cx43Y286A with Cx43 reduced its half-life from 5.5 to 3 hours. (B) Cos-7 cells transfected with Cx43Y286A either alone or together with Cx43-Ub, were incubated with 50 μg/ml cyclohexamide for 0, 1 and 2 hours. Cells were then subjected to cell surface protein biotinylation and the biotinylated fraction of the cell lysates was precipitated with Neutravidin beads. Precipitates were then analyzed by Western blot using polyclonal antibodies against Cx43. The intensity of the bands was measured and plotted in a graph (Squares = Cx43Y286A; Triangles = Cx43Y286A + Cx43-Ub). Co-expression of Cx43Y286A with Cx43-Ub reduced its surface half-life from 2 to less than 1 hour. The values are the average of three individual experiments ± SD. Asterisks indicate statistically significant differences from controls (* $p < 0.05$; ** $p < 0.01$).

for GFP-Cx43, the incorporation of Cx43-Ub into GFP-Cx43Y286A hemichannels results in a

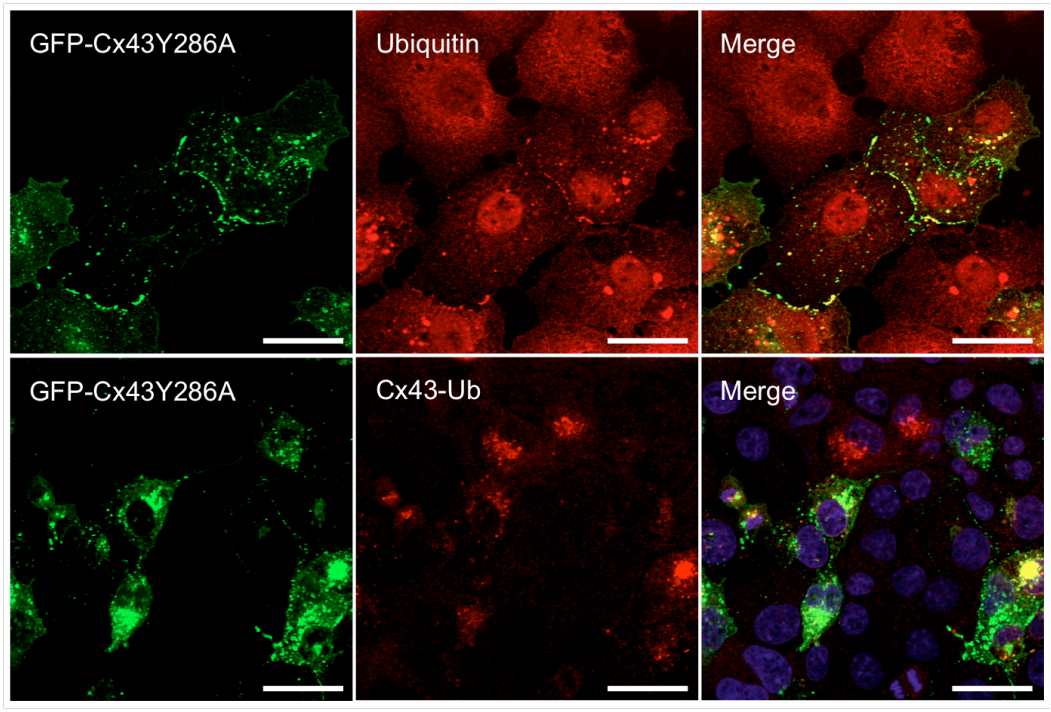

Figure 38 - Ubiquitination induces the internalization of Cx43Y286A gap junction plaques. Cos-7 cells were transfected with GFP-Cx43Y286A either alone (upper panels) or together with myc-Cx43-Ub (lower panels) and then fixed and stained with polyclonal antibodies against ubiquitin (upper panels) or monoclonal antibodies against myc (lower panels). Nuclei were stained with DAPI. Co-expression of Cx43-Ub induces the internalization of the large gap junction plaques formed by Cx43Y286A. Scale bars, 100 μm.

decrease of GFP-Cx43Y286A at the plasma membrane (Figure 38, lower panels). Interestingly, while GFP-Cx43 shows weak colocalization with ubiquitin at the plasma membrane, the large gap junction plaques formed by the GFP-Cx43Y286A construct show an extensive colocalization with ubiquitin (Figure 38, upper panels).

The current model of Cx43 gap junction plaque renewal states that newly formed channels are accrued to the edges of the plaque, while older channels are internalized from the centre of the plaque (Falk et al, 2009; Gaietta et al, 2002). Although the co-expression of Cx43-Ub impairs the formation of large gap junction plaques by GFP-Cx43Y286A, some are still observed. When the large plaques formed by GFP-Cx43Y286A are analyzed at a higher magnification, it is possible to visualize small holes in the plaques (Figure 39). These may correspond to locations where part of the gap junction plaque has internalized. Interestingly, small vesicles containing both GFP-Cx43Y286A and Cx43-Ub, that may correspond to internalized sections of the plaque, are also detected near these holes. Taken together, these results indicate that the incorporation of Cx43-Ub into gap junction plaques can induce the destabilization of plaques formed both by the wild type form of Cx43, as well as by the mutant Cx43Y286A that harbours an internalization defect. Furthermore, the data also supports a model in which the destabilizing effect of Cx43-Ub expression is due to increased internalization of gap junction plaques.

Treatment of cells with PMA is known to induce the phosphorylation of Cx43, inducing its ubiquitination and subsequent internalization (Lampe, 1994; Leithe & Rivedal, 2004b; Pitts & Burk,

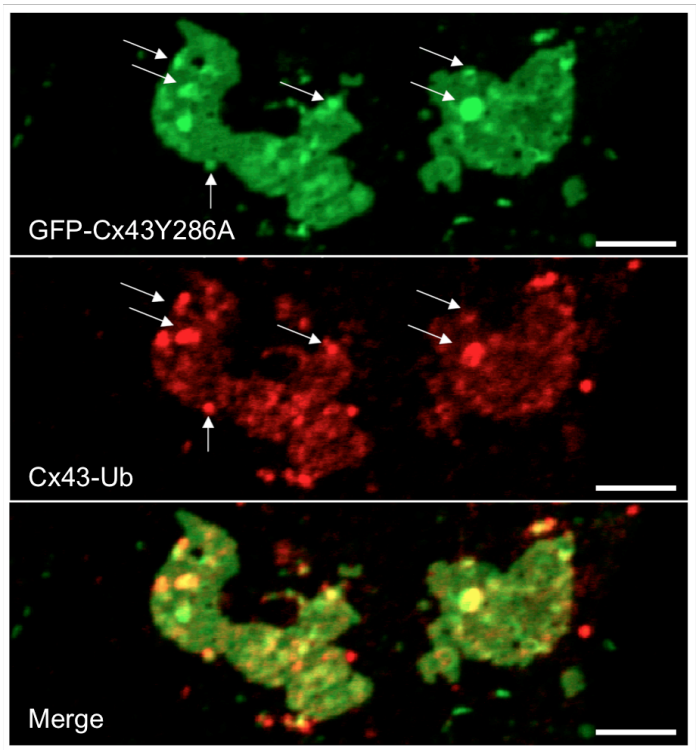

Figure 39 - Ubiquitination induces the internalization of small sections of gap junction plaques. Cos-7 cells co-transfected with GFP-Cx43Y286A and myc-Cx43-Ub were fixed and stained with monoclonal antibodies directed against myc. Small intracellular vesicles positive for GFP-Cx43Y286A and Cx43-Ub can be found in close vicinity to holes in the gap junction plaque. Scale bars, 1 μm.

1987). To investigate the effect of PMA treatment upon GFP-Cx43Y286A subcellular distribution, Cos-7 cells were transfected with plasmids encoding GFP-Cx43Y286A and treated with 50nM PMA for several lengths of time. Cells were then fixed and imaged by fluorescence microscopy. Figure 40 shows that treatment with 50 nM PMA during 5 minutes induces an increase in the number and size of the small holes present on gap junction plaques. Furthermore, after 45 minutes of treatment, the size of the plaques was greatly reduced, with only very few holes still present. This data suggests that PMA treatment, which is known to induce Cx43 ubiquitination and gap junction plaque internalization, leads to gap junction internalization through a process that appears to be similar to what is observed when GFP-Cx43Y286A is co-expressed with Cx43-Ub.

Internalization of Cx43 triggered by ubiquitin is dependent on the interaction with Eps15

In the previous chapter we showed that Eps15 interacts with ubiquitinated Cx43 and that Eps15 depletion resulted in the accumulation of both Cx43 at the cell surface and gap junction plaques. Having shown that the expression of a chimeric Cx43 fused to ubiquitin induces the destabilization of gap junction plaques and reduces the surface stability of non-ubiquitinated Cx43, we attempted to ascertain if this results from an increased interaction with Eps15. To address this question, Cos-7 cells were transfected with Eps15 and wild type Cx43 either in the presence or absence of Cx43-

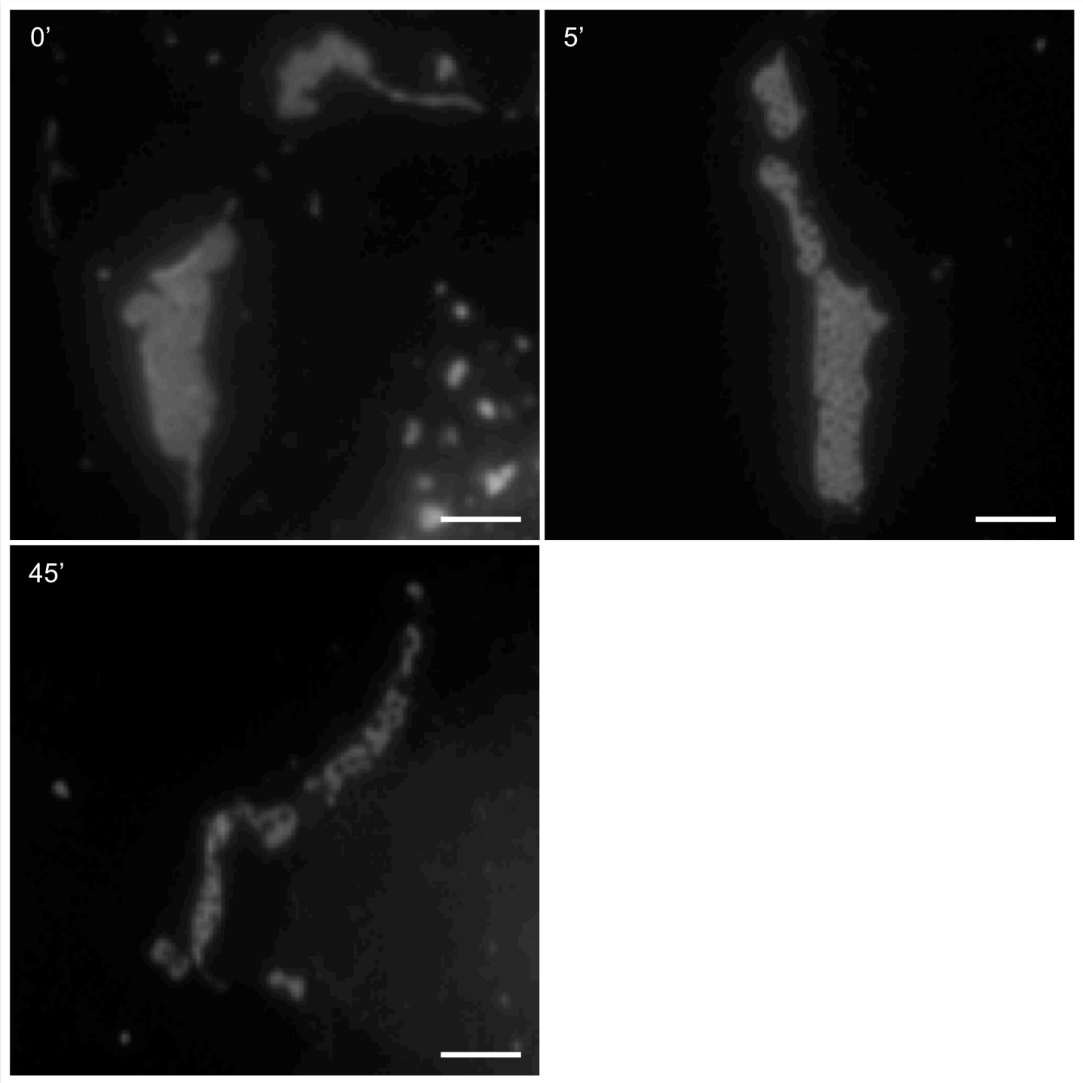

Figure 40 - PMA induces the internalization of small sections of gap junction plaques. Cos-7 cells transfected with GFP-Cx43Y286A were treated with 50 nM of PMA for 0, 5 or 45 minutes before being fixed and imaged by confocal microscopy. 5 minutes of PMA treatment led to a substantial increase in the size and number of holes present in gap junction plaques. After 45 minutes of treatment few gap junction plaques still remained. Scale bars 1.3 μm.

Ub. Cx43 was then selectively immunoprecipitated using polyclonal antibodies against Cx43, followed by Western blot and probing with antibodies against Eps15 (Figure 41A). The results obtained clearly show that, in cells co-expressing Cx43-Ub, the amount of Eps15 co-immunoprecipitated with anti-Cx43 antibodies is higher than in cells expressing Cx43 wild type alone.

To demonstrate that Eps15 is important for Cx43-Ub-triggered internalization of Cx43, endogenous Eps15 was depleted using siRNA, after which the levels of Cx43 at the plasma membrane, either in the presence or absence of Cx43-Ub, were determined by biotinylation assays. Cos-7 cells were first transfected with siRNA targeted against Eps15. Following 24 hours, cells were then transfected with either wild type Cx43, Cx43-Ub or both. After another 24 hours, cells were subjected to cell surface protein biotinylation. Biotinylated proteins from cell lysates were isolated with Neutravidin beads and the samples analyzed by Western blot using polyclonal antibodies

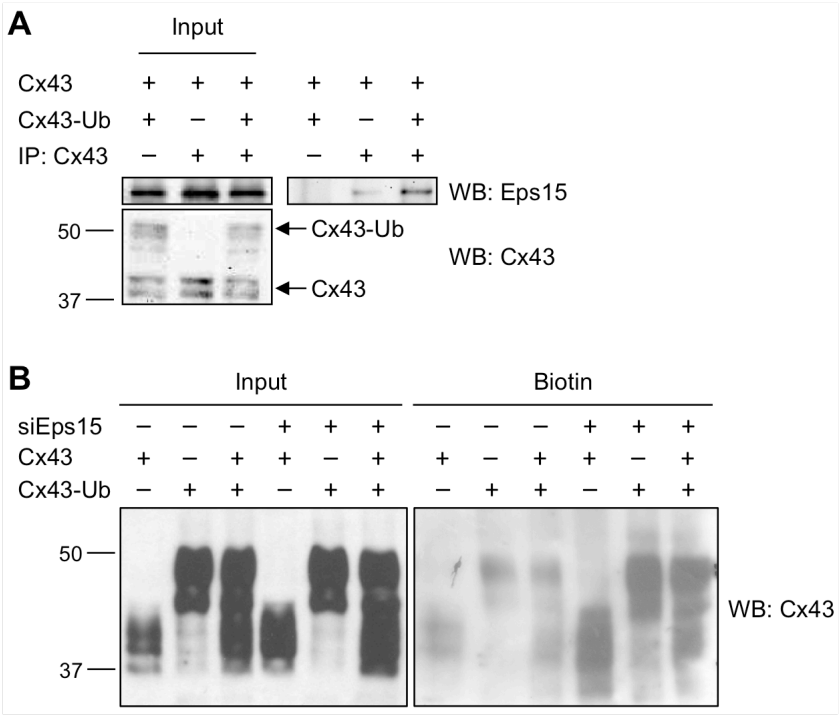

Figure 41 - Internalization of Cx43 induced by Cx43-Ub depends on the interaction with Eps15. (A) Cos-7 cells were co-transfected with Cx43 and Eps15 either alone or together with Cx43-Ub. Lysates were then immunoprecipitated with polyclonal antibodies against Cx43 and the samples analyzed by Western blot using antibodies against Eps15 (upper panels) or Cx43 (lower panel). Eps15 displays increased interaction with Cx43 when Cx43-Ub is co-expressed. (B) Cos-7 cells were transfected with Cx43 and/or Cx43-Ub alone or together with siRNA for Eps15. Cells were then subjected to cell surface protein biotinylation and the biotinylated fraction of the cell lysates was precipitated with Neutravidin beads. Precipitates were analyzed by Western blot using polyclonal antibodies against Cx43. Cx43 and Cx43-Ub both accumulate at the plasma membrane in cells depleted of Eps15 when compared to control cells.

directed against Cx43. Given that Eps15 was previously shown to be important for the internalization of Cx43, and that Cx43 ubiquitination was shown to be important for the recognition of the protein by Eps15 (Chapter 5), we predicted that the depletion of Eps15 would lead to the accumulation of Cx43-Ub at the plasma membrane. Indeed, data depicted in Figure 41B confirms this hypothesis as Eps15 depletion induced the accumulation of both Cx43 and Cx43-Ub at the plasma membrane.

Depletion of Nedd4 leads to the accumulation of Cx43-Ub at the plasma membrane

Leykauf et al. have shown that Nedd4 depletion leads to the accumulation of gap junction plaques (Leykauf et al, 2006). Furthermore, in the previous chapter it was also shown that mutation of the PY-motif in Cx43 or impairment of Nedd4 function (through the expression of a ligase dead form of the protein or siRNA depletion) reduced the levels of ubiquitinated Cx43. Similar conditions were also shown to impair the interaction of Cx43 with Eps15. Thus we attempted to evaluate whether Nedd4 was still required for the internalization of Cx43 when in the presence of Cx43-Ub. Cos-7 cells were first transfected with siRNA targeted against Nedd4. Following 24 hours, cells were then transfected with either wild type Cx43, Cx43-Ub or both. After another 24 hours, cells were

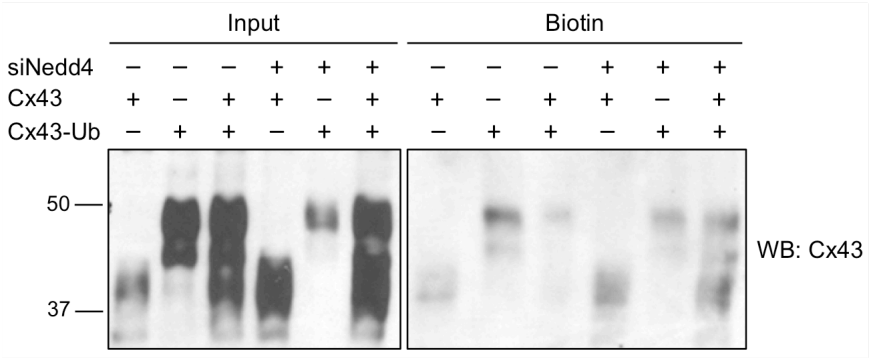

Figure 42 - Nedd4 depletion stabilizes ubiquitinated Cx43 at the plasma membrane. Cos-7 cells were transfected with Cx43 and/or Cx43-Ub alone or together with siRNA for Nedd4. Cells were then subjected to cell surface protein biotinylation and the biotinylated fraction of the cell lysates was precipitated with Neutravidin beads. Precipitates were analyzed by Western blot using polyclonal antibodies against Cx43. Cx43 and Cx43-Ub both accumulate at the plasma membrane in cells depleted of Nedd4 when compared to control cells.

subjected to cell surface protein biotinylation. Biotinylated proteins from cell lysates were isolated with Neutravidin beads and the samples analyzed by Western blot using polyclonal antibodies directed against Cx43. Interestingly, data depicted in Figure 42 shows that the depletion of Nedd4 also leads to the accumulation of Cx43 and Cx43-Ub at the plasma membrane.

Given that in the previous chapter Eps15 was shown to be important for the internalization of Cx43, and that Cx43 ubiquitination was shown to be important for Eps15 recognition of the protein, it was not surprising that depletion of Eps15 led to the accumulation of Cx43-Ub at the plasma membrane (Figure 41B). On the other hand, the similar effect resulting from Nedd4 depletion is harder to explain. Nedd4 is suggested to mediate Cx43 internalization by ubiquitinating the protein. As such, we expected that the expression of Cx43-Ub would bypass the necessity of Nedd4 ubiquitination of the protein, however, Nedd4 depletion not only induced the accumulation of Cx43 at the plasma membrane, it also resulted in the accumulation of the Cx43-Ub chimera. Previous reports have suggested that a single ubiquitin moiety is insufficient to signal the internalization of plasma membrane proteins, since the UBD domains present in the necessary endocytic adaptors bind poorly to monoubiquitin (Barriere et al, 2006; Hawryluk et al, 2006). Thus, it is possible that the single ubiquitin present in Cx43-Ub is not sufficient to drive the internalization of the protein, even when multiple Cx43-Ub monomers are oligomerized into the same connexon, and that Nedd4 activity is still required for further Cx43 ubiquitination. Alternatively, and given the role of Nedd4 in ubiquitinating multiple other plasma membrane proteins and endocytic adaptor proteins, it is possible that impairing Nedd4 function has other negative effects on Cx43 internalization, that are independent of Cx43 ubiquitination.

Ubiquitinated Cx43 is internalized through both clathrin-dependent and clathrin-independent pathways

Although the role of clathrin-mediated endocytosis in Cx43 internalization has been extensively

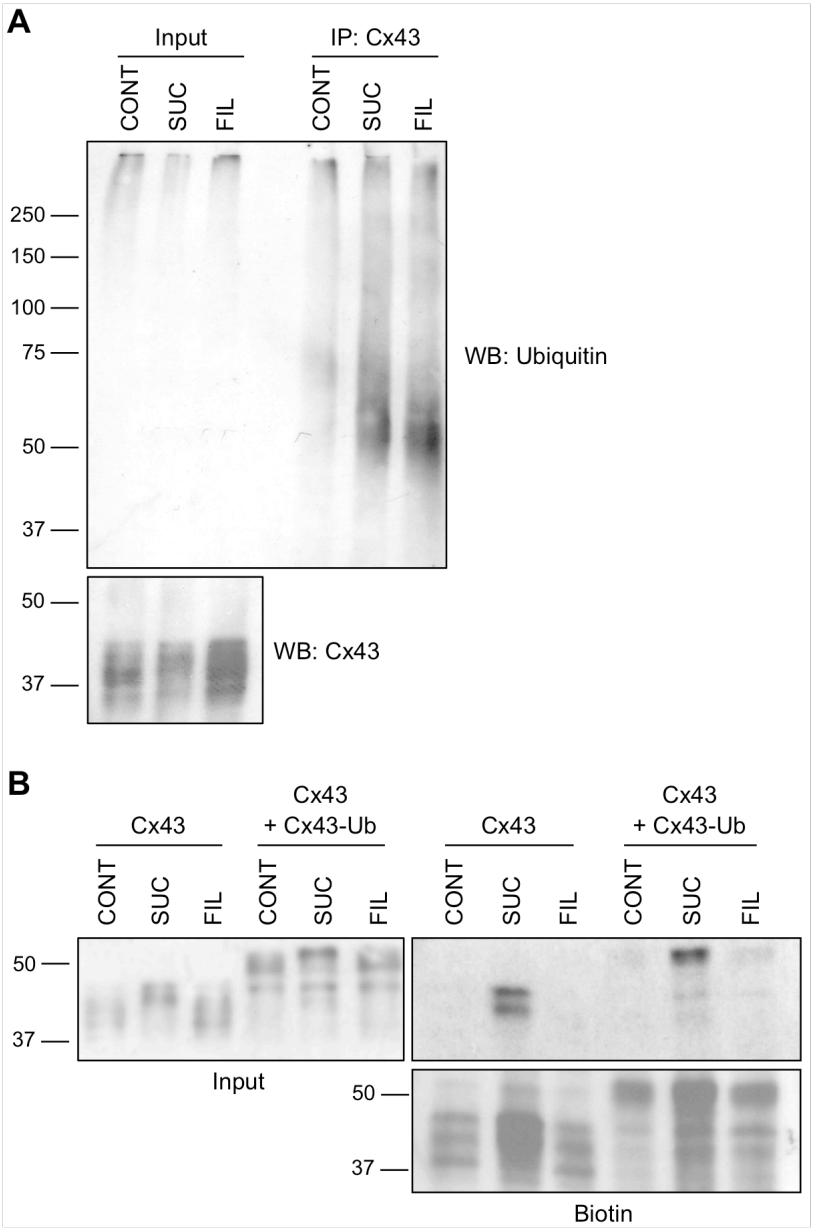

Figure 43 - Ubiquitinated Cx43 is internalized though both CME and CIE. (A) HEK293FT cells transfected with Cx43 were treated with 0.45 M of sucrose or 5 μg/mL of Filipin III for 1 hour. Lysates were then immunoprecipitated using polyclonal against Cx43 and the immunoprecipitates analyzed by Western blot using monoclonal antibodies against ubiquitin (P4D1). Both inhibition of CME with sucrose and inhibition of CIE with Filipin III, lead to an increase in ubiquitinated Cx43 levels. (B) Cos-7 cells transfected with Cx43 and/or Cx43-Ub were treated with 0.45 M of sucrose or 5 μg/mL of Filipin III for 1 hour before being subjected to cell surface protein biotinylation. The biotinylated fraction of the cell lysates was precipitated with Neutravidin beads and the precipitates analyzed by Western blot using polyclonal antibodies against Cx43. Inhibition of CME leads to the accumulation of both Cx43 and Cx43-Ub at the cell surface. However, inhibition of CIE only leads to the accumulation of Cx43 at the cell surface when Cx43-Ub is present.

characterized, several reports suggest that Cx43 can also be internalized through a clathrin-independent mechanism that may involve caveolae (Langlois et al, 2008; Schubert et al, 2002). To assess the internalization pathway followed by ubiquitinated Cx43, HEK293FT cells were transfected with Cx43 and then treated for 1 hour with either 0.45 M sucrose, which creates hypertonic conditions that inhibit CME, or with 5 μg/ml of Filipin III, an antibiotic that sequesters membrane cholesterol and inhibits clathrin-independent endocytosis. Cell lysates were then

precipitated with polyclonal antibodies directed against Cx43 and samples were subsequently analyzed by Western blot using monoclonal antibodies against Cx43 or ubiquitin (P4D1). As shown in Figure 43A, inhibition of both clathrin-dependent and clathrin-independent pathways led to the accumulation of ubiquitinated Cx43 when compared to controls. This data suggests that both pathways are responsible for the internalization of ubiquitinated Cx43.

To further characterize the importance of each pathway in the internalization of ubiquitinated Cx43, experiments were performed using the Cx43-Ub chimera. Cos-7 cells were transfected with wild type Cx43, either alone or alongside Cx43-Ub, and then treated during 1 hour with either 0.45 M of sucrose or with 5 μg/ml of Filipin III. Cells were then subjected to cell surface protein biotinylation and biotinylated proteins were precipitated using Neutravidin beads. Samples were subsequently analyzed by Western blot using polyclonal antibodies directed against Cx43. As depicted in Figure 43B, inhibition of CME led to the accumulation of Cx43 at the plasma membrane both in the presence and absence of Cx43-Ub. Interestingly, inhibition of clathrin-independent endocytosis only led to an accumulation of Cx43 at the cell surface when the Cx43-Ub chimera was present. Thus, although clathrin-dependent mechanisms appear to be involved in the internalization of both Cx43 and ubiquitinated Cx43, clathrin-independent mechanisms appear to be more specific towards the latter.

Cx43 colocalizes with the ESCRT components Hrs and Tsg101

Both the proteasome and the lysosome have been implicated in Cx43 degradation (Laing et al, 1997). However, the effect of proteasome inhibition upon Cx43 stability is likely to result from the depletion of free ubiquitin, which would impede Cx43 ubiquitination and subsequent internalization, rather than any role of the proteasome in directly degrading the protein. Thus, the lysosome is the main site of Cx43 degradation. The bulk of internalized plasma membrane proteins are normally routed back to the plasma membrane after reaching the early endosome. For internalized membrane proteins to be directed to the lysosome, they must first be sorted away from the default recycling pathway. Furthermore, to enable specificity to this sorting mechanism, proteins require a sorting signal. Ubiquitination has been shown to function as one such signal, being recognized by the ESCRT sorting machinery, which directs ubiquitinated proteins to the interior of MVBs where they are eventually delivered to the lysosomal compartment. Given how we have shown that Cx43 is ubiquitinated and that this modification functions as an internalization signal, we decided to investigate whether Cx43 interacts with subunits of the ESCRT machinery. Hrs is a component of ESCRT-0, while Tsg101 is a component of ESCRT-I; both proteins contain UBD domains and are thought to be involved in the recognition and binding to ubiquitinated substrates. As such, they are prime targets for interaction with Cx43. To evaluate the subcellular distribution of Cx43 and Hrs, Cos-7 cells were transfected with plasmids encoding FLAG tagged Hrs (Hrs-FLAG). Cells were

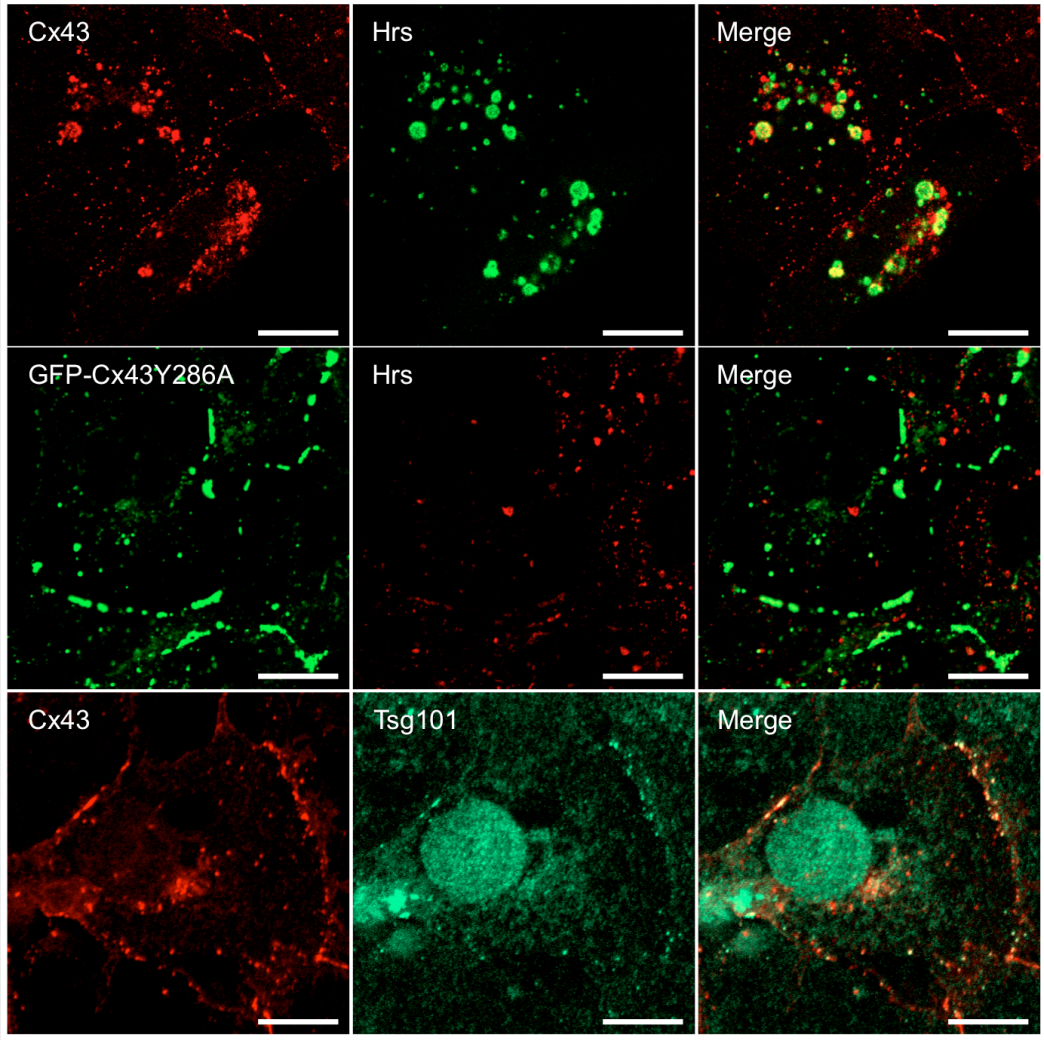

Figure 44 - Cx43 colocalizes with the ESCRT components Hrs and Tsg101. Cos-7 cells were transfected with Hrs-FLAG alone (upper panels) or with GFP-Cx43Y286A (middle panels). Cells were fixed and stained either with monoclonal antibodies against Cx43 (upper and lower panels), polyclonal antibodies against FLAG (upper and middle panels) or polyclonal antibodies against Tsg101 (lower panels). Cx43 colocalizes with Hrs and Tsg101 in intracellular vesicles and also at the plasma membrane. Colocalization of Hrs with gap junction plaques is significantly increased in the presence of Cx43Y286A. Scale bars, 50μm.

then fixed and stained with monoclonal antibodies directed against Cx43 and polyclonal antibodies directed against the FLAG tag. Cells were subsequently imaged by immunofluorescence confocal microscopy. Figure 44 (top panels) shows that Cx43 colocalizes with Hrs mainly in endocytic vesicles, presumably MVBs, with some colocalization in regions near the plasma membrane. The subcellular distribution of Hrs was also compared to that of Cx43Y286A using Cos-7 cells co-transfected with both GFP-Cx43Y286A and Hrs-FLAG. As seen in Figure 44 (middle panels), Hrs colocalization with Cx43 at the plasma membrane is substantially increased in cells overexpressing the mutant Cx43 with impaired internalization. Furthermore, colocalization in intracellular vesicles is substantially decreased. Taken together with the data presented in Figure 38, showing that expression of GFP-Cx43Y286A led to a substantial increase in the staining of ubiquitin at gap junction plaques, the increase in Hrs localization at gap junction plaques may be

due to the increased presence of ubiquitinated proteins at the plasma membrane.
To evaluate the subcellular distribution of Cx43 and Tsg101, Cos-7 cells grown on glass coverslips were fixed and stained with monoclonal antibodies directed against Cx43 and polyclonal antibodies directed against Tsg101. Cells were then imaged by immunofluorescence confocal microscopy. As seen in Figure 44 (bottom panels), Tsg101 colocalizes with Cx43 both at the plasma membrane and in intracellular vesicles, presumably MVBs.
Taken together, these results suggest that Cx43 may be sorted to the lysosome in early endosomes through interactions mediated by the ESCRT machinery. It should be noted that during the time in which this work was carried out, other authors have published studies demonstrating that Hrs and Tsg101 are important for the internalization and endosomal sorting of Cx43 to late endosomes and lysosomes (Auth et al, 2009; Leithe et al, 2009).

The DUB UBPY modulates the intracellular sorting of Cx43

Although several reports indicate that Cx43 can be ubiquitinated, as of yet, no studies have focused on the reversal of this modification carried out by DUBs. The DUB UBPY has been shown to interact with several components of the ESCRT machinery (Clague & Urbe, 2006; Raiborg & Stenmark, 2009), and as such we decided to investigate the importance of UBPY for Cx43 intracellular trafficking. As a first approach, the subcellular distribution of both Cx43 and UBPY was evaluated using Cos-7 cells co-transfected with RFP-tagged Cx43 (RFP-Cx43) and V5-tagged UBPY (V5-UBPY). Cells were subsequently fixed and stained with monoclonal antibodies directed against the V5 tag. As shown in Figure 45A, Cx43 colocalizes with UBPY mainly in intracellular vesicles.
Next, the effect of UBPY overexpression and depletion upon Cx43 levels and subcellular distribution was evaluated in immunoprecipitation and immunofluorescence experiments. Cos-7 cells were co-transfected with Cx43 and either plasmids encoding UBPY, or shRNA directed against UBPY. Cell lysates were then precipitated with antibodies directed against Cx43 and the samples analyzed by Western blot using monoclonal antibodies directed against Cx43 or ubiquitin. Alternatively, cells were fixed and stained with monoclonal antibodies directed against Cx43 and polyclonal antibodies directed against ubiquitin. Data presented in Figure 45B shows that overexpression of UBPY slightly reduces the levels of both immunoprecipitated Cx43 and ubiquitinated Cx43 when compared to controls. Conversely, shRNA depletion of UBPY slightly increases the levels of both Cx43 and ubiquitinated Cx43. Furthermore, immunofluorescence data presented in Figure 46 shows that overexpression of UBPY leads to the disappearance of intracellular vesicles containing Cx43, while the shRNA depletion of UBPY induces the accumulation of Cx43 in intracellular vesicles that colocalize with ubiquitin. Taken together, this data suggests that UBPY is important for the intracellular sorting of Cx43 to the endolysosomal

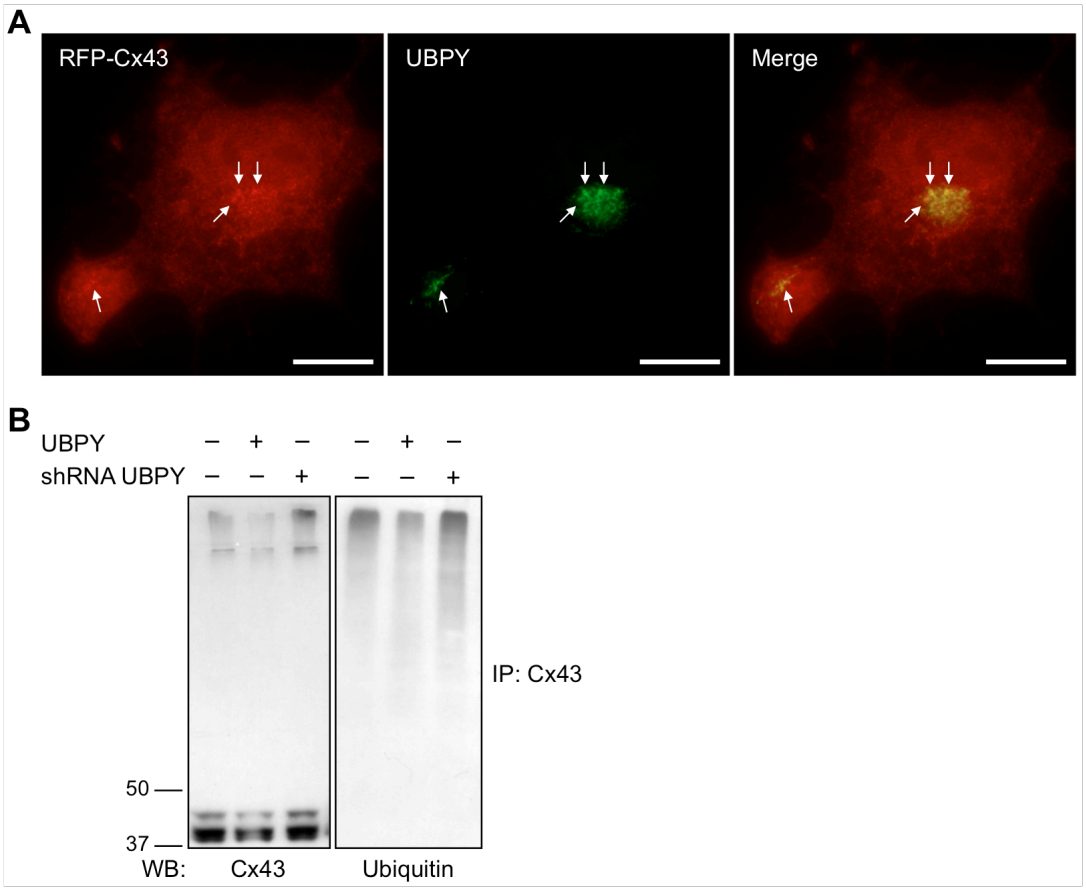

Figure 45 - UBPY colocalizes with Cx43 and modulates its ubiquitinated state. (A) Cos-7 cells transfected with RFP-Cx43 and V5-UBPY were fixed and stained with monoclonal antibodies against V5. UBPY colocalizes with Cx43 in intracellular vesicles. Scale bars, 100 μm. (B) Lysates from Cos-7 cells transfected with Cx43 and either UBPY or shRNA against UBPY, were immunoprecipitated using polyclonal antibodies against Cx43. Precipitates were then analyzed by Western blot with monoclonal antibodies against Cx43 and ubiquitin (P4D1). UBPY overexpression leads to a decrease in both Cx43 and ubiquitinated Cx43 levels, conversely, shRNA depletion of UBPY leads to a slight increase in both Cx43 and ubiquitinated Cx43 levels.

compartment. Although the UBPY overexpression data showing diminished levels of ubiquitinated Cx43 may suggest that UBPY directly deubiquitinates Cx43, taken together with the immunoprecipitation data showing a decrease in overall Cx43 levels and the immunofluorescence data showing a decrease in intracellular Cx43 levels without any apparent changes in surface expression, it is likely that UBPY promotes Cx43 sorting through the activation of monoubiquitinated ESCRT components. Nevertheless, the possibility that UBPY promotes Cx43 sorting by deubiquitinating the protein at late stages of ESCRT sorting cannot be discarded.

Cx43 interacts and colocalizes with markers of recycling endosomes

Most plasma membrane proteins that are internalized through constitutive endocytic mechanisms are often recycled back to the plasma membrane due to the absence of sorting signals, such as ubiquitination, directing them to the endolysosomal pathway. As the majority of Cx43 gap junctions are internalized in a constitutive manner in the absence of ubiquitination, it is likely that gap

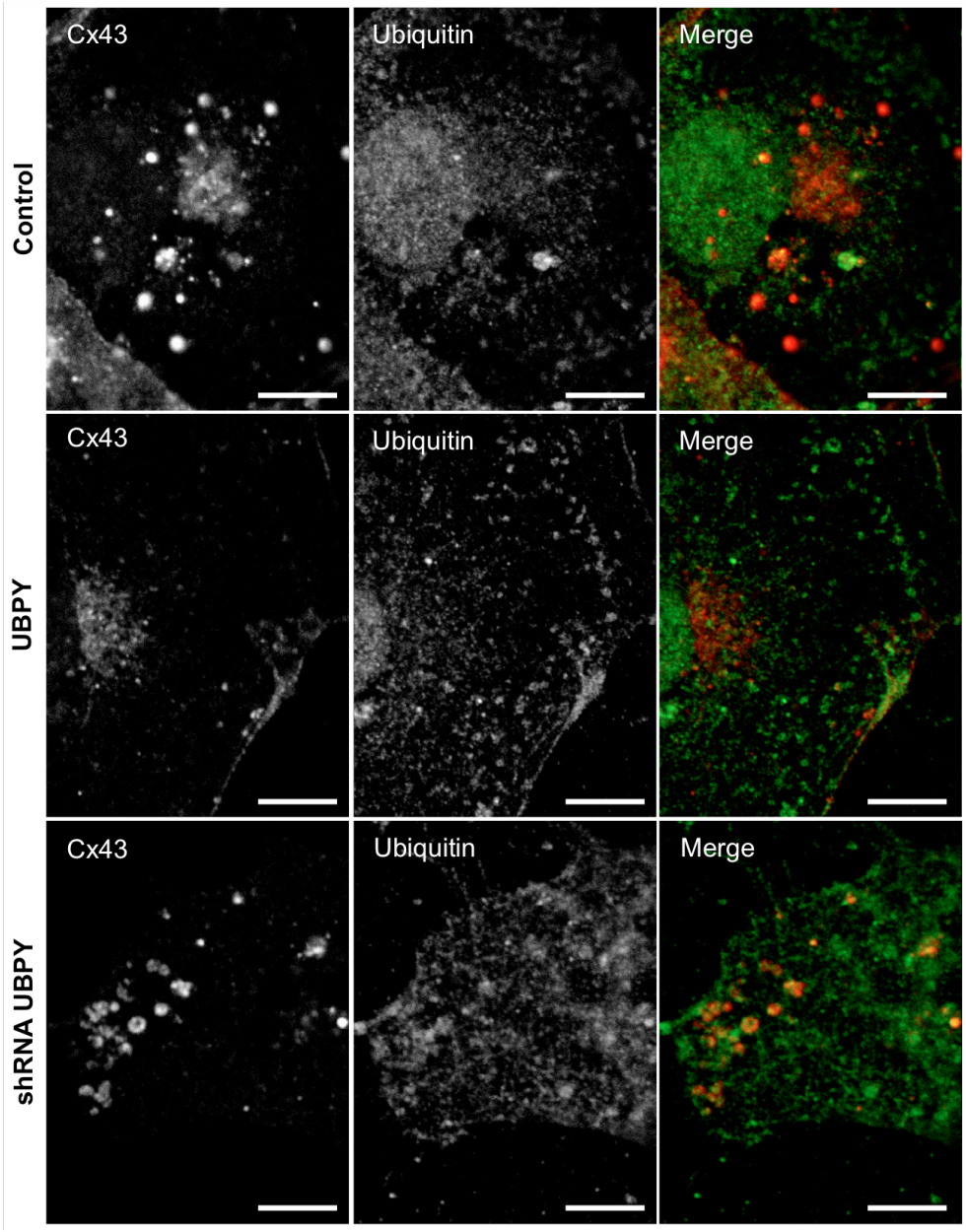

Figure 46 - UBPY modulates the intracellular trafficking of Cx43. Cos-7 cells transfected with either UBPY or shRNA against UBPY were fixed and stained with monoclonal antibodies against Cx43 (BD Transduction) or polyclonal antibodies against ubiquitin. Overexpression of UBPY leads to the disappearance of intracellular vesicles containing Cx43, while shRNA depletion of UBPY induces the accumulation of Cx43 in intracellular vesicles that colocalize with ubiquitin. Scale bars, 25 μm.

junctions can also be recycled back to the plasma membrane following endocytosis. To investigate whether or not Cx43 is a substrate for endocytic recycling pathways, subcellular colocalization experiments were carried out using Rab4 and Rab11 as markers for recycling vesicles. Cos-7 cells were transfected with plasmids encoding either YFP-tagged Rab4 (YFP-Rab4) or GFP-tagged Rab11 (GFP-Rab11). Cells were subsequently fixed and stained with monoclonal antibodies directed against Cx43. As depicted in Figure 47, Cx43 present in intracellular vesicles colocalizes extensively with Rab4, showing a more moderate colocalization with Rab11, indicating that Cx43 can be found in endocytic recycling vesicles, and thus, may be recycled back to the plasma

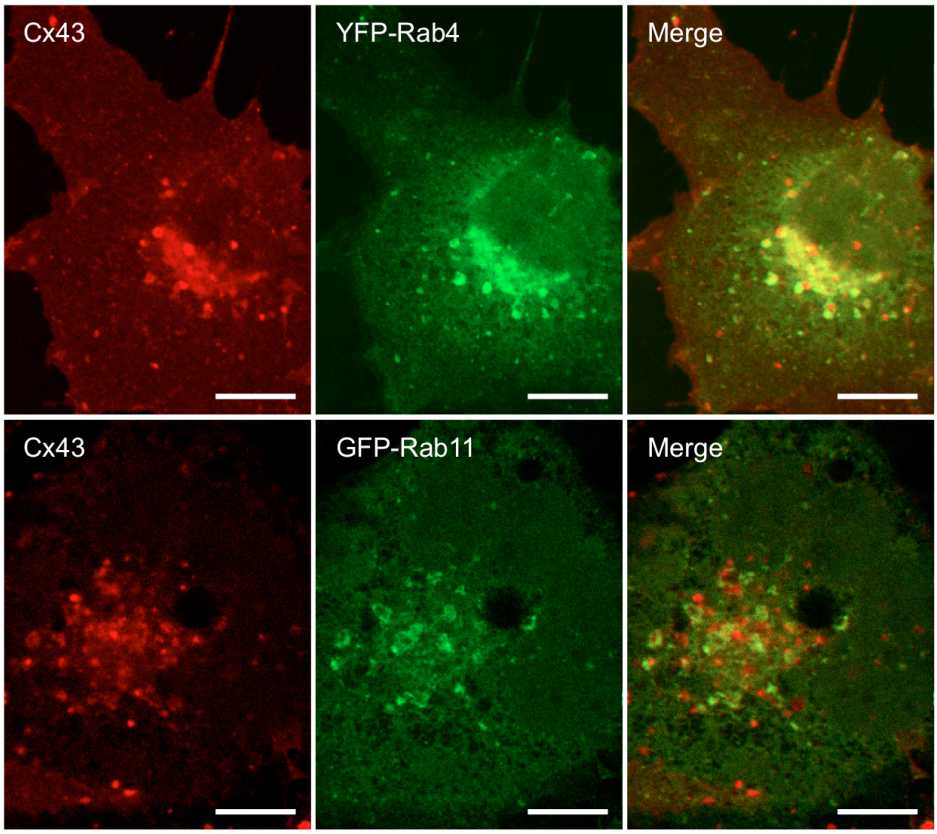

Figure 47 - Cx43 colocalizes with Rab4 and Rab11. Cos-7 cells transfected with either YFP-Rab4 or GFP-Rab11 were fixed and stained with monoclonal antibodies against Cx43 (BD Transduction). Cx43 can be found in intracellular vesicles positive for Rab4 or Rab11. Scale bars, 50 μm.

membrane.

To further characterize the presence of Cx43 in these vesicles, Cos-7 cells were co-transfected with Cx43 and either YFP-Rab4 or GFP-Rab11. Lysates were precipitated with polyclonal antibodies directed against Cx43 and the shuamples analyzed by Western blot using monoclonal antibodies directed against GFP. Alternatively, following transfection cells were subjected to cell surface protein biotinylation. Biotinylated proteins were then precipitated using Neutravidin beads and the samples analyzed by Western blot using polyclonal antibodies directed against Cx43. As seen in Figure 48, both Rab4 and Rab11 co-immunoprecipitate with Cx43 (Figure 48A). Furthermore, cells overexpressing either Rab protein display increased levels of Cx43 at the cell surface (Figure 48B). This data strongly suggests that Cx43 can be recycled back to the plasma membrane through both the rapid recycling pathway (Rab4) and the slow recycling pathway (Rab11).

Discussion

Although it was previously shown that Cx43 is ubiquitinated, the role of this post-translational modification on gap junction internalization and degradation has never been clearly demonstrated.

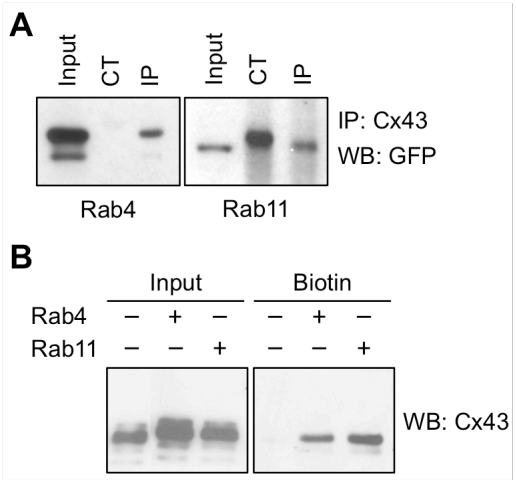

Figure 48 - Rab4 and Rab11 stabilize Cx43 at the plasma membrane. (A) Lysates of Cos-7 cells co-transfected with Cx43 and either YFP-Rab4 or GFP-Rab11 were immunoprecipitated with polyclonal antibodies against Cx43 and the precipitates analyzed by Western blot using monoclonal antibodies against GFP. Cx43 interacts with Rab4 and Rab11. (B) Cos-7 cells co-transfected with Cx43 and either YFP-Rab4 or GFP-Rab11 were subjected to cell surface protein biotinylation. The biotinylated fraction of the cell lysates was precipitated using Neutravidin beads and the precipitates analyzed by Western blot using monoclonal antibodies against Cx43. Overexpression of both Rab4 or Rab11 induces an accumulation of Cx43 at the cell surface.

The results presented here show, for the first time, that the attachment of ubiquitin to Cx43 is sufficient to trigger its internalization and subsequent degradation. Moreover, we demonstrate that the internalization triggered by ubiquitin is independent of the endocytic YXXØ sorting signal present on the carboxyl-terminal of Cx43. Indeed, expression of a Cx43-Ub chimera is sufficient to drive the internalization and degradation of both wild type Cx43 and of Cx43Y286A, a mutant form of the protein that presents a defect in CME due to a mutation in the YXXØ sorting motif. Using CHX-chase assays we showed that expression of Cx43-Ub decreases the half-life of both Cx43 and Cx43Y286A by about 50%. Consistently, the half-life of both forms of Cx43 at the plasma membrane surface also decreases in the presence of Cx43-Ub. Data obtained with the Cx43Y286A mutant is particularly important since the main internalization pathway of Cx43, that requires an intact YXXØ sorting motif, is impaired. In this particular situation, ubiquitination would constitute the main internalization signal able to trigger the internalization of mutant Cx43 present at gap junction plaques. We also demonstrate that clathrin-independent endocytosis of Cx43 is mainly involved in the internalization of ubiquitinated Cx43, although ubiquitinated Cx43 can also be internalized through clathrin-dependent mechanisms. Furthermore, we partially characterize the intracellular route followed by Cx43 following internalization, demonstrating that the protein can be found in intracellular vesicles that colocalize with Hrs and Tsg101. Moreover, it is also demonstrated that UBPY modulates the intracellular trafficking of Cx43. Lastly, Cx43 was shown to be present in intracellular vesicles positive for Rab4 and Rab11, which are common markers of rapid recycling endosomes and of the endocytic recycling compartment respectively.

The monoubiquitination of plasma membrane proteins is suggested to target them for internalization, in a process mediated by endocytic adaptors containing ubiquitin-binding domains

(UBDs). However, recent studies have challenged this notion since it was shown that UBD domains have a rather low affinity for monoubiquitin (Barriere et al, 2006; Hawryluk et al, 2006). Instead, UBD domains were shown to have a higher affinity for Lys63-linked polyubiquitin chains. Additionally, Barriere et al. have shown that the monoubiquitination of several subunits in an oligomeric membrane protein, but not the monoubiquitination of a single monomeric protein, could also function as an internalization signal. It was suggested that multiple monoubiquitin moieties in close spatial proximity, either due to the multimonoubiquitination of a single protein or due to the monoubiquitination of several subunits in an oligomeric complex, would provide UBD domains with multiple interaction points, allowing them to bind to these signals with similar affinities as to the binding of polyubiquitin chains (Barriere et al, 2006). We hypothesized that the Cx43-Ub chimera induces the internalization of Cx43 by oligomerizing into the same connexon channels, directly increasing the ubiquitin internalization signal present at these channels. Indeed, immunoprecipitation experiments demonstrated that Cx43 oligomerizes into the same channels as the Cx43-Ub chimera, since the selective precipitation of one form of the protein also induced the co-precipitation of the other form. Further evidence supporting that both proteins can be found within the same hemichannels stems from the observation that Cx43-Ub colocalizes with both Cx43 and Cx43Y286A. Additionally, our data demonstrates that the presence of ubiquitin in Cx43-containing hemichannels leads to an increased interaction with Eps15. Altogether, we suggest that the increased internalization and degradation of Cx43 induced by the Cx43-Ub chimera is due to the ubiquitin moiety.

Plasma membrane proteins containing YXXØ sorting signals are recognized by endocytic adaptor proteins, such as AP2, and internalized through clathrin-mediated endocytosis (Doherty & McMahon, 2009). Accordingly, previous reports have demonstrated that the tyrosine-based motif present in Cx43 regulated the turnover and surface stability of the protein (Thomas et al, 2003). Moreover, the YXXØ sorting motif of Cx43 also overlaps with a PPXY-motif that is important for Cx43 interaction with Nedd4 and ubiquitination (Chapter 5). Indeed, we showed that mutation of Tyr286, a critical residue of both the PPXY- and YXXØ-motifs, impairs Cx43 ubiquitination and stabilizes the protein at the cell surface, leading to the formation of large gap junction plaques. Moreover, and similar to what happens with wild type Cx43, we also demonstrate that the overexpression of Cx43-Ub induces the destabilization of the large plaques formed by Cx43Y286A. Close analysis of the plaques formed by Cx43Y286A reveal that, following expression of Cx43-Ub, intracellular vesicles positive for both Cx43Y286A and Cx43-Ub can be found in close proximity to small holes present on the gap junction plaques. Presumably these vesicles correspond to sections of the gap junction plaque that have been recently internalized. Furthermore, treatment of cells with PMA, which is known to induce the ubiquitination and subsequent internalization of Cx43 gap junction plaques (Leithe & Rivedal, 2004b), was shown to dramatically increase the number of these holes on Cx43Y286A gap junction plaques, ultimately

leading to the reduction of gap junction plaque size. Interestingly, although the Y286A mutation significantly decreases Cx43 ubiquitination, the large gap junction plaques formed by Cx43Y286A show increased colocalization with ubiquitin when compared to plaques formed by wild type Cx43. If Cx43 ubiquitination indeed functions as an internalization signal, as the expression of Cx43-Ub seems to indicate, why is the internalization of Cx43Y286A compromised when its gap junctions seemingly present increased colocalization with ubiquitin? The immunofluorescence microscopy data does not rule out the possibility that the increased ubiquitin signal present at Cx43Y286A gap junction plaques is due to the accumulation of ubiquitinated proteins proximal to gap junction sites, rather than the direct conjugation of ubiquitin to gap junction channels. In this case, Cx43Y286A accumulated at the plasma membrane would act as a scaffold that sequesters ubiquitinated proteins at gap junction plaques. Known Cx43 binding partners that are substrates for ubiquitination include E-cadherin (Fujita et al, 2002), occludin (Traweger et al, 2002) and β-catenin (Aberle et al, 1997).

Following endocytosis, plasma membrane proteins are usually delivered to early endosomes where protein sorting occurs. At early endosomes, ubiquitinated membrane proteins interact with the ESCRT machinery and are sorted to endolysosomal compartments, where they are eventually degraded (Raiborg & Stenmark, 2009; Williams & Urbe, 2007). While Cx43 gap junctions plaques are known to internalize as large double membrane vesicles termed annular gap junctions (Jordan et al, 2001), a more recent study demonstrated that these structures are broken down from double membrane structures to connexin-enriched multivesicular endosomes prior to reaching the lysosome (Leithe et al, 2006), indicating that internalized annular gap junctions follow the conventional sorting route to the lysosome. Through immunofluorescence imaging experiments, we show here that Cx43 can be found in intracellular vesicles that are positive for Hrs and Tsg101, which are important components of the ESCRT machinery. These results are in accordance with recently published reports that demonstrated that both Hrs and Tsg101 are important for the internalization and sorting of Cx43 to late endosomes and lysosomes (Auth et al, 2009; Leithe et al, 2009). As such, we hypothesized that the oligomerization of Cx43-Ub into Cx43 hemichannels would also increase the degradation rate of Cx43 by enhancing the interaction with sorting machinery. Indeed, CHX-chase experiments demonstrated that expression of the Cx43-Ub chimera reduced the half-life of both wild type and mutant Y286A forms of Cx43. We also show that the presence of lysosome inhibitors leads to the accumulation of Cx43, even in the presence of Cx43-Ub. Moreover, we show that the Cx43-Ub chimera colocalizes with the endosomal markers Rab5 and Rab7 in intracellular vesicles, further reinforcing that the increased internalization induced by ubiquitin results in Cx43 degradation.

Several authors have already shown that Cx43 is a substrate for ubiquitination and that a small population of ubiquitinated Cx43 can be found at the plasma membrane within gap junction plaques (Laing & Beyer, 1995; Rutz & Hulser, 2001). Although for some receptors ubiquitin is not

required for internalization, but rather for signalling intracellular sorting to lysosomes, this does not appear to be the case for Cx43. Data obtained in this study with the Cx43Y286A mutant, together with those obtained by others (Thomas et al, 2003), show that constitutive internalization of Cx43 occurs mainly through the canonical AP2 dependent clathrin-mediated endocytosis. However, the presence of ubiquitin in both wild type and mutant Cx43 hemichannels accelerates the internalization and degradation of the proteins, thus suggesting that ubiquitin is likely to be important in the internalization of Cx43. Leithe et al. have shown that phosphorylation of Cx43 induced by EGF or PMA treatment leads to Cx43 ubiquitination and internalization (Leithe & Rivedal, 2004a; Leithe & Rivedal, 2004b). Furthermore, data from Leykauf et al. shows that phosphorylated Cx43 has a higher binding affinity for the ubiquitin ligase Nedd4 and that Nedd4 depletion leads to the accumulation of Cx43 at the plasma membrane (Leykauf et al, 2006). In the previous chapter we demonstrated that Nedd4-mediated ubiquitination of Cx43 is important for the subsequent interaction of the protein with Eps15, and that this interaction occurred through the UIM domains of Eps15. Depletion of Eps15 also led to the accumulation of Cx43 at the cell surface. As such, depletion of Eps15, but not of Nedd4, was expected to prevent the destabilization effects of Cx43-Ub expression upon the surface expression of Cx43. Indeed, siRNA depletion of Eps15 was shown to stabilize both Cx43 and Cx43-Ub at the plasma membrane. Surprisingly, the same results were obtained when Nedd4 levels were depleted by siRNA. Although the expression of Cx43-Ub was expected to bypass the necessity of Nedd4-mediated Cx43 ubiquitination, it is possible that the single ubiquitin moiety present on Cx43-Ub is still insufficient to signal Cx43 for internalization, even when more than one Cx43-Ub monomer is present within the same hemichannel. As such, Nedd4 activity would be necessary to further ubiquitinate Cx43, in order to allow the protein to be recognized by endocytic adaptors. In this situation, expression of the Cx43-Ub chimera would increase Cx43 internalization by increasing the local concentration of ubiquitin signals, thus lowering the rounds of ubiquitination that must occur before Cx43 can be recognized by endocytic adaptors. However, we cannot exclude that Nedd4 activity may be necessary for Cx43 internalization, through a mechanism that is independent of Cx43 ubiquitination. Importantly, several endocytic proteins, including Eps15, are regulated by Nedd4-mediated ubiquitination (Hoeller et al, 2006). Thus, the observed effect of Nedd4 depletion may be due to an indirect effect upon Eps15 or other endocytic adaptors.

Although the role of clathrin-mediated endocytosis in Cx43 internalization has been extensively characterized, data from several reports suggest that CME is only responsible for 55% of internalized annular gap junctions (Gumpert et al, 2008; Piehl et al, 2007). Additionally, Cx43 has been shown to interact with both Cav-1 and Cav-2 and to target to lipid raft domains, suggesting that the protein can be internalized through a clathrin-independent mechanism that may involve caveolae (Langlois et al, 2008; Schubert et al, 2002). As demonstrated in the previous chapter, Eps15 is involved in the internalization of ubiquitinated Cx43; and while Eps15 has been

traditionally linked to clathrin-dependent endocytosis, the endocytic adaptor has also been shown to mediate the clathrin-independent endocytosis of ubiquitinated EGFR (Sigismund et al, 2005). Thus, and in accordance with the model proposed for the internalization of ubiquitinated EGFR, we hypothesized that ubiquitinated Cx43 could be internalized through a clathrin-independent mechanism. Indeed, although inhibition of CME was shown to increase the surface expression of both Cx43 and Cx43-Ub, inhibition of CIE only led to the accumulation of Cx43 at the plasma membrane in the presence of the Cx43-Ub chimera, indicating that this pathway may have a role in the ubiquitin triggered internalization of Cx43. Thus, CME is involved in both the constitutive internalization of Cx43, via the YXXØ-motif, and in the ubiquitin-mediated internalization of the protein, while CIE is mainly involved in the internalization of ubiquitinated Cx43.

Equally important in the regulation of cellular ubiquitination are deubiquitinating enzymes (DUBs), which are proteolytic enzymes that can cleave post-translationally formed branched peptide bonds in mono or multiubiquitinated conjugates, thus having the potential to regulate any ubiquitin-mediated cellular process, including proteolysis and protein trafficking/endocytosis. However, their regulation and contribution to the control of endocytosis remains very vague. Recent reports suggest that ubiquitinating and deubiquitinating activities compete along the endocytic pathway. Additionally, it has been shown that different DUBs have crucial but distinct roles in the endocytic pathway, which most likely reflect their distinct specificities for different types of polyubiquitin chains (Clague & Urbe, 2006). As such, DUBs and Cx43 deubiquitination are likely to have different roles in modulating the intracellular trafficking of Cx43 depending on where the protein is deubiquitinated: 1) Cx43 deubiquitination at the plasma membrane would stabilize the protein at the cell surface, thus inhibiting its degradation; 2) Cx43 deubiquitination at early endosomes would rescue the protein from endolysomal sorting, redirecting the protein back to the plasma membrane; 3) Cx43 deubiquitination at late steps of endolysomal sorting would direct the protein for lysomal degradation. The DUB UBPY has been shown to interact with components of both the ESCRT-0 and the ESCRT-III complexes (Mizuno et al, 2006; Row et al, 2006), suggesting that it may be involved in the rescue of membrane proteins from being sorted into MVBs and directed for endolysosomal degradation. However, UBPY has also been shown to promote the endosomal sorting of EGFR, presumably by reversing the autoinhibitory monoubiquitination of the ESCRT-0 components Hrs and STAM (Clague & Urbe, 2006; Raiborg & Stenmark, 2009). As mentioned above, Cx43 was found to colocalize with the ESCRT components Hrs and Tsg101 in intracellular vesicles. Ubiquitination of the protein was also found to be important for Cx43 internalization and sorting to the lysosome. Thus we decided to investigate whether UBPY was involved in rescuing Cx43 from endolysosomal sorting or rather if, and as suggested for EGFR, UBPY promoted Cx43 sorting to the lysosome. Although Cx43 was shown to colocalize with UBPY and overexpression of the DUB led to diminished levels of ubiquitinated Cx43, suggesting that ubiquitinated Cx43 may be a substrate for UBPY, immunoprecipitation and immunofluorescence data suggest that UBPY

promotes the lysosomal sorting of Cx43. Indeed, overexpression of UBPY was shown to decrease the total levels of Cx43, while depletion of the protein led to a slight increase in Cx43 levels. Furthermore, immunofluorescence data showed that expression of UBPY decreased the number of intracellular vesicles positive for both Cx43 and ubiquitin. Conversely, shRNA depletion of UBPY led to the accumulation of intracellular vesicles positive for Cx43 and ubiquitin. As such, in this study we demonstrate for the first time that the DUB UBPY modulates the intracellular sorting of Cx43.

The presence of a YXXØ sorting motif on the carboxyl-terminal of Cx43 suggests that the protein is constitutively internalized in a clathrin-dependent manner. This constitutive internalization is also most likely independent of Cx43 ubiquitination. Considering that proteins without any specific sorting signal are generally recycled back to the plasma membrane, this suggests that Cx43 is also likely to be directed to endocytic recycling pathways. Furthermore, it is also possible that even ubiquitinated Cx43 may be salvaged from endolysomal degradation pathways through the action of DUBs, similar to how AMSH-mediated deubiquitination rescues EGFR from being sorted to the lysosome (Bowers et al, 2006; McCullough et al, 2004). Indeed, immunofluorescence experiments show that Cx43 can be found in intracellular vesicles that are positive for Rab4, a marker of rapid recycling endosomes, and in vesicles positive for Rab11, a marker of the endocytic recycling compartment involved with slow recycling pathways. Furthermore, both Rab proteins were shown to co-precipitate with Cx43 and overexpression of both Rab proteins led to the accumulation of Cx43 at the cell surface. This data suggests that Cx43 can be recycled back to the plasma membrane through both the rapid and slow recycling routes.

Conclusions

Although the main constitutive internalization pathway of Cx43 occurs through the canonical AP2 dependent clathrin-mediated endocytosis, ubiquitin may play an important role in regulated signal-induced gap junction internalization. Therefore, we propose a model in which under basal conditions Cx43 internalization occurs through a pathway that does not depend on ubiquitin, while internalization of Cx43 in response to changes in the cellular environment may require the prior ubiquitination of the protein. Furthermore, ubiquitination of the protein is also suggested to signal its intracellular sorting to the endolysosomal compartment, through a mechanism involving the ESCRT machinery and the DUB UBPY. Lastly, we provide evidence that Cx43 can be recycled back to the plasma membrane through both rapid and slow recycling pathways. Although we partially characterize the internalization and intracellular trafficking routes followed by Cx43, many questions are still unresolved, such as the molecular mechanisms that determine the internalization pathway followed by ubiquitinated Cx43 or whether Cx43 is indeed a substrate for deubiquitination.

Chapter 7

Chapter 7: General Conclusions

Gap junction intercellular communication is critical to ensure the correct function of tissues and organs in mammals. The extent of gap junction intercellular communication is a direct result of the number and functionality of connexin-based pores. Therefore, processes that influence the stability of connexin at the plasma membrane are critical for regulating intercellular communication. Data presented in this study begins by assessing the critical role of some destabilizing factors, including cholesterol oxidation, in GJIC and proceeds by investigating in greater detail the molecular mechanisms involved in the regulation of GJIC. The results presented in this study provide new and important insights on the mechanisms and molecular players involved in regulating several aspects of Cx43 internalization and intracellular trafficking.

The oxysterol 7-ketocholesterol was shown to stabilize Cx43 at the plasma membrane in lens epithelial cells, thus leading to an increase in gap junction channel assembly and gap junction intercellular communication. This observation is particularly important in the lens for several reasons: the lens is continuously exposed to oxidative insults; lens plasma membranes contain the highest cholesterol content of any biological membrane; and intercellular communication in the lens relies on an extensive network of gap junctions which is essential for the maintenance of lens transparency. Thus, upregulation of gap junction intercellular communication due to accumulation of 7-ketocholesterol, may disrupt a variety of highly regulated events, including the differentiation of lens epithelial cells into fibres, which may ultimately compromise lens transparency leading to cataract formation.

The molecular mechanisms associated with Cx43 internalization and degradation has only recently begun to be investigated in some detail. In this manuscript we showed that Cx43 ubiquitination requires, at least in part, the activity of the E3 ligase Nedd4. Additionally, we showed that Cx43 ubiquitination is important for the interaction of Cx43 with the endocytic adaptor Eps15, through a mechanism that requires the ubiquitin-interacting motifs present on Eps15. Eps15 was also shown to mediate the internalization of Cx43. Furthermore, we show that Cx43 ubiquitination leads to a decrease in the half-life of the protein at the plasma membrane and induces the internalization of Cx43. Significantly, data presented in this study shows that ubiquitination of some Cx43 subunits in a connexon is sufficient to trigger internalization of ubiquitinated as well as non-ubiquitinated Cx43. This mechanism was shown to be independent on the tyrosine sorting signal, YXXØ, present on Cx43, which mediates the constitutive internalization of the protein. Interestingly, the internalization of Cx43 was shown to be mediated both by clathrin-dependent and clathrin-independent mechanisms. These observations led us to propose a new model for Cx43 internalization (Figure 49) in which Nedd4-mediated ubiquitination of Cx43 would recruit Eps15 to gap junction plaques, which would then direct Cx43 for internalization through both clathrin-mediated and clathrin-independent endocytosis.

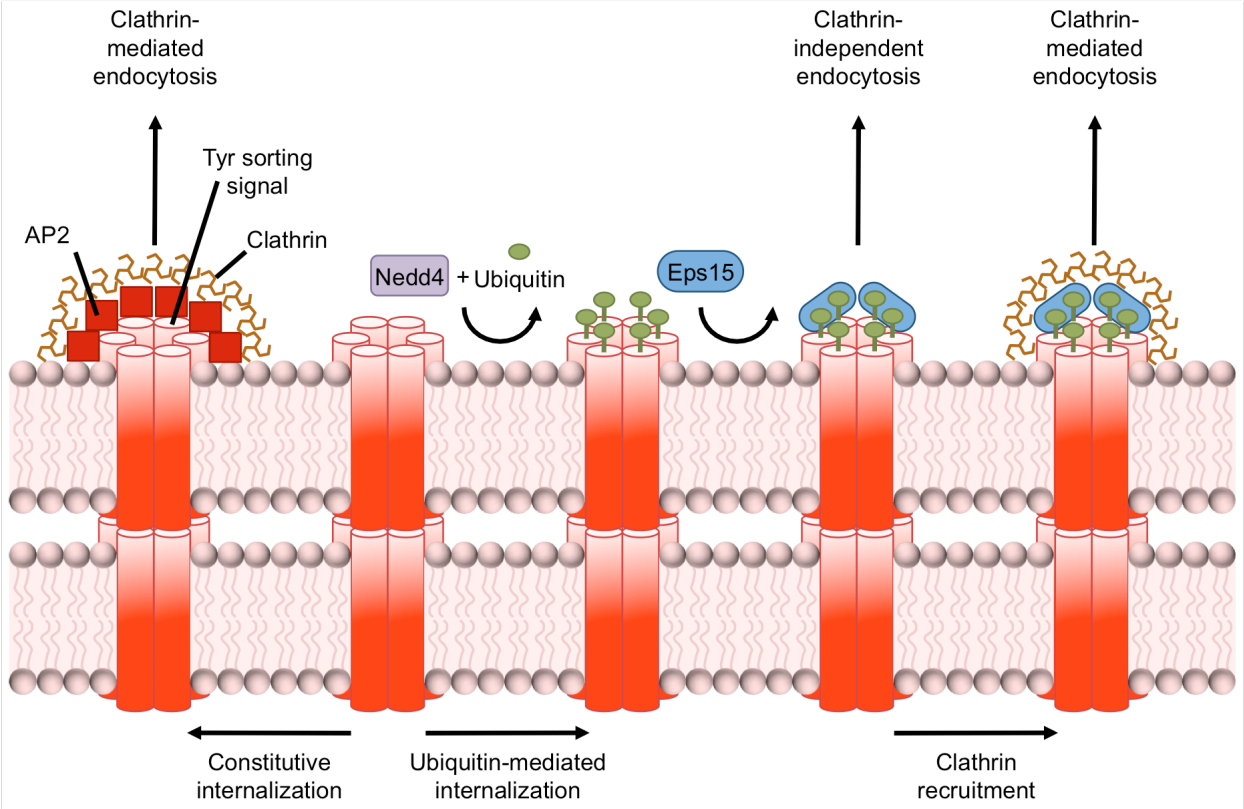

Figure 49 - Internalization of Cx43. Proposed model for the internalization of Cx43. Constitutive internalization of Cx43 is mediated by the tyrosine sorting signal present on the protein, which recruits the endocytic adaptor AP2 complex. The AP2 complex in turn induces the formation of a clathrin coat, leading to the clathrin-mediated internalization of gap junctions. Alternatively, ubiquitin-mediated internalization is initiated through the conjugation of ubiquitin to Cx43 catalyzed by the ubiquitin ligase Nedd4. Monoubiquitination of several Cx43 subunits, or multimonoubiquitination of a single subunit, would provide the polyubiquitin signal necessary to recruit Eps15 to gap junctions. The ubiquitin moieties present on Cx43 then bind to the UIM domains of Eps15, which in turn mediates the internalization of Cx43. Internalization of ubiquitinated Cx43 can then proceed through a clathrin-independent mechanism, or Eps15 can recruit components of the clathrin coat with internalization then proceeding through a clathrin-dependent mechanism.

Once established that ubiquitin targets Cx43 for internalization, we further investigated whether ubiquitin would also be involved in directing the intracellular sorting of the protein. Cx43 ubiquitination was shown to decrease the half-life of the protein, indicating a role for ubiquitin in directing the protein for lysosomal degradation. Consistently, Cx43 was shown to colocalize with the ESCRT components Hrs and Tsg101 in intracellular vesicles. As both Hrs and Tsg101 are involved in the intracellular sorting of ubiquitinated membrane proteins from early endosomes to the lysosome, this suggests that ubiquitin is involved in the intracellular sorting of Cx43. Additionally, the deubiquitinating enzyme UBPY, which is known to interact with components of the ESCRT machinery and is thought to deubiquitinate proteins before their sorting into multivesicular bodies, was shown to modulate the levels of ubiquitinated Cx43 and the endolysosomal sorting of the protein. Altogether, these results show, for the first time, that the levels of ubiquitin attached to Cx43, through a mechanism that involves the orchestrated activity of both the ubiquitin ligase Nedd4 and the deubiquitinating enzyme UBPY, provides an additional level of regulation of Cx43 degradation, and consequently, of gap junction intercellular communication. Finally, Cx43 was also

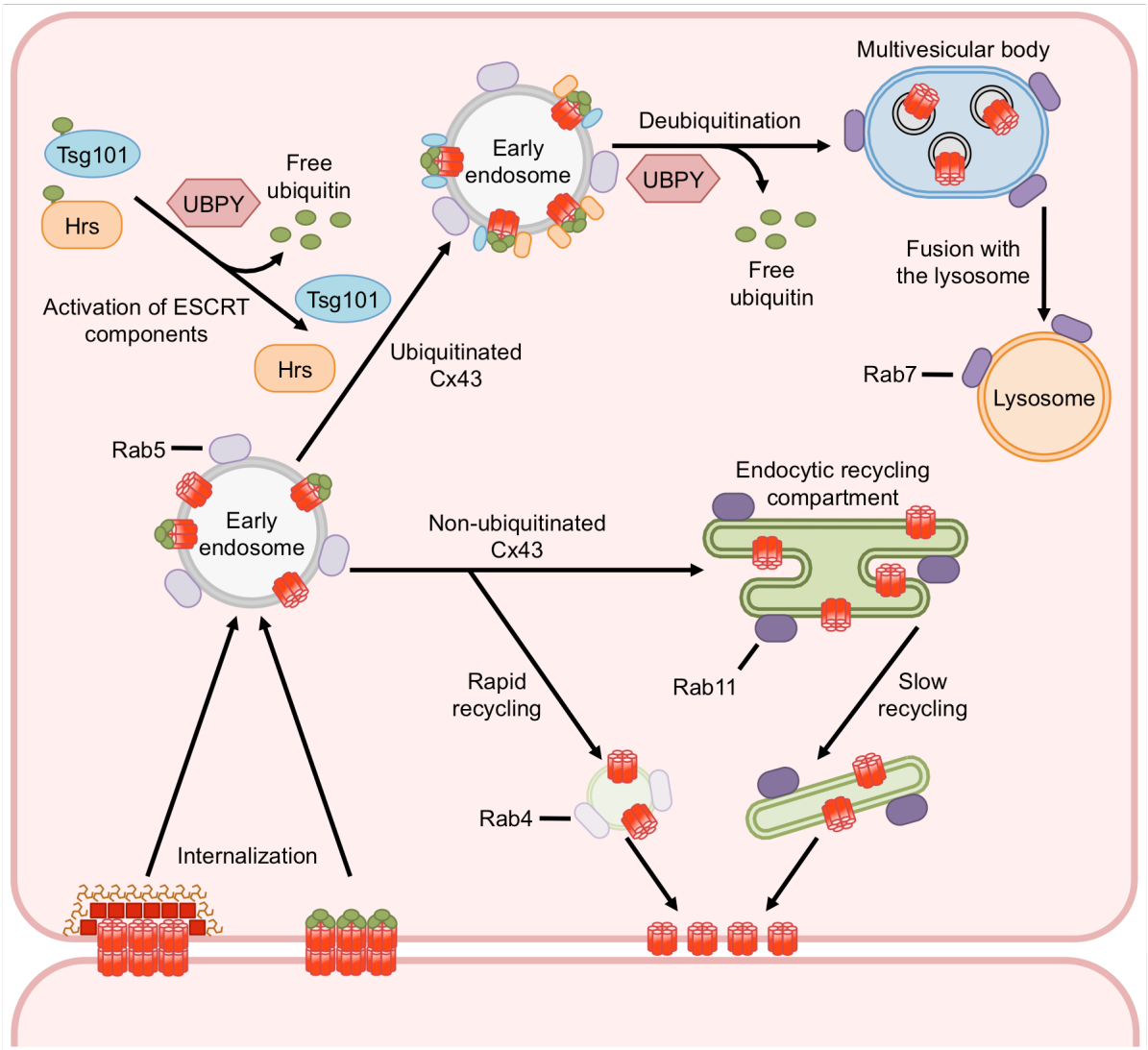

Figure 50 - Intracellular sorting of Cx43. Proposed model for the intracellular trafficking of internalized Cx43. Following internalization, endocytic vesicles containing non-ubiquitinated or ubiquitinated Cx43 converge at the early endosome. Ubiquitinated Cx43 is recognized by the ESCRT machinery components Hrs and/or Tsg101, which then sorts the protein to the interior of multivesicular bodies, which eventually delivers the protein to the lysosome for degradation. The deubiquitinating enzyme UBPY promotes the endolysosomal sorting of Cx43, either through the activation of ESCRT components, by releasing them from their autoinhibitory monoubiquitination, or/and through the direct deubiquitination of Cx43 at the ESCRT-III complex, the last step before proteins are sorted into intralumenal vesicles. Non-ubiquitinated Cx43 present in the early endosome, is either sorted directly back to the plasma membrane in Rab4 positive recycling vesicles (rapid recycling pathway), or sorted to the Rab11 positive endocytic recycling compartment before being transported to the plasma membrane (slow recycling pathway).

shown to colocalize and interact with Rab4, a marker of rapid recycling vesicles, and Rab11, a marker of the endocytic recycling compartment that is involved in slow recycling pathways. Results presented in this study further indicate that internalized Cx43 is recycled back to the plasma membrane through both rapid and slow recycling pathways. These observations led us to propose a new model for the intracellular trafficking of internalized Cx43 (Figure 50) in which endocytosed Cx43 is first delivered to early endosomes where the protein is sorted to the endolysosomal compartment through the interaction of ubiquitinated Cx43 with the ESCRT components Hrs and Tsg101. UBPY would also promote the lysosomal targeting of Cx43, either by releasing

components of the ESCRT machinery from their autoinhibitory monoubiquitination, or by deubiquitinating Cx43 at the ESCRT-III complex, allowing for the recycling of free ubiquitin and committing Cx43 to lysosomal degradation. Moreover, constitutively internalized Cx43, or ubiquitinated Cx43 that was rescued from endolysosomal sorting through deubiquitination, would be recycled back to the plasma membrane through both the rapid and slow recycling routes.

The results obtained in this study provide new insights for the role of ubiquitin in modulating the internalization and intracellular trafficking of Cx43, providing a basis for future studies aiming at identifying the molecular players involved in Cx43 internalization, intracellular sorting, recycling and degradation.

References

References

Aberle H, Bauer A, Stappert J, Kispert A, Kemler R (1997) beta-catenin is a target for the ubiquitin-proteasome pathway. *EMBO J* **16**(13)**:** 3797-3804

Abraham V, Chou ML, DeBolt KM, Koval M (1999) Phenotypic control of gap junctional communication by cultured alveolar epithelial cells. *Am J Physiol* **276**(5 Pt 1)**:** L825-834

Acquaviva C, Pines J (2006) The anaphase-promoting complex/cyclosome: APC/C. *J Cell Sci* **119**(Pt 12)**:** 2401-2404

Ahmad S, Diez JA, George CH, Evans WH (1999) Synthesis and assembly of connexins in vitro into homomeric and heteromeric functional gap junction hemichannels. *Biochem J* **339 (Pt 2):** 247-253

Al-Hakim AK, Zagorska A, Chapman L, Deak M, Peggie M, Alessi DR (2008) Control of AMPK-related kinases by USP9X and atypical Lys(29)/Lys(33)-linked polyubiquitin chains. *Biochem J* **411**(2)**:** 249-260

Altevogt BM, Kleopa KA, Postma FR, Scherer SS, Paul DL (2002) Connexin29 is uniquely distributed within myelinating glial cells of the central and peripheral nervous systems. *J Neurosci* **22**(15)**:** 6458-6470

Anselmi F, Hernandez VH, Crispino G, Seydel A, Ortolano S, Roper SD, Kessaris N, Richardson W, Rickheit G, Filippov MA, Monyer H, Mammano F (2008) ATP release through connexin hemichannels and gap junction transfer of second messengers propagate Ca2+ signals across the inner ear. *Proc Natl Acad Sci U S A* **105**(48)**:** 18770-18775

Anumonwo JM, Taffet SM, Gu H, Chanson M, Moreno AP, Delmar M (2001) The carboxyl terminal domain regulates the unitary conductance and voltage dependence of connexin40 gap junction channels. *Circ Res* **88**(7)**:** 666-673

Archard HO, Denys FR (1979) Development of annular gap junctions in guinea pig epithelia. *J Oral Pathol* **8**(4)**:** 187-197

Armstrong CM, Hille B (1998) Voltage-gated ion channels and electrical excitability. *Neuron* **20**(3)**:** 371-380

Atkinson MM, Lampe PD, Lin HH, Kollander R, Li XR, Kiang DT (1995) Cyclic AMP modifies the cellular distribution of connexin43 and induces a persistent increase in the junctional permeability of mouse mammary tumor cells. *J Cell Sci* **108 (Pt 9):** 3079-3090

Auth T, Schluter S, Urschel S, Kussmann P, Sonntag S, Hoher T, Kreuzberg MM, Dobrowolski R, Willecke K (2009) The TSG101 protein binds to connexins and is involved in connexin degradation. *Exp Cell Res* **315**(6)**:** 1053-1062

Baker RT, Board PG (1987) The human ubiquitin gene family: structure of a gene and pseudogenes from the Ub B subfamily. *Nucleic Acids Res* **15**(2)**:** 443-463

Banach K, Weingart R (2000) Voltage gating of Cx43 gap junction channels involves fast and slow current transitions. *Pflugers Arch* **439**(3)**:** 248-250

Barker RJ, Price RL, Gourdie RG (2002) Increased association of ZO-1 with connexin43 during remodeling of cardiac gap junctions. *Circ Res* **90**(3)**:** 317-324

Barriere H, Nemes C, Lechardeur D, Khan-Mohammad M, Fruh K, Lukacs GL (2006) Molecular basis of oligoubiquitin-dependent internalization of membrane proteins in Mammalian cells. *Traffic* **7**(3)**:** 282-297

Bastiaanse EM, Jongsma HJ, van der Laarse A, Takens-Kwak BR (1993) Heptanol-induced decrease in cardiac gap junctional conductance is mediated by a decrease in the fluidity of membranous cholesterol-rich domains. *J Membr Biol* **136**(2)**:** 135-145

Bays NW, Gardner RG, Seelig LP, Joazeiro CA, Hampton RY (2001) Hrd1p/Der3p is a membrane-anchored ubiquitin ligase required for ER-associated degradation. *Nat Cell Biol* **3**(1)**:** 24-29

Beardslee MA, Lerner DL, Tadros PN, Laing JG, Beyer EC, Yamada KA, Kleber AG, Schuessler RB, Saffitz JE (2000) Dephosphorylation and intracellular redistribution of ventricular connexin43 during electrical uncoupling induced by ischemia. *Circ Res* **87**(8)**:** 656-662

Beblo DA, Veenstra RD (1997) Monovalent cation permeation through the connexin40 gap junction channel. Cs, Rb, K, Na, Li, TEA, TMA, TBA, and effects of anions Br, Cl, F, acetate, aspartate, glutamate, and NO3. *J Gen Physiol* **109**(4)**:** 509-522

Bergoffen J, Scherer SS, Wang S, Scott MO, Bone LJ, Paul DL, Chen K, Lensch MW, Chance PF, Fischbeck KH (1993) Connexin mutations in X-linked Charcot-Marie-Tooth disease. *Science* **262**(5142)**:** 2039-2042

Berthoud VM, Beyer EC, Kurata WE, Lau AF, Lampe PD (1997) The gap-junction protein connexin 56 is phosphorylated in the intracellular loop and the carboxy-terminal region. *Eur J Biochem* **244**(1)**:** 89-97

Bevans CG, Kordel M, Rhee SK, Harris AL (1998) Isoform composition of connexin channels determines selectivity among second messengers and uncharged molecules. *J Biol Chem* **273**(5)**:** 2808-2816

Beyer EC, Davis LM, Saffitz JE, Veenstra RD (1995) Cardiac intercellular communication: consequences of connexin distribution and diversity. *Braz J Med Biol Res* **28**(4)**:** 415-425

Beyer EC, Kistler J, Paul DL, Goodenough DA (1989) Antisera directed against connexin43 peptides react with a 43-kD protein localized to gap junctions in myocardium and other tissues. *J Cell Biol* **108**(2)**:** 595-605

Beyer EC, Paul DL, Goodenough DA (1987) Connexin43: a protein from rat heart homologous to a gap junction protein from liver. *J Cell Biol* **105**(6 Pt 1)**:** 2621-2629

Bolon ML, Peng T, Kidder GM, Tyml K (2008) Lipopolysaccharide plus hypoxia and reoxygenation synergistically reduce electrical coupling between microvascular endothelial cells by dephosphorylating connexin40. *J Cell Physiol* **217**(2)**:** 350-359

Bonifacino JS, Traub LM (2003) Signals for sorting of transmembrane proteins to endosomes and lysosomes. *Annu Rev Biochem* **72:** 395-447

Borissenko L, Groll M (2007) Diversity of proteasomal missions: fine tuning of the immune response. *Biol Chem* **388**(9)**:** 947-955

Borsum T, Henriksen T, Reisvaag A (1985) Oxidized low density lipoprotein can reduce the pinocytic activity in cultured human endothelial cells as measured by cellular uptake of [14C]sucrose. *Atherosclerosis* **58**(1-3)**:** 81-96

Boswell BA, Le AC, Musil LS (2009) Upregulation and maintenance of gap junctional communication in lens cells. *Exp Eye Res* **88**(5)**:** 919-927

Bowers K, Piper SC, Edeling MA, Gray SR, Owen DJ, Lehner PJ, Luzio JP (2006) Degradation of endocytosed epidermal growth factor and virally ubiquitinated major histocompatibility complex class I is independent of mammalian ESCRTII. *J Biol Chem* **281**(8)**:** 5094-5105

Brooks P, Fuertes G, Murray RZ, Bose S, Knecht E, Rechsteiner MC, Hendil KB, Tanaka K, Dyson J, Rivett J (2000) Subcellular localization of proteasomes and their regulatory complexes in mammalian cells. *Biochem J* **346 Pt 1:** 155-161

Bruzzone R, White TW, Paul DL (1994) Expression of chimeric connexins reveals new properties of the formation and gating behavior of gap junction channels. *J Cell Sci* **107 (Pt 4):** 955-967

Bucci C, Parton RG, Mather IH, Stunnenberg H, Simons K, Hoflack B, Zerial M (1992) The small GTPase rab5 functions as a regulatory factor in the early endocytic pathway. *Cell* **70**(5)**:** 715-728

Bukauskas FF, Jordan K, Bukauskiene A, Bennett MV, Lampe PD, Laird DW, Verselis VK (2000) Clustering of connexin 43-enhanced green fluorescent protein gap junction channels and functional coupling in living cells. *Proc Natl Acad Sci U S A* **97**(6)**:** 2556-2561

Bukauskas FF, Verselis VK (2004) Gap junction channel gating. *Biochim Biophys Acta* **1662**(1-2)**:** 42-60

Burra S, Jiang JX (2009) Connexin 43 hemichannel opening associated with Prostaglandin E(2) release is adaptively regulated by mechanical stimulation. *Commun Integr Biol* **2**(3)**:** 239-240

Butkevich E, Hulsmann S, Wenzel D, Shirao T, Duden R, Majoul I (2004) Drebrin is a novel connexin-43 binding partner that links gap junctions to the submembrane cytoskeleton. *Curr Biol* **14**(8)**:** 650-658

Castro C, Gomez-Hernandez JM, Silander K, Barrio LC (1999) Altered formation of hemichannels and gap junction channels caused by C-terminal connexin-32 mutations. *J Neurosci* **19**(10)**:** 3752-3760

Chanson M, White MM, Garber SS (1996) cAMP promotes gap junctional coupling in T84 cells. *Am J Physiol* **271**(2 Pt 1)**:** C533-539

Chastagner P, Israel A, Brou C (2006) Itch/AIP4 mediates Deltex degradation through the formation of K29-linked polyubiquitin chains. *EMBO Rep* **7**(11)**:** 1147-1153

Chetrit D, Ziv N, Ehrlich M (2009) Dab2 regulates clathrin assembly and cell spreading. *Biochem J* **418**(3)**:** 701-715

Chow SE, Lee RS, Shih SH, Chen JK (1998) Oxidized LDL promotes vascular endothelial cell pinocytosis via a prooxidation mechanism. *FASEB J* **12**(10)**:** 823-830

Christ GJ, Brink PR (1999) Analysis of the presence and physiological relevance of subconducting states of Connexin43-derived gap junction channels in cultured human corporal vascular smooth muscle cells. *Circ Res* **84**(7)**:** 797-803

Churchill GC, Lurtz MM, Louis CF (2001) Ca(2+) regulation of gap junctional coupling in lens epithelial cells. *Am J Physiol Cell Physiol* **281**(3)**:** C972-981

Clague MJ, Urbe S (2003) Hrs function: viruses provide the clue. *Trends Cell Biol* **13**(12)**:** 603-606

Clague MJ, Urbe S (2006) Endocytosis: the DUB version. *Trends Cell Biol* **16**(11)**:** 551-559

Cooper CD, Lampe PD (2002) Casein kinase 1 regulates connexin-43 gap junction assembly. *J Biol Chem* **277**(47)**:** 44962-44968

Cottrell GT, Lin R, Warn-Cramer BJ, Lau AF, Burt JM (2003) Mechanism of v-Src- and mitogen-activated protein kinase-induced reduction of gap junction communication. *Am J Physiol Cell Physiol* **284**(2)**:** C511-520

Courbard JR, Fiore F, Adelaide J, Borg JP, Birnbaum D, Ollendorff V (2002) Interaction between two ubiquitin-protein isopeptide ligases of different classes, CBLC and AIP4/ITCH. *J Biol Chem* **277**(47)**:** 45267-45275

Creighton TE (1993) *Proteins : structures and molecular properties*, 2nd edn. New York: W.H. Freeman.

Crow DS, Beyer EC, Paul DL, Kobe SS, Lau AF (1990) Phosphorylation of connexin43 gap junction protein in uninfected and Rous sarcoma virus-transformed mammalian fibroblasts. *Mol Cell Biol* **10**(4)**:** 1754-1763

da Fonseca PC, Morris EP (2008) Structure of the human 26S proteasome: subunit radial displacements open the gate into the proteolytic core. *J Biol Chem* **283**(34)**:** 23305-23314

Das S, Smith TD, Sarma JD, Ritzenthaler JD, Maza J, Kaplan BE, Cunningham LA, Suaud L, Hubbard MJ, Rubenstein RC, Koval M (2009) ERp29 restricts Connexin43 oligomerization in the endoplasmic reticulum. *Mol Biol Cell* **20**(10)**:** 2593-2604

Das Sarma J, Meyer RA, Wang F, Abraham V, Lo CW, Koval M (2001) Multimeric connexin interactions prior to the trans-Golgi network. *J Cell Sci* **114**(Pt 22)**:** 4013-4024

Dbouk HA, Mroue RM, El-Sabban ME, Talhouk RS (2009) Connexins: a myriad of functions extending beyond assembly of gap junction channels. *Cell Commun Signal* **7:** 4

Deckert V, Duverneuil L, Poupon S, Monier S, Le Guern N, Lizard G, Masson D, Lagrost L (2002) The impairment of endothelium-dependent arterial relaxation by 7-ketocholesterol is associated with an early activation of protein kinase C. *Br J Pharmacol* **137**(5)**:** 655-662

Decrock E, De Vuyst E, Vinken M, Van Moorhem M, Vranckx K, Wang N, Van Laeken L, De Bock M, D'Herde K, Lai CP, Rogiers V, Evans WH, Naus CC, Leybaert L (2009) Connexin 43 hemichannels contribute to the propagation of apoptotic cell death in a rat C6 glioma cell model. *Cell Death Differ* **16**(1)**:** 151-163

Defamie N, Mograbi B, Roger C, Cronier L, Malassine A, Brucker-Davis F, Fenichel P, Segretain D, Pointis G (2001) Disruption of gap junctional intercellular communication by lindane is associated with aberrant localization of connexin43 and zonula occludens-1 in 42GPA9 Sertoli cells. *Carcinogenesis* **22**(9)**:** 1537-1542

Deffenbaugh AE, Scaglione KM, Zhang L, Moore JM, Buranda T, Sklar LA, Skowyra D (2003) Release of ubiquitin-charged Cdc34-S - Ub from the RING domain is essential for ubiquitination of the SCF(Cdc4)-bound substrate Sic1. *Cell* **114**(5)**:** 611-622

Derangeon M, Bourmeyster N, Plaisance I, Pinet-Charvet C, Chen Q, Duthe F, Popoff MR, Sarrouilhe D, Herve JC (2008) RhoA GTPase and F-actin dynamically regulate the permeability of Cx43-made channels in rat cardiac myocytes. *J Biol Chem* **283**(45)**:** 30754-30765

Deshaies RJ, Joazeiro CA (2009) RING domain E3 ubiquitin ligases. *Annu Rev Biochem* **78:** 399-434

Deutsch C (2003) The birth of a channel. *Neuron* **40**(2)**:** 265-276

Di Fiore PP, Polo S, Hofmann K (2003) When ubiquitin meets ubiquitin receptors: a signalling connection. *Nat Rev Mol Cell Biol* **4**(6)**:** 491-497

Doherty GJ, McMahon HT (2009) Mechanisms of endocytosis. *Annu Rev Biochem* **78:** 857-902

Duffy HS, Sorgen PL, Girvin ME, O'Donnell P, Coombs W, Taffet SM, Delmar M, Spray DC (2002) pH-dependent intramolecular binding and structure involving Cx43 cytoplasmic domains. *J Biol Chem* **277**(39)**:** 36706-36714

Dunham B, Liu S, Taffet S, Trabka-Janik E, Delmar M, Petryshyn R, Zheng S, Perzova R, Vallano ML (1992) Immunolocalization and expression of functional and nonfunctional cell-to-cell channels from wild-type and mutant rat heart connexin43 cDNA. *Circ Res* **70**(6)**:** 1233-1243

Ek JF, Delmar M, Perzova R, Taffet SM (1994) Role of histidine 95 on pH gating of the cardiac gap junction protein connexin43. *Circ Res* **74**(6)**:** 1058-1064

Ek-Vitorin JF, Calero G, Morley GE, Coombs W, Taffet SM, Delmar M (1996) PH regulation of connexin43: molecular analysis of the gating particle. *Biophys J* **71**(3)**:** 1273-1284

Etienne-Manneville S, Hall A (2002) Rho GTPases in cell biology. *Nature* **420**(6916)**:** 629-635

Evans WH, De Vuyst E, Leybaert L (2006) The gap junction cellular internet: connexin hemichannels enter the signalling limelight. *Biochem J* **397**(1)**:** 1-14

Falk MM, Baker SM, Gumpert AM, Segretain D, Buckheit RW, 3rd (2009) Gap junction turnover is achieved by the internalization of small endocytic double-membrane vesicles. *Mol Biol Cell* **20**(14)**:** 3342-3352

Feng Y, Press B, Wandinger-Ness A (1995) Rab 7: an important regulator of late endocytic membrane traffic. *J Cell Biol* **131**(6 Pt 1)**:** 1435-1452

Fernandes R, Girao H, Pereira P (2004) High glucose down-regulates intercellular communication in retinal endothelial cells by enhancing degradation of connexin 43 by a proteasome-dependent mechanism. *J Biol Chem* **279**(26)**:** 27219-27224

Finley D (2009) Recognition and processing of ubiquitin-protein conjugates by the proteasome. *Annu Rev Biochem* **78:** 477-513

Flick K, Raasi S, Zhang H, Yen JL, Kaiser P (2006) A ubiquitin-interacting motif protects polyubiquitinated Met4 from degradation by the 26S proteasome. *Nat Cell Biol* **8**(5)**:** 509-515

Fujimoto K, Nagafuchi A, Tsukita S, Kuraoka A, Ohokuma A, Shibata Y (1997) Dynamics of connexins, E-cadherin and alpha-catenin on cell membranes during gap junction formation. *J Cell Sci* **110 (Pt 3):** 311-322

Fujita Y, Krause G, Scheffner M, Zechner D, Leddy HE, Behrens J, Sommer T, Birchmeier W (2002) Hakai, a c-Cbl-like protein, ubiquitinates and induces endocytosis of the E-cadherin complex. *Nat Cell Biol* **4**(3)**:** 222-231

Gaietta G, Deerinck TJ, Adams SR, Bouwer J, Tour O, Laird DW, Sosinsky GE, Tsien RY, Ellisman MH (2002) Multicolor and electron microscopic imaging of connexin trafficking. *Science* **296**(5567)**:** 503-507

Giepmans BN, Moolenaar WH (1998) The gap junction protein connexin43 interacts with the second PDZ domain of the zona occludens-1 protein. *Curr Biol* **8**(16)**:** 931-934

Giepmans BN, Verlaan I, Hengeveld T, Janssen H, Calafat J, Falk MM, Moolenaar WH (2001a) Gap junction protein connexin-43 interacts directly with microtubules. *Curr Biol* **11**(17)**:** 1364-1368

Giepmans BN, Verlaan I, Moolenaar WH (2001b) Connexin-43 interactions with ZO-1 and alpha- and beta-tubulin. *Cell Commun Adhes* **8**(4-6)**:** 219-223

Gilleron J, Fiorini C, Carette D, Avondet C, Falk MM, Segretain D, Pointis G (2008) Molecular reorganization of Cx43, Zo-1 and Src complexes during the endocytosis of gap junction plaques in response to a non-genomic carcinogen. *J Cell Sci* **121**(Pt 24)**:** 4069-4078

Girao H, Mota MC, Ramalho J, Pereira P (1998) Cholesterol oxides accumulate in human cataracts. *Exp Eye Res* **66**(5)**:** 645-652

Girao H, Pereira P (2003) Phosphorylation of connexin 43 acts as a stimuli for proteasome-dependent degradation of the protein in lens epithelial cells. *Mol Vis* **9:** 24-30

Girao H, Pereira P (2007) The proteasome regulates the interaction between Cx43 and ZO-1. *J Cell Biochem* **102**(3)**:** 719-728

Girao H, Pereira P, Ramalho J, Quinlan R, Prescott A (2003a) Cholesterol oxides mediated changes in cytoskeletal organisation involves Rho GTPases small star, filled. *Exp Cell Res* **291**(2)**:** 502-513

Girao H, Shang F, Pereira P (2003b) 7-ketocholesterol stimulates differentiation of lens epithelial cells. *Mol Vis* **9:** 497-501

Glickman MH (2000) Getting in and out of the proteasome. *Semin Cell Dev Biol* **11**(3)**:** 149-158

Glickman MH, Ciechanover A (2002) The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol Rev* **82**(2)**:** 373-428

Glickman MH, Rubin DM, Fried VA, Finley D (1998) The regulatory particle of the Saccharomyces cerevisiae proteasome. *Mol Cell Biol* **18**(6)**:** 3149-3162

Goldberg AL, Cascio P, Saric T, Rock KL (2002a) The importance of the proteasome and subsequent proteolytic steps in the generation of antigenic peptides. *Mol Immunol* **39**(3-4)**:** 147-164

Goldberg GS, Moreno AP, Lampe PD (2002b) Gap junctions between cells expressing connexin 43 or 32 show inverse permselectivity to adenosine and ATP. *J Biol Chem* **277**(39)**:** 36725-36730

Goldberg GS, Valiunas V, Brink PR (2004) Selective permeability of gap junction channels. *Biochim Biophys Acta* **1662**(1-2)**:** 96-101

Gong X, Li E, Klier G, Huang Q, Wu Y, Lei H, Kumar NM, Horwitz J, Gilula NB (1997) Disruption of alpha3 connexin gene leads to proteolysis and cataractogenesis in mice. *Cell* **91**(6)**:** 833-843

Gonzalez D, Gomez-Hernandez JM, Barrio LC (2007) Molecular basis of voltage dependence of connexin channels: an integrative appraisal. *Prog Biophys Mol Biol* **94**(1-2)**:** 66-106

Goodenough DA, Goliger JA, Paul DL (1996) Connexins, connexons, and intercellular communication. *Annu Rev Biochem* **65:** 475-502

Gossman DG, Zhao HB (2008) Hemichannel-mediated inositol 1,4,5-trisphosphate (IP3) release in the cochlea: a novel mechanism of IP3 intercellular signaling. *Cell Commun Adhes* **15**(4)**:** 305-315

Grant BD, Donaldson JG (2009) Pathways and mechanisms of endocytic recycling. *Nat Rev Mol Cell Biol* **10**(9)**:** 597-608

Groll M, Bajorek M, Kohler A, Moroder L, Rubin DM, Huber R, Glickman MH, Finley D (2000) A gated channel into the proteasome core particle. *Nat Struct Biol* **7**(11)**:** 1062-1067

Groll M, Ditzel L, Lowe J, Stock D, Bochtler M, Bartunik HD, Huber R (1997) Structure of 20S proteasome from yeast at 2.4 A resolution. *Nature* **386**(6624)**:** 463-471

Gros DB, Jongsma HJ (1996) Connexins in mammalian heart function. *Bioessays* **18**(9)**:** 719-730

Gu S, Yu XS, Yin X, Jiang JX (2003) Stimulation of lens cell differentiation by gap junction protein connexin 45.6. *Invest Ophthalmol Vis Sci* **44**(5)**:** 2103-2111

Gumpert AM, Varco JS, Baker SM, Piehl M, Falk MM (2008) Double-membrane gap junction internalization requires the clathrin-mediated endocytic machinery. *FEBS Lett* **582**(19)**:** 2887-2892

Haglund K, Sigismund S, Polo S, Szymkiewicz I, Di Fiore PP, Dikic I (2003) Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation. *Nat Cell Biol* **5**(5)**:** 461-466

Hansen SH, Sandvig K, van Deurs B (1993) Clathrin and HA2 adaptors: effects of potassium depletion, hypertonic medium, and cytosol acidification. *J Cell Biol* **121**(1)**:** 61-72

Hanson PI, Roth R, Lin Y, Heuser JE (2008) Plasma membrane deformation by circular arrays of ESCRT-III protein filaments. *J Cell Biol* **180**(2)**:** 389-402

Harris AL (2001) Emerging issues of connexin channels: biophysics fills the gap. *Q Rev Biophys* **34**(3)**:** 325-472

Hatakeyama S, Yada M, Matsumoto M, Ishida N, Nakayama KI (2001) U box proteins as a new family of ubiquitin-protein ligases. *J Biol Chem* **276**(35)**:** 33111-33120

Hawryluk MJ, Keyel PA, Mishra SK, Watkins SC, Heuser JE, Traub LM (2006) Epsin 1 is a polyubiquitin-selective clathrin-associated sorting protein. *Traffic* **7**(3)**:** 262-281

Henzl MT, Thalmann I, Larson JD, Ignatova EG, Thalmann R (2004) The cochlear F-box protein OCP1 associates with OCP2 and connexin 26. *Hear Res* **191**(1-2)**:** 101-109

Hershko A, Ciechanover A (1998) The ubiquitin system. *Annu Rev Biochem* **67:** 425-479

Herve JC, Bourmeyster N, Sarrouilhe D, Duffy HS (2007) Gap junctional complexes: from partners to functions. *Prog Biophys Mol Biol* **94**(1-2)**:** 29-65

Heuser JE, Anderson RG (1989) Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation. *J Cell Biol* **108**(2)**:** 389-400

Hicke L, Dunn R (2003) Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. *Annu Rev Cell Dev Biol* **19:** 141-172

Hicke L, Schubert HL, Hill CP (2005) Ubiquitin-binding domains. *Nat Rev Mol Cell Biol* **6**(8)**:** 610-621

Hochstrasser M (2009) Origin and function of ubiquitin-like proteins. *Nature* **458**(7237)**:** 422-429

Hoeller D, Crosetto N, Blagoev B, Raiborg C, Tikkanen R, Wagner S, Kowanetz K, Breitling R, Mann M, Stenmark H, Dikic I (2006) Regulation of ubiquitin-binding proteins by monoubiquitination. *Nat Cell Biol* **8**(2)**:** 163-169

Houghton FD, Thonnissen E, Kidder GM, Naus CC, Willecke K, Winterhager E (1999) Doubly mutant mice, deficient in connexin32 and -43, show normal prenatal development of organs where the two gap junction proteins are expressed in the same cells. *Dev Genet* **24**(1-2)**:** 5-12

Huang L, Kinnucan E, Wang G, Beaudenon S, Howley PM, Huibregtse JM, Pavletich NP (1999) Structure of an E6AP-UbcH7 complex: insights into ubiquitination by the E2-E3 enzyme cascade. *Science* **286**(5443)**:** 1321-1326

Huang XD, Horackova M, Pressler ML (1996) Changes in the expression and distribution of connexin 43 in isolated cultured adult guinea pig cardiomyocytes. *Exp Cell Res* **228**(2)**:** 254-261

Hume AN, Tarafder AK, Ramalho JS, Sviderskaya EV, Seabra MC (2006) A coiled-coil domain of melanophilin is essential for Myosin Va recruitment and melanosome transport in melanocytes. *Mol Biol Cell* **17**(11)**:** 4720-4735

Hunter AW, Barker RJ, Zhu C, Gourdie RG (2005) Zonula occludens-1 alters connexin43 gap junction size and organization by influencing channel accretion. *Mol Biol Cell* **16**(12)**:** 5686-5698

Isakson BE, Duling BR (2005) Heterocellular contact at the myoendothelial junction influences gap junction organization. *Circ Res* **97**(1)**:** 44-51

Isakson BE, Kronke G, Kadl A, Leitinger N, Duling BR (2006) Oxidized phospholipids alter vascular connexin expression, phosphorylation, and heterocellular communication. *Arterioscler Thromb Vasc Biol* **26**(10)**:** 2216-2221

Jiang JX, Goodenough DA (1996) Heteromeric connexons in lens gap junction channels. *Proc Natl Acad Sci U S A* **93**(3)**:** 1287-1291

John S, Cesario D, Weiss JN (2003) Gap junctional hemichannels in the heart. *Acta Physiol Scand* **179**(1)**:** 23-31

John SA, Kondo R, Wang SY, Goldhaber JI, Weiss JN (1999) Connexin-43 hemichannels opened by metabolic inhibition. *J Biol Chem* **274**(1)**:** 236-240

Johnston MF, Ramon F (1981) Electrotonic coupling in internally perfused crayfish segmented axons. *J Physiol* **317:** 509-518

Johnstone S, Isakson B, Locke D (2009) Biological and biophysical properties of vascular connexin channels. *Int Rev Cell Mol Biol* **278:** 69-118

Jongen WM, Fitzgerald DJ, Asamoto M, Piccoli C, Slaga TJ, Gros D, Takeichi M, Yamasaki H (1991) Regulation of connexin 43-mediated gap junctional intercellular communication by Ca2+ in mouse epidermal cells is controlled by E-cadherin. *J Cell Biol* **114**(3)**:** 545-555

Jordan K, Chodock R, Hand AR, Laird DW (2001) The origin of annular junctions: a mechanism of gap junction internalization. *J Cell Sci* **114**(Pt 4)**:** 763-773

Kaiser P, Flick K, Wittenberg C, Reed SI (2000) Regulation of transcription by ubiquitination without proteolysis: Cdc34/SCF(Met30)-mediated inactivation of the transcription factor Met4. *Cell* **102**(3)**:** 303-314

Kanemitsu MY, Lau AF (1993) Epidermal growth factor stimulates the disruption of gap junctional communication and connexin43 phosphorylation independent of 12-0-tetradecanoylphorbol 13-acetate-sensitive protein kinase C: the possible involvement of mitogen-activated protein kinase. *Mol Biol Cell* **4**(8)**:** 837-848

Kelly BT, McCoy AJ, Spate K, Miller SE, Evans PR, Honing S, Owen DJ (2008) A structural explanation for the binding of endocytic dileucine motifs by the AP2 complex. *Nature* **456**(7224)**:** 976-979

Kim DY, Kam Y, Koo SK, Joe CO (1999) Gating connexin 43 channels reconstituted in lipid vesicles by mitogen-activated protein kinase phosphorylation. *J Biol Chem* **274**(9)**:** 5581-5587

Kirkin V, Dikic I (2007) Role of ubiquitin- and Ubl-binding proteins in cell signaling. *Curr Opin Cell Biol* **19**(2)**:** 199-205

Kiss AL, Botos E (2009) Endocytosis via caveolae: alternative pathway with distinct cellular compartments to avoid lysosomal degradation? *J Cell Mol Med* **13**(7)**:** 1228-1237

Kisselev AF, Akopian TN, Woo KM, Goldberg AL (1999) The sizes of peptides generated from protein by mammalian 26 and 20 S proteasomes. Implications for understanding the degradative mechanism and antigen presentation. *J Biol Chem* **274**(6)**:** 3363-3371

Kitagawa M, Hatakeyama S, Shirane M, Matsumoto M, Ishida N, Hattori K, Nakamichi I, Kikuchi A, Nakayama K (1999) An F-box protein, FWD1, mediates ubiquitin-dependent proteolysis of beta-catenin. *EMBO J* **18**(9)**:** 2401-2410

Kitson N, Van Lennep EW, Young JA (1978) Gap junctions in human sebaceous glands. *Cell Tissue Res* **190**(1)**:** 115-121

Koegl M, Hoppe T, Schlenker S, Ulrich HD, Mayer TU, Jentsch S (1999) A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. *Cell* **96**(5)**:** 635-644

Komada M, Kitamura N (2005) The Hrs/STAM complex in the downregulation of receptor tyrosine kinases. *J Biochem* **137**(1)**:** 1-8

Kouranti I, Sachse M, Arouche N, Goud B, Echard A (2006) Rab35 regulates an endocytic recycling pathway essential for the terminal steps of cytokinesis. *Curr Biol* **16**(17)**:** 1719-1725

Koval M (2006) Pathways and control of connexin oligomerization. *Trends Cell Biol* **16**(3)**:** 159-166

Koval M, Geist ST, Westphale EM, Kemendy AE, Civitelli R, Beyer EC, Steinberg TH (1995) Transfected connexin45 alters gap junction permeability in cells expressing endogenous connexin43. *J Cell Biol* **130**(4)**:** 987-995

Koval M, Harley JE, Hick E, Steinberg TH (1997) Connexin46 is retained as monomers in a trans-Golgi compartment of osteoblastic cells. *J Cell Biol* **137**(4)**:** 847-857

Krutovskikh V, Yamasaki H (2000) Connexin gene mutations in human genetic diseases. *Mutat Res* **462**(2-3)**:** 197-207

Kumar NM, Gilula NB (1986) Cloning and characterization of human and rat liver cDNAs coding for a gap junction protein. *J Cell Biol* **103**(3)**:** 767-776

Kumar NM, Gilula NB (1996) The gap junction communication channel. *Cell* **84**(3)**:** 381-388

Kuras L, Rouillon A, Lee T, Barbey R, Tyers M, Thomas D (2002) Dual regulation of the met4 transcription factor by ubiquitin-dependent degradation and inhibition of promoter recruitment. *Mol Cell* **10**(1)**:** 69-80

Kwak BR, Saez JC, Wilders R, Chanson M, Fishman GI, Hertzberg EL, Spray DC, Jongsma HJ (1995) Effects of cGMP-dependent phosphorylation on rat and human connexin43 gap junction channels. *Pflugers Arch* **430**(5)**:** 770-778

Laing JG, Beyer EC (1995) The gap junction protein connexin43 is degraded via the ubiquitin proteasome pathway. *J Biol Chem* **270**(44)**:** 26399-26403

Laing JG, Tadros PN, Westphale EM, Beyer EC (1997) Degradation of connexin43 gap junctions involves both the proteasome and the lysosome. *Exp Cell Res* **236**(2)**:** 482-492

Laird DW (2006) Life cycle of connexins in health and disease. *Biochem J* **394**(Pt 3)**:** 527-543

Laird DW (2009) The gap junction proteome and its relationship to disease. *Trends Cell Biol*

Lampe PD (1994) Analyzing phorbol ester effects on gap junctional communication: a dramatic inhibition of assembly. *J Cell Biol* **127**(6 Pt 2)**:** 1895-1905

Lampe PD, Lau AF (2004) The effects of connexin phosphorylation on gap junctional communication. *Int J Biochem Cell Biol* **36**(7)**:** 1171-1186

Lampe PD, TenBroek EM, Burt JM, Kurata WE, Johnson RG, Lau AF (2000) Phosphorylation of connexin43 on serine368 by protein kinase C regulates gap junctional communication. *J Cell Biol* **149**(7)**:** 1503-1512

Langlois S, Cowan KN, Shao Q, Cowan BJ, Laird DW (2008) Caveolin-1 and -2 interact with connexin43 and regulate gap junctional intercellular communication in keratinocytes. *Mol Biol Cell* **19**(3)**:** 912-928

Larsen WJ, Tung HN, Murray SA, Swenson CA (1979) Evidence for the participation of actin microfilaments and bristle coats in the internalization of gap junction membrane. *J Cell Biol* **83**(3)**:** 576-587

Lauf U, Giepmans BN, Lopez P, Braconnot S, Chen SC, Falk MM (2002) Dynamic trafficking and delivery of connexons to the plasma membrane and accretion to gap junctions in living cells. *Proc Natl Acad Sci U S A* **99**(16)**:** 10446-10451

Le AC, Musil LS (1998) Normal differentiation of cultured lens cells after inhibition of gap junction-mediated intercellular communication. *Dev Biol* **204**(1)**:** 80-96

Le AC, Musil LS (2001) A novel role for FGF and extracellular signal-regulated kinase in gap junction-mediated intercellular communication in the lens. *J Cell Biol* **154**(1)**:** 197-216

Lee JR, Derosa AM, White TW (2009) Connexin mutations causing skin disease and deafness increase hemichannel activity and cell death when expressed in Xenopus oocytes. *J Invest Dermatol* **129**(4)**:** 870-878

Leithe E, Brech A, Rivedal E (2006) Endocytic processing of connexin43 gap junctions: a morphological study. *Biochem J* **393**(Pt 1)**:** 59-67

Leithe E, Kjenseth A, Sirnes S, Stenmark H, Brech A, Rivedal E (2009) Ubiquitylation of the gap junction protein connexin-43 signals its trafficking from early endosomes to lysosomes in a process mediated by Hrs and Tsg101. *J Cell Sci* **122**(Pt 21)**:** 3883-3893

Leithe E, Rivedal E (2004a) Epidermal growth factor regulates ubiquitination, internalization and proteasome-dependent degradation of connexin43. *J Cell Sci* **117**(Pt 7)**:** 1211-1220

Leithe E, Rivedal E (2004b) Ubiquitination and down-regulation of gap junction protein connexin-43 in response to 12-O-tetradecanoylphorbol 13-acetate treatment. *J Biol Chem* **279**(48)**:** 50089-50096

Leithe E, Rivedal E (2007) Ubiquitination of gap junction proteins. *J Membr Biol* **217**(1-3)**:** 43-51

Leverson JD, Joazeiro CA, Page AM, Huang H, Hieter P, Hunter T (2000) The APC11 RING-H2 finger mediates E2-dependent ubiquitination. *Mol Biol Cell* **11**(7)**:** 2315-2325

Leykauf K, Salek M, Bomke J, Frech M, Lehmann WD, Durst M, Alonso A (2006) Ubiquitin protein ligase Nedd4 binds to connexin43 by a phosphorylation-modulated process. *J Cell Sci* **119**(Pt 17)**:** 3634-3642

Li LK, So L, Spector A (1985) Membrane cholesterol and phospholipid in consecutive concentric sections of human lenses. *J Lipid Res* **26**(5)**:** 600-609

Li W, Bengtson MH, Ulbrich A, Matsuda A, Reddy VA, Orth A, Chanda SK, Batalov S, Joazeiro CA (2008) Genome-wide and functional annotation of human E3 ubiquitin ligases identifies MULAN, a mitochondrial E3 that regulates the organelle's dynamics and signaling. *PLoS One* **3**(1)**:** e1487

Li W, Ye Y (2008) Polyubiquitin chains: functions, structures, and mechanisms. *Cell Mol Life Sci* **65**(15)**:** 2397-2406

Lin D, Boyle DL, Takemoto DJ (2003) IGF-I-induced phosphorylation of connexin 43 by PKCgamma: regulation of gap junctions in rabbit lens epithelial cells. *Invest Ophthalmol Vis Sci* **44**(3)**:** 1160-1168

Lin D, Takemoto DJ (2005) Oxidative activation of protein kinase Cgamma through the C1 domain. Effects on gap junctions. *J Biol Chem* **280**(14)**:** 13682-13693

Lin R, Warn-Cramer BJ, Kurata WE, Lau AF (2001) v-Src phosphorylation of connexin 43 on Tyr247 and Tyr265 disrupts gap junctional communication. *J Cell Biol* **154**(4)**:** 815-827

Lipardi C, Mora R, Colomer V, Paladino S, Nitsch L, Rodriguez-Boulan E, Zurzolo C (1998) Caveolin transfection results in caveolae formation but not apical sorting of glycosylphosphatidylinositol (GPI)-anchored proteins in epithelial cells. *J Cell Biol* **140**(3)**:** 617-626

Liu L, Li Y, Lin J, Liang Q, Sheng X, Wu J, Huang R, Liu S (2009) Connexin43 interacts with Caveolin-3 in the heart. *Mol Biol Rep*

Liu S, Taffet S, Stoner L, Delmar M, Vallano ML, Jalife J (1993) A structural basis for the unequal sensitivity of the major cardiac and liver gap junctions to intracellular acidification: the carboxyl tail length. *Biophys J* **64**(5)**:** 1422-1433

Lo CW, Cohen MF, Huang GY, Lazatin BO, Patel N, Sullivan R, Pauken C, Park SM (1997) Cx43 gap junction gene expression and gap junctional communication in mouse neural crest cells. *Dev Genet* **20**(2)**:** 119-132

Locke D, Liu J, Harris AL (2005) Lipid rafts prepared by different methods contain different connexin channels, but gap junctions are not lipid rafts. *Biochemistry* **44**(39)**:** 13027-13042

Lopes VS, Ramalho JS, Owen DM, Karl MO, Strauss O, Futter CE, Seabra MC (2007) The ternary Rab27a-Myrip-Myosin VIIa complex regulates melanosome motility in the retinal pigment epithelium. *Traffic* **8**(5)**:** 486-499

Luders J, Demand J, Hohfeld J (2000) The ubiquitin-related BAG-1 provides a link between the molecular chaperones Hsc70/Hsp70 and the proteasome. *J Biol Chem* **275**(7)**:** 4613-4617

Lurtz MM, Louis CF (2003) Calmodulin and protein kinase C regulate gap junctional coupling in lens epithelial cells. *Am J Physiol Cell Physiol* **285**(6)**:** C1475-1482

Lurtz MM, Louis CF (2007) Intracellular calcium regulation of connexin43. *Am J Physiol Cell Physiol* **293**(6)**:** C1806-1813

Maldonado-Baez L, Wendland B (2006) Endocytic adaptors: recruiters, coordinators and regulators. *Trends Cell Biol* **16**(10)**:** 505-513

Malewicz B, Kumar VV, Johnson RG, Baumann WJ (1990) Lipids in gap junction assembly and function. *Lipids* **25**(8)**:** 419-427

Mao Y, Senic-Matuglia F, Di Fiore PP, Polo S, Hodsdon ME, De Camilli P (2005) Deubiquitinating function of ataxin-3: insights from the solution structure of the Josephin domain. *Proc Natl Acad Sci U S A* **102**(36)**:** 12700-12705

Marquart KH (1977) So-called annular gap junctions in bone cells of normal mice. *Experientia* **33**(2)**:** 270-272

Martin PE, George CH, Castro C, Kendall JM, Capel J, Campbell AK, Revilla A, Barrio LC, Evans WH (1998) Assembly of chimeric connexin-aequorin proteins into functional gap junction channels. Reporting intracellular and plasma membrane calcium environments. *J Biol Chem* **273**(3)**:** 1719-1726

Martinez-Wittinghan FJ, Sellitto C, White TW, Mathias RT, Paul D, Goodenough DA (2004) Lens gap junctional coupling is modulated by connexin identity and the locus of gene expression. *Invest Ophthalmol Vis Sci* **45**(10)**:** 3629-3637

Maxfield FR, McGraw TE (2004) Endocytic recycling. *Nat Rev Mol Cell Biol* **5**(2)**:** 121-132

McCullough J, Clague MJ, Urbe S (2004) AMSH is an endosome-associated ubiquitin isopeptidase. *J Cell Biol* **166**(4)**:** 487-492

McDonough H, Patterson C (2003) CHIP: a link between the chaperone and proteasome systems. *Cell Stress Chaperones* **8**(4)**:** 303-308

McGrath JP, Jentsch S, Varshavsky A (1991) UBA 1: an essential yeast gene encoding ubiquitin-activating enzyme. *EMBO J* **10**(1)**:** 227-236

Meacham GC, Patterson C, Zhang W, Younger JM, Cyr DM (2001) The Hsc70 co-chaperone CHIP targets immature CFTR for proteasomal degradation. *Nat Cell Biol* **3**(1)**:** 100-105

Mellman I (1996) Endocytosis and molecular sorting. *Annu Rev Cell Dev Biol* **12:** 575-625

Meresse S, Gorvel JP, Chavrier P (1995) The rab7 GTPase resides on a vesicular compartment connected to lysosomes. *J Cell Sci* **108 (Pt 11):** 3349-3358

Meyer R, Malewicz B, Baumann WJ, Johnson RG (1990) Increased gap junction assembly between cultured cells upon cholesterol supplementation. *J Cell Sci* **96 (Pt 2):** 231-238

Meyer RA, Laird DW, Revel JP, Johnson RG (1992) Inhibition of gap junction and adherens junction assembly by connexin and A-CAM antibodies. *J Cell Biol* **119**(1)**:** 179-189

Meyerholz A, Hinrichsen L, Groos S, Esk PC, Brandes G, Ungewickell EJ (2005) Effect of clathrin assembly lymphoid myeloid leukemia protein depletion on clathrin coat formation. *Traffic* **6**(12)**:** 1225-1234

Miranda M, Sorkin A (2007) Regulation of receptors and transporters by ubiquitination: new insights into surprisingly similar mechanisms. *Mol Interv* **7**(3)**:** 157-167

Mizuno E, Kobayashi K, Yamamoto A, Kitamura N, Komada M (2006) A deubiquitinating enzyme UBPY regulates the level of protein ubiquitination on endosomes. *Traffic* **7**(8)**:** 1017-1031

Moreno AP (2004) Biophysical properties of homomeric and heteromultimeric channels formed by cardiac connexins. *Cardiovasc Res* **62**(2)**:** 276-286

Moreno AP, Chanson M, Elenes S, Anumonwo J, Scerri I, Gu H, Taffet SM, Delmar M (2002) Role of the carboxyl terminal of connexin43 in transjunctional fast voltage gating. *Circ Res* **90**(4)**:** 450-457

Moreno AP, Fishman GI, Spray DC (1992) Phosphorylation shifts unitary conductance and modifies voltage dependent kinetics of human connexin43 gap junction channels. *Biophys J* **62**(1)**:** 51-53

Moreno AP, Lau AF (2007) Gap junction channel gating modulated through protein phosphorylation. *Prog Biophys Mol Biol* **94**(1-2)**:** 107-119

Morley GE, Taffet SM, Delmar M (1996) Intramolecular interactions mediate pH regulation of connexin43 channels. *Biophys J* **70**(3)**:** 1294-1302

Mukhopadhyay D, Riezman H (2007) Proteasome-independent functions of ubiquitin in endocytosis and signaling. *Science* **315**(5809)**:** 201-205

Murata S, Sasaki K, Kishimoto T, Niwa S, Hayashi H, Takahama Y, Tanaka K (2007) Regulation of CD8+ T cell development by thymus-specific proteasomes. *Science* **316**(5829)**:** 1349-1353

Murray SA, Williams SY, Dillard CY, Narayanan SK, McCauley J (1997) Relationship of cytoskeletal filaments to annular gap junction expression in human adrenal cortical tumor cells in culture. *Exp Cell Res* **234**(2)**:** 398-404

Musil LS, Goodenough DA (1991) Biochemical analysis of connexin43 intracellular transport, phosphorylation, and assembly into gap junctional plaques. *J Cell Biol* **115**(5)**:** 1357-1374

Musil LS, Goodenough DA (1993) Multisubunit assembly of an integral plasma membrane channel protein, gap junction connexin43, occurs after exit from the ER. *Cell* **74**(6)**:** 1065-1077

Musil LS, Le AC, VanSlyke JK, Roberts LM (2000) Regulation of connexin degradation as a mechanism to increase gap junction assembly and function. *J Biol Chem* **275**(33)**:** 25207-25215

Myers SJ, Stanley KK (1999) Src family kinase activation in glycosphingolipid-rich membrane domains of endothelial cells treated with oxidised low density lipoprotein. *Atherosclerosis* **143**(2)**:** 389-397

Nagasawa K, Chiba H, Fujita H, Kojima T, Saito T, Endo T, Sawada N (2006) Possible involvement of gap junctions in the barrier function of tight junctions of brain and lung endothelial cells. *J Cell Physiol* **208**(1)**:** 123-132

Nagy JI, Ochalski PA, Li J, Hertzberg EL (1997) Evidence for the co-localization of another connexin with connexin-43 at astrocytic gap junctions in rat brain. *Neuroscience* **78**(2)**:** 533-548

Nakagawa S, Maeda S, Tsukihara T (2010) Structural and functional studies of gap junction channels. *Curr Opin Struct Biol*

Naus CC, Hearn S, Zhu D, Nicholson BJ, Shivers RR (1993) Ultrastructural analysis of gap junctions in C6 glioma cells transfected with connexin43 cDNA. *Exp Cell Res* **206**(1)**:** 72-84

Niessen H, Harz H, Bedner P, Kramer K, Willecke K (2000) Selective permeability of different connexin channels to the second messenger inositol 1,4,5-trisphosphate. *J Cell Sci* **113 (Pt 8):** 1365-1372

Nishikawa H, Ooka S, Sato K, Arima K, Okamoto J, Klevit RE, Fukuda M, Ohta T (2004) Mass spectrometric and mutational analyses reveal Lys-6-linked polyubiquitin chains catalyzed by BRCA1-BARD1 ubiquitin ligase. *J Biol Chem* **279**(6)**:** 3916-3924

Nossal R (2001) Energetics of clathrin basket assembly. *Traffic* **2**(2)**:** 138-147

Nussbaum AK, Dick TP, Keilholz W, Schirle M, Stevanovic S, Dietz K, Heinemeyer W, Groll M, Wolf DH, Huber R, Rammensee HG, Schild H (1998) Cleavage motifs of the yeast 20S proteasome beta subunits deduced from digests of enolase 1. *Proc Natl Acad Sci U S A* **95**(21)**:** 12504-12509

Olk S, Zoidl G, Dermietzel R (2009) Connexins, cell motility, and the cytoskeleton. *Cell Motil Cytoskeleton* **66**(11)**:** 1000-1016

Owen DJ (2004) Linking endocytic cargo to clathrin: structural and functional insights into coated vesicle formation. *Biochem Soc Trans* **32**(Pt 1)**:** 1-14

Oyamada M, Oyamada Y, Takamatsu T (2005) Regulation of connexin expression. *Biochim Biophys Acta* **1719**(1-2)**:** 6-23

Ozkaynak E, Finley D, Solomon MJ, Varshavsky A (1987) The yeast ubiquitin genes: a family of natural gene fusions. *EMBO J* **6**(5)**:** 1429-1439

Page AM, Hieter P (1999) The anaphase-promoting complex: new subunits and regulators. *Annu Rev Biochem* **68:** 583-609

Pak Y, Glowacka WK, Bruce MC, Pham N, Rotin D (2006) Transport of LAPTM5 to lysosomes requires association with the ubiquitin ligase Nedd4, but not LAPTM5 ubiquitination. *J Cell Biol* **175**(4)**:** 631-645

Paul DL (1986) Molecular cloning of cDNA for rat liver gap junction protein. *J Cell Biol* **103**(1)**:** 123-134

Paul DL, Ebihara L, Takemoto LJ, Swenson KI, Goodenough DA (1991) Connexin46, a novel lens gap junction protein, induces voltage-gated currents in nonjunctional plasma membrane of Xenopus oocytes. *J Cell Biol* **115**(4)**:** 1077-1089

Paulson AF, Lampe PD, Meyer RA, TenBroek E, Atkinson MM, Walseth TF, Johnson RG (2000) Cyclic AMP and LDL trigger a rapid enhancement in gap junction assembly through a stimulation of connexin trafficking. *J Cell Sci* **113 (Pt 17):** 3037-3049

Paznekas WA, Boyadjiev SA, Shapiro RE, Daniels O, Wollnik B, Keegan CE, Innis JW, Dinulos MB, Christian C, Hannibal MC, Jabs EW (2003) Connexin 43 (GJA1) mutations cause the pleiotropic phenotype of oculodentodigital dysplasia. *Am J Hum Genet* **72**(2)**:** 408-418

Paznekas WA, Karczeski B, Vermeer S, Lowry RB, Delatycki M, Laurence F, Koivisto PA, Van Maldergem L, Boyadjiev SA, Bodurtha JN, Jabs EW (2009) GJA1 mutations, variants, and connexin 43 dysfunction as it relates to the oculodentodigital dysplasia phenotype. *Hum Mutat* **30**(5)**:** 724-733

Peng J, Schwartz D, Elias JE, Thoreen CC, Cheng D, Marsischky G, Roelofs J, Finley D, Gygi SP (2003) A proteomics approach to understanding protein ubiquitination. *Nat Biotechnol* **21**(8)**:** 921-926

Peracchia C (1990a) Effects of caffeine and ryanodine on low pHi-induced changes in gap junction conductance and calcium concentration in crayfish septate axons. *J Membr Biol* **117**(1)**:** 79-89

Peracchia C (1990b) Increase in gap junction resistance with acidification in crayfish septate axons is closely related to changes in intracellular calcium but not hydrogen ion concentration. *J Membr Biol* **113**(1)**:** 75-92

Peracchia C (2004) Chemical gating of gap junction channels; roles of calcium, pH and calmodulin. *Biochim Biophys Acta* **1662**(1-2)**:** 61-80

Peracchia C, Bernardini G, Peracchia LL (1983) Is calmodulin involved in the regulation of gap junction permeability? *Pflugers Arch* **399**(2)**:** 152-154

Peracchia C, Wang XG, Peracchia LL (2000) Chemical gating of gap junction channels. *Methods* **20**(2)**:** 188-195

Petroski MD, Kleiger G, Deshaies RJ (2006) Evaluation of a diffusion-driven mechanism for substrate ubiquitination by the SCF-Cdc34 ubiquitin ligase complex. *Mol Cell* **24**(4)**:** 523-534

Pfeffer SR (2005) Structural clues to Rab GTPase functional diversity. *J Biol Chem* **280**(16)**:** 15485-15488

Pickart CM, Eddins MJ (2004) Ubiquitin: structures, functions, mechanisms. *Biochim Biophys Acta* **1695**(1-3)**:** 55-72

Pickart CM, Fushman D (2004) Polyubiquitin chains: polymeric protein signals. *Curr Opin Chem Biol* **8**(6)**:** 610-616

Pickart CM, Rose IA (1985) Ubiquitin carboxyl-terminal hydrolase acts on ubiquitin carboxyl-terminal amides. *J Biol Chem* **260**(13)**:** 7903-7910

Piehl M, Lehmann C, Gumpert A, Denizot JP, Segretain D, Falk MM (2007) Internalization of large double-membrane intercellular vesicles by a clathrin-dependent endocytic process. *Mol Biol Cell* **18**(2)**:** 337-347

Pitts JD, Burk RR (1987) Mechanism of inhibition of junctional communication between animal cells by phorbol ester. *Cell Tissue Kinet* **20**(2)**:** 145-151

Plemper RK, Bohmler S, Bordallo J, Sommer T, Wolf DH (1997) Mutant analysis links the translocon and BiP to retrograde protein transport for ER degradation. *Nature* **388**(6645)**:** 891-895

Plotkin LI, Manolagas SC, Bellido T (2002) Transduction of cell survival signals by connexin-43 hemichannels. *J Biol Chem* **277**(10)**:** 8648-8657

Qin H, Shao Q, Igdoura SA, Alaoui-Jamali MA, Laird DW (2003) Lysosomal and proteasomal degradation play distinct roles in the life cycle of Cx43 in gap junctional intercellular communication-deficient and -competent breast tumor cells. *J Biol Chem* **278**(32)**:** 30005-30014

Raiborg C, Stenmark H (2009) The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins. *Nature* **458**(7237)**:** 445-452

Reaume AG, de Sousa PA, Kulkarni S, Langille BL, Zhu D, Davies TC, Juneja SC, Kidder GM, Rossant J (1995) Cardiac malformation in neonatal mice lacking connexin43. *Science* **267**(5205)**:** 1831-1834

Reggiori F, Klionsky DJ (2005) Autophagosomes: biogenesis from scratch? *Curr Opin Cell Biol* **17**(4)**:** 415-422

Revilla A, Bennett MV, Barrio LC (2000) Molecular determinants of membrane potential dependence in vertebrate gap junction channels. *Proc Natl Acad Sci U S A* **97**(26)**:** 14760-14765

Revilla A, Castro C, Barrio LC (1999) Molecular dissection of transjunctional voltage dependence in the connexin-32 and connexin-43 junctions. *Biophys J* **77**(3)**:** 1374-1383

Reyes-Turcu FE, Ventii KH, Wilkinson KD (2009) Regulation and cellular roles of ubiquitin-specific deubiquitinating enzymes. *Annu Rev Biochem* **78:** 363-397

Reyes-Turcu FE, Wilkinson KD (2009) Polyubiquitin binding and disassembly by deubiquitinating enzymes. *Chem Rev* **109**(4)**:** 1495-1508

Richard G, Brown N, Rouan F, Van der Schroeff JG, Bijlsma E, Eichenfield LF, Sybert VP, Greer KE, Hogan P, Campanelli C, Compton JG, Bale SJ, DiGiovanna JJ, Uitto J (2003) Genetic heterogeneity in erythrokeratodermia variabilis: novel mutations in the connexin gene GJB4 (Cx30.3) and genotype-phenotype correlations. *J Invest Dermatol* **120**(4)**:** 601-609

Robinson MS (2004) Adaptable adaptors for coated vesicles. *Trends Cell Biol* **14**(4)**:** 167-174

Rosenzweig R, Osmulski PA, Gaczynska M, Glickman MH (2008) The central unit within the 19S regulatory particle of the proteasome. *Nat Struct Mol Biol* **15**(6)**:** 573-580

Rothberg KG, Heuser JE, Donzell WC, Ying YS, Glenney JR, Anderson RG (1992) Caveolin, a protein component of caveolae membrane coats. *Cell* **68**(4)**:** 673-682

Rotin D, Kumar S (2009) Physiological functions of the HECT family of ubiquitin ligases. *Nat Rev Mol Cell Biol* **10**(6)**:** 398-409

Row PE, Prior IA, McCullough J, Clague MJ, Urbe S (2006) The ubiquitin isopeptidase UBPY regulates endosomal ubiquitin dynamics and is essential for receptor down-regulation. *J Biol Chem* **281**(18)**:** 12618-12624

Russell SJ, Steger KA, Johnston SA (1999) Subcellular localization, stoichiometry, and protein levels of 26 S proteasome subunits in yeast. *J Biol Chem* **274**(31)**:** 21943-21952

Rutz ML, Hulser DF (2001) Supramolecular dynamics of gap junctions. *Eur J Cell Biol* **80**(1)**:** 20-30

Saez JC, Berthoud VM, Branes MC, Martinez AD, Beyer EC (2003) Plasma membrane channels formed by connexins: their regulation and functions. *Physiol Rev* **83**(4)**:** 1359-1400

Saksena S, Wahlman J, Teis D, Johnson AE, Emr SD (2009) Functional reconstitution of ESCRT-III assembly and disassembly. *Cell* **136**(1)**:** 97-109

Saliez J, Bouzin C, Rath G, Ghisdal P, Desjardins F, Rezzani R, Rodella LF, Vriens J, Nilius B, Feron O, Balligand JL, Dessy C (2008) Role of caveolar compartmentation in endothelium-derived hyperpolarizing factor-mediated relaxation: Ca2+ signals and gap junction function are regulated by caveolin in endothelial cells. *Circulation* **117**(8)**:** 1065-1074

Schnitzer JE, Oh P, McIntosh DP (1996) Role of GTP hydrolysis in fission of caveolae directly from plasma membranes. *Science* **274**(5285)**:** 239-242

Schubert AL, Schubert W, Spray DC, Lisanti MP (2002) Connexin family members target to lipid raft domains and interact with caveolin-1. *Biochemistry* **41**(18)**:** 5754-5764

Seki A, Duffy HS, Coombs W, Spray DC, Taffet SM, Delmar M (2004) Modifications in the biophysical properties of connexin43 channels by a peptide of the cytoplasmic loop region. *Circ Res* **95**(4)**:** e22-28

Sellitto C, Li L, White TW (2004) Connexin50 is essential for normal postnatal lens cell proliferation. *Invest Ophthalmol Vis Sci* **45**(9)**:** 3196-3202

Seol JH, Feldman RM, Zachariae W, Shevchenko A, Correll CC, Lyapina S, Chi Y, Galova M, Claypool J, Sandmeyer S, Nasmyth K, Deshaies RJ (1999) Cdc53/cullin and the essential Hrt1 RING-H2 subunit of SCF define a ubiquitin ligase module that activates the E2 enzyme Cdc34. *Genes Dev* **13**(12)**:** 1614-1626

Sharon M, Taverner T, Ambroggio XI, Deshaies RJ, Robinson CV (2006) Structural organization of the 19S proteasome lid: insights from MS of intact complexes. *PLoS Biol* **4**(8)**:** e267

Shaw RM, Fay AJ, Puthenveedu MA, von Zastrow M, Jan YN, Jan LY (2007) Microtubule plus-end-tracking proteins target gap junctions directly from the cell interior to adherens junctions. *Cell* **128**(3)**:** 547-560

Shembade N, Harhaj NS, Parvatiyar K, Copeland NG, Jenkins NA, Matesic LE, Harhaj EW (2008) The E3 ligase Itch negatively regulates inflammatory signaling pathways by controlling the function of the ubiquitin-editing enzyme A20. *Nat Immunol* **9**(3)**:** 254-262

Shestopalov VI, Panchin Y (2008) Pannexins and gap junction protein diversity. *Cell Mol Life Sci* **65**(3)**:** 376-394

Shigematsu S, Watson RT, Khan AH, Pessin JE (2003) The adipocyte plasma membrane caveolin functional/structural organization is necessary for the efficient endocytosis of GLUT4. *J Biol Chem* **278**(12)**:** 10683-10690

Sigismund S, Woelk T, Puri C, Maspero E, Tacchetti C, Transidico P, Di Fiore PP, Polo S (2005) Clathrin-independent endocytosis of ubiquitinated cargos. *Proc Natl Acad Sci U S A* **102**(8)**:** 2760-2765

Simon AM, Goodenough DA, Li E, Paul DL (1997) Female infertility in mice lacking connexin 37. *Nature* **385**(6616)**:** 525-529

Singh D, Solan JL, Taffet SM, Javier R, Lampe PD (2005) Connexin 43 interacts with zona occludens-1 and -2 proteins in a cell cycle stage-specific manner. *J Biol Chem* **280**(34)**:** 30416-30421

Sirnes S, Leithe E, Rivedal E (2008) The detergent resistance of Connexin43 is lost upon TPA or EGF treatment and is an early step in gap junction endocytosis. *Biochem Biophys Res Commun* **373**(4)**:** 597-601

Skowyra D, Koepp DM, Kamura T, Conrad MN, Conaway RC, Conaway JW, Elledge SJ, Harper JW (1999) Reconstitution of G1 cyclin ubiquitination with complexes containing SCFGrr1 and Rbx1. *Science* **284**(5414)**:** 662-665

Sleer LS, Brown AJ, Stanley KK (2001) Interaction of caveolin with 7-ketocholesterol. *Atherosclerosis* **159**(1)**:** 49-55

Solan JL, Lampe PD (2009) Connexin43 phosphorylation: structural changes and biological effects. *Biochem J* **419**(2)**:** 261-272

Sowa G, Pypaert M, Fulton D, Sessa WC (2003) The phosphorylation of caveolin-2 on serines 23 and 36 modulates caveolin-1-dependent caveolae formation. *Proc Natl Acad Sci U S A* **100**(11)**:** 6511-6516

Spray DC, Burt JM (1990) Structure-activity relations of the cardiac gap junction channel. *Am J Physiol* **258**(2 Pt 1)**:** C195-205

Staub O, Dho S, Henry P, Correa J, Ishikawa T, McGlade J, Rotin D (1996) WW domains of Nedd4 bind to the proline-rich PY motifs in the epithelial Na+ channel deleted in Liddle's syndrome. *EMBO J* **15**(10)**:** 2371-2380

Steinacher R, Schar P (2005) Functionality of human thymine DNA glycosylase requires SUMO-regulated changes in protein conformation. *Curr Biol* **15**(7)**:** 616-623

Steinman RM, Mellman IS, Muller WA, Cohn ZA (1983) Endocytosis and the recycling of plasma membrane. *J Cell Biol* **96**(1)**:** 1-27

Stenmark H (2009) Rab GTPases as coordinators of vesicle traffic. *Nat Rev Mol Cell Biol* **10**(8)**:** 513-525

Stolt PC, Bock HH (2006) Modulation of lipoprotein receptor functions by intracellular adaptor proteins. *Cell Signal* **18**(10)**:** 1560-1571

Sue Menko A (2002) Lens epithelial cell differentiation. *Exp Eye Res* **75**(5)**:** 485-490

Swaminathan S, Amerik AY, Hochstrasser M (1999) The Doa4 deubiquitinating enzyme is required for ubiquitin homeostasis in yeast. *Mol Biol Cell* **10**(8)**:** 2583-2594

Takens-Kwak BR, Jongsma HJ (1992) Cardiac gap junctions: three distinct single channel conductances and their modulation by phosphorylating treatments. *Pflugers Arch* **422**(2)**:** 198-200

Tang Z, Li B, Bharadwaj R, Zhu H, Ozkan E, Hakala K, Deisenhofer J, Yu H (2001) APC2 Cullin protein and APC11 RING protein comprise the minimal ubiquitin ligase module of the anaphase-promoting complex. *Mol Biol Cell* **12**(12)**:** 3839-3851

Theiss C, Meller K (2002) Microinjected anti-actin antibodies decrease gap junctional intercellular commmunication in cultured astrocytes. *Exp Cell Res* **281**(2)**:** 197-204

Thomas MA, Zosso N, Scerri I, Demaurex N, Chanson M, Staub O (2003) A tyrosine-based sorting signal is involved in connexin43 stability and gap junction turnover. *J Cell Sci* **116**(Pt 11)**:** 2213-2222

Thomas T, Jordan K, Laird DW (2001) Role of cytoskeletal elements in the recruitment of Cx43-GFP and Cx26-YFP into gap junctions. *Cell Commun Adhes* **8**(4-6)**:** 231-236

Torok K, Stauffer K, Evans WH (1997) Connexin 32 of gap junctions contains two cytoplasmic calmodulin-binding domains. *Biochem J* **326 (Pt 2):** 479-483

Toyofuku T, Akamatsu Y, Zhang H, Kuzuya T, Tada M, Hori M (2001) c-Src regulates the interaction between connexin-43 and ZO-1 in cardiac myocytes. *J Biol Chem* **276**(3)**:** 1780-1788

Toyofuku T, Yabuki M, Otsu K, Kuzuya T, Hori M, Tada M (1998) Direct association of the gap junction protein connexin-43 with ZO-1 in cardiac myocytes. *J Biol Chem* **273**(21)**:** 12725-12731

Traub LM (2009) Tickets to ride: selecting cargo for clathrin-regulated internalization. *Nat Rev Mol Cell Biol* **10**(9)**:** 583-596

Traub LM, Lukacs GL (2007) Decoding ubiquitin sorting signals for clathrin-dependent endocytosis by CLASPs. *J Cell Sci* **120**(Pt 4)**:** 543-553

Traweger A, Fang D, Liu YC, Stelzhammer W, Krizbai IA, Fresser F, Bauer HC, Bauer H (2002) The tight junction-specific protein occludin is a functional target of the E3 ubiquitin-protein ligase itch. *J Biol Chem* **277**(12)**:** 10201-10208

Turin L, Warner A (1977) Carbon dioxide reversibly abolishes ionic communication between cells of early amphibian embryo. *Nature* **270**(5632)**:** 56-57

Ulrich HD (2004) How to activate a damage-tolerant polymerase: consequences of PCNA modifications by ubiquitin and SUMO. *Cell Cycle* **3**(1)**:** 15-18

Unno M, Mizushima T, Morimoto Y, Tomisugi Y, Tanaka K, Yasuoka N, Tsukihara T (2002) The structure of the mammalian 20S proteasome at 2.75 A resolution. *Structure* **10**(5)**:** 609-618

Urschel S, Hoher T, Schubert T, Alev C, Sohl G, Worsdorfer P, Asahara T, Dermietzel R, Weiler R, Willecke K (2006) Protein kinase A-mediated phosphorylation of connexin36 in mouse retina results in decreased gap junctional communication between AII amacrine cells. *J Biol Chem* **281**(44)**:** 33163-33171

Valiunas V, Bukauskas FF, Weingart R (1997) Conductances and selective permeability of connexin43 gap junction channels examined in neonatal rat heart cells. *Circ Res* **80**(5)**:** 708-719

van Deurs B, Roepstorff K, Hommelgaard AM, Sandvig K (2003) Caveolae: anchored, multifunctional platforms in the lipid ocean. *Trends Cell Biol* **13**(2)**:** 92-100

van Leuken R, Clijsters L, Wolthuis R (2008) To cell cycle, swing the APC/C. *Biochim Biophys Acta* **1786**(1)**:** 49-59

van Rijen HV, van Veen TA, Hermans MM, Jongsma HJ (2000) Human connexin40 gap junction channels are modulated by cAMP. *Cardiovasc Res* **45**(4)**:** 941-951

van Veen TA, van Rijen HV, Jongsma HJ (2000) Electrical conductance of mouse connexin45 gap junction channels is modulated by phosphorylation. *Cardiovasc Res* **46**(3)**:** 496-510

VanSlyke JK, Musil LS (2002) Dislocation and degradation from the ER are regulated by cytosolic stress. *J Cell Biol* **157**(3)**:** 381-394

VanSlyke JK, Musil LS (2005) Cytosolic stress reduces degradation of connexin43 internalized from the cell surface and enhances gap junction formation and function. *Mol Biol Cell* **16**(11)**:** 5247-5257

Vanslyke JK, Naus CC, Musil LS (2009) Conformational maturation and post-ER multisubunit assembly of gap junction proteins. *Mol Biol Cell* **20**(9)**:** 2451-2463

Veenstra RD, Wang HZ, Beblo DA, Chilton MG, Harris AL, Beyer EC, Brink PR (1995) Selectivity of connexin-specific gap junctions does not correlate with channel conductance. *Circ Res* **77**(6)**:** 1156-1165

Veenstra RD, Wang HZ, Beyer EC, Brink PR (1994) Selective dye and ionic permeability of gap junction channels formed by connexin45. *Circ Res* **75**(3)**:** 483-490

Verdecia MA, Joazeiro CA, Wells NJ, Ferrer JL, Bowman ME, Hunter T, Noel JP (2003) Conformational flexibility underlies ubiquitin ligation mediated by the WWP1 HECT domain E3 ligase. *Mol Cell* **11**(1)**:** 249-259

Verhagen JC, ter Braake P, Teunissen J, van Ginkel G, Sevanian A (1996) Physical effects of biologically formed cholesterol oxidation products on lipid membranes investigated with fluorescence depolarization spectroscopy and electron spin resonance. *J Lipid Res* **37**(7)**:** 1488-1502

Visintin R, Prinz S, Amon A (1997) CDC20 and CDH1: a family of substrate-specific activators of APC-dependent proteolysis. *Science* **278**(5337)**:** 460-463

Wall ME, Otey C, Qi J, Banes AJ (2007) Connexin 43 is localized with actin in tenocytes. *Cell Motil Cytoskeleton* **64**(2)**:** 121-130

Walz J, Erdmann A, Kania M, Typke D, Koster AJ, Baumeister W (1998) 26S proteasome structure revealed by three-dimensional electron microscopy. *J Struct Biol* **121**(1)**:** 19-29

Wang HZ, Veenstra RD (1997) Monovalent ion selectivity sequences of the rat connexin43 gap junction channel. *J Gen Physiol* **109**(4)**:** 491-507

Wang Z, Schey KL (2009) Phosphorylation and truncation sites of bovine lens connexin 46 and connexin 50. *Exp Eye Res* **89**(6)**:** 898-904

Warn-Cramer BJ, Cottrell GT, Burt JM, Lau AF (1998) Regulation of connexin-43 gap junctional intercellular communication by mitogen-activated protein kinase. *J Biol Chem* **273**(15)**:** 9188-9196

Watanabe H, Washioka H, Tonosaki A (1988) Gap junction and its cytoskeletal undercoats as involved in invagination-endocytosis. *Tohoku J Exp Med* **156**(2)**:** 175-190

Wei CJ, Francis R, Xu X, Lo CW (2005) Connexin43 associated with an N-cadherin-containing multiprotein complex is required for gap junction formation in NIH3T3 cells. *J Biol Chem* **280**(20)**:** 19925-19936

White TW (2002) Unique and redundant connexin contributions to lens development. *Science* **295**(5553)**:** 319-320

White TW (2003) Nonredundant gap junction functions. *News Physiol Sci* **18:** 95-99

White TW, Bruzzone R, Goodenough DA, Paul DL (1992) Mouse Cx50, a functional member of the connexin family of gap junction proteins, is the lens fiber protein MP70. *Mol Biol Cell* **3**(7)**:** 711-720

White TW, Bruzzone R, Wolfram S, Paul DL, Goodenough DA (1994) Selective interactions among the multiple connexin proteins expressed in the vertebrate lens: the second extracellular domain is a determinant of compatibility between connexins. *J Cell Biol* **125**(4)**:** 879-892

White TW, Paul DL, Goodenough DA, Bruzzone R (1995) Functional analysis of selective interactions among rodent connexins. *Mol Biol Cell* **6**(4)**:** 459-470

Wiborg O, Pedersen MS, Wind A, Berglund LE, Marcker KA, Vuust J (1985) The human ubiquitin multigene family: some genes contain multiple directly repeated ubiquitin coding sequences. *EMBO J* **4**(3)**:** 755-759

Williams RL, Urbe S (2007) The emerging shape of the ESCRT machinery. *Nat Rev Mol Cell Biol* **8**(5)**:** 355-368

Williams TM, Lisanti MP (2004) The caveolin proteins. *Genome Biol* **5**(3)**:** 214

Woelk T, Oldrini B, Maspero E, Confalonieri S, Cavallaro E, Di Fiore PP, Polo S (2006) Molecular mechanisms of coupled monoubiquitination. *Nat Cell Biol* **8**(11)**:** 1246-1254

Wu X, Zhao X, Baylor L, Kaushal S, Eisenberg E, Greene LE (2001) Clathrin exchange during clathrin-mediated endocytosis. *J Cell Biol* **155**(2)**:** 291-300

Xu X, Francis R, Wei CJ, Linask KL, Lo CW (2006) Connexin 43-mediated modulation of polarized cell movement and the directional migration of cardiac neural crest cells. *Development* **133**(18)**:** 3629-3639

Yap KL, Kim J, Truong K, Sherman M, Yuan T, Ikura M (2000) Calmodulin target database. *J Struct Funct Genomics* **1**(1)**:** 8-14

Yaron A, Hatzubai A, Davis M, Lavon I, Amit S, Manning AM, Andersen JS, Mann M, Mercurio F, Ben-Neriah Y (1998) Identification of the receptor component of the IkappaBalpha-ubiquitin ligase. *Nature* **396**(6711)**:** 590-594

Yin X, Liu J, Jiang JX (2008) Lens fiber connexin turnover and caspase-3-mediated cleavage are regulated alternately by phosphorylation. *Cell Commun Adhes* **15**(1)**:** 1-11

Yu H, Peters JM, King RW, Page AM, Hieter P, Kirschner MW (1998) Identification of a cullin homology region in a subunit of the anaphase-promoting complex. *Science* **279**(5354)**:** 1219-1222

Zampighi GA, Planells AM, Lin D, Takemoto D (2005) Regulation of lens cell-to-cell communication by activation of PKCgamma and disassembly of Cx50 channels. *Invest Ophthalmol Vis Sci* **46**(9)**:** 3247-3255

Zhou L, Griffin JW (2003) Demyelinating neuropathies. *Curr Opin Neurol* **16**(3)**:** 307-313

Zhou L, Kasperek EM, Nicholson BJ (1999) Dissection of the molecular basis of pp60(v-src) induced gating of connexin 43 gap junction channels. *J Cell Biol* **144**(5)**:** 1033-1045

Zhou Y, Yang W, Lurtz MM, Ye Y, Huang Y, Lee HW, Chen Y, Louis CF, Yang JJ (2007) Identification of the calmodulin binding domain of connexin 43. *J Biol Chem* **282**(48)**:** 35005-35017