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HIF & CHIPs
A NEW AUTOPHAGIC PATHWAY FOR THE DEGRADATION HIF-1α

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Abbreviations

ADM	Adrenomedulin
ADP	Adenosine Diphosphate
Aha1	Activator of 90 kDa Heat Shock Protein ATPase Homolog 1
AMP	Adenosine Monophosphate
AMPK	5′-AMP-activated Protein Kinase
APAF1	Apoptotic Peptidase Activating Factor 1
APF-1	ATP-dependent Proteolysis Factor 1
ATG	Autophagy-related Genes
ATP	Adenosine Triphosphate
BAG	Bcl-2-associated Athogene
BAX	Bcl-2–associated X protein
BCC3	Bcl-2 Binding Component 3
Bcl	B-cell Lymphoma
BH3	Bcl-2 Homology
bHLH	Basic Helix-loop-helix
BNip3	Bcl-2/Adenovirus EIB 19-kDa Interacting Protein 3
BrdU	5-bromo-2′-deoxyuridine
CASA	Chaperone Assisted Selective Autophagy
CBP	CREB-binding Protein
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CHIP	Carboxy Terminus of Hsc70-interacting Protein
CMA	Chaperone-Mediated Autophagy
CP	Core Particle
DAPk	Death-associated Protein Kinases
DNA	Deoxyribonucleic Acid
DUB	Deubiquitinating Enzyme
E1	Ubiquitin-activating Enzyme
E2	Ubiquitin Carrier Protein

E3	Ubiquitin-protein Ligase
EE	Early Endosome
EF1α	Elongation Factor 1α
EGF	Epidermal Growth Factor
eIF2α	Eukaryotic Initiation Factor 2α
ER	Endoplasmic Reticulum
ERAD	Endoplasmic Reticulum Associated Degradation
ErbB2	Erythroblast Leukemia Viral Oncogene Homolog 2
Erk	Extracellular-signal-regulated Kinase
ESCRT	Endosomal Sorting Complex Required for Transport
ET1	Endothelin 1
Fas	TNF Receptor Superfamily Member 6
FIH-1	Factor Inhibiting HIF-1
GABARAP	Gamma-aminobutyric Acid Receptor Associated Protein
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase
GATE-16	Golgi-associated ATPase enhancer of 16kDa
GFAP	Glial Fibrillary Acidic Protein
GLUT	Glucose Transporter
GR	Glucocorticoid Receptor
GTP	Guanine Triphosphate
HECT	Homologous to the E6-AP Carboxyl Terminus
HIF-1	Hypoxia Inducible Factor 1
Hip	Hsc70-interacting Protein
HLF	HIF-like Factor
Hop	Hsc70-Hsp90 organizing Protein
HRE	Hypoxia Responsive Element
HRF	HIF-related Factor
Hsc	Heat Shock Cognate
Hsp	Heat Shock Protein
IGF	Insulin-like Growth Factor
IkappaB	Inhibitor of Kappa B

IP3	Inositol-trisphosphate
Ire	ER-membrane-associated Protein
LAMP	Lysosome Associated Protein
LC3	Light Chain 3
LE	Late Endosome
LMP	Lysosomal Membrane Protein
M6PR	Mannose-6-phosphate Receptor
MAPK	Mitogen-activated Protein Kinase
Mdm	Mourine Double Minute
MGO	Methylglyoxal
MMP	Matrix Metalloproteinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium Bromide
MVB	Multivesicular Bodies
NBR1	Neighbor of BRAC1 gene 1
NCOA2	Nuclear Receptor Coactivator 2
NOS2	Nitric Oxide Synthase
NSM	Nonselective Microautophagy
ODDD	Oxygen-dependent Degradation Domain
PAI	Plasminogen Activator Inhibitor
PAS	Pre-autophagosomal Structure
PE	Phosphatidylethanolamine
PHD	Prolyl Hydroxylase
PI3K	Phosphoinositol 3-kinase
PK	Piruvate Kinase
PM	Plasma Membrane
PMAIP1	Phorbol-12-myristate-13-acetate-induced Protein 1
PPQC	Peripheral Protein Quality Control
RCAN1	Regulator of Calcineurin 1
RCC	Renal Cell Carcinoma
RING	Really Interesting New Gene
RNA	Ribonucleic Acid

RP	Regulatory Particle
RPE	Retinal Pigmented Epithelium
SENP	Sentrin Specific Peptidase
SM	Selective Microautophagy
SRC-1	Steroid Receptor Coactivator-1
SSAT	Spermidine/Spermine N(1)-acetyltransferase
Stv	Starvin
SUMO	Small Ubiquitin-like Modifier
TAD	Transactivation Domain
TCA	Tricarboxylic Acid
TGF-β	Transforming Growth Factor
TGN	Trans-Golgi Network
TOR	Target of Rapamycin
TPR	Tetratricopeptide Repeat
TSE	Tubular Sorting Endosome
Ub	Ubiquitin
UBA	Ubiquitin Associated
Ubc	Ubiquitin-conjugating Enzyme
UBL	Ubiquitin Like
UCH	Ubiquitin Carboxy-terminal Esterase
Uev	Ubiquitin-conjugating Enzyme E2 Variant
UPS	Ubiquitin–proteasome System
USP	Ubiquitin Specific Peptidase
VEGF	Vascular Endothelial Growth Factor
VHL	von Hippel-Lindau Tumor Supressor
Vps	Vacuolar Protein Sorting Proteins

Resumo

O factor de transcrição HIF-1 (*Hypoxia Inducible Factor 1*) é maioritariamente regulado pela degradação proteassomal da sua subunidade lábil, o HIF-1α, num mecanismo que depende de oxigénio. Neste estudo demonstramos que o HIF-1α também é degradado no lisossoma, através da Autofagia Mediada por Chaperones (AMC). O dominio KFERQ, identificado em todos os substratos da AMC, é necessário para o direccionamento do HIF-1α para o lisossoma, assim como para a sua interacção com o receptor da AMC, a LAMP2A. O chaperone Hsc70, responsável pela entrega de substratos à AMC, também interage com o domínio KFERQ do HIF-1α. Neste trabalho demonstramos ainda que o HIF-1α se localiza e é degradado em lisossomas isolados de fígado de rato positivos para a AMC, assim como compete com substratos canónicos desta via pelo acesso aos lisossomas. Por outro lado, neste trabalho demonstramos, pela primeira vez, que o co-chaperone CHIP é necessário para o direccionamento do HIF-1α para a AMC. Este novo mecanismo para a degradação do HIF-1α não depende da presença de oxigénio mas é activado pela deprivação de nutrientes. De facto, os níveis de HIF-1α presente nos lisossomas positivos para a AMC aumentam significativamente em animais em jejum. Ao mesmo tempo, a interacção entre o HIF-1α e a LAMP2A também aumenta em células submetidas a deprivação de nutrientes. A degradação excessiva de HIF-1α, induzida pela sobreactivação desta via, compromete a resposta adaptativa das células à hipóxia. Este facto sugere que esta via pode ter importância fisiopatológica em situações que combinem a hipóxia com a falta de nutrientes.

Abstract

The transcription factor HIF-1 (Hypoxia Inducible Factor 1) is mostly regulated by the oxygen dependent proteasomal degradation of the labile subunit HIF-1α. In this study we show that HIF-1α is degraded in the lysosome and, more importantly, it is a new substrate for Chaperone-Mediated Autophagy (CMA). The KFERQ-like motive that has been identified in all CMA substrates is required to direct HIF-1α to the lysosomes and for the subsequent interaction with the CMA receptor LAMP2A. The chaperone Hsc70, that targets the substrates for CMA, also interacts with the KFERQ-like motive of HIF-1α. Moreover, we show that HIF-1α binds to and is translocated into intact CMA positive lysosomes, isolated from rat liver, competing with canonical CMA substrates. In addition we show, for the first time, that the co-chaperone CHIP is required to direct HIF-1α for CMA. This new pathway for degradation of HIF-1α does not depend on the presence of oxygen and is activated in response to nutrient deprivation such that the levels of HIF-1α bound to CMA+ lysosomes significantly increase in starved animals and the binding of HIF-1α to LAMP2A clearly increases in response to nutrient deprivation. Excessive degradation of HIF-1α by CMA compromises cells ability to respond to and survive under hypoxia, suggesting that this pathway might be of pathophysiological importance in conditions that combine hypoxia with nutrient deprivation.

Chapter 1. Introduction

1.1 Proteolysis

The physicist Erwin Schrödinger wrote in his book "***What is Life?***" that "*The chromosome structures…are instrumental in bringing about the development they foreshadow. They are law-code and executive power or, to use another simile, they are architect's plan and builder's craft-in one.*" (Schrödinger, 1962). He believed that chromosomes were made up of an "*aperiodic crystal*" that contained genetic information in the form of a code. Paradoxically, in his time, the crystals were considered to be proteins rather than nucleic acids. Yet, these and other considerations of his on several subjects regarding biological sciences were pivotally influential in the future works of pioneering molecular biologist. (Dronamraju, 1999). The groundbreaking discoveries made thereafter on DNA structure, code and replication spawned an era where biologists focused their attention on how the genetic code was transcribed into RNA and then translated onto proteins, or in other words, how proteins were made (Ciechanover, 2006). Proteins were essentially regarded as very stable cellular constituents, either subjected to minor "wear and tear" that could eventually hamper their function, or as energy-providing fuel. The paradigm in the field at that time was, (i) ingested proteins are completely digested and the products are excreted and (ii) the structural proteins were stable and static (Ciechanover, 2006). Paradigmatically, the concept of protein turnover had its embryo in the 1930's, through Rudolf Scheonheimer seminal studies. Using ^{15}N-labed tyrosine in rats Scheonheimer realized that only ~50% of the administered heavy amino acid was recovered from urine, while the rest was incorporated to tissue proteins (Schoenheimer et al., 1939). They further discovered that only a fraction was attached to the original carbon chain, namely to tyrosine, while the bulk was distributed over other nitrogenous groups of the proteins (Schoenheimer et al., 1939). These experiments demonstrated unequivocally that the body structural

proteins are in a dynamic state of synthesis and degradation, and that even amino acids are in a state of dynamic interconvertion (Schoenheimer et al., 1939)

1.2 Different pathways, the same end: Lysosome vs Proteasome

By the mid-1950's several independent experiments had substantiated the idea that cellular proteins are in a constant state of synthesis and degradation (Ciechanover, 2006), so the discovery by Christian de Duve and his coworkers of an organelle that contains a broad array of isolated proteases with different specificities provided, for the first time, a machinery that could potentially mediate intracellular proteolysis (de Duve, 2005). As a concept, a membrane sealed compartment that prevented the actual proteases from escaping into the cytoplasm was attractive. It confined proteolysis to a well-defined space, preventing random degradation of protein substrates throughout the cell. De Duve called the newly discovered compartment lysosome, Greek for "*digestive body*".

The discovery of the lysosome happened by chance. Part of the biochemical work that de Duve and coworkers had to perform consisted in distinguishing and separating glucose 6-phosphatase from another liver phosphatase, the non-specific acid phosphatase. This acid phosphatase showed abnormally low activities in the homogenate and in the recovered fractions. When re-assayed, after the preparations had been kept in a refrigerator for 5 days, the activity had risen to normal value (de Duve, 2005). Further experiments indicated that acid phosphatase was enclosed within a sac-like particle surrounded by a membrane that prevented the enzyme from getting out and the substrate from getting in (Berthet et al., 1951). The mere ageing at low temperature, allowed the enzyme and substrate to interact, suppressing the latency of the enzyme.

The definition of lysosome has evolved over the years. As early as 1963 the scientific community had recognized the presence of lysosomes in several cell types and had found them to be involved in the digestion of extracellular material taken up by endocytosis and of intracellular material segregated by autophagy (de Duve, 2005). Nowadays we recognize that the lysosomal system is a much more complex digestive system that involves numerous stages of lysosomal maturation together

with structures devoid of hydrolases as endosomes, multivesicular bodies and autophagic vesicles. They mediate four different digestive processes, receptor mediated-endocytosis, non-specific engulfment of cytosolic droplets containing extracellular fluid or pinocytosis, phagocytosis and autophagy (micro-, macro- and chaperone mediated) (Ciechanover, 2006).

After the initial discovery of the lysosome and throughout the first findings about its functioning, the assumption was made that all intracellular protein degradation was mediated by the lysosome. It seemed logical that, since proteolysis results from direct interaction between substrate and protease, it would be impossible for proteases to float freely in the cytosol. However this general theory for proteolysis, even though gradually, was eventually challenged. More than the observations that different proteins had different half-lifes (Goldberg and St John, 1976; Schimke and Doyle, 1970) and that the alteration of cellular physiological conditions heterogeneously influenced the rates of degradation of different proteins, there was the evidence that general or specific lysosomal inhibitors had different effects on different protein populations (Ciechanover, 2006). Lysosome inhibition had a strong inhibitory effect on the proteolysis of endocytosed/pynocytosed extracellular proteins, a partial inhibitory effect on long-lived cytosolic proteins and almost no effect on short-lived cytosolic proteins (Knowles and Ballard, 1976; Neff et al., 1979). Moreover proteolysis also requires energy, in the form of ATP, for the degradation of proteins (Mandelstam, 1958; Simpson, 1953; Steinberg and Vaughan, 1956).

Proteolysis is an exergonic process and the thermodynamically paradoxical energy requirement for intracellular proteolysis made researchers believe that energy cannot be consumed directly by proteases or the proteolytic process *per se*, and was instead used indirectly (Simpson, 1953). The prevailing theory argued that energy was required for the transport of substrates into the lysosome or for the maintenace of low intralysosomal pH (Steinberg and Vaughan, 1956). Nevertheless bacteria (Goldberg and Dice, 1974; Goldberg and St John, 1976) and then reticulocytes (Ciehanover et al., 1978; Etlinger and Goldberg, 1977), which lack lysosomes, were still able to degrade proteins and still met the requirement for energy. In the 1970s Hershko, Ciechanover and Rose managed to isolate two different fractions of these extracts which were both required to reconstitute the

energy-dependent proteolytic activity that was found in the crude extract (Ciehanover et al., 1978; Etlinger and Goldberg, 1977). They purified the active component from fraction I and found a small 8.5 kDa heat stable protein that was designated ATP-dependent Proteolysis Factor 1 (APF-1) (Ciehanover et al., 1978). APF-1 was later identified as ubiquitin. Further studies showed that fraction I contained other components necessary for the degradation of substrates, but these were not necessary for the reconstitution of the system at that time. Importantly, multiple moieties of ubiquitin were covalently conjugated to the target substrate when incubated in the presence of fraction II, a modification that required ATP (Ciechanover et al., 1980; Hershko et al., 1980). It was also found that the modification was reversible, and ubiquitin could be removed from the substrate or its degradation products (Hershko et al., 1980). These revealing first findings led, in 1980, to the proposal of a model according to which protein substrate modification by multiple moieties of ubiquitin targeted it for degradation by a downstream, and at that time yet unidentified protease, that couldn't recognize the unmodified substrate. Furthermore, following degradation, reusable ubiquitin was released (Hershko et al., 1980).

Goldknopf and Busch (Goldknopf and Busch, 1975, 1977) as well as Hunt and Dayhoff (Hunt and Dayhoff, 1977), found that ubiquitin was bound to the substrate by a fork-like, branched isopeptide bond between the carboxy-terminal glycine of ubiquitin (Gly76) and the ε-NH_2 group of an internal lysine (Lys119). After the addition of the first ubiquitin moiety the extension of the polyubiquitin chain was made between Gly76 of one ubiquitin moiety and internal Lys48 of the previously conjugated moiety. This conjugation reaction is mediated by three different enzymes: (i) E1, the ubiquitin-activating enzyme, (ii) E2, the ubiquitin carrier protein, and (iii) E3, the ubiquitin-protein ligase (Ciechanover et al., 1982; Hershko et al., 1983). Soon after, Hough and colleagues partially purified and characterized a high-molecular mass alkaline protease that degraded ubiquitin adducts of lysozyme but not untagged lysozyme, in an ATP-dependent mode (Hough et al., 1986). This protease was later called the 26S proteasome and provided all the necessary criteria for being the specific proteolytic arm of the ubiquitin system.

1.2.1 Ubiquitin-Proteasome System

Degradation of a protein via the ubiquitin-proteasome pathway involves two discrete and successive steps: *1*) tagging of the substrate by covalent attachment of multiple ubiquitin molecules and *2*) degradation of the tagged protein by the 26S proteasome complex with release of free and reusable ubiquitin (Glickman and Ciechanover, 2002). The proteasome is a large, 26S, multicatalytic protease that degrades polyubiquitinated proteins to small peptides. It is composed of two subcomplexes: a 20S core particle (CP) that carries the catalytic activity and a 19S regulatory particle (RP). The 20S CP is a barrel-shaped structure composed of four stacked rings, two identical outer-rings and two identical inner-rings. The function of the 19S RP is to recognize ubiquitinated proteins and other potential substrates of the proteasome (Glickman, 2000; Groll et al., 1997; Lowe et al., 1995).

Ubiquitin-mediated proteolysis of a variety of cellular proteins plays an important role in many basic cellular processes. Among these are regulation of cell cycle and division, differentiation and development, involvement in the cellular response to stress and extracellular effectors, morphogenesis of neuronal networks, modulation of cell surface receptors, ion channels and the secretory pathway, DNA repair, transcriptional regulation, transcriptional silencing, long-term memory, circadian rhythms, regulation of the immune and inflammatory responses, and biogenesis of organelles (Glickman and Ciechanover, 2002).

The tagging of substrates requires what is designated as the ubiquitin conjugation machinery, composed of the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2) and the ubiquitin-protein ligase (E3) (Hershko et al., 1983). E1 activates ubiquitin, via a two-step intramolecular and ATP-dependent reaction, to generate a high-energy E1-thiol-ester ubiquitin intermediate. The activated ubiquitin moiety is then transferred to E2 (Hershko et al., 1983). E2s catalyze covalent attachment of ubiquitin to target proteins, or, when acting along with HECT domain E3s, transfer of the activated ubiquitin moiety to a highenergy E3 ubiquitin intermediate (Hershko et al., 1983). They all share an active-site ubiquitin-binding Cys residue and are distinguished by the presence of a UBC domain required for binding of distinct E3s. Many E2s have been described in higher organisms.

Typically, each E2 interacts with a number of ligases, thus being involved in targeting numerous substrates. The E3s, which are responsible for the specific recognition of the multitude substrates of the ubiquitin system, are the least defined components of the pathway and display the greatest variety among its different components (Glickman and Ciechanover, 2002).

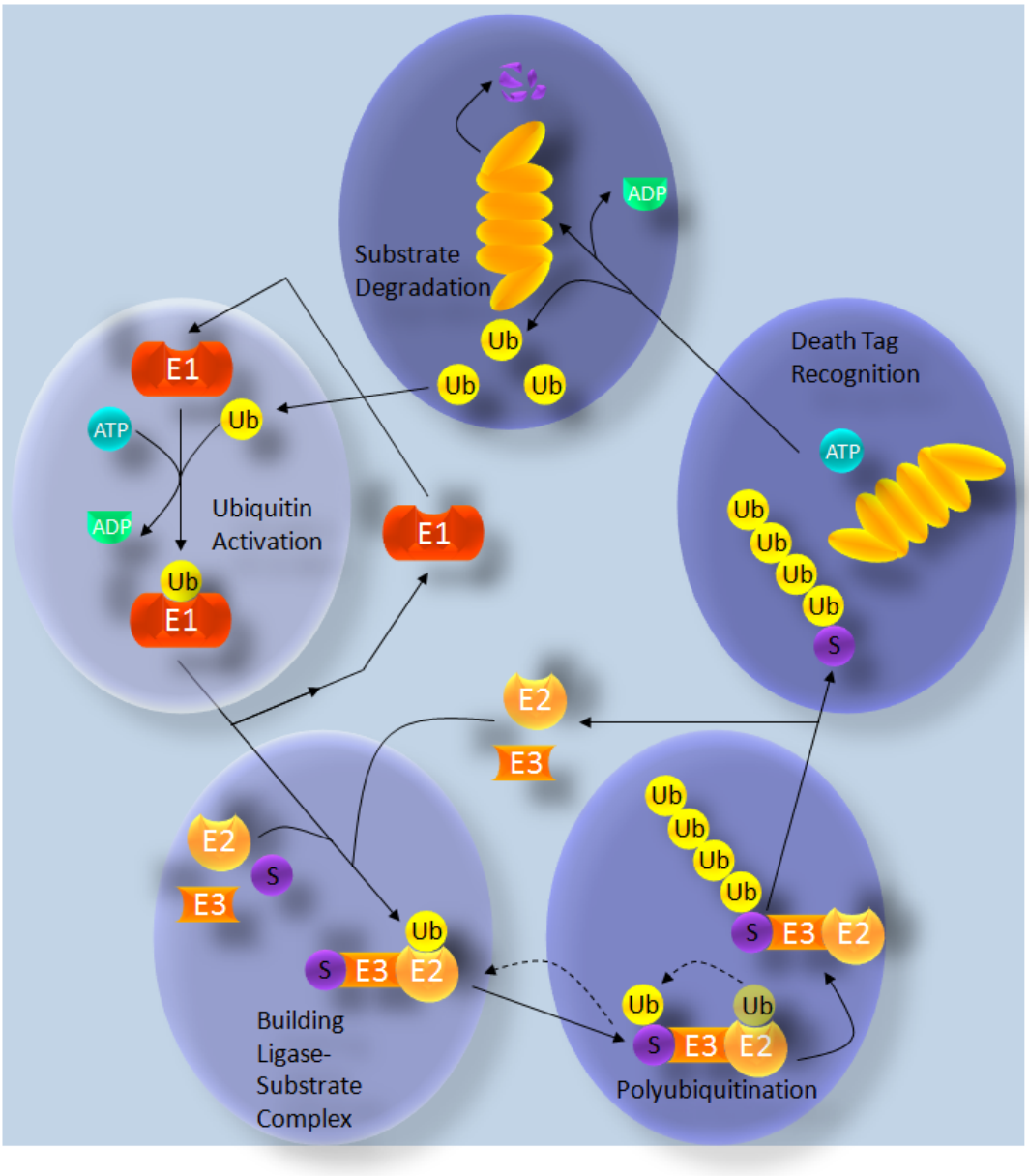

Figure 1. Schematic of the Ubiquitin-Proteasome System. Adapted from (Nalepa et al., 2006)

The ubiquitin ligase is a protein or a protein complex that binds to both the E2 and the substrate. Interaction with the substrate can be direct or via ancillary proteins. In the RING finger domain E3s, the E3 serves as a scaffold that brings together the E2 and the substrate to the proximity, allowing for efficient transfer of

the activated ubiquitin moiety from the E2 to the substrate (Jackson et al., 2000; Joazeiro and Weissman, 2000; Lorick et al., 1999; Yewdell and Bennink, 2001).
In other cases (HECT domain E3s), the activated ubiquitin is transferred from E2 to an internal Cys residue on the E3 before conjugation of ubiquitin to an NH_2 group in the target. Here, the E3 has a catalytic role. An additional subset of E3s (U-box domain), much like RING E3s, serve as a scaffold aiding in the transfer of ubiquitin from the E2 to a previously conjugated ubiquitin moiety, in effect elongating polyubiquitin chains (Scheffner et al., 1993). Finally, ubiquitin is also a product of the proteasome; ubiquitin, or ubiquitin attached to a residual peptide chain, is released from the proteasome and recycled back into the ubiquitin pathway (Hough et al., 1986; Hough and Rechsteiner, 1986; Swaminathan et al., 1999). A deubiquitinating enzyme (DUB) must be associated with the RP to remove or edit these polyubiquitin chains. The RP from a number of sources has been shown to contain an ubiquitin hydrolase activity that can serve to edit these ubiquitin chains, or remove ubiquitin from protein substrates. DUBs can also be associated with the E3-substrate complex an edit ubiquitin chains (Eytan et al., 1993; Lam et al., 1997a; Laroia et al., 1999; Layfield et al., 1999; Liakopoulos et al., 1998).

1.2.2 Lysosomes

1.2.2.1 Biogenesis

The knowledge gathered in the last 60 years on the lysosomal system allowed the concept of protein degradation on this compartment to develop and expand. We know now that lysosomes receive their substrates through endocytosis, phagocytosis and autophagy. But what we also know is that lysosome formation, or more accurately lysosome biogenesis, is a collaborative effort of both the cellular biosynthetic pathway and the endocytic pathway. The degradative endocytic pathway is a gradual step-by-step process that starts at the endocytic vesicles and ends in the lysosomes. This step-by-step process determines that the endocytosed cargo passes through a range of endosomal intermediates that are distinguished by their content, molecular makeup, morphology, pH and the kinetics by which endocytic tracers reach them (Sachse et al., 2002). From beginning to end, lysosome

biogenesis requires the constant exchange of incoming and outgoing membranes and multiple fusion events. The net result is a gradual remodelling of an endosomal intermediate into a later-stage endosome (Dunn and Maxfield, 1992; Futter et al., 1996; Stoorvogel et al., 1991). Though it oversimplifies the complexity of the endocytic pathway, endosomes are widely subdivided in early endosomes (EEs) and late endosomes (LEs) (Sachse et al., 2002). The EEs are the main recipients of incoming endocytic vesicles from the plasma membrane. Cargo received by the EEs can both recycle back to the plasma membrane, as well as be transported further along the endocytic pathway. At this early stage EEs will already start receiving endogenous proteins from the Trans-Golgi Network (TGN), such as mannose-6-phosphate receptors (M6PRs) carrying the lysosomal hydrolases (Klumperman et al., 1993; Waguri et al., 2003). In the mildly acidic environment of EEs, lysosomal hydrolases dissociate from M6PRs and remain in the endosomal lumen (Bonifacino and Rojas, 2006). Moreover EEs contain a vacuolar part (also referred to as the sorting endosome) from which a reticulum of multibranching tubules emerges (Conus and Simon, 2008; Tooze and Hollinshead, 1991), referred to as the tubular sorting endosome (TSE) (Peden et al., 2004; van Meel and Klumperman, 2008) or tubular endosomal network (Bonifacino and Rojas, 2006; van Meel and Klumperman, 2008), that enables them to recycle proteins to the plasma membrane or to the TGN. The EEs also have low levels of lysosomal membrane proteins LMPs (for example, LAMP1, LAMP2 and CD63) that are destined for the lysosome (Peden et al., 2004). The cargo that remains in the endosomal compartments is thereby destined to get degraded in the lysosomal system. As the endosomal compartments maturate they will contain almost no cargo to recycle back to the plasma membrane but instead will be more enriched in lysosomal hydrolases (Geuze et al., 1988; Griffiths et al., 1988), form fewer and less extensive tubular extensions and communicate further with the TGN and other endosomal compartments rather than the plasma membrane (Luzio et al., 2007). At this point the endosomal compartment will be filled of globular vacuoles, forming what is designated as LE intermediates or multivesicular bodies (MVBs) (Rink et al., 2005; Stoorvogel et al., 1991). As the sorting events of the LEs and the TGN increase these compartments will progressive attain an increased degradative capacity as well as a lower pH (Lubke et al., 2009;

Woodman and Futter, 2008).

Hence, as a concept, the separation of the endocytic pathway from lysosome biogenesis seems to be inaccurate. The endocytic pathway is best regarded as a spatiotemporal continuum of vesicular intermediates, which continuously exchange their content, while undergoing gradual molecular and structural remodelling and functional transformation (Saftig and Klumperman, 2009). The other face of lysosome biogenesis relies on the input, from the biosynthetic pathway, of newly synthesized lysosomal proteins. As for the study of the pathway this adds further complexity to the system. In fact, as lysosomal proteins are synthesized in the endoplasmic reticulum (ER) and transported through the Golgi complex to the TGN, they can follow the constitutive secretory pathway to the plasma membrane and subsequently reach lysosomes by endocytosis. The best-characterized direct intracellular pathway is the clathrin-dependent transport of lysosomal hydrolases by M6PRs (Saftig and Klumperman, 2009). On the other hand, lysosomal proteins can move from the TGN to the endo-lysosomal system. The available literature suggests that there are multiple pathways for both lysosomal hydrolases and lysosomal membrane proteins (for example, lysosomal integral membrane protein 2-mediated transport of β-glucocerebrosidase), which may enter the endo-lysosomal pathways at distinct stages of maturation. Hence the delivery of lysosomal proteins to the endosomal system is a multiple step process where the biosynthetic pathway intersects the endocytic pathway in different endosomal intermediates (Saftig and Klumperman, 2009).

1.2.2.2 Cargo delivery

As soon as the endosomes were identified it became well established that many endocytosed macromolecules, such as low-density lipoprotein, were delivered to lysosomes. What was surprising at that time was the sequential passage through early and late endosomes towards the final destination, the lysosomes (Mellman, 1996; Storrie and Desjardins, 1996). Since that time other ligands such as epidermal growth factor (EGF), that remain bound to their receptors and are soon after endocytosed, showed us that receptors, and other plasma membrane proteins are

internalized from the surface of the endosome into lumenal vesicles, go through late endosomes and ended up in the lysosomes. This is how, to the best of our knowledge, endocytosed cargo is delivered to the lysosome for degradation (White et al., 2006). This sorting mechanism has become one of great interest for scientist in the field and has been well studied throughout the years. Using yeast as a model, researches were able to identify 12 soluble vacuolar protein sorting (Vps) proteins organize into four ESCRT complexes (endosomal sorting complexes required for transport) — ESCRT-0, -I, -II and -III — that are required for protein trafficking to the vacuole (Bowers and Stevens, 2005; Gruenberg and Stenmark, 2004; Hurley and Emr, 2006; Kostelansky et al., 2007; Slagsvold et al., 2006). One surprising observation was that some of the endocytosed cargo required ubiquitination for the intracellular sorting to the endosomal compartments, which was still mediated by the ESCRT machinery (Bowers et al., 2006; Haglund et al., 2003; Huang et al., 2006). This configured one of the first examples of substrate ubiquitination, were the substrate was not delivered to the proteasome. It also showed that the formation of Lys48 chains, or even chain formation for that matter (the first examples of ubiquitinated endocytosed cargo, from the plasma membrane, were of covalent attachment of single ubiquitin moieties to different amino acids or "multi-monoubiquitination") were not the only ubiquitin based post-transductional modifications occurring.

On the other hand, cargo delivery to the lysosome can also occur by lysosomal fusion with phagosomes (phagocytosis) and autophagosomes (autophagy). Phagocytosis is an essential process by which specialized cells engulf invading pathogens, apoptotic cells and other foreign particles that are >0.5 μm in diameter. This often occurs by a zippering mechanism, in which pseudopods (actin-driven protrusions of the plasma membrane) engulf a target by repeated receptor–ligand interactions (Luzio et al., 2007). Thought canonically regarded as a simple phagossome-to-lysosome fusion it is now widely accepted that the phagosome 'matures' by multiple transient interactions with endosomal compartments, including lysosomes, to form a hybrid-like organelle termed the phagolysosome (Desjardins, 1995). Hence phagosomes are capable of fusing with both early and late endosomes and with lysosomes (Jahraus et al., 1998; Mayorga et al., 1991), as

shown by various different assays that demonstrate these capabilities (Jahraus et al., 1998; Mayorga et al., 1991; Peyron et al., 2001; Stockinger et al., 2006). The primary function of the phagolysosome is to degrade the phagocytosed particle.

1.2.3 Autophagy

Autophagy — or to be more specific macroautophagy, microautophagy and chaperone-mediated autophagy — are important mechanisms for the degradation of cytoplasmic components, including organelles, and has long been known to involve degradation by lysosomal enzymes. It is important in many physiological processes (Levine and Klionsky, 2004), including the response to starvation, cell growth and innate immunity. In macroautophagy, double-membrane vesicles called autophagosomes sequester part of the cytoplasm and then fuse with lysosomes to form hybrid-like organelles called autolysosomes. In microautophagy the limiting/sequestering membrane is the lysosomal membrane itself, which invaginates to form tubules or vesicles that pinch off into the lysosomal lumen (Ahlberg and Glaumann, 1985). Studies on microautophagy during recent years mainly focused on its characterization in yeast, where in addition to some genes shared with macroautophagy, a separate subset of genes also participates exclusively in this process (Tuttle and Dunn, 1995). However, microautophagy is still very poorly understood in mammals, and in fact, it is possible that this process actually takes place in late endosomes rather than in lysosomes and occurs through the formation of the multivesicular bodies in this compartment (Fader and Colombo, 2009). Chaperone-mediate autophagy is a direct pathway for transporting cytosolic proteins over the lysosomal limiting membrane and into the lysosome lumen for degradation. The C-terminal tail of LAMP2A is implicated in the transport of cytosolic substrates across the lysosomal limiting membrane as well as different chaperones and co-chaperones being considered the most important amongst them Hsc70 (Cuervo and Dice, 1996). CMA is important for different biological processes, such as the presentation of cytoplasmic antigens by MHC class II molecules (Zhou et al., 2005), cellular ageing (Zhang and Cuervo, 2008) and neurodegeneration (Cuervo et al., 2004).

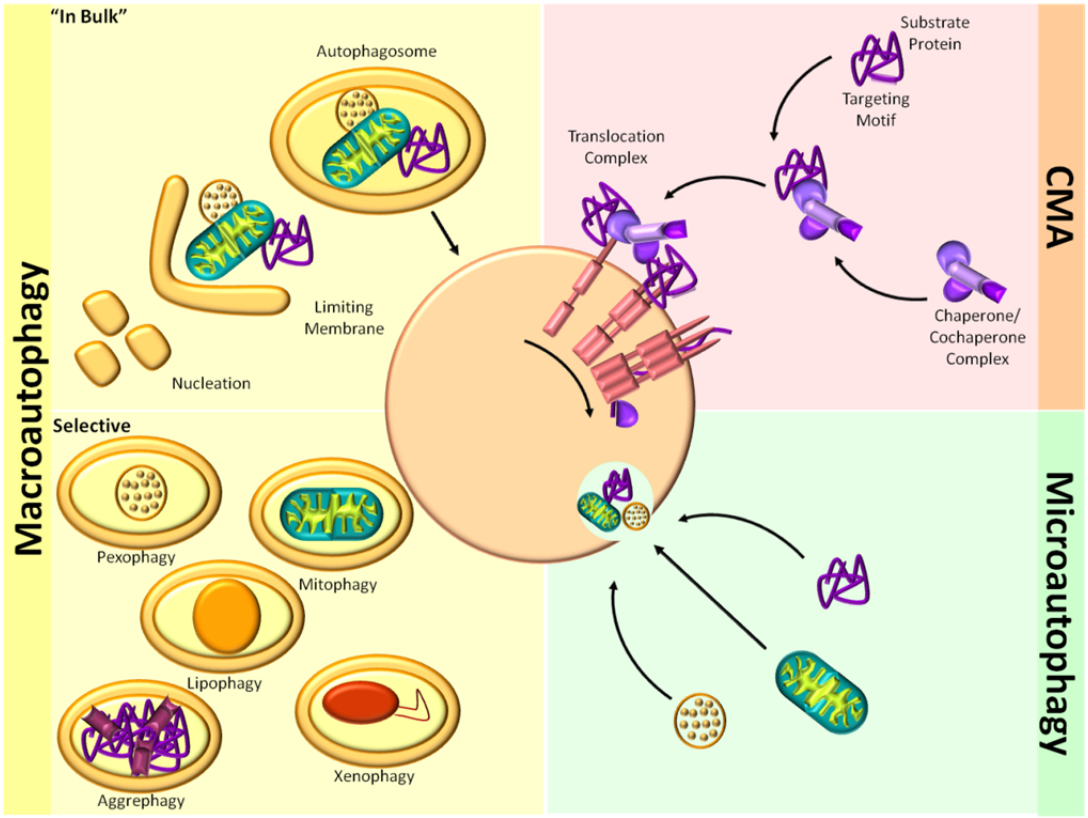

Figure 2. Schematic of the Autophagy pathways. Adapted from (Bejarano and Cuervo, 2010)

1.2.3.1 Macroautophagy

In macroautophagy, a small part of the cytoplasm is sequestered by a membrane sac, the so-called isolation membrane (also termed the phagophore), which results in the formation of a double-membrane structure, the autophagosome. The autophagosome matures and finally fuses with lysosomes. Following fusion, the inner membrane and enclosed cytoplasmic materials are degraded by lysosomal enzymes. The resultant degradation products, such as amino acids, can be reused for many purposes (Mizushima et al., 2011). In addition to canonical macroautophagy, special types of macroautophagy have been discovered. These include autophagy directed to organelles, such as mitochondria (mitophagy) and peroxisomes (pexophagy), to intracellular bacteria (xenophagy) (Klionsky et al., 2007) and to lipid storages (lipophagy) (Singh et al., 2009). It is now also clear that autophagosomes can even recognize certain soluble proteins, such as p62 (Johansen and Lamark, 2011; Kraft et al., 2010). Through these diverse modes, autophagy is involved in

many physiological processes: generation of amino acids during starvation, quality control of intracellular proteins and organelles (related to suppression of cellular degeneration and tumorigenesis), regulation of expression levels of selective substrates, degradation of pathogens, and antigen presentation (Cecconi and Levine, 2008; Deretic and Levine, 2009; Levine and Kroemer, 2008; Menzies et al., 2011; Mizushima and Levine; Mizushima et al., 2008; Virgin and Levine, 2009; White et al.; Wong and Cuervo).

The autophagosome is a unique organelle both in its structure and in its dynamic regulation. The autophagosome is highly inducible; numbers can increase by more than tenfold during starvation. Autophagosomes are rapidly consumed by fusion with lysosomes, having a half-life of 10–25 minutes in the liver (Hailey et al., 2010; Pfeifer, 1978; Schworer et al., 1981).

Elongation of the isolation membrane generates autophagosomes. However, the source of this membrane, how it elongates, and how it completes sealing remain unknown (Tooze and Yoshimori, 2010). As the ER is frequently observed in close proximity to autophagosomes, to this day the ER is still regarded as the most probable source of the autophagosome or the platform for autophagosome formation. Nevertheless other sources for the autophagosome, like mitochondria or the plasma membrane, are now been considered (Rubinsztein et al., 2012).

The molecular machinery responsible for the autophagossome formation in autophagy came from yeast genetic studies, in which 35 autophagy-related (*ATG*) genes have been identified (Nakatogawa et al., 2009). ATG genes consist of several functional units: Atg1 kinase and its regulators, the PI3K complex, Atg9, the Atg2-Atg18 complex, and two ubiquitin-like conjugation systems (Mizushima et al., 2011) Mammals contain counterparts for most yeast Atg proteins as well as some additional factors that are specific to higher eukaryotes (Mizushima et al., 2011). The hierarchical analysis among yeast Atg proteins suggested that two ubiquitin-like conjugation systems function at a late step of autophagosome formation (Mizushima et al., 2011). The two conjugation systems are Atg12–Atg5 and Atg8–PE (phosphatidylethanolamine), Atg8 mammalian homologs are LC3, GABARAP, and GATE-16 (GABARAPL2). These proteins are ubiquitin-like proteins that are synthesized as precursors with additional sequences at their C termini, which are

processed by the cysteine protease Atg4 (Kabeya et al., 2004; Kirisako et al., 2000). The resulting C-terminal glycine-exposed form of Atg8 is activated by Atg7 (E1-like enzyme), transferred to Atg3 (E2-like enzyme), and finally covalently linked to an amino group of PE, a major membrane phospholipis (Ichimura et al., 2000). Atg4 functions also as a deconjugating enzyme and may regulate the level of free Atg8 (Kirisako et al., 2000). Atg8–PE localizes on both the isolation membrane and the autophagosome (Kirisako et al., 1999). On the other hand, in the Atg12 conjugation system, Atg12 is synthesized as a C-terminal glycine exposed form, is activated by Atg7, is transferred to Atg10 (E2-like enzyme), and finally forms a conjugate with the sole target protein Atg5 (Mizushima et al., 1998). The Atg12–Atg5 conjugate interacts with Atg16 (Atg16L in mammals) to form a complex with a 2:2:2 stoichiometry via homodimerization of Atg16/Atg16L1 (Fujioka et al., 2010; Fujita et al., 2008). The Atg12 system has no deconjugating enzyme, and the Atg12–Atg5-Atg16 complex is formed constitutively irrespective of nutrient conditions. In yeast, Atg12–Atg5 conjugates reside on the pre-autophagossomal structure (PAS) but not on the complete autophagosome (Suzuki et al., 2001b). Likewise, in mammals, Atg12–Atg5-Atg16L1 predominantly localizes on the outer surface of the isolation membrane and dissociates from the membrane immediately before or after the completion of autophagosome formation. Both conjugation systems serve as good markers for the detection of membrane structures during autophagy; the Atg12-Atg5 localizes specifically to the isolation membrane, whereas the Atg8-PE is useful in tracing the whole process of autophagy including the formation of the autophagosome and its fusion with lysosomes/vacuoles (Kabeya et al., 2000; Kirisako et al., 1999; Mizushima et al., 2001)

Autophagosomes were thought to sequester cytosolic material nonspecifically. Nonetheless, as a response to starvation, there is ample evidence for selective autophagic degradation of various cellular structures, including protein aggregates, mitochondria, and microbes (Xie and Klionsky, 2007). The mechanism of selective autophagy is not well understood; however, the involvement of ubiquitin in this process is evident: analogous to the proteasome, where ubiquitinated cargo is delivered by Ub receptors (Elsasser and Finley, 2005; Husnjak et al., 2008), autophagic clearance of protein aggregates requires the Ub-binding receptors p62

and NBR1 (Kirkin et al., 2009; Komatsu et al., 2007; Pankiv et al., 2007). It is envisaged that by simultaneous binding to both ubiquitin and the autophagosome associated Ub-like (UBL) proteins (i.e., LC3/GABARAP proteins), these molecules can mediate docking of ubiquitinated protein aggregates to the autophagosome, thereby ensuring their selective degradation. The UBA domain of p62 can bind both K48-linked and K63-linked Ub chains but with a higher affinity for K63 chains (Long et al., 2008; Wooten et al., 2008). NBR1 binds Ub via its UBA domain with a bias toward the K63-linked polyUb chains (Kirkin et al., 2009). Hence, rather surprinsingly, the attachment of Ub moieties to various cellular cargos constitutes a universal degradation signal, recognized by two major intracellular proteolytic systems: the proteasome and macroautophagy.

In relation to its regulation, macroautophagy can be rapidly upregulated when cells need to generate intracellular nutrients and energy, for example, during starvation, growth factor withdrawal, or high bioenergetic demands. Autophagy is also upregulated when cells are preparing to undergo structural remodeling such as during developmental transitions or to rid themselves of damaging cytoplasmic components, for example, during oxidative stress, infection, or protein aggregate accumulation. Nutritional status, hormonal factors, and other cues like temperature, oxygen concentrations, and cell density are important in the control of autophagy. (Klionsky et al., 2007; Maiuri et al., 2007; Mizushima and Klionsky, 2007; Rubinsztein et al., 2007). One of the key regulators of autophagy is the target of rapamycin, TOR kinase, which is the major inhibitory signal that shuts off autophagy in the presence of growth factors and abundant nutrients. The class I PI3K/Akt signaling molecules link receptor tyrosine kinases to TOR activation and thereby repress autophagy in response to insulin-like and other growth factor signals (Lum et al., 2005). Some of the other regulatory molecules that control autophagy include 5′-AMP-activated protein kinase (AMPK), which responds to low energy; the eukaryotic initiation factor 2α (eIF2α), which responds to nutrient starvation, double-stranded RNA, and endoplasmic reticulum (ER) stress; BH3-only proteins that contain a Bcl-2 homology-3 (BH3) domain and disrupt Bcl-2/Bcl-XL inhibition of the Beclin 1/class III PI3K complex; the tumor suppressor protein, p53; death-associated protein kinases (DAPk); the ER-membrane-associated protein, Ire-1; the stress-activated kinase, c-

Jun-N-terminal kinase; the inositol-trisphosphate (IP3) receptor (IP3R); GTPases; Erk1/2; ceramide and calcium (Criollo et al., 2007; Maiuri et al., 2007; Meijer and Codogno, 2006; Rubinsztein et al., 2007).

1.2.3.2 Microautophagy

The term microautophagy was first proposed by de Duve and Wattiaux more than 40 years ago (De Duve and Wattiaux, 1966). At the time it referred to the hypothetical notion that tiny portions of cytoplasm in mammalian cells could be directly sequestered and subsequently engulfed by lysosomes. By contrast with the more morphologically obvious process of macroautophagy, in microautophagy the lysosomal membrane itself is envisaged as undergoing local deformation/rearrangement to directly engulf portions of cytoplasm and any constituents (Mijaljica et al., 2011). In subsequent investigations spanning some 30 years, other research groups have used the term microautophagy to describe lysosomal uptake but without providing much, if any, mechanistic insight into the process observed. (Ahlberg and Glaumann, 1985; Ahlberg et al., 1982; Mortimore et al., 1973; Mortimore et al., 1989; Sakai et al., 1989) It is thus unclear whether these investigators were describing the same process at a mechanistic level.

In the past two decades, the growth in our understanding of microautophagic processes has come almost entirely from studies carried out in baker's yeast, *Saccharomyces cerevisiae*, and the methylotrophic yeasts, *Pichia pastoris* and *Hansenula polymorpha* (Cuervo, 2004b; Dunn et al., 2005; Farre et al., 2009; Farre and Subramani, 2004; Kiel; Kissova et al., 2007; Kunz et al., 2004; Manjithaya et al., 2010; Roberts et al., 2003; Sakai et al., 1998; Sakai et al., 2006; Uttenweiler and Mayer, 2008). Microautophagy-mediated degradation in yeasts can either be nonselective or selective. (Kunz et al., 2004) Nonselective microautophagy (NSM) can be regarded as being involved in degradation of randomly sequestered portions of cytosol, whereas selective microautophagy (SM) is involved in the degradation of specific organelles, (Farre et al., 2009) namely: (1) mitochondria (micromitophagy) (Bhatia-Kissova and Camougrand, 2010; Farre et al., 2009; Kissova et al., 2007; Nowikovsky et al., 2007; Tolkovsky, 2009) (2) the nucleus (piecemeal

microautophagy of the nucleus (PMN) or micronucleophagy) (Krick et al., 2008; Krick et al., 2009; Kvam and Goldfarb, 2007; Roberts et al., 2003) and (3) peroxisomes (micropexophagy) (Dunn et al., 2005; Farre et al., 2009; Farre et al., 2008; Krick et al., 2008; Krick et al., 2009; Kvam and Goldfarb, 2007; Manjithaya et al., 2010; Roberts et al., 2003; Sakai et al., 1998; Sakai et al., 2006).

Little information from the studies done so far can be derived to establish how the process of microautophagy is regulated. There are many unknowns regarding the regulation of microautophagy and its mechanistic nature in mammalian cells, which needs to be further investigated. Microautophagy in mammalian cells is unresponsive to most commonly used autophagy induction conditions such as amino acid and glucagons deprivation (Cuervo, 2004a, b; Mortimore et al., 1988). Interestingly, some other stimuli such as short-term starvation (12h) induce changes in the morphology of the lysosomes (e.g., intralysosomal vesicle sequestration; arm-like membrane extension formation) in hepatocytes, but without clear consequences in the overall rate of protein turnover (Cuervo, 2004a, b; Mortimore et al., 1988).

Textbook entries concerning autophagy in mammalian cells often present electron microscopy images showing organelles such as mitochondria and peroxisomes within lysosomes. What is not clear is the autophagic pathway(s) by which such organelles were delivered to lysosomes, but presumably such organelle delivery occurs by macroautophagy. Thus, there is little direct evidence for microautophagy of peroxisomes or mitochondria in mammalian cells.

In face of the lack of evidence gathered so far it is obvious that currently we know very little about the mechanisms and physiological relevance of microautophagy in mammalian cells and that is hard to judge just how relevant the yeast findings are to mammals.

1.2.3.3 Chaperone-mediated autophagy

In contrast to the in-bulk sequestration of cytosolic components characteristic of macro and microautophagy, soluble cytosolic proteins can be targeted selectively for degradation in lysosomes by CMA. In fact what distinguishes CMA from other forms

of autophagy is the selective recognition of cargo by cytosolic chaperones and the fact that substrates are not engulfed, but, instead, translocate across the lysosomal membrane in a receptor-mediated manner (Chiang et al., 1989). As in the case of macroautophagy, basal CMA activity can be detected in most types of mammalian cells, but maximal activation of this pathway is triggered in response to stressors, such as long-term starvation, oxidative stress, or exposure to toxic compounds that induce abnormal conformational changes in cytosolic proteins (Massey et al., 2006)

Selectivity in CMA is conferred by the presence of a pentapeptide motif in the amino acid sequence of the substrate proteins biochemically related to KFERQ that, when recognized by a cytosolic chaperone, results in the targeting of substrates to lysosomes (Dice, 1990). CMA substrates are recognized first in the cytoplasm by the heat shock cognate protein of 70 kD (Hsc70), the constitutively expressed member of the 70-kD family of chaperones (Chiang et al., 1989). This is actually the same chaperone responsible for disassembly of clathrin from coated vesicles and for folding of unfolded cytosolic proteins upon recognition of exposed hydrophobic regions. It is unknown what determines the multiplicity of functions of the chaperones, but the particular array of cochaperones that bind to Hsc70 in each condition is probably behind the final fate of the substrate protein. A subset of cochaperones, Hsp90, Hsp40, Bcl-2 associate athanogene 2 (Bag-1), Hsc70-Hsp90 organizing protein (Hop), and Hsc70-interacting (Hip), protein has been shown to interact with the CMA substrate–chaperone complex at the lysosomal membrane. Some of the cochaperones may not be directly involved in substrate targeting, but rather participate in the unfolding step required before the substrate can translocate across the lysosomal membrane (Dice, 2007).

Once at the lysosomal surface, the substrate–chaperone complex binds to the membrane, and, after unfolding the substrate, is translocated into the lumen. At the lysosomal membrane the lysosome-associated membrane protein type 2A (LAMP2A) acts as the resident a CMA “receptor” (Cuervo and Dice, 1996). LAMP2A is a single-span membrane protein with a very heavily glycosylated luminal region and a short (12–amino acid) C-terminus tail exposed on the surface of the lysosomes, where substrate proteins bind. LAMP2A is one of the three splice variants of the lamp2 gene, all of which contain identical luminal regions, but different transmembrane

and cytosolic tails (Gough et al., 1995). The mechanisms behind the translocation of substrate proteins across the lysosomal membrane are, as yet, poorly understood. Much evidence supports direct translocation across the lysosomal membrane, rather than engulfment by invaginations of the membrane. Invaginations have never been observed when this transport is reproduced *in vitro*. On the other hand conjugation or cross-linking of substrate proteins to bigger structures, such as gold particles, prevents their uptake, and substrate proteins need to be completely unfolded before reaching the lysosomal lumen (Cuervo and Dice, 1996; Cuervo et al., 1995a; Cuervo et al., 1994; Terlecky and Dice, 1993). By analogy with other protein translocation systems, involvement of a multispan membrane protein to create a discontinuity in the lysosomal membrane is expected. However, to date, proteomic analysis of proteins associated with LAMP2A at the lysosomal membrane has not rendered any such multispan membrane proteins. Nevertheless evidences point to the existence of a unique mechanism for translocation of substrate proteins across the lysosomal membrane via CMA that involves multimerization of LAMP2A (Bandyopadhyay et al., 2008). In fact, binding of substrate proteins to the cytosolic tail of monomeric forms of LAMP2A drives its multimerization to form a 700-kD complex at the lysosomal membrane (Bandyopadhyay et al., 2008). Moreover, the presence of a lysosome-specific form of Hsp90 on the luminal side of the lysosomal membrane is essential to preserve the stability of LAMP2A while it undergoes these conformational changes at the lysosomal membrane (Bandyopadhyay et al., 2008). Also, it seems that the CMA translocation complex forms only transiently and that, once the substrate crosses the membrane, LAMP2A rapidly disassembles in a process mediated by the Hsc70 present on the cytosolic side of the lysosomal membrane (Bandyopadhyay et al., 2008). Cytosolic and lysosomal chaperones only associate with lower-order complexes of substrate and LAMP2A, but are no longer present in the 700-kD complex required for translocation. The regulation of CMA through changes in lysosomal LAMP2A highlights the importance of lateral mobility within the membrane, which has been shown to be determined by its dynamic association with lysosomal lipid microdomains (Kaushik et al., 2006). In this context, upon conditions of low CMA activity, part of LAMP2A is recruited into regions of defined lipid composition, whereas the number of LAMP2A molecules in these lipid

microdomains is markedly reduced when CMA is activated. Accordingly, an increase in microdomain size, by augmenting lysosomal cholesterol results in reduced CMA, whereas cholesterol-extracting drugs increase membrane levels of LAMP2A activating CMA (Kaushik et al., 2006). In fact, the regulated degradation of LAMP2A described above occurs in these lipid microdomains, as luminal cathepsin A preferentially associates to the lysosomal membrane in these regions. By contrast, binding of substrates to LAMP2A and its assembly into and disassembly from the multimeric CMA translocation complex only pertains to LAMP2A molecules outside these microdomains (Kaushik et al., 2006). Also, CMA activation includes not only the relocation of LAMP2A outside the lipid microdomains as well as a luminal pool of intact LAMP2A that can be retrieved to the lysosomal membrane upon CMA stimulation (Cuervo and Dice, 2000). Intrinsic properties of LAMP2A are required to modulate its membrane dynamics. In addition to the GxxG motif required for multimerization (Bandyopadhyay et al., 2008), a proline residue that is present at the interface between its transmembrane and luminal regions is absolutely required for the mobilization of LAMP2A into the lipid microdomains (Kaushik et al., 2006). Other components at the lysosomal membrane that modulate LAMP2A dynamics are the intermediate filament protein glial fibrillary acidic protein (GFAP) and elongation factor 1α (EF1α), a pair of interacting proteins that modify the stability of the multimeric LAMP2A complex and the association of LAMP2A with the lipid microdomains in a GTP-dependent manner (Bandyopadhyay et al., 2010). A lysosome specific variant of GFAP associates with LAMP2A multimers enhancing the stability of the complex and counteracting the disassembly-promoting effect of Hsc70. Lysosomal GFAP partitions into two subpopulations; unphosphorylated GFAP that binds to multimers of LAMP2A and phosphorylated GFAP (GFAP-P), the latter of which is usually bound to the GTP-binding protein EF1α. Unphosphorylated GFAP has higher affinity for GFAP-P than for LAMP2A, but formation of GFAP–GFAP-P dimers is usually prevented by the presence of EF1α bound to GFAP-P. In the presence of GTP, EF1α is released from the lysosomal membrane allowing the dissociation of GFAP from the translocation complex and its binding to GFAP-P (Bandyopadhyay et al., 2010). This dissociation favors the rapid disassembly of the LAMP2A multimeric complex and its active mobilization to lipid microdomains for degradation. Changes

in the levels of GFAP–GFAP-P, EF1α present at the lysosomal membrane, as well as of intracellular GTP or intra-lysosomal Ca^{2+} (facilitating association of cathepsin A to lipid microdomains) can all contribute to modulation of CMA activity.

The final step in substrate translocation into the lysosome appears to involve a form of Hsc70 resident in the lysosomal lumen (lys-Hsc70) (Agarraberes et al., 1997). Only those lysosomes containing Hsc70 in their lumen are competent for uptake of CMA substrates. Interestingly, the percentage of Hsc70-containing lysosomes, which is no more than 40% under resting conditions, escalates to 80% in liver under conditions in which CMA is up-regulated, such as during prolonged starvation or mild oxidative stress (Cuervo et al., 1997; Kiffin et al., 2004). The mechanism by which lys-Hsc70 mediates substrate translocation remains unclear. This chaperone can act either actively, by facilitating substrate internalization in an energy-dependent manner, or passively, by binding the portion of substrate already translocated and preventing its retrograde movement to the cytoplasm. Also unknown is the pathway followed by lys-Hsc70 to reach the lysosomal lumen. It is possible that Hsc70 reaches the lysosome through fusion with late endocytic compartments, where Hsc70 has also been detected. Whether other luminal chaperones are required for substrate translocation is currently unknown.

RNase A, the earliest CMA substrate identified (Dice et al., 1986), has a pentapeptide shown to be critical in its degradation. This pentapeptide (KFERQ) is shared by all substrate proteins identified to date. The pentapeptide motif is, as most intracellular targeting motifs, relaxed and does not rely on the exact amino acid composition, but rather on the charge of the residues in the sequence. Sequence analysis of the cytosolic proteome has revealed that about 30% of cytosolic proteins might be potential substrates for CMA (Dice, 1990). However, it is possible that this amount is an underestimation, because particular post-translational modifications, such as deamidation, phosphorylation, acetylation, etc., could provide the charge missing in a four–amino acid sequence (Dice, 2007). This possibility of modulating chaperone recognition of the substrates by post-translational modifications adds an additional level of regulation to CMA. Another interesting fact is the existence of substrates that can be degraded either by the proteasome or by the lysosome, through CMA. Indeed, several proteasomal substrates were shown to have KFERQ

motifs and, consequently, get degraded in the lysosome by CMA. These include Huntingtin (Thompson et al., 2009), RCAN1 (Liu et al., 2007), α-Synuclein (Cuervo et al., 2004) and IkappaB (Cuervo et al., 1998).

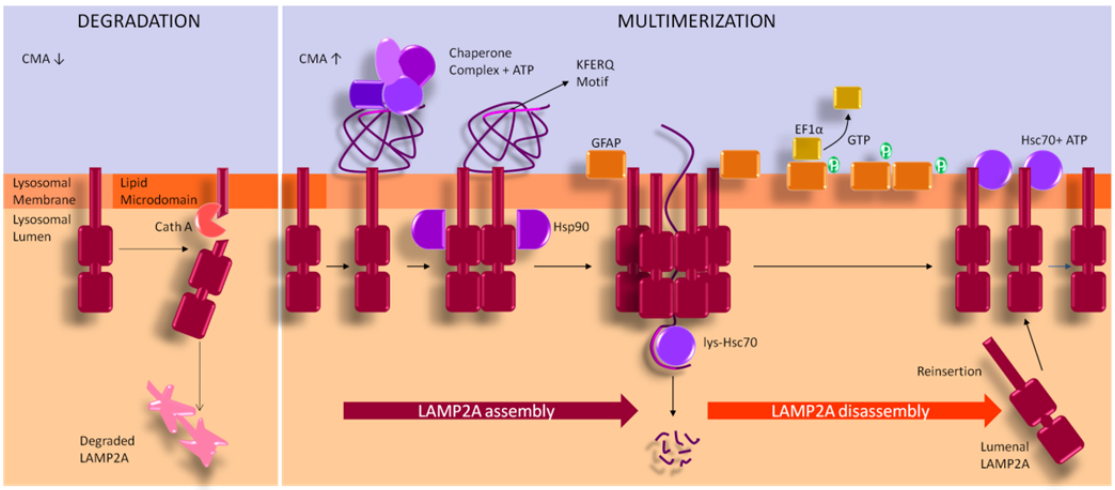

Figure 3. Schematic of the Chaperone-Mediated Autophagy pathway. Adapted from (Cuervo and Macian, 2012)

1.3-Protein Folding

Mammalian cells are crowded with proteins, tipically expressing around 10,000 different protein species (Bartlett and Radford, 2009; Dobson and Ellis, 1998). Nascent proteins generally acquire a specific conformation, by "folding" into a three dimentional architecture, or "native state" (Dunker et al., 2008). Only afterwards are proteins able to perform their function. How this is accomplished and how cells ensure the conformational integrity of their proteome in the face of acute and chronic challenges constitute one of the most fundamental problems in biology. Central to this problem is that proteins must retain conformational flexibility to function, and thus are only marginally thermodynamically stable in their physiological environment (Hartl et al., 2011). Strikingly, 20–30% of all the proteins in a mammalian cell seem to be inherently devoid of any ordered three-dimensional structure, adopting folded conformations only after interaction with binding partners (Dunker et al., 2008). Thus, protein quality control and the maintenance of proteome homeostasis (known as proteostasis) is crucial for cellular functioning. Proteostasis is achieved by an integrated network of several hundred proteins

(Powers et al., 2009), including, most prominently, molecular chaperones and their regulators, which assist in *de novo* folding or refolding, and the ubiquitin–proteasome system (UPS) and autophagy system, which mediate the timely removal of irreversibly misfolded and aggregated proteins. Deficiencies in proteostasis have been shown to facilitate the manifestation or progression of numerous diseases, such as neurodegeneration and dementia, type 2 diabetes, peripheral amyloidosis, lysosomal storage disease, cystic fibrosis, cancer and cardiovascular disease (Hartl et al., 2011).

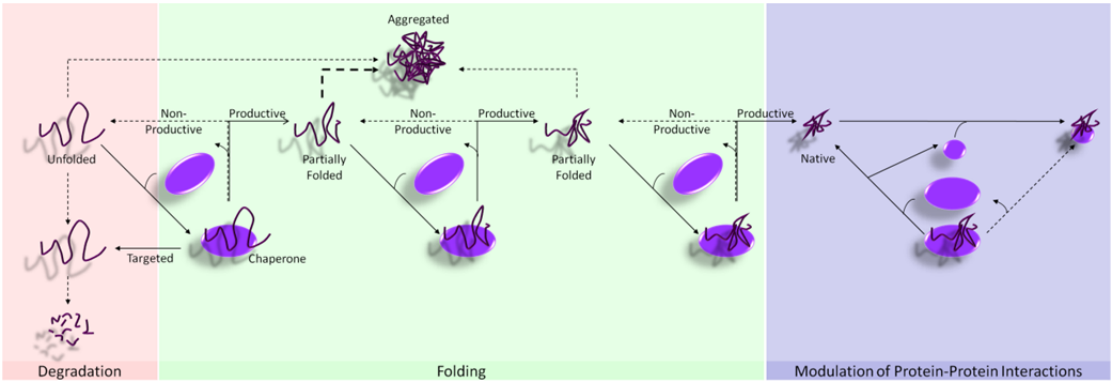

Figure 4. Schematic representation of protein folding and degradation through the client protein-chaperone binding and release cycle. Adapted from (Kampinga and Craig, 2010)

Due to the vast number of possible conformations, proteins (specially the large ones) can adopt folding reactions that are highly complex and heterogeneous, relying on the cooperation of many weak, non-covalent interactions. In the case of soluble proteins, hydrophobic forces are particularly important in driving chain collapse and the burial of non-polar amino-acid residues within the interior of the protein (Skach, 2009). Therefore, it seems likely, that the fundamental requirement for molecular chaperones arose very early during the evolution of densely crowded cells, owing to the need to minimize protein aggregation during folding and maintain proteins in soluble, yet conformationally dynamic states. Moreover, as mutations often disrupt the ability of a protein to adopt a stable fold (Tokuriki and Tawfik, 2009), it follows that the chaperone system provides a crucial buffer, allowing the evolution of new protein functions and phenotypic traits (Rutherford and Lindquist, 1998; Tokuriki and Tawfik, 2009).

1.3.1 Major Chaperone Classes

We define a molecular chaperone as any protein that interacts with, stabilizes or helps another protein to acquire its functionally active conformation, without being present in its final structure (Hartl, 1996; Hartl and Hayer-Hartl, 2009). Several different classes of structurally unrelated chaperones exist in cells, forming cooperative pathways and networks. Members of these protein families are often known as stress proteins or heat-shock proteins (Hsps), as they are upregulated under conditions of stress in which the concentrations of aggregation-prone folding intermediates increase. Chaperones are usually classified according to their molecular weight (Hsp40, Hsp60, Hsp70, Hsp90, Hsp100 and the small Hsps). They are involved in a multitude of proteome-maintenance functions, including *de novo* folding, refolding of stress-denatured proteins, oligomeric assembly, protein trafficking and assistance in proteolytic degradation. The chaperones that participate broadly in *de novo* protein folding and refolding, such as the Hsp70s, Hsp90s and the chaperonins (Hsp60s), are multicomponent molecular machines that promote folding through ATP and cofactor-regulated binding and release cycles. They typically recognize hydrophobic amino-acid side chains exposed by non-native proteins and may functionally cooperate with ATP-independent chaperones, such as the small Hsps, which function as 'holdases', buffering aggregation.

1.3.2 The Hsp70 and Hsp90 Systems

The constitutively expressed Hsc70 (also known as HspA8) and stress-inducible form, Hsp70, are central players in protein folding and proteostasis control. Increasing Hsp70 levels has also proven effective in preventing toxic protein aggregation in disease models (Hartl et al., 2011). The ATP-dependent reaction cycle of Hsp70 is regulated by chaperones of the Hsp40 (also known as DnaJ) family and nucleotide-exchange factors. Some of these factors are also involved in linking chaperone functions with the UPS and autophagy for the removal of misfolded proteins (Hartl et al., 2011). Binding and release by Hsp70 is achieved through the allosteric coupling of a conserved amino-terminal ATPase domain with a carboxy-

terminal peptide-binding domain. These chaperones recognize extended five to seven-residue segments enriched in hydrophobic amino acids, preferentially when they are framed by positively charged residues. Such segments occur on average every 50−100 amino acids in proteins, and the exposure of these fragments correlates with the aggregation propensity of the protein (Hartl et al., 2011). In the ATP-bound state, the chaperone adopts an open conformation. Hydrolysis of ATP to ADP is strongly accelerated by Hsp40, leading to chaperone closure and stable peptide binding (Hartl et al., 2011). After ATP hydrolysis, a nucleotide-exchange factor binds to the Hsp70 ATPase domain and catalyses ADP−ATP exchange, resulting in substrate release (Hartl et al., 2011).

On the other hand, Hsp90 forms a proteostasis hub that controls numerous important signalling pathways in eukaryotic cells (Taipale et al., 2010). These pleiotropic functions include, among others, cell-cycle progression, telomere maintenance, apoptosis, mitotic signal transduction, vesicle-mediated transport, innate immunity and targeted protein degradation. Indeed, the evolution and maintenance of these functional networks is thought to depend on the ability of Hsp90 to buffer the effects of structurally destabilizing mutations in the underlying protein complexes, thereby allowing the acquisition of new traits (Rutherford and Lindquist, 1998). Hsp90 functions downstream of Hsp70 in the structural maturation and conformational regulation of numerous signal-transduction molecules, such as kinases and steroid receptors (McClellan et al., 2007; Taipale et al., 2010). It cooperates in this process with several regulators and co-chaperones, many of which use tetratricopeptide repeat (TPR) domains to dock onto Hsp90. For example, the TPR protein Hop provides a direct link between Hsp70 and Hsp90, allowing substrate transfer (Scheufler et al., 2000).

Hsp90 functions as a dimer of subunits that are assembled by their C-terminal domains. An N-terminal domain binds and hydrolyses ATP and is joined to the C-terminal domain by a middle domain. Similar to other chaperones, the Hsp90 dimer undergoes an ATP-driven reaction cycle that is accompanied by considerable structural rearrangement (Mayer, 2010). ATP binding leads to the dimerization of the N-terminal domains, forming the Hsp90 ‘molecular clamp’. After hydrolysis, the ATPase domains dissociate, and the Hsp90 monomers separate N-terminally.

How Hsp90 recruits different types of substrate proteins with the help of various co-chaperones remains enigmatic. Hsp90 appears to have several substrate-interaction regions, and the binding strength seems to be strongly influenced by the structural flexibility of the substrate (Wandinger et al., 2008), in line with the proposed role of Hsp90 as an evolutionary capacitor in protecting mutated protein variants from degradation (Rutherford and Lindquist, 1998).

1.4 CHIP: Coupling folding with degradation

Molecular chaperones and proteolytic pathways have for long been viewed as opposing forces that control protein biogenesis. A common assumption was that molecular chaperones are specialized in protein folding, whereas energy-dependent proteases such as the proteasome and the lysosome, mediate efficient protein degradation. Data collected over the years, however, suggests that molecular chaperones directly cooperate with the ubiquitin/proteasome system and the lysosomal system during protein quality control in eukaryotic cells. In the center of this axis of protein refolding/degradation is the carboxyl terminus of Hsp70 interacting protein (CHIP). CHIP cDNA encodes a 34.5-kDa protein containing three 34-amino acid TPR domains (involved in interactions with several heat shock factors) at its N-terminus (Ballinger et al., 1999) and a ‘‘U-box’’ domain (with E3-ligase activity) at its C-terminus (Murata et al., 2001b). Separating the TPR and U-box domains in CHIP is a central domain rich in charged residues and also containing two possible nuclear localizing signals. This protein is nowadays considered to act as a tilt from the folding-refolding machinery toward the degradative pathways as well as serving as a link between the two.

1.4.1 CHIP chaperone-binding activity

CHIP is a *bona fide* interaction partner with the major cytoplasmic chaperones Hsc70 and Hsp70, based on their interactions with CHIP in a yeast 2-hybrid screen and *in vitro* binding assays (Ballinger et al., 1999). The TPR domain and an adjacent charged region of CHIP (amino terminus residues 1 to 197) are necessary for its

interaction with Hsp70 and Hsc70 (Ballinger et al., 1999). CHIP interacts with the C-terminal domain of Hsc70 (residues 540 to 650 of Hsc70), which is known to contain a TPR-acceptor site that also interacts with the TPR domain-containing cochaperone Hop (Demand et al., 1998). Although the carboxy-terminal domain of the 70-kDa heat shock proteins is the interaction domain for CHIP's amino terminal TPR domain, it is the amino terminal ATP-binding domain of Hsp70-Hsc70 that regulates substrate binding in a nucleotide-dependent fashion. The molecular cochaperones Hip and Hsp40 promote substrate binding by stabilizing the adenosine diphosphate (ADP)-bound conformation and by enhancing the ATPase activity of Hsp70-Hsc70, respectively (Hohfeld et al., 1995; Minami et al., 1996), On the other hand BAG-1 promotes substrate release by exchanging ATP for ADP (Hohfeld and Jentsch, 1997) (Fig 1). In contrast, CHIP inhibits ATP hydrolysis of Hsp70-Hsc70 and, therefore, attenuates substrate binding and refolding, resulting in inhibition of the ''forward'' Hsp70-Hsc70 substrate folding-refolding pathway, at least in *in vitro* assays (Hohfeld and Jentsch, 1997). The cellular consequences of this ''antichaperone'' function are not yet clear. This activity may provide a mechanism to slow the Hsc70 reaction cycle under stressful conditions, or it may assist in ''loading'' misfolded proteins into the ubiquitin-proteasome machinery, as described below.

Another chaperone that contains a TPR-acceptor site to interact with cochaperones and that interacts with CHIP is Hsp90. Indeed, CHIP does interact with Hsp90 with approximately equivalent affinity to its interactions with Hsp70 (Connell et al., 2001). This interaction results in remodeling of Hsp90 chaperone complexes. For example, the cochaperone p23 (which is required for the appropriate activation of several Hsp90-dependent steroid receptors such as the glucocorticoid receptor [GR]) is excluded. The mechanism for this activity is unclear—p23 and CHIP bind Hsp90 through different sites—yet, the consequence of this action is predictable: CHIP should inhibit the function of proteins that require Hsp90 for conformational activation. Indeed, CHIP inhibits GR substrate binding and steroid-dependent transactivation ability. Interistingly, this effect of CHIP is accompanied by decreased steady-state levels of GR. Indeed, CHIP induceed ubiquitination of the GR *in vivo* and *in vitro* and its proteasome-dependent degradation. This effect is both U-box and TPR-domain dependent, suggesting that CHIP's effect on GR require direct

interaction with Hsp90 and direct ubiquitylation of GR and delivery to the proteasome. These observations are not limited to GR. Other well studied example is ErbB2, another Hsp90 client, that is also degraded by CHIP in a proteasome-dependent fashion (Xu et al., 2002). Nor are they limited to Hsp90 clients; the cystic fibrosis transmembrane conductance receptor, an Hsp70 client, undergoes CHIP-dependent degradation that is Hsp70 dependent (Meacham et al., 2001), and luciferase undergoes CHIP dependent ubiquitination *in vitro* when it is misfolded and bound by Hsp70 (Murata et al., 2001a). In each case, the effects of CHIP are dependent on both the TPR domain, indicating a necessity for interactions with molecular chaperones, and the U-box, which suggests that the U-box is most likely the ‘‘business end’’ with respect to ubiquitination. Hence, the studies are consistent in supporting a role for CHIP as a key component of the chaperone-dependent quality control mechanism. CHIP efficiently targets client proteins, particularly when they are partially unfolded (as is the case for most Hsp90 clients when bound to the chaperone) or frankly misfolded (as is the case for most proteins binding to Hsp70 through exposed hydrophobic residues).

1.4.2 CHIP ubiquitination activity

Once the ubiquitination activity of CHIP was recognized, it was logical to speculate that its U-box might function in a manner analogous to that of RING fingers, which have been appreciated rather recently as key components of the largest family of ubiquitin ligases. Indeed, CHIP utilizes its U-box for binding to E2 ubiquitin-conjugating enzymes of the Ubc4/5 family and acts as an E3 ubiquitin ligase during the ubiquitination of known chaperone substrates (Demand et al., 2001; Hatakeyama et al., 2001; Jiang et al., 2001; Murata et al., 2001b; Pringa et al., 2001). As a consequence, elevating the cellular concentration of CHIP results in an increased degradation of chaperone substrates by the proteasome (Connell et al., 2001; Meacham et al., 2001). *In vitro* reconstitution of CHIP-mediated ubiquitination confirmed this conclusion. Efficient ubiquitination of heat-denatured firefly luciferase by the CHIP/UbcH5 conjugation machinery was dependent on the presence of either Hsp70 or Hsp90 and did not occur with native luciferase (Murata

et al., 2001b). The chaperones apparently select substrates for CHIP-mediated ubiquitination. However, a direct interaction between CHIP and the protein substrate may contribute to the selection process, as CHIP is able to ubiquitinate at least some protein substrates even in the absence of chaperones (Demand et al., 2001) by directly recognizing certain regions of the substrate (He et al., 2004). Moreover CHIP was also found to interact with the Ubc13-Uev1a E2 complex *in vitro*, to essentially produce K63 linked chains, in contrast with the K48 polyubiquitination mediated by the CHIP-UbcH5 complex (Xu et al., 2008; Zhang et al., 2005). The function and/or destination of these K63 polyubiquitin chains is still unknown. In any case, the chaperone/CHIP complex may be viewed as a multi-subunit ubiquitin ligase that contains either Hsp70 or Hsp90 as the main substrate recognition factor. The complex thus resembles other multi-subunit ligases that are characterized by the association of certain ubiquitin ligases and ubiquitin-conjugating enzymes with substrate recognition factors, for example the Skp1/cullin/F-box protein (SCF) complex (Jackson et al., 2000; Zheng et al., 2002). During sorting of chaperone substrates to the proteasome, CHIP may cooperate with the other chaperones or cochaperones. For example, the nucleotide exchange factor of Hsp70 BAG-1, in addition to the BAG domain that mediates Hsp70 binding and regulation, possesses an ubiquitin-like domain within its primary structure. The presence of such a domain is the defining feature of a family of diverse proteins, termed ubiquitin domain proteins (UDPs) (Jentsch and Pyrowolakis, 2000). Similar to other UDPs, BAG-1 utilizes the integrated ubiquitin domain for an association with the proteasome (Alberti et al., 2002; Luders et al., 2000a). Because the domain does not overlap with the chaperone binding site of the cofactor, BAG-1 can act as a coupling factor between Hsp70 and the proteasome. As a consequence, elevating BAG-1 levels induced a proteasomal association of Hsp70 in cell culture experiments. The isolation of CHIP/Hsp70 complexes also lead to the identification of cochaperones that have an inhibitor effect on CHIP activity. Both BAG-2 and HspBP1 attenuate the ubiquitin ligase activity of CHIP when complexed with Hsc70. As a consequence, they interfer with the CHIP-induced degradation of specific substrates (Alberti et al., 2004; Arndt et al., 2005). These findings indicate that the loading of chaperone-bound substrates to the proteasome, mediated by CHIP, is a tightly regulated event

that most probably will not only rely on CHIP and its ubiquitinating activity, but also on the particular binding partners present on these complexes.

1.4.3 Quality control

The characterization of the CHIP cofactor challenges previous concepts regarding the role of molecular chaperones in protein quality control. So far, a kinetic partitioning of non-native polypeptides between chaperones and proteases was proposed to underlie quality control (Wickner et al., 1999). Chaperone binding would direct the non-native polypeptide towards the folded state, whereas an association with components of the degradation machinery would result in polypeptide destruction. This model infers a competition between chaperones and the degradation machinery in substrate binding. The identification of CHIP now suggests a different concept. Through association with the cofactor, molecular chaperones are directly turned into protein degradation factors (Cyr et al., 2002; Sha et al., 2000). Hence, the fate of the chaperone bound polypeptide would be determined to a significant extent by the cofactors that associate with the chaperone/substrate complex. Conceptually this means that the intracellular balance of the competing and cooperating cofactors may therefore set the threshold between folding and degradation.

Binding of CHIP and BAG-1 would lead to degradation, whereas attempts to fold the bound polypeptide would occur upon binding of folding cofactors, such as Hip and Hop. The diverse cofactors apparently define functionally distinct Hsp70 chaperone machines. For example, CHIP and Hop compete in chaperone binding as both cofactors seem to utilize the same docking site at the carboxyl terminus of Hsp70 and Hsp90, respectively (Brinker et al., 2002; Scheufler et al., 2000). A similar competition of cofactors is observed at the amino-terminal ATPase domain of Hsp70, where the proteasome recruitment factor BAG-1 competes with the folding-stimulating cofactor Hip (Hohfeld and Jentsch, 1997; Takayama et al., 1999).

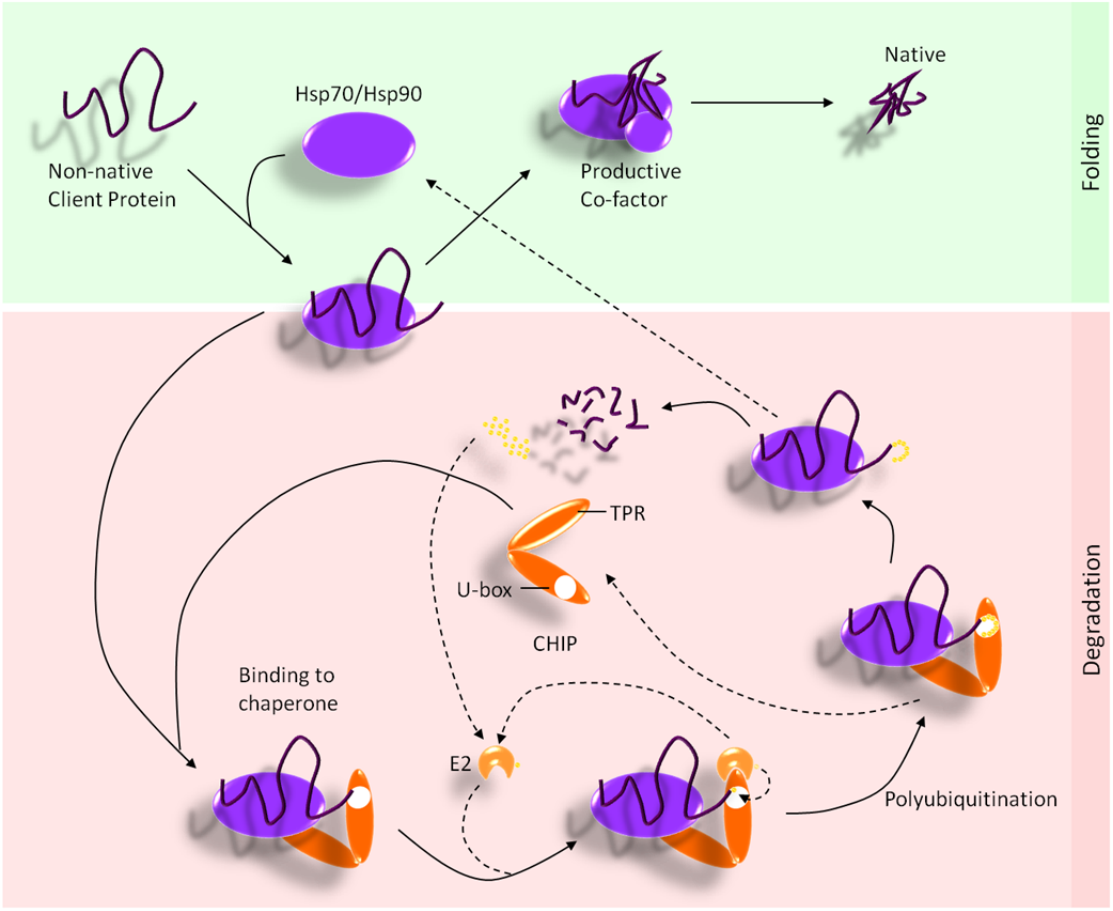

Figure 5. Schematic representation of CHIPs role in folding versus degradation Adapted from (Hohfeld et al., 2001; Kampinga and Craig, 2010).

1.4.4 New roles for CHIP

The number of cellular events where chaperones are found to be involved has expanded over the years. Thus, not surprisingly, CHIP has surfaced as a binding partner in previously unanticipated molecular mechanisms. These new roles substantiate CHIP as more than a molecular switch between the folding and degradation machinery. Most probably CHIP is also an important bridge between different degradation pathways. CHIP was found to be a component of Lewy bodies in the human brain, where it colocalizes with α-synuclein and Hsp70 (Shin et al., 2005). α-Synuclein is a major component of Lewy bodies, the pathological hallmark of Parkinson disease, dementia with Lewy bodies, and related disorders (Dickson and Yen, 1989; Spillantini et al., 1998a; Spillantini et al., 1998b). In this model overexpression of CHIP inhibits α-synuclein inclusion formation and reduces α-

synuclein protein levels (Shin et al., 2005). Also authors demonstrated that CHIP could mediate α-synuclein degradation by two discrete mechanisms that can be dissected using deletion mutants; the tetratricopeptide repeat domain was critical for proteasomal degradation, whereas the U-box domain was sufficient to direc α-synuclein toward the lysosomal degradation pathway (Shin et al., 2005). Though the authors do not discart the traditional model of a functional interaction between CHIP and Hsp70, which combines chaperone-mediated refolding and degradation in the proteasome, to modulate α-synuclein aggregation, they also observed that CHIP could degrade α-synuclein when the E3 ubiquitin ligase activity-conferring domain is deleted (Shin et al., 2005). This suggests that an additional degradation pathway for CHIP-mediated degradation of α-synuclein exists. This hypothesis was supported by the fact that CHIP-mediated deagradation of α-synuclein was not reversed by proteasome inhibitors (Shin et al., 2005). In fact CHIP could mediate α-synuclein degradation via the lysosome in a process where the U-box domain was required. Hence the CHIP E3 ubiquitin ligase activity was involved in the lysosomal degradation pathway. Authors also hypothesize that probably CHIP was ubiquitinating the substrates with K63 linked chains (Shin et al., 2005), that at the time were already known to target proteins to the lysosome (Aguilar and Wendland, 2003; Hicke and Dunn, 2003).

Several years later, based on the characterization of the Drosophila melanogaster cochaperone Starvin (Stv), the ortholog of human co-chaperone BAG-3, a conserved chaperone machinery was found to be involved in Z disk maintenance in the flies muscle tissue (Arndt et al., 2010). This machinery, instead of keeping Z disk proteins in a folded conformation, facilitated the degradation of damaged components, such as filamin, not by the proteasome but through what authors defined as chaperone assisted selective autophagy (CASA) (Arndt et al., 2010). Stv coordinated with Hsc70, the small heat shock protein HspB8 and CHIP, during disposal of the Z disks, a protein assembly essential for actin anchoring in striated muscles, which is subjected to mechanical, thermal, and oxidative stress during contraction (Frank et al., 2006). Furthermore, CHIP mediated ubiquitination was recognized by the autophagy adaptor p62, that binds both LC3 and ubiquitin.

Impaired CASA resulted in Z disk disintegration and progressive muscle weakness in flies, mice, and men (Arndt et al., 2010).

On the other hand, CHIP seems to be involved in the degradation of misfolded proteins in the plasma membrane in what was coined as the peripheral protein quality control (PPQC) (Okiyoneda et al., 2010). Using the F508 mutant, the most prevalent mutation in the cystic fibrosis transmembrane conductance regulator (CFTR), that causes a temperature-sensitive folding of immature protein, it was possible to separate F508 CFTR degraded in the ERAD process, which is almost all the synthesized protein, of the mutant protein degraded after going to the plasma membrane (PM) (Okiyoneda et al., 2010). In fact, CHIP has for long been identified as one of the key components of the ERAD machinery as well as on the ERAD mediated degradation of F508 CFTR (Meacham et al., 2001; Younger et al., 2004). The arrival of the folding defective protein to the PM was achieved by decreasing the temperature to 26ºC and them increasing it again to 37ºC. At 26ºC the mutant CFTR folding defect is rescued and the protein can then travel to the PM. At 37ºC the cytoplasmic region of conformationally defective PM CFTR was selectively recognized by Hsc70 in concert with DNAJA1 (JA1) and, possibly by the Hsp90/HOP/Aha1 machinery (Okiyoneda et al., 2010). Prolonged interaction with the chaperone/co-chaperone complex recruited CHIP-UbcH5, leading to ubiquitination of conformationally damaged PM CFTR (Okiyoneda et al., 2010). Ubiquitinated non-native PM CFTR was rapidly endocytosed, possibly by clathrin-mediated internalization upon recruitment of Ub-binding endocytic adaptors (Okiyoneda et al., 2010). Depending on the folding propensity of the cargo molecule and the proteostasis network state, dynamic interaction with chaperones and co-chaperones may favor the client protein refolding, deubiquitination and subsequent recycling to the PM. Alternatively, irreversible unfolding of the PM CFTR would lead to persistent ubiquitination by CHIP-UbcH5, providing efficient sorting signals for ESCRT-dependent cargo concentration, intraluminal budding and multivesicular endosome formation for delivery into the degradative lysosomal compartment.

1.5 Cellular adaptation to hypoxia: Hypoxia Inducible Factor

1.5.1 Hypoxia Inducible Factor Family

Adaptation to low oxygen tension (hypoxia) in cells and tissues leads to the transcriptional induction of a series of genes that participate in angiogenesis, iron metabolism, glucose metabolism, and cell proliferation/survival. The primary factor mediating this response is the hypoxia inducible factor-1 (HIF-1) (Ke and Costa, 2006). HIF-1 is a heterodimer composed of two subunits: HIF-1α (or its paralogs HIF-2α and HIF-3α) and HIF-1β (Gradin et al., 1996). While HIF-1β is constitutively expressed, the other subunits are oxygen regulated

HIF-1 was discovered by the identification of a hypoxia response element (HRE; 5′-RCGTG-3′) in the 3′ enhancer of the gene for erythropoietin (EPO), a hormone that stimulates erythrocyte proliferation and undergoes hypoxia-induced transcription (Goldberg et al., 1988; Semenza et al., 1991). These proteins belong to the basic helix-loop-helix-Per-ARNT-Sim (bHLH-PAS) protein family (Wang et al., 1995). The bHLH and PAS motifs are required for heterodimer formation between the HIF-1α and HIF-1β subunits, and the downstream basic region affords specific binding to the HRE DNA sequence (Crews, 1998). Two transactivation (stimulation of transcription) domains, N-terminal (N-TAD) and C-terminal (C-TAD), were identified later (Ruas et al., 2002). The C-TAD in particular has been shown to interact with coactivators such as CBP/p300 to activate gene transcription (Lando et al., 2002b). HIF-1α also contains an oxygen-dependent degradation domain (ODDD) that mediates oxygen-regulated stability (Pugh et al., 1997). Later work revealed that HIF-1α is ubiquitously expressed in human and mouse tissues and has a general role in multiple physiological responses to hypoxia, such as erythropoiesis, glycolysis and angiogenesis, which quickly counteract oxygen deficiency (Semenza, 1998).

Shortly after the cloning of HIF-1α, a closely related protein, HIF-2α [also termed endothelial PAS protein, HIF-like factor (HLF), HIF-related factor (HRF), and member of the PAS superfamily 2 (MOP2)] was identified and cloned (Ema et al., 1997; Flamme et al., 1997; Hogenesch et al., 1997; Tian et al., 1997). HIF-2α shares 48%

amino acid sequence identity with HIF-1α and accordingly shares a number of structural and biochemical similarities with HIF-1α (for instance, heterodimerization with HIF-1β and binding HREs). In contrast to ubiquitously expressed HIF-1α, HIF-2α is predominantly expressed in the lung, endothelium, and carotid body (Ema et al., 1997; Tian et al., 1998; Tian et al., 1997).

HIF-3α, which was discovered later, is also expressed in a variety of tissues, dimerizes with HIF-1β, and binds to HREs (Gu et al., 1998). In addition, a splice variant of HIF-3α, inhibitory PAS (IPAS), which is predominantly expressed in the Purkinje cells of the cerebellum and corneal epithelium, was subsequently discovered (Makino et al., 2001). IPAS possesses no endogenous transactivation activity. Rather, it interacts with the amino-terminal region of HIF-1α and prevents its DNA binding, acting as a dominant-negative regulator of HIF-1 (Makino et al., 2001). However, IPAS can also be induced by hypoxia in the heart and lung, contributing to a negative feedback loop for HIF-1 activity in these tissues (Makino et al., 2002). HIF-1α and HIF-2α have been more extensively studied, whereas research on HIF-3α and other HIF isoforms is relatively scarce.

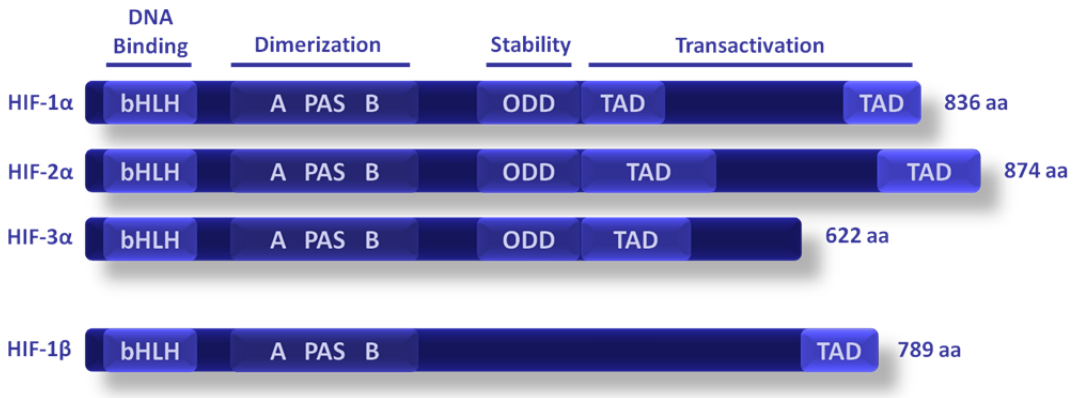

Figure 6. .Schematic of HIF family members and their respective domains. Adapted from (Rankin and Giaccia, 2008)

1.5.2 HIF-1 regulation

Although HIF-1α protein stability is regulated in an oxygen-dependent manner, stabilization alone is not sufficient for full transcriptional activation of HIF-1. The second major mechanism controlling HIF-1α activity is through the modulation of its

transactivation domains N-TAD and C-TAD. These domains function by recruiting transcriptional coactivators such as CBP/p300, SRC-1, and NCOA2 (Arany et al., 1996; Carrero et al., 2000; Ebert and Bunn, 1998; Ema et al., 1999; Kallio et al., 1997). Under normal oxygen tension, hydroxylation of the asparagines residue 803 (Asn803) in the C-TAD of HIF-1α by factor inhibiting HIF-1α (FIH-1) prevented the interaction of HIF-1α with CBP/p300 (Hewitson et al., 2002; Lando et al., 2002b; Sang et al., 2002). Hypoxia abrogates asparagine hydroxylation, which allowes the C-TAD of HIF-1α to efficiently interact with CBP/p300 therein, activating the transcription of the respective target genes (Lando et al., 2002a).

In addition, it has been reported that FIH-1 binds VHL, forming a ternary complex with HIF-1α (Mahon et al., 2001). Although interaction with VHL was not required for FIH-1 activity, histone deacetylases recruited by VHL interfered with the transcription processes, facilitating FIH-1 to modulate HIF-1α (Hewitson et al., 2002; Sang et al., 2002). FIH-1 is mainly located in the cytoplasm, but a fraction of it is likely to reside in the nucleus as well (Metzen et al., 2003). The transcription of FIH-1 is independent of the oxygen concentration, and it does not influence HIF-1α stability (Metzen et al., 2003). Like the PHDs, the asparaginyl hydroxylase FIH-1 is a 2-OG-dependent dioxygenase that also requires Fe^{2+} and ascorbate as cofactors (Lando et al., 2002a). Utilization of oxygen as a substrate allows FIH-1 to serve as a second oxygen sensor.

Furthermore, phosphorylation of HIF-1α by the mitogen-activated protein kinase (MAPK) pathway has been reported to play a role in the transcription factor regulation (Minet et al., 2001; Richard et al., 1999; Sodhi et al., 2000). It has been shown that the MAP kinases p42/44 and p38 kinase phosphorylated HIF-1α *in vitro* (Richard et al., 1999; Sodhi et al., 2000). In addition, inhibitors of p42/44 and p38 blocked HIF-1α-mediated reporter gene expression (Hur et al., 2001). Moreover, HIF-1α transactivation during hypoxia required p42/44 MAPKs (Conrad et al., 1999; Hofer et al., 2001; Hur et al., 2001) Taking these findings into account, it could be the case that HIF-1β binds preferentially to the phosphorylated form of HIF-1α (Suzuki et al., 2001a).

In addition to the post-translational modification of HIF-1α described above,

SUMOylation also negatively regulates the transcriptional activity of HIF-1α, because SUMO-deficient HIF-1α increases HRE-dependent transcription (Berta et al., 2007). This contradicts an earlier report claiming that SUMOylation increases HIF-1α-mediated transcription in the same cell line. The earlier finding, however, appears questionable, in that global overexpression of *SUMO-1* was used to examine the impact of SUMO modification of HIF-1α (Bae et al., 2004). In yet another case, *S*-nitrosation on cysteine 800 of HIF-1α has been shown to increase its transactivation through its interaction with CBP/p300 (Yasinska and Sumbayev, 2003).

Besides hypoxia, HIF-1α is also regulated in an oxygen-independent manner. Cytokines, growth factors, environmental stimuli, and other signaling molecules have been implicated in controlling HIF-1α under normoxic conditions (Feldser et al., 1999; Gorlach et al., 2001; Hellwig-Burgel et al., 1999; Richard et al., 2000; Salnikow et al., 2000). Although complex and cell-type dependent, some have been shown to stimulate HIF-1α transactivation or synthesis by activation of the MAPK or the phosphatidylinositol 3-kinase (PI3K) signaling pathways (Li et al., 2004; Zelzer et al., 1998).

1.5.3 HIF-1 transcriptional activity

Since cells, tissues and organs need to adapt to changes in oxygen supply, it is not surprising that a significant amount of the HIF-1α target genes are regulated in a tissue-specific manner. To date, there are more than one hundred HIF-1α downstream genes identified. Moreover, DNA microarrays found that more than 2% of all human genes are regulated by HIF-1α in arterial endothelial cells, directly or indirectly (Manalo et al., 2005). Also in response to hypoxia, the capacity of red blood cells to transport oxygen is up-regulated by the expression of genes involved in erythropoiesis and iron-metabolism. For example hypoxia increases the expression of EPO, which is required for the formation of red blood cells (Semenza et al., 1991). Since iron-metabolizing is a rate-limiting step of heme production, it is logical that hypoxia also up-regulates transferrin, which transports Fe^{3+} into cells (Rolfs et al., 1997); the transferring receptor, which binds transferring and enables cellular transferrin uptake (Bianchi et al., 1999; Lok and Ponka, 1999; Ponka and Lok,

1999; Tacchini et al., 1999); and ceruloplasmin (also known as a ferroxidase), which is required to oxidize ferrous (Fe^{2+}) to ferric (Fe^{3+}) iron (Lok and Ponka, 1999; Mukhopadhyay et al., 2000). Increasing the transcription of these genes supports iron supply to erythroid tissues (Rolfs et al., 1997).

In the same way, not only HIF-1α increases the production of red blood cells but also enhances the transport of oxygen to tissues through angiogenesis. A large number of genes involved in different steps of angiogenesis have been shown to increase by hypoxia challenge (Berra et al., 2000; Bunn and Poyton, 1996; Forsythe et al., 1996; Giordano and Johnson, 2001; Levy et al., 1995; Semenza, 2002). Among them, the vascular endothelial cell growth factor (VEGF), which is the most potent endothelial-specific mitogen, directly participates in angiogenesis by recruiting endothelial cells into hypoxic and avascular areas and stimulates their proliferation (Conway et al., 2001; Josko et al., 2000; Neufeld et al., 1999). Therefore, the induction of VEGF and various other proangiogenic factors leads to an increase in the vascular density and consequently to a decrease in the oxygen diffusion distance. In addition, HIF-1α regulates genes involved in governing the vascular tone such as nitric oxide synthase (NOS2) (Melillo et al., 1995), heme oxygenease 1 (Lee et al., 1997), endothelin 1 (ET1) (Hu et al., 1998), adrenomedulin (ADM) (Nguyen and Claycomb, 1999), and the 1β-adrenergic receptor (Eckhart et al., 1997). Moreover, hypoxia induces genes involved in matrix metabolism and vessel maturation such as matrix metalloproteinases (MMPs) (Ben-Yosef et al., 2002), plasminogen activator receptors and inhibitors (PAIs) (Kietzmann et al., 1999), and collagen prolyl hydroxylase (Takahashi et al., 2000).

Low oxygen supply poses a challenge for cellular metabolism. To adapt to this condition cells often switch their glucose metabolism pathway away from the oxygen-dependent tricarboxylic acid (TCA) cycle to the oxygen-independent glycolysis (Dang and Semenza, 1999; Seagroves et al., 2001). With only 2 ATP molecules from each glucose molecule produced by glycolysis, instead of 38 ATP provided by TCA cycle, hypoxic cells elevate their ability to generate ATP by increasing the glucose uptake. This is achieved by up-regulating the expression of glycolytic enzymes and glucose transporters (Wenger, 2002). Hypoxia and HIF-1α

increase virtually all the enzymes in the glycolytic pathway, as well as the glucose transporters 1 and 3 (GLUT1, GLUT3) (Chen et al., 2001). Furthermore, the glycolysis metabolic products, such as lactate and pyruvate, have been reported to cause HIF-1α accumulation under normoxia and at the same time regulate hypoxia-inducible gene expression, establishing a potential positive feedback loop (Lu et al., 2002).

Under these challenging conditions it is somewhat counterintuitive the existence of evidences showing that HIF-1α can induce growth factors, such as insulin-like growth factor-2 (IGF2) and transforming growth factor (TGF-β) (Feldser et al., 1999; Krishnamachary et al., 2003). Binding of such growth factors to their cognate receptors activates signal transduction pathways that lead to cell proliferation and/or survival and stimulates the expression of HIF-1α itself (Semenza, 2003). It is, nevertheless, also true that hypoxia can lead not only to cell survival but also to cell death, though in very specific circumstances. For example hypoxia has been shown to induce apoptosis, where HIF-1α plays a complex role (Carmeliet et al., 1998). Genetic studies using embryonic stem cells harboring a deletion of HIF-1α showed decreased apoptosis compared with wild type when challenged with low oxygen (Carmeliet et al., 1998). Activation of caspase-3 and Apaf-1- mediated caspase-9, and the release of cytochrome *c*, have been reported in several cell types under hypoxic conditions (Brunelle and Chandel, 2002; McClintock et al., 2002). It has also been demonstrated that the expression of HIF-1α significantly correlated with apoptosis and the proapoptotic factors, such as caspase-3, Fas, and Fas ligand (Volm and Koomagi, 2000). Moreover, hypoxia depressed the antiapoptotic protein Bcl-2 (Carmeliet et al., 1998), whereas the proapoptotic protein Bcl-2/adenovirus EIB 19-kDa interacting protein 3 (BNip3) and its homolog Nip3-like protein X (NIX) were up-regulated in a HIF-dependent manner (Bruick, 2000). Some genes involved in cell cycle control, such as p53 and p21, were also found to be HIF-dependent (Carmeliet et al., 1998). In addition, p53 has been implicated in regulating hypoxia-induced apoptosis through induction of apoptosis-related genes such as Bax, NOXA and PUMA (Schuler and Green, 2001).

To make the overall picture even more confusing, in addition to the above classes of genes, HIF-1α is also regulates many other target genes implicated in

diverse processes such as adipogenesis (Yun et al., 2002), carotide body formation (Kline et al., 2002), B lymphocyte development (Kojima et al., 2002), and immune reactions (Hellwig-Burgel et al., 2005).

1.5.4 Proteolysis of HIF-1α

HIF-1α is virtually undetectable under normoxia, since it is a substrate for the ubiquitin-proteasome system (UPS) (Salceda and Caro, 1997). Under normoxia, HIF-1α is hydroxylated on proline residues 402/564 by specific prolyl hydroxylases (PHD 2 mainly), that use oxygen and α-ketoglutarate as substrates (Ivan et al., 2001; Jaakkola et al., 2001). Once hydroxylated, HIF-1α is recognized by the Von Hippel Lindau (VHL) protein. VHL interacts with Elongin C and thereby recruits an ubiquitin ligase complex (Kamura et al., 2000; Kelly et al., 2003). The protein SSAT2, which interacts with HIF-1α, VHL, and Elongin C, stabilizes the interaction of VHL with Elongin C, thereby facilitating ubiquitination of HIF-1α (Baek et al., 2007a). Ubiquitination marks HIF-1α for degradation by the proteasome (Salceda and Caro, 1997). When oxygen becomes limiting HIF-1α hydroxylation is inhibited, the protein escapes degradation and is translocated into the nucleus, where it dimerizes with HIF-1β and binds to hypoxia-responsive elements (HRE) (Semenza et al., 1996). HIF-1α activates the expression of numerous hypoxia responsive genes that help cells to survive under low oxygen (Semenza, 1999).

Although the PHD2-VHL pathway is the critical mechanism regulating HIF-1α stability in response to changes in O_2 concentration, studies have revealed that there are O_2-independent mechanisms for the degradation of HIF-1α. The RACK1 protein can bind to HIF-1α and interact with Elongin C, thereby recruiting an E3 ubiquitin-protein ligase complex (Liu et al., 2007). RACK1 can substitute VHL to promote ubiquitination and degradation of HIF-1α, with the critical distinction that RACK1-HIF-1α interaction is not O_2-regulated. Furthermore, heat shock protein 90 (Hsp90) which is known to bind and stabilize HIF-1α, competing with RACK1 for binding to the transcription factor (Liu et al., 2007).

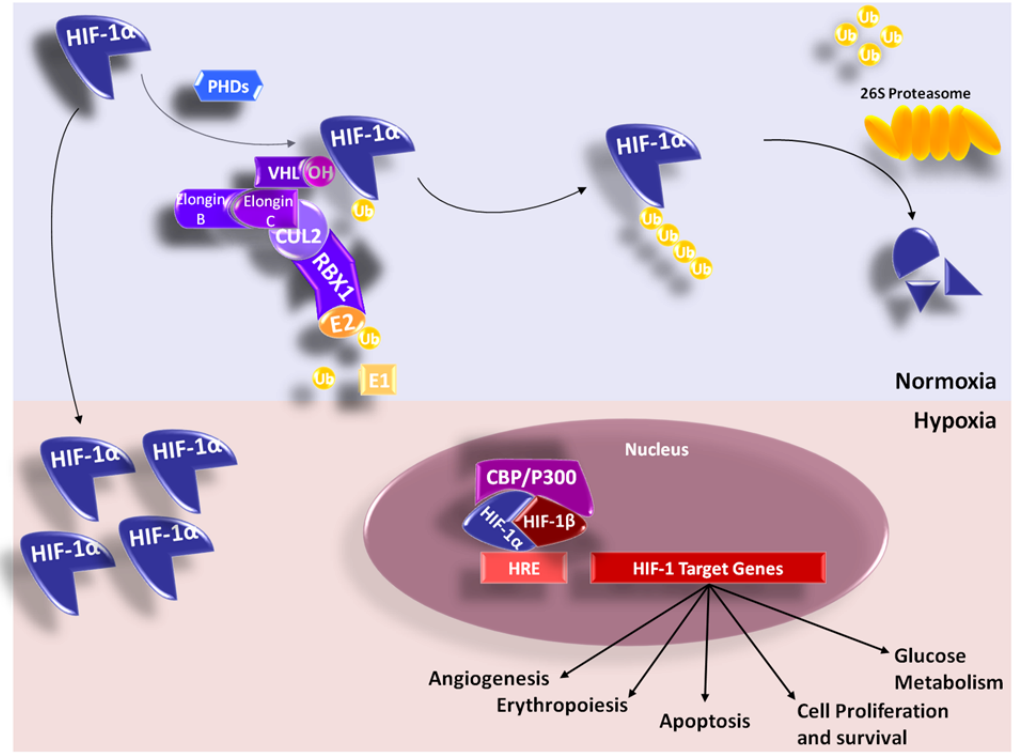

Figure 7. **Schematic representation of canonical HIF-1 pathway.** Adapted from (Harris, 2002)

In turn Hsp90 inhibitors, which have been shown to inhibit tumor growth, induce proteasomal degradation of HIF-1α, even in cells lacking VHL (Isaacs et al., 2002). The protein SSAT1, which shares 46% amino acid identity with SSAT2, also binds to HIF-1α and promotes its ubiquitination and degradation (Baek et al., 2007b). However, in contrast to SSAT2, which stabilizes the interaction of VHL and Elongin C and thereby promotes O_2-dependent ubiquitination (Baek et al., 2007b), SSAT1 acts by stabilizing the interaction of HIF-1α with RACK1. Thus the paralogs SSAT1 and SSAT2 play complementary roles in promoting O_2-independent and O_2-dependent degradation of HIF-1α, respectively.

CHIP (carboxyl terminus of Hsc70-interacting protein), has also been found to be involved in the degradation of HIF-1α. HIF-1α interacts with Hsp70 that, through recruiting the ubiquitin ligase CHIP, promotes the ubiquitination and proteasomal degradation of the transcription factor, thereby inhibiting HIF-1α-dependent gene expression. Disruption of Hsp70-CHIP interaction blocks HIF-1α degradation and attenuates the decay of HIF-1α levels during prolonged hypoxia (Luo et al., 2010). On the other hand, CHIP was also involved in a mechanism whereby methylglyoxal (MGO), which accumulates in high-glucose conditions, led to a rapid proteasome-

dependent degradation of HIF-1α under hypoxia (Bento et al., 2010). Data showed that increased association of Hsp40/70 with HIF-1α led to recruitment of CHIP, which promoted polyubiquitination and degradation of HIF-1α. Moreover, MGO-induced destabilization of HIF-1α led to a dramatic decrease in HIF-1 transcriptional activity, suggesting that accumulation of MGO is likely to be the link between high glucose and the loss of cell response to hypoxia in diabetes. There have been also reports of P53 regulation of HIF-1α stability. Loss of p53 in tumor cells enhances HIF-1α levels and augments HIF-1-dependent transcriptional activatity. In this case Mdm2 (murine double minute 2) mediates ubiquitination and proteasomal degradation of the HIF-1α (Ravi et al., 2000).

On the other side of ubiquitin-mediated degradation is deubiquitination. The pVHL-interacting deubiquitinating enzyme 2 (USP20) (Li et al., 2002a; Li et al., 2002b) interacts with HIF-1α (Li et al., 2005). USP20 can specifically deubiquitinate and stabilize HIF-1α. These findings suggest that ubiquitination of HIF-1α is a dynamic process and that ubiquitinated HIF-1α might be rescued from degradation by USP20 through deubiquitination. Although pVHL functions as a master control for HIF-1α stabilization, as pVHL-E3 ligase mediates the ubiquitination of both HIF-1α and USP20 (Li et al., 2002a), the balance between the pVHL-mediated ubiquitination and USP20-mediated deubiquitination of HIF-1α provides another level of control for HIF-1α stabilization.

Another important pathway in HIF-1α stability is SUMOylation. HIF-1α can undergo modification with SUMO-1, -2 and -3. Interestingly, hypoxia upregulates the expression of SUMO-1, which participates in HIF-1α sumoylation (Shao et al., 2004). SUMOylation was reported to promote HIF-1α degradation *via* a proteosomal pathway, suggesting cooperation between SUMOylation and ubiquitination (Cheng et al., 2007). In stark contrast, several other groups demonstrated that SUMO modification stabilizes HIF-1α (Bae et al., 2004) (Carbia-Nagashima et al., 2007) or even that HIF-1α turnover rate was not affected by sumoylation *in vivo* (Berta et al., 2007). Inversely, in the presence of the sumo-specific isopeptidase SENP1, HIF-1α is deSUMOylated and escapes degradation (Cheng et al., 2007). In the presence of such facts, one can only conclude that more detailed analyses are required to resolve these controversies.

Chapter 2. Objectives

Selective protein degradation has emerged as a key regulatory mechanism involved in virtually every aspect of cell biology. For many years, the ubiquitin-proteasome system (UPS) remained the single pathway for selective degradation of soluble proteins in cells. More recently, a new form of selective protein degradation was identified, which can degrade soluble substrates in the lysosome. Substrates targeted to chaperone mediated autophagy (CMA) are first recognized by a cytosolic chaperone, the heat shock cognate protein of 70kDa (Hsc70), and subsequently targeted to the lysosomal membrane where they bind to lysosome associated membrane protein type 2A (LAMP2A), that acts as a receptor for this pathway. The target protein is then translocated into the lysosomal lumen where it is completely degraded by lysosomal proteases. A generalized assumption has prevailed where proteolytic pathways are mechanistically separated events. However, it is becoming apparent that proteolytic systems rely upon complex molecular machines and functional supramolecular assemblages that may, through specific players, crosstalk in unexpected ways. This study explores the possibility that a crosstalk exists between two proteolytic systems: UPS and CMA, enabling degradation of specific substrates through both pathways. Data will show that HIF-1α, a typical substrate of UPS, can be diverted to CMA by virtue of its ability to bind to and be ubiquitinated by CHIP. CHIP (carboxyl terminus of the Hsc70-interacting protein) is a unique regulator since it has an ubiquitin-ligase domain (U-box) and is able to bind chaperones through its TPR motif. Because of this dual function, CHIP is able to switch chaperone-activity from protein folding to protein degradation. This study shows that CHIP and its associated supramolecular-complexes can shuttle HIF-1α to CMA. Data will also show that HIF-1α degradation is more complex than anticipated and that different stimuli may influence HIF-1α stability and consequently HIF-1α activity in previously unexpected ways.

Chapter 3. Methods

3.1 Animals and Cells: We used male wistar rats (200-250g). We also used the ARPE-19 cells (LGC Promochem, Teddington, UK), the renal carcinoma cell line RCC4 VHL-/- (American Type Culture Collection (ATCC), Manassas, VA, USA), the NIH-3T3 cell line, kindly provided by Dr. A. M. Cuervo (Albert Einstein Coledge of Medicine, Yeshiva University, New York, USA) and both HIF-1α +/+ and HIF-1α -/- MEFs, kindly provided by Dr. Roland H Wenger (University of Zurich, Switzerland)

3.2 Cell culture and Treatments: The ARPE-19 cells (LGC Promochem, Teddington, UK) were cultured in Dulbecco's modified Eagle's medium/ Ham's F12 (DMEM:F12; 1:1) supplemented with 10% fetal bovine serum (FBS), antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml amphotericin B) and glutamine. The renal carcinoma cell line RCC4 VHL-/- (American Type Culture Collection (ATCC), Manassas, VA, USA), was grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS, antibiotics and glutamine. The NIH-3T3 cell line was grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS, antibiotics and glutamine. HIF-1 +/+ and HIF-1 -/- MEFs, were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS, antibiotics and glutamine All media and glutamine were purchased from Invitrogen (Carlsbad, CA, USA). When appropriate, cells were treated with the following agents: 300 µM cobalt chloride ($CoCl_2$, Sigma-Aldrich, St. Louis, MO, USA), 20 µM MG132 or Z-LLL-CHO (Calbiochem, San Diego, CA, USA), 200 µM chloroquine (Sigma-Aldrich, St. Louis, MO, USA), 100 µM leupeptin (Sigma-Aldrich, St. Louis, MO, USA), 20 mM ammonium chloride (NH_4Cl, Sigma-Aldrich, St. Louis, MO, USA), 10 mM 3-methyladenine (3-MA, Sigma-Aldrich, St. Louis, MO, USA). An incubator Nuair N4950E (Nuaire, Plymouth, MN, USA) was used to perform the hypoxic treatments (2% O_2, 5% CO_2, 37ºC) Transient transfection of cells was performed with lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to manufacturer's recommendations.

3.3 Antibodies and reagents: The following antibodies were used: mouse anti-HIF-1α clone mgc3, dilution of 1:500 (western blot) 1:100 (immunocytochemestry) (Thermo Scientific, Waltham, MA, USA); rabbit anti-HIF-1α clone PA1-16601, dilutions of 1:1000 (Thermo Scientific, Waltham, MA, USA); rabbit anti-HIF-2α/EPAS1 (PAB12124), dilution of 1:200 (Abnova, Taipei City, Taiwan); rabbit anti-Hydroxyp564 (PAB9964), 1:200 (Abnova, Taipei City, Taiwan); rabbit antiHIF-1α Hydroxyproline 402 (Q16665), dilution of 1:200 (Millipore, Billerica, MA, USA); rabbit anti-LC3 (PA1-16931), dilution of 1:1000 (Thermo Scientific, Waltham, MA, USA); mouse anti-actin clone C4, dilutions of 1:1000 (Millipore-Chemicon, Billerica, MA, USA); rat anti-Hsc70 clone 1B5, dilution of 1:1000 (Stressgen, Ann Arbor, MI, USA); mouse anti-Hsc70 clone 13D3, dilution of 1:4000 (Novus Biologicals, Cambridge, UK); mouse anti-c-myc clone 9E10, dilution of 1:500 (Zymed-Invitrogen, Carlsbad, CA, USA); rabbit anti-LAMP2A, dilution of 1:2000 (lgp96), dilution of 1:1000 (Zymed-Invitrogen, Carlsbad, CA, USA); rabbit anti-LAMP2A (ab18528), rabbit anti-LAMP2A (ab18528) dilution of 1:500 (western blot) dilution of 1:100 (immunocytochemestry) (Abcam, Cambridge, UK); mouse anti-LAMP2 clone H4B4, dilution of 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse anti-LAMP1 clone LY1C6 (Stressgen, Ann Arbor, MI, USA); rabbit anti-P21 clone H-164, dilution of 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse anti-GAPDH clone 6C5, dilution of 1:2000 (Abcam, Cambridge, UK), goat anti-Cathepsin B clone S-12, dilution of 1:500, and horseradish peroxidase-conjugated secondary goat anti-mouse, goat anti-rabbit and rabbit anti-goat, dilution of 1:5,000 (Bio-Rad, Hercules, CA, USA). Alexa Fluor 568-conjugated goat anti-mouse and green-conjugated Alexa Fluor anti-rabbit dilution of 1:100 (Invitrogen, Carlsbad, CA, USA). Protein G–Sepharose (GE Healthcare Bio-Sciences, Uppsala, Sweden). Polyvinylidene fluoride (PVDF) membranes (GE Healthcare Bio-Sciences, Uppsala, Sweden). ECL (GE Healthcare Bio-Sciences, Uppsala, Sweden). Glycergel (Dako, Glostrup, Denmark). Hydroxyprobe TM-1 Plus Kit (Millipore, Billerica, MA, USA). Cell Proliferation ELISA, Brdu (colorimetric) (Penzberg, Upper Bavaria, Germany). Quantikine mouse VEGF immunoassay (ELISA) (R&D Systems, Minneapolis, MN, USA.). MTT Formazan Powder M2003 (Sigma-Aldrich, St. Louis, MO, USA).

3.4 Plasmids: For this work we used the following plasmids: pcDNA3.1 c-myc-CHIP wt, pcDNA3.1 c-myc-CHIP K30A, pcDNA3.1 c-myc-CHIP H260Q (Xu et al., 2002); pcDNA3 HIF-1α wt-V5 and pcDNA3 HIF-1α (P402A and P564A)-V5 (Hagen et al., 2003). To originate the KFERQ mutant HIF-1α we used the pcDNA3 HIF-1α wt-V5. We mutated the aminoacids N529 and E530 both to A using the 5'-ACTATCCACATAAAAACAATATTCACT-3' and the 5'-GATATGGTCGCTGCATTCAAGTTGGAA-3' primers. The mutation was verified by DNA sequencing.

3.5 Isolation of Lysosomal Fractions: Male Wistar rats were fasted for 48 hr before sacrifice for lysosomal isolation. Lysosomes were isolated from a light mitochondrial fraction in a discontinuous metrizamide density gradient (Wattiaux et al., 1978) by the shorter method (Aniento et al., 1993). Fractions from the top layer (fraction 1) and the 26.3/19.8% metrizamide interface (fraction 2) were collected separately, diluted five times with 0.3 M sucrose, and sedimented at 37,000 × *g* for 10 min in a Sorvall centrifuge (rotor SS-34; DuPont Instruments, Herts, UK). Lysosomes from fractions 1 and 2 were resuspended in 0.3 M sucrose and centrifuged again at 10,000 × *g* for 5 min in a Heraeus Biofuge 28RS (rotor HFA 22.1; Heraeus Sepatech, Osterode, Germany) to separate pellets (P1 and P2, respectively). For the isolation of the cytosolic and the endoplasmic reticulum fractions liver homogenates were centrifuged at 6800g for 5 minutes and the supernatant was centrifuged at 77000g for 1 hour (rotor TLA-110, Beckman-Coulter, Brea, CA, USA).

3.6 Binding and uptake assay. Following isolation, lysosomal integrity was verified by measuring the activity of ß-hexosaminidase (Cuervo et al., 1997). Preparations with more than 10% broken lysosomes after isolation were discarded. Substrate proteins were incubated with freshly isolated rat liver lysosomes 10 mM 3-(N-morpholino) propanesulfonic acid (MOPS), pH 7.3, 0.3 M sucrose for 20 min at 37°C in the presence or absence of a cocktail of proteinase inhibitors (Cuervo et al.,

1997). At the end of the incubation lysosomes were collected by centrifugation, washed and subjected to SDS-PAGE.

3.7 Viral shRNA Production and Infection: For shRNA targeting of human CHIP, the oligonucleotides were annealed and ligated into pENTR/U6 according to manufacturers' instructions. The following oligonucleotides were used: shRNA1 forward 5'-CACCGGAGATGGAGAGCTATGATGACGAATCATCATAGCTCTCC ATCTCC-3'; shRNA1 reverse 5'- AAAAGGAGATGGAGAGCTATGATGATTCGTC ATCATAGCTCTCCATCTCC-3'; shRNA2 forward 5'-CACCGGCTATGAAGGAGG TTATTGACGAATCAATAACCTCCTTCATAGCC-3'; shRNA2 reverse 5'-AAAAG GCTATGAAGGAGGTTATTGATTCGTCAATAACCTCCTTCATAGCC-3'. All plasmids were verified by DNA sequencing. Site-specific recombination between pENTR286 attL sites and pAd/BLOCKiT-DEST attR sites was performed using LR clonase II (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Pac1-digested recombinant packaging vector was then transfected into a producer cell line HEK293A and recombinant viral particles were harvested after lysis of infected cells 8-10 days later (Hume et al., 2006). The term "mock" in the gene silencing figures refers to a control with a scrambled shRNA sequence. The lentiviral vectors containing shRNA targeting mouse LAMP2A (Kaushik et al., 2008), ATG7 and the control empty vector were kindly provided by Dr. A. M. Cuervo (Albert Einstein College of Medicine, Yeshiva University, New York, USA).

3.8 Measurement of 20S proteasome activity. Cells were washed twice with PBS, lysed with a Tris buffer (50 mM Tris pH 7.4, 1 mM DTT) and sonicated. After centrifugation (16,000 x g for 10 minutes at 4ºC), protein concentration was determined using the Coomassie method and 40 µg of protein was incubated with the following fluorogenic substrates: 100 µM Suc-LLVY-MCA for the chymotrypsin-like activity (Biomol-Enzo Life Sciences, Farmingdale, NY, USA); 25 µM Boc-LRR-MCA for the trypsin-like activity (Biomol-Enzo Life Sciences, Farmingdale, NY, USA); 150 µM Z-LLE-MCA for the caspase-like activity (Calbiochem, San Diego, CA, USA). The proteasome activities were monitored during 1 hour at 37ºC, for periods of 5 minutes (excitation wavelength at 380 nm; emission wavelength at 460 nm).

Absorbance was measured on a Biotek Synergy HT spectrophotometer (Biotek, Winooski, VT, USA), using the Gen 5 software to monitor the results (Biotek, Winooski, VT, USA).

3.9 Quantitative Real-Time PCR. Following the relevant treatments, total RNA was purified with Qiagen RNeasy mini kit (Qiagen, Valencia, CA, USA), according to manufacturer's specifications, and treated with RNase-free DNase I (GE Healthcare Bio- Sciences, Uppsala, Sweden). SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexadeoxynucleotide primers were used to synthesize cDNA. For the cDNA-real-time PCR the SYBR Green PCR master mix (Bio-Rad, Hercules, CA, USA) was used according to the manufacturer's instructions, and the cDNA amplification was performed using the following sets of primers:

- hHIF-1alpha forward 5'-CTACTAGTGCCACATCATCAC-3';
- hHIF-1alpha reverse 5'-CTAGTATCTTTGGATTTAGTTCTT-3';
- mHIF-1alpha forward 5'-CAGAAATGGCCCAGTGAGAAAAGG G-3';
- mHIF-1alpha reverse 5'-ATGGCCCGTGCAGTGAAGCAC-3';
- mGLUT-1 forward 5'-ATCCCAGCAGCAAGAAGGTGACG-3';
- mGLUT1 reverse 5'-TGGTGGATGGGATGGGCTCTCC-3';
- mVEGF forward 5'-ATGCCAAGTGGTCCCAGGCTGC-3';
- mVEGF reverse 5'-ATCGGACGGCAGTAGCTTCGC-3';
- 18S rRNA forward 5'-GTCTGCCCTATCAACTTTC-3';
- 18S rRNA reverse 5'-TTCCTTGGATGTGGTAGC-3'.

The real-time PCR analyses were conducted on an ABI Prism 7000 quantitive PCR system (Applied Biosystems, Foster City, CA, USA).

3.10 Morphological analysis. MEF cells were trypsinized and centrifuged at 200×g for 5 min. 0.3×10^6 cells were then resuspended in 30 µl of FBS and placed on a slide for microscopic analysis. The cells were stained upon incubation for 5 min with May–Grünwald solution (0.3% v/v in methanol) (Sigma, St. Louis, MO, USA), diluted in distilled water at 1:1 (v/v) ratio and then stained with Giemsa solution (0.75% w/v in glycerol/methanol 1:1) (Sigma, St. Louis, MO, USA) previously diluted in distilled

water (8×). After rinsing with distilled water, the cells were left to dry at room temperature. The cells were imaged by confocal microscopy using a Zeiss LSM710 system (Carl Zeiss AG, Oberkochen, Germany).

3.11 Determination of VEGF concentration in the media The concentration of diffusible VEGF in the cell culture supernatants was measured by Quantikine enzyme-linked immunosorbent (ELISA) assay kits using monoclonal antibodies directed against mouse VEGF, according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA). Briefly, cell culture supernatant samples or standards were added to a 96-well plate, previously coated with a mouse monoclonal antibody against VEGF. Wells were then incubated with a horseradish-peroxidase (HRP)-conjugated polyclonal antibody against VEGF or Ang-2 and incubated with the substrate solution. Absorbance was measured at 450 nm, with wavelength correction at 570 nm, on a Biotek Synergy HT spectrophotometer (Biotek, Winooski, VT, USA), using the Gen 5 software to monitor the results (Biotek, Winooski, VT, USA).

3.12 Brdu colorimetric proliferation assay. Cells seeded onto a 96-well plate were incubated with 5-bromo-2'-deoxyuridine labeling solution to a final concentration of 10 µM for 6 hours at 37ºC. Subsequently, labeling FixDenat was added to the cells for 30 minutes at room temperature. Thirty minutes later, FixDenat was removed and 100 µl of peroxidase-conjugated anti-BrdU antibody (1:100 dilution) was added per well. Antibody was incubated for 90 minutes at room temperature. Subsequently, substrate solution tetramethyl-benzidine (TMB) was added. Cells were incubated at room temperature until color development was sufficient for photometric detection (5 - 30 minutes). Absorbance was measured at 370 nm (reference wavelength at 492 nm) on a Biotek Synergy HT spectrophotometer (Biotek, Winooski, VT, USA).

3.13 MTT cell viability assay. After the treatments, cells seeded onto 24-well plates were washed twice with PBS and incubated with 0.5 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Invitrogen, Carlsbad, CA,

USA] in Krebs buffer (130 mM NaCl, 4 mM KCl, 1.5 mM MgCl2, 1 mM CaCl2, 6 mM glucose, 10 mM HEPES, pH 7.4) for 2 hours at 37°C. Subsequently, supernatants were removed and the precipitated dye was dissolved in 300 µl 0.04 M HCl (in isopropanol) and quantified at a wavelength of 570 nm, with wavelength correction at 620 nm, using a Biotek Synergy HT spectrophotometer (Biotek, Winooski, VT, USA).

Chapter 4. HIF-1α is a substrate for Chaperone-Mediated Autophagy

4.1 Introduction

Hypoxia-Inducible Factor 1 (HIF-1) is a heterodimer composed of two subunits: HIF-1α and HIF-1β (Gradin et al., 1996). While HIF-1β is constitutively expressed, HIF-1α is virtually undetectable under normoxia, since it is a substrate for the ubiquitin-proteasome system (UPS) (Salceda and Caro, 1997). Under normoxia, HIF-1α is hydroxylated on proline residues 402/564 by specific prolyl hydroxylases, that use oxygen and α-ketoglutarate as substrates (Ivan et al., 2001; Jaakkola et al., 2001). Once hydroxylated, HIF-1α is recognized by the Von Hippel Lindau (VHL) protein, which is part of an ubiquitin ligase complex that targets HIF-1α for polyubiquitinylation and subsequent degradation by the proteasome (Maxwell et al., 1999). When oxygen becomes limiting HIF-1α hydroxylation is inhibited, the protein escapes degradation and is translocated into the nucleus, where it dimerizes with HIF-1β and binds to hypoxia-responsive elements (HRE). HIF-1 activates the expression of numerous hypoxia-responsive genes that help cells to survive under low oxygen (Semenza, 1999).

Recent evidence suggests that the degradation of HIF-1α may also occur through alternative pathways. For example the RACK1/Elongin-C/Elongin-B complex (Liu et al., 2007) and the co-chaperone CHIP (Bento et al., 2010; Luo et al., 2010) were both shown to mediate the ubiquitinylation and subsequent proteasomal degradation of HIF-1α in an oxygen-independent manner. Moreover, a recent report (Olmos et al., 2009) began to unveil a role for the lysosome in the degradation of HIF-1α in a renal carcinoma cell line after incubation with 15-Deoxy-Delta (12,14)-prostaglandin-J(2) (Olmos et al., 2009).

A putative crosstalk between the UPS and the lysosomal Chaperone-Mediated Autophagy (CMA) remains poorly understood. Although the two pathways share common traits of specificity towards substrates, they are mechanistically different.

The UPS relies upon a series of enzymes (E1, E2 and E3) that conjugate ubiquitin to the substrate targeting it for proteasomal degradation (Hershko et al., 1983). CMA, on the other hand is a form of selective autophagy (Massey et al., 2004) whereby substrates containing a targeting motif biochemically related to the pentapeptide KFERQ are recognized by the chaperone, Heat Shock Cognate of 73 kDa (cyt-Hsc70) (Chiang et al., 1989) and degraded in the lysosome (Cuervo and Dice, 1996). At the lysosomal membrane, the substrate interacts with the Lysosomal Associated Membrane Protein 2A (LAMP2A) (Cuervo and Dice, 1996) which acts as a CMA receptor.

The major goal in this study is to investigate the molecular mechanism whereby the canonical UPS substrate HIF-1α is degraded by CMA, thus helping to elucidate the putative crosstalk between these two proteolytic pathways.

4.2 Results

4.2.1 Lysosomal inhibitors, but not macroautophagy inhibitors, induce the accumulation of HIF-1α

To investigate the role of the lysosome in the degradation of HIF-1α, we first assessed the effect of lysosome inhibitors on the stabilization of the transcription factor. Human retinal pigmented epithelial cells (ARPE-19) were treated with the lysosomal inhibitors leupeptin and the weak bases chloroquine and NH_4Cl. Data presented in Fig.8A shows that HIF-1α accumulates in the presence of the lysosomal inhibitors, with both chloroquine and NH_4Cl having a stronger effect than leupeptin. Furthermore, treatment with both chloroquine and MG-132 showed a cumulative effect in the stabilization of HIF-1α (Fig.8B). However, incubation of cells with chloroquine failed to stabilize HIF-2α (Fig.8C), indicating that lysosomal degradation of HIF-1α is specific and does not occur for all HIF-α isoforms.

Based on these results, chloroquine was used as the lysosome inhibitor of choice for most experiments. To exclude that the accumulation of HIF-1α results from a

putative inhibitory effect of chloroquine on the proteasome, cells were incubated with chloroquine for 8 hours and the proteasome activity was assessed by using specific fluorogenic substrates. Results show that chloroquine does not significantly inhibit any of the three proteolytic activities of the proteasome (Fig.9A).

Moreover, RT-PCR experiments show that neither MG-132 nor chloroquine significantly altered the mRNA levels of HIF-1α (Fig.9B).

To exclude that chloroquine might act by inhibiting the activity of prolyl hydroxylases (PHDs), both RCC4 (a renal carcinoma cell line deficient in VHL) (Fig.9D) and ARPE-19 cell lysates (Fig.9C) were incubated with chloroquine and no changes were observed in the activity of PHDs, as revealed by their ability to hydroxylate HIF-1α proline residues 402 and 564.

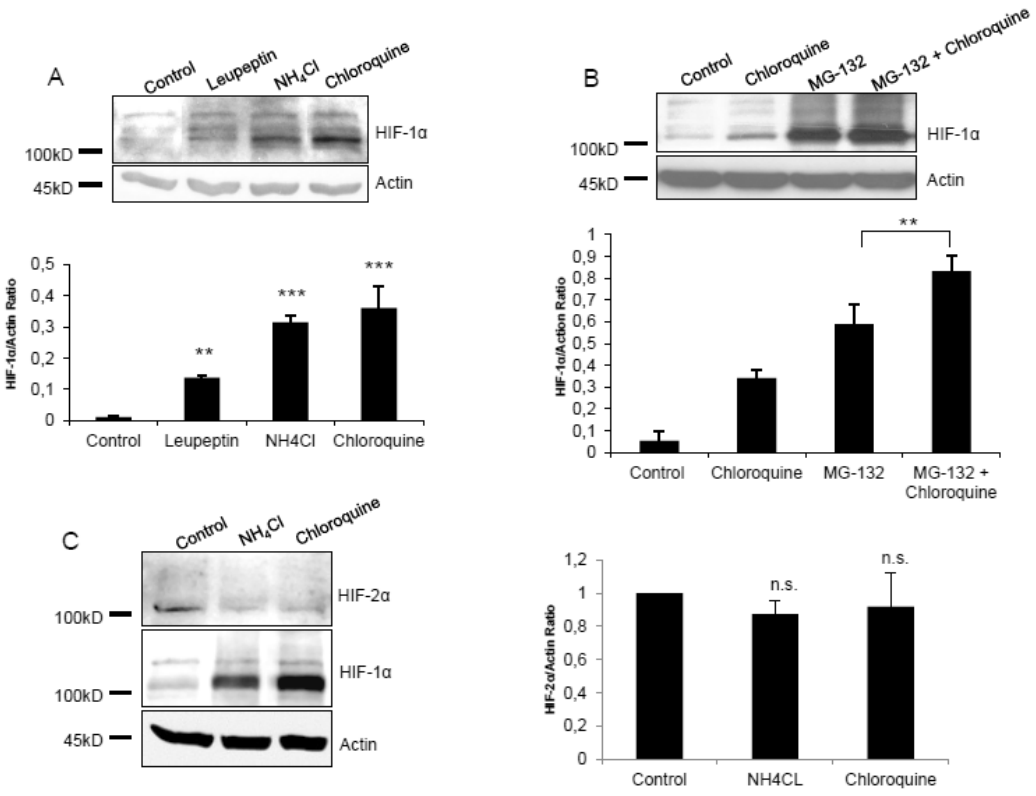

Figure 8. HIF-1α is stabilized by lysosome inhibitors. (A,B,C) ARPE-19 cells incubated either in the presence or absence of 100 μM leupeptin, 20 mM NH_4Cl, 200 μM chloroquine, 20 μM MG-132 for 8 hours (A) Western blot for HIF-1α and actin. All lysosome inhibitors tested stabilized HIF-1α in ARPE-19 cells. (B) Western blot for HIF-1α and actin. Incubation of ARPE-19 cells with both MG-132 and choloroquine had a cumulative effect in HIF-1α stabilization. (C) Western blot for HIF-2α, HIF-1α and actin. Lysosomal inhibitors failed to stabilize HIF-2α. (D) All the results represent the mean ± SD of at least three independent experiments. (n.s. non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$)

This was true even when chloroquine was used at a concentration ten times higher that the standard concentration to treat cells. Furthermore, incubation of ARPE-19 cells with chloroquine and with the hypoxia probe pimidazole (Fig.10A), allowed to exclude that chloroquine is inducing an ”acidic hypoxia”, as demonstrated by the absence of pimidazole adducts in cells incubated with chloroquine. (Haq et al., 2005). There are a number of pathways through which substrates can be targeted for lysosomal degradation, being macroautophagy one of the best characterized. To assess whether macroautophagy is the mechanism involved in HIF-1α degradation, cells were incubated with the macroautophagy inhibitor 3-methyladenine (3-MA). Alternatively, macroautophagy was inhibited in a mouse cell line by silencing ATG7 (Autophagy-Related protein 7), which is essential for the formation of the autophagosome membrane (Singh et al., 2009). Incubation of ARPE-19 cells with 3-MA, or silencing of ATG7 in NIH-3T3 cells, both failed to stabilize HIF-1α when compared to controls (Fig.10B, C and D), suggesting that macroautophagy is not the mechanism responsible for the lysosome-dependent degradation of HIF-1α.

Alltogether the results clearly demonstrate that HIF-1α is degraded by the lysosome through a mechanism that is not mediated by macroautophagy. These observation led us to hypothesize that CMA could be the proteolytic pathway involved in the lysosomal mediated degradation of HIF-1α.

4.2.2 The KFERQ-like motif of HIF-1α is required for the lysosomal degradation of the protein

All CMA substrates identified so far have a pentapeptide consensus sequence, often referred to as a KFERQ-like motif. Significantly, following recently established criteria (Cuervo, 2010), we identified the non-canonical KFERQ motifs, $_{529}$NEFKL$_{533}$ and $_{627}$VTKDN$_{630}$, in human and rat HIF-1α respectively (Fig.11A, highlighted in pink) and two canonic KFERQ motifs in mouse HIF-1α: $_{512}$ERLLQ$_{516}$ and $_{684}$RVIEQ$_{689}$ (Fig.11A, highlighted in yellow). However, the presence of a KFERQ motif on a substrate is not sufficient to prove that it is a *bona fide* CMA substrate. To show that the pentapeptide sequence found in HIF-1α is indeed required for its lysosomal

degradation, the first two aminoacids of the sequence of human HIF-1α were mutated to alanine (AAHIF-1α).

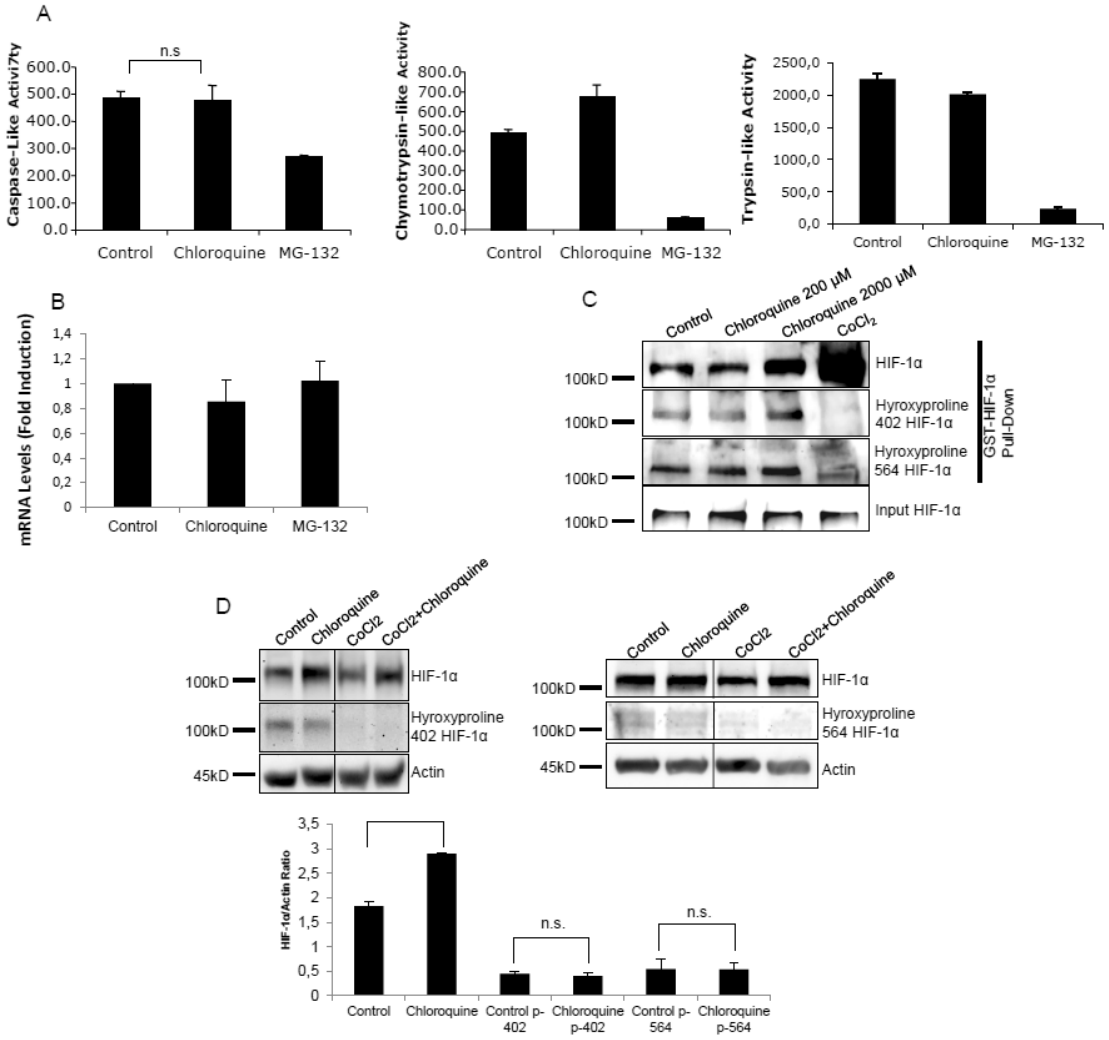

Figure 9. Chloroquine does not change HIF-1α mRNA levels nor does it inhibit proteasome activity or HIF-1α hydroxylation. ARPE-19 or RCC4 VHL -/- cells were incubated either in the presence or absence of 200µM of chloroquine, 20µM of MG-132 and either 300µM of $CoCl_2$ or 2% O_2 for 8 h and harvested. (A) Cell extracts were homogenized and protein concentration was determined. 40 µg of each sample were incubated with the following fluorogenic substrates, in order to assess the different proteolytic activities of the proteasome: Z-LLE-MCA for monitoring the caspase-like activity, Suc-LLVY-MCA for the chymotrypsin-like activity, and Boc-LRR-MCA for the trypsin-like activity. Chloroquine does not inhibit any of the three proteasomal activities. (B) Cells were lysed and total RNA was purified. Quantitative Real-Time RT-PCR analysis was performed and levels of HIF-1α mRNA were determined for the different experimental conditions. Neither chloroquine nor MG-132 altered the levels of mRNA of HIF-1α. (C) Pull-down of recombinant GST-HIF-1α incubated in ARPE-19 cell lysates in the presence or absence of 300 µM $CoCl_2$, 200 µM chloroquine and/or 2000 µM chloroquine and probed for HIF-1α hydroxyprolines 402 and 564. Incubation of cell lysates at a concentration of chloroquine of 2000 µM did not inhibit PHD activity. (D) Western blot for HIF-1α, actin and HIF-1α hydroxyproline 402 or 564 in RCC4 cells. Chloroquine does not induce any obvious change in the

hydroxylation of either prolines 402 or 564. All the results represent the mean ± SD of at least three independent experiments. (n.s. non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

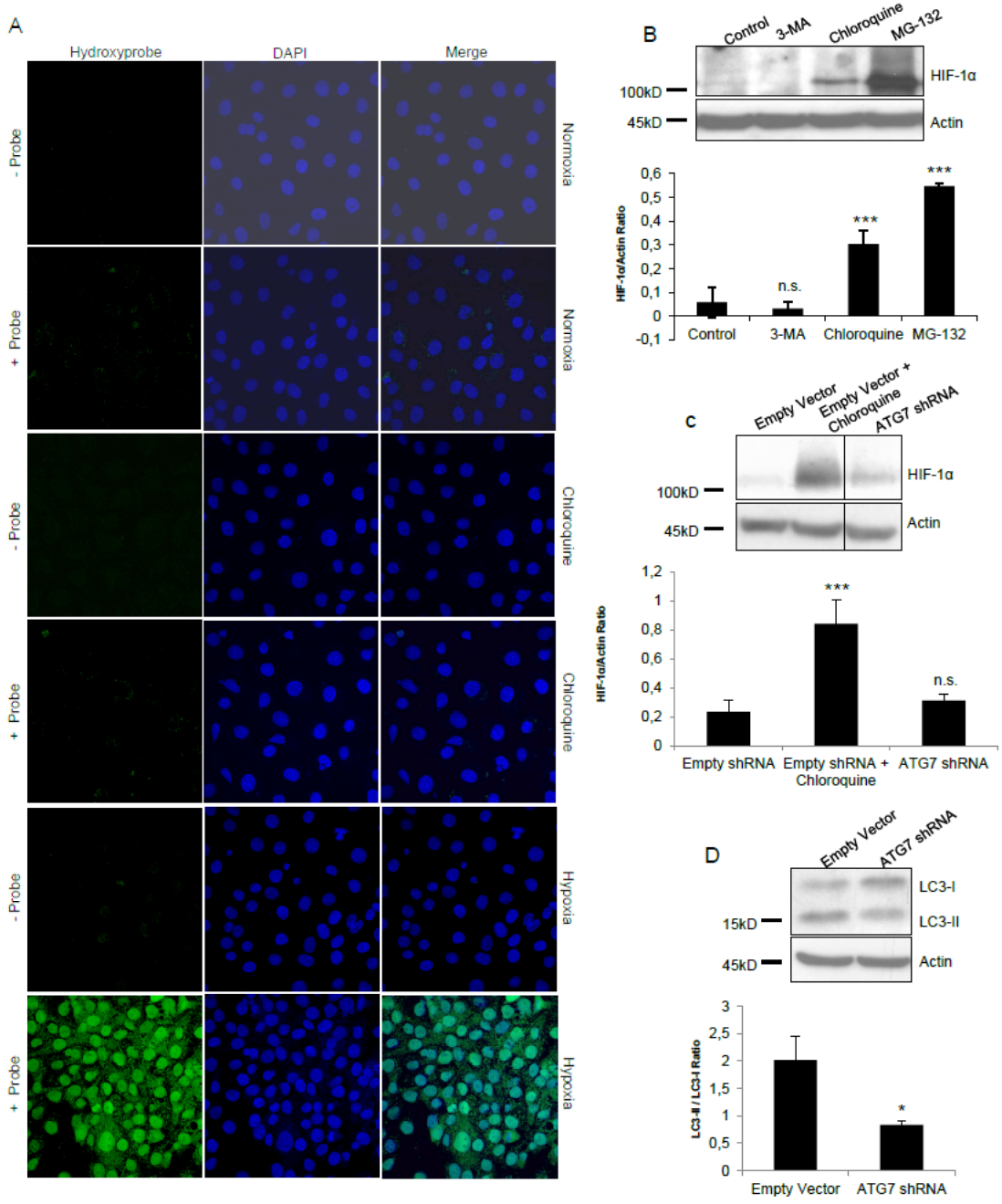

Figure 10. Chloroquine does not induce an "acidic hypoxia" and macroautophagy inhibition fails to stabilize HIF-1α. (A) Immunocytochemistry for the hypoxia probe pimidazole. ARPE-19 cells were incubated in the presence or absence of 200μM of chloroquine or 2% O_2 for 8 hours and with 200μM pimidazole for the last 2 hours of incubation. The samples were probed with an antibody specific for protein-pimidazole adducts (FITC-Mab-1). Chloroquine does not induce hypoxia as detected by the hypoxia probe. (B) ARPE-19 cells incubated either in the presence or absence of 100 μM leupeptin, 20 mM NH_4Cl, 200 μM chloroquine, 20 μM MG-132 or 10 mM 3-MA for 8 hours. Western blot for HIF-1α and actin. Pharmacological inhibition of macroautophagy failed to stabilize HIF-1α. (C,D) NIH-3T3 cells transduced with an empty lentiviral vector or a lentiviral vector encoding for ATG7 shRNA for 7

days at 37ºC and incubated in the presence or absence of 100 μM chloroquine for 8 hours. Cell extracts were probed for HIF-1α and for actin (C). Alternatively the ratio of LC3-II/LC3-I was determined (D). Silencing of ATG7 did not lead to stabilization of HIF-1α. All the results represent the mean ± SD of at least three independent experiments. (n.s. non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Data presented in Fig.11B shows that this mutation rendered the protein insensitive to lysosomal inhibition, indicating that a functional pentapeptide motif is necessary for targeting HIF-1α to the lysosome. Incubation of cells under hypoxia (2% O_2) clearly shows stabilization of both wild type and mutant HIF-1α (Fig.11C). Moreover, the levels of AAHIF-1α mutant that accumulate in response to hypoxia are about two times higher than the wild type form of HIF-1α, reflecting the increased stabilization of the protein following mutation of the CMA-targeting pentapeptide (Fig.11C).

The stabilization of AAHIF-1α under hypoxia also suggets that the mutant AAHIF-1α retains the ability to be hydroxylated under normoxia. To further confirm this possibility V5-HIF-1α was immunoprecipitated and probed for hydroxylated P402/P564. Fig.11D shows that both wtHIF-1α and AAHIF-1α are hydroxylated to a comparable extent. Since wtHIF-1α degradation in normoxia is known to be preceded by hydroxylation, data in Fig.11C and D discards the possibility of the HIF-1α KFERQ mutation affecting HIF-1α hydroxylation.

4.2.3 The KFERQ-like motif is required for HIF-1α interaction with Hsc70 and the CMA receptor LAMP2A

A requirement for all CMA substrates is the ability to bind to the CMA receptor LAMP2A and, through the KFERQ-like motif, to Hsc70. Thus, the ability of HIF-1α to interact with LAMP2A in ARPE-19 cells was evaluated by co-immunoprecipitation experiments. Because all the available antibodies against LAMP2A recognize an epitope in the C-terminus of the protein, which can be masked by the binding of substrates as they reach the lysosome (Bandyopadhyay et al., 2008), we chose to use an antibody raised against all LAMP2 isoforms.

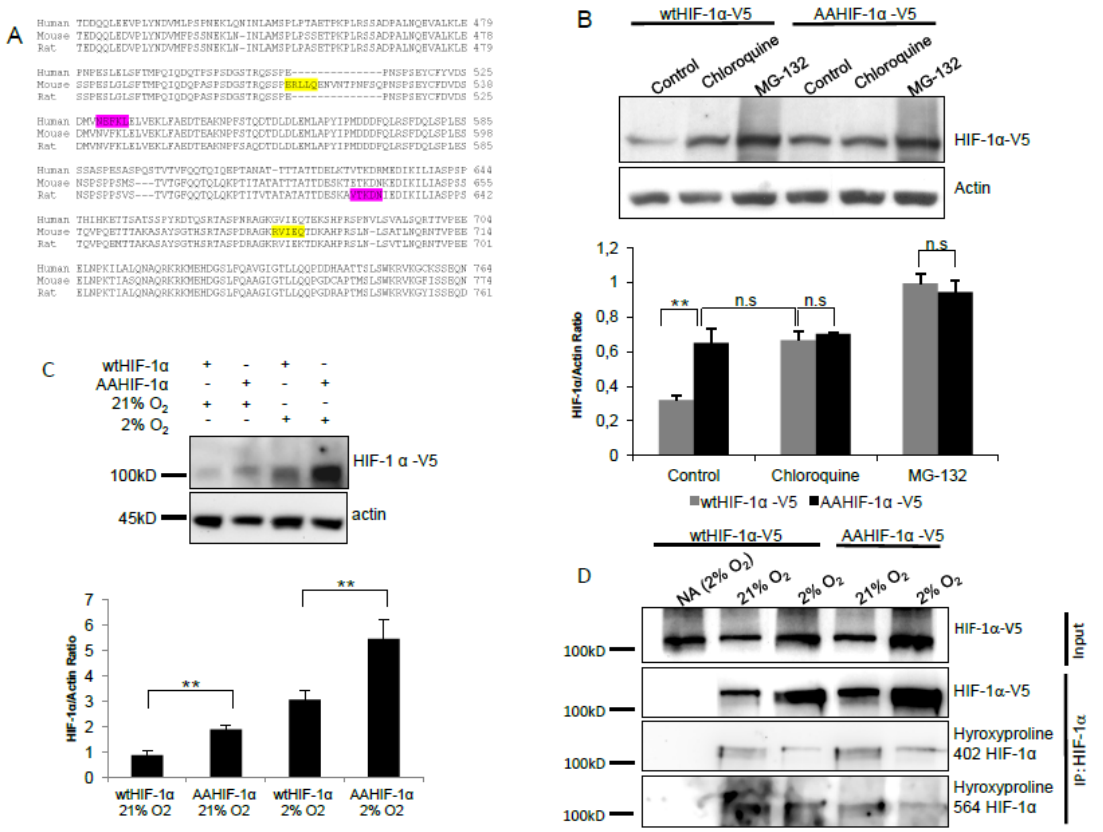

Figure 11. HIF-1α has a KFERQ-like pentapeptide sequence. (A) Alignment of human, mouse and rat HIF-1α. The human HIF-1α contains a non-canonical KFERQ-like pentapeptide $_{529}$NEFKL$_{533}$ (purple box). The mouse HIF-1α contains two canonical KFERQ-like pentapeptide sequences $_{512}$ERLLQ$_{516}$ and $_{684}$RVIEQ$_{689}$ (yellow boxes). The rat HIF-1α contains one non-canonical KFERQ-like pentapeptide $_{627}$VTKDN$_{630}$ (purple box). (B,C,D) Western blot for V5-tag and actin of ARPE-19 cells transfected either with wtHIF-1α or AAHIF-1α (both tagged with V5) and incubated either in the presence or absence of 200 μM chloroquine, 20 μM MG-132 or 2% O_2. (B) The AAHIF-1α mutant was more stable than wtHIF-1α and it was insensitive to lysosome inhibitiors. (C) Similarly to wtHIF-1α the mutant AAHIF-1α is stabilized under hypoxia. (D) Immunoprecipitation of V5-tagged wtHIF-1α and AAHIF-1α. AAHIF-1α is hydroxylated in prolines 402/564 similarly to wtHIF-1α and, in both cases, hypoxia prevents its hydroxylation. The results represent the mean ± SD of at least three independent experiments. (n.s. non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Data presented in Fig.12A shows that immunoprecipitation of LAMP2 resulted in the co-precipitation of HIF-1α. Consistently, immunoprecipitation of HIF-1α also resulted in the co-precipitation of LAMP2A (Fig.12B). Interestingly, not only lysosome inhibition, but also proteasome inhibition resulted in increased co-precipitation of HIF-1α with LAMP2 (Fig.12A and B). This interaction is specific for LAMP2A, since neither the LAMP2B nor the LAMP2C isoforms co-immunoprecipitate with HIF-1α (Fig-5E).

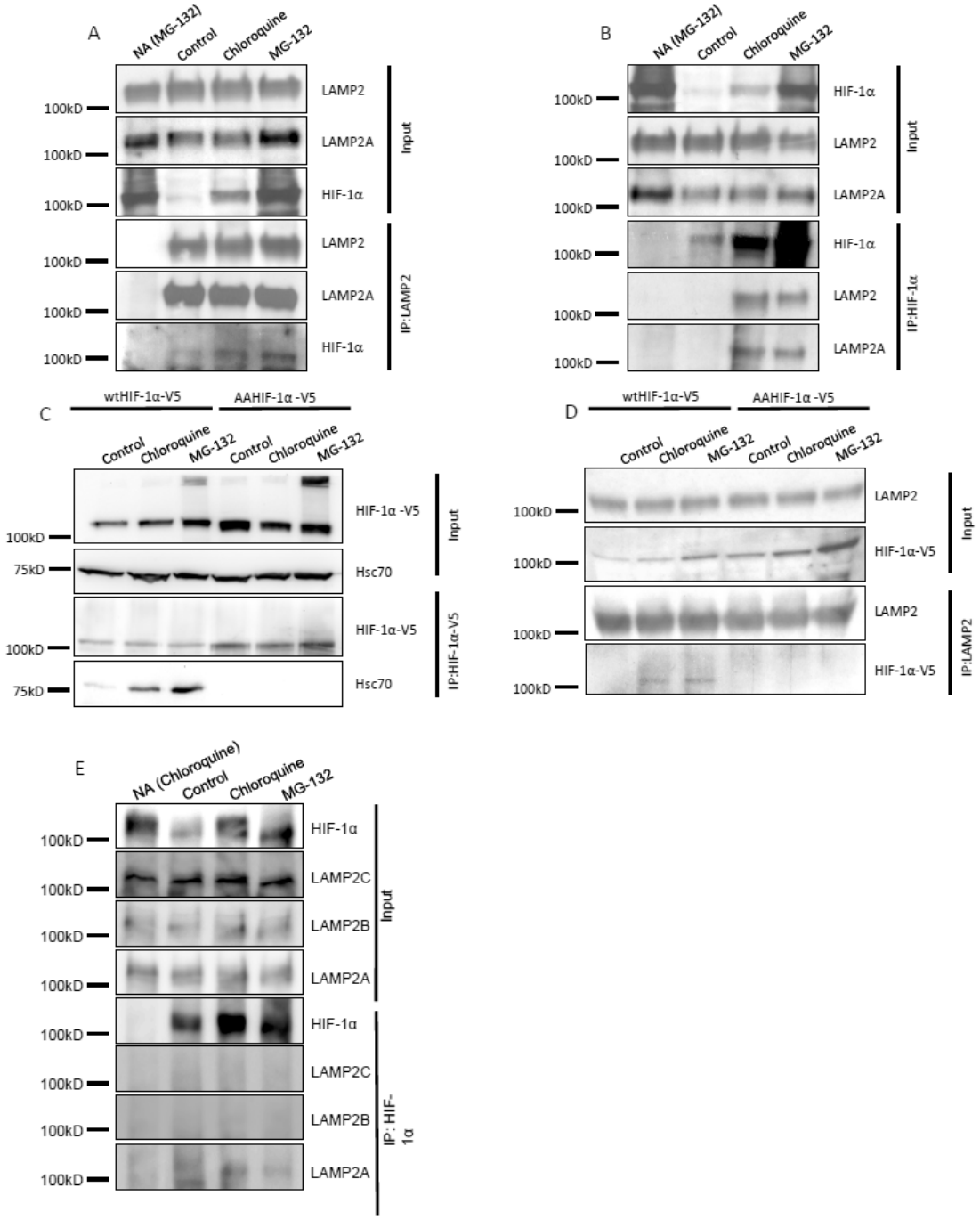

Figure 12. HIF-1α has a KFERQ-like pentapeptide sequence and interacts with both LAMP2A and Hsc70. (A,B) ARPE-19 cells were incubated either in the presence or absence of 200 μM chloroquine or 20 μM MG-132 for 8 hours. (A) Immunoprecipitation of LAMP2 resulted in the co-precipitation of the LAMP2A isoform and wtHIF-1α. (B) The LAMP2A isoform co-precipitated with wtHIF-1α. (C,D) Immunoprecipitation of HIF-1α-V5. (C) Hsc70 co-precipitated with wtHIF-1α-V5 but not with the mutant AAHIF-1α-V5. (D) wtHIF-1α-V5 but not AAHIF-1α-V5 co-precipitated with LAMP2. (E) Immunoprecipitation of HIF-1α. NIH 3T3 cells were incubated either in the presence or absence of 200μM chloroquine or 20μM MG-132 for 8 h and harvested. Immunoprecipitates were blotted for all three LAMP2 isoforms. HIF-1α only interacts with the LAMP2A isoform. (NA) no antibody.

To assess whether Hsc70 interacts with the KFERQ-like motif of HIF-1α ARPE-19 cells were transfected with V5-tagged wild-type or mutant HIF-1α (AAHIF-1α) and subsequently treated with either lysosome or proteasome inhibitors. Data presented in Fig.12C shows that mutation of the KFERQ-like sequence of HIF-1α abrogated its interaction with Hsc70. Consistently, data presented in Fig.12D shows that mutation of the KFERQ-like motif also prevents the association of HIF-1α with the CMA receptor LAMP2A, as revealed by the failure of AAHIF-1α to co-immunoprecipitate with LAMP2A.

4.2.4 HIF-1α localizes in lysosomes positive for CMA and is transported into intact lysosomes

Immunofluorescence of ARPE-19 cells (Fig.13A) shows increased co-localization of LAMP2A with HIF-1α in the presence of proteasome inhibitors (MG-132), and even more so in the presence of the lysosome inhibitor chloroquine. To prove that HIF-1α localizes specifically to CMA competent lysosomes we isolated lysosomal fractions.

As previously reported (Cuervo et al., 1994), not all lysosomes support CMA and it is possible to isolate CMA positive lysosomes (containing lys-Hsc70) that show higher efficiency in degrading CMA substrates such as glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and Ribonuclease A. In Fig.13B we show that HIF-1α localizes only in the lysosomal fraction positive for Hsc70 and for the CMA substrate GAPDH (CMA+ lysosomes). Consistently, purified HIF-1α was translocated into intact isolated lysosomes and degraded, since the concomitant addition of protease inhibitors (PI) increased the levels of recombinant HIF-1α present in the lysosomes (Fig.13C).

Furthermore, within 20 minutes of addition, more than 80% of the total recombinant HIF-1α was degraded by the lysosomes (Fig.13C). To test if the binding and uptake of HIF-1α was CMA-dependent we added a 2 M excess of the CMA substrate GAPDH. GAPDH competed with the transcription factor and inhibited the

binding and uptake of the protein into intact lysosomes whereas ovalbumin, which is not a CMA substrate, failed to do so (Fig.14).

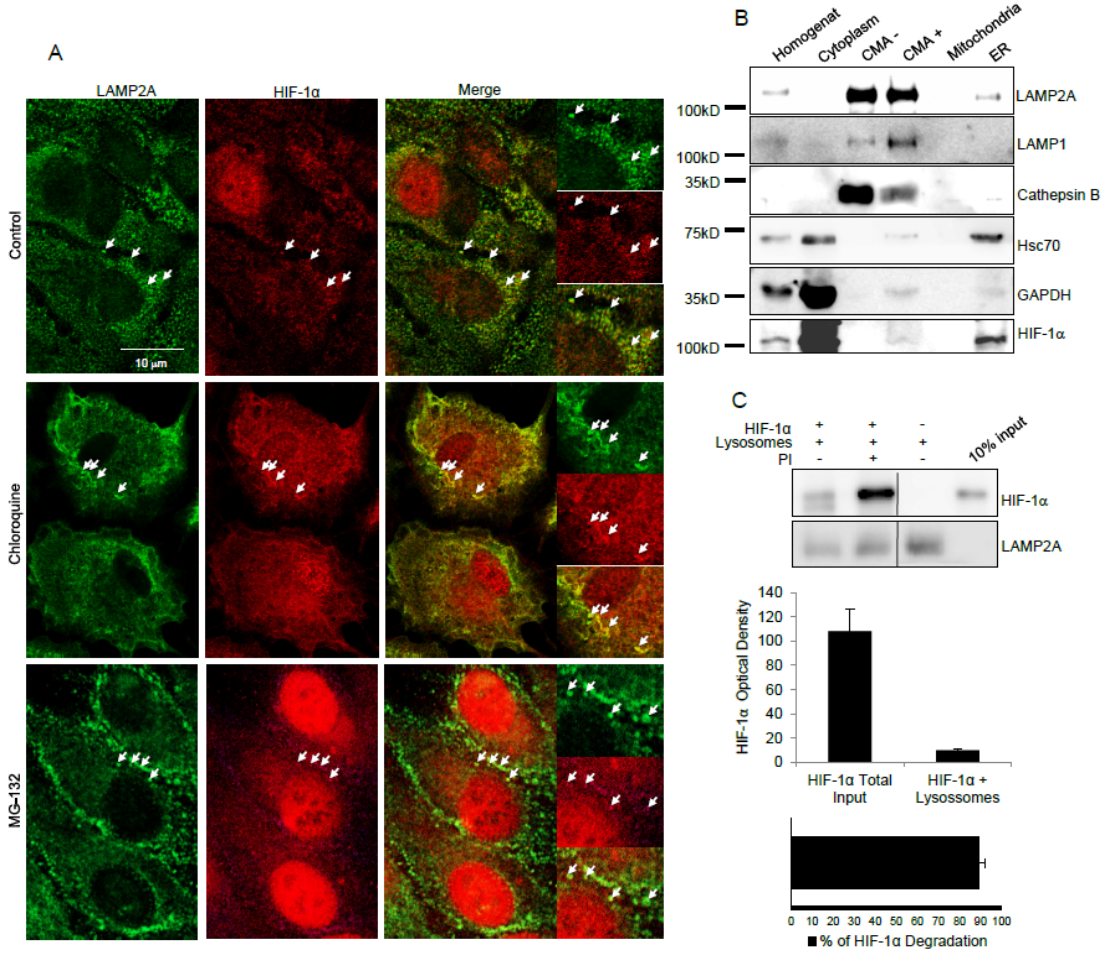

Figure 13. HIF-1α localizes to CMA+ lysosomes and is transported into intact lysososmes. (A) ARPE-19 cells were incubated either in the presence or absence of 200 μM chloroquine or 20 μM MG-132 for 8 hours. Cells were fixed and immunostained with anti-LAMP2A (green) and anti-HIF-1α (red) antibodies. Images are representative of at least three independent experiments. (B) Western blot of lysosomal and other sub-cellular fractions obtained from liver of starved rat. HIF-1α localizes into the same lysosomal fraction (CMA positive) as Hsc70 and GAPDH. (C) Western blot of lysosomes incubated either in the presence or absence of recombinant HIF-1α, GAPDH, ovalbumin and protease inhibitor cocktail. Values represent the mean ± SD of at least three independent experiments. HIF-1α binds to the lysosomal membrane (in the absence of protease inhibitors we measured the binding of HIF-1α to the lysosomal membrane) and is translocated into lysosomes (in the presence of protease inhibitors we measured the binding and uptake of HIF-1α by the lysosome). Lysosomes degrade ≈80% of recombinant HIF-1α.

4.2.5 Silencing of the CMA receptor LAMP2A stabilizes HIF-1α

Silencing of the CMA receptor LAMP2A acts as a specific form of inhibiting CMA. LAMP2A was depleted from mouse NIH-3T3 cells by infection with lentiviral vectors containing a shRNA directed against the mouse LAMP2A sequence (Kaushik et al.,

2008). shRNAs against ATG7 were used as a control for macroautophagy inhibition. As shown in Fig.15A, after seven days of infection, LAMP2A levels were significantly reduced. In addition, depletion of LAMP2A, but not of ATG7, led to a substantial stabilization of HIF-1α, further reinforcing that HIF-1α is a specific substrate for CMA, but not for macroautophagy. As shown in Fig.15B, while depletion of ATG7 decreased the LC3-II/LC3-I ratio, depletion of LAMP2A had no effect on this ratio, indicating that silencing of LAMP2A did not affect macroautophagy.

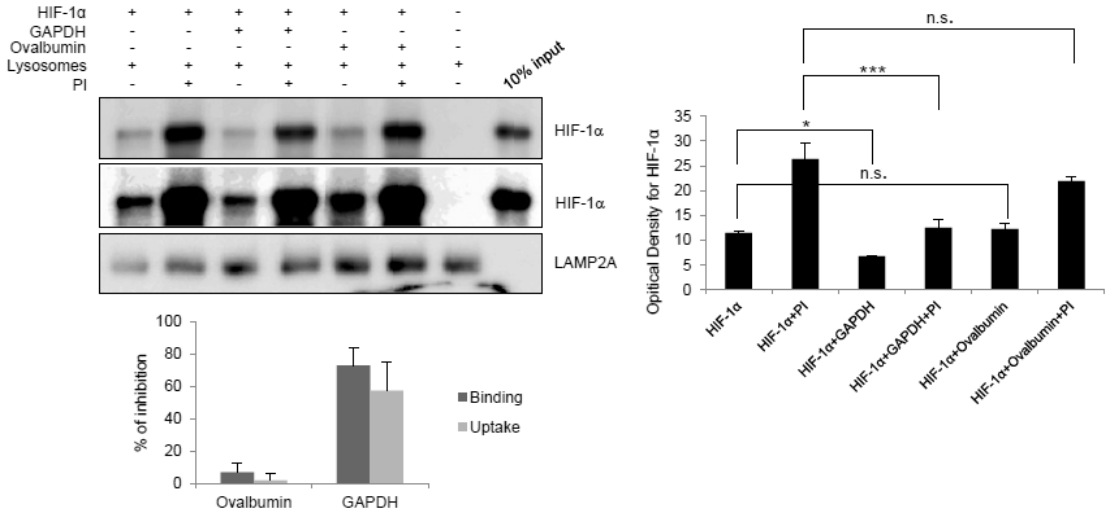

Figure 14. HIF-1α is transported into intact lysososmes. Incubation of lysosomes with 2M excess of GAPDH inhibits HIF-1α binding and uptake, whereas incubation with ovalbumin fails do to so. Uptake was calculated by subtracting the protein associated to lysosomes in the presence (protein bound to the lysosomal membrane and taken up by lysosomes) and absence (protein bound to the lysosomal membrane) of inhibitors of lysosomal proteases. Results are represented as percentage of inhibition. The results represent the mean ± SD of at least three independent experiments. (n.s. non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Depletion of LAMP2A, however, induced a significant decrease in the levels of HIF-1α mRNA (Fig.15C), in agreement with previous reports showing that accumulation of HIF-1α during prolonged hypoxia induces the expression of a natural antisense against HIF-1α (Uchida et al., 2004) ultimately leading to the degradation of the mRNA. Altogether data is consistent with a model where HIF-1α is a specific substrate for CMA.

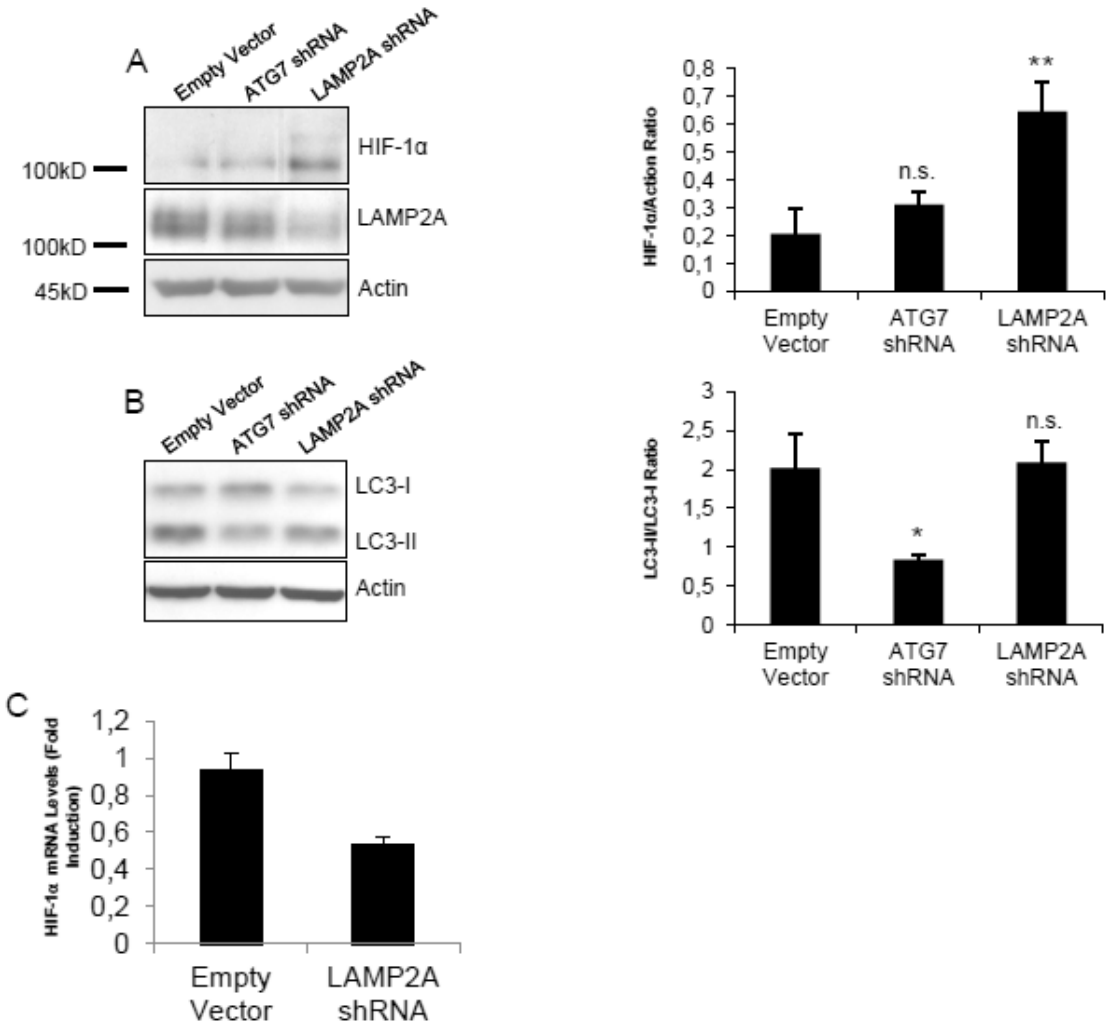

Figure 15. Silencing of LAMP2A stabilizes HIF-1α. (A,B) Western blot of HIF-1α, LAMP2A, LC3 and actin of NIH-3T3 cells transduced with an empty vector, ATG7 shRNA or LAMP2A shRNA and maintained for 7 days at 37ºC. (A) Depletion of LAMP2A, but not of ATG7, induced the accumulation of HIF-1α. (B) The ratio LC3-II/LC3-I was determined as an indicator of macroautophagy. Depletion of LAMP2A had no effect on macroautophagy activation. (C) Quantitative Real-Time RT-PCR analysis was performed and levels of HIF-1α mRNA were determined for the different experimental conditions. Cells depleted of LAMP2A show a decrease in HIF-1α mRNA levels. The results represent the mean ± SD of at least three independent experiments. (n.s. non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

4.2.6 Inhibition of the proteasome up-regulates CMA-dependent degradation of HIF-1α

Several studies have suggested the existence of a crosstalk between proteasomal and lysosomal degradation. One example is the up-regulation of autophagy when the proteasome is inhibited (Pandey et al., 2007). To investigate the effect proteasomal inhibition on CMA activity, ARPE-19 cells treated with proteasomal inhibitors were immunoprecipitated with antibodies directed against LAMP2. Data

presented in Fig.16A shows that inhibition of the proteasome led to a time-dependent increase in the amount of HIF-1α and Hsc70 that co-precipitated with LAMP2.

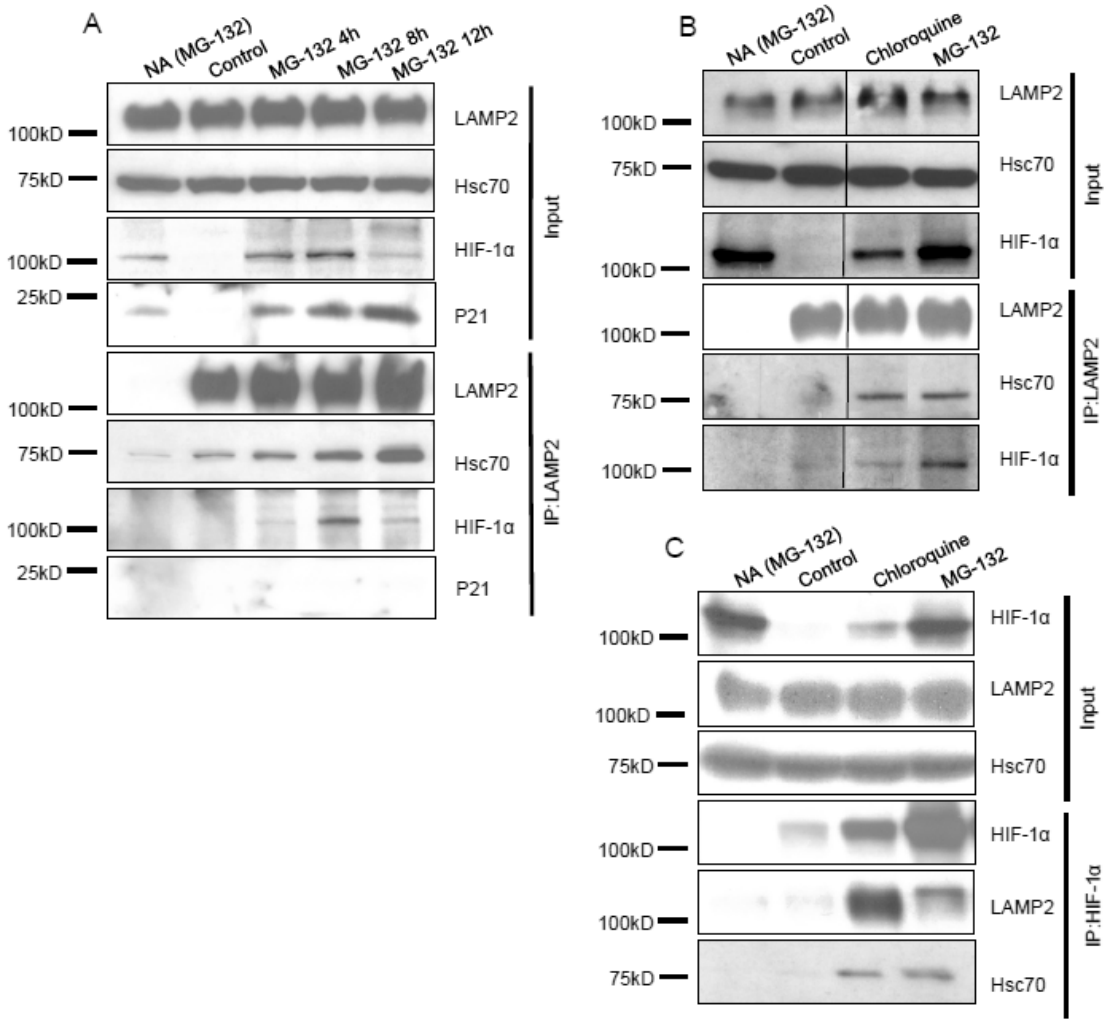

Figure 16. Inhibition of the proteasome up-regulates CMA-dependent degradation of HIF-1α. (A,B,C) ARPE-19 were incubated either in the presence or absence of 20 μM MG-132 or 200 μM chloroquine for the depicted time periods. (A) Immunoprecipitation of LAMP2. Proteasome inhibition led to a time-dependent increase in the interaction of Hsc70 and HIF-1α. NA (no antibody). Immunoprecipitation of LAMP2 (B) or HIF-1α (C). Both proteasome and lysosome inhibitiors increased Hsc70 and HIF-1α interaction with LAMP2A.

Moreover, inhibition of the lysosome for 8h increased the co-precipitation of Hsc70 with both HIF-1α and LAMP2 (Fig.16B and C). To verify that proteasome inhibition specifically leads to CMA-dependent HIF-1α degradation we assessed the interaction of LAMP2A with P21 (cyclin dependent kinase P21/WAF). As with HIF-1α, P21 is also an UPS substrate (Cayrol and Ducommun, 1998) but does not have a KFERQ-like pentapeptide sequence, being an unlikely substrate for CMA. Data presented in

Fig.16A clearly shows that while P21 readily accumulated in the presence of proteasome inhibitors, co-precipitation of P21 with LAMP2 was virtually undetectable

Chapter 5. CHIP as a molecular switch of HIF-1α for Chaperone-Mediated Autophagy

5.1 Introduction

Selective protein degradation has emerged as a key factor in the regulation of virtually every aspect of cell biology. The molecular regulation of protein degradation is currently an area of very active research, however much remains to be clarified. Indeed, it appears that many recent observations do not entirely fit into currently accepted models for regulation of protein degradation. For example, a generalized assumption has prevailed, where proteolytic pathways are mechanistically separated events, each pathway acting upon specific substrates. However, recently it was shown that blockage of one-degradation pathway leads to functional alterations in other proteolytic pathways (Massey et al., 2008). This suggests the occurrence of an unanticipated crosstalk between different proteolytic pathways in the cell. The ubiquitin-proteasome system (UPS) and Chaperone-mediated autophagy (CMA) are major pathways for selective degradation of proteins, in eukaryotic cells.

Although the prevailing idea establishes that cells rely on two separate proteolytic pathways to target and selectively degrade specific substrates recent studies have shown evidences that a crosstalk exists between the two pathways. In support of this hypothesis is the observation that early blockage of CMA leads to a transient decrease in proteasome activity (Massey et al., 2008). Also, CMA can specifically degrade some subunits of the 20S proteasome (Cuervo et al., 1995b). Moreover, results presented in the previous chapter of this work suggest that proteasome inhibition can direct HIF-1α to CMA. Thus it is conceivable that following inactivation of one pathway and/or other specific conditions, substrates can be shuttled from one pathway to the other. We further hypothesize that chaperone and co-chaperone complexes containing CHIP (Carboxyl terminus of the Hsc70-Interacting Protein) are more likely to be shuttled between the two pathways. CHIP is an E3 ubiquitin ligase containing three TPR domains at its amino-terminal and an

U-box domain at its carboxy-terminal. The U-box domain plays a key role in targeting proteins for ubiquitination and subsequent proteasome-dependent degradation. The ubiquitin ligase activity of CHIP depends on interactions with specific E2 ubiquitin-conjugating enzymes, the UBC4/UBC5 family (Xu et al., 2008; Zhang et al., 2005), which are "stress-activated" ubiquitin-conjugating enzymes. On the other hand, CHIP is known to be a *bona fide* interaction partner of the major cytoplasmic chaperones Hsc70 and Hsp70, through its TPR domain (Goldberg, 2003; Murata et al., 2001b). CHIP has been shown to target several substrates for the UPS, including immature CFTR, GR, ErbB2 and α-synuclein. In the case of α-synuclein, it was shown to be a target protein of CHIP-induced ubiquitination and CHIP is able to mediate degradation decisions, between proteasomal and lysosomal pathways for this protein (Shin et al., 2005). Thus, data suggests that CHIP is likely to be a molecular switch between UPS and other proteolytic pathways, shuttling substrates between them.

5.2 Results

5.2.1 The co-chaperone CHIP is required to target HIF-1α for CMA-dependent degradation

Recently it was reported that the E3 ligase CHIP is involved in the proteasomal degradation of HIF-1α (Bento et al., 2010; Luo et al., 2010). Furthermore, CHIP is known to interact with elements of the molecular chaperone machinery (including Hsc70) (Ballinger et al., 1999; Connell et al., 2001; Petrucelli et al., 2004). To address if CHIP is involved in the shuttling of HIF-1α to CMA degradation, ARPE-19 cells were transfected with plasmids encoding CHIP fused to a myc tag. Data presented in Fig. 10A and B indicates that CHIP associates with HIF-1α in control conditions and that this interaction is increased in the presence of either proteasome or lysosome inhibitors (compare lane 2 with lanes 3 and 4 of Fig.17A and B). Furthermore, the amount of LAMP2 that co-precipitated with either HIF-1α (Fig. 17A) or CHIP (Fig.17B) also increased in the presence of proteasome or lysosome inhibitors. In Fig.17C we

show that CHIP, altought it does not have a KFERQ-like motif, localizes only in the lysosomal fraction positive for Hsc70 and for the CMA substrate GAPDH (CMA+ lysosomes). The data above is consistent with a model in which CHIP and Hsc70, are part of a macromolecular complex directing HIF-1α for CMA-dependent degradation, by a process that requires subsequent interaction with LAMP2A. To further address this hypothesis, we evaluated the levels of interaction between HIF-1α and LAMP2A in ARPE-19 cells infected with a shRNA directed against CHIP.

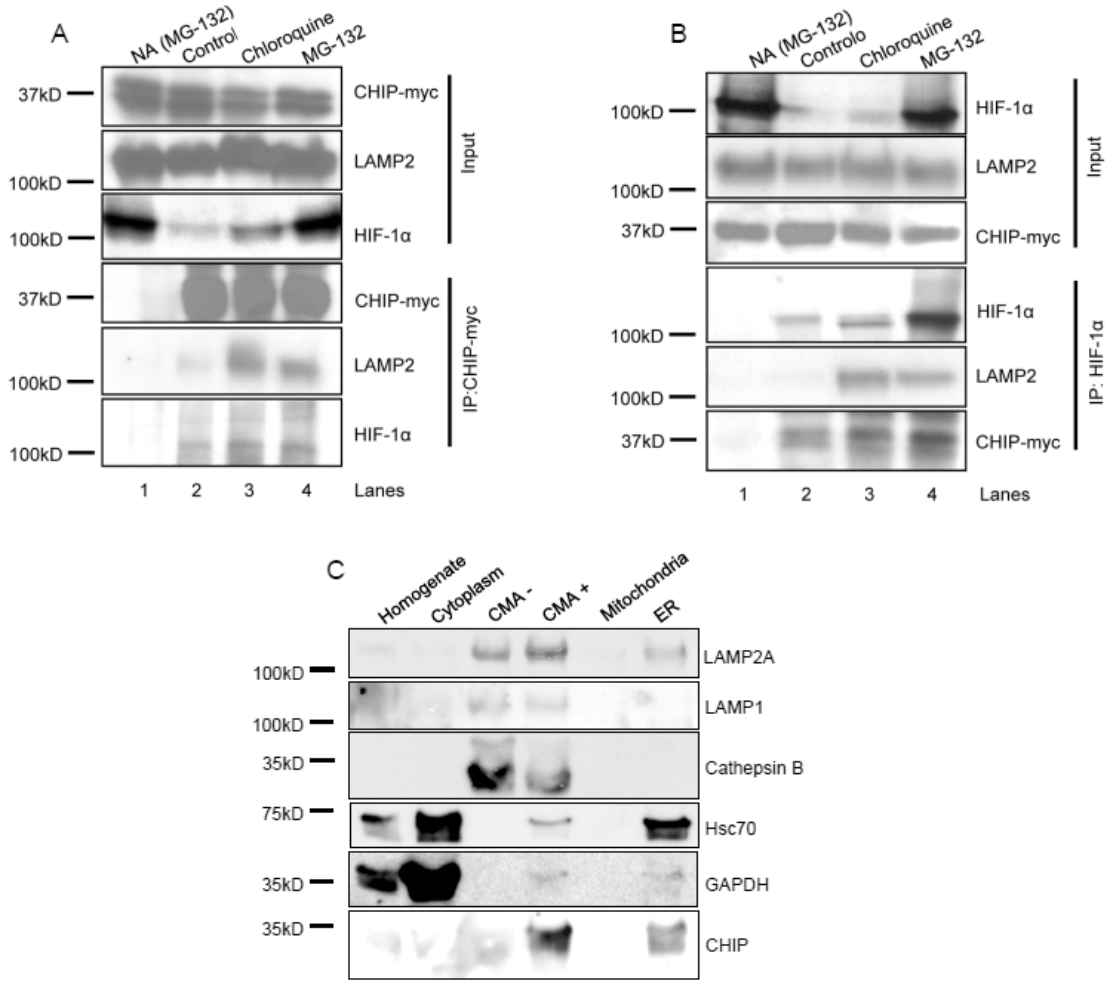

Figure 17. CHIP interacts both with LAMP2A and HIF-1α and localizes in CMA+ lysosomes. (A,B) ARPE-19 cells were transfected with CHIP-myc and incubated either in the presence or absence of 200 μM chloroquine, 20 μM MG-132 (A) Immunoprecipitation of myc-tagged CHIP. ARPE-19 cells were transfected with CHIP-myc. CHIP interacted with LAMP2A and with HIF-1α. (B) Immunoprecipitation for HIF-1α with a specific antibody. HIF-1α interacted with LAMP2A and with CHIP. (C) Western blot of lysosomal and other sub-cellular fractions obtained from liver of starved rat. CHIP localizes into the same lysosomal fraction (CMA positive) as Hsc70 and GAPDH.

Silencing of CHIP results in an increase in the total levels of HIF-1α both under normoxia (compare lane 1 with lanes 2 and 3 in Fig.18A) or chemical hypoxia (compare lane 4 with lanes 5 and 6 in Fig.18A).

Moreover, treatment of cells with either MG-132 or chloroquine, following silencing of CHIP, did not produce any obvious change in the levels of HIF- 1α (Fig. 11B). Interestingly, and perhaps more importantly, silencing of CHIP dramatically reduced the levels of HIF-1α that co-immunoprecipitate with LAMP2 (Fig.18A and B), suggesting that CHIP is required for the interaction between LAMP2A and HIF-1α. To further investigate the mechanism involved in CHIP-dependent targeting of HIF-1α to CMA, ARPE-19 cells were transfected with two mutant forms of CHIP: one that does not have a functional TPR domain (K30A-CHIP), and therefore is unable to bind chaperones, and a second mutant that does not have ubiquitin ligase activity (H260Q-CHIP) (Xu et al., 2002).

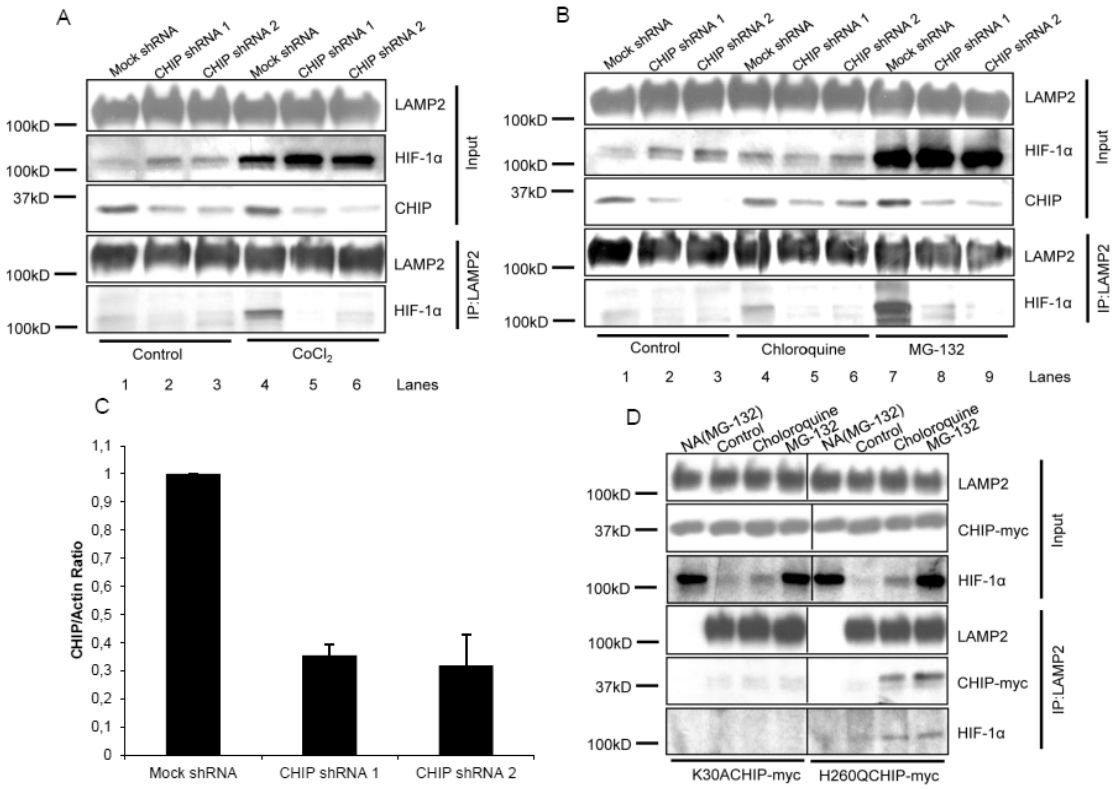

Figure 18. CHIP is a critical player in the CMA-dependent degradation of HIF-1α. (A,B) Immunoprecipitation of LAMP2. ARPE-19 cells were transduced with two different adenoviral vectors encoding shRNAs targeting CHIP or mock shRNA for 48 hours. Silencing of CHIP decreased HIF-1α interaction with LAMP2A. (C) ARPE-19 cells were transduced with two different adenoviral vectors encoding shRNAs targeting CHIP or with an adenoviral vector coding for a mock shRNA. Tranduction proceeded for 48 h after which the cells were harvested and the whole cell extracts were analyzed by Western blot, using anti-CHIP and anti-actin antibodies. CHIP levels were normalized for actin

expression and the results were plotted. The results represent the mean ± SD of at least three independent experiments. (D) Immunoprecipitation of LAMP2. ARPE-19 cells were transfected either with the K30ACHIP-myc mutan or the H260QCHIP-myc mutant. Chaperone-binding activity of CHIP was required for the interaction between HIF-1α and LAMP2. NA (no antibody).

Data presented in Fig.18D shows that the K30A mutant of CHIP, but not the H260Q mutant, fails to co-immunoprecipitate with LAMP2. Similarly, overexpression of K30A CHIP mutant abrogated the interaction of HIF-1α with LAMP2 (Fig.18D), while with the H260Q mutant is still possible to detect some interaction. Nevertheless, the mutant H260Q-CHIP did not completely abrogate the interaction between HIF-1α and LAMP2 but largely reduced it (Fig.19A), suggesting that CHIP mediated ubiquitination might be involved in the degradation of HIF-1α by CMA. To further address the role of ubiquitinylation on targeting of HIF-1α to CMA, cell lysates enriched in ubiquitinylated proteins were obtained using recombinant GST bound Tandem Ubiquitin Binding Entities (GST-TUBEs).

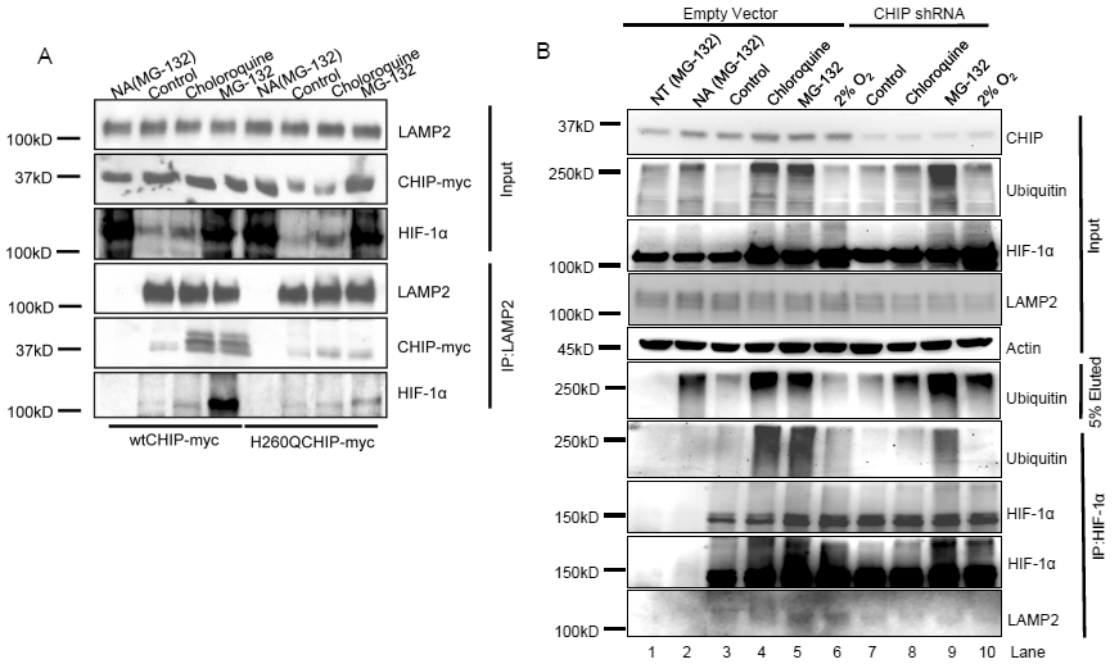

Figure 19. CHIP ubiquitination activity has a role in the CMA-dependent degradation of HIF-1α. ARPE-19 cells were transfected with CHIP-myc and incubated either in the presence or absence of 200 μM chloroquine, 20 μM MG-132 or 2% O_2 for 8 hours or. (A) Immunoprecipitation of LAMP2. ARPE-19 cells were transfected either with the H260QCHIP-myc mutant or with wtCHIP. The ubiquitin ligase activitiy of CHIP only partially inhibited the interaction between HIF-1α and LAMP2. (B) HIF-1α immunoprecipitation of GST-TUBEs-bound ubiquitinated proteins. ARPE-19 cells were transduced for 48 hours with an adenoviral vector encoding shRNAs targeting CHIP. Ubiquitinylated proteins were pulled-down with GST-TUBEs. After elution from the beads, ubiquitinated HIF-1α was immunoprecipitated. CHIP depletion inhibited the ubiquitination of HIF-1α, stabilized by chloroquine

or by hypoxia as well as prevented the co-precipitation of LAMP2A with HIF-1α. NT (no TUBEs), NA (no antibody).

The ubiquitinated fraction of HIF-1α was further evaluated following immunoprecipitation of HIF-1α HIF-1α was subsequently immunoprecipitated from this fraction. Data on Fig.19B (Lane 4) shows that incubation not only with MG-132 or 2% O_2, but also with chloroquine increased levels of ubiquitinylated HIF-1α, whereas depletion of CHIP strongly reduced the levels of ubiquitinylated HIF-1α for all treatments (compare Lanes 3, 4, 5 and 6 with Lanes 7, 8, 9 and 10). Furthermore, levels of LAMP2A that co-precipitate with HIF-1α are reduced in cells depleted of CHIP. Interestingly, both the chaperone binding activity and the ubiquitin ligase activity of CHIP are required for an effective interaction of HIF-1α with LAMP2A, suggesting that CHIP might also act as a molecular switch, directing HIF-1α towards CMA-mediated degradation.

Chapter 6. Biological relevance of CMA-dependent degradation of HIF-1α

6.1 Introduction

Accumulation of HIF-1α under hypoxia and consequent activation of HIF-1 transcriptional activity are critical to ensure cell survival under low oxygen. Thus, it is not surprising that downregulation of HIF-1α response and destabilization of HIF-1α will have a great impact on a variety of biological processes that critically affect a number of human diseases. In some circumstances, excessive accumulation of HIF-1α and the resulting neovascularization are vital for cell and tissue survival, such as in myocardial infarction and diabetic critical limb ischemia. On other circumstances, accumulation of HIF-1α can have severe noxious effects by stimulating pathological neovascularization, such as in diabetic retinopathy, or by supporting growth and progression of many types of solid tumors. The formation of new microvascular networks, in solid tumors, display a broad range of structural and functional abnormalities that in turn lead to irregular and poor blood flow, which culminates in deficient supply of oxygen to the tumor cells and the formation of hypoxic or even anoxic areas. This reduces oxygen availability and induces HIF-1, which regulates the transcription of genes that help cells to survive under low oxygen by regulating cell immortalization, glucose and energy metabolism, vascularization, invasion and metastasis, resistance to chemotherapy and radiation therapy (Harris, 2002; Semenza, 2010). Indeed, HIF-1α induction allows tumor cells to successfully adapt to or overcome their oxygen-deprived state and to survive in a hostile environment. Consistently, mutations in VHL that are associated with renal cell carcinoma (RCC) and cerebral haemangiogblastomas render the E3 ligase complex incapable of ubiquitinate HIF-1α, resulting in an accumulation of HIF-1α and continuous activation of hypoxia responsive genes (Harris, 2002; Semenza, 2010).

In this section we aimed at evaluating the biological relevance of CMA-dependent degradation of HIF-1α.

6.2 Results

6.2.1 CMA-dependent degradation of HIF-1α is independent on oxygen

Previous results presented in this work showed that inhibition of CMA by LAMP2A depletion resulted in HIF-1α stabilization. Furthemore, results suggested that HIF-1α degradation by CMA was did not required oxygen or VHL mediated proteasomal degradation. To further assess if proline hydroxylation was required for HIF-1α degradation through CMA we used a mutant HIF-1α, in which prolines 402/564 were mutated to alanines (PPHIF-1α). Data presented in Fig.20A and Fig.20B shows that the mutant HIF-1α accumulates under normoxia which is likely to reflect the inability of VHL to target the protein for proteasomal degradation.

However, the mutant PPHIF-1α still co-precipitated with LAMP2A (Fig.20A) and still retained the ability to interact with Hsc70 (Fig. 13B). Moreover, incubation of ARPE-19 cells in hypoxia (2% O_2) (Fig.20C and Fig.20D) or the hypoxia mimetic agent $CoCl_2$ (Fig.20E and F) induced accumulation of endogenous HIF-1α but did not disrupt the interaction of HIF-1α with LAMP2A, as revealed by co-immunoprecipitation experiments (Fig.20C, D, E and F), suggesting that CMA might mediate degradation of HIF-1α in hypoxic conditions, when VHL-mediated proteasomal degradation of HIF-1α is not involved. The above data also indicates that CMA-dependent degradation of HIF-1α is not dependent on the hydroxylation of prolines 402/564 of HIF-1α.

6.2.2 Starvation is the stimuli that activates CMA-dependent degradation of HIF-1α

It is widely accepted that starvation is an activator of macroautophagy and that prolonged starvation can also activate CMA (Cuervo et al., 1995a). Hence we incubated NIH-3T3 cells (either transduced with an empty vector or with the shRNA for LAMP2A) under hypoxia (2% O_2) in the presence or absence of serum.

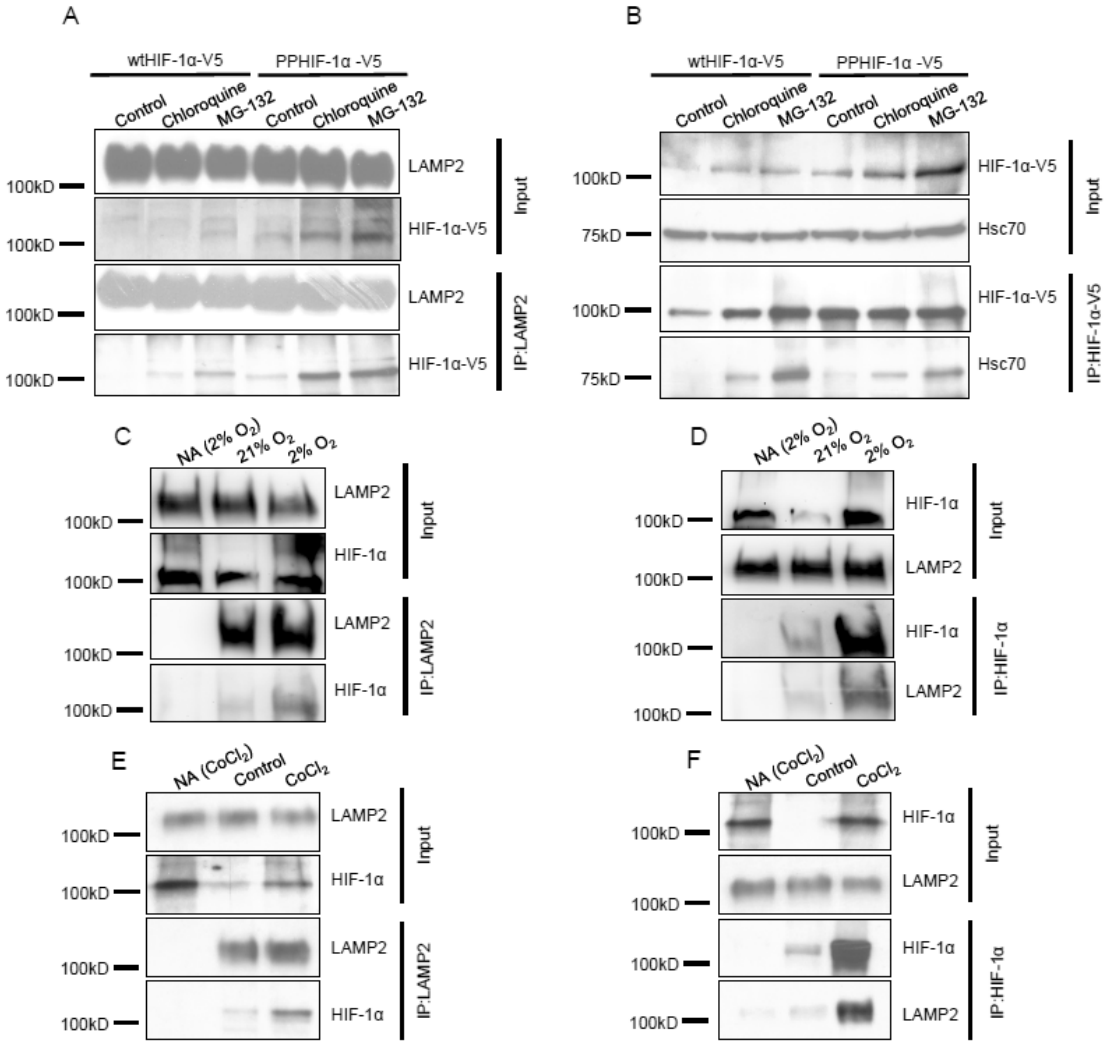

Figure 20. CMA-dependent degradation of HIF-1α is independent on oxygen. ARPE-19 cells were incubated either in the presence or absence of 200 μM chloroquine, 20 μM MG-132, 2% O_2 or 300 μM of $CoCl_2$ for 8 hours. (A, B) Immunoprecipitation of LAMP2 (A) or V5 tag (B). ARPE-19 cells were transfected either with a V5-tagged wtHIF-1α or PPHIF-1α. Both wtHIF-1α and PPHIF-1α interacted with LAMP2A and Hsc70. Immunoprecipitation of LAMP2 (C) or HIF-1α (D). Hypoxia did not alter the ability of HIF-1α to interact with LAMP2A. NA (no antibody). (E,F) Inhibition of HIF-1α proline hydroxylation by $CoCl_2$ did not alter the ability of HIF-1α to interact with LAMP2A. NA (no antibody).

Depletion of LAMP2A did not affect activity of PHDs (Fig.21A) nor did it affect the activity of the proteasome (Fig.21B). Likewise, serum deprivation did not affect proteasome activity (Fig.21B). In cells expressing normal levels of LAMP2A, 6 hours of serum deprivation was sufficient to induce a decrease in the levels of HIF-1α that could be reverted by inhibiting the lysosome (Fig.22A). Conversely, in cells where LAMP2A was silenced, the levels of HIF-1α did not change significantly following prolonged starvation (Fig.22A). The same results were obtained with chemical hypoxia by using $CoCl_2$ (Fig.22B and C).

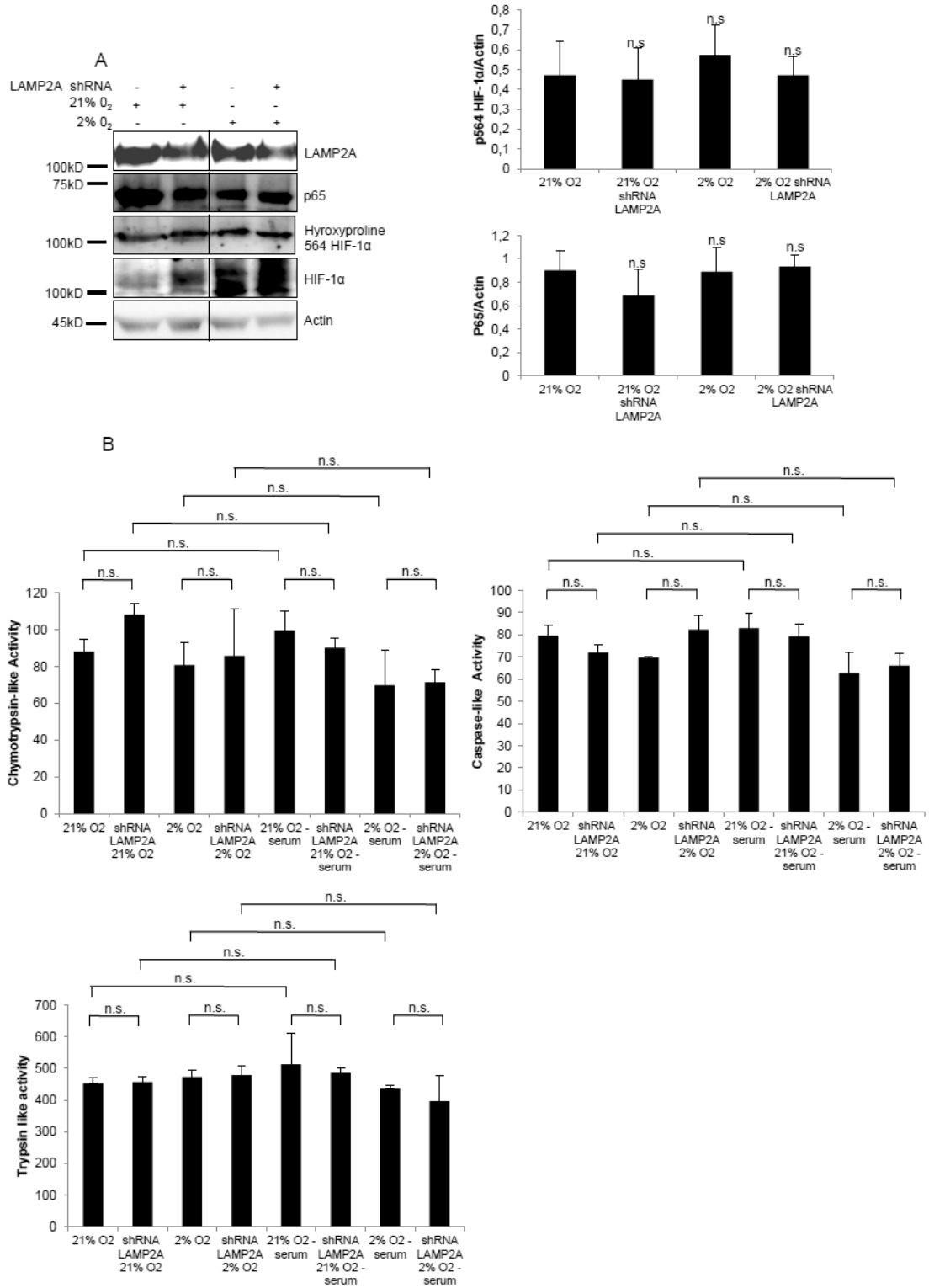

Figure 21. **LAMP2A depletion does not inhibit PHDs or the proteasome.** NIH-3T3 cells were transduced either with an empty vector or LAMP2A shRNA and maintained for 1 month at 37ºC. Subsequently cells were incubated in the presence or absence of 2% O_2 and serum for 24 hours. (A) Western blot for HIF-1α, actin and HIF-1α hydroxyproline 564 and p65. Depletion of LAMP2A does not induce alterations in the hydroxylation of proline 564 nor on the stabilization of p65. (B) Cell extracts were homogenized and protein concentration was determined. Samples of each extract were

incubated with the following fluorogenic substrates, in order to assess the different proteolytic activities of the proteasome: Suc-LLVY-MCA for the chymotrypsin-like activity, Z-LLE-MCA for monitoring the caspase-like activity and Boc-LRR-MCA for the trypsin-like activity. Neither LAMP2A depletion nor serum deprivation inhibited any of the proteasomal activities. The results represent the mean ± SD of at least three independent experiments. (n.s. non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

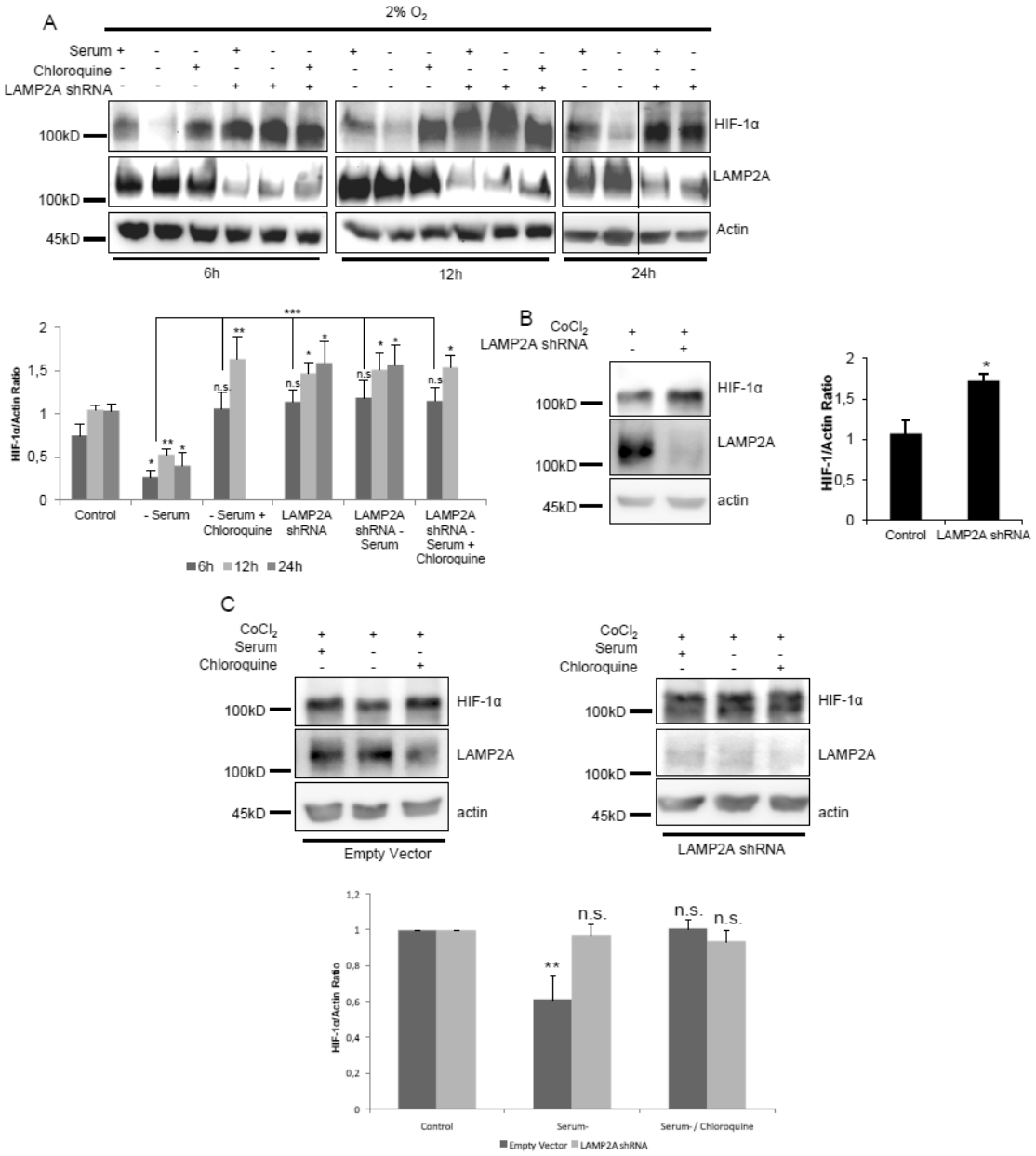

Figure 22. Starvation acts as a stimuli for CMA-dependent degradation of HIF-1α and is independent on prolyne hydroxylation. (A) Western blot of HIF-1α, LAMP2A and actin. NIH-3T3 cells were transduced with either an empty vector or LAMP2A shRNA and maintained for one month at 37ºC. Subsequently, cells were incubated in 2% of O_2 for 6, 12 and 24 hours in the presence or absence of serum and 200 μM chloroquine. Serum deprivation decreases the levels of HIF-1α and this decrease

can be reverted by lysosomal inhibition (200 μM of chloroquine). HIF-1α is stabilized upon starvation in cells depleted of LAMP2A. (B,C) Western blot of HIF-1α, LAMP2A and actin. NIH-3T3 cells were transduced either with an empty vector or LAMP2A shRNA and maintained for 1 month at 37ºC. Subsequently cells were incubated in the presence of 300 μM $CoCl_2$ for 6 hours in the presence or absence of serum. (C) LAMP2A knock-down further increases the stabilization of HIF-1α. (D) Serum deprivation decreases the levels of HIF-1α and this decrease can be reverted by lysosomal inhibitors (200 μM of chloroquine). HIF-1α does not decrease upon starvation in cells depleted for LAMP2A. The results represent the mean ± SD of at least three independent experiments, (n.s. non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

These results demonstrate that degradation of HIF-1α upon serum deprivation is mediated by CMA. A comparable decrease in HIF-1α levels after serum deprivation was also observed in RCC4 cells (compare lane lane 1 and lane 3 in Fig.16B), suggesting that this pathway is not cell type specific. Furthermore, NIH-3T3 cells depleted of LAMP2A under hypoxia (2% of O_2) showed increased stabilization of HIF-1α at 12 and 24 hours (Fig.22A).

To further confirm CMA-dependent degradation of HIF-1α under starvation, we evaluated the effect of serum deprivation on the KFERQ mutated HIF-1α. Consistently, we showed that AAHIF-1α is insensitive to serum deprivation under hypoxia (Fig.23A). In agreement with the data gathered so far, we also showed that serum deprivation increased the co-precipitation of HIF-1α with LAMP2A, both in normoxia (21% O_2) and in hypoxia (2% of O_2) (Lane 3 and Lane 8 in Fig.23B). Furthermore, we evaluated the physiological relevance of starvation-induced degradation of HIF-1α at an organism level. For this purpose we determined the levels of HIF-1α localized in CMA+ lysosomes obtained from fed or starved livers. The results presented in FIG.23C show that HIF-1α was increased in lysosomes isolated from starved rat livers (Fig.23C) indicating that CMA-dependent degradation of HIF-1α occurs in living animals and not only in cell cultures. Importantly, also GAPDH, a well stablished CMA substrate, localizes only in the CMA+ lysosomes of starved animals.

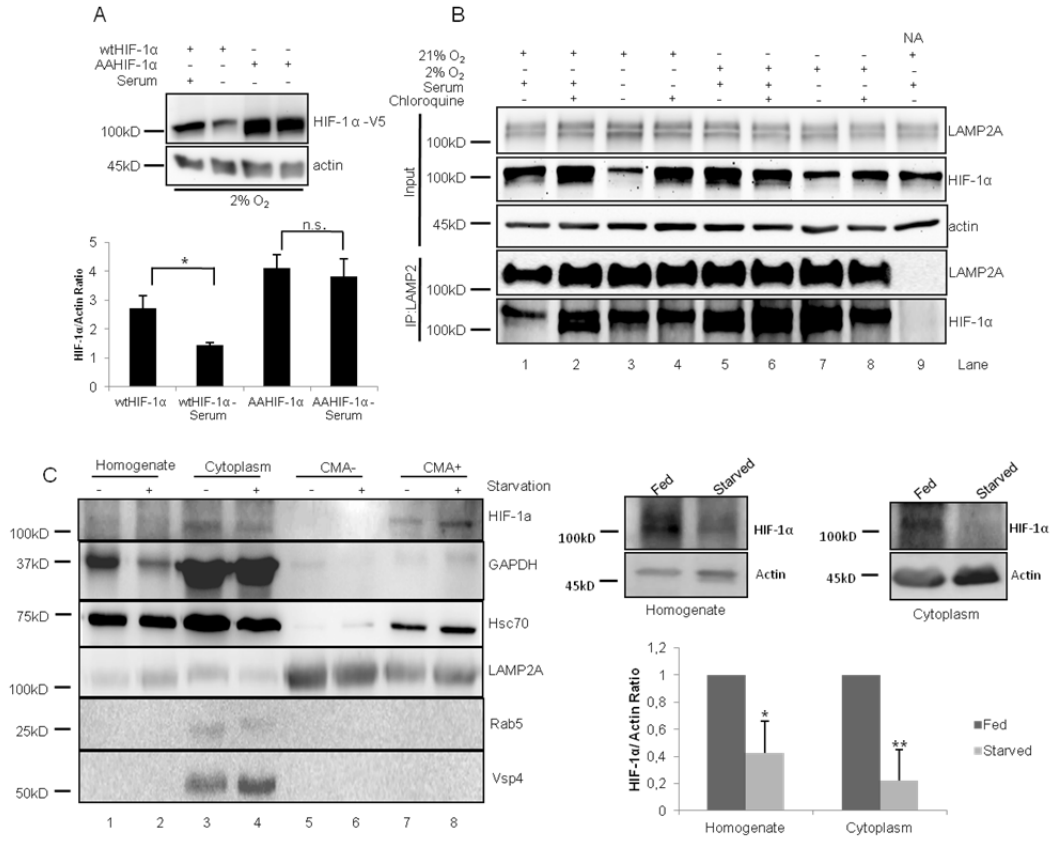

Figure 22. Starvation induces HIF-1α degradation in the liver and HIF-1α localization only in CMA+ lysosomes. (A) Western blot for V5-tag and actin of ARPE-19 cells transfected either with wtHIF-1α or AAHIF-1α (both tagged with V5). Cells were incubated in 2% O_2 in the presence or absence of serum for 8 hours. AAHIF-1α is insensitive to serum deprivation. (B) Immunoprecipitation of LAMP2. RCC4 cells were incubated either in normoxia (21% O_2) or under hypoxia (2% O_2) in the presence or absence of serum and 200 μM chloroquine for 8 hours and harvested. Serum deprivation decreases total levels of HIF-1α but increases the co-precipitation of HIF-1α with LAMP2A, both in normoxia and in hypoxia. (C) Subcellular fractions of rat liver probed for relevant proteins as indicated in the figure. HIF-1α localizes into the same lysosomal fraction (CMA positive fraction) as Hsc70 and GAPDH only in starved rats. Starvation decreases the levels of HIF-1α in the the cytosolic fraction. The results represent the mean ± SD of at least three independent experiments, (n.s. non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Moreover, the increased association of HIF-1α with lysosomes isolated form rat livers does not result from increased synthesis of HIF-1α but rather appears to be due to the selective targeting of cytosolic HIF-1α to the lysosomes as indicated by the decrease on levels of HIF-1α from the total liver homogenates and the cytosolic fraction of starved animals (Fig.23C).

Overall, data is consistent with a model in which CMA can be activated by starvation in animals (rat) and by serum deprivation in various cell lines leading, in both cases, to increased lysosomal degradation of HIF-1α.

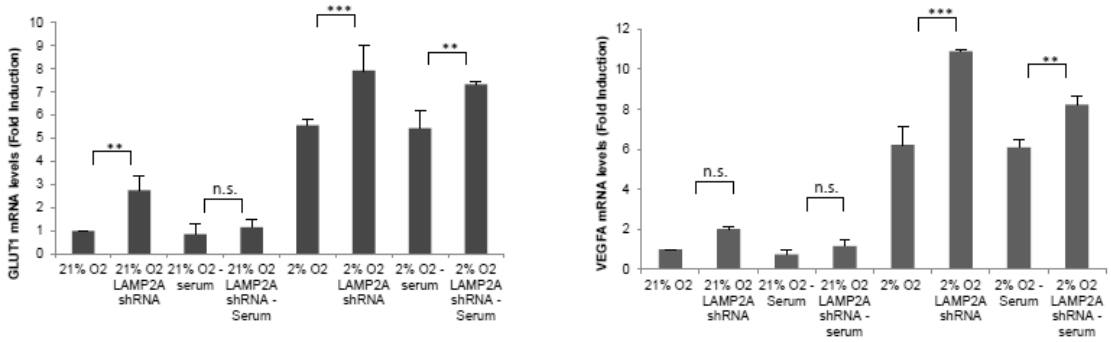

Figure 23. Inhibition of CMA results in the increase of GLUT-1 and VEGF mRNA levels. NIH-3T3 or HIF-1α +/+ or HIF-1α-/- MEFs cells were transduced with an empty vector or a vector containing LAMP2A shRNA and maintained for one month at 37ºC. Subsequently cells were incubated in the presence or absence of 2% O_2 for 6, 12 or 24 hours. Quantitative Real-Time RT-PCR analysis of GLUT-1 and VEGF in normal or LAMP2A depleted NIH-3T3 cells. The mRNA levels of both GLUT-1 and VEGF in LAMP2A depleted cells significantly increase in hypoxic conditions, in the presence or absence of serum. mRNA levels of GLUT-1 are also increase in LAMP2A depleted cells in normoxic conditions. The results represent the mean ± SD of at least three independent experiments. (n.s. non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

6.2.3 Degradation of HIF-1α by CMA contributes to poor cell response to hypoxia and increased cell death

To address the biological implications of degradation of HIF-1α by CMA, we began by asking whether the HIF-1α that accumulates in cells that are incompetent for CMA (depleted of LAMP2A) is transcriptionally active. We focused on two major genes that are well established to be regulated by HIF-1α: the vascular endothelial growth factor (VEGF) (Levy et al., 1995) and the glucose transporter-1 (GLUT-1) (Ebert et al., 1995). NIH-3T3 cells depleted of LAMP2A exhibit an increased expression of VEGF and GLUT-1 mRNA, both in hypoxic or hypoxic and serum deprived cells (Fig.24). Furthermore, and consistent with the increased levels of mRNA, in cells depleted of LAMP2A, GLUT-1 protein levels are further increased in hypoxia, with the levels of GLUT-1 protein peaking at 12 hours of hypoxia (Fig.25A).

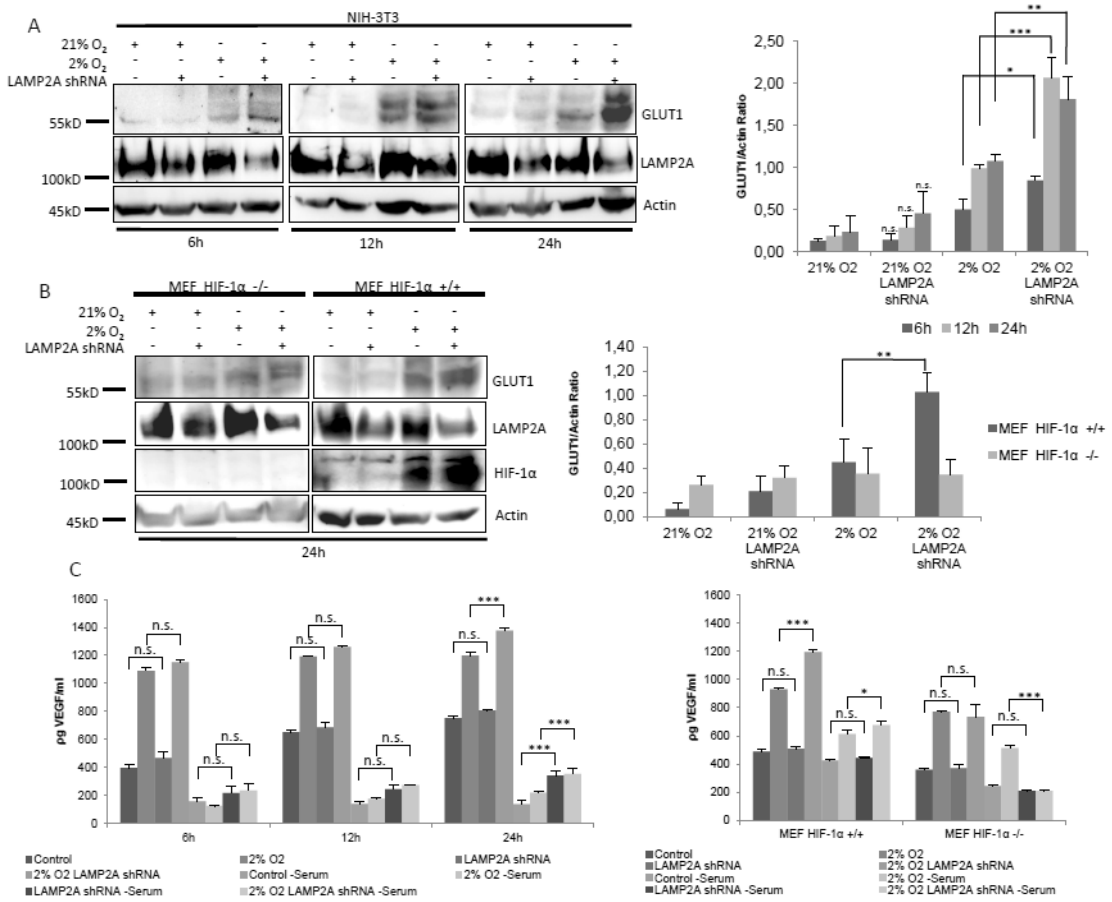

Figure 24. **Inhibition of CMA results in the stabilization of GLUT-1 and VEGF.** NIH-3T3 or HIF-1α +/+ or HIF-1α-/- MEFs cells were transduced with an empty vector or a vector containing LAMP2A shRNA and maintained for one month at 37ºC. Subsequently cells were incubated in the presence or absence of 2% O_2 for 6, 12 or 24 hours (A) NIH-3T3 or (C) HIF-1α +/+ or HIF-1α-/- MEFs. (B) LAMP2A depleted cells show a more significant increase in the levels of GLUT-1 in hypoxic conditions at 6, 12 and 24 hours. (B) HIF-1α +/+, but not HIF-1α-/- MEFs depleted of LAMP2A show increased GLUT-1 protein levels when compared to cells transduced with an empty vector. (C) ELISA for the secreted VEGF of NIH-3T3 and HIF-1α +/+ or HIF-1α-/- MEFs. NIH-3T3 cells depleted of LAMP2A show increased secretion of VEGF only at 24 hours of hypoxia, with or without serum as well as in normoxia without serum. HIF-1α +/+, but not HIF-1α-/-, MEFs depleted of LAMP2A show increased secretion of VEGF in to the media in hypoxic conditions with or without serum. The results represent the mean ± SD of at least three independent experiments. (n.s. non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

As a control experiment, to confirm that the observed effects are mediated by HIF-1α, we used either wild-type (HIF-1α +/+) or HIF-1α knocked-out (HIF-1α -/-) mouse embryonic fibroblasts (MEFs), that have a severely impaired cellular response to hypoxia (Carmeliet et al., 1998; Hu et al., 2006). Consistent with a role of HIF-1α in the accumulation of GLUT-1 in cells with compromised CMA, HIF-1α -/- MEFs depleted of LAMP2A failed to up-regulate the expression of GLUT-1, whereas HIF-1α +/+ depleted of LAMP2A further up-regulated GLUT-1 in response to hypoxia

(Fig.25B) indicating that by knocking down CMA, cells were able to accumulate transcriptionally active forms of HIF-1α.

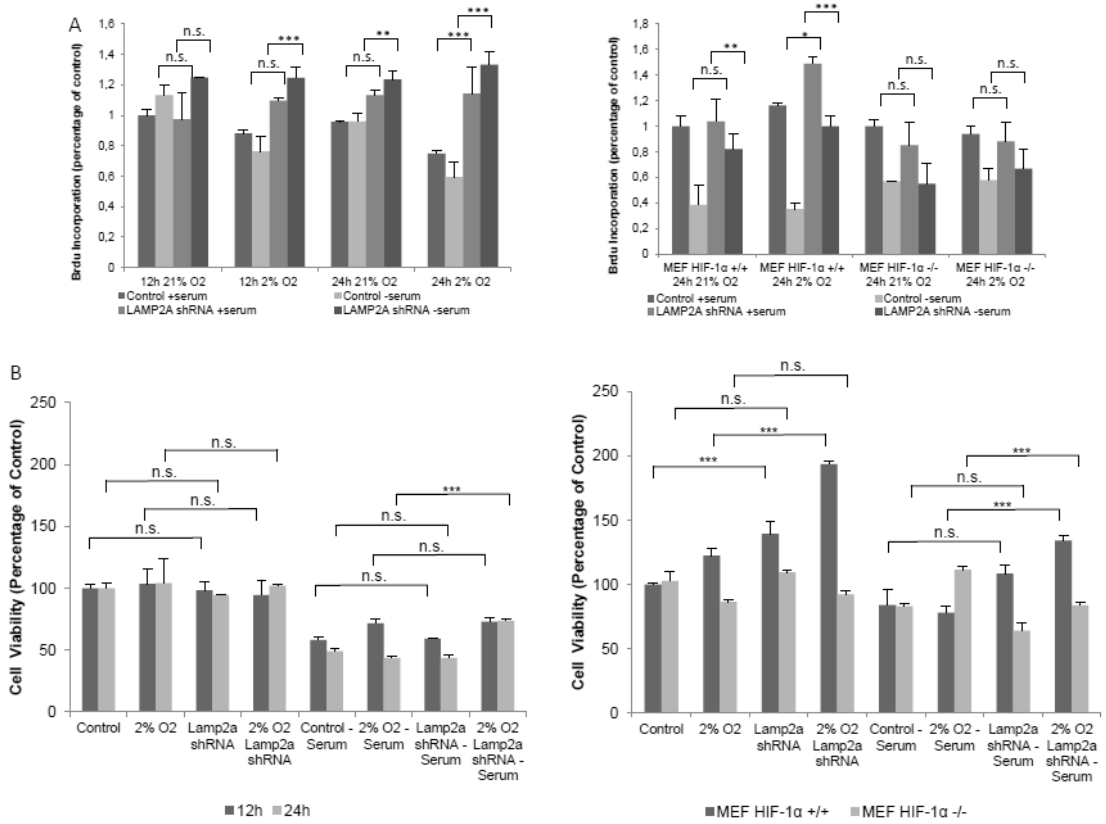

Figure 26. Inhibition of CMA results in the stabilization of HIF-1α and improved cell response and survival to hypoxia. (A) Bromodeoxyuridine DNA incorpotation assay (Brdu) for NIH-3T3 and HIF-1α +/+ or HIF-1α-/- MEFs. NIH-3T3 cells depleted for LAMP2A display increased proliferation at 12 hours in hypoxia -Serum, and at 24 hours in hypoxia +Serum as well as hypoxia and normoxia –Serum. HIF-1α +/+, but not HIF-1α-/- MEFs depleted for LAMP2A at 24 hours of treatment show increases in the proliferation rate similar to NIH-3T3s cells in the same conditions. (B) MTT cell viability assay for NIH-3T3, HIF-1α +/+ or HIF-1α-/- MEFs. Cells were transduced either with an empty vector or LAMP2A shRNA and maintained for one month at 37ºC and incubated in normoxia or hypoxia and with or without serum for 12 and 24 hours (NIH-3T3) or 24 hours (MEFs). NIH-3T3 show a decrease in cell viability after serum deprivation that was partially reverted in LAMP2A depleted cells incubated with 2% O_2 for 24 hours. HIF-1α +/+ MEFs depleted for LAMP2A show increased viability with no treatments as well as in hypoxia either in the presence or absence of serum. Cells that do not express HIF-1α (HIF-1α -/-) show poor viability . The results represent the mean ± SD of at least three independent experiments. (n.s. non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Consistent with these observations, NIH-3T3 cells depleted of LAMP2A showed increased secretion of VEGF at 24 hours of hypoxia. As observed for GLUT-1, silencing of LAMP2A in HIF-1α +/+ MEFs increased VEGF secretion under hypoxia. On the other hand, HIF-1α -/- LAMP2A depleted cells failed to further up-regulate levels

of VEGF in response to hypoxia when compared with the HIF-1α -/- MEFs transduced with empty vector (Fig.25C).

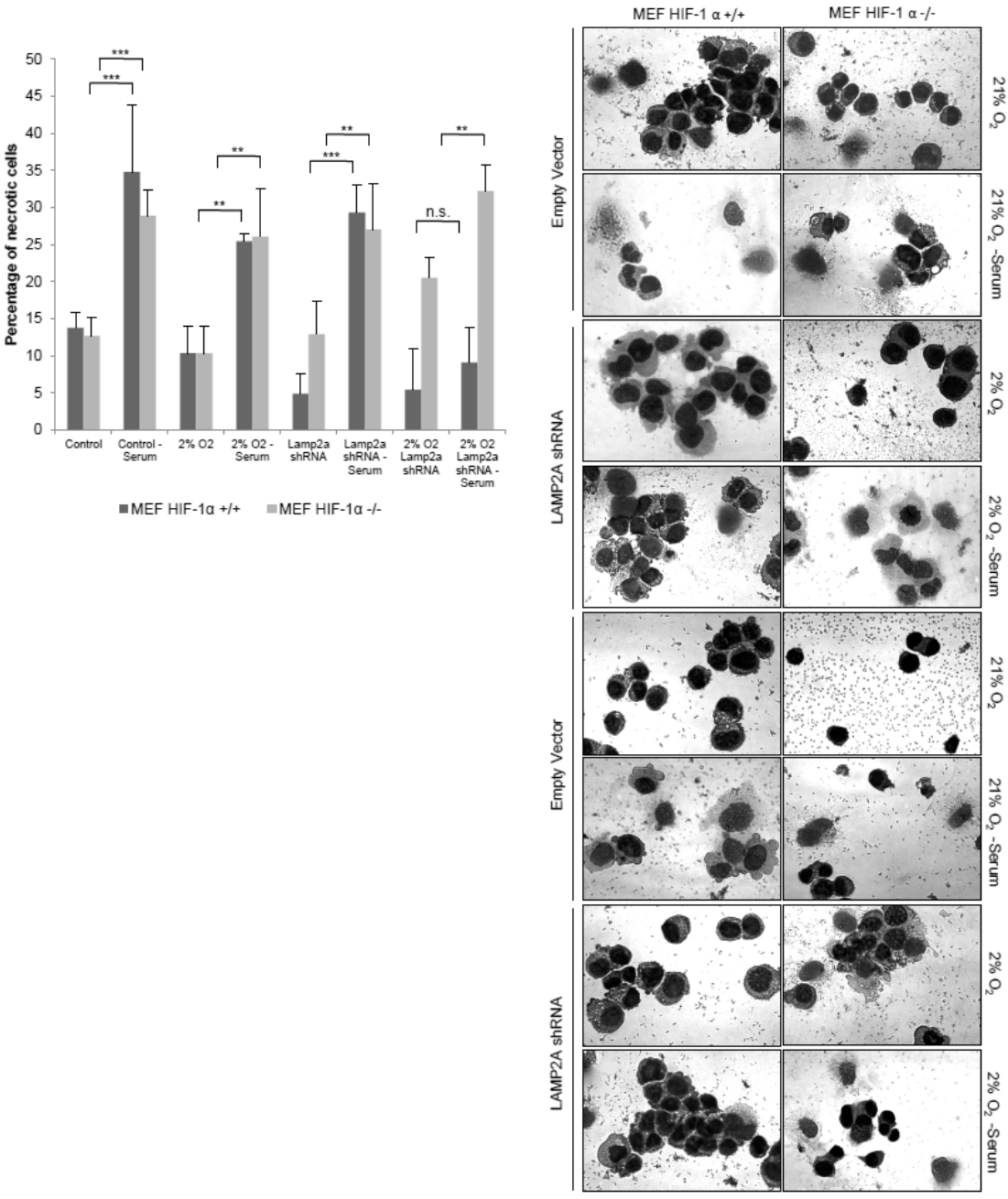

Figure 25. Stabilization of HIF-1alpha, by inhibiting CMA, results in increased cell survival. May–Grünwald and Giemsa solution staining of HIF-1α +/+ or HIF-1α-/- MEFs. Cells were transduced either with an empty vector or LAMP2A shRNA and maintained for 1 month at 37ºC and incubated under hypoxia (2% O_2) or normoxia (21% O_2) in the presence or absence of serum for 24 hours. Both HIF-1α +/+ and HIF-1α -/- MEFs show increased cell death by necrosis, characterized by loss of plasma membrane integrity, cell and organelles swelling, and frequent release of cytosolic content into the extracellular space after serum deprivation. Depletion of LAMP2A in HIF-1α +/+, but not in HIF-1α -/-,

MEFs rescues necrotic cell death in hypoxic conditions. The results represent the mean ± SD of at least three independent experiments. (n.s. non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Both NIH-3T3 and HIF-1α +/+ MEF cells depleted of LAMP2A show increased DNA incorporation of the proliferation marker bromodeoxyuridine (Brdu), the more robust effect being observed in hypoxic cells (Fig.26A). Conversely, depletion of LAMP2A on HIF-1α -/- MEFs does not have a significant effect on incorporation of Brdu and cell proliferation. On the other hand, depletion of LAMP2A in hypoxic NIH-3T3 cells deprived of serum for 24 hours, significantly increased cell viability (Fig.26B). In HIF-1α +/+ MEFs, reduced levels of LAMP2A improve cell viability in hypoxia or normoxia, but serum deprived cells only show increased viability when incubated in 2% O_2. HIF-1α -/- MEFs depleted for LAMP2A show no increase in cell viability (Fig.26B). In addition, after 24 hours of hypoxia and serum deprivation, necrotic cell death of HIF-1α +/+ LAMP2A-depleted MEFs is reduced by 25% compared to HIF-1α -/- LAMP2A-depleted MEFs, as assessed by May–Grünwald and Giemsa solution staining (Fig.27).

Chapter 7. Discussion

A number of sparse and indirect evidences suggest that lysosome might indeed be involved in regulation of HIF-1α. For example, it was shown that cathepsin B, a lysosomal protease, can regulate the levels of HIF-1α protein in primary endothelial cells (Im et al., 2005) as well as in a renal carcinoma (RCC4) cell line deficient in VHL (Olmos et al., 2009). In this study we show, for the first time, that HIF-1α, a well-established substrate of the UPS, can also be targeted for degradation in the lysosome by CMA. In fact, data shows that HIF-1α is stabilized by lysosome inhibitors. In addition, the human, mouse and rat HIF-1α all have the pentapeptide sequence biochemically related to the KFERQ motif, which is a well established requirement for a protein to be a substrate for CMA (Cuervo, 2010). Significantly, the mutation of the non-canonical KFERQ motif of human HIF-1α, $_{529}$NEFKL$_{533}$, is sufficient to render the protein insensitive to lysosome inhibitors. Moreover, KFERQ-mutated HIF-1α does not associate with LAMP2A nor does it associate with Hsc70, two key molecular players in CMA. We further show that HIF-1α is selectively present in rat liver lysosomes that are positive for CMA and that it is selectively transported into intact lysosomes, as described for other canonical CMA substrates, including GAPDH and Ribonuclease A. Interestingly, the human and rat KFERQ-like motif and at least one of the mice KFERQ-liKe motifs ($_{512}$ERLLQ$_{516}$) of HIF- 1α localize within the oxygen-dependent degradation (ODD) domain. This domain consists of about 200 amino acids and is thought to be responsible for the oxygen-dependent degradation of HIF-1α by the proteasome (Huang et al., 1998). Indeed, it was reported that the HIF-ODD domain, under physiological conditions, is highly unstructured. This could contribute to the versatility of this region and its ability to interact with various proteins (Sanchez-Puig et al., 2005), namely Hsc70.

Data presented in this study shows that proteasome inhibition leads to an increased association between the CMA receptor LAMP2A and both HIF-1α and Hsc70. However indirect, these and other observations suggest that inhibition of the proteasome might up-regulate CMA such that some UPS substrates (e.g. HIF-1α) are targeted for degradation in the lysosome. It was shown that blockage of CMA by

silencing LAMP2A can lead to an accumulation of polyubiquitinated substrates (Massey et al., 2008), suggesting that a crosstalk may exist between proteasomal degradation and CMA.

Reports on VHL independent pathways for degradation of HIF-1α are not without precedent. However, data presented here shows that regulation of HIF-1α degradation is likely to be more complex than anticipated and that the lysosome plays an important role in this process. An exciting possibility, consistent with this hypothesis, is that cells might have a number of "dual-pathway substrates" that can be degraded either by the proteasome or by the lysosome, through CMA. Indeed, several proteins were shown to be degraded both by the proteasome and the lysosome. These include Huntingtin (Thompson et al., 2009), RCAN1 (Liu et al., 2007), EGFR and ErbB2 (Sha et al., 2009), α-Synuclein (Cuervo et al., 2004) and IkappaB (Cuervo et al., 1998). By analogy, it is conceivable that HIF-1α may also be a "dual-pathway" substrate, being degraded both by the UPS and CMA.

The molecular players involved in targeting HIF-1α for CMA are not yet fully elucidated, although they appear to involve the chaperone (Hsc70) and CMA-receptor (LAMP2A), as previously described for other CMA substrates. In addition, we show, for the first time, that the co-chaperone CHIP is also involved in targeting HIF-1α for CMA. Indeed, data presented in this work suggests that CHIP is likely to be a key player in targeting HIF-1α for CMA. Whether or not CHIP participates in the targeting of other substrates for lysosomal degradation remains to be elucidated. CHIP is a unique protein, in that it has both ubiquitin ligase activity and the ability to bind chaperones, making it a putative molecular switch between CMA and UPS. Consistently, CHIP was shown to direct α–synuclein for degradation both in the proteasome and in the lysosome (Shin et al., 2005). More recently, CHIP was reported to assist in the disposal of damaged Z disk proteins through autophagy (Arndt et al., 2010). Data presented here shows that the ability of CHIP to bind to chaperones is strictly required for the targeting of HIF-1α for CMA-dependent degradation whereas the ubiquitin ligase activity of CHIP contributes, to a lesser extent, to the targeting of HIF-1α to the lysosome through CMA.

Indeed, our data also provides clues to suggest that the ubiquitin ligase activity of CHIP is likely to play a role in directing substrates to CMA. The role of ubiquitination

in such a process is still unknown, however it should be noted that a specific deubiquitinating enzyme (UCH-L1) is present at the surface of the lysosome, presumably bound to the CMA receptor LAMP2A (Kabuta et al., 2008). The function of this enzyme remains to be elucidated however it might serve to increase the affinity of conjugated substrates to the lysosomal membrane, acting as a docking point for ubiquitinated substrates. If CMA degrades ubiquitinated substrates perhaps deubiquitinating enzymes would have here a function similar to that present in the proteasome caps, editing ubiquitin chains before the substrates are degraded (Lam et al., 1997a; Lam et al., 1997b; Voges et al., 1999). Moreover, a recent report showed that CHIP ability to bind chaperones and to ubiquitinate substrates is important in the degradation on filamin by macroautophagy, supporting a model where CHIP participates in the degradation of substrates not only through the proteasome but also in the lysosome (Arndt et al., 2010).

CHIP complexes typically contain the chaperones Hsc70, Hsp90, the co-chaperones BAG1, BAG2 and HspBP1 and the proteasomal subunits S1 and C8 (Arndt et al., 2005). BAG1, BAG2 and HspBP1 are present in ternary complexes with Hsc70 and CHIP. The cochaperone BAG1 was shown to stimulate the CHIP-mediated degradation of the glucocorticoid hormone receptor (Demand et al., 2001) and has the ability to associate with the proteasome through its ubiquitin-like domain and to act as a substrate release factor of Hsc70 in the vicinity of the proteasome (Alberti et al., 2002; Luders et al., 2000a; Luders et al., 2000b). On the other hand, BAG2 inhibits ubiquitin ligase activity of CHIP by abrogating the CHIP/E2 cooperation (Arndt et al., 2005). Moreover, HspBP1 acts by inducing conformational changes of the chaperone complex, which interfere with CHIP-mediated ubiquitination (Alberti et al., 2004). Thus, a cooperation of CHIP with other co-chaperones of Hsc70 emerged as a regulatory principle. Although BAG1 and BAG2 might be viewed as antagonistic regulators of the CHIP ubiquitin ligase, differences in substrate specificity have to be taken into account. For example, BAG2 is an essential component in CFTR maturation, whereas BAG1 does not affect the CHIP-mediated degradation of the ion channel (Meacham et al., 2001). The two co-chaperones may thus cooperate with Hsc70 in the biogenesis of distinct sets of chaperone substrates. Importantly, co-chaperone cooperation not only provides a means to stimulate

chaperone assisted degradation but also to interfere with it (Arndt et al., 2005). Taking into account our data it is reasonable to hypothesize that complexes that predominantly contain, in addition to CHIP and Hsc70, the co-chaperones HspBP1 and BAG2, are likely to divert complexes to the CMA pathway. Conversely, CHIP-associated complexes containing BAG1 would preferentially divert targets to UPS.

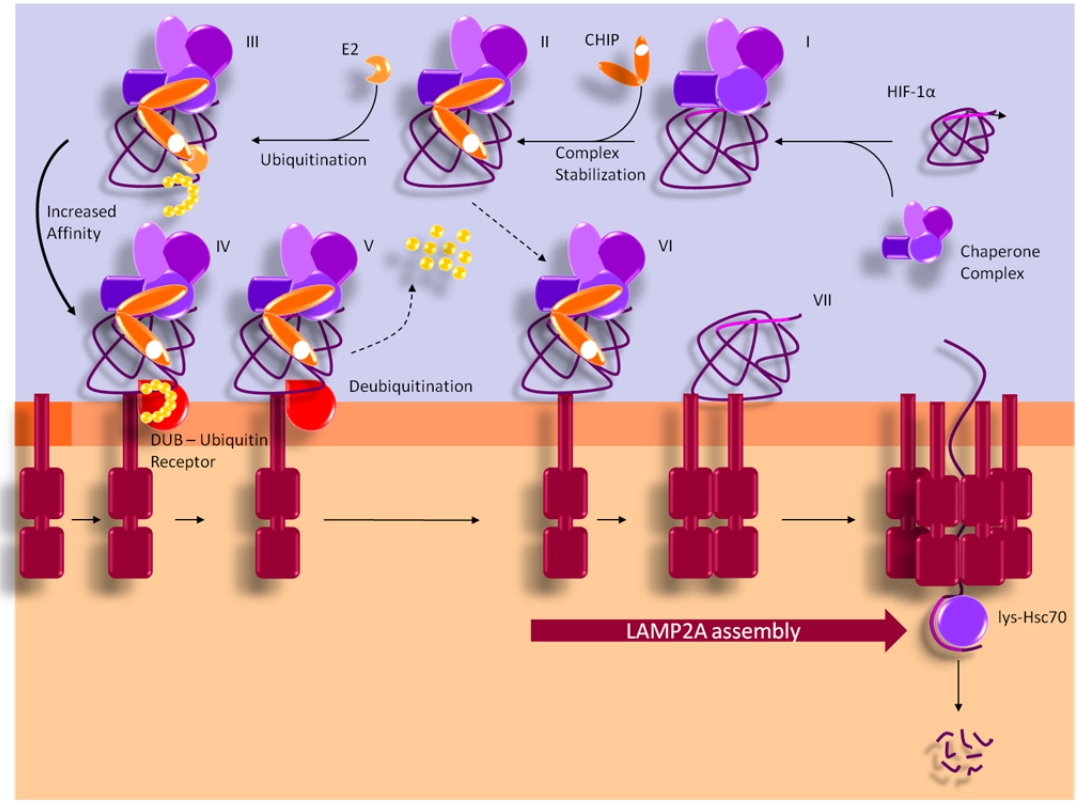

Figure 26. Tentative model for the degradation of HIF-1α by CMA. I) HIF-1α KFERQ-like motif is recognized by a complex of chaperones and co-chaperones. II) The co-chaperone CHIP interacts with the complex through its TPR domain. This interaction is essential for HIF-1α degradation by CMA and can account, at least, for HIF-1α interaction with LAMP2A. III) Nevertheless, CHIP can also ubiquitinate HIF-1α, an event that increases HIF-1α affinity for LAMP2A. IV) At the lysososmal membrane HIF-1α interacts with LAMP2A and the ubiquitin chain is recognized by a DUB enzyme that acts as a receptor, increasing the affinity of the complex for the lysosomal membrane. V) After recognition of the ubiquitin chain, the DUB enzyme removes it from HIF-1α. VI) The chaperone and co-chaperone complex separates from HIF-1α. VII) LAMP2A oligomerization allows the transfer of HIF-1α into the lysosomal lumen with the subsequent degradation of the transcription factor.

Data also suggests that HIF-1α undergoes lysosomal degradation in both normoxic and hypoxic conditions and that both prolonged serum deprivation or starvation act as a stimuli triggering CMA-dependent degradation of HIF-1α in cell cultures and *in vivo* respectively. Prolonged serum deprivation and starvation were both shown to activate CMA in cultured cell lines and in liver hepatocytes (Cuervo

and Dice, 1996; Cuervo et al., 1997). Data presented here show that after 48h of starvation the levels of HIF-1α decrease in rat livers and that the transcription factor is targeted to CMA positive lysosomes for degradation. We also show that serum deprivation, a well established stimuli for CMA activation in cell lines, also induced a decrease in protein levels of HIF-1α. Moreover, cells that were depleted of LAMP2A failed to degrade HIF-1α following serum deprivation.

Although serum deprivation, in cell culture, reproduces the hallmarks of CMA activation following long-term starvation in animals (Bandyopadhyay et al., 2008; Cuervo and Dice, 2000; Cuervo et al., 1997), there are other stimuli that might activate CMA in the liver, resulting in increased lysosomal degradation of HIF-1α. For example, ketone bodies were also show to induce CMA in fibroblasts in a manner that is similar to serum deprivation (Finn and Dice, 2005). Interestingly, this is likely to be of physiological relevance in the liver as synthesis and accumulation of ketone bodies was shown to occur following prolonged starvation (Laffel, 1999; Lowell and Goodman, 1987; Russell et al., 1997). The ketone bodies-induced activation of CMA would lead to increased degradation of HIF-1α. The effect of ketone bodies on cell types that do not utilize ketone bodies for energy, as the hepatocytes, is of increasing protein breakdown in times of nutritional stress (Finn and Dice, 2005) and as we observed, of decreasing HIF-1α protein levels. In further support of our hypothesis, that activation of CMA leads to increased lysosomal degradation of HIF-1α, is the report showing that food restriction leads to a decrease of both the protein levels of HIF-1α and the mRNA levels of the hypoxia-responsive genes HO-1, VEGF, EPO and iNOS in the liver (Kang et al., 2005). Many of these genes lead to increased glucose uptake (Park et al., 2007; Roth et al., 2004a; Roth et al., 2004b) and glycolysis (Semenza, 1999). In this context HIF-1α, by activating the expression of numerous glycolisis related genes, as well as increasing the expression of proteins that help cells capture glucose, is an inducer of glycolisis. Since the liver is the major contributor of blood glucose, it is reasonable to hypothesize that the decrease in HIF-1α protein levels in the liver under starvation underlie a liver adaption to a gluconeogenic state (Yabaluri and Bashyam, 2010). Hence, our findings are consistent with a model in which CMA has an important contribution in starvation-induced liver metabolic stress.

On an opposing model, high fat diet was recently reported to have an inhibitory effect in CMA activity (Rodriguez-Navarro et al., 2012). Interestingly, high fat diet also induces accumulation of HIF-1α on mouse livers (Ochiai et al., 2011). Furthermore, mice lacking HIF-1α in hepatocytes show aggravated glucose intolerance when fed with high fat diet, as well as elevated blood glucose and decreased levels of glucokinase (GK) (Ochiai et al., 2011). GK is known to facilitate glucose uptake from the blood and induce its accumulation in the liver as glycogen (Roth et al., 2004a). Taking this into consideration, it is conceivable that HIF-1α accumulation in the liver upon high fat diet is, at least in part, due to a decreased degradation of HIF-1α by CMA. Thus, it is possible that the regulation of HIF-1α by CMA has an important role in controlling blood glucose levels. Moreover, lipidomic analysis of lysosomal membranes of high fat diet fed animals showed substantial quantitative and qualitative changes in the lipid composition of liver lysosomes, which in turn decreased LAMP2A stability (Rodriguez-Navarro et al., 2012). Apparently, it is the reduction in the levels of LAMP2A that leads to the inhibition of CMA (Rodriguez-Navarro et al., 2012). Similar alterations on the lipid membranes of liver lysosomes are observed in aged animals, concomitantly with a decrease in LAMP2A stability and CMA activity (Rodriguez-Navarro et al., 2012; Zhang and Cuervo, 2008). In accordance with these observations and with our data, aged rats show increased levels of HIF-1α in hepatocytes as well as increased expression of the hypoxia-responsive genes HO-1, VEGF, EPO and iNOS, but no increase in the mRNA levels of HIF-1α (Kang et al., 2005). Consistently and in further support of our observations, a restriction in food intake of 60% was shown to decrease both the protein levels of HIF-1α and the mRNA levels of the above hypoxia-responsive genes (Kang et al., 2005).

On the other hand, this new pathway for the degradation of HIF-1α is likely to impact on a variety of biological processes and on a number of pathophysiological conditions where cells are under both prolonged hypoxia and nutrient deprivation. For example, solid tumors often have a core characterized by a hypoxic environment and increased transcriptional activity of HIF-1α. In addition to hypoxia, many solid tumours also endure nutrient deprivation due to limited blood supply (Jain, 2005). The biological consequences of the stabilization of HIF-1α by inhibiting CMA, under

hypoxia and serum deprivation, include increased cell viability, increased cell proliferation and decreased cell death. The findings described in this study are likely to greatly impact on our understanding of the molecular mechanisms that regulate tumour cell survival. In fact, our data is consistent with previous reports showing a decrease of HIF-1α upon serum removal, as well as an absence of HIF-1α in the hypoxic perinecrotic regions of solid tumours (Sobhanifar et al., 2005). Although the authors did not identify the mechanism through which serum removal decreases HIF-1α levels, they clearly relate the loss of HIF-1α of hypoxic regions with the lack of nutrients in the core of the solid tumours (Sobhanifar et al., 2005). In addition, a recent report shows that the embryonic M2 isoform of pyruvate kinase (PKM2), a pyruvate kinase isoform commonly expressed in tumors, is a substrate for CMA (Lv et al., 2011). Also, very recently it was also shown that CMA may have an important role in tumorigenesis affecting tumor growth, survival and metastatic potential (Kon et al., 2011). However, the exact impact of this pathway on tumorigenesis most likely depends on the type of tumor and on the specific substrates that are targeted for degradation. Data presented here support a model where CMA could be responsible for the degradation of HIF-1α in nutrient deprived tissues, albeit the low oxygen concentrations these tissues might be enduring. These findings may also provide clues on new molecular targets, including components of CMA, for innovative therapies in a variety of solid tumours that heavily rely on HIF-1α for survival.

References

Agarraberes, F.A., Terlecky, S.R., and Dice, J.F. (1997). An intralysosomal hsp70 is required for a selective pathway of lysosomal protein degradation. J Cell Biol *137*, 825-834.

Aguilar, R.C., and Wendland, B. (2003). Ubiquitin: not just for proteasomes anymore. Curr Opin Cell Biol *15*, 184-190.

Ahlberg, J., and Glaumann, H. (1985). Uptake--microautophagy--and degradation of exogenous proteins by isolated rat liver lysosomes. Effects of pH, ATP, and inhibitors of proteolysis. Exp Mol Pathol *42*, 78-88.

Ahlberg, J., Marzella, L., and Glaumann, H. (1982). Uptake and degradation of proteins by isolated rat liver lysosomes. Suggestion of a microautophagic pathway of proteolysis. Lab Invest *47*, 523-532.

Alberti, S., Bohse, K., Arndt, V., Schmitz, A., and Hohfeld, J. (2004). The cochaperone HspBP1 inhibits the CHIP ubiquitin ligase and stimulates the maturation of the cystic fibrosis transmembrane conductance regulator. Mol Biol Cell *15*, 4003-4010.

Alberti, S., Demand, J., Esser, C., Emmerich, N., Schild, H., and Hohfeld, J. (2002). Ubiquitylation of BAG-1 suggests a novel regulatory mechanism during the sorting of chaperone substrates to the proteasome. J Biol Chem *277*, 45920-45927.

Aniento, F., Roche, E., Cuervo, A.M., and Knecht, E. (1993). Uptake and degradation of glyceraldehyde-3-phosphate dehydrogenase by rat liver lysosomes. J Biol Chem *268*, 10463-10470.

Arany, Z., Huang, L.E., Eckner, R., Bhattacharya, S., Jiang, C., Goldberg, M.A., Bunn, H.F., and Livingston, D.M. (1996). An essential role for p300/CBP in the cellular response to hypoxia. Proc Natl Acad Sci U S A *93*, 12969-12973.

Arndt, V., Daniel, C., Nastainczyk, W., Alberti, S., and Hohfeld, J. (2005). BAG-2 acts as an inhibitor of the chaperone-associated ubiquitin ligase CHIP. Mol Biol Cell *16*, 5891-5900.

Arndt, V., Dick, N., Tawo, R., Dreiseidler, M., Wenzel, D., Hesse, M., Furst, D.O., Saftig, P., Saint, R., Fleischmann, B.K*., et al.* (2010). Chaperone-assisted selective autophagy is essential for muscle maintenance. Curr Biol *20*, 143-148.

Bae, S.H., Jeong, J.W., Park, J.A., Kim, S.H., Bae, M.K., Choi, S.J., and Kim, K.W. (2004). Sumoylation increases HIF-1alpha stability and its transcriptional activity. Biochem Biophys Res Commun *324*, 394-400.

Baek, J.H., Liu, Y.V., McDonald, K.R., Wesley, J.B., Hubbi, M.E., Byun, H., and Semenza, G.L. (2007a). Spermidine/spermine-N1-acetyltransferase 2 is an essential

component of the ubiquitin ligase complex that regulates hypoxia-inducible factor 1alpha. J Biol Chem *282*, 23572-23580.

Baek, J.H., Liu, Y.V., McDonald, K.R., Wesley, J.B., Zhang, H., and Semenza, G.L. (2007b). Spermidine/spermine N(1)-acetyltransferase-1 binds to hypoxia-inducible factor-1alpha (HIF-1alpha) and RACK1 and promotes ubiquitination and degradation of HIF-1alpha. J Biol Chem *282*, 33358-33366.

Ballinger, C.A., Connell, P., Wu, Y., Hu, Z., Thompson, L.J., Yin, L.Y., and Patterson, C. (1999). Identification of CHIP, a novel tetratricopeptide repeat-containing protein that interacts with heat shock proteins and negatively regulates chaperone functions. Mol Cell Biol *19*, 4535-4545.

Bandyopadhyay, U., Kaushik, S., Varticovski, L., and Cuervo, A.M. (2008). The chaperone-mediated autophagy receptor organizes in dynamic protein complexes at the lysosomal membrane. Mol Cell Biol *28*, 5747-5763.

Bandyopadhyay, U., Sridhar, S., Kaushik, S., Kiffin, R., and Cuervo, A.M. (2010). Identification of regulators of chaperone-mediated autophagy. Mol Cell *39*, 535-547.

Bartlett, A.I., and Radford, S.E. (2009). An expanding arsenal of experimental methods yields an explosion of insights into protein folding mechanisms. Nat Struct Mol Biol *16*, 582-588.

Bejarano, E., and Cuervo, A.M. (2010). Chaperone-mediated autophagy. Proc Am Thorac Soc *7*, 29-39.

Ben-Yosef, Y., Lahat, N., Shapiro, S., Bitterman, H., and Miller, A. (2002). Regulation of endothelial matrix metalloproteinase-2 by hypoxia/reoxygenation. Circ Res *90*, 784-791.

Bento, C.F., Fernandes, R., Ramalho, J., Marques, C., Shang, F., Taylor, A., and Pereira, P. (2010). The chaperone-dependent ubiquitin ligase CHIP targets HIF-1alpha for degradation in the presence of methylglyoxal. PLoS One *5*, e15062.

Berra, E., Milanini, J., Richard, D.E., Le Gall, M., Vinals, F., Gothie, E., Roux, D., Pages, G., and Pouyssegur, J. (2000). Signaling angiogenesis via p42/p44 MAP kinase and hypoxia. Biochem Pharmacol *60*, 1171-1178.

Berta, M.A., Mazure, N., Hattab, M., Pouyssegur, J., and Brahimi-Horn, M.C. (2007). SUMOylation of hypoxia-inducible factor-1alpha reduces its transcriptional activity. Biochem Biophys Res Commun *360*, 646-652.

Berthet, J., Berthet, L., Appelmans, F., and De Duve, C. (1951). Tissue fractionation studies. II. The nature of the linkage between acid phosphatase and mitochondria in rat-liver tissue. Biochem J *50*, 182-189.

Bhatia-Kissova, I., and Camougrand, N. Mitophagy in yeast: actors and physiological roles. FEMS Yeast Res *10*, 1023-1034.

Bhatia-Kissova, I., and Camougrand, N. (2010). Mitophagy in yeast: actors and physiological roles. FEMS Yeast Res *10*, 1023-1034.

Bianchi, L., Tacchini, L., and Cairo, G. (1999). HIF-1-mediated activation of transferrin receptor gene transcription by iron chelation. Nucleic Acids Res *27*, 4223-4227.

Bonifacino, J.S., and Rojas, R. (2006). Retrograde transport from endosomes to the trans-Golgi network. Nat Rev Mol Cell Biol *7*, 568-579.

Bowers, K., Piper, S.C., Edeling, M.A., Gray, S.R., Owen, D.J., Lehner, P.J., and Luzio, J.P. (2006). Degradation of endocytosed epidermal growth factor and virally ubiquitinated major histocompatibility complex class I is independent of mammalian ESCRTII. J Biol Chem *281*, 5094-5105.

Bowers, K., and Stevens, T.H. (2005). Protein transport from the late Golgi to the vacuole in the yeast Saccharomyces cerevisiae. Biochim Biophys Acta *1744*, 438-454.

Brinker, A., Scheufler, C., Von Der Mulbe, F., Fleckenstein, B., Herrmann, C., Jung, G., Moarefi, I., and Hartl, F.U. (2002). Ligand discrimination by TPR domains. Relevance and selectivity of EEVD-recognition in Hsp70 x Hop x Hsp90 complexes. J Biol Chem *277*, 19265-19275.

Bruick, R.K. (2000). Expression of the gene encoding the proapoptotic Nip3 protein is induced by hypoxia. Proc Natl Acad Sci U S A *97*, 9082-9087.

Brunelle, J.K., and Chandel, N.S. (2002). Oxygen deprivation induced cell death: an update. Apoptosis *7*, 475-482.

Bunn, H.F., and Poyton, R.O. (1996). Oxygen sensing and molecular adaptation to hypoxia. Physiol Rev *76*, 839-885.

Carbia-Nagashima, A., Gerez, J., Perez-Castro, C., Paez-Pereda, M., Silberstein, S., Stalla, G.K., Holsboer, F., and Arzt, E. (2007). RSUME, a small RWD-containing protein, enhances SUMO conjugation and stabilizes HIF-1alpha during hypoxia. Cell *131*, 309-323.

Carmeliet, P., Dor, Y., Herbert, J.M., Fukumura, D., Brusselmans, K., Dewerchin, M., Neeman, M., Bono, F., Abramovitch, R., Maxwell, P.*, et al.* (1998). Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. Nature *394*, 485-490.

Carrero, P., Okamoto, K., Coumailleau, P., O'Brien, S., Tanaka, H., and Poellinger, L. (2000). Redox-regulated recruitment of the transcriptional coactivators CREB-binding protein and SRC-1 to hypoxia-inducible factor 1alpha. Mol Cell Biol *20*, 402-415.

Cayrol, C., and Ducommun, B. (1998). Interaction with cyclin-dependent kinases and PCNA modulates proteasome-dependent degradation of p21. Oncogene *17*, 2437-2444.

Cecconi, F., and Levine, B. (2008). The role of autophagy in mammalian development: cell makeover rather than cell death. Dev Cell *15*, 344-357.

Chen, C., Pore, N., Behrooz, A., Ismail-Beigi, F., and Maity, A. (2001). Regulation of glut1 mRNA by hypoxia-inducible factor-1. Interaction between H-ras and hypoxia. J Biol Chem *276*, 9519-9525.

Cheng, J., Kang, X., Zhang, S., and Yeh, E.T. (2007). SUMO-specific protease 1 is essential for stabilization of HIF1alpha during hypoxia. Cell *131*, 584-595.

Chiang, H.L., Terlecky, S.R., Plant, C.P., and Dice, J.F. (1989). A role for a 70-kilodalton heat shock protein in lysosomal degradation of intracellular proteins. Science *246*, 382-385.

Ciechanover, A. (2006). Intracellular protein degradation: from a vague idea thru the lysosome and the ubiquitin-proteasome system and onto human diseases and drug targeting. Exp Biol Med (Maywood) *231*, 1197-1211.

Ciechanover, A., Elias, S., Heller, H., and Hershko, A. (1982). "Covalent affinity" purification of ubiquitin-activating enzyme. J Biol Chem *257*, 2537-2542.

Ciechanover, A., Heller, H., Elias, S., Haas, A.L., and Hershko, A. (1980). ATP-dependent conjugation of reticulocyte proteins with the polypeptide required for protein degradation. Proc Natl Acad Sci U S A *77*, 1365-1368.

Ciehanover, A., Hod, Y., and Hershko, A. (1978). A heat-stable polypeptide component of an ATP-dependent proteolytic system from reticulocytes. Biochem Biophys Res Commun *81*, 1100-1105.

Connell, P., Ballinger, C.A., Jiang, J., Wu, Y., Thompson, L.J., Hohfeld, J., and Patterson, C. (2001). The co-chaperone CHIP regulates protein triage decisions mediated by heat-shock proteins. Nat Cell Biol *3*, 93-96.

Conrad, P.W., Freeman, T.L., Beitner-Johnson, D., and Millhorn, D.E. (1999). EPAS1 trans-activation during hypoxia requires p42/p44 MAPK. J Biol Chem *274*, 33709-33713.

Conus, S., and Simon, H.U. (2008). Cathepsins: key modulators of cell death and inflammatory responses. Biochem Pharmacol *76*, 1374-1382.

Conway, E.M., Collen, D., and Carmeliet, P. (2001). Molecular mechanisms of blood vessel growth. Cardiovasc Res *49*, 507-521.

Crews, S.T. (1998). Control of cell lineage-specific development and transcription by bHLH-PAS proteins. Genes Dev *12*, 607-620.

Criollo, A., Vicencio, J.M., Tasdemir, E., Maiuri, M.C., Lavandero, S., and Kroemer, G. (2007). The inositol trisphosphate receptor in the control of autophagy. Autophagy *3*, 350-353.

Cuervo, A.M. (2004a). Autophagy: in sickness and in health. Trends Cell Biol *14*, 70-77.

Cuervo, A.M. (2004b). Autophagy: many paths to the same end. Mol Cell Biochem *263*, 55-72.

Cuervo, A.M. (2010). Chaperone-mediated autophagy: selectivity pays off. Trends Endocrinol Metab *21*, 142-150.

Cuervo, A.M., and Dice, J.F. (1996). A receptor for the selective uptake and degradation of proteins by lysosomes. Science *273*, 501-503.

Cuervo, A.M., and Dice, J.F. (2000). Regulation of lamp2a levels in the lysosomal membrane. Traffic *1*, 570-583.

Cuervo, A.M., Dice, J.F., and Knecht, E. (1997). A population of rat liver lysosomes responsible for the selective uptake and degradation of cytosolic proteins. J Biol Chem *272*, 5606-5615.

Cuervo, A.M., Hu, W., Lim, B., and Dice, J.F. (1998). IkappaB is a substrate for a selective pathway of lysosomal proteolysis. Mol Biol Cell *9*, 1995-2010.

Cuervo, A.M., Knecht, E., Terlecky, S.R., and Dice, J.F. (1995a). Activation of a selective pathway of lysosomal proteolysis in rat liver by prolonged starvation. Am J Physiol *269*, C1200-1208.

Cuervo, A.M., and Macian, F. (2012). Autophagy, nutrition and immunology. Mol Aspects Med *33*, 2-13.

Cuervo, A.M., Palmer, A., Rivett, A.J., and Knecht, E. (1995b). Degradation of proteasomes by lysosomes in rat liver. Eur J Biochem *227*, 792-800.

Cuervo, A.M., Stefanis, L., Fredenburg, R., Lansbury, P.T., and Sulzer, D. (2004). Impaired degradation of mutant alpha-synuclein by chaperone-mediated autophagy. Science *305*, 1292-1295.

Cuervo, A.M., Terlecky, S.R., Dice, J.F., and Knecht, E. (1994). Selective binding and uptake of ribonuclease A and glyceraldehyde-3-phosphate dehydrogenase by isolated rat liver lysosomes. J Biol Chem *269*, 26374-26380.

Cyr, D.M., Hohfeld, J., and Patterson, C. (2002). Protein quality control: U-box-containing E3 ubiquitin ligases join the fold. Trends Biochem Sci *27*, 368-375.

Dang, C.V., and Semenza, G.L. (1999). Oncogenic alterations of metabolism. Trends Biochem Sci *24*, 68-72.

de Duve, C. (2005). The lysosome turns fifty. Nat Cell Biol *7*, 847-849.

De Duve, C., and Wattiaux, R. (1966). Functions of lysosomes. Annu Rev Physiol *28*, 435-492.

Demand, J., Alberti, S., Patterson, C., and Hohfeld, J. (2001). Cooperation of a ubiquitin domain protein and an E3 ubiquitin ligase during chaperone/proteasome coupling. Curr Biol *11*, 1569-1577.

Demand, J., Luders, J., and Hohfeld, J. (1998). The carboxy-terminal domain of Hsc70 provides binding sites for a distinct set of chaperone cofactors. Mol Cell Biol *18*, 2023-2028.

Deretic, V., and Levine, B. (2009). Autophagy, immunity, and microbial adaptations. Cell Host Microbe *5*, 527-549.

Desjardins, M. (1995). Biogenesis of phagolysosomes: the 'kiss and run' hypothesis. Trends Cell Biol *5*, 183-186.

Dice, J.F. (1990). Peptide sequences that target cytosolic proteins for lysosomal proteolysis. Trends Biochem Sci *15*, 305-309.

Dice, J.F. (2007). Chaperone-mediated autophagy. Autophagy *3*, 295-299.

Dice, J.F., Chiang, H.L., Spencer, E.P., and Backer, J.M. (1986). Regulation of catabolism of microinjected ribonuclease A. Identification of residues 7-11 as the essential pentapeptide. J Biol Chem *261*, 6853-6859.

Dickson, D.W., and Yen, S.H. (1989). Beta-amyloid deposition and paired helical filament formation: which histopathological feature is more significant in Alzheimer's disease? Neurobiol Aging *10*, 402-404; discussion 412-404.

Dobson, C.M., and Ellis, R.J. (1998). Protein folding and misfolding inside and outside the cell. EMBO J *17*, 5251-5254.

Dronamraju, K.R. (1999). Erwin Schrodinger and the origins of molecular biology. Genetics *153*, 1071-1076.

Dunker, A.K., Silman, I., Uversky, V.N., and Sussman, J.L. (2008). Function and structure of inherently disordered proteins. Curr Opin Struct Biol *18*, 756-764.

Dunn, K.W., and Maxfield, F.R. (1992). Delivery of ligands from sorting endosomes to late endosomes occurs by maturation of sorting endosomes. J Cell Biol *117*, 301-310.

Dunn, W.A., Jr., Cregg, J.M., Kiel, J.A., van der Klei, I.J., Oku, M., Sakai, Y., Sibirny, A.A., Stasyk, O.V., and Veenhuis, M. (2005). Pexophagy: the selective autophagy of peroxisomes. Autophagy *1*, 75-83.

Ebert, B.L., and Bunn, H.F. (1998). Regulation of transcription by hypoxia requires a multiprotein complex that includes hypoxia-inducible factor 1, an adjacent transcription factor, and p300/CREB binding protein. Mol Cell Biol *18*, 4089-4096.

Ebert, B.L., Firth, J.D., and Ratcliffe, P.J. (1995). Hypoxia and mitochondrial inhibitors regulate expression of glucose transporter-1 via distinct Cis-acting sequences. J Biol Chem *270*, 29083-29089.

Eckhart, A.D., Yang, N., Xin, X., and Faber, J.E. (1997). Characterization of the alpha1B-adrenergic receptor gene promoter region and hypoxia regulatory elements in vascular smooth muscle. Proc Natl Acad Sci U S A *94*, 9487-9492.

Elsasser, S., and Finley, D. (2005). Delivery of ubiquitinated substrates to protein-unfolding machines. Nat Cell Biol *7*, 742-749.

Ema, M., Hirota, K., Mimura, J., Abe, H., Yodoi, J., Sogawa, K., Poellinger, L., and Fujii-Kuriyama, Y. (1999). Molecular mechanisms of transcription activation by HLF and HIF1alpha in response to hypoxia: their stabilization and redox signal-induced interaction with CBP/p300. EMBO J *18*, 1905-1914.

Ema, M., Taya, S., Yokotani, N., Sogawa, K., Matsuda, Y., and Fujii-Kuriyama, Y. (1997). A novel bHLH-PAS factor with close sequence similarity to hypoxia-inducible factor 1alpha regulates the VEGF expression and is potentially involved in lung and vascular development. Proc Natl Acad Sci U S A *94*, 4273-4278.

Etlinger, J.D., and Goldberg, A.L. (1977). A soluble ATP-dependent proteolytic system responsible for the degradation of abnormal proteins in reticulocytes. Proc Natl Acad Sci U S A *74*, 54-58.

Eytan, E., Armon, T., Heller, H., Beck, S., and Hershko, A. (1993). Ubiquitin C-terminal hydrolase activity associated with the 26 S protease complex. J Biol Chem *268*, 4668-4674.

Fader, C.M., and Colombo, M.I. (2009). Autophagy and multivesicular bodies: two closely related partners. Cell Death Differ *16*, 70-78.

Farre, J.C., Krick, R., Subramani, S., and Thumm, M. (2009). Turnover of organelles by autophagy in yeast. Curr Opin Cell Biol *21*, 522-530.

Farre, J.C., Manjithaya, R., Mathewson, R.D., and Subramani, S. (2008). PpAtg30 tags peroxisomes for turnover by selective autophagy. Dev Cell *14*, 365-376.

Farre, J.C., and Subramani, S. (2004). Peroxisome turnover by micropexophagy: an autophagy-related process. Trends Cell Biol *14*, 515-523.

Feldser, D., Agani, F., Iyer, N.V., Pak, B., Ferreira, G., and Semenza, G.L. (1999). Reciprocal positive regulation of hypoxia-inducible factor 1alpha and insulin-like growth factor 2. Cancer Res *59*, 3915-3918.

Finn, P.F., and Dice, J.F. (2005). Ketone bodies stimulate chaperone-mediated autophagy. J Biol Chem *280*, 25864-25870.

Flamme, I., Frohlich, T., von Reutern, M., Kappel, A., Damert, A., and Risau, W. (1997). HRF, a putative basic helix-loop-helix-PAS-domain transcription factor is closely related to hypoxia-inducible factor-1 alpha and developmentally expressed in blood vessels. Mech Dev *63*, 51-60.

Forsythe, J.A., Jiang, B.H., Iyer, N.V., Agani, F., Leung, S.W., Koos, R.D., and Semenza, G.L. (1996). Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol *16*, 4604-4613.

Frank, D., Kuhn, C., Katus, H.A., and Frey, N. (2006). The sarcomeric Z-disc: a nodal point in signalling and disease. J Mol Med (Berl) *84*, 446-468.

Fujioka, Y., Noda, N.N., Nakatogawa, H., Ohsumi, Y., and Inagaki, F. Dimeric coiled-coil structure of Saccharomyces cerevisiae Atg16 and its functional significance in autophagy. J Biol Chem *285*, 1508-1515.

Fujioka, Y., Noda, N.N., Nakatogawa, H., Ohsumi, Y., and Inagaki, F. (2010). Dimeric coiled-coil structure of Saccharomyces cerevisiae Atg16 and its functional significance in autophagy. J Biol Chem *285*, 1508-1515.

Fujita, N., Itoh, T., Omori, H., Fukuda, M., Noda, T., and Yoshimori, T. (2008). The Atg16L complex specifies the site of LC3 lipidation for membrane biogenesis in autophagy. Mol Biol Cell *19*, 2092-2100.

Futter, C.E., Pearse, A., Hewlett, L.J., and Hopkins, C.R. (1996). Multivesicular endosomes containing internalized EGF-EGF receptor complexes mature and then fuse directly with lysosomes. J Cell Biol *132*, 1011-1023.

Geuze, H.J., Stoorvogel, W., Strous, G.J., Slot, J.W., Bleekemolen, J.E., and Mellman, I. (1988). Sorting of mannose 6-phosphate receptors and lysosomal membrane proteins in endocytic vesicles. J Cell Biol *107*, 2491-2501.

Giordano, F.J., and Johnson, R.S. (2001). Angiogenesis: the role of the microenvironment in flipping the switch. Curr Opin Genet Dev *11*, 35-40.

Glickman, M.H. (2000). Getting in and out of the proteasome. Semin Cell Dev Biol *11*, 149-158.

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

Goldberg, A.L. (2003). Protein degradation and protection against misfolded or damaged proteins. Nature *426*, 895-899.

Goldberg, A.L., and Dice, J.F. (1974). Intracellular protein degradation in mammalian and bacterial cells. Annu Rev Biochem *43*, 835-869.

Goldberg, A.L., and St John, A.C. (1976). Intracellular protein degradation in mammalian and bacterial cells: Part 2. Annu Rev Biochem *45*, 747-803.

Goldberg, M.A., Dunning, S.P., and Bunn, H.F. (1988). Regulation of the erythropoietin gene: evidence that the oxygen sensor is a heme protein. Science *242*, 1412-1415.

Goldknopf, I.L., and Busch, H. (1975). Remarkable similarities of peptide fingerprints of histone 2A and nonhistone chromosomal protein A24. Biochem Biophys Res Commun *65*, 951-960.

Goldknopf, I.L., and Busch, H. (1977). Isopeptide linkage between nonhistone and histone 2A polypeptides of chromosomal conjugate-protein A24. Proc Natl Acad Sci U S A *74*, 864-868.

Gorlach, A., Diebold, I., Schini-Kerth, V.B., Berchner-Pfannschmidt, U., Roth, U., Brandes, R.P., Kietzmann, T., and Busse, R. (2001). Thrombin activates the hypoxia-inducible factor-1 signaling pathway in vascular smooth muscle cells: Role of the p22(phox)-containing NADPH oxidase. Circ Res *89*, 47-54.

Gough, N.R., Hatem, C.L., and Fambrough, D.M. (1995). The family of LAMP-2 proteins arises by alternative splicing from a single gene: characterization of the avian LAMP-2 gene and identification of mammalian homologs of LAMP-2b and LAMP-2c. DNA Cell Biol *14*, 863-867.

Gradin, K., McGuire, J., Wenger, R.H., Kvietikova, I., fhitelaw, M.L., Toftgard, R., Tora, L., Gassmann, M., and Poellinger, L. (1996). Functional interference between hypoxia and dioxin signal transduction pathways: competition for recruitment of the Arnt transcription factor. Mol Cell Biol *16*, 5221-5231.

Griffiths, G., Hoflack, B., Simons, K., Mellman, I., and Kornfeld, S. (1988). The mannose 6-phosphate receptor and the biogenesis of lysosomes. Cell *52*, 329-341.

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

Gruenberg, J., and Stenmark, H. (2004). The biogenesis of multivesicular endosomes. Nat Rev Mol Cell Biol *5*, 317-323.

Gu, Y.Z., Moran, S.M., Hogenesch, J.B., Wartman, L., and Bradfield, C.A. (1998). Molecular characterization and chromosomal localization of a third alpha-class hypoxia inducible factor subunit, HIF3alpha. Gene Expr *7*, 205-213.

Hagen, T., Taylor, C.T., Lam, F., and Moncada, S. (2003). Redistribution of intracellular oxygen in hypoxia by nitric oxide: effect on HIF1alpha. Science *302*, 1975-1978.

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

Hailey, D.W., Rambold, A.S., Satpute-Krishnan, P., Mitra, K., Sougrat, R., Kim, P.K., and Lippincott-Schwartz, J. Mitochondria supply membranes for autophagosome biogenesis during starvation. Cell *141*, 656-667.

Hailey, D.W., Rambold, A.S., Satpute-Krishnan, P., Mitra, K., Sougrat, R., Kim, P.K., and Lippincott-Schwartz, J. (2010). Mitochondria supply membranes for autophagosome biogenesis during starvation. Cell *141*, 656-667.

Haq, I.U., Ali, S., and Qadeer, M.A. (2005). Influence of dissolved oxygen concentration on intracellular pH for regulation of Aspergillus niger growth rate during citric acid fermentation in a stirred tank bioreactor. Int J Biol Sci *1*, 34-41.

Harris, A.L. (2002). Hypoxia--a key regulatory factor in tumour growth. Nat Rev Cancer *2*, 38-47.

Hartl, F.U. (1996). Molecular chaperones in cellular protein folding. Nature *381*, 571-579.

Hartl, F.U., Bracher, A., and Hayer-Hartl, M. (2011). Molecular chaperones in protein folding and proteostasis. Nature *475*, 324-332.

Hartl, F.U., and Hayer-Hartl, M. (2009). Converging concepts of protein folding in vitro and in vivo. Nat Struct Mol Biol *16*, 574-581.

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

He, B., Bai, S., Hnat, A.T., Kalman, R.I., Minges, J.T., Patterson, C., and Wilson, E.M. (2004). An androgen receptor NH2-terminal conserved motif interacts with the COOH terminus of the Hsp70-interacting protein (CHIP). J Biol Chem *279*, 30643-30653.

Hellwig-Burgel, T., Rutkowski, K., Metzen, E., Fandrey, J., and Jelkmann, W. (1999). Interleukin-1beta and tumor necrosis factor-alpha stimulate DNA binding of hypoxia-inducible factor-1. Blood *94*, 1561-1567.

Hellwig-Burgel, T., Stiehl, D.P., Wagner, A.E., Metzen, E., and Jelkmann, W. (2005). Review: hypoxia-inducible factor-1 (HIF-1): a novel transcription factor in immune reactions. J Interferon Cytokine Res *25*, 297-310.

Hershko, A., Ciechanover, A., Heller, H., Haas, A.L., and Rose, I.A. (1980). Proposed role of ATP in protein breakdown: conjugation of protein with multiple chains of the polypeptide of ATP-dependent proteolysis. Proc Natl Acad Sci U S A *77*, 1783-1786.

Hershko, A., Heller, H., Elias, S., and Ciechanover, A. (1983). Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown. J Biol Chem *258*, 8206-8214.

Hewitson, K.S., McNeill, L.A., Riordan, M.V., Tian, Y.M., Bullock, A.N., Welford, R.W., Elkins, J.M., Oldham, N.J., Bhattacharya, S., Gleadle, J.M*., et al.* (2002). Hypoxia-inducible factor (HIF) asparagine hydroxylase is identical to factor inhibiting HIF (FIH) and is related to the cupin structural family. J Biol Chem *277*, 26351-26355.

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

Hofer, T., Desbaillets, I., Hopfl, G., Gassmann, M., and Wenger, R.H. (2001). Dissecting hypoxia-dependent and hypoxia-independent steps in the HIF-1alpha activation cascade: implications for HIF-1alpha gene therapy. FASEB J *15*, 2715-2717.

Hogenesch, J.B., Chan, W.K., Jackiw, V.H., Brown, R.C., Gu, Y.Z., Pray-Grant, M., Perdew, G.H., and Bradfield, C.A. (1997). Characterization of a subset of the basic-helix-loop-helix-PAS superfamily that interacts with components of the dioxin signaling pathway. J Biol Chem *272*, 8581-8593.

Hohfeld, J., Cyr, D.M., and Patterson, C. (2001). From the cradle to the grave: molecular chaperones that may choose between folding and degradation. EMBO Rep *2*, 885-890.

Hohfeld, J., and Jentsch, S. (1997). GrpE-like regulation of the hsc70 chaperone by the anti-apoptotic protein BAG-1. EMBO J *16*, 6209-6216.

Hohfeld, J., Minami, Y., and Hartl, F.U. (1995). Hip, a novel cochaperone involved in the eukaryotic Hsc70/Hsp40 reaction cycle. Cell *83*, 589-598.

Hough, R., Pratt, G., and Rechsteiner, M. (1986). Ubiquitin-lysozyme conjugates. Identification and characterization of an ATP-dependent protease from rabbit reticulocyte lysates. J Biol Chem *261*, 2400-2408.

Hough, R., and Rechsteiner, M. (1986). Ubiquitin-lysozyme conjugates. Purification and susceptibility to proteolysis. J Biol Chem *261*, 2391-2399.

Hu, C.J., Iyer, S., Sataur, A., Covello, K.L., Chodosh, L.A., and Simon, M.C. (2006). Differential regulation of the transcriptional activities of hypoxia-inducible factor 1 alpha (HIF-1alpha) and HIF-2alpha in stem cells. Mol Cell Biol *26*, 3514-3526.

Hu, J., Discher, D.J., Bishopric, N.H., and Webster, K.A. (1998). Hypoxia regulates expression of the endothelin-1 gene through a proximal hypoxia-inducible factor-1 binding site on the antisense strand. Biochem Biophys Res Commun *245*, 894-899.

Huang, F., Kirkpatrick, D., Jiang, X., Gygi, S., and Sorkin, A. (2006). Differential regulation of EGF receptor internalization and degradation by multiubiquitination within the kinase domain. Mol Cell *21*, 737-748.

Huang, L.E., Gu, J., Schau, M., and Bunn, H.F. (1998). Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. Proc Natl Acad Sci U S A *95*, 7987-7992.

Hunt, L.T., and Dayhoff, M.O. (1977). Amino-terminal sequence identity of ubiquitin and the nonhistone component of nuclear protein A24. Biochem Biophys Res Commun *74*, 650-655.

Hur, E., Chang, K.Y., Lee, E., Lee, S.K., and Park, H. (2001). Mitogen-activated protein kinase kinase inhibitor PD98059 blocks the trans-activation but not the stabilization or DNA binding ability of hypoxia-inducible factor-1alpha. Mol Pharmacol *59*, 1216-1224.

Hurley, J.H., and Emr, S.D. (2006). The ESCRT complexes: structure and mechanism of a membrane-trafficking network. Annu Rev Biophys Biomol Struct *35*, 277-298.

Husnjak, K., Elsasser, S., Zhang, N., Chen, X., Randles, L., Shi, Y., Hofmann, K., Walters, K.J., Finley, D., and Dikic, I. (2008). Proteasome subunit Rpn13 is a novel ubiquitin receptor. Nature *453*, 481-488.

Ichimura, Y., Kirisako, T., Takao, T., Satomi, Y., Shimonishi, Y., Ishihara, N., Mizushima, N., Tanida, I., Kominami, E., Ohsumi, M.*, et al.* (2000). A ubiquitin-like system mediates protein lipidation. Nature *408*, 488-492.

Im, E., Venkatakrishnan, A., and Kazlauskas, A. (2005). Cathepsin B regulates the intrinsic angiogenic threshold of endothelial cells. Mol Biol Cell *16*, 3488-3500.

Isaacs, J.S., Jung, Y.J., Mimnaugh, E.G., Martinez, A., Cuttitta, F., and Neckers, L.M. (2002). Hsp90 regulates a von Hippel Lindau-independent hypoxia-inducible factor-1 alpha-degradative pathway. J Biol Chem *277*, 29936-29944.

Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J.M., Lane, W.S., and Kaelin, W.G., Jr. (2001). HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. Science *292*, 464-468.

Jaakkola, P., Mole, D.R., Tian, Y.M., Wilson, M.I., Gielbert, J., Gaskell, S.J., Kriegsheim, A., Hebestreit, H.F., Mukherji, M., Schofield, C.J*., et al.* (2001). Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science *292*, 468-472.

Jackson, P.K., Eldridge, A.G., Freed, E., Furstenthal, L., Hsu, J.Y., Kaiser, B.K., and Reimann, J.D. (2000). The lore of the RINGs: substrate recognition and catalysis by ubiquitin ligases. Trends Cell Biol *10*, 429-439.

Jahraus, A., Tjelle, T.E., Berg, T., Habermann, A., Storrie, B., Ullrich, O., and Griffiths, G. (1998). In vitro fusion of phagosomes with different endocytic organelles from J774 macrophages. J Biol Chem *273*, 30379-30390.

Jain, R.K. (2005). Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science *307*, 58-62.

Jentsch, S., and Pyrowolakis, G. (2000). Ubiquitin and its kin: how close are the family ties? Trends Cell Biol *10*, 335-342.

Jiang, J., Ballinger, C.A., Wu, Y., Dai, Q., Cyr, D.M., Hohfeld, J., and Patterson, C. (2001). CHIP is a U-box-dependent E3 ubiquitin ligase: identification of Hsc70 as a target for ubiquitylation. J Biol Chem *276*, 42938-42944.

Joazeiro, C.A., and Weissman, A.M. (2000). RING finger proteins: mediators of ubiquitin ligase activity. Cell *102*, 549-552.

Johansen, T., and Lamark, T. (2011). Selective autophagy mediated by autophagic adapter proteins. Autophagy *7*, 279-296.

Josko, J., Gwozdz, B., Jedrzejowska-Szypulka, H., and Hendryk, S. (2000). Vascular endothelial growth factor (VEGF) and its effect on angiogenesis. Med Sci Monit *6*, 1047-1052.

Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y., and Yoshimori, T. (2000). LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J *19*, 5720-5728.

Kabeya, Y., Mizushima, N., Yamamoto, A., Oshitani-Okamoto, S., Ohsumi, Y., and Yoshimori, T. (2004). LC3, GABARAP and GATE16 localize to autophagosomal membrane depending on form-II formation. J Cell Sci *117*, 2805-2812.

Kabuta, T., Furuta, A., Aoki, S., Furuta, K., and Wada, K. (2008). Aberrant interaction between Parkinson disease-associated mutant UCH-L1 and the lysosomal receptor for chaperone-mediated autophagy. J Biol Chem *283*, 23731-23738.

Kallio, P.J., Pongratz, I., Gradin, K., McGuire, J., and Poellinger, L. (1997). Activation of hypoxia-inducible factor 1alpha: posttranscriptional regulation and conformational

change by recruitment of the Arnt transcription factor. Proc Natl Acad Sci U S A *94*, 5667-5672.

Kampinga, H.H., and Craig, E.A. (2010). The HSP70 chaperone machinery: J proteins as drivers of functional specificity. Nat Rev Mol Cell Biol *11*, 579-592.

Kamura, T., Sato, S., Iwai, K., Czyzyk-Krzeska, M., Conaway, R.C., and Conaway, J.W. (2000). Activation of HIF1alpha ubiquitination by a reconstituted von Hippel-Lindau (VHL) tumor suppressor complex. Proc Natl Acad Sci U S A *97*, 10430-10435.

Kang, M.J., Kim, H.J., Kim, H.K., Lee, J.Y., Kim, D.H., Jung, K.J., Kim, K.W., Baik, H.S., Yoo, M.A., Yu, B.P.*, et al.* (2005). The effect of age and calorie restriction on HIF-1-responsive genes in aged liver. Biogerontology *6*, 27-37.

Kaushik, S., Massey, A.C., and Cuervo, A.M. (2006). Lysosome membrane lipid microdomains: novel regulators of chaperone-mediated autophagy. EMBO J *25*, 3921-3933.

Kaushik, S., Massey, A.C., Mizushima, N., and Cuervo, A.M. (2008). Constitutive activation of chaperone-mediated autophagy in cells with impaired macroautophagy. Mol Biol Cell *19*, 2179-2192.

Ke, Q., and Costa, M. (2006). Hypoxia-inducible factor-1 (HIF-1). Mol Pharmacol *70*, 1469-1480.

Kelly, B.D., Hackett, S.F., Hirota, K., Oshima, Y., Cai, Z., Berg-Dixon, S., Rowan, A., Yan, Z., Campochiaro, P.A., and Semenza, G.L. (2003). Cell type-specific regulation of angiogenic growth factor gene expression and induction of angiogenesis in nonischemic tissue by a constitutively active form of hypoxia-inducible factor 1. Circ Res *93*, 1074-1081.

Kiel, J.A. Autophagy in unicellular eukaryotes. Philos Trans R Soc Lond B Biol Sci *365*, 819-830.

Kietzmann, T., Roth, U., and Jungermann, K. (1999). Induction of the plasminogen activator inhibitor-1 gene expression by mild hypoxia via a hypoxia response element binding the hypoxia-inducible factor-1 in rat hepatocytes. Blood *94*, 4177-4185.

Kiffin, R., Christian, C., Knecht, E., and Cuervo, A.M. (2004). Activation of chaperone-mediated autophagy during oxidative stress. Mol Biol Cell *15*, 4829-4840.

Kirisako, T., Baba, M., Ishihara, N., Miyazawa, K., Ohsumi, M., Yoshimori, T., Noda, T., and Ohsumi, Y. (1999). Formation process of autophagosome is traced with Apg8/Aut7p in yeast. J Cell Biol *147*, 435-446.

Kirisako, T., Ichimura, Y., Okada, H., Kabeya, Y., Mizushima, N., Yoshimori, T., Ohsumi, M., Takao, T., Noda, T., and Ohsumi, Y. (2000). The reversible modification

regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway. J Cell Biol *151*, 263-276.

Kirkin, V., Lamark, T., Sou, Y.S., Bjorkoy, G., Nunn, J.L., Bruun, J.A., Shvets, E., McEwan, D.G., Clausen, T.H., Wild, P.*, et al.* (2009). A role for NBR1 in autophagosomal degradation of ubiquitinated substrates. Mol Cell *33*, 505-516.

Kissova, I., Salin, B., Schaeffer, J., Bhatia, S., Manon, S., and Camougrand, N. (2007). Selective and non-selective autophagic degradation of mitochondria in yeast. Autophagy *3*, 329-336.

Kline, D.D., Peng, Y.J., Manalo, D.J., Semenza, G.L., and Prabhakar, N.R. (2002). Defective carotid body function and impaired ventilatory responses to chronic hypoxia in mice partially deficient for hypoxia-inducible factor 1 alpha. Proc Natl Acad Sci U S A *99*, 821-826.

Klionsky, D.J., Cuervo, A.M., Dunn, W.A., Jr., Levine, B., van der Klei, I., and Seglen, P.O. (2007). How shall I eat thee? Autophagy *3*, 413-416.

Klumperman, J., Hille, A., Veenendaal, T., Oorschot, V., Stoorvogel, W., von Figura, K., and Geuze, H.J. (1993). Differences in the endosomal distributions of the two mannose 6-phosphate receptors. J Cell Biol *121*, 997-1010.

Knowles, S.E., and Ballard, F.J. (1976). Selective control of the degradation of normal and aberrant proteins in Reuber H35 hepatoma cells. Biochem J *156*, 609-617.

Kojima, H., Gu, H., Nomura, S., Caldwell, C.C., Kobata, T., Carmeliet, P., Semenza, G.L., and Sitkovsky, M.V. (2002). Abnormal B lymphocyte development and autoimmunity in hypoxia-inducible factor 1alpha -deficient chimeric mice. Proc Natl Acad Sci U S A *99*, 2170-2174.

Komatsu, M., Waguri, S., Koike, M., Sou, Y.S., Ueno, T., Hara, T., Mizushima, N., Iwata, J., Ezaki, J., Murata, S.*, et al.* (2007). Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice. Cell *131*, 1149-1163.

Kon, M., Kiffin, R., Koga, H., Chapochnick, J., Macian, F., Varticovski, L., and Cuervo, A.M. (2011). Chaperone-mediated autophagy is required for tumor growth. Sci Transl Med *3*, 109ra117.

Kostelansky, M.S., Schluter, C., Tam, Y.Y., Lee, S., Ghirlando, R., Beach, B., Conibear, E., and Hurley, J.H. (2007). Molecular architecture and functional model of the complete yeast ESCRT-I heterotetramer. Cell *129*, 485-498.

Kraft, C., Peter, M., and Hofmann, K. (2010). Selective autophagy: ubiquitin-mediated recognition and beyond. Nat Cell Biol *12*, 836-841.

Krick, R., Muehe, Y., Prick, T., Bremer, S., Schlotterhose, P., Eskelinen, E.L., Millen, J., Goldfarb, D.S., and Thumm, M. (2008). Piecemeal microautophagy of the nucleus requires the core macroautophagy genes. Mol Biol Cell *19*, 4492-4505.

Krick, R., Muhe, Y., Prick, T., Bredschneider, M., Bremer, S., Wenzel, D., Eskelinen, E.L., and Thumm, M. (2009). Piecemeal microautophagy of the nucleus: genetic and morphological traits. Autophagy *5*, 270-272.

Krishnamachary, B., Berg-Dixon, S., Kelly, B., Agani, F., Feldser, D., Ferreira, G., Iyer, N., LaRusch, J., Pak, B., Taghavi, P*., et al.* (2003). Regulation of colon carcinoma cell invasion by hypoxia-inducible factor 1. Cancer Res *63*, 1138-1143.

Kunz, J.B., Schwarz, H., and Mayer, A. (2004). Determination of four sequential stages during microautophagy in vitro. J Biol Chem *279*, 9987-9996.

Kvam, E., and Goldfarb, D.S. (2007). Nucleus-vacuole junctions and piecemeal microautophagy of the nucleus in S. cerevisiae. Autophagy *3*, 85-92.

Laffel, L. (1999). Ketone bodies: a review of physiology, pathophysiology and application of monitoring to diabetes. Diabetes Metab Res Rev *15*, 412-426.

Lam, Y.A., DeMartino, G.N., Pickart, C.M., and Cohen, R.E. (1997a). Specificity of the ubiquitin isopeptidase in the PA700 regulatory complex of 26 S proteasomes. J Biol Chem *272*, 28438-28446.

Lam, Y.A., Xu, W., DeMartino, G.N., and Cohen, R.E. (1997b). Editing of ubiquitin conjugates by an isopeptidase in the 26S proteasome. Nature *385*, 737-740.

Lando, D., Peet, D.J., Gorman, J.J., Whelan, D.A., Whitelaw, M.L., and Bruick, R.K. (2002a). FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. Genes Dev *16*, 1466-1471.

Lando, D., Peet, D.J., Whelan, D.A., Gorman, J.J., and Whitelaw, M.L. (2002b). Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. Science *295*, 858-861.

Laroia, G., Cuesta, R., Brewer, G., and Schneider, R.J. (1999). Control of mRNA decay by heat shock-ubiquitin-proteasome pathway. Science *284*, 499-502.

Layfield, R., Franklin, K., Landon, M., Walker, G., Wang, P., Ramage, R., Brown, A., Love, S., Urquhart, K., Muir, T*., et al.* (1999). Chemically synthesized ubiquitin extension proteins detect distinct catalytic capacities of deubiquitinating enzymes. Anal Biochem *274*, 40-49.

Lee, P.J., Jiang, B.H., Chin, B.Y., Iyer, N.V., Alam, J., Semenza, G.L., and Choi, A.M. (1997). Hypoxia-inducible factor-1 mediates transcriptional activation of the heme oxygenase-1 gene in response to hypoxia. J Biol Chem *272*, 5375-5381.

Levine, B., and Klionsky, D.J. (2004). Development by self-digestion: molecular mechanisms and biological functions of autophagy. Dev Cell *6*, 463-477.

Levine, B., and Kroemer, G. (2008). Autophagy in the pathogenesis of disease. Cell *132*, 27-42.

Levy, A.P., Levy, N.S., Wegner, S., and Goldberg, M.A. (1995). Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia. J Biol Chem *270*, 13333-13340.

Li, J., Davidson, G., Huang, Y., Jiang, B.H., Shi, X., Costa, M., and Huang, C. (2004). Nickel compounds act through phosphatidylinositol-3-kinase/Akt-dependent, p70(S6k)-independent pathway to induce hypoxia inducible factor transactivation and Cap43 expression in mouse epidermal Cl41 cells. Cancer Res *64*, 94-101.

Li, Z., Na, X., Wang, D., Schoen, S.R., Messing, E.M., and Wu, G. (2002a). Ubiquitination of a novel deubiquitinating enzyme requires direct binding to von Hippel-Lindau tumor suppressor protein. J Biol Chem *277*, 4656-4662.

Li, Z., Wang, D., Messing, E.M., and Wu, G. (2005). VHL protein-interacting deubiquitinating enzyme 2 deubiquitinates and stabilizes HIF-1alpha. EMBO Rep *6*, 373-378.

Li, Z., Wang, D., Na, X., Schoen, S.R., Messing, E.M., and Wu, G. (2002b). Identification of a deubiquitinating enzyme subfamily as substrates of the von Hippel-Lindau tumor suppressor. Biochem Biophys Res Commun *294*, 700-709.

Liakopoulos, D., Doenges, G., Matuschewski, K., and Jentsch, S. (1998). A novel protein modification pathway related to the ubiquitin system. EMBO J *17*, 2208-2214.

Liu, Y.V., Baek, J.H., Zhang, H., Diez, R., Cole, R.N., and Semenza, G.L. (2007). RACK1 competes with HSP90 for binding to HIF-1alpha and is required for O(2)-independent and HSP90 inhibitor-induced degradation of HIF-1alpha. Mol Cell *25*, 207-217.

Lok, C.N., and Ponka, P. (1999). Identification of a hypoxia response element in the transferrin receptor gene. J Biol Chem *274*, 24147-24152.

Long, J., Gallagher, T.R., Cavey, J.R., Sheppard, P.W., Ralston, S.H., Layfield, R., and Searle, M.S. (2008). Ubiquitin recognition by the ubiquitin-associated domain of p62 involves a novel conformational switch. J Biol Chem *283*, 5427-5440.

Lorick, K.L., Jensen, J.P., Fang, S., Ong, A.M., Hatakeyama, S., and Weissman, A.M. (1999). RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination. Proc Natl Acad Sci U S A *96*, 11364-11369.

Lowe, J., Stock, D., Jap, B., Zwickl, P., Baumeister, W., and Huber, R. (1995). Crystal structure of the 20S proteasome from the archaeon T. acidophilum at 3.4 A resolution. Science *268*, 533-539.

Lowell, B.B., and Goodman, M.N. (1987). Protein sparing in skeletal muscle during prolonged starvation. Dependence on lipid fuel availability. Diabetes *36*, 14-19.

Lu, H., Forbes, R.A., and Verma, A. (2002). Hypoxia-inducible factor 1 activation by aerobic glycolysis implicates the Warburg effect in carcinogenesis. J Biol Chem *277*, 23111-23115.

Lubke, T., Lobel, P., and Sleat, D.E. (2009). Proteomics of the lysosome. Biochim Biophys Acta *1793*, 625-635.

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

Luders, J., Demand, J., Papp, O., and Hohfeld, J. (2000b). Distinct isoforms of the cofactor BAG-1 differentially affect Hsc70 chaperone function. J Biol Chem *275*, 14817-14823.

Luo, W., Zhong, J., Chang, R., Hu, H., Pandey, A., and Semenza, G.L. (2010). Hsp70 and CHIP selectively mediate ubiquitination and degradation of hypoxia-inducible factor (HIF)-1alpha but Not HIF-2alpha. J Biol Chem *285*, 3651-3663.

Luzio, J.P., Pryor, P.R., and Bright, N.A. (2007). Lysosomes: fusion and function. Nat Rev Mol Cell Biol *8*, 622-632.

Lv, L., Li, D., Zhao, D., Lin, R., Chu, Y., Zhang, H., Zha, Z., Liu, Y., Li, Z., Xu, Y*., et al.* (2011). Acetylation targets the M2 isoform of pyruvate kinase for degradation through chaperone-mediated autophagy and promotes tumor growth. Mol Cell *42*, 719-730.

Mahon, P.C., Hirota, K., and Semenza, G.L. (2001). FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. Genes Dev *15*, 2675-2686.

Maiuri, M.C., Zalckvar, E., Kimchi, A., and Kroemer, G. (2007). Self-eating and self-killing: crosstalk between autophagy and apoptosis. Nat Rev Mol Cell Biol *8*, 741-752.

Makino, Y., Cao, R., Svensson, K., Bertilsson, G., Asman, M., Tanaka, H., Cao, Y., Berkenstam, A., and Poellinger, L. (2001). Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression. Nature *414*, 550-554.

Makino, Y., Kanopka, A., Wilson, W.J., Tanaka, H., and Poellinger, L. (2002). Inhibitory PAS domain protein (IPAS) is a hypoxia-inducible splicing variant of the hypoxia-inducible factor-3alpha locus. J Biol Chem *277*, 32405-32408.

Manalo, D.J., Rowan, A., Lavoie, T., Natarajan, L., Kelly, B.D., Ye, S.Q., Garcia, J.G., and Semenza, G.L. (2005). Transcriptional regulation of vascular endothelial cell responses to hypoxia by HIF-1. Blood *105*, 659-669.

Mandelstam, J. (1958). Turnover of protein in growing and non-growing populations of Escherichia coli. Biochem J *69*, 110-119.

Manjithaya, R., Nazarko, T.Y., Farre, J.C., and Subramani, S. Molecular mechanism and physiological role of pexophagy. FEBS Lett *584*, 1367-1373.

Manjithaya, R., Nazarko, T.Y., Farre, J.C., and Subramani, S. (2010). Molecular mechanism and physiological role of pexophagy. FEBS Lett *584*, 1367-1373.

Massey, A., Kiffin, R., and Cuervo, A.M. (2004). Pathophysiology of chaperone-mediated autophagy. Int J Biochem Cell Biol *36*, 2420-2434.

Massey, A.C., Follenzi, A., Kiffin, R., Zhang, C., and Cuervo, A.M. (2008). Early cellular changes after blockage of chaperone-mediated autophagy. Autophagy *4*, 442-456.

Massey, A.C., Zhang, C., and Cuervo, A.M. (2006). Chaperone-mediated autophagy in aging and disease. Curr Top Dev Biol *73*, 205-235.

Maxwell, P.H., Wiesener, M.S., Chang, G.W., Clifford, S.C., Vaux, E.C., Cockman, M.E., Wykoff, C.C., Pugh, C.W., Maher, E.R., and Ratcliffe, P.J. (1999). The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature *399*, 271-275.

Mayer, M.P. (2010). Gymnastics of molecular chaperones. Mol Cell *39*, 321-331.

Mayorga, L.S., Bertini, F., and Stahl, P.D. (1991). Fusion of newly formed phagosomes with endosomes in intact cells and in a cell-free system. J Biol Chem *266*, 6511-6517.

McClellan, A.J., Xia, Y., Deutschbauer, A.M., Davis, R.W., Gerstein, M., and Frydman, J. (2007). Diverse cellular functions of the Hsp90 molecular chaperone uncovered using systems approaches. Cell *131*, 121-135.

McClintock, D.S., Santore, M.T., Lee, V.Y., Brunelle, J., Budinger, G.R., Zong, W.X., Thompson, C.B., Hay, N., and Chandel, N.S. (2002). Bcl-2 family members and functional electron transport chain regulate oxygen deprivation-induced cell death. Mol Cell Biol *22*, 94-104.

Meacham, G.C., Patterson, C., Zhang, W., Younger, J.M., and Cyr, D.M. (2001). The Hsc70 co-chaperone CHIP targets immature CFTR for proteasomal degradation. Nat Cell Biol *3*, 100-105.

Meijer, A.J., and Codogno, P. (2006). Signalling and autophagy regulation in health, aging and disease. Mol Aspects Med *27*, 411-425.

Melillo, G., Musso, T., Sica, A., Taylor, L.S., Cox, G.W., and Varesio, L. (1995). A hypoxia-responsive element mediates a novel pathway of activation of the inducible nitric oxide synthase promoter. J Exp Med *182*, 1683-1693.

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

Menzies, F.M., Moreau, K., and Rubinsztein, D.C. Protein misfolding disorders and macroautophagy. Curr Opin Cell Biol *23*, 190-197.

Menzies, F.M., Moreau, K., and Rubinsztein, D.C. (2011). Protein misfolding disorders and macroautophagy. Curr Opin Cell Biol *23*, 190-197.

Metzen, E., Berchner-Pfannschmidt, U., Stengel, P., Marxsen, J.H., Stolze, I., Klinger, M., Huang, W.Q., Wotzlaw, C., Hellwig-Burgel, T., Jelkmann, W.*, et al.* (2003). Intracellular localisation of human HIF-1 alpha hydroxylases: implications for oxygen sensing. J Cell Sci *116*, 1319-1326.

Mijaljica, D., Prescott, M., and Devenish, R.J. (2011). Microautophagy in mammalian cells: revisiting a 40-year-old conundrum. Autophagy *7*, 673-682.

Minami, Y., Hohfeld, J., Ohtsuka, K., and Hartl, F.U. (1996). Regulation of the heat-shock protein 70 reaction cycle by the mammalian DnaJ homolog, Hsp40. J Biol Chem *271*, 19617-19624.

Minet, E., Michel, G., Mottet, D., Raes, M., and Michiels, C. (2001). Transduction pathways involved in Hypoxia-Inducible Factor-1 phosphorylation and activation. Free Radic Biol Med *31*, 847-855.

Mizushima, N., and Klionsky, D.J. (2007). Protein turnover via autophagy: implications for metabolism. Annu Rev Nutr *27*, 19-40.

Mizushima, N., and Levine, B. Autophagy in mammalian development and differentiation. Nat Cell Biol *12*, 823-830.

Mizushima, N., Levine, B., Cuervo, A.M., and Klionsky, D.J. (2008). Autophagy fights disease through cellular self-digestion. Nature *451*, 1069-1075.

Mizushima, N., Noda, T., Yoshimori, T., Tanaka, Y., Ishii, T., George, M.D., Klionsky, D.J., Ohsumi, M., and Ohsumi, Y. (1998). A protein conjugation system essential for autophagy. Nature *395*, 395-398.

Mizushima, N., Yamamoto, A., Hatano, M., Kobayashi, Y., Kabeya, Y., Suzuki, K., Tokuhisa, T., Ohsumi, Y., and Yoshimori, T. (2001). Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells. J Cell Biol *152*, 657-668.

Mizushima, N., Yoshimori, T., and Ohsumi, Y. (2011). The role of Atg proteins in autophagosome formation. Annu Rev Cell Dev Biol *27*, 107-132.

Mortimore, G.E., Lardeux, B.R., and Adams, C.E. (1988). Regulation of microautophagy and basal protein turnover in rat liver. Effects of short-term starvation. J Biol Chem *263*, 2506-2512.

Mortimore, G.E., Neely, A.N., Cox, J.R., and Guinivan, R.A. (1973). Proteolysis in homogenates of perfused rat liver: responses to insulin, glucagon and amino acids. Biochem Biophys Res Commun *54*, 89-95.

Mortimore, G.E., Poso, A.R., and Lardeux, B.R. (1989). Mechanism and regulation of protein degradation in liver. Diabetes Metab Rev *5*, 49-70.

Mukhopadhyay, C.K., Mazumder, B., and Fox, P.L. (2000). Role of hypoxia-inducible factor-1 in transcriptional activation of ceruloplasmin by iron deficiency. J Biol Chem *275*, 21048-21054.

Murata, S., Herman, P., and Lakowicz, J.R. (2001a). Texture analysis of fluorescence lifetime images of nuclear DNA with effect of fluorescence resonance energy transfer. Cytometry *43*, 94-100.

Murata, S., Minami, Y., Minami, M., Chiba, T., and Tanaka, K. (2001b). CHIP is a chaperone-dependent E3 ligase that ubiquitylates unfolded protein. EMBO Rep *2*, 1133-1138.

Nakatogawa, H., Suzuki, K., Kamada, Y., and Ohsumi, Y. (2009). Dynamics and diversity in autophagy mechanisms: lessons from yeast. Nat Rev Mol Cell Biol *10*, 458-467.

Nalepa, G., Rolfe, M., and Harper, J.W. (2006). Drug discovery in the ubiquitin-proteasome system. Nat Rev Drug Discov *5*, 596-613.

Neff, N.T., DeMartino, G.N., and Goldberg, A.L. (1979). The effect of protease inhibitors and decreased temperature on the degradation of different classes of proteins in cultured hepatocytes. J Cell Physiol *101*, 439-457.

Neufeld, G., Cohen, T., Gengrinovitch, S., and Poltorak, Z. (1999). Vascular endothelial growth factor (VEGF) and its receptors. FASEB J *13*, 9-22.

Nguyen, S.V., and Claycomb, W.C. (1999). Hypoxia regulates the expression of the adrenomedullin and HIF-1 genes in cultured HL-1 cardiomyocytes. Biochem Biophys Res Commun *265*, 382-386.

Nowikovsky, K., Reipert, S., Devenish, R.J., and Schweyen, R.J. (2007). Mdm38 protein depletion causes loss of mitochondrial K+/H+ exchange activity, osmotic swelling and mitophagy. Cell Death Differ *14*, 1647-1656.

Ochiai, D., Goda, N., Hishiki, T., Kanai, M., Senoo-Matsuda, N., Soga, T., Johnson, R.S., Yoshimura, Y., and Suematsu, M. (2011). Disruption of HIF-1alpha in hepatocytes

impairs glucose metabolism in diet-induced obesity mice. Biochem Biophys Res Commun *415*, 445-449.

Okiyoneda, T., Barriere, H., Bagdany, M., Rabeh, W.M., Du, K., Hohfeld, J., Young, J.C., and Lukacs, G.L. (2010). Peripheral protein quality control removes unfolded CFTR from the plasma membrane. Science *329*, 805-810.

Olmos, G., Arenas, M.I., Bienes, R., Calzada, M.J., Aragones, J., Garcia-Bermejo, M.L., Landazuri, M.O., and Lucio-Cazana, J. (2009). 15-Deoxy-Delta(12,14)-prostaglandin-J(2) reveals a new pVHL-independent, lysosomal-dependent mechanism of HIF-1alpha degradation. Cell Mol Life Sci *66*, 2167-2180.

Pandey, U.B., Nie, Z., Batlevi, Y., McCray, B.A., Ritson, G.P., Nedelsky, N.B., Schwartz, S.L., DiProspero, N.A., Knight, M.A., Schuldiner, O*., et al.* (2007). HDAC6 rescues neurodegeneration and provides an essential link between autophagy and the UPS. Nature *447*, 859-863.

Pankiv, S., Clausen, T.H., Lamark, T., Brech, A., Bruun, J.A., Outzen, H., Overvatn, A., Bjorkoy, G., and Johansen, T. (2007). p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J Biol Chem *282*, 24131-24145.

Park, S.K., Haase, V.H., and Johnson, R.S. (2007). von Hippel Lindau tumor suppressor regulates hepatic glucose metabolism by controlling expression of glucose transporter 2 and glucose 6-phosphatase. Int J Oncol *30*, 341-348.

Peden, A.A., Oorschot, V., Hesser, B.A., Austin, C.D., Scheller, R.H., and Klumperman, J. (2004). Localization of the AP-3 adaptor complex defines a novel endosomal exit site for lysosomal membrane proteins. J Cell Biol *164*, 1065-1076.

Petrucelli, L., Dickson, D., Kehoe, K., Taylor, J., Snyder, H., Grover, A., De Lucia, M., McGowan, E., Lewis, J., Prihar, G*., et al.* (2004). CHIP and Hsp70 regulate tau ubiquitination, degradation and aggregation. Hum Mol Genet *13*, 703-714.

Peyron, P., Maridonneau-Parini, I., and Stegmann, T. (2001). Fusion of human neutrophil phagosomes with lysosomes in vitro: involvement of tyrosine kinases of the Src family and inhibition by mycobacteria. J Biol Chem *276*, 35512-35517.

Pfeifer, U. (1978). Inhibition by insulin of the formation of autophagic vacuoles in rat liver. A morphometric approach to the kinetics of intracellular degradation by autophagy. J Cell Biol *78*, 152-167.

Ponka, P., and Lok, C.N. (1999). The transferrin receptor: role in health and disease. Int J Biochem Cell Biol *31*, 1111-1137.

Powers, E.T., Morimoto, R.I., Dillin, A., Kelly, J.W., and Balch, W.E. (2009). Biological and chemical approaches to diseases of proteostasis deficiency. Annu Rev Biochem *78*, 959-991.

Pringa, E., Martinez-Noel, G., Muller, U., and Harbers, K. (2001). Interaction of the ring finger-related U-box motif of a nuclear dot protein with ubiquitin-conjugating enzymes. J Biol Chem *276*, 19617-19623.

Pugh, C.W., O'Rourke, J.F., Nagao, M., Gleadle, J.M., and Ratcliffe, P.J. (1997). Activation of hypoxia-inducible factor-1; definition of regulatory domains within the alpha subunit. J Biol Chem *272*, 11205-11214.

Rankin, E.B., and Giaccia, A.J. (2008). The role of hypoxia-inducible factors in tumorigenesis. Cell Death Differ *15*, 678-685.

Ravi, R., Mookerjee, B., Bhujwalla, Z.M., Sutter, C.H., Artemov, D., Zeng, Q., Dillehay, L.E., Madan, A., Semenza, G.L., and Bedi, A. (2000). Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1alpha. Genes Dev *14*, 34-44.

Richard, D.E., Berra, E., Gothie, E., Roux, D., and Pouyssegur, J. (1999). p42/p44 mitogen-activated protein kinases phosphorylate hypoxia-inducible factor 1alpha (HIF-1alpha) and enhance the transcriptional activity of HIF-1. J Biol Chem *274*, 32631-32637.

Richard, D.E., Berra, E., and Pouyssegur, J. (2000). Nonhypoxic pathway mediates the induction of hypoxia-inducible factor 1alpha in vascular smooth muscle cells. J Biol Chem *275*, 26765-26771.

Rink, J., Ghigo, E., Kalaidzidis, Y., and Zerial, M. (2005). Rab conversion as a mechanism of progression from early to late endosomes. Cell *122*, 735-749.

Roberts, P., Moshitch-Moshkovitz, S., Kvam, E., O'Toole, E., Winey, M., and Goldfarb, D.S. (2003). Piecemeal microautophagy of nucleus in Saccharomyces cerevisiae. Mol Biol Cell *14*, 129-141.

Rodriguez-Navarro, J.A., Kaushik, S., Koga, H., Dall'armi, C., Shui, G., Wenk, M.R., Di Paolo, G., and Cuervo, A.M. (2012). Inhibitory effect of dietary lipids on chaperone-mediated autophagy. Proc Natl Acad Sci U S A.

Rolfs, A., Kvietikova, I., Gassmann, M., and Wenger, R.H. (1997). Oxygen-regulated transferrin expression is mediated by hypoxia-inducible factor-1. J Biol Chem *272*, 20055-20062.

Roth, U., Curth, K., Unterman, T.G., and Kietzmann, T. (2004a). The transcription factors HIF-1 and HNF-4 and the coactivator p300 are involved in insulin-regulated glucokinase gene expression via the phosphatidylinositol 3-kinase/protein kinase B pathway. J Biol Chem *279*, 2623-2631.

Roth, U., Jungermann, K., and Kietzmann, T. (2004b). Modulation of glucokinase expression by hypoxia-inducible factor 1 and upstream stimulatory factor 2 in primary rat hepatocytes. Biol Chem *385*, 239-247.

Ruas, J.L., Poellinger, L., and Pereira, T. (2002). Functional analysis of hypoxia-inducible factor-1 alpha-mediated transactivation. Identification of amino acid residues critical for transcriptional activation and/or interaction with CREB-binding protein. J Biol Chem *277*, 38723-38730.

Rubinsztein, D.C., Gestwicki, J.E., Murphy, L.O., and Klionsky, D.J. (2007). Potential therapeutic applications of autophagy. Nat Rev Drug Discov *6*, 304-312.

Rubinsztein, D.C., Shpilka, T., and Elazar, Z. (2012). Mechanisms of autophagosome biogenesis. Curr Biol *22*, R29-34.

Russell, R.R., 3rd, Cline, G.W., Guthrie, P.H., Goodwin, G.W., Shulman, G.I., and Taegtmeyer, H. (1997). Regulation of exogenous and endogenous glucose metabolism by insulin and acetoacetate in the isolated working rat heart. A three tracer study of glycolysis, glycogen metabolism, and glucose oxidation. J Clin Invest *100*, 2892-2899.

Rutherford, S.L., and Lindquist, S. (1998). Hsp90 as a capacitor for morphological evolution. Nature *396*, 336-342.

Sachse, M., Ramm, G., Strous, G., and Klumperman, J. (2002). Endosomes: multipurpose designs for integrating housekeeping and specialized tasks. Histochem Cell Biol *117*, 91-104.

Saftig, P., and Klumperman, J. (2009). Lysosome biogenesis and lysosomal membrane proteins: trafficking meets function. Nat Rev Mol Cell Biol *10*, 623-635.

Sakai, M., Araki, N., and Ogawa, K. (1989). Lysosomal movements during heterophagy and autophagy: with special reference to nematolysosome and wrapping lysosome. J Electron Microsc Tech *12*, 101-131.

Sakai, Y., Koller, A., Rangell, L.K., Keller, G.A., and Subramani, S. (1998). Peroxisome degradation by microautophagy in Pichia pastoris: identification of specific steps and morphological intermediates. J Cell Biol *141*, 625-636.

Sakai, Y., Oku, M., van der Klei, I.J., and Kiel, J.A. (2006). Pexophagy: autophagic degradation of peroxisomes. Biochim Biophys Acta *1763*, 1767-1775.

Salceda, S., and Caro, J. (1997). Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. J Biol Chem *272*, 22642-22647.

Salnikow, K., Blagosklonny, M.V., Ryan, H., Johnson, R., and Costa, M. (2000). Carcinogenic nickel induces genes involved with hypoxic stress. Cancer Res *60*, 38-41.

Sanchez-Puig, N., Veprintsev, D.B., and Fersht, A.R. (2005). Binding of natively unfolded HIF-1alpha ODD domain to p53. Mol Cell *17*, 11-21.

Sang, N., Fang, J., Srinivas, V., Leshchinsky, I., and Caro, J. (2002). Carboxyl-terminal transactivation activity of hypoxia-inducible factor 1 alpha is governed by a von Hippel-Lindau protein-independent, hydroxylation-regulated association with p300/CBP. Mol Cell Biol *22*, 2984-2992.

Scheffner, M., Huibregtse, J.M., Vierstra, R.D., and Howley, P.M. (1993). The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell *75*, 495-505.

Scheufler, C., Brinker, A., Bourenkov, G., Pegoraro, S., Moroder, L., Bartunik, H., Hartl, F.U., and Moarefi, I. (2000). Structure of TPR domain-peptide complexes: critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine. Cell *101*, 199-210.

Schimke, R.T., and Doyle, D. (1970). Control of enzyme levels in animal tissues. Annu Rev Biochem *39*, 929-976.

Schoenheimer, R., Ratner, S., and Rittenberg, D. (1939). The Process of Continuous Deamination and Reamination of Amino Acids in the Proteins of Normal Animals. Science *89*, 272-273.

Schrödinger, E. (1962). What is life? The physical aspect of the living cell (Cambridge [Eng.]

New York,, The University Press;

The Macmillan company).

Schuler, M., and Green, D.R. (2001). Mechanisms of p53-dependent apoptosis. Biochem Soc Trans *29*, 684-688.

Schworer, C.M., Shiffer, K.A., and Mortimore, G.E. (1981). Quantitative relationship between autophagy and proteolysis during graded amino acid deprivation in perfused rat liver. J Biol Chem *256*, 7652-7658.

Seagroves, T.N., Ryan, H.E., Lu, H., Wouters, B.G., Knapp, M., Thibault, P., Laderoute, K., and Johnson, R.S. (2001). Transcription factor HIF-1 is a necessary mediator of the pasteur effect in mammalian cells. Mol Cell Biol *21*, 3436-3444.

Semenza, G.L. (1998). Hypoxia-inducible factor 1: master regulator of O2 homeostasis. Curr Opin Genet Dev *8*, 588-594.

Semenza, G.L. (1999). Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. Annu Rev Cell Dev Biol *15*, 551-578.

Semenza, G.L. (2002). Involvement of hypoxia-inducible factor 1 in human cancer. Intern Med *41*, 79-83.

Semenza, G.L. (2003). Targeting HIF-1 for cancer therapy. Nat Rev Cancer *3*, 721-732.

Semenza, G.L. (2010). HIF-1: upstream and downstream of cancer metabolism. Curr Opin Genet Dev *20*, 51-56.

Semenza, G.L., Jiang, B.H., Leung, S.W., Passantino, R., Concordet, J.P., Maire, P., and Giallongo, A. (1996). Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. J Biol Chem *271*, 32529-32537.

Semenza, G.L., Nejfelt, M.K., Chi, S.M., and Antonarakis, S.E. (1991). Hypoxia-inducible nuclear factors bind to an enhancer element located 3' to the human erythropoietin gene. Proc Natl Acad Sci U S A *88*, 5680-5684.

Sha, B., Lee, S., and Cyr, D.M. (2000). The crystal structure of the peptide-binding fragment from the yeast Hsp40 protein Sis1. Structure *8*, 799-807.

Sha, Y., Pandit, L., Zeng, S., and Eissa, N.T. (2009). A critical role for CHIP in the aggresome pathway. Mol Cell Biol *29*, 116-128.

Shao, R., Zhang, F.P., Tian, F., Anders Friberg, P., Wang, X., Sjoland, H., and Billig, H. (2004). Increase of SUMO-1 expression in response to hypoxia: direct interaction with HIF-1alpha in adult mouse brain and heart in vivo. FEBS Lett *569*, 293-300.

Shin, Y., Klucken, J., Patterson, C., Hyman, B.T., and McLean, P.J. (2005). The co-chaperone carboxyl terminus of Hsp70-interacting protein (CHIP) mediates alpha-synuclein degradation decisions between proteasomal and lysosomal pathways. J Biol Chem *280*, 23727-23734.

Simpson, M.V. (1953). The release of labeled amino acids from the proteins of rat liver slices. J Biol Chem *201*, 143-154.

Singh, R., Xiang, Y., Wang, Y., Baikati, K., Cuervo, A.M., Luu, Y.K., Tang, Y., Pessin, J.E., Schwartz, G.J., and Czaja, M.J. (2009). Autophagy regulates adipose mass and differentiation in mice. J Clin Invest *119*, 3329-3339.

Skach, W.R. (2009). Cellular mechanisms of membrane protein folding. Nat Struct Mol Biol *16*, 606-612.

Slagsvold, T., Pattni, K., Malerod, L., and Stenmark, H. (2006). Endosomal and non-endosomal functions of ESCRT proteins. Trends Cell Biol *16*, 317-326.

Sobhanifar, S., Aquino-Parsons, C., Stanbridge, E.J., and Olive, P. (2005). Reduced expression of hypoxia-inducible factor-1alpha in perinecrotic regions of solid tumors. Cancer Res *65*, 7259-7266.

Sodhi, A., Montaner, S., Patel, V., Zohar, M., Bais, C., Mesri, E.A., and Gutkind, J.S. (2000). The Kaposi's sarcoma-associated herpes virus G protein-coupled receptor up-regulates vascular endothelial growth factor expression and secretion through mitogen-activated protein kinase and p38 pathways acting on hypoxia-inducible factor 1alpha. Cancer Res *60*, 4873-4880.

Spillantini, M.G., Crowther, R.A., Jakes, R., Cairns, N.J., Lantos, P.L., and Goedert, M. (1998a). Filamentous alpha-synuclein inclusions link multiple system atrophy with Parkinson's disease and dementia with Lewy bodies. Neurosci Lett *251*, 205-208.

Spillantini, M.G., Crowther, R.A., Jakes, R., Hasegawa, M., and Goedert, M. (1998b). alpha-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with lewy bodies. Proc Natl Acad Sci U S A *95*, 6469-6473.

Steinberg, D., and Vaughan, M. (1956). Observations on intracellular protein catabolism studied in vitro. Arch Biochem Biophys *65*, 93-105.

Stockinger, W., Zhang, S.C., Trivedi, V., Jarzylo, L.A., Shieh, E.C., Lane, W.S., Castoreno, A.B., and Nohturfft, A. (2006). Differential requirements for actin polymerization, calmodulin, and Ca2+ define distinct stages of lysosome/phagosome targeting. Mol Biol Cell *17*, 1697-1710.

Stoorvogel, W., Strous, G.J., Geuze, H.J., Oorschot, V., and Schwartz, A.L. (1991). Late endosomes derive from early endosomes by maturation. Cell *65*, 417-427.

Storrie, B., and Desjardins, M. (1996). The biogenesis of lysosomes: is it a kiss and run, continuous fusion and fission process? Bioessays *18*, 895-903.

Suzuki, H., Tomida, A., and Tsuruo, T. (2001a). Dephosphorylated hypoxia-inducible factor 1alpha as a mediator of p53-dependent apoptosis during hypoxia. Oncogene *20*, 5779-5788.

Suzuki, K., Kirisako, T., Kamada, Y., Mizushima, N., Noda, T., and Ohsumi, Y. (2001b). The pre-autophagosomal structure organized by concerted functions of APG genes is essential for autophagosome formation. EMBO J *20*, 5971-5981.

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

Tacchini, L., Bianchi, L., Bernelli-Zazzera, A., and Cairo, G. (1999). Transferrin receptor induction by hypoxia. HIF-1-mediated transcriptional activation and cell-specific post-transcriptional regulation. J Biol Chem *274*, 24142-24146.

Taipale, M., Jarosz, D.F., and Lindquist, S. (2010). HSP90 at the hub of protein homeostasis: emerging mechanistic insights. Nat Rev Mol Cell Biol *11*, 515-528.

Takahashi, Y., Takahashi, S., Shiga, Y., Yoshimi, T., and Miura, T. (2000). Hypoxic induction of prolyl 4-hydroxylase alpha (I) in cultured cells. J Biol Chem *275*, 14139-14146.

Takayama, S., Xie, Z., and Reed, J.C. (1999). An evolutionarily conserved family of Hsp70/Hsc70 molecular chaperone regulators. J Biol Chem *274*, 781-786.

Terlecky, S.R., and Dice, J.F. (1993). Polypeptide import and degradation by isolated lysosomes. J Biol Chem *268*, 23490-23495.

Thompson, L.M., Aiken, C.T., Kaltenbach, L.S., Agrawal, N., Illes, K., Khoshnan, A., Martinez-Vincente, M., Arrasate, M., O'Rourke, J.G., Khashwji, H.*, et al.* (2009). IKK phosphorylates Huntingtin and targets it for degradation by the proteasome and lysosome. J Cell Biol *187*, 1083-1099.

Tian, H., Hammer, R.E., Matsumoto, A.M., Russell, D.W., and McKnight, S.L. (1998). The hypoxia-responsive transcription factor EPAS1 is essential for catecholamine homeostasis and protection against heart failure during embryonic development. Genes Dev *12*, 3320-3324.

Tian, H., McKnight, S.L., and Russell, D.W. (1997). Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. Genes Dev *11*, 72-82.

Tokuriki, N., and Tawfik, D.S. (2009). Chaperonin overexpression promotes genetic variation and enzyme evolution. Nature *459*, 668-673.

Tolkovsky, A.M. (2009). Mitophagy. Biochim Biophys Acta *1793*, 1508-1515.

Tooze, J., and Hollinshead, M. (1991). Tubular early endosomal networks in AtT20 and other cells. J Cell Biol *115*, 635-653.

Tooze, S.A., and Yoshimori, T. (2010). The origin of the autophagosomal membrane. Nat Cell Biol *12*, 831-835.

Tuttle, D.L., and Dunn, W.A., Jr. (1995). Divergent modes of autophagy in the methylotrophic yeast Pichia pastoris. J Cell Sci *108 (Pt 1)*, 25-35.

Uchida, T., Rossignol, F., Matthay, M.A., Mounier, R., Couette, S., Clottes, E., and Clerici, C. (2004). Prolonged hypoxia differentially regulates hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha expression in lung epithelial cells: implication of natural antisense HIF-1alpha. J Biol Chem *279*, 14871-14878.

Uttenweiler, A., and Mayer, A. (2008). Microautophagy in the yeast Saccharomyces cerevisiae. Methods Mol Biol *445*, 245-259.

van Meel, E., and Klumperman, J. (2008). Imaging and imagination: understanding the endo-lysosomal system. Histochem Cell Biol *129*, 253-266.

Virgin, H.W., and Levine, B. (2009). Autophagy genes in immunity. Nat Immunol *10*, 461-470.

Voges, D., Zwickl, P., and Baumeister, W. (1999). The 26S proteasome: a molecular machine designed for controlled proteolysis. Annu Rev Biochem *68*, 1015-1068.

Volm, M., and Koomagi, R. (2000). Hypoxia-inducible factor (HIF-1) and its relationship to apoptosis and proliferation in lung cancer. Anticancer Res *20*, 1527-1533.

Waguri, S., Dewitte, F., Le Borgne, R., Rouille, Y., Uchiyama, Y., Dubremetz, J.F., and Hoflack, B. (2003). Visualization of TGN to endosome trafficking through fluorescently labeled MPR and AP-1 in living cells. Mol Biol Cell *14*, 142-155.

Wandinger, S.K., Richter, K., and Buchner, J. (2008). The Hsp90 chaperone machinery. J Biol Chem *283*, 18473-18477.

Wang, G.L., Jiang, B.H., Rue, E.A., and Semenza, G.L. (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci U S A *92*, 5510-5514.

Wattiaux, R., Wattiaux-De Coninck, S., Ronveaux-dupal, M.F., and Dubois, F. (1978). Isolation of rat liver lysosomes by isopycnic centrifugation in a metrizamide gradient. J Cell Biol *78*, 349-368.

Wenger, R.H. (2002). Cellular adaptation to hypoxia: O2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O2-regulated gene expression. FASEB J *16*, 1151-1162.

White, E., Karp, C., Strohecker, A.M., Guo, Y., and Mathew, R. Role of autophagy in suppression of inflammation and cancer. Curr Opin Cell Biol *22*, 212-217.

White, I.J., Bailey, L.M., Aghakhani, M.R., Moss, S.E., and Futter, C.E. (2006). EGF stimulates annexin 1-dependent inward vesiculation in a multivesicular endosome subpopulation. EMBO J *25*, 1-12.

Wickner, S., Maurizi, M.R., and Gottesman, S. (1999). Posttranslational quality control: folding, refolding, and degrading proteins. Science *286*, 1888-1893.

Wong, E., and Cuervo, A.M. Autophagy gone awry in neurodegenerative diseases. Nat Neurosci *13*, 805-811.

Woodman, P.G., and Futter, C.E. (2008). Multivesicular bodies: co-ordinated progression to maturity. Curr Opin Cell Biol *20*, 408-414.

Wooten, M.W., Geetha, T., Babu, J.R., Seibenhener, M.L., Peng, J., Cox, N., Diaz-Meco, M.T., and Moscat, J. (2008). Essential role of sequestosome 1/p62 in

regulating accumulation of Lys63-ubiquitinated proteins. J Biol Chem *283*, 6783-6789.

Xie, Z., and Klionsky, D.J. (2007). Autophagosome formation: core machinery and adaptations. Nat Cell Biol *9*, 1102-1109.

Xu, W., Marcu, M., Yuan, X., Mimnaugh, E., Patterson, C., and Neckers, L. (2002). Chaperone-dependent E3 ubiquitin ligase CHIP mediates a degradative pathway for c-ErbB2/Neu. Proc Natl Acad Sci U S A *99*, 12847-12852.

Xu, Z., Kohli, E., Devlin, K.I., Bold, M., Nix, J.C., and Misra, S. (2008). Interactions between the quality control ubiquitin ligase CHIP and ubiquitin conjugating enzymes. BMC Struct Biol *8*, 26.

Yabaluri, N., and Bashyam, M.D. (2010). Hormonal regulation of gluconeogenic gene transcription in the liver. J Biosci *35*, 473-484.

Yasinska, I.M., and Sumbayev, V.V. (2003). S-nitrosation of Cys-800 of HIF-1alpha protein activates its interaction with p300 and stimulates its transcriptional activity. FEBS Lett *549*, 105-109.

Yewdell, J.W., and Bennink, J.R. (2001). Cut and trim: generating MHC class I peptide ligands. Curr Opin Immunol *13*, 13-18.

Younger, J.M., Ren, H.Y., Chen, L., Fan, C.Y., Fields, A., Patterson, C., and Cyr, D.M. (2004). A foldable CFTR{Delta}F508 biogenic intermediate accumulates upon inhibition of the Hsc70-CHIP E3 ubiquitin ligase. J Cell Biol *167*, 1075-1085.

Yun, Z., Maecker, H.L., Johnson, R.S., and Giaccia, A.J. (2002). Inhibition of PPAR gamma 2 gene expression by the HIF-1-regulated gene DEC1/Stra13: a mechanism for regulation of adipogenesis by hypoxia. Dev Cell *2*, 331-341.

Zelzer, E., Levy, Y., Kahana, C., Shilo, B.Z., Rubinstein, M., and Cohen, B. (1998). Insulin induces transcription of target genes through the hypoxia-inducible factor HIF-1alpha/ARNT. EMBO J *17*, 5085-5094.

Zhang, C., and Cuervo, A.M. (2008). Restoration of chaperone-mediated autophagy in aging liver improves cellular maintenance and hepatic function. Nat Med *14*, 959-965.

Zhang, M., Windheim, M., Roe, S.M., Peggie, M., Cohen, P., Prodromou, C., and Pearl, L.H. (2005). Chaperoned ubiquitylation--crystal structures of the CHIP U box E3 ubiquitin ligase and a CHIP-Ubc13-Uev1a complex. Mol Cell *20*, 525-538.

Zheng, N., Schulman, B.A., Song, L., Miller, J.J., Jeffrey, P.D., Wang, P., Chu, C., Koepp, D.M., Elledge, S.J., Pagano, M*., et al.* (2002). Structure of the Cul1-Rbx1-Skp1-F boxSkp2 SCF ubiquitin ligase complex. Nature *416*, 703-709.

Zhou, D., Li, P., Lin, Y., Lott, J.M., Hislop, A.D., Canaday, D.H., Brutkiewicz, R.R., and Blum, J.S. (2005). Lamp-2a facilitates MHC class II presentation of cytoplasmic antigens. Immunity *22*, 571-581.