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The nucleolar protein Vito/Nol12 *is required for the growth and differentiation progression activities of the* Dpp *pathway during* Drosophila *eye development.*

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ABSTRACT

Drosophila Decapentaplegic (Dpp), a member of the BMP2/4 class of the TGF-βs, is required for organ growth, patterning and differentiation. However, much remains to be understood about the mechanisms acting downstream of these multiple roles. Here we investigate this issue during the development of the *Drosophila* eye. We have previously identified *vito* as a dMyc-target gene encoding a nucleolar protein that is required for proper tissue growth in the developing eye. By carrying out a targeted *in vivo* double-RNAi screen to identify genes and pathways functioning with Vito during eye development, we found a strong genetic interaction between *vito* and members of the Dpp signaling pathway including the TGF-β receptors *tkv* (type I), *put* (type II), and the co-Smad *medea* (*med*). Analyzing the expression of the Dpp receptor Tkv and the activation pattern of the pathway's transducer, P-Mad, we found that *vito* is required for a correct signal transduction in Dpp-receiving cells. Overall, we validate the use of double RNAi to find specific genetic interactions and, in particular, we uncover a link between the Dpp pathway and Vito, a nucleolar component. *vito* would act genetically downstream of Dpp, playing an important role in maintaining a sufficient level of Dpp activity for the promotion of eye disc growth and regulation of photoreceptor differentiation in eye development.

INTRODUCTION

Tissue-specific integration of growth and patterning signals must occur for the control of the basic events necessary for cell and organ growth, but the mechanisms underlying this integration are still poorly understood. The progressive nature of *Drosophila* eye development makes it an excellent model to study how cellular growth, proliferation, and apoptosis events control organ size and patterning (reviewed in (Amore and Casares, 2010).

In a recent study we identified *viriato (vito)*, the single *Drosophila* member of the Nol12/Nop25 gene family, as a crucial regulator of nucleolar architecture, cell proliferation and cell survival during *Drosophila* development (Marinho et al., 2011). *vito* is a novel transcriptional target of *Drosophila* Myc, and it has an important function in ensuring a coordinated nucleolar response to dMyc-induced growth, thereby allowing normal tissue growth (Marinho et al., 2011). The ability of Myc to stimulate nucleolar hypertrophy, with increased pre-rRNA transcription and ribosome biogenesis, has been shown to be crucial for Myc-dependent growth in both *Drosophila* and mammalian cells (reviewed in (van Riggelen et al., 2010)). Interestingly, in the eye imaginal disc, *vito* function is required for cell proliferation and cell survival anterior to the morphogenetic furrow (MF) in a domain where both progenitor and precursor cells are found (Marinho et al., 2011). At the molecular level, Vito function has yet to be characterized in detail. However, the fact that requirement for Vito function depends on the cellular differentiation state is particularly intriguing given the accepted function for Nol12-containing proteins. In the budding yeast, the single Vito homologue, Rrp17p/Ydr412p, was shown to be a 5'-3'exonuclease required for processing of pre-

60S ribosomal RNA and other nucleolar non-coding RNA processing events (Li et al., 2009; Oeffinger et al., 2009; Peng et al., 2003; Sahasranaman et al., 2011). Importantly, human Nol12 was shown to rescue both *Rrp17* mutants (Oeffinger et al., 2009) and *vito* knockdown (Marinho et al., 2011), strongly implying a conservation of the molecular function of the Nol12 domain, the single identifiable domain in this family of small proteins. Thus, a further characterization of *vito* function in eye development might reveal novel genetic links between the signaling pathways controlling dynamic progression of differentiation and the modulation of Vito's putative 5'-3' exonuclease activity and targets of action.

Genome-wide RNAi screens have been important to study novel gene functions in defined cellular contexts (Mohr et al., 2010). Furthermore, to explore the complex relationships between genes and signaling pathways, large-scale systematic pairwise genetic interactions have been widely used in yeast and are now starting to be used in mammalian and *Drosophila* cell culture systems (Bakal et al., 2008).

In this work we performed an eye-targeted double RNAi screen with the goal of searching for Vito partners and interacting signaling pathways. To our knowledge, although not genome-wide, this is the first *in vivo* double-RNAi screen to study synthetic genetic interactions during *Drosophila* development. Moreover, although quantifying genetic interactions has previously been a challenge, here we took advantage of the recently used multiplicative model to quantitatively score our genetic interactions using a phenotype rather than the fitness of a cell (Baryshnikova et al., 2010; Horn et al., 2011). Of the twelve genes we identified as interacting with *vito*, eleven are predicted to function in nervous system development, thus clearly establishing an important role for *vito* in developmental control. Interestingly, the strongest aggravating synthetic interactions were found to occur between *vito* and

members of the Dpp signaling pathway. Our detailed characterization of these interactions showed that *vito* has a positive role in Dpp signaling, acting downstream of Dpp. When Dpp signaling is attenuated, Vito is indispensable for Dpp to promote tissue growth during early eye development and later for Dpp-dependent triggering of retina differentiation. Thus, our results demonstrate the strength of *in fly* screens based on the generation of double-RNAi sensitized background for the identification of subtle modulation of signaling pathway activity and novel genetic links.

RESULTS

An eye-targeted double-RNAi screen to identify *vito* interactors during eye development

In order to identify genes that cooperate with *vito* during eye development, we took advantage of the identification of a set of 188 target genes of the Eyeless (Ey) transcription factor to perform an eye-targeted double-RNAi genetic screen (Fig. 1A) (see Materials and Methods). The set of 188 *ey*-target genes was previously identified as genes with preferential expression in the eye primordium that were also induced by *ey* in ectopic locations (Ostrin et al., 2006). In a first step, we targeted by RNAi 148 genes, out of the 188, including genes functionally classified as being involved in eye development, cell cycle, transcription, or translation. For the majority of these genes (36%) the function and/or biological process has not been characterized yet (Fig. 1B). Using Gene Ontology (GO) annotations to identify common and enriched properties for this list, we found a significant statistical enrichment in genes related to eye-antennal disc development, eye development and neuron differentiation (Fig. 1C). Furthermore, we also included several members of signaling pathways important for growth and

patterning during eye development, such as TGF-β, Hedgehog (Hh), Notch and Wingless (Wg) pathways in the double-RNAi test group, so that a total of 162 genes were screened (Fig. 1A, Supplementary Table 1).

Two genes are said to interact if the phenotype produced by the lack of both genes deviates from the expected combination of the individual phenotypes (Mani et al., 2008; Phillips, 2008). Therefore, genetic interactions were identified by comparing the phenotype of experimental double-RNAi with the expected phenotype of double-RNAis based on individual single RNAis, and the adult retina size was the scored phenotype. To score the expected double-RNAi phenotypes we used a model that assumes that the expected double mutant phenotype can be the result of the multiplicative combination of the single mutant phenotypes (Baryshnikova et al., 2010; Costanzo et al., 2011). The multiplicative model, widely used in yeast, has recently been implemented in higher organisms and tested using phenotypes other than fitness (Horn et al., 2011; Jonikas et al., 2009). Thus, to determine if *vito* interacts with a candidate gene, genetic interactions scores (π) were calculated based on the differences between the expected and the observed double-RNAi adult retina sizes (Fig 1D).

Identification of twelve genes interacting with *vito* in eye development

After performing the eye-targeted double-RNAi screen for *vito* in combination with the 162 gene set we identified 12 genetic interactions (Table I and Fig. 2A,B). All these displayed aggravating or synergistic effects (positive π scores), i.e., exhibited a more severe eye phenotype than expected based on the multiplicative combination of the single RNAi phenotypes. To overcome potential off-target effects we tested a second UAS-*vito*RNAi and further available RNAi lines for *vito* interactors, obtaining very similar results (Supplementary Table 1, and data not shown).

A network of genetic and protein interactions for *vito* and its 12 gene interactors reveals direct connections between them (Fig. 2C), and a GO term analysis shows an enrichment for genes involved in nervous system development (11 out of 12 interactors) and eye development (8 out of 12 interactors). Not unexpectedly, these enrichments suggest that Vito might be involved in processes related to nervous system development in the eye, such as neuronal photoreceptor differentiation. Examples of interactors include Fasciclin 2 (Fas2) that was recently found to be expressed in a dynamic pattern during eye imaginal disc development, and to act in the inhibition of epidermal growth factor receptor (EGFR) signaling in the developing eye (Mao and Freeman, 2009); and Delta, a Notch ligand that is important to establish dorsal and ventral compartments and regulate growth in the developing eye (Kumar, 2011). Semaphorin-5c (Sema-5c), although not apparently related to eye development, was shown to regulate Dpp signaling in a *Drosophila* tumor metastasis (*l(2)gl* tumors) (Woodhouse et al., 2003). Interestingly, the genes that interact most strongly with *vito* (highest π score, Table I) are either essential Retinal Determination (RD) genes (like *eya*, *so* and *ey*) or components of the Dpp/TGF-β signal transduction pathway (Fig. 2). Furthermore, we observed that *vito* also interacts with members of the Activin branch of the TGF-β signaling pathway, such as the type-I receptor Babo and the R-Smad Smox (Brummel et al., 1999) (Table I, Fig. 2A,B).

vito genetically interacts with Dpp signaling pathway during eye development

Our screen for Vito interactors during eye development identified a strong genetic interaction with the Dpp branch of the TGF-β signaling pathway. To investigate further this interaction we reduced Dpp signaling using RNAi for the type-I receptor *tkv* or the co-SMAD *med*, which resulted in a mild reduction in adult eye size (Fig. 3 A,B). A

slight delay in the progression of differentiation at the eye imaginal disc margins is observed, resembling the phenotype caused by hypomorphic Dpp signaling mutants (Chanut and Heberlein, 1997) (Fig. 3A). Interestingly, the differentiation defects in the eye disc caused by RNAi against *tkv* or *med* are strongly enhanced by *vito*RNAi, as the double-RNAi eye discs are reduced in size and lack differentiation (Fig. 3 A,B). Importantly, the reductions in eye disc sizes, seen after co-expression of *vito*RNAi and *tkv* or *med* RNAi, are not statistically different from the reductions seen for *vito*RNAi-only expressing discs (44% reduction for *vito*RNAi against 52% or 53% reduction for co-expression of *vito*RNAi with *tkv*RNAi or *med*RNAi, respectively). This result strongly suggests that, in these experimental conditions, the lack of differentiation was not simply due to a strong reduction in tissue growth (Fig. 3 A,B). An analysis of the interaction between *vito* and the type-II receptor *put* revealed further details on the role of Vito in Dpp signaling during eye disc growth and patterning. Flies expressing a weak RNAi against *put* (*put*RNAi1; Supplementary Table 1) do not show any visible defects in adult eyes or eye discs (Fig. 4 A,B). However, eye discs co-depleted of Vito and Put show a clear delay in MF progression, resembling the phenotype of a weak *dpp* loss-of-function allele dpp^{blk} (Fig. 4C-F) (Chanut and Heberlein, 1997). Interestingly, a strong downregulation of Dpp signaling that was achieved using a more efficient RNAi line against *put* (*put*RNAi2) produces eye discs that attain a normal size but that completely lack retinal differentiation (Fig. 4G,H). In turn, co-depletion of *put*RNAi2 and *vito*RNAi results in a strong eye disc growth deficit, lack of differentiation and adult lethality (Fig. 4I,J). Overall these results indicate a dual role of Vito during eye development: early in development Vito is required for tissue growth together with Dpp, and in later stages of eye disc development Vito modulates Dpp signaling to promote the progression of the morphogenetic furrow in the eye imaginal disc.

In the eye disc, *vito* is expressed in a dynamic pattern with high expression in the anterior region, while in the wing, and in other imaginal discs, *vito* appears to be transcribed at low levels without a specific pattern of expression (Marinho et al., 2011). To evaluate the tissue specificity of the *vito*/Dpp interaction we knocked down both *vito* and *tkv* and scanned for growth and patterning defects in the wing. Downregulation of Dpp signaling (by *tkv*RNAi and *med*RNAi), driven by several distinct Gal4 lines, leads to expected wing size reductions and/or patterning defects (Fig. S1). Interestingly, the additional knocking-down of *vito* did not alter *tkv*RNAi or *med*RNAi phenotypes in the wing (Fig. S1). Therefore, the interactions we observed in the eye seem to be tissue-specific.

The Vito-Dpp signaling interaction is not explained by an increase in apoptosis

We have shown previously that Vito is required for cell survival, particularly at the anterior region of the eye disc, next to the MF (Marinho et al., 2011). Therefore, we next investigated whether the absence of retinal differentiation seen in double-RNAi for *vito* and *tkv* could be explained by a generalized induction of apoptosis. As expected, *vito*RNAi eye discs had a significant number of cells positive for activated cleaved caspase-3, an apoptosis effector (Fig. 5A,B). Some apoptosis was also detected after knocking-down *tkv*, particularly in the anterior domain close to the disc margins, the area where some delay in the morphogenetic furrow is detected (Fig. 5A,B). However, when *vito*RNAi is co-expressed with *tkv*RNAi the number of cells undergoing apoptosis is not statistically different from those seen in *vito*RNAi eye discs (Fig. 5A,B), and we could not detect cells undergoing apoptosis in the posterior margin of the eye disc, where retinal differentiation would normally initiate (Fig. 5A). Therefore, the absence of retinal differentiation is not explained by an increase in apoptosis.

***vito* is a positive regulator of the Dpp signaling pathway**

Next, we decided to address whether *vito* could modulate phenotypes resulting from Dpp overexpression. When we overexpressed *dpp* under the *ey*-Gal4 driver, 100% of the flies did not survive to adulthood, dying as pupae (Fig 6 A,B). We observed that ectopic Dpp expression resulted in smaller eye discs with generalized photoreceptor differentiation, even in regions of the eye disc that would normally give rise to head cuticle (Fig. 6C), which is consistent with the known role of Dpp in MF initiation and progression. Interestingly, we found that depletion of Vito was able to rescue excessive activation of Dpp, reverting lethality in 30% of the flies, and partially recovering normal eye disc size (70% increase) and differentiation (Fig. 6A-D). Additionally, depletion of Vito increases the undifferentiated area of the eye disc (from 15.7% to 38.9%) in Dpp overexpression conditions (Fig. 6E). The area that remains undifferentiated in UASdpp^{D}+*vito*RNAi corresponds mainly to the dorsal part of the eye where Orthodenticle (Otd) is expressed (Fig. 6C). Otd is a Wg target that is expressed in an anterior dorsal region (head primordia) and in the developing ommatidia (Royet and Finkelstein, 1996).

Furthermore, Vito's capacity to modulate Dpp signaling seems to be important for eye but not for wing development, as depletion of Vito failed to modify Dpp overexpression phenotypes in the adult wing (Fig. 6F). Overall these results suggest that Vito modulates the cellular response downstream of Dpp, and that the Vito/Dpp interaction is not exclusively due to the role of Vito in tissue growth.

***vito* regulates uniform Dpp signaling at the morphogenetic furrow**

To investigate the mechanism by which Vito modulates Dpp signaling, we studied *dpp* transcription upon knocking-down *vito*. In wild-type discs, *dpp* is expressed along the posterior and lateral margins before furrow initiation (Fig. 7A), but afterwards, *dpp* expression progresses with the furrow and is shut down at the margins (Fig. 7A'1-A''2, and (Blackman et al., 1991)). In *vito*RNAi discs we observed an anterior expansion of the *dpp* expression pattern during dynamic differentiation progression, and this expansion is already detected in early eye development (Fig. 7B-B''2, white arrows). Therefore, *vito* appears not to be required to activate *dpp* expression, although it plays an important role to regulate its expression pattern.

To further explore which steps of the Dpp pathway are affected by a reduction in *vito* function, we decided to study the expression pattern of the Dpp pathway's type-I receptor Tkv, one of the strongest interactors of *vito* (Table I). With that purpose we assessed the levels and the expression pattern of Tkv using a YFP exon trap insertion that reflects Tkv endogenous expression (Yuva-Aydemir et al., 2011). In control discs, Tkv is expressed in the differentiated photoreceptors zone and in the medial anterior region of the eye disc (Fig 7C). Depletion of *vito* does not change the Tkv expression pattern, however, Tkv levels are reduced at the MF and adjacent anterior regions (Fig 7D). This reduction became even more evident when a 3D-histogram representing quantitatively Tkv expression levels across the eye disc was created (Fig. C'1, D'1). Tkv expression anteriorly to the MF seems to be dependent on Dpp signaling because when we knockdown *med* or *put* the levels of Tkv were decreased (to levels comparable with *vito*RNAi) only close to the margins, the region where MF progression is delayed (Fig. 7E, E'1 and not shown). Knocking-down *vito* or *med* failed to affect the expression levels of other unrelated plasma membrane proteins (Fig. S2 and not shown).

These results suggest that Dpp signaling and *vito* expression are required for *tkv* basal expression in the anterior region of the disc. It is interesting to contrast these results with the observed down-regulation of *tkv* expression in clones expressing Dpp or activated TkvQD in the wing disc (Crickmore and Mann, 2006; Lecuit and Cohen, 1998) and in TkvQD-expressing clones in the eye disc (not shown). These results could imply that the modulation of Tkv levels has a biphasic response where a minimum level of *dpp* signaling might be required for basal expression, and high levels of Dpp signaling repress Tkv expression.

Next, we analyzed the activation pattern of the Dpp pathway transcriptional effector Mad. When *vito*RNAi was driven by *ey*-Gal4, a weaker and irregular pattern of active phosphorylated Mad (p-Mad) was observed near the furrow, even though it was accompanied by a slightly broader activation of p-Mad anteriorly in the disc (compare Fig. 7F-F''2 with 7G-G''2). Furthermore, eye imaginal discs expressing RNAi for *med* or *tkv* present a p-Mad pattern delayed at the eye disc margins, correlating well with the delay in morphogenetic furrow progression in these genotypes (Fig. 7H-H''2 and data not shown). Importantly, co-depletion of *vito* together with *tkv* or *med* resulted in a very strong reduction of p-Mad staining (Fig. 7I,J), a phenotype similar to the one observed with a strong RNAi for Dpp signaling (*put*RNAi2) (Fig. 7K). A uniform MF propagation and ommatidial differentiation requires a re-initiation (reincarnation) of the MF along the margins, with *dpp* function being essential for this localized process (Chanut and Heberlein, 1997; Pignoni and Zipursky, 1997; Wiersdorff et al., 1996). Thus, the significant decrease of Mad activation we observed at the lateral margins of *vito*RNAi eye discs suggests that Vito could be required for the activation of Dpp signaling predominantly at the disc margins (Fig. 7G'1).

vito overexpression partially rescues retina differentiation in *punt* knockdown

Overall, our results show that normal *vito* expression levels are required for Dpp signaling, in particular if Dpp signaling function is compromised. Thus, we decided to test whether an increase in Vito expression levels would be sufficient to rescue Dpp signaling in an RNAi-driven hypomorphic-like situation. Upon *put* knockdown *(ey>put*RNAi2*),* no photoreceptor differentiation was observed in the eye disc and the majority of these flies ecloded without both retinas (77%), or exhibiting only one small retina (23%) (Fig. 8). Remarkably, low-level overexpression of Vito partially rescued the *put*RNAi2 phenotype, being sufficient to promote significant photoreceptor differentiation in the eye disc, and consequently the majority of the adults (65%) ecloded with both retinas (Fig. 8). Overall, these results are consistent with the idea that *vito* is a positive regulator of the Dpp pathway, downstream of Dpp, and that raising Vito levels can partially compensate for a reduction in the activity of Dpp signaling.

DISCUSSION

Different genetic relationships are uncovered by the detection of aggravating synthetic interactions. A pair of genes could act in parallel pathways converging on the same biological process ('between-pathway' interaction), or could either act at the same level or different levels of one pathway ('within pathway' interaction). Ultimately, it is also possible that each gene may act in unrelated processes revealing an indirect interaction, even though the breakdown of the system occurs when both genes are compromised (Costanzo et al., 2011). Within this conceptual framework, here we report the first *in vivo* double-RNAi screen to study genetic interactions during *Drosophila* development, providing evidence for the usefulness of tissue-targeted RNAi screens for the detection

of aggravating synthetic genetic interactions. We performed an *in vivo* double-RNAi screen to uncover genes and pathways functioning with the nucleolar regulator Vito during eye development and twelve interactor genes were identified. Eleven out of the twelve Vito interactor genes identified have been described, or predicted, to be involved in the development of the nervous system. Furthermore, we also identified a significant interaction between *vito* and the Retinal Determination genes *ey*, *eya*, and *so*. However these interactions are weaker (lower interaction scores, Table I) than the interactions between *vito* and Dpp signaling genes. Dpp and Eya (this latter partnering with So, the Six2 homologue) are both required downstream of Hh for retinogenesis (Pappu et al., 2003). Both Dpp and Eya/So are then required for further differentiation of the retina and the repression of the *hth*, a transcription factor that maintains the progenitor state (Bessa et al., 2002; Pichaud and Casares, 2000). Dpp and Hh are also required redundantly to establish So expression (Firth and Baker, 2009). Thus, the interaction between *vito* and the Retinal Determination genes could potentially be a consequence of the significant modulation of Dpp signaling by *vito* hereby described. Interestingly, we observed that *vito* interacts with members of the TGF-β signaling pathway, including the Dpp signaling pathway receptors *tkv* and *put*, but also with members of the Activin signaling branch, such as the R-Smad *smox/dSmad2* and the receptor type-I *baboon*. Recent knowledge about the cross-talk between the Activin and Dpp branches is arising as it was shown that Smox (the Activin dedicated R-Smad) has a role in wing disc growth that requires the function of Mad (Sander et al., 2010). Moreover, it was also reported that Baboon, the type-I activin receptor, is able to phosphorylate the Dpp branch dedicated Mad in a Smox-concentration dependent manner (Peterson et al., 2012). These reports hint at the complex inter-regulation between both branches of the TGF-β signaling, which complicates the detailed analysis of the exact contribution of

vito to the signaling activities of the two branches (Brummel et al., 1999; Lecuit et al., 1996; Nellen et al., 1996; Spencer et al., 1982; Zecca et al., 1995).

Taken together, the data presented here demonstrates that Vito acts downstream of Dpp, having a dual role during eye development: Vito cooperates with Dpp in growth stimulation during early stages of eye disc development and also in later stages during the process of eye disc patterning. Vito/Dpp interaction does not seem to be based on Vito's requirement for survival in the developing eye, because no increase in the number of apoptotic cells was detected when Vito was depleted together with the Dpp receptor *tkv*. We show that the *vito*-Dpp interaction specifically takes place in the context of eye development where we have also shown that *vito* is required for Myc-stimulated growth (Marinho et al., 2011). To assess a potential interaction between the Dpp pathway and Myc in the developing eye, we carried out double-RNAi experiments that revealed a synthetic interaction between *dMyc* and *Med*. However, the interaction observed after co-depleting *vito* and *med* is even stronger (data not shown). These results point to a specific and direct interaction of *vito* with the Dpp signaling pathway that is not simply an indirect effect from the previously described *dMyc*-*vito* interaction. Overall, our data is consistent with a role of Vito in positively regulating Dpp signaling since depletion of Vito partially reverted Dpp overexpression phenotypes, and the phenotype of depleting *vito* in a Dpp weak RNAi background resembled a strong RNAi for a Dpp pathway component. Remarkably, overexpression of low levels of Vito could rescue the absence of differentiation caused by a strong reduction in Dpp activity by *put* RNAi. Moreover, *vito*RNAi eye discs showed a delay in MF progression and an irregular activation of p-Mad within the furrow, which was accompanied with a reduction in Tkv levels. Whether Vito regulates Dpp signaling by direct modulation of Tkv levels, or whether this downregulation of Tkv is an indirect effect due to a

decreased signaling output from Dpp signaling remains an open question. In conclusion, our genetic data reveal that Vito, a nucleolar putative RNA 5'-3' exonuclease, modulates Dpp signaling during fly eye development. Extensively known for its role in ribosome biogenesis, recent studies suggest that the nucleolar sub-nuclear compartment is also linked to cell-cycle and developmental decisions. As an example, Nucleostemin (Tsai and McKay, 2002) is a nucleolar GTP-binding protein with both ribosomal and non-ribosomal roles, and was recently shown to maintain self-renewal of embryonic stem cells (Qu and Bishop, 2012) and to play a role in injured-induced liver regeneration (Shugo et al., 2012). As a further example, differentiation of primary spermatocytes into mature spermatids was shown to require the nucleolar sequestration of Polycomb Repression Complex 1 factors (Chen et al., 2005). Provocatively, one of the reports by The Encyclopedia of DNA Elements (ENCODE) project that surveyed the transcriptome of nuclear subcompartments in the K562 cell line revealed that a small fraction of transcripts with distinct GO-enrichment was unique to the nucleolar compartment (Djebali et al., 2012). Although our genetic data do not reveal the molecular mechanism underlying the Vito/Dpp interaction, it is interesting to note that we have previously shown that alterations of Vito expression levels have profound effects on nucleolar architecture (Marinho et al., 2011). In the face of the dynamic and potentially important role of the nucleolus in the control of gene expression, the interaction between Dpp and *vito* could result from altered expression or sub-nuclear localization of yet to be identified regulators of Dpp signaling when *vito* expression is knocked-down. Thus, further experiments are necessary to elucidate the mechanisms underlying the role of Vito in the modulation of Dpp functions in *Drosophila* eye development.

Materials and Methods

Fly strains and genotypes

All crosses were raised at 25ºC under standard conditions. The following stocks (described in FlyBase, unless stated otherwise) were used: *dpp*-Gal4, dpp^{blk}, *MS1096*-Gal4, *en*-Gal4:UASGFP, sal^{EPv}-Gal4, *ey*-Gal4, UAS-*lacZ*, UAS-dpp^{D}, UAS-*vito*, *dpp3.0-lacZ*, Tkv-YFP (CPTI-002487; Flannotator) and SqhGFP (Sisson et al., 2000). Eye-targeted RNAi knockdown of *vito* was induced by crossing *eyeless*-Gal4 with UAS-*vito*RNAiKK, VDRC #102513. A second UAS-*vito*RNAi transformant (named UAS-*vito*RNAi (VDRC #34548)) observed to generate a very similar eye phenotype was also used throughout experiments.

Double-RNAi screen and genetic interaction scores

All 209 UAS-RNAi lines used in our screen (supplementary table 1) were obtained from VDRC, NIG-Fly stock center (http:/www.shigen.nig.ac.jp/fly/nigfly/index.jsp) and Transgenic RNAi Project (TRiP) at Harvard Medical School. Eye-targeted RNAi knockdown was induced by crossing males from the RNAi stocks carrying an inducible UAS-RNAi construct to virgins of the *eyeless*-Gal4 driver line or *eyeless*-Gal4, UAS-*vito*RNAiKK. All crosses were done at 25ºC. The flies were examined under a stereomicroscope (Stemi 2000, Zeiss) equipped with a digital camera (Nikon Digital Sight DS-2Mv), and several representative pictures for each transgenic line were taken, if significant alterations in eye size were detected. In a primary analysis eye phenotypes with *ey*-Gal4 driver were qualitatively classified as positive (if an eye size reduction was observed), negative (if no phenotype in the eye was observed) or lethal if no progeny was observed. For the crosses with *ey*-Gal4, UAS-*vito*RNAiKK, genetic

interactions were identified by comparing the phenotype of the single RNAis to that of the combined double RNAis. Interactions were classified as small (+) if the phenotype observed was slightly severe than the independent single RNAi phenotypes, medium (++) if there was a significant reduction in the eye size and strong (+++) if the eye was absent. The medium and strong interactions detected in the screen were further analyzed and quantified by measuring the size of the adult retinas in single RNAis and the observed adult size retina obtained in the double RNAi using the using the Polygon selection tool of ImageJ 1.46J (NIH, Bethesda, MA, USA). Genetic interactions scores (π) were calculated based on the differences between the adult retina size observed in the double RNAi and the estimation of the expected double RNAi adult retina size, which was calculated based on a previously described model (Baryshnikova et al., 2010; Dixon et al., 2009). Consequently the expected adult retina size in the double RNAi is the result of the product between the two individual adult retina size values for the single RNAis. In our screen, deviations between the expected and the experimentally observed double RNAi phenotype revealed only negative genetic interactions (aggravating).

Interaction Map

Vito interaction map was done using Cytoscape 2.7 (http://www.cytoscape.org/) and interaction data generated using the DroID-Plugin for Cytoscape (Murali et al., 2011), which provides access to the *Drosophila* Interactions Database (DroID) from within the Cytoscape environment.

Immunostaining

Eye-antennal imaginal discs and salivary glands were prepared for

immunohistochemistry using standard protocols. Primary antibodies used were: rabbit anti-cleaved Caspase-3 at 1:200 (Cell Signaling), mouse anti-Armadillo N27A1 at 1:100 (Developmental Studies Hybridoma Bank, DSHB), rabbit anti-β-galactosidase at 1:1000 (Cappel), rat anti-Elav 7E8A10 at 1:100 (DSHB), rabbit anti-p-Mad at 1:100 (gift from Ginés Morata), guinea-pig anti-Otd at 1:750 (Ranade et al., 2008). Appropriate Alexa-Fluor conjugated secondary antibodies were from Molecular Probes. Images were obtained with the Leica SP2 confocal system and processed with Adobe Photoshop.

3D histograms

Tkv-YFP 3D histograms were done using the SurfacePlot_3D plugin from the ImageJ 1.46J software (NIH, Bethesda, MA, USA) (http://rsb.info.nih.gov/ij/plugins/surface-plot-3d.html). This plug-in creates interactive surface plots where the luminance of each pixel in the image is interpreted as the height for the plot.

Size measurements and statistics

Eye disc areas were measured using the Polygon selection tool of ImageJ 1.46J software (NIH, Bethesda, MA, USA), considering only the eye disc from the eye-antennal imaginal disc from at least 20 discs for each genotype. GraphPad Prism 5.0 was used for statistical analysis and generating the graphical output. Statistical significance was determined using an unpaired, two-tailed Student's *t*-test, with a 95% confidence interval, after assessing the normality distribution of the data with D'Agostino-Pearson normality test.

Figure legends

Figure 1 – Targeted double-RNAi screen design to identify Vito genetic interactions. (A) Eye-targeted double-RNAi screen methodology. **(B)** Functional classification of the 148 *ey*-induced genes tested, based on Gene Ontology (GO) annotations with further manual Flybase data mining. **(C)** Significantly enriched Gene Ontology terms (*P*-value <0.01) for the 148 *ey*-induced genes tested. The number of genes belonging to each annotation is shown. **(D)** A multiplicative model was used to classify genetic interactions as either neutral or enhancement according to the genetic interaction score (π). The π-score is the difference between the expected double-RNAi retina size (the product of the individual-RNAi retina sizes) and the measured double-RNAi retina size. Retina sizes are expressed as fractions of control retina size. Genetic interactions (positive enhancements) are the ones displaying positive π-scores.

Figure 2 – Analysis of the Vito genetic interactions. (A) Adult eye phenotypes of the individual and double RNAis for interactions identified in the screen**.** For Dpp and Activin signaling pathways only an example is shown. **(B)** *vito* interacts strongly with Retinal Determination genes and Dpp signaling members. Synthetic genetic interactions are revealed by significant differences between experimental adult retinal sizes (red bars) and expected values (blue bars). Sizes are displayed as fractions relative to control retinal size values. Asterisks indicate absent eyes. **(C)** Cytoscape network of direct interactions between all the Vito interactor genes and Vito itself. Genes/Proteins are indicated by circular nodes and interactions by lines or edges. In red, Retinal determination proteins; in dark blue, proteins belonging to the TGF-β signaling pathway; and in light blue the remaining Vito interactors. When compared to the entire

Drosophila genome, this group of genes has significant GO term enrichment in nervous system development (P=2.97X10^{-11}) (orange), eye development (P=1.45X10^{-7}), and/or eye-antennal disc development (P=2.75X10^{-8}).

Figure 3 – *vito* genetically interacts with the Dpp pathway. (A) Lateral views of adult eyes and the corresponding eye imaginal discs of the indicated genotypes stained for cellular membrane (anti-Armadillo, red) and photoreceptors (anti-Elav, green). Downregulation of *vito* (*ey*-Gal4/UAS-*vito*RNAi;+) causes a reduced eye phenotype and affects the size of the imaginal disc when compared to control. RNAi knockdown of *tkv* (*ey*-Gal4/UAS-*tkv*RNAi1; +) or *med* (*ey*-Gal4/+; UAS-*med*RNAi1/+) causes a small reduction in adult eye size and a delay in retinal differentiation at the lateral margins of the eye disc. Retinal differentiation is absent in discs or retinas co-expressing an RNAi against *tkv* and *vito* (*ey*-Gal4, UAS-*vito*RNAi/UAS-*tkv*RNAi1), or *med* and *vito* (*ey*-Gal4, UAS-*vito*RNAi/+; UAS-*med*RNAi1/+). **(B)** Eye disc sizes of the indicated genotypes were measured and represented as a distribution. Dots represent individual measurements and horizontal bars show mean values.

Figure 4 – Vito is required for eye disc growth and patterning when Dpp signaling is compromised. (A, B) Downregulation of Dpp signaling using a weak RNAi line against *put* (*ey*-Gal4/UAS-*put*RNAi1), does not produce any visible phenotype in adult eyes or L3 eye discs; **(C, D)** Co-expression of RNAis against *put* and *vito* (*ey*-Gal4, UAS-*vito*RNAi/UAS-*put*RNAi1) induces a reduction in adult retina size and a delay in retinal differentiation; **(E, F)** dpp^{blk} mutant fly present a reduced adult eye, as a result of an impairment of morphogenetic furrow progression at eye imaginal disc margins; **(G, H)** A strong downregulation of Dpp signaling using *put*RNAi2 (*ey*-Gal4/+; UAS-

*put*RNAi2/+) results in adult flies without retinas. Despite *put*RNAi2 eye imaginal discs can growth similar to controls, they were not able to start and progress differentiation; **(I, J)** Co-expression of *vito*RNAi in these flies (*ey*-Gal4, UAS-*vito*RNAi/+; UAS-*put*RNAi2/+) leads to synthetic lethality, with the flies reaching the pupal phase without head formation. A strong tissue growth deficit can be observed in the corresponding eye imaginal discs. All eye imaginal discs were stained for photoreceptors (anti-Elav, green) and cellular membrane (anti-Armadillo, red).

Figure 5 –*vito*/Dpp genetic interaction is not explained by an increase in apoptosis in the eye imaginal disc. (A) Third-instar *Drosophila* eye imaginal discs of the indicated genotypes stained for Armadillo (red) and cleaved Caspase-3 (green). Depleting *vito* (*ey*-Gal4, UAS-*vito*RNAi/+) causes significant cell death in the anterior domain of the eye disc when compared to control discs (*ey*-Gal4/UAS-*lacZ*;+). Reduced Dpp signaling by expression of *tkv*RNAi (*ey*-Gal4/UAS-*tkv*RNAi1;+) also caused cell death anteriorly in the eye disc. In discs co-expressing *tkv*RNAi and *vito*RNAi (*ey*-Gal4, UAS-*vito*RNAi/UAS-*tkv*RNAi1) no significant differences in the number of apoptotic cells are seen comparing to *vito*RNAi. **(B)** Quantification of cell death assessed by the number of cleaved Caspase-3 positive cells in the eye discs in A. Data are presented as the mean + s.e.m (n=19-24).

Figure 6 – Vito depletion rescues lethality and ectopic differentiation induced by Dpp overexpression. (A) Head dorsal views of: control (*ey*-Gal4; UASmyrRFP/ UAS-*lacZ*); overexpression of Dpp (*ey*-Gal4; UASmyrRFP/+; UAS-dpp^{D}/+); overexpression of Dpp in the absence of *vito* (*ey*-Gal4, UAS-*vito*RNAi/+; UAS-dpp^{D}/+); **(B)** Percentage of lethality after Dpp overexpression together with an unrelated UAS or after

overexpression of Dpp jointly with *vito* depletion. **(C)** Eye discs of the above genotypes were stained for photoreceptors (anti-Elav, red) and DNA (DAPI, blue) (top row), or stained for Otd (green) and Armadillo (red) (bottom row). **(D)** Eye disc areas of the indicated genotypes were measured and represented as a distribution using as reference the mean value of the *ey>dpp^{D}* eye discs area. Dots represent individual measurements and horizontal bars show mean values (n=14-15). (***, $P<1x10^{-4}$ relative to overexpression of Dpp and myrRFP). **(E)** The percentage of undifferentiated area in the eyes discs was measured for the corresponding total eye disc area. Dots represent individual measurements and horizontal bars show mean values (n=14-15). (***, $P<1x10^{-4}$ relative to overexpression of Dpp). **(F)** Vito depletion does not change Dpp overexpression phenotype in the wing**.** Adult wings of control (*MS1096*; UAS-*lacZ*/+), Dpp overexpressing (*MS1096*;;UAS-*dpp^{D}*/+), and Dpp overexpression without Vito (*MS1096*;UAS-*vito*RNAi/+;UAS-*dpp^{D}*/+) flies.

Figure 7 – Vito is required to maintain Tkv receptor levels and to restrict the dpp signal to the morphogenetic furrow. (A-A''2) Wild-type eye imaginal disc development from 2nd- to 3rd-instar larvae reporting *dpp* expression and photoreceptor differentiation (dppZ in green and ELAV in red). **(B-B''2)** Dynamic progression of *dpp* expression in *vito*-depleted eye imaginal discs during larvae development. Similarly to wild type, a *dpp* expressing stripe initiates from the posterior margin and progresses throughout the eye disc to the anterior region, however *vito*RNAi discs fail to shutdown *dpp* expression at the eye imaginal disc lateral margins (B'1-B''2, white arrows). **(C-E)** A Tkv-YFP protein trap exposes Tkv endogenous levels in 3rd instar larva imaginal discs (TkvYFP is represented in green). **(C'1-E'1)** 3D histograms depict the pixel intensity of the receptor Tkv at eye imaginal disc of the referred genotypes (The colours

represent a thermal scale: blue is the lower pixel intensity value and red the higher pixel intensity value). **(C, C'1)** In a control situation Tkv is particularly enriched at the differentiated region and at the medial anterior region. **(D, D'1)** *ey>vito*RNAi imaginal disc presents a decrease in Tkv levels anteriorly to the MF that can be observed even more clear in the 3D histogram. **(E, E'1)** *ey>med*RNAi shows a decrease in Tkv levels at the eye imaginal disc margins, the region where the differentiation is delayed. **(F-K)** Activation of the Dpp signaling cascade represented by phosphorylation of Mad (p-Mad is represented in green and Armadillo in red). **(F)** Control discs show a clear p-Mad staining with two major bands, a more sharp and intense closer to the MF and a broader with a less intense signal at the anterior region. **(F'1)** Higher magnification 3D histogram shows the regular p-Mad staining at the control eye imaginal disc margin. **(F''2)** Higher magnification 3D histogram of the eye imaginal disc medial plane presents a continuous p-Mad staining. **(G)** Absence of *vito* results in an irregular signal of p-Mad. **(G'1)** 3D histogram representing a higher magnification of *ey>vito*RNAi imaginal disc margin shows that p-Mad signal is irregular and virtually absent. **(G''2)** In the absence of *vito*, p-Mad staining 3D histogram reveals a low and irregular intensity of p-Mad staining at an eye imaginal disc medial plane. **(H)** Depletion of *tkv* by RNAi results in a p-Mad staining delayed at the eye imaginal disc margins. **(H'1)** Higher magnification 3D histogram shows the delayed p-Mad staining at the *tkv*-depleted eye imaginal disc margin. **(H''2)** Higher magnification 3D histogram of the eye imaginal disc medial plane presents an uneven p-Mad staining in the absence of *tkv*.**(I)** Co-depletion of *vito* and *tkv* completely suppresses the p-Mad staining, likewise a strong RNAi for *put* (*put*RNAi2) **(K)**. **(J)** Simultaneously depletion of *vito* and *med* also suppresses Mad phosphorylation.

Figure 8 – Overexpression of Vito restores retina differentiation in *put* loss-of-function flies. (A) Lateral views of adult eyes and the corresponding eye imaginal discs of the indicated genotypes stained for Armadillo (red) and Elav (green). Flies overexpressing Vito do not exhibit eye phenotype. Loss of *put* flies lack differentiation that is partially restored by Vito overexpression. **(B)** Percentage of adult flies presenting 2 retinas, 1 retina or without (w/o) retinas (n=57-81). >UAS*vito* (*ey*-Gal4/+; UAS-*vito*/+); >*put*RNAi (*ey*-Gal4/+; *put*RNAi2/+); >*put*RNAi + UAS*vito* (*ey*-Gal4/+; UAS-*vito*/ *put*RNAi2)**.**

Table I – List of Vito genetic interactions. This table shows information about the genes identified in the screen as Vito interactors, as well as quantifications of retina sizes of the individual RNAis, expected phenotypes based on the multiplicative model, and the measured retina sizes in the double RNAis. Genetic interaction scores (π) were calculated as described in materials and methods. * Expected retina sizes were calculated based on the value for *vito*RNAi retina size of 0.70. n.d. not determined.

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Highlights:

We performed an eye-targeted double-RNAi screen for Vito interactors.

vito interacts genetically with members of the Dpp signaling pathway.

Dpp-dependent eye disc growth and differentiation is modulated by Vito.

Nucleolar Vito is required for correct Dpp signaling in eye primordium.

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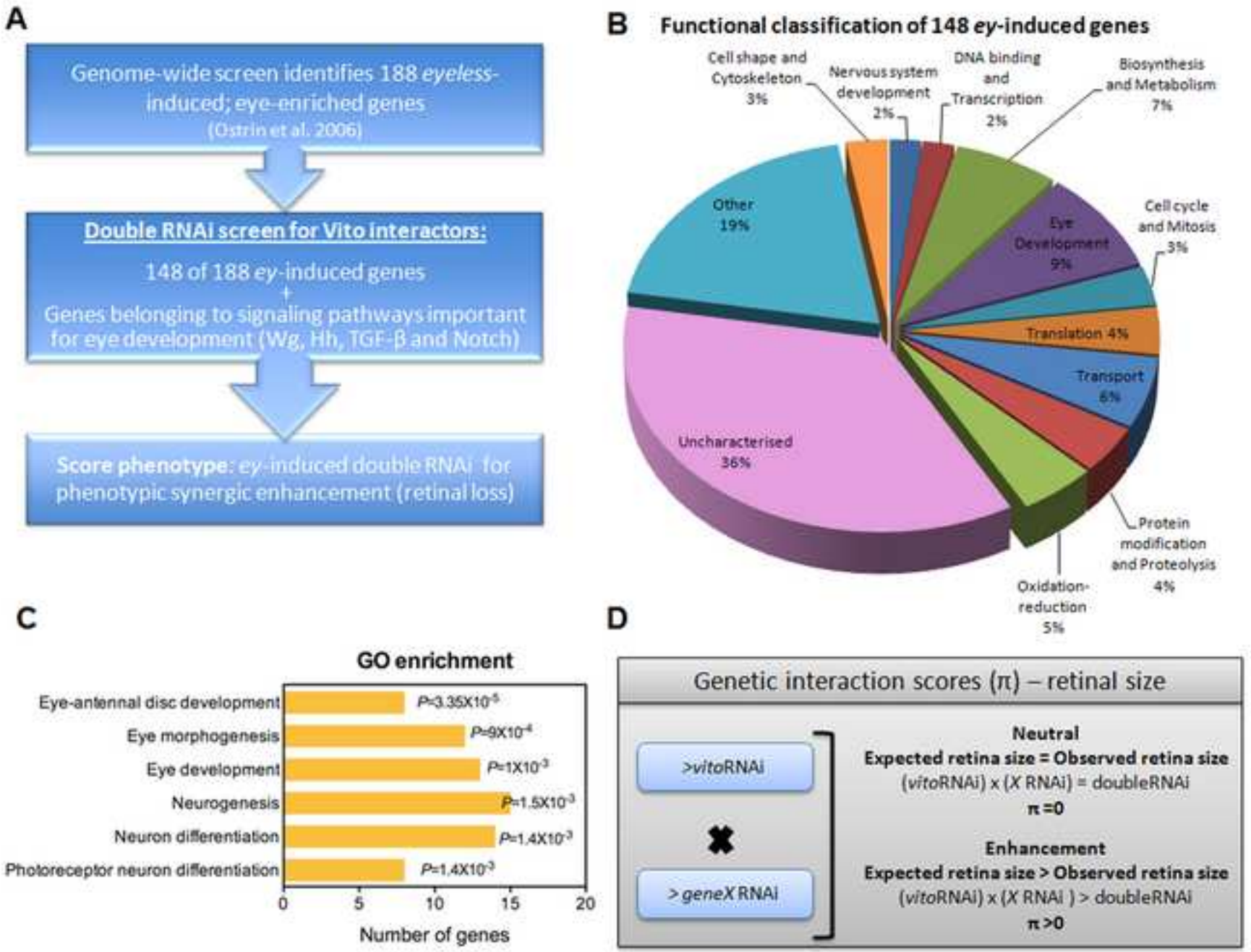
Figure 1



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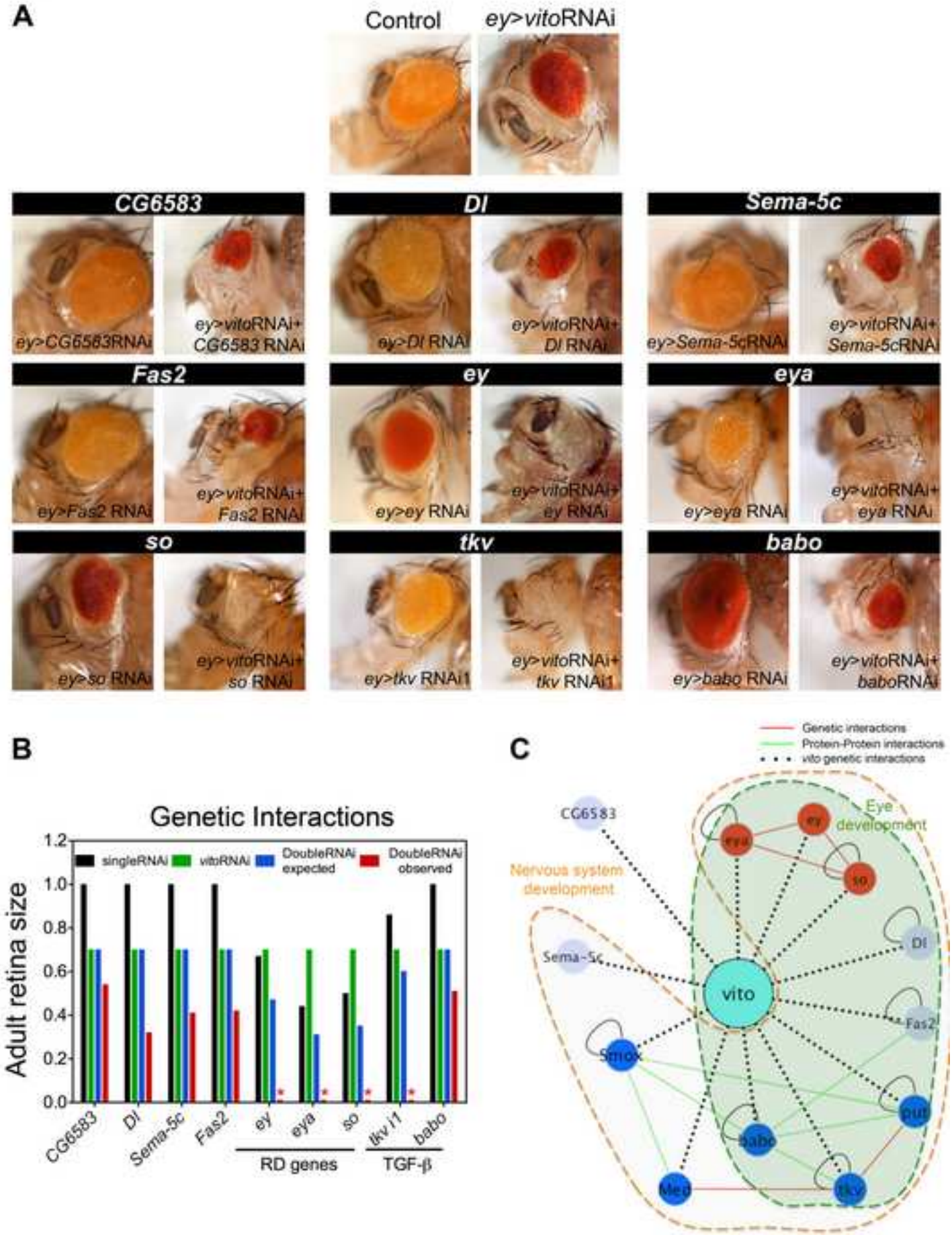

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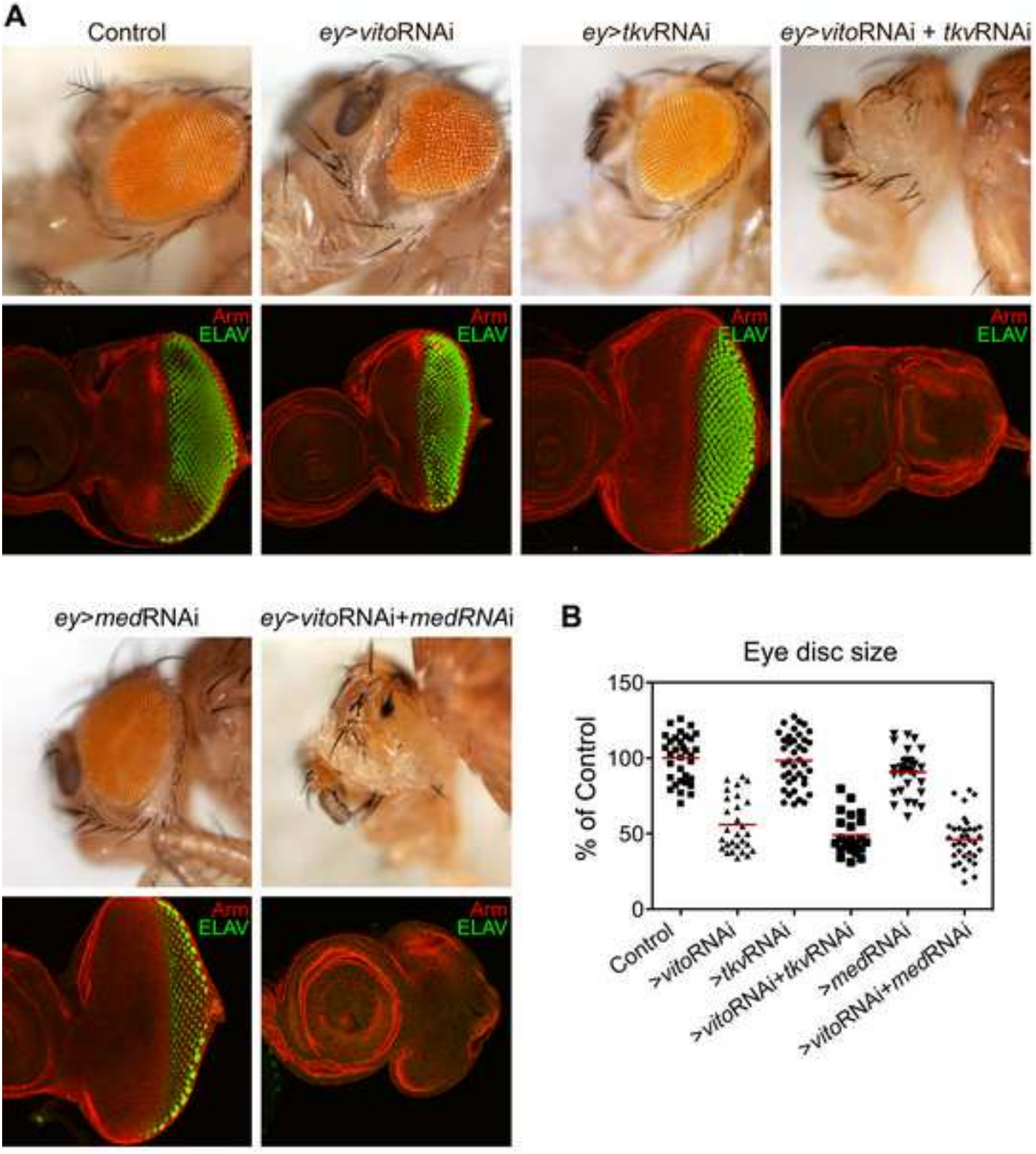

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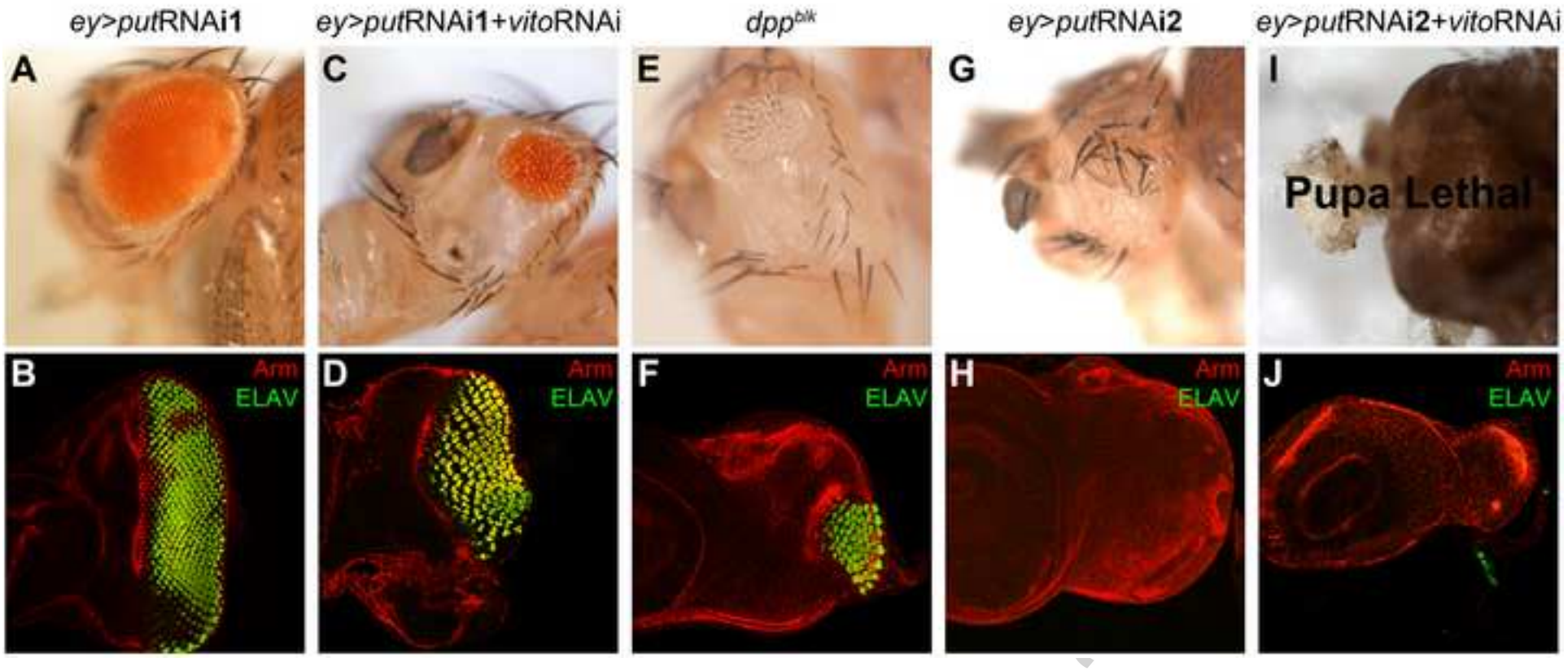

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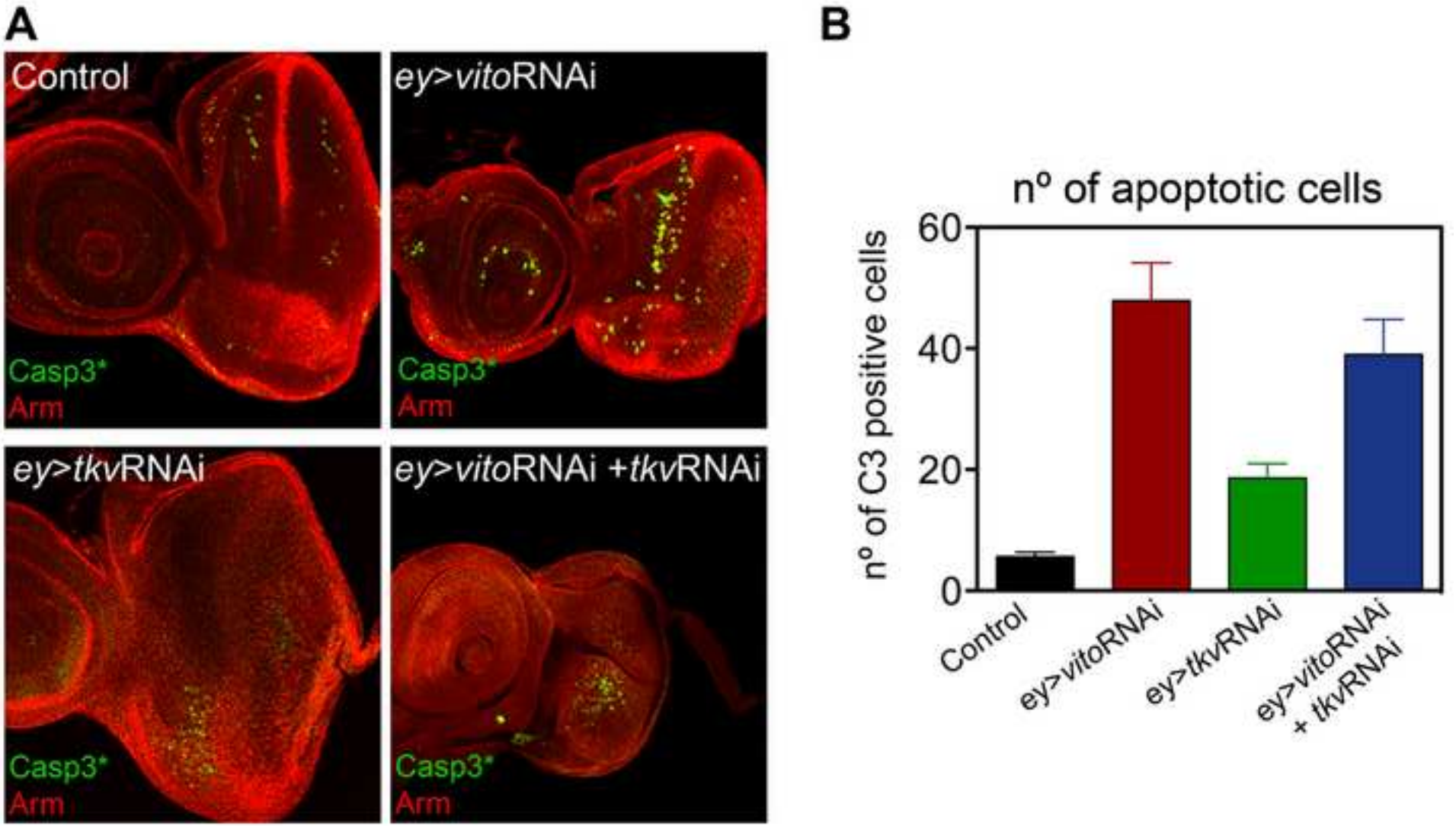

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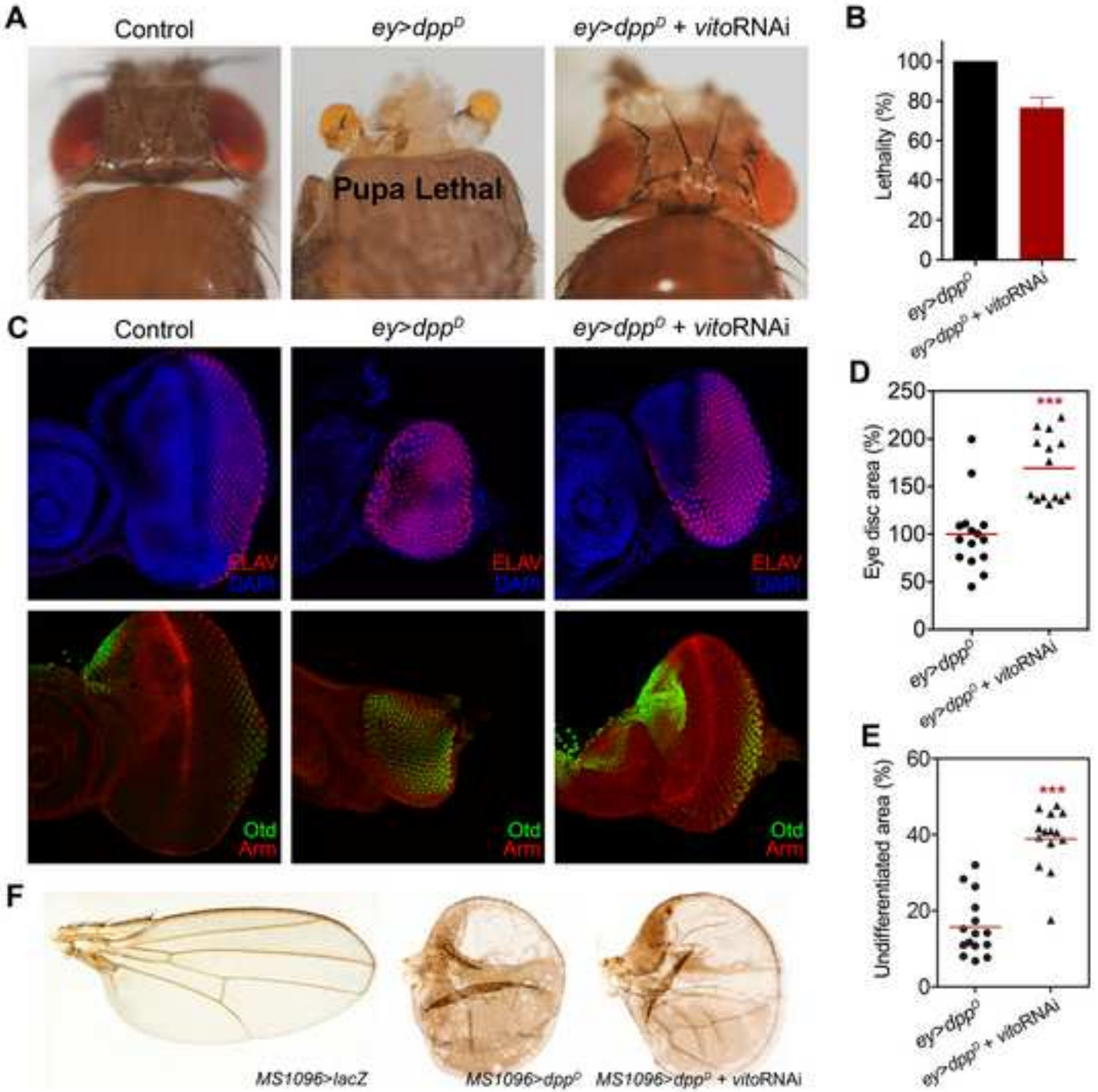

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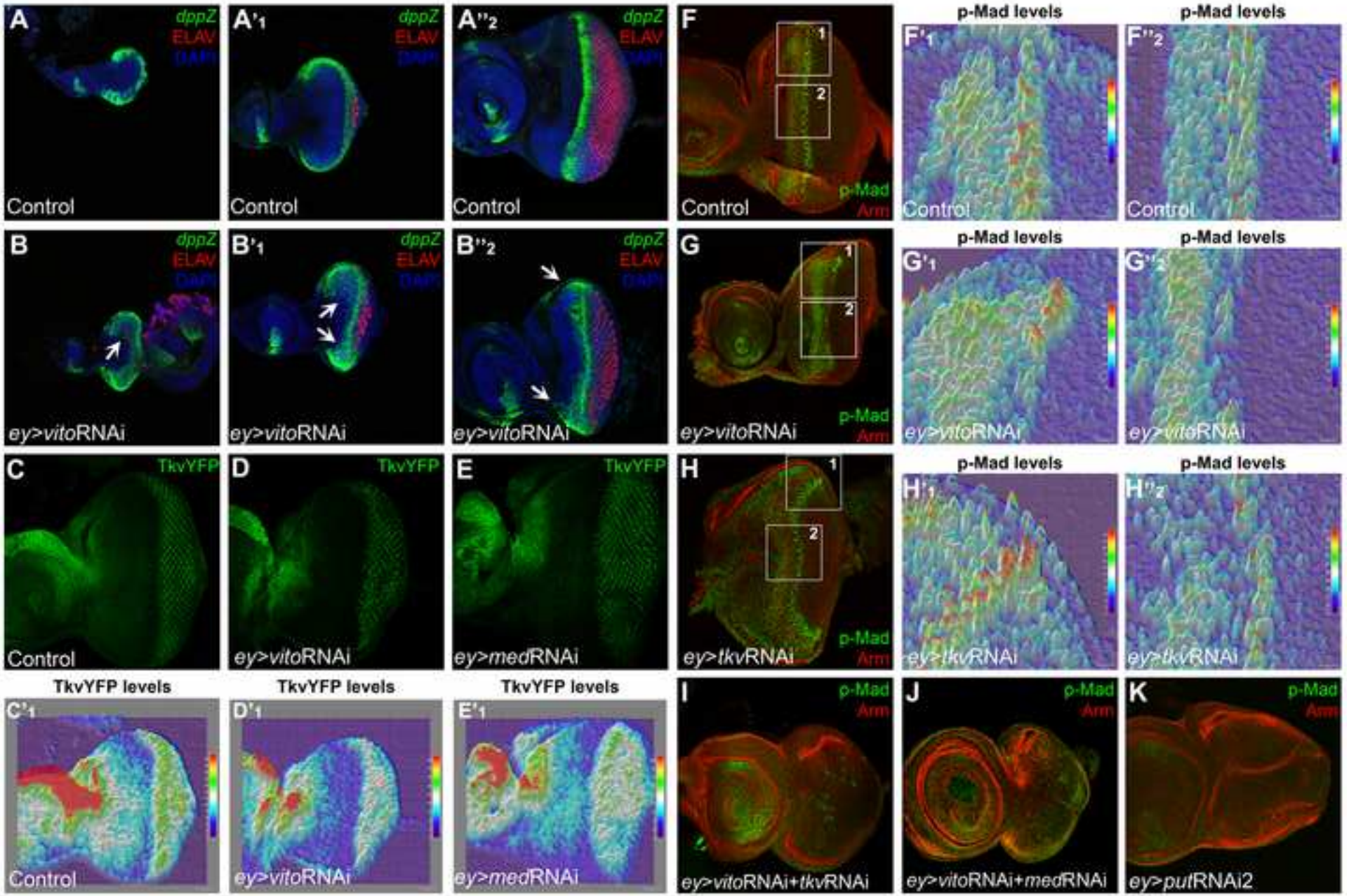

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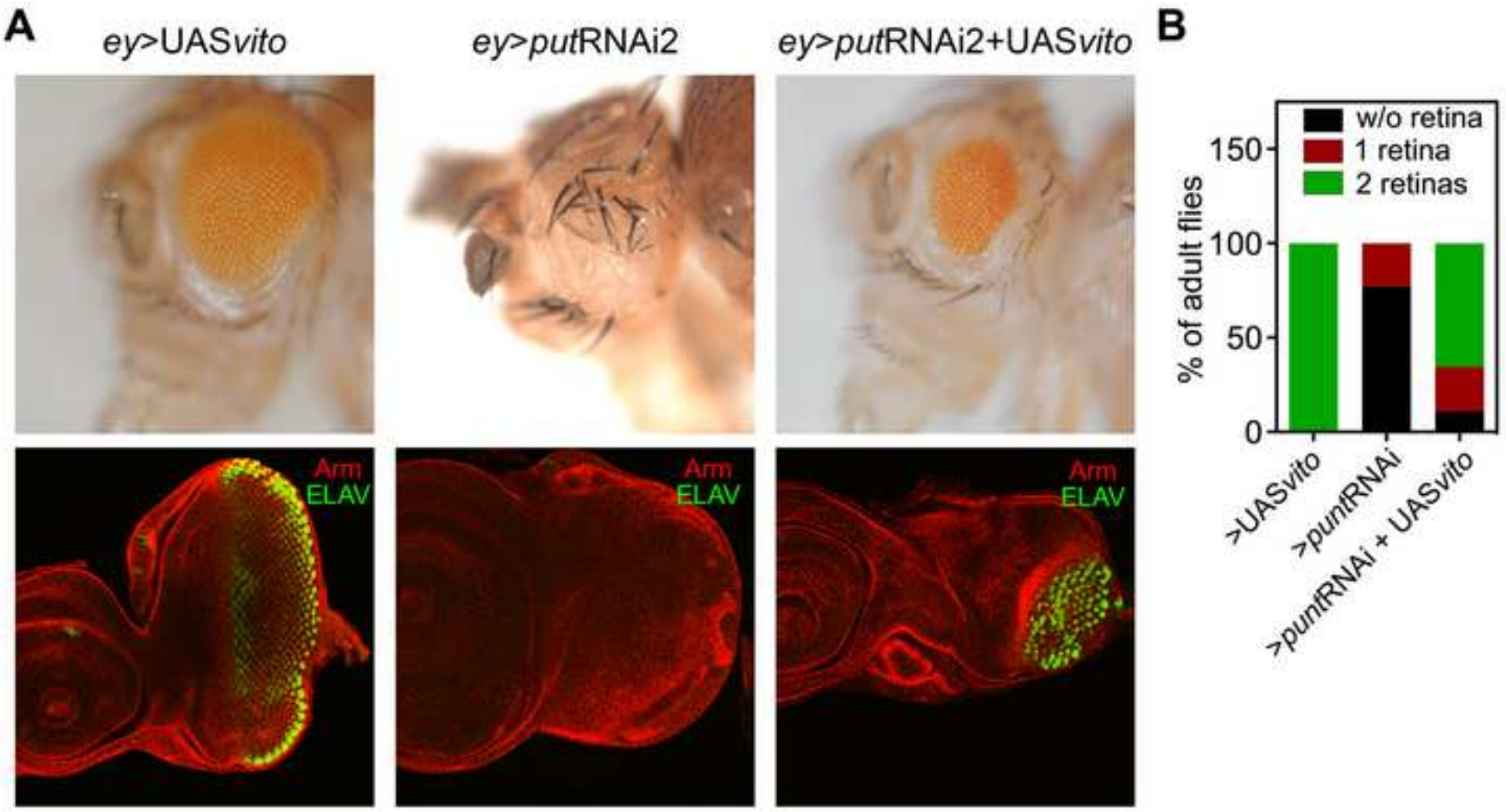

Table1

Table I

		Gene ID	Symbol	Gene name	Function	Single RNAi (retina size)	Double RNAi *vito* RNAi X singleRNAi (expected retina size)*	Double RNAi (observed retina size)	Interaction score (π)
eyeless-induced, eye-enriched genes		CG6583	CG6583		Unknown	1.00	0.70	0.54	0.16
		CG3619	Dl	Delta	Notch binding	1.00	0.70	0.32	0.38
		CG5661	Sema-5c	Semaphorin-5c	Receptor activity	1.00	0.70	0.41	0.29
		CG3665	Fas2	Fasciclin 2	Protein binding	1.00	0.70	0.42	0.28
		CG1464	ey	eyeless	DNA binding/transcriptional regulator	0.67	0.47	0.00	0.47
		CG9554	eya	eyes absent	Protein tyrosine phosphatase activity	0.44	0.31	0.00	0.31
		CG11121	so	sine oculis	DNA binding	0.50	0.35	0.00	0.35
TGF-β signalling pathway	Dpp pathway	CG14026	tkv	thickveins	Transforming growth factor beta receptor activity, type I	0.74	0.52	0.00	**0.52**
		CG7904	put	punt	Transforming growth factor beta receptor activity, type II	0.00	0.00	Synthetic lethal	n.d.
		CG1775	Med	Medea	Sequence-specific DNA binding	0.95	0.67	0.00	**0.67**
	Act-β	CG8224	babo	baboon	Activin receptor activity, type I	1.00	0.70	0.51	0.19
		CG2262	Smox	Smad on X	Sequence-specific DNA binding	1.00	0.70	0.51	0.19