Ligand-independent activation of the Hedgehog pathway displays non-cell autonomous proliferation during eye development in *Drosophila*

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Abstract

Deregulation of the Hedgehog (Hh) signaling pathway is associated with the development of human cancer including medullobastoma and basal cell carcinoma. Loss of Patched or activation of Smoothened in mouse models increases the occurrence of tumors. Likewise, in a *Drosophila* eye model, deregulated Hedgehog signaling causes overgrowth of eye and head tissues. Surprisingly, we show that cells with deregulated Hh signaling do not or only little contribute to the tissue overgrowth. Instead, they become more sensitive to apoptosis and may eventually be eliminated. Nevertheless, these mutant cells increase proliferation in the adjacent wild-type tissue, i.e., in a non-cell autonomous manner. This non-cell autonomous effect is position-dependent and restricted to mutant cells in the anterior portion of the eye. We also observe precocious non-cell autonomous differentiation in genetic mosaics with deregulated Hh signaling. Together, these non-cell autonomous growth and differentiation phenotypes in the *Drosophila* eye model reveal another strategy by which oncogenes may generate a supportive micro-environment for tumor growth.

Keywords

Hedgehog signaling; Costal-2; Patched; Non-cell autonomous overgrowth

1. Introduction

The Hedgehog (Hh) pathway is an important cell/cell signaling pathway in both vertebrates and invertebrates (reviewed in (Huangfu and Anderson, 2006; Ingham, 2008; Jiang and Hui, 2008). It was initially discovered in *Drosophila melanogaster* where it is required for embryonic segmentation (Nusslein-Volhard and Wieschaus, 1980). Since then, the Hh

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pathway has been shown to be involved in many biological processes including patterning, cell proliferation and cell fate specification as well as morphogenesis and homeostasis (Huangfu and Anderson, 2006; Jiang and Hui, 2008; Kalderon, 2005). In humans, deregulated, i.e., increased Hh signaling is associated with various cancers including basal cell carcinoma, medulloblastoma, rhabdomyosarcoma, glioma as well as breast, colorectal, pancreatic and prostate cancer (Jiang and Hui, 2008; Teglund and Toftgard, 2010). Therefore, a comprehensive understanding of the biology and pathology of the Hh pathway is essential for the development of treatments of these diseases.

The Hh pathway controls the activity of the transcription factor Cubitus interruptus (Ci; Gli in mammals) (Aza-Blanc et al., 1997). In the absence of Hh, the transmembrane protein Patched (Ptc; Ptch1 in mammals) keeps another transmembrane protein, Smoothened (Smo), in intracellular vesicles (Denef et al., 2000; Ingham et al., 2000; Nakano et al., 2004; Stegman et al., 2004). The absence of Smo enables several kinases including PKA, GSK-3 and CK1 to phosphorylate Ci (Chen et al., 1998; Price and Kalderon, 1999; Price and Kalderon, 2002; Sisson et al., 2006; Zhang et al., 2005) and mark it for ubiquitylation by the Slimb ubiquitin ligase (Jia et al., 2005; Jiang and Struhl, 1998; Noureddine et al., 2002; Ou et al., 2002; Smelkinson and Kalderon, 2006; Smelkinson et al., 2007). Ubiquitylation triggers partial degradation of newly synthesized full length Ci of 155kD (Ci155) to a 75kD protein (Ci75) that acts as a transcriptional repressor of Hh gene targets (Aza-Blanc et al., 1997; Methot and Basler, 1999; Wang and Price, 2008). This proteolysis occurs in a protein complex composed of Ci, the protein kinase Fused and Costal-2 (Cos2) which is a kinesin-like protein with similarity to Kif-7 in mammals (Cheung et al., 2009; Endoh-Yamagami et al., 2009; Farzan et al., 2008; Ogden et al., 2004; Robbins et al., 1997; Ruel et al., 2007; Sisson et al., 1997; Wang and Holmgren, 2000). Upon binding of Hh to its receptor Ptc, Smo translocates to the plasmamembrane and interacts with Cos2 to release full length Ci (Ci155). The release of Ci155 is triggered by phosphorylation of Cos2 and Smo by Fused (Jia et al., 2003; Liu et al., 2007; Lum et al., 2003; Nybakken and Perrimon, 2002; Nybakken et al., 2002; Ruel et al., 2007; Ruel et al., 2003; Zhu et al., 2003). Ci155 can now act as a transcriptional activator of Hh target genes.

Genetically, *ptc*, *cos2* and *PKA* are negative regulators of the Hh pathway. Loss of these genes results in accumulation of Ci155 and promotes ligand-independent, deregulated Ci activity (Chen and Struhl, 1996; Jiang and Struhl, 1995; Li et al., 1995; Pan and Rubin, 1995; Sisson et al., 1997; Thomas and Ingham, 2003; Wang and Holmgren, 1999). Similarly, in humans, ligand-independent Gli-induced tumors are caused by loss of *Ptch1* or by gain-of-function mutations of *Smo*. Gain-of-function mutations of *Gli* transcription factors can also contribute to tumors, most notably glioma (Jiang and Hui, 2008; Teglund and Toftgard, 2010).

Hh signaling is crucial for development of the *Drosophila* compound eye, which depends on a changing balance of proliferation and differentiation (Baker, 2007; Carthew, 2007; Roignant and Treisman, 2009). During the first two larval stages, the eye-antennal imaginal disc proliferates extensively (Carthew, 2007; Wolff and Ready, 1991a). In the 3rd larval stage (L3), cells at the posterior edge of the eye disc form a groove, called the morphogenetic furrow (MF) (Wolff and Ready, 1991a). For the following two days, the MF moves anteriorly across the eye disc. Cells at the MF arrest proliferation and the first five photoreceptor neurons per ommatidium begin to differentiate (Baker, 2007; Carthew, 2007; Roignant and Treisman, 2009). While the MF moves on, the remaining cells undergo one additional round of proliferation (second mitotic wave), before they permanently arrest proliferation and differentiate into additional photoreceptor neurons, cone, pigment and bristle cells (Baker, 2007; Carthew, 2007; Roignant and Treisman, 2009). After the MF stops in the early pupal stage, the cells anterior to the MF differentiate into head cuticle.

Hh signaling is required for movement of the MF across the eye disc (Heberlein et al., 1995; Heberlein et al., 1993). Photoreceptor neurons posterior to the MF express Hh, which induces *decaplentaplegic* (*dpp*) expression (Greenwood and Struhl, 1999; Heberlein et al., 1993). Both Hh and Dpp diffuse to anteriorly located cells which Dpp arrests in G1 (Firth and Baker, 2005; Horsfield et al., 1998). In turn, these cells start to differentiate and produce Hh, just pushing the MF further anteriorly. Posterior to the MF, Hh promotes the second mitotic wave through expression of the Notch ligand Delta and thus Notch signaling (Baonza and Freeman, 2005; Firth and Baker, 2005). Thus, the Hh pathway is needed for the transition from proliferating to differentiating state of the eye disc, making it a critical target for homeostasis.

Hh signaling is also known to regulate proliferation (Chanut and Heberlein, 1995; Duman-Scheel et al., 2002; Heberlein et al., 1995). Consistently, we show here that deregulated, ligand-independent Hh signaling due to loss of the negative regulators *cos2* and *ptc* causes overgrowth phenotypes of mosaic eyes and heads. Paradoxically, however, *cos2* and *ptc* mutant cells have a growth-disadvantage and are eventually eliminated by apoptosis. In mosaic discs, proliferation is increased at the border to adjacent $cos2^+$ tissue suggesting that the overgrowth is mediated through induction of non-cell autonomous proliferation. This effect is position-dependent and restricted to *cos2* clones in or anterior to the MF. Finally, we demonstrate that *cos2* clones not only cause non-cell autonomous precocious proliferation, but also non-cell autonomous differentiation. Together, these non-cell autonomous growth and differentiation phenotypes in the *Drosophila* eye model reveal another strategy by which oncogenes may generate a supportive micro-environment for tumor growth.

2. Results

2.1. *cos2* mosaics display non-cell autonomous overgrowth

In a mutagenesis screen (see Experimental Procedures), we isolated three independent alleles of the Hh pathway gene *costal-2* (*cos2*). In mosaics induced by the *ey-FLP/FRT* system, the three alleles behaved similarly and generated overgrowth of the eye (shown for one allele in Fig. 1A, B). Surprisingly, when comparing the relative representation of *cos2* mutant tissue (marked in white due to loss of the $white^+$ (w^+) pigment transgene) and the wild-type or heterozygous tissue (referred to as $cos2^+$ and marked in red due to the presence of the w^+ pigment transgene), we noted that nearly the entire overgrown eye was red, i.e., $cos2^+$ (Fig. 1 A, B), suggesting that this overgrowth was non-cell autonomous. Often, the red eyes had small portions of white *cos2* mutant tissue, indicating that the *cos2* clones were viable, but had a growth disadvantage over $cos2^+$ tissue. In these mosaic eyes, the ommatidia were frequently roughened and expanded along the anterior margin even when this region was red (and therefore $cos2^+$, Fig. 1A, B).

In addition to the effect on the eye itself, overgrowth was also seen in head cuticle and the antennae (Fig. 1E, F). Like the eye, the head and antennal structures developed (often with duplication of structures), indicating that differentiation occurred in these mosaics. Indeed, similar overgrowths with pattern duplications were seen in leg tissues (Suppl. Fig. S1) as well as wing tissues (Sisson et al., 1997) when those developing tissues were mosaic for *cos2*, indicating that the overgrowth was not an eye-specific effect.

2.2. The Hh pathway modulates proliferation non-cell autonomously

Overgrowth phenotypes of the eye have been seen with mutants that increase Hh signaling including *ptc* (Chanut and Heberlein, 1995; Ma and Moses, 1995; Wehrli and Tomlinson, 1995), *pka-C1* (Johnson et al., 1995; Pan and Rubin, 1995; Shyamala and Bhat, 2002; Strutt

and Mlodzik, 1997; Strutt et al., 1995) and ectopic expression of *hh* (Heberlein et al., 1995), while mutations that reduce Hh signaling such as *smo* have the opposite effect (Strutt and Mlodzik, 1997). These phenotypes have been attributed to altered regulation of the formation of the MF in the eye imaginal disc and the subsequent precocious formation of ommatidia. However, the potential non-cell autonomy of these overgrowth phenotypes as observed in *cos2* mosaics has not been reported.

To determine whether the non-cell autonomy was a *cos2*-specific effect or general phenotype of deregulated Hh signaling, we tested mutants of other negative regulators of the Hh pathway for similar non-cell autonomous phenotypes. Like *cos2*, *ey-FLP*-induced mosaics of *ptc* and *pka-C1* display non-cell autonomous overgrowth as seen by the predominantly red mosaic eye with the greatest effect at the anterior midline of the eye (Fig. 1C, D). Despite the overgrowth of *ptc* mosaic eyes, we never recovered any *ptc* mutant clones in adult flies indicating a strong non-autonomous effect. These mosaics also generated overgrowth of the head cuticle and antennae similar to *cos2* (shown for *ptc* in Fig. 1G).

In contrast to these mutants, mosaic loss of *smoothened* (*smo*), a gene required for Hh signaling, in the developing eye formed eyes that were smaller with over-represented smo^+ (red) tissue (Fig. 1H). *smo* mutant tissue is present, but small presumably due to increased cell death (Vrailas and Moses, 2006). However, eyes less than half the size of wild type suggests that the loss of *smo* mutant cells cannot be compensated for by new proliferation as it does in mosaics with cell-lethal mutations (see for example Fig. 3B). This observation implies that, in *smo* mosaics, a Hh-derived signal is missing that is required to increase proliferation of the adjacent tissue. In summary, these observations support the notion that Hh signaling controls cell proliferation non-cell autonomously during eye development. Because *cos2* mosaics showed the strongest non-cell autonomous effects, we largely focus on *cos2* for the characterization of the non-cell autonomous phenotypes.

2.3. *cos2* acts via the canonical Hh pathway to promote non-cell autonomous proliferation

Next, we asked if *cos2* regulates non-cell autonomous proliferation through the canonical Hh pathway, i.e., through Cubitus interruptus (Ci). Activation of the Hh signaling pathway normally blocks the proteolytic processing of this transcription factor, retaining the active, full-length Ci protein (Ci155), while preventing the formation the transcriptional repressor form (Ci75; see Introduction). Recent work in vertebrate systems has indicated that Hh, Ptc and their homologues can act independently of Gli transcription, i.e., in a non-canonical manner (Jenkins, 2009). We examined whether the overgrowth phenotype of *cos2* mosaics is mediated through the canonical pathway, i.e., through Ci155. First, it has been reported that Ci155 accumulates in *cos2* clones (Methot and Basler, 2000; Sisson et al., 1997; Wang et al., 2000; Wang and Holmgren, 1999; Wang and Holmgren, 2000) suggesting that proteolytic processing of Ci155 is blocked due to loss of Cos2 function.

Second, we determined if expression of the repressor form Ci75 can suppress the overgrowth phenotype of *cos2* mosaics using the MARCM system (Lee and Luo, 2001) (Experimental Procedures). Expression of Ci75 in otherwise wild-type clones did not significantly affect eye size, although some disruption of the patterning of the ommatidia is apparent (Fig. 1J). In contrast, expression of Ci75 in *cos2* mutant clones suppresses the overgrowth phenotype of *cos2* mosaics (compare Fig. 1K and 1L). These results demonstrates that overexpression of Ci75 can overcome the accumulation of Ci155 and implies that the overgrowth of *cos2* mosaics is due to the failure to convert Ci155 to the repressor form Ci75 in the mutant tissue. Thus, *cos2* acts via the canonical Hh/Ci pathway to regulate growth.

2.4. *cos2* mosaics induce proliferation at and anterior to the MF

We examined *ey-FLP*-induced *cos2* mosaic eye-antennal imaginal discs from 3rd instar larvae. *ey-FLP* induces clones throughout the disc, i.e., in the most anterior region that forms the antenna, in the central region that forms the head capsule, and in the posterior region that represent the eye field (Fig. 2A, B). In *cos2* mosaics, mutant clones are visible in all three regions of the disc, and all three regions are clearly overgrown relative to control discs of matched age (Fig. 2C). Noticeably, this overgrowth is predominantly GFP^+ and therefore consists of mostly $cos2^+$ cells, indicating that the mosaic loss of *cos2* has a non-cell autonomous effect on inducing overgrowth.

We examined *cos2* clones in eye discs using BrdU incorporation as marker for proliferation to determine if *cos2* could non-cell autonomously alter proliferation in $cos2^+$ cells. Normally, proliferation occurs in two waves of cells – a broad asynchronous wave anterior to the MF and a synchronous wave posterior to the MF (second mitotic wave, SMW) (Fig. 2D). Cells in the MF are cell-cycle arrested in G1 (Firth and Baker, 2005; Horsfield et al., 1998). *cos2* clones crossing the MF are associated with additional BrdU incorporation at the boundary of the clones (Fig. 2E, E'; yellow arrows). Within mutant clones located anterior to the MF, there is a marked decrease in BrdU labeling, indicating that many of these cells are not synthesizing DNA (Fig. 2E, E'; white arrows). However, there may be an increase in DNA synthesis in the $cos2^+$ tissue adjacent to these clones as well, suggesting a non-cell autonomous effect on proliferation in *cos2* mosaic discs. Clones crossing the SMW do not show changes in BrdU labeling.

In summary, cells within the *cos2* clones have reduced levels of proliferation, while $cos2^+$ cells adjacent to them increase their proliferation rate providing further support for the non-cell autonomous effects on proliferation seen in the *cos2* mosaic flies.

2.5. Eyes predominantly mutant for *cos2* and *ptc* have a growth disadvantage

To further characterize the non-cell autonomous phenotypes of *cos2* and *ptc* mutants, we generated predominantly mutant eye discs by eliminating all wild-type and heterozygous cells using the *ey-FLP* cell-lethal method (Stowers and Schwarz, 1999). This method eliminates potential cell-cell signaling between mutant and non-mutant tissue which is necessary for non-cell autonomous interactions. Specifically, eyes are generated that are predominantly composed of mutant tissue because the homozygous twin spot dies due to a cell-lethal mutation and heterozygous tissue is removed using the dominant expression of the pro-apoptotic gene *hid* from an eye-specific driver (*GMR-hid*) (Stowers and Schwarz, 1999). The remaining tissue is homozygous for the chromosome arm which carries the mutation of interest. If this chromosome is wild-type, the tissue loss by the cell-lethal mutation and *GMR-hid* is completely compensated by new proliferation, generating a normal eye that is phenotypically $white^-$ (w^-) due to loss of the cell-lethal clones (phenotypically w^+) and heterozygous *GMR-hid* tissue (w^+) (Fig. 3B). However, when that chromosome arm carries a mutation in either *cos2* or *ptc*, the adult eye is reduced in size, which is particularly dramatic for *ptc* (Fig. 3C, D) and similar to viable *ptc* heteroallelic combinations with reduced levels of *ptc* (Thomas and Ingham, 2003). Thus, autonomously, *cos2* and *ptc* are growth-impaired. In contrast, overgrowth of head cuticle and antennae is still observed (Fig. 3C, D). Because *GMR-hid* is not expressed in head and antenna, heterozygous cells survive in these tissues and can receive the signal for proliferation by mutant cells (*cos2*, *ptc*). Therefore, this analysis further supports the notion that the overgrowth of *cos2* and *ptc* mosaics is due to non-cell autonomous interactions between mutant and non-mutant tissue which occurs in the antennae and head cuticle, but not in the eye in this mutant background.

We analyzed both *cos2* and *ptc* mosaics for changes in gene expression associated with cell death. Protein levels of the pro-apoptotic protein Hid (Grether et al., 1995) are elevated posterior to the MF in both *cos2* and *ptc* clones (Fig. 3F,G). Interestingly, Hid levels are not or only weakly increased in mutant clones anterior to the MF (Fig. 3F,G). In summary, the reduction of the size of predominantly mutant *ptc* and *cos2* eyes (Fig. 3C,D) and the underrepresentation of mutant clones in mosaic eyes (Fig. 1A–D) is likely due to a combination of Hid-induced apoptosis and reduced proliferation (Fig. 2E).

2.6. *cos2* mosaics induces precocious autonomous and non-cell autonomous differentiation

We also examined *cos2* clones for changes in differentiation using antibodies to ELAV as markers for the developing photoreceptor neurons, which form in and posterior to the MF (Fig. 4A). In *cos2* clones crossing the MF, an expansion of ELAV staining anterior to the furrow was observed (Fig. 4B'), suggesting that these cells were differentiating precociously. This expansion was attributed to altered and ectopic formation of the MF in the more anterior part of the disc as seen with other mutants in Hh signaling (Chanut and Heberlein, 1995; Ma and Moses, 1995; Ma et al., 1993; Pan and Rubin, 1995; Strutt and Mlodzik, 1997; Strutt et al., 1995; Wehrli and Tomlinson, 1995). However, in addition to the precocious autonomous differentiation in *cos2* clones, ELAV staining was detected outside of the clonal boundary in regions adjacent to the MF (Fig. 4B', see inset), indicating that the *cos2* mutant cells were also able to promote differentiation non-cell autonomously. Evidence for non-cell autonomous precocious differentiation has previously been provided in *pka* mosaics (Strutt et al., 1995). *cos2* clones located posterior to the MF differentiated normally and formed ELAV-positive clusters of developing photoreceptors independent of *cos2* levels.

Because the precocious differentiation was attributed to the altered and ectopic formation of the MF, we examined how the MF was modified in *cos2* mosaics. We used the *dpp-lacZ* reporter, which is expressed along the developing MF in wild type discs (Fig. 4C). In *cos2* mosaics, *dpp-lacZ* expression was significantly altered in a position-dependent manner in eye-antennal disc (Fig. 4D,D'). As predicted, the expression of *dpp-lacZ* was shifted towards the anterior by *cos2* clones that cross the MF (yellow arrow in Fig. 4D',D''). In these clones, there is little to no *dpp-lacZ* expression detectable (white arrow in Fig. 4D',D''). However, in clones located anterior to the MF including in the antenna portion, loss of *cos2* led to ectopic autonomous expression of *dpp-lacZ* in the mutant tissue (asterisks in Fig. 4D',D''). Ectopic expression of *dpp-lacZ* in these clones was not associated with ELAV expression, indicating that the levels of *dpp* expression in clones not in contact with the MF are usually not high enough for precocious differentiation. *cos2* clones posterior to the MF do not induce *dpp-lacZ* expression.

3. Discussion

3.1. Mosaics with deregulated Hh signaling cause non-autonomous overgrowth

Signal-independent, deregulated Hh signaling causes cancer in humans and overgrowth in *Drosophila* (Chanut and Heberlein, 1995; Heberlein et al., 1995; Ma and Moses, 1995; Pan and Rubin, 1995; Strutt and Mlodzik, 1997; Strutt et al., 1995; Teglund and Toftgard, 2010; Wehrli and Tomlinson, 1995). However, the autonomous/non-cell autonomous nature of this overgrowth has not been determined. We show here that in mosaics with deregulated Hh signaling through loss of *cos2*, *ptc* or *PKA-C1*, the overgrown eye and head tissues are largely composed of non-mutant (heterozygous and wild-type) cells, i.e., non-cell autonomously, while the mutant cells themselves do not or only weakly contribute to the overgrowth. Instead, *cos2* and *ptc* mutant clones do not proliferate and are sensitive to

apoptosis as evidenced by increased Hid protein levels within clones and the small size of the eye in the cell-lethal background.

Non-cell autonomous overgrowth has also been observed for mosaics of *hyperplastic discs*, which encodes an ubiquitin ligase. In this case, the overgrowth has been attributed to a combined deregulation of Hh and Dpp signaling (Lee et al., 2002). Mutants outside of Hh signaling that cause non-cell autonomous overgrowth in genetic mosaics affect genes involved in ESCRT (endosomal sorting complex required for transport) function such as *vacuolar sorting protein (vps) 20*, *vps22*, *vps23*, *vps25*, *vps28* and *vps32* as well as the E1 ubiquitin-activating enzyme *Uba1* (Herz and Bergmann, 2009; Herz et al., 2006; Herz et al., 2009; Lee et al., 2008; Moberg et al., 2005; Pfleger et al., 2007; Thompson et al., 2005; Vaccari and Bilder, 2005; Vaccari et al., 2009). Loss of ESCRT function results in accumulation of endocytosed cell surface proteins at the endosome resulting in deregulation of many signaling pathways (Herz et al., 2006; Moberg et al., 2005; Thompson et al., 2005; Vaccari and Bilder, 2005; Vaccari et al., 2009). *Uba1* mutations affect every ubiquitin-dependent process in the cell including inactivation of cell signaling receptors. What these mutants all have in common is that they cause deregulation of cell signaling pathways.

Our data are in apparent contrast to a previous study which showed that cells with increased Ci activity regulate growth autonomously (Duman-Scheel et al., 2002). However, the experimental conditions used in that study are significantly different from ours. These authors strongly overexpressed Ci155 using the binary Gal4/UAS system. In our study, we use *cos2* mutants which also causes accumulation of Ci155, but these levels are dependent on expression from the endogenous *ci* gene and do not reach the unphysiologically high levels resulting from binary expression of a *Ci155* transgene.

3.2. Position-dependent effects in *cos2* mosaics

Our results demonstrate that *cos2*, a regulator of the Hh pathway, acts in several distinct ways in the eye imaginal disc. Normally, as previously shown for the Hh pathway (Heberlein et al., 1995; Heberlein et al., 1993), *cos2* regulates the progression of the MF as shown by precocious photoreceptor differentiation and *dpp-lacZ* expression associated with *cos2* clones (Fig. 4). Furthermore, *cos2* mutant clones promote proliferation non-cell autonomously as seen by the predominantly wild type tissue in the adult mosaic eyes (Fig. 1) and the changes in BrdU labeling (Fig. 2). *cos2* is also required for the viability of cells in the developing eye (Fig. 3).

The regulation of these processes is position-dependent within the developing eye disc and involves both autonomous and non-cell autonomous effects. Our analysis shows that increased proliferation is detectable at the border with neighboring non-mutant cells when *cos2* mutant clones are located either at or anterior to the MF. We did not detect any effect on proliferation in *cos2* clones posterior to the MF. Therefore, because under normal developmental conditions, Hh signaling is required for MF progression anterior to the MF, the position-dependence of deregulated Hh activity at and anterior to the MF implies that only tissue in which Hh signaling is normally activated responds to deregulation of it, which was already noted by (Heberlein et al., 1995; Lebovitz and Ready, 1986; Ma et al., 1993). Other tissues are inert to deregulated Hh signaling. These findings explain why in the resulting adult mosaic flies, the overgrowth extends preferentially anteriorly of the eye and the head cuticle which is specified anterior to the MF (Fig. 1).

While proliferation and differentiation occurs anterior to the MF, *cos2* and *ptc* mutant clones posterior to the MF accumulate levels of the pro-apoptotic Hid protein in an autonomous manner. *cos2* mutant cells can survive to adulthood (Fig. 1A), but are strongly underrepresented compared to non-mutant cells. *ptc* mutant cells are completely absent in

mosaic eyes (Fig. 1C). Therefore, these mutant cells are sensitive to apoptotic signaling and many of them are eliminated. This elimination could be due to cell competition (Baker, 2011; Johnston, 2009; Tamori and Deng, 2011). However, even by removing the cell competitive environment using the cell-lethal method, predominantly mutant *cos2* and *ptc* eyes are strongly reduced in size (Fig. 3C, D) suggesting that the underrepresentation of *cos2* and *ptc* mutant clones is not solely due to cell competition. It is more likely that the autonomous loss of *cos2* and *ptc* is a combination of reduced proliferation and increased sensitivity to apoptotic signals. However, the same can be said about cells that have lost Hh activity: they are also upregulating *hid* and die by cell death (Werz et al., 2005; Vrailas and Moses, 2006). It is not uncommon for cells with the incorrect developmental information to increase expression of *hid* and die both in embryos and imaginal discs (Werz et al., 2005; Wolff and Ready, 1991b).

3.3. Significance for Hh-induced human cancers

Ligand-independent Hh signaling is associated with a subset of human tumors such as basal cell carcinoma, medulloblastoma, rhabdomyosarcoma, glioma as well as breast, colorectal, pancreatic and prostate cancer (reviewed in (Teglund and Toftgard, 2010). Many of the affected tissues in these tumors require sonic Hh (Shh) signaling for normal homeostasis. For example, in skin, Shh signaling is required for maintaining stem cell population, and for regulating hair f ollicle and sebaceous gland development (Athar et al., 2006). In the cerebellum, Shh controls proliferation of cerebellar cortical cells and therefore the overall size of the tissue (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). Deregulation of Shh signaling in these tissues by losing Ptch1 or increasing Smo and Gli functions can cause basal cell carcinoma and medulloblastoma (Athar et al., 2006; Teglund and Toftgard, 2010). However, it is unknown if these human malignancies are caused by autonomous or non-cell autonomous interactions. Therefore, our findings in *Drosophila* that ligand-independent Hh signaling causes non-cell autonomous proliferation may be significant for understanding of these tumors in humans. For example, ligand-independent signaling in these tumors may be needed to promote a supportive microenvironment for tumor growth. It would also be interesting to determine why tissues are inert to deregulated Hh signaling if they do not require Hh signaling for normal homeostasis such as the tissue posterior to the MF in the fly eye. Future studies may help identifying such principles and aid in developing therapies for treatment of these diseases.

4. Experimental Procedures

4.1. Fly stocks

The new *cos2* (*cos2^{H29}, cos2^{L51}, cos2^{P50}*) and *ptc^C* alleles were isolated in a mutagenesis screen for modifiers of a cell death phenotype and will be published elsewhere (manuscript in preparation). Other mutants and transgenes used are: *pka-C1^{B3}, pka-C1^{K2}, UAS-Ci^{75}* (kind gift of Dan Kalderon); *smo^3* (kind gift of Mardelle Atkins); *dpp-lacZ, ptc^{S2}* and all *ey-FLP/FRT, hs-FLP/FRT, MARCM* and *FRT cell-lethal* stocks were obtained from the Bloomington *Drosophila* stock center in Bloomington, IN.

4.2. Mosaics

Mosaics were induced using several techniques. Generally, we used the FLP/FRT system with *hs-FLP* (Xu and Rubin, 1993) or *ey-FLP* (Newsome et al., 2000) as the enzymatic source and marking the non-mutant tissue using either *ubi-GFP* to express GFP in the larval tissue or *P[w$^+$]* to generate red eye pigment in adults in a *w$^−$* background. Mosaics were also generated using the MARCM (mosaic analysis using a repressible cell marker) technique which allows expression of transgenes such as *UAS-Ci75* in mutant clones (Lee and Luo,

2001). Mosaics were induced by heat shocking 1st instar larvae 1 hour at 37°C. For MARCM analysis, a second heat shock in 2nd instar was added.

4.3. Immunohistochemistry

Imaginal discs were dissected from 3rd instar larvae and stained using standard protocols. Antibodies to the following primary antigens were used: BrdU (BD Biosciences); ELAV and Ci (Developmental Studies Hybridoma Bank); HID (kind gift from Hong Dong Ryoo); β-GAL (Promega). Cy3-conjugated anti-guinea pig and anti-mouse (Jackson ImmunoResearch) and AlexaFluor 546-conjugated anti-mouse and anti-rabbit (Invitrogen) were used as secondary antibodies.

4.4. Imaging

Adult eyes, heads and legs were imaged using a Zeiss AxoImager using CZ projection software. Legs were collected in ethanol:glycerol then mounted in isopropanol for imaging. GraphPad Prism 5 was used for graphing in combination with Image J software. Confocal images were taken using either an Olympus Fluoview 500 or Fluoview 1000 Laser Confocal Microscope and digital images processed using the associated software. Figures were assembled using Adobe Photoshop.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Signal-independent, deregulated Hh signaling inhibits apoptosis non-autonomously

- Notch signaling is used as a relay mechanism

- Hh-induced Notch signaling induces expression of an IAP non-autonomously

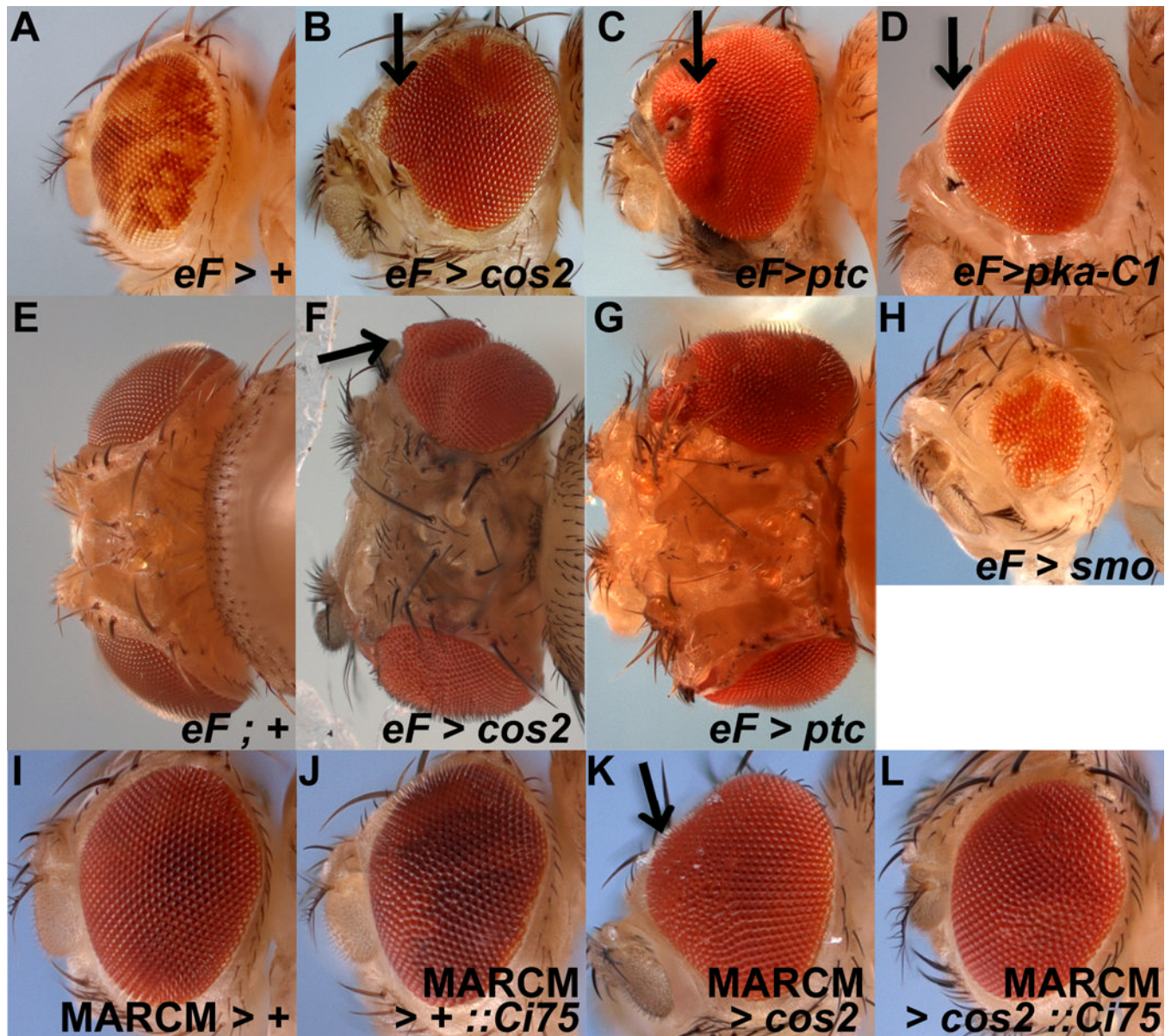- Non-autonomous control of apoptosis by increased Hh activity is position-dependent

Figure 1. Non-cell autonomous overgrowth of eye and head cuticle in mosaics with deregulated Hh signaling

Anterior is to the left and images are from females. Mosaics were obtained by *ey-FLP* (*eF*) induced mitotic recombination, symbolized by >. MARCM-induced expression of transgenes such as *Ci75* is symbolized by ::.

(**A–H**) Wild-type tissue is marked with red (w^+) eye pigment while mutant tissue is white (w^-). In wild type mosaics (A), the eye is a mixture of red and white eye tissue. In *cos2* (B,C) and *pka-C1* mosaics (D), not only are the eyes overgrown, but most of the pigment is red, i.e. *cos2$^+$* and *pka-C1$^+$*, indicating that mostly wild type tissue contributes to the overgrown tissue. The overgrowth is most apparent at the anterior margin especially at the midline (arrows) of the eye (see also K). Some white tissue is apparent in the eye as well, indicating that the clones can survive, but have a growth disadvantage. Compared to controls (E), head cuticle also overgrows in *cos2* (F) and *ptc* (G) mosaics. Reduced Hh signaling in *smo* mosaics (H) results in eyes of reduced size.

(**I–L**) Expression of *Ci75* in *cos2* mutant clones using the MARCM system suppresses the overgrowth in *cos2* mosaics (K,L). Ectopic expression of *Ci75* by itself in wild-type clones does not alter the size of the eye (J).

Genotypes:

A. *y w ey-FLP; FRT42D P[w⁺] / FRT42D.*

B. *y w ey-FLP; FRT42D P[w⁺] / FRT42D cos2^{P50}.*

C. *y w ey-FLP; FRT42D P[w⁺] / FRT42D ptc^{S2}.*

D. *y w ey-FLP; P[w⁺] FRT40 / P[y⁺] pka-C1^{K2} FRT40.*

E. *y w ey-FLP; FRT42D P[w⁺] / CyO.*

F. *y w ey-FLP; FRT42D P[w⁺] / FRT42D cos2^{H29}.*

G. *y w ey-FLP; FRT42D P[w⁺] / FRT42D ptc^{S2}.*

H. *y w ey-FLP; P[w⁺] FRT40 / P[y⁺] smo^{3} FRT40.*

I. *y w hs-FLP; FRT42 P[tubP-GAL80]/FRT42; P[tubP-GAL4]/+.*

J. *y w hs-FLP; FRT42 P[tubP-GAL80]/FRT42; P[tubP-GAL4]/UAS-Ci^{75}.*

K. *y w hs-FLP; FRT42 P[tubP-GAL80]/FRT42 cos2^{H29} ; P[tubP-GAL4]/+.*

L. *y w hs-FLP; FRT42 P[tubP-GAL80]/FRT42 cos2^{H29} ; P[tubP-GAL4]/UAS-Ci^{75}.*
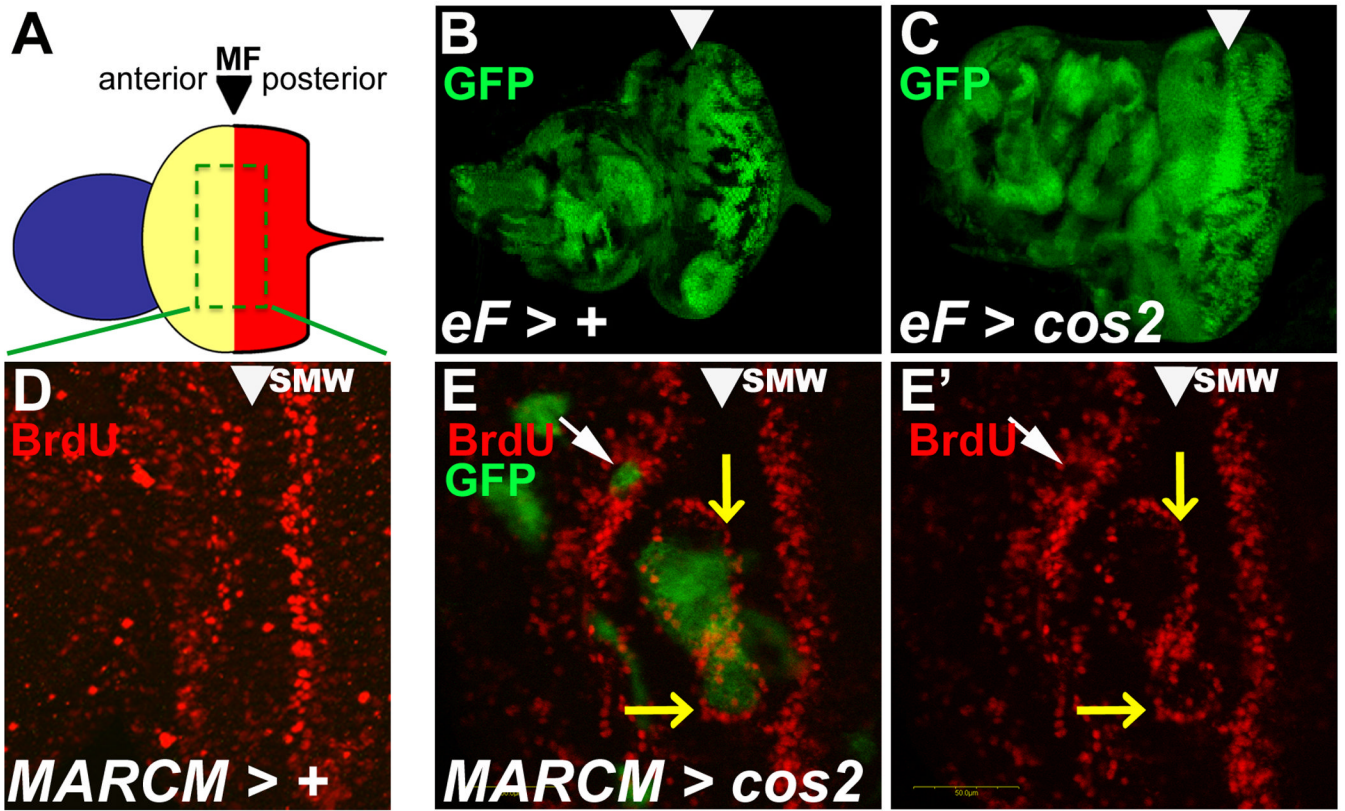
Figure 2. Ectopic proliferation in *cos2* mosaics eye disc at or anterior to the MF
Shown are eye-antennal imaginal discs from 3rd instar larvae. The MF is marked by arrowheads. Clones were induced by *ey-FLP* (*eF*) induced mitotic recombination, symbolized by >.

(**A**) Schematic view of the eye-antennal disc. Anterior (blue) forms the antenna. Central (yellow) forms the head capsule, and posterior (red) forms the eye field which are separated by the morphogenetic furrow (MF).

(**B,C**) Size comparison of age-matched control (B) and *cos2* mosaic discs (C). Although mutant clones (marked by the absence of GFP) are smaller in *cos2* mosaics, the mosaic discs are larger and often deformed compared to control discs.

(**D**) The normal BrdU pattern in wild-type discs. SMW – second mitotic wave.

(**E,E'**) *cos2* clones, positively marked by GFP due to MARCM, located in the MF stimulate BrdU incorporation at the boundary of the clone (white arrowhead). Clones anterior to the MF (yellow arrow) have reduced BrdU labeling.

Genotypes:

(**B**) *y w ey-FLP; FRT42 ubi-GFP/FRT42*

(**C**) *y w ey-FLP; FRT42 ubi-GFP/FRT42 cos2^{H29}*

(**D**) *y w hs-FLP UAS-mCD8-GFP; FRT42 cos2^{H29} /CyO; tubP[GAL4]/+*

(**E,E'**) *y w hs-FLP UAS-mCD8-GFP; FRT42 tubP[GAL80]/FRT42 cos2^{H29} ; tubP[GAL4]/+*
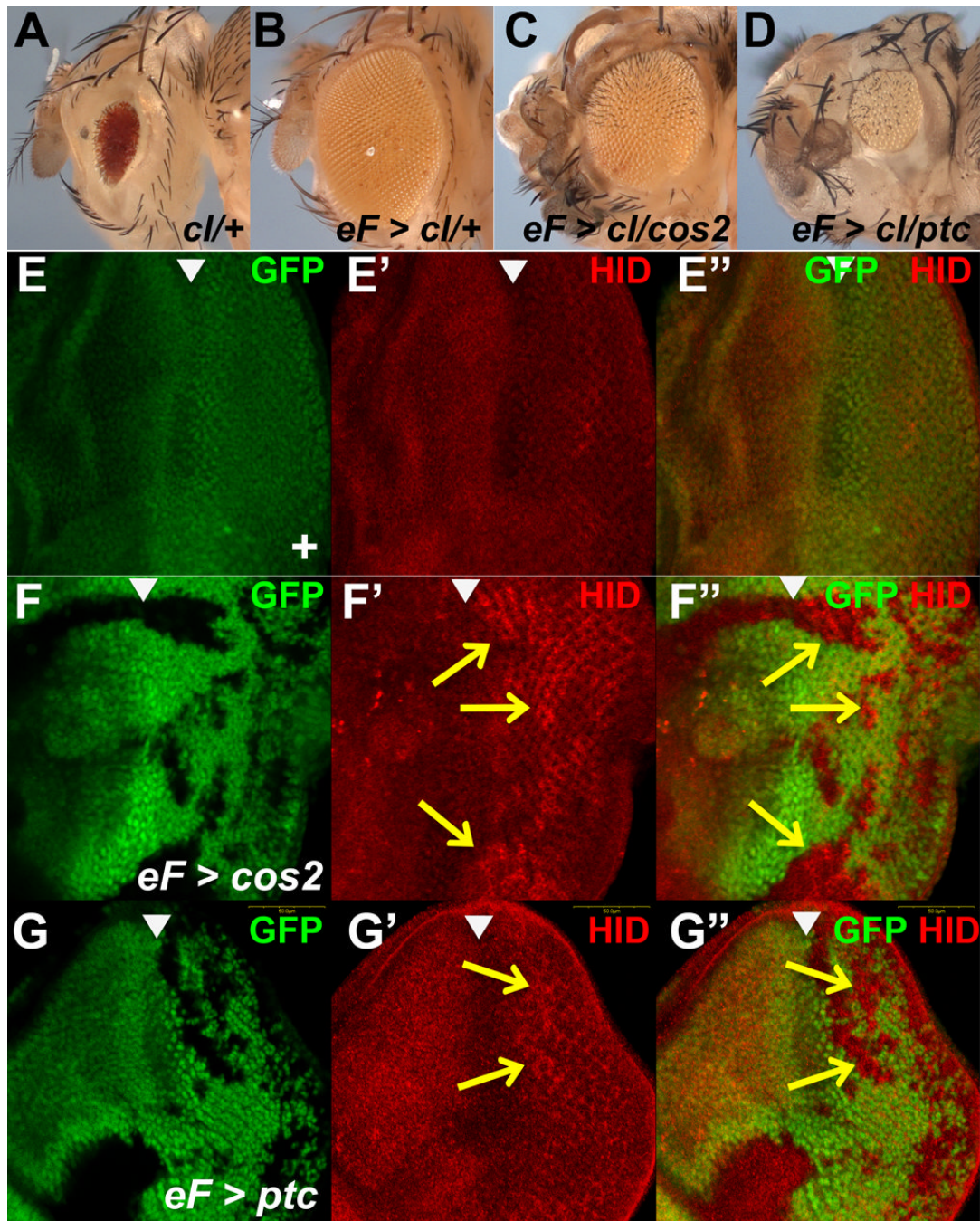
Figure 3. Neither *cos2* nor *ptc* mutant tissue is sufficient for overgrowth
(**A–D**) Shown are fly eyes obtained by the cell-lethal method. (A) In the unflipped control, the *GMR-hid* transgene ablates most heterozygous cells and provides red eye pigment. (B) In *trans* to a wild type chromosome, the twin spot cells survive and re-populate the eye with a few red surviving cells from the dominant marker (*GMR-hid*). (C,D) Neither *cos2* nor *ptc* mutant tissue generate a full sized adult eye. Especially *ptc* mutant eyes are significantly reduced in size (D).

(**E–G**) Anti-Hid antibody labelings of control (E), *cos2* (F) and *ptc* (G) mosaic eye discs obtained by *ey-FLP*-induced mitotic recombination. In *cos2* and *ptc* mutant clones posterior to the MF, Hid levels are elevated (arrows).

Genotypes:

(**A**) *y w; FRT42 GMR-hid l(2)CL-R/+; ey-GAL4 UAS-FLP*

(**B**) *y w; FRT42 GMR-hid l(2)CL-R/FRT42; ey-GAL4 UAS-FLP*

(**C**) *y w; FRT42 GMR-hid l(2)CL-R/FRT42 cos2^{H29} y$^+$; ey-GAL4 UAS-FLP*

(**D**) *y w; FRT42 GMR-hid l(2)CL-R/FRT42 ptc^C y$^+$; ey-GAL4 UAS-FLP*

(**E**) *y w ey-FLP; FRT42 ubi-GFP/CyO*

(**F**) *y w ey-FLP; FRT42 cos2^{H29} /FRT42 ubi-GFP*

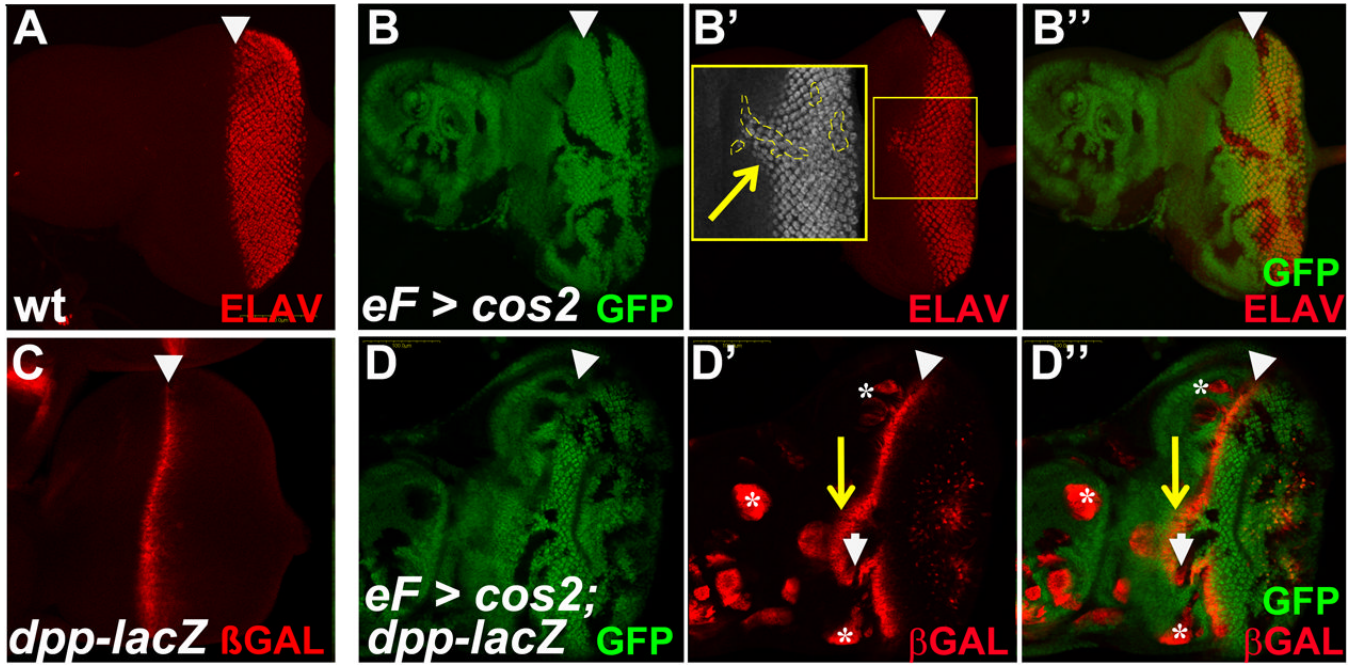(**G**) *y w ey-FLP; FRT42 ptc^C /FRT42 ubi-GFP*

Figure 4. *cos2* mutants generate precocious, autonomous and non-autonomous differentiation in the eye imaginal disc
(**A**) ELAV marks the differentiating neuronal tissue posterior to the MF in control discs.
(**B**) In *ey-FLP*-induced *cos2* mosaics, ELAV expression does extend anterior to the MF. This precocious expansion is associated with mutant clones, but is also seen in adjacent *cos2*+ tissue (shown in inset outlined in dashed line). ELAV-labeling is not grossly altered in *cos2* clones posterior to the MF.
(**C**) *dpp-lacZ* marks the MF in wild-type eye discs.
(**D**) In *ey-FLP*-induced *cos2* mosaics, multiple changes in *dpp-lacZ* expression are observed. The MF is shifted anterior around clones crossing the MF (arrow in D'). These clones reduce expression of *dpp-lacZ* (arrowhead). In contrast, clones further anterior and not associated with the MF autonomously induce *dpp-lacZ* expression (asterisk). β-GAL staining posterior to the MF is due to a weak *GMR-lacZ* reporter on the *ey-FLP* chromosome.

Genotypes:

(**A**) *y w ey-FLP; FRT42 ubi-GFP/CyO*

(**B**) *y w ey-FLP; FRT42 cos2^{H29} /FRT42 ubi-GFP*

(**C**) *y w ey-FLP; ubi-GFP FRT40/CyO; dpp-lacZ*

(**D**) *y w ey-FLP GMR-lacZ; FRT42 cos2^{H29} /FRT42 ubi-GFP; dpp-lacZ*