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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.

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 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;

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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, 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, 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, 1993). Photoreceptor neurons posterior to the MF express Hh, which induces *decapentaplegic* (*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 and 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. 1A and 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 and B).

In addition to the effect on the eye itself, overgrowth was also seen in head cuticle and the antennae (Fig. 1E and 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.