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Salvador-Warts-Hippo pathway in a developmental checkpoint monitoring Helix-Loop-Helix proteins

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Abstract

The E-proteins and Id-proteins are, respectively, the positive and negative heterodimer partners for the basic-helix-loop-helix protein family, and as such contribute to a remarkably large number of cell fate decisions. E-proteins and Id-proteins also function to inhibit or promote cell proliferation and cancer. Using a genetic modifier screen in Drosophila, we show that the Id-protein Extramacrochaetae enables growth by suppressing activation of the Salvador-Warts-Hippo pathway of tumor suppressors, activation that requires transcriptional activation of the expanded gene by the E-protein Daughterless. Daughterless protein binds to an intronic enhancer in the expanded gene, both activating the SWH pathway independently of the transmembrane protein Crumbs, and bypassing the negative feedback regulation that targets the same expanded enhancer. Thus the Salvador-Warts-Hippo pathway has a cell-autonomous function to prevent inappropriate differentiation due to transcription factor imbalance, and monitors the intrinsic developmental status of progenitor cells, distinct from any responses to cell-cell interactions.

Introduction

The coordination of differentiation with growth and proliferation is important so that organs develop with proper organization and size. Failures of this coordination may cause organ malformations or tumors. We describe how certain defective cells are recognized and prevented from causing neural defects by the Salvador-Warts-Hippo (SWH) pathway.

Transcription factors of the basic helix-loop-helix (bHLH) family contribute to a remarkably large number of cell fate decisions. E-proteins are bHLH transcription factors that bind

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DNA at E-box (CANNTG) consensus sequences. E proteins appear to be expressed in every cell, and heterodimerize with tissue-specific bHLH proteins to play specific roles in differentiation (Massari and Murre, 2000). *Drosophila* has a single E-protein, Daughterless (Da), which in combination with heterodimer partners is required for neurogenesis, sex determination, and mesoderm development (Murre et al., 1989; Goulding et al., 2000; Huang et al., 2000; Massari and Murre, 2000). The 4 mammalian E-proteins E12 and E47 (also known as TCF3), E2-2 (TCF4), and HEB (TCF12) play crucial roles in the regulation of commitment, cell growth and differentiation in lymphocytes, muscle cells, neurons, and other cells (Massari and Murre, 2000; Slattery et al., 2008; Kee, 2009).

By contrast to E-proteins, Id-proteins prevent DNA binding and function when they heterodimerize with either E-proteins or tissue-specific bHLH proteins like the muscle specific protein MyoD (Benezra et al., 1990; Lassar et al., 1991) or proneural genes of the Achaete-Scute Complex (AS-C) (Van Doren et al., 1991). Levels of the only *Drosophila* Id-protein, Extramacrochaetae (Emc), set thresholds for differentiation in response to positively-acting bHLH heterodimers. Accordingly, hypomorphic mutations that reduce *emc* function lead to ectopic neural differentiation, whereas null alleles are lethal to the embryo (Van Doren et al., 1992; Cubas and Modolell, 1992).

It has been known for many years that clones of cells completely lacking *emc* cannot easily be recovered in growing imaginal disc tissues (Garcia Alonso and Garcia-Bellido, 1988; de Celis et al., 1995). This implies a role for *emc* in cell proliferation or survival as well as in differentiation, but the mechanism of this growth contribution was not known. Mammalian Id genes are also growth regulators and can act as proto-oncogenes (Iavarone et al., 1994; Lasorella et al., 1996; Norton, 2000; Hasskarl and Munger, 2002; Perk et al., 2005).

The Salvador-Warts-Hippo (SWH) pathway has emerged as a new growth pathway, largely conserved between *Drosophila* and mammals (Bossuyt et al., 2014). The core components are the cytoplasmic Hpo and Wts kinases that, together with their accessory proteins Sav and Mats, phosphorylate and retain Yorkie (Yki)/YAP in the cytoplasm (Tapon et al., 2002; Harvey et al., 2003; Jia et al., 2003; Pantalacci et al., 2003; Udan et al., 2003; Wu et al., 2003; Huang et al., 2005; Lai et al., 2005). Yki/YAP is a transcriptional coactivator that lacks DNA-binding activity but interacts with proteins such as Scalloped/TEAD family transcription factors to mediate Yki/YAP-induced gene expression (Wu et al., 2008; Goulev et al., 2008; Zhao et al., 2008; Zhang et al., 2008). Yki/YAP can promote cell proliferation, particularly in progenitors and stem cells. Deregulation of Yki/YAP promotes tissue overgrowth and tumor formation, whereas Yki/Yap activity is required for normal growth and for tissue regeneration (Ramos and Camargo, 2012). Mutations in the upstream Hpo pathway genes lead to overgrowth phenotypes in *Drosophila* and are implicated in various human cancers (Pan, 2010; Grusche et al., 2010; Zhao et al., 2011; McCaffrey and Macara, 2011; Genevet and Tapon, 2011; Staley and Irvine, 2012; Schroeder and Halder, 2012).

Hpo and Wts activity depend on more upstream proteins that suggest the SWH pathway is regulated by cell-cell interactions. These include the apical membrane protein Crumbs (Crb), the protocadherin Fat/Fat4, the FERM domain proteins Expanded (Ex)/FRMD6, Merlin (Mer)/NF2 and Kibra (Hamaratoglu et al., 2006; Willecke et al., 2006; Feng and Irvine,

2007; Baumgartner et al., 2010; Ling et al., 2010; Robinson et al., 2010; Yu et al., 2010; Chen et al., 2010; Genevet et al., 2010). Fat can interact with another protocadherin, Dachsous, expressed on neighboring cells, providing a potential cell-cell signaling pathway influencing growth (Cho et al., 2006; Rogulja et al., 2008; Willecke et al., 2008; Schwank et al., 2011). Crb seems to regulate SWH through homophilic interactions (Ling et al., 2010; Robinson et al., 2010; Chen et al., 2010). Evidence is increasing that SWH activity may be influenced mechanically, perhaps through the actin cytoskeleton as well as through cell junctions (Boggiano and Fehon, 2012; Schroeder and Halder, 2012).

The physiological roles of the SWH pathway are not yet fully understood (Lawrence and Casal, 2013). Although it is hypothesized that the SWH pathway controls organ growth, direct evidence is lacking that normal growth terminates due to an increase in SWH activity. On the other hand, loss of SWH regulation may underlie tissue expansion that occurs in wound healing and regeneration (Cai et al., 2010; Karpowicz et al., 2010; Shaw et al., 2010; Zhao et al., 2011; Grusche et al., 2011; Barry et al., 2013), and in the expansion of the wing primordium during *Drosophila* development (Zecca and Struhl, 2010). While reduced SWH pathway activity is implicated in growth stimulation, evidence that SWH hyperactivity blocks growth and cell survival is mainly found in experimental situations (Jia et al., 2003; Udan et al., 2003; Bennett and Harvey, 2006; Dong et al., 2007; Tyler and Baker, 2007). The SWH pathway is thought to mediate contact inhibition in mammalian cells (Lallemand et al., 2003; Zhao et al., 2007) as well as the distinction between trophectoderm and inner cell mass in mouse embryogenesis (Nishioka et al., 2009). The core of the SWH pathway is also required for selection of rhodopsin expression in the postmitotic retina, another process that depends on cell-cell interactions (Jukam and Desplan, 2011).

In this study we report that unbalanced bHLH expression activates the SWH pathway through the direct transcriptional regulation of the *ex* gene by the E-protein Daughterless. Our findings indicate that SWH activity prevents *emc* mutant cells from causing inappropriate ectopic neurogenesis. Our findings therefore identify a role for the SWH pathway in the cell-autonomous recognition and elimination of certain mis-specified cells during development.

Results

Emc regulates growth through Da

Like mammalian Id-proteins, the *Drosophila* Id-protein Extramacrochaetae (Emc) is a HLH protein lacking a basic domain whose heterodimers are unable to bind DNA (Benezra et al., 1990; Campuzano, 2001). The *emc* gene promotes cell growth and survival, and it is hard to recover clones of progenitor cells in imaginal discs that lack *emc* (Garcia Alonso and Garcia-Bellido, 1988; de Celis et al., 1995). Because Emc expression is regulated by the E-protein Daughterless (Da), and serves as a negative feedback regulator of *daughterless* expression and activity, *emc* null mutant cells express high levels of Da proteins that are responsible for their growth defect (Bhattacharya and Baker, 2011). Consistent with this finding, Da overexpression during growth under the control of *eyg-Gal4* can reduce the size of adult organs and larval tissues including the eye and the salivary gland (Figures 1A–B and 1H–K, and Figure S1A–B). Therefore, excess Da reduced both diploid, mitotic growth

of imaginal discs and endoreplicative growth of the salivary gland. As expected if *eyg>da* mimics the growth effects of *emc* loss, the small eye was further reduced by loss of one copy of the endogenous *emc* gene, but partially restored by loss of one copy of the endogenous *da* gene, and eye size was also reduced by RNA interference of *emc* by a dsRNA (Figure S1D–F and data not shown).

Da functions with the Salvador-Warts-Hippo pathway

To elucidate the mechanism by which Da affects cell survival and growth, we performed a dominant genetic modifier screen using a series of chromosomal deletions (Parks et al., 2004; Ryder et al., 2004; Roote and Russell, 2012; Cook et al., 2012) to identify loci that were dose-sensitive for growth effects of high Da (Table S1). The size of the *eyg>da* eye was modified by reduced gene dose of multiple cell death and cell cycle regulators, including *head involution defective*, *grim*, *reaper*, *sickle*, *string*, *wee1*, *Rb*, *E2f1*, *Cyclin A*, and *Cyclin E*, and was rescued by co-expression of the anti-apoptotic proteins baculovirus p35, Diap1, or dominant-negative Dronc (Table S2). Two of the regions with strong dominant suppression of Da-induced growth inhibition were cytological intervals 56D10-56E2 and 99F8-100A5, which uncovered *hippo* (*hpo*) and *warts* (*wts*) respectively. Multiple independent point mutants of *hpo* and *wts* were all then found to suppress the Da overexpression phenotype (Figures 1C–D). For comparison, the eyg^{CD}>da small eye phenotype was not modified by heterozygosity for $pten^{MGH1}$ (data not shown), a null allele of a negative regulator of the Insulin pathway, indicating that the genetic interaction with *hpo* and *wts* was not shared by all growth regulators. By contrast, when Da was over-expressed in the differentiating, post-mitotic eye using *GMR-GAL4*, the effects on differentiation that resulted were not modified by *hpo* or *wts* gene dose, showing that *hpo* and *wts* primarily affected Da function during growth (Figures S1G–H). This latter finding also argued that *hpo* and *wts* did not simply modify transcriptional activation by Gal4. Taken together, these results suggested that Da acted through Hpo and Wts, two components of the SWH pathway that coordinately regulates growth and survival.

To establish how much of the SWH pathway was implicated in the growth response to high Da, point mutations for components that were not uncovered by the deficiency collections were tested. Growth suppression in Da-overexpressing eyes also depended on the gene dose of Sav, Yki, and the adaptor protein *ex* (Figures 1G and 1L). By contrast, *eyg>da* was not modified by dosage of the transmembrane protein genes *ft* or *crb* (Figures 1E–F).

The SWH pathway is epistatic to Da in growth control

To establish how quantitatively growth suppression by Da was dependent on the SWH pathway, Da was over-expressed specifically in clones of cells that also lacked particular SWH components. Mosaic analyses with a repressible cell marker (MARCM) (Lee and Luo, 1999) was used to generate null clones of *ex* that also over-express Da. The sizes of ex^{e1}*; tub>Da* clones were indistinguishable from ex^{e1} clones (Figures 2A–D and 2I), indicating that Da over-expression (*tub>Da*) did not inhibit clone growth in the absence of *ex*. Similar results were found in wts^{X1} mutant clones (Figures 2J and S2A–D). Since Da protein level was not affected in *wts* clones (Figure S2J), these findings indicated that *wts* and *ex* were epistatic to high Da, and required for high Da to affect growth. By contrast, clones

overexpressing Da and mutant for *crb* rarely survived in imaginal discs, indicating that Da over-expression continued to inhibit growth in the absence of *crb* (Figures 2E–H and 2K). High Da did not affect growth in the absence of *ft* (Figures S2E–I), perhaps because Ft can affect Wts independently of parts of the pathway (Cho et al., 2006). Together, the results indicated that Da required the core SWH pathway to inhibit growth, but did not require the transmembrane protein Crb.

Da regulates *ex* transcription

Since Da is a transcription factor, it might regulate transcription of SWH genes. Quantitative RT-PCR analysis was performed to identify potential targets. The mRNA levels of *ex* were increased in Da-overexpressing tissue when compared to wild-type, while *hpo* and *mer* mRNA levels did not show significant changes (Figure 3A). Since *ex* was also the most upstream SWH component that acted as a genetic modifier of Da, the transcription of *ex* was examined in situ using an enhancer trap line, *ex-LacZ*. When Da was over-expressed under Actin-Gal4 control using a Flp-out method, *ex* transcription was cell-autonomously elevated as indicated by the *ex-LacZ* reporter (Figures 3C–D). Similar results were seen in both diploid imaginal disc cells, and in polytene salivary gland cells that grow by endoreplication. The anti-apoptotic baculovirus p35 was co-expressed with Da in these experiments to preserve cells over-expressing Da. *ex-LacZ* was not affected in otherwise wild type cells expressing p35 (Figures S3A–B). To investigate *ex* transcription in another way, the *Nubbin-Gal4* driver (*Nub-Gal4*) was used to over-express Da. *Nub-Gal4* is active specifically in the wing pouch (Figure 3E), and *Nub>da* clearly elevated *ex* transcription in most such cells as shown both by the *ex-LacZ* reporter and antibody staining for the Ex protein (Figures 3F–G; Figures S3D–E). *Nub>da* leads to cell death in the wing disc and a great reduction in adult wing size that is partially rescued by co-expression of the antiapoptotic protein baculovirus p35 (Figures S3F–L). Finally, the *ex-LacZ* reporter was also up-regulated in clones of *emc* mutant cells in the wing disc, confirming that elevated Da expression mimics the effects of mutating *emc* (Figure 3B). These findings indicate that high levels of Da activate *ex* transcription. Elevated *ex* transcription is known to be sufficient to activate the SWH pathway, epistatically to certain transmembrane receptors (Hamaratoglu et al., 2006; Tyler and Baker, 2007).

Identification of cis-regulatory elements for the *ex* gene

To determine whether *ex* was a direct transcriptional target of Da, we first sought the Da response element of the *ex* gene. A ChIP-chip database of early embryogenesis (MacArthur et al., 2009) reported modest association of Da protein with two regions. These putative Da-response elements were tested in vivo for enhancer activity (Figure 4A). The region within the third *ex* intron drove GFP reporter gene expression in patterns identical to *ex-LacZ* (Figures 4B–D). To determine whether Intron 3 enhancer ($Enh^{Intron3}$) confers response to Da, Da was over-expressed in the wing pouch, eye disc or salivary glands. GFP expression was elevated in Da over-expressing cells (Figure 4E and not shown), indicating that $Enh^{Intron3}$ is a Da-responsive enhancer. By contrast, the proximal promoter region did not respond to Da overexpression in wing imaginal discs (Figures 4J–K).

To assess whether Da directly associates with $Enh^{Intron3}$ in larval tissues, chromatin immunoprecipitation (ChIP) experiments were conducted with chromatin isolated from wing imaginal discs in which *Nub-GAL4* drove HA-tagged Da. DNA amplified from chromatin immunoprecipitated with anti- HA was significantly enriched in regions C and E within the identified enhancer element (Figure 4H). Other parts of the enhancer showed less enrichment or were not enriched compared to negative controls (amplicon A near the transcription start site, B in the first intron, and G in the 3′ UTR). None of the regions was enriched when immunoprecipitated with control IgG antiserum, or in HA-immunoprecipitated chromatin prepared from wing discs of *Nub-GAL4* flies (Figures 4H–I), although *Nub-GAL4* chromatin could be precipitated with other antibodies (Figure S4F). Da-responsiveness was retained by a 658 bp fragment covering the regions with enriched Da association, that contains three E-box sequences that are putative binding sites for bHLH proteins (Figures 4L–N). Da-responsiveness was lost when these E-boxes were mutated (Figures 4L and 4O–P). Thus, Da protein is bound to the Da-responsive element of *ex* in wing discs in vivo, consistent with direct binding as the mechanism of activation by high Da levels.

Da affected *ex* expression in cells that are not known to express any bHLH heterodimer partners (Figures 3B–C and 4E). Although Da functions as a heterodimer with proneural bHLH proteins where they are expressed, and ectopic expression of the proneural proteins Achaete and Scute could activate the enhancer (Figures S4D–E). Da can also homodimerize and Da homodimers can bind to DNA (Murre et al., 1989; Jarman et al., 1993; Huang et al., 2000; Jafar-Nejad et al., 2003). Indeed, we found that $Enh^{Intron3}$-GFP was upregulated in the wing imaginal epithelium when a covalent Da-Da homodimer was expressed (Figure 4F), indicating that SWH activity could be regulated by Da homodimers and that this can occur outside of proneural regions (Figures S4D, E). Covalent Da-Da dimer expression led to phenotypes similar to over-expression of Da monomer, but more severe (Fig. S4A–C and data not shown).

In addition to its essential role in SWH signaling, *ex* is also a transcriptional target of Yki. Since Yki is inhibited by SWH activity, regulated *ex* transcription constitutes a negative feedback loop for the SWH pathway, whereby diminished SWH activity disinhibits Yki, upregulating *ex* transcription and restoring SWH activity (Hamaratoglu et al., 2006). Since the expression pattern of $Enh^{Intron3}$-GFP mimicked *ex-lacZ* expression in multiple imaginal discs even in the absence of Da over-expression, we determined whether $Enh^{Intron3}$ also encoded the feedback of Yki on *ex* expression. Indeed, co-expressing Scalloped (Sd: the DNA-binding protein partner for Yki during wing development (Goulev et al., 2008; Zhang et al., 2008; Pan, 2010; Zhao et al., 2011) and Yki elevated $Enh^{Intron3}$-GFP in the wing discs (Figure 4G). In sum, these data demonstrate that $Enh^{Intron3}$ is a *cis*-regulatory element of *ex* regulated by the bHLH protein Da as well as by the SWH pathway through Sd/Yki.

Da over-expression reduces Yki activity

Our data show that Da requires SWH activity to affect growth (Figures 1, 2 and S2). If elevated *ex* expression and SWH activity is the relevant mechanism, then Yki target gene expression should be affected by Da. To test this, we monitored the miRNA *bantam* (*ban*)

using the *ban-GFP* sensor (Brennecke et al., 2003). When Da was over-expressed, either clonally or using *Nub-GAL4*, a significant increase of *ban-GFP* sensor was revealed in wing discs (Figures 5A–C, S5B). Because *ban* destabilizes the *ban-GFP* sensor, this indicated reduction of *ban* miRNA levels and Yki activity in Da over-expressing cells. Another assay examined the *four-joint* enhancer trap (*fj-lacZ*) or *diap1* reporter (*diap1-lacZ*) as readouts of Yki activity (Cho et al., 2006; Wu et al., 2008). Expression of *fj-lacZ* and *diap1-lacZ* were decreased in *emc* clones or Da overexpressing cells (Figures 5D, S5C, S5F), indicating reduced Yki activity when Da is upregulated. This effect of Da was dependent on *ex* (Figure S5E). Collectively, these data confirmed that high Da both activated *ex* transcription and reduced Yki activity, as expected if this is the mechanism of growth control by high Da.

The physiological role of SWH regulation by Da

During normal development, Da levels are uniform in most uncommitted imaginal disc cells (Cronmiller and Cline, 1987; Bhattacharya and Baker, 2011). If these normal levels contribute to *ex* transcription and SWH activity, then *da* null mutant cells would have enhanced growth, like clones mutant for SWH components. When the size of clones homozygous for *da* null alleles was compared with the reciprocal 'twin-spot' clones induced by the same mitotic recombination, however, no difference was seen (Figures 6A–B). Consistent with this finding, $Enh^{Intron3}$-GFP reporter activity was unaffected in clones of *da* null mutant cells (Figures 6C and S6A). Therefore *da* did not appear to act as a brake on the growth rate of normal imaginal disc cells, although we cannot exclude that *da* may regulate growth through SWH in some other tissue.

Clones of cells lacking *emc* are lost from imaginal discs during growth, because of their high Da levels and SWH activity (Garcia Alonso and Garcia-Bellido, 1988; Bhattacharya & Baker, 2011;_Figures 3, 5 in this study). Therefore, the normal level of Emc protein is required for imaginal disc growth, and the signal of inadequate *emc* activity is high Da. To assess the potential significance of the pathway, we removed *ex* from Da over-expressing cells to prevent SWH hyperactivity. In ex^{e1}; tub>*da* clones, numerous ectopic sensory organ precursors (SOPs) were detected that were not present in ex^{e1} clones (Figures 6D–E). Previous studies show that *da* is an especially potent driver of ectopic neurogenesis (Jafar-Nejad et al., 2003). Consistent with this, ectopic bristle sensory organs differentiated in pharate adults (Figures 6F and S6B). We also found hypomorphic *ex* mutant genotypes that survive with minimal growth defects (Figure 6G). Such genotypes often differentiate supernumerary sensory bristles even without mutation of *emc* or targeted expression of *da* (Figure 6G). These data indicate that *ex* plays a role in fine-tuning neural patterning, and that SWH activity prevents excess neuronal differentiation of cells with inappropriate HLH gene expression by eliminating these cells (Figure 6H).

Discussion

We describe a process that prevents certain mis-specified cells from differentiating into malformed organs. This process creates a requirement for the *emc* gene in imaginal disc cell growth, since *emc* loss results in high Da levels that trigger the pathway through transcriptional activation of the *ex* gene, an upstream regulator of the SWH tumor

suppressor pathway. If *ex* or the downstream SWH genes are mutated, then cells with high Da levels not only survive and grow but produce numerous ectopic neuronal structures. This surveillance function for SWH signalling does not require cell-cell signalling and is distinct from potential roles for SWH in limiting organ growth or preventing tumorigenesis. It may represent an adaptive function for SWH pathway hyperactivity.

Selecting against Progenitor Cells with Incorrect Fate Specification

The heterodimer partners of Da and Emc include proneural bHLH protein that define proneural regions and neural progenitor cells and which are highly regulated in space and time (Modolell and Campuzano, 1998; Quan and Hassan, 2005). Da, by contrast, is expressed ubiquitously and controlled by *emc* (Bhattacharya and Baker, 2011). Inadequate *emc* expression permits higher levels of *da* expression and of Da/bHLH heterodimers, leading to ectopic neural differentiation (Van Doren et al., 1992; Cubas and Modolell, 1992; Bhattacharya and Baker, 2011). Mammalian Id genes are similar feedback regulators of mammalian E proteins (Bhattacharya and Baker, 2011; Schmitz et al., 2012). We have shown here that even if *emc* expression or its regulation is defective, abnormal neurogenesis is still restrained by SWH signaling that restricts the proliferation and survival of cells with abnormal Da expression. High Da levels directly activate transcription of the *ex* gene, thereby activating the SWH pathway of tumor suppressors in a cell-autonomous fashion (Figure 6H). Because *ex* is a feedback inhibitor of SWH signaling that is transcriptionally activated by Yki (Hamaratoglu et al., 2006), *ex* activation by high Da has the added effect of bypassing feedback control of SWH signaling (Figure 6H), which likely contributes to the efficiency of removal of cells with high Da. Indeed, when *ex* is removed, cells with high Da are not removed but produce dramatic neural hyperplasia, in which ectopic bristles almost cover a clone in the thoracic epidermis (Figure 6E–F). All these neurogenic defects would be maladaptive in nature, where the pattern of sensory bristles is highly selected (Simpson and Marcellini, 2006).

Our findings suggest that the Da/Emc balance is permissive for normal growth and we have not found evidence for regulation that determines normal organ size or growth rate (Figure 6A, B). By contrast, Da/Emc imbalance outside the normal range in mutant cells triggers the SWH pathway to block growth and remove cells that will otherwise perturb developmental patterning. SWH activation in abnormal development might be analogous to the p53 tumor suppressor, which is inactive in most normal cells, but activated by DNA damage and other stresses (Brady and Attardi, 2010). Interestingly a recent study reported that *emc* hypomorphic cells, which are less severely affected that *emc* null cells and can survive in imaginal discs, nevertheless exhibit a growth deficit caused by repression of the cell cycle gene *string*/cdc25, and that *string*/cdc25 is repressed directly by abnormally high Da (Andrade-Zapata and Baonza, 2014). Thus there may be multiple, Da-dependent pathways that converge to select against progenitor cells with incorrect cell fate specification.

Implications for development and cancer

Mammalian E proteins and Id proteins are well-established tumor suppressors and proto-oncogenes (Yan et al., 1997; Norton, 2000; Hasskarl and Munger, 2002; Sikder et al., 2003; Perk et al., 2005; Murre, 2005; Iavarone and Lasorella, 2006; Slattery et al., 2008; Kee,

2009). In normal development, E-proteins and Id proteins regulate the coordination of differentiation with cell cycle arrest (Jen et al., 1992; Peverali et al., 1994), and the expansion of mammary epithelial cells in response to pregnancy and lactation (Mori et al., 2000; Parrinello et al., 2001; Itahana et al., 2008; Dong et al., 2011). At least in part, these growth controls relate to the transcriptional activation of cyclin-dependent kinase inhibitor genes by E proteins, such that E proteins are required for cellular senescence, counteracted by Id proteins (Van der Put et al., 2004; Zheng et al., 2004). The senescence mechanisms may not be conserved between mammalian and *Drosophila* cells (Simcox et al., 2008), but other pathways of tumor suppression by mammalian E proteins exist (Niola et al., 2013), and in certain contexts E proteins can be tumor promoting, and Id proteins tumor suppressive (Schmitz et al., 2012).

The distinctive phenotype of SWH pathway mutations is dramatically enhanced growth and organ size (Pan, 2007; Saucedo and Edgar, 2007). The normal biological functions of the pathway are still debated (Lawrence and Casal, 2013). Reduced SWH activity is implicated in wound healing and regenerative growth (Cai et al., 2010; Karpowicz et al., 2010; Shaw et al., 2010; Zhao et al., 2011; Grusche et al., 2011; Barry et al., 2013). Mice mutant for *Mst1*, *Mst2*, *Lats1* or *Lats2* are tumour prone, suggesting that tumor growth could mimic wound healing or regeneration. Epigenetic silencing of these genes has been reported in human cancer (Takahashi et al., 2005; Jiang et al., 2006; Seidel et al., 2007), where other SWH components are mutated, such as *NF2* in neurofibromatosis (Zender et al., 2006; Evans, 2009). Yap is amplified in cancers of liver, colon, lung, ovary (Overholtzer et al., 2006; Steinhardt et al., 2008).

Clearly, SWH activity is normally maintained between a low threshold necessary to prevent hyperplasia, and a high threshold that blocks growth and kills cells. Reduced SWH activity is associated with regenerative responses. In principle, increased SWH might be hyper-activated to eliminate potential tumors, perhaps because of imbalanced expression of E proteins and Id proteins; tumor cells might evolve to evade such a checkpoint. Microarray data from E2A deficiency mice that exhibit high incidence of T-cell leukemia suggest that FRMD6, a mammalian homolog of *ex*, is an E2A target, which would be consistent with this hypothesis (supplementary data in (Welinder et al., 2011)).

Our work shows directly that in *Drosophila* hyperactivation of the SWH tumor suppressor pathway can select against cells that express certain developmental errors, which may be adaptive for development. It will be interesting to discover whether SWH signaling can be hyperactivated to remove other kinds of dysfunctional cells besides those expressing inappropriate bHLH protein levels, whether in development or in cancer.

Experimental procedures

Clonal Induction and Clone Size Measurement

Fly culture and cross were performed according to standard procedures at 25 °C except where noted. Immunochemistry and scanning electron microscopy were performed as described (Firth et al., 2006; Baker et al., 2014). Flp-on expression clones were generated by crossing *UAS*-lines to hs-FLP^{122}; Act5C>CD2> GAL4 UAS-GFP. Mutant clones were

made by FLP/FRT-mediated mitotic recombination. FLP was induced by heat shock at 36–48 hr AEL and animals were dissected at late third instar. MARCM was conducted to express UAS-transgenes in mitotic clones of wts^{X1}, $crb^{82\text{-}04}$ or ex^{e1} mutant cells using ActGAL4 or TubGAL4. Heat shocks were performed at 24–48 hr AEL and incubated at 26.5°C until animals were dissected. *Nub>da* experiments were performed at 18°C to minimize lethality. Clone sizes and perimeters were measured in microns using NIH Image J.

Quantitative RT-PCR

Eyg-GAL4>UAS-GFP (control) and *eyg-GAL4>* UAS-Da salivary gland were dissected in PBS. Total RNA was isolated using the Zymo Research Micro RNA isolation kit and DNA-Free RNA kit (Zymo Research). First Strand cDNA Synthesis Kit for RT-PCR (AMV; Roche) and random hexamer oligo-p$(dT)_{15}$ primers were used to produce cDNAs from the extracted total RNA (1 μg). To measure mRNA levels, Real-time qPCR was performed with SYBR Green Master PCR Mix (ABI) using the ABI 7900HT Detection System. All reactions were performed three times. The relative amount of specific mRNAs under each condition was calculated after normalization to the *rp49* transcript. rp49-5′: TACAGGCCCAAGATCGTGAA, rp49-3′: ACGTTGTGCACCAGGAACTT; ex-5′: CAGCAGCAGCCGAAAACCT, ex-3′: TTGGGCCATATTTTGAGAGC; mer-5′: AAGCACGACCTGGAGAAGAA, mer-3′:AGGCTATCCGTGGAGGACTT; hpo-5′:GGAGTCGAACTTGGGCACTA, hpo-3′: GCTGCTGCTGTTGTTGTTGT. The statistical significance of the difference was assessed using a Student's *t*-test with significance at $P<0.05$.

Chromatin Immunoprecipitation

Wing imaginal discs were dissected from late third instar larvae in cold PBS and fixed for 20 min at room temperature in 1.8% formaldehyde. After quenching in 0.125M Glycine, the discs were washed twice in cold PBS containing 0.01% Triton and complete protease inhibitor cocktail (Roche). Incubation of the discs with cold cell lysis buffer (5 mM PIPES, 85 mM KCl, 0.5% NP-40) for 10 min on ice and centrifugation 5 min at 4°C. Pellet was resuspended with nuclei lysis buffer (50 mM Tris-Cl, 10 mM EDTA, 1% SDS and complete protease inhibitor cocktail) to perform sonication. Soluble chromatin was transfer to a new tube after centrifugation, and 10% was removed for input. Chromatin was precleared with protein A/G agarose beads (Santa Cruz) for 1 hr, then incubated overnight with HA probe (F-7) antibody (Santa Cruz) or control IgG (normal mouse IgG, Santa Cruz) in ChIP dilution buffer (16.7 mM Tris-Cl, 167 mM NaCl, 1.2 mM EDTA, 0.01% SDS, 1.1% Triton X-100, complete protease inhibitor cocktail). Antibody-chromatin complexes were pulled down with beads (Invitrogen) for 3 hr at 4°C. Beads were washed five times in high salt wash buffer (50 mM HEPES, 500 mM NaCl, 1 mM EDTA, 0.1%SDS, 1% Triton X-100, 0.1% deoxycholate), and twice in TE buffer. Chromatin was eluted in ChIP elution buffer (1% SDS, 0.1M $NaHCO_3$) supplemented with 1 uL of proteinase K (20 ug/uL) and RNase. To reverse crosslinks, eluted materials were incubated at 65°C over 6 hr. PCR purification kit (Qiagen) was used to clean up DNA. Real-time PCR analysis was performed on ABI 7900HT instrument using SYBR Green Master PCR Mix (ABI). Results were quantified using the delta Ct method, normalizing to the input samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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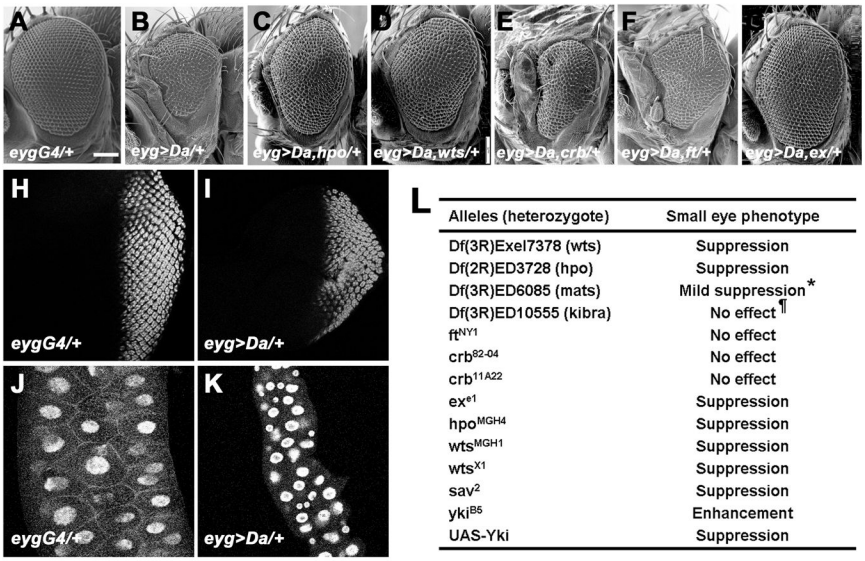

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Alleles (heterozygote)	Small eye phenotype
Df(3R)Exel7378 (wts)	Suppression
Df(2R)ED3728 (hpo)	Suppression
Df(3R)ED6085 (mats)	Mild suppression*
Df(3R)ED10555 (kibra)	No effect¶
ft^{NY1}	No effect
crb^{82-04}	No effect
crb^{11A22}	No effect
ex^{e1}	Suppression
hpo^{MGH4}	Suppression
wts^{MGH1}	Suppression
wts^{X1}	Suppression
sav^{2}	Suppression
yki^{B5}	Enhancement
UAS-Yki	Suppression

Figure 1. SWH Pathway Components Are Required for Excess Da-induced Growth and Survival Defect

(A–G) Scanning electron micrographs of adult eyes. Scale bar, 100 μm. (A) Control eyg^{CD}-*GAL4/+* flies. (B) eyg^{CD}-*GAL4* driving *UAS-Da* flies (abbreviated as *eyg>Da*). (C) *eyg>Da*, hpo^{MGH4}/+ flies. (D) *eyg>Da*, wts^{X1}/+ flies. (E) *eyg>Da*, crb^{82-04}/+ flies. (F) *eyg>Da*, ft^{NY1}/+ flies. (G) eyg^{CD}*>Da*, ex^{e1}/+ flies. (H–I) Third instar eye imaginal disc of eyg^{CD}-*GAL4* (H) or *eyg>da* (I) staining for anti-Elav (red). (J–K) Third instar salivary gland of eyg^{CD}-*GAL4* (J) or *eyg>da* (K) staining for DAPI (blue). (L) A subset of SWH pathway genes genetically interact with *eyg>Da*. *Results with *mats* were equivocal. The other independent mutant of mats, $mats^{e235}$, did not suppress *eyg>Da*. ¶Using TRiP RNA against *kibra* partially suppressed *eyg>Da*. See also Figure S1 and Tables S1–S2.

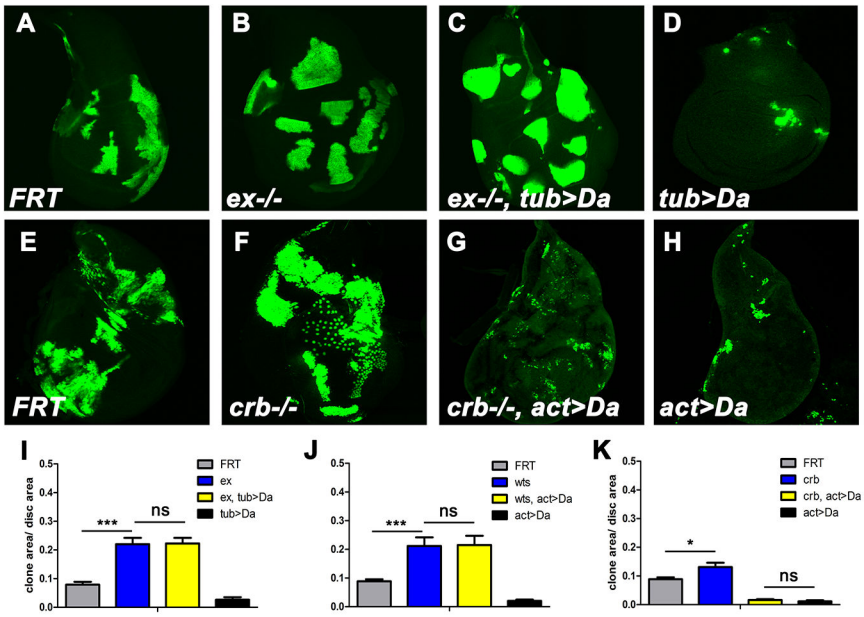

Figure 2. Da Over-expression acts upstream of *ex*

(A–D) Third instar wing imaginal discs with positively marked MARCM clones stained for GFP (green, to mark the clones). *wild-type* control (A), ex^{e1} (B), ex^{e1}, *tub>Da* (C), and *tub>Da* (D). Although clones of ex^{e1} or ex^{e1}, *tub>Da* grew well, *tub>Da* clones were rarely recovered in the disc proper. (E–H) Third instar wing imaginal discs with *wild-type* control (E), $crb^{82\text{-}04}$ (F), $crb^{82\text{-}04}$, *act>Da* (G), and *act>Da* (H). Loss of *crb* did not affect clone loss when Da was over-expressed. (I–K) Quantification results of A–D, E–H and Figure S2A–D, respectively. 10 discs of each genotype are analyzed. Areas of clones are normalized to the total wing disc area. Mean±SEM is shown, significance assessed with Student's t-test; *** P<0.001; ** P<0.01; * P<0.05; NS, not significant. See also Figure S2.

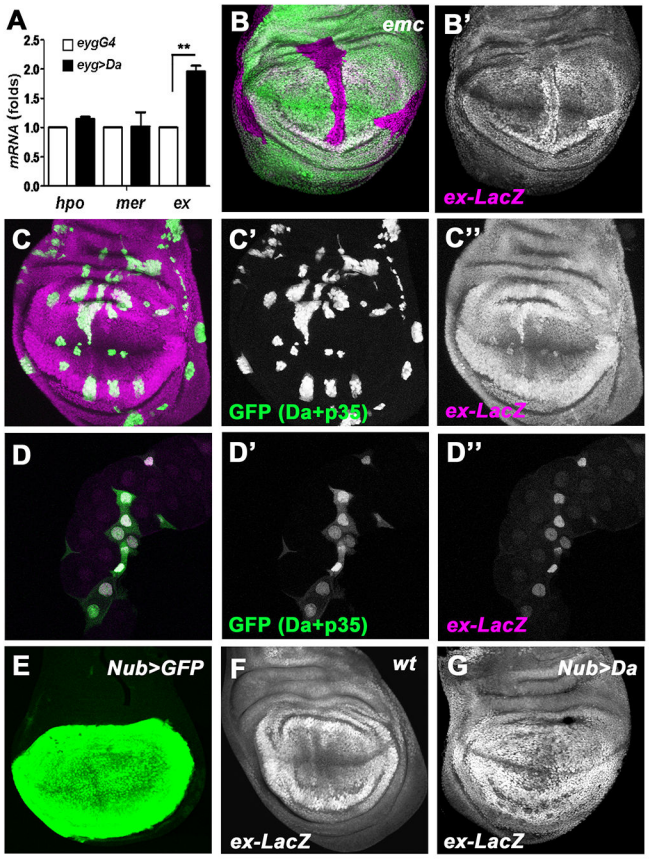

Figure 3. *ex* Is a Transcriptional Target of Da

(A) A graph comparing *hpo*, *mer* and *ex* mRNA levels in wild type and Da-overexpressing salivary glands, as measured by qRT-PCR. Results represent mean±SEM (n=3). ** P<0.01. The mRNA levels of *ex* are increased 1.96±0.17 fold. (B) A wing disc containing *emc* mutant clones (GFP negative) stained for *ex-lacZ* reporter expression (magenta). Transcriptional activity of the *ex* gene is reported by detecting β-galactosidase expression. The increased levels of *ex-lacZ* in *emc* mutant cells are most obvious in the wing pouch. (C) Wing imaginal discs containing Da-overexpressing clones (ActGal4>GFP+Da+P35, GFP positive, green) stained for *ex-lacZ* reporter expression (magenta). The anti-apoptotic baculovirus p35 was co-expressed with Da in these experiments to preserve cells over-expressing Da. The *ex-LacZ* reporter was elevated by high Da cell-autonomously. (D) Salivary glands containing Da-overexpressing clones (ActGal4>GFP+Da+P35, GFP positive, green) and stained for *ex-lacZ* reporter expression (magenta). Note *ex-lacZ* was elevated in Da-overexpressing clones. Salivary glands from Da-overexpression animals are often smaller, in addition to the cell-autonomous effect of Da-overexpression. (E) UAS-GFP under the control of *Nub-GAL4* in the wing imaginal discs. Note GFP is detected in the wing pouch region. (F, G) Wing imaginal discs of *wild type* (F) and *Nub>Da* (G) containing *ex-LacZ* (magenta). Note the elevation of *ex-LacZ* reporter in Da-overexpressing wing pouch cells. See also Figure S3.

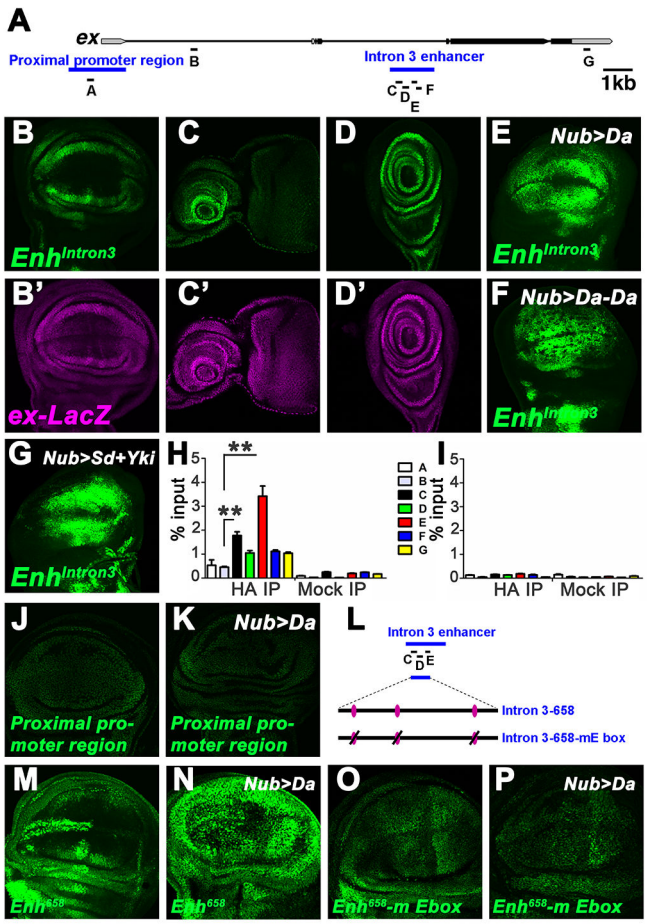

Figure 4. Characterization of *ex* Enhancers

(A) Schematic representation of the *ex* locus. Exons are represented as black arrows, introns are represented by lines. Non-coding regions are shown as grey arrows. Blue bars label Proximal promoter region and Intron 3 enhancer (Enh^{intron3}). Bars labeled A–G indicated regions amplified by PCR in ChIP experiments. (B–D) Expression patterns of Enh ^{intron3}-GFP and *ex-LacZ* in the wing (B, B′), eye (C, C′), or leg (D, D′) discs. (E–G) Wing discs of *Nub>Da* (E), *Nub>Da-Da* homodimer (F), and *Nub>Sd+Yki* (G) staining for Enh ^{intron3}-GFP reporter expression. Note the upregulation of Enh ^{intron3}-GFP levels in all these wing discs. (H) ChIP analysis of the Enh^{Intron3} enhancer. Anti-HA (HA IP) or IgG antibodies are used to precipitate chromatin from wing discs of *Nub-Gal4>UAS-HA-Da*. Quantitative PCR was done on regions A–G (see panel A). Graph shows the percentage of signal relative to input. Results represent mean±SEM (n=3). **P<0.01 (t-test). (I) Control HA ChIP. Anti-HA antibody is used to precipitate chromatin from *Nub-Gal4* wing discs. (J) *Wild-type* wing disc staining for *Proximal promoter region-GFP* expression. (K) Wing disc of *Nub>Da* staining for *Proximal promoter region-GFP* expression. The Proximal promoter region-GFP expression was unaffected by Da over-expression. (L) Schematic representation of the Intron 3 enhancer. Bars labeled C, D, E indicate ChIP amplicons. Subfragment (658 bp) covering C to E regions contains 3 putative E-box elements (purple ovals). (M) *Wild-type* wing disc staining for Intron 3-658-GFP (abbreviated as *Enh^{658}*) expression. Unlike the

complete enhancer, this construct drives more GFP expression in cells at the anterior wing margin. (N) Wing disc of *Nub>Da* staining for Enh^{658} expression. Note the elevation of Enh ^{658}-GFP levels throughout the Nub-Gal4 domain. (O) Intron 3-658-GFP loses expression with three mutated E-box elements (abbreviated as Enh^{658}*-mE box*). (P) Enh^{658}*-mE box* expression in *Nub>Da* wing disc. Note that the Enh^{658}*-mE box* did not respond to Da.

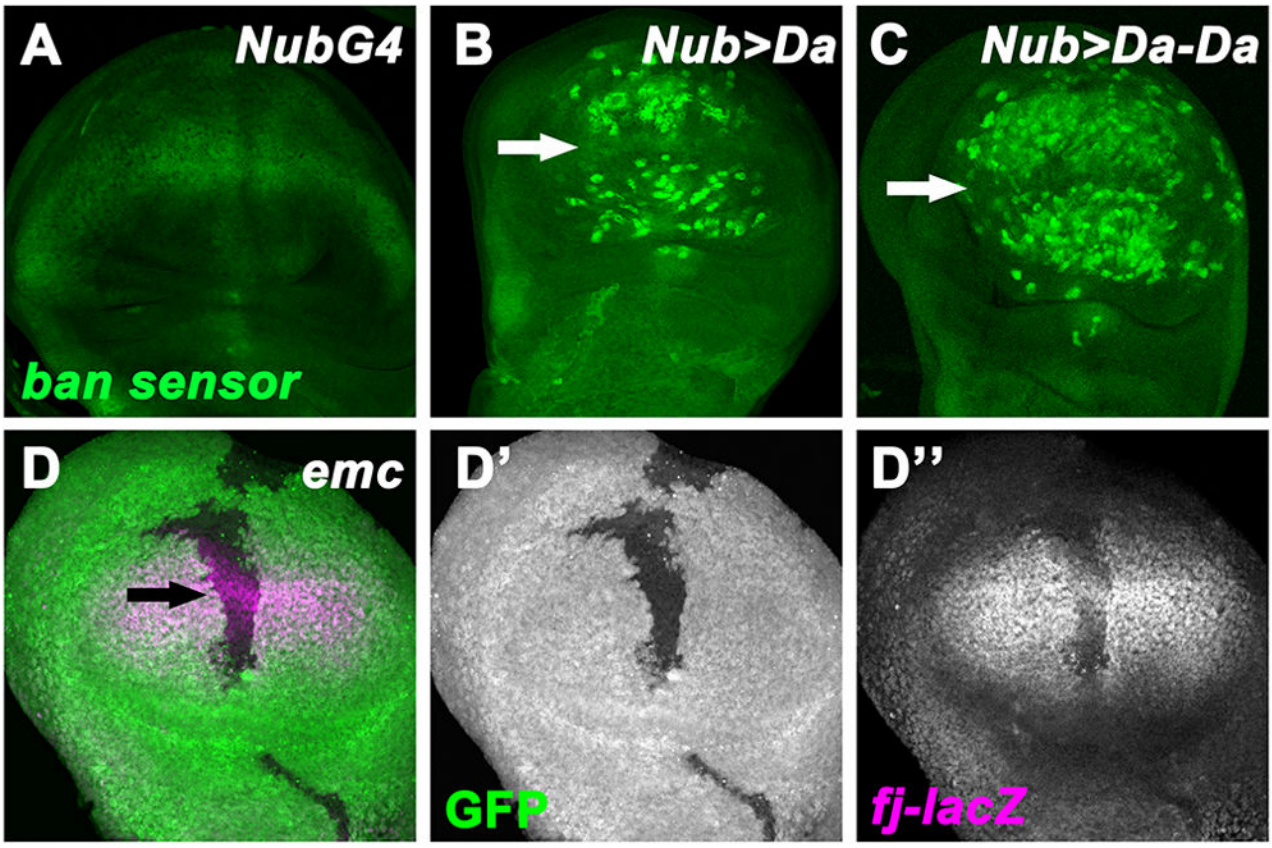

Figure 5. High Da affects multiple SWH pathway genes

(A–C) Wing imaginal discs of *Nub-GAL4* (A), *Nub>Da* (B), and *Nub>Da-Da* (C) staining for *ban-GFP* sensor (green). Activity of *ban* is negatively reported by the *ban-GFP* sensor. Note the elevation of *ban-GFP* sensor in Da-overexpressing cells (wing pouch), except cells near the dorsal-ventral boundary (proneural region, indicated by white arrow). (D–D″) An early-mid third instar wing disc containing *emc* mutant cells (GFP negative) is visualized for *fj-lacZ* reporter expression (D″, magenta). Note the decrease levels of *fj-lacZ* in *emc* mutant clones. Some *emc* mutant cells close to the proneural region retain a residual *fj-lacZ* expression (black arrow).

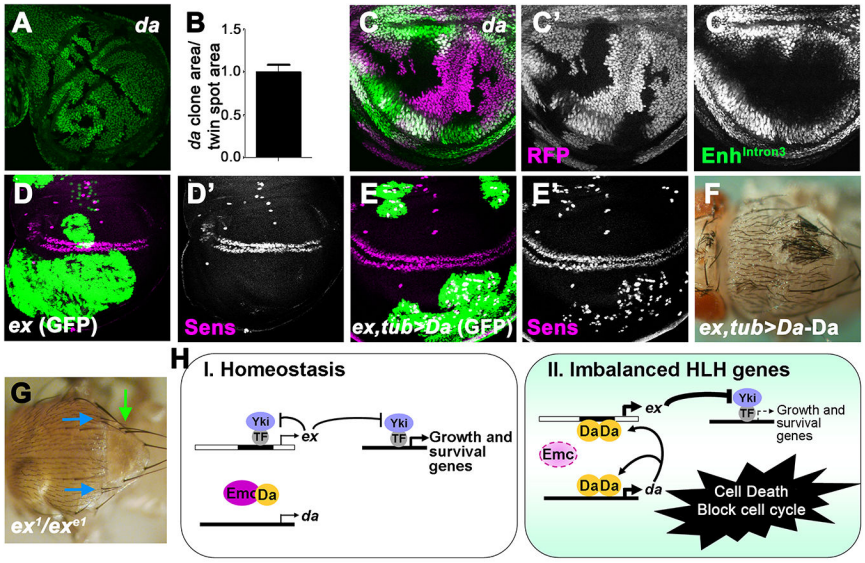

Figure 6. Ex removes ectopic High-Da neurons

(A) Third instar wing imaginal discs containing da^{10} mutant cells (GFP negative) and their reciprocal twin spot clones (2X GFP staining). (B) Quantification of clone area relative to twin spot area of 10 discs. (C–C′) Third instar wing imaginal discs containing da^{3} mutant cells (RFP (megenta) negative) visualized for $Enh^{intron3}$-GFP reporter expression (green). (D–E′) Third instar wing imaginal discs of ex^{e1} (D–D′) and ex^{e1}, *tub>Da* (E–E′) positively marked MARCM clones stained for GFP (green, to mark the clones) and stained for the Senseless (Sens) protein in megenta. Note that ectopic Sens staining in ex^{e1}, *tub>Da* MARCM clones (blue outline in panel E′). (F) Ectopic bristles (white arrow) in adult thorax containing ex^{e1}, *tub>Da-Da* clones. Note that only the clone expressing Da-Da is homozygous for the *ex* mutant allele. (G) The allelic combination ex^{1}/ex^{e1} can survive to adulthood with minimal growth defects. More than 40% of this genotype differentiate supernumerary thoracic macrochaetae (blue arrows: post-alar bristles; green arows: scutellar bristle). (H) Model. (I) In the normal situation, unspecified progenitor cells proliferate in the imaginal discs. Both da expression and SWH activity maintain basal levels through independent regulatory mechanisms. (II) When emc−/− cells or Da over-expressing cells arise outside the cell-cycle arrested neural regions, elevated levels of Da take over regulation of *ex* transcription, leading to the activation of the SWH pathway to antagonize Yki activity. Growth and proliferation are blocked, while apoptosis is promoted, preventing such cells contributing to abnormal patterns of neurogenesis. See also Figure S6.