Oncogenic Ras stimulates Eiger/TNF exocytosis to promote growth

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ABSTRACT
Oncogenic mutations in Ras deregulate cell death and proliferation to cause cancer in a significant number of patients. Although normal Ras signaling during development has been well elucidated in multiple organisms, it is less clear how oncogenic Ras exerts its effects. Furthermore, cancers with oncogenic Ras mutations are aggressive and generally resistant to targeted therapies or chemotherapy. We identified the exocytosis component Sec15 as a synthetic suppressor of oncogenic Ras in an in vivo Drosophila mosaic screen. We found that oncogenic Ras elevates exocytosis and promotes the export of the pro-apoptotic ligand Eiger (Drosophila TNF). This blocks tumor cell death and stimulates overgrowth by activating the JNK-JAK-STAT non-autonomous proliferation signal from the neighboring wild-type cells. Inhibition of Eiger/TNF exocytosis or interfering with the JNK-JAK-STAT non-autonomous signaling at various steps suppresses oncogenic Ras-mediated overgrowth. Our findings highlight important cell-intrinsic and cell-extrinsic roles of exocytosis during oncogenic growth and provide a new class of synthetic suppressors for targeted therapy approaches.

KEY WORDS: Eiger, TNF, Exocytosis, Oncogenic Ras, Tumors

INTRODUCTION
Activating mutations of the oncogenic Ras gene are highly prevalent in human cancers (Bos, 1989). However, targeted therapy strategies have not yielded the desired effects and the available chemotherapy regimens are not effective at treating the most aggressive Ras cancers (Asghar et al., 2010; Lièvre et al., 2010). Genetic studies in fruit flies and worms have led to the identification of Ras effectors and the characterization of the Ras/Raf/mitogen activated protein kinases (Ras/MAPK) signaling cascade during normal development (Nishida and Gotoh, 1993; Simon et al., 1991; Sternberg and Horvitz, 1991). This pathway was found to be conserved in mammals (Alessi et al., 1994; Covley et al., 1994; Kolch et al., 1991; Mansour et al., 1994; Qiu et al., 1995). However, targeting components of the Ras/MAPK signaling cascade only partly inhibits overgrowth (Blum and Kloog, 2005), suggesting that oncogenic Ras drives tumorigenesis via additional signaling events. Moreover, blocking the Ras/MAPK pathway causes death of normal cells, thus making this approach not suitable for targeted therapy and placing an impetus on the need to identify the additional signaling events that oncogenic Ras specifically triggers to promote tumor development. Identification of mutations that synthetically suppress Ras tumor growth could not only broaden our understanding of cancer biology, but could also lead to the discovery of novel therapeutic targets. Indeed, several laboratories have conducted RNA interference (RNAi)-based synthetic suppressor screens in tissue culture settings and have identified important vulnerabilities of oncogenic Ras cells (Barbie et al., 2009; Luo et al., 2009; Sarthy et al., 2007; Scholl et al., 2009; Steckel et al., 2012; Strebhardt and Ullrich, 2006). Additional whole-animal synthetic suppressor screens could be particularly informative for revealing the role of oncogenic Ras in processes that are important for regulating growth in vivo.

Expression of oncogenic Ras (RasV12) in Drosophila imaginal discs gives rise to overgrowth (Karim and Rubin, 1998). Generating patches of labeled RasV12-expressing cells surrounded by wild-type cells allows putative host-tumor cell interactions to occur and permits genetic screens to identify mutations that can either enhance or suppress the growth of RasV12-expressing tumors (Chi et al., 2010; Pagliarini and Xu, 2003). Here, we report the characterization of one of the synthetic suppressor mutations, sec15, a mutation in a gene that encodes a component of the exocytosis machinery. Sec15 is a subunit of the evolutionarily conserved multiprotein complex termed the Sec6/Sec8 complex or the exocyst complex, which was originally identified in yeast (Finger et al., 1998; Finger and Novick, 1999; Guo et al., 1999; Heider and Munson, 2012; Novick et al., 1980; TerBush and Novick, 1995). The exocyst complex regulates secretion in eukaryotes by controlling the delivery of vesicles to the cell plasma membrane (Grindstaff et al., 1998; Guo et al., 1999; Heider and Munson, 2012; Jafari-Nejad et al., 2005; Mehta et al., 2005; TerBush and Novick, 1995). In contrast to core exocyst components, which are broadly required for normal exocyst function and cell viability, Sec15 regulates the delivery of specific cargo proteins and is dispensable for cell viability in Drosophila (Mehta et al., 2005).

In addition to being essential for cellular organization in all eukaryotes, vesicle transport has recently been found to play important roles in regulating signal transduction. For example, transport of endocytosed cell surface molecules to signaling targets on endosomes allows signal transduction to occur, whereas targeting these molecules to the lysosome for degradation attenuates or suppresses signaling (Seth et al., 2002). Transcytosis of vesicles facilitates the establishment of morphogen gradients, which are crucial for conveying proliferation and cell fate specification cues during development (Seth et al., 2002). Exocytosis has been previously found to mediate signal transduction by sending signaling molecules including neurotransmitters and ligands to neighboring cells (Li and Chin, 2003). By studying how sec15 suppresses RasV12, we show that exocytosis also regulates signal transduction within a cell by clearing signaling ligands. We found that RasV12 cells clear Eiger (also known as TNF) by exocytosis to downregulate pro-apoptotic Janus NH2-terminal kinase (JNK, also known as Bsk – FlyBase) signaling (Igaki et al., 2009; Moreno et al., 2002) and thus evade cell death. We have previously shown that JNK activation triggered by cell polarity defects could stimulate non-autonomous JAK-STAT signaling for proliferation (Wu et al., 2010).
Here, we show that oncogenic Ras elevates exocytosis to hijack this process in order to promote overgrowth. Exocytosis-dependent accumulation of Eiger/TNF results in JNK activation in surrounding wild-type cells, which in turn, non-autonomously stimulates JAK-STAT signaling to promote the proliferation of RasV12 cells. These findings provide new mechanistic insights into the long known ability of oncogenic Ras cells to avoid cell death and promote growth, and also highlight the importance of exocytosis in signal transduction and cancer biology.

RESULTS
sec15 synthetically interacts with oncogenic Ras
In Drosophila, GFP-marked mosaic clones of cells expressing RasV12 overgrow to develop into tumors (Pagliarini and Xu, 2003). The overgrowth phenotype can be readily ascertained by visualizing fluorescent signal intensity in third instar whole larvae (Fig. 1A,C) or by examining clone size in dissected eye-antenna imaginal discs (Fig. 1E,G). Furthermore, RasV12 tumors caused pupal lethality (98.4%, N=62; Fig. 1K). We induced sec15 or RasV12 single mutant clones or RasV12, sec15 double mutant clones and examined the growth of these mutant clones in similarly aged third instar eye-antenna discs. We found that the sec15 mutation did not disrupt cell proliferation (supplementary material Fig. S1A,B), and the size of sec15 mutant clones was comparable to that of wild-type clones (Fig. 1A,B,E,F), consistent with the reported cell viability of the sec15 null mutation (Mehta et al., 2005). In addition, sec15 null mutant cells persisted into the adult eye (Fig. 1J). The viability of sec15 mutant cells is not due to maternal protein deposition, as Sec15 protein level was dramatically reduced in mutant clone cells (supplementary material Fig. S2A). However, the sec15 mutation dramatically suppressed the overgrowth phenotype of RasV12 clones (Fig. 1C,D,G,H; 77.4% of the double mutants showed strong suppression similar to that shown in Fig. 1D,H; N=53). Furthermore, the sec15 mutation rescued the lethality of the animals bearing RasV12 tumors (76% viable, N=125, compared with 1.6% viable, N=62 for RasV12 animals; Fig. 1L). Moreover, RNA interference (RNAi)-mediated knockdown of Sec15 in RasV12, scrib+ cells suppressed tumor growth and invasion (supplementary material Fig. S8A-D). Finally RNAi knockdown of two core exocyst components, Sec6 and Sec8 showed a similar effect on RasV12-mediated overgrowth (supplementary material Fig. S1C-N). The sec15 mutant or Sec15 or Sec8 RNAi alone had no detectable effect on growth, whereas Sec6 RNAi alone showed a reduction in clone sizes (supplementary material Fig. S1C-N). Taken together, we conclude that the sec15 mutation synthetically suppresses RasV12 tumor growth.

Oncogenic Ras stimulates the exocyst
Interestingly, it was previously observed that RNAi depletion of exocytosis proteins also suppresses HrasV12-mediated tumor growth in human cells (Issaq et al., 2010). We thus investigated the mechanism underlying this phenomenon. The observation that sec15 can selectively suppress RasV12 tumor growth suggested that oncogenic Ras could regulate the exocyst to promote growth. We first analyzed the abundance of exocyst proteins in RasV12 tumor clones relative to that of wild-type cells. We examined Sec15 protein levels in RasV12 tumors and extended this analysis to include Sec6, Sec8 and Rab11. Rab11 interacts with Sec15 to initiate assembly of the exocyst and the exocytosis of endocytosed molecules (Wu et al., 2005; Zhang et al., 2004). The previously published Sec15, Sec6 and Sec8 antibodies showed negligible staining in corresponding mutant and RNAi knockdown clones (supplementary material Fig. S2A-C). Sec15, Sec6 and Sec8 proteins were specifically upregulated in RasV12 clones (Fig. 2A,C,E,G). Similarly, Rab11 was upregulated in RasV12 cells (Fig. 2D,F). The increased levels of exocyst proteins in RasV12 cells were subsequently confirmed by western blotting (Fig. 2H). Exocyst proteins displayed no obvious subcellular localization defects in RasV12 cells (Fig. 2E-G), indicating that oncogenic Ras stimulates exocyst proteins but does not affect their respective subcellular localization.

The observation that Rab11 is upregulated along with core exocyst proteins suggested that oncogenic Ras elevates the secretion of endocytosed molecules. To begin to address this possibility, we sought to examine the transport of endocytosed molecules in the mutant cells by performing Dextran dye pulse-chase experiments. After the dye pulse phase we found that RasV12 cells had no problem
whether Eiger/TNF and Sec15 proteins interact in TNF signaling from directly killing tumor cells. We first examined to accelerate cellular clearance of Eiger/TNF, hence preventing Eiger/TNF-overgrowth, we hypothesized that oncogenic Ras could promote RasV12 et al., 2009). Because endocytosis is not generally inhibited in endocytosis is not generally inhibited in endosomes, which endocytosed to activate JNK signaling on endosomes, which Eiger/TNF is soluble ligand (Black et al., 1997; Brandt et al., 2004; Jue et al., 2002). Similar to vertebrate TNFs, Eiger is produced as a crude measure of secretion within these cells. Interestingly, RasV12 cells were significantly more efficient at dye clearance after the dye chase phase. RasV12 cells showed fewer dye-positive punctae compared with wild-type cells (the pulse/chase ratios of dye-positive punctae for RasV12 and wild-type cells were 4.63±0.27 and 1.53±1.12, respectively; Fig. 3D,E,G). The sec15 mutation abrogated the increased dye clearance effect of oncogenic Ras [2.74±1.98 (after the pulse), 1.26±0.29 (after chase) and 2.18±0.34 (pulse/chase ratio); Fig. 3C,F,G]. Taken together, the above data indicate that oncogenic Ras stimulates the exocyst.

Oncogenic Ras promotes the interaction of Eiger/TNF with the exocyst

We next sought to investigate the significance of exocyst stimulation vis-à-vis tumor growth. One way in which Ras activation contributes to tumorigenesis is by allowing the cell to evade cell death (Cox and Der, 2003; Downward, 1998; Wolfman et al., 2002; Wu et al., 2009). In Drosophila, the tumor necrosis factor (TNFα) homolog Eiger triggers cell death (Igaki et al., 2009; Moreno et al., 2002). Similar to vertebrate TNFs, Eiger is produced as a transmembrane protein and is subsequently cleaved to yield a soluble ligand (Black et al., 1997; Brandt et al., 2004; Jue et al., 1990; Kauppila et al., 2003; Moss et al., 1997). Eiger/TNF is endocytosed to activate JNK signaling on endosomes, which triggers cell death (Igaki et al., 2009; Moreno et al., 2002). Preventing endocytosis impinges on Eiger/TNF signaling (Igaki et al., 2009). Because endocytosis is not generally inhibited in RasV12 cells and sec15 suppresses oncogenic Ras-mediated overgrowth, we hypothesized that oncogenic Ras could promote an interaction between the exocyst and Eiger/TNF in order to accelerate cellular clearance of Eiger/TNF, hence preventing Eiger/TNF signaling from directly killing tumor cells. We first examined whether Eiger/TNF and Sec15 proteins interact in RasV12 cells using two independent and complementary approaches, immunostaining and biochemistry. We found that Eiger/TNF colocalized with Sec15 in RasV12 cells (Fig. 4A-C). Sectioning of isosurface-rendered images from confocal projections revealed that indeed Sec15 coats Eiger/TNF-positive vesicles (Fig. 4D), suggesting that Eiger/TNF is a Sec15 cargo protein. Moreover, we performed anti-Eiger or anti-GFP control pulldown experiments on lysate prepared from eye-antenna discs bearing RasV12 clones and found that Sec15 and other exocyst proteins, Sec5 and Sec8, specifically co-purified with Eiger/TNF (Fig. 4M). We conclude that oncogenic Ras promotes the interaction of the exocyst with Eiger/TNF.

Oncogenic Ras promotes Eiger/TNF exocytosis to prevent tumor cell death

Endocytosed molecules destined for secretion are shuttled to the cell cortex in Rab11-positive endosomes (Dollar et al., 2002; Sonnichsen et al., 2000; Ullrich et al., 1996). We thus tested whether Eiger/TNF localizes to early (Rab5-positive) endosomes and to recycling (Rab11-positive) endosomes. We found that Eiger/TNF colocalized with both Rab5 and Rab11 endosomes (Fig. 4E-L). Together with the Eiger/TNF-exocyst interaction data above, these observations supported the notion that Eiger/TNF is being cleared out of RasV12 tumors by exocytosis. To directly test this, we examined Eiger/TNF protein distribution in similarly aged RasV12 or RasV12, sec15 mutant clones. We found that Eiger/TNF accumulated at the margins of RasV12 clones (Fig. 5A-C), consistent with the Eiger/TNF protein being exocytosed. Importantly, examination of RasV12, sec15 double mutant clones revealed that Eiger/TNF no longer accumulated at the boundary, but rather accumulated within the clones (Fig. 5D,E). The sec15 mutant clones showed a moderate retention of Eiger/TNF (Fig. 5V,W). We then depleted Eiger/TNF specifically in the RasV12 cells and found that this prevented the accumulation of Eiger/TNF that is otherwise seen as a ring around the clones (Fig. 5F,G).

Furthermore, the cellular release of soluble TNF from its precursor transmembrane form and its resulting activity are mediated by the TNF-converting enzyme, Tace (Black et al., 1997; Blobel, 1997; Moss et al., 1997). Eiger/TNF contains a Tace cleavage site
equivalent to TNFα cleavage site in vertebrates (Kauppila et al., 2003; Narasimamurthy et al., 2009). We first verified that Drosophila Tace plays a similar role in Eiger/TNF signaling. First, we found that RNAi knockdown of the sole Drosophila Tace in the developing eye suppressed the Eiger/TNF-mediated small-eye phenotype (supplementary material Fig. S3A-D). Similar results were obtained in wing imaginal discs (data not shown). We subsequently tested for a role for Tace in Eiger/TNF non-autonomous signaling using the hanging-eye phenotype model (Narasimamurthy et al., 2009). We found that coexpression of eiger and Tace in cells rescued by expression of the dominant-negative JNK allele bsk-DN in the developing eye (GMR>egr,Tace,bsk-DN) produced a hanging-eye phenotype (supplementary material Fig. S3F,F'). The majority of GMR>egr,Tace,bsk-DN animals died in late pupal stages (73%, N=157) and show a ring of necrotic tissue around the eyes (supplementary material Fig. S3F'). Interestingly, the GMR>egr,bsk-DN animals did not have a dramatic hanging-eye phenotype (supplementary material Fig. S3E). These data indicate that Tace plays an important role in the secretion and signaling of Eiger. Finally, we knocked down Tace by RNAi in the RasV12 cells and found that this abolished Eiger/TNF accumulation around the RasV12 clones (supplementary material Fig. S4). Collectively, these data indicate that the pool of Eiger/TNF seen around RasV12 clones originates from RasV12 cells, and Sec15 plays an important role in Eiger/TNF protein export.

Eiger/TNF normally activates JNK signaling to trigger cell death (Igaki et al., 2009; Moreno et al., 2002). Correspondingly, we found that JNK activity was elevated inside the RasV12, sec15 double mutant clones compared with that of RasV12 or sec15 mutant clones (Fig. 5H,L,X,Y). Increased JNK signaling correlated with increased ectopic cell death inside the RasV12, sec15 double mutant clones compared with RasV12 single mutant clones, as measured by activated caspase-3 immunoreactivity, a cell death indicator (58.74±19.48%; N=257 versus 6.99±4.32%; N=699 caspase-positive cells, respectively; mean±s.d.; Fig. 5O-Q versus Fig. 5R,S; Fig. 5Z). Furthermore, concomitant suppression of JNK signaling, by expressing the dominant-negative JNK allele bsk-DN, restored viability to RasV12, sec15 clones (3.85±5.8%; N=408 caspase-
positive cells; Fig. 5T-U,Z). We conclude that Sec15-dependent exocytosis of Eiger/TNF downregulates JNK to protect RasV12 cells from apoptosis.

Eiger/TNF activates JAK-STAT signaling non-autonomously
Although blocking JNK signaling in RasV12, sec15 double mutant clones suppresses cell death, it fails to fully restore RasV12 tumor growth (supplementary material Fig. S5A,B). This suggests that Sec15 contributes to RasV12 tumor growth by an additional mechanism. Similar to vertebrate TNF, Eiger can act non-autonomously (Cordero et al., 2010; Pérez-Garijo et al., 2013). In addition, it has been shown that JNK signaling can non-autonomously stimulate the proliferation of adjacent cells (Ryoo et al., 2004; Wu et al., 2010), thus we wondered whether the Sec15-dependent accumulation of Eiger/TNF in the surrounding wild-type cells activates JNK to promote RasV12 tumor growth. We tested whether JNK is activated in the wild-type cells surrounding RasV12 clones. JNK activity was elevated in wild-type cells surrounding RasV12 tumors (Fig. 5H-J), but not in wild-type cells surrounding the Eiger/TNF-secretion-defective RasV12, sec15 double mutant clones or the Eiger/TNF RNAi-depleted RasV12 clones (Fig. 5K-N). In agreement with this and previous findings (Karim and Rubin, 1998), we could detect activated caspase-positive cells around RasV12 tumors (Fig. 5O-Q). Therefore, increased Eiger/TNF secretion by RasV12 cells leads to Eiger/TNF accumulation and activation of JNK signaling in surrounding wild-type cells. This is consistent with earlier reports of Eiger/TNF activating JNK non-autonomously (Cordero et al., 2010; Pérez-Garijo et al., 2013).

We have previously shown that JNK activation triggered by cell polarity defects can induce the expression of JAK-STAT ligands encoded by the unpaired (upd) genes. Upd ligands cooperate with RasV12 through non-autonomous JAK-STAT activation (Wu et al., 2010). Blocking JNK signaling in RasV12 cells has no obvious effect on their proliferation (Igaki et al., 2006), supporting the possibility that the observed JNK activation in surrounding wild-type cells could promote RasV12 tumor growth by a non-cell autonomous mechanism. We thus wondered whether Eiger/TNF-exocytosis-mediated JNK activation in the surrounding cells promotes the growth of RasV12 cells. We first examined Upd expression levels using the Upd-lacZ reporter line (Sun et al., 1995) and found that Upd was particularly up-regulated in wild-type cells surrounding RasV12 clones (supplementary material Fig. S6D). Next, we used a JAK-STAT activity GFP reporter (Bach et al., 2007), which includes the promoter fragment of Socs36E, a transcriptional target of JAK-STAT signaling (Karsten et al., 2007), which includes the promoter fragment of Socs36E, a transcriptional target of JAK-STAT signaling (Karsten et al., 2002), to examine JAK-STAT activity level in RasV12 cells. JAK-STAT was upregulated in RasV12 cells and in the surrounding Upd-producing wild-type cells (Fig. 6A). This increase was more robust in larger RasV12 clones than in smaller ones (supplementary material Fig. S6B, arrow versus boxed area). Similarly, Stat92E immunoreactivity, which is an indicator of JAK-STAT pathway activation (Chen et al., 2002; Johansen et al., 2003), was elevated in RasV12 discs (supplementary material Fig. S6F). In a complementary experiment, we found that the transcription level of Socs36E was elevated in RasV12 discs compared with that of wild-type control discs (Fig. 6F), consistent with the STAT-GFP and Stat92E results. This indicates that JAK-STAT signaling is activated in RasV12 cells.

To test whether the observed activation of JAK-STAT in RasV12 cells is due to increased Eiger/TNF exocytosis to neighboring wild-
type cells and the resulting JNK activation there, we first blocked Eiger/TNF exocytosis to the surrounding wild-type cells and examined JAK-STAT activation. We found that depleting Eiger/TNF specifically in RasV12 cells (RasV12, egr-RNAi clones) or preventing the secretion of Eiger/TNF (RasV12, sec15 double mutant cells) or reducing the protein dosage of the Eiger/TNF receptor (Wengen) all suppressed JAK-STAT activity compared to that of RasV12 cells (Fig. 6F; supplementary material Fig. S6E-G). Consistent with these results, wild-type cells surrounding RasV12, sec15 double mutant clones show elevated phospho-JNK levels. 

Next, we tested whether directly blocking JNK activation specifically in the surrounding wild-type cells would suppress JAK-STAT activation in RasV12 clones. To prevent JNK activation specifically in wild-type cells surrounding RasV12 clones, we induced RasV12 clones in JNKKK dtak1 (Tak1–FlyBase) mutant discs, but rescued JNKKK/dTAK1 function with a UAS-dTAK1 transgene only in the RasV12 clones (i.e. clones of cells co-expressing RasV12 and dTAK1 in dtak1-null mutant discs). These clones, hereafter referred to as dtak1//RasV12 clones, did not show STAT-GFP expression (Fig. 6B), mimicking RasV12, egr-RNAi clones. Finally, we removed the surrounding wild-type cells by expressing RasV12 throughout the developing eye discs [Eyeless-Gal4>RasV12, (ey>RasV12)] and examined JAK-STAT activation. These discs did not show broad STAT-GFP induction. Instead, we detected isolated patches of STAT-GFP-positive cells near caspase-positive cells (supplementary material Fig. S6C). Taken together, these findings indicate that it is the activation of JNK signaling specifically in the surrounding wild-type cells that non-cell autonomously stimulates JAK-STAT signaling in RasV12 cells. 

JAK-STAT signaling promotes oncogenic Ras-mediated overgrowth

Finally, we sought to investigate whether this Eiger/TNF exocytosis-triggered JNK-JAK-STAT signaling relay contributes to RasV12 tumor growth. If this is the case, then it should be expected that (1) depleting Eiger/TNF in RasV12 cells (RasV12, egr-RNAi), (2)
inducing RasV12 clones in eiger mutant animals, (3) directly blocking JNK in wild-type cells surrounding RasV12 cells (RasV12//dtak1, juxtaposed dtak1 and RasV12 mutant cells) or (4) preventing JAK-STAT activation in RasV12 cells would result in suppression of overgrowth. Indeed, RasV12, eiger-RNAi or RasV12 clones induced in eiger mutant animals showed reduced growth compared with that of mosaic RasV12 clones (Fig. 7A-C,G). Moreover, RasV12, Tace-RNAi clones, which display no Eiger/TNF accumulation around the JAK-STAT activation (supplementary material Fig. S3C,G-J). Similarly, activation in RasV12 clones killed the animal during pupal stages (Fig. 1K; supplementary material Fig. S6K). We then tested whether preventing JAK-STAT clones kills the animal during pupal stages (Fig. 1K; supplementary material Fig. S6K). This suggests that the patches of dying cells contribute to the growth of RasV12 cells. Taken together, the above findings indicate that the accumulation of Eiger/TNF and the resulting activation of JNK signaling in the surrounding wild-type cells non-autonomously promote RasV12 tumor growth through JAK-STAT activation (Fig. 8).
Sec15 transcription is increased in RasV12 tissues (supplementary material Fig. S7A). Furthermore, activated Rals interact with and stimulate the exocyst through Exo84 and Sec5 (Hamad et al., 2002; Moskalenko et al., 2002), suggesting that RalGEF-Ral signaling could be the underlying means by which RasV12 cells generally stimulate exocytosis. We cannot rule out the possibility that the exocytosis of other molecules is elevated.

Eiger/TNF exocytosis is mediated by the Sec15-Eiger/TNF interaction. Consistent with this, sec15 mutant cells retain Eiger/TNF. In contrast to RasV12, sec15 double mutant cells, sec15 mutant cells do not undergo apoptosis. This is likely owing to the fact that oncogenic Ras stimulates Eiger/TNF expression (supplementary material Fig. S7B) in addition to promoting Eiger/TNF exocytosis. The Sec15-Eiger/TNF interaction is interesting as it might represent a novel role of the exocyst in cargo selection. It is tempting to speculate that the cytosolic domain of Eiger/TNF could mediate its interaction with the exocyst. In the future it will be interesting to investigate the nature of this interaction, map out the relevant domains and elucidate a sorting mechanism. The finding that oncogenic Ras hijacks the exocytosis machinery to clear up pro-apoptotic ligands provides a new way by which tumor cells can evade cell death and an additional explanation of how exocytosis can modulate signal transduction.

Oncogenic Ras usurps the JNK-JAK-STAT non-autonomous signal to promote overgrowth

We have previously shown that JNK activation triggered by cell polarity defects can stimulate non-autonomous JAK-STAT signaling for proliferation and that JAK-STAT signaling can cooperate with RasV12 (Wu et al., 2010). Here, we discovered that one of the effects of oncogenic Ras itself is to hijack this non-autonomous JNK-JAK-STAT proliferation signaling cascade. We propose that Eiger/TNF, including Eiger/TNF from the nearby fast-secreting RasV12 cells, is endocytosed but rapidly cleared out of RasV12 cells by the exocyst. Elevated exocytosis in RasV12 cells creates an imbalance such that the amount of secreted Eiger/TNF is higher than that being internalized. This not only causes a RasV12...
cell to retain less Egger/TNF, but the endocytosis and exocytosis cycle also permits neighboring RasV12 cells to relay Egger/TNF, resulting in the eventual accumulation of Egger/TNF in the surrounding wild-type cells. Consistent with this, depleting Egger/TNF or interfering with the exocyst specifically in RasV12 cells abolished Egger/TNF accumulation around RasV12 clones. Egger/TNF from RasV12 cells activates JNK signaling in the surrounding wild-type cells. JNK activation stimulates JAK-STAT signaling in RasV12 cells to promote tumor overgrowth. Congruent with this model, JNK activity and the JAK-STAT ligand Udp are upregulated in wild-type cells surrounding RasV12 clones. Preventing Egger/TNF secretion (RasV12, sec13 double mutant clones or RasV12, egr-RNAi clones) suppresses JNK activation, Udp stimulation and JAK-STAT activation in the surrounding wild-type cells. Moreover, blocking JNK signaling specifically in wild-type cells surrounding RasV12 clones prevents JAK-STAT activation in RasV12 cells and suppresses tumor growth.

Interestingly, oncogenic Ras or wound-induced JNK activation does not cause invasion, in contrast to JNK induced by the cell polarity mutation scrib. This could be due to context-dependent JNK activation. However, we found that Sec13-RNAi suppresses the growth and invasion phenotypes of RasV12, scrib double mutant clones (supplementary material Fig. S8A-D) and rescues animal lethality (supplementary material Fig. S8E-F), highlighting the importance of exocytosis in promoting tumor growth and invasion.

Another striking result of our study is the realization of the importance of the interaction between RasV12 and the surrounding non-RasV12 cells in oncogenic Ras-mediated growth. This is best illustrated by the fact that a tissue in which oncogenic Ras is uniformly expressed grows less than a tissue containing both wild-type and oncogenic Ras-expressing cells. This is consistent with accumulating data from rodents and patients indicating that signaling events emanating from host/stromal cells promote tumor development (Brennes et al., 2011; Lu et al., 2013; Mueller and Fusenig, 2004; Sounni and Noel, 2012). Additional significance is derived from recent studies of various solid tumors showing heterogeneity within each tumor (Diaz et al., 2012; Gerlinger et al., 2012; Gerstung et al., 2011; Marusyk and Polyak, 2010).

Our study reveals that an important effect of oncogenic Ras is the increased exocytosis of Egger/TNF. Indeed, the fact that blocking exocytosis of Egger/TNF can suppress oncogenic Ras-mediated growth highlights the importance of Egger/TNF exocytosis in cancer biology. Exocytosis proteins are conserved and have been implicated in diverse human cancers types, including Ras cancers (Cheng et al. 2004, 2005; Issaq et al., 2010; Neel et al., 2011; Palmer et al., 2002), suggesting that a similar tumor-promoting mechanism could be at play in vertebrates. Because sec15 null mutation does not cause cell lethality and the gene is evolutionarily conserved, it suggests a new type of potential therapeutic targets.

**MATERIALS AND METHODS**

**Fly lines**

Animals were aged at 25°C on standard medium. The following fly lines were used in this study: (1) y w; FRT82B; (2) y w; FRT82B, UAS-RasV12/TM6B; (3) y w; FRT82B, UAS-RasV12; (4) y w; FRT82B, sec15; (H. Bellen, Baylor College of Medicine, Houston, TX, USA); (5) y w; UAS-RasV12/TM6B; (6) y w; UAS-rib-RNAi (VDRC); (7) FRT40A, UAS-STAT-65; (8) y w; UAS-RasV12/TM6B; (9) w; UAS-RasV12; (10) FRT82B, Stat922/TM6B; (11) w; UAS-dTak1; (12) y w; GAL4,UAS-myrRFP,FRT82B,tub-GAL80; (13) y w; ey-Flp5;act>y+>GAL4,UAS-myrRFP,FRT82B,tub-GAL80; (14) y w; ey-Flp5, act>y+>GAL4,UAS-STAT-65; (15) y w; ey-Flp5, act>y+>GAL4; UAS-GFP/Cyo; FRT82B, Tub-Gal80/TM6B; (16) y w; dTak1; ey-Flp5, act>y+>GAL4,UAS-GFP,FRT82B,tub-GAL80; (17) y w; ey-Flp5, act>y+>GAL4; UAS-myrRFP,FRT82B,tub-GAL80; (18) y w; UAS-dTak1; FRT82B, UAS-RasV12; (19) upd-lacZ; UAS-RasV12; sec15; FRT82B/Sh; (20) UAS-RasV12; UAS-sec15-RNAi; 10XSTAT-65F; FRT82B/tub; (21) ey-Flp5, act>y+>GAL4,UAS-GFP; (22) sec6-RNAi (VDRC); (23) sec8-RNAi (VDRC); (24) Wgn (P. Barker, McGill University, Montreal, Canada); (25) UAS-TACE-RNAi; (26) UAS-TACE; (27) UAS-Rab5-GFP; (28) GMR-Gal4; (29) UAS-Eiger; and (30) UAS-Bsk-DN.

**Staining and imaging**

Eye-antenna discs were dissected, fixed and stained as described previously (Igaki et al., 2006; Pagliarini and Xu, 2003; Wu et al., 2010). Tumor and adult eye size analyses were carried out on a Leica MZ FLIII fluorescence stereomicroscope equipped with a camera. Samples were examined by confocal microscopy with a Zeiss LSM510 Meta system. Images were analyzed and processed with Imaris (Bitplane) and Illustrator (Adobe) software, respectively. The following primary antibodies were used: guinea pig anti-Sec6 at 1:1000 [U. Tesap (University of Toronto, Canada) and H. Bellen]; guinea pig anti-Sec6 at 1:1000 (U. Tesap and H. Bellen); guinea pig anti-Sec15 at 1:1000 (H. Bellen); rat anti-Rab11 at 1:1000 (S. Cohen, University of Kansas, USA); rabbit anti-Rab11 at 1:1000 (D. Ready, Purdue University, IN, USA); rabbit anti-Stat92E at 1:1000 (S. Hou); rabbit anti-phospho-histone-3 at 1:1000; rabbit anti-cleaved caspase-3 at 1:500; rabbit anti-Eiger polyclonal antibody R1 at 1:50 (T. Igaki, Kobe University, Japan); mouse anti-Phospho-JNK monoclonal antibody G9 at 1:250. Secondary antibodies were from Invitrogen. TUNEL staining was performed using the Apoptag Red kit from Chemicon.

**Isosurface rendering**

Projections of serial confocal sections were used to generate three-dimensional graphical images using the image analysis software Imaris. The clipping plane function was used to section across the resulting graphical images.

**Vesicle trafficking studies**

The whole central nervous system, including the attached eye discs, was dissected in Schneider medium and incubated in Schneider medium supplemented with 0.5 mg/ml Dextran-Alexa-546 for 2 h. Half of the samples were immediately washed and fixed. Eye discs were separated and mounted in DAPI-Vectashield. The remaining half of the samples were washed and incubated in Schneider medium for an additional 2 h (chase), rinsed in PBS, fixed and mounted as above.

**Western blots and immunoprecipitation**

Third instar imaginal discs bearing wild-type or RasV12-expressing cells were homogenized in lysis buffer (50 mM HEPES pH 7.5, 150 mM KCl, 5 mM MgCl2, supplemented with protease inhibitor tablets; Roche). Samples were separated by SDS-PAGE, transferred onto nitrocellulose membrane and blotted with anti-Stat15, anti-Stat6, anti-Stat4. Lysates were diluted 1:100 and blotted with anti-α-Tubulin antibodies as a loading control. For immunoprecipitation experiments, 500 μl of lysate obtained from discs containing RasV12 clones was pre-cleared with Protein A-agarose beads for 1 h at 4°C, split equally into two tubes and incubated either with 2 μl of anti-Eiger or 2 μl of anti-GFP antibodies for 4 h at 4°C. Lysates were then incubated with Protein A-agarose beads for 1 h at room temperature. For pull downs, beads were precipitated and washed three times in modified lysis buffer containing 0.5% Triton X-100. Samples were separated by SDS-PAGE, transferred onto nitrocellulose membrane and blotted with antibodies against Sec15, Sec6, Sec5 and α-Tubulin.

**Real-time polymerase chain reaction**

Total RNA from eye-antenna imaginal discs containing wild-type or mutant clones was isolated using a Trizol RNA extraction method. The SuperScript II Transcriptase kit (Invitrogen) was used to synthesize cDNA. Real-time PCR was carried out on an Applied Biosystems machine using the SYBR Green fast kit following the manufacturer’s instructions. Relative gene expression was obtained from triplicate runs normalized to Rp49
immunoprecipitation experiments were performed as described previously. Phenotype experiments were carried out at 29°C. Immunostaining and standard MARCM system (Lee and Luo, 2001) at 25°C. Hanging-eye TGCTGTCCATCAACACCTT-3′; TGCGACAGCTTAGCATATC-3′; CAA-3′.


