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 proliferation 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.

KEYWORDS: 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; Cowley 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 Ulrich, 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; Jafar-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 (Soto 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 (Soto 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-triggered cell death (Igaki et al., 2009; Moreno et al., 2002). Importantly, examination of Ras<sup>V12</sup> tumors by exocytosis. To directly test this, we took up the dye as they contained more dye (dye-positive punctae) than control cells [4.05±1.47 for Ras<sup>V12</sup> versus 2.29±0.65 for wild-type cells (means±s.d.); Fig. 3A,B,G], indicating that endocytosis is generally not inhibited in Ras<sup>V12</sup> cells. Examining dye abundance in mutant versus control cells after the dye chase period provided a crude measure of secretion within these cells. Interestingly, Ras<sup>V12</sup> cells were significantly more efficient at dye clearance after the dye chase phase. Ras<sup>V12</sup> cells showed fewer dye-positive punctae compared with wild-type cells (the pulse/chase ratios of dye-positive punctae for Ras<sup>V12</sup> and wild-type cells were 4.63±0.27 and 1.53±1.12, respectively; Fig. 3D,E,G). The <i>sec15</i> 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 <i>Drosophila</i>, 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; Blobel, 1997; Kauppila et al., 2003; Moss et al., 1997). Eiger/TNF is a soluble ligand (Black et al., 1997; Brandt et al., 2004; Jue et al., 2002). Similar to vertebrate TNFs, Eiger is produced as a 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 suppressed the ability of RasV12 cells to clear out the dye (C,F). (G) Quantification of A-F. The number of dye-positive vesicles after each experimental phase (pulse and chase) was scored in multiple clones for each genotype. Pulse:chase ratios were derived from averages of dye-positive vesicle numbers after each experimental phase. Error bars represent standard deviation from the mean for each genotype analyzed. In this and all subsequent figures, numbers within bars indicate the number of cells analyzed per corresponding genotype.

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-
secretion by polarity defects can induce the expression of JAK-STAT ligands autonomously (Cordero et al., 2010; Pérez-Garijo et al., 2013). Consistent with earlier reports of Eiger/TNF activating JNK non-activation of JNK signaling in surrounding wild-type cells. This is RasV12 clones. JNK activity was elevated in wild-type cells surrounding 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 (Baeh 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 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 oncocgenic 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, egr-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 in RasV12//dtak1 (supplementary material Fig. S3C,G-J). We then tested whether preventing JAK-STAT
activity in RasV12//dtak1 clones kills the animal during pupal stages (Fig. 1K; supplementary
material Fig. S6K). We then tested whether preventing JAK-STAT
clones (Fig. 7A-C,G). Moreover, RasV12, Tace-RNAi
clones, which display no Eiger/TNF accumulation around the
JAK-STAT activation in RasV12//dtak1 (supplementary material Fig. S3C,G-J). Similarly, RasV12//dtak1 mutant cells showed reduced growth compared to that of controls
(Fig. 7E-G).

Furthermore, removing the surrounding wild-type cells by expressing RasV12 throughout the eye discs using eyFLP, Act>y>Gal4, UAS-RasV12 or ey>RasV12 results only in moderate
growth compared to the more pronounced overgrowth of mosaic
RasV12 tissues (supplementary material Fig. S6J and see figure S1 in
Wu et al., 2010). Consistent with reduced growth, ey>RasV12 discs do
cause animal lethality, whereas the overgrowth of mosaic RasV12 clones kills the animal during pupal stages (Fig. 1K; supplementary
material Fig. S6K). We then tested whether preventing JAK-STAT
activation in RasV12 cells, by co-expressing an RNAi transgene against
the JAK-STAT receptor, Domeless. (RasV12, dom-RNAi clones)
suppresses RasV12 tumor growth. RasV12, dom-RNAi clones showed a
reduced mitotic potential as determined by the percentage of
phosphorylated-histone-3-positive cells (6.3±5%, N=214 for
RasV12, dom-RNAi clones and 49.5± 29.5%, N=214 for RasV12;
mean±s.d.; Fig. 6C,D,E) and correspondingly showed reduced tumor
growth (Fig. 7A,D,G). Similarly, blocking JAK-STAT activity in ey>RasV12 (ey>RasV12, dom-RNAi) suppresses growth
(supplementary material Fig. S5K,L). 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).

DISCUSSION

Earlier efforts to understand Ras signaling during normal development in model organisms led to the elucidation of the
Ras/MAPK signaling cascade (Nishida and Gotoh, 1993; Simon
et al., 1991; Sternberg and Horvitz, 1991). However, the signaling
events that are elicited by oncogenic Ras to drive overgrowth are
complex and not fully understood (reviewed by Mitin et al., 2005).
Here, we used a Drosophila model and searched for mutations that
can block RasV12 overgrowth in an in vivo synthetic screen. We
identified sec15, a mutation in a gene encoding a component of the
exocytosis machinery, as a synthetic suppressor of RasV12 tumor
growth. Studying the underlying cause of this phenomenon led to
the unexpected discovery of both cell-intrinsic and cell-extrinsic
mechanisms that promote RasV12 overgrowth.

Oncogenic Ras stimulates Eiger/TNF exocytosis

We found that RasV12 tumors elevate exocytosis to promote rapid
clearance of Eiger/TNF and hence avert JNK-mediated cell death.
Oncogenic Ras could stimulate the exocytosis through the Ras-specific
guanine nucleotide exchange factors (RalGEFs). It has been shown
that oncogenic Ras activates RalGEFs, which in turn activate the
Ras-like small GTPases RaLA and RaLB (Lim et al., 2005; Urano
et al., 1996; White et al., 1996; Wolthuis et al., 1998). Moreover,
RalGEF-Ral signaling stimulates gene expression (Neel et al.,
2011). It is therefore likely that Ras mediates the observed increased
levels of exocyst proteins in RasV12 cells. Consistent with this,
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-induced cell death (green inhibition arrow) by promoting (top green arrow) the exocytosis of Eiger to the surrounding wild-type cells (top red arrow). This results in JNK activation in the surrounding wild-type (WT) cells, which non-autonomously activates the JAK-STAT pathway (bottom red arrow) to promote oncogenic Ras tumor growth.

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**Fig. 7. Eiger/TNF exocytosis activation of JAK-STAT signaling promotes RasV12 tumor overgrowth.** (A-F) Clones (green) from similarly aged intact third instar larvae (A-D), dissected cephalic regions (E,F) or dissected eye discs (lower panels). RasV12 clones overgrow (A). eiger/Tnf RNAi knockdown specifically in the RasV12 clones (B) or generating RasV12 clones in eiger mutant animals (C) or blocking JAK-STAT signaling by using Domeless RNAi (dom-RNAi) (D) suppresses the overgrowth of RasV12 clones. Dissected cephalic regions (E,F) or dissected eye discs (lower panels) show that dTAK1-rescued RasV12 clones induced in dtak1 hemizygous males (RasV12/dtak1−/−) (F), exhibit reduced growth compared with dTAK1-rescued RasV12 clones induced in the siblings (dtak1 heterozygous females; RasV12/dtak1+/−) (E). (G) Quantification of A-F. Confocal stacks from similarly aged animals of the indicated genotypes and the image analysis software Imaris were used to measure clone (green) sizes. Error bars show s.d. The numbers in the bars indicate the sample size for the corresponding genotype.

**Fig. 8. Oncogenic Ras promotes overgrowth by stimulating Eiger/TNF exocytosis and activating JNK-JAK-STAT non-autonomous proliferation signaling.** Eiger normally activates JNK to trigger cell death. JAK-STAT signaling on the other end promotes cell proliferation (black arrows). Sec15 blocks Eiger-induced cell death (green inhibition arrow) by promoting (top green arrow) the exocytosis of Eiger to the surrounding wild-type cells (top red arrow). This results in JNK activation in the surrounding wild-type (WT) cells, which non-autonomously activates the JAK-STAT pathway (bottom red arrow) to promote oncogenic Ras tumor growth.
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. Convergent with this model, JNK activity and the JAK-STAT ligand Upd are upregulated in wild-type cells surrounding RasV12 clones. Preventing Egger/TNF secretion (RasV12, sec15 double mutant clones or RasV12, egr-RNAi clones) suppresses JNK activation, Upd 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 Sec15-RNAi suppresses the growth and invasion phenotypes of RasV12, scrib double mutant clones (supplementary material Fig. S8-A-D) and rescues animal lethality (supplementary material Fig. S8-E), 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 Fusseneg, 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; Neil 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; FRT40A, UAS-RasV12; (4) y w; FRT82B, sec15 (H. Bellen, Baylor College of Medicine, Houston, TX, USA); (5) y w; UAS-RasV12; FRT82B, sec15/TM6B; (6) UAS- Sec15-RNAi (VDRC); (7) FRT40A, UAS-RasV12; UAS-eiger-RNAi/TM6B; (8) w; UAS-RasV12; FRT82B, Stat92E6340/TM6B; (9) w; UAS-RasV12; FRT82B, UAS- dim (IR) TM6B; (10) y w; ey-Flip1; act> y-GAL4, UAS-GFP,S657; FRT82B, tub-GAL80; (11) y w; ey-Flip1; act>y-GAL4, UAS-myRFP,FRT82B, tub-GAL80; (12) y w; ey-Flip1; tub-GAL80, FRT40A; act>y-GAL4, UAS-GFP,S657; (13) w; 10XSTAT-GFP,1; (14) UAS-UAS-BskDN; ey-Flip5, act>y-GAL4, UAS-GFP; FRT82B, tub-GAL80; (15) Eyless-GAL4 (Bloomington); (16) w; dTak1; ey-Flip5, act>y-GAL4; UAS-myrRFP,FRT82B, tub-GAL80; (17) w; dTak1; ey-Flip5, act>y-GAL4, UAS-myRFP,FRT82B, tub-GAL80; (18) UAS-dTak1; FRT82B, UAS-RasV12, (19) w; upd-lacZ, UAS-RasV12, sec15, FRT82B/Sh; (20) UAS-RasV12; UAS- Sec15-RNAi; 10XSTAT-GFP,1/TM6B; (21) ey-Flip5, act>y-GAL4, UAS-GFP,S657; (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 (Bloomington); (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. Tepass (University of Toronto, Canada) and H. Bellen]; guinea pig anti-Sec6 at 1:1000 (U. Tepass 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 2h. 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 2h (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-sec5, anti-Sec6, anti-Sec8. Lysates were diluted 1:100 and blotted with anti-α-Tubulin antibodies as a loading control. For immunoprecipitation experiments, 500 µl of lystate obtained from discs containing RasV12 clones was pre-cleared with Protein A-agarose and blotted with anti-sec5, anti-sec6, anti-sec8, anti-Rab5, anti-α-Tubulin. Lysates were diluted 1:100 and blotted with anti-α-Tubulin antibodies for 4 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 Sec5, Sec8, Sec6 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 First-Strand Synthesis System kit was used to synthesize cDNA. Real-time PCR was performed using the Apoptag Red kit from Chemicon.

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immunoprecipitation experiments were performed as described previously. The standard MARCM system (Lee and Luo, 2001) at 25°C. Hanging-eye

TGCTGTCCATCAACACCTT-3

TGCGACAGCTTAGCATATC-3

CATT-3

used:


4738

Alessi, D. R., Saito, Y., Campbell, D. G., Cohen, P., Sithanandam, G., Rapp, 12 months.


**Supplementary Figure 1**

*sec15* mutant cells show normal proliferation potential while RNAi knockdown of *Sec15*, *Sec6*, and *Sec8* suppresses Ras tumor growth.
A and B) Eye disc showing sec15 mutant clones (red) across the proliferative layer of cells (brackets). Discs were treated with the S-phase marker 5-ethynyl-2'-deoxyuridine (EDU, green) and DAPI to detect cells nuclei (blue).

C-H) Comparable confocal projections of eye-antenna discs showing RFP-labeled wild-type, sec6-RNAi, sec8-RNAi, Ras\textsuperscript{V12}, Ras\textsuperscript{V12}, sec6-RNAi, Ras\textsuperscript{V12}, sec8-RNAi clones.

sec6-RNAi clones (D) grow less than wild-type clones (C). sec8-RNAi (E) and wild-type clones (C) are similar in size. Ras\textsuperscript{V12} clones overgrow (F). RNAi knockdown of Sec6 or Sec8 suppresses Ras\textsuperscript{V12} overgrowth (G and H).

I-N) Adult eyes showing exocyst proteins knockdown suppresses Ras\textsuperscript{V12} pupal lethality. RNAi knockdown of Sec6 or Sec8 results in viable adult animals (I-K). RNAi knockdown of Sec6 or Sec8 or Sec15 suppresses pupal lethality caused by Ras\textsuperscript{V12} clones overgrowth (L-N).
Supplementary Figure 2

Sec15, Sec6, and Sec8 antibodies are specific.

A-C) sec15, sec6-RNAi, and sec8-RNAi clones stained with the corresponding antibodies (Sec15, Sec6, Sec8,) and co-stained with DAPI to mark nuclei. Bottom panels show Sec15, Sec6, Sec8, channels alone. Dotted green lines denote clones boundaries.

Staining of Sec15 (A), Sec6 (B), and Sec8 (C) are reduced to negligible levels in the corresponding mutant or RNAi cells.
Supplementary Figure 3

*Drosophila* Tace regulates Eiger/TNF secretion and signaling.

A-F') Adult eyes showing wild-type (A) or *GMR-Gal4* driven transgenes (B-F'). Eiger’s expression produces a smaller eye (B). Expression of *Tace-RNAi* alone (C). *Tace-RNAi, Eiger* co-expression suppresses the small eye phenotype (D). Co-expression of *Eiger* and *bsk-DN* produce a moderate hanging eye phenotype (E). The hanging eye phenotype is more pronounced in adult eyes co-expressing *Eiger, bsk-*
$DN$, and a wild-type version of Tace (F). F’ shows a rotated view of F. (F’). The majority of these animal die in late pupal stages and show a ring of necrotic tissue around the eyes as shown in the image of a dissected pupal eye (F’’).

G-J) Equivalent confocal projections showing dissected eye discs expressing $Ras^{V12}$ (G), $Ras^{V12}$, Tace-RNAi (H), Tace-RNAi (I), and wild-type clones (green) (J) stained with DAPI (blue).
Supplementary Figure 4

*Tace* knockdown suppresses the accumulation of Eiger/TNF around the *Ras*\(^{V12}\) clones and suppresses overgrowth.

*Ras*\(^{V12}\), *Tace-RNAi* double mutant clones (green) stained with DAPI (blue) and Eiger (red). The bottom channel is Eiger alone. The dotted green lines delimit the clone boundary.
Supplementary Figure 5

**Additional mechanism contributes to sec15 suppression of Ras\(^{V12}\) tumor growth.**

A and B) Dissected eye discs showing Ras\(^{V12}\) or Ras\(^{V12}\), sec15, bsk-DN triple mutant clones (green) stained with DAPI (blue) to mark DNA. bsk-DN expression in Ras\(^{V12}\), sec15 double mutant clones (B) fails to fully restore Ras\(^{V12}\) tumor growth (A)
Supplementary Figure 6

Surrounding wild-type cells promote Ras\textsuperscript{V12} tumor growth via JAK-STAT activation.

A-C) JAK-STAT signaling (GFP) reporter expression in wild-type (A), in mosaic Ras\textsuperscript{V12} clones (B), and in discs homogenously expressing Ras\textsuperscript{V12} under the eyGal4
driver \((ey>\text{Ras}^{V12})\) (C). Mosaic \text{Ras}^{V12} \text{clones} \((ey\text{Flp, ActGal4}>y^+>UAS-GFP, UAS-\text{Ras}^{V12}, \text{FRT82B/TubGal80, FRT82B})\) strongly activate JAK-STAT (B) compared to control (A). JAK/STAT activation is more robust in larger \text{Ras}^{V12} \text{clones} than it is in smaller ones (B; arrow v.s boxed area).

JAK-STAT is not uniformly expressed in \(ey>\text{Ras}^{V12}\) discs. Instead activation is restricted to patches of cells neighboring capsapse3-positive dying cells (C).

D and H) \text{Upd-LacZ} expression around \text{Ras}^{V12} \text{clones} (D) and \text{Ras}^{V12}, \text{sec15} \text{double mutant clones} (H). Discs were stained with DAPI (blue) to mark cell nuclei and \(\beta\)-galactosidase (red) to detect \text{Upd} expression. Middle panel of H shows clone channel alone and the bottom panel is \text{Upd-LacZ} alone. Dotted green lines mark clones boundaries.

E-G) Wild-type discs bearing wild-type clones (E) or mosaic \text{Ras}^{V12} \text{clones} (F) or mosaic \text{Ras}^{V12} \text{clones} in discs heterozygous for the Wengen mutation \((\text{wgn}^{+/-})\) (G) stained for Stat92e and DAPI. Stat92e immune-reactivity is increased in \text{Ras}^{V12} \text{clones} (F) in the wild-type background (F) but not in \text{Ras}^{V12} \text{clones} induced in the \text{wgn}^{+/-} discs (G).

I and J) Dissected eye-antenna discs containing mosaic \text{Ras}^{V12} \text{clones} \((ey\text{Flp, ActGal4}>y^+>UAS-GFP, UAS-\text{Ras}^{V12}, \text{FRT82B/TubGal80, FRT82B})\) or uniformly expressing \text{Ras}^{V12} \((ey\text{Flp, ActGal4}>y^+>UAS-GFP, UAS-\text{Ras}^{V12})\) in the eye field fixed and stained with DAPI. Mosaic \text{Ras}^{V12} (J) \text{clones} cause significantly more tissue overgrowth than discs uniformly expressing \text{Ras}^{V12} (I).

K and L) Uniform expression of \text{Ras}^{V12} \((ey>\text{Ras}^{V12})\) or \text{dom-RNAi} co-expression \((ey>\text{Ras}^{V12}, \text{dom-RNAi})\) in the developing eye. Contrary to mosaic \text{Ras}^{V12} \text{tumors}, which are large and kill the animal, eye discs uniformly expressing \text{Ras}^{V12} in the developing eye field yields viable adults (K). Blocking JAK-STAT signaling \((\text{dom-RNAi})\) results in a smaller eye (L).
Supplementary Figure 7

**A**

Fold difference in SEC15 expression relative to Rp49.

**B**

Fold difference in EGER expression relative to Rp49.
Supplementary Figure 7

Oncogenic Ras transcriptionally stimulates Sec15 and Eiger.

RT-PCR experiment measuring Sec15 (A) and Eiger (B) expression levels relative to the expression levels of the housekeeping gene Rp49. Normalized fold differences of Sec15 and Eiger expressions levels between RasV12 and wild-type are shown. Experiments were performed in triplicates and standard deviations were derived from the coefficient variations of experimental and control samples.
Supplementary Figure 8

sec15-RNAi suppresses the overgrowth and invasiveness of Ras\textsuperscript{V12}, scrib\textsuperscript{−} mutant clones

A and B) Dissected cephalic complexes from similarly aged larvae showing GFP-labeled mutant clones. Brackets designate the ventral nerve cord (VNC). Ras\textsuperscript{V12}, scrib\textsuperscript{−} double mutant clones overgrow and invade the VNC (A). Growth and VNC invasion are suppressed in the Ras\textsuperscript{V12}, scrib\textsuperscript{−}, sec15-RNAi triple mutant clones (B).

C and D’) Intact similarly aged animal showing RFP-labeled mutant clones. Ras\textsuperscript{V12}, scrib\textsuperscript{−} double mutant clones overgrow and kill the animal in larval stages(C). sec15-RNAi suppresses tumor overgrowth and allows animals to pupate (D, D’).

E) Lethality rescue quantification.