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Tumor suppressors Sav/Scrib and oncogene Ras regulate stem cell transformation in adult *Drosophila* Malpighian Tubules

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Abstract

An increasing body of evidence suggests that tumors might originate from a few transformed cells that share many properties with normal stem cells. However, it remains unclear how normal stem cells are transformed into cancer stem cells. Here, we demonstrated that mutations causing the loss of tumor suppressor Sav or Scrib or activation of the oncogene Ras transform normal stem cells into cancer stem cells through a multistep process in the adult *Drosophila* Malpighian Tubules (MTs). In wild-type MTs, each stem cell generates one self-renewing and one differentiating daughter cell. However, in flies with loss-of-function *sav* or *scrib* or gain-of-function *Ras* mutations, both daughter cells grew and behaved like stem cells, leading to the formation of tumors in MTs. Ras functioned downstream of Sav and Scrib in regulating the stem cell transformation. The Ras-transformed stem cells exhibited many of the hallmarks of cancer, such as increased proliferation, reduced cell death, and failure to differentiate. We further demonstrated that several signal transduction pathways (including MEK/MAPK, RhoA, PKA, and TOR) mediate Ras function in the stem cell transformation. Therefore, we have identified a molecular mechanism that regulates stem cell transformation, and this finding may lead to strategies for preventing tumor formation in certain organs.

Keywords

Drosophila; Malpighian Tubules; Stem cell; Cancer stem cell; Tumor suppressor; Sav; Scrib; Oncogene; Ras

Introduction

Recent studies suggest that tumors may originate from a few transformed cells with stem-cell characteristics, called cancer stem cells (Reya *et al.*, 2001; Kim and Dirks, 2008). This hypothesis is based on observations that some solid tumors and leukemias contain small numbers of self-renewing cells that can re-generate an entire tumor mass when transplanted into mice (Al-Hajj *et al.*, 2003; Lapidot *et al.*, 1994; O'Brien *et al.*, 2007; Ricci-Vitiani *et al.*, 2007; Singh *et al.*, 2004). In the brain, these cancer stem cells often express markers of normal stem cells and remain dependent on the niche microenvironments that regulate normal stem cells (Calabrese *et al.*, 2007). In mouse intestine, normal stem cells are the cells-of-origin for cancer and are susceptible to neoplastic transformation (Barker *et al.*, 2009; Zhu *et al.*, 2009). Conventional cancer therapies designed to block cell proliferation may not always affect these stem cells, due to their unique properties (Michor *et al.*, 2005).

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Therefore, understanding the molecular mechanisms of stem cell regulation and transformation is an important prerequisite for developing new therapeutic strategies.

Besides the germline stem cells systems, the *Drosophila* adult stem cells such as intestinal stem cells (ISCs) are emerging as an important model to study genes and signal regulating the tissue homeostasis in steady-state and challenging condition (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Buchon et al., 2009; Jiang et al., 2009; Cronin et al., 2009; Amcheslavsky et al., 2009; Chatterjee and Ip, 2009.) We identified that the adult Malpighian Tubules (MT) is maintained by multipotent RNSC in *Drosophila* (Singh *et al.*, 2007). The Malpighian Tubules (MTs) of *Drosophila*, which function as the fly kidney, is another model system for studying adult stem cell regulation and transformation in a genetic model organism (Singh et al., 2007). The adult *Drosophila* has two pairs of MTs, a long anterior pair and a short posterior pair, which converge onto the alimentary canal through common ureters (Pugatcheva and Mamon, 2003; Sözen *et al.*, 1997; Wessing and Eichelberg, 1978). Each tubule of the longer anterior pair can be divided into four compartments: initial, transitional, main (secretory), and proximal (reabsorptive). The last of these can be further divided into a lower tubule and a ureter. We recently identified multipotent renal and nephric stem cells (RNSCs) among the “tiny” cells in the lower tubules and ureters of the MTs (Singh *et al.*, 2007). An RNSC can produce a new RNSC and an immature daughter, a renablast (RB), through asymmetric division. The RB has two potential fates. In the region of the lower tubules and ureters, the RB can become a mature renalcyte (RC) in about 5 days, through endoreplication. Alternatively, the RB can move toward the distal upper tubules and differentiate into a type I or II cell in the transitional and initial segments. In addition, autocrine JAK-STAT signaling regulates the self-renewal of these stem cells (Singh *et al.*, 2007).

Here, we screened for mutants that affect RNSC self-renewal and differentiation, using the positively marked mosaic lineage (PMML) labeling technique (Kirilly *et al.*, 2005) and the MARCM (mosaic analysis with a repressible cell marker) system (Lee and Luo, 1999). These screens revealed that mutations causing the loss of tumor suppressor Scribble (Scrib) or Salvador (Sav) function or the constitutive activation of the Ras oncogene caused RNSC overproliferation and resulted in tumor growth. Genetic experiments suggested that Ras functions downstream of Scrib and Sav in regulating stem cell transformation. We further analyzed the genes and signal transduction pathways that might mediate Ras^o function in RNSC transformation. Our results suggested that Scrib/Sav and Ras transform RNSCs by a multistep process involving several signal transduction pathways.

Materials and Methods

Drosophila Stocks

All crosses were done at 25°C unless otherwise indicated. The following fly stocks used in this study were described in FlyBase unless otherwise specified: *hs-FLP UAS-srcEGFP*, *FRT^{52B} (y) (yellow-FRT-Gal4)*, and *FRT^{52B} (w) (white-Actin5C-FRT) UAS-EGFP* were provided by T. Xie; *UAS-DaPKC^{CAAXWT}* and *UAS-DaPKC^{ΔN}* were provided by C. Doe and S. Campuzano; *Cyclin E-LacZ* and *hpo⁴²⁻⁴⁷* were provided by D. J. Pan; *UAS-Raf^{K497M}* was provided by M. Stern; *Dco^{B3}* was provided by D. Kalderon; *FRT^{82B}-Scrib¹* and *FRT^{82B}-Scrib²* were provided by D. Bilder; *FRT^{82B}-Sav³* and *FRT^{82B}-wts^{latsX1}* were provided by I. Hariharan; *sav^{shrp-5}* and *sav^{shrp-6}* were provided by G. Halder; *UAS-Ras^{V12}*, *UAS-Raf^{Gof}*, *UAS-PI3K^{DN}*, *UAS-RhoA^{N19}*, *UAS-RhoA^{V14}*, *Pnt-LacZ*, *Diap-LacZ*, *DaPKC^{k06403}*, *Igf⁴*, *Tor^{ΔP}*, *Stat92E^{6C8}*, *FRT^{82B} piM*, *AyGal4 UAS-GFP*, *FRT^{40A}-tub-Gal80*, *FRT^{G13}-tub-Gal80*, *FRT^{82B}-tub-Gal80*, *SM6*, *hs-Flp*, and *TM3, Sb, hs-Flp* were obtained from Bloomington stock center; All *UAS-RANi* lines were obtained from the VDRC stock center.

Generate GFP-marked RNSC clones

To generate GFP-marked mutant clones using the MARCM system, flies with the following genotypes were used:

FRT^{40A} UAS-Ras^{V12}, Tor^{ΔP}/FRT^{40A}-tub-Gal80; AyGal4 UAS-GFP/TM3, Sb, hs-Flp

FRT^{40A} UAS-Ras^{V12}, Dco^{B3}/FRT^{40A}-tub-Gal80; AyGal4 UAS-GFP/TM3, Sb, hs-Flp

FRT^{G13} DaPKC^{K06403}/FRT^{G13}-tub-Gal80; AyGal4 UAS-GFP/TM3, Sb, hs-Flp

FRT^{G13} UAS-Ras^{V12}, DaPKC^{K06403}/FRT^{G13}-tub-Gal80; AyGal4 UAS-GFP/TM3, Sb, hs-Flp

SM6, hs-Flp/ AyGal4 UAS-GFP, FRT^{82B}-tub-Gal80/ FRT^{82B} piM

SM6, hs-Flp/ AyGal4 UAS-GFP; FRT^{82B}-tub-Gal80/ FRT^{82B} Ras^{C40b}

SM6, hs-Flp/ AyGal4 UAS-GFP; FRT^{82B}-tub-Gal80/ FRT^{82B} Scrib¹

SM6, hs-Flp/ AyGal4 UAS-GFP; FRT^{82B}-tub-Gal80/ FRT^{82B} Sav³

SM6, hs-Flp/ AyGal4 UAS-GFP; FRT^{82B}-tub-Gal80/ FRT^{82B} UAS-Ras^{V12}

SM6, hs-Flp/ AyGal4 UAS-GFP; FRT^{82B}-tub-Gal80/ FRT^{82B} UAS-Ras^{V12}, Stat92E^{6C8}

SM6, hs-Flp/ AyGal4 UAS-GFP; FRT^{82B}-tub-Gal80/ FRT^{82B} Ras^{C40b}, Scrib¹

SM6, hs-Flp/ AyGal4 UAS-GFP; FRT^{82B}-tub-Gal80/ FRT^{82B} Ras^{C40b}, Sav³

To generate GFP-marked clones that overexpressed certain genes using the PMML system, flies with the following genotypes were used:

hs-Flp UAS-srcEGFP/+; FRT^{52B}(w) UAS-EGFP/ FRT^{52B}(y)

hs-Flp UAS-srcEGFP/+; FRT^{52B}(w) UAS-EGFP/ UAS-Ras^{V12} FRT^{52B}(y)

hs-Flp UAS-srcEGFP/+; FRT^{52B}(w) UAS-EGFP/ FRT^{52B}(y); UAS-Raf^{Gof/+}

hs-Flp UAS-srcEGFP/+; FRT^{52B}(w) UAS-EGFP/ FRT^{52B}(y); UAS-DaPKC^{CAAXWT/+}

hs-Flp UAS-srcEGFP/+; FRT^{52B}(w) UAS-EGFP/ UAS-RhoA^{V14} FRT^{52B}(y)

hs-Flp UAS-srcEGFP/+; FRT^{52B}(w) UAS-EGFP/ UAS-Ras^{V12} FRT^{52B}(y); UAS-RhoA^{N19/+}

hs-Flp UAS-srcEGFP/+; FRT^{52B}(w) UAS-EGFP/ UAS-Ras^{V12} FRT^{52B}(y); UAS-Raf^{K497M/+}

hs-Flp UAS-srcEGFP/+; FRT^{52B}(w) UAS-EGFP/ UAS-Ras^{V12} FRT^{52B}(y); UAS-PI3K^{DN/+}

hs-Flp UAS-srcEGFP/+; FRT^{52B}(w) UAS-EGFP/ UAS-Ras^{V12} FRT^{52B}(y); UAS-Dsor1 RNAi/+

hs-Flp UAS-srcEGFP/+; FRT^{52B}(w) UAS-EGFP/ UAS-Ras^{V12} FRT^{52B}(y); UAS-other RNAi/+

One- or two-day-old adult females with the correct genotype were heat-shocked once (for PMML) or six times (for MARCM) (37°C, 60 min) for three consecutive days, with an interval of 8–12 hours between heat shocks. The flies were transferred to fresh food daily after heat shock, and their MTs were processed for staining at the indicated times.

Immunofluorescence staining and microscopy

The MTs were dissected in cold PBS and fixed for 30 min in 4% formaldehyde in PBS. After several washes with PBT (PBS plus 0.1% Triton X-100), the samples were preabsorbed in 5% normal goat serum in PBT overnight at 4°C. The MTs were then incubated in primary antibody diluted in PBT for 2 hours at room temperature. After several washes with PBT, the MTs were incubated with secondary antibody diluted in PBT for 1 hour at room temperature. After several more washes with PBT, the MTs were mounted in Vectashield mounting medium with DAPI. Confocal images were obtained using a Zeiss LSM510 system, and processed using Adobe Photoshop CS3.

The following antibodies were used: rabbit polyclonal anti-STAT92E antibody (1:1000); rabbit polyclonal anti-dMyc antibody (1:2000; gift from D. Stein); rabbit polyclonal anti-DaPKC antibody (1:200; Santa Cruz, Santa Cruz, CA); rabbit polyclonal anti-Baz antibody (1:1000; gift from A. Wodarz); rabbit polyclonal anti-Scrib antibody (1:1000; gift of C. Doe); rabbit polyclonal anti-YKI antibody (1:1000; gift of D. J. Pan); rabbit polyclonal anti-pCdc2 antibody (1:500; Cell Signaling, Danvers, MA); rabbit polyclonal anti-pH3 antibody (1:500; Upstate, Lake Placid, NY); Chicken polyclonal anti-GFP antibody (1:3000; Abcam, Cambridge, MA); mouse monoclonal anti- β -Gal antibody (1:200; Clontech, Mountain View, CA); rat monoclonal anti-DE-cadherin antibody (1:50; gift from T. Uemura); mouse monoclonal anti-Armadillo N7A1 (1:20; DSHB); mouse monoclonal anti-Cyc E (1:5; gift from H. Richardson); mouse monoclonal anti-DIAP (1:50; gift from B. Hay); mouse monoclonal anti-D-pERK (1:50, Sigma, St Louis, MO); mouse monoclonal anti- γ -tubulin (1:50, Sigma). Secondary Abs were goat anti-mouse, goat anti-rat, goat anti-rabbit, and goat anti-chicken IgG conjugated to Alexa 488, Alexa 568, or Alexa 633 (1:500; Invitrogen, Carlsbad, CA).

Results

Tumor suppressors Scrib and Sav control the proliferation and fate determination of stem cells in adult *Drosophila* MTs

To identify genes that regulate stem cell self-renewal and differentiation in adult *Drosophila* MTs and posterior midgut, we screened genes that are known to play important roles in cell-fate regulation in other systems, using both the PMML (Kirilly *et al.*, 2005) and the MARCM system (Lee and Luo, 1999). These methods allow the generation of an individual homozygous mutant (the MARCM system) or the overexpression (the PMML technique) of stem cell clones that express GFP in an otherwise heterozygous, GFP-negative background. From these screens, we found that the loss-of-function mutation of Scrib or Sav or the activation of Ras caused RNSCs/RBs overproliferation and resulted in tumor growth.

The RNSCs in the adult *Drosophila* MTs are small diploid cells in the region of the lower tubules and ureters (Singh *et al.*, 2007). In wild-type MTs, an RNSC can produce a new RNSC and an immature diploid daughter RB, through asymmetric division. The RB can become a mature RC in the lower tubules and ureters. The RCs are polyploid, with a large nucleus. Both RNSCs and RBs are diploid with a small nucleus, express Stat92E protein, and are Armadillo (Arm) and DE-Cadherin (DE-Cad) positive. The only known detectable difference between RNSCs and RBs is that the JAK-STAT signaling (measured by a Stat92E-GFP reporter) is stronger in RNSCs than in RBs (Singh *et al.*, 2007). However,

since it is still very difficult to distinguish RNSCs from RBs precisely, we will discuss them together as RNSCs/RBs.

We generated GFP-positive marked clones in the MTs of wild-type controls or *scrib* (*scrib*¹) or *sav* (*sav*³) strong loss-of-function mutants using the MARCM system (Lee and Luo, 1999; Figure 1). In the wild-type MT, GFP-marked clones were rare, and usually only a few clusters appeared in each pair of MTs (Figure 1A). Each control GFP⁺ clone usually but not always had one RNSC and one RB (i.e., small nucleus, diploid, Arm and Stat92E positive; Figure 1B and C, white arrow) and several RCs (i.e., large nucleus, polyploid, Arm and Stat92E negative; Figure 1B and C, red arrows; Figure S1D and G) 6 days after clonal induction (ACI). In contrast, there were many GFP-marked clones in the *scrib* and *sav* mutants, and usually lots of clusters were seen in each pair of MTs (Figure 1D and G). These MTs were significantly enlarged (supporting information (SI) Figure S1B and C), and their mitotic index was significantly increased compared to wild-type clones (0.76 for n=17 MTs with wild-type clones compared to 4.57 for n=14 MTs with *scrib* clones and 4.33 for n=12 MTs with *sav* clones). Each GFP⁺ mutant cluster typically contained multiple RNSCs/RBs (diploid, Arm and Stat92E positive; Figure 1E, F, H and I; Figure S1E, F, H and I) and a few RCs (polyploid, Arm and Stat92E negative; Figure 1E, F, H, and I). The proportion of GFP⁺ cells that were RNSCs/RBs was significantly increased in both the *scrib* and *sav* mutant clones compared with wild-type clones. The average ratio of RNSCs/RBs to RCs was 11.1:1 (611/55) in 6 MTs with *scrib* mutant clones, 10.8:1 (522/51) in 6 MTs with *sav* mutant clones, and 0.88:1 (50/57) in 5 MTs with wild-type clones. We conclude that, while wild-type RNSCs go through an asymmetric division to generate one RNSC and one RB, *scrib* and *sav* mutant RNSCs often result in increased RNSC/RB production, and that while wild-type RNSCs are relatively quiescent and only divide infrequently, *scrib* and *sav* mutant RNSCs are more active and divide frequently.

We also analyzed mutant clones of other components in the Scrib and Sav pathways. In the Scrib pathway, we analyzed either strong or null mutant alleles (Bilder *et al.*, 2000) of *lethal giant larvae* (*lgl*^Δ) and *disc-large* (*dlg*^{m52}); in the Sav pathway, we analyzed either strong or null mutant alleles (Harvey *et al.*, 2003; Wu *et al.*, 2003) of *warts* (*wts*^{latsX1}) and *hippo* (*hpo*⁴²⁻⁴⁷). Among these mutants, only RNSC clones of *wts*^{latsX1} significantly overproliferate as seen in RNSC clones of *scrib*¹ and *sav*³ (Figure S2A, B, and data not shown), suggesting that either only selective members in these two pathways regulate RNSC self-renewal or other genes compensate loss-of function of *lgl*, *dlg*, and *hpo* in regulating RNSCs.

We also examined protein expression of Scrib and Yorkie (YKI, a downstream transcriptional coactivator in the Sav pathway). Scrib is mainly expressed in differentiated RCs while YKI is expressed in both RNSCs/RBs and RCs (Figure S2C and D). The Scrib and Sav pathways may function to block RNSC differentiation.

The oncogene Ras functions downstream of Scrib and Sav to regulate RNSC transformation

Mutations that activate the oncogene Ras have been identified in more than 30% of all types of human tumors (Barbacid, 1987). We found that activation of the oncogene Ras caused RNSC overproliferation and resulted in tumorous growth in MTs (Figure 2). We generated GFP-positive marked clones in wild-type control MTs or in MT cells expressing a constitutively activate form of Ras (Ras^{V12}) using the MARCM technique (Lee and Luo, 1999; Figure 2). In the wild type MTs, the GFP⁺ clones were rare and usually only a few clusters were seen in the lower tubules and ureters of each pair of MTs (Singh *et al* 2007; Figure 2A) 6 days ACI; each cluster usually contained one RNSC and one RB (both are diploid, Arm-positive, DE-Cad-positive, and Stat92E-positive) and several RCs (polyploid,

Arm-negative, DE-Cad-negative, and Stat92E-negative) (Figure 2A, 3B, and 3D). In contrast, GFP⁺ cells in MTs expressing constitutively activate Ras, formed large tumorous masses in the lower tubules, ureters, and main segments (Figure 2B, 2C, 3A, and 3C). All the cells in the *Ras*^{V12} clones had a small nucleus (diploid) and coexpressed Arm, DE-Cad, and Stat92E (Figure 2B, 2C, 3A, and 3C). The mitotic index of the *Ras*^{V12} clones was significantly greater than that of the wild-type clones (0.96 for n=14 MTs with wild-type clones compared to 20.44 for n=16 MTs with *Ras*^{V12} clones). No differentiated RCs were detected in the *Ras*^{V12} clones (Figure 2B, 2C, 3A, and 3C). These observations suggest that activation of the Ras oncogene results in RNSCs that overproliferate and fail to differentiate.

To determine Ras^p function in regulating RNSCs under physiological conditions, we generated clones in a *Ras*-null mutant (*Ras*^{C40b}; Hou *et al.*, 1995) using the MARCM system (Lee and Luo, 1999). Marked clones homozygous for the *Ras*-null mutants were generated in the MTs and identified by GFP expression. Only a few differentiated RCs (4.86 RCs and 0.29 RNSC/RBs for n=7 MT with *Ras*^{C40b} clone compared to 11.40 RCs and 10.00 RNSC/RBs for n=5 MT with wild-type clone) (large nucleus and Arm negative) were detected in the *Ras*-null mutant MTs (Figure 2D). Together with the above results from mutants with activated Ras, these *Ras*-null phenotypes suggested that normal Ras signaling promotes RNSC proliferation and blocks stem-cell differentiation.

To determine the epistatic relationships between *Ras* and the tumor suppressors *scrib* and *sav*, we generated double-mutant clones of *Ras*^{C40b} *scrib*¹ or of *Ras*^{C40b} *sav*³ using the MARCM system. All of these double-mutant clones had only a few differentiated RCs (Figure 2E and F; larger nucleus and Arm negative), like the single *Ras*-null mutant, suggesting that Ras functions downstream or parallel of the two tumor suppressors in regulating RNSC fate.

Scrib cooperates with a Ras gain-of-function mutation to promote tumor progression and cell invasion in *Drosophila* imaginal tissues (Brumby and Richardson, 2003; Pagliarini and Xu, 2003), and human Scrib cooperates with H-Ras to promote cell invasion in mammalian cultured cells (Dow *et al.*, 2008). To examine whether the constitutively-active *Ras*^{V12} would cooperate with loss-of-function Scrib or Sav mutants in regulating RNSC transformation, we generated double mutant clones of *Ras*^{V12} *scrib*¹ or *Ras*^{V12} *sav*³, using the MARCM system. All these double mutant clones looked exactly like the *Ras*^{V12} mutant clones, and no additional phenotypes were observed (data not shown). We also transplanted the GFP⁺ double-mutant RNSCs into the abdomen of wild-type adult hosts and found that the transformed stem cells (just like the *Ras*^{V12}-transformed RNSCs) rapidly proliferated, and never migrated to other organs, suggesting that they are not metastatic tumors. Together, these findings suggested that the two tumor suppressors function upstream or parallel of Ras and do not cooperate with Ras to promote tumor progression or cell invasion in MTs.

The Ras-transformed stem cells exhibit hallmarks of cancer

We assayed additional markers to distinguish the normal RNSCs and transformed RNSCs caused by the loss-of-function mutation of *scrib* or *sav* or activated *Ras*. We found that regulators of the cell cycle and proliferation, Cyclin E (Cyc E), phosphorylated Cdc2 (pCdc2), *Drosophila* Myc (dMyc), and phosphorylated ERK (pERK), were barely expressed in normal RNSCs but were very strongly expressed in the *Ras*^{V12}-transformed RNSCs (Figure 3E-H, Fig. 4A-B, and Figure S3A); however, a transcriptional reporter of *cyc E* (*cycE-lacZ*; Duman-Scheel *et al.*, 2002) was not expressed in either normal or *Ras*^{V12}-transformed RNSCs (Figure S3B; data not shown), suggesting that the elevated Cyc E in the transformed RNSCs was not due to an increase in *cyc E* transcription.

Reduced cell death is another hallmark of human cancer. We observed that the apoptosis inhibitor DIAP and its transcriptional reporter *diap1-lacZ* (Ryoo *et al.*, 2002) were dramatically elevated in the Ras^{V12}-transformed RNSCs compared with their levels in wild-type RNSCs (compare Figure 4C with D and 4E with F). We also directly examined cell death in MTs containing GFP+ clones homozygous for *Ras*^{V12}, using an Apoptag kit, and did not detect any cell death (data not shown).

In *Drosophila* larval neuroblasts, disrupting the cell polarity impairs fate specification during stem-cell division and eventually drives the neuroblasts into malignancy (Gonzalez, 2007). We examined the distribution of two cell-polarity markers, Bazooka (Baz) and *Drosophila* atypical protein kinase C (DaPKC), and found that all of the Ras-transformed RNSCs showed uniform cortical Baz and DaPKC expression, but in the wild-type RNSCs, these proteins formed an apical crescent (compare Fig. 5C and D with A and B; compare Fig. S4A with B), indicating that the activated Ras disrupted RNSC polarity and maybe asymmetric division as well.

In *Drosophila* imaginal discs, loss of the class I tumor suppressor Sav results in increased transcription of *cyc E* and *diap1*, leading to increased proliferation and reduced apoptosis (Brumby and Richardson, 2005; Saucedo and Edgar, 2007), whereas loss of the class II tumor suppressor Scrib mostly disrupts the apical-basal polarity of the epithelial cells, leading to increased proliferation and failure to differentiate (Brumby and Richardson, 2005; Bilder, 2004). We examined all of the above markers, and found no significant changes in the Cyc E, dMyc, pCdc2, pERK, or DIAP levels in the mutant *scrib* or *sav* clones, compared with wild-type RNSCs (data not shown). However, the two cell-polarity markers, Baz and DaPKC, showed uniform cortical distribution in the *scrib* and *sav* mutant RNSCs (Figure 5E and F; Baz data not shown).

In summary, Ras^{V12}-transformed RNSCs show increased proliferation, reduced apoptosis, disrupted cell polarity, and a failure to differentiate, while the *scrib* and *sav* mutant RNSCs show only increased proliferation and disrupted cell polarity, and perhaps ectopic self-renewing divisions that generate more stem cells. Thus, the Ras^{V12}-transformed RNSCs share many of the hallmarks of human cancer.

A simple change in RNSC polarity is not sufficient for their transformation

Apically localized DaPKC is an important regulator of the asymmetric division of larval neuroblasts. Overexpression of a constitutively active form, DaPKC-CAAX (a membrane-targeted version of DaPKC) causes the ectopic localization of DaPKC to both the apical and basal cortex, disrupting the neuroblasts' asymmetric division, and resulting in stem-cell tumor formation (Lee *et al.*, 2006). In wild-type RNSCs, DaPKC formed an apical crescent (Figure 5A and B), but was uniformly distributed in the cortex of *scrib* and *sav* mutant RNSCs (Figure 5E and F) and in RNSCs expressing constitutively active Ras (Figure 5C and D). These observations raised the interesting possibility that the tumorous growth of RNSCs with these mutations might result from the ectopic localization of DaPKC on the cortex. To examine this possibility, we first generated *DaPKC* mutant clones using the MARCM system. We found that all the *DaPKC* mutant RNSCs differentiated into RCs (large nucleus and Arm negative; Figure 5G). We also generated clones that expressed two constitutively active forms of DaPKC (DaPKC^{CAAXWT} and DaPKC^{ΔN}), using the PMML technique, and observed only a few GFP-positive RNSCs/RBs (diploid and Arm positive; Figure 5H and data not shown) in clones of the two constitutively active forms of DaPKC. Since the loss of DaPKC resulted in RNSC differentiation, and the constitutive activation of DaPKC blocked RNSC proliferation and differentiation, these results suggest that DaPKC may regulate RNSC asymmetric division. However, unlike its function in neuroblasts, the constitutive activation of DaPKC did not result in RNSC overproliferation and tumor

formation. We also generated *DaPKC Ras^{V12}* double mutant clones, and found that the loss of *DaPKC* did not suppress the tumors generated by RNSCs expressing constitutively active Ras (Figure 5I).

We further examined Baz expression during the cell cycle of RNSCs (Figure S4). Baz formed an apical crescent in non-dividing RNSCs (Figure S4C and L). During metaphase and early anaphase, Baz showed uniform cortical distribution (Figure S4D-F). During late anaphase and cytokinesis, Baz concentrated at the junctions that separate the two newly-formed cells (Figure S4G-I). In larval neuroblasts, Baz formed an apical crescent at metaphase (Figure S4J and K) and is directly co-related with asymmetric cell division. In RNSCs, Baz distribution changes according to cell activation and is not directly co-related with asymmetric cell division.

The above data together suggest that the expression of DaPKC and Baz proteins in RNSCs may serve as an indicator of RNSC activation and is not directly co-related with stem cell asymmetric division. Ras transforms RNSCs by a mechanism independent of DaPKC.

Signaling Downstream of Ras regulates RNSC Transformation

Ras has many downstream effectors that regulate complex signaling networks (Repasky *et al.*, 2004). To identify the signal transduction pathways that mediate Ras^{V12} function in RNSC transformation, we screened UAS-transgenes available from public stock centers that express either a dominant-negative form or RNAi of a known signal transduction component. We found that the expression of a dominant-negative form of Raf or Rho A or an RNAi of MEK (Dsor1) suppressed the Ras^{V12} phenotypes (Figure 6A-C). In contrast, the expression of a dominant-negative form of Ral, PI3K, Bsk, or Rac1, or an RNAi of *cyc E* or *dMyc* did not affect the Ras^{V12} phenotypes (Figure 6D; data not shown). We also found that the loss of *PKA* or *Tor* blocked the Ras^{V12}-induced RNSC tumor formation but the loss of *Stat92E* did not affect the Ras^{V12} phenotype (Figure 6E-G). We further found that the expression of a dominant-negative form of Rho A alone blocked RNSC proliferation (Figure S5C) and the expression of an RNAi of MEK (Dsor1) alone promoted RNSC differentiation (Figure S5D; 9.50 RCs and 1.25 RNSC/RBs for n=4 MT). The expression of an RNAi of MEK (Dsor1) also suppressed RNSC overproliferation phenotype associated with *unpaired* (*upd*) overexpression (compare Figure S5B with 5A), further suggesting that the Ras/MEK pathway functions downstream of the JAK-STAT pathway in regulating RNSC self-renewal.

To check the effectiveness of RNAi of *cyc E* or *dMyc* in knocking down the corresponding gene's activity, we examined expression of dMyc and CycE in RNSC clones of Ras^{V12} alone (Figure S3C and E) or Ras^{V12} with RNAi of *dMyc* (Figure S3D) or Ras^{V12} with RNAi of *cyc E* (Figure S3F). We once again observed that RNAi of *cyc E* or *dMyc* did not affect the Ras^{V12} phenotypes although both RNAi of *dMyc* and RNAi of *cyc E* significantly blocked the corresponding protein overexpression associated with Ras^{V12} mutation (compare Figure S3D with 3C and 3F with 3E), further suggesting that Ras either transforms RNSCs by a mechanism independent of dMyc and Cyc E or the residual activity of dMyc and Cyc E is still sufficient to mediate Ras function.

Finally, we expressed constitutively active Raf, RhoA, or ERK (rl^{Sem}) in RNSCs, using the PMML technique. We found that only the constitutively active Raf induced stem-cell tumor formation (Fig. 6H and I; data not shown), which is consistent with previous report that Raf was the primary effector of Ras in other *Drosophila* systems (Prober and Edgar, 2000).

In summary, the above results suggest that the MEK, RhoA, Tor, and PKA pathways function downstream of Ras/Raf in transforming RNSCs, and that Ras functions

downstream of JAK-STAT, Scrib, and Sav. The activation of RhoA and MEK/ERK are necessary but not sufficient to induce RNSC tumor formation. The RNAi of *cyc E* or *dMyc* was insufficient to suppress the Ras^{V12} phenotypes, suggesting that other downstream factors besides Cyc E and dMyc are critical for mediating Ras^{V12} function in RNSC transformation.

Discussion

There is now compelling evidence that most cancers are generated from cancer stem cells (CSCs) (Reya *et al.*, 2001; Kim and Dirks, 2008). Drugs that specifically target CSCs could prove to be highly effective treatments for cancer. However, because CSCs often express markers of normal stem cells and remain dependent upon the niche microenvironments that regulate normal stem cells (Calabrese *et al.*, 2007; Barker *et al.*, 2009; Zhu *et al.*, 2009), anti-CSC drugs may also damage normal stem cells and possess significant toxicities. Therefore, understanding the molecular mechanisms underlying how normal stem cells are transformed into CSCs and identifying the differences between normal stem cells and CSCs in different tissues are important prerequisites for developing anti-CSC therapeutic strategies.

In this study, we demonstrated that the mutation of tumor suppressors Scrib or Sav or activation of the oncogene Ras promotes stem-cell transformation in adult *Drosophila* MTs, and that Ras functions downstream or parallel of Scrib and Sav in the regulation of stem-cell transformation. The Ras-transformed stem cells exhibited many of the hallmarks of cancer, including increased proliferation, reduced cell death, and failure to differentiate. Downstream of Ras/Raf, several signal transduction pathways, including MEK/MAPK, RhoA, PKA, and TOR, cooperatively mediate Ras^{V12} function in regulating stem-cell transformation. These findings could be helpful for us to understand the stem cell transformation.

Function of the tumor suppressors Scrib and Sav in RNSC transformation

Our results showed that loss-of-function mutations of Scrib or Sav in MTs caused an increased proliferation of RNSCs. However, the RNSCs remained capable of differentiating into daughter cells, although the proportion of stem cells/differentiated cells and the mitotic index were higher in the mutant than in wild-type MTs. Similar phenotypes are reported for *lethal giant larvae* (*lgl*) mutant larval neuroblasts in *Drosophila* (Lee *et al.*, 2006) and *patched* (*ptc*) mutant neural stem cells (NSCs) in the mouse (Yang *et al.*, 2008). The sum of the evidence indicates that stem cells bearing mutant tumor-suppressor genes occasionally undergo ectopic self-renewal to generate two stem cell daughters. This may lead to early expansion of the stem-cell pool and, in turn, to a significant increase in direct downstream precursors, which continue to expand and ultimately contribute to tumorigenesis.

Relationship between the tumor suppressors Scrib/Sav and the Ras oncogene

The polarity regulator Scrib cooperates with a Ras gain-of function mutant (Ras^{V12}) to promote tumor progression and cell invasion in *Drosophila* imaginal tissues (Brumby and Richardson, 2003; Pagliarini and Xu, 2003). *Scrib* mutant clones in the eye disc exhibit loss of cell polarity, defective differentiation capacity and ectopic cell proliferation, but do not overgrow due to increased cell death, while Ras^{V12} in the eye disc promote differentiation, cell survival and cell proliferation (Brumby and Richardson, 2003). When oncogenic Ras is expressed within the scrib mutant clones, the differentiation is attenuated, cell death is prevented and cell proliferation is further enhanced, these functions together promote neoplastic tumor development. However, our data suggest that Scrib acts upstream of Ras (because the lack of Ras prevented the Scrib mutant phenotypes) in regulating RNSC transformation, and the loss of Scrib did not further enhance the Ras^{V12} mutant phenotype.

The lacking cooperation of Scrib and Ras in RNSCs maybe due to the unique properties of RNSCs. In RNSCs, the *scrib* mutant clones did not exhibit increased cell death (data not shown) and the Ras^{V12} did not promote differentiation (Figure 2B and C), therefore *scrib*⁻ and Ras^{V12} do not compensate each other in transforming RNSCs.

In *Drosophila* imaginal tissues, Sav is a tumor suppressor of a recently described *Drosophila* signaling pathway, the Hippo/Sav/Warts (Wts) pathway; Hippo and Wts are protein kinases, and Sav is a scaffold protein that helps activate their kinase activities (Saucedo and Edgar, 2007). Sav mutations have been found in human kidney cancer cell lines (Tapon *et al.*, 2002). Activation of the Hippo pathway leads to cell-cycle arrest and/or apoptosis. The Hippo family kinases bind to members of the Ras association family (RASSF) in both mammalian and *Drosophila* cells (Saucedo and Edgar, 2007; Polesello *et al.*, 2006). *Drosophila* RASSF (dRASSF) restricts the tumor suppressor activity of Hippo (an oncogenic role) by competing with Sav for binding to Hippo (Polesello *et al.*, 2006). However, the loss of dRASSF also partially rescues the Ras-loss phenotype (a tumor-suppressing role) in the developing eye (Polesello *et al.*, 2006). Our data for RNSCs suggest that Sav, like Scrib, acts upstream of Ras. It will be interesting to determine whether dRASSF also bridges the interaction between Sav and Ras in the regulation of the RNSCs, in future studies.

Ras-transformed CSCs exhibit many of the hallmarks of cancer

In their advanced stages, human cancers exhibit several hallmarks (Hanahan and Weinberg, 2000), including supplying their own growth/proliferation signals, insensitivity to anti-proliferative signals, evasion of apoptosis, failure to differentiate, invasion/metastasis, activation of a telomerase to allow unlimited replicative potential, and increased angiogenesis. Only the first four hallmarks are conserved in the *Drosophila* model of cancer, because the fly uses a system different from mammalian telomere maintenance to control its DNA replication, and continued tumor growth does not depend on angiogenesis, because of the fly's open circulation system (Brumby and Richardson, 2005). However, the Ras-transformed RNSCs displayed the first three hallmarks, suggesting that they are like cancer stem cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The tumor suppressors *Scrib* and *Sav* regulate RNSC proliferation.

MTs with GFP-marked wild-type (A-C), *scrib*¹ (D-F), and *sav*³ (G-I) MARCM clones, were stained with the indicated antibodies. White arrows indicate RNSCs, while red arrows indicate the large RCs in (B, E, and H). Dashed arrows in (A, D, G) point from the ureter to the upper tubules. Scale bars in (A, D, G) represent 10 μ m; in all other panels represent 5 μ m.

Figure 2.

The oncogene Ras functions downstream of Scrib and Sav in regulating RNSC transformation.

GFP⁺ clones were generated in the MTs of flies of the indicated genotypes by the MARCM technique and stained with the indicated antibodies. A, Wild-type control clones generated in the *FRT*^{82B} *piMM* MTs. B and C, *UAS-Ras*^{V12} mutant clones. D, *Ras*^{C40b} mutant clones. E, *Ras*^{C40b}, *scrib*¹ mutant clones. F, *Ras*^{C40b}, *sav*³ mutant clones. Green: GFP; red: Arm; blue: DAPI. White arrows indicate RNSCs; red arrows indicate the large RCs. Dashed arrows in (A, B, D, E, F) point from the ureter to the upper tubules. Scale bars in (A, B, D, E, F) represent 10 μ m; in C represents 5 μ m.

Figure 3.

Activated Ras blocks the differentiation and promotes the proliferation of RNSCs. GFP⁺ clones were generated in the MTs of *Ras^{V12}* (A, C, E, G) or wild-type (B, D, F, H) flies by the PMML technique and stained with the indicated antibodies. Scale bars in A, C, E, and G represent 7.5 μm; Scale bars in B, D, F, and H represent 10 μm.

Figure 4.

Activated Ras blocks the cell death and promotes the migration of RNSCs. GFP+ clones were generated in the MTs of *Ras^{V12}* (A, C, E) or wild-type (B, D, F) flies by the PMML technique and stained with the indicated antibodies. Scale bars in A, C, and E represent 7.5 μm ; Scale bars in B, D, and F represent 10 μm .

Figure 5.

Change in cell polarity alone is insufficient to transform RNSCs.

GFP⁺ clones were generated in the MTs of flies of the indicated genotypes by the MARCM (B-G and I) or the PMML (H) technique and stained with the indicated antibodies. A and B, Wild-type MT and control clones generated in the *FRT^{82B} piMMTs*. C and D, *UAS-Ras^{V12}* mutant clones. E, *scrib¹* mutant clones. F, *sav³* mutant clones. G, *DaPKC^{k06403}* mutant clones. H, *UAS-DaPKC^{CAAXWT}* mutant clones. I, *UAS-Ras^{V12}, DaPKC^{k06403}* mutant clones. White arrows in H indicate RNSCs; red arrows in G indicate the large RCs. Dashed arrows in (G, H, I) point from the ureter to the upper tubules. Scale bars in (A-F) represent 5 μ m; in (G-I) represent 10 μ m.

Figure 6.

Several signal transduction pathways cooperatively mediate Ras ρ function in RNSC transformation.

GFP $^{+}$ clones were generated in the MTs of flies of the indicated genotypes by the PMML (A-D and F-I) or the MARCM (E) technique and stained with anti-GFP (green) and anti-Arm (red) antibodies, and DAPI (blue). A, *UAS-Ras^{V12}*, *UAS-RhoA^{N19}* mutant clones. B, *UAS-Ras^{V12}*, *UAS-Raf^{K497M}* mutant clones. C, *UAS-Ras^{V12}*, *UAS-Dsor1 RNAi* mutant clones. D, *UAS-Ras^{V12}*, *UAS-PI3K^{DN}* mutant clones. E, *UAS-Ras^{V12}*, *Stat92E^{j6C8}* mutant clones. F, *UAS-Ras^{V12}*, *Pka-C^{I^{B3}}* mutant clones. G, *UAS-Ras^{V12}*, *Tor^{ΔP}* mutant clones. H, *UAS-Raf^{Gof}* mutant clones. I, *UAS-RhoA^{V14}* mutant clones. Dashed arrows point from the ureter to the upper tubules. Scale bars represent 10 μ m.