The *Drosophila* RASSF Homolog Antagonizes the Hippo Pathway

Cedric Polesello,1 Sven Huelsmann,2 Nicholas H. Brown,2 and Nicolas Tapon1,*

1 Apoptosis and Proliferation Control Laboratory
Cancer Research UK
London Institute
44 Lincoln’s Inn Fields
London
United Kingdom
2 The Gurdon Institute and
Department of Physiology, Development, and
Neuroscience
University of Cambridge
Tennis Court Road
Cambridge
United Kingdom

Summary

Correct organ size is determined by the balance between cell death and proliferation. Perturbation of this delicate balance leads to cancer formation [1]. Hippo (Hpo), the *Drosophila* ortholog of MST1 and MST2 (Mammalian Sterile 20-like 1 and 2) is a key regulator of a signaling pathway that controls both cell death and proliferation [2, 3]. This pathway is so far composed of two Band 4.1 proteins, Expanded (Ex) and Merlin (Mer), two serine/threonine kinases, Hpo and Warts (Wts), the scaffold proteins Salvador (Sav) and Mats, and the transcriptional coactivator Yorkie (Yki). It has been proposed that Ex and Mer act upstream of Hpo, which in turn phosphorylates and activates Wts. Wts phosphorylates Yki and thus inhibits its activity and reduces expression of Yki target genes such as the caspase inhibitor DIA1 and the micro RNA *bantam* [4–6]. However, the mechanisms leading to Hpo activation are still poorly understood. In mammalian cells, members of the Ras association family (RASSF) of tumor suppressors have been shown to bind to MST1 and modulate its activity [7]. In this study, we show that the *Drosophila* RASSF ortholog (dRASSF) restricts Hpo activity by competing with Sav for binding to Hpo. In addition, we observe that dRASSF also possesses a tumor-suppressor function.

Results and Discussion

The mammalian RASSF family comprises six different loci encoding a variety of splice variants. Most transcripts encode proteins that contain a Ras association domain (RA), an N-terminal C1-type zinc finger, and a C-terminal SARAH (Sav RASSF Hippo) domain [8–13] and Figure S1A). RASSF family members, most notably RASSF1A, are frequently silenced in a variety of solid tumors [14], mainly by promoter methylation [15]. Thus, it has been proposed that RASSF genes act as tumor suppressors.

The biological function of these genes is not well understood. RASSF1A and Nore1A have both been shown to interact with MST1 via its SARAH domain [7]. Overexpression of RASSF1A or Nore1A inhibits MST1 activation, but coexpression of these RASSF proteins with Ras enhanced MST1 activity [16]. RASSF1A knockout mice have mildly increased tumor susceptibility [17], confirming that RASSF genes can act as tumor suppressors. The weakness of the mouse phenotype, which is at odds with the frequency of RASSF1A inactivation in human tumors, can be ascribed to redundancy with other family members.

By contrast, *Drosophila melanogaster* has a single RASSF family member, which is encoded by the CG4656 gene and which we will refer to as dRASSF. Like its vertebrate counterparts, dRASSF encodes a protein bearing an RA and SARAH domain at its C terminus (Figure S1A in the Supplemental Data available online). It also possesses a LIM domain that shares some similarities with C1 zinc fingers at its N terminus.

We generated mutant alleles of dRASSF by imprecise excision of two nearby transposons, GE23517 and EY2800 (see Supplemental Experimental Procedures). We obtained multiple alleles, which delete up to the fourth intron, including the initiating ATG (Figure S1B). Some transcript was still detected in dRASSF44.2, but a strong reduction was found in dRASSFX36, which lacks the transcription start (Figure S1C). However, antibodies raised against the C terminus (amino acids 792–806) and a nonconserved region (amino acids 294–308) of dRASSF showed that full-length dRASSF is absent in lysates from all mutant lines, suggesting our *dRASSF* mutants are indeed loss-of-function mutations for the locus (Figure S1D and data not shown). All of these alleles were viable and behaved identically in subsequent assays. In addition, dRASSF staining was severely reduced in FLP/FRT-generated *dRASSF* mutant clones in the eye-imaginal disc, the larval precursor to the adult eye (Figure S1E).

Although the *dRASSF* mutant flies are viable, they present a clear growth defect in comparison to wild-type animals when reared in carefully controlled conditions (Figure 1A). *dRASSF* mutant flies were 15% lighter than their wild-type counterparts (Figure 1D), a phenotype which was significantly rescued by introduction of a single copy of a *dRASSF* rescue construct, although wild-type levels of dRASSF were not fully restored (see Figure S1D). dRASSF mutant flies were fully fertile and normally proportioned (not shown) but sensitive to γ-irradiation (Figure S1F). Wing surface area was reduced by 8% in *dRASSF* mutant flies, whereas wing hair density was unaffected (Figures 1B, 1C, 1E, and 1F). This suggests that the growth defect of *dRASSF* mutant flies is due to a reduction in cell number and not a defect in cell size.

In mammals, members of the RASSF family are known to interact with MST1 and thus to modulate its
pro-apoptotic activity [7]. We therefore tested whether dRASSF can interact with Hpo. We performed coimmunoprecipitation (Co-IP) experiments in Drosophila Kc cells with dRASSF antibodies to immunoprecipitate endogenous protein. As expected, dRASSF robustly coimmunoprecipitated with Hpo (Figure 2A). The association between Hpo and Sav is mediated by these proteins’ shared SARAH domains. Likewise, Hpo’s SARAH domain is required for its association with dRASSF, as shown by the fact that a truncated form of Hpo (Hpo\(^{DC}\)) [18] lacking this domain fails to bring down dRASSF (compare Figures 2B and 2C). Thus, the Hpo SARAH domain can associate with both Sav and dRASSF.

Sav is stabilized by the presence of Hpo ([18, 19] and Figure 2D, lane 2). We therefore tested whether dRASSF levels are modulated by Hpo. dRASSF immunostaining was reduced in clones mutant for a hpo allele that lacks the SARAH domain (Figure 2D, lane 2). This weight defect is partially rescued in the presence of the genomic rescue construct (GR), *p < 0.05 (white n = 120, dRASSF\(^{XT6/X16}\) n = 120, dRASSF\(^{xt6/x36}\) n = 90, dRASSF\(^{xt6/x44.2}\) n = 90, GR;dRASSF\(^{xt6/x44.2}\) n = 90).

Figure 2A. The association between Hpo and Sav is mediated by these proteins’ shared SARAH domains. Likewise, Hpo’s SARAH domain is required for its association with dRASSF, as shown by the fact that a truncated form of Hpo (Hpo\(^{DC}\)) [18] lacking this domain fails to bring down dRASSF (compare Figures 2B and 2C). Thus, the Hpo SARAH domain can associate with both Sav and dRASSF.
is analogous to the situation for Sav, which is also stabilized by a kinase-dead form of Hpo [18].

Because Hpo, Sav, and dRASSF all contain a SARAH domain, we speculated that dRASSF might also bind Sav. To test this, we investigated whether dRASSF interacts with Sav by co-IP but repeatedly failed to detect such an interaction (Figure S2 C and data not shown). Because the possibility of a ternary complex had been raised by Scheel and Hofmann [13], we then tested whether the three proteins could be found in the same complex. Coexpressing Hpo, Sav, and dRASSF in cultured Kc cells. As expected, Hpo was able to bind Sav and dRASSF (Figure 3A). However, Sav immunoprecipitates only contained Hpo and not dRASSF, and dRASSF immunoprecipitates contained Hpo but not Sav (Figure 3A). We obtained identical results with endogenous IPs by using dRASSF and Sav antibodies (Figure S2C). These data support the notion that Sav and dRASSF are not present in the same complex but are in two different Hpo complexes.

Sav has been shown to be a positive regulator of the Hpo pathway, whereas our genetic results suggest that dRASSF might antagonize Hpo activity. We were therefore interested in determining whether complexing with Sav or dRASSF might influence Hpo activity. We probed our immunoprecipitates with a phospho-MST1 antibody that recognizes phosphorylated (active) Hpo [20]. Interestingly, although Hpo that was coimmunoprecipitated with dRASSF showed barely detectable levels of phosphorylation, the Sav-associated fraction was highly phosphorylated (Figure 3A). Thus, Hpo can exist as two pools, a highly active Sav-associated pool and an inactive dRASSF-associated pool. This correlates with data showing that Nore1 can repress MST1 activity in mammalian cells [16]. This also suggests that Sav can promote Hpo activation and provides
the first direct evidence of a function for the Hpo/Sav interaction.

Next, we wanted to test our prediction that drassf depletion would promote Hpo activation. Like that of Hpo’s mammalian counterparts, phosphorylation of endogenous Hpo can be potently stimulated by the drug Staurosporine (STS) in Kc cells ([16, 20, 21] and Figure 3B, lane 1). Although RNAi depletion of drassf alone was not able to induce Hpo phosphorylation (Figure 2D, compare lanes 4 and 5), drassf depletion markedly potentiated STS-induced Hpo activation (Figure 3B, compare lanes 1 and 2). Thus, drassf restricts Hpo activation in cultured cells.

Given their opposing effects on Hpo activation, we investigated the relationship between Sav and drassf. Depletion of drassf in Kc cells gives rise to an increase in Sav protein levels (Figure 2D lines 1 and 4). Although drassf levels were unaltered in sav mutant clones (Figure S2A), overexpression of sav in the wing disc results in a robust decrease of drassf staining (Figure 3E). We then tested whether drassf and sav compete to bind Hpo. To address this question, because Sav and drassf repress each other’s expression and drassf has reduced affinity for phosphorylated Sav, we mixed separate Kc cell lysates expressing a kinase-dead form of Hpo (HpoK205-Flag), Sav-HA, and HA-drassf and performed IPs after the proteins were allowed to bind overnight. Both Sav and drassf were able to interact with Hpo (Figure 3C). In these conditions, increasing the amount of Sav was able to displace the drassf fraction bound to Hpo, showing that Sav and drassf are competing to bind Hpo. The outcome of the competition probably determines the stability of Sav and drassf; both proteins are downregulated when Hpo is depleted by RNAi (Figure 2D). Thus, we suggest that interplay between the inhibitor drassf and the activator Sav determines the level of Hpo activation and therefore affects body size.

We tested this model by performing genetic-interaction experiments. We crossed a mutant allele of hpo into the drassf mutant background and measured the adult body mass (Figure 3D). The body-mass reduction of drassf mutant flies (15% reduction) was substantially reduced by removal of just one copy of Hpo (8% reduction). Flies overexpressing Sav showed a reduction of 10% in weight and 5% in wing area, mimicking drassf loss of function (Figure S3). This wing defect was significantly increased in a drassf mutant background (Figures S3B–S3D). In addition, misexpression of drassf was able to robustly rescue the rough-eye phenotype elicited by coexpression of sav and wts (Figures 3F–3I). These data support the notion that drassf can antagonize Sav-mediated Hpo activation in vivo.

Though our results are consistent with biochemical data on mammalian RASSF family members [7, 16], they are at odds with the fact that RASSF genes are commonly silenced in tumor cells. Avruch and colleagues have proposed that one RASSF protein, Nore1, possesses a tumor-suppressor function that is independent of MST1 and MST2 [22]. We found two lines of evidence to support this notion. First, we made clones that are mutant for two hpo hypomorphic alleles, hpo42–48 (19) compare Figures 4C and 4F, hpo42–48 (22) not shown), that remove the SARAH domain in a drassf mutant background in the head by using the eyeless FLP system [24]. Interestingly, the overgrowth phenotype elicited by these hpo alleles was strongly enhanced by loss of drassf. By contrast, a hpo allele (hpo42–47 [19] bearing an inactivating deletion in the kinase domain but an intact SARAH domain was barely if at all enhanced by drassf loss of function (Figures 4B and 4E). This suggests that drassf may possess a tumor-suppressor function, which may be uncovered when the Hpo function is compromised.

In addition, we examined the relationship between Ras1 and drassf because the mammalian RASSF proteins have all been shown to bind Ras proteins [8–10, 25]. In Drosophila imagoal tissues, Ras1 mutant clones grow poorly and are eliminated by apoptosis (26, 27) and Figure 4G). When we made double-mutant clones for Ras1 and drassf in the developing eye, we observed a substantial rescue of the growth defect observed in clones mutant for Ras1 alone (Figure 4H). This rescue of Ras loss of function was the result of both increased proliferation (Figure S4) quantified with phosphorylated Histone 3 staining and a reduction of apoptosis visualized with a cleaved-Caspase 3 antibody (Figure S5). Thus, drassf appears to antagonize Ras1 signaling in growth control, which is again suggestive of

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Figure 3. drassf Antagonizes Hpo Activity

(A) Hpo/Sav and Hpo/drassf are two distinct complexes. Hpo-Myc, Sav-HA, and GFP-drassf were cotransfected in Kc cells. Anti-Myc, anti-HA, anti-drassf, or control (anti-Flag) immunoprecipitates were blotted for GFP-drassf, Sav-HA, Hpo-Myc, and phospho-MST1. Hpo interacts with Sav and drassf (lane 2). Sav and drassf interact only with Hpo and not with each other (lanes 3 and 4). The Hpo fraction bound to drassf (lane 3) and that bound to drassf is not (lane 4).

(B) drassf inhibits Hpo phosphorylation. Kc cells were treated for 4 days with eGFP, drassf, or Hpo dsRNAi. The addition of STS 3 hr prior to lysis induced Hpo phosphorylation (lane 1). Western blots were probed with drassf, Hpo34, and phospho-MST1 antibodies. In the presence of drassf dsRNAi, Hpo phosphorylation increased (lane 2). As expected, the Hpo band disappeared upon Hpo dsRNAi treatment (lane 3).

(C) Sav competes with drassf to bind Hpo. Kc cell lysates expressing, respectively, HpoK205-Flag, Ha-drassf, Sav-HA (200 ng), and Sav-HA (400 ng) were mixed, incubated overnight, and immunoprecipitated with Flag antibody. Blots were probed with HA and Flag antibodies. Increasing the amount of Sav displaced drassf from Hpo (compare lanes 5 and 6).

(D) The drassf phenotype is sensitive to hpo loss of function. The histogram represents the total body weight as a percent of control flies (white). The reduction in body size in drassf flies can be partially rescued by removal of one copy of hpo (hpo42–48 allele). p < 0.05 (white n = 80, drassfX16/X16 n = 80, FRT42D, hpo42–48/+ n = 80, and FRT42D, hpo42–48 n = 80).

(E–F) Sav controls drassf protein level. GFP (green) and Sav were expressed in the posterior half of the wing disc by the engrailed-GAL4 (en-GAL4) driver. A robust reduction of drassf staining (in red [E]) was observed in the en domain. [E] shows Sav overexpression in a separate disc. (F–I) drassf reduces apoptosis induced by sav and wts coexpression. Shown are electron micrographs of Drosophila heads from GMR::Gal4 control animals (F) or from GMR::Gal4/HUAS::drassf (G), GMR::Gal4/GMR::sav+wts (H), or GMR::Gal4/UAS::drassf/GMR::sav+wts (I). Overexpression of drassf inhibits the rough-eye phenotype generated by coexpression of Sav and Wts.

See Supplemental Experimental Procedures for exact genotypes. Error bars correspond to standard deviations.
a "tumour-suppressing" effect distinct from its "onco-

genic" role in opposing the Hpo pathway. However,
Aoyama et al. suggest that NORE1 may also have both
Ras- and MST-independent functions [22]. Future
experiments will therefore be aimed at gaining a better
understanding of the RASSFs' growth-restricting func-
tions. The fact that the dRASSF mutations are viable
might therefore reflect the facts that its ability to regulate
the Hpo pathway may be redundant with other modes of
regulation and that loss of dRASSF's tumor-suppressive
activity is balanced by loss of its growth-promoting
activity. O'Neill et al. have proposed that MST2 may be
inactivated by binding to Raf-1. It will be interesting to
determine whether this mode of regulation is redundant
with RASSF [28].

In summary, we have generated mutant alleles of the
sole Drosophila ortholog of the RASSF family of tumor
suppressors. Surprisingly, dRASSF mutant flies are
smaller than control flies. This growth defect can proba-
bly be ascribed in part to dRASSF's ability to antagonize
Hpo signaling by competing with Sav for binding to Hpo.
In addition, we have shown that dRASSF also possesses
a tumor-suppressor activity, which is uncovered when
hpo or Ras1 function is compromised. It will be interest-
ing to investigate whether some mammalian RASSF
proteins share these properties.

Supplemental Data
Supplemental Data include Experimental Procedures and Five Fig-
ures and are available online at http://www.current-biology.com/
full/16/24/2459/DC1/.

Acknowledgments
We thank S. Leevens, I. Hariharan, D. Pan, G. Haider, and the Bloo-
mington Drosophila Stock Center for fly stocks. We are grateful to
T. Gilbank, S. Murray, and F. Earl for transgenic generation, A.
Weston at the London Research Institute EM facility for SEMs, and
D. Andersen, J. Colombani, and F. Josué for comments on the man-
uscript. We thank J. Overton, B. Aeme, F. Josué, and J. Batut for
technical help. Work in N.H.B's laboratory was supported by the
Welcombe Trust grant 69943. Work in N.T.'s laboratory was sup-
ported by Cancer Research UK. C.P. is supported by a FEBS long-
term fellowship.

Received: June 23, 2006
Revised: October 19, 2006
Accepted: October 20, 2006
Published: December 18, 2006

References
1. Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of can-
cer. Cell 100, 57–70.
2. Edgar, B.A. (2006). From cell structure to transcription: Hippo
forges a new path. Cell 124, 267–273.

Figure 4. Tumour-Suppressor Function of
dRASSF
(A–C, E, and F) Scanning Electron Micro-
graphs of Drosophila heads from control
animals (A), animals bearing hpo42–47 clones
(B), hpo42–48 clones (C), hpo42–47 clones in
a dRASSF loss-of-function background (E),
or hpo42–48 clones in a dRASSF loss-of-func-
tion background (F). The overgrowth pheno-
type elicited by the loss of hpo is enhanced
by the removal of dRASSF. See Supplemental
Experimental Procedures for genotypes.
(D) Schematic representation of Hpo protein
showing the different mutations used. The
hpo42–47 allele causes a deletion of six amino
acids in the kinase domain, and this deletion
probably inhibits Hpo-ATP binding. The
hpo42–48 allele is a deletion of 20 bp and gives
rise to a premature stop codon. hpoKC203
changes G to A at the 5’ splicing site and
the translation run into a stop codon in the
intron.
(G–H) dRASSF rescues Ras loss of func-
tion. (G–G) Ras1c40b clones (marked by a
lack of GFP) are small. (H–H) Ras1c40b
dRASSFX36 clones (marked by a lack of GFP)
are larger than Ras1c40b clones. dRASSF stain-
ing is in red (G’ and H’).
3. Hergovich, A., Stegert, M.R., Schmitz, D., and Hemmings, B.A. (2006). NDR kinases regulate essential cell processes from yeast to humans. Nat. Rev. Mol. Cell Biol. 7, 253–264.

4. Thompson, B.J., and Cohen, S.M. (2006). The Hippo pathway regulates the bantam microRNA to control cell proliferation and apoptosis in Drosophila. Cell 126, 767–774.

5. Nolo, R., Morrison, C.M., Tao, C., Zhang, X., and Halder, G. (2006). The bantam MicroRNA is a target of the Hippo tumor-suppressor pathway. Curr. Biol. 16, 1895–1904.

6. Huang, J., Wu, S., Barrera, J., Matthews, K., and Pan, D. (2005). The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the Drosophila Homolog of YAP. Cell 122, 421–434.

7. Khokhlatchev, A., Rabizadeh, S., Xavier, R., Nedwidek, M., Chen, T., Zhang, X.F., Seed, B., and Avruch, J. (2002). Identification of a novel Ras-regulated proapoptotic pathway. Curr. Biol. 12, 253–265.

8. Vavvas, D., Li, X., Avruch, J., and Zhang, X.F. (1998). Identification of Nore1 as a potential Ras effector. J. Biol. Chem. 273, 5439–5442.

9. Eckfeld, K., Hesson, L., Vos, M.D., Bieche, I., Latif, F., and Clark, G.J. (2004). RASSF4/AD037 is a potential ras effector/tumor suppressor of the RASSF family. Cancer Res. 64, 8686–8693.

10. Vos, M.D., Ellis, C.A., Elam, C., Ulk, A.S., Taylor, B.J., and Clark, G.J. (2003). RASSF2 is a novel K-Ras-specific effector and potential tumor suppressor. J. Biol. Chem. 278, 28045–28051.

11. Tommasi, S., Dammann, R., Jin, S.G., Zhang Xi, X.F., Avruch, J., and Pfeifer, G.P. (2002). RASSF3 and NORE1: Identification and cloning of two human homologues of the putative tumor suppressor gene RASSF1. Oncogene 21, 2713–2720.

12. Dammann, R., Li, C., Yoon, J.H., Chin, P.L., Bates, S., and Pfeifer, G.P. (2000). Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. Nat. Genet. 25, 315–319.

13. Scheel, H., and Hofmann, K. (2003). A novel interaction motif, SARAH, connects three classes of tumor suppressor. Curr. Biol. 13, R899–R900.

14. Agathangelou, A., Cooper, W.N., and Latif, F. (2005). Role of the Ras-association domain family 1 tumor suppressor gene in human cancers. Cancer Res. 65, 3497–3508.

15. Pfeifer, G.P., Yoon, J.H., Liu, L., Tommasi, S., Wilczynski, S.P., and Dammann, R. (2002). Methylation of the RASSF1A gene in human cancers. Biol. Chem. 383, 907–914.

16. Praskova, M., Khokhlatchev, A., Ortiz-Vega, S., and Avruch, J. (2004). Regulation of the MST1 kinase by autophosphorylation, by the growth inhibitory proteins, RASSF1 and NORE1, and by Ras. Biochem. J. 381, 453–462.

17. Tommasi, S., Dammann, R., Zhang, Z., Wang, Y., Liu, L., Tsark, W.M., Wilczynski, S.P., Li, J., You, M., and Pfeifer, G.P. (2005). Tumor susceptibility of Rassf1a knockout mice. Cancer Res. 65, 92–98.

18. Pantalacci, S., Tapon, N., and Leopold, P. (2003). The Salvador partner Hippo promotes apoptosis and cell-cycle exit in Drosophila. Nat. Cell Biol. 5, 921–927.

19. Wu, S., Huang, J., Dong, J., and Pan, D. (2003). hipo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with Salvador and warts. Cell 114, 445–456.

20. Colombani, J., Polesello, C., Josue, F., and Tapon, N. (2006). Dmp53 activates the Hippo pathway to promote cell death in response to DNA damage. Curr. Biol. 16, 1453–1458.

21. Glantschnig, H., Rodan, G.A., and Reszka, A.A. (2002). Mapping of MST1 kinase sites of phosphorylation. Activation and auto-phosphorylation. J. Biol. Chem. 277, 42987–42996.

22. Aoyama, Y., Avruch, J., and Zhang, X.F. (2004). Nore1 inhibits tumor cell growth independent of Ras or the MST1/2 kinases. Oncogene 23, 3426–3433.

23. Udan, R.S., Kango-Singh, M., Nolo, R., Tao, C., and Halder, G. (2003). Hippo promotes proliferation arrest and apoptosis in the Salvador/Warts pathway. Nat. Cell Biol. 5, 914–920.

24. Newsome, T.P., Asling, B., and Dickson, B.J. (2000). Analysis of Drosophila photoreceptor axon guidance in eye-specific mosaics. Development 127, 851–860.