A Gain-of-Function Germline Mutation in Drosophila ras1 Affects Apoptosis and Cell Fate during Development

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Abstract

The RAS/MAPK signal transduction pathway is an intracellular signaling cascade that transmits environmental signals from activated receptor tyrosine kinases (RTKs) on the cell surface and other endomembranes to transcription factors in the nucleus, thereby linking extracellular stimuli to changes in gene expression. Largely as a consequence of its role in oncogenesis, Ras signaling has been the subject of intense research efforts for many years. More recently, it has been shown that milder perturbations in Ras signaling during embryogenesis also contribute to the etiology of a group of human diseases. Here we report the identification and characterization of the first gain-of-function germline mutation in Drosophila ras1 (ras1R68Q), the Drosophila homolog of human K-ras, N-ras and H-ras. A single amino acid substitution (R68Q) in the highly conserved switch II region of Ras causes a defective protein with reduced intrinsic GTPase activity, but with normal sensitivity to GAP stimulation. The ras1R68Q mutant is homozygous viable but causes various developmental defects associated with elevated Ras signaling, including cell fate changes and ectopic survival of cells in the nervous system. These biochemical and functional properties are reminiscent of germline Ras mutants found in patients afflicted with Noonan, Costello or cardio-facio-cutaneous syndromes. Finally, we used ras1R68Q to identify novel genes that interact with Ras and suppress cell death.

Introduction

Multicellular organisms must extensively coordinate the activities of many diverse and highly specialized cells, requiring effective and flexible signaling mechanisms for both development and tissue homeostasis. Many inter-cellular signals are transmitted by receptor tyrosine kinases (RTKs), which control key aspects of cellular growth, differentiation, metabolism and cell death [1,2]. On the other hand, mutations that lead to abnormal activation of RTKs can generate oncogenes that promote tumorigenesis [3,4]. Ras proteins are guanine nucleotide binding proteins that act as molecular switches to transduce RTK-signals from the outside to the interior of the cell [5]. Remarkably, ~20% of all tumors contain an activating point mutation in Ras [6,7]. Consequently, this pathway has been extensively studied both in the context of normal signal transduction, and oncogenic growth. Because the RTK/RAS signaling network is highly conserved among animals, genetic model systems have made major contributions for elucidating this pathway [8,9,10,11].

One major physiologic function of the RTK/RAS pathway during development is the transmission of anti-apoptotic signals that suppress the activation of an intrinsic cell death program [12,13]. In Drosophila, as in mammals, cells are over-produced during development and compete for limiting amounts of extracellular survival factors in order to suppress the induction of apoptosis [14,15]. This strategy permits appropriate matching of different cell types in a tissue and allows for the elimination of any superfluous and potentially dangerous cells. A conserved mechanism for survival signaling involves activation of receptor tyrosine kinases (RTKs) at the cell surface, which in turn stimulates the antiapoptotic activity of Ras [13]. Active Ras promotes its anti-apoptotic effect via several effector pathways, including the mitogen-activated protein kinase p42/44 (MAPK) of the ERK-type (extracellular signal-related kinase) via Raf [16,17,18], and the Akt kinase via Phosphoinositide 3-kinase [19]. In Drosophila, one major target for the anti-apoptotic activity of Ras is the pro-apoptotic Hid protein, which is inactivated via phosphorylation by MAPK [20,21,22]. Active Hid induces apoptosis by binding to and inhibiting Drosophila Inhibitor of Apoptosis Protein-1 (Diap1), an essential inhibitor of caspases in Drosophila [23,24,25,26,27]. In living cells, Diap1 inhibits both initiator and effector caspases, and its function is required for the survival of virtually all somatic cells [23,25,26,28,29,30,31]. In response to apoptotic stimuli, Diap1 is inactivated by natural IAP-antagonists, including Reaper, Hid and Grim (RHG proteins). The active forms of RHG proteins are generated in doomed cells by a combination of transcriptional induction and post-transcriptional regulation [32,33,34,35]. Once active, RHG proteins form complexes that both disrupt binding of Diap1 to caspases and also stimulate auto-ubiquitination and degradation of Diap1, thereby removing the “brakes on death” [27,36]. One important role of Hid is to recruit Reaper to the outer mitochondrial membrane, which is important for efficient inactivation Diap1 and apoptosis induction [27]. Survival signals, such as Spitz in the case of midline glia, inhibit the pro-apoptotic...
activity of Hid via activation of EGFR, Ras and MAPK, leading to direct phosphorylation of Hid by MAPK and inhibition of Hid pro-apoptotic activity [21,33].

We previously conducted large-scale dominant modifier screens in Drosophila to identify genetic modifiers of Hid-induced apoptosis [20]. These screens identified several loss-of-function alleles in sprouty and gap1, both negative regulators of the RAS/MAPK signaling pathway and helped define the mechanism by which MAPK signaling inactivates a critical component of the apoptotic machinery [22,37]. Here we report the identification and characterization of another Hid-modifier mutation, which maps to the switch II region of ras1 (also known as ras85D), the Drosophila homologue of mammalian N-ras, K-ras and H-ras. Although many loss-of-function alleles have been described for Drosophila ras1, this mutation is the first endogenous gain-of-function allele reported for this gene. We demonstrate biochemically that this viable hypermorphic, ras1R78Q, produces a defective Ras protein with reduced intrinsic GTPase activity, but normal sensitivity to GAP stimulation. These biochemical features are reminiscent of those recently described for mutant human H-ras and K-ras proteins known to underlie a group of related developmental disorders that includes Noonan syndrome, Costello syndrome and cardio-facio-cutaneous syndrome [38,39,40]. Flies mutant for ras1R78Q exhibit a number of developmental defects that are characteristic of abnormally elevated RTK/RAS/MAPK signaling, including enhanced resistance to apoptosis, supernumerary R7 cells in the eye and ectopic wing vein formation, demonstrating that the mutant Ras protein has enhanced signaling capacity in vivo. This allele should be a useful tool to study the physiological consequences of modest activation of Ras signaling in vivo. Finally, we used this mutant to identify novel interactors of Ras that suppressers cell death.

Materials and Methods

Fly stocks

The following fly stocks were used: GMR-rpr6 [41], GMR-rpr32 CyO/Sco, GMR-hid1A, GMR-hid16 and GMR-hid16/1 [20], GMR-hid16 and hs-hid1 [24], GMR-egr [42], GMR-phys [43], GMR- rho1 [44], Ig-GALA (F.M. Hoffmann, unpublished), UAS-hid [45], argl [46], EGFR = f65C [47], v8-C [48], seo-ras1f1 [49], P[sh1-1.0-lacZ] [50], Hml-GAL4, 2xUAS-EGFP (J.A. Rodriguez, unpublished). Stocks for mitotic recombination mapping (u4 h16 t1 sc1 cu1 sc1 and u4 h16 t0 sc1 cu1 sc1) and P-element insertion lines were obtained from the Bloomington Stock Center (Bloomington, IN). All other lines were generated by meiotic recombination of the appropriate alleles.

Genetic screens

Dominant modifier screens were performed as described in Fig. S1. Approximately 170,000 F1 progeny from ENU and EMS mutagenized GMR-rpr6 flies were screened for modification of a GMR-rpr6 induced rough eye phenotype, yielding 25 enhancers and 3 suppressors (Table S1). Similarly, 300,000 F1 progeny from ENU, EMS and x-ray mutagenized flies were screened for suppression of a GMR-hid10 induced rough eye phenotype, resulting in the recovery of 128 additional suppressors (Table S2). In sum total, 158 dominant modifiers of GMR-rpr or GMR-hid were isolated in these screens.

Complementation analyses using phenotype and map information placed 133 of these modifiers into 13 complementation groups, while the remaining mutants represent single hits or have no recessive phenotype. To further enrich for mutants that are cell death specific, we eliminated general modulators of GMR promoter expression or eye development by testing modifiers against GMR-phys and GMR-rho induced rough eye phenotypes, which are unrelated to cell death [43,44]. In addition, reasoning that mutants involving apoptosis genes should be able to modify cell death phenotypes in contexts other than the eye, suppressors from the GMR-hid screen were tested for their ability to suppress hs-hid induced embryonic lethality and zg-GALA, UAS-hid induced wing ablation. On the basis of these secondary screens, we eliminated several complementation groups including gls, which encodes the transcription factor that drives GMR expression, Su(GMR)2A and su(GMR)2A, which are known to indirectly and non-specifically affect GMR promoter expression, and Su(GMR)3A and Su(GMR)2B, complementation groups that have not been assigned to a previously characterized gene [51,52]. We also eliminated 4 alleles linked to the parental GMR-rpr transgene. Our cell death enriched subset of modifiers therefore consists of 58 mutants in total, 40 that fall into 6 complementation groups and 18 single alleles.

All crosses and suppression experiments were carried out at 25 °C except crosses with zg-GALA and UAS-hid, which were performed at both 18 °C and 25 °C. Suppression experiments with hs-hid were done by heat shocking 1st instar larvae at 37 °C for 15 minutes.

Biochemistry

A cDNA clone encoding Drosophila ras1 was obtained from the Drosophila Genomics Resource Center (clone ID: RE53955) and the entire ras1 ORF was subcloned into pBluescript (Stratagene). Mutant ras1R78Q was generated using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene). The Ras ORFs were then subcloned into pET-28a (Novagen) in frame for an N-terminal His tag. Catalytic human p120-Gap (GAP-285, amino acids 714–998, IMAGE Clone: 4892173, Open Biosystems) was subcloned into the pET41a vector (Novagen) to generate an N-terminal GST tag. Fusion proteins were expressed in BL21(DE3) E. coli (Invitrogen) and affinity purified on an AKTA Purifier (Pharmacia) using a HisTrap FF column (GE Healthcare) for Ras proteins and a GSTTrap FF column (GE Healthcare) for GAP-285. Ras purification was done according to the procedure described for human H-Ras [53]. GAP-285 was expressed by inducing cells for 16 hours at 30 °C with 0.2 mM IPTG.

Intrinsic GTPase activities were measured using [gamma-32P]GTP (3000 Ci/mmol, NEN) and the EasyRad Phosphate Assay (Cytoskeleton) [54]. GAP-stimulated GTPase activities were measured with a real-time assay using the fluorescent substrate MDCC-PBP (Invitrogen) and 2 μM Ras protein, with or without, 0.02 μM GAP-285 [55].

Phenotypic Analyses

To visualize larval hemocytes, wandering 3rd instar larva expressing UAS-EGFP driven by Hml-GAL4 were collected and immobilized on ice prior to imaging [56]. MG cells in stage 17 embryos were visualized using P[sh1-1.0-lacZ] and β-gal immunohistochemistry as previously described [57]. The number of MG was averaged for segments T2 to A5. Tangential sections (1 μm) of adult eyes were prepared according to standard protocols for analysis of ommatidia [38].

Results

Genetic screens for dominant modifiers of apoptosis in Drosophila

Dominant modifier screens are designed to detect pathway components for which small perturbations in gene dosage can alter
a sensitized phenotype, thus allowing for the recovery of both loss-of-function and gain-of-function mutations. We used eye-specific expression of the Drosophila cell death genes hid or rpr under control of the GMR promoter to generate a dosage sensitive eye ablation phenotype and then screened for dominant modifiers of this phenotype to identify regulatory components of the intrinsic cell phenotype and then screened for dominant modifiers of this phenotype. Although several genes identified in this way have been reported, details of these screens have not been previously published and are provided in the supplementary material (Fig. S1, Tables S1 and S2).

We identified a subset of 58 mutants that specifically affect rpr or hid-induced cell death. 40 of these correspond to 6 complementation groups, while 18 represent single alleles. Of the 6 complementation groups identified, 3 correspond to genes that regulate EGFR receptor (EGFR) signaling. Five loss-of-function alleles each of gap1 and sprouty, both negative regulators of EGFR/MAPK signaling, were recovered as strong, hid specific suppressors. These mutants have been characterized elsewhere [20]. We also isolated five loss-of-function Star alleles as enhancers of GMR-rpr. Star is required for the correct processing of Spitz, a stimulatory ligand of EGFR [59]. Ten alleles of diap1, the major biochemical target for the pro-apoptotic activity of RHG proteins were isolated, including loss-of-function alleles that enhance rpr-, hid-and grn-induced cell death and two distinct classes of gain-of-function alleles [23,28,31]. The fifth complementation group, consisting of 12 alleles, displayed a differential modulation of cell death phenotypes reminiscent of diap1 RING mutants and was found to encode dbruce, the Drosophila ortholog of mouse Bruce and human Apollon [60,61]. This very large (4852 amino acid) BIR-containing protein is cytoprotective against caspases and required for spermatid survival [62,63,64]. Finally, 5 single alleles from the GMR-rpr screen likely represent weak hypomorphs of diap1 as they map close to the diap1 locus, and a sixth allele was identified as an allele of Delta.

The remaining complementation group, Su(GMR-hid)2A, and 12 additional single alleles were previously not characterized. We chose one allele, Su(21-3s) for further analysis based on its ability to potentially suppress hid-induced phenotypes (eye/wing/organismal lethality) without non-specifically affecting GMR-phyl (Table S2). Su(21-3s) mutants are homozygous viable and do not have overt abnormalities. This mutant was mapped by meiotic recombination to the right arm of chromosome 3, near the viable marker curled.

Characterization of Su(21-3s) suppressor phenotypes

We first examined more rigorously the suppressor phenotypes of Su(21-3s) in the eye by testing the modifier effects of one or two copies of Su(21-3s) against various GMR expression constructs. This analysis confirmed that Su(21-3s) potently suppresses GMR-hid induced cell death in a dosage dependent manner (Fig. 1A,B). We found suppression of GMR-rpr and GMR-grn phenotypes, however, to be extremely weak, even with two copies of Su(21-3s) (Fig. 1E,F). Given that hid is highly expressed in the developing eye, we believe the small effect exerted by Su(21-3s) on GMR-rpr and GMR-grn is due primarily to a suppression of endogenous Hid activity and not that of Rpr or Grm [24]. We conclude from these data that Su(21-3s) is a specific suppressor of hid-induced cell death.

The activity of Hid is regulated by the EGF/MAPK pathway in a manner that depends on intact MAPK phosphorylation sites in Hid. Our analysis here reveals that Su(21-3s) readily suppresses GMR-hidinduced cell death, a hid allele lacking 5 of 5 predicted MAPK phosphorylation sites, but fails to suppress GMR-Induced cell death, which is missing all 5 MAPK sites (Fig. 1C,D) [37]. This requirement for one or two of the predicted MAPK phosphorylation sites in Hid (Ser-121 and Thr-228), along with the observed specificity for GMR-hid suppression, strongly suggested that Su(21-3s) might be mediating its suppressive effects through the EGF/MAPK pathway.

We further extended analysis of the Su(21-3s) suppression phenotypes to the developmental context of larval hemocytes, an important model system for the study of vertebrate haematopoiesis [65,66]. Drosophila hemocytes require trophic signaling from multiple pathways for their survival and in its absence undergo caspase dependent cell death [67,68]. Larval hemocytes also undergo caspase dependent cell death in response to ectopic hid expression [69]. Using a hemocyte specific promoter to drive expression of UAS-EGFP, we are readily able to visualize hemocytes in wandering 3rd instar larvae (Fig. 1G) [56]. Ectopically expressing UAS-hid using the same driver results in near complete ablation of hemocytes by the 1st instar larval stage (data not shown) and generates 3rd instar larvae that are completely devoid of hemocytes (Fig. 1H). We found that the Su(21-3s) mutation is able to partially suppress this cell death such that EGFP expressing hemocytes are clearly visible anteriorly in the lymph glands of 3rd instar larvae (Fig. 1I). Circulating hemocytes, however, appear to remain susceptible to hid-induced cell death and are missing, even in the presence of two copies of the Su(21-3s) allele. It may be that Su(21-3s) is a weak suppressor of cell death in hemocytes, sufficient to suppress Hid activity in young hemocytes localized to the supportive environment of a lymph gland, but insufficient in the context of a mature circulating hemocyte.

Su(21-3s) is a viable gain-of-function allele of ras1 (ras85D)

In order to identify the gene responsible for the Su(21-3s) phenotype, we mapped it by a second, finer round of meiotic recombination to a 1 Mb interval between 85A and 85E, then further localized the mutation by P-element mediated male recombination to a 270 Kb interval between 85D11 and 85E1 (Fig. 2A). Given that Su(21-3s) differentially suppresses hid, but not grn or rpr in a manner reminiscent of EGFR/MAPK pathway mutants, we suspected that Su(21-3s) might be a rare hypermorphic allele of ras1, or ras85D as it is otherwise known, because it is located within this interval. Indeed, when we sequenced ras1 in a candidate gene approach, a G to A transition in exon3 was identified. This mutation results in an amino acid substitution at position 68 of the Ras protein, replacing a positively charged arginine within the universally conserved switch II region of Ras with a neutral glutamine (Fig. 3).

The switch regions of Ras have been defined as regions that undergo a large conformational change when Ras transitions from the GTP- to the GDP-bound state [70]. Detailed biochemical analysis and crystal structures have revealed that residues in the switch II region of Ras contact and are stabilized by the GTPase Activating Protein (GAP), allowing them to participate up to a 1000 times more efficiently in the catalysis of GTP [71]. As a consequence, mutations in the switch II region of Ras interfere with its catalytic GTPase activity and prolong the time Ras remains bound to GTP. Mutations that reduce the GTPase activity of Ras are hypermorphic since it is the GTP-bound form of Ras that engages and activates downstream signaling effectors. The signaling activities of Ras are terminated when GTP bound by Ras is converted to GDP, explaining why the most frequently occurring oncogenic mutations in Ras, at amino acids 12,13 and 61, also render Ras biochemically inert as a GTPase (Fig. 3) [72,73]. It seemed feasible, therefore, that the R68Q mutation identified in Su(21-3s) flies could similarly result in a Ras protein.
with reduced GTPase activity, leading to a prolonged RAS/MAPK signal that suppresses cell death induced by GMR-hid.

We reasoned that if the Su(21-3s) phenotype is due to a gain-of-function mutation in ras1, it should be revertible by introduction of a second, intragenic loss-of-function mutation. To test this, we conducted a reversion screen for loss of the Su(21-3s) suppression phenotype and successfully recovered two mutants containing intragenic loss-of-function ras1 mutations (Figs. 2 and S1). One revertant contains a 31 bp deletion in ras1 that results in a Ras protein truncated at amino acid 87. The second revertant contains an in frame 18 bp deletion of ras1 that removes amino acids 87–92 which are essential for Ras function [74]. These revertants have genetic...
properties of ras1 null alleles and fail to complement the known null alleles ras1<sup>−/−</sup> and ras1<sup>−/−</sup> and supporting the idea that the Su(21-3s) phenotype is due to a revertible gain-of-function mutation in ras1.

As an allele of ras1, Su(21-3s) should interact genetically with other members of the MAPK signaling pathway in a predictable manner. We crossed GMR-hid<sup>698</sup> flies in a Su(21-3s) background to mutants of MAPK signaling and observed the degree of cell death in the eye (Fig. S2). MAPK signaling mutants tested include argos, ras1, rolled and EGFR. In this analysis, we found that the Su(21-3s) mutant behaves in a manner consistent with that expected for a gain-of-function ras1 allele. For example, Su(21-3s) is not much affected by loss-of-function mutations in upstream components of MAPK signaling, such as argos or EGFR but is strongly ameliorated by loss of downstream components, such as rolled. Additionally, when a dominant negative form of Ras1 (yn-ras<sup>1177</sup>) is expressed in the eye, the suppressive effects of Su(21-3s) are completely abrogated. On the basis of our mapping data, the sequence data and the genetic interaction data presented above, we conclude that Su(21-3s) is a hypermorphic allele of ras1, which we rename here, ras1<sup>R68Q</sup>.

**Biochemical analysis of recombinant Ras1<sup>R68Q</sup> protein**

To test the hypothesis that exchanging a positively charged arginine with a neutral glutamine at position 68 of Ras results in a protein with deficient GTPase activity, the intrinsic GTPase rates of wildtype and mutant Drosophila Ras1 proteins were measured. Full-length wildtype Ras1 and mutant Ras1<sup>R68Q</sup> were bacterially expressed and purified as His-tagged fusion proteins, yielding large amounts of pure, catalytically active enzyme. Intrinsic GTPase activity rates were measured with a kinetic phosphate assay employing [γ<sup>32</sup>P]GTP as substrate. This sensitive assay revealed that Ras1<sup>R68Q</sup> has an intrinsic GTPase activity that is approximately 1/3 that of wildtype Ras1, with enzymatic rates ([k<sub>cat</sub>] of 0.020 min<sup>−1</sup> and 0.063 min<sup>−1</sup>, respectively (Fig. 4C). Since many activating Ras mutations also result in an enzyme that is insensitive to GTPase activating proteins (GAPs), the ability of Ras1<sup>R68Q</sup> to be stimulated by GAP was also assessed. Recombinant human GAP-285 protein was purified by affinity chromatography and its ability to stimulate wildtype and mutant Ras1 proteins was tested using a real-time fluorescent assay. These experimental data show that Ras1<sup>R68Q</sup> remains amenable to GAP stimulation (Fig. 4D). This means that in contrast to constitutively active Ras mutants such as oncogenic RasV<sup>12</sup>, whose GTPase activity is completely refractory to stimulation by GAPs, Ras1<sup>R68Q</sup> can be regulated and is able to cycle between on and off states [75]. Together, these biochemical data support the hypothesis that Ras1<sup>R68Q</sup> has a reduced basal level of GTPase activity, remains in its active GTP-bound form for longer and thus has an enhanced activity.
with an increase of Ras activity in
(n = 448) whereas embryos contained an average of 2.8 MG cells per segment
to wildtype embryos (Fig. 5E,F). Stage 17 wildtype
Drosophila
the EGFR/Ras/MAPK pathway [21,76]. During formation of the embryonic development depends on survival signals mediated by ras1R68Q apoptosis in a RHG-dependent manner such that by stage 17, only ras1R68Q significant by an unpaired t-test (p < 0.0001) and is consistent with nearly normal cellular function and organismal signaling capacity, but is still amenable to regulation, making it compatible with nearly normal cellular function and organismal development. These biochemical features are highly reminiscent of those recently described for germline H-ras and K-ras mutants found in the developmental disorders Noonan, Costello and CFC syndromes and the most frequent cancer-associated somatic mutations (labeled in red). R68Q indicates the mutation characterized in this study, a non-conserved arginine to glutamine amino acid substitution within the switch II region of Drosophila Ras1 (dashed red box). hs, H. sapiens; dm, D. melanogaster; mm, M. musculus; ce, C. elegans.

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Ras1R68Q promotes survival of midline glia (MG) The survival of Drosophila midline glia (MG) cells during embryonic development depends on survival signals provided by the EGFR/Ras/MAPK pathway [21,76]. During formation of the Drosophila central nervous system, there are initially approximately ten MG cells per segment at stage 13. Most of these undergo apoptosis in a RHG-dependent manner such that by stage 17, only three MG per segment survive [45,77]. We tested the effect of ras1V12 in this system. MG cells were visualized in wildtype and ras1R68Q embryos using the MG-specific pslit-lacZ reporter, and marked MG cells were carefully counted. This analysis revealed an increase in the number of MG cells in ras1R68Q embryos as compared to wildtype embryos (Fig. 5E,F). Stage 17 wildtype embryos contained an average of 2.8 MG cells per segment (n = 448) whereas ras1R68Q embryos contained an average of 3.3 MG cells per segment (n = 420). This difference is statistically significant by an unpaired t-test (p < 0.0001) and is consistent with an increase of Ras activity in ras1R68Q flies (Fig. 5G).

Ras1R68Q causes supernumerary R7 cells in the eye The adult Drosophila eye comprises about 800 ommatidia, each with a precise, reproducible structure consisting of eight photoreceptors and 12 accessory cells [78,79]. Adoption of a neuronal cell fate by the precursor of the R7 photoreceptor requires an inductive signal from the neighboring R8 cell and is dependent on EGFR/MAPK signaling [80,81,82]. Furthermore, the cone cell precursors are capable of acquiring an R7 cell fate if MAPK signaling is ectopically activated in these cells, resulting in extra R7 cells that are easily visualized [83]. To determine if the ras1R68Q mutation exerts effects in a paradigm other than apoptosis, semi-thin plastic sections of adult eyes were prepared and analyzed for defects in ommatidia formation. We observed two types of defect in ras1R68Q flies typical for mutations that increase Ras/MAPK signaling during eye development. First, we detected ommatidia with supernumery R7 cells (Fig. 5B,C). Second, we noticed occasional ommatidia missing outer photoreceptor cells, also a phenotypic consequence of elevated MAPK signaling (Fig. 5D) [83]. The developmental defects in retinal cell differentiation observed here further supports our hypothesis that ras1R68Q is a gain-of-function ras1 allele.

The wings of ras1R68Q flies contain ectopic vein material In addition to defects in the eye and midline glial cells, ras1R68Q flies also show abnormalities in adult wing tissues. Homozygous ras1R68Q flies have an extra longitudinal “veinlet” branching off the posterior crossvein (Fig. 5I). Additionally, an ectopic longitudinal vein was seen beneath the posterior crossvein and an ectopic crossvein appeared between the L4 and L5 wing veins near the hinge. These defects are remarkably similar to those observed in the wings of nemo and DER conditional flies, which have elevated levels of MAPK signaling [84]. When UAS-ras1R68Q was overexpressed in the wing using en-Gal4, extensive ectopic wing vein material and blisters developed (Fig. 5J). Overexpression of wildtype Ras1 gave a similar but less severe phenotype. Finally, we attempted to express ras1P172T in the wing using en-Gal4 but found this to cause organismal lethality.

Figure 3. Amino acid alignment of fly, worm and mammalian Ras1. These homologs have extensive primary sequence homology. Drosophila Ras1 (dmRas1), for example, is 87% identical to human K-ras (hsKras) at the amino acid level when C-terminal membrane-targeting sequences are excluded. Conserved regions are shaded in grey, with residues identical to the consensus sequence represented by a grey dot, while non-conserved residues remain unshaded. Five highly conserved signature motifs named “G box” sequences, labeled G1-G5 and boxed in red, are found in all residues remaining. Conserved regions are shaded in grey, with residues identical to the consensus sequence represented by a grey dot, while non-conserved residues remain unshaded. Five highly conserved signature motifs named “G box” sequences, labeled G1-G5 and boxed in red, are found in all homologs. Conserved regions are shaded in grey, with residues identical to the consensus sequence represented by a grey dot.

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Overexpression of ras1R68Q in the eye induces both severe overgrowth and cell death

It was previously shown that overexpression of wildtype Ras1 in the Drosophila eye, even at the high levels obtained by transgene expression, results in little or no observable phenotypic effect [83]. For this reason, studies of elevated Ras signaling in Drosophila regularly rely on a very strong, constitutively active RasV12 mutant allele. We similarly observed that wild-type UAS-ras1 expressed by GMR-Gal4 had little effect on eye development in 11 independent transgenic lines (Fig. S4). In striking contrast, seven independent transgenic lines expressing UAS-ras1R68Q resulted in highly distorted eyes that exhibited both hyperplastic tissue overgrowth and widespread cell death ablation phenotypes. For purposes of comparison, we attempted to express two different UAS-ras1V12 alleles in the same manner, but again found this induced organismal lethality (likely due to the fact that GMR drives some expression in tissues other than the eye and Ras1V12 induces non-cell autonomous cell death when overexpressed) [85]. We generated many more than seven UAS-ras1R68Q transgenic lines but similarly found many of them to be lethal in combination with GMR-Gal4. This lethality was not observed in any of the 16 independent UAS-ras1 transgenic lines tested. These experiments further support the view that Ras1R68Q is an activated form of Ras that nevertheless remains amenable to regulation and therefore is less biologically potent than the constitutively active Ras1V12 protein.

Identification of Novel Ras Interactors and Suppressors of Cell Death

In the reversion screen described above we were also able to recover additional suppressors of GMR-hid. We collected a number
of strong dominant suppressors of the GMR-hid eye phenotype and mapped them using deletions on the 3rd chromosome. As indicated in Table 1, we successfully recovered 14 suppressors that fall into 8 complementation groups. In most cases, we were able to identify a single gene that appears to be responsible for the suppressors phenotype (indicated in bold). In two cases, the mutations were narrowed to a small region, but we were unable to unequivocally identify a single candidate. We also recovered mutations in the glass gene, which affects expression from the GMR-driver [86]. We recovered mutations in Gap1 and Delta, both of which were identified in our original hid suppressor screen, indicating an overlap in the mutational spectrum between the two screens [20]. Based on previous reports, mutations in Gap1 are expected to suppress GMR-hid, and Delta/Notch signaling is known to intersect and cooperate with the Ras/Mapk pathway [87]. Interestingly, we also isolated a number of novel ras interactors, including four alleles of the predicted transcription elongation factor Su[Tpl] and an allele of notum, a component of the Wnt/Wingless signaling pathway. These results indicate that use of the rasR68Q allele in screens may indeed uncover novel regulatory interactions that have been missed with other strategies, including those that make use of the constitutively active, non-regulatable rasV12 hypermorph.

Discussion

We have conducted genetic screens for dominant modifiers of cell death induced by the Drosophila IAP-antagonists, hid and rpr. From
Table 1. Suppressors of GMR-hid recovered from the reversion screen.

| Complementation Group | Location       | Candidate Gene |
|-----------------------|----------------|----------------|
| SupX3/SupX6           | 67C10          | Gap1           |
| SupE6.1/SupE6.2        | 66E6-67B1      | Argk2          |
| SupE8.1/SupE8.2        | 66B6-66C1      | ERB1           |
| SupX9/SupE10.1/SupE10.2/SupX13 | 76D3    | Su(Tpl)        |
| SupX8                  | 72C3           | Notum          |
| SupE7.2                | 99E4           | Hdc            |
| rasR68Q interactor1    | 62B1           | Drpr           |
| rasR68Q interactor2    | 92A1           | DI             |

In most cases, a single mutant gene corresponding to these suppressors could be identified (indicated in bold). In two cases, the mutations were narrowed to a small region but a single gene could not be unequivocally identified; in these cases, we list the most likely candidate gene (with an "?" based on mapping data and published literature.

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over 150 mutants initially isolated, secondary screens allowed us to identify 58 cell death specific modifiers. Of these, 40 alleles were placed into six complementation groups that define both known and unknown genes. These include Star, gap and sprouty involved in EGF/MAPK signaling, the known cell death regulator diap1, the very large BIR and UBC containing dbruce, and an unknown gene, Su(GMRhid)24 that remains unidentified. Here we focused on a previously uncharacterized cell death suppressor originally termed Su(21-3s). Using a combination of meiotic and P-element induced male recombination, genetic reversion, biochemistry and in vivo analysis, we demonstrate that this mutant is a gain-of-function mutation in ras1 (ras65D), the Drosophila homolog of human K-ras, N-ras and H-ras. We also show that this allele affects cell fate decisions and the pattern of normal, developmental apoptosis in paradigms known to depend on Ras-signaling.

One important role of Ras signaling during development is the transmission of an anti-apoptotic signal [13,20,22]. As previously reported, the pro-apoptotic protein Hid contains 5 potential MAPK phosphorylation sites that are essential for its sensitivity to Ras-mediated inhibition [20]. A Hid protein with either 3/5 or 5/5 mutant MAPK sites (HidW12 and HidW12, respectively) was refractory to suppression by the gain-of-function MAPK allele r1G02a (a very mild suppression by r1G02a is due to phosphorylation of the endogenous wildtype Hid protein). In contrast, there was still some suppression of HidW12 and HidW12 by RasV12. It was postulated that this might be due to the ability of Ras, unlike MAPK, to exert additional anti-apoptotic effects through activation of the PI3-K/Akt-kinase effector branch. In the current study, we found that r1G02a was able to partially suppress HidW12 but not HidW12 (Fig. 1C,D). Because HidW12 retains two phosphorylation sites, it appears that partial phosphorylation of Hid is sufficient for a mild inhibitory effect, and that all five phospho-acceptor sites need to be eliminated in order for Hid to become refractory to inhibition by MAPK. Furthermore, it appears that RasV12, unlike RasV12, is unable to exert an additional suppressive effect via PI3-K/Akt-kinase. Perhaps the enhanced signaling activity of RasG02Q is able to activate the MAPK effector branch, but does not reach a required threshold to engage the PI3-K/Akt-kinase pathway [59,88,89,90,91]. This may also help to explain the organismal viability of RasG02Q as compared to RasV12. Along the same line, RasG02Q was able to suppress Hid-induced cell death of lymphocytes within the protective environs of the lymph gland but not of those that were circulating (Fig. 1G-I). In sharp contrast, over-expression of RasV12 in hemocytes not only leads to survival of circulating hemocytes but in fact results in a massive overproliferation of hemocytes (Rodriguez and Steller, unpubl. data) These results serve to highlight the exquisite sensitivity of biological systems to the degree of Ras signaling and suggest that between the extremes of wildtype Ras and constitutively active RasV12 lies a large spectrum of biological responsiveness.

Ras is highly conserved among metazoans and a number of Ras structures have been published that make it possible predict how mutations in specific regions might affect function. In the case of RasG02Q, we considered that this change may affect the transition state of Ras. According to the “arginine-finger hypothesis” proposed by Schelland and colleagues, GTPase-activating-proteins (GAPs) dramatically accelerate the GTPase reaction of Ras by supplying an arginine side chain (arginine-789 in the case of GAP-334) into the active site of Ras to neutralize developing charges in the transition state [71]. A detailed analysis of the interactions between Ras and GAP-334 showed no role for R68 of Ras, explaining why RasG02Q can be stimulated by GAP [71,74,92,93]. However, a close inspection of the Ras catalytic site (Fig. 4B) shows that R68 extends its side chain towards the catalytic center [94]. Mutating R68 to glutamine removes a stabilizing positive charge from the transition state and, according to the arginine-finger hypothesis, would be expected to result in less efficient hydrolysis of GTP. We tested this prediction biochemically and indeed found that RasG02Q hydrolyzes GTP intrinsically at a reduced rate, approximately 30% of that of wild type GTP (Fig. 4C).

Oncogenic mutations in Ras occur most frequently at codons 12,13 or 61 and result in an enzyme with deficient GTPase activity. This renders Ras inactive because Ras is ‘‘on’’ when bound to GTP and switches off by hydrolyzing bound GTP to GDP. Inhibition of Ras GTPase activity therefore stabilizes Ras in its active conformation, prolonging its recruitment and activation of downstream signaling components [5,7,10,70,95]. The reduced GTPase activity of RasG02Q means that it would remain in its active GTP-bound conformation for longer periods of time allowing for enhanced signaling to downstream effector pathways. As noted above, however, RasG02Q may not remain in an active state sufficiently long to engage the catalytic p110 subunit of PI3K. An interesting alternative possibility however may be that R68 is directly involved in an interaction with PI3K and a mutation in R68 negatively affects this interaction. This raises the intriguing possibility that some of the phenotypes described for RasG02Q (Fig. 5) may actually be due to a loss, rather than a gain of PI3K activity.

During the initial mapping and characterization of rasG02Q, we conducted a reversion screen in order to provide genetic evidence for our hypothesis that we had identified a rare gain-of-function allele in ras65D (Fig. 2). While searching for revertants, we also recovered several mutants that were strong suppressors of GMR-hid. Recognizing that these mutants might be synergizing with r1G02Q to produce such a strong suppression, we successfully recovered and mapped 14 of these suppressors. As indicated in Table 1, most were mapped to a single candidate gene. Since these mutants were essentially derived from a dominant modifier screen for suppression of GMR-hid induced cell death, but within a sensitized r1G02Q background, we expected the mutational spectrum to be overlapping, yet distinct from that of previous GMR-hid or UAS-RasV12 based screens. Indeed several suppressors turned out to overlap with ones identified previous screens. However, we also isolated two novel interactors: one allele of notum and four alleles of Su(Tpl). This demonstrates the utility of r1G02Q to identify novel genetic interactions. While notum affects the Wingless signaling pathway, Su(Tpl) is thought to function in the regulation of transcription in response to stress [96,97,98].
Much of our understanding of Ras-mediated signaling is derived from a combination of biochemical experiments conducted in mammalian tissue culture, and genetic studies in model organisms [10]. For example, Ras-mediated signaling regulates the specification and differentiation of R7 photoreceptors in the Drosophila eye ([80,81,99]). However, until now, studies on the physiological consequences of elevated Ras in Drosophila have relied on overexpression of the activated ras1F12 allele [83,85,100]. The viable hypermorphic ras1 allele described here, ras1R68Q, represents the first endogenous gain-of-function mutation in Drosophila Ras and hence offers a new tool for the analysis of Ras biology in situ. In particular, certain aspects of Ras biology have remained largely inaccessible to the use of constitutively active versions of this protein. This is because mutants, such as ras1F12, do not cycle normally between off and on states, are insensitive to regulatory upstream components of MAPK signaling such as EGFR (A vs D) is not much affected by loss of function mutations in Ras1 (F), but is strongly ameliorated by loss of downstream components, ras1R68Q overexpression of the activated ras1F12 allele [83,85,100]. The viable hypermorphic ras1 allele described here, ras1R68Q, offers the potential for a refined understanding of the normal physiological roles of this important protein. Significantly, the ras1R68Q allele described here shares overall biochemical properties with recently discovered mutations in k-ras that underlie human developmental disorders, such as Noonan, Costello and CFC syndromes.

Supporting Information

Figure S1 Genetic schemes for dominant modifier and reversion screens. (A) GMR-epy screen, yac; GMR-epYIII homoygous males were fed a solution of sucrose and 0.25 mg/ml ENU or 25 mM EMS and mated to females of the same strain. F1 progeny were screened for suppression or enhancement of the parental rough eye phenotype. Of the 170,000 F1 progeny screened, ~95% derived from ENU treated males, while 5% were from EMS treated males (B) GMR-hid screen, yac males were treated as above or with 4500 rad x-rays and then crossed to GMR-hid10 homoygous females. F1 progeny were screened for suppression of the GMR-hid10 rough eye phenotype. Of the 300,000 F1 progeny screened, ~49% derived from EMS treated males, ~49% from x-ray treated males and 2% from ENU treated males. (C) Reversion screen. Homozygous Su(21-3s) males were treated with 4000 rad x-rays and crossed to GMR-hid10; Sh/TM6B females. 80,000 F1 progeny were screened for loss of the Su(21-3s) suppression phenotype. (TIF)

Figure S2 The Su(21-3s) mutant differentially interacts with components of the EGFR/MAPK pathway. Suppression of the GMR-hid10 induced eye ablation phenotype by Su(21-3s) (A vs D) is not much affected by loss of function mutations in upstream components of MAPK signaling such as egf (E) or argos (F), but is strongly ameliorated by loss of downstream components, such as rolded (B). Additionally, when a dominant negative form of Ras1 (sev-ras1N17/) is expressed in the eye, the suppressive effects of Su(21-3s) are completely abrogated (C). Genotypes: (A) GMR-hid10/+; (B) GMR-hd10/+; Su(21-3s)/+; (C) GMR-hd10/+; sev-ras1N17/++; (D) GMR-hd10/+; Su(21-3s)/+; (E) GMR-hid10/egf; Su(21-3s)/+; (F) GMR-hd10/+; Su(21-3s)/+egf. (TIF)

Figure S3 Overexpression of ras1 in the wing induces ectopic vein material. Overexpression of either wildtype ras1 (C) or mutant ras1R68Q (D) using the en-Gal4 driver results in the deposition of significant amounts of ectopic wing vein material. This phenotype is much more severe with ras1R68Q however, which frequently also results in wing blisters. Panels (A) and (B) are included for comparison only and are the same images shown in Figure 5. (TIF)

Figure S4 Overexpression of ras1 in the eye induces developmental defects. Both overgrowth and cell death phenotypes are observed when Ras is overexpressed in the fly eye. Flies overexpressing wildtype ras1 (B,G) exhibit relatively minor disruptions in eye patterning and in the case of sev-Gal4 driven expression, a small but significant amount of overgrowth occurs in the anterior part of the eye (G). In contrast, overexpression of ras1R68Q with GMR-Gal4 (C-F) causes severe overgrowth and patterning disruptions. An example from each of four independent transgenic lines is shown to illustrate the range of phenotypes. Likewise, overexpression of ras1R68Q with sev-Gal4 elicits a much more pronounced overgrowth phenotype in the anterior part of the eye (H) compared to that of wildtype ras1 (G). Genotypes: (A) GMR-Gal4/+; (B) GMR-Gal4/+;UAS-ras1R68Q/+; (C-F) GMR-Gal4/+;UAS-ras1R68Q/+; (G) sev-Gal4/+;UAS-ras1/+; (H) sev-Gal4/+;UAS-ras1R68Q/+. (TIF)

Table S1 GMR-ery modifiers: Summary of genetic interactions. Complementation groups are named for the known gene to which they correspond. The group named “other” consists of mutants that could not be placed into complementation groups. -th-st- indicates that the mutation was roughly mapped by meiotic recombination around the markers th and st and may be located on either side, whereas sr-e indicates that the mutation maps between st and e. Alleles with the same map position and similar phenotypes are grouped together for simplicity. Rep, reduced eye pigmentation; Sup, suppressor; Enh, enhancer; −, no effect; ND, not done. (TIF)

Table S2 GMR-hid suppressors: Summary of genetic interactions. Legend is as described in Table S1. -th-st-, -cu- and -sr- indicate that the mutation was roughly mapped by meiotic recombination around the designated markers and may be located on either side, whereas st-cu, cu-sr and sr-e indicate that the mutation maps between the designated markers. The mutation characterized in this study, Su(21-3s), is highlighted in yellow. Rep, reduced eye pigmentation; Ro, rough eye; Wv, extra wing veins; Wk, weak; Sup, suppressor; Enh, enhancer; -, no effect; ND, not done. (TIF)

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Author Contributions

Conceived and designed the experiments: CG HS. Performed the experiments: CG. Analyzed the data: CG HS. Contributed reagents/materials/analysis tools: CG HS. Wrote the paper: CG HS.

References

1. Cantley LC, Auger KR, Carpenter C, Dukworth B, Graziani A, et al. (1991) Oncogenes and signal transduction. Cell 64: 281–302.
2. Blume-Jensen P, Hunter T (2001) Oncogenic kinase signalling. Nature 411: 355–365.
38. Aoki Y, Niihori T, Kawame H, Kurosawa K, Ohashi H, et al. (2005) Germline
37. Bergmann A, Agapite J, Steller H (1998) Mechanisms and control of
34. Kornbluth S, White K (2005) Apoptosis in Drosophila: neither fish nor fowl
33. Bilak A, Su TT (2009) Regulation of Drosophila melanogaster pro-apoptotic
32. Bader M, Steller H (2009) Regulation of cell death by the ubiquitin-proteasome
29. Zachariou A, Tenev T, Goyal L, Agapite J, Steller H, et al. (2003) IAP-
26. Wang SL, Hawkins CJ, Yoo SJ, Muller HA, Hay BA (1999) The Drosophila
25. Lisi S, Mazzon I, White K (2000) Diverse Domains of THREAD/DIAP1 Are
24. Grether ME, Abrams JM, Agapite J, White K, Steller H (1995) The head
22. Kurada P, White K (1998) Ras promotes cell survival in Drosophila by
20. Bergmann A, Agapite J, McCall K, Steller H (1998) The Drosophila gene hid is
19. Yao R, Cooper GM (1995) Requirement for phosphatidylinositol-3 kinase in
18. Parrizas M, Saltiel AR, LeRoith D (1997) Insulin-like growth factor 1 inhibits
17. Gardner AM, Johnson GL (1996) Fibroblast growth factor-2 suppression of
16. Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME (1995) Opposing
15. Steller H, Grether ME (1994) Programmed cell death in Drosophila. Neuron
13. Downward J (1998) Ras signalling and apoptosis. Curr Opin Genet Dev 8:
12. Wada T, Penninger JM (2004) Mitogen-activated protein kinases in apoptosis
11. Rebay I (2002) Keeping the receptor tyrosine kinase signaling pathway in
8. Vidal M, Cagan RL (2006) Drosophila models for cancer research. Curr Opin
7. Bo JL (1989) ras oncogenes in human cancer: a review. Cancer Res 49: 4602–4609.
6. Vidal M, Cagan RL (2006) Drosophila models for cancer research. Curr Opin
5. Colicelli J (2004) Human RAS superfamily proteins and related GTPases. Sci
4. Hunter T (1991) Cooperation between oncogenes. Cell 64: 249–270.
3. Bishop JM (1991) Molecular themes in oncogenesis. Cell 64: 235–248.
2. Hunter T (1991) Cooperation between oncogenes. Cell 64: 249–270.
1. Nordstrom M, Baracid M (2003) RAS oncogenes: the first 30 years. Nat Rev

44. Hariharan IK, Hu KQ, Asha A, Quintanilla A, Ezzell RM, et al. (1995) Characterization of Rho Gtpase Family Homologs in Drosophila-Melanogaster - Overexpressing Rho in Retinal Cells Causes a Late Developmental Defect. Embo Journal 14: 292–302.
43. Chang HC, Solomon NN, Wood W, Steller H, et al. (2005) RhoGDPase and GTPase activities control Drosophila ommatidium development. Dev Cell 12: 487–497.
42. Solomon NN, Kronenberg M, Wood W, Steller H, et al. (2005) A Germline Hypermorph in Drosophila ras1 gene. EMBO J 24: 6624–6632.
41. White K, Tahaoglu E, Steller H (1996) Cell killing by the Drosophila gene reaper. Science 271: 805–807.
40. Chen P, Nordstrom W, Bisht B, Abrams JM (1996) A germ, a novel cell death gene in Drosophila. Genes Dev 10: 1773–1792.
39. Schubbert S, Bollag G, Lubynska N, Nguyen H, Kretz CP, et al. (2007) Biochemical and functional characterization of germ line KRAS mutations. Mol Cell Biol 27: 7765–7770.
38. Schubbert S, Zenker M, Rowe SL, Boll S, Klein C, et al. (2006) Germline KRAS mutations cause Noonan syndrome. Nat Genet 38: 331–336.
37. Bergmann A, Agapite J, Steller H (1998)Mechanisms and control of programmed cell death in Drosophila. Oncogene 17: 3215–3223.
36. Aoki Y, Niihori T, Kawame H, Kurosawa K, Ohashi H, et al. (2005) Germline mutations in HRAS proto-oncogene cause Costello syndrome. Nat Genet 37: 1038–1040.
69. Charroux B, Roijer J (2009) Elimination of plasmatocytes by targeted apoptosis reveals their role in multiple aspects of the Drosophila immune response. Proc Natl Acad Sci U S A 106: 9797–9802.
70. Millburn MV, Tong L, deVos AM, Brungen A, Yamazaki Z, et al. (1990) Molecular switch for signal transduction: structural differences between active and inactive forms of protooncogenic ras proteins. Science 247: 939–945.
71. Scheffzek K, Ahmadian MR, Kabsch W, Wiesmüller L, Lautwein A, et al. (1997) The Ras-RasGAP complex: structural basis for GT-Pase activation and its loss in oncogenic Ras mutants. Science 277: 335–338.
72. Brose MS, Volpe P, Feldman M, Kumar M, Rishi I, et al. (2002) BRAF and RAS mutations in human lung cancer and melanoma. Cancer Res 62: 6997–7000.
73. Lee SH, Lee JW, Soong YH, Kim HS, Park WS, et al. (2003) BRAF and KRAS mutations in stomach cancer. Oncogene 22: 6942–6945.
74. Willeumier BM, Papageorge AG, Kung HF, Bekesi E, Robins T, et al. (1986) Mutational analysis of a ras catalytic domain. Mol Cell Biol 6: 2646–2654.
75. Tzahay M, McCormick F (1987) A cytoplasmic protein stimulates normal N-ras p21 GT-Pase, but does not affect oncogenic mutants. Science 230: 542–545.
76. Stemberlink C, Jacobs JR (1997) Argos and Spitz group genes function to regulate midline glial cell number in Drosophila embryos. Development 124: 3767–3796.
77. Sonnenfeld MJ, Jacobs JR (1995) Apoptosis of the midline glia during Drosophila embryogenesis: a correlation with axon contact. Development 121: 569–578.
78. Cagan RL, Ready DF (1989) The emergence of order in the Drosophila pupal retina. Dev Biol 136: 346–362.
79. Morante J, Desplan C, Celik A (2007) Generating patterned arrays of photoreceptor cells in the Drosophila retina. Dev Biol 136: 346–362.
80. Gaul U, Mardon G, Rubin GM (1992) A putative Ras GTPase activating protein acts as a negative regulator of signaling by the Sevenless receptor tyrosine kinase. Cell 68: 1007–1019.
81. Simon MA, Bowtell DD, Dodson GS, Laverty TR, Dodson GS, et al. (1991) Ras1 and a putative guanine nucleotide exchange factor perform crucial steps in signaling by the sevenless protein tyrosine kinase. Cell 67: 701–716.
82. Yang L, Baker NE (2003) Role of the EGFR/Ras/Raf pathway in specification of photoreceptor cells in the Drosophila retina. Development 128: 1183–1191.
83. Forer SA, Simon MA, Rubin GM (1992) Signalling by the sevenless protein tyrosine kinase is mimicked by Ras1 activation. Nature 355: 559–561.
84. Brummer D, Oellers N, Szabad J, Biggs WH 3rd, Zipursky SL, et al. (1994) A gain-of-function mutation in Drosophila MAP kinase activates multiple receptor tyrosine kinase signaling pathways. Cell 76: 875–888.
85. Karim FD, Rubin GM (1996) Ectopic expression of activated Ras1 induces hyperplastic growth and increased cell death in Drosophila imaginal tissues. Development 125: 1–9.
86. Ellis MC, O’Neill EM, Rubin GM (1993) Expression of Drosophila glass protein and evidence for negative regulation of its activity in non-neuronal cells by another DNA-binding protein. Development 118: 855–863.
87. Mittal S, Subramanyam D, Dey D, Kumar RV, Rangarajan A (2009) Cooperation of Notch and Ras/MAPK signaling pathways in human breast carcinogenesis. Mol Cancer 8: 128.
88. Halfar K, Rönnel C, Stocker H, Hafen E (2001) Ras controls growth, survival and differentiation in the Drosophila eye by different thresholds of MAP kinase activity. Development 128: 1687–1696.
89. Yang L, Baker NE (2003) Cell cycle withdrawal, progression, and cell survival regulation by EGFR and its effectors in the differentiating Drosophila eye. Dev Cell 4: 359–369.
90. Prober DA, Edgar BA (2002) Interactions between Ras1, dMyc, and dPI3K signaling in the developing Drosophila wing. Genes Dev 16: 2286–2299.
91. Rodríguez-Viciana P, Warne PH, Dhand R, Vanhaesebroek B, Gout I, et al. (1994) Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature 370: 527–532.
92. Ahmadian MR, Stege P, Scheffzek K, Wittinghofer A (1997) Confirmation of the arginine-finger hypothesis for the GAP-stimulated GTP-hydrolysis reaction of Ras. Nat Struct Biol 4: 606–609.
93. Scheffzek K, Lautwein A, Kabsch W, Ahmadian MR, Wittinghofer A (1996) Crystal structure of the GTPase-activating domain of human p120GAP and implications for the interaction with Ras. Nature 381: 591–596.
94. Chen J, Anderson JB, DeVerre-Scott C, Fedorova ND, Geer LY, et al. (2003) MMDB: Entrez’s 3D-structure database. Nucleic Acids Res 31: 474–477.
95. Bourne HR, Sanders DA, McCormick F (1991) The GTPase superfamily: conserved structure and molecular mechanism. Nature 349: 117–127.
96. Smith ER, Winter B, Eisenberg JC, Shultafard A (2000) Regulation of the transcriptional activity of poised RNA polymerase II by the elongation factor ELL. Proc Natl Acad Sci U S A 103: 8575–8579.
97. Piddini E, Vincent JP (2009) Interpretation of the wingless gradient requires signaling-induced self-inhibition. Cell 136: 296–307.
98. Giráldez AJ, Copley RR, Cohen SM (2002) HSPG modification by the secreted enzyme Notum shapes the Wingless morphogen gradient. Dev Cell 2: 667–676.
99. Rebay I, Chen F, Hsiao F, Kolejadzic PA, Kung BH, et al. (2000) A genetic screen for novel components of the Ras/Mitogen-activated protein kinase signaling pathway that interact with the yan gene of Drosophila identifies split ends, a new RNA recognition motif-containing protein. Genetics 154: 527–532.
100. Giraldez AJ, Copley RR, Cohen SM (2002) HSPG modification by the secreted enzyme Notum shapes the Wingless morphogen gradient. Dev Cell 2: 667–676.
101. Belden WJ, Larrodo LF, Froehlich AC, Shi M, Chen CH, et al. (2007) The Germline Hypermorph in Drosophila ras1.