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Rabex-5 ubiquitin ligase activity restricts Ras signaling to establish pathway homeostasis *in vivo* in *Drosophila*

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SUMMARY

The Ras signaling pathway allows cells to translate external cues into diverse biological responses. Depending on context and the threshold reached, Ras signaling can promote growth, proliferation, differentiation, or cell survival. Failure to maintain precise control of Ras can have adverse physiological consequences. Indeed, excess Ras signaling disrupts developmental patterning and causes developmental disorders [1–2], while in mature tissues, it can lead to cancer [3–5]. We identify *Rabex-5* as a new component of Ras signaling crucial for achieving proper pathway outputs in multiple contexts *in vivo*. We show that *Drosophila Rabex-5* restricts Ras signaling to establish organism size, wing vein pattern, and eye-versus-antennal fate. *Rabex-5* has both Rab5 GEF activity that regulates endocytic trafficking [6] and also ubiquitin ligase activity [7–8]. Surprisingly, over-expression studies demonstrate that *Rabex-5* ubiquitin ligase activity, not its Rab5 GEF activity, is required to restrict wing vein specification and to suppress the eye phenotypes of oncogenic Ras expression. Furthermore, genetic interaction experiments indicate that *Rabex-5* acts at the step of Ras, and tissue culture studies show that *Rabex-5* promotes Ras ubiquitination. Together, these findings reveal a new mechanism for attenuating Ras signaling *in vivo* and suggest an important role for *Rabex-5*-mediated Ras ubiquitination in pathway homeostasis.

Key words/phrases

Rabex-5; RabGEF1; Ras; ERK; A20; ubiquitin ligase; tumor suppressors; oncogenes; overgrowth

RESULTS AND DISCUSSION

Rabex-5 loss causes increased size

We characterized the gene *Rabex-5* (also called *RabGEF1*, annotated *CG9139*, Fig. S1) in *Drosophila*. *Rabex-5* was first identified as a Rab5 guanine nucleotide exchange factor (GEF) with a highly conserved VPS9 domain [6]. Studies of Rab5 and other VPS proteins indicate that they play a role in growth control [9–10]. We used gal4/UAS expression [11] of an inverted repeat allele of *Rabex-5*, *Rabex-5*^{IR} [12], to perform tissue-specific RNAi, and we generated deletion alleles *Rabex-5*^{ex9}, *Rabex-5*^{ex40} and *Rabex-5*^{ex42} (Fig. S1, Supplemental Experimental Procedures).

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Deletion allele homozygotes, heterozygotes with one copy of *Rabex-5*^{ex9}, *Rabex-5*^{ex40} or *Rabex-5*^{ex42} over the large deletion *Df(3L)BSC250*, and flies undergoing strong, constitutive *Rabex-5* RNAi died as giant larvae or giant pupae often containing melanotic tumors (Fig. 1A–B; not shown). Using RNAi conditions to reduce but not eliminate *Rabex-5* resulted in viable adults whose length, weight, and wing size increased by over 10% (Fig. 1C–E).

Loss of Rabex-5 causes ectopic wing veins and eye/antennal effects

Constitutive or wing-specific *Rabex-5* RNAi resulted in extra posterior crossveins (PCV) and extra longitudinal veins (LV) (Fig. 1G, 1I–K, Fig. S1) compared to controls (Fig. 1F, 1H). Removing one copy of *Rabex-5* enhanced *Rabex-5* RNAi in the wing (Fig. 1J), and homozygous *Rabex-5*^{ex42} mutant wing tissue also formed extra veins (Fig. S1). Thus, it is unlikely this phenotype resulted from RNAi off-target effects.

Strong *Rabex-5* RNAi in the early eye led to 10–25% of eyes with severe phenotypes including overgrown eyes, outgrowths, small or lost eyes, and extra antennae (Fig. 1M–P; Fig. S1) that were not observed in controls (Fig. 1L). Similar phenotypes were seen in eyes containing primarily *Rabex-5*^{ex42} mutant tissue (Fig. S1).

Rabex-5 mutant larvae exhibit increased ERK activity

These phenotypes are consistent with failure to regulate Ras. Increased Ras signaling causes melanotic tumors [13] as seen upon loss of *Rabex-5*. Ras activity is required for wing vein specification and in excess causes ectopic veins [14–15] as seen upon *Rabex-5* reduction. Ras is also important in the eye. Increased Ras signaling early in eye development induces hyperplastic growth [16] while exceeding certain thresholds may promote antennal fates instead of eye fates [17]. Eye phenotypes from *Rabex-5* loss are consistent with Ras activity near the threshold between driving hyperplasia and promoting an eye-to-antenna switch. To evaluate Ras signaling, we assessed dually phosphorylated ERK (dpERK), a downstream Ras effector. Western analysis showed 2.5 to 5-fold increased ERK activity in mutant larvae compared to controls when normalized for total ERK (Fig. 1Q,Fig. S1). Previous *in vitro* studies reported that *Rabex-5* loss in mast cells in culture extended the duration of ERK activation when triggered by aggregation of FcεR1 receptors or by stem cell factor [18–19], consistent with our *in vivo* findings.

Size, eye, and wing phenotypes are Ras-dependent

Ras mutations dominantly suppressed the extra wing veins caused by constitutive and wing-specific *Rabex-5* RNAi (Fig. 1K, Fig. S1), reduced the percentage of flies with severe eye phenotypes upon *Rabex-5* RNAi (Fig. 1P), and suppressed the increased body size, body length, and wing area of constitutive *Rabex-5* RNAi (Fig. 1D, Fig. S1). Body weight of all flies and wing area in female flies were no longer statistically different from controls. Mutations in *Egfr*, *sos*, Raf, MEK, and ERK dominantly suppressed the extra PCV RNAi phenotype (Fig. S1). Mutation in ERK also dominantly suppressed homozygous *Rabex-5*^{ex42} phenotypes (Fig. S1).

These data indicate that *Rabex-5* restricts Ras signaling through ERK to achieve appropriate organism size, to specify wing vein pattern, and to establish eye versus antennal fate in *Drosophila*.

Loss of *Rabex-5* enhances activated Ras and *Egfr* but not Raf

If *Rabex-5* restricts Ras signaling, mutations activating the pathway in an otherwise wild-type background may be somewhat inhibited by endogenous *Rabex-5*. Therefore, loss of *Rabex-5* might exacerbate the consequence of expressing activated pathway components. Activated *Egfr* (*Egfr*^{A887T}), *RasV12S35* (oncogenic Ras signaling primarily through ERK),

and activated *Raf* (*Raf^{gof}*) [20] (details in Supplemental Experimental Procedures) expression in differentiating eye cells each caused cause rough eyes of reduced size (*Egfr^{A887T}*, Fig. 2A, Fig. S2; *RasV12S35*, Fig. 2B, Fig. S2; *Raf^{gof}*, not shown). As these phenotypes worsened, we observed black tissue and lethality (Fig. S2). Loss of *Rabex-5* significantly enhanced the number of eyes with black tissue and significantly increased lethality of *Egfr^{A887T}* or *RasV12S35* expression (Fig. 2A–C, Fig. S2). *Rabex-5* RNAi in differentiating eye cells resulted in no obvious abnormalities (Fig. S2), so phenotypic enhancement was unlikely due to additive effects of combining two visible phenotypes. Unlike its enhancement of *Egfr^{A887T}* and *RasV12S35* phenotypes, reducing *Rabex-5* in differentiating eye cells did not enhance the phenotype of *Raf^{gof}* expression (Fig. 2C; not shown).

Expressing *RasV12S35* in the early eye results in eye overgrowth and other abnormalities; loss of *Rabex-5* dramatically enhanced these phenotypes (Fig. 2G–H, Fig. S2), notably the overgrowth, extra antennae, and ectopic eye phenotypes in larval eye discs (Fig. 2G–H, Fig. S2) compared to control discs (Fig. 2D–F).

The ubiquitin ligase domain of *Rabex-5* restricts wing vein formation

Rabex-5 displays GEF activity for Rab5 [8]. Rab5 regulates endocytosis and can influence receptor trafficking, so *Rabex-5* could inhibit Ras via Rab5. In addition, *Rabex-5* has a ubiquitin ligase domain [9–10]. We engineered *Drosophila* transgenic lines to express myc-tagged wild-type and mutant forms of *Rabex-5*. *Rabex-5^{DPYT}* was designed to inactivate Rab5 GEF activity and *Rabex-5^{FY}* to inactivate ubiquitin ligase activity within full-length protein (Supplemental Experimental Procedures). Both *Rabex-5^{WT}* and *Rabex-5^{DPYT}* expressed in the posterior wing resulted in loss of crossveins and loss of the distal part of longitudinal vein L5 (Fig. 3B–C, 3E, Fig. S3) compared to a control wing (Fig. 3A). In contrast, *Rabex-5^{FY}* did not alter wing vein pattern (Fig. 3D–E). These results indicate that the ubiquitin ligase domain restricts wing vein formation. Clones of *Rabex-5^{DPYT}* in wing discs showed loss of dpERK staining (Fig. 3F–H) establishing that wing vein loss likely resulted from decreased Ras signaling.

Rabex-5 ubiquitin ligase domain blocks activated Ras in the eye

If *Rabex-5* inhibits Ras, it could antagonize oncogenic Ras. To assay suppression, we expressed *RasV12S35* in differentiating eye cells using conditions resulting in black tissue in 30% of eyes. *Rabex-5^{WT}* and *Rabex-5^{DPYT}* each reduced roughness, eye size, and black tissue phenotypes of *RasV12S35* expressing eyes (Fig. 4A–B, 4D, Fig. S4). Change in roughness was subtle; eyes were scored double-blind by other lab members to confirm suppression in an unbiased manner. By quantitation, *Rabex-5^{WT}* and *Rabex-5^{DPYT}* statistically significantly suppressed the reduced eye size and black tissue phenotypes compared to *RasV12S35* eyes (Fig. 4D, Fig. S4). Rescued eyes were statistically no different in size from controls. *Rabex-5^{FY}* did not suppress *RasV12S35* phenotypes (Fig. 4C–D, Fig. S4). Consistent with Fig. 2C, *Rabex-5^{WT}* did not suppress appearance of black tissue in *Raf^{gof}* eyes (Fig. S4). Enhancement of *RasV12S35* by *Rabex-5* loss (Fig. 2) and its suppression by the *Rabex-5* ubiquitin ligase domain (Fig. 4) together with failure of *Rabex-5* to suppress the *Raf^{gof}* phenotype and its loss to enhance *Raf^{gof}* (Fig. 2, Fig. 4, not shown) indicates that *Rabex-5* restricts Ras signaling through its ubiquitin ligase domain most likely at the step of Ras.

Rabex-5 promotes Ras ubiquitination

The importance of a functional ubiquitin ligase domain for Ras inhibition suggests that ubiquitination of one or more substrates mediates effects on Ras signaling. Previously, we and others showed Ras ubiquitination reduces Ras signaling to ERK [21–22]. In S2 cells,

Rabex-5 promoted Ras ubiquitination via its ligase domain (Fig. 4E,Fig. S4) but not Rab5 ubiquitination, showing specificity for Ras (Fig. S4). F25 Y26 mutation impairs ubiquitin binding [7], so *Rabex-5* could bind ubiquitinated Ras to block downstream signaling, or *Rabex-5* may affect Ras by targeting other substrates. However, the simplest model is that *Rabex-5* constrains Ras signaling by direct Ras ubiquitination. In fact, mammalian *Rabex-5* promotes Ras ubiquitination in a purified system [23].

CONCLUSIONS

Our findings represent the first evidence that *Rabex-5* attenuates Ras signaling *in vivo*. We show that inhibition occurs at the step of Ras possibly by direct Ras ubiquitination, and such inhibition plays a critical role in control of size and tissue fate in *Drosophila*. Mammalian *Rabex-5* promotes ubiquitination of HRas and NRas [23] suggesting that *Rabex-5* restriction of Ras is highly conserved. Interestingly, we did not see supernumary R7 cells upon *Rabex-5* loss (not shown), indicating that *Rabex-5* antagonizes Ras in some but not all developmental contexts. Germline mutations increasing Ras signaling occur in "Rasopathies" [1–2] which are associated with patterning defects making *Rabex-5* an attractive candidate to evaluate in developmental disorders. Indeed, *Rabex-5* wing vein phenotypes resemble those seen in a *Drosophila* model of Noonan Syndrome [24]. Somatic mutations causing higher Ras activity are common in cancer [3–5]. *Rabex-5* overgrowth phenotypes resemble those of mutation in many *Drosophila* tumor suppressors [25–26]. Thus, our study suggests that *Rabex-5* ubiquitin ligase activity could serve as a novel means of tumor suppression.

EXPERIMENTAL PROCEDURES

Student unpaired t-tests compared length, weight, wing area (Fig. 1D) and eye size (Fig. 4D). Chi-square or Fisher's exact tests compared PCV, LV, or eye phenotypes (Fig. 1K, 1P, 2C, 2H, 3E, 4D). S2 cells cultured at 25°C were transfected with *pIE$^{1–4}$-Flag-6HisRas*, *Actin-Gal4*, *UAS-HA-Ubiquitin* and *UAS-myc-Rabex-5* using Cellfectin (Invitrogen). Ubiquitin conjugates were isolated from cell lysates as described [21]. Flies were raised on standard media at 25°C unless otherwise stated. Larvae were dissected, stained, and imaged as before [21]. Extracts were prepared from individual larvae and Westerns were visualized with the Li-Cor Odyssey as before [21]. For genotypes and additional details, see Supplemental Experimental Procedures.

Highlights

- Rabex-5 loss causes Ras-dependent increased size, extra wing veins, and eye effects
- The Rabex-5 ubiquitin ligase domain restricts Ras signaling *in vivo*
- Rabex-5 promotes Ras ubiquitination

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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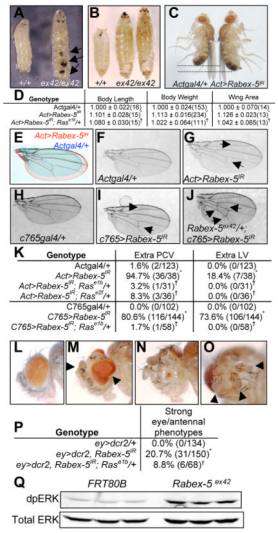


Figure 1. Loss of *Rabex-5* causes increased size, ectopic wing veins, and eye/antennal phenotypes

A-B) *FRT80B* control, left. Homozygous *Rabex-5* deletion (*ex42/ex42*) resulted in giant larvae (A, right) and pupae (B, right) often containing melanotic masses (arrows, A). C) Constitutive *Rabex-5* RNAi (Act>*Rabex-5*^{IR}, right) increased adult size compared to *Actgal4/+* controls (left, males shown). D) Table of *Act>Rabex-5*^{IR} phenotypes. Length, weight, wing area normalized to 1.000 for controls and shown as a ratio to controls ± standard deviation; number of samples in parentheses. E) Overlay of *Act>Rabex-5*^{IR} (red) and control *Actgal4/+* (blue) wings. F) Control *Actgal4/+* wing. G) Act>*Rabex-5*^{IR} wings contain extra PCV (lower arrow) and extra LV (upper arrow). H) Control *c765gal4/+* wing. I) *Rabex-5* RNAi in the wing at 30°C using *c765gal4* caused extra veins (arrows) and was enhanced (J) by deletion allele *Rabex-5*^{ex42}. K) Table of wing phenotypes. Wings with ectopic veins over total wings in parentheses. L) Control *ey>dcr2* eye. M–O) Strong *Rabex-5* RNAi in the early eye (*ey>dcr2, Rabex-5*^{IR}) produced (M) overgrown eyes, (N) small eyes, (O) extra antennae (overhead view of N) and other abnormalities (Fig. S2). P) Table of eye results. *statistically significant difference compared to control ($p<0.05$); †statistically significant suppression by *Ras* alleles ($p<0.05$). Images and quantitation in D–O from females; quantitation in P of males. Q) Western blot of larvae homozygous for control *FRT80B* or *Rabex-5*^{ex42}. dpERK (upper panel) and total ERK (lower panel) staining showed 3.9 ± 0.23 (standard deviation) fold increased ERK activation in mutant larvae compared to controls. Additional controls in Fig. S1.

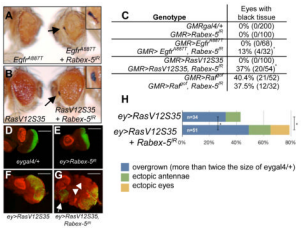
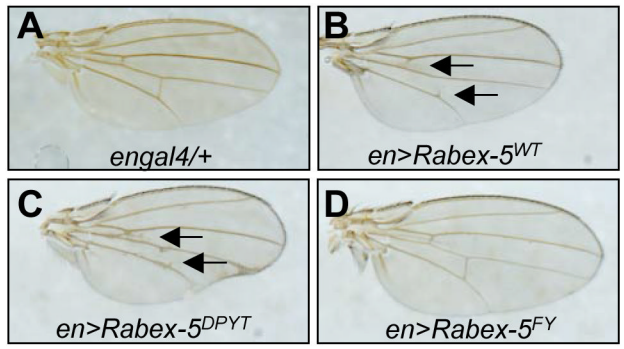


Figure 2. Loss of *Rabex-5* enhances activated *Egfr* and oncogenic Ras

A–C) Activated *Egfr*, $Egfr^{A887T}$ (left, A) or *RasV12S35* (left, B) expressed in differentiating eye cells caused small, rough eyes. A–B) *Rabex-5* RNAi (right) enhanced *GMR>*$Egfr^{A887T}$ (A) and *GMR>RasV12S35* (B), evident by appearance of black tissue (arrows; enlarged in insets) and lethality (Fig. S2). C) Table quantifying effects of *Rabex-5* loss on $Egfr^{A887T}$, *RasV12S35*, and Raf^{gof}. Female eyes are shown and quantified. In Raf^{gof} eyes, *Rabex-5* reduction did not statistically significantly change the frequency of black tissue compared to control ($p>0.05$). (D–G) ELAV (green) stains presumptive photoreceptors; *Distal-less* (red) stains presumptive antennae in larval discs. D) *eygal4/+* control. E) *ey>*$Rabex\text{-}5^{IR}$ control. F) Larval disc expressing *RasV12S35* in the early eye. G) *RasV12S35* discs with reduced *Rabex-5* showed increased overgrowth, extra antennae (arrowheads), and ectopic eyes (arrow). Scale bar, 200μm. H) Graph quantifying phenotypes in D–E. *statistically significant difference compared to control ($p<0.05$). Additional controls and quantitation in Fig. S2.



E

Genotype	Partial to complete PCV Loss	Complete PCV loss	Distal L5 loss
engal4/+ (males)	0.0% (0/54)	0.0% (0/54)	0.0% (0/54)
engal4/+ (females)	0.0% (0/51)	0.0% (0/51)	0.0% (0/51)
en>Rabex-5^{WT} (males)	65.0%(13/20)*	0.0%(0/20)	15%(3/20)*
en>Rabex-5^{WT} (females)	57.9%(44/76)*	6.6%(5/76)	10.5%(8/76)*
en>Rabex-5^{DPYT} (males)	100%(11/11)*	9.1%(1/11)	45.5%(5/11)*
en>Rabex-5^{DPYT} (females)	83.3%(30/36)*	16.7%(6/36)*	11.1%(4/36)*
en>Rabex-5^{FY} (males)	0.0%(0/18)	0.0%(0/18)	0.0%(0/18)
en>Rabex-5^{FY} (females)	0.0%(0/20)	0.0%(0/20)	0.0%(0/20)

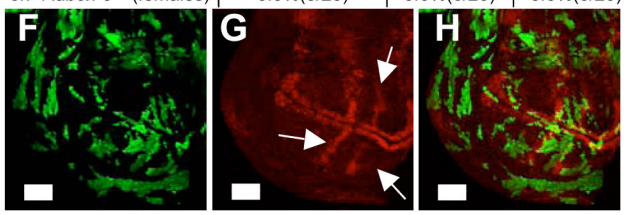
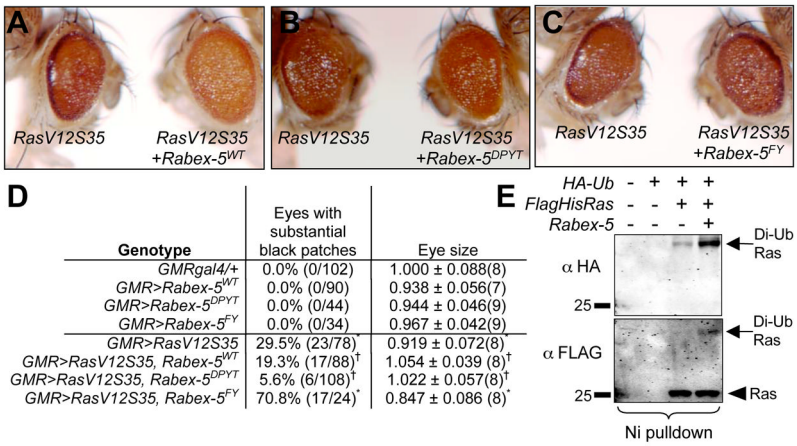


Figure 3. *Rabex-5* regulates wing vein specification via its ubiquitin ligase domain

A) Control *engal4/+* wing. B) Expressing *Rabex-5^{WT}* or (C) *Rabex-5^{DPYT}* using *engal4* resulted in crossvein loss (arrows). D) Expressing *Rabex-5^{FY}* did not alter wing vein pattern. Female wings are shown. (E) Table of results from A–D. *statistically significant difference for transgenes compared to control ($p<0.05$). F–H) Wing clones expressing *Rabex-5^{DPYT}* (green) show loss of dpERK staining (red in G–H, arrows in G). H) Merge of F, G. Scale bar, 50μm. Additional controls in Fig. S3.



Genotype	Eyes with substantial black patches	Eye size
GMRgal4/+	0.0% (0/102)	1.000 ± 0.088(8)
GMR>Rabex-5^{WT}	0.0% (0/90)	0.938 ± 0.056(7)
GMR>Rabex-5^{DPYT}	0.0% (0/44)	0.944 ± 0.046(9)
GMR>Rabex-5^{FY}	0.0% (0/34)	0.967 ± 0.042(9)
GMR>RasV12S35	29.5% (23/78)*	0.919 ± 0.072(8)*
GMR>RasV12S35, Rabex-5^{WT}	19.3% (17/88)†	1.054 ± 0.039 (8)†
GMR>RasV12S35, Rabex-5^{DPYT}	5.6% (6/108)†	1.022 ± 0.057(8)†
GMR>RasV12S35, Rabex-5^{FY}	70.8% (17/24)*	0.847 ± 0.086 (8)*

Figure 4. *Rabex-5* ubiquitin ligase domain suppresses oncogenic Ras

A–C) Co-expressing *Rabex-5^{WT}* (A, *RasV12S35+Rabex-5^{WT}*, right) or *Rabex-5^{DPYT}* (B, *RasV12S35+Rabex-5^{DPYT}*, right) in differentiating eye cells suppressed *RasV12S35* (left in A–C). C) Co-expressing *Rabex-5^{FY}* (*RasV12S35+Rabex-5^{FY}*, right) did not suppress *RasV12S35*. D) Summary table. Eye size was normalized to 1.000 for *GMRgal4/+* controls and shown as a ratio to controls ± standard deviation. *statistically significant difference for *Rabex-5* and/or *RasV12S35* transgene expression compared to *GMRgal4/+* control ($p<0.05$).† statistically significant suppression of *GMR>RasV12S35* ($p<0.05$). L) Nickel pulldown of lysates from cells expressing tagged Ras, Ub, and *Rabex-5*. *Rabex-5* expression increased Ras ubiquitination (right lane). Anti-HA (upper panel), anti-FLAG (lower panel) staining; di-ubiquitinated Ras, arrows; unconjugated Ras, arrowhead. Additional controls in Fig. S4.