An Activating Mutation in ERK Causes Hyperplastic Tumors in a *scribble* Mutant Tissue in *Drosophila*

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ABSTRACT Receptor tyrosine kinase signaling plays prominent roles in tumorigenesis, and activating oncogenic point mutations in the core pathway components Ras, Raf, or MEK are prevalent in many types of cancer. Intriguingly, however, analogous oncogenic mutations in the downstream effector kinase ERK have not been described or validated *in vivo*. To determine if a point mutation could render ERK intrinsically active and oncogenic, we have assayed in *Drosophila* the effects of a mutation that confers constitutive activity upon a yeast ERK ortholog and has also been identified in a few human tumors. Our analyses indicate that a fly ERK ortholog harboring this mutation alone (Rolled^{R80S}), and more so in conjunction with the known *sevenmaker* mutation (Rolled^{R80S+D334N}), suppresses multiple phenotypes caused by loss of Ras-Raf-MEK pathway activity, consistent with an intrinsic activity that is independent of upstream signaling. Moreover, expression of Rolled^{R80S} and Rolled^{R80S+D334N} induces tissue overgrowth in an established *Drosophila* cancer model. Our findings thus demonstrate that activating mutations can bestow ERK with pro-proliferative, tumorigenic capabilities and suggest that *Drosophila* represents an effective experimental system for determining the oncogenicity of ERK mutants and their response to therapy.

KEYWORDS *Drosophila*; ERK; hyperplastic tumors; oncogenic mutations; rolled RTK signaling; *sevenmaker*

DYSREGULATED Receptor tyrosine kinase (RTK)-mediated signaling has been implicated in diverse human diseases, primarily cancer (Porter and Vaillancourt 1998; McCormick 1999; Blume-Jensen and Hunter 2001; Yarden 2001; Bache *et al.* 2004; Zandi *et al.* 2007; Klein and Levitzki 2009; Hanahan and Weinberg 2011). Correspondingly, constitutive RTK pathway activity is prevalent in a high percentage of cancers, mainly due to activating mutations in

constituents of the Ras-Raf-MEK axis (Bos 1989; Brose *et al.* 2002; Karnoub and Weinberg 2008; Bollag *et al.* 2012). This signal transduction cascade converges on the downstream effector Extracellular signal regulated kinases (ERK1/2), which accordingly become abnormally phosphorylated and activated in many human cancers, implying that their activity is critical for tumorigenesis (Blume-Jensen and Hunter 2001; Kohno and Pouyssegur 2006; Samatar and Poulidakos 2014; Sanchez-Vega *et al.* 2018). Intriguingly, however, oncogenic mutations in ERK1/2 themselves have not been identified as yet, and activating ERK mutations have not been shown to play a causative role in cancer.

The reason for the lack of established activating mutations in ERK1/2 in cancer is unclear. Given the unique dual phosphorylation, catalyzed by MEK on neighboring Thr and Tyr residues, which is required for their activation, it has been proposed that in the course of evolution ERKs acquired a

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conformation that is protective against such mutations (Canagarajah *et al.* 1997). Additionally, unlike other protein kinases, ERKs normally do not possess a natural autophosphorylation/autoactivation capability (Beenstock *et al.* 2016), such that overexpression or gene amplification do not cause enhanced activity. Hence, perhaps ERKs cannot undergo conversion to become oncogenic. Nevertheless, a few intrinsically active, MEK-independent ERKs have been experimentally identified (Emrick *et al.* 2001, 2006; Levin-Salomon *et al.* 2008; Goetz *et al.* 2014; Brenan *et al.* 2016; Smorodinsky-Atias *et al.* 2016). Moreover, recent large-scale genome sequencing of patient-derived tumors uncovered several mutations in ERKs (Zehir *et al.* 2017), although whether such mutations represent cancer driver mutations or mere nonpathogenic passenger mutations is not known. Here, we use *Drosophila* as an *in vivo* system with which to explore whether a given ERK mutant, isolated in patients or via basic research, is an oncoprotein.

As proof of concept, we focused on a particular mutation initially identified in the yeast ERK, Mpk1 (Levin-Salomon *et al.* 2008). This mutation, R68S, which renders Mpk1 intrinsically active, maps to a residue that is conserved in both mammalian ERK1 (R84) and ERK2 (R65), as well as in the single *Drosophila* ERK ortholog Rolled (R80) (Biggs and Zipursky 1992; Biggs *et al.* 1994). We reasoned that this mutation might be clinically relevant, as mutations at the same residue were also found in large-scale genome sequencing of human tumors (R84H in ERK1: catalogue of somatic mutations in cancer (COSMIC) ID: 4875437; and R67I in ERK2: COSMIC ID: 6946407) (Zehir *et al.* 2017). We also revisited the *sevenmaker* gain-of-function Rolled mutation (D334N; referred to herein as Rolled^{D334N}), which was discovered in a genetic screen carried out in flies and also found in cancer patients (D321N in ERK2: COSMIC ID: 98175) (Brunner *et al.* 1994; Zehir *et al.* 2017). While the catalytic activity of Rolled^{D334N} still depends on phosphorylation by its upstream MEK (*Drosophila* Downstream of Raf; DSor), it undergoes ineffective dephosphorylation and inactivation by its cognate phosphatases, and thus remains in a prolonged active state (Bott *et al.* 1994; Chu *et al.* 1996). Notably, activating mutations in ERK have not been tested to date for their oncogenic potential in a multicellular model organism.

Correspondingly, we have generated transgenic flies expressing the ERK variants Rolled^{R80S}, Rolled^{D334N}, and Rolled^{D334N+R80S}, as well as control Rolled^{WT}, and evaluated their activities using three *in vivo* readouts: (i) the effects they exert on wing venation, a process that is controlled by the Epidermal growth factor receptor (EGFR)-Ras-Raf-MEK-ERK signaling pathway (de Celis *et al.* 1997; Roch *et al.* 2002; de Celis 2003; Hasson *et al.* 2005; Hasson and Paroush 2006; Ajuria *et al.* 2011); (ii) their ability to rescue lethality and eye phenotypes brought about by RNA interference (RNAi)-mediated knockdown of MEK (*DSor*^{RNAi}); and (iii) their tumorigenic potential in a *Drosophila* cancer model (Brumby and Richardson 2003; Pagliarini and Xu 2003). Using these assays, we find that the active Rolled variants, but not

Rolled^{WT}, lead to phenotypes characteristic of ERK activation, likely due to their capability to undergo spontaneous autophosphorylation. Significantly, expression of these variants in cells mutant for the epithelial polarity gene *scribble* (*scrib*) facilitates cellular overgrowth and formation of hyperplastic tumors (Bilder 2004; Gonzalez 2013). Taken together, these data indicate that activating mutations can endow ERK with pro-proliferative, tumorigenic capabilities, and that *Drosophila* provides an effective platform with which to evaluate the activity and oncogenicity of ERK mutations.

Materials and Methods

Fly culture

Flies were cultured and crossed on yeast-cornmeal-molasses-malt extract-agar medium at 25°, or at 18° where indicated.

Fly stocks

Transgenic lines were generated by cloning complementary DNAs of either native *rolled* or its mutant derivatives into the *pUAST-attB* vector, and all constructs were integrated at the *attP40* site (Genetic Services Inc., Boston, MA) to ensure comparable expression levels (Markstein *et al.* 2008). The following mutant stocks and GAL4 drivers were used: *UAS-DSor*^{RNAi} (Dietzl *et al.* 2007), *UAS-Ras*^{DN} (Lee *et al.* 1996), *UAS-Raf*^{DN} (de Celis 1997), *UAS-LacZ* (Brand and Perrimon 1994), and *UAS-GFP* (Yeh *et al.* 1995). Expression in the wing was attained using the wing-specific *MS1096-GAL4* driver (Park and Edwards 2004), and that in the eye using *eyless* (*ey*)-*GAL4* (Hazelett *et al.* 1998).

To ectopically express Rolled variants in a *scrib* mutant tissue (Bilder and Perrimon 2000) in the developing larval eye imaginal disc, we used a Flipase/FRT-mediated mitotic recombination driven by the *ey* promoter (*eyFLP1*). Recombination leads to clonal, eye-specific GAL4 expression, which drives expression of both a given Rolled derivative and GFP. Clones were generated by crossing *eyFLP1*; *Act > y⁺>GAL4*, *UAS-GFP*; *P[FRT82B]*, *Tub-GAL80* virgin females to *w*; *UAS-Rolled*; *P[FRT82B]*, *scrib*¹/*TM6B* males (Brumby and Richardson 2003; Pagliarini and Xu 2003).

Mounting of fly wings

Flies were soaked in 1:3 glycerol:ethanol overnight. Wings were then removed and dehydrated three times in 100% ethanol. Once the ethanol evaporated, wings were placed on slides in a drop of Euparal (Electron Microscopy Sciences, Hatfield, PA) and covered with a glass coverslip.

Microscopy

Light microscope images were attained using a Nikon TE2000 microscope and Olympus DP70 digital camera, and confocal images using a Zeiss LSM710 confocal microscope. For imaging GFP in adult flies, a Nikon SMZ18 stereomicroscope and Nikon DS-Qi2 digital camera were used. Images were processed using Adobe Photoshop software, and ImageJ software was used to quantify wing area, vein length, and GFP intensity.

Immunofluorescence antibody staining

Larvae were dissected in phosphate buffer saline (PBS) and fixed in 4% paraformaldehyde in PBS + 0.1% Triton X-100 for 20 min at room temperature. For antibody staining, samples were blocked in 1% normal goat serum in PBT (1XPBS containing 0.5% Triton-X-100) for 1 hr before incubation with mouse anti-Bs (1:100; kind gift of Seth Blair) overnight at 4°. Alexa 488-conjugated donkey anti mouse (1:400; Jackson ImmunoResearch Laboratories, Westgrove, PA) served as secondary antibody. Samples were mounted in Vectashield medium (Vector Laboratories, Peterborough, UK). Finally, Alexa Fluor 555 phalloidin (1:100; Invitrogen, Waltham, MA) was used for visualization of F-actin.

Protein expression in bacteria

DNA sequences encoding native and mutant forms of Rolled were subcloned in the pET28a vector (Novagen, Darmstadt, Germany). To express the Rolled derivatives, constructs were transformed into BL21(DE3)pLysS competent cells (Invitrogen) and cell cultures were grown at 37° in the presence of kanamycin (34 µg/ml) and chloramphenicol (25 µg/ml) until they reached an A600 of 0.3–0.4. Protein expression was induced using 0.2 mM isopropyl-1-thio-D-galactopyranoside for 3 hr at 30° (OD 600 ~0.85). Then, 250 µl were removed, centrifuged, and resuspended in 50 µl Laemmli buffer, and immediately heated at 100° for 5 min. Finally, 5 µl were subsequently loaded on each lane of a 10% SDS-PAGE minigel and subjected to immunoblotting.

Wing imaginal disc lysates

Wing imaginal discs from ~10 wandering third instar larvae were dissected and collected within 15 min and immediately placed in cold lysis buffer supplemented with protease and phosphatase inhibitors. The wing discs were subsequently mixed with 3 × cracking buffer containing 0.25 M Tris pH 6.8, 25% glycerol, 6% β-mercaptoethanol, 4% SDS, and few grains of bromophenol blue, pipetted thoroughly and heated up to 100° for 10 min, then placed on ice until loaded (without freezing and thawing cycles).

Immunoblotting

Primary antibodies used for immunoblotting were rabbit anti-ERK (1:1000; Cell Signaling Technology, Danvers, MA), rabbit anti-ERK (1:1000; Cell Signaling Technology), mouse anti-ERK (1:5000; Cell Signaling Technology), and mouse anti-actin (1:1000; Abcam, Cambridge, UK). Secondary antibodies were anti-rabbit Alexa Fluor 680 (1:5000; Thermo Fisher Scientific Inc., Rockford, IL) and anti-mouse IRDye-800 (1:10,000 Rockland Immunochemicals, Limerick, PA).

Data availability

Plasmids and fly stocks are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the manuscript and figures. Supplemental material available at figshare: <https://doi.org/10.25386/genetics.8851346>.

Results

Expression of Rolled variants harboring the R80S mutation phenocopies excessive Ras-Raf-MEK-ERK pathway activity

To determine whether the R80S mutation renders Rolled constitutively active *in vivo*, we first tested the effects of this mutation on wing vein formation, an EGFR-regulated process that defines the stereotypical array of veins on the wing surface (Figure 1, A and B) (Diaz-Benjumea and Garcia-Bellido 1990; Clifford and Schüpbach 1992; Queenan *et al.* 1997; Martin-Blanco *et al.* 1999; Abouchar *et al.* 2014). Toward this end, we generated transgenic flies expressing *Rolled*^{R80S}, as well as flies expressing the native form of the kinase (*Rolled*^{WT}) as control. To rule out positional effects and to ensure comparable expression levels, the transgenes were precisely targeted to a common genomic integration site (see *Materials and Methods*). The GAL4/UAS system was then used to express the two variants in the larval wing disc, the tissue which eventually gives rise to the adult wing (Brand and Perrimon 1993). Indeed, when expressed under the regulation of the wing-specific *MS1096-GAL4* driver, *Rolled*^{R80S} brings about modest yet consistent formation of extra vein material in distal wing positions, a feature of excessive Ras-Raf-MEK-ERK signaling activity (Figure 1D, arrowheads; cf. *GFP*-expressing control in Figure 1A; quantified in Figure 1E) (Diaz-Benjumea and Hafen 1994). In comparison, expression of *Rolled*^{WT} only weakly promoted ectopic venation in intervein regions (Figure 1C; cf. control in Figure 1A; quantified in Figure 1E).

Next, we hypothesized that a stronger effect would be exerted by a doubly mutated Rolled derivative, harboring a mutation that stimulates its catalytic activity (R80S) in combination with one that extends the duration of its activity (D334N). Hence two additional transgenes, expressing *Rolled*^{D334N} and *Rolled*^{R80S+D334N}, were similarly generated. As previously reported, expression of *Rolled*^{D334N}, driven by *MS1096-GAL4*, causes formation of extra vein material and enhances vein branching (Figure 1F, asterisks) (Brunner *et al.* 1994). Notably, however, expression of *Rolled*^{R80S+D334N} leads to more pronounced effects, including excessive vein formation in intervein territories and vein thickening (Figure 1G, asterisks). These results indicate that the R80S mutation enhances Rolled activity and augments the *sevenmaker* phenotype.

The activity of Rolled^{R80S} and Rolled^{R80S+D334N} is independent of upstream Ras-Raf-MEK signaling

We reasoned that if the above Rolled variants are intrinsically active, then their expression should suppress phenotypes caused by genetic inactivation of the Ras-ERK pathway (Clifford and Schupbach 1989; Diaz-Benjumea and Garcia-Bellido 1990; Diaz-Benjumea and Hafen 1994). In support of this idea, expression of the Rolled variants under *MS1096-GAL4* regulation rescues, to varying degrees, severe rudimentary wing phenotypes induced by RNAi-mediated knockdown of MEK/DSor (*DSor*^{RNAi}; Figure 2, A and B). Thus, although

Figure 1 Expression of Rolled variants harboring activating mutations leads to the formation of extra vein material. (A, C, D, F, and G) Wings of transgenic male flies, expressing GFP or distinct Rolled derivatives under *MS1096-GAL4* regulation. (A) Control, GFP-expressing wing. Note the five typical longitudinal wing veins, labeled L1–5, whose formation is induced by the (B) EGFR pathway (Clifford and Schüpbach 1989; Diaz-Benjumea and Garcia-Bellido 1990; Clifford and Schüpbach 1992). (C and D) Expression of *Rolled^{WT}* hardly has an effect on venation (C), whereas that of *Rolled^{R80S}* (D) causes formation of ectopic veins mainly in distal regions of the wing (multicolor arrowheads). (E) Bar graph showing the number of ectopic veins that develop in wings expressing *Rolled^{WT}* (open bars) or *Rolled^{R80S}* (filled bars) under *MS1096* regulation, classified by size: large, medium, or small (orange, green, or purple, respectively; see corresponding arrowheads in panels C and D). Forty-five male wings from each definitive genotype were scored. (F and G) Expression of *Rolled^{D334N}* leads to dramatic vein hypertrophy (F; asterisks), whereas an even stronger extravein phenotype, discernable by relatively darker pigmentation, is observed upon expression of *Rolled^{R80S+D334N}* (G; asterisks).

wings coexpressing *DSor^{RNAi}* together with *Rolled^{R80S}* are still undeveloped, their size is nevertheless larger than that of those co-expressing *Rolled^{WT}* (Figure 2, C and D; quantified in Figure 2G). A more dramatic effect is seen when expression of *Rolled^{R80S+D334N}* is compared to that of *Rolled^{D334N}* in a *DSor^{RNAi}* background (Figure 2, E and F; quantified in Figure 2G). Additionally, coexpression of the Rolled derivatives partly rescues vein patterning defects brought about by dominant-negative forms of Ras (*Ras^{DN}*) and Raf (*Raf^{DN}*) (Supplemental Material, Figures S1 and S2, respectively). Thus, expression of these Rolled derivatives ameliorates, to different extents, the loss of vein material and reduced wing size derived from disrupted EGFR-mediated signal transduction.

Consistent with the above results, the constitutively active Rolled variants also rescue to various degrees the lethality caused by *DSor^{RNAi}* expression in the eye, a tissue in which sequential EGFR-mediated signal transduction is essential (Freeman 1996). Specifically, *DSor^{RNAi}* expression, under *eyeless (ey)*-*GAL4* regulation, results in 100% pupal lethality even at 18° (a temperature at which *GAL4* drives relatively low expression) (Duffy 2002). When we monitored the number of emerging adults, we found that this lethality is scarcely

rescued by the coexpression of *Rolled^{WT}* (4% viability), whereas coexpression of the other *Rolled* derivatives brings about more substantial survival (ranging from 38 to 86% viability; Figure 3A). The degree of phenotypic suppression (i.e., mild, moderate, or strong) was also evident when the eye size of surviving flies was quantified (Figure 3, B and C). In this respect, the suppression of the *DSor^{RNAi}*-induced eye phenotype by *Rolled^{R80S}* was substantially higher than by *Rolled^{WT}*, and *Rolled^{R80S+D334N}* scored better than *Rolled^{D334N}* (Figure 3C).

The ability of *Rolled^{D334N}* to suppress phenotypes caused by impaired Ras-Raf-MEK signaling to the same extent as *Rolled^{R80S}* is somewhat surprising. However, when *Rolled^{D334N}* is phosphorylated by MEK *in vitro*, it possesses a higher level of activity than *Rolled^{R80S}* (Levin-Salomon *et al.* 2008), suggesting that residual pathway activity in these backgrounds (mirrored, for example, by the incomplete loss of veins in the *Ras^{DN}*- and *Raf^{DN}*-expressing wings) partially activates *Rolled^{D334N}*, which is then sustained in its active state.

Taken together, our results indicate that *Rolled^{R80S}* and *Rolled^{R80S+D334N}* can compensate for defective Ras-Raf-MEK signaling to different extents. They thus appear to function

Figure 2 Expression of intrinsically active Rolled variants partially compensates for severe wing phenotypes caused by the RNAi-mediated knockdown of *DSor*. (A–F) Wings of transgenic females expressing *DSor^{RNAi}* alone, *DSor^{RNAi}* together with *LacZ* (to exclude the possibility that the increased number of UAS-based transgenes dilutes the GAL4 protein), or *DSor^{RNAi}* jointly with distinct Rolled derivatives, under *MS1096-Gal4* regulation. (A–C) Expression of *DSor^{RNAi}* results in underdeveloped vestigial wings (A), a phenotype that is not improved by the coexpression of either *LacZ* (B) or *Rolled^{WT}* (C). (D) Coexpression of *Rolled^{R80S}* exerts a subtle effect, reflected by a larger wing size. (E and F) Coexpression of *DSor^{RNAi}* along with *Rolled^{R80S+D334N}* (F) brings about wing development and differentiation that is more significant than that caused by *Rolled^{D334N}* alone (E). (G) Quantification of the rescue of wing phenotypes caused by *DSor^{RNAi}* expression. Scatterplot showing wing area (in millimeters squared) of flies coexpressing *DSor^{RNAi}* together with *Rolled^{WT}*, *Rolled^{R80S}*, *Rolled^{D334N}*, and *Rolled^{R80S+D334N}*, all under *MS1096-GAL4* regulation. Data represents the mean $\pm$ SD derived from 50 female wings per genotype. *** $P < 0.0001$ compared to wings expressing *Rolled^{WT}* or *Rolled^{D334N}*, respectively (Mann–Whitney U-test).

independently of upstream signaling in the context of the *Drosophila* wing and eye (and likely in other tissues as well), suggesting that they are intrinsically active *in vivo*.

Intrinsically active Rolled variants suppress *DSor^{RNAi}*-mediated effects at the level of gene expression

To evaluate the ability of intrinsically active Rolled variants to suppress *DSor^{RNAi}*-induced phenotypes at the molecular level, we followed the expression pattern of the *Drosophila* Serum response factor protein, encoded by the *blistered* (*bs*) gene. *bs* is expressed in presumptive intervein regions of the developing wing and, consequently, gaps in its expression correspond to future veins L3–5 (Figure 4A, red arrowheads) (Fristrom *et al.* 1993; Montagne *et al.* 1996). Since Ras-ERK pathway activity inhibits *bs* expression within presumptive vein territories (Roch *et al.* 1998), *bs* expression is derepressed in veins of *MS1096-DSor^{RNAi}* wing discs, most noticeably in the dorsal compartment, where *MS1096-GAL4* reaches maximal expression (Figure 4B, red arrowheads). Coexpression of *Rolled^{WT}* together with *DSor^{RNAi}* does not alleviate this phenotype (Figure 4C). In contrast, coexpression of either *Rolled^{R80S}* or *Rolled^{D334N}* partially restores the gaps in *bs* expression caused by *DSor^{RNAi}*, whereas that of

Rolled^{R80S+D334N} fully rescues the normal pattern of *bs* expression (Figure 4, D–F, red arrowheads). Thus, the intrinsic activity of the Rolled variants is also evident at the level of pathway target gene expression.

Intrinsically active Rolled variants undergo spontaneous autophosphorylation

We next assessed the degree of activation of the intrinsically active Rolled variants *in vivo*, using immunoblotting with antibodies specifically recognizing dually phosphorylated ERK (anti-pERK) (Gabay *et al.* 1997a,b). As shown in Figure 5A, the level of phosphorylated ERK (pERK) in lysates extracted from wing discs of *Rolled^{R80S}*-expressing larvae persistently surpasses that observed in larvae expressing *Rolled^{WT}*, whereas the level in *Rolled^{R80S+D334N}*-expressing larvae exceeds that of larvae expressing *Rolled^{D334N}* (Figure 5A), consistent with the results presented above. The level of pERK observed in *Rolled^{R80S+D334N}*-expressing larvae is ~6-fold lower in comparison to that observed in larvae expressing a constitutively activated form of Ras85D (*Ras^{V12}*) (Figure 5B; see Discussion).

What could be the molecular mechanism underlying the ability of the Rolled derivatives to function in the absence of

Figure 3 Intrinsically active Rolled variants rescue lethality and eye phenotypes brought about by *DSor^{RNAi}* expression. (A) Percentage of flies co-expressing *DSor^{RNAi}* together with *LacZ* or with distinct Rolled derivatives, under *ey-GAL4* regulation at 18°C, that exhibit pupal lethality (red) or adult viability (green). *n* indicates the number of flies from each definitive genotype that were scored. Note that coexpression of *DSor^{RNAi}* together with either *LacZ* or *Rolled^{WT}* results in lethality, whereas coexpression of *DSor^{RNAi}* with the intrinsically active Rolled isoforms greatly improves viability. *** $P < 0.001$ viability of flies expressing *Rolled^{R80S}* or *Rolled^{R80S+D334N}* compared to flies expressing *Rolled^{WT}* or *Rolled^{D334N}*, respectively (Fisher's exact test). (B) Eyes of surviving flies were classified according to the degree of suppression (from left to right): strong, moderate, or mild. (C) Percentage of eyes scored in each category of suppression for the different genetic backgrounds: strong (blue), moderate (yellow), and mild (red). *n* indicates the number of eyes from each definitive genotype that were scored. Note that sample number of survivors expressing *Rolled^{WT}*; *DSor^{RNAi}* is small due to massive lethality (96%). * $P < 0.05$ strong suppression in flies expressing *Rolled^{R80S}* or *Rolled^{R80S+D334N}* compared to strong suppression in flies expressing *Rolled^{WT}* or *Rolled^{D334N}*, respectively (Fisher's exact test).

their upstream activators? Normally, ERK family members are activated by their respective MEKs, which dually phosphorylate them on Thr and Tyr residues in the TEY motif within their activation loop (Payne *et al.* 1991). Possibly, the MEK-independent intrinsic activity of the Rolled variants is due to an acquired spontaneous autophosphorylation capacity. However, ERKs do not normally possess autophosphorylation or autoactivation capabilities (Beenstock *et al.* 2016). Hence, it is also possible that these mutations somehow enforce an active conformation without the need of prior activation loop phosphorylation. To distinguish between these possibilities, we expressed the different Rolled variants in *Escherichia coli*, in which MEKs do not exist (Heise and Cobb 2006), and monitored the extent of their TEY motif phosphorylation using anti-pERK staining. As Figure 5C shows, Rolled^{WT} is not

phosphorylated in this system and the autophosphorylation of Rolled^{D334N} is barely visible (Figure 5B). By contrast, the phosphorylation levels of Rolled^{R80S} and Rolled^{R80S+D334N} are significantly higher (Figure 5C), and clearly correlate with their effects *in vivo*.

We conclude that the inherent ability of Rolled^{R80S} to undergo autophosphorylation at its activation loop is the mechanism at the basis of its intrinsic activity. Notably, Emrick *et al.* showed that an active ERK variant undergoes autophosphorylation via an intramolecular mechanism *in cis* (Emrick *et al.* 2001), and our own analysis of human ERK2^{R65S} gave a similar outcome [Karina Smorodinsky-Atias and David Engelberg, unpublished results; reviewed in Beenstock *et al.* (2016)]. Thus, it is unlikely that the intrinsically active Rolled variants *trans*-phosphorylate and activate endogenous Rolled.

Figure 4 Expression of intrinsically active Rolled variants rescues the Blistered expression pattern. (A–F) Confocal images of third instar larval wing discs of transgenic flies expressing *LacZ* alone, *DSoxRNAi* together with *LacZ*, or *DSoxRNAi* together with distinct Rolled derivatives, under *MS1096-GAL4* regulation, all stained for the Bs protein (green). (A) Control, *LacZ*-expressing imaginal wing disc. Note that Bs is expressed only in intervein cells and, accordingly, the three gaps in its expression correspond to the presumptive L3–5 vein territories (demarcated by red arrowheads in this and all other panels). Expression of *DSoxRNAi* together with *LacZ* results in expanded Bs pattern (B), a phenotype that is barely improved by the coexpression of *Rolled*^{WT} (C). Coexpression of *Rolled*^{R80S} (D) or *Rolled*^{D334N} (E) leads to partial rescue, reflected by a reduction of Bs staining within the presumptive vein territories, whereas co-expression of *Rolled*^{R80S+D334N} rescues the Bs pattern (F).

Intrinsically active Rolled variants induce epithelial tumorigenesis in vivo

The autoactivation capability of *Rolled*^{R80S} and *Rolled*^{R80S+D334N} *in vitro*, coupled with their effects on the wing, eye, and viability *in vivo*, suggests that they have intrinsic catalytic activity that is independent of upstream control. To test whether these mutants can exert phenotypes associated with oncogenic transformation, we utilized an established *Drosophila* cancer model (Brumby and Richardson 2003; Pagliarini and Xu 2003). This system was previously used to demonstrate the oncogenic properties of *Ras*^{V12}, which was shown to induce large metastatic tumors when expressed in cells harboring an additional mutation in the conserved apico-basal polarity gene, *scrib* (Bilders *et al.* 2000; Brumby and Richardson 2003; Pagliarini and Xu 2003; Humbert *et al.* 2008). Accordingly, we asked whether activating mutations in ERK could stimulate tumor growth when introduced into a *scrib* mutant tissue. To this end, GFP-labeled clones of cells, expressing intrinsically active Rolled variants (or *Rolled*^{WT}, as control) and concurrently mutant for *scrib*, were induced in the eye disc (Figure 6A; see *Materials and Methods*). As shown in Figure 6, *Rolled*^{WT}; *scrib*^{−/−} GFP-positive clones formed in the eye field are notably larger than *scrib*^{−/−} clones alone (Figure 6, B and C; quantified in Figure S3). This effect was more pronounced in clones analogously expressing *Rolled*^{R80S}, *Rolled*^{D334N}, and *Rolled*^{R80S+D334N} (Figure 6, D–F; quantified in Figure S3). Remarkably, whereas marked *Rolled*^{WT}; *scrib*^{−/−} clones were hardly detectable in the adjacent antenna disc, sizable *Rolled*^{R80S}; *scrib*^{−/−} clones were readily identifiable in this location, as were *Rolled*^{D334N}; *scrib*^{−/−} and *Rolled*^{R80S+D334N};

scrib^{−/−} clones (Figure 6, C–F; quantified in Figure S3). Thus, all Rolled derivatives tested (including the native form) are capable of triggering overgrowth of *scrib*^{−/−} eye cells; however, only clones expressing intrinsically active Rolled variants survive and/or excessively proliferate in the antennal disc. Furthermore, we find that induction of *Rolled*^{R80S}; *scrib*^{−/−} and *Rolled*^{R80S+D334N}; *scrib*^{−/−} clones at the larval stage subsequently gives rise to overproliferation of GFP-positive cells in the abdomen of a significantly higher number of adult flies than when *Rolled*^{D334N}; *scrib*^{−/−} or *scrib*^{−/−} clones are induced (Figure 6G; quantified in Figure 6H). Collectively, our data demonstrate that the R80S mutation in ERK, which confers intrinsic kinase activity, is a pro-proliferative, oncogenic mutation that induces hyperplastic tumor formation in a *scrib* mutant tissue.

Discussion

We previously used yeast genetic screens to isolate intrinsically active ERK variants and subsequently characterized them biochemically and in cultured cells (Levin-Salomon *et al.* 2008; Goshen-Lago *et al.* 2016, 2017; Smorodinsky-Atias *et al.* 2016). Here we demonstrate that one such variant, *Rolled*^{R80S}, is constitutively active in *Drosophila*. Importantly, since expression of the above variant partially compensates for the loss of *Ras*, *Raf*, and *MEK*, and given its capacity to autophosphorylate, it represents a *bona fide* gain-of-function kinase whose enzymatic activity is largely independent of upstream signaling.

Combining the R80S mutation with the *sevenmaker* mutation (*i.e.*, *Rolled*^{R80S+D334N}) further amplifies its effects in all assays employed in this study. While the autophosphorylation

Figure 5 Rolled^{R80S} and Rolled^{R80S+D334N} undergo spontaneous autophosphorylation. (A) Western blot analysis of wing disc lysates prepared from larvae of the indicated genotypes, immunoblotted with anti-pERK or anti-total ERK antibodies. Relative levels of ERK activation were calculated based on the ratio between pERK and total ERK, normalized to those of Rolled^{WT}. Note that, in three independent replicates, the Rolled^{R80S} variant shows consistently higher phosphorylation than Rolled^{WT}, whereas phosphorylation of Rolled^{R80S+D334N} always surpasses that of Rolled^{D334N} (by 1.56 ± 0.32 and 1.66 ± 0.37 , respectively). We attribute the substantial variability in the signals between experiments to the low ratio between pERK and total ERK levels in the Rolled^{WT} background, to which we normalize the results. (B) Western blot analysis of whole wing disc lysates prepared from larvae expressing either Rolled^{R80S+D334N} or Ras^{V12}, immunoblotted with anti-pERK or anti-Actin antibodies. Relative levels of ERK activation were calculated based on the ratio between pERK and Actin, normalized to that of Rolled^{R80S+D334N}.

Note that level of pERK observed in Ras^{V12}-expressing larvae is ~6-fold higher in comparison to that observed in larvae expressing Rolled^{R80S+D334N} (5.96 ± 0.79 , derived from four independent biological repeats; $P < 0.03$) (Mann–Whitney U-test). (C) Rolled^{WT}, Rolled^{R80S}, Rolled^{D334N}, and Rolled^{R80S+D334N} were expressed as recombinant proteins in *E. coli*. Following induction, cell lysates were prepared and immunoblotted using anti-pERK and anti-ERK antibodies. Relative levels denote the ratio of anti-pERK to anti-ERK staining in each case, normalized to those of Rolled^{WT}.

of Rolled^{D334N} is negligible, that of Rolled^{R80S} and, especially, of Rolled^{R80S+D334N} are clearly detectable in our assays. Since ERK phosphatases are absent in bacteria, the effect of the D334N mutation on the autocatalytic activity of Rolled^{R80S+D334N} *in vitro* was unexpected. We speculate that in addition to reducing the affinity to phosphatases (Bott *et al.* 1994; Chu *et al.* 1996), the *sevenmaker* mutation also confers a conformational change that predisposes Rolled to autophosphorylation induced by the R80S mutation (Beenstock *et al.* 2016). Further studies will be required to discern the structural basis for this synergistic effect.

As shown here, intrinsically active ERK mutants are oncoproteins that can induce tissue overgrowth in a *scrib* mutant tissue, *in vivo*, raising the question why activating mutations in ERK1/2 have not been identified as cancer drivers. From a structural-functional perspective, it is conceivable that ERK1/2 are relatively insensitive to single activating mutations due to their distinctive mode of regulation and/or their unique regulatory domains, which might obstruct self-activation (Beenstock *et al.* 2014, 2016; Tesker *et al.* 2016). The relative activity of intrinsically active ERK1/2 is, indeed, lower in comparison to MEK-activated ERK1/2, both *in vitro* as well as in cultured cells (Emrick *et al.* 2001, 2006; Levin-Salomon *et al.* 2008; Goshen-Lago *et al.* 2016). Accordingly, the level of autophosphorylation may be enough to promote hyperproliferation, but insufficient to facilitate full-scale tumor growth. Consequently, activated ERK1/2 are less aggressive than the oncogenic Ras and Raf mutants (Brumby and Richardson 2003; Pagliarini and Xu 2003; Uhlir *et al.* 2005; Dow *et al.* 2008). Future studies will determine whether the tumor-like growth in the abdomen of adult Rolled^{R80S} and Rolled^{R80S+D334N} flies (Figure 6G) stems

merely from leaky expression driven by *ey-GAL4*, or whether intrinsically active ERK mutations actually do cause some degree of tissue invasion and metastasis.

Regardless, our genetic and biochemical characterization of the R80S mutant strongly suggest that an ERK harboring this mutation will remain intrinsically active *in vivo* even in the presence of pharmacological Raf and/or MEK inhibitors. The anticipated resistance to therapeutic strategies involving Raf/MEK inhibition stresses the necessity to monitor somatic mutations in ERK and their sensitivity to available ERK inhibitory agents, some of which are currently in clinical trials. In fact, different ERK mutations have been tested *in vitro* and in tissue culture for their sensitivity to an assortment of ERK inhibitors (Goetz *et al.* 2014; Wagle *et al.* 2014); however, their responsiveness to drug treatment has not been established at the organismal level. The fly assays described here should facilitate a fast evaluation of the susceptibility of any given ERK mutation to candidate inhibitors. Moreover, our findings emphasize the need to develop ERK inhibitors that target the enzymatic activity of ERK itself and would thus proficiently inhibit the autophosphorylation propensity of intrinsically active ERK mutants.

The fly cancer model with which we demonstrate the tumorigenic capacity of the R80S mutation was previously used to study the effects caused by oncogenic mutations in Ras or Raf, jointly with *scrib*-instigated polarity dysfunction. However, in those cases the aggressiveness of the ensuing tumors caused lethality at the larval stage, restricting any insights gained to this developmental stage. By contrast, induction of Rolled^{R80S}; *scrib*^{-/-} and Rolled^{R80S+D334N}; *scrib*^{-/-} clones does not impede eclosion of adults. Although the difference in aggressiveness between the Ras- and ERK-induced

Figure 6 Expression of intrinsically active Rolled variants in a *scrib*^{-/-} mutant clone induces hyperplastic tumors. (A) Schematic representation of an eye-antenna imaginal disc, the primordium that will eventually give rise to the adult eye (yellow) and antenna (blue). GFP-labeled clones (green), expressing *Rolled*^{WT} or intrinsically active Rolled variants and mutant for *scrib*, were induced predominantly in cells located posteriorly to the morphogenetic furrow in eye discs (gray line) (see *Materials and Methods*). (B–F) Confocal images of third instar eye-antenna imaginal discs, dissected from flies expressing different Rolled derivatives in clones of cells that are homozygous mutant for *scrib*, labeled with GFP (green). Discs were dissected simultaneously and counterstained for phalloidin (red), to illuminate their contours and to reveal respective morphogenetic furrow progression, which is comparable in all cases. The following genotypes were induced in clones: (B) *scrib*^{-/-}; (C) *Rolled*^{WT}; *scrib*^{-/-}; (D) *Rolled*^{R80S}; *scrib*^{-/-}; (E) *Rolled*^{D334N}; *scrib*^{-/-}; and (F) *Rolled*^{R80S+D334N}; *scrib*^{-/-}. (G) Adult fly (in this case, *ey > Rolled*^{R80S}; *scrib*^{-/-}) with mass of GFP-positive cells in its abdomen (white arrowheads). (H) Bar graph showing the percentage of adults with GFP-positive cell mass in their abdomens. *n* indicates the number of flies from each definitive genotype that were scored. *** *P* < 0.001; * *P* < 0.05, n.s. *P* > 0.05 compared to flies with *scrib*^{-/-} clones (Fisher's exact test).

tumors could reflect stronger overactivation of the pathway in the former case (Figure 5B), oncogenic Ras also stimulates other pathways such as Jun N-terminal kinase, Notch and PI3K (Brumby and Richardson 2003; Uhlirova *et al.* 2005; Willecke *et al.* 2011). The induction of *Rolled*^{R80S}; *scrib*^{-/-} and *Rolled*^{R80S+D334N}; *scrib*^{-/-} clones thus provides an advantageous platform with which to study the specific contribution of the Ras-ERK axis to tumorigenesis.

A recent study described transgenic mice expressing an ERK variant equivalent to *Rolled*^{R80S} (ERK1^{R84S}) specifically in the heart. Those mice develop modest cardiac hypertrophy

that protects the heart from further pressure overload (Mutlak *et al.* 2018). Thus, activating mutations in ERK could be clinically relevant not only for cancer, as our study indicates, but also for other human conditions.

There is clearly a great urgency in developing assays that would enable the distinction between tumor-causing driver mutations and nonpathogenic bystander variants. Here we utilized a relatively simple, inexpensive, and timesaving *Drosophila* cancer model as a platform with which to evaluate the oncogenic potential of an activating ERK mutation. Using this *in vivo* system, we specifically demonstrated the

pro-proliferative capacity of the R80S mutation in the context of the whole organism. Importantly, our findings indicate that human mutations analogous to R80S in Rolled, which have been recently identified in cancer patients (*i.e.*, R84H in ERK1 and R67I in ERK2), could endow ERK with intrinsic activity that potentially drives tumor growth in cooperation with mutations in tumor suppressor genes such as *scrib*. They, furthermore, raise the possibility that, besides R80S, additional *gain-of-function* mutations in ERK play a significant role in human cancer.

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