Inhibition of ERK-MAP kinase signaling by RSK during *Drosophila* development

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Although p90 ribosomal S6 kinase (RSK) is known as an important downstream effector of the ribosomal protein S6 kinase/extracellular signal-regulated kinase (Ras/ERK) pathway, its endogenous role, and precise molecular function remain unclear. Using gain-of-function and null mutants of RSK, its physiological role was successfully characterized in *Drosophila*. Surprisingly, RSK-null mutants were viable, but exhibited developmental abnormalities related to an enhanced ERK-dependent cellular differentiation such as ectopic photoreceptor- and vein-cell formation. Conversely, overexpression of RSK dramatically suppressed the ERK-dependent differentiation, which was further augmented by mutations in the Ras/ERK pathway. Consistent with these physiological phenotypes, RSK negatively regulated ERK-mediated developmental processes and gene expressions by blocking the nuclear localization of ERK in a kinase activity-independent manner. In addition, we further demonstrated that the RSK-dependent inhibition of ERK nuclear migration is mediated by the physical association between ERK and RSK. Collectively, our study reveals a novel regulatory mechanism of the Ras/ERK pathway by RSK, which negatively regulates ERK activity by acting as a cytoplasmic anchor in *Drosophila*.

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Introduction

The p90 ribosomal protein S6 kinase (RSK) is well conserved among the metazoan systems (reviewed in Blenis, 1993; Nebreda and Gavin, 1999). RSK was originally identified as a direct target of the ribosomal protein S6 kinase/extracellular signal-regulated kinase (Ras/ERK)-MAP kinase signaling

pathway, which regulates the most critical cellular responses including cell proliferation, differentiation, and metabolism (reviewed in Blenis, 1993; Pearson *et al*, 2001). RSK specifically binds to ERK (Scimeca *et al*, 1992) and is activated through phosphorylation by activated ERK (Sturgill *et al*, 1988; Chung *et al*, 1991). As the activity of RSK tightly correlates with that of ERK, RSK has been thoroughly studied as one of the critical downstream effectors of ERK. Indeed, various physiologically important molecules such as lamin-C, glycogen synthase kinase 3, cAMP-responsive binding-element protein (CREB), histone 3B, anaphase-promoting complex (APC), C/EBP beta, Bub1, c-Fos, filamin A, and tuberous sclerosis complex (TSC) were suggested as putative targets mediating the molecular function of RSK (Schwab *et al*, 2001; Roux *et al*, 2004; Woo *et al*, 2004; reviewed in Frodin and Gammeltoft, 1999). Although these targets seem to appropriately explain the roles of RSK as a downstream effector of the Ras/ERK signaling pathway, there has been insufficient evidence to consider them as the actual downstream targets of RSK *in vivo*. Furthermore, some recent studies suggest that RSK has an inhibitory role in the Ras/ERK pathway while neither the physiological relevance nor the molecular mechanism has yet been sufficiently addressed (Myers *et al*, 2004).

The existence of many RSK isoforms (RSK 1–4) in the mammalian genome has hampered extensive genetic researches on the physiological functions of RSK (Alcorta *et al*, 1989; Moller *et al*, 1994; Yntema *et al*, 1999). In this study, we took advantage of the *Drosophila* system, which has only a single RSK gene in the genome (Wassarman *et al*, 1994). We generated null flies for *Drosophila* RSK and succeeded in characterizing its *in vivo* function. Surprisingly, we found that RSK is devoted in negatively regulating nuclear ERK function, restraining ERK in the cytoplasm during *Drosophila* eye and wing development, by physical association with ERK.

Results

RSK null flies survive to the adult stage with some developmental abnormalities

To understand the role of RSK at the organism level, we generated a null mutant for *Drosophila* RSK, a single orthologue of the mammalian RSK gene family (Wassarman *et al*, 1994). Surprisingly, the RSK null flies (*RSK^{D1}*) that completely lack RSK transcripts and proteins (Figure 1B–D) survived to the adult stage. However, *RSK^{D1}* mutants displayed several developmental abnormalities including some developmental delay, reduced fertility, and shortened longevity, as well as previously reported learning defects (Supplementary Figure 1 and data not shown; Putz *et al*, 2004). Since RSK is ubiquitously expressed throughout all developmental stages (Figure 1E) and tissues (Figure 1F), we inferred that RSK is involved in general developmental processes that are not essential for the survival of the organism.

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Figure 1 Ectopic differentiation of retinal and wing vein cells in *RSK* null mutant. (A) Genomic organization of the *Drosophila* *RSK* locus and the molecular status of the *RSK* mutants. The EP element insertion in EP(X)7363 (triangle) and 3.1 kb genomic deletion in *RSK^{D1}* are indicated. (B) *RSK* expression in *RSK^{D1}* mutant. The amount of *RSK* transcripts was visualized through RT-PCR. *rp49* was used as a control. (C) Northern blot analyses of *RSK^{D1}* mutant. 18S rRNA was used as a control. (D) Lysates from flies with indicated genotypes were immunoprecipitated using guinea-pig anti-*RSK* antibody. The immune complexes were analyzed by immunoblot using rabbit anti-*RSK* antibody. Tubulin (tub) was used as a loading control. (E) RT-PCR analysis of *RSK* expression in wild-type flies at various developmental stages (*rp49*, loading control). (F) *In situ* hybridization of wild-type embryo with *RSK* antisense and sense probes. (G–K) SEM images (left panels) and tangential cross sections (right panels) of *Drosophila* adult eye. Left bottom panels are magnified versions ($\times 600$) of SEM images. Arrowheads point to ectopic photoreceptor-containing ommatidia. (L) Quantified data of (I) to (K). The mean number of photoreceptor cells per ommatidium was calculated as described in Materials and Methods. Error bar indicates the standard deviation (s.d.) between ommatidia. (M, N) Pupal eyes were stained with anti-Arm antibody and rhodamine-labeled phalloidin (green and red, respectively), or with anti-Dlg antibody (green). Boxed area contains a cluster of ommatidial precursor cells, which is magnified on the right panels. Genotypes are: WT (*w¹¹¹⁸*, G, I, M), *RSK^{D1}* (*RSK^{D1}/Y*, H, J, N), and *RSK^{D1}; da>RSK^{WT}* (*RSK^{D1}/Y; UAS-RSK^{WT}/+; da-Gal4/+*, K). Flies of (I–K) and (M, N) were cultured at 29°C.

***RSK* is a negative regulator of the retinal cell fate determination**

To find out more about endogenous *RSK* function, we mainly focused on the eye differentiation phenotypes of *RSK^{D1}* mutants since these phenotypes are highly correlated with the Ras/ERK pathway (Dickson *et al*, 1992; Brunner *et al*, 1994). As a result, we unexpectedly found *RSK^{D1}* adult flies

to have disarrayed eye structure and some ommatidia with increased number of photoreceptor rhabdomeres (R cells) (Figure 1H). When *RSK^{D1}* mutants were grown at a higher temperature, the number of R cells was further increased and other eye defects were also exacerbated (compare Figure 1J with I). To further examine these phenotypes in earlier developmental stages, we stained the pupal retinal cells

with anti-Armadillo (Arm, *Drosophila* β -catenin) antibodies to mark adherens junctions. A characteristic staining pattern of seven 'dots' forming a circle at the center of each ommatidium was observed in the wild-type eye (Figure 1M; Hong *et al*, 2003). However, in *RSK^{D1}* mutants, we found some pupal ommatidia aberrantly containing extra anti-Arm antibody-stained dots (Figure 1N), consistent with the extra R-cell phenotype of the adult ommatidia in the mutant (Figure 1H and J). These results strongly suggested that RSK plays a negative role in photoreceptor cell differentiation during eye development.

Additionally, the *RSK*-null mutants displayed severe irregularities in ommatidial spacing (Figure 1J), presumably reflecting defects in the differentiation of non-neuronal retinal cells such as cone and pigment cells. To analyze the non-neuronal retinal cells, we utilized anti-Discs large (Dlg) antibodies to stain the membrane structure of cone and pigment cells (Woods *et al*, 1997). In the wild-type pupal retina, the regular arrangement of four cone cells and 11 pigment cells was discernable in each ommatidium (Figure 1M). However, *RSK*-null flies displayed an increased number of cone and pigment cells with highly disrupted structures (Figure 1N) under the same developmental conditions (34 h after puparium formation). These results strongly supported that RSK regulates not only the neuronal photoreceptor cell differentiation but also the non-neuronal retinal cell development during *Drosophila* eye morphogenesis.

To provide further evidence for our hypothesis, the transallelic mutants of *RSK^{D1}* with *Df(1)R8A*, an RSK deficiency

allele, also displayed identical phenotypes to *RSK^{D1}* mutants (data not shown). Furthermore, weak ubiquitous expression of the wild-type RSK (*RSK^{WT}*) transgene by the *da-Gal4* driver (Supplementary Figure 2) fully rescued the eye phenotypes of *RSK^{D1}* mutants (Figure 1K and L), demonstrating that the defects in *Drosophila* retinal cell differentiation indeed resulted from the absence of RSK.

As null mutation of *RSK* ectopically induced differentiation of both neuronal and non-neuronal retinal cells, we hypothesized RSK to be a general negative regulator of retinal cell fate determination. To further confirm this possibility, we expressed *RSK^{WT}* by using eye-specific *Gal4* drivers. As expected, expression of *RSK^{WT}* in *Drosophila* eye by the *gmr-Gal4* (Supplementary Figure 2) induced a roughened eye phenotype with dramatically reduced number of photoreceptor cells (Figure 2B, compared to Figure 2A). Moreover, *RSK^{WT}*-overexpressing pupal eyes further revealed inhibited differentiation of both neuronal and non-neuronal retinal cells (Figure 2D and F).

Collectively, these *RSK* null and overexpression experiments consistently demonstrated that RSK is a negative regulator of retinal cell differentiation in *Drosophila*.

RSK is a negative regulator of the wing vein differentiation

To address the possibility of the involvement of RSK in differentiation processes other than retinal cell development, we carefully observed other adult structures such as the thorax, abdomen, legs, and wings. Notably, some *RSK^{D1}*

Figure 2 Inhibition of retinal cell differentiation by RSK. (A, B) SEM image (left panels) and tangential cross section (right panels) of *Drosophila* adult eye. The numbers indicate the mean number of photoreceptor cells per ommatidium $\pm$ s.d. (C–F) Pupal eyes stained with anti-Arm antibody (C, D) and with anti-Dlg antibody (E, F). Boxed area contains a cluster of ommatidial precursor cells. Right panels are magnified views of the boxed areas, respectively. Genotypes are: *gmr-Gal4* (*gmr-Gal4*/+, A, C, E) and *gmr>RSK^{WT}* (*gmr-Gal4* UAS-*RSK^{WT}*/+, B, D, F).

Figure 3 Inhibition of wing vein cell differentiation by RSK. (A–E, G–J) Microscopic views of *Drosophila* adult wings. Longitudinal veins (L1–L5) as well as anterior (AC) and posterior (PC) cross veins were indicated in wild-type wings. Right panels of (B) and (D) are magnified views of the boxed areas, respectively. (F) Quantified data of (C) to (E). More than 100 wings for each genotype were used for quantification. Flies of (C–E) were cultured at 29°C. Details of all indicated genotypes are described in Supplementary data.

mutants (~16%) had ectopic veins in various regions of the wing (Figure 3B), suggesting that differentiation of wing vein cells is also promoted by RSK null mutation. The number of ectopic veins was significantly increased when the flies were grown at 29°C (Figure 3D and F). Moreover, RSK^{D1} allele over *Df(1)R8A* also led to identical wing vein phenotypes to those of RSK-null flies (data not shown), and weak ubiquitous expression of RSK^{WT} transgene by the *da-Gal4* driver completely rescued the ectopic wing vein phenotypes of RSK^{D1} flies (Figure 3E and F), verifying that the ectopic differentiation of wing vein cells resulted from the absence of RSK.

Consistently, overexpression of RSK^{WT} by the *e16E-Gal4* (induces gene expression in the posterior part of wing) or the *MS1096-Gal4* (induces gene expression in the whole wing) dramatically induced compartment-specific vein-loss phenotypes (Figure 3H and J). In sum, our data demonstrated that RSK negatively regulates vein cell differentiation during *Drosophila* wing development.

Genetic interaction between RSK and the Ras/ERK pathway

As the Ras/ERK pathway positively regulates the developmental processes of retinal and wing vein cell formation (Dickson *et al*, 1992; Brunner *et al*, 1994), and because all of our genetic studies consistently suggested RSK as a negative regulator of retinal and wing vein cell differentiation, we doubted the reliability of the established hypothesis on the downstream role of RSK in the Ras/ERK pathway in *Drosophila*. Moreover, RSK-null embryos frequently displayed partial deletion of the abdominal denticle belts (Supplementary Figure 3), which is highly similar to those caused by gain-of-function mutation of ERK (Brunner *et al*, 1994) and Torso (Klingler *et al*, 1988). Therefore, we sus-

pected that RSK may have an antagonizing function against the Ras/ERK pathway in *Drosophila*.

To substantiate whether RSK indeed suppresses the Ras/ERK signaling activity in *Drosophila*, we performed various genetic interaction assays between RSK and the components of the Ras/ERK pathway. First, we tested whether downregulation of Ras/ERK signaling enhances the RSK-overexpression phenotypes. Although the eyes with heterozygotic mutation of Ras, Raf, MEK, or ERK itself showed no change in the number of R cells (Kim *et al*, 2004 and data not shown), overexpression of RSK^{WT} with heterozygotic mutation of Ras (Figure 4A), Raf (Figure 4B), MEK (Figure 4C), or ERK (Figure 4D) resulted in more severe roughened-eye phenotypes with further decreased number of R cells, when compared to the eyes overexpressing RSK^{WT} in a wild-type genetic background (Figure 2B). Next, we examined whether RSK-null mutation enhances gain-of-function phenotypes of the Ras/ERK pathway components. As previously reported, ectopic expression of constitutively active Ras (Ras^{V12}) or Raf (Raf^{F179}) caused roughened eye phenotypes with extra R cells in some ommatidia (Supplementary Figure 4D, F, H, and J). Interestingly, these phenotypes were further enhanced in heterozygotic or hemizygotic backgrounds of RSK^{D1} (Supplementary Figure 4E, G, I, and K). These two series of experiments consistently suggested that RSK negatively regulates the Ras/ERK pathway.

We next questioned whether downregulation of Ras/ERK signaling suppresses the RSK-null phenotypes. Interestingly, RSK^{D1} phenotypes were significantly suppressed by heterozygotic mutation of Ras or ERK (Supplementary Figure 4B and C), demonstrating that RSK-null phenotypes result from upregulation of the Ras/ERK pathway. Conversely, we hypothesized that if RSK-null mutation indeed upregulates the

Figure 4 Genetic interaction between RSK and the components of the Ras/ERK pathway. (A–N) SEM images (upper panels) and tangential cross sections (lower panels) of *Drosophila* adult eyes. Detailed genotypes of (A–N) are described in Supplementary data. The numbers indicate the mean number of photoreceptor cells per ommatidium $\pm$ s.d. (O) RSK null mutation rescued the lethality of hemizygote *Raf^{HM7}* males at 25°C. Adult viability was scored as previously described (Kim *et al*, 2004).

Ras/ERK pathway, it should relieve the phenotypes caused by a low activity in the Ras/ERK signaling pathway. As expected, *RSK^{D1}* mutation strongly suppressed the eye roughness and R-cell number decrease phenotypes in a hypomorphic *Raf* mutant (*Raf^{HM7}*; Figure 4N, compared to Figure 4M), and even rescued the temperature-sensitive lethality (although *Raf^{HM7}* hemizygote males are viable at 18°C, they are lethal at 25°C; Melnick *et al*, 1998) of *Raf^{HM7}* mutants at 25°C

(Figure 4O). Collectively, these results coherently showed that RSK is a negative regulator of the Ras/ERK pathway *in vivo*.

Finally, we assessed whether RSK overexpression suppresses the phenotypes caused by the upregulation of the Ras/ERK pathway. Strikingly, the gain-of-function phenotypes induced by a receptor tyrosine kinase (*sev^{S11}*; Figure 4E), Ras (*sev>Ras^{V12}*; Figure 4F), and Raf (*sev>Raf^{F179}*; Figure 4G) were almost completely suppressed by expression of *RSK^{WT}* (Figure 4I–K, respectively), supporting that RSK inhibits the Ras/ERK pathway at the downstream of these molecules. However, interestingly, *RSK^{WT}* (Figure 4L) entirely failed to suppress the gain-of-function phenotypes of ERK (*sev>ERK^{Sem}*; Figure 4H), showing the epistatic relationship between RSK and ERK.

RSK inhibits *in vivo* activities of ERK by preventing its nuclear localization

Next, we questioned how RSK inhibits the Ras/ERK pathway. From our genetic results, we found that RSK is epistatic to hyperactive RTK, Ras, and Raf, but not ERK. Hence, we presumed the direct regulation of ERK activity by RSK, without involving upstream components. Interestingly, it has been previously demonstrated that mammalian ERK is constitutively associated with RSK in quiescent cells and that the key residues of the docking domains of the two proteins play a crucial role in the interaction (mutation in the conserved Asp³¹⁹ to Asn in the common docking (CD) domain of ERK (Dimitri *et al*, 2005; corresponding to ERK^{Sem} mutation in *Drosophila*) or a mutation in the conserved Arg⁷⁴² (Arg⁹⁰² in *Drosophila* RSK; Figure 6B) of RSK to Ala in the ERK-docking (D) domain (Roux *et al*, 2003) nullified their interaction in mammals). We predicted that the physical association between ERK and RSK also occurs in *Drosophila* since their interaction domains are highly conserved and both ERK and RSK are colocalized in the cytoplasm of larval eye discs (Figure 5A). Indeed, our hypothesis was confirmed by the fact that RSK physically associated with ERK under overexpressed (Figure 5B) or endogenous (Figure 5C) conditions. Moreover, ERK^{Sem} (ERK^{D334N}) and RSK^{R902A} mutants failed to bind RSK and ERK, respectively (Figures 5B and 6C, respectively). These results demonstrated that RSK–ERK interaction in *Drosophila* occurs via key residues in the D domain of RSK and the CD domain of ERK, as in mammals.

As various ERK-binding molecules modulate ERK activity by altering its subcellular localization (Kwon *et al*, 2002; Burack and Shaw, 2005; reviewed in Tanoue and Nishida, 2003) and because retinal cell fate determination is fully dependent on the nuclear localization of activated ERK in *Drosophila* (Kumar *et al*, 2003), we questioned whether RSK affects the intracellular localization pattern of ERK during *Drosophila* eye development. Thus, we expressed a hemagglutinin (HA)-tagged form of *Drosophila* ERK in the eye disc to detect ERK localization using anti-HA antibody. HA-ERK was predominantly distributed in the cytoplasm (Figure 5F), but hyperactive Ras strongly induced migration of HA-ERK into the nucleus (Figure 5D). Surprisingly, co-expression of *RSK^{WT}* dramatically suppressed the Ras-induced nuclear localization of HA-ERK (Figures 5D and 7K), while co-expression of *RSK^{WT/RA}* completely failed to generate the same result as *RSK^{WT}* (Figure 7H and K). Consistently, even though *RSK^{WT}* and *RSK^{WT/RA}* were expressed at similar levels in the

eye and wing, expression of RSK^{WT/RA} could not inhibit retinal and wing vein cell differentiation (Figure 6E and F), showing that RSK-ERK association is essential for the physiological functions of RSK.

Next, to determine whether endogenous RSK controls ERK localization, we examined the subcellular localization of HA-ERK in RSK-null mutants. Surprisingly, in RSK^{D1} mutant eye discs, ERK migrated into the nucleus in a substantial portion of the *Gal4*-expressing retinal cells unlike those in the control eye discs (Figure 5F and H), implicating that endogenous RSK plays a critical role in preventing ERK nuclear localization. To further examine whether endogenous ERK is indeed regulated by RSK, we monitored the localization of endogenous ERK using anti-phospho-ERK (anti-dpERK) antibodies. Although the endogenous ERK activated by the hyperactive Ras migrated into the nucleus (Figure 5E), RSK^{WT} overexpression dramatically inhibited this nuclear translocation by retaining dpERK in the cytoplasm without significantly diminishing its concentration (Figures 5E and 7K), suggesting that RSK-dependent control of ERK localization does not affect the phosphorylation of ERK. Furthermore, we observed that endogenous dpERK is predominantly cytoplasmic in the morphogenetic furrow of the wild-type eye discs as previously reported (Kumar *et al*, 2003), but RSK-null mutation strongly induced its nuclear translocation (Figure 5G and H). Taken together, we clearly confirmed that RSK suppresses the nuclear translocation of activated ERK molecules *in vivo*.

With the nuclear migration of ERK being prerequisite for phosphorylation of its nuclear targets such as transcription factors (reviewed in Tanoue and Nishida, 2003), we speculated that RSK may downregulate the activities of nuclear ERK targets by preventing ERK nuclear localization. To test this, we examined the expression levels of ERK-dependent genes, *rhomboid* (*rho*) and *pointed-P1* (*pnt-P1*). The transcription of *rho* can be visualized by anti- β -galactosidase staining using the *rho-lacZ* reporter, as previously described (Martin-Blanco *et al*, 1999). As expected, *rho-lacZ* expression was dramatically decreased by co-expression of RSK^{WT} in eye discs (Figure 5I) but not by RSK^{WT/RA} (Figure 7I), compared to the control. Consistently, RSK-null flies showed an increased expression of endogenous *pnt-P1* transcripts (Gabay *et al*, 1996; Rawlins *et al*, 2003), comparable to the flies overexpressing Ras^{V12} (*hs>Ras^{V12}*; Figure 5J). Therefore, we concluded that RSK reduces the transcription of ERK downstream targets by retaining the ERK protein in the cytosol.

The catalytic activity of RSK is dispensable for its physiological function during *Drosophila* eye and wing development

Previous mammalian studies indicated that the kinase activity of RSK is critical for the molecule's functions *in vivo*. However, our biochemical and genetic data suggested that RSK does not act as a signal transducer of ERK, but as a spatial regulator of ERK by blocking the nuclear localization of activated ERK in *Drosophila*. Hence, we doubted whether the kinase activity of RSK is crucial for its physiological function. To address this issue, we generated transgenic fly lines expressing a kinase-dead mutant of RSK (RSK^{KR}, K231R) as well as a kinase-dead and binding-defective mutant of RSK (RSK^{KR/RA}) (Figure 6A). The K231R mutation was expected to nullify the kinase activity of RSK by disrupting the conserved lysine residue in the ATP-binding site on the N-terminal

kinase (NTK) domain, which is mainly responsible for the phosphorylation of its downstream substrates (Roux *et al*, 2003; Woo *et al*, 2004). Through immunohistochemical and immunoblot analyses, we confirmed that RSK^{KR} and RSK^{KR/RA} transgenes are expressed efficiently to a similar level as that of RSK^{WT} and RSK^{WT/RA} (Figure 6C, E, and F). As expected, RSK^{KR} was completely incapable of phosphorylating its substrate (transphosphorylation) as well as the kinase itself (autophosphorylation), while RSK^{WT} was capable of performing both phosphorylating activities (Figure 6D), showing that RSK^{KR} is indeed catalytically inactive. However, surprisingly, RSK^{KR} prevented the differentiation of retinal and vein cells as did RSK^{WT}, while RSK^{KR/RA} did not (Figure 6E and F). Moreover, weak transgenic expression of RSK^{KR} using the *da-Gal4* driver completely suppressed the ectopic retinal and vein cell differentiation phenotypes of RSK^{D1} homozygous flies (Supplementary Figure 6), while RSK^{KR/RA} did not (data not shown). Furthermore, RSK^{K231R/K597R} (RSK^{KR/KR}) and RSK^{K231M/K597M} (RSK^{KM/KM}) mutants, whose N-terminal kinase and C-terminal kinase activities are completely eliminated, also inhibited retinal and vein cell differentiation (Supplementary Figure 5), and rescued the RSK-null eye and wing phenotypes (Supplementary Figure 6). Thus, we deduced that the ERK-binding activity of RSK, but not the phosphotransferase activity, is essential for the *in vivo* function of RSK in *Drosophila* eye and wing development.

To further examine whether RSK^{KR} displays an identical molecular function as RSK^{WT}, we conducted the same genetic and histological analyses as performed for RSK^{WT}. Expectedly, RSK^{KR} genetically interacted with various loss-of-function mutants of the Ras/ERK pathway to induce severer roughened-eye phenotypes with dramatically reduced R cells (Figure 7C and D), compared to the eyes overexpressing RSK^{KR} in a wild-type genetic background (Figure 7B). Moreover, RSK^{KR} suppressed the gain-of-function phenotypes induced by a receptor tyrosine kinase (*sev^{s11}*; data not shown), Ras (*sev>Ras^{V12}*; Figure 7E), and Raf (*sev>Raf^{F179}*; Figure 7F), but not by ERK (*sev>ERK^{Sem}*; Figure 7G). Furthermore, while RSK^{KR} was able to prevent the nuclear localization of HA-ERK (Figure 7H and K) or endogenous dpERK (Figure 7J and K) and to suppress the transcriptional induction of *rho* (Figure 7I), RSK^{KR/RA} was neither able to suppress the nuclear localization of HA-ERK (Figure 7H and K) or endogenous dpERK (Figure 7J and K) nor able to reduce the expression of *rho* (Figure 7I). These results consistently demonstrated that kinase-dead RSK functions through a similar molecular mechanism with wild-type RSK during eye development. Collectively, we concluded that RSK negatively regulates Ras/ERK-dependent eye differentiation via direct physical association with ERK in a kinase-independent manner.

Discussion

RSK, a negative regulator of the Ras/ERK pathway *in vivo*

Here, we investigated the physiological role of RSK using *Drosophila* model system. Overexpression of RSK strongly suppressed retinal (both in neuronal and non-neuronal cells) and wing vein cell differentiation, which is controlled by the Ras/ERK pathway. Conversely, RSK-null flies displayed ecto-

pic differentiation of retinal and wing vein cells and deletion of abdominal denticle belts in embryos, which are reminiscent of the phenotypes caused by gain-of-function mutations of the Ras/ERK pathway components (Klingler *et al*, 1988; Dickson *et al*, 1992; Brunner *et al*, 1994). Furthermore, various genetic interaction assays consistently indicated

that the *RSK*-null phenotypes were caused by hyperactivation of the Ras/ERK pathway, and that the *RSK* overexpression phenotypes were caused by decreased activities of the Ras/ERK pathway. These genetic experiments strongly support *RSK* as a negative regulator of ERK-dependent cellular differentiation *in vivo*.

Many negative regulators of the Ras/ERK pathway including various dual-specificity phosphatases are transcriptionally induced by activation of the Ras/ERK pathway to form a negative feedback loop (Muda *et al*, 1996; Leever, 1999; Reffas and Schlegel, 2000). Since RSK acts as a negative regulator of the Ras/ERK pathway, it is possible to hypothesize that expression of RSK may be induced by Ras/ERK signaling activity. However, our results clearly showed that RSK is ubiquitously expressed in all developmental stages (Figure 1E), while Ras/ERK signaling is activated in a specific region and at specific times (Gabay *et al*, 1997). Furthermore, although the expression of *pnt-P1* was highly induced by hyperactive Ras (Ras^{V12}) and silenced by dominant-negative Ras (Ras^{N17}), *RSK* gene expression was not altered by Ras at all (Supplementary Figure 8), suggesting the Ras/ERK signaling pathway does not transcriptionally induce RSK.

A dispensable role of the protein kinase activity of RSK in *Drosophila*

Genetic and biochemical analyses using kinase-dead mutants of RSK suggested that the kinase activity of RSK is dispensable for its role during *Drosophila* eye and wing development. This is in stark contrast to previous assertions on RSK as an important kinase that controls many crucial downstream targets of the Ras/ERK pathway through phosphorylation in mammals (Frodin and Gammeltoft, 1999). Supporting our results, there were no differences in the phosphorylation level of histone H3, a well-known target of RSK, between wild-type and *RSK*-null eye and wing discs (data not shown; Sassone-Corsi *et al*, 1999). Therefore, we believe that the substrate phosphorylation by RSK is largely unnecessary for its function in *Drosophila*. However, as some phenotypes including life span reduction, fertility reduction and growth retardation shown in *RSK*-null flies were not significantly rescued by expressing kinase-dead mutants of RSK by the *da-* or *hs-Gal4* driver (data not shown), we cannot entirely exclude the possibility that the kinase function plays a role in developmental processes other than eye and wing development. In addition, as only one RSK isoform exists in *Drosophila*, the physiological function of RSK shown in this study may not satisfactorily represent more specialized physiological roles of all the RSK isoforms (RSK1-4) in mammals.

Through a biochemical study using rat PC12 cell line, it has been claimed that RSK negatively regulates the Ras/ERK pathway by phosphorylating the Son of sevenless (Sos)

Ras-GEF protein, an upstream activator of Ras (Douvillat and Downward, 1997). However, our genetic analyses using *Drosophila* did not coincide with this result. Expression of RSK strongly suppressed the phenotypes of the constitutively active forms of Ras and Raf which are downstream signaling molecules of Sos (Figure 4), suggesting that RSK-mediated inhibition of the Ras/ERK pathway does not occur through Sos in *Drosophila*. Moreover, kinase-dead RSK also completely inhibited Ras/ERK-dependent signaling in a similar manner to wild-type RSK (Figure 7), which further undermined the possibility of phosphorylation-dependent inhibition of Sos by RSK in *Drosophila* eye development.

RSK as a cytoplasmic anchor of ERK

As the phosphorylation of ERK was thought as a prerequisite for its nuclear entry (Khokhlatchev *et al*, 1998; Lenormand *et al*, 1998), we also determined whether RSK negatively regulates ERK phosphorylation by inducing gene expression of MAP kinase-specific phosphatases (MKP). Interestingly, although RSK dramatically inhibited the nuclear migration of ERK, it did not affect the status of ERK phosphorylation (Figures 5 and 7) and the level of MKP gene expression (Supplementary Figure 7). Rather, direct protein-protein association between RSK and ERK is the essential mechanism to inhibit ERK signaling by RSK, as the mutant forms of RSK defective in binding ERK completely failed to rescue the phenotypes of *RSK*-null flies (data not shown) and as wild-type RSK failed to suppress the phenotypes of ERK^{Sem} (Figures 4, 5 and 7).

Interestingly, recent reports demonstrated that ERK enters the nucleus by diffusion in a temperature-dependent manner (Whitehurst *et al*, 2002; Burack and Shaw, 2005), which may explain the temperature-sensitive phenotypes of *RSK^{D1}* flies (Figures 1 and 3). This suggests that the binding partner of ERK is necessary for the tight regulation of the ERK nuclear localization. As RSK is constitutively cytoplasmic even in the presence of upstream activators such as Ras^{V12} (Figures 5 and 7), it is very likely that RSK appropriately maintains ERK activity by restraining ERK in a cytoplasmic compartment, which would prevent ERK from activating its nuclear targets. Consistent with our argument, the nuclear entry of activated ERK was dramatically increased by the loss of RSK (Figure 5).

Collectively, our studies demonstrate that RSK is a critical negative regulator of ERK in *Drosophila* by acting as a cytoplasmic anchor.

Figure 5 Inhibition of ERK activity and its nuclear localization by RSK. (A) The eye discs of *gmr>ERK + RSK^{WT}* (*gmr-Gal4 UAS-RSK^{WT}/UAS-ERK*) flies co-expressing Myc-tagged RSK and HA-tagged ERK were stained with anti-Myc (red) or anti-HA (green) antibody. (B) Physical interaction between RSK and ERK. Lysates from ~600 adult fly heads of the *gmr-Gal4* (*gmr-Gal4/+*), *gmr>RSK^{WT}* (*gmr-Gal4 UAS-RSK^{WT}/+*), *gmr>RSK^{WT} + ERK^{Sem}* (*gmr-Gal4 UAS-RSK^{WT}/UAS-ERK^{Sem}*), and *gmr>RSK^{WT} + ERK* (*gmr-Gal4 UAS-RSK^{WT}/UAS-ERK*) flies were subjected to co-immunoprecipitation assay using anti-HA antibody. The anti-HA immune complexes were analyzed by immunoblot using anti-Myc antibody. Anti-Myc and -HA immunoblots were also performed using whole-cell lysates (WCL). Myc-tagged RSK^{WT}, HA-tagged ERK and HA-tagged ERK^{Sem} were used. (C) Lysates of *RSK^{D1}* (*RSK^{D1}*), WT (*w¹¹¹⁸*), and *hs>RSK^{WT}* (*hs-Gal4/UAS-RSK^{WT}*) flies were subjected to co-immunoprecipitation assay using guinea-pig anti-RSK antibody. The anti-RSK immune complexes were analyzed by immunoblot using rabbit anti-ERK antibody or rabbit anti-RSK antibody. Anti-ERK immunoblots were also performed using WCL. Tubulin (tub) was used as a loading control. (D, E) Subcellular localization of RSK and ERK was determined in *Drosophila* eye discs. Eye discs with indicated genotypes were stained with anti-HA (D) or anti-dpERK (E) antibody (green), anti-Myc antibody (red), and Hoechst 33258 (blue). Detailed genotypes are described in Supplementary data. Each magnified image ($\times 60\,000$) of the eye discs was shown as an inset. (F) The eye discs of *sev>ERK* (*sev-Gal4 UAS-ERK/+*) and *RSK^{D1}; sev>ERK* (*RSK^{D1}; sev-Gal4 UAS-ERK/+*) were stained with anti-HA antibody (green) and Hoechst 33258 (blue). (G) The eye discs of WT (*w¹¹¹⁸*) and *RSK^{D1}* (*RSK^{D1}*) flies were stained with anti-dpERK antibody (green) and Hoechst 33258 (blue). (H) Nuclear localization rates of HA-ERK (left graph, quantified from (F)) and dpERK (right graph, quantified from (G)) were presented as bar graphs ($*P = 6.83 \times 10^{-5}$; $**P = 6.71 \times 10^{-5}$). Error bars indicate the s.d. between eye discs. (I) Anti- β -galactosidase antibody staining of *gmr-Gal4 rho-lacZ* (*gmr-Gal4/+; rho-lacZ/+*) and *gmr>RSK^{WT} rho-lacZ* (*gmr-Gal4 UAS-RSK^{WT}/+; rho-lacZ/+*) eye discs. (J) Northern blot showing the transcription level of the *pnt-P1* gene in WT (*w¹¹¹⁸*), *RSK^{D1}* (*RSK^{D1}*) and *hs>Ras^{V12}* (*hs-Gal4/UAS-Ras^{V12}*) flies. 18S rRNA was used as a loading control.

Figure 6 Essential roles of the physical association between RSK and ERK. (A) Various RSK mutants were generated as described in Materials and methods. Each black box indicates the domain containing a mutation, which changes the key amino-acid residue in the N-terminal kinase domain (NTKD) or the D domain. (B) Alignments of the D domain of *Drosophila* (d) RSK, human (h) RSK1-4, and avian (av) RSK1. The black boxes indicate the highly conserved residues among species. The asterisk indicates the key ERK-docking residue, Arg⁹⁰² in *Drosophila*. This arginine was mutated to alanine in order to obtain RSK^{RA}. (C) Co-immunoprecipitation assay was performed using lysates from ~600 adult fly heads with indicated genotypes. The anti-HA immune complexes were analyzed by immunoblot using anti-Myc antibody. Anti-Myc and -HA immunoblots were also performed using whole-cell lysates (WCL). All RSK proteins were Myc-tagged, and ERK was HA-tagged. (D) RSK from flies with indicated genotypes was subjected to kinase assays using S6 protein as a substrate (Kim *et al*, 2000). The autophosphorylated RSK protein (**) and transphosphorylated S6 protein (*) were visualized by autoradiography. Anti-Myc and -tubulin (tub) immunoblots were performed using WCL. (E) Tangential cross sections of adult eyes (upper panels) and anti-Myc immunostaining of larval eye discs (lower panels). The numbers indicate the mean number of photoreceptor cells per ommatidium ± s.d. (F) Microscopic views of adult wings (upper panels) and larval wing discs stained with anti-Myc antibody (lower panels). Detailed genotypes of (C–F) are described in Supplementary data.

Materials and methods

Fly strains

Ras^{e1b}, Raf^{Δ1M7}, Sor^{LH110}, r1^{10a}, sev^{s11}, UAS-Ras^{V12}, UAS-Ras^{N17}, UAS-Raf^{F179}, sev-Ras^{V12}, y/w; CyO, P[Δ2-3]/Bc Egfr, rho^{AA69}, Df(X)/R8A,

UAS-GFP, da-Gal4, hs-Gal4, gmr-Gal4, sev-Gal4, e16E-Gal4 and MS1096-Gal4 are described elsewhere (Wodarz *et al*, 1995; Kim *et al*, 2004; Lee *et al*, 2006). Myc-tagged wild-type *Drosophila* RSK, kinase-dead forms (K231R, K231R/K597R, or K231M/K597M) of *Drosophila* RSK, ERK-binding mutant form (R902A) of *Drosophila* RSK, and kinase-dead and ERK-binding mutant form (K231R/

Figure 7 Kinase-independent inhibition of ERK by RSK. (A–G) RSK^{KR} functionally interacts with components of the Ras/ERK pathway. Upper panels show SEM images. Lower panels show tangential cross sections of adult eyes. The numbers indicate the mean number of photoreceptor cells per ommatidium ± s.d. (H) Eye discs stained with anti-HA antibody (green), anti-Myc antibody (red), and Hoechst 33258 (blue). Each magnified image (× 60 000) of the eye discs was shown as an inset. SEM images of *Drosophila* adult eyes were shown in bottom panels. (I) Anti-β-galactosidase antibody staining of eye discs. (J) The eye discs stained with anti-dpERK antibody (green), anti-Myc antibody (red), and Hoechst 33258 (blue). (K) Quantification of HA-ERK nuclear localization in H and 5D (* $P = 1.32 \times 10^{-4}$; ** $P = 1.19 \times 10^{-4}$), and dpERK nuclear localization in J and 5E (* $P = 4.32 \times 10^{-3}$; ** $P = 3.21 \times 10^{-3}$). Error bars indicate the s.d. between eye discs. Details of all briefly indicated genotypes are described in Supplementary data.

R902A) of *Drosophila* RSK were cloned into pUAST vector. HA-tagged wild-type *Drosophila* ERK and constitutive active form of *Drosophila* ERK (D334N; ERK^{sem}) were cloned into pUAST vector. All *Drosophila* stocks were grown on standard medium at 25°C except some designated experiments.

Generation of RSK null flies

From the GenExel fly library, we found an EP line, RSK^{EP(X)7363}, containing an EP-element insertion at 320 base pairs (bp) upstream of the translation start site. The P-element from RSK^{EP(X)7363} was excised by crossing with flies containing Δ2–3 transposase. More than 100 excision lines (scored by the loss of eye color) were established and analyzed through PCR using the primers flanking *Drosophila* RSK. Subsequently, we isolated one deletion line whose RSK gene was disrupted, and named the allele 'RSK^{D1}' after its isolation number. In the RSK^{D1} genome, the first exon of the RSK gene (95 440th–98 535th nucleotides of AE003574) as well as some parts of the putative promoter region and the first intron was deleted.

Analyses of adult fly phenotypes

Scanning electron micrograph (SEM) images were obtained by LEO1455VP in a variable pressure secondary electron (VPSE) mode. Histological sections of adult eyes and imaging of adult wing blades were performed as previously described (Kim *et al*, 2004). To quantify the eye phenotypes, we calculated the mean value of the number of photoreceptor cells per ommatidium for each genotype. All of these experiments were independently performed by examining ~800 ommatidia from more than five different eyes with the same genotype.

Histology and molecular analyses

Immunostaining and RNA *in situ* hybridization were carried out as previously described (Kim *et al*, 2004). A 778 bp fragment (nucleotide 550–1328) of RSK-coding sequence was used as a

probe for RNA *in situ* hybridization. To quantify the nuclear localization of ERK, we calculated the proportion of the number of cells (or ommatidial clusters) with nuclear ERK to the total number of cells (or ommatidial clusters) with the ERK signals from three different eye discs of the same stage in a blind fashion. *P*-values were calculated by one-way ANOVA analysis. For the induction of the *hs-Gal4* driver, flies were heat-shocked at 37°C for 2 h and allowed to recover at 25°C for 1 h before processing. Total protein extractions, protein immunoblots, co-immunoprecipitation, and kinase assays were performed as previously described (Kim *et al*, 2000, 2003). Northern blot and RT-PCR analysis was performed as previously described (Lee *et al*, 2001). A 784 bp fragment (nucleotide 544–1328) of RSK-coding sequence was used as a probe for Northern analysis. Following antibodies were used for immunoblot, immunoprecipitation, and immunostaining: mouse anti-Myc (9E10, DSHB), rabbit anti-Myc (Cell Signaling), rabbit anti-ERK (Sigma M5670), mouse anti-β-galactosidase (40-1a, DSHB), mouse anti-Arm (N2 7A1, DSHB), mouse anti-tubulin (E7, DSHB), mouse anti-Dlg (4F3, DSHB), mouse anti-phosphospecific-ERK (Sigma M8159), and rat anti-HA (3F10, Roche). Rabbit and guinea-pig anti-RSK antibodies were raised against the N-terminal fragment (amino acids 1–172) of *Drosophila* RSK.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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