

The *Drosophila radish* gene encodes a protein required for anesthesia-resistant memory

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Communicated by Susumu Tonegawa, Massachusetts Institute of Technology, Cambridge, MA, September 28, 2006 (received for review December 8, 2005)

Long-term memory in *Drosophila* is separable into two components: consolidated, anesthesia-resistant memory and long-lasting, protein-synthesis-dependent memory. The *Drosophila* memory mutant *radish* is specifically deficient in anesthesia-resistant memory and so represents the only molecular avenue to understanding this memory component. Here, we have identified the *radish* gene by positional cloning and comparative sequencing, finding a mutant stop codon in gene *CG15720* from the *Drosophila* Genome Project. Induction of a wild-type *CG15720* transgene in adult flies acutely rescues the mutant's memory defect. The *phospholipase A2* gene, previously identified as *radish* [Chiang *et al.* (2004) *Curr. Biol.* 14:263–272], maps 95 kb outside the behaviorally determined deletion interval and is unlikely to be *radish*. The Radish protein is highly expressed in the mushroom bodies, centers of olfactory memory. It encodes a protein with 23 predicted cyclic-AMP-dependent protein kinase (PKA) phosphorylation sequences. The Radish protein has recently been reported to bind to Rac1 [Formstecher *et al.* (2005) *Genome Res.* 15:376–384], a small GTPase that regulates cytoskeletal rearrangement and influences neuronal and synaptic morphology.

immunolocalization | long-term memory | memory consolidation | transformation rescue

Historically, long-term memory has been defined by two experimental criteria: (i) resistance to treatments such as anesthesia or electroconvulsive shock that disrupt the patterned activity of the brain (1), and (ii) requirement for new protein synthesis (2, 3). In *Drosophila*, these two criteria define two independent forms of memory. Anesthesia-resistant memory (ARM) is deficient in the memory mutant *radish*, whereas long-lasting protein-synthesis-dependent memory is eliminated by overexpressing the blocking form of the transcription factor cAMP-response element-binding protein or by feeding cycloheximide (4, 5, 6). ARM and long-lasting protein-synthesis-dependent memory may interact at times, at least in anatomically mutant flies (7).

These forms of long-term memory appear to be conserved across species, and there are indications of mechanistic conservation as well. cAMP-response element-binding protein is involved in long-term memory in *Drosophila*, *Aplysia*, and mice (6, 8, 9). Memory in bees, as in rodents, is sensitive to electroconvulsive shock immediately after training and resistant to it at later times (1, 10).

Results

The specific defect of the *radish* mutant, diminished ARM, normal long-lasting protein-synthesis-dependent memory, provides a key to obtaining mechanistic information about ARM. Here, we report the molecular identification of the *radish* gene. The *radish* mutant was isolated in a screen for learning-defective flies after chemical mutagenesis. Genetic recombination and deletion mapping of the memory defect placed the gene in a 140-kb stretch in region 11D of the X chromosome (4). Our transcript analysis and information from the *Drosophila* genome project indicated 17 genes in this interval. We PCR-amplified

105 kb of the genomic 11D region DNA from *radish* flies and from the parental wild-type Canton-S (C-S) flies, sequenced the products, and compared the sequences. The sequences analyzed included the ORFs of all identified genes in or near the interval (Fig. 1A). In this comparison, we detected only a 1-nt difference between mutant and parent strains, a C-to-T transition in the ORF of the *CG15720* (11) predicted gene. This mutation converts a glutamine codon in the wild-type ORF into an amber stop codon in the mutant *radish* DNA.

Our previous mapping of the memory phenotype revealed that the *radish* mutation is uncovered by the Df(1)N105 X-chromosome deficiency (4). However, the *CG15720* ORF lies proximal of the Df(1)N105 breakpoint, but close enough so that the deletion could plausibly eliminate its transcription. To verify that this was the case, we used RT-PCR to isolate the *CG15720* transcript from C-S/Df(1)N105, *radish*/Df(1)N105, and *radish*/C-S flies. We further amplified the region of the mutation and sequenced the products. The results were all consistent with Df(1)N105 *cis*-acting elimination of *radish* gene transcription, C-S/Df(1)N105 gave only wild-type sequence; *radish*/Df(1)N105 gave only mutant sequence; *radish*/C-S gave 50:50 wild-type and mutant sequence (Fig. 1B).

The *CG15720* gene is expressed in the fly head. Northern blot analysis revealed a single 6-kb transcript (Fig. 2A). We amplified this transcript by RT-PCR, sequenced its ORF, and found that it spanned five exons (Fig. 2B). The *radish* mutation is at the 3' end of the fourth exon, which should truncate the protein in *radish* mutant flies. The inferred mutant protein lacks the C-terminal 64 aa.

To ascertain whether *CG15720* is, in fact, the *radish* gene, we reestablished wild-type *CG15720* gene expression in *radish* mutant flies and assayed ARM. We subcloned the wild-type *CG15720* ORF DNA into the germ-line transformation vector *hs-CaSpeR* under the control of the inducible promoter for heat-shock protein 70. We injected the *hs-CG15720* construct into mutant *radish* embryos (12) and bred two resulting transformant flies into homozygous populations (*hs-rsh-1*, *hs-rsh-2*). We induced the *hs-CG15720* transgene with a 25-min heat shock (37°C), waited 1 h for transgene expression, and trained the flies in the odor-discrimination paradigm of Tully and Quinn (13). Two hours later, we anesthetized the flies by cooling on ice for 2 min, allowed them to recover, and tested memory 1 h thereafter.

Expression of the *hs-CG15720* transgene in mutant *radish* flies restored normal ARM (Fig. 3A). Without heat-shock induction of the *hs-CG15720* transgene, the flies remembered as poorly as mutant *radish* flies (Fig. 3B). Western blot analysis indicated that

Author contributions: E.F., S.W., and W.G.Q. designed research; E.F. and S.W. performed research; E.F., S.W., and W.G.Q. analyzed data; and E.F., S.W., and W.G.Q. wrote the paper.

The authors declare no conflict of interest.

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Abbreviations: ARM, anesthesia-resistant memory; C-S, Canton-S, MB, mushroom body; PKA, cyclic-AMP-dependent protein kinase.

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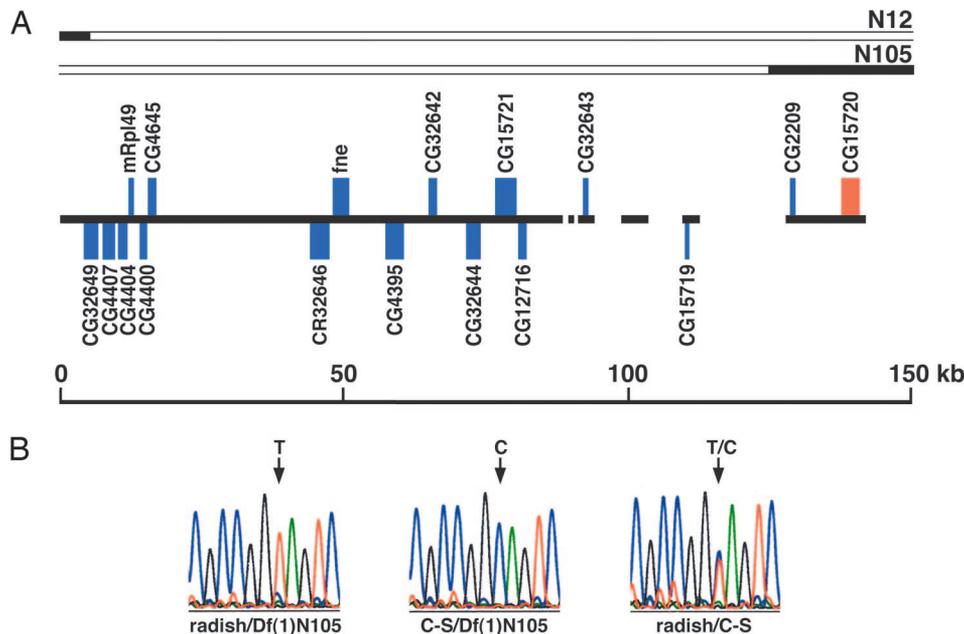


Fig. 1. Identification of the *radish* gene. (A) Identification of an amber mutation by comparing wild-type and mutant *radish* genomic DNA. The *radish* interval is defined by the centromere-distal breakpoint of the deletion Df(1)N12 and the proximal breakpoint of the deletion Df(1)N105 (upper two bars). Information from the *Drosophila* Genome Project indicates 17 genes in or adjoining the interval. The rectangles attached to the third bar show the extent of the ORFs of these genes (rectangles above, genes transcribed rightward; rectangles below, genes transcribed leftward). We PCR-amplified, sequenced, and compared a total of 105-kb DNA of each genotype (third, interrupted, heavy bar). We found a single nucleotide difference in *radish* DNA at position 1549 of the CG15720 ORF. The mutation converts a glutamine codon into an amber stop codon. (B) The deletion Df(1)N105 eliminates transcription of CG15720 on that chromosome, although its ORF lies outside the deletion. We RT-PCR amplified the CG15720 transcript from flies with the genotypes *radish*/Df(1)N105, *C-S*/Df(1)N105, and *radish*/*C-S*, further amplified the region containing the *radish* mutation, and sequenced the product. *radish*/Df(1)N105 had only *radish* (TAG) sequence, and *C-S*/Df(1)N105 had only wild-type (CAG) sequence. *radish*/*C-S* flies gave both sequences, with C and T peaks of equal height.

the transgene was expressed after heat shock and not in its absence (Fig. 3C). (We also observed transgenic rescue of memory without anesthesia, Fig. 6, which is published as supporting information on the PNAS web site). These results strongly indicate that the CG15720 gene is *radish*.

Induction of this transgene in *radish*⁺ flies elicited no additional ARM, indicating that this transgene specifically rescues the *radish* memory defect. When ARM was assayed after heat shock, exactly as in Fig. 3A, *w, rsh*⁺ scores were 0.15 ± 0.05 ($n = 7$); scores for transformed *w, rsh*⁺[*hs-rsh-1*] flies were 0.15 ± 0.03 ($n = 8$). Without heat-shock treatment, scores for *w, rsh*⁺ were 0.18 ± 0.04 ($n = 7$); scores for transformed *w, rsh*⁺[*hs-rsh-1*] flies were 0.19 ± 0.04 ($n = 8$).

Chiang *et al.* (14) have reported that the *radish* gene encodes a phospholipase A2 gene (*PLA2*) with the designation CG4346. However, we found that this gene is far outside the deletion, Df(1)N105, that defines the proximal end of the *radish* interval (4). PCR-amplification of Df(1)N105 DNA indicates that the proximal breakpoint of this deletion is 95 kb distal of the *PLA2*

gene (Fig. 4). Recently Dubnau and colleagues have done additional experiments, including repeating their complementation crosses with reciprocal parental genotypes. Their recent results indicate that *PLA2* and *radish* are different genes (J. Dubnau, personal communication).

The Radish protein is similar to a predicted protein from the mosquito *Anopheles gambiae* (66% identity) and to one from the honey bee *Apis mellifera* (45% identity). It has no striking homology to proteins with known function (www.ncbi.nlm.nih.gov/blast). Sequence comparison with mammalian proteins reveals low homology (25%) to Arg/Ser-rich splicing factors (15). 29% of amino acids in the Radish protein are either arginines (14%) or serines (15%).

The inferred Radish amino acid sequence has 23 potential PKA and 14 protein PKC phosphorylation sites (<http://ca.expasy.org/prosite>). The Radish sequence also has five bipartite nuclear localization signals (NLSs) (<http://ca.expasy.org/prosite>); and each NLS overlaps with a PKA target site. The truncated protein in the *radish* mutant lacks two PKA target sequences and two NLSs.

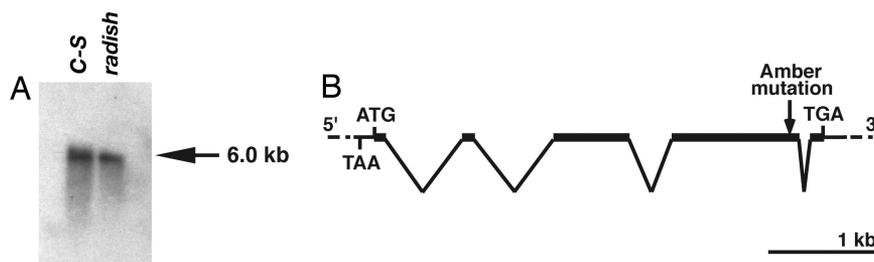


Fig. 2. The *radish* transcript. (A) On a Northern blot with poly[A] RNA (3 μ g per lane) from wild-type *C-S* and *radish* fly heads CG15720 DNA hybridized to a single 6-kb transcript. (B) The CG15720 ORF has five exons (wide horizontal bars). The amber mutation was at the 3' end of the fourth exon (vertical arrow).

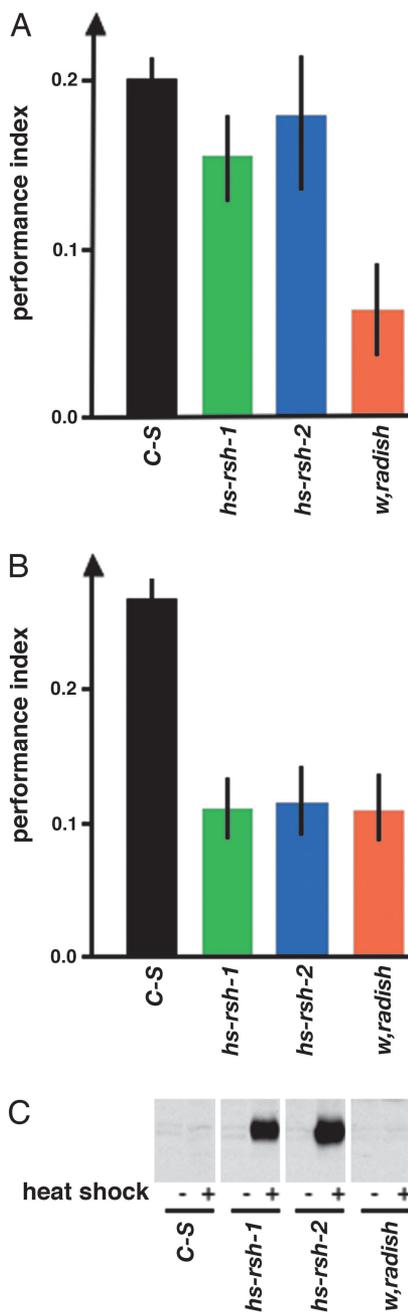


Fig. 3. Transformation rescue of *radish* mutant's deficit in ARM with wild-type *CG15720* DNA. We transformed mutant *w⁻, radish⁻* flies with the wild-type *CG15720* ORF DNA under control of the heat-shock promoter *hsp70* and measured memory of two independent transformant lines (*hs-rsh-1* and *hs-rsh-2*). (A) Heat-shock induction of wild-type *CG15720* rescues ARM in mutant *radish* flies. We heat-shocked the transformants (and as controls, the parental stock *w⁻, radish⁻*, and wild-type C-S flies) at 37°C for 25 min, kept them at 25°C for 1 h, trained them in an olfactory discrimination paradigm, anesthetized them by cooling (for 2 min in ice water) 2 h after training, and tested them 1 h thereafter. ARM of both transformant lines (*hs-rsh-1*: number of experiments, $n = 9$; *hs-rsh-2*: $n = 9$) is statistically indistinguishable from that of wild-type C-S flies ($n = 12$) and significantly higher ($P < 0.05$) than that of *w⁻, radish⁻* flies ($n = 8$). All statistical comparisons were done by post hoc ANOVA (Student–Newman–Keuls test). (B) We saw no rescue of ARM without *CG15720* transgene induction. In the absence of heat-shock, the transformants (*hs-rsh-1*: $n = 12$; *hs-rsh-2*: $n = 12$) perform as poorly in ARM as the mutant *w⁻, radish⁻* ($n = 14$) flies and significantly worse ($P < 0.05$) than wild-type C-S flies ($n = 14$). (C) Western blots hybridized with a Radish antibody showed heat-shock-dependent induction of an immunoreactive protein band of 85 kDa.

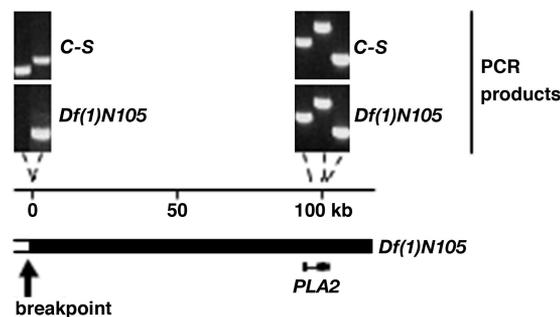


Fig. 4. Localization of the phospholipase A2 (*PLA2*) gene relative to the *radish* interval by PCR amplification. We had behaviorally mapped the proximal end of the *radish* interval to the proximal breakpoint of the deletion *Df(1)N105* (4). We used genomic *Df(1)N105* DNA as a template for PCRs with primers corresponding to the *PLA2* gene and to the DNA distal of it. As a control, we PCR-amplified wild-type C-S DNA in parallel. The size and locus of the PCR products are shown as the short horizontal bars that are connected with dashed lines to the corresponding gel lanes with their PCR products. The scale is below them. The absence of the most distal *Df(1)N105* PCR product indicates the beginning of the deletion. The broad black horizontal bar depicts *Df(1)N105* DNA, with the arrow indicating the breakpoint. The deletion starts left of the arrow. *Df(1)N105* PCR products with three pairs of primers from the *PLA2* gene indicate that the *PLA2* gene [right cluster of connected vertical lines (exons) at the bottom of the figure], is 95 kb outside the deletion, and the PCR products do not appear altered in the deletion flies.

To localize the Radish protein in the fly brain we raised a polyclonal rabbit antibody to a synthetic peptide corresponding to amino acids 522–542 of the inferred Radish protein. (This region is deleted in the Radish mutant protein.) We used this antibody to immunostain wild-type and *radish* mutant brains. Radish immunoreactivity was evident throughout the neuropil of wild-type brains and was particularly strong in the calyx, peduncle, and lobes of the mushroom bodies (MBs) and ellipsoid body of the central complex (Fig. 5 A, B, and D). Critically, this staining was absent in brains of *radish* mutant flies (Fig. 5C).

Discussion

The *radish* mutant defines ARM, one type of long-term memory. We have identified this gene by detecting a mutation and by transformation rescue. The Radish protein functions acutely in the adult fly to engender ARM, because its expression 1 h before training is sufficient to rescue memory in mutant *radish* flies. PKA-dependent phosphorylation of the Radish protein, as suggested by numerous PKA target sites, would logically link ARM to the cAMP pathway that functions in short-term memory in *Drosophila*. The idea is inconsistent with the findings that *rutabaga* flies have substantial ARM (4, 7, 16), although the reduction in cAMP levels caused by the mutation is partial (17, 18).

The high Arg/Ser content of the inferred amino acid sequence and the observed homology to Arg/Ser-rich splicing factors both suggest a role in RNA processing for the Radish protein. This notion appears to be contradicted by the finding that the translation inhibitor cycloheximide does not affect ARM (5). However, protein synthesis in these experiments was reduced by only 50%.

Finding the Radish protein in the MBs is consistent with the known importance of the MBs in olfactory memory, the expression of many other memory-relevant genes (including cAMP cascade components) there, and the observation that blocking MB output abolishes ARM (7, 19, 20). Staining in the MB calyx and lobes indicates the presence of the Radish protein in neurites. Nuclear localization is not apparent, in current immunostains, but it may be present under other conditions, e.g., on activation of PKA.

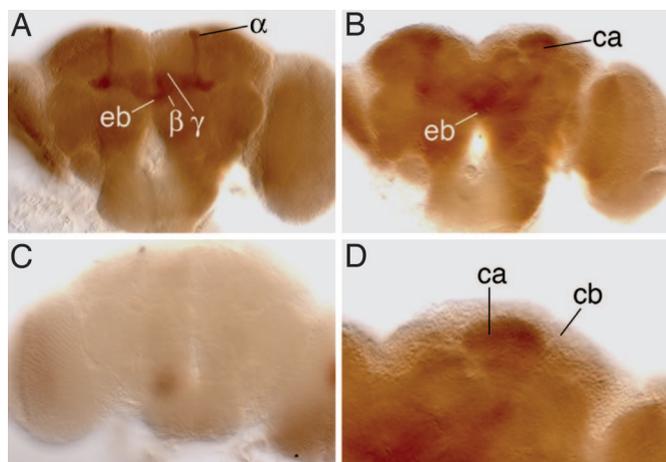


Fig. 5. Radish is strongly expressed in the MB neuropil. (A) Radish-antibody immunostain of a wild-type brain at the level of MB lobes. We raised an antibody against a peptide with sequences from the C-terminal end of the Radish protein that is deleted in the mutant. This antibody stained the α , β , and γ MB lobes of wild-type brains. The ellipsoid body (eb) of the central complex (cc) is also immunostained. (B) A deeper focal plane at the level of MB calyces (ca) of the same brain shown in A. (C) No immunostaining with the Radish antibody of a mutant *radish* brain is visible. (D) A higher magnification of the calyx region from B. cb indicates the cell bodies of the Kenyon cells of the MB.

Long-term memory in *Aplysia* and mice is correlated with changes in synaptic morphology (22, 23). One last property of the Radish protein argues for its role in synaptic morphology. Formstecher *et al.* (24) carried out an extensive high-throughput yeast two-hybrid screen for interacting *Drosophila* gene products using 102 proteins as bait. Intriguingly, this analysis identified an interaction between CG15720 (Radish) and Rac1. Rac1 is a small GTPase of the Rho family that regulates cytoskeletal assembly to influence neuronal and synaptic morphology in *Drosophila* and mammals (25–27). Furthermore, Rac1 lies in the same cell-signaling pathways as Pak1 and fragile-X protein (FMRP), both of which have documented effects on synaptic and behavioral plasticity (23, 28). Strikingly, amino acids 538–579 of the Radish protein are sufficient for its binding to Rac1 (24), and these residues are all deleted in the mutant Radish protein. Therefore, it is plausible that the critical function of Radish in ARM is to change synaptic morphology through Rac1 interaction.

Materials and Methods

Fly Stocks. Mutant *radish* had been derived from wild-type *C-S* flies by chemical (EMS) mutagenesis. The deficiency stocks Df(1)N12 and Df(1)N105 were from the Bloomington Stock Center (Indiana University, Bloomington, IN). The flies were reared at 25°C and 60% relative humidity with a 14/10-h light/dark cycle.

Memory Assays. Flies were trained and tested in an odor-discrimination paradigm with electric-shock reinforcement as in Tully and Quinn (13).

DNA Preparation. For sequence comparisons, we isolated genomic DNA from wild-type and mutant *radish* flies. Each of these DNA preparations was done from 50 female flies. PCRs were carried out with *Taq*, *Pfu* (Stratagene), or *Ex-Taq* (TaKaRa) according to the manufacturer's protocols. Sequencing was done by the Biopolymers Laboratory at The Howard Hughes Medical Institute at the Massachusetts Institute of Technology.

Hemizygous Df(1)N105 flies die as embryos. We therefore collected embryos from a cross of Df(1)N105/FM6 females with

FM6 males. We isolated DNA from single embryos and tested the genotype of each embryo by PCRs with primers corresponding to DNA that is deleted in Df(1)N105. The absence of a PCR product indicated that the DNA was from a Df(1)N105 male.

RNA Preparation. We isolated total RNA as described in Drain *et al.* (29) or with the TRIzol reagent from Invitrogen (Carlsbad, CA) according to the manufacturer's protocol. mRNA was isolated with the Poly (A) Quik mRNA isolation kit (Stratagene, La Jolla, CA).

RT-PCR. RT-PCRs were done with the Pro-STAR Ultra-HF RT-PCR System (Stratagene) according to the manufacturer's protocol. The ORF of our RT-PCR product was as predicted by the *Drosophila* Genome Project, except that our wild-type and *radish* CG15720 ORF is nine nucleotides shorter (GCAGCACCA; position 351–360).

Hs-CG15720 Construct. We RT-PCR amplified the ORF of the CG15720 gene, added *Xba*I restriction enzyme sequences to the ORF by primer extension, and used these *Xba*I sites to subclone the CG15720 DNA into the hs-CaSpeR germ-line transformation vector. This vector contains the *miniwhite* gene as an eye-color marker. The DNA for the transformation experiments was purified with a plasmid purification kit according to the manufacturer's protocol (Qiagen, Valencia, CA).

Transformation Experiments. Transformation was carried out as described by Rubin and Spradling (12). Mutant *w⁻*, *radish⁻* embryos injected with the *hs-CG15720* construct at a concentration of 1 $\mu\text{g}/\mu\text{l}$, together with $\pi 25.7\text{wc}$ (0.5 $\mu\text{g}/\mu\text{l}$) as transposase source. Surviving larvae were transferred to vials with food, and the resulting adult flies were mated to *w⁻*, *radish⁻* flies. Their offspring were screened for successful transformation by eye color. We crossed single independent transformants to *w⁻*, *radish⁻* flies and generated stocks homozygous for the transgene. Later, to test specificity of rescue, we transferred our *radish⁺* transgene that was autosomal, [*hs-rsh-1*], into a *w⁻*, *rsh⁺* background by conventional genetic crosses.

Antibody Production and Purification. Rabbit polyclonal anti-Radish antibodies were generated by Invitrogen. Rabbits were injected with an HPLC-purified synthetic peptide, CRNFRQSSQSDDDVCR YRGRR, representing amino acids 522–542 from the C terminus of the inferred Radish (CG15720) protein. Anti-Radish antisera were affinity-purified on immobilized GST:Radish (amino acids 468–576) fusion protein. One milligram of bacterially produced GST:Radish was electrophoresed on a 9% SDS/PAGE gel and electrophoretically transferred to nitrocellulose. Protein was visualized by Ponceau S staining, and the relevant strip of nitrocellulose with the GST:Radish protein was excised. Membrane was washed twice for 2 min in TBS (20 mM Tris, pH 7.4/500 mM NaCl/0.05% Tween 20), blocked with 3% BSA in TBS for 1 h, and washed twice for 2 min in TBS. One milliliter of raw serum was diluted with 4 ml of TBS and incubated with the membrane overnight at 4°C. The filter was washed twice for 5 min with TBS and twice for 5 min with PBS (20 mM sodium phosphate, pH 7.2/150 mM NaCl), and antibody was eluted in 1 ml of glycine. Tris, pH 8.0 (1 M), was added to a final pH of 7.0. Sodium azide (5 mM) was added, and aliquots were frozen at -80°C .

Western Blot Analysis. For Western blot analysis, 10 female and 10 male flies of each genotype were mashed in 200 μl of 2 \times Laemmli buffer. Twenty microliters of extract was loaded per lane on a 10–20% gradient Tris-Cl SDS/PAGE gel (Bio-Rad, Hercules, CA) and electrophoretically transferred to nitrocellulose. The filter was processed by using standard protocols with

a 1:500 dilution of purified anti-Radish antibody, followed by a 1:3,000 dilution of secondary HRP-coupled goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA). Chemiluminescence was detected with Supersignal reagent (Pierce, Rockford, IL) and autoradiography.

Immunohistochemistry. Adult brains were fixed for 30 min in 4% paraformaldehyde in sodium phosphate buffer, blocked for 30 min in PBT+NGS (PBS with 0.1% Triton X-100/0.1% BSA/5% normal goat serum), and incubated overnight with affinity-purified Radish polyclonal antibody (1:1,000) at 4°C. Brains were washed six times with PT (1.86 mM NaH₂PO₄/8.41 mM

Na₂HPO₄/175 mM NaCl/0.1% Triton X-100) and incubated for 2 h with secondary HRP-coupled goat anti-rabbit IgG (1:500). After six washes with PT, peroxidase activity was visualized with Pierce DAB in stable peroxide buffer, cleared in glycerol, and photographed on a Zeiss Axioskop2 plus microscope (Zeiss, Thornwood, NY).

We thank Ruth Brain for excellent immunohistochemistry, Sean M. Perry for help with figures, and Troy Littleton for helpful discussions and, in particular, for bringing the published CG15720-Rac1 interaction to our attention. This work was supported by National Institutes of Health Grants GM56843 (to W.G.Q.) and MH09883 (to S.W.).

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