Requirement for *Drosophila 14-3-3ζ* in Raf-dependent photoreceptor development

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Based on biochemical and functional data obtained with tissue culture cells and yeast, 14-3-3 proteins have been implicated in a number of different signal transduction processes, in particular in the signal-dependent activation of protein kinases. We performed a functional analysis of 14-3-3 in a multicellular organism, initiated by the cloning of a *14-3-3ζ* homolog of *Drosophila melanogaster,* termed *D14-3-3ζ*. *D14-3-3ζ* transcripts are strongly enriched in the developing central nervous system. In addition, they are predominantly expressed in the region posterior to the morphogenetic furrow of the eye imaginal disc where cells differentiate as photoreceptors. In these cells D14-3-3ζ is localized apically. Both the expression pattern and the subcellular localization are consistent with the proposed function of 14-3-3 proteins in Ras/Raf/MAPK signaling. D14-3-3ζ mutant analysis combined with rescue experiments involving gain-of-function alleles of Raf and Ras indicate that D14-3-3ζ is an essential component of the Raf/Ras signaling pathway and necessary for photoreceptor differentiation. It acts upstream of Raf and downstream of Ras.

[*Key Words:* 14-3-3; *Drosophila;* eye development; Raf; Ras; signal transduction]

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As implied by their interactions with important signaling proteins including Raf, PKC, BCR/ABL, BAD, CDC 25, and others (Freed et al. 1994; Irie et al. 1994; Braselmann and McCormick 1995; Conklin et al. 1995; Aitken 1996; Zha et al. 1996), 14-3-3 proteins appear to participate in a broad spectrum of biological signal transduction processes. In a number of well-studied cases, the contact between 14-3-3 and its target protein centers around a serine or threonine residue, and association occurs only when these side chains are phosphorylated (Michaud et al. 1995; Muslin et al. 1996; Zha et al. 1996). Provided that phosphorylation of the 14-3-3 contact site on the target protein occurs in response to a signal-regulated event, the 14-3-3 proteins would participate in signal-dependent protein–protein interactions as shown recently for SH2-containing signal transduction proteins, which recognize tyrosine-phosphorylated target proteins (Pawson et al. 1993).

One of the most intensely studied cases of possible 14-3-3-mediated signal transduction is the activation of the mitogen-activated-protein kinase kinase kinase (MAPKKK) c-Raf by Ras. The mechanistic understanding of Raf activation by extracellular ligands is still fragmentary. It appears, however, that upon cell stimulation, cytoplasmic Raf is transferred to the inner aspect of the cell membrane (Leevers et al. 1994). This translocation, which occurs in a Ras-dependent fashion (Leevers et al. 1994), is a prerequisite for the subsequent activation of the kinase activity of Raf, a process thought to be mediated by the phosphorylation of Raf (Daum et al. 1994). Candidate Raf kinases include protein kinase Cα (PKCα) and ceramide-activated protein kinase (Kölch et al. 1993; Yao et al. 1995). In addition, experimentally induced dimerization of Raf results in Raf kinase activity, suggesting that autophosphorylation is a further possible mechanism that results in Raf activation (Farrar et al. 1996; Luo et al. 1996).

Recently, a number of studies have identified 14-3-3 proteins as Raf-binding partners. Several lines of evidence have documented a physical interaction between Raf and 14-3-3 (Marais and Marshall 1995; Aitken 1996). In addition, there are a number of in vivo and in vitro experiments in support of a function of 14-3-3 in the Ras-dependent activation of the kinase activity of Raf (Fantl et al. 1994; Irie et al. 1994; Li et al. 1995). However, such an assignment of 14-3-3 function also has been doubted (Michaud et al. 1995; Suen et al. 1995), because the interaction between these two proteins per se is not sufficient to activate Raf's kinase (Michaud et al. 1995; Suen et al. 1995). Based on this, it has been

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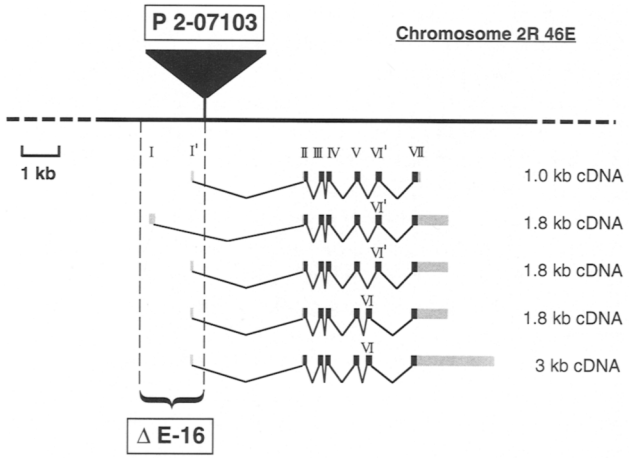

Figure 1. Genomic organization, cDNAs, and mutants of *D14-3-3ζ*. Five different types of *D14-3-3ζ* cDNAs have been isolated that fall into three size classes of 1.0 1.8, and 3.0 kb, consistent with data from Northern blot analyses (Swanson and Ganguly 1992; data not shown). The transcripts differ by the alternative use of exons I or I′ and VI or VI′ as well as by usage of three different poly(A) addition sites. P-element l(2)-07103 is integrated in the first intron 1633 bp downstream of the splice donor site of exon I′. Imprecise excision E-16 of P-element l(2)-07103 induced a 1957-bp deletion spanning exons I and I′.

speculated that 14-3-3 acts as a bridging factor between Raf and a hypothetical Raf activator, that it functions as a chaperone (Braselmann and McCormick 1995; Marais and Marshall 1995), or that it protects Raf from inactivation by phosphatases (Dent et al. 1995; Jelinek et al. 1996). Furthermore, the stimulatory effects of 14-3-3 proteins on Raf protein kinase have so far been demonstrated when they were overexpressed in yeast and in *Xenopus* oocytes, respectively (Fantl et al. 1994; Irie et al. 1994; Li et al. 1995). Thus, the role of 14-3-3 proteins and their necessity for activating the kinase activity of Raf are unclear.

In *Drosophila*, the developmental decision of cells in the eye imaginal disc to either differentiate into photoreceptors or to adopt an alternative non-neuronal fate depends on receptor tyrosine kinase (RTK)–Ras–Raf signaling (Simon 1994; Zipursky and Rubin 1994). Two RTKs, Sevenless and Drosophila EGF receptor (DER), have been placed in this pathway upstream of Ras. Sevenless is required only for the differentiation of the UV-sensitive R7 photoreceptor, whereas DER also controls the fate of the outer photoreceptors R1–R6 (Simon 1994; Wassarman et al. 1995). Genetics combined with molecular studies on photoreceptor differentiation have therefore been instrumental to unravel the RTK–Ras–Raf signaling pathway (Dickson et al. 1992). We have used this system to characterize the in vivo function of a *Drosophila* 14-3-3 gene, termed *D14-3-3ζ*. We show that this member of the 14-3-3 family of proteins is indeed an integral component of the Ras/Raf signaling pathway that mediates cell–cell communication events in multicellular organisms. *D14-3-3ζ* is necessary for both cell proliferation and photoreceptor differentiation during eye development and its gene product acts upstream of Raf but downstream of Ras.

Results

The Drosophila 14-3-3ζ *gene*.

The previously identified *Drosophila melanogaster 14-3-3* locus at chromosomal position 46 E (Swanson and Ganguly 1992) encodes a protein that is most homologous to mammalian 14-3-3ζ. Thus, we refer to this gene as *D14-3-3ζ*. Physical characterization of genomic DNA fragments and several cDNAs established that *D14-3-3ζ* codes for at least five alternative mRNAs (Fig. 1). These different transcripts arise by use of multiple promoters and polyadenylation sites, as well as by alternative splicing (Fig. 1). Switching of exons VI and VI′ affects the open reading frame and causes sequence differences in the respective translation products (Fig. 2). The variant amino acids are predicted to lie in α-helix 6 on the outside of the groove-shaped 14-3-3 dimer (Liu et al. 1995; Xiao et al. 1995). Interestingly, helix 6 is composed of the sequences that are least conserved throughout the 14-3-3 protein family (Aitken et al. 1992). This suggests that helix 6 might confer specificity to 14-3-3 interactions with target proteins. Both exons VI and VI′ encode a potential phosphorylation site characterized previously in mammalian 14-3-3 β and ζ (Fig. 2) (Aitken et al. 1995).

D14-3-3ζ *expression pattern*

Northern blot analysis showed that *D14-3-3ζ* is expressed throughout all stages of embryonic and larval development (Swanson and Ganguly 1992; data not shown). Whole-mount in situ hybridizations to embryos and larvae, with probes common to all splice forms of *D14-3-3ζ* mRNAs, revealed a strong enrichment of *D14-3-3ζ* in the central nervous system (Fig. 5, below; data not shown). Similarly, high levels of 14-3-3 have been detected in the developing mouse brain (McConnell et al. 1995), suggesting that 14-3-3 could have an evolutionarily conserved function in brain development and/or physiology.

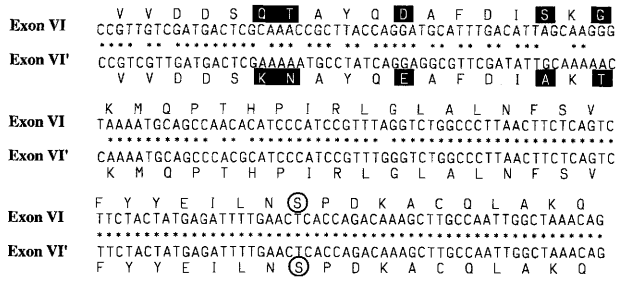

Figure 2. Exons VI and VI′ encode different peptide sequences. The DNA sequences of exons VI and VI′ were conceptually translated and aligned. Nucleotide identities are indicated with asterisks. The variant amino acids are shown in white on a black background. A serine residue that is homologous to in vivo phosphorylation sites in the mammalian 14-3-3ζ and ζ sequences is circled.

In addition to the strong 14-3-3 expression in the central nervous system, we also noted an enrichment of the transcripts in the region posterior to the progressing morphogenetic furrow of the developing eye imaginal disc (data not shown). This location is consistent with a role of D14-3-3ζ in eye development and Raf-mediated photoreceptor differentiation. In order to visualize how the D14-3-3ζ protein is distributed in the eye imaginal disc, we raised antibodies directed against bacterially expressed D14-3-3ζ protein. As shown in Figure 3a, these antibodies specifically recognize a doublet with an apparent molecular mass ~29 kD on immunoblots of crude larval protein extracts. Antibody staining of whole-mount preparations of eye imaginal discs illustrates that D14-3-3ζ is expressed in most, if not all cells of the disc (Fig. 3b–n; red channel). Strong D14-3-3ζ antibody staining levels were found in the region posterior to the morphogenetic furrow where cells undergo neuronal induction and differentiation as photoreceptors (as visualized by ELAV staining; green channel in Fig. 3). Within these cells, the distribution of D14-3-3ζ protein appears to be concentrated apically (Fig. 3c–n), showing a subcellular pattern similar to the distribution of proteins that act upstream of Raf, such as Boss, Sevenless, D-EGF receptor, Drk, Sos, and Dos (Tomlinson et al. 1987; Krämer et al. 1991; Zak and Shilo 1992; Olivier et al. 1993; Karlovich et al. 1995; Raabe et al. 1996).

14-3-3 function is required for photoreceptor differentiation

The subcellular colocalization of components known to stimulate Raf in response to neurogenic signaling is consistent with the proposed function of D14-3-3 within the Ras/Raf signaling cascade required for photoreceptor differentiation. To obtain experimental evidence for this inference, we examined the phenotype of flies that carry a mutation in the *D14-3-3ζ* locus. P-element insertion l(2)-07103, which causes a lethal phenotype when homozygous, was mapped to the first intron of the *D14-3-3ζ* gene (Fig. 1). The lethality is rescued by expression of a 1.0-kb *D14-3-3ζ* cDNA fragment under the control of a ubiquitous promoter (data not shown). This indicates that the P-element insertion causes a specific defect in *D14-3-3ζ*. We refer to this allele, which causes a hypomorphic mutation (see below), as $D14\text{-}3\text{-}3\zeta^{07103}$.

To analyze the effect of the $D14\text{-}3\text{-}3\zeta^{07103}$ allele on eye development, we generated homozygous cell clones by mitotic recombination (see Materials and Methods). Such clones, albeit small and recovered at low frequency, show loss of photoreceptors in many mutant ommatidia (Fig. 4a). All photoreceptors can be affected by loss of 14-3-3ζ function. Ommatidia lacking outer as well as inner photoreceptors can be found within $14\text{-}3\text{-}3\zeta^{07103}$ homozygous mutant tissue. This phenotype is reminiscent of clones homozygous for *Drosophila ras* or *raf* hypomorphic alleles in which photoreceptors of all classes are similarly affected (Simon et al. 1991; Dickson et al. 1992; Lu et al. 1994). The clonal phenotype argues for a function of *D14-3-3ζ* in the control of photoreceptor induction and/or differentiation. To further corroborate this conclusion and to confirm that the mutant phenotype is caused by a defect in photoreceptor differentiation, and is not an indirect consequence of poor cell proliferation, we performed an antisense experiment. For this, *D14-3-3ζ* antisense RNA was expressed from a transgene under the control of the *sevenless* enhancer that drives expression in postmitotic photoreceptor pre-

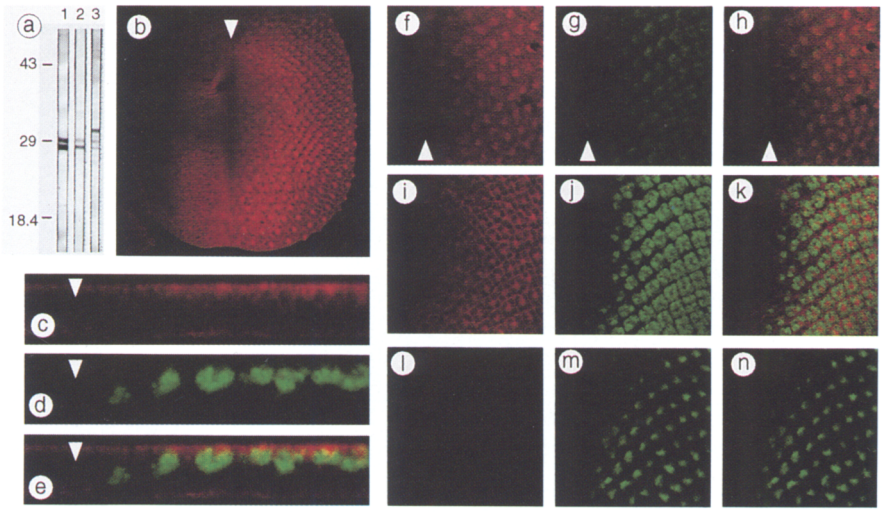

Figure 3. D14-3-3ζ is localized apically in differentiating photoreceptor cells. (*a*) Immunoblot of crude extracts from wild-type *Drosophila* larvae probed with anti D14-3-3ζ serum (strip 1 dilution, 1:1000; strip 2 dilution, 1:10,000). Strip 3 was probed with a commercial antibody raised against a conserved peptide in 14-3-3ζ (Santa Cruz, SC629). Note that the doublet at 29 kD recognized by the anti D14-3-3ζ serum is also stained by the anti-peptide antibody. Molecular sizes of protein standards in kilodaltons are indicated. (*b*) Third instar eye imaginal disc stained for D14-3-3ζ expression. The position of the morphogenetic furrow (MF) is indicated in this and the following panels by a white arrowhead. Anterior is to the *left*. (*c–n*) Confocal images of a third instar eye imaginal disc stained for D14-3-3ζ (visualized in red, *c,f,i,l*) and the neuronal nuclear marker ELAV (visualized in green, *d,g,j,m*). Overlaid images for both antigens are shown in *e,h,k,* and *n*. (*c–e*) A medial section along the apical–basolateral axis of the imaginal disc. Note the appearance of ELAV-positive nuclei posterior to the MF and their migration to the apical (*top*) side of the disc. Apical D14-3-3ζ staining increases posterior to the MF in neuronal cells (as verified by costaining with an anti-HRP antibody, data not shown). (*f–n*) Tangential sections through the apical surface of the eye disc (*f–h*) and progressively more basal sections at the level of the R1–R7 nuclei (*i–k*) and the R8 nuclei (*l–n*).

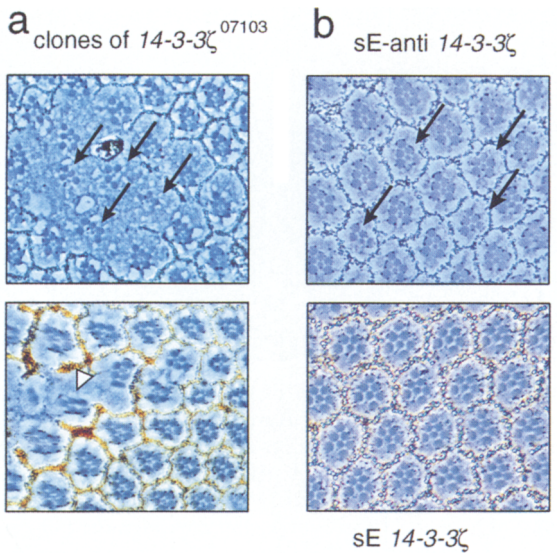

Figure 4. Reduced *D14-3-3ζ* function causes defects in photoreceptor differentiation. (*a*) Homozygous mutant clones of *D14-3-3ζ*^{07103}, marked by lack of pigment. Mutant ommatidia frequently lack outer photoreceptors (some indicated by arrows) and R7 cells (arrowhead) compared with the surrounding pigmented wild-type tissue. The differentiation of outer photoreceptor cells appears more sensitive to 14-3-3ζ antisense expression than that of R7 cells. The reasons for this are not clear. (*b*) Tangential section through the eye of a transgenic fly carrying two copies of *sE anti-D14-3-3ζ* (*top*). Ommatidia with reduced numbers of photoreceptors are indicated by arrows. The same phenotype was observed in both independent transgenic lines tested. The equivalent sense construct, *sE D14-3-3ζ*, did not cause any mutant eye phenotype in three independent lines (*bottom*).

cursor cells undergoing neuronal induction and differentiation. Consistent with the phenotype caused by the *14-3-3*^{07103} allele, the eyes of the fly strains expressing the antisense transcript display a weakly penetrant but reproducible loss-of-photoreceptor phenotype (Fig. 4b). Corresponding transgenic lines expressing the *D14-3-3ζ* sense transcript have a wild-type appearance (Fig. 4b, bottom). We therefore conclude that the lack of *D14-3-3ζ* activity directly interferes with photoreceptor differentiation.

Strong mutant alleles of D14-3-3ζ *cause cell lethality*

Because the 07103 P-element insertion in the first intron of *D14-3-3ζ* does not affect coding sequences and thus might not be a null mutation, we generated additional *D14-3-3ζ* mutant alleles by imprecise excision of the P-element (see Materials and Methods). In one of these alleles, *D14-3-3ζ*$^{E-16}$, both alternatively spliced first exons and the putative RNA initiation sites are deleted (Fig. 1). No *D14-3-3ζ* RNA could be detected by in situ hybridization on homozygous embryos (Fig. 5), indicating that *14-3-3*$^{E-16}$ represents a strong, possibly an RNA null allele. The lethality could be rescued by a transgene that carries 13.5 kb of genomic DNA spanning the *D14-3-3ζ* transcription unit and flanking sequences (data not shown). The rescue fragment was almost entirely sequenced and contains no other conspicuous transcription units in addition to *14-3-3ζ*. This result provides strong evidence that no other genes were affected by the E-16 mutation. In contrast to the case of *14-3-3ζ*^{07103} mutants (see above), the lethality of homozygous *14-3-3ζ*^{E16} alleles cannot be recued by ubiquitous expression of a *14-3-3ζ* cDNA. This finding may be explained by a requirement of all *14-3-3ζ* splice variants for viability.

As an initial attempt to genetically dissect potential regulatory interactions between components of the RTK–Ras–Raf signaling pathway and 14-3-3ζ, we tested whether the lack of one functional allele in 14-3-3ζ heterozygotes modifies the phenotypic effects of loss-of-function or gain-of-function alleles of components of the pathway, such as *sevenless, Sos, raf,* and *ras.* In none of the tested sensitized backgrounds were significant effects of 14-3-3ζ heterozygosity measurable. A possible explanation for this result is the relatively high expres-

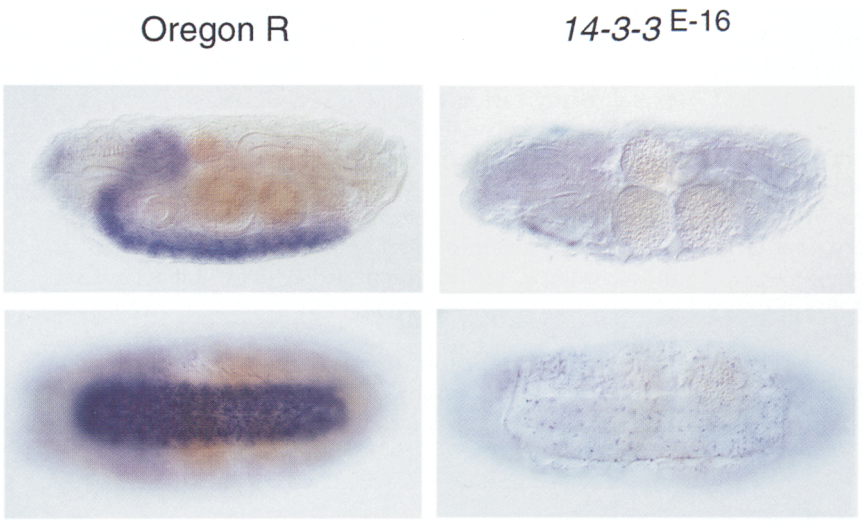

Figure 5. Lack of *D14-3-3ζ* mRNA in embryos carrying the *D14-3-3ζ*^{E16} mutant allele. D14-3-3ζ mRNA expression in terminally developed wild-type embryos and embryos homozygous for *D14-3-3ζ*^{E16} as revealed by whole-mount in situ hybridization. A probe common to all spliced forms of D14-3-3ζ was used. Orientation of embryos is anterior to the *left* and dorsal to the *top;* shown are lateral views (*top*) and ventral views (*bottom*).

sion levels of 14-3-3ζ in the developing eye that can be reduced by 50% without making this component limiting.

Attempts to generate mutant clones of cells that are homozygous for *14-3-3ζ*$^{E-16}$ failed, even though the mutant chromosome underwent somatic recombination, as monitored by the appearance of wild-type twin clones (data not shown). We conclude that lack of *D14-3-3ζ* is incompatible with cell proliferation or survival.

Activated alleles of raf rescue 14-3-3 deficiency

One of the functions of the Ras–Raf–MAP kinase pathway in higher eukaryotes is the control of cell proliferation (Simon et al. 1991; Dickson et al. 1992; Daum et al. 1994). To investigate whether the inability of *D14-3-3ζ*-deficient cells to grow was a consequence of defective Ras signaling, we attempted to rescue this defect by locally expressing activated versions of either Ras or Raf from transgenes driven by the *sevenless* enhancer. Such vectors direct expression predominantly in differentiating photoreceptor cells and ubiquitously at low levels (data not shown). In addition to showing that the lack of cell survival is indeed caused by defective Ras signaling, rescue of the 14-3-3-deficient mutant phenotype by activated Ras or Raf would place 14-3-3 in the signaling cascade.

ras^{Val12} encodes a variant of *Drosophila ras1* bearing the dominant activating Val12 mutation (Fortini et al. 1992). The *raf*^{torY9} allele encodes a gain-of-function version of Raf in which the amino terminus and the CR1 domain were replaced by the transmembrane domain of a constitutively active Torso mutant (Dickson et al. 1992). These gain-of-function alleles cause a characteristic mutant appearance of the eye attributable to the differentiation of ectopic photoreceptor cells (Dickson et al. 1992; Fortini et al. 1992). We induced somatic recombination to generate clones of cells that are homozygous for *D14-3-3ζ*$^{E-16}$ and contain one copy of either *ras*^{Val12} or *raf*^{torY9} (see Materials and Methods). Whereas no *D14-3-3ζ*-deficient clones were ever detected in the *ras*^{Val12} expressing eyes, clones containing ommatidia and photoreceptors were observed in eyes of flies carrying the activated Raf allele *raf*^{torY9} (Fig. 6). Thus, the artificial activation of Raf rescues the nonviability caused by a *D14-3-3ζ* mutation and permits photoreceptor development. These results indicate that *D14-3-3ζ* acts downstream of Ras and upstream of Raf in the signaling pathway that controls cell proliferation in the *Drosophila* eye imaginal disc. Because of the very disorganized appearance of the eye both in and outside of the clone, it was not possible to quantitatively evaluate the effect of *D14-3-3ζ*$^{E-16}$ on photoreceptor differentiation in the background of activated Raf. Importantly, however, in the rescued clonal area, inner as well as outer photoreceptors can be discerned. As in the surrounding 14-3-3ζ heterozygous tissue, *raf*^{torY9}-induced supernumerary R7 cells have differentiated inside the clone.

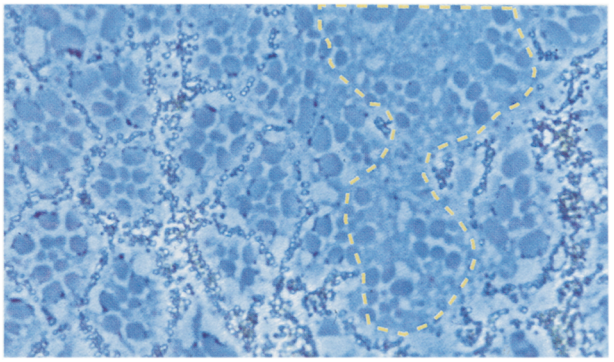

Figure 6. Tangential section through a clone of *D14-3-3ζ*^{E16} homozygous tissue in an eye expressing Raf^{torY9}. Viable clones of cells that are homozygous for *D14-3-3ζ*^{E16} can be obtained only in transgenic *Drosophila* strains expressing Raf^{torY9}. The mutant clone (within the dotted yellow line) is marked by the absence of pigment. In an equivalent experiment carried out in strains that express Ras^{Val12}, a constitutively active form of Ras1 (Fortini et al. 1992), no homozygous clones for *D14-3-3ζ*^{E16} were detected (data not shown). Clones of 14-3-3ζ homozygous tissue could be induced at a low but significant frequency (2 per 100 offspring from radiated F_0) when one copy of sE–*raf*^{torY9} was present in the fly stock. No clones were ever observed (0 per 100) in otherwise identical strains lacking the sE-*raf*^{torY9} transgene or carrying an sE–*ras*^{Val12} transgene.

Discussion

The serine/threonine kinase Raf relays information in a number of different signal transduction systems. Raf signaling is involved in the control of cell proliferation and transformation, as well as in the control of cell differentiation processes, for example, during the development of the *Drosophila* eye or the terminal structures of the early embryo (Ambrosio et al. 1989; Daum et al. 1994; Zipursky and Rubin 1994; Dickson et al. 1995). Here we provide evidence that *D14-3-3ζ* acts as an integral component of the Ras/Raf signaling pathway required for proper photoreceptor cell development in the *Drosophila* eye. Defects in the *Drosophila D14-3-3ζ* gene interfere with cell proliferation and photoreceptor differentiation that can be rescued in response to activated Raf but not by activated Ras. These findings are most easily explained by assuming that *D14-3-3ζ* activity is necessary for Raf activation by acting downstream of Ras. However, we cannot exclude more complicated scenarios in which 14-3-3 and Raf act in parallel pathways. In conjunction with previous work in which the functional relationship of Raf and 14-3-3 was examined in other systems, a requirement of 14-3-3 for Raf activation and the biological relevance of it is now firmly established.

The requirement for *D14-3-3ζ* is not restricted to eye development. The finding that a strong *D14-3-3ζ* mutation results in lethality indicates additional and essential functions for *D14-3-3ζ* that were not addressed by our analysis. Furthermore, a recent study showed that the

reduction of the *D14-3-3ζ* gene product, in this case termed Leonardo, decreases the ability of olfactory learning without affecting sensory modalities or brain anatomy that are a prerequisite for conditioning (Skoulakis and Davis 1996). This function of D14-3-3ζ is consistent with its role in protein kinase C-mediated processes because their disruption was also shown to result in learning and memory deficits (for a detailed discussion, see Skoulakis and Davis 1996). Our results put forward the possibility that D14-3-3ζ-mediated Ras/Raf signaling may participate in learning and memory processes. How does it come then, that the reduction of the *D14-3-3ζ* gene product in *leonardo* mutants does not cause proliferation or differentiation defects in the mushroom bodies (Skoulakis and Davis 1996)? *D14-3-3ζ* codes for an adult-specific 2.9-kb splicing variant that is strongly enriched or even exclusively expressed in the head (Swanson and Ganguly 1992). Thus, it might be possible that the different splice variants operate in different signal transduction pathways not necessarily linked to Raf function. Alternatively, the strong reduction of *D14-3-3ζ* expression in the mushroom bodies may affect only acquisition of memory, whereas low residual levels of D14-3-3ζ are sufficient to mediate the cellular aspects of differentiation and proliferation, processes that are severely affected in the lack-of-function mutation used in our study.

Although we were able to demonstrate that D14-3-3ζ is an integral component of Raf signaling required for cell differentiation and viability or proliferation in a multicellular organism, the exact molecular role of 14-3-3 in Ras signaling is still undetermined. Further genetic studies in *Drosophila* will help to elucidate the process of Raf activation and to distinguish between the possible different biological functions of 14-3-3. In this regard it should be useful that the *D14-3-3ζ* mutants presented here can be phenotypically rescued by transgenes constructed from 14-3-3 cDNA or genomic fragments. This provides an experimental basis for a detailed mutational analysis of 14-3-3 function in vivo and to establish whether the different isoforms that derive from splicing of specific protein domains participate in different biological pathways such as information processing and storage and cell differentiation and proliferation, respectively.

The biological role of 14-3-3 proteins in higher organisms is poorly understood. Recently, however 14-3-3 immunoreactivity in spinal fluid has been identified as a premortem diagnostic marker for bovine spongiform encephalopathy and Creutzfeld–Jacob disease (Hsich et al. 1996). Whether the appearance of 14-3-3 in spinal fluid is an indication for a direct role of 14-3-3 in the pathology of these neuro-degenerative diseases or whether this effect is indirect is not yet clear. Further information about the role of 14-3-3 in neuronal function and differentiation is required to answer this question. Studies in genetically accessible systems such as *Drosophila* might make valuable contributions toward this goal.

Materials and methods

P-element, cDNA, and genomic characterization

The P-element l(2)-07103 was obtained from the Berkeley *Drosophila* Genome Project as part of the Spradling collection. DNA adjacent to the integration site was isolated by plasmid rescue (Wilson et al. 1989) and by screening a *D. melanogaster* genomic library in λEMBL4 (generously provided by M. Noll). Using genomic probes, *D14-3-3ζ* cDNAs were isolated from embryonic libraries (Brown and Kafatos 1988). The breakpoint of $14\text{-}3\text{-}3^{E16}$ was cloned by PCR. The E-16 allele was generated by remobilization of the P-element l(2)07103 using standard methods (Robertson et al. 1988). Genomic and cDNA clones were sequenced. Complete genomic and cDNA sequences are accessible in the European Molecular Biology Labortory (EMBL) databank under accession no. Y12573.

Analysis of expression pattern

D14-3-3ζ antibodies were raised in rabbits against bacterially expressed histidine-tagged full-length D14-3-3ζ protein, which was purified using ProBond Resin (Invitrogen). The immunoblot was developed with a secondary antibody coupled to alkaline phosphatase. Antibody staining of eye imaginal discs was carried out by standard methods (Tomlinson and Ready 1987). Peripodial membranes of eye imaginal discs were removed. Embryonic lethal, abnormal vision (ELAV) was detected with a rat monoclonal antibody (gift from G. Rubin). Secondary antibodies were from Jackson labs. Optical sections of fluorescently labeled eye imaginal discs were obtained by using the EMBL confocal microscope. Pictures were processed with Adobe Photoshop. Homozygous mutant clones for *14-3-3* alleles were generated by irradiating first instar larvae with 1000 Rad. Preparation and microscopic analysis of eye sections were performed as described (Tomlinson and Ready 1987).

In situ hybridization of whole-mount embryos was performed according to Tautz and Pfeifle (1989) with a digoxigenin-labeled genomic fragment containing the coding region of all 14-3-3ζ splice variants. Similar results were obtained when the 1.8-kb cDNA was used. Embryos homozygous for the $14\text{-}3\text{-}3^{E16}$ allele were identified using a CyO balancer marked with *lacZ* expressed in the *hunchback* domain.

Transgenic fly strains

sE anti-14-3-3 and *sE 14-3-3* were generated by inserting a 1.0-kb cDNA (*Eco*RI–*Bgl*II) fragment spanning the complete *D14-3-3ζ* open reading frame into the P-element transformation vector KB 267 (Basler et al. 1991). For the cDNA derived rescue construct the same *Eco*RI–*Bgl*II fragment was cloned into a P-element expression vector driven by the ubiquitously active *armadillo* promoter (Vincent et al. 1994). The genomic rescue construct contained a 13.5-kb genomic DNA that spans the whole transcription unit coding for all splice variants and 1.7 kb of upstream and downstream sequences. Transgenic lines were obtained by standard procedures (Spradling and Rubin 1982). The genomic DNA contained in the rescue construct was sequenced except for 500 bp at the extreme 3′ end, and contains no apparent transcription units in addition to *14-3-3ζ*. One copy of this construct restored viability in $14\text{-}3\text{-}3\zeta^{E\text{-}16}$ homozygotes with normal Mendelian distribution. No phenotypic abnormalities were observed in the rescued lines.

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Note added in proof

A second *Drosophila 14-3-3* gene, *14-3-3ϵ*, has been identified by Chang and Rubin (this issue) in a screen for modifiers of activated Ras.

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