A role for Ebi in neuronal cell cycle control

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Mutations in *ebi* were isolated as enhancers of an over-proliferation phenotype generated by elevated E2F/DP activity in the *Drosophila* eye. *ebi* alleles also strongly suppress a phenotype caused by the cyclin-dependent kinase inhibitor p21, restoring S phases in the second mitotic wave of the developing eye disk. *ebi* mutant embryos display ectopic S phases within the peripheral nervous system and central nervous system at a time in development when neuronal precursor cells would normally begin to differentiate. Consistent with this, we find that *ebi* mutants have a reduced capacity to undergo neuronal differentiation, that Ebi physically interacts with Sina and phyllopod, and that Ebi promotes Ttk88 degradation *in vitro* and in S2 cells. Ectopic expression of Ttk88 inhibited differentiation in embryos and eye discs; however, this block to differentiation was insufficient to promote S phase entry in either of the situations where *ebi* mutations gave this effect. We conclude that Ebi has two distinct functions; it promotes the degradation of a repressor of neuronal differentiation (Ttk88), and has a second independent function that limits S phase entry.

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Introduction

The developing *Drosophila* eye has provided an extremely powerful model system for the study of pathways that control cellular differentiation. In particular, the role of the EGF receptor pathway in initiating photoreceptor differentiation has been analyzed in great detail. Following activation of receptor tyrosine kinases (RTKs), newly activated MAPK has been shown to phosphorylate Yan and pointed (Pnt), two ETS-domain transcription factors that together regulate a common set of critical target genes (Lai and Rubin, 1992; Brunner *et al.*, 1994; O'Neill *et al.*, 1994). A third ETS-transcription factor, Tramtrack (Ttk), functions downstream of Yan and Pnt to repress genes required for R7 differentiation (Lai *et al.*, 1996). In undifferentiated cells, Pnt is found in an inactive form whereas Yan and Ttk actively repress their target genes

(Lai and Rubin, 1992; Chang *et al.*, 1995; Dickson *et al.*, 1995). Phosphorylation of Yan blocks its ability to repress transcription and targets it for nuclear export and subsequent ubiquitin-dependent degradation (Rebay and Rubin, 1995). In contrast, phosphorylation of Pnt stimulates its ability to activate transcription of YAN/PNT target genes. One such transcriptional target is Phyllopod (Phyl). When expressed, Phyl and seven in absentia (Sina) form a complex that targets Ttk for degradation (Carthew and Rubin, 1990; Li *et al.*, 1997; Tang *et al.*, 1997). Therefore, prior to RTK activation, Yan and Ttk repress genes required for differentiation. Following activation, Yan and Ttk are targeted for degradation, allowing the initiation of the R7 cell fate.

Recently, Zipursky and co-workers have shown that Ebi is needed for EGFR-regulated differentiation in *Drosophila* (Dong *et al.*, 1999). Mutant alleles of *ebi* genetically interact with components of the EGFR pathway, and *ebi* mutant embryos possess many of the phenotypes previously described for *egfr* mutant embryos. Studies of Ebi function in the eye revealed that it is required for R7 differentiation. Ebi has been proposed to facilitate neuronal differentiation by promoting the down-regulation of TTK88 (Dong *et al.*, 1999).

Elimination of EGFR function in the developing eye disk has demonstrated that this RTK is also required for normal control of cell proliferation. Indeed, EGFR gain-of-function mutations cause excess proliferation and tissue growth in the disk (Baker *et al.*, 1992; Dominguez *et al.*, 1998). Since cell cycle exit and differentiation must be coordinated in order for the developmental program to proceed, it makes biological sense that the pathways controlling differentiation may also participate in the events that culminate in cell cycle withdrawal. Precisely how the EGFR pathway controls cell proliferation in some settings, and stimulates differentiation in others, remains unclear.

In *Drosophila*, S phase entry is severely reduced in both *cyclin E* and *de2f* mutant embryos (Knoblich *et al.*, 1994; Duronio *et al.*, 1995). Conversely, the ectopic expression of either cyclin E or dE2F/dDP, in either the embryo or imaginal disks, is sufficient to drive cells into S phase (Duronio and O'Farrell, 1994; Knoblich *et al.*, 1994; Richardson *et al.*, 1995; Asano *et al.*, 1996; Brook *et al.*, 1996). During normal development, the activities of cyclin E and dE2F/dDP are constrained, in part, by Dacapo (Dap) and the retinoblastoma homolog, Rbf. In several cell types Dap, a *Drosophila* homolog of the p21/p27 CDK inhibitors, is important for timely cell cycle exit. During embryogenesis, a pulse of Dap expression is needed for epidermal cells to introduce a G₁ phase following mitosis of cell cycle 16 (de Nooij *et al.*, 1996; Lane *et al.*, 1996). As a consequence, *dap* mutants fail to arrest the cell cycle and instead continue through a 17th

Fig. 1. Mutations in Ebi act as dominant enhancers of the GMR-dE2F-dDP-p35 rough eye phenotype. Scanning electron micrographs (SEMs) of adult eyes showing 200 and 600 $\times$ magnification of wild-type (A and D), GMR-dE2F-dDP-p35/+ (B and E) and GMR-dE2F-dDP-p35/ebi eyes (C and F). Note the general increase in roughness of the GMR-dE2F-dDP-p35/ebi eye, with a single allele of *ebi* removed (C and F), compared with GMR-dE2F-dDP-p35/+ (B and E). At the 600 $\times$ magnification, bristle cell duplications are evident in the GMR-dE2F-dDP-p35/ebi eye (F) when compared with GMR-dE2F-dDP-p35/+ (E).

cell cycle before arresting in G₁ of cell cycle 18. In contrast to *dap* mutants, Rbf-deficient embryos briefly arrest following mitosis of cell cycle 16, but fail to maintain the arrest and instead re-enter the cell cycle (Du and Dyson, 1999). These results suggest that Dap expression initiates the G₁ arrest by directly inhibiting cyclin E/Cdc2c, whereas Rbf functions to maintain the G₁ phase, possibly by limiting the expression of E2F targets such as cyclin E.

Here we describe observations that link *ebi* to cell cycle control and provide a different perspective on Ebi function to that described by Dong *et al.* (1999). We show that *ebi* mutations genetically interact with a number of key cell cycle regulators and demonstrate a requirement for Ebi in cell cycle exit during the developmental program. Although the inability to exit the cell cycle correlates with a reduction in the capacity to differentiate, our results suggest that an inability to degrade Ttk88 is not responsible for the cell cycle phenotypes of the *ebi* mutants. These results show that the loss of Ebi function perturbs both cell cycle exit and the onset of differentiation, indicating that Ebi is ideally situated to regulate these two processes coordinately.

Results

ebi alleles are dominant enhancers of the GMR-dE2F-dDP-p35 rough eye phenotype

Previously, we have used a dE2F/dDP overexpression phenotype in the *Drosophila* eye to screen randomly generated EMS and X-ray-induced mutations for alleles

that are modifiers of E2F activity (Staehling-Hampton *et al.*, 1999). As the initial screen was not saturated, we used the same GMR-dE2F-dDP-p35 chromosome to screen a P-element collection for additional alleles that are important for this E2F-dependent phenotype. From this screen, the P-element P[w+; LacZ]K16213 was isolated as a dominant enhancer of the GMR-dE2F-dDP-p35 phenotype (Figure 1). K16213 enhanced the general roughness of the eye, increasing the irregular arrangement of the ommatidia relative to the GMR-dE2F-dDP-p35 phenotype alone (Figure 1), and generating a large number of bristle duplications. K16213 is a lethal insertion that maps to position 21C on the left arm of chromosome II. K16213 was tested against mutant alleles that had been mapped to the 21C region and a complementation group of three alleles was found that failed to complement the P-element (CC1, CC3 and CC4). No escapers were observed from any of the possible trans-heterozygous combinations of K16213, CC1, CC3 and CC4, indicating that the mutated gene is essential for *Drosophila* development. Each of the three EMS alleles enhanced the GMR-dE2F-dDP-p35 to a similar degree to that observed for the P-element (data not shown).

Excision of the P-element from K16213 reverted the lethality associated with this chromosome and eliminated its ability to modify the GMR-dE2F-dDP-p35 phenotype (data not shown). Database searches using K16213 flanking sequence identified a putative open reading frame (ORF) that encodes a 700 amino acid protein with a C-terminal WD-repeat domain and a divergent N-terminal F-box domain possessing many, but not all

Fig. 2. The domain structure of Ebi and the location of the mutations identified in the four non-complementing lines. The top panel shows a schematic representation of the N-terminal divergent F-box and the C-terminal WD-repeats. The position and nature of the P-element, P[w+;LacZ] K16213, *ebi*^{CC1} (L16Q), *ebi*^{CC3} (S602F) and *ebi*^{CC4} (an 11 bp deletion at amino acid 313) are marked. A sequence alignment of the F-box domains of yeast Cdc4, human Skp2, *Drosophila* Slimb and human, *Drosophila* and yeast Ebi is shown in the bottom panel. Identical residues are marked by reverse shading and similar residues are shaded in gray. The stars mark the residues conserved between F-box domains (Bai *et al.*, 1996). The arrow marks the tryptophan residue (white residues on gray) that is conserved in Skp1 binding proteins (Cdc4, Skp2 and Slimb) but not in Ebi. The numbers indicate the amino acid positions in Cdc4.

of the residues present in bona fide F-box proteins such as Cdc4 (Figure 2). Sequencing of this ORF from flies carrying the CC1, CC3 and CC4 alleles identified distinct mutations corresponding to CC1 (L16Q), CC3 (S602F) and CC4 (an 11 bp deletion at amino acid 313) (Figure 2). During the course of our investigation, Dong *et al.* (1999) reported the isolation of mutations in this gene as dominant enhancers of the *roughex* mutation. Dong *et al.* (1999) have named this gene Ebi, and we, therefore, refer to the alleles described here as *ebi*^{K16213}, *ebi*^{CC1}, *ebi*^{CC3} and *ebi*^{CC4} (Figure 2).

Mutant alleles of *ebi* dominantly suppress the GMR-p21 rough eye and restore the second mitotic wave

As we had isolated *ebi* alleles as enhancers of a dE2F-dependent phenotype that is characterized by increased cell proliferation, we examined whether Ebi gene dosage could suppress eye phenotypes caused by a reduction in cell proliferation. de Nooij and Hariharan (1995) have shown that expression of the human cyclin-dependent kinase inhibitor p21 strongly inhibits S phase entry in the developing eye disk, giving a rough eye phenotype characterized by reduced numbers of pigment cells and fused ommatidia. The GMR-p21 phenotype is dominantly suppressed by mutations in Ebi (Figure 3). The number of ommatidia in the pGMR-p21/*ebi* adult eye (Figure 3C and F) are similar to wild type and, other than minor bristle defects, the pGMR-p21/*ebi* adult eye is morphologically normal (Figure 3; data not shown).

Wild-type eye disks exhibit a band of S phases, posterior to the morphogenetic furrow that constitutes

the second mitotic wave (Wolff and Ready, 1993). This wave of S phase is required to generate the full complement of cells that will be needed for subsequent differentiation events. GMR driven expression of p21 abolishes the second mitotic wave of S phases, causing the dramatic reduction in the number of cells observed in the adult eye (de Nooij and Hariharan, 1995; Figure 3). To examine the effect of reducing the dosage of Ebi on this process, 3rd instar larval eye disks were isolated from the various genotypes and S phase cells labeled by BrdU incorporation. Consistent with the adult phenotype, the second mitotic wave of S phase in pGMR-p21/*ebi* disks is partially restored and in some cases returns to levels similar to that observed in the wild type (Figure 3). Thus, the normal function of Ebi is necessary in these G₁ phase cells for p21 to block S phase entry efficiently.

***ebi* mutants display ectopic S phases in the PNS and CNS at stage 13/14 of embryogenesis**

The ability of *ebi* alleles to enhance an eye phenotype resulting from increased cell proliferation, and to suppress a phenotype resulting from reduced cell proliferation raised the possibility that Ebi might act to limit proliferation during normal development. To test this we examined whether loss of Ebi function would lead to inappropriate S phase entry. Stocks carrying the *ebi* alleles were intercrossed to generate trans-heterozygous mutants, and BrdU incorporation was used to determine the patterns of embryonic S phases (see Materials and methods). Following germ band retraction in wild-type embryos, cells within the peripheral nervous system (PNS) have completed S phase of cell cycle 16 and arrest in G₁ of cell

Fig. 3. *ebi* dominantly suppresses the GMR-p21 rough eye phenotype by restoring the second mitotic wave of S phases in the developing eye disk. Shown are SEMs of wild-type (A and D), GMR-p21/+ (B and E) and GMR-p21/ebi (C and F) eyes demonstrating that removal of one copy of *ebi* suppresses the GMR-p21 adult eye phenotype to approximately wild type. At the 600 $\times$ magnification, a small number of missing bristles are evident in the GMR-p21/ebi eye. (G, H and I) Shown are the S phase patterns in 3rd instar larval eye disks as determined by BrdU incorporation from wild-type (G), GMR-p21/+ (H) and GMR-p21/ebi (I) eye disks. In the wild-type eye disk (G), a star marks the second mitotic waves of S phases. Expression of p21 from the GMR promoter blocks the second mitotic wave in the GMR-p21/+ eye (H). In contrast, removal of one copy of *ebi* reduces the inhibitory effect of p21 and partially restores the second mitotic wave.

cycle 17. In contrast, cells within the fore and hindgut enter a period of endo-reduplication. In *ebi* mutants, the endocycles in the gut (consistent with stage 13 embryos) occur as normal (Figure 4). However, cells within the PNS fail to exit the cell cycle and continue into a 17th S phase (Figure 4). This PNS phenotype is very similar to that observed in *dap* mutant embryos, although *ebi* mutants do not display the ectopic S phases seen in the epidermis of *dap* mutant embryos (de Nooij *et al.*, 1996; Lane *et al.*, 1996). In addition to ectopic S phases in the PNS, *ebi* mutants display aberrant S phase entry within the central nervous system (CNS) (Figure 4). The ectopic S phases in the CNS are clearly visible as an expansion of the BrdU incorporation in the CNS of the mutant embryos relative to the incorporation observed in the wild type (Figure 4). The cell cycle defects observed in the *ebi* mutant could arise through a defect in cell cycle withdrawal or might reflect a

delay in the normal cell cycle program. It is not possible from the above observations to distinguish definitively between these two possible models. However, if the extra S phases we see in the *ebi* mutants occur due to a delay in the S phase program we would expect to see a reduction in the number of S phases in the mutant at earlier stages in development. To address this possibility we performed BrdU pulse labeling experiments on wild-type and *ebi* mutant embryos to analyze S phases at developmental times prior to stage 13/14. Consistent with a defect in cell cycle exit at later stages, we find no evidence of a reduction in the number of S phases in the *ebi* mutant at developmental times prior to stage 13/14, when compared with the wild type (data not shown). Thus, it seems most likely that the additional S phases observed in the *ebi* mutant arise from a defect in cell cycle exit and not from a delay in the S phase program.

Fig. 4. *Ebi* mutant embryos display ectopic S phases in the PNS and CNS at stage 13 of embryogenesis. Shown are the pattern of S phases determined by BrdU incorporation from wild type (A, C and E) and *ebi* mutant embryos (B, D and F). (A–D) show the pattern of S phases in wild type and *ebi* mutants following a 10 min BrdU pulse. (E and F) show the pattern of S phases following a 30 min BrdU pulse. In stage 13 wild-type embryos (A, C and E), cells within the PNS are not in S phase and consequently fail to incorporate BrdU. In contrast, a large number of S phases are evident within the PNS at this stage in the *ebi* mutants. The embryos shown are of an equivalent stage (stage 13) as determined by the characteristic S phase pattern of the endocycles in the fore and hindgut (E and F).

Fig. 5. *Ebi* mutants display defects in embryonic PNS development. Shown are wild-type (A and C) and *ebi* mutant embryos (B and D) stained with the monoclonal antibody Mab22C10. The severity of the phenotype varies and in a majority of cases only a small number of PNS neurons are missing, as had been reported for the *phyl* mutation.

The neuronal-specific antibody Mab22C10 (Zipursky *et al.*, 1984) was used to analyze the integrity of neuronal lineages in *ebi* mutants. Consistent with the cell cycle defects described, we observe a number of defects in neuronal differentiation including a reduction in peripheral neuron staining and an absence of a large number of Mab22C10-positive cells within the CNS (Figure 5). This phenotype has also been observed in *phyllopod* mutants (Chang *et al.*, 1995) and in embryos that over-express Ttk69 and Ttk88 from heat shock transgenes (Guo *et al.*, 1995).

One possible interpretation of the experiments described above is that Ebi has a function that limits S phase entry, and that the defects in neuronal differentiation are an indirect effect of a failure to arrest the cell cycle

completely. The observations of Dong *et al.* (1999), showing that Ebi regulates Ttk88 degradation, suggest an alternative interpretation. If the failure to degrade Ttk88 interferes with neuronal differentiation, then the additional S phases described above might reflect the continued proliferation of cells that have failed to differentiate, rather than a direct role in cell cycle control. A third possibility is that Ebi function is important for both cell cycle exit and for the onset of differentiation. We first looked for biochemical evidence to support the idea that Ebi promotes Ttk88 degradation and then, in order to distinguish between these models, we examined whether simply blocking neuronal differentiation by elevating Ttk88 was sufficient to cause the increase in S phase cells that occurs when Ebi levels are reduced.

Ebi promotes the degradation of Ttk88

We found three lines of evidence that link Ebi to Sina and Phyl-dependent degradation of Ttk88. The first line of evidence comes from transient transfection experiments. Previously, Li *et al.* (1997) have demonstrated that Sina and Phyl are able to target Ttk88 for ubiquitin-dependent degradation when expressed in transient transfection experiments in S2 cells. S2 cells contain high levels of endogenous Ebi (data not shown). To interfere with the activity of this protein, we expressed an N-terminal fragment (EbiN) that was previously used as a dominant-negative mutant to interfere with Ttk88 degradation in the eye disk (Dong *et al.*, 1999). Cells were co-transfected with pIZT vectors (Invitrogen; see Materials and methods) constitutively expressing either Cat or EbiN from the OpIE2 promotor, in the presence of metallothioneine-inducible vectors containing Phyl, Sina and Ttk88-myc (Tang *et al.*, 1997). Following transfection and copper induction, cells were metabolically labeled with [³⁵S]methionine and then chased with cold methionine for various time points to monitor Ttk88 degradation (Li *et al.*, 1997). As has been previously reported, in the presence of Sina and Phyl, Ttk88 is degraded with a half life of ~25 min, a process that can be blocked by the proteosome inhibitor MG132 (Li *et al.*, 1997; Figure 6). We observed that Ttk88 degradation was blocked in cells expressing the EbiN dominant-negative from pIZT, whereas a control pIZT vector that constitutively expressed Cat gave no effect (Figure 6A).

The second line of evidence stems from an *in vitro* degradation assay. To investigate whether Ebi is linked to Ttk88 degradation we attempted to reconstitute Ttk88 degradation *in vitro*. Initial experiments showed that Ttk88 was not degraded when co-expressed with Sina and Phyl in rabbit reticulocyte lysate (data not shown). In an attempt to stimulate the degradation activity, we incubated GST (glutathione *S*-transferase) or GST-Ebi beads together with ubiquitin, an ATP regeneration system and Sina-Phyl-Ttk co-expressed in reticulocyte lysate. Neither GST nor GST-Ebi was able to promote Ttk88 degradation in this setting (Figure 6B). We speculated that Ebi may function as a bridge between the Sina-Phyl-Ttk88 complex and an activity required for ubiquitylation and subsequent degradation of Ttk88. In order to provide this missing activity, we pre-incubated GST or GST-Ebi beads with S2 extracts (now referred to as loaded beads) prior to performing the degradation reaction. Loaded GST-Ebi beads were unable to degrade Ttk88 when expressed alone. However, when Sina and Phyl were co-translated with Ttk88, loaded GST-Ebi beads were able to promote degradation of Ttk88 by a mechanism that was blocked by the proteosome inhibitor, LLnL, whereas loaded GST beads could not (Figure 6B and C). The need for Sina and Phyl for Ttk degradation mirrors their requirement *in vivo* (Li *et al.*, 1997; Tang *et al.*, 1997). *In vitro*-translated dE2F, dDP or cyclin E was not degraded in any of the experiments described (Figure 6C; data not shown). In order to map the region of Ebi that associates with the activity required for Ttk88 degradation, we incubated loaded GST-EbiN and GST-EbiC beads and then performed the degradation assay. Interestingly, neither half of Ebi alone was capable of targeting Ttk88 for degradation (data not shown). These data suggest that the full-length

Fig. 6. Ebi promotes the ubiquitin-dependent degradation of Ttk88.

(A) Shown is a pulse-chase experiment in S2 cells over a 40 min time frame that monitors the ubiquitin-dependent degradation of Ttk88, which requires Sina and Phyl (see Materials and methods for details). In the presence of constitutively expressed Cat-HisV5, Ttk88 is degraded with a half life of ~25 min. Ttk88 degradation is inhibited by the proteosome inhibitor MG132. Expression of the dominant-negative N-terminal domain of Ebi (EbiN-HisV5) blocks this process resulting in the stabilization of Ttk88. (B and C) Ebi targets Ttk88 for ubiquitin-dependent degradation *in vitro* through a Sina and Phyl-dependent mechanism that requires an additional activity from S2 extracts. (B) GST-Ebi, but not GST alone, can associate with an activity from S2 extracts that is able to degrade Ttk88 in the presence of Sina and Phyl (lane 4), but not when Ttk88 is expressed alone (lane 2). (C) When GST-Ebi is pre-incubated with S2 extracts, Ebi is able to target Ttk88 for degradation (lane 5). Ttk88 degradation by Ebi can be blocked by incubating the reaction in the presence of the proteosome inhibitor, LLnL (lane 6). GST had no effect on Ttk88 (B, lanes 1 and 2; C, lanes 1–3).

Ebi protein may act to bring the Sina-Phyl-Ttk88 complex and a ubiquitylation activity into close proximity.

The results of the *in vitro* degradation assay suggested that Ebi might physically associate with Ttk88, Sina and Phyl. This was tested using the *in vitro* translated Sina, Phyl and Ttk88, and the GST-Ebi fusion proteins described above. GST-Ebi was found to interact strongly with Sina and Phyl, and weakly with Ttk88, when these proteins were expressed and assayed individually (Figure 7A). The GST control showed no association (Figure 7A). Interestingly, when Sina, Phyl and Ttk88 are

Fig. 7. Ebi can physically associate with Ttk88, Sina and Phyl through its C-terminal WD-repeat domain. (A) GST-Ebi, but not GST alone, is able to associate with Ttk88, Sina and Phyl expressed *in vitro*. Mapping of the interaction in Ebi shows that binding to Ttk88, Sina and Phyl occurs in the C-terminus (GST-EbiC). GST, GST-Ebi (full length), GST-EbiN (N-terminal fragment) and GST-EbiC (C-terminal fragment) fusion proteins are used. (B) When Ttk88, Sina and Phyl are co-translated, the Ebi-Ttk88 association is stimulated compared with when Ttk88 is expressed alone (A). I is 1/10th of the input into each pull down. (C) Ebi coprecipitates with Sina and Phyl from transfected S2 cells. Sina-HisV5 and/or Phyl-HisV5, in cell lysates from the indicated transfected populations, were purified by Ni-NTA beads and Ebi association was detected by western blotting with the monoclonal antibody 12E6.

co-expressed in the same lysate, GST-Ebi is able to pull down Ttk88 with a much higher affinity compared with when Ttk88 is expressed and bound alone (Figure 7B). This suggests that the Ebi-Ttk interaction is indirect and may require Sina and Phyl to facilitate association. To define the region of Ebi required for Sina, Phyl and Ttk association, we tested GST-EbiN (N-terminal domain) and GST-EbiC (C-terminal WD-repeat domain) fusion proteins for binding. GST-EbiN does not associate with any of the proteins, whereas GST-EbiC is able to bind to

all three proteins, although with a slightly reduced affinity when compared with the full-length protein (Figure 7A). Since the β -propeller structure formed by WD-repeat domains provides a surface for many protein-protein interactions, it is possible that this domain in Ebi provides a scaffold for Sina, Phyl and Ttk88 association. To provide further evidence for these interactions, we transiently co-transfected pIZT-Ebi with pIZT-V5His-Sina or pIZT-V5His-Phyl, or all three constructs into S2 cells. Sina and Phyl (His₆- and V5-tagged) were purified from lysates from the various transfected populations by Ni-NTA agarose chromatography. The beads were then subjected to western blotting using a monoclonal antibody to Ebi. Ebi was found to co-precipitate with Sina and Phyl but was not observed in the untransfected control (Figure 7C).

Increased Ttk levels do not phenocopy the effects of *ebi* alleles on S phase entry

If stabilization of Ttk88 is responsible for the cell cycle phenotypes associated with Ebi, we expected to observe a consistent pattern of genetic interactions between GMR-p21 or GMR-E2F-DP-p35 and mutations in the EGFR pathway that function to regulate Ttk88 levels. However, unlike mutations in Ebi, we found that loss-of-function alleles of *egfr*, *gap1*, *raf1*, *ras1*, *mapk*, *yan*, *sina*, *phyl* and *ttk* had no strong effect on either the GMR-p21 or the GMR-E2F-DP-p35 phenotype (see Materials and methods for details). Mutations in the Ets-domain transcription factor Pointed (*pnt*) enhanced the GMR-E2F-DP-p35 phenotype but failed to modify the GMR-p21 phenotype (Staehling-Hampton *et al.*, 1999). While it is possible that Ebi is the only dosage-sensitive component of the EGFR pathway whose levels effect cell proliferation, these results suggested that Ebi might have an activity that is independent of Ttk88 degradation.

To test directly whether stabilization of Ttk88 is likely to be responsible for the changes in cell cycle control caused by *ebi* mutant alleles, we examined the effects of elevating the levels of Ttk88. As previously described, ectopic expression of Ttk88 was induced by heat shock for 30 min at 39°C in embryos carrying a hs-Ttk88 (Guo *et al.*, 1995; see Materials and methods for details). Following recovery, embryos were either pulsed with BrdU or aged for immunohistochemistry. As previously reported, the ectopic expression of Ttk88 from a heat shock-regulated transgene is sufficient to disrupt neuronal differentiation in stage 13–14 embryos (Figure 8; Guo *et al.*, 1995). Unlike the *ebi* mutant embryos, BrdU incorporation demonstrated that the block to differentiation in hs-Ttk88 embryos does not result in a failure to exit the cell cycle in the PNS and CNS (Figure 8). Furthermore, expression of Ttk88 from a GMR transgene inhibits differentiation in the eye disk (Li *et al.*, 1997; Tang *et al.*, 1997; data not shown). However, the GMR-Ttk88 transgene failed to suppress the GMR-p21 eye phenotype and was unable to restore S phases in the GMR-p21 eye disk (Figure 9). In contrast, halving the dosage of Ebi or expressing cyclin E from the GMR promoter was sufficient to restore the second mitotic wave of S phases (Figure 9). We conclude that increasing Ttk88 protein to a level where differentiation is perturbed in either the embryo or the eye disk is insufficient to promote S phase entry in either of the situations where *ebi* mutations gave this effect (Figures 8 and 9). We infer

Fig. 8. Increased Ttk88 levels do not phenocopy the effects of *ebi* alleles on S phase entry. Heat shock-induced expression of Ttk88 from a heat shock-regulated transgene (hs-Ttk88) is sufficient to disrupt neuronal differentiation as determined by MAb22C10 staining (**D**) but failed to alter the normal pattern of S phases as determined by BrdU incorporation (**B**). In contrast, heat shock of wild-type embryos lacking the transgene displayed the normal pattern of neuronal differentiation (**C**) and proliferation (**A**).

that Ebi must have a second function, independent of Ttk88 degradation, that is important for regulating cell cycle exit.

Discussion

Modifier screens provide a relatively unbiased way to identify genes that are functionally important for a biological process. Mutant alleles of *ebi* were first isolated by Dong *et al.* (1999) as enhancers of a *roughex* (*rux*) mutation. Although Rux is known to act as a cyclin-dependent kinase inhibitor (Thomas *et al.*, 1994, 1997; Dong *et al.*, 1997; Foley *et al.*, 1999), Dong *et al.* (1999) noted that *ebi* mutations enhanced the differentiation defects of the *rux* phenotype rather than the earlier cell cycle defects. Consistent with this interpretation they observed that *ebi* mutations dominantly suppress differentiation phenotypes induced by constitutive activation of the EGFR pathway, and that Ebi regulates Ttk88 protein levels in the eye imaginal disk. Ttk88 is an ETS-domain transcription factor that functions to repress genes required for photoreceptor differentiation. The idea that Ebi normally functions during neuronal differentiation is further supported by the observation that the expression of neuronal markers is reduced in *ebi* mutant embryos.

In this study, we have independently isolated mutant alleles of *ebi* as dominant enhancers of a rough eye phenotype induced by elevated dE2F activity. We find that *ebi* alleles are dominant suppressors of the GMR-p21 phenotype, and that this suppression occurs because halving the gene dosage of Ebi reduces the ability of the p21 CDK inhibitor to block the second mitotic wave. The second mitotic wave represents the synchronous division of cells that have not yet become committed to a cell fate; therefore, neither the p21-induced arrest, nor its suppression by *ebi* alleles appears to be tied to neuronal differentiation. These results suggest that the function provided by Ebi is important for some aspects of cell cycle control. Consistent with this, we observed inappropriate BrdU incorporation in the PNS and ectopic BrdU incorporation in the CNS of *ebi* mutant embryos.

Fig. 9. Increased Ttk88 levels do not suppress the GMR-p21 phenotype. Ectopic expression of Ttk88 from the GMR promoter does not alter the pattern of S phases in the developing eye disk in either the presence (**E**) or absence (**B**) of GMR-p21 as compared with the wild type (**A**). In contrast, removing one copy of *ebi* (**D**) or increasing cyclin E levels (**F**; GMR-CycE) is sufficient to restore partially the second mitotic wave of S phases in the GMR-p21 eye disks as compared with the GMR-p21/+ eye disk alone (**C**). The S phase pattern in 3rd instar eye disks was determined by BrdU incorporation.

Although cell cycle exit and the induction of differentiation are tightly linked processes, it is evident that the role of Ebi in cell cycle control is distinct from its previously reported role in the regulation of Ttk88. Ectopic expression of Ttk88 is able to disrupt neuronal differentiation in both the embryo and the eye disk, yet

Ttk88 was insufficient to promote S phase entry or to suppress the p21-induced cell cycle arrest. Thus Ebi appears to have at least two distinct functions; Ebi promotes EGFR-induced down-regulation of Ttk88, and independently it also promotes G₁ arrest in certain cell types. In the light of these results, we suggest that, in some cell types, Ebi could serve to coordinate cell exit with the onset of differentiation.

What is the molecular basis of Ebi function? Closely related homologs of Ebi are found in diverse species. Two regions of these proteins that are most highly conserved, and that define this group of proteins, include an N-terminal domain that resembles an F-box sequence and the C-terminal domain that contains WD40-repeats. The conservation of a divergent F-box suggests that Ebi homologs may target associated proteins for degradation. If so, Ttk88 appears to be the most likely substrate. The expression of an N-terminal fragment of Ebi blocks the Sina-Phyllopod-induced degradation of Ttk88 in tissue culture cells and in eye imaginal disks, and the C-terminus of Ebi can physically interact with Sina-Phyl-Ttk88. Moreover, *in vitro* assays show that Ebi-associated proteins can promote the degradation of Ttk88. Since F-box proteins can target multiple substrates for degradation [for examples see Cdc4 (Drury *et al.*, 1997; Feldman *et al.*, 1997) and Slimb (Jiang and Struhl, 1998)], this would provide a simple model to explain how Ebi could independently regulate multiple processes. The results described here could be explained simply if Ebi targets Ttk88 and a positive cell cycle regulator such as dE2F or cyclin E (Clurman *et al.*, 1996; Marti *et al.*, 1999). However, we have been unable to detect a physical association between Ebi and either cyclin E or dE2F/DP using the *Drosophila* proteins, or their human homologs, in either *in vitro* assays or by transient transfection. In addition, GST-Ebi beads failed to stimulate the degradation of cyclin E, dE2F or dDP in the *in vitro* degradation assays described above. While the genetic interactions suggested that cyclin E and dE2F might be the most obvious candidates, it is clear that a more systematic approach is needed to identify a suitable cell cycle regulator.

Several observations suggest that Ebi may not act simply as a component of the conventional SCF complex. The N-terminal sequences of Ebi proteins contain many, but not all of the residues that are conserved in F-box proteins that interact with Skp1 (Figure 2). *Drosophila* Ebi binds very weakly to GST-Skp1, but we have been unable to recover Ebi-Skp1 complexes from transfected cells. The *Drosophila* genome contains at least nine genes that are related to Skp1 and it is possible that Ebi interacts with a Skp-like protein. It is intriguing that, in addition to the connections between Ebi and Ttk88, Ebi homologs have also been physically linked with transcriptional repressors in two other species. Sif2, the yeast ortholog of Ebi, has been found to interact with Sir4, a protein implicated in transcriptional silencing (Cockell *et al.*, 1998). The recent purification of the SMRT repressor complex revealed that it contains TBL1, a human homolog of Ebi (Guenther *et al.*, 2000). While the role of the Ebi-related proteins in these complexes is uncertain, these studies raise the possibility that Ebi's role in G₁ arrest may be attributable to its association with repressor complexes. One attractive

model, which combines all of these studies, is the idea that the divergent F-box in the N-terminus of Ebi recruits a specialized Skp-Cul complex to chromatin in order to selectively ubiquitylate repressor proteins. Clearly, further work is needed to test this and other models of Ebi function.

Materials and methods

Genetics and *Drosophila* strains

Flies were cultured as previously described (Staehling-Hampton *et al.*, 1999). *ebi* alleles (CC1, CC3 and CC4) were isolated following EMS mutagenesis by virtue of their failure to complement a deficiency that removes the 21C region. GMR-p21 flies were a gift from I. Hariharan. GMR-dE2F-dDP-p35 flies were described previously (Staehling-Hampton *et al.*, 1999). Sina alleles were kindly provided by A. Travers and M. Freeman. All other fly stocks used were obtained from the Bloomington Indiana stock center. GMR-p21 genetic interaction tests: genetic interactions were performed as described previously (Staehling-Hampton *et al.*, 1999). The following mutations in members of the EGFR and sevenless pathways were tested for their ability to modify the GMR-p21 and GMR-dE2F-dDP-p35 phenotypes: E(sev)3C^{E2F} (ras hypomorph); E(sev)3C^{elb} (ras hypomorph); Gap1^{A13D}; Yan^p; aop¹ (yan allele); Egfr E1 (ellipse); sev^{d2}; r1^{K162}; raf^{E110}; r1^{sem}; sina²; ttk¹. The four Ebi alleles (*ebi*^{K16213}, *ebi*^{CC1}, *ebi*^{CC3} and *ebi*^{CC4}) were balanced over a Cyo-wglacZ balancer. Trans-heterozygous *ebi* mutant embryos were identified by performing LacZ antibody staining in combination with either anti-BrdU or Mab22C10 antibodies. *ebi* mutants were unambiguously identified by a lack of the characteristic wingless pattern of LacZ expression.

Plasmids and molecular biology

Full-length Ebi cDNA was obtained from ATCC (Clone #: LD13813). Plasmid construction was performed by PCR using the following primers: (A) 5'-TGAGTTTTCAGCGACG-3' plus 5' *Eco*RI or *Bam*HI linkers; (B) 5'-TCAGAAATTGCGAGGTCC-3' plus 5' *Hind*III or *Spe*I linkers; (C) 5'-GAACAAGAGCTGCGGAGTC-3' plus 5' *Hind*III or *Spe*I linkers; (D) 5'-GGGACTCCCGCAGCTCTTGTTC-3' plus 5' *Eco*RI or *Bam*HI linkers. Full-length Ebi: PCR, primers A + B; EbiN: PCR, primers A + C; EbiC: PCR, primers B + D. GST-Ebi fusions were constructed in the pGEX-2T vector (Pharmacia Biotech) and were expressed in JM109 cells for 3 h at 30°C following induction. His₆-tagged Ebi fusion constructs were made in pQE-30 (Qiagen). S2 transfection constructs were made using the pIZT insect select system (Invitrogen). Protein expression and purification was performed following the manufacturer's instructions. Full-length His₆-Ebi was used to immunize mice for monoclonal antibody production. Hybridomas were screened against GST-EbiN and GST-EbiC fusion proteins by ELISA. Positive clones were screened for their ability to recognize endogenous Ebi on westerns from S2 and embryo extracts, to immunoprecipitate endogenous Ebi, to recognize Ebi in immunofluorescence in S2 cells and in immunohistochemistry in embryos. *In vitro* transcription/translation (pBS-SK+) and pIZT-His₆-V5 (Invitrogen) constructs for Ttk88-myc, Sina and Phyl were derived from pRmHa3 constructs kindly provided by A. Tang and G. Rubin (Tang *et al.* 1997).

BrdU analysis of eye imaginal disks

Isolation, preparation and analysis of eye imaginal disks were performed as described previously (Staehling-Hampton *et al.*, 1999). Briefly, eye disks from third instar larvae were dissected in Schneider's media and then incubated in BrdU (0.5 mg/ml in Schneider's) for 1 h. Disks were washed in Schneider's, washed in phosphate-buffered saline (PBS), and then fixed for 30 min in 4% formaldehyde in PEM buffer (100 mM PIPES pH 7.0, 2 mM EGTA and 1 mM MgSO₄). After fixation the disks were washed in PBS and 0.3% Triton X-100 for 10 min, PBS and 0.6% Triton X-100 for 30 min, and washed in methanol for 30 min. Disks were then re-hydrated through a methanol/PBS series and transferred to PBS, 0.3% Triton X-100, 2 M HCl for 30 min. The disks were washed twice in PBS for 10 min and then blocked for 30 min in PBS, 0.3% Triton X-100, 10% NGS. After blocking, the disks were incubated overnight at 4°C in a 1:100 dilution of mouse anti-BrdU antibody (Becton Dickinson) in PBS, 0.3% Triton X-100, 10% NGS. The disks were then washed in PBS and 0.3% Triton X-100, and the blocking step repeated. Secondary antibody incubations were performed at room temperature for 2 h using a 1:100

dilution of HRP-goat anti-mouse (Bio-Rad) in PBS, 0.3% Triton X-100, 10% NGS. The disks were washed in PBS and developed using diaminobenzidine (DAB).

Embryo analysis

For BrdU staining, staged embryos were harvested in 0.7% NaCl and 0.01% Triton X-100, washed extensively in dH₂O, then dechorionated in 50% Chlorox for 2 min. Embryos were permeabilized in octane for 6 min and then incubated in 1 mg/ml BrdU (Sigma) in Schneider's medium for 30 min. Embryos were fixed in an equal volume of 4% formaldehyde and heptane. BrdU incorporation was detected using a mouse anti-BrdU antibody (Becton Dickinson, 1:100). For hs-Ttk88 expression, embryos were collected, aged for 6 h at 25°C, then heat shocked for 30 min at 39°C. Following a 1 h recovery period at 25°C, embryos were either aged to stage 16 for immunohistochemistry with Mab22C10 antibody or pulsed with BrdU to determine the pattern of S phases. Wild-type flies were heat shocked in parallel for control. Antibody staining was performed as previously described (Guo *et al.*, 1995).

Binding assays

Radiolabeled *in vitro* translation products (TNT) were synthesized using the TNT-coupled transcription/translation system as per the manufacturer's instructions (Promega). Five hundred nanograms of the GST fusion protein on Sepharose beads were pre-incubated for 10 min at room temperature with 200 µl of Z' buffer [25 mM HEPES pH 7.5, 12.5 mM MgCl₂, 20% (v/v) glycerol, 0.1% NP-40, 150 mM KCl] with 1 µl of 200 mM dithiothreitol (DTT), 6 µl of 5 mg/ml bovine serum albumin and 50 µg/ml ethidium bromide. Two microliters of IVT product were added and the reaction incubated, with gentle rocking for 1 h at room temperature. Matrices were washed three times with 1 ml of NETN buffer (20 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40) without ethidium bromide prior to electrophoresis and autoradiography.

S2 cells were transiently transfected with pIZT-Ebi, pIZT-His₆-V5-Sina and/or pIZT-His₆-V5-Phyl constructs. Seventy-two hours post-transfection, cells were harvested and lysed in ELB250 [50 mM HEPES pH 7.0, 250 mM NaCl, 0.5% NP-40, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM NaF, 0.1 mM Na₂VO₃, 0.5 mM DTT and protease inhibitors] prior to clarification of extracts using a 42K spin. Extracts were incubated with Ni-NTA agarose beads for 30 min at 4°C before washing three times with ELB250. Proteins associated with the beads were subjected to electrophoresis and then western blotting with V5 (Invitrogen) and Ebi antibodies.

Pulse-chase experiments

Experiments were performed as previously described by Li *et al.* (1997). Briefly, S2 cells were transfected by CellFectin (Gibco-BRL) as per the manufacturer's instructions. Forty-eight hours post-transfection, cells were washed in Graces-M (methionine-free media) supplemented with 10% dialysed FBS (Gibco-BRL) and 0.7 mM CuSO₄. Cells were then incubated with 100 µCi of [³⁵S]methionine (Amersham) in 0.5 ml Graces-M with 10% FBS and 0.7 mM CuSO₄ for 4 h. The medium was then replaced with Graces plus 10% FBS, 0.7 mM CuSO₄ and 3 mg/ml methionine, and then cells were incubated for the indicated periods of time. Cells were harvested as per Li *et al.* (1997). Ttk88myc was then immunoprecipitated using 9E10 antibodies before SDS-PAGE and autoradiography.

In vitro degradation assays

Five hundred nanograms of GST fusion protein on Sepharose beads were incubated with S2 extracts for 1 h at 4°C. Matrices were washed three times with 1 ml of ELB250 before performing the degradation reaction. Reactions consisted of the GST fusion protein (with or without pre-incubation in S2 extracts), radiolabeled protein (1 µl of radiolabeled Ttk88-myc, Sina, Phyl co-expressed in the same TNT reaction, or 0.25 µl of Ttk88-myc expressed alone, per reaction), 6 µg of ubiquitin, 1.5 µl of 10× ATP regeneration system (20 mM HEPES pH 7.0, 10 mM ATP, 10 mM magnesium acetate, 300 mM creatine phosphate, 0.5 mg/ml creatine phosphokinase) and 1.5 µl of 10× reaction buffer (40 mM magnesium acetate, 10 mM DTT and 1 mM PMSF). The proteolysis inhibitor LLnL (Sigma), dissolved in dimethylsulfoxide (DMSO), was added to the reactions indicated to a final concentration of 50 µM. DMSO was added to reactions without LLnL as a control. The total reaction volume was 15 µl, not including the bead volume. Reactions were incubated at 30°C for 2 h prior to SDS-PAGE on 8% gels followed by fluorography and autoradiography.

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References

- Asano,M., Nevins,J.R. and Wharton,R.P. (1996) Ectopic E2F expression induces S phase and apoptosis in *Drosophila* imaginal discs. *Genes Dev.*, **10**, 1422–1432.
- Bai,C., Sen,P., Hofmann,K., Ma,L., Goebel,M., Harper,J.W. and Elledge,S.J. (1996) Skp1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. *Cell*, **86**, 263–274.
- Baker,N. and Rubin,G.M. (1992) Ellipse mutations in the *Drosophila* homologue of the EGF receptor affect pattern formation, cell division and cell death in eye imaginal discs. *Dev. Biol.*, **150**, 381–396.
- Brook,A., Xie,J.E., Du,W. and Dyson,N. (1996) Requirements for dE2F function in proliferating cells and in post-mitotic differentiating cells. *EMBO J.*, **15**, 3676–3683.
- Brunner,D., Ducker,K., Oellers,N., Hafen,E., Scholz,H. and Klammt,C. (1994) The ETS domain protein Pointed-P2 is a target of MAP kinase in the sevenless signal transduction pathway. *Nature*, **370**, 386–389.
- Carthew,R.W. and Rubin,G.M. (1990) *seven in absentia*, a gene required for specification of R7 cell fate in the *Drosophila* eye. *Cell*, **63**, 561–577.
- Chang,H.C., Solomon,N.M., Wasserman,D.A., Karim,F.D., Therrien,M., Rubin,G.M. and Wolff,T. (1995) *phyllpopod* functions in fate determination of a subset of photoreceptors in *Drosophila*. *Cell*, **80**, 463–472.
- Clurman,B.E., Sheaff,R.J., Thress,K., Groudine,M. and Roberts,J.M. (1996) Turnover of cyclin E by the ubiquitin-proteasome pathway is regulated by cdk2 binding and cyclin phosphorylation. *Genes Dev.*, **10**, 1979–1990.
- Cockell,M., Renauld,H., Watt,P. and Gasser,S.M. (1998) Sif2p interacts with Sir4p amino-terminal domain and antagonizes telomeric silencing in yeast. *Curr. Biol.*, **8**, 787–790.
- de Nooij,J.C. and Hariharan,I.K. (1995). Uncoupling cell fate determination from patterned cell division in the *Drosophila* eye. *Science*, **270**, 983–985.
- de Nooij,J.C., Letendre,M.A. and Hariharan,I. (1996) A cyclin-dependent kinase inhibitor, Dacapo, is necessary for timely exit from the cell cycle during *Drosophila* embryogenesis. *Cell*, **87**, 1237–1247.
- Dickson,B.J., Dominguez,M., Van der Straten,A. and Hafen,E. (1995) Control of *Drosophila* photoreceptor cell fates by Phyllopopod, a novel nuclear protein acting downstream of the Raf kinase. *Cell*, **80**, 453–462.
- Dominguez,M., Wasserman,J.D. and Freeman,M. (1998) Multiple function of the EGF receptor in *Drosophila* eye development. *Curr. Biol.*, **8**, 1039–1048.
- Dong,X., Zavitz,K.H., Thomas,B.J., Lin,M., Campbell,S. and Zipursky,S.L. (1997) Control of G₁ in the developing *Drosophila* eye: rca1 regulates Cyclin A. *Genes Dev.*, **11**, 94–105.
- Dong,X., Tsuda,L., Zavitz,K.H., Lin,M., Li,S., Carthew,R.W. and Zipursky,S.L. (1999) *ebi* regulates epidermal growth factor receptor signaling pathways in *Drosophila*. *Genes Dev.*, **13**, 954–965.
- Drury,L.S., Perkins,G. and Diffley,J.F. (1997) The Cdc4/34/53 pathway targets Cdc6p for proteolysis in budding yeast. *EMBO J.*, **16**, 5966–5976.
- Du,W. and Dyson,N. (1999) The role of RBF in the introduction of G₁ regulation during *Drosophila* embryogenesis. *EMBO J.*, **18**, 916–925.
- Duronio,R.J. and O'Farrell,P.H. (1994) Developmental control of a G₁–S transcriptional program in *Drosophila*. *Development*, **120**, 1503–1515.
- Duronio,R.J., O'Farrell,P.H., Xie,J.E., Brook,A. and Dyson,N. (1995) The transcription factor E2F is required for S phase during *Drosophila* embryogenesis. *Genes Dev.*, **9**, 1445–1455.
- Feldman,R.M., Correll,C.C., Kaplan,K.B. and Deshaies,R.J. (1997) A

- complex of Cdc4p, Skp1p and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. *Cell*, **91**, 221–230.
- Foley,E., O'Farrell,P.H. and Sprenger,F. (1999) Rux is a cyclin-dependent kinase inhibitor (CKI) specific for mitotic cyclin–Cdk complexes. *Curr. Biol.*, **9**, 1392–1402.
- Guenther,M.G., Lane,W.S., Fischle,W., Verdin,E., Lazar,M.A. and Shiekhattar,R. (2000) A core SMRT corepressor complex containing HDAC3 and TBL1, a WD40-repeat protein linked to deafness. *Genes Dev.*, **14**, 1048–1057.
- Guo,M., Bier,E., Jan,L.Y. and Jan,Y.N. (1995) *tramtrack* acts downstream of *numb* to specify distinct cell fates during asymmetric cell divisions in the *Drosophila* PNS. *Neuron*, **14**, 913–925.
- Jiang,J. and Struhl,G. (1998) Regulation of the Hedgehog and wingless signaling pathways by the F-box/WD40 repeat protein Slimb. *Nature*, **391**, 493–496.
- Knoblich,J.A., Sauer,K., Jones,L., Richardson,H., Saint,R. and Lehner,C.F. (1994) Cyclin E controls S phase progression and its down-regulation during *Drosophila* embryogenesis is required for the arrest of proliferation. *Cell*, **77**, 107–120.
- Lai,Z.C. and Rubin,G.M. (1992) Negative control of photoreceptor development in *Drosophila* by the product of the *yan* gene, an ETS domain protein. *Cell*, **70**, 609–620.
- Lai,Z.C., Harrison,S.D., Karim,F., Li,Y. and Rubin,G.M. (1996) Loss of *tramtrack* gene activity results in ectopic R7 cell formation, even in a *sina* mutant background. *Proc. Natl Acad. Sci. USA*, **93**, 5025–5030.
- Lane,M.E., Sauer,K., Wallace,K., Jan,N.Y., Lehner,C.F. and Vaessin,H. (1996) Dacapo, a cyclin-dependent kinase inhibitor, stops cell proliferation during *Drosophila* development. *Cell*, **87**, 1225–1235.
- Li,S., Li,Y., Carthew,R.W. and Lai,Z.C. (1997) Photoreceptor cell differentiation requires regulated proteolysis of the transcriptional repressor Tramtrack. *Cell*, **90**, 469–478.
- Marti,A., Wirbelauer,C., Scheffner,M. and Krek,W. (1999) Interaction between ubiquitin-protein ligase SCF-Skp2 and E2F-1 underlies the regulation of E2F-1 degradation. *Nature Cell Biol.*, **1**, 14–19.
- O'Neill,E.M., Rebay,I., Tijan,R. and Rubin,G.M. (1994) The activities of two Ets-related transcription factors required for *Drosophila* eye development are modulated by the Ras/MAPK pathway. *Cell*, **78**, 137–147.
- Rebay,I. and Rubin,G.M. (1995). *Yan* functions as a general inhibitor of differentiation and is negatively regulated by activation of the Ras1/MAPK pathway. *Cell*, **81**, 857–866.
- Richardson,H., O'Keefe,L.V., Marty,T. and Saint,R. (1995) Ectopic cyclin E expression induces premature entry into S phase and disrupts pattern formation in the *Drosophila* eye imaginal disc. *Development*, **121**, 3371–3379.
- Staehling-Hampton,K., Ciampa,P.J., Brook,A. and Dyson,N. (1999) A genetic screen for modifiers of E2F in *Drosophila melanogaster*. *Genetics*, **153**, 275–287.
- Tang,A.H., Neufeld,T.P., Kwan,E. and Rubin,G.M. (1997) PHYL acts to down regulate TTK88, a transcriptional repressor of neuronal cell fates, by a SINA-dependent mechanism. *Cell*, **90**, 459–467.
- Thomas,B.J., Gunning, D.A., Cho,J. and Zipursky,L. (1994) Cell cycle progression in the developing *Drosophila* eye: roughex encodes a novel protein required for the establishment of G₁. *Cell*, **77**, 1003–1014.
- Thomas,B.J., Zavitz,K.H., Dong,X., Lane,M.E., Weigmann,K., Finley,R.L., Brent,R., Lehner,C.F. and Zipursky,S.L. (1997) Roughex down-regulates G₂ cyclins in G₁. *Genes Dev.*, **11**, 1289–1298.
- Wolff,T. and Ready,D.F. (1993) Pattern formation in the *Drosophila* retina. In Bate,M. and Martinez Arias,A. (eds), *The Development of Drosophila melanogaster*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 1277–1326.

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