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Synthetic Lethality of Retinoblastoma Mutant Cells in the Drosophila Eye by Mutation of a Novel Peptidyl Prolyl Isomerase Gene

Kyle A. Edgar,*,[1] Marcia Belvin,*,[1,2,3] Annette L. Parks,*,[4] Kellie Whittaker,* Matt B. Mahoney,*,[5] Monique Nicoll,* Christopher C. Park,*,[6] Christopher G. Winter,* Feng Chen,*,[7] Kim Lickteig,*,[8] Ferhad Ahmad,* Hanife Esengil,*,[9] Matthew V. Lorenzi,† Amanda Norton,*,[10] Brent A. Rupnow,† Laleh Shayesteh,* Mariano Tabios,* Lynn M. Young,*,[11] Pamela M. Carroll,‡,[12] Casey Kopczynski,*,[13] Gregory D. Plowman,*,[3] Lori S. Friedman*,[3] and Helen L. Francis-Lang*

**Exelixis, South San Francisco, California 94083 and †Oncology Drug Discovery and ‡Applied Genomics, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey 08543*

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ABSTRACT

Mutations that inactivate the retinoblastoma (Rb) pathway are common in human tumors. Such mutations promote tumor growth by deregulating the G1 cell cycle checkpoint. However, uncontrolled cell cycle progression can also produce new liabilities for cell survival. To uncover such liabilities in Rb mutant cells, we performed a clonal screen in the Drosophila eye to identify second-site mutations that eliminate Rbf^- cells, but allow Rbf^+ cells to survive. Here we report the identification of a mutation in a novel highly conserved peptidyl prolyl isomerase (PPIase) that selectively eliminates Rbf^- cells from the Drosophila eye.

An important goal of novel cancer therapy is to elicit the death of mutant tumor cells in the patient, while allowing normal cells to survive. The identification of gene products required for tumor cell survival can provide highly validated drug targets for the development of therapeutic inhibitors. Ideally, targets could be identified that would kill cancer cells while sparing normal cells. A synthetic lethal screen is one method of identifying such targets. In this type of screen, cells are genetically altered to model tumor cells and one then screens for mutations that eliminate the model tumor cells but have little or no effect on wild-type cells.

One way to model tumor cells is to functionally inactivate the RB1 gene. In addition to being mutated in retinoblastomas, where it was initially discovered, RB1 is mutated in many other cancers including prostate (Kubota *et al.* 1995), bladder (Miyamoto *et al.* 1995), parathyroid (Cryns *et al.* 1994), and 90% of small cell lung cancers (SCLCs) (Minna *et al.* 2002). RB1 is also functionally inactivated in tumors that do not harbor mutations in the RB1 locus itself, but do carry mutations that target the pathway through the loss of cyclin-dependent kinase (Cdk) inhibitors or overexpression of Cyclin D1 or Cdk4 (reviewed in Sherr and McCormick 2002). Additionally, the transforming activities of DNA tumor virus oncoproteins are mediated via their interaction with RB1 (Helt and Galloway 2003).

The RB1 protein acts as a critical regulator of G1/S phase progression by binding to members of the E2F family of transcription factors (Dyson 1998; Nevins 2001). E2F-RB1 complexes prevent entry into S phase by actively repressing transcription through the recruitment of histone deacetylases and other chromatin modifiers to E2F-responsive promoters (Harbour and Dean 2000; Ogawa *et al.* 2002). Progression from G1 through S phase occurs when RB1 is inactivated through phosphorylation by the Cdk complexes Cyclin D/Cdk4 or Cyclin D/Cdk6 and Cyclin E/Cdk2 (Lundberg and Weinberg 1998). Phosphorylation relieves transcrip-

[1]These authors contributed equally to this work.

[2]*Corresponding author:* Genentech, 1 DNA Way, Bldg. 11, MS215, South San Francisco, CA 94080. E-mail: mbelvin@gene.com

[3]*Present address:* Genentech, South San Francisco, CA 94080.

[4]*Present address:* Department of Biology, Boston College, Chestnut Hill, MA 02467.

[5]*Present address:* EnVivo Pharmaceuticals, Watertown, MA 02472.

[6]*Present address:* Department of Molecular and Medical Pharmacology, UCLA School of Medicine, University of California, Los Angeles, CA 90095.

[7]*Present address:* DOE Joint Genome Institute, Walnut Creek, CA 94598.

[8]*Present address:* Celera Genomics, South San Francisco, CA 94080.

[9]*Present address:* Department of Molecular Pharmacology, Stanford University, Stanford, CA 94305.

[10]*Present address:* Pediatrics Department, University of California, UCSF School of Medicine, San Francisco, CA 94143.

[11]*Present address:* Institute for Genomic Research, Rockville, MD 20850.

[12]*Present address:* Merck Research Laboratories, Boston, MA 02115.

[13]*Present address:* Biotech Initiative, Chapel Hill, NC 27516.

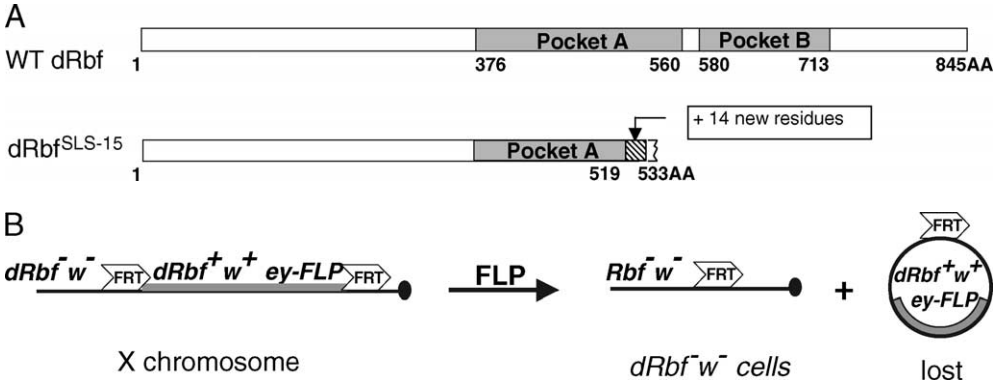

FIGURE 1.—Schematics of the Rbf protein and Rbf rescue construct. (A) Diagram of the wild-type Rbf and $Rbf^{SLS\text{-}15}$ mutant proteins. The mutation analysis of the $Rbf^{SLS\text{-}15}$ transcripts revealed an 11-bp deletion resulting in a frameshift at amino acid residue 519, followed by the addition of 14 novel residues and truncation of the Rbf protein at residue 533. The truncated protein lacks Pocket B, a highly conserved RBF domain that is required for interactions with partner proteins and the execution of RBF function. (B) Diagram of the Rbf rescue construct and Rbf^- clone generation. The $Rbf^{SLS\text{-}15}$ mutation combined with a Rbf rescue construct allows for the generation of Rbf^- clones specifically in the eye, due to eye-specific FLP expression followed by recombination between the FRT sites and subsequent loss of the Rbf^+ and w^+ genes. All other tissues, which do not express FLP, remain Rbf^+, resulting in a rescue of the organismal lethality normally associated with *Rbf*-deficient flies.

tional repression and allows E2F-dependent transcription of target genes required for S phase progression, such as Cyclin E (MORRIS *et al.* 2000) as well as enzymes required for DNA synthesis and metabolism (STEVAUX and DYSON 2002). In addition to its effects on cell proliferation, loss of RB1 predisposes cells to apoptosis through the actions of E2F on p53 (reviewed in CHAU and WANG 2003), thereby creating a selective pressure for tumors to accumulate mutations in p53.

Components of the RB1 pathway are being investigated as potential anticancer targets. These include the upstream kinases, Cdk2, Cdk4, and Cdk6, and the downstream effector of retinoblastoma (Rb), E2F (MCLAUGHLIN *et al.* 2003; VERMEULEN *et al.* 2003). These targeted approaches could lead to therapies with an improved profile of efficacy *vs.* toxicity compared to conventional treatment. It would also be of interest to identify novel targets involved in RB1 biology, especially those necessary for the viability of cells mutant for RB1. We therefore carried out a synthetic lethal screen in Drosophila to look for RB1-interacting genes.

Like its mammalian counterpart, Drosophila Rbf (CG7413) binds to E2F1 and regulates E2F target gene expression (DU *et al.* 1996; DU and DYSON 1999; DATAR *et al.* 2000; DICK and DYSON 2003) and is regulated by the Cdk complexes Cyclin D/Cdk4 and Cyclin E/Cdc2c (XIN *et al.* 2002), indicating that the function of RB1 is conserved between Drosophila and mammals.

To identify novel therapeutic targets in the RB1 pathway, we performed a synthetic lethal genetic screen in Drosophila to identify recessive mutations that result in the loss of cells that lack dRB1 (Rbf^-), but allow wild-type cells (Rbf^+) to survive. The synthetic lethal approach is commonplace in unicellular organisms such as yeast, where synthetic lethality is scored via organismal death. In multicellular organisms, however, synthetic lethality cannot be scored simply by organismal lethality, because desired mutations may cause organismal lethality on their own due to their function in essential tissues or cell types. An additional complication in the case of *Rbf* is that it itself is required for embryonic survival. To circumvent this issue, we generated mosaic animals that carry clones of Rbf^- tissue in the eye, whereas the rest of the animal is Rbf^+. We then generated overlapping clones of homozygous induced mutations in the eye and screened for potential synthetic lethality by scoring for the absence of clones carrying both the induced mutation and the Rbf^- mutation. We report the identification of a mutation in a novel highly conserved peptidyl prolyl isomerase that preferentially eliminates *Rbf* mutant cells.

MATERIALS AND METHODS

Drosophila stocks and handling: All fly stocks and crosses were handled using standard procedures at 25°. *Rbf* alleles and rescue lines used in this study have been deposited at the Bloomington Drosophila Stock Center. $Rbf^{SLS\text{-}15}$ (Figure 1A) was generated in a suppressor screen as being able to reverse the G1 arrest conferred by the overexpression of human p21 in the Drosophila eye (data not shown). *PExp{FRT2.1 [Rbf⁺, w+, 3.5ey-FLP]}* was inserted on the X chromosome and recombined onto the $Rbf^{SLS\text{-}15}$ chromosome to rescue the embryonic lethal phenotype while generating Rbf^- cells in the eye. The subsequent $Rbf^{SLS\text{-}15}$, *PExp{FRT2.1[Rbf⁺, w⁺, 3.5ey-FLP]}* chromosome was crossed to *Minute*-FRT, w^+ lines for each individual chromosome arm (MFRT2R, MFRT2L, MFRT3R, and MFRT3L) to generate the female "screening stocks" (RbfSS2R, RbfSS2L, RbfSS3R, and RbfSS3L) that allowed the generation of marked homozygous clones in a single generation (full stock genotypes used in the screen are provided in Table 1). The screening males used in mutagenesis were constructed by recombining an unmarked isogenic chromosome arm onto each FRT arm to facilitate the creation of *w* homozygous clones when crossed to screening stock females. This was done by recombining a *P*-element insertion from the Exelixis collection, which was inserted in an isogenic chromosome just proximal to the FRT, onto [P-*ry* FRT]. The presence of the *P* element was identified using w^+, and the presence of the FRT was monitored by PCR using primers Neo2F (ATC

TABLE 1

List of strains constructed for use in dRbf synthetic lethal screen

Stock description	Abbreviation	Genotype
dRbf alleles	CAS-21	*Su(p21)CAS-21/FM7c; +/CyO, P{p21-pGMR-33B}*
	SLS-15	*w, Su(p21)SLS-15, P{ry[+t7.2] = neopFRT}18A/FM7c, ftz lacZ*
Rescue construct	RbfR	*y, w, P{dRbf-pExP-FRT2.1-3.5ey-FLP-5}/FM7c*
Rescued dRbf alleles	CAS-21+dRbR	*w, Su(p21)CAS-21, PExp{FRT2.1[dRbf$^+$, w$^+$, 3.5ey-FLP]}; sp; e*
	SLS-15+dRbfR	*w, Su(p21)S* RbfSS2L *LS-15, PExp{FRT2.1[dRbf$^+$, w$^+$, 3.5ey-FLP]}; sp; e*
dRbf screening stocks	RbfSS2L	*w, Su(p21)SLS-15, PExp{FRT2.1[dRbf$^+$, w$^+$, 3.5ey-FLP]}; M(2)24F[1]P{w[+mC] = piM}36F P{ry[+t7.2] = neoFRT}40A/CyO*
	RbfSS2R	*w, Su(p21)SLS-15, PExp{FRT2.1[dRbf$^+$, w$^+$, 3.5ey-FLP]}; P{ry[+t7.2] = neoFRT}42D P{w[+mC] = piM}45F M(2)53[1]/CyO*
	RbfSS3R	*w, Su(p21)SLS-15, PExp{FRT2.1[dRbf$^+$, w$^+$, 3.5ey-FLP]}; P{ry[+t7.2] = neoFRT}82B P{w[+mC] = piM}87E RpS3[*]/TM6B, Tb[1]*
	RbfSS3L	*w, Su(p21)SLS-15, PExp{FRT2.1[dRbf$^+$, w$^+$, 3.5ey-FLP]}; Dp(1;3)sc[j4], y$^+$, P{w$^+$}, M(3)67C, pi75c, P[ry$^+$, hs-neo, FRT]80B; ry/TM6B*
Male stocks	IsoFS2L	*iso2 P{ry[+t7.2] = neoFRT}40A; P{ry[+7.2] = ey-FLP.N}6, ry[506]*
	IsoFS2R	*P{ry[+t7.2] = neoFRT}42D iso2; P{ry[+7.2] = ey-FLP.N}6, ry[506]*
	IsoFS3L	*P{ry[+7.2] = ey-FLP.N}5/CyO; iso3 P[ry+, hs-neo, FRT]80B/TM6B*
	IsoFS3R	*P{ry[+7.2] = ey-FLP.N}5; P{ry[+t7.2] = neoFRT}82B iso3*
dRbf$^+$ MFRT lines for counterscreen	MFRT2L	*w[*]; M(2)24F[1] P{w[+mC] = piM}36F P{ry[+t7.2] = neoFRT}40A/CyO*
	MFRT2R	*w[*]; P{ry[+t7.2] = neoFRT}42D P{w[+mC] = piM}45F M(2)53[1]/CyO*
	MFRT3L	*yw; Dp(1;3)sc[j4], y$^+$, P{w$^+$}, M(3)67C, pi75c, P[ry$^+$, hs-neo, FRT]80B; ry/TM6B*
	MFRT3R	*w[*]; P{ry[+t7.2] = neoFRT}82B P{w[+mC] = piM}87E RpS3[*]/TM6B, Tb[1]*
ey-flp lines	EFL2	*P{ry[+t7.2] = ey-FLP.N}6, ry[506]*
	EFL3	*P{ry[+t7.2] = ey-FLP.N}5, ry[506]*
CyO-GFP source	CyO-GFP	*w; L[2] Pin[1]/CyO, P{w[+mC] = GAL4-Kr.C}DC3, P{w[+mC] = UAS$^-$ GFP.S65T}DC7*
KE1 alleles	KE1-1	*yw, FRT(2R)KE1-1/CyO*
	KE1-2	*yw, FRT(2R)KE1-2/CyO*

TGGACGAAGAGCATCAGGG) and Neo2Ra (CGATACCG TAAAGCACGAGGAAG). The isogenic arm was then recombined onto the FRT line by monitoring the absence of w^+ and the presence of the FRT by PCR. Males also carried an exogenous source of *ey*-FLP on the non-FRT autosome to create more robust homozygous clones than those produced by the *PExp{FRT2.1[Rbf$^+$, w$^+$, 3.5ey-FLP]}* construct alone.

Primary genetic screen: Males were mutagenized by feeding them 5 mM EMS for 20–24 hr (in a 1% sucrose solution) after a 4-hr starvation period. Batches of 40 mutagenized males were mated to 30–50 virgin females (Figure 2A). The low EMS concentration was determined to induce only 0.8 lethal mutations per autosomal arm, which was essential to the success of identifying synthetic loci, since any additional mutations that caused cell lethality would have led us to discard the hit during the counterscreen. The mutagenesis rates for each round were confirmed by monitoring the segregation of X-linked lethals in the F_1 generation: these were 2L = 0.289, 2R = 0.289, 3L = 0.141, and 3R = 0.221, respectively. Additional mutagenesis was performed via gamma-ray irradiation at 1.625 krad using a Cobalt-60 source Gammacell 220 Irradiator. Crosses were flipped daily for 3 consecutive days. Progeny were scored for the absence of *w* tissue in the eye, leaving the w^+ (*Minute*) tissue to populate the eye. Candidate mutations that resulted in the elimination of 90% of the *w* tissue were selected for further testing and crossed to balancer stocks. Five of the resulting progeny were subsequently retested to ensure the passage of the mutation and the validity of the phenotype.

Counterscreen: Individual modifiers were subsequently mated to a corresponding counterscreen stock (*Rbf$^+$, Minute-FRT, w$^+$* lines: MFRT2R, MFRT2L, MFRT3R, and MFRT3L) and assayed for *w* tissue viability in the eye to demonstrate a specific interaction dependent on Rbf$^-$ (Figure 2B). Confirmed synthetic modifiers were stocked over *CyO* or *TM6B* balancer chromosomes.

Genetic mapping of modifiers: Only synthetic lethal modifiers that were also homozygous organismal lethal were mapped. Recombination mapping of the synthetic lethal phenotype was conducted using $al^1\ dp^{ov1}\ b^1\ pr^1\ cn^1\ c^1\ px^1\ sp^1$ for hits on the second chromosome or $ru^1\ h^1\ th^1\ st^1\ cu^1\ sr^1\ e^s\ ca^1$ for hits on the third chromosome and selecting for recombinants that retained a FRT. A copy of *ey*-FLP (EFL2 or EFL3) was crossed in and recombinants were scored for organismal

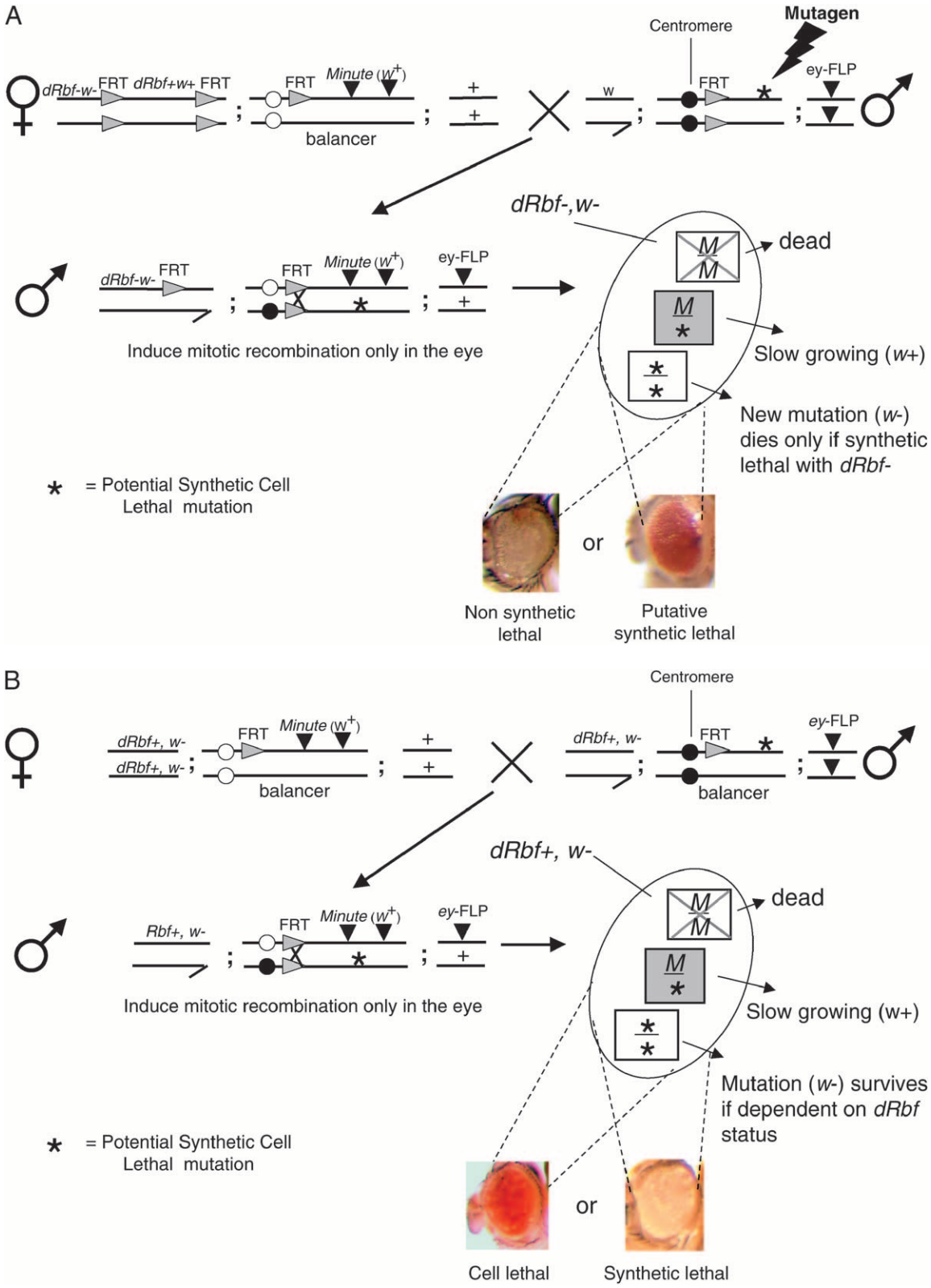

FIGURE 2.—Schematic of the primary screen and counterscreen. (A) Schematic of the primary screen. Rbf^+ screening-stock virgin females were crossed to mutagenized male stocks. Male progeny were assayed for mutations that resulted in the loss of *w* eye clones, causing the eyes to be w^+. Two separate FLP/FRT recombination events are initiated by the *eyeless* promoter. First, the FRTs flanking the Rbf rescue construct recombine in *cis*, eliminating the Rbf^+ and w^+ genes, resulting in a large Rbf^-, *w* clone in the eye. Second, the *trans* recombination between the two autosomal FRTs results in the generation of three different cell types:

lethality and synthetic lethality (Table 3). The organismal lethal phenotype was further mapped using deficiencies obtained from the Bloomington Stock Center and deficiencies created by Exelixis (Parks *et al.* 2004) that span the region identified by the recombination mapping (Table 4). Homozygous lethal transposons residing within interacting deficiencies were assayed for lethality in conjunction with our screen hits. Candidate loci within the mapped regions were analyzed by DNA sequencing.

F_2 lethal noncomplementation screen for additional KE1 alleles: *FRT(42D); ey-FLP* males were mutagenized via gamma-ray irradiation at 2.0 krad. Batches of 40 mutagenized males were mated to 30 *yw; Sp/CyO; ey-FLP* virgin females. Individual male progeny were mated to *+; FRT [KE1-1]/ CyO-GFP* virgin females and progeny were scored for the absence of straight wings. Putative KE1 allele-carrying males were crossed to the 2R screening stock (RbfSS2R) to ensure the absence of *w* clones and crossed to the 2R counterscreening stock (MFR-T2R) to ensure the presence of *w* clones and confirm synthetic lethality. We scored 5000 individual male crosses and isolated one new allele of *KE1* (referred to as *KE1-2*), which was lethal in *trans* to KE1-1, irrespective of the presence of ey-FLP.

Mutation detection of KE1 alleles: Staggered sequencing primers, spaced at 120- to 150-bp intervals and facing both directions, were designed for all open reading frames and their flanking regions throughout the genomic region of interest: coordinates 20047526–20093250 (FlyBase release 4.0). The selected forward and reverse PCR primer pairs were then used to amplify the regions of interest, using genomic DNA prepared from five individual larvae (large larvae in the case of homozygous mutants or the parental mutagenized strain for controls). Using this procedure, we were able to obtain high-quality fragments of genomic DNA up to 10 kb in length, although the usual product length was ~7 kb. Products were amplified for 30 cycles using a modified long-range PCR protocol with Takara (Berkeley, CA) LA Taq polymerase, checked on agarose gels, and purified with the Millipore (Bedford, MA) MultiScreen PCR cleanup kit. Purified PCR products were used as templates for sequencing, using the above-designed staggered sequencing primers and primer walking in both directions to obtain full-length sequence. ABI (Columbia, MD) BigDye sequencing reactions were performed according to manufacturer's protocol using 20–80 ng PCR product. Reactions were ethanol precipitated and loaded onto an ABI 3700 sequencer. Sequencing traces were uploaded to a Unix workstation, assembled with the PhredPhrap package, and viewed and analyzed with Consed. Of the nine currently annotated open reading frames in this region (FlyBase release 4.0), five were sequenced in entirety: CG3511, CG12252, Nurf-38, CG12252, and CG3522. Additionally, in KE1-2 mutants, we sequenced the entire upstream region of CG3511, through to the adjacent locus of CG12252.

Taqman analysis of transcripts: Both *KE1-1* and *KE1-2* were stocked over marked *CyO-GFP* balancer chromosomes (Table 1). Triplicate groups of 10 third instar larvae negative for GFP were collected from *isoFS2R*, *KE1-1*, and *KE1-2* animals (Table 1). Total RNA was collected using QIAGEN's (Valencia, CA) RNeasy kit for total RNA isolation from animal tissue. The RNA was reverse transcribed into cDNA [Applied Biosystems (Foster City, CA) Multiscribe reverse transcriptase—random hexamer primed]. TaqMan primer/probe assays were carried out for 18S ribosomal RNA, CG3511, and the adjacent locus CG3522. Relative quantity values were obtained for each sample compared to a cDNA standard curve. Standard cDNA was created by reverse transcribing total RNA from an isogenic *w* fly strain (Exelixis strain A5001, BL-6326). TaqMan assays were run on the ABI PRISM 7900HT sequence detection system. Normalized values for the quantity of CG3511 transcript levels were generated by dividing the CG3511 values by the 18S values for each sample.

Protein sequence data mining: Protein sequences related to the CG3511 protein were found by a combination of BLAST and Smith-Waterman pairwise analyses against human sequence databases and all sequence databases from the National Center for Biotechnology Information. Sequences were additionally mined solely on the basis of being predicted to contain the Pfam domain models found in CG3511; sequences containing the prolyl isomerase domain (model PF00160) either alone or following three to four WD domains (model PF0400) were identified and analyzed. Only sequences with Pfam scores >0 and *E*-values <1 were used in the analyses. All sequences data mined were analyzed against the fly genome to select those with top BLAST scores to CG3511 and not to another fly protein sequence. Those meeting BLAST requirements were termed orthologs. All mined sequences that conserved the PF00400 and PF00160 domain organization met orthology criteria, while none of the PF00160 only sequences did. Sequence alignments were performed using Clustal W and visualized by a tree diagram for multiple sequence alignments or by BOXSHADE for pairwise alignments.

RESULTS

Stock generation and synthetic lethal screen: Inactivating mutations in *Rbf* were isolated in a suppressor screen for genes able to overcome the G1 arrest caused by the overexpression of human p21 in the Drosophila eye [*Su(p21)SLS-15* and *Su(p21)CAS-21*; data not shown]. *Su(p21)SLS-15* ($Rbf^{SLS\text{-}15}$) mutant flies were subsequently used as the starting point for a *Rbf* synthetic lethal screen. Sequencing of the mutant chromosome and RT-PCR analysis of $Rbf^{SLS\text{-}15}$ transcripts revealed an 11-bp

1. *Minute/Minute* (*M/M*): This cell type is cell lethal because *M/M* cells die, regardless of the *Rbf* status of the cell.
2. *Minute/mutation* (*M/**): This cell type is viable and marked with w^+. When cells are heterozygous for Minute they are slow growing and are easily outcompeted.
3. *mutation/mutation* (*/*): This cell type is viable if the mutation is not synthetic lethal with Rbf^-, since this outcompetes the *M/** clone, resulting in a 90–95% *w* eye. When there is a synthetic lethal interaction with Rbf^-, the clone is unable to populate the eye and *M/** is the only cell type that survives, resulting in a w^+ eye.

(B) Schematic of the counterscreen. To eliminate those mutations that are not dependent upon *Rbf* status, hits from the primary screen were crossed to Rbf^+ *MFRT* line virgins. The FRT/FLP recombination events under the direction of the *eyeless* promoter result in the generation of three different cell types: (1) *M/M*, as described above; (2) *M/**, as described above; and (3) */*, if the previously observed synthetic lethal phenotype is indeed Rbf^- specific, this cell type will be able to populate the eye in a Rbf^+ background, resulting in a *w* eye. Conversely, if these cells are absent, resulting in a w^+ eye, then there is no Rbf^- synthetic interaction and the previously observed phenotype was due to nonspecific cell lethality.

TABLE 2

Summary of screen hit rates

Chromosome arm screened	Mutant chromosomes scored	Primary screen hits	Confirmed synthetic lethal hits
2L	132,708	222	0
2R	49,216	220	2
3L	43,621	896	5
3R	116,915	247	3
Total	342,560	1,585	10

TABLE 3

Visible recombination mapping of *KE1-1*

Recombinant	Synthetic lethal	Organismal lethal	Large larvae
al, dp, b, FRT(42D) [*]	Y	Y	Y
FRT(42D), [*], c, px, sp	N	N	N
FRT(42D), [*], px, sp	N	N	N
FRT(42D), [*], sp	N	N	N
FRT(42D), c, [*]	Y	Y	Y
FRT(42D), c, px, [*]	Y	Y	Y

Recombinants bearing the visible chromosomal markers shown in column 1 were scored for synthetic lethality with Rbf$^-$ in eye clones (column 2), organismal lethality as homozygotes (column 3), and the presence of the large larva phenotype as homozygotes (column 4). Y, the phenotype is present; N, phenotype absent. *[*]*, portion of mutant chromosome.

deletion resulting in a frameshift mutation at amino acid residue 519 and the addition of 14 novel residues before ending at residue 533 (Figure 1A). This generates a truncated protein lacking the highly conserved Rbf-binding pocket, which is required for interactions with partner proteins and RBF function (Helt and Galloway 2003). Like reported null alleles of *Rbf* (Du and Dyson 1999; Datar *et al.* 2000), our alleles confer embryonic lethality as homozygous mutations.

To circumvent the requirement for *Rbf* during development, we constructed a transgenic Rbf$^+$ screening strain bearing a FLP-FRT rescue transgene to provide wild-type Rbf to all cells and to mark Rbf$^+$ cells in the developing eye with w^+ (Figure 1B, Table 1). This transgenic strain is rescued to complete viability and fertility and generates marked viable clones of Rbf$^-$, *w* cells where FLP recombinase is expressed. To generate homozygous clones of newly induced mutations in the F_1 progeny, these flies also carried a FRT at the base of one of the autosomal chromosomal arms in *cis* to a Minute mutation (MFRTs) (Figure 2; Lambertsson 1998) to generate the *Rbf* screening stocks (Table 1). For the screen, a low frequency of mutations was induced by EMS in *w* males carrying an autosomal FRT chromosome plus *ey*-FLP. These flies were then crossed to the transgenic *Rbf* screening stock females. *ey*-FLP generates overlapping clones of both Rbf$^-$, *w* (from the screening stock females) and the mutagenized FRT autosome (from males) in the eyes of the F_1 progeny, thereby enabling us to screen for recessive synthetic lethal mutations in a single generation. Putative synthetic lethal progeny were identified by the presence of solid red eyes (Rbf$^-$, *M*, w^+), indicating that the mutant cells (Rbf$^-$, *w*) are absent. We screened through individual progeny from crosses generating mitotic clones on the second and third autosomes, which constitute ~80% of the genome. We screened 342,000 mutagenized chromosomes and initially identified 1585 chromosomes bearing putative synthetic lethal mutations in combination with Rbf$^-$ (Table 2), for retest and counterscreening in the following generation.

To eliminate those mutations that cause cell lethality independent of *Rbf* status, we counterscreened the 1585

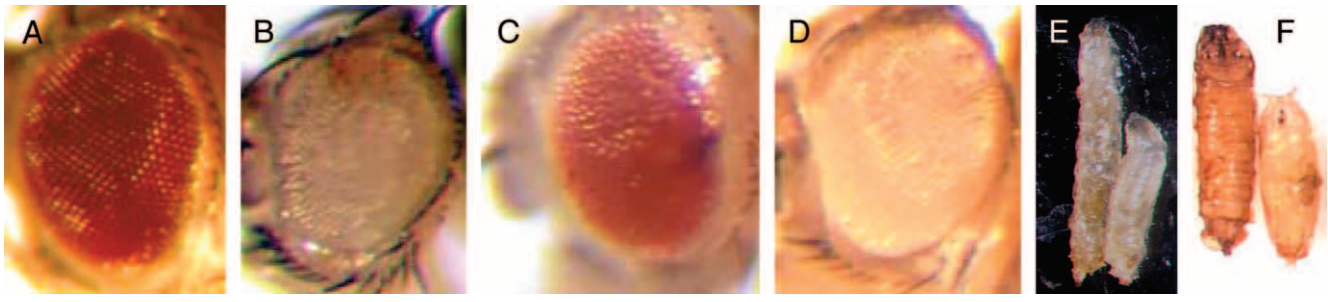

Figure 3.—Phenotypes of *KE1-1* eye clones, mutant larvae, and pupae. (A) Wild-type Drosophila eye. (B) Rbf$^-$, *w* clone generated in the screening stock. (C) Clone of *KE1-1* generated in the Rbf$^-$ *w* screening stock. The *KE1-1*, Rbf$^-$, *w* cells die due to synthetic lethality, leaving the eye populated with Rbf$^-$, *M*, w^+ cells. (D) Clone of *KE1-1* generated in the Rbf$^+$ counterscreen stock. The *KE1-1*, Rbf$^+$, *w* cells are viable, demonstrating that *KE1-1* is not cell lethal on its own. (E) Large larva phenotype of a *KE1-1/KE1-1* wandering third instar larva (left) compared to a *KE1-1/+* larva (right). (F) Rare *KE1-1/KE1-1* escaper pupae (left) are also large compared to KE1-1/+ pupae (right). Full genotypes of flies shown in B–D are: (B) *Rbf^{SLS-15}, PExp{FRT2.1[Rbf$^+$, w^+, 3.5ey-FLP]}; P{ry[+t7.2] = neoFRT}42D P{w[+mC] = piM}45F M(2)53[1]/P{ry[+t7.2] = neoFRT}42D iso2; P{ry[+7.2] = ey-FLP.N}6, ry[506]}*; (C) *w, Rbf^{SLS-15}, Pexp{FRT2.1 [Rbf$^+$, w^+, 3.5ey-FLP]}; P{ry[+t7.2] = neoFRT}42D, P{w[+mC] = piM}45F, M(2)53[1]/ P{ry[+t7.2] = neoFRT}42D, iso2[KE1-1]; P{ry[+7.2] = ey-FLP.N}6, ry[506]/+*; (D) *w; P{ry[+t7.2] = neoFRT}42D, P{w[+mC] = piM}45F, M(2)53[1]/P{ry[+t7.2] = neoFRT}42D, iso2[KE1-1]; P{ry[+7.2] = ey-FLP.N}6, ry[506]/+*.

TABLE 4

Mapping of *KE1* organismal lethality using chromosomal deficiencies

Deficiency stock	Left end	Right end	Viability with KE1-1 and KE1-2
BL-1682	59D5–10	60B3–8	Viable
BL-2355	59D8–11	60A7	Viable
BL-1587	59E2	60B1	Viable
Df(2R)Exel7180	59E3	59F6	Viable
Df(2R)Exel7182	60A13	60A16	Viable
Df(2R)Exel9024	60A16	60A16	Viable
Df(2R)Exel6080	60A6	60B5	Viable
Df(2R)Exel7184	60B12	60C4	Viable
Df(2R)Exel6082	60B4	60C6	Viable
Df(2R)Exel6281	60C4	60C7	Viable
BL-1473	60C5–6	60D1	Viable
BL-2604	60C6	60D9–10	Lethal
BL-3157	60E6	60F1–2	Viable
BL-2471	60E6–9	60E11	Viable
BL-2528	60E9	60F1	Viable

Deficiency name or stock number tested is given in column 1. The left- and right-hand cytogenetic locations are given in columns 2 and 3, and the lethality or viability when the deficiency was scored with *KE1-1* and *KE1-2* is given in column 4.

TABLE 5

Fine-scale mapping of *KE1* organismal lethality using custom-generated deficiencies

Deficiency stock	Left end	Right end	Viability with KE1-1 and KE1-2
BL-2604	60C6	60D9–10	Lethal
Df(2R)Exel6278	60C7	60D4	Viable
Df(2R)Exel6278	60C7	60D4	Viable
Df(2R)Exel9043	60C7	60C7	Viable
Df(2R)Exel7185	60C8	60D3	Viable
Df(2R)Exel7186[a]	60D10	60E1	Viable
Df(2R)Exel8091[a]	60D4	60D14	Lethal

Deficiency name or stock number tested is given in column 1. The left- and right-hand cytogenetic locations are given in columns 2 and 3, and the lethality or viability when the deficiency was scored with *KE1-1* and *KE1-2* is given in column 4.

[a] Df was not permanently stocked.

chromosomes in Rbf^+ eye clones induced under similar conditions (Figure 2B) and reconfirmed their ability to reduce the viability of Rbf^- cells. Ten of the 1585 mutations were found to be *bona fide* synthetic lethals, reducing the viability of Rbf^-, but not Rbf^+, cells (Table 2). Nine of these were developmentally lethal and complemented one another. One of these 9, on the right arm of the second chromosome, was designated *KE1-1*. When homozygous *KE1-1* mutant clones are induced in the developing eye in a Rbf^- background, the resulting adult eyes lack the Rbf^-, *w* clonal tissue (Figure 3, B and C). However, when *KE1-1* mutant clones are generated in a Rbf^+ background, the tissue is viable (Figure 3D), demonstrating that *KE1-1* is homozygous viable in cells in the presence of wild-type *Rbf*. Thus, the lethal interaction is specific to Rbf^- cells, and *KE1-1* is a true synthetic lethal mutation. When *KE1-1* is homozygous in all tissues throughout development, homozygous larvae display an enlarged body phenotype compared to their heterozygous *KE1-1*/+ siblings (Figure 3E). These "large larvae" wander for an extended period before death, although rare escapers can progress to giant pupae that fail to eclose as adults (Figure 3F).

To identify the *KE1-1* locus, we defined the chromosomal region sufficient to confer synthetic lethality in eye clones using standard recombination mapping with visible markers (Table 3). This analysis defined a region at the tip of 2R distal to *sp* at 60C as necessary and sufficient to confer the Rbf^- synthetic lethal phenotype. When homozygous, this chromosomal region also produced a lethal phenotype with large larvae and delayed pupation. This demonstrated that the Rbf^--dependent synthetic lethality, large larval phenotype, developmental delay, and organismal lethality all cosegregate with the region distal to 60C and suggested that a single locus might be responsible for all the observed phenotypes. Organismal lethality was used for further mapping and revealed that *KE1-1* failed to complement an existing chromosomal deletion spanning 60C6 to 60D9–10 (Table 4, BL-2604). Using the targeted deletion strategy previously described (Parks *et al.* 2004), this large deficiency was then subdivided into five small overlapping deletions with molecularly defined endpoints. Only one of the small deletions generated, Df(2)Exel8091 (60D4–60D14), failed to complement the organismal lethality present on the *KE1-1* chromosome (Table 5), placing the locus responsible for homozygous lethality between genomic coordinates 20047526 and 20093250 (FlyBase v4.0).

Confirmation that CG3511 mutations confer the Rbf^- synthetic interaction phenotype: We sequenced several candidate open reading frames between these coordinates and identified lesions in one open reading frame, CG3511, which is predicted to encode a previously uncharacterized protein with similarities to cyclophilins. The predicted KE1-1 cDNA contains a pair of missense mutations at nucleotides 569 and 570, followed by a single-base-pair deletion at nucleotide 572 (Figure 4A). These changes are predicted to cause a frameshift at amino acid 133 and the early truncation of the protein at residue 158 (Figure 4, B and C). While the mutations in CG3511 confer organismal lethality, proof that this mutation alone was sufficient to cause the synthetic interaction with Rbf^- in eye clones remained to be shown. We therefore conducted a noncomplementation screen to identify additional mutations in CG3511 and tested their ability to prevent the survival of Rbf^- clones (Figure 5, materials and methods). From this screen we isolated *KE1-2*, which also

A

```
CG3511 KE1-1 545  AGTACCCATCAAATCACTGACCACTGA-GACAGCGGCACGCTGCTCTGCTCGGCGG  600
CG3511-RA    545  AGTACCCATCAAATCACTGACCACCAACGACAGCGGCACGCTGCTCTGCTCGGCGG  600
```

B

```
Dm_CG3511       1 MSDKIKEDLK-----RSAPEE--------ENEPQEESVSSEKEAEGEEDAWIGPMPSEQSA
Hs_KIAA0073     1 MAAESGSDFQQRRRRRRDPEEPEKTELSERELAVAVAVSQENDEENEERWVGPLPVEATL

Dm_CG3511      49 PAPAKKKKVLPYEHIYLENLPNAESYERSYMHRDVITHLVCTKTDFVVTASLDGHIKFWK
Hs_KIAA0073    61 ---AKKRKVLEFERVYLDNLPSASMYERSYMHRDVITHVVCTKTDFIITASHDGHVKFWK

Dm_CG3511     109 KGELGIEFVKHFRSHLVPIKSLTTNDSGTLLCSAATDQTAKVFDVVNFDMINIIRLGYTP
Hs_KIAA0073   118 KIEEGIEFVKHFRSHLGVIESIAVSSEGALFCSVGDDKAMKVFDVVNFDMINMLKLGYFP

Dm_CG3511     169 QCSEWINGPGDAVQALAISDSESSRIHIYDGQGGGEALHTLEKLHSAPVVAMCLNVAMET
Hs_KIAA0073   178 GQCEWIYCPGDAISSVAASEKSTGKIFIYDGRGDNQPLHIFDKLHTSPLTQIRLNPVYKA

Dm_CG3511     229 VISVDFNGILEYWQNSKYDYKFPQRLVNFDSKLDTSLFEFAKQKTQVTGLAATPDGKRFA
Hs_KIAA0073   238 VVSSDKSGMIEYWTGPPHEYKFPKN-VNWEYKTDTDLYEFAKCKAYPTSVCFSPDGKKIA

Dm_CG3511     289 AISTDRKVRVFQFNTGKLIRVFDEALSTYTQMQCTKHALPNMEFGRRMAAERDLEKTAQN
Hs_KIAA0073   297 TIGSDRKVRIFRFVTGKLMRVFDESLSMFTELQQMFQQLPDMEFGRRMAVERELEKVDAV

Dm_CG3511     349 ATLNILFDSTGNFLLYPTMLGIKVINVVTNRCVTILGKTDNIRPLQVALFQGRIKRQKAA
Hs_KIAA0073   357 RLINIVFDETGHFVLYGTMLGIKVINVETNRCVRILGKQENIRVMQLALFQGIAKKHRAA

Dm_CG3511     409 ITMEQEASENPALQNILNDPTAFCTAYKKSRFYLYSRRLPSDLQ--DVDRDIFNEKPSKE
Hs_KIAA0073   417 TTIEMKASENPVLQNIQADPTIVCTSFKKNRFYMFTKREPEDTKSADSDRDVFNEKPSKE

Dm_CG3511     467 DIIAVPEASVVQRIYENVVLHTTKGDIHMRLFFKEVPKTVENFCVHAKNGYYNGHIFHRV
Hs_KIAA0073   477 EVMAATQAEGPKRVSDSAIIHTSMGDIHTKLFPVECPKTVENFCVHSRNGYYNGHTFHRI

Dm_CG3511     527 IKGFMVQTGDPTGTGTGGKSIWGSDFKDEFVPSLKHDRPYTVSMANAGPNTNGSQFFITV
Hs_KIAA0073   537 IKGFMIQTGDPTGTGMGGESIWGGEFEDEFHSTLRHDRPYTLSMANAGSNTNGSQFFITV

Dm_CG3511     587 LPTPWLDNKHTVFGRVYRGMEVVLNICNSKANPKTDKPYDDIKIISIHLSN
Hs_KIAA0073   597 VPTPWLDNKHTVFGRVTKGMEVVQRISNVKVNPKTDKPYEDVSIINITVK-
```

C

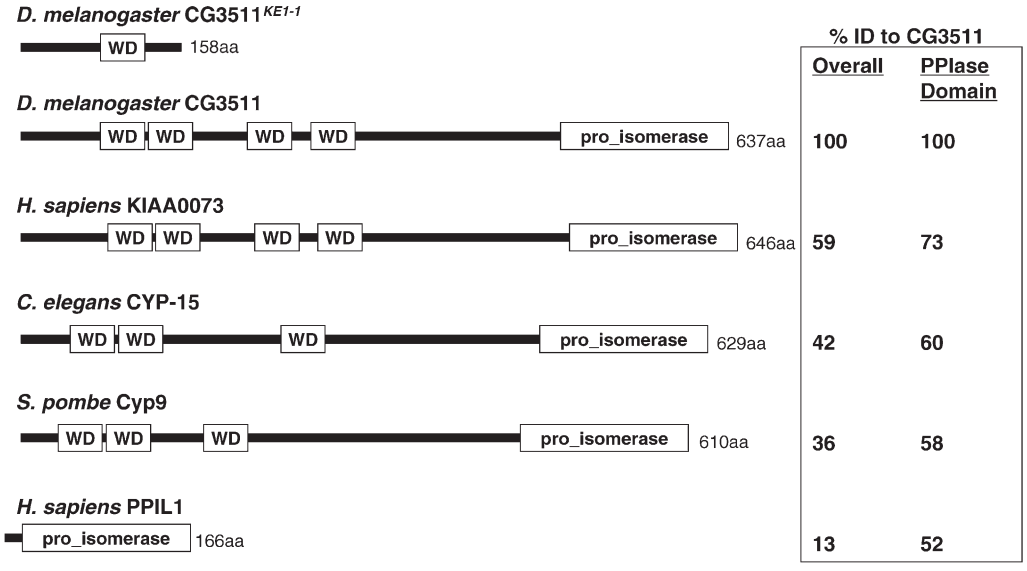

FIGURE 4.—CG3511 encodes a unique and highly conserved peptidyl prolyl isomerase protein. (A) The KE1-1 mutant contains a two-nucleotide substitution and a single-base-pair deletion in the transcript of CG3511-RA, when compared to wild type. A partial sequence of the transcript between nucleotides 545 and 600 is shown, with the changes present in the KE1-1 mutant given in boldface type. (B) Protein sequence alignment of CG3511 and its predicted human ortholog KIAA0073. Identical residues are shaded in black, similar residues are shaded gray. The WD domains and prolyl isomerase domain predictions are graphically represented above the alignment by hatched bars and solid bars, respectively. An asterisk denotes the location of the first frameshifted residue in the *KE1-1* mutant. (C) Conservation of predicted proteins and domains encoded by the *KE1-1* allele, wild-type CG3511, and selected eukaryotic orthologs. PPIL1 represents the next closest PPIase to CG3511 and is shown for comparison. The organization of WD motifs and the peptidyl prolyl isomerase within the proteins is depicted by boxes. Percentage sequence identities throughout the proteins and within the conserved peptidyl prolyl isomerase domains are shown.

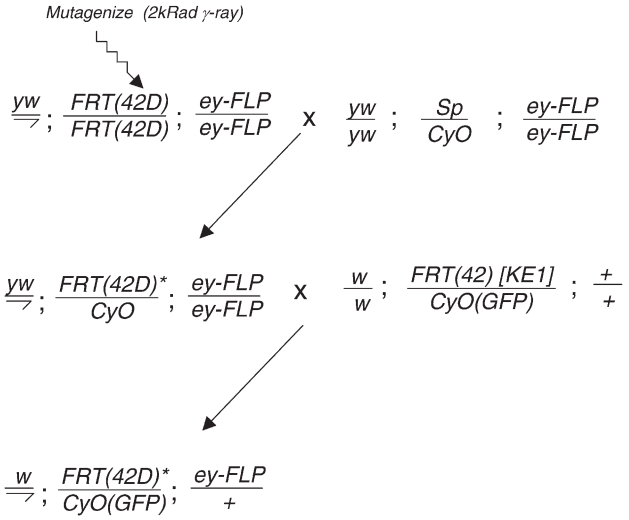

FIGURE 5.—F_2 lethal noncomplementation screen for additional *KE1* alleles. Mutagenized *yw*; *FRT(42D); ey-FLP* males were mated to females bearing additional copies of *ey-FLP*. Single male F_1 progeny, heterozygous for the newly induced mutations, were mated to *KE1-1* females and the F_2 progeny were scored for the absence of *[FRT(42D)*/KE1-1]* flies.

displayed the large larva phenotype when homozygous or when in *trans* to *KE1-1* (data not shown). *KE1-2* was also lethal over Df(2)Ex8091 and BL-2604 (Table 5), confirming that *KE1-2* likely represents a second allele of CG3511. Sequencing of the open reading frame of CG3511 and its adjacent 5′ region (into CG12252) did not reveal any mutations, so we analyzed transcript levels in mutant larvae by RT-PCR. Transcript levels of CG3511 were reduced >90% in *KE1-2* larvae compared to the parental strain (Figure 6). A reduction in transcript levels of ~50% was also observed in homozygous *KE1-1* larvae. This reduction in mRNA levels in mutants was specific to CG3511, since transcript levels of adjacent genes were present at normal levels (data not shown). A likely explanation is that the *KE1-2* mutant contains an aberration in a distant *cis*-regulatory element controlling the transcript levels of CG3511. *KE1-2* was introduced into our screening and counterscreening strains to test its interactions with *Rbf* in the eye. Clones homozygous for *KE1-2* failed to survive in Rbf^- but not Rbf^+ eyes, confirming that the mutation on the *KE1-2* chromosome is sufficient to confer the Rbf^- synthetic phenotype (data not shown). As with *KE1-1*, recombination mapping using visible markers demonstrated that the Rbf^--dependent synthetic lethality, large larvae phenotype, and organismal lethality of *KE1-2* all cosegregated with the region distal to 60C, containing CG3511. Thus, even though we were unable to define the nucleotide changes in *KE1-2* mutants, these mapping data suggest that the *KE1-2* chromosome contains a lesion that cosegregates with the same narrowly defined region containing CG3511 and that causes a reduction in the levels of this transcript. The most plausible explanation is that the KE1-2 mutant chromosome bears a lesion in a *cis*-regulatory element in CG3511, and that the observed reduction in transcript levels is sufficient to confer the Rbf^--dependent phenotype.

The product of CG3511 is predicted to encode a protein of 637 amino acids. The N terminus of the protein contains four WD domains, which often impart protein interaction and scaffolding functions to proteins (SMITH *et al.* 1999). At the carboxyl terminus is a cyclophilin-type peptidyl prolyl isomerase (PPIase) domain. Sequence analysis reveals that CG3511 is 59% identical overall to its predicted human ortholog, KIAA0073, and 73% identical within its prolyl isomerase domain (Figure 4, B and C). The peptidyl prolyl isomerase-like (PPIL) proteins are the next most closely related PPIases, but they lack WD domains and are predicted to be orthologs of other fly proteins. Single CG3511 orthologs are found throughout eukaryotes, including nematode CYP-15 and fission yeast Cyp9. The inclusion of multiple WD domains distinguishes these unique PPIases from others described to date.

DISCUSSION

We have designed and carried out a screen in which overlapping clones of mutant cells are generated in the eye in such a way as to allow screening of recessive mutations for synthetic lethality in the F_1 generation. This scheme made it possible to screen through large numbers of mutations without having to set up individ-

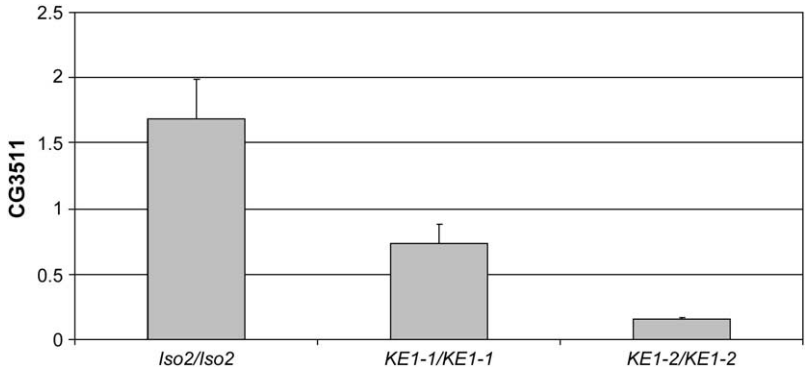

FIGURE 6.—CG3511 is underexpressed in *KE1* mutants. Quantitative analysis of CG3511 transcript levels in larvae is shown. The *y*-axis shows normalized CG3511 transcript levels (see MATERIALS AND METHODS) present in wild-type (*IsoFSR*), *KE1-1*, and *KE1-2* mutant third instar larvae. The reduction in transcript levels observed in the *KE1-2* larvae is >10-fold.

ual lines and therefore allowed for the isolation of the very rare Rbf synthetic lethal mutations.

Peptidyl prolyl isomerases belong to an extended protein superfamily whose members all catalyze the *cis-trans* isomerization of proline imidic bonds in polypeptides. The superfamily includes the cyclophilin-like peptidyl prolyl isomerases (Cyp), the FK-506-binding proteins (immunophilin/FKBP), and the parvulin/Pin proteins (Shaw 2002). In addition to sequence and structural divergence, differences in substrates and sensitivity to inhibitors distinguish members within these families (Harrison and Stein 1990; Hennig *et al.* 1998). Mechanistically, interconversion of x-Pro bond *cis-trans* conformation can alter protein folding and the conformation of the native state, leading to potential effects on protein function and regulation of serine/threonine phosphorylation events (Andreotti 2003; Weiwad *et al.* 2004). PPIases have been shown to play diverse functional roles in the cell and some, like Pin1, have been implicated in cellular transformation and human cancer (Bao *et al.* 2004; Yeh *et al.* 2004).

There is considerable evidence in the literature to support a mechanistic link between the PPIase Pin1 and its regulation of the cell cycle and apoptosis (Lu 2003; Urist and Prives 2004). Pin1 alters the conformation of the p53 family members p53 and p73 and is required for them to induce the DNA damage checkpoint in response to genotoxic stress (Zacchi *et al.* 2002; Zheng *et al.* 2002; Urist and Prives 2004). Pin1 has also been shown to interact with Cdc25 and Plk1 and to modulate Cyclin D1 expression levels and activity and Rb phosphorylation (Liou *et al.* 2002; Shaw 2002; You *et al.* 2002). In turn, Pin1 itself is a direct target of E2F activity, participating in a positive feedback loop involving cyclin D1/Cdks, E2F, and RB1 (Ryo *et al.* 2002). Loss of Pin1 in mouse embryonic fibroblasts causes cell cycle defects and decreases the levels of cyclinD1 and phosphorylated RB1 (You *et al.* 2002). Similarly, Pin1 knockout mice display a range of proliferative defects, many of which are attributed to its effects on Cyclin D1 (Liou *et al.* 2002). Although KIAA0073, the human ortholog of CG3511, has not been studied as extensively as Pin1, it is possible that KIAA0073 and other PPIases aside from Pin1 might also interact with components of the cell cycle and checkpoint pathways, as was previously suggested from the comparatively mild knockout phenotype observed for Pin1 (Liou *et al.* 2002).

In summary, we describe a novel conserved gene, CG3511, which when mutated (as in *KE1-1*) or when its transcript levels are reduced in abundance (as in *KE1-2*) results in the specific loss of Rbf^- cells in the Drosophila eye. Future experiments will elucidate how the PPIase protein family may interact with RB1 to regulate cell survival and/or proliferation. KIAA0073 may represent an efficacious and novel anti-cancer drug target whose inhibition might result in the specific death of RB1 mutant cells. Such a synthetic lethal target would have applications in several RB1 pathway-dependent cancers, such as SCLC (Sherr and McCormick 2002), and may represent a unique opportunity for targeted therapeutics.

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