dE2F2-Independent Rescue of Proliferation in Cells Lacking an Activator dE2F1[▽]

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In *Drosophila melanogaster*, the loss of activator *de2f1* leads to a severe reduction in cell proliferation and repression of E2F targets. To date, the only known way to rescue the proliferation block in *de2f1* mutants was through the inactivation of dE2F2. This suggests that dE2F2 provides a major contribution to the *de2f1* mutant phenotype. Here, we report that in mosaic animals, in addition to *de2f2*, the loss of a DEAD box protein Belle (Bel) also rescues proliferation of *de2f1* mutant cells. Surprisingly, the rescue occurs in a dE2F2-independent manner since the loss of Bel does not relieve dE2F2-mediated repression. In the eye disc, *bel* mutant cells fail to undergo a G₁ arrest in the morphogenetic furrow, delay photoreceptor recruitment and differentiation, and show a reduction of the transcription factor Ci155. The down-regulation of Ci155 is important since it is sufficient to partially rescue proliferation of *de2f1* mutant cells. Thus, mutation of *bel* relieves the dE2F2-mediated cell cycle arrest in *de2f1* mutant cells through a novel Ci155-dependent mechanism without functional inactivation of the dE2F2 repressor.

Cell proliferation and differentiation are precisely coordinated during the development of a multicellular organism. The loss of such control may ultimately lead to defects in development and cancer. E2F transcription factors are important regulators of the cell cycle and critical downstream targets of the retinoblastoma (pRB) tumor suppressor protein (12). In spite of remarkable progress, dissecting the pRB pathway in mammalian cells remains a challenging task. One issue arises from functional redundancy and compensation among E2F and pRB family members. In the past years, *Drosophila melanogaster* has been recognized as a valuable tool for understanding various aspects of E2F biology. This is mainly due to the high conservation in cell cycle regulation between flies and mammals and the relative simplicity of the E2F/pRB module in *Drosophila*. In *Drosophila*, there are two E2F genes. As mammalian counterparts, their products can be classified as repressors (dE2F2) and activators (dE2F1).

Analysis of dE2F single- and double-mutant animals has provided insights into the normal function of dE2Fs during development. *de2f1* mutant larva are severely retarded in larval growth and show a strong reduction in S phases and the expression of E2F targets. Unexpectedly, these defects are largely suppressed in *de2f1 de2f2* double mutants. This suggests that the *de2f1* mutant phenotype is not entirely due to the absence of activator dE2F1 but rather, to some extent, is due to the presence of the “unchecked” repressor dE2F2 (18). This result is particularly striking since *de2f2* mutants are viable and develop normally (5, 18). Why “unchecked” dE2F2 has such a strong effect on cell proliferation in the absence of dE2F1 is not clear. A possible explanation, supported by gene expression profiling (11), is that, in *de2f1* mutant cells, dE2F2 inap-

propriately represses dE2F1-specific genes that are critical for cell proliferation. Until now, the only known way to rescue the *de2f1* mutant phenotype in vivo was through mutations in *de2f2* or *rbf1* (13, 18, 41). This may indicate that *de2f1* mutant cells are not capable of proliferating unless dE2F2/RBF is inactivated. However, this idea was not rigorously tested, and it is not clear whether cell cycle arrest in *de2f1* mutant cells can be overridden in a dE2F2-independent manner.

These puzzling properties of the *de2f1* mutant phenotype prompted us to take a genetic approach to address some of the unresolved questions about the mechanism of cell cycle arrest in these cells. Using a mosaic genetic screen in *Drosophila*, we found that the loss of DEAD box protein Belle (Bel) rescues the cell cycle defects of *de2f1* mutant cells. Surprisingly, dE2F2-mediated repression is not relieved in *bel* mutants, suggesting that proliferation in *de2f1 bel* double-mutant cells occurs in the presence of a functional dE2F2/RBF. The results show that Bel specifically affects Hedgehog (Hh) signaling, and this effect is important for the rescue of the *de2f1* mutant phenotype. Thus, cell cycle arrest in *de2f1* mutant cells can be relieved not only in a dE2F2-dependent manner but also in a dE2F2-independent manner.

MATERIALS AND METHODS

Generation of the *de2f2 FRT40A* chromosome. The *de2f2* gene is located at 39B in close proximity to the centromere. Therefore, a *de2f2* mutation cannot be combined with *FRT40A* by standard meiotic recombination. Additionally, since *de2f2* mutant alleles are viable, new *de2f2* mutations cannot be easily induced on the *FRT40A*-containing chromosome. To overcome these problems, we employed *P* element-induced male recombination. With low frequency, a *P* element transposase generates crossovers in the *P* element in the male germ line. Since *FRT40A* is a *P* element insertion, we used it to recover rare crossover events on *FRT40A*. The *de2f2*^{c03344} mutant allele is a *PB* insertion into the *de2f2* gene. The advantage in using this allele is that *PB* is stable in the presence of the *P* element transposase. Briefly, a *dp de2f2*^{c03344} *bw* chromosome that carries recessive markers to identify the recombinants was constructed. Potential recombination events were revealed in the progeny of *w; dp de2f2*^{c03344} *bw/dp*⁺ *FRT40A bw*⁺; Δ 2-3 *Sb/TM6 Tb* males by segregation among *dp* and *bw* markers. Two independent stocks of *w; dp de2f2*^{c03344} *FRT40A* flies were recovered.

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Fly stocks. The following mutant alleles were used: *de2f1* mutant, *de2f1*⁷²⁹ (16); *smo* mutant, strong hypomorphic mutation *smo*^{11G26} (46); *bel* mutant, *bel*^{L4740}; *de2f2* mutants, *de2f2*^{c03344}; *dDP* mutant, *dDP*^{u3}.

bel^{7d19} was isolated in the FLP/FLP recombination target (FRT) screen for suppressors of the *de2f1* mutant phenotype. *FRT82B de2f1*⁷²⁹ males were mutagenized with ethyl methanesulfonate and crossed to *w; TM3/MKRS* females, and then male progeny were crossed individually to *y w ey-FLP; FRT82B P[mini-w]90E 1(3)cl-R3¹/TM6B Tb* females. Since clones of *de2f1* mutant cells are very small, the majority of flies had no visible white spots marking *de2f1* mutant cells. Exceptional flies, whose eyes contained large white patches indicating that the block in *de2f1*⁷²⁹ mutant cells is overridden, were retained. Forty thousand chromosomes were screened in the F2 screen. Mapping was performed by crossing *7d19* flies to flies with a set of deficiencies uncovering *3R*. *7d19* fails to complement deficiency *Df(3)Exel6149* and several alleles of the gene *bel*; the gene *bel* was uncovered by the deficiency.

For the eye disc analysis, clones of homozygous mutant cells were generated in the animals of the following genotypes: *ey-FLP; FRT82B bel*^{7d19} *de2f1*⁷²⁹/*FRT82B P[Ubi-GFP]*, *ey-FLP; FRT82B bel*^{L4740} *de2f1*⁷²⁹/*FRT82B P[Ubi-GFP]*, *ey-FLP; FRT82B bel*^{L4740}/*FRT82B P[Ubi-GFP]*, *ey-FLP; FRT40A de2f2*^{c03344}/*FRT40A P[Ubi-GFP]*, *ey-FLP; FRT40A de2f2*^{c03344}/*FRT40A P[Ubi-GFP]*; *FRT82B de2f1*⁷²⁹/*FRT82B P[Ubi-GFP]*, *ey-FLP; FRT40A smo*^{11G26}/*FRT40A P[Ubi-GFP]*; *FRT82B de2f1*⁷²⁹/*FRT82B P[Ubi-GFP]*, *ey-FLP; 2x PCNA-GFP; FRT82B bel*^{7d19} *de2f1*⁷²⁹/*FRT82B P[Ubi-GFP]*, and *ey-FLP; FRT42D dDP*^{u3}/*FRT42D P[Ubi-GFP]*.

Immunofluorescence. Eye imaginal discs were dissected in 10% fetal bovine serum-Schneider's insect medium and then fixed in 4% formaldehyde-phosphate-buffered saline (PBS). Washes were then done using 0.3% Triton X-100-PBS. Discs were then blocked in 0.1% Triton X-100-10% normal donkey serum-PBS for 1 h before addition of appropriate primary antibodies. Washes were then done using 0.1% Triton X-100-PBS. Discs were then incubated in blocking solution containing the appropriate secondary antibodies. The secondary conjugated antibodies used in this work were anti-mouse Alexa Fluor 405 (1:500; Invitrogen), anti-rabbit Alexa Fluor 405 (1:500; Invitrogen), anti-mouse Cy3 (1:100), anti-rabbit Cy3 (1:100), anti-rabbit fluorescein isothiocyanate (1:200), and anti-rat Cy3 (1:50) from Jackson Immunolaboratories. Washes were then done using 0.1% Triton X-100-PBS. Bromodeoxyuridine (BrdU) labeling of eye imaginal discs was performed as previously described (17, 41). Images were collected on a Zeiss LSM510 confocal microscope. The primary antibodies used in this work were rabbit anti-β-galactosidase (β-Gal; 1:500; Cappel), mouse anti-BrdU (1:50) (BD Bioscience), rabbit anti-green fluorescent protein (GFP; 1:1,000) (Invitrogen), rabbit anti-phospho-H3 (1:150) (Upstate), mouse anti-RBFI (14), mouse anti-cyclin E (1:5) (42), rabbit anti-dE2F2 (1:100) (18), rabbit anti-phosphorylated Mothers against decapentaplegic (pMad; 1:5,000) (39), rat anti-Ci155 (1:1) (36), rabbit anti-Atonal (Ato; 1:50) (25), and rabbit anti-Bel (1:500) (26). Mouse antibodies anti-cyclin B (1:10), anti-Notch (1:100), anti-Delta (DI; 1:50), anti-Pros (1:20), anti-Patched (Ptc; 1:50), anti-Cut (1:10), and anti-β-Gal (1:20) and rat anti-Elav (1:50) are from the Developmental Studies Hybridoma Bank.

Real-time PCR. Quantitative real-time PCR on RNA isolated from S2 cells was performed as previously described (41). To measure the expression of *Arp53D* in the eye imaginal discs, total RNA was isolated from wild-type and mutant discs of different genotypes. Since *Arp53D* is not expressed in wild-type cells, we expect that the presence of RNA from these cells in the sample would not obscure derepression of *Arp53D* in the mutant cells. This was confirmed by using RNA prepared from the eye discs containing clones of *dDP* mutant cells. *dDP* represents a valid control since *Arp53D* is fully derepressed in *dDP*-deficient cells (11) and clones of *dDP* mutant cells are small (19). RNA was prepared from the eye imaginal discs dissected from wild-type larvae, *dDP* mutant larvae (in which essentially all cells are *dDP* mutant), and larvae in which clones of *dDP* mutant cells were induced using *ey-FLP* (where approximately 10% of all cells are mutant and 90% are wild type [19]). The results of real-time PCR are shown (see Fig. 4B). The *Arp53D* signal is increased by a factor of several thousand in RNA isolated from the eye imaginal discs of *dDP* homozygous mutant larvae and is increased by a factor of several hundred in an RNA sample isolated from the mosaic eye imaginal discs carrying clones of *dDP* mutant cells. Thus, this technique is sensitive and reliable to detect the expression of *Arp53D* in a small subset of cells.

Manipulations with S2 cells. RNA interference (RNAi), transfections, and Western blotting were done as previously described (41). The primary antibodies used in this work were rabbit anti-Bel (1:6,000) (26), rabbit anti-dE2F2 (1:2,000) (18), and mouse anti-RBFI (DX5; 1:5) (14).

Using the pIEX-7 Ek/LIC vector (Novagen), we cloned in the wild-type Bel cDNA open reading frame (ORF; Drosophila Genomics Resource Center). The

FIG. 1. Rescue of proliferation in clones of *de2f1* mutant cells by mutation of *de2f2*. Eye imaginal discs were dissected from third-instar larva. Clones of mutant cells were induced with *ey-FLP* and are identified by the lack of GFP (green). Posterior is to the right. Genotypes are shown. (A) Control experiment showing clones of homozygous tissue with the wild-type chromosome. In the absence of mutations, there are approximately equal amounts of tissue marked by GFP and by the lack of GFP. (B) Clones of *de2f2*^{c03344} mutant cells (GFP negative) do not express dE2F2 protein. (C, C*, D, and D*) Induction of clones of cells that are homozygously mutant for *de2f2*^{c03344} and *de2f1*⁷²⁹. Double-mutant cells fail to express GFP. Cells that are homozygously mutant for the *de2f1*⁷²⁹ allele express β-Gal (red) from the *PZ[lacZ]* element inserted into the *de2f1* gene. Note that large clones of *de2f1* homozygous mutant cells (red) can be recovered only in *de2f2* homozygous mutant cells (lack of GFP), while, in the presence of *de2f2* (GFP positive), clones of *de2f1* mutant cells do not exceed one to three cells (yellow). (C*) GFP clone marker alone of the same image as in panel C. (D and D*) Higher magnification of the same image as in panels C and C*. Two clones of *de2f1 de2f2* double-mutant cells are outlined. Arrows point to *de2f1* single-mutant cells that carry wild-type *de2f2*. Staining with DNA dye 4',6'-diamidino-2-phenylindole confirms that these are individual cells. (D*) GFP clone marker of the same image as in panel D.

construct features a C-terminal S tag for protein detection. This expression construct was sequenced to confirm accurate insertion of the Bel ORF.

RESULTS

The loss of *de2f2* rescues the proliferative defect of *de2f1* mutant cells in mosaic animals. As has been previously shown, clones of *de2f1* mutant cells are extremely small (3, 37, 41). Although the larval phenotype of the *de2f1* mutant is largely rescued by mutation of *de2f2*, it has not been tested whether and to what extent the loss of *de2f2* suppresses the small clone

FIG. 2. The loss of *bel* rescues proliferation of *de2f1* mutant cells. Wild-type cells are marked by the presence of GFP (green), while mutant tissue lacks GFP. Clones of mutant cells were induced with *ey-FLP* and are identified by the lack of GFP (green). (A) Clones of *bel*^{7d19} *de2f1*⁷²⁹ double-mutant cells. (B and B*) The loss-of-function *bel*^{L4740} mutant allele makes no Bel protein (red), as revealed by the absence of staining with anti-Bel antibody in clones of *bel*^{L4740} mutant cells (absence of green). (B*) The same image as in panel B, showing expression of Bel (red) without GFP. (C) The known loss-of-function *bel*^{L4740} mutant allele rescues proliferation of *de2f1* mutant cells. Note the appearance of patches of double-mutant, GFP-negative tissue. (D) The eye discs were labeled with BrdU (red) to visualize S phases. The MF is marked by the arrowhead. Wild-type cells asynchronously cycle anterior to the MF, are arrested in G₁ within the MF, and synchronously enter S phase in the SMW posterior to the MF. The loss of *bel* partially rescues the SMW in *de2f1* mutant cells, as evident by the appearance of *bel* *de2f1* double-mutant BrdU-positive cells in the SMW. (E) Distribution of Bel protein in a wild-type eye disc. Bel is shown in red, and DAPI staining is shown in blue. Bel exhibits a predominantly cytoplasmic distribution.

size of *de2f1* mutant cells. To date, a *de2f2* FRT mutant chromosome has not been reported. We used a novel strategy to create a *de2f2* FRT40A mutant chromosome that carries loss-of-function allele *de2f2*^{c03344}. In the eye discs, clones of *de2f2* mutant cells can be distinguished by the lack of a GFP marker while the wild-type tissue expresses GFP. As expected, the dE2F2 protein is undetectable in clones of *de2f2*^{c03344} mutant cells (Fig. 1B and B*).

Next, we induced clones of *de2f1* *de2f2* double-mutant cells. Since *de2f1* and *de2f2* are on different chromosomes, partially overlapping clones of *de2f1* single-, *de2f2* single-, and *de2f1* *de2f2* double-mutant cells are recovered. Cells that are *de2f1* homozygous mutants are marked by β -Gal, while *de2f2* *de2f1* double mutants can be distinguished by the lack of GFP signal. Strikingly, relatively large clones of *de2f1* mutant cells (β -Gal positive) can be recovered only when *de2f2* is inactivated (GFP negative) (Fig. 1C, C*, D, and D*). In contrast, clones of *de2f1* mutant cells (β -Gal positive) are extremely small in the presence of the wild-type copy of *de2f2* (GFP positive). Thus, proliferation of *de2f1* mutant cells is rescued by the loss of *de2f2* in mosaic animals. We note that the rescue is not complete and that the clones of *de2f1* *de2f2* double-mutant cells are smaller than clones of wild-type cells (compare Fig. 1C and A). Thus, E2F-deficient cells are at a proliferative disadvantage when directly compared with wild-type cells. This observation supports the idea that E2F-deficient cells are not completely normal (19, 35) and further underscores the importance of E2F regulation for normal proliferation.

The loss of *bel* rescues proliferation of the *de2f1* mutant cells. We have used the FLP/FRT system to systematically screen the *Drosophila* eye for genes that when mutated rescue proliferation of *de2f1* mutant cells. One mutant isolated in the screen was named *7d19*. The small size of clones of *de2f1* mutant cells (3, 37) was rescued by *7d19*, and numerous clones of *7d19* *de2f1*⁷²⁹ double-mutant cells were recovered (Fig. 2A). By standard mapping techniques, *7d19* has been mapped to the *bel* gene. *bel* encodes a DEAD box RNA helicase that is highly conserved from yeast to humans. DEAD box proteins have been implicated in RNA-processing events such as splicing, nuclear export, degradation, and translation (9, 29, 31). Sequencing of the *bel* ORF from the *7d19* chromosome identifies a point mutation that results in a glycine-to-serine substitution at position 414. This mutation affects the highly conserved “GG doublet” of the DEAD box protein family (9). Mutation of either of these glycines completely abolishes the function of the related DEAD box helicase eukaryotic initiation factor 4A (eIF4A) in yeast (9). Thus, the *bel*^{7d19} mutant appears to be a strong loss-of-function allele and accordingly it fails to complement known *bel* mutant alleles. To confirm the identity of *bel*, we tested whether a known loss-of-function allele of *bel*, *bel*^{L4740}, rescues the *de2f1* mutant phenotype. In the *bel*^{L4740} mutant allele, a P element is inserted into the *bel* gene and no protein is produced (Fig. 2B and B*). Numerous clones of *bel*^{L4740} *de2f1*⁷²⁹ double-mutant cells were recovered (Fig. 2C). Thus, the genetic interaction between *de2f1* and *bel* mutant

alleles strongly suggests that the loss of *bel* relieves the cell cycle arrest of *de2f1* mutant cells.

In the eye disc, the patterns of S phases can be visualized by BrdU labeling. During larval development, a morphogenetic furrow (MF) sweeps from posterior to anterior across the eye imaginal disc. As asynchronously dividing cells enter the MF, they undergo a G₁ arrest. Posterior to the MF uncommitted cells enter a final synchronous cell division called the second mitotic wave (SMW), visualized as a sharp band of BrdU-positive cells. The loss of *de2f1* prevents entry into S phase in the SMW (13). Mutation of *bel* partially rescues this defect. As shown in Fig. 2D, *bel de2f1* double-mutant cells enter S phase in the SMW although the number of BrdU-positive cells and the overall level of BrdU labeling are somewhat reduced compared to those for the wild type.

As an initial step in characterizing Bel, we immunostained eye discs with anti-Bel antibody. In the wild-type tissue, Bel protein is expressed ubiquitously throughout the disc. This staining is highly specific because it is absent in cells homozygous for the *bel*^{L4740} mutant allele, which is expected to make no Bel protein (Fig. 2B and B*). High-magnification confocal images show that Bel is absent from the nucleus and localized to the cytoplasm, as would be expected for an RNA helicase acting posttranscriptionally (Fig. 2E). We repeatedly observed a slight but reproducible increase of Bel protein in cells preceding the MF, while its expression is somewhat reduced in the more posterior differentiating cells.

The effect of *belle* on dE2F2-mediated repression. One of the hallmarks of the *de2f1* mutant phenotype is a reduction of E2F-dependent transcription, which is partially due to dE2F2-mediated repression. Therefore, we asked whether *bel* affects dE2F2-mediated repression. The trivial explanation that the levels of RBF1 or dE2F2 are down-regulated by mutation of *bel* is unlikely since both proteins are expressed normally in *bel* mutant cells and in RNAi-treated tissue culture cells (Fig. 3). In the eye discs, a *PCNA*-GFP reporter construct is commonly used as a sensitive readout of E2F transcription (45). This reporter reflects the normal expression of *PCNA*, a well-characterized E2F target, and has been previously validated to assess E2F transcriptional activity (2, 45). We examined the expression of the reporter in the eye imaginal discs following induction of clones of *bel de2f1* double-mutant cells. The reporter is expressed at a high level ahead of the MF and in a stripe of cells immediately posterior in wild-type cells. However, the *PCNA*-GFP reporter remains repressed in *bel de2f1* double-mutant cells (Fig. 4A). Thus, although mutation of *bel* rescues the proliferation of *de2f1* mutant cells, it appears that the loss of *bel* fails to restore the expression of the E2F reporter in *de2f1* mutant cells. This raises the possibility that dE2F2-mediated repression is not affected in *bel* mutant cells. This was confirmed in two ways. First, we measured the expression of a known dE2F2-specific target, *Arp53D*, in the eye imaginal discs by real-time reverse transcription-PCR (for details see Materials and Methods). In wild-type cells, *Arp53D* is expressed at very low levels but is strongly derepressed in cells lacking dE2F2 or DDP both in vivo and in vitro (11). In *bel* mutant cells, the level of *Arp53D* transcripts is as low as in wild-type cells, indicating that *Arp53D* remains fully repressed (Fig. 4B). Similarly, depletion of Bel by RNAi in *Drosophila* S2 tissue culture cells does not relieve the repression of *Arp53D*

FIG. 3. Levels of dE2F2 and RBF1 are normal in *bel* mutant cells. Clones of mutant cells were induced with *ey-FLP* and are identified by the lack of GFP (green). (A, A*, B, and B*) Clones of *bel* mutant cells were induced in the eye discs, and the levels of RBF1 and dE2F2 were determined by immunofluorescence. Clones of mutant cells are distinguished by the lack of GFP. (A and A*) RBF1 (red) is expressed ubiquitously in the eye disc, with a slight increase anterior to the MF. Staining with anti-phospho-H3 antibody (blue in panel A) marks the positions of the MF (arrowhead) and the SMW. (A*) The same image as in panel A, showing phospho-H3 (white) and RBF1 (red) expression. (B and B*) dE2F2 (red) is expressed normally in clones of *bel* mutant cells (lack of GFP). (B*) The same image as in panel B, showing dE2F2 expression (red). (C) Bel was depleted by RNAi in S2 cells, and the levels of RBF1, cyclin A (CycA), CycB, and dE2F2 were determined by Western blot analysis. A nonspecific band for dE2F2 is marked by an arrow. Tubulin was used as a loading control.

(data not shown). Second, we examined the effect of Bel on dE2F2-mediated repression in transient transfections. In agreement with the previous results, dE2F2 represses an E2F reporter equally well in both control and Bel-depleted cells (Fig. 4C). Accordingly, the overexpression of Bel in transient transfections fails to repress the E2F reporter (Fig. 4D).

Taken together, these data indicate that mutation of *bel* does not generally relieve E2F-mediated repression. Although we cannot exclude the possibility that some E2F targets are derepressed in *bel* mutants, the explanation that the loss of *bel* rescues the *de2f1* mutant phenotype through a global release of E2F-dependent repression is unlikely. The inability of *bel* mutations to restore the transcriptional defects in *de2f1* mutant cells may explain why the loss of *bel* provides only a partial rescue of the *de2f1* mutant phenotype.

FIG. 4. Effect of Bel on dE2F2-mediated repression. (A) The loss of *bel* fails to restore the normal level of *PCNA*-GFP reporter (green) in the SMW inhibited in *de2f1* mutant cells. *bel de2f1* double-mutant cells are marked by the presence of β -Gal (red), and wild-type cells are distinguished by the lack of β -Gal. The position of the MF is shown by the arrowhead. (B) A well-known dE2F2-specific target, *Arp53D*, remains fully repressed in the eye discs containing clones of *bel* single-mutant and *bel de2f1* double-mutant cells. *Arp53D* is strongly derepressed in *dDP* mutant eye discs (DP) and in the eye discs containing clones of *dDP* mutant cells (FLP DP). The level of *Arp53D* was determined by real-time reverse transcription-PCR. The means of the three experiments are shown above the bars. (C) Depletion of Bel in tissue culture cells has no effect on the ability of dE2F2 to repress a *PCNA-luc* reporter in transient transfections. Cells were incubated with control (solid bars) or Bel (open bars) double-stranded RNA (dsRNA). After 4 days, cells were transfected with a *PCNA-luc* reporter and with either an empty vector or pIE4-dE2F2 expression plasmids in triplicate. To normalize for the efficiency of transfection, a β -Gal expression plasmid was included. Depletion of Bel was verified by Western blotting. (D) Bel does not repress the E2F reporter in transient transfections. Cells were cotransfected with a *PCNA-luc* reporter; a β -Gal expression plasmid; and vector alone or a pIE-dE2F2, pIE4-dE2F1, or pIE-S-Bel expression construct. As expected, dE2F2 represses the E2F reporter while dE2F1 activates it. In contrast, overexpression of Bel does not repress the *PCNA-luc* reporter. Expression of exogenous Bel was verified by Western blotting as shown below.

***bel* is required for G_1 arrest in the MF in the developing eye.**

The results described above suggest that *bel* rescues the *de2f1* mutant phenotype in a dE2F2-independent manner. To gain insights into the mechanism of the rescue, we examined *bel* mutant cells in greater detail. In particular, we focused on the region of the eye disc known as the MF, where wild-type cells are arrested in G_1 prior to entering the SMW (Fig. 5A and B). Strikingly, while neighboring wild-type cells synchronously undergo G_1 arrest, *bel* mutant cells continue cycling. This is evident by the presence of *bel* mutant cells, which ectopically incorporate BrdU (Fig. 5C), show an abnormally high level of mitotic cyclin B (Fig. 5D) and cyclin A (data not shown), and undergo mitoses in the anterior part of the MF (Fig. 5E and E*). Double staining with cyclin B and phospho-H3 revealed the presence of cells that have a high level of cyclin B but were phospho-H3 negative, cells that have high level of cyclin B and phospho-H3, and cells that had already degraded cyclin B but retained a high level of phospho-H3 (Fig. 5E and E*). The random distribution of the cell cycle markers suggests that *bel* mutant cells continue to asynchronously cycle in the MF. However, *bel* mutant cells do eventually arrest in the posterior part of the MF. These results indicate that *bel* promotes a G_1 arrest,

at least in this developmental context. In an attempt to test this in overexpression experiments, we used an EP element located immediately upstream of the *bel* gene to drive ectopic Bel expression with different Gal4 drivers. However, the level of exogenous Bel protein was low and insufficient to affect the cell cycle progression, presumably due to a high level of the endogenous Bel protein.

We sought additional evidence that *bel* mutant cells asynchronously proliferate in the MF, instead of being arrested randomly in the cell cycle. In the wild-type eye imaginal disc, proliferating cells respond to gamma irradiation by arresting in G_2/M in response to a DNA damage checkpoint. This arrest can be visualized by complete disappearance of a mitotic phospho-H3 marker 1 h after irradiation (Fig. 5F). We reasoned that, if *bel* mutant cells are cycling in the MF, then there will be no phospho-H3-positive cells following gamma irradiation. Conversely, if, in the MF, *bel* mutant cells are arrested randomly within the cell cycle, then we expect to detect the persistence of cells expressing phospho-H3 after gamma irradiation. After 1 h of irradiation, phospho-H3-positive cells were completely absent among mutant and wild-type cells (Fig. 5G and F). From this result we come to two conclusions. First, a

FIG. 5. *bel* mutant cells fail to arrest in G_1 in the MF. Clones of mutant cells are identified by the lack of GFP (green). (A and B) In wild-type discs, cells are arrested in G_1 in the MF (arrowhead). This is evident by the lack of cells that are BrdU positive (red) and express mitotic marker phospho-H3 (blue) (A) and the absence of cells that express the G_2 marker cyclin B (CycB; red) (B) within the MF. The image shown in panel A is at a higher magnification than the image in panel B. Anterior to the MF, the number of mitotic cells is increased. In the SMW, a synchronous wave of S phases is followed by a wave of mitoses. (C, D, E, and E*) *bel* mutant cells (GFP negative) fail to arrest in the anterior region of the MF and continue cycling, as evident by the appearance of mutant cells that show ectopic markers of S phase (BrdU), G_2 (CycB), and mitosis (phospho-H3). (C) *bel* mutant cells ectopically incorporate BrdU (red) in the MF. (D) *bel* mutant cells ectopically express CycB (red; arrow) in the MF. (E) *bel* mutant cells show ectopic CycB (red) and phospho-H3 (blue) in the MF. Double staining with CycB and phospho-H3 reveals cells that express only CycB (red; arrowhead), CycB, and phospho-H3 (magenta; thick arrow) and cells that degraded CycB but still express phospho-H3 (blue; thin arrow). Random distribution of the cell cycle markers indicates that *bel* mutant cells are asynchronously cycling. (E*) The same image as in panel E, showing the GFP clone marker (green) and CycB (red). The position of a clone is outlined. (F and G) *bel* mutant cells arrest normally following gamma irradiation. Clones of cells homozygous for wild-type (F) and *bel*^{L-4740} mutant (G) chromosomes were induced with *ey-FLP*. Larvae were irradiated and kept at 25°C for 1 h, and mitotic cells were visualized with anti-phospho-H3 antibody.

DNA damage checkpoint remains intact in *bel* mutants. Second, *bel* mutant cells fail to arrest in G_1 in the MF and indeed asynchronously cycle.

The Cubitus interruptus transcription factor, Ci155, is down-regulated in *bel* mutant cells. To gain insight into the normal function of *bel* in wild-type cells, we focused on characterization of the failure of *bel* mutant cells to arrest within the MF. The G_1 arrest in the MF has been shown to be dependent on the partially redundant Hh and decapentaplegic (Dpp) pathways (2, 17, 23, 47). Therefore, we examined whether these pathways are deregulated in *bel* mutant cells. First, we determined the expression of pMad, an activated form of a downstream transcription factor of Dpp signaling in the eye (1, 40). In wild-type cells, pMad is expressed at a low level near the MF, with a characteristic granular distribution and increases in regular groups of posterior cells (Fig. 6A and A*) (47). In clones of *bel* mutant cells, both high- and low-level expression of pMad antigen is normal.

The full-length form of transcription factor Ci155 is a downstream nuclear effector of the Hh pathway and commonly used as a readout of activation of Hh signaling (33, 36, 47). In wild-type cells, Ci155 accumulates to a high level anterior to

the MF. However, the level of Ci155 is strongly reduced in clones of *bel* mutant cells (Fig. 6B and B*). Ptc is a well known target of Ci155. In the eye imaginal disc, Ptc is expressed at a low level in a thin stripe in the MF and at a higher level in differentiating photoreceptors (43). In agreement with the reduced level of Ci155, Ptc is strongly down-regulated in the MF in clones of *bel* mutant cells (Fig. 6C and C*). The reduction of Ci155 is not simply a consequence of ectopic proliferation of *bel* mutant cells in the MF since Ci155 has been previously shown to be expressed normally in *thick veins* mutant cells that also fail to arrest in the MF (17, 47).

We next tested whether the loss of *bel* specifically reduces Ci155 or whether it has a more general effect on other developmental pathways. We focused on Notch and epidermal growth factor receptor (EGFR) signaling due to their critical role in cell proliferation, differentiation, and survival during eye development. In wild-type cells, Notch is expressed in the MF and the SMW. As shown in Fig. 6D, D*, E, and E*, the pattern of Notch expression and that of its ligand D1 is unaffected in *bel* mutant cells. To assess activation of the EGFR pathway, we looked at the distribution of phospho-extracellular signal-regulated kinase (p-ERK). p-ERK is expressed in the

FIG. 6. Mutation of *bel* specifically reduces the level of Ci155. Clones of *bel* mutant cells were induced with *ey-FLP* are marked by the absence of GFP (green). Panels marked with an asterisk lack the GFP signal, and clones of *bel* mutant cells are outlined. (A and A*) A marker of the Dpp pathway, pMad (red in panel A and white in panel A*), is expressed in *bel* mutant cells. (B and B*) Expression of Ci155 (red in panel A and white in panel A*) is strongly reduced in *bel* mutant cells. (C and C*) Ptc is expressed in differentiating ommatidia and at a low level in the MF (marked with a bar under the image). Ptc (red in panel C and white in panel C*) is strongly down-regulated in the MF in *bel* mutant cells. (D and D*) Expression of Notch (N) (red in panel D and white in panel D*) is not altered in *bel* mutant cells. (E and E*) Expression of the Notch ligand, DI (red in panel E and white in panel E*), is normal in *bel* mutant cells. (F and F*) Expression of p-ERK, a marker of activated EGFR (red in panel F and white in panel F*), is unaffected by a mutation in *bel*. (G) Mutation of *smo* partially rescues the small clone size of *de2f1* mutant cells. *smo de2f1* double-mutant cells are distinguished by the lack of GFP (green). (H and I) The effect of *Bel* on transcriptional activation of a Ptc reporter gene in *Drosophila* S2 cells. (H) Ci155 fails to efficiently activate the reporter in *Bel*-deficient cells. Control cells (solid bars) or cells treated with *Bel* double-stranded RNA (dsRNA) (open bars) were transfected with an empty vector or pDΔ136-Luc (Ci) together with a luciferase reporter plasmid under the control of either a wild-type (pDΔ136-Luc) or mutant (pDΔ136-mut) Ptc promoter. To determine transfection efficiency, a β-Gal expression plasmid was cotransfected in each well. The experiments were done in triplicate to calculate the averages and standard errors. (I) *Bel* potentiates Ci155-dependent activation of the Ptc reporter construct. Transfections were done essentially as in panel H but without an RNAi treatment, and an S-Tag-Bel expression plasmid was included. Expression of S-Tag-Bel was confirmed by Western blotting.

MF in a group of cells from which the R8 photoreceptor cell is selected. p-ERK staining was observed in clones of *bel* mutant cells, indicating that the loss of *bel* does not affect the EGFR pathway (Fig. 6F and F*). Thus, the loss of *bel* specifically reduces Ci155, the effector of Hh signaling, but has no effect on several other tested pathways.

Taken together these data raise a question as to whether the reduced level of Ci155 in *bel* mutant cells is important for the rescue of cell proliferation in *de2f1* mutant cells. To test this idea,

we introduced a *smoothened* (*smo*) mutation into *de2f1* mutants since *smo* mutant cells fail to accumulate Ci155. The mutation of *smo* rescues the proliferation of *de2f1* mutant cells, as evident by the appearance of multiple clones of *smo de2f1* double-mutant cells, although the rescue is slightly less efficient than in the case of *bel* mutations (Fig. 6G). Thus, we concluded that the reduction of Ci155 by *bel* mutations is an important mechanism in relieving the cell cycle block in *de2f1* mutant cells.

Next, we examined the effect of *Bel* on Ci155-mediated

FIG. 7. The loss of *bel* delays the onset of differentiation. Clones of *bel* mutant cells were induced with *ey-FLP* and are distinguished by the lack of GFP (green). Cells are stained with an R8 marker anti-Ato (A), neuronal marker anti-Elav (B), and the cone cell markers anti-Prospero (C), and anti-Cut (D), shown in red. Panels marked with an asterisk lack the GFP signal.

transcriptional activation in transient transfections in S2 cells. We used a *ptc* Δ 136-Luc reporter, which contains a *patched* promoter fused to luciferase. Although Ci155 is not expressed endogenously in S2 cells, the cells retain a normal response to Hh signaling once Ci155 is provided exogenously (20, 34). In control-treated cells, transfection of the Ci155 expression construct activates the reporter (Fig. 6H). This activation is dependent on a Ci155 binding site because a mutant reporter construct, *ptc* Δ 136-mut, does not respond to Ci155. In Bel-depleted cells, Ci155 activation is significantly reduced. This is not due to an effect of Bel on the basal level of the reporter since the expression of a mutant reporter is unaffected in Bel-depleted cells. These results are consistent with the reduced level of Ptc in clones of *bel* mutant cells (Fig. 6C). In a converse experiment, overexpression of Bel cooperates with Ci155 and increases the expression of the reporter (Fig. 6I). It has been reported that the known components of the Hh pathway modulate the expression of the *ptc* Δ 136-Luc reporter to a similar magnitude in transient transfections (20). Thus, our data indicate the importance of Bel in Hh signaling.

Differentiation is delayed in *bel* mutant cells in the eye imaginal disc. Next, we examined photoreceptor differentiation in *bel* mutant cells. Ato is a transcription factor that determines the first photoreceptor, R8, to differentiate (25). In *bel* mutant cells, both up-regulation and resolution of Ato expression are delayed (Fig. 7A and A*). We used markers for different cell types to further define the differentiation defects in *bel* mutant cells. We find that expression of a neuronal marker, Elav, is delayed and displaced slightly posteriorly in *bel* mutant cells (Fig. 7B and B*). This is in agreement with the delay in R8 recruitment, as revealed by Ato expression. In more posteriorly situated clones, most of the *bel* mutant cells

eventually express Elav, although there are some cells that remain Elav negative. Staining for cone cell markers Prospero and Cut revealed that there are very few cone cells present in clones of *bel* mutant cells and that cone cell differentiation is severely delayed (Fig. 7C, C*, D, and D*). Thus, we concluded that *bel* mutant cells delay, but still undergo, differentiation and that this delay is likely to be a consequence of delayed recruitment of ommatidial cells.

DISCUSSION

In an unbiased mosaic genetic screen, we have identified *bel* as a suppressor of the *de2f1* mutant phenotype. Although several “cell cycle” screens in *Drosophila* have been conducted, the *bel* gene was not among the isolated modifiers (4, 27, 44, 48). This may be due to the limitations of the previous screening strategies, which relied mostly upon overexpression of dE2F or RBF1 to generate the phenotype. In this work, we combined the FLP/FRT technique with the *de2f1* loss-of-function phenotype. This allowed us to rapidly screen for randomly induced recessive suppressor mutations while avoiding the unwanted effects provoked by the proteins present at abnormally high levels.

Previously, the *de2f1* larval phenotype was shown to be rescued by mutating the *de2f2* gene. Similarly, in mosaic animals, the cell cycle block in *de2f1* mutant cells is also dependent on *de2f2*, and the clones of cells lacking dE2Fs can be recovered even when competing with wild-type cells. Surprisingly, *bel* rescues the *de2f1* mutant phenotype through a novel, dE2F2-independent mechanism. In *bel* mutant cells, the levels of dE2F2 and RBF1 are normal and *bel* mutations do not generally relieve dE2F2-mediated repression. To our knowledge,

this is the first example of proliferation of *de2f1* mutant cells occurring in vivo in the presence of a functional dE2F2/RBF1 complex. In spite of the inability to relieve dE2F2-mediated repression, the loss of *bel* rescues the cell proliferation defects of *de2f1* mutant cells. In the eye discs, *bel* mutations partially restore S phase entry in the SMW, which is inhibited in *de2f1* mutant cells. However, mutant cells proliferate slower than their wild-type neighbors since the clones of *de2f1 bel* double-mutant cells are relatively small. This is consistent with the idea that dE2F-deficient cells are defective in their proliferation potential (19; this work). Alternatively, the loss of *bel* might not fully rescue all of the defects in *de2f1* mutant cells, including those that are dE2F2 dependent, since *bel* mutants fail to relieve the dE2F2-mediated repression. Nevertheless, *de2f1 bel* double-mutant cells progress through the cell cycle and differentiate, indicating that the mutant cells are largely normal in their response to developmental cues.

How does *bel* rescue the cell cycle defects in *de2f1* mutant cells? *bel* mutant cells fail to arrest in G₁ in the anterior part of the MF. Previous work has clearly established the central role of Dpp and Hh signaling in promoting cell cycle arrest in the MF (2, 17, 23, 47). In *bel* mutant cells, the expression of the downstream effector of Hh signaling Ci155 is strongly reduced. Consistently, mutation of *smo*, which prevents accumulation of Ci155, partially rescues proliferation defects in *de2f1* mutant cells. Thus, the reduced expression of Ci155 in *bel* mutant cells appears to be important for the rescue of the *de2f1* mutant phenotype. Since Ci155 is generally used as a marker of Hh pathway activity, it is possible that Hh signaling is reduced in *bel* mutant cells. This effect of *bel* is highly specific since other signaling pathways such as the Notch, Dpp, and EGFR pathways appear to be unaffected. It is worth noting that *bel* was independently isolated in a screen for genes required for photoreceptor differentiation, which also identified a number of Hh pathway components (24). However, the loss of *bel* does not completely phenocopy the loss of Hh signaling. In particular, the entry into the SMW that is indirectly dependent on Hh signaling (2, 15, 17) is relatively normal in *bel* mutant cells. One possible explanation is that the residual Ci155 present in *bel* mutant cells might be sufficient to promote the SMW but insufficient for cell cycle arrest in the MF. Interestingly, *bel* has been identified in an initial genome-wide RNAi screen as a potential candidate involved in the Hh signaling pathway (38). Our analysis of the *bel* mutant phenotype adds new significance to this finding, and we further expand these initial studies by examining the consequences of *bel* depletion and *bel* overexpression on an Hh reporter in a different cell line. However, although our data support the idea that the loss of *bel* rescues the *de2f1* mutant phenotype at least partially through down-regulation of Ci155, the inability of the *smo* mutant allele to fully suppress the *de2f1* mutant phenotype, to the extent of *bel* mutations, implies the existence of another, currently unidentified mechanism by which *bel* rescues the *de2f1* mutant phenotype.

Based on sequence homology and its ability to functionally substitute for the yeast protein Ded1p, Bel has been assigned to the Ded1p subfamily of DEAD box proteins (26). Although Ded1p was implicated in the regulation of pre-mRNA splicing and mRNA export, the mostly well-defined role of Ded1p is in translational initiation (8, 10, 30). We disfavor the explanation

that the loss of *bel* leads to a general reduction in protein synthesis because *Minute* mutants, which are characterized by a reduction in protein translation, proliferate more slowly than *bel* mutant cells. Additionally, mutations in eIF4A, a DEAD box protein that acts as a subunit of eIF4F in translational initiation, inhibits DNA replication (21). Since *bel* mutant cells proliferate normally and the loss of *bel* actually rescues the proliferation block of *de2f1* mutant cells, it is unlikely that *bel* mutations reduce the capacity of the cell to translate proteins. Interestingly, mutations in *ded1*, the *Schizosaccharomyces pombe* homolog of *bel*, specifically affect translation of mRNAs encoding Cig2 and Cdc13 (22). Ded1 has been isolated in a multicopy screen of a cold-sensitive mutant of the fission yeast Cdc2 and interacted with checkpoint kinase Chk1 (32). This raises the possibility that Bel may also be involved in translational regulation of specific mRNAs, for example, those encoding components or regulators of the Hh signaling pathway. Such a model is supported by the findings that Bel is almost exclusively localized to the cytoplasm. Alternatively, Bel may exert its effect independently of regulation of translation. It has been recently shown that eIF4A inhibits Dpp signaling by promoting ubiquitination and degradation of two downstream targets of Dpp in *Drosophila*, Mad and Medea (28). Thus, DEAD box proteins may have diverse and unexpected functions in the cell.

The genetic rescue of the *de2f1* mutant phenotype by the loss of *bel* underscores the potency of mutation of *bel* in overriding the cell cycle arrest in *de2f1* mutant cells. These findings closely parallel studies of mammalian cells since DDX3, the mammalian homolog of Bel, has been recently implicated in negative regulation of cell proliferation. Intriguingly, DDX3 is down-regulated at a high frequency in hepatocellular carcinoma patients, suggesting that DDX3 might be a candidate tumor suppressor (6, 7). Given that Hh signaling is reduced in *bel* mutant cells, it would be interesting to determine if DDX3 also affects this developmental pathway in mammals. In conclusion, the results described here illustrate the advantages of a genetic approach in studying the *de2f1* mutant phenotype, which may provide novel insights into the in vivo mechanisms of cell cycle regulation by dE2F and RBF.

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