# Repression of *dMyc* expression by Wingless promotes Rbf-induced $G_1$ arrest in the presumptive *Drosophila* wing margin

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Little is known about how patterns of cell proliferation and arrest are generated during development, a time when tight regulation of the cell cycle is necessary. In this study, the mechanism by which the developmental signaling molecule Wingless (Wg) generates $G_1$ arrest in the presumptive *Drosophila* wing margin is examined in detail. Wg signaling promotes activity of the *Drosophila* retinoblastoma family (Rbf) protein, which is required for $G_1$ arrest in the presumptive wing margin. Wg promotes Rbf function by repressing expression of the $G_1$-S regulator *Drosophila myc* (*dmyc*). Ectopic expression of dMyc induces expression of Cyclin E, Cyclin D, and Cdk4, which can inhibit Rbf and promote $G_1$-S progression. Thus, $G_1$ arrest in the presumptive wing margin depends on the presence of Rbf, which is maintained by the ability of Wg signaling to repress *dmyc* expression in these cells. In addition to advancing the understanding of how patterned cell-cycle arrest is generated by the Wg signaling molecule during development, this study indicates that components of the Rbf/E2f pathway are targets of dMyc in *Drosophila*. Although Rbf/E2f pathway components mediate the ability of dMyc to promote $G_1$ progression, dMyc appears to regulate growth independently of the RBF/E2f pathway.

Despite many advances in our understanding of the molecules that regulate the cell cycle, comparatively little is known about how patterns of cell proliferation or cell-cycle arrest are generated during development (1). Current studies indicate that some of the same molecules responsible for regulating patterning and differentiation also regulate cell proliferation and growth. For example, in addition to regulating expression of target genes that mediate its ability to promote cell patterning and differentiation, the developmental signaling molecule Hedgehog (Hh) induces expression of *Cyclins D* and *E* during *Drosophila* development. Induction of these $G_1$-S cyclins mediates the ability of Hh to drive cell growth and proliferation (2).

Wingless (Wg), a member of the Wnt family of secreted signaling proteins, functions as both a patterning molecule and a cell-cycle regulator during *Drosophila* development. The presumptive wing margin of the third-instar wing disk consists of a strip of cells located at the dorso-ventral boundary. Patterning of the margin, which will eventually contain an organized array of mechano- and chemosensory bristles, takes place during the third instar. This patterning process is regulated in part by Wg, which induces expression of proneural genes, such as *achaete* (*ac*) and *scute* (*sc*) (3, 4).

Before their differentiation as bristles, presumptive wing margin cells undergo cell-cycle arrest (5, 6). Because of the fact that this arrest occurs while most other wing disk cells are still cycling, the presumptive wing margin is often referred to as the zone of nonproliferating cells (ZNC). Notch promotes this arrest by sustaining Wg expression at the dorsal/ventral boundary. Wg-induced expression of *ac* and *sc* in the dorsal- and ventral-anterior regions of the ZNC induces $G_2$ arrest (6). Ac and Sc promote $G_2$ arrest in these cells by down-regulating

expression of *string*, the *Drosophila* homologue of the mitosis-inducing phosphatase Cdc25. Thus, through induction of proneural gene expression, Wg functions to promote both patterning and $G_2$ arrest in the dorsal and ventral anterior domains of the ZNC (6).

Although dorsal and ventral anterior ZNC cells arrest in $G_2$, other ZNC cells, including central anterior and all posterior cells, arrest in $G_1$ (ref. 6; Fig. 1*A*). In the anterior central region, Notch activity prevents $G_2$ arrest by repressing *ac* and *sc*. Notch-dependent Wg expression is required to promote $G_1$ arrest in the anterior central and posterior regions. Thus, although Wg is often associated with induction of cell proliferation (7, 8), in the wing, it can promote cell-cycle arrest (6). Recent studies also have indicated that Wg can constrain growth during *Drosophila* wing development (9).

The mechanism by which Wg induces $G_1$ arrest in the ZNC is not understood. Interestingly, Wg signaling inhibits expression of the growth inducer *Drosophila myc* (*dmyc*) in the ZNC (ref. 10; Fig. 2*G*). Myc is a transcription factor that heterodimerizes with Max; Myc-Max heterodimers promote transcription of genes with proximal E box consensus binding sites (11–13). dMyc is known to promote $G_1$-S progression in *Drosophila* (10). However, the transcriptional targets that mediate dMyc's ability to promote $G_1$ progression in flies have not yet been identified. Identification of such targets could lead to a better understanding of how Wg-mediated $G_1$ arrest is obtained in the ZNC, where dMyc expression is normally inhibited.

The retinoblastoma (Rb) pathway, a key regulator of the $G_1$-S phases of the cell cycle (14, 15), is a potential Wg target that could function to regulate the cell cycle in the ZNC. In its hypophosphorylated state, Rb proteins [such as *Drosophila* Retinoblastoma family (Rbf)] bind to and inhibit E2f, a heterodimer composed of an E2f and a DP subunit. E2f proteins, such as *Drosophila* E2f1 (dE2f1), regulate the transcription of a number of genes that function to promote S phase, such as *DNA polymerase* and *Cyclin E*. Binding of Rbf to dE2f1 inhibits the transcription activating function of dE2f1 and mediates $G_1$ arrest. Rbf could therefore mediate the ability of Wg to promote $G_1$ arrest in the ZNC. The role of Rbf in the ZNC, as well as the potential relationship between dMyc and the Rbf/E2f pathway, is examined here.

## Materials and Methods

**Fly Strains.** Fly strains used in this study include: FRT[18A]; MKRS,Hs-Flp/TM6b (16); *Rbf[14]* (17); *UAS-dmyc[42]* (18); *UAS-Rbf-*

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*280* (19); *UAS-dap* (20); UAS-dTCFΔN$^1$ (21); C96>Gal4, UAS-GFP$^{NLS}$S65T (6, 22); and en>Gal4, UAS-GFP$^{NLS}$S65T (23).

**Generation of Clones.** The FLP/FRT system (16) was used to generate *Rbf$^{-/-}$* clones in larvae of the following genotype: *Rbf$^{14}$, FRT$^{18A}$/FRT$^{18A}$; MKRSHs-Flp/+*. Heat shocks (38°C for 1 h) were given to generate FLP expression ≈48 h after the midpoint of the egg collection.

**Immunohistochemistry and *in Situ* Hybridization.** The following antibodies were used in this investigation: anti-BrdUrd mouse monoclonal (Becton Dickinson); FITC-conjugated anti-BrdUrd mouse monoclonal (Boehringer Mannheim); anti-Rbf (17); and anti-Digoxygenin mouse monoclonal (Roche Diagnostics). Secondary antibodies were obtained from Jackson ImmunoResearch. Discs were removed from wandering third-instar larvae and fixed for 15–30 min in 4% paraformaldehyde. Antibody staining was performed generally according to the procedure described by Patel (24). *In situ* hybridization was performed by using a modified version of the Patel (25) protocol.

**BrdUrd Incorporation.** Third-instar larvae were dissected in M3 culture media (Sigma), and discs were transferred immediately to M3 containing 0.3 mg/ml BrdUrd (U.S. Biologicals, Swampscott, MA). The discs were incubated in BrdUrd for 60 ± 10 min, rinsed with M3 for 15 min, rinsed with PBS for 15 min, and then fixed in 4% paraformaldehyde for 30 min. After fixation, discs were rinsed with PBS plus 0.1% Triton X-100 (PT) and then treated with 4 M HCl for 30 min. After the acid treatment, discs were rinsed, and primary antibody was applied.

**Flow Cytometry.** Wing discs expressing UAS-transgenes under C96>Gal4; UAS-GFP (22) control were dissociated as described (10, 23). GFP expression marked cells under control of C96>Gal4. Analysis of cell-cycle phasing and cell size [by forward scatter (FSC)] was carried out by using a Beckman Coulter Ultra Hypersort. Seventy wing discs were examined per experiment. Data analysis was done with CELLQUEST (BD Biosciences) software. Cell-cycle phasing was estimated by gating the G$_1$ and G$_2$ peaks in CELLQUEST. Gates were imposed on the G$_1$ and G$_2$ peaks in controls and then used to estimate the fraction of experimental cells in each phase of the cell cycle. Values reported in Fig. 4 represent a typical experiment. Cell size was measured by examining FSC distributions as described (23). Mean FSC values of experimental cells marked with GFP were normalized to the mean FSC value of control cells expressing GFP alone.

## Results

**Rbf Is Required for G$_1$ Arrest in the ZNC.** Cells in the posterior and anterior central region of the ZNC arrest in G$_1$ during the third instar, as illustrated through their failure to incorporate BrdUrd (ref. 6; Fig. 1*B*). Wg is required for this G$_1$ arrest, as well as for the G$_2$ arrest of a subset of cells in the anterior ZNC (ref. 6; Fig. 1*A*). In the ZNC of *wg$^{ts}$* mutants, or in presumptive wing margins in which dTCFΔN, a dominant-negative form of the Wg-transducer *Drosophila TCF* (21), has been expressed, cells that normally undergo cell-cycle arrest fail to do so (ref. 6; Fig. 1*C*). Although it is known that Wg induces G$_2$ arrest in the anterior dorsal and ventral domains of the ZNC through inhibition of *string* (6), the mechanism by which Wg induces G$_1$ arrest in the anterior central and posterior regions of the ZNC (Fig. 1*A*) is not well understood.

Inhibition of dE2f1 target genes such as *RNR-2* (6) and *PCNA* (Fig. 2*A*) in the wild-type ZNC suggests that Wg signaling might function to inhibit the transcription factor dE2f1, which is expressed in the ZNC (data not shown). To
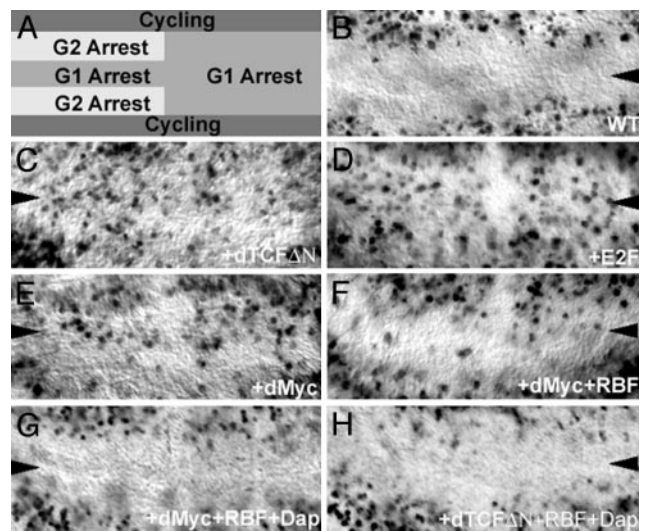


**Fig. 1.** Rbf regulates G$_1$ arrest in the ZNC. (*A*) Patterns of cell-cycle arrest induced by Wg in the *Drosophila* ZNC, adapted from ref. 6. (*B–H*) BrdUrd incorporation (black in *B–H*) marks cells in S phase. Cell-cycle arrest in the ZNC (wild type shown in *B*) is inhibited when a dominant-negative form of dTCF (dTCFΔN) is expressed in the ZNC with the C96>Gal4 wing-margin-specific driver (*C*). Overexpression of dE2f1 (*D*) or dMyc (*E*) with the C96>Gal4 driver promotes G$_1$ progression in regions of the ZNC that are normally arrested in G$_1$. Coexpression of Rbf-280 with dMyc (*F*) slightly inhibits the ability of dMyc to promote G$_1$ progression in the ZNC. Overexpression of Dap and Rbf-280 with dMyc (*G*) or with dTCFΔN (*H*) promotes G$_1$ arrest. Third-instar wing discs oriented anterior left and dorsal up are shown here and in all subsequent figures.

study this, dTCFΔN was expressed ectopically by using the C96>Gal4 (22) driver, which is specific to the presumptive wing margin; previous studies show that expression of dTCFΔN in the ZNC mimics results obtained with the *wg$^{ts}$* mutant (6). Expression of dTCFΔN leads to ectopic *PCNA* expression in the ZNC (Fig. 2*B*), suggesting that loss of Wg signaling results in activation of dE2f1. When Rbf-280, a constitutively active form of Rbf (19), is coexpressed with dTCFΔN in the ZNC, the normal inhibition of *PCNA* expression is restored (Fig. 2*D*). These observations suggest that Wg signaling might promote G$_1$ arrest in the ZNC by activating Rbf, which would then inhibit dE2f1.

To examine whether Rbf is required to induce cell-cycle arrest in the ZNC, the effect of *Rbf* loss on ZNC G$_1$ arrest was investigated. BrdUrd incorporation was examined in clones of cells that lack a functional copy of *Rbf*. Cells lacking Rbf fail to arrest in G$_1$ in both the anterior central and posterior regions of the ZNC (Fig. 3). Overexpression of dE2f1 in these G$_1$ arrested regions results in a similar loss of arrest (Fig. 1*D*). These data indicate that Rbf is, in fact, required for G$_1$ arrest in the ZNC.

**Repression of *dmyc* Expression by Wg Is Required for Rbf Function in the ZNC.** The mechanism by which Wg promotes Rbf function in the ZNC was examined. Expression of dTCFΔN in the wing margin has no impact on levels of Rbf (data not shown), indicating that Wg does not regulate Rbf expression. However, previous studies have shown that Wg signaling does function to inhibit expression of *dmyc* in the presumptive wing margin (ref 10; Fig. 2*G*). When Wg signaling is inhibited through expression of dTCFΔN, *dmyc* is expressed ectopically in the ZNC (10). Expression of dMyc, like overexpression of dE2f1 (Fig. 1*D*) or loss of Rbf (Fig. 3), is sufficient to prevent the block to G$_1$-S phase transition in the ZNC, as evidenced through BrdUrd
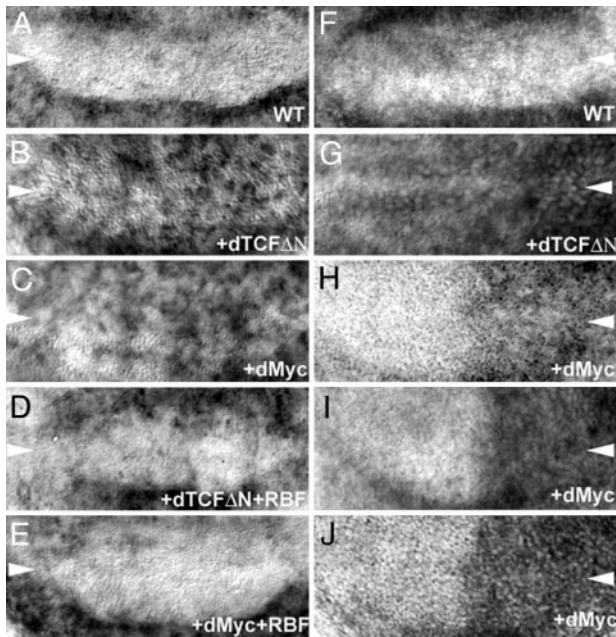
**Fig. 2.** Regulation of gene expression in the ZNC. Expression of the dE2f1 target gene *PCNA* is normally inhibited in the ZNC (*A*). Expression of either dTCFΔN (*B*) or dMyc (*C*) with the C96>Gal4 driver induces ectopic *PNCA* expression. Using the C96>Gal4 driver to coexpress Rbf-280 with dTCFΔN (*D*) or dMyc (*E*) restores repression of *PCNA* expression. Expression of *dmyc* is normally inhibited by Wg in the ZNC (*F*). When Wg signaling is disrupted by expression of dTCFΔN with the C96>Gal4 driver, *dmyc* expression is no longer repressed (*G*). Expression of dMyc with the En>Gal4 driver (*H–J*), which drives gene expression in the posterior of the wing, induces higher *Cyclin E* (*H*), *Cyclin D* (*I*), and *cdk4* (*J*) levels (compare to wild-type RNA levels in the anterior of each disk). Expression levels were analyzed through *in situ* hybridization.

incorporation (Fig. 1*E*) and fluorescence-activated cell sorting (Fig. 4*C*) experiments (10). These observations suggest that inhibition of *dMyc* expression in the ZNC is critical for Rbf-mediated $G_1$ arrest.

To examine whether dMyc activates the E2f pathway in the ZNC, the effect of dMyc expression on dE2f1 target gene transcription was examined. When dMyc is expressed in the presumptive wing margin with C96>Gal4, *PCNA* expression is induced (Fig. 2*C*). Thus, ectopic expression of dMyc, like inhibition of Wg signaling, activates the Rbf/E2f pathway in the ZNC. These observations indicate that during normal development of the wing margin, repression of *dmyc* expression by Wg is required for Rbf-mediated $G_1$ arrest.

**dMyc Can Inactivate Rbf Through Induction of $G_1$-S Cyclin and Cdk4 Expression in the ZNC.** To better understand why repression of dMyc expression by Wg is required for Rbf-mediated $G_1$ arrest in the ZNC, the mechanism by which dMyc activates dE2f1 was examined in more detail. Cyclins D and E, inhibitors of Rbf (14), both are expressed at low levels in the ZNC (see anterior portion of presumptive wing margins in Fig. 2 *H* and *I*). The effects of dMyc on expression of these $G_1$ cyclins were examined. When dMyc is expressed by using the En>Gal4 driver (which promotes *UAS*-transgene expression in the posterior of the wing), high levels of *Cyclin E* mRNA (Fig. 2*H*) expression are induced in the posterior of the wing disk, including the ZNC. Similarly, expression of dMyc promotes ectopic expression of *Cyclin D* mRNA in the presumptive wing margin (Fig. 2*I*). Higher levels of Cyclin E and D proteins also were noted in the ZNC when dMyc was expressed with either the En>Gal4 or C96>Gal4 drivers (data not shown). Furthermore, dMyc can induce expression of *Cyclin-*
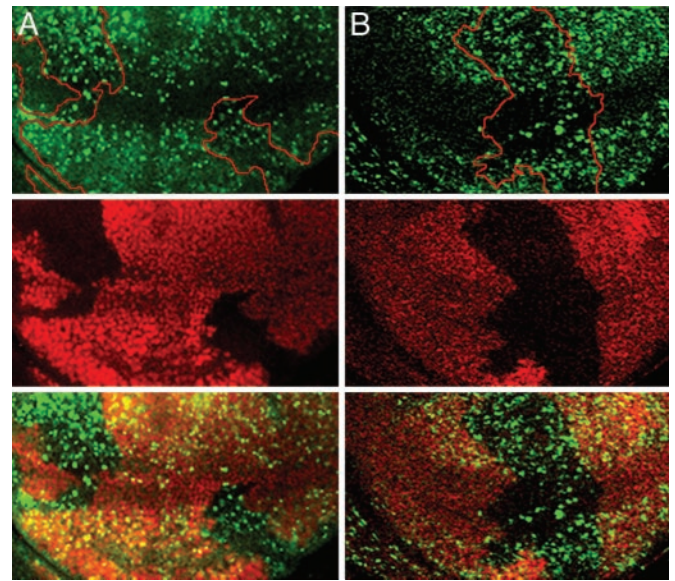


**Fig. 3.** Rbf is required for $G_1$ arrest in the ZNC. Loss of Rbf (indicated by loss of Rbf staining, red, in the circled *Rbf*$^{-/-}$ clones shown in *Middle*) results in S-phase entry in regions of the ZNC that are normally arrested in $G_1$ (BrdUrd in green, *Top*; overlay shown in the *Bottom*). Two clones are visible in *A*, and a single clone is visible in *B*.

*dependent kinase 4* (*cdk4*) mRNA, Cyclin D's kinase partner (Fig. 2*J*). These observations suggest that the ability of dMyc to promote $G_1$ progression in the ZNC (Fig. 1*E*) occurs through induction of *cdk4*, *Cyclin D*, and *Cyclin E*, known inhibitors of Rbf (14).

To test this hypothesis, constitutively active Rbf-280, which cannot be regulated by $G_1$-S cyclins (19), was coexpressed with
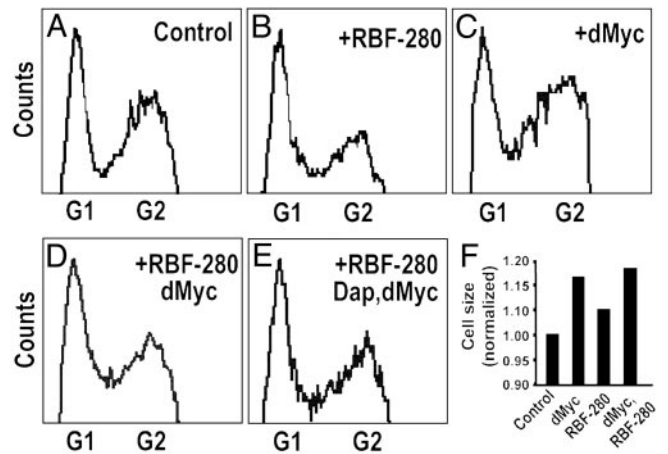


**Fig. 4.** dMyc can promote cell-cycle progression and growth in the wing margin. (*A–E*) Cell-cycle phasing. The indicated genes were expressed with the C96>Gal4 driver and marked by coexpression of GFP. GFP-expressing ZNC cells were analyzed through fluorescence-activated cell sorting. Estimated percentages of cells in each phase of the cell cycle are as follows. Control: 35.8% $G_1$, 8.8% S, 55.4% $G_2$; +Rbf-280: 39.7% $G_1$, 8.7% S, 51.6% $G_2$; +dMyc: 28.6% $G_1$, 10.3% S, 61.1% $G_2$; +Rbf-280, dMyc: 35.1% $G_1$, 10.3% S, 54.6% $G_2$; +Rbf-280, dMyc, Dap: 33.9% $G_1$, 7.1% S, 59.0% $G_2$. dMyc promotes $G_1$-S phase progression; Rbf-280 and Dap block this progression. (*F*) Cell size. GFP again was used to mark ZNC cells of the indicated genotypes. FSC distributions of GFP-positive experimental cells/GFP-positive control cells are shown. Expression of dMyc with C96>Gal4 increases cell size, but coexpression of Rbf-280 with dMyc or Rbf-280 plus Dap plus dMyc (data not shown) does not block induction of growth by dMyc.

dMyc in the presumptive wing margin. Overexpression of the dE2f1 target gene *PCNA* is observed when dMyc is expressed ectopically in the ZNC (Fig. 2*C*). This induction of *PCNA* expression can be inhibited by coexpression of constitutively active Rbf-280 with dMyc (Fig. 2*E*). These studies suggest that when dMyc is expressed ectopically in the ZNC, cells express high levels of Cyclin D, Cyclin E, and Cdk4, which can inactivate Rbf, resulting in dE2f1-activated transcription.

Because coexpression of Rbf-280 with dMyc inhibits the ability of dMyc to induce *PCNA* expression, its ability to inhibit dMyc-promoted $G_1$ progression was tested. Coexpression of Rbf-280 with dMyc does not completely restore $G_1$ arrest in the ZNC (Fig. 1*F*). Similarly, coexpression of Rbf-280 with dTCFΔN does not completely restore $G_1$ arrest in the ZNC (data not shown). The inability of Rbf-280 to completely block dMyc-promoted $G_1$-S progression is not surprising, because Cyclin E appears to promote S phase in the presence of Rbf-280 (2). Thus, increased levels of Cyclin E that are still observed when dMyc plus Rbf-280 are coexpressed in the wing margin (data not shown; this is also true of Cyclin D levels) might be capable of inducing S phase independently of Rbf in the ZNC. To test this idea, Dacapo (Dap), the *Drosophila* P21/27 homolog and inhibitor of CyclinE/Cdk2 (20, 26), was coexpressed with dMyc. Although Dap alone cannot block dMyc-promoted $G_1$ progression (data not shown), when Rbf-280 and Dap are coexpressed with dMyc in the ZNC, no ectopic S phases are observed (Figs. 1*G* and 4*E*). Furthermore, coexpression of Rbf-280 and Dap with dTCFΔN restores $G_1$ arrest in the ZNC (Fig. 1*H*). These results suggest that inhibition of both Cyclin D/Cdk 4 activity (by Rbf-280) and Cyclin E/Cdk2 activity (by Dap and Rbf-280) are required to block dMyc promoted $G_1$-S progression. Thus, during normal development, inhibition of dMyc expression in the ZNC is necessary to prevent accumulation of high levels of Cyclin D/Cdk4 and Cyclin E, which could prevent $G_1$ arrest.

### dMyc Promotes Growth Independently of the Rbf/E2f Pathway. Exclusion of *dMyc* expression from the ZNC is a prerequisite for the growth arrest of these cells (growth is defined here as an increase in cell mass and cell size). Overexpression of dMyc in the ZNC results in a visible increase in cell size, which also can be detected through FSC analysis (ref. 10; Fig. 4*F*). Recent studies indicate that overexpression of Rbf-280 inhibits Cyclin D/Cdk4-induced cell growth and proliferation in the wing disk (19). These results suggested that dMyc, which induces cell proliferation in part through inhibition of Rbf, also might induce growth by inhibiting Rbf. To test this hypothesis, Rbf-280 was coexpressed with dMyc, and cell size was evaluated. Visual inspection of cells coexpressing Rbf-280 plus dMyc indicates that dMyc can still induce cellular growth in the presence of Rbf-280 (data not shown). This observation was confirmed with FSC analysis. The average size of cells expressing dMyc is higher than that of control cells (ref. 10; Fig. 4*F*), and this size is not reduced when dMyc plus Rbf-280 are coexpressed (Fig. 4*F*) or when Rbf-280 plus Dap are expressed in conjunction with dMyc (data not shown). These data suggest that dMyc induces cellular growth independently of the Rbf/E2f pathway.

### Discussion
**Rbf-Induced Cell-Cycle Arrest in the ZNC.** This investigation examines the mechanism by which Wg signaling promotes $G_1$ arrest in the presumptive *Drosophila* wing margin. It was postulated that Rbf might mediate the ability of Wg to induce $G_1$ arrest, as loss of Wg signaling promotes expression of dE2f1 target genes (Fig. 2*B*). Overexpression of Rbf can block this induction of dE2f1 target gene expression (Fig. 2*D*). Strikingly, loss of Rbf in the ZNC prevents $G_1$ arrest, as evidenced by ectopic BrdUrd incorporation in *Rbf* mutant clones (Fig. 3). This

requirement for Rbf in the ZNC is notable. Surprisingly few developing fly tissues display such an absolute requirement for Rbf to promote $G_1$ arrest. To date, Rbf has been shown to be required to limit DNA replication in the embryo (17) and in the ovary (27). However, in many tissues, loss of Rbf does not result in ectopic S phase (W.D., unpublished observation); a likely explanation for this finding is that in other developing tissues, Rbf may function as one of several redundant mechanisms that function to promote $G_1$ arrest. Such redundancy would help to ensure that the cell cycle is regulated tightly during development.

**Interactions Between Rbf/E2f and dMyc in *Drosophila*.** In an attempt to better understand the mechanism by which Wg promotes Rbf function, this investigation uncovered interactions between dMyc and components of the Rbf/E2f pathway. Wg signaling normally inhibits dMyc expression in the ZNC (ref. 10; Fig. 2*G*). Ectopic expression of dMyc in the ZNC can induce expression of dE2f1 target genes (Fig. 2*C*), which can be blocked by the addition of Rbf-280 (Fig. 2*E*). Thus, overexpression of dMyc, which results from loss of Wg signaling in the ZNC, must somehow inactivate Rbf. These data indicate that inhibition of dMyc expression in the ZNC is critical for Rbf function.

The results presented in Fig. 2 *H–J* indicate why exclusion of dMyc from the ZNC is necessary for Rbf activity. Overexpression of dMyc leads to high levels of *Cyclin E*, *Cyclin D*, and *Cdk4* transcripts. dMyc also regulates Cyclin E posttranscriptionally in *Drosophila* (28). $G_1$-S Cyclins/Cdks function to phosphorylate and inhibit Rbf, suggesting that dMyc blocks Rbf activity through activation of $G_1$-S Cyclins/Cdks. Thus, inhibition of dMyc by Wg helps to ensure that $G_1$-S Cyclins/Cdks do not activate S phase. This idea is supported by the results shown in Fig. 1 *G* and *H*, which indicate that only a combination of both Dap and constitutively active Rbf (that cannot be regulated by Cyclins/Cdks) can restore $G_1$ arrest when Wg signaling is blocked or when dMyc is expressed. These results are summarized in Fig. 5. These data suggest that either Cyclin D or Cyclin E activity can mediate the ability of dMyc to promote S phase in the ZNC. Coexpressing Dap alone with dMyc, which would block only Cyclin E/Cdk2 activity, does not restore $G_1$ arrest (data not shown). Furthermore, overexpression of dMyc in a *cdk4* mutant background still results in ectopic S phases (data not shown), suggesting that Cyclin E/Cdk2 also are sufficient to mediate dMyc's ability to promote $G_1$ progression. Thus, either Cyclin D/Cdk4 or Cyclin E/Cdk2 is sufficient to mediate the ability of dMyc to promote $G_1$ progression. The ability of Wg to inhibit dMyc expression is thus critical for RBF activation and $G_1$ arrest in the ZNC. Still, it is possible that Wg promotes $G_1$ arrest through other mechanisms that have not yet been uncovered. Our observation that overexpression of dTCFΔN with C96>Gal4 can promote S phase even in a *dmyc* mutant background (data not shown) supports this idea.

It is likely that dMyc/dMax directly up-regulate transcription of *Cyclin D* and *cdk4* in *Drosophila*. Myc/Max heterodimers regulate transcription by binding to various consensus sequences, such as the E box (11–13, 29). Previous studies indicated that cMyc induces *Cyclin D2* expression in mice by binding to two consensus E boxes in the *Cyclin D2* promoter (30). *cdk4* also was identified as a transcriptional target of c-Myc (31). Furthermore, Orian *et al.* (29), who recently used the DamID method to carry out global genomic mapping of the *Drosophila* Myc, Max, and Mad/Mnt proteins, have suggested that *cdk4* is a transcriptional target of dMyc and *Cyclin D* is a transcriptional target of dMax. Although future studies should analyze the *Drosophila Cyclin D* and *Cdk 4* regulatory regions in more detail, these results suggest that the observed ability of dMyc to induce *Cyclin D* and *Cdk4* expression in the ZNC most likely occurs through transcriptional regulation of these proteins by dMyc/
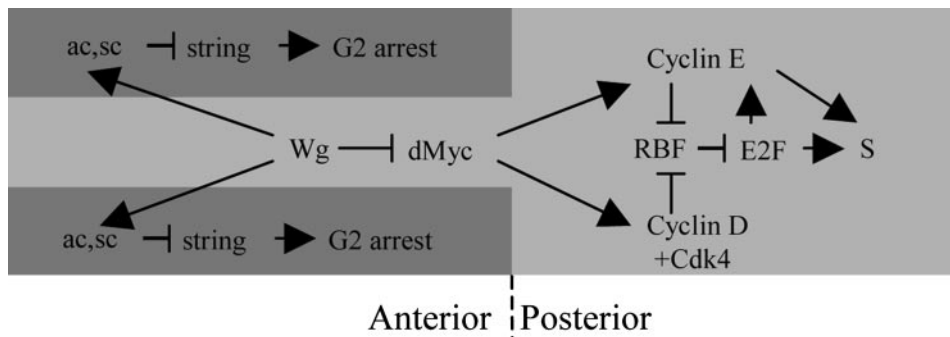
**Fig. 5.** Model for the induction of $G_1$ arrest by Wg in the ZNC. The genetic interactions for induction of $G_2$ arrest by Wg were described previously (6). This study indicates that induction of $G_1$ arrest by Rbf depends on the inhibition of dMyc expression by Wg.

dMax. In contrast, *Cyclin E* was not identified as a target of dMyc or dMax (29). It is more likely that the ability of dMyc to induce growth in the wing (see below) indirectly leads to increased *Cyclin E* transcript levels.

**dMyc Regulates Growth Independently of the Rbf Pathway.** Recent studies indicate that both dMyc (10) and Rbf (19) can regulate cellular growth in the *Drosophila* wing. dMyc induces cellular growth, whereas Rbf inhibits cellular growth and proliferation. dMyc can promote cellular growth in the presence of constitutively active Rbf (Fig. 4*F*), suggesting that dMyc can induce growth independently of the Rbf/E2f pathway. Such results are consistent with previous studies that indicate that Ras, which can induce growth by increasing levels of dMyc protein (28), also is capable of inducing growth in the presence of Rbf (19). It is likely that dMyc regulates growth through induction of genes encoding regulators of protein synthesis, such as ribosomal proteins and the DEAD-box helicase Pitchoune, as well as other proteins that regulate cellular metabolism (18, 29).

**Activation and Repression of Cell Proliferation by Wnt Signaling.** Wnt signaling is generally associated with the stimulation of cell proliferation during development and in tumor cells (7, 8). However, in the ZNC, Wnt/Wg signaling actually promotes cell-cycle arrest (6). Ironically, in the ZNC, Wg signaling suppresses expression of *dmyc* (10); however, a *cMyc* reporter was found to be directly up-regulated by Tcf4 in a colon carcinoma cell line (32). Thus, Wg appears to be able to up-regulate *Myc* expression in some tissues and to repress it in others.

The ability of Wg signaling to either activate or repress the same target gene in different situations has been observed in other cases. For example, in the developing *Drosophila* midgut, low levels of Wg signaling, in conjunction with Dpp, stimulate expression of *Ubx* and *lab*; high levels of Wg signaling result in the repression of *Ubx* and *lab* by means of the transcriptional repressor Teashirt (33, 34). Thus, expression of Wnt target genes can be turned on or off in response to the modulation of Wg levels as well as by the presence or absence of the various proteins that can regulate transcription in conjunction with, or in response to, Wg signaling. Such flexibility is advantageous to a developing organism.

**Developmental Morphogens: Patterning, Differentiation, and Cell Cycle Regulation.** Wg signaling can be modulated to affect expression of the same target gene differently in various situations. Moreover, Wg signaling can be modulated to promote or inhibit the different, somewhat conflicting cellular processes of patterning, growth, proliferation, and differentiation. The same is true for Hh signaling, which also regulates all of these cellular processes (2). Thus, it seems, at least in the case of Hh and Wg, that one signaling molecule can regulate many different types of cellular and developmental events. In order for various cellular programs to be implemented and coordinated during development, the way that a particular cell type responds to Wg or Hh signaling at any given time must be tightly regulated. The delicate balance between various processes that can occur in response to Hh or Wg signaling is likely maintained through tight control of the temporal and spatial expression patterns of Hh and Wnt targets and the molecules that regulate them.

1. Skaer, H. (1998) *Trends Genet.* **14,** 337–339.
2. Duman-Scheel, M., Weng, L., Xin, S. & Du, W. (2002) *Nature* **417,** 299–304.
3. Cubas, P., de Celis, J. F., Campuzano, S. & Modolell, J. (1991) *Genes Dev.* **5,** 996–1008.
4. Skeath, J. B. & Carroll, S. B. (1991) *Genes Dev.* **5,** 984–995.
5. O'Brochta, D. A. & Bryant, P. J. (1985) *Nature* **313,** 138–141.
6. Johnston, L. A. & Edgar, B. A. (1998) *Nature* **394,** 82–84.
7. Serrano, N. & O'Farrell, P. H. (1997) *Curr. Biol.* **7,** R186–R195.
8. Taipale, J. & Beachy, P. A. (2001) *Nature* **411,** 349–354.
9. Johnston, L. A. & Sanders, A. L. (2003) *Nat. Cell. Biol.* **5,** 827–833.
10. Johnston, L. A., Prober, D. A., Edgar, B. A., Eisenman, R. N. & Gallant, P. (1999) *Cell* **98,** 779–790.
11. Blackwood, E. M., Kretzner, L. & Eisenman, R. N. (1992) *Curr. Opin. Genet. Dev.* **2,** 227–235.
12. Amati, B. & Land, H. (1994) *Curr. Opin. Genet. Dev.* **4,** 102–108.
13. Henriksson, M. & Luscher, B. (1996) *Adv. Cancer Res.* **68,** 109–182.
14. Weinberg, R. A. (1995) *Cell* **81,** 323–330.
15. Dyson, N. (1998) *Genes Dev.* **12,** 2245–2262.
16. Xu, T. & Rubin, G. M. (1993) *Development (Cambridge, U.K.)* **117,** 1223–1237.
17. Du, W. & Dyson, N. (1999) *EMBO J.* **18,** 916–925.
18. Zaffran, S., Chartier, A., Gallant, P., Astier, M., Arquier, N., Doherty, D., Gratecos, D. & Semeriva, M. (1998) *Development (Cambridge, U.K.)* **125,** 3571–3584.
19. Xin, S., Weng, L., Xu, J. & Du, W. (2002) *Development (Cambridge, U.K.)* **129,** 1345–1356.
20. Lane, M. E., Sauer, K., Wallace, K., Jan, Y. N., Lehner, C. F. & Vaessin, H. (1996) *Cell* **87,** 1225–1235.
21. van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A., *et al.* (1997) *Cell* **88,** 789–799.
22. Gustafson, K. & Boulianne, G. L. (1996) *Genome* **39,** 174–182.

23. Neufeld, T. P., de la Cruz, A. F., Johnston, L. A. & Edgar, B. A. (1998) *Cell* **93,** 1183–1193.
24. Patel, N. H. (1994) *Methods Cell Biol.* **44,** 445–487.
25. Patel, N. (1996) *In Situ Hybridization to Whole Mount Drosophila Embryos* (Wiley–Liss, New York).
26. de Nooij, J. C., Letendre, M. A. & Hariharan, I. K. (1996) *Cell* **87,** 1237–1247.
27. Bosco, G., Du, W. & Orr-Weaver, T. L. (2001) *Nat. Cell. Biol.* **3,** 289–295.
28. Prober, D. A. & Edgar, B. A. (2000) *Cell* **100,** 435–446.
29. Orian, A., van Steensel, B., Delrow, J., Bussemaker, H. J., Li, L., Sawado, T., Williams, E., Loo, L. W., Cowley, S. M., Yost, C., *et al.* (2003) *Genes Dev.* **17,** 1101–1114.
30. Bouchard, C., Thieke, K., Maier, A., Saffrich, R., Hanley-Hyde, J., Ansorge, W., Reed, S., Sicinski, P., Bartek, J. & Eilers, M. (1999) *EMBO J.* **18,** 5321–5333.
31. Hermeking, H., Rago, C., Schuhmacher, M., Li, Q., Barrett, J. F., Obaya, A. J., O'Connell, B. C., Mateyak, M. K., Tam, W., Kohlhuber, F., *et al.* (2000) *Proc. Natl. Acad. Sci. USA* **97,** 2229–2234.
32. He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B. & Kinzler, K. W. (1998) *Science* **281,** 1509–1512.
33. Yu, X., Riese, J., Eresh, S. & Bienz, M. (1998) *EMBO J.* **17,** 7021–7032.
34. Waltzer, L., Vandel, L. & Bienz, M. (2001) *EMBO J.* **20,** 137–145.

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