

Identification of Domains Responsible for Ubiquitin-Dependent Degradation of dMyc by Glycogen Synthase Kinase 3 β and Casein Kinase 1 Kinases^{∇‡}

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In the present study, we report that ubiquitin-mediated degradation of dMyc, the *Drosophila* homologue of the human c-myc proto-oncogene, is regulated in vitro and in vivo by members of the casein kinase 1 (CK1) family and by glycogen synthase kinase 3 β (GSK3 β). Using *Drosophila* S2 cells, we demonstrate that CK1 α promotes dMyc ubiquitination and degradation with a mechanism similar to the one mediated by GSK3 β in vertebrates. Mutation of *ck1 α* or *- ϵ* or *sgg/gsk3 β* in *Drosophila* wing imaginal discs results in the accumulation of dMyc protein, suggesting a physiological role for these kinases in vivo. Analysis of the dMyc amino acid sequence reveals the presence of conserved domains containing potential phosphorylation sites for mitogen kinases, GSK3 β , and members of the CK1 family. We demonstrate that mutations of specific residues within these phosphorylation domains regulate dMyc protein stability and confer resistance to degradation by CK1 α and GSK3 β kinases. Expression of the dMyc mutants in the compound eye of the adult fly results in a visible defect that is attributed to the effect of dMyc on growth, cell death, and inhibition of ommatidial differentiation.

Myc is a member of a family of transcription factors that plays a pivotal role in the regulation of proliferation, growth and apoptosis. Myc expression is regulated by multiple signaling events acting at transcriptional, translational, and post-translational levels (6, 50). Phosphorylation is one of the most relevant posttranslational mechanisms by which Myc stability is regulated. Activation of the Ras extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK), c-Jun kinase (JNK), and cyclin-dependent kinase 1 (CDK1) (36, 44) rapidly stabilizes c-Myc protein by phosphorylation on serine 62 (46). This event primes Myc for phosphorylation by glycogen synthase kinase 3 β (GSK3 β) on threonine 58 (45). Dephosphorylation of serine 62 by the phosphatase PP2A/B56 α allows the ubiquitin ligase SCF (for Skip1-Cullin-F box) Fbw7 (SCF^{Fbw7}) to bind c-Myc on threonine 58, hence promoting its degradation by the proteasome pathway (54). In addition, the extent to which c-Myc exerts its effects is modulated by the SCF-SKP2 ubiquitin ligase, which enhances c-Myc transcriptional activity before triggering proteasomal degradation (25, 51). Coordination between phosphorylation and ubiquitination is a conserved feature in mammals and *Drosophila*, since mutations in *archipelago* (*ago*), encoding a ubiquitin ligase that is the *Drosophila* homologue of the mammalian Fbw7 ubiquitin ligase, result in dMyc protein accumulation (32), as does phosphorylation of dMyc by activated Ras (41).

In addition to phosphorylation at its N terminus, c-Myc protein stability is regulated by phosphorylation in a PEST domain (amino acids 253 to 266) located near the conserved acidic box (AB). Deletion of the c-Myc PEST domain enhances c-Myc protein stability (16).

Although the *Drosophila* Myc protein (dMyc) is only 26% identical to human c-Myc over its length, the homology increases up to 57% in critical functional regions such as the AB and the PEST domain (13). Moreover, dMyc and c-Myc proteins share similar functions; human c-Myc can rescue lethal *dmyc* mutations to viability (5), and dMyc is able to partially substitute for the proliferation defect of *c-myc*-deficient mouse embryo fibroblasts (49).

Analysis of the dMyc protein reveals the presence of evolutionarily conserved phosphorylation sites that correspond to optimal amino acid consensus sequences for phosphorylation by members of the casein kinase 1 (CK1) family and by the GSK3 β kinases. GSK3 β phosphorylation requires a priming event (10) that for some proteins, such as β -catenin/Armadillo or Cubitus interruptus (Ci), is carried out by members of the CK1 family (21, 28, 40, 53).

In the present study, we report that members of the CK1 kinases act as novel components of the process regulating dMyc protein stability through a mechanism that favors its

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ubiquitination and degradation, similar to the action of GSK3 β . In vivo, decreasing levels of *ck1 α* or *- ϵ* , or *sgg/gsk3 β* in wing imaginal disc cells favor dMyc protein accumulation, preferentially in the presumptive hinge and notum territories of the disc. Through site-directed mutagenesis, we identified novel phosphorylation domains that are responsible for dMyc degradation induced by the action of members of the CK1 and GSK3 β family of kinases. Expression of these dMyc phosphorylation mutants in vivo in the compound eyes of the adult fly (4) results in strong defects, which, at the cellular level, shows that expression of a proteasome-resistant form of dMyc inhibits ommatidial differentiation and increases cell death during the development of the eye imaginal discs.

MATERIALS AND METHODS

Fly husbandry and strains. Fly cultures and crosses were maintained on standard fly medium at 25°C. UAS-DCO and *FRT82B dco³/TM6B* stocks were a gift from M. Noll and E. Frei, *UAS-RNAi-CK1 α* (where *RNAi-CK1 α* denotes the *CK1 α* gene modified by RNA interference [RNAi]) stock was a gift from D. Kalderon, and *yw hs-Flp; Act5C-FRT y⁺FRT-Gal4* stock was a gift from A. Yamamoto. *UAS-HA-dMyc* stock was described in reference 4; *UAS-Sgg* stock was a gift from M. Milán. We generated the *dmyc^{P0}tub FRT-(dmyc cDNA)-FRT-Gal4-ey-FLP/Y* line (hereafter called *ey>dmyc^{P0}*) that constitutively expresses the *flippase (FLP-ase)* under the control of the *eyeless* promoter (*ey-FLP*), allowing the *dmyc* cDNA construct to be flipped out, rendering the eye mutant for *dmyc^{P0}*. This line also expresses the Gal4 reporter. We used the UAS/Gal4 system to express UAS transgene in the *eyeless* domain. Line *ey>yw* carries the same construct as in *ey>dmyc^{P0}* but in the *yw* background.

Cell culture and transfection of S2 cells. *Drosophila* Schneider S2 cells and the S2-HA-dMyc cell line (4) were grown at 25°C using Schneider medium (Gibco) supplemented with 10% heat-inactivated fetal calf serum and 100 IU of penicillin-streptomycin (Gibco). S2 cells were transfected by using Cellfectin reagent (Invitrogen) as described in reference 3. The S2-*tub-Gal4* line was generated in the present study by stable transfection of the *tubulin Gal4* plasmids. The *tubulin Gal4* construct was cotransfected with the pcP4 plasmid encoding the α -amanitin resistance gene, and cells were selected using 5 μ g of α -amanitin/ml for 3 weeks (Sigma) (3).

Western blotting and chemical treatments. Cells were lysed in 50 mM HEPES buffer (pH 7.5) containing 0.5% Triton X-100, 150 mM NaCl, 1 mM EDTA, and protease and phosphatase inhibitors. Lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, subjected to Western blot analysis, and visualized by using enhanced chemiluminescence (Amersham Pharmacia Biotech). For the ubiquitination analysis, *N*-ethylmaleimide, an inhibitor of deubiquitinating enzymes, was added to the lysis buffer, and immunoprecipitation was performed with rat anti-HA antibody bound to protein G-Sepharose (CL-4B; Amersham Pharmacia Biotech). We used the antibodies anti-HA (Roche), antiubiquitin (FK2-BioMol), antiactin and antivinculin (Sigma), and anti-dMyc (41). Chemicals were used at final concentrations of 50 μ M for MG132, 10 mM for okadaic acid (OA), 10 μ M for cycloheximide (CHX; Sigma), and 50 mM for LiCl and 10 μ M CK1-7 (U.S. Biologicals).

Clonal analysis. Clones mutant for *dco³* were induced by mitotic recombination using the FLP-FRT method (52). *yw hs-Flp; FRT82 Ubi-GFP/TM6B* flies were crossed to *yw; FRT82B dco³/TM6B* flies, and clones were induced by a 20-min heat shock at 37°C at 65 \pm 2 h after egg laying (AEL). *sgg/gsk3 β* mutant clones were generated by mitotic recombination as described previously (21). For ectopic expression of UAS-DCO, UAS-Sgg, and *UAS-RNAi-ck1 α* , Flp-out clones were induced using the *yw hs-Flp; Act5C>FRTy⁺FRT>Gal4; UAS-GFP* line and larvae were heat shocked at 37°C for 8 min at 65 \pm 2 h AEL.

Immunofluorescence analysis of imaginal discs and antibody staining. Discs were fixed in 4% paraformaldehyde in phosphate-buffered saline, permeabilized using 0.5% Triton X-100, and then blocked in 5% bovine serum albumin in phosphate-buffered saline. The antibodies used were anti-dMyc (1:5 dilution) (42), rat anti-ELAV (1:1,000 dilution; Developmental Study Hybridoma Bank), and rabbit anti-active caspase-3 (1:200 dilution; Invitrogen). The secondary antibodies, anti-mouse antibody-Alexa-555 and anti-rat antibody-fluorescein isothiocyanate (Invitrogen), were used at a 1:200 dilution in 5% bovine serum albumin, together with DAPI (4',6-diamidino-2-phenylindole) at 1 μ g/ml. After a washing step, discs were mounted in Vectashield and analyzed under a Leica

microscope. The images were processed by using Open Lab and Adobe Photoshop software.

Site-directed mutagenesis. Site-directed mutagenesis was carried out by using the QuikChange mutagenesis kit (Stratagene). Primer sequences are available in the supplemental material.

Determination of cell number and cell size in adult eyes. Flies were reared under identical growth conditions and age matched (3-day-old males). To determine the ommatidial number, ommatidia were counted from scanning electron micrographs of eyes from different animals. From the same photographs, the sizes of the ommatidia were calculated by measuring the area of 20 ommatidia located in the center of the eye using Adobe Photoshop.

RESULTS

CK1 and GSK3 β activity induces dMyc protein degradation. Analysis of the dMyc amino acid sequence reveals the presence of conserved domains (Fig. 1A and Table 1) containing consensus sites for phosphorylation by members of the CK1 (S/T-XX-S/T) family and by GSK3 β (S/T-XXX-S/T) (37). In vitro kinase assays demonstrated that dMyc and c-Myc proteins were directly phosphorylated by CK1 α and GSK3 β kinases (see Fig. S1 in the supplemental material), suggesting that Myc protein is a substrate for these kinases. In order to analyze whether CK1 α and GSK3 β affect dMyc protein stability, *Drosophila* S2 cells were treated with specific inhibitors of these kinases, and the dMyc protein levels were analyzed by Western blotting. As shown in Fig. 1B, treatment of the cells with either CK1-7, an inhibitor of the CK1 kinases, or LiCl, a specific inhibitor of GSK3 β , led to the accumulation of dMyc protein, suggesting that both CK1 kinases and GSK3 β act in the pathway of dMyc protein degradation. Since it was reported that GSK3 β induces c-Myc degradation through the proteasomal pathway (45), we blocked proteasome activity with the drug MG132 and verified the effects on dMyc protein levels. As shown in Fig. 1C, treatment of the cells with MG132 resulted in the accumulation of two forms of immunoreactive dMyc protein (arrowhead, compare lane 1 to lane 2). Treatment of cells with the kinase inhibitor LiCl or CK1-7, in the presence of MG132, blocked the appearance of the slower-migrating dMyc band (compare lane 1 with lane 5 to lane 6), suggesting that the upper band of the doublet represents a hyperphosphorylated form of dMyc. The tumor suppressor protein phosphatase type 2A (PP2A) favors c-Myc degradation by dephosphorylating c-Myc at serine 62 (54). Since electrophoretic mobility shift has been used as an indication of the phosphorylation status of a protein, we used OA to inhibit PP2A and determine whether treatment with OA changes the dMyc electrophoretic mobility. As shown in Fig. 1C, OA caused an upward electrophoretic mobility shift of dMyc protein (compare lanes 1 and 3), indirectly suggesting that inhibition of PP2A affects dMyc dephosphorylation. In order to test whether the upward electrophoretic shift observed in the presence of OA was due to phosphorylation of dMyc exclusively by GSK3 β , we treated cells with LiCl and OA together. LiCl only partially decreased the dMyc mobility shift induced by OA (compare lane 3 to lane 4), suggesting that another kinase, insensitive to LiCl, is responsible for the residual accumulation of the hyperphosphorylated form of dMyc (lane 4).

CK1 α and GSK3 β induce dMyc ubiquitination and degradation through the proteasome pathway. We then analyzed whether expression of CK1 α , GSK3 β , or their kinase-dead mutant forms (KD) would affect dMyc protein levels in S2

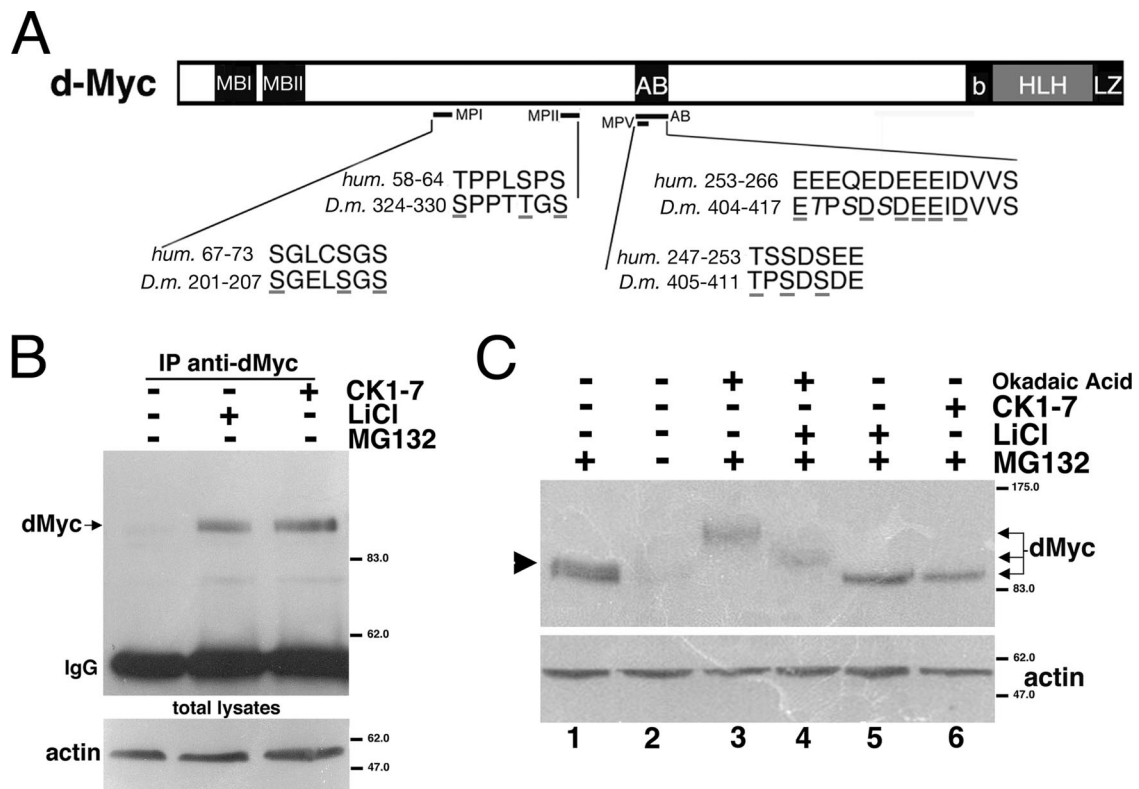


FIG. 1. Inhibition of GSK3 β and CK1 kinases increases dMyc protein level in S2 cells. (A) Schematic representation of dMyc protein with the amino acid sequences of dMyc phosphorylation mutants and homologous sequences in c-Myc. The amino acids changed by site-directed mutagenesis are underlined. (B) Treatment of S2 cells with LiCl and CK1-7 kinase inhibitors stabilizes endogenous dMyc protein. Cells were treated with the indicated inhibitors for 4 h; dMyc was immunoprecipitated from the cell extracts by using anti-dMyc antiserum, and its expression level was analyzed by Western blotting with anti-dMyc antiserum. The position of immunoglobulins is marked on the left. Total lysates were blotted with antiactin for control loading. (C) S2 cells were treated with MG132 (proteasome inhibitor), OA (an inhibitor of PP2A), LiCl (an inhibitor of GSK3 β), and CK1-7 (an inhibitor of CK1s). Endogenous dMyc protein was analyzed in total lysates by Western blotting with anti-dMyc antibodies; actin was used as a loading control. The arrowhead on the left represents a dMyc doublet visible in cells treated with MG132. The arrows on the right point to dMyc forms of different electrophoretic mobilities. Molecular mass markers are shown on the right.

cells. HA-tagged kinases cloned under the control of the copper-inducible pMT promoter (53) were transfected into S2 cells, and their expression was induced by using CuSO₄. dMyc protein level was analyzed in lysates by Western blotting. Figure 2A shows that the dMyc protein levels were considerably reduced in the presence of CK1 α or GSK3 β kinases. Conversely, expression of the kinase-dead mutants caused the opposite effect and stabilized the dMyc protein. CK1 ϵ , another member of the CK1 family of kinases, which sometimes acts as a substitute to CK1 α in controlling protein degradation (53), was shown to have an effect similar to that of CK1 α or GSK3 β on reducing endogenous dMyc protein stability (see Fig. S2 in the supplemental material). This regulation was not exerted at the transcriptional level since *dmyc* mRNA did not significantly change in the presence of the kinases or their inactive forms (see Fig. S3 in the supplemental material). To further assess the contribution of these kinases to dMyc protein turnover, we used the protein synthesis inhibitor CHX to measure the dMyc half-life in the presence of CK1 α and GSK3 β or of their kinase-dead forms. S2 cells were treated with CHX, and the level of dMyc protein was analyzed at the indicated time points by Western blotting. The intensity of the respective bands was quantified and is reported in the graph shown in Fig. 2B. CK1 α

or GSK3 β activity decreased the endogenous dMyc half-life from 35 min to ~15 min. Conversely, expression of the kinase-dead forms of GSK3 β or CK1 α increased dMyc half-life to 80 and 75 min, respectively. These experiments were repeated at least three times, with similar results.

Since it was demonstrated that phosphorylation of c-Myc by GSK3 β allows Myc ubiquitination by the ligase Fbw7, we tested whether the presence of GSK3 β or CK1 α kinase would also promote dMyc protein ubiquitination. We performed these experiments with the cell line S2-HA-dMyc, which expresses dMyc under the control of the inducible *hsp70* promoter (4) and in the presence of MG132 to avoid rapid dMyc degradation. S2-HA-dMyc cells were transfected with the various wild-type kinases or the inactive mutants, and their expression was induced with CuSO₄. Subsequently, a 1-h heat shock was applied to induce expression of HA-dMyc. Cells were lysed, dMyc was immunoprecipitated with anti-dMyc monoclonal antibodies, and ubiquitination levels were analyzed by Western blotting. Expression of CK1 α or GSK3 β kinases favored dMyc ubiquitination, whereas expression of the kinase-dead mutants blocked this process. Taken together, our data indicate that members of the CK1 family are novel components of the Myc degradation pathways,

TABLE 1. Conserved phosphorylation domains in Myc proteins from *D. melanogaster* and humans

| Mutant | Domain (aa) or mutation ^a | Sequence homology ^b | % Identity ^c | Stability (min) ^d |
|----------|--|---|-------------------------|------------------------------|
| dMyc-PI | <i>D. melanogaster</i> mutation <i>D. melanogaster</i> (201–207) Human (67–73) | A--A-A SGELSGS <u>SGLCSPS</u> | 50 | <30 |
| dMyc-PII | <i>D. melanogaster</i> mutation <i>D. melanogaster</i> (324–330) Human (58–64) | A--A-A <i>SPPTTGS</i> <u>TPPLSPS</u> | 43 | 60 |
| dMyc-PV | <i>D. melanogaster</i> mutation <i>D. melanogaster</i> (405–411) Human (247–253) | A-A-A-- <i>TPSDSDE</i> <u>TSSDSEE</u> | 75 | 180 |
| AB | <i>D. melanogaster</i> mutation <i>D. melanogaster</i> (404–417) Human (253–266) | Q--N-NQQ-N-- ETPSDSDDEEIDVVS <u>EEQEDEEEIDVVS</u> | 54 | 180 |

^a aa, amino acids.

^b Amino acid substitutions in *D. melanogaster* Myc protein are represented in boldface. Conserved substituted amino acids in the human Myc sequence are underlined. The residues in dMyc-PII and dMyc-PV that are putative substrates for GSK3 β are in italics, and those that are putative substrates for CK1 α are double underlined.

^c That is, the percentage of identical amino acids between *D. melanogaster* and human Myc protein sequences, calculated using DNA-Strider 1.3.

^d The stability of dMyc mutants was compared to that of dMyc-WT, which, in our biochemical experiments, was estimated to be 30 min (Fig. 6).

which, similar to GSK3 β , regulate Myc protein stability through the ubiquitin-proteasome signaling.

GSK3 β , CK1 α , and CK1 ϵ control dMyc protein expression in vivo. To assess a possible role of GSK3 β and CK1 kinases in the control of dMyc protein expression in vivo, we analyzed whether reduction or ectopic expression of these kinases affects dMyc protein levels in the clones of cells within the wing imaginal discs of *Drosophila* third-instar larvae. At this stage of development, dMyc is expressed within all cells of the presumptive notum, hinge, and wing pouch territories (Fig. 3B and 4A to A''), except for a stripe of cells located along the dorsal-ventral boundary, an area also known as the zone of nonproliferative cells (ZNC), where it is transcriptionally repressed by Wg signaling (Fig. 3) (12, 19, 22).

To reduce gene function in clones of cells, we used either mutant alleles (for GSK3 β and CK1 ϵ) or overexpression of RNAi transgenes (for CK1 α) (40). To study ectopic kinase function, we used a combination of the UAS/Gal4 and Flp-out techniques (7, 15, 55) to create clones ectopically expressing a given kinase. The same method was used to ectopically express a given RNAi transgene. All overexpression clones were marked by coexpression of green fluorescent protein (GFP), and all types of clones were monitored for dMyc protein levels by using immunostaining with anti-dMyc antisera (41).

In *Drosophila*, GSK3 β is encoded by the gene *shaggy* (*sgg*) (also called *zeste-white3* [*zw3*] and referred to here as *sgg*). *sgg* acts in vivo as an inhibitory component of the Wnt/Wingless (Wg) signaling pathway, which regulates a wide variety of developmental processes, ranging from pattern formation to cell fate determination and proliferation (8).

sgg mutant clones were recognized either by GFP expression (Fig. 4D and E) or by increased levels of full-length Ci (Ci155), a component of the Hedgehog (Hh) signaling pathway that is known to accumulate autonomously and at high levels within *sgg* mutant clones generated within the anterior compartment (Fig. 4A', B', C', D'', and E'') (21). As shown in the figure, accumulation of dMyc protein is observed in *sgg* mutant clones

located within the presumptive notum and hinge regions (Fig. 4B, C, D', and E', arrows), confirming that Sgg/GSK3 β activity is required to regulate dMyc protein expression in vivo. Moreover, our data exclude a role for Hh signaling in the regulation of dMyc expression, since higher dMyc protein levels are also observed in *sgg* clones located within the posterior compartment, where Hh signaling is not operating due to the absence of Ci (Fig. 4C, C'', and E', asterisk) (31). Within the wing pouch, peak activation of the Wg signaling pathway has been shown to repress *dmyc* transcription (12, 19, 22). Consistent with this, we find that *sgg* mutant clones generated within this specific region of the disc are not associated with increased levels of dMyc protein (Fig. 4B, B', C, and C', arrowhead).

To understand how overexpression of Sgg affects dMyc protein stability in vivo, we measured the size of clones of cells that ectopically express UAS-Sgg and compared it to the size of control wild-type clones that only express UAS-GFP (see Fig. S4 in the supplemental material; Table 1). UAS-Sgg clones were much smaller than control wild-type clones, suggesting that they experience a growth disadvantage. This effect can be explained if overexpression of Sgg reduces dMyc protein levels, thereby causing the cells to be eliminated by "cell competition." Cell competition is a phenomenon arising when two populations of cells with different growth properties are apposed (34). Cells expressing lower dMyc levels grow poorly and are outcompeted and eventually eliminated by cells expressing wild-type dMyc levels (48) (11, 35). Consistent with this effect, we found that coexpression of dMyc and Sgg partially rescues the growth disadvantage of clones overexpressing Sgg alone (see Fig. S4 in the supplemental material; Table 1). Nevertheless, these UAS-Sgg; UAS-dMyc clones were smaller in size than control wild-type or UAS-dMyc clones, further suggesting that overexpression of Sgg reduces dMyc protein expression.

Next, we analyzed how CK1s regulate dMyc expression in vivo. CK1s are believed to modulate the Wingless signaling cascade (26); however, their effect on this pathway has been subject to controversy.

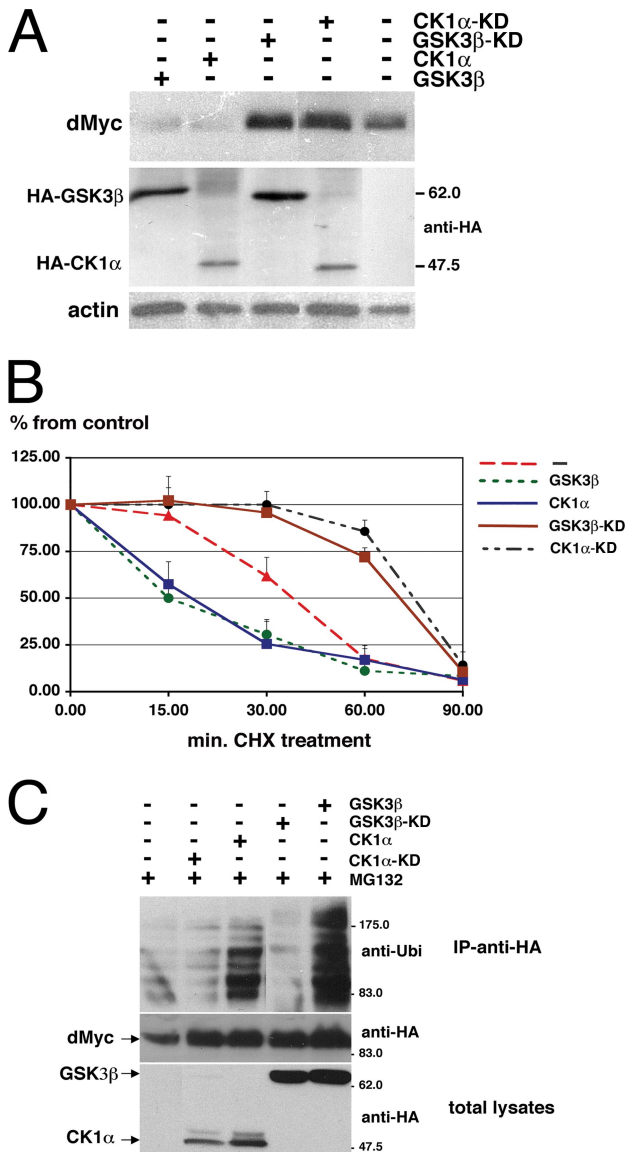


FIG. 2. CK1 α and GSK3 β kinases induce ubiquitin-dependent degradation of dMyc. (A) CK1 α and GSK3 β regulate endogenous dMyc protein stability. S2 cells were transfected with the indicated HA-tagged kinase mutants and protein expression was induced by using copper sulfate. Their levels of expression were quantified by immunoblotting with anti-HA. Endogenous dMyc protein was quantified by using anti-dMyc antiserum. Actin was used as loading control. Molecular markers are indicated on the right. (B) Half-life of dMyc in the presence of CK1 α and GSK3 β or their KD mutants. The graph represents the quantification of endogenous dMyc levels after the addition of CHX. Cells were lysed at the indicated time points after CHX treatment, and dMyc expression was visualized by Western blotting with anti-dMyc antibodies. The intensity of the dMyc bands was quantified in pixels from the scanned X-ray film by using Adobe Photoshop. The background was calculated from an equivalent area in each lane and subtracted from the value for dMyc in the respective lanes. Time zero was set at 100. The data are plotted as percentages over the control for each point. These experiments were repeated three times. (C) GSK3 β and CK1 α kinases mediate dMyc ubiquitination. The stable cell line S2-HA-dMyc (3) was transfected with the various kinases, and expression was induced by using copper ions (3). In the upper panel, HA-dMyc was immunoprecipitated from cell extracts using anti-HA antiserum, and its ubiquitinated forms were visualized using antiubiquitin antiserum. In the lower panel, the expression of HA-dMyc and HA-kinases was visualized using anti-HA antiserum. MG132 was added to the medium at heat shock to avoid Myc degradation.

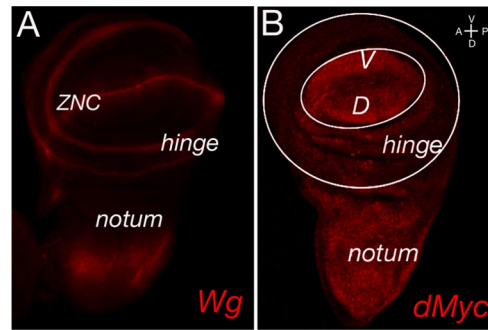


FIG. 3. Wg and dMyc protein expression in late third-instar wing imaginal discs. (A) Subdivision of the *Drosophila* late third-instar wing imaginal disc relative to the Wg expression pattern (red). Two rings of Wg expression mark the presumptive hinge territory; the wing pouch primordium lies within the inner ring, while the notum is formed from tissue outside of the outer ring. Furthermore, Wg is expressed in a thin stripe of cells straddling the boundary between dorsal (D) and ventral (V) compartments, subdividing the prospective wing pouch into dorsal and ventral halves. (B) Late third-instar wing imaginal disc stained for dMyc protein (red). A white circle roughly outlines the boundary between prospective wing pouch and hinge, while a larger white circle distinguishes the prospective hinge area from the prospective notal area. dMyc protein is highly expressed within all cells of the presumptive notum and wing pouch domains except for a stripe of cells extending along the dorsal-ventral boundary. This area of reduced dMyc protein expression corresponds to the ZNC, where *dmyc* has been shown to be transcriptionally repressed by peak Wg activity associated with the dorsal-ventral boundary. Discs are oriented ventral up and anterior to the left.

CK1 α acts as a negative regulator of Wnt/Wg signaling by phosphorylating β -catenin/Armadillo, thus priming it for phosphorylation by GSK3 β and ensuing degradation (1).

CK1 ϵ , on the other hand, has been identified as a positive regulator of Wnt/Wg signaling in mammals and *Xenopus* since it destabilizes the β -catenin degradation complex through the phosphorylation of Dishevelled (9, 14, 38).

Zhang et al. demonstrated that the combination of a hypomorphic *ck1 ϵ* mutation and ectopic expression of RNAi-*ck1 α* enhances ectopic Wg signaling during *Drosophila* limb formation, suggesting a synergistic interaction between the two kinases (56).

The *Drosophila* homologue of *ck1 ϵ* is called *doubletime* (*dbt*) for its effect on the degradation of Period, a regulator of the circadian clock (39), or *disc overgrown* (*dco*) because of the hyperplastic growth of *dco*³ mutant imaginal discs (57). First, we analyzed clones of cells mutant for the *dco*³ allele. We observed that *dco*³ clones (marked by the absence of GFP) were larger than their associated wild-type sibling clones (marked by strong GFP expression) (Fig. 5). In particular, clones generated with the presumptive notum/hinge territory autonomously accumulate high levels of dMyc protein (right panel), indicating that CK1 ϵ activity negatively regulates dMyc expression within this region. The same was also suggested by the behavior of clones of cells that ectopically express DCO/CK1 ϵ : they were significantly smaller than control wild-type clones that ectopically expressed only GFP (see Fig. S4 in the supplemental material; Table 1), and were associated with reduced levels of endogenous dMyc protein (data not shown). Next, we generated clones of cells ectopically expressing UAS-DCO/CK1 ϵ , together with UAS-dMyc, and compared them

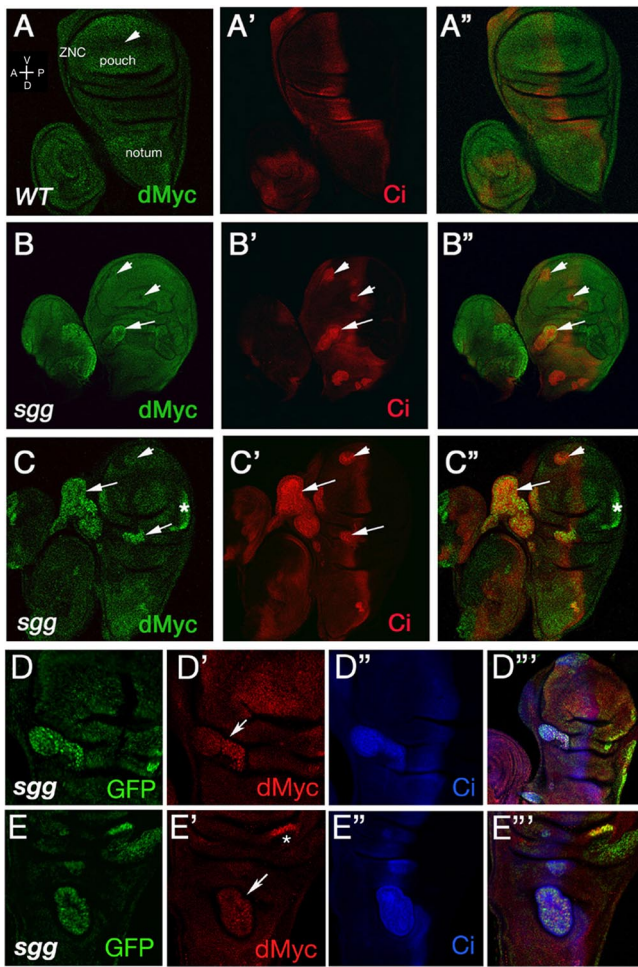


FIG. 4. dMyc is upregulated in *sgg/gsk3* mutant cells. *sgg* mutant clones from the third-instar wing imaginal disc were marked by the expression of GFP (D and E) or the protein Ci155, which is expressed autonomously in the anterior compartment of the disc and is upregulated in *sgg* mutant cells (A' to C', D'', and E'). Immunostaining for endogenous dMyc (panels A to C in green and panels D' and E' in red) was performed using anti-dMyc antisera. Endogenous dMyc levels are higher in the wing pouch and notum but reduced along the ZNC by a peak activation of the Wg signaling pathway that has been shown to repress *dmyc* transcription (A). Consistent with this effect, the *sgg* mutant clones generated within this specific region of the disc are not associated with increased levels of dMyc protein (B, B', C, and C', arrowhead). However, accumulation of the dMyc protein is observed in *sgg* mutant clones located within the presumptive notum and hinge regions (B, C, D', and E', arrows). Higher dMyc protein levels are also observed in *sgg* clones located within the posterior compartment, marked by the absence of Ci (C and E', asterisk). Discs are oriented ventral up and anterior to the left.

to clones ectopically expressing UAS-DCO/CK1 ϵ alone or UAS-dMyc alone. The sizes of the clones were measured, and their dMyc protein levels were examined. UAS-DCO/CK1 ϵ ; UAS-dMyc clones were larger than UAS-DCO/CK1 ϵ clones but smaller than UAS-dMyc clones. In addition, UAS-DCO/CK1 ϵ ; UAS-dMyc clones revealed less ectopic dMyc protein than UAS-dMyc clones (see Fig. S4 in the supplemental material; Table 1), further confirming that DCO/CK1 ϵ downregulates dMyc protein expression.

Finally, we generated clones of cells ectopically expressing *RNAi-ck1 α* (40) and tested them for dMyc protein levels. *UAS-RNAi-ck1 α* clones, when located within the presumptive notum and hinge domains, showed autonomous upregulation of dMyc protein levels (Fig. 5, right panel, arrow). In contrast, dMyc protein levels were reduced in clones that were generated with the presumptive wing pouch, in agreement with the loss of *ck1 α* causing ectopic activation of Wg signaling and thus the repression of *dmyc* transcription within this region (12, 19, 22).

Taken together, our data suggest that within tissues in which Wg signaling is not involved in the repression of *dmyc* transcription (as in presumptive hinge and notum), both CK1 ϵ and CK1 α , as well as Sgg/GSK3 β , are directly implicated in the downregulation of dMyc protein.

In vitro characterization of dMyc phosphorylation mutants reveals that dMyc-PII and AB mutants are resistant to CK1 α and GSK3 β degradation. Analysis of the dMyc amino acid sequence reveals the presence of conserved motifs that are potential substrates for phosphorylation by CK1s and GSK3 β (Fig. 1A). In order to understand the contribution of these domains to dMyc protein stability, we performed site-directed mutagenesis and replaced serine and threonine residues within the dMyc-PI, -PII, and -PV boxes with alanine. We also converted the AB glutamic acid and aspartate residues into glycine and asparagine, respectively (Table 1). All dMyc mutants were tagged with the hemagglutinin (HA) epitope at their N termini and expressed in S2 cells using the UAS/Gal4 method. The dMyc mutants' half-life was measured biochemically in Western blot experiments using the protein synthesis inhibitor CHX. Cells were transfected with dMyc-WT, the various mutants were treated with CHX, and extracts were prepared at the indicated times after treatment. The expression level of the dMyc mutants was quantified by Western blotting with anti-HA antibodies. Mutation of the residues within the dMyc-PI domain shortened dMyc half-life from 30 to 15 min (Fig. 6, upper panel). Conversely, mutations within the dMyc-PII and dMyc-PV domains lengthened dMyc half-life up to 60 min for PII and to over 180 min for PV. Similarly, substitution of acidic amino acids in the AB region increased the Myc protein half-life to more than 180 min (Fig. 6, lower panel), suggesting an important role for the acidic domain in the regulation of dMyc stability.

Next, we analyzed whether the newly identified dMyc domains associated with its protein stability were sensitive to CK1 α and GSK3 β kinase-induced degradation. dMyc-WT, dMyc-PI, dMyc-PII, dMyc-PV, and dMyc-AB were coexpressed in S2 cells, together with increasing concentrations of CK1 α or GSK3 β kinases. Expression of the kinases was induced with copper sulfate, and the dMyc protein level was analyzed by Western blotting with anti-HA antiserum. As shown in Fig. 7, dMyc-PII and dMyc-AB were resistant to degradation induced by CK1 α and GSK3 β kinases, while dMyc-PI and dMyc-PV presented degradation kinetics similar to those of dMyc-WT.

These data suggest that dMyc-PII and the AB contain functional domains necessary for the regulation of dMyc protein stability, and this effect is regulated by CK1 α and GSK3 β kinases.

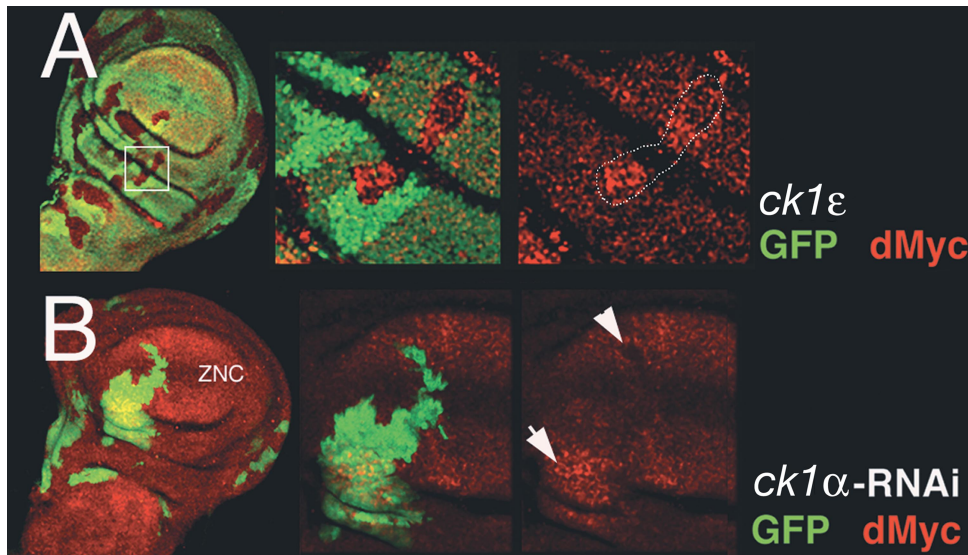


FIG. 5. dMyc is upregulated in cells with reduced levels of *dco3/ck1ε* and *ck1α*. (A) Expression of dMyc protein (red) in *dco3/ck1ε* mitotic clones from wing imaginal discs. Clones were induced at 65 ± 2 h AEL by mitotic recombination. dMyc protein was analyzed in *dco3/ck1ε* mutant clones (GFP negative) and compared to levels observed in sibling clones (marked with GFP₂). A higher magnification of the area (on the right) shows an increase in dMyc protein level in *dco3/ck1ε* clones (marked with a white line). (B) Flp-out clones in the wing imaginal discs expressing *UAS-RNAi-ck1α* were induced at 52 ± 2 h AEL. Expression of endogenous dMyc in these clones (marked by GFP) was visualized by immunostaining with anti-dMyc antiserum (red). Expression of dMyc is higher in clones located in the hinge region (arrow) and lower in the ZNC (arrowhead). Discs are oriented ventral up and anterior to the left.

Increased dMyc stability inhibits ommatidial differentiation and induces cell death during eye development. To characterize the physiological relevance of dMyc mutants in vivo, we used Gal4 promoter lines to express various UAS-dMyc transgenes in different tissues. dMyc-PI-overexpressing flies, and to lesser extent, dMyc-WT-overexpressing flies were viable with all of the tested promoters, while expression the dMyc-II, dMyc-PV, or dMyc-AB mutants resulted in developmental lethality (see Table S2 in the supplemental material). To overcome the lethality of our transgenes, we restrict their expression to the eye, using flies with reduced *dmyc* levels (*dmyc^{P0}*), which allows for expression of the transgenes under the *eyeless* compartment (4).

Since expression of dMyc during compound eye formation modulates the growth and the number of ommatidia (22), we performed genetic epistasis experiments to assess the contri-

bution of various UAS-dMyc mutants to the size and number of ommatidia. Expression of dMyc-PI increased ommatidial size, but to a lesser extent than expression of dMyc-WT ($317 \mu\text{m}^2$ in *ey>dmyc^{P0}/Y*; dMyc-PI/+ and $341 \mu\text{m}^2$ in *ey>dmyc^{P0}/Y*; dMyc-WT/+) (Fig. 8B). In addition, the total number of ommatidia in *ey>dmyc^{P0}/Y*; dMyc-PI/+ flies ($n = 756$) was higher than that in *ey>dmyc^{P0}/Y*; dMyc-WT/+ animals ($n = 633$), suggesting that the expression of dMyc-PI is less effective than the expression of dMyc-WT for inducing apoptosis (33). Conversely, expression of dMyc-II, dMyc-PV, and dMyc-AB increased the ommatidial size of *ey>dmyc^{P0}/Y* flies (Fig. 8B), with the noteworthy observation that the number of ommatidia was substantially reduced specifically when dMyc-PV and dMyc-AB transgenes were expressed, suggesting that the presence of the stable dMyc mutant proteins resulted in a strong eye defect accompanied by a reduction of the tissue of the head

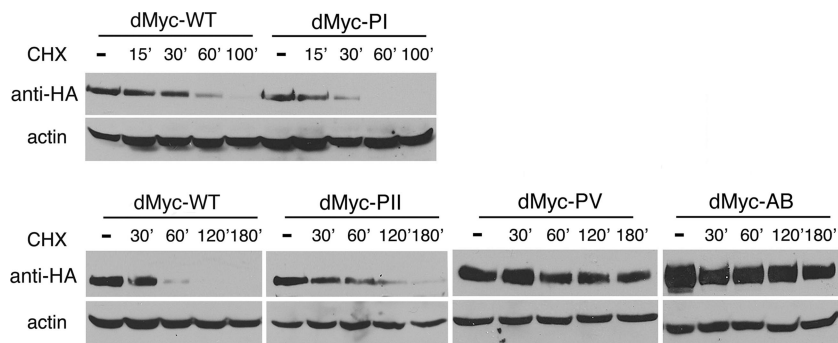


FIG. 6. Stability of dMyc phosphorylation mutants. The half-life of dMyc mutant protein was determined in S2 cells transfected with the indicated constructs and treated with CHX for various lengths of time. Protein levels were quantified by Western blotting with anti-HA antibody. Actin was used as a loading control.

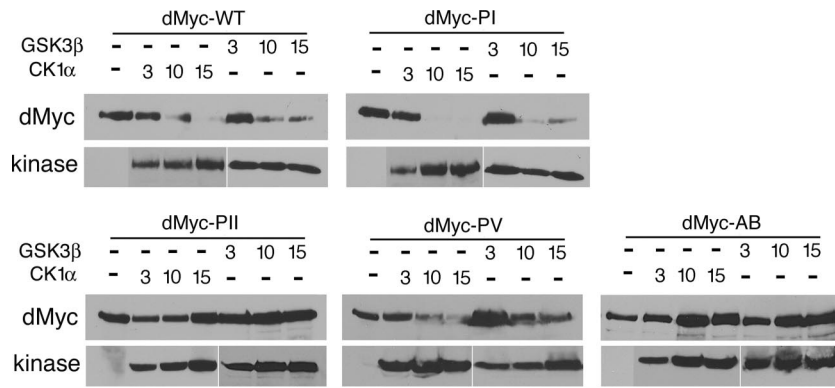


FIG. 7. Stability of dMyc-WT and mutants in the presence of GSK3β and CK1α kinases. HA-dMyc-WT and mutants were transfected in S2 cells together with the indicated concentrations (in μg) of plasmids encoding HA-CK1α or HA-GSK3β kinases. Proteins expression was analyzed in the cell extracts by Western blotting with anti-HA antiserum. Actin was used as loading control.

capsule (Fig. 8A). To understand, at the cellular level, the mechanism responsible for these defects, we monitored how the expression of dMyc mutants affected apoptosis (33, 47) and photoreceptor differentiation (2) during the eye development. Eye imaginal discs of the indicated genotypes were collected from third-instar larvae and stained by immunofluorescence for the neuronal markers ELAV (Fig. 9A to F), for dMyc (Fig. 9A' to F'), and for the cleaved form of caspase-3 (A'' to F''). The expression of dMyc-PV or dMyc-AB significantly reduced the number of differentiated photoreceptors (ELAV positive) and increased the number of apoptotic cells (caspase-3 positive). Conversely, expression of dMyc-PI affected neither photoreceptor differentiation nor apoptosis, further confirming that dMyc-PI has a lower transcriptional activity than that of dMyc-WT.

Taken together, the in vivo data and the biochemical analysis in S2 cells indicate that dMyc-PII, dMyc-PV, and dMyc-AB mutants are more stable than dMyc-WT, and they also illustrate how the modulation of dMyc stability critically impacts patterning in vivo.

DISCUSSION

Myc is a member of a family of transcription factors and plays a pivotal role in the regulation of proliferation, growth, and apoptosis. Myc protein expression is tightly regulated, and its stability is modulated by phosphorylation events downstream of mitotic signals. Here, we showed that members of the CK1 family are novel regulatory components of Myc protein stability. Our biochemical data demonstrated that CK1α induces dMyc ubiquitination and degradation through the proteasome pathway. Further, we showed that the specific proteasome degradation pathway induced by CK1α-mediated dMyc phosphorylation is the same as the one induced by GSK3β phosphorylation (Fig. 1 and 2), a kinase that was previously identified as a key regulator of c-Myc protein degradation (46).

In vivo downregulation of GSK3β and CK1α or CK1ε kinases in wing imaginal discs results in the accumulation of dMyc protein, an effect particularly visible in the hinge and notum regions but not in cells adjacent to the ZNC (Fig. 4 and

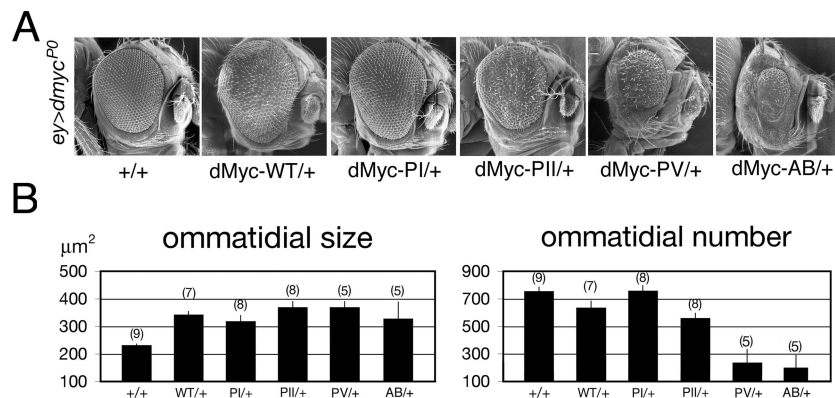


FIG. 8. Expression of dMyc mutants in vivo affects ommatidial size and number. (A) Representative scanning electron micrographs of adult eyes from *ey>dmyc^{P0}/Y* expressing the transgenes UAS-dMyc-WT, UAS-dMyc-PI, UAS-dMyc-PII, UAS-dMyc-PV, and UAS-dMyc-AB. (B) Graphics show the quantification of ommatidial size and number of the indicated genotypes. Standard deviations were calculated based on the number of animals analyzed and are indicated in parentheses.

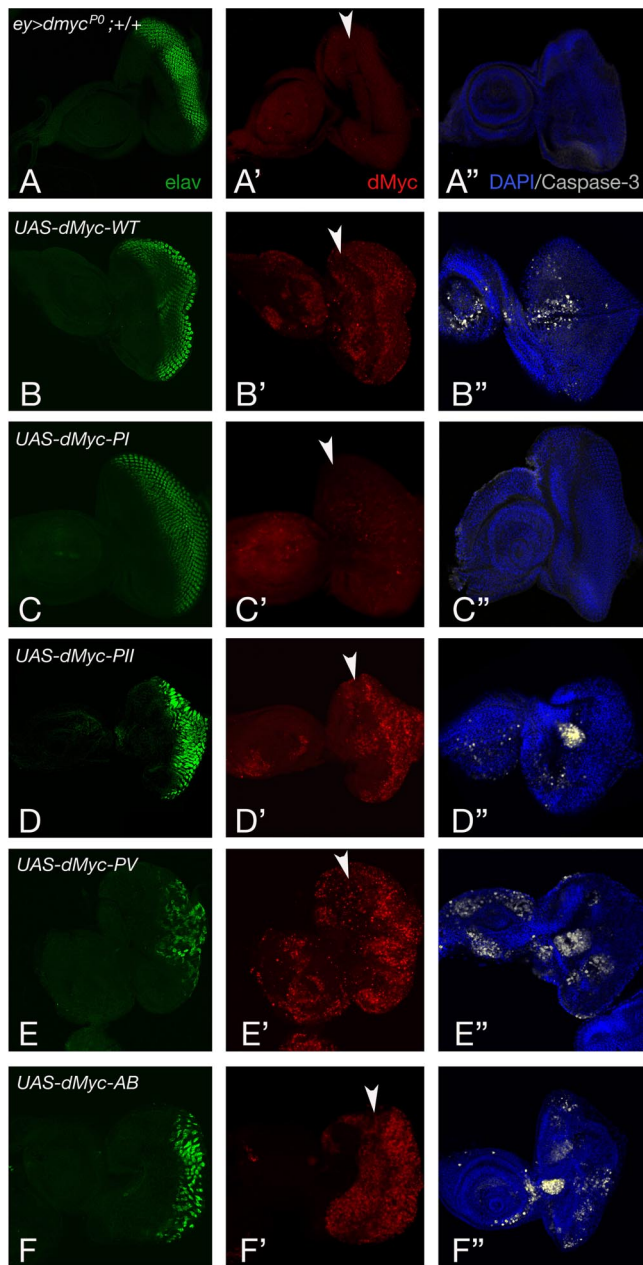


FIG. 9. Expression of dMyc-PV and dMyc-AB inhibits ommatidial differentiation and increases apoptosis in eye imaginal discs. Photographs of eye imaginal discs from third-instar larvae of the indicated genotypes were stained for ELAV (green) and dMyc (red) expression (A to F). Apoptosis is visualized by using anti-active caspase 3 antibodies (white) and nuclei stained with DAPI (A'' to F''). Photographs were taken at $\times 20$ magnification. The morphogenetic furrow is indicated by an arrowhead.

5). Reduction of GSK3 β and CK1 α activates *Wingless* (Wg) signaling (28), which in turn negatively regulates *dmyc* RNA in the ZNC (12, 19, 22). This functional relationship might explain the lack of expression of dMyc protein in clones falling in the wing pouch area and in the ZNC. This positional effect also suggests that dMyc activity is regulated by patterning signals active during the development of the wing imaginal discs.

Our analysis of the dMyc amino acid sequence uncovered novel conserved domains, which serve as potential phosphorylation substrates for CK1s or GSK3 β kinases. Biochemical characterization of these domains indicated that a combination of amino acid substitutions (S201A, S205A, and S207A) in the dMyc-PI sequence produces a protein with a shorter half-life than dMyc-WT (Table 1). In vivo expression of the dMyc-MPI mutant did not confer the typical ommatidial roughness that is induced by the expression of dMyc-WT. Moreover, expression of dMyc-PI failed to induce apoptosis in the eye imaginal discs, an effect normally associated with dMyc-WT overexpression (Fig. 9). In conclusion, our data suggest that dMyc-PI produces a protein that is less stable than dMyc-WT. In vertebrates, phosphorylation of c-Myc on Ser-62 by MAPK/ERK, JNK N-terminal kinase, or CDK4 increases its stability (18, 46). The dMyc-PI sequence does not contain a bona fide ERK phosphorylation site (PXSP). However, Ser-201 lies in a favorable context for phosphorylation by the ribosomal S6 kinase-p90 RSK (37). RSK-p90 belongs to a class of Ser/Thr kinases, activated by ERK (20) and insulin signaling (23, 43), that phosphorylates the S6 protein component of the 40S ribosomal subunit in response to mitogenic stimulation, resulting in enhanced translation (24). Interestingly, it has been reported that RSK-p90 activation by ERK is capable of switching on mTOR signaling via inactivation of the TSC1/2 complex (27), suggesting a role for this kinase in protein synthesis and mass accumulation. No evidence for this regulatory mechanism has been described thus far in *Drosophila*. We hypothesize that growth factors may stabilize Myc protein, possibly through phosphorylation by the RSK-p90 kinase, and promote ribosomal biogenesis, in accordance with the prominent role played by dMyc in the production of mass and growth regulation (17).

Biochemical analysis of the protein stability of dMyc-PII, dMyc-PV, and dMyc-AB showed an increased half-life of these mutants compared to dMyc-WT. The sequence within the dMyc-PII domain (S324A-T328A-S330A) contains potential targets for phosphorylation by GSK3 β at Ser-324 [324-S/T-XXX-S/T-(PO₄)₄], which requires a priming event of phosphorylation at the +4 position (Thr-330) (10). This phosphorylation event also acts as priming for other kinases (i.e., CK1s) and creates an optimum consensus site for phosphorylation by CK1s at Thr-330 [S/T-(PO₄)-XX-330-S/T] (30). We found that alanine substitutions of amino acids 324, 328, and 330 conferred resistance to dMyc protein degradation upon phosphorylation by the CK1 α and GSK3 β kinases (Fig. 7). Previously, Moberg et al. proposed Ser-324 to be a phosphorylation target for GSK3 β and to act as a potential binding site for the ubiquitin ligase Ago, the *Drosophila* homologue of the human Fbw7 ligase (32). These authors found that mutation at Ser-324 did not decrease the capacity of dMyc to bind Ago, nor did it influence dMyc protein stability (32). Our experiments show that mutation of the residues S324, T328, and S330 confers to the dMyc-PII mutant a resistance to degradation mediated by the ubiquitin ligase Ago. Moreover, we found that dMyc-MPV, which is degraded by CK1 α and GSK3 β kinases, is somewhat resistant to degradation by Ago (see Fig. S5 in the supplemental material), suggesting that CK1 α - and GSK3 β -mediated phosphorylation of dMyc is not sufficient to induce its degradation by Ago but perhaps by another unknown ubiquitin ligase.

Our data also demonstrate that the dMyc-AB plays an important role in the regulation of dMyc protein stability. Mutation of acidic amino acids imparted to dMyc resistance to degradation primed by CK1 α and GSK3 β kinases (Fig. 7). It was recently proposed that acidic domains act as docking sites for the CK1 and CK2, enabling proper positioning of the kinases to recognize their substrates. We speculate that the conserved acidic amino acid stretch in Myc protein helps the binding of CK1 and CK2 kinases and favors Myc phosphorylation (30). In support of this hypothesis, we found the dMyc-PV amino acid sequence (residues 405, 407, and 409), located within the AB (amino acids 404 to 414), to be highly homologous to the PEST domain of c-Myc (amino acids 226 to 270). This domain was previously demonstrated to be relevant for c-Myc stability and to act as a potential substrate for CK2 phosphorylation (29). Our biochemical data show that mutations of the dMyc-PV and the AB domains confer increased stability to dMyc protein and suggest that the acidic sequence functions similarly to the PEST domain to control dMyc stability. Notably, Ser-407 constitutes an optimum consensus site for phosphorylation by CK2 (S/T-407-XX-D/E) (37). This observation agrees with the hypothesis that in mammals CK2 is involved in the regulation of c-Myc degradation by targeting the PEST domain (29).

In vivo expression of the stable mutants dMyc-PII, dMyc-PV, and dMyc-AB resulted in a visible eye defect (Fig. 8), accompanied by a reduction of the head capsule and a diminution of the number of the ommatidia. This was particularly visible for dMyc-PV and -AB. Cellular analysis of third-instar larvae eye imaginal discs revealed that expression of these mutants induced apoptosis during disc development. Apoptosis was detected not only within the compartment of dMyc expression (cell autonomous) but also in the neighboring cells (non-cell autonomous; Fig. 9). This is a well-documented phenomenon and illustrates the role of dMyc in cell competition, where cells expressing high dMyc kill slower-proliferating neighboring cells nonautonomously through an unidentified mechanism (11, 35, 48).

In conclusion, multiple phosphorylation events may work hierarchically to prime Myc phosphoamino acids for binding by multiple kinases. We propose here that different kinases respond to a "phosphorylation code" that is required to properly control Myc protein stability. This code will depend on an upstream program that in turn activates these kinases. The identification of other phosphorylation residues in dMyc will help in drawing a complete map of phosphorylation activities and will elucidate the events necessary for robust regulation of Myc protein stability. For example, we speculate that components of growth signaling pathways, such as *ras* or insulin, may influence the activities of different combinations of kinases, thus affecting phosphorylation at different amino acids to control dMyc protein stability. In support of this hypothesis, we produced preliminary data showing that activation of the DILP (for *Drosophila* insulin-like peptides) pathway increases dMyc protein stability in vivo through the inactivation of GSK3 β kinase (F. Parisi, unpublished data), suggesting that the metabolic and nutrient pathways affect growth by partially controlling dMyc protein expression.

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