Developmental Control of Cell Cycle Regulators: A Fly's Perspective

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During early development in many species, maternally supplied gene products permit the cell cycle to run at maximum velocity, subdividing the fertilized egg into smaller and smaller cells. As development proceeds, zygotic controls are activated that first limit divisions to defined spatial and temporal domains, coordinating them with morphogenesis, and then halt proliferation altogether, to allow cell differentiation. Analysis of the regulation of cyclin-dependent kinases (Cdks) in *Drosophila* has provided insights into how this embryonic program of cell proliferation is controlled at the molecular level and how it is linked to developmental cues. Recent studies have also begun to reveal how cell proliferation is controlled during the second phase of *Drosophila* development, which occurs in imaginal tissues. In contrast to their embryonic progenitors, imaginal cells proliferate with a cycle that requires cell growth and is linked to patterning processes controlled by secreted cell signaling molecules. The functions of these signaling molecules appear to be nearly as conserved between vertebrates and invertebrates as the cell cycle control apparatus itself, suggesting that the mechanisms that coordinate growth, patterning, and cell proliferation in developing tissues have ancient origins.

During the development of multicellular creatures, up to 10^{15} cells can be generated. How is such massive cell proliferation coordinated with morphogenesis? Present models are based largely on experiments with cells in culture, but a full understanding clearly requires analyses in whole organisms. Here we relate recent progress concerning this topic in *Drosophila* and discuss its relevance to proliferation control in vertebrates. We focus on insights based on the identification of cyclin-dependent kinases (Cdks) as pivotal regulators of the eukaryotic cell cycle. Since this breakthrough to a molecular understanding of cell cycle control was accomplished in studies of yeast and frog extracts a few years ago, investigations of how these complexes are regulated during development have progressed rapidly.

The inventory of cyclins and Cdks appears to be conserved among multicellular eukaryotes, but distinct in yeast. Cyclins with proven roles in cell cycle control (A-, B-, D-, and E-types) as well as their kinase partners (Cdk1, Cdk2, Cdk4, or Cdk6) are present in both *Drosophila* and vertebrates. D-type Cyclins in complexes with Cdk4 or Cdk6 regulate progression through the G1 phase of the cell cycle, cyclin E-Cdk2 regulates entry into S phase, cyclin A–Cdk2 regulates progression through S phase, and cyclins A and B in association with Cdk1 (Cdc2) regulate entry into M phase (*1*).

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The activation of these kinases is carefully controlled at multiple levels. Concentrations of the activating cyclin subunits are modulated both transcriptionally and through periodic, ubiquitin-dependent proteolysis, and the kinase subunits are subject to both activating and inhibiting phosphorylation. Two families of Cdk inhibitors also modulate Cdk activity in vertebrates (*2*), and at least one such inhibitor is present in *Drosophila* (*3*). Physiological targets of cyclin-Cdk complexes like the retinoblastoma protein (pRB) and E2F, which modulate transcription of cell cycle genes, are also present in both *Drosophila* and vertebrates (*4*–*6*).

Embryology often forces a perspective opposite to that engendered by studies in cell culture. Thus while the question of how cell proliferation is stimulated has been extensively addressed in culture, how proliferation is terminated appropriately has presented itself as a more relevant question for early embryogenesis. For embryos that develop in large eggs, nutrients and cell cycle factors are stockpiled during oogenesis. The requirement for cell growth is thus alleviated, allowing early embryonic cell cycles to proceed rapidly as they partition the egg into smaller and smaller cells. In addition, pattern formation in many large eggs operates in a field the size of the final organism from the outset, and so does not have to be coordinated with growth. This "large egg" strategy contrasts with an alternative process found in mammals in which the embryo is continuously nurtured by the mother, cells double their mass during each division cycle, and patterning occurs in parallel with growth. In *Drosophila* the large egg, growth-independent strategy is used during embryogenesis, whereas the alternative, growth-linked process is used to generate the adult fly, which develops from imaginal cells nurtured by the larva.

Controlling Maternally Driven Cell Cycles

At least one of the maternal cell cycle regulators present in an egg must be kept inactive to maintain cell cycle arrest before fertilization. The arrest point (either before or during the various stages of female meiosis) varies in different organisms, and various molecular mechanisms for arrest appear to have evolved. In vertebrates, the kinase c-Mos effects arrest in metaphase of the second meiotic division (*7*). In *Drosophila*, the importance of mechanical tension exerted by the spindle on paired meiotic chromosomes during the arrest in the first meiotic division has been demonstrated with elegant genetic experiments (*8*) though the molecular mechanism remains unknown.

Fertilization (or egg activation in *Drosophila*) releases the meiotic arrest, freeing maternal gene products to drive an exponential proliferation of cells. Eventually the maternal cell cycle oscillator is checked by the degradation of Cdk activators, with different activators disappearing at specific developmental stages. After degradation of an essential activator, subsequent cell cycle progression becomes dependent on its zygotic re-expression, allowing the activator to be used to differentially regulate the cycle according to cell type (Fig. 1A). The maternal activator first removed in *Drosophila*, Cdc25, illustrates this principle clearly. Cdc25 is a dual specificity phosphatase that removes inhibitory phosphates from the adenosine triphosphate (ATP)–binding site of Cdk1. This activates Cyclin A–Cdk1 and Cyclin B–Cdk1 kinases, triggering mitosis. Initially, Cdc25 is expressed in large amounts from abundant maternal mRNA stored in the *Drosophila* egg, as are all other proteins required for cell cycle progression. These maternal regulators set the extreme pace of the initial embryonic cycles, which have a cycle time of less than 10 min. Degradation of maternal Cdc25 stops this explosive production of nuclei. The mechanism that triggers this degradation appears to be a chain reaction that is sensitive not to developmental time or the total number of cell cycles, but to the ratio of nuclei cytoplasm (*9*, *13*).

This proposed chain reaction starts with the progressive depletion of a maternal cell

cycle factor by the proliferating embryonic nuclei, and a consequent lengthening of interphases. Although the identity of the critical depleted factor remains unknown, recent studies of a maternal effect mutant, *grapes*, provide a tantalizing clue. The *grapes* mutant embryos fail to lengthen interphases and, catastrophically, enter mitosis before the completion of DNA replication. This suggests that S-phase lengthening may result from depletion of factors required for DNA replications, and that the delay of mitosis is normally enforced through a *grapes*-dependent checkpoint control (*10*). As a result of cell cycle lengthening, zygotic transcriptional activation occurs (*11*). This may occur because transcription is mechanically suppressed during the earliest cycles by unrelenting DNA replication and chromosome condensation, because transcriptional repressors are titrated out of the embryonic cytoplasm by the proliferating nuclei, or because Cdk1, which is continuously active during the first eight cycles, represses transcription by phosphorylating components of the transcription apparatus (*12*). In any case, transcription produces new gene products that promote the degradation of many maternal mRNAs including *string* and *twine* (*13*), which encode partially redundant Cdc25 protein phosphatases. These mRNAs are degraded to undetectable amounts during interphase 14, and as the Cdc25^{string} protein (and presumably also the Cdc25^{twine} protein) is degraded during each mitosis just like the mitotic A- and B-type cyclins, maternal Cdc25 protein is also destroyed (*13*). The result is that Cdk1 is inhibited by phosphorylation, and the embryonic cells arrest in G2. The linkage between the increasing ratio of nuclei to cytoplasm, slowing of the cell cycle, and transcriptional activation may explain how cell cycle arrest is always achieved at the correct cell number regardless of how much time, or how many cycles, are required.

Similar chain reactions probably slow down the cleavage cycles in other organisms with large eggs rich in maternal components. Experiments with *Xenopus* have also emphasized the role of the increasing ratio of nuclei to cytoplasm in cell cycle slowing at the midblastula transition, and have suggested that a maternal DNA replication factor may be what is depleted (*14*). As in *Drosophila* cell cycle alterations during this transition involve the activation of zygotic transcription, inhibitory phosphorylation of Cdk1, and the degradation of maternal cell cycle regulators such as cyclins A and E (*15*). Although the regulatory significance of these changes in *Xenopus* has not been tested, some of them are presumably required for the slowing and desynchronization of the cycle and the acquisition of G1 phases, which occur as development progresses (*16*).

Patterning and Terminating Zygotically Driven Cell Cycles

The cycles that follow inactivation of maternal Cdc25 in *Drosophila* are differentially regulated at G2 to M phase transitions through precisely regulated pulses of transcription of the zygotic Cdc25^{string} gene (Fig. 1A). Because regulators of G1 to S phase transitions are still expressed constitutively, these cycles lack G1 altogether (*17, 18*). Cells with the same developmental fate express Cdc25^{string} at the same time and divide synchronously, whereas different cell types have distinct temporal programs of division (*19, 20*). The well-known set of genes that specifies cell fate in *Drosophila* must therefore also regulate transcription of Cdc25^{string}. Many of these genes encode transcription factors that are expressed in spatially restricted domains, and these appear to act directly and combinatorially on the regulatory region of the Cdc25^{string} gene, a large (>30 kb) array of many tissue-specific enhancer elements (*20, 21*). These regulatory elements integrate pattern information in much the same way as the control elements of developmental regulators like the homeobox genes. It is not known whether transcriptional control of vertebrate Cdc25 genes has a comparable complexity, but the expression patterns these genes exhibit in vivo make this seem likely (*22*). Although studies of cultured fibroblasts have emphasized a simpler G2-specific expression mechanism shared by Cdc25C, Cdk1, and Cyclin A (*23*), analyses in vivo are required to ascertain the full range of inputs to which these genes respond. Cell type– and stage-specific transcriptional controls may help to explain not only developmental regulation of the cell cycle, but also why malignant transformation requires different genetic alterations in different cell types.

Terminating zygotically driven cell proliferation. During most of *Drosophila*'s embryonic divisions S phases occur immediately af-

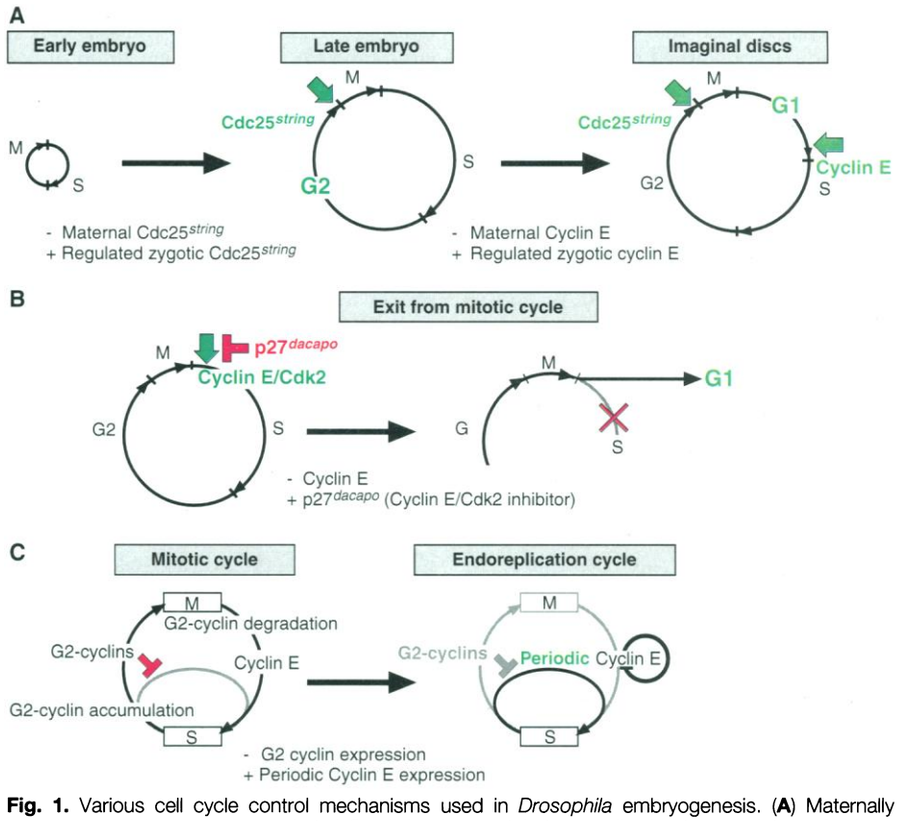

Fig. 1. Various cell cycle control mechanisms used in *Drosophila* embryogenesis. (**A**) Maternally provided regulators are removed at defined developmental stages, allowing the regulation of subsequent cell cycles by zygotic re-expression. Regulation of Cdc25 expression results in the acquisition of a G2-phase and directs the program of embryonic divisions according to developmental fate. Regulation of Cyclin E results in the acquisition of a G1 phase. (**B**) Exit from the mitotic cycle is achieved by decreased expression of Cyclin E in parallel with increased expression of a Cyclin E–Cdk2 inhibitor. (**C**) Cells are switched from mitotic cycles to an endoreplication cycle by turning off expression of the G2-Cyclins (A- and B-type) and expressing Cyclin E periodically.

ter mitoses, without intervening G1 periods. After the final embryonic mitosis, however, cells arrest for the first time in G1. This arrest requires the timely inactivation of Cyclin E–Cdk2, which promotes DNA replication, and is achieved by decreased transcription of the *Cyclin E* gene on the one hand (*24*), and increased transcription of a Kip-type inhibitor of Cyclin E–Cdk2 on the other (*3*) (Fig. 1B). Embryos with too much Cyclin E–Cdk2 activity sustain one extra cell cycle and die from hyperplasia. Forced expression of the dE2F-dDP transcription factor can induce *Cyclin E* transcription and postpone G1 arrest, suggesting that downregulation of dE2F might also play a role here (*25, 26*). What transiently induces expression of the Cdk inhibitor during cell cycle exit in the fly embryo, and how Cyclin E transcription ceases at the same stage, are intriguing and perhaps generally important mysteries.

Functional characterization of another *Drosophila* gene, *roughex*, has indicated that entry into G1 requires inactivation of Cyclin A as well as Cyclin E. Mutations in *roughex* were identified because they cause a rough eye phenotype that results from an inability to synchronize cells in G1 during a critical phase when the regular pattern of ommatidia is established in the developing eye (*27*). Instead of arresting in G1, cells in *roughex* mutants progress into S phase and continue proliferating, apparently because Cyclin A–dependent Cdk activity is not suppressed sufficiently during G1 (*27–29*). Recent screens for suppressors of *rux*'s phenotype have identified another gene, *rca*-1, which appears to be a dosage-sensitive regulator of Cyclin A activity (*30*). A failure to arrest cell proliferation at the correct developmental stage has also been described for a *Caenorhabditis elegans* mutant, *cul-1* (*31*). The *cul-1* gene has several vertebrate homologs and also a yeast homolog, *CDC53*, which is required for degradation of G1 cyclins by the ubiquitin-dependent pathway (*31, 32*). The phenotype of *cul-1* mutants is entirely consistent with the idea that G1 Cyclins are not eliminated fast enough to execute a timely G1 arrest.

The biochemical properties and in vivo expression patterns of vertebrate Cdk inhibitors suggest that these genes, like the *Drosophila* inhibitor, could help cells exit the proliferative cycle before differentiation (*33*). However, mice lacking $p21^{Cip1}$ show no developmental defects, making a requirement for this inhibitor in arresting the cell cycle at differentiation doubtful (*34*). Deletion of murine $p27^{Kip1}$, in contrast, results in a post-natal increase in cell number in many organs, and thus appears to result from a cell-autonomous failure to terminate proliferation on time (*35*). Eventually, normal cell differentiation occurs in $p27^{Kip1}$-deficient mice, just as it does in mutant flies and the similarly affected *cul-1* mutant nematodes. That cells in vivo should have multiple means for exiting proliferation as well as stimulating it should come not as a surprise, but as a comforting indication that developmental controls are multiply insured against things going awry.

Switching from mitotic to endoreplication cycles. Many cells in invertebrates do not stop cycling when they become post-mitotic, but switch to an endoreplication cycle in which repeated S phases occur without intervening mitoses (*36*). While endoreplication is restricted to a few cell types in vertebrates, it is very important for growth in many other animals and plants (*37*). In *Drosophila*, the switch to endoreplication appears to be accomplished by loss of the mitotic Cyclins A and B while periodic expression of the S phase–promoter Cyclin E continues (Fig. 1C) (*18, 38*). Similar observations have been made in endoreplicating maize endosperm and in several mutants in yeast (*39, 40*). The increase in Cdk activity that triggers DNA replication in yeast also makes replication origin complexes incapable of re-initiation (*41*). This block to re-initiation is preserved during late S and G2 by the accumulation of the mitosis-promoting cyclin-Cdks, and removed when these complexes are inactivated by cyclin degradation at mitosis (*40–42*). Thus, the absence of mitotic cyclins in endoreplicating cells may explain both the lack of mitosis and why re-initiation is no longer dependent on mitosis, whereas periodic Cyclin E expression provides an explanation for how multiple rounds of DNA replication are triggered. Consistent with these ideas, inactivation of Cdk1 complexes in *Drosophila* does not just arrest cells in G2, but forces them into an endoreplication cycle (*18, 43*). Moreover, although the pulses of Cyclin E expression that normally precede each S phase during endoreduplication are necessary and sufficient to trigger S phases, periodic endoreplication can be inhibited by simply over-expressing Cyclin E continuously (*44*).

Imaginal Cell Proliferation in *Drosophila*: A Model for Vertebrates?

The stereotyped division programs seen in *Drosophila* and *Caenorhabditis* embryos are quite unlike those of vertebrates, in which cell cycles are neither synchronous nor obviously patterned into spatial domains. However, some tissues in *Drosophila*—the imaginal discs that produce adult appendages—exhibit a proliferation behavior much like that seen in vertebrates. Unlike their embryonic progenitors, imaginal cells proliferate with a cycle that requires cell growth, incorporates a G1 phase, and is linked to patterning processes controlled by secreted signaling molecules.

Imaginal cells arrest in G1 during mid-embryogenesis and remain quiescent until after the larva hatches. Reactivation of their cell cycle requires the influx of nutrition from feeding, and occurs after a substantial (sixfold) increase in cell mass (*45*). This and the fact that these cells maintain a constant size as they proliferate indicates that cell growth is a limiting parameter in their division cycle. Each parcel of imaginal cells (a disc) has 10 to 50 cells in the newly hatched larva, and these proliferate to as many as 100,000 cells before they differentiate into an adult structure such as a wing, leg, or eye. Because very little cell death is observed during disc growth (*46*), it appears that cell number in these adult structures is dictated primarily, though not exclusively, by cell division. Proliferation occurs throughout the discs, with a cell cycle that averages 8 hours and includes substantial G1 and G2 periods (*45, 47*). Curiously, DNA replication and mitosis in growing discs occur in small, nonclonal clusters of cells, suggesting that local cell-cell communication may be an important parameter in cycle regulation (*48, 49*).

What drives imaginal cell proliferation and terminates it when the disc has reached its final size and shape? Immature discs transplanted into adult hosts grow until the disc reaches its normal size and then stop, indicating that size regulation of the disc is largely autonomous (*50*). A similar size control phenomenon has been noted with anlagen of some vertebrate organs (*51*). Regeneration experiments with imaginal discs and insect and amphibian limbs have suggested a formal explanation—the polar coordinate model—for how growth and patterning might be linked in appendage development (*52–54*). According to this concept cell proliferation stops when a complete map of positional values, or developmental fates, is established in a given anlage. Discontinuities in positional information in immature anlagen, or in mature anlagen after surgical manipulation, are recognized and trigger the localized cell proliferation (*55*) needed to generate cells expressing the missing positional values.

Some of the earliest clues to how positional information is generated and interpreted came from studies of *Drosophila* wings that were clonally mosaic for normal and *Minute* cells. *Minute* mutations cripple protein synthesis, and consequently have a dominant, cell-autonomous effect to slow growth. These studies revealed the phenomenon of cell competition, in which clones of slow growing *Minute* cells are

eliminated by their faster growing *Minute*$^+$ neighbors (56) (Fig. 3). A seminal observation from the early clonal studies was that even fast-growing (*Minute*$^+$) clones, although they can take over large regions of tissue, are unable to cross an invisible line between the anterior and posterior halves of the wing, labeled the anterior-posterior (A-P) compartment boundary (57). This clonal boundary is established during embryogenesis. Additional boundaries that distinguish dorsal and ventral compartments and finer spatial restrictions subdivide the wing further during the larval stages (57, 58).

Cell signaling drives growth and patterning in the imaginal discs. A flurry of recent work has addressed the molecular basis of compartment boundaries and their significance for pattern formation and tissue growth (59). A-P patterning in the wing is established by the homeodomain protein Engrailed, expressed exclusively in posterior compartments beginning in the early embryonic stages. Expression of Engrailed is clonally inherited, and defines cell mixing properties such that cells expressing Engrailed cannot intermingle with those that do not. Expression of Engrailed also promotes posterior expression of the secreted molecule Hedgehog, a short-range signal that diffuses into the anterior compartment to trigger expression of a second, longer range secreted signal, Decapentaplegic (DPP), a homolog of the transforming growth factor–β (TGF-β) family member bone morphogenetic protein-4 (BMP-4). The *dpp* signal is expressed in a stripe along the A-P border (60–62), received on both sides of the border by receptors [Thickveins (TKV), Saxophone, and Punt] and transduced to the nucleus to regulate a still largely unknown set of target genes (62–66). A similar signaling cascade sets up the dorsal-ventral compartment boundary in the wing, but uses a distinct set of genes. Here the players are the homeodomain protein Apterous, the transmembrane proteins Fringe, Notch, Serrate, and Delta; the transcription factor Vestigial (VG); and the secreted signal Wingless (WG) (67–70, 72).

The importance of these signaling pathways in controlling cell proliferation is illustrated by the striking alterations that result when virtually any of the gene functions mentioned above are altered. Loss of DPP or WG activity results in extreme reductions in cell numbers in specific pattern elements (Fig. 2A) (60, 62, 69, 73, 74), and clones of cells lacking signal transducers like TKV, Vestigial, and MAD show a cell-autonomous failure to grow (66, 69, 74). Conversely, ectopic expression of DPP results in A-P pattern duplications that involve massive increases in cell numbers (Fig. 2A), and clonal expression of an activated DPP receptor causes overproliferation in a cell-autonomous fashion (60, 64, 65, 74, 75). Similarly, inappropriate activation of WG signaling induces ectopic dorsal-ventral boundaries that promote extensive overproliferation (67, 76).

How DPP and WG stimulate cell proliferation is not yet understood. One simple model, in which DPP- and WG-responsive transcription factors directly control the ex-

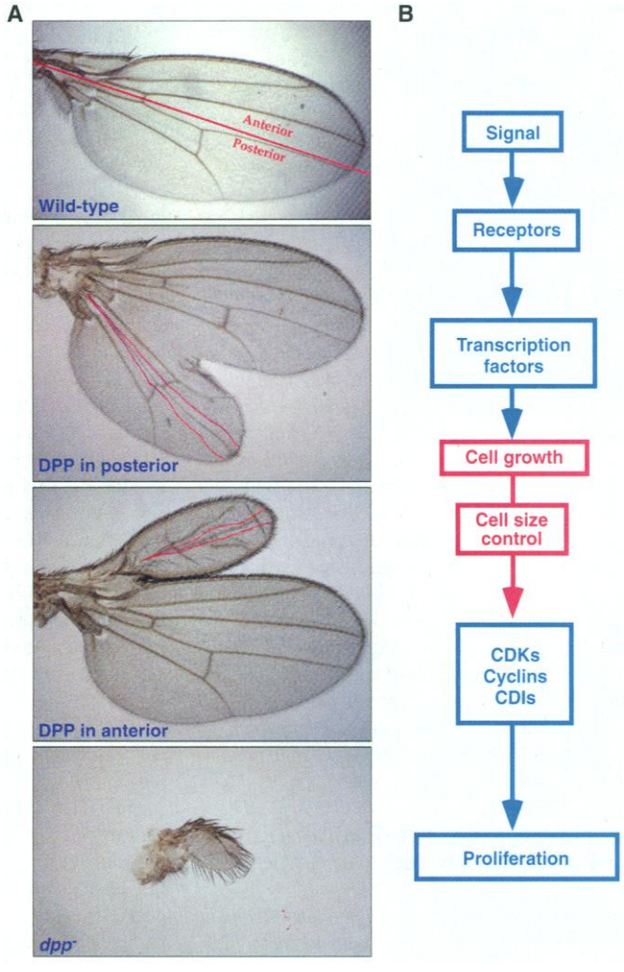

Fig. 2. Patterning and proliferation in *Drosophila* wings. (**A**) DPP signaling has profound effects on wing size and pattern. In developing wild-type wings (WT, top) DPP is expressed in a narrow stripe along the A/P border (red). When DPP is ectopically expressed elsewhere duplications arise that involve massive overproliferation. The regions of ectopic expression (middle two panels) are outlined in red. When DPP function is lost throughout the wing, cell proliferation is severely curtailed (bottom panel). Modified with permission from (62) (**B**) A proposal for how *dpp* and *wg* signaling might promote cell proliferation in the imaginal discs, in which cell cycle genes are regulated indirectly through mechanisms that stimulate and monitor cell growth (red).

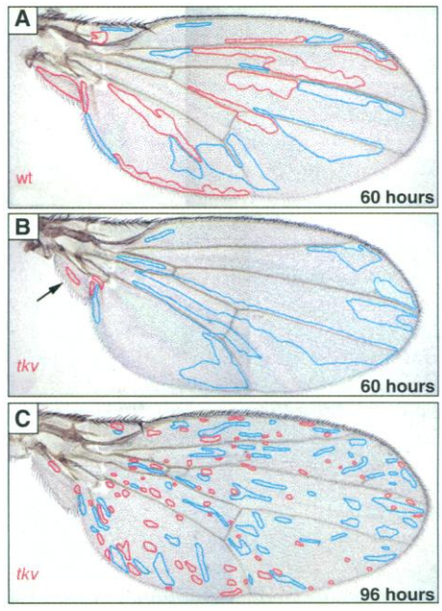

Fig. 3. Cell competition in the fly wing. (**A**) A wing in which clones of cells expressing neutral markers were induced by mitotic recombination early in disc development (60 hours). Marked clones are outlined in red and their sister clones (twin spots) are outlined in blue. (**B**) A wing in which clones of cells lacking the DPP receptor TKV were induced early in development (60 hours). The *tkv*$^-$ clones are outlined in red and their wild-type twin spots in blue. The *tkv*$^-$ cells have been out-competed by their wild-type sisters in most of the wing, and survive only in a small region where dpp signaling is not required (arrow). (**C**) A wing in which *tkv*$^-$ clones (red) were induced somewhat later in development (96 hours). In this case many clones survive, but they are smaller than their wild-type twinspots (blue). This suggests that their growth has been compromised by their ability to receive the DPP signal. Modified with permission from (*74*).

pression of cell cycle regulators such as Cyclins, Cdks, and Cdk inhibitors, seems unlikely because few correlations have been found between patterns of proliferation and the sources of the known patterning signals. Nevertheless, the existence of several overlapping gradients of mitogens, and the possibility that such gradients modulate rates of cell proliferation rather than simply starting or stopping the cycle might explain why patterned proliferation has been difficult to detect. A mitogen gradient model is consistent with the finding that cell clones lacking DPP transducers like TKV, MAD, or VG survive more frequently and grow to a larger size when located far from the source of DPP (Fig. 3) (*60*, *69*). This suggests that other signals—perhaps WG—may fulfill DPP's mitogenic role in cells far from the anterior-posterior boundary. Moreover, spatial zones of cell cycle synchronization which correlate with DPP and WG expression patterns do occur in wing discs (*49*, *77*) and eye discs (*27*) during the last few cycles before cell differentiation. Genetic manipulations have demonstrated that these signaling pathways have profound effects on cell cycle progression in these zones (*78*) and thus lend some credence to the idea that, in this specific context, DPP and WG might modulate expression of cell cycle regulators fairly directly.

One alternative to a system of overlapping mitogen gradients supposes that proliferation is stimulated by the interaction of cells exposed to different concentrations of DPP or WG, and stops when these concentration gradients are too subtle to be sensed. Though attractive in the context of the polar coordinate model, this idea is problematic because it requires that cells measure very small concentration differences. Another important point to keep in mind here is that although DPP and WG are essential for patterning in all of the discs, they effect very different degrees of proliferation in discs with different identities (for example, wing as compared to leg). These differential responses to signaling derive at least in part from states of expression of homeobox-type selector genes that are established in the embryo before disc growth, and are thus cell-intrinsic.

Another reason that the connections between signaling and cell cycle genes have been elusive may be that they are very indirect. Several findings suggest that the targets of DPP and WG signaling may not be cell cycle genes at all, but genes that control cell metabolism and growth. For instance, clones of *tkv*$^-$ cells are handicapped for proliferation and cell competition, properties curiously similar to those of *Minute* cells, which simply have reduced rates of protein synthesis (Fig. 3). When *tkv*$^-$ cells are given a growth advantage by making them *Minute*$^+$ in a *Minute* background, they do proliferate and can give rise to large clones in some regions of the developing wing where *dpp* seems normally to be required (*74*). A parsimonious, though undoubtedly simplistic interpretation of this result is that DPP signaling may enhance protein synthesis and thereby stimulate cell growth, and that increased proliferation results because cells are programmed to maintain a constant size (Fig. 2B). How cell growth and size control are coupled to the cell cycle is still an obscure topic, but there are some clues. For instance, loss of the mitotic inducer, Cdc2, blocks cell division without arresting growth (*43*), whereas clones of cells lacking the dE2F transcription factor fail to grow and are frequently lost through cell competition (*79*), just like *Minute* cells. Experiments in the same vein in vertebrates show that cells lacking the E2F repressor, pRB, have a cell cycle that is relatively resistant to inhibitors of protein synthesis (*80*), and suggest that pRB could restrain cell growth by repressing RNA polymerase I and III transcription (*81*). Vertebrate cyclin D1, a cell cycle activator and repressor of pRB, also appears to be linked to translational control, because its amount (and presumably activity) can be increased dramatically by overexpression of eIF4E, a translational initiation factor whose activity is normally stimulated by growth factor signaling (*82*). Results like this suggest that the Cyclin D–pRB–E2F pathway might have a role in linking protein synthesis and cell growth to proliferation, and open the possibility that the *Drosophila* homologs of these molecules (*6*, *83*) could be relayers of *dpp*- and *wg*-signaling.

Insights into cell proliferation control can also be expected from the molecular analysis of mutations that cause overproliferation of the imaginal cells. A number of genes required to arrest proliferation at the correct disc size have been identified and cloned (*84*). Oddly, cell proliferation in many of these mutants is actually slowed, compared to that in wild-type cells, and overproliferation in the discs occurs during an extended larval period. In contrast, mutation in the *lats* or *warts* genes promotes increased cell proliferation that cannot be arrested at the correct developmental stage (*85*). This interesting gene encodes a Ser-Thr protein kinase with close relatives in vertebrates.

How Similar Are Proliferation Controls in Insects and Mice?

It should no longer come as a surprise that growth and patterning in vertebrates is regulated by many of the same signaling pathways as in the fly. In vertebrate limbs Sonic hedgehog (Shh, a HH homolog) and the fibroblast growth factors FGF4 and FGF8 act to set up the anterior-posterior and the proximal-distal axes respectively, and Wnt-7a (a WG homolog) acts in establishing the dorsal-ventral axis (*86*). As in the fly, Shh induces DPP relatives—the bone morphogenetic proteins (BMPs, *87*), and Wnt-7a induces LMX1, a homolog of *Drosophila*'s Apterous (*88*). These factors and their relatives function in growth and patterning not only in the limbs but in virtually all other tissues that have been studied. In some cases ectopic expression of a signaling molecule (such as FGF-4 or Shh) can induce duplicated or additional limbs requiring massive extra cell proliferation (*89*), just as in flies.

Many of these signaling genes have been knocked out in transgenic mice, and in several cases tissues known to express a particular product suffer retarded growth. For instance, deletion of FGF-4 blocks development of the inner cell mass, deletion of the platelet-derived growth factor receptor causes growth deficiencies in many mesodermal tissues, deletion of the epidermal growth factor receptor causes deficiencies in epithelial development, deletion of the signaling molecule Wnt-1 causes underdevelopment of the cerebellum, and disruption of Shh causes deletion of distal limbs and many other structures (*90*). As in *Drosophila*, it is not clear what the targets of these signals are, or how they interface with genes controlling growth, cell proliferation, or cell survival. Work from the last 2 years, however, has begun to chip away at this problem.

The three murine D-type cyclins, thought from cell culture studies to connect growth factors to cell cycle control, are differently expressed in vivo and thus might confer cell-type specificity to proliferation (*91*). This possibility seems to be supported by studies showing that disruption of Cyclin D1 suppresses proliferation in the retina and mammary epithelium, both regions where expression is normally high, whereas disruption of Cyclin D2 suppresses proliferation of ovarian granulosa cells, which normally activate Cyclin D2 expression and proliferate in response to follicle stimulating hormone (*92*). The primary targets of cyclin D–Cdk complexes are believed to be the pocket proteins pRB, p107, and p130, which repress E2F-type transcription factors (*4*, *93*). Although the tumor suppressor function of pRB suggested that it might mediate cell cycle exit, the phenotype of Rb$^-$ mice failed to support this simple hypothesis. Rb$^-$ mice die young, but most of their cells proliferate and arrest normally,

and Rb^- cells can contribute normally to many tissues in chimeric mice (*71*). The lack of profound proliferation defects in Rb^- cells in vivo could result from functional overlap with other members of this gene family. In support of this idea, knockouts of p107 and p130 cause negligible developmental defects, but mice lacking both genes exhibit severe bone defects that are associated with over-proliferation of chondrocytes (*94*). Synthetic effects have also been observed in Rb^+/Rb^-; $p107^-$ mice, suggesting that all three pocket proteins might have some overlap in function (*95*). Although many of the mouse knock-outs seem at least superficially consistent with paradigms generated in vitro, recent reports describing the phenotypes of E2F-1 mutant mice underscore the inadequacy of these models. E2F's well-substantiated role in triggering S-phase gene expression led to the expectation that $E2F^-$ mice would lack tissues because of a loss of cell proliferation. Although this expectation was supported by the cell cycle arrest phenotype of *Drosophila* E2F mutants (*25*), E2F-1 mutant mice develop normally but suffer a wide range of tumors later in life (*96*). Knockouts of the other four murine E2F genes and their DP subunits should clarify the roles of these factors in cell proliferation, but it may be a long while before the connections between these genes and patterning signals are understood in the mouse.

In conclusion, phenomenal progress has been made recently in delineating the signaling pathways that organize pattern formation and promote cell proliferation, and in identifying the proximal regulators of cell cycle progression that may respond to these signals. An exciting, and perhaps more difficult puzzle for the future is to clarify the connection between these two highly conserved sets of genes, and the role cell growth plays in this linkage.

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How Proteolysis Drives the Cell Cycle

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Oscillations in the activity of cyclin-dependent kinases (CDKs) promote progression through the eukaryotic cell cycle. This review examines how proteolysis regulates CDK activity—by degrading CDK activators or inhibitors—and also how proteolysis may directly trigger the transition from metaphase to anaphase. Proteolysis during the cell cycle is mediated by two distinct ubiquitin-conjugation pathways. One pathway, requiring CDC34, initiates DNA replication by degrading a CDK inhibitor. The second pathway, involving a large protein complex called the anaphase-promoting complex or cyclosome, initiates chromosome segregation and exit from mitosis by degrading anaphase inhibitors and mitotic cyclins. Proteolysis therefore drives cell cycle progression not only by regulating CDK activity, but by directly influencing chromosome and spindle dynamics.

The periodicity of DNA replication and mitosis in eukaryotes contrasts with the continuous nature of most metabolic reactions that produce cellular growth. The eukaryotic chromosome cycle is composed of an ordered series of discrete events; the periods of replication and chromosome segregation do not overlap as they do in prokaryotes. Interposition of a chromosome-alignment step between replication and segregation completes the set of events that constitute the basic eukaryotic chromosome cycle. The steps in this cycle are initiated in sequence by the cell cycle regulatory machinery, which also controls centrosome duplication and cell division (cytokinesis), and coordinates these discontinuous events with cell growth. In this review, we explore how specific protein degradation provides direction, order, and proper timing to the key events of the chromosome cycle.

Biologists have long grappled with the problem of how cell division is controlled. Early models postulated the existence of an initiator that would accumulate during the cell cycle, inducing DNA replication (*1*) or mitosis (*2*) when it reached a critical concentration. The process of mitosis would then abruptly inactivate the initiator, resetting the cycle. This model proved to be remarkably prescient, for today we know these initiators include the mitotic cyclins, which accumulate during interphase to drive entrance into mitosis and are degraded at the end of mitosis to reset the cycle (*3–5*). Subsequent work has shown that proteolysis has a pervasive role in regulating cell cycle progression: Protein degradation is required for multiple processes in mitosis and also for the onset of DNA replication (Fig. 1).

To understand how proteolysis regulates transitions through the cell cycle, we must explain how proteolytic activity is controlled and how substrate specificity is achieved. Although there are many proteolytic processes inside and outside of cells, the ones known to be important for cell cycle progression rely on the assembly of a ubiquitin chain on the substrate, which targets it for degradation by the 26S proteasome (*6*). Ubiquitin, a small, highly conserved protein, is first activated at its COOH-terminus by formation of a thioester bond with the ubiquitin-activating enzyme, E1. Ubiquitin is subsequently transesterified to one member of a family of E2 or UBC (ubiquitin-conjugating) enzymes, for which there are 13 genes in the budding yeast genome (*7*). Finally, ubiquitin is transferred from the E2 to a lysine residue of the target protein, either directly or with the assistance of a ubiquitin-protein ligase (E3). An E3 is generally required for the formation of multiubiquitin chains on the substrate, a step that facilitates efficient recognition of the substrate by the proteasome. The rate and specificity of ubiquitin-mediated proteolysis may also be controlled by the disassembly of ubiquitin chains, which is catalyzed by a large and poorly characterized family of deubiquitinating enzymes (UBPs). There are more genes for UBPs (16) than for E2s (13) in budding yeast (*7*), and perturbations to deubiquitinating enzyme activity can profoundly alter cell physiology (*8*).

The enzymes of the ubiquitin system were first defined as eluates from a ubiquitin-affinity column (hence the letter E in their name). Whereas E1 and E2 enzymes formed covalent bonds with the ubiquitin column, the first E3 characterized could be eluted with high concentrations of salt or increased pH (*9*). An E3 was functionally defined as an activity that was both necessary and sufficient for the transfer of ubiquitin to the substrate in the presence of a ubiquitin-charged E2 enzyme, indicating that it participated in the final step of ubiquitination (*9*). In addition to facilitating multiubiquitination of substrates, E3s appear to be the primary source of substrate specificity in the ubiquitination cascade, as some E3s have been shown to directly bind substrates (*10, 11*). Two E3s, E6-AP (*12*) and UBR1 (*11*), may function catalytically, forming a thioester with ubiquitin as an intermediate in the transfer reaction (*13, 14*). Despite the similarity in reaction mechanism, UBR1 and E6-AP do not share significant sequence similarity. As relatively few E3s have been mechanistically characterized, it remains to be seen whether all

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