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Cdks and the *Drosophila* cell cycle

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Abstract

Cyclin-dependent kinases play essential roles in driving the cell cycle. Much progress has been made in *Drosophila* over the past year in identifying the specific requirements for individual cyclins in particular cell cycle events. These studies encompass many aspects of the cell cycle, from the addition of a G₁ phase to the cell cycle during embryogenesis to the role of cyclin degradation in progression through anaphase.

Introduction

Drosophila provides a unique experimental system in which to study the cell cycle, effectively bridging the gap between vertebrate and unicellular systems. It serves as an excellent model for vertebrate systems — there being many homologous molecules between them — because of the relative lack of redundancy in such molecules in *Drosophila* and also because *Drosophila* offers us the ability to manipulate them genetically. The developmental complexity here is, in its own way, advantageous: the numerous restrictions to proliferation are added successively, so that each type of cell cycle control is developmentally isolated and can thus be exposed to experimental analysis [1–3].

Cyclins act as the regulatory subunits of key cell cycle regulators called cyclin-dependent kinases (cdks). Although there is some overlap in the proposed functions of each cyclin, individual cyclins are required for specific cell cycle phenomena. These requirements may reflect differences in the potential activity of the cyclin-dependent kinases — for example, their substrate specificity or subcellular localization — or the timing of their activation. This activation can be regulated in multiple ways, such as by cyclin expression or stabilization, or by the presence of inhibitors specific to individual cdks. Some of these requirements exist in every cell cycle but others only appear in particular developmental contexts and change with the changing cell cycle. We describe here the introduction of a G₁ phase into the cell cycle during embryogenesis, which is achieved in part through the inactivation of the S phase cyclin, Cyclin E. We also discuss the ability of Cyclin E and Cyclin A to promote DNA replication during S phase and to inhibit it during G₂ phase. Finally, we address the importance of sequential cyclin degradation during mitosis.

The addition of a G₁ phase to the embryonic cell cycle

The classic cell fusion experiments of Rao and Johnson [4] defined two basic levels of control over entry into S phase. One level is mediated by *trans*-acting S phase factors: when an S and a G₁ phase cell are fused, the G₁ nucleus enters S phase precociously, presumably driven by diffusible, positively acting factors present within the cytoplasm of the S-phase cell. A second

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level of control acts within each nucleus: when an S and G₂ cell are fused, the G₂ nucleus does not enter S, as if it were unable to replicate despite the presence of *trans*-acting S phase factors. Replication competence is restored at mitosis, which separates G₂ and G₁ phases.

In *Drosophila*, the *trans*-acting S phase factors analogous to those identified by Rao and Johnson [4] appear to be supplied by E2F-driven transcription, which leads to the expression of numerous replication functions [5–7]. Among these is Cyclin E, a key regulator of S phase [6,8,9]. In *Drosophila*, both E2F and Cyclin E are required for S phase, as mutant embryos fail to incorporate BrdU in their absence. Additionally, because of mutual activation, ectopic expression of either is capable of driving cells from G₁ into S phase [9,10•,11•–13•].

The *trans*-acting S phase factors appear to be available constantly during the first 16 cell cycles of *Drosophila* embryogenesis: E2F is constitutively active [6], Cyclin E is always expressed [14], and S phase begins immediately upon completion of mitosis [15]. We suggest that these early S phases are limited only by the restoration of replication competence which occurs at mitosis. Following mitosis 16, the nature of S phase control changes and passage through mitosis is no longer the sole limitation for S phase. Instead, both E2F and Cyclin E are downregulated earlier in cycle 16, thereby limiting the supply of *trans*-acting factors and preventing immediate entry into S phase upon completion of mitosis [6,9].

The downregulation of Cyclin E expression during cycle 16 is, however, not sufficient to eliminate Cyclin E activity by the end of mitosis. Instead, the establishment of a G₁ phase also requires the presence of the recently discovered p21/p27-type cdk-inhibitor Dacapo [16•, 17•]. Dacapo — which is expressed for the first time late in cell cycle 16 close to the time of Cyclin E and E2F downregulation — inhibits Cyclin-E-associated kinase activity *in vitro* and co-immunoprecipitates from embryonic extracts with Cyclin E. Dacapo plays an essential role in the creation of a G₁ phase: in *dacapo* mutants, many cells that would normally enter a G₁ phase following mitosis 16 instead enter S phase. In addition, premature expression of Dacapo is sufficient to establish a G₁ phase earlier in embryogenesis.

The addition of a G₁ phase to the cell cycle during fly embryogenesis thus involves the depletion of *trans*-acting S phase factors by at least three mechanisms: downregulation of E2F; inhibition of Cyclin E expression; and expression of Dacapo (Fig. 1). As they follow the same complex spatial and temporal pattern, we suspect that these events are coordinated. Given the extensive potential for crossregulation among the factors — Cyclin E and E2F can activate each other, and Dacapo can inhibit Cyclin E — this coordination could be facilitated by mutual interaction; alternatively, each of these events could be driven independently by an upstream regulator. As none of the events precedes the others significantly, we suspect that an upstream regulator is acting at some level.

Whereas the above description of G₁ establishment in the *Drosophila* embryo provides an example of how entry into S phase can be prevented, resulting in a G₁ phase, studies in the eye imaginal disc have demonstrated that not all G₁ phases are maintained similarly. For example, Dacapo is only expressed in those G₁ cells in the eye which are in the process of exiting the cell cycle and undergoing differentiation; in other cells Dacapo is not expressed during G₁ [16•,17•]. In addition, some eye cells in G₁ phase do not enter S phase following ectopic expression of Cyclin E or E2F [11•–13•]. Different G₁ phases thus appear to be regulated differently; it will be interesting to see if the logic of their creation (i.e. limitation of *trans*-acting factors) is conserved in all G₁ cells.

The role of Cyclins E and A in controlling DNA replication

How does Cyclin-E-associated kinase activity induce entry into S phase? One possibility, suggested by work in mammalian systems, is that Cyclin E activates E2F by inactivating the

E2F-inhibiting retinoblastoma protein (Rb [18]; a homolog of which was identified recently in *Drosophila* [19•]). Although Cyclin E is in fact required for E2F activity in certain cells of the *Drosophila* embryo, E2F is activated independently of Cyclin E in other cells — in fact, in those cells, Cyclin E expression is dependent on E2F [8•]. Nevertheless, Cyclin E is required for S phase in all cells, suggesting an E2F-independent S phase requirement for Cyclin E. This idea was tested directly by Duronio *et al.* [10••], who supplied E2F throughout wild-type and cyclin E mutant embryos by heat shock induction. Although E2F was clearly active following heat shock in both wild-type and *cyclin E* mutants, S phase was only induced in wild-type embryos. Cyclin E is thus required for S phase even in the presence of active E2F; the nature of this requirement remains an important issue for future investigation.

Is Cyclin E required continuously during S phase or only for its initiation? Studies of Cyclin E in endocycle S phases — cycles that consist of multiple rounds of S phase without intervening mitoses — in *Drosophila* nurse cells suggest that Cyclin E is required continuously [20••]. These S phases are incomplete, resulting in under-replication of certain regions of the genome [21]. Lilly and Spradling [20••] have identified a mutation in *cyclin E* that alters its normal fluctuations (but not its overall levels), resulting in an apparent increase in its persistence and causing a complete round of replication. They then tested the idea that Cyclin E is thus limiting for a complete round of replication in wild-type nurse cells by supplementing the endogenous Cyclin E with Cyclin E produced by heat shock induction. This additional expression resulted in the complete replication of the genome. Thus, Cyclin E is limiting for DNA replication in these cells; the simplest interpretation of these results is that, in wild-type nurse cells, Cyclin E disappears prior to the complete replication of the genome for which its continuous presence would be required.

Cyclin A has also been implicated in S phase control but its role in promoting DNA replication is less clear than that of Cyclin E. Studies of mammalian tissue culture cells have identified a requirement for Cyclin A in S phase there [22,23]. Consistent with this work, there is evidence from *Drosophila* that Cyclin-A-associated kinase activity can induce DNA replication. For example, ectopic Cyclin A expression in the eye imaginal disc — resulting from the *roughex* mutation — leads to ectopic S phase [24]; in addition, heat shock expression of Cyclin A can induce S phase in the embryonic amnioserosa [25]. But *Drosophila* endocycles occur in the absence of Cyclin A, indicating that Cyclin A is not required for all replication. One possible resolution of this apparent contradiction is that Cyclin A may be required for the completion of replication specifically in those mitotic cells with a G₁ phase; in these cells, Cyclin E is downregulated prior to the completion of DNA replication — apparently similar to endocycling nurse cells, described above — in preparation for the next G₁ phase (Fig. 2).

Once replication is complete, Cyclin A plays a negative role in controlling DNA replication as it is required during G₂ phase to prevent rereplication of the genome. Mutants of *cyclin A* are blocked from entering mitosis of cycle 16 [26] but do not arrest the cell cycle altogether: post cell cycle 16, S phases occur normally in mutant embryos [25,27•]. As post-replicative cells must usually pass through mitosis to regain the ability to replicate, mutation of *cyclin A* appears to confer upon cells the ability to enter S phase without passage through mitosis. Mutations in *Drosophila cdc2*, the kinase partner of Cyclin A, also lead to endoreplication in otherwise diploid cells in the larval CNS [28•]. This ability of Cyclin A to regulate DNA replication both positively and negatively can be explained by a model in which fluctuations of cdk activity are required for multiple rounds of S phase [29] and are consistent with extensive work in yeasts demonstrating a requirement for cdk activity in preventing rereplication during G₂ phase in those organisms [30–32]. Also consistent with result, continuous expression of Cyclin E in *Drosophila* endocycles can prevent endoreplication (PJ Follette, RJ Duronio, PH O'Farrell, unpublished data; C Lehner, personal communication). The ability of Cyclin A, the

fluctuations of which are dependent on mitosis, to block rereplication has led to the suggestion that fly endocycles are created at least in part through the downregulation of Cyclin A [27•].

Cyclin degradation and progression through mitosis

The end of G₂ phase is marked by the activation of G₂ cyclin-associated kinases which drive cells into mitosis; but Cdk activity also plays a negative role in mitosis, as its disappearance is required for progression through anaphase. Recent results in *Drosophila* suggest that normal mitotic progression depends not simply on the elimination of cdk activity but more specifically on the correct order of disappearance of specific cyclin–CDK complexes.

The G₂/M cyclins are regulated in part by their stability and are degraded in a specific order during mitosis. These cyclins can be stabilized by the removal of the protein domain responsible for their ubiquitination and subsequent degradation, the 'destruction box' [33]. When these stabilized forms of the cyclins are expressed, cells arrest in mitosis (the other, non-stabilized cyclins are still degraded, however). There is a striking correlation in *Drosophila* between the timing of disappearance of each of the cyclins and the particular arrest point associated with expression of its stabilized form [34••]. Cyclin A — which normally disappears during metaphase — causes a metaphase arrest when stabilized. Stabilization of Cyclin B — which is normally degraded at about the time of the metaphase–anaphase transition — causes an arrest during early anaphase, after sister chromatid separation. Finally, the stabilization of the last-disappearing Cyclin B3 results in a late anaphase arrest.

Although the degradation of cyclins has been shown to be a necessary element of anaphase progression in flies, expression of stable forms of cyclins in *Xenopus* extracts or in yeasts is generally insufficient to inhibit passage through anaphase [35–37]. However, overexpression of the destruction box alone — inhibiting the ubiquitination and degradation machinery — results in a metaphase arrest, suggesting that proteins other than those cyclins, the stabilized forms of which have been expressed experimentally, must be degraded for certain anaphase events to occur. These proteins may be additional cyclins, or, alternatively, proteins other than cyclins.

These results suggest a model (Fig. 3) in which numerous proteins, including cyclins, are each responsible for maintaining a subset of the functions required to enter mitosis (e.g. chromosome condensation, spindle assembly, or nuclear envelope breakdown) and that the order of the loss of each function may be essential for normal progression through anaphase. If the functions maintained by each protein were overlapping, then the loss of a particular factor may not eliminate a particular function — it may still be maintained by another protein — but instead may disrupt the proper timing of its disappearance, resulting in a defective but still viable mitosis. For example, Cyclin-B-deficient cells in *Drosophila* continue to divide but cytokinesis initiates prior to the complete separation of the chromosomes [38].

Mutations have been identified in *Drosophila* in several genes required for normal progression through mitosis. Cells deficient in *fizzy* function arrest just prior to the metaphase–anaphase transition [34••,39], and the *pimples* and *three rows* mutations block sister-chromatid separation [40•]. Analysis of these genes will likely contribute to our understanding of anaphase progression.

Conclusions

Whereas *Drosophila* is an excellent system in which to investigate the developmental control of the cell cycle — how the cell cycle is both regulated and modified to fulfill the requirements of development — it has also yielded many secrets about the basic machinery of the cell cycle in all cells. We have discussed here the requirement for the presence or absence of several

cyclin-associated kinases in driving various cell cycle events. Although the cell cycle responsibilities of the individual cyclins are becoming clearer, the specific function of the cyclins in cell cycle events and the nature of their regulation are still largely mysterious. Research in *Drosophila* will surely continue to contribute to the illumination of these and other secrets of the cell cycle.

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Abbreviations

cdk	cyclin-dependent kinase
CNS	central nervous system
Rb	retinoblastoma protein

Figure 1.

A schematic depiction of the three events that must occur for the creation of a G₁ phase during *Drosophila* embryogenesis: E2F inactivation, downregulation of Cyclin E, and expression of the Cyclin E inhibitor Dacapo. In the absence of Dacapo expression, Cyclin E protein levels are sufficient to allow entry into S phase following mitosis 16 (M16); Dacapo inhibits the Cyclin E that is present, however, thereby preventing entry into S phase and creating a G₁ phase.

Figure 2.

The ability of Cyclins E and A to induce DNA replication is consistent with models in which their respective requirements for S phase change during development. **(a)** In endocycles, Cyclin A is not expressed and Cyclin E is solely responsible for S phase. S phase occurs apparently only when Cyclin E is present and ends upon the disappearance of Cyclin E, even though the genome is not completely replicated by that time. **(b)** In mitotic cycles that lack a G₁ phase – such as the first 16 cycles of *Drosophila* embryogenesis – Cyclin E is present at constantly high levels and entry into S phase is limited only by the restoration of replication competence that accompanies mitosis. As a result of perpetually high levels of Cyclin E, Cyclin A may not be required for S phase in these cells. **(c)** In mitotic cycles with a G₁ phase, Cyclin E is

downregulated prior to the end of S phase in preparation for the following cell cycle. In these cells, the absence of Cyclin E late in S phase may create a requirement for Cyclin A for the completion of replication.

Figure 3.

A model in which the order of degradation of cyclins and other proteins during anaphase determines the order of events required for anaphase progression. Molecules X, Y, and Z are degraded in a certain order during anaphase (the order as drawn here is determined by differences in the kinetics of their disappearance, rather than the alternatively possible presence of separate triggers for the degradation of each molecule) and act to specifically inhibit various events required for anaphase progression (e.g. sister chromatid separation, chromosome segregation), represented here as events 1–5. The molecules are schematized here as inhibiting overlapping subsets of the events but it is also possible that each molecule prevents a specific assortment of activities.