Cyclins: **Growing pains for *Drosophila***
Edan Foley and Frank Sprenger

Tradition holds that cyclin D is required for the initiation of cell division; recent studies in *Drosophila*, however, suggest that cyclin D has a separate function in governing growth.

Address: Department of Genetics, University of Cologne, Weyertal 121, D-50931, Cologne, Germany.

Events such as development, patterning and cancer progression are necessarily tied in to cell division. It is thus no surprise that the initial step in cell division is tightly regulated. Our current understanding of this process is that a cyclin-dependent kinase, either Cdk4 or Cdk6, partnered with its regulatory subunit cyclin D, responds to mitogenic signals by phosphorylating 'pocket' proteins, such as the retinoblastoma protein (Rb). Phosphorylation of Rb leads to release of the transcription factor E2F, which is then free to activate the transcription of several genes involved in DNA replication [1]. Indeed, mutations in the genes encoding cyclin D, Rb and the cyclin D inhibitor p16 are observed in many human cancers [2].

The fruitfly *Drosophila* presents an ideal organism for investigating the function of cyclin D. The recently completed sequence of the *Drosophila* genome has revealed that the fruitfly has only one *cycD* gene and only one gene encoding a cyclin D kinase partner, Cdk4/6. For simplicity, we shall refer to Cdk4/6 as Cdk4. Two groups [3,4] have recently investigated the consequences of mutations in Cdk4 or overexpression of the genes for Cdk4 and cyclin D in *Drosophila*, and have come up with some surprising findings. Cyclin D does not appear to be essential for cell-cycle progression; rather, it promotes growth. Moreover, cyclin D-dependent regulation of growth is at least partly independent of Rb.

An impressive amount of data has accumulated in the last decade in support of the view that cyclin D has a pivotal role in governing progression through G1 phase of the cell cycle. The genes for cyclin D and its kinase partner Cdk4 are both transcribed in response to serum in cultured mammalian cells [5]. Cyclin D–Cdk4 complexes efficiently phosphorylate Rb *in vitro* and *in vivo* [6]. Hypophosphorylated Rb inhibits E2F in a simple stoichiometric fashion, as well as by recruiting histone deacetylase activity to E2F-responsive genes [1,7]. Phosphorylated Rb no longer associates with or inhibits E2F activity, and free E2F has been shown to activate the transcription of numerous genes required for S-phase initiation and DNA replication. Thus, a model consisting of a linear cascade of gene activities with cyclin D at the top, liberating E2F at the bottom to irreversibly initiate cell division has developed over the last few years.

This understanding of cyclin D function has, however, evolved primarily from *in vitro* studies or work done with cell cultures. Very little evidence exists from whole organism studies to support the proposed *in vivo* functions of cyclin D. In knockout mice where individual cyclin D genes have been disrupted, only modest defects are observed [8,9]. These studies have been confounded by the fact that mice have three different genes for cyclin D, and two that encode potential Cdk partners for cyclin D. Thus, a functional redundancy between the separate genes has often been proposed to explain the mild phenotype in the different knockout mice. Against this background it seemed a good idea to turn to a different model organism, in particular *Drosophila*. As mentioned above, the *Drosophila* genome has only gene each for cyclin D and its kinase partner. Furthermore, *Drosophila* cyclin D does not associate with other cyclin-dependent kinases, and cyclin D–Cdk4 can phosphorylate Rb, indicating that the proposed functions for cyclin D are conserved in *Drosophila* [3].

Slow growers

The two new papers [3,4] report the consequences of mutations in the *Drosophila* gene for Cdk4, or of coordinately overexpressing the genes for Cdk4 and cyclin D. The most surprising revelation is that these genes are dispensable for cell-cycle progression. Cells divide in the absence of Cdk4 activity and overexpressing cyclin D–Cdk4 does not accelerate G1 phase. The only apparent phenotypic manifestation of manipulating cyclin D–Cdk4 activity is a disturbance in organ or organism growth. A second twist to the tale is that, although cyclin D–Cdk4 can phosphorylate mouse Rb and does interact with the *Drosophila* homologue of Rb, RBF, the effects of cyclin D on growth appear to be independent of RBF.

Datar *et al.* [4] created transgenic flies in which the genes for cyclin D and Cdk4 were overexpressed in distinct regions or at specific times, and looked at the consequences for growth and cell-cycle progression. It has previously been demonstrated that increasing the rates of cell division in specific compartments or clones of the developing wing disc does not increase compartmental or clonal area because cells compensate by becoming smaller [10]. Conversely, decreasing rates of cell division does not decrease clonal area, because cells within the clone become larger [10].

Datar *et al.* [4] found, however, that in the developing *Drosophila* wing, clones of cells overexpressing cyclin D–Cdk4 were larger than control clones, because they contained a larger population of normal-size cells. This indicates that overproduction of cyclin D–Cdk4 reduces cell doubling time. Cells within the clones had the same cell-cycle profile as control cells, indicating that cyclin D–Cdk4 overexpression accelerates proliferation throughout the entire cell cycle. As these cells did not have a reduced size to compensate for their accelerated rate of division, it follows that they must have also experienced an accelerated growth rate. Excessive cellular growth was also seen in post-mitotic cells of the differentiating eye on cyclin D–Cdk4 overexpression, indicating that the growth observed upon cyclin D–Cdk4 overexpression is not necessarily linked to cell-cycle progression.

Equally telling is the approach taken by Meyer *et al.* [3], who produced *cdk4* mutant flies. Interestingly, the homozygous mutants were viable, showing normal development and eclosing at the expected time. Mutant adults displayed moderate fertility defects and were able to produce progeny. The *cdk4* gene is therefore dispensable for organism survival in general, and cell-cycle progression in particular. But the mutant adults were smaller than wild-type, with a reduction in body weight of about 20%. The *cdk4* mutant clones produced in the wing were also smaller than their wild-type twin clones. Individual cells in mutant flies or inside a mutant clone were of normal size. Mutant cells did not show an increased rate of apoptosis.

Figure 1
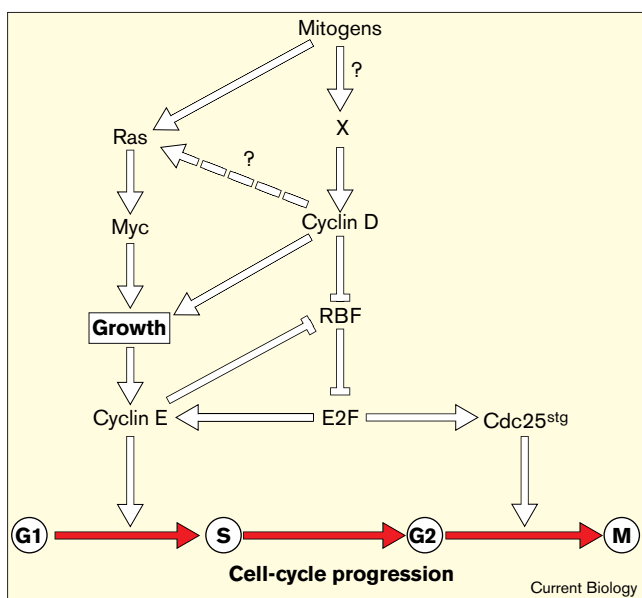
A simplified model of how cyclin D may coordinately regulate growth and cell-cycle progression.

The reduction in body or clonal size must then be caused by a slower proliferation rate. The mutant cells did not, however, have a prolonged G1 phase, as might have been expected. Instead all phases of the cell cycle were equally prolonged. As clones occupy a smaller area then their twin wild-type clones, growth and proliferation are equally affected in *cdk4* mutant cells.

Rb or not Rb?

As in earlier work on mammalian systems, both groups [3,4] observed numerous genetic and biochemical interactions between cyclin D–Cdk4 and RBF. Stimulation of growth by cyclin D appears, however, to be independent of its ability to phosphorylate RBF [4]. For example, the post-mitotic growth of the developing eye produced upon overexpression of cyclin D–Cdk4 could not be suppressed by coordinately overexpressing RBF. Furthermore, if cyclin D–Cdk4 were to function solely by inactivating RBF, one would expect RBF mutants to have the same phenotype as cells overexpressing cyclin D–Cdk4, whereas actually their phenotypes are quite different. Clones of cells overexpressing cyclin D–Cdk4 grew and divided faster than normal, but RBF mutant clones were no larger than wild-type and their individual cells had a reduced size.

In contrast to its mammalian homologues, Cdk4 does not seem to be the principal RBF kinase *in vivo* [3]. Meyer *et al.* [3] analyzed the phosphorylation status of mouse Rb expressed in *Drosophila* embryos. Overproduction of cyclin D–Cdk4 slightly enhanced Rb phosphorylation. A more dramatic enhancement of Rb phosphorylation, however, was seen upon overexpression of the gene for cyclin E. Furthermore, overexpression of *dacapo*, the *Drosophila* homologue of the cyclin E inhibitor p21, greatly reduced Rb phosphorylation. These results suggest that cyclin E–Cdk2 is the principal Rb/RBF kinase in *Drosophila*.

The new studies [3,4] suggest the existence of two distinct cyclin D pathways in *Drosophila*. The first conforms to the traditional function ascribed to cyclin D: cyclin D–Cdk4 phosphorylates RBF, allowing a cell to progress through the cell cycle. RBF has been proposed to be a negative regulator of both G1 and G2 in *Drosophila* [10]. This is presumably why the entire cell cycle is slowed in *cdk4* mutants, and why the rate of proliferation is accelerated in clones of cells overproducing both Cdk4 and cyclin D. Cyclin D is not, however, essential for cell-cycle progression, perhaps because of redundancy between cyclin D–Cdk4 and cyclin E–Cdk2 in RBF inactivation. Cyclin D also acts, apparently independently of RBF, in the cell-autonomous control of cell growth. This is a new development in our understanding of cyclin D and may explain the hypotrophic phenotypes observed in some *cyclin D* knockout mice [9]. It will be important to dissect the function of cyclin D in cell-growth control and determine to what extent this process is tied in with cell division.

To boldly grow where no fly has grown before...

For these further investigations it will be essential to have mutants deficient in cyclin D. Such mutants are not yet available, but the *cdk4* mutant *Drosophila* phenotype gives a good idea as to what to expect — sublethality with a general drop in organism size. The availability of cyclin D mutants should facilitate the next step forward — isolating the downstream targets of cyclin D required for growth and also for identifying the upstream 'growth sensor'.

In the budding yeast *Saccharomyces cerevisiae*, the G1 cyclin Cln3 is proposed to perform such a function. Cln3 is highly unstable and has several upstream open reading frames in its mRNA, which are proposed to delay ribosome access to the start codon for translation of Cln3 [11]. During robust times of protein synthesis, as one would expect to find in periods of growth, ribosome levels increase, raising the likelihood of Cln3 translation. Eventually a critical threshold level of Cln3 is reached which triggers cell-cycle initiation. This provides an elegant way of coupling cell division to growth-friendly circumstances. The mRNA for cyclin D has a complicated 5′ untranslated region and has been suggested to act as a growth sensor. This does not seem to be the case, however, as *cdk4* mutants do not have a prolonged G1 phase and overproduction of cyclin D–Cdk4 does not shorten G1. The picture could also become more complicated as completion of the *Drosophila* genome sequence uncovered a second Rb-like protein, RBF2. How this RBF2 fits into the story remains to be determined.

There has been a large number of papers in the past few years that significantly advance our understanding of how growth may be regulated in a developing organism (reviewed in [12]). The results from these papers indicate the existence of two growth regulatory pathways. In the first pathway, the insulin receptor responds to extracellular signals by activating intracellular molecules such as phosphatidylinositol 3-kinase and S6 kinase [13–15]. One consequence of this is increased synthesis of proteins coded by a special class of mRNAs [16]. Most of these proteins are involved in biosynthesis, indicating that insulin signaling may upregulate general protein synthesis, an obvious prerequisite for growth. It will be interesting to see if there is some crosstalk between this signaling pathway and cyclin D activity.

A second growth regulatory pathway involves the activity of at least two other proteins originally believed to be involved only in cell-cycle progression, Ras and Myc [17,18]. *Drosophila* Ras upregulates the activity of Myc, which stimulates growth and also upregulates cyclin E protein levels, enhancing progress through S phase. It is attractive to think that cyclin D may belong in this hierarchy of proteins, especially as E2F is known to activate transcription of the gene for cyclin E. If this is the case, then cyclin D acts either upstream of, or parallel to, Ras (see the model illustrated in Figure 1), as cyclin D enhances progression through the cell cycle in general, whereas Ras and Myc only enhance S phase entry.

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