***Drosophila* Cdk4 is required for normal growth and is dispensable for cell cycle progression**

Claas A.Meyer, Henning W.Jacobs, Sanjeev A.Datar¹, Wei Du², Bruce A.Edgar¹ and Christian F.Lehner³

Department of Genetics, University of Bayreuth, 95440 Bayreuth, Germany, ¹Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA 98109 and ²Ben May Institute for Cancer Research, University of Chicago, Chicago, IL 60637, USA

³Corresponding author
e-mail: chle@uni-bayreuth.de

C.A.Meyer and H.W.Jacobs contributed equally to this work

Complexes of D-type cyclins and cdk4 or 6 are thought to govern progression through the G₁ phase of the cell cycle. In *Drosophila*, single genes for Cyclin D and Cdk4 have been identified, simplifying genetic analysis. Here, we show that *Drosophila* Cdk4 interacts with Cyclin D and the Rb homolog RBF as expected, but is not absolutely essential. Flies homozygous for null mutations develop to the adult stage and are fertile, although only to a very limited degree. Overexpression of inactive mutant Cdk4, which is able to bind Cyclin D, does not enhance the *Cdk4* mutant phenotype, confirming the absence of additional Cyclin D-dependent cdk's. Our results indicate, therefore, that progression into and through the cell cycle can occur in the absence of Cdk4. However, the growth of cells and of the organism is reduced in *Cdk4* mutants, indicating a role of D-type cyclin-dependent protein kinases in the modulation of growth rates.

Keywords: Cdk4/cell growth/cell proliferation/cyclin D/*Drosophila*

Introduction

D-type cyclins, their kinase partners cdk4 and 6, the INK inhibitors and the kinase substrate retinoblastoma protein (Rb) are all known for their crucial importance in human tumorigenesis (Weinberg, 1995; Sherr, 1996; Sherr and Roberts, 1999). At the cellular level, Rb has been shown to regulate progression through the G₁ phase of the mammalian cell cycle, predominantly by binding to E2F transcription factors, which control a large number of genes involved in cell proliferation and DNA replication (Dyson, 1998). Rb represses expression of E2F target genes by recruiting histone deacetylase activity and by inhibiting the E2F transcriptional activation domain (Harbour *et al.*, 1999). The ability of Rb to block progression through G₁ is abolished by Rb hyperphosphorylation, which is initiated by D-type cyclin-dependent kinases during G₁ (Sherr, 1994; Sherr and Roberts, 1999). In mammalian cells, the synthesis of D-type cyclins is controlled by extracellular signals. Mitogens induce a rapid accumulation of D-type cyclins. Conversely, anti-

mitogens or withdrawal of mitogens result in a rapid decline. D-type cyclins are therefore thought to function as a functional link between extracellular signals and the cell cycle machinery. Accordingly, D-type cyclin–cdk complexes might be predicted to play an important role in the regulation of cell proliferation during development.

In mice, a number of genes of the INK (Serrano *et al.*, 1996; Franklin *et al.*, 1998), cyclin D (Fantl *et al.*, 1995; Sicinski *et al.*, 1995, 1996), cdk4 (Rane *et al.*, 1999; Tsutsui *et al.*, 1999), Rb (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992, 1996; Cobrinik *et al.*, 1996), E2F (Field *et al.*, 1996; Yamasaki *et al.*, 1996) pathway have been knocked out. In general, cell proliferation during early embryonic development is normal in the absence of these genes. In addition, mouse embryo fibroblasts derived from homozygous mutants proliferate in cell culture. It is often not clear to what extent functional redundancies explain the absence of severe cell proliferation defects in these mutants, because multiple related pathway components encoded by small gene families are present in mammals. The three mammalian D-type cyclins, for instance, can bind to either cdk4 or cdk6 and the different complexes might have largely overlapping functions (Sherr, 1994). While genetic inactivation of either cyclin D1, D2 or cdk4 does not block cell cycle progression, overexpression of INK inhibitors (Guan *et al.*, 1994; Koh *et al.*, 1995; Lukas *et al.*, 1995b; Medema *et al.*, 1995) and microinjection of antibodies against D-type cyclins (Baldin *et al.*, 1993; Quelle *et al.*, 1993; Lukas *et al.*, 1994, 1995a) have indicated that Rb⁺ cells fail to progress into S-phase when D-type cyclin–cdk complexes are inhibited.

In *Drosophila*, single genes for Cyclin D and its kinase partner Cdk4 (previously designated Cdk4/6) have been described (Finley *et al.*, 1996; Sauer *et al.*, 1996), as well as in the nematode *Caenorhabditis elegans* (Park and Krause, 1999). Extensive screening of genomic libraries at low stringency has not revealed additional cdk4-like genes in *Drosophila* (Sauer *et al.*, 1996). Additional cdk4 homologs also cannot be identified in the large set of *Drosophila* EST sequences and in the published genome sequence (Adams *et al.*, 2000). Therefore, the analysis of the *Cdk4* mutant phenotype, which we describe here, can be expected to provide a definitive answer to the question of whether cdk4 activity represents an obligatory requirement for progression through the G₁ phase. In addition, the cell proliferation program of wild-type *Drosophila* development is very well known and the effects of mutations can be studied at the cellular level within the organism.

Our results demonstrate that *Cdk4* interacts genetically with the *Drosophila* Rb family member RBF, as expected from the analyses in mammals. However, *Cdk4* is not essential for progression through the cell cycle or for

Fig. 1. Cyclin D but not Dacapo is bound to *Drosophila* Cdk4 *in vivo*. Immunoprecipitates (IP) were isolated with anti-myc antibodies from extracts of embryos expressing either myc-tagged Cdk1 (Cdk1-myc), Cdk2 (Cdk2-myc), Cdk4 (Cdk4-myc) or mutant Cdk4 (Cdk4^{D175N}-myc) and analyzed in immunoblot experiments (IB) with antibodies against the myc epitope (myc), Cyclin D (CycD), Cyclin E (CycE), Cyclin A (CycA), Cyclin B (CycB), Cyclin B3 (CycB3) or Dacapo (DAP). Immunoblot signals resulting from the binding of secondary antibodies to mouse immunoglobulin used for immunoprecipitation are indicated by dots, while arrowheads mark signals reflecting the presence of Cyclin D and Cyclin B. Two independent experiments are shown in the panels on the right and left sides.

development to the adult stage. Nevertheless, *Cdk4* is clearly required for normal fertility and growth of cells and organism.

Results

Drosophila Cdk4 binds to Cyclin D *in vivo*

Analysis of full-length cDNAs indicated that the characteristic pRb-binding motif LXCXL, which is not encoded by the sequence described previously (Finley *et al.*, 1996), is also present in *Drosophila* Cyclin D. Co-immunoprecipitation experiments confirmed that *Drosophila* Cyclin D associates with Cdk4 *in vivo* (Figure 1). Immunoblotting with a monoclonal antibody revealed the presence of Cyclin D in anti-myc immunoprecipitates isolated from extracts of Cdk4-myc-expressing embryos. In contrast, Cyclin D was not detected in immunoprecipitates from Cdk1-myc or Cdk2-myc extracts. Moreover, while Cyclins A, B, B3 and E were clearly present in either of these two latter immunoprecipitates, they were not detected in Cdk4-myc immunoprecipitates. Our results indicate therefore that Cyclin D binds specifically to Cdk4.

Mammalian cdk4 and cdk6 bind to INK- and CIP/KIP-type cdk inhibitors. While INK inhibitors have not been identified in *Drosophila*, the *dacapo* gene has been shown

Fig. 2. Molecular characterization of wild-type and mutant *Cdk4* alleles. (A) *Cdk4* exon sequences are indicated by boxes. Filled boxes indicate translated regions. Putative transcriptional start sites are indicated by arrows and the structure of alternative transcripts is illustrated. Triangles indicate insertion sites of P elements. While the line *EP(2)0844* carries a P element in the 5' region, the following lines have insertions clustered around the position indicated by the second triangle: *l(2)05428*, *l(2)s4639*, *l(2)k06503*, *EP(2)2192*, *EP(2)2358*. The size and position of the intragenic deletion in *Cdk4*³ resulting from an imprecise excision of *l(2)s4639* is indicated by the black bar. The positions of the primers 1 and 2 used for the PCR experiment (B) are indicated by the open arrows. The regions used as probes 3 and 4 for the Southern blot (C) are indicated by boxes and relevant *Bam*HI restriction sites are indicated. (B) Genomic DNA from wild-type (+/+), *Cdk4*³/CyO (+/-) and *Cdk4*³ (-/-) flies was analyzed by PCR for the presence of *Cdk4* sequences. The primers 1 and 2 (A) result in the amplification of a 2.1 kb fragment from the wild-type allele and a 0.3 kb fragment from the *Cdk4*³ allele. (C) Genomic DNA from *Cdk4*³/CyO and *Cdk4*³ flies was digested with *Bam*HI and analyzed on Southern blots using *Cdk4* fragments 3 and 4 (A) as probes. (D) 0–2 h embryo extracts from either wild type (+/+) or *Cdk4*³ (-/-) were analyzed for the presence of Cdk4 by immunoblotting with anti-Cdk4 antisera (CDK4). Immunoblotting with anti-tubulin (TUB) was used as a loading control.

to encode a CIP/KIP-type inhibitor, which binds to Cyclin E–Cdk2 complexes (De Nooij *et al.*, 1996; Lane *et al.*, 1996). While Dacapo was readily observed in Cdk2-myc immunoprecipitates as expected, it could not be detected in Cdk4-myc immunoprecipitates (Figure 1).

Cdk4 is required for normal growth and fertility but is not essential for cell cycle progression

By mobilizing a P element [*l(2)s4639*] we isolated an intragenic deletion *Cdk4*³ eliminating essential kinase domains (Figure 2). Surprisingly, homozygous *Cdk4*³ progeny from heterozygous parents developed into adult flies which eclosed without a developmental delay. While the fertility of homozygous females was severely reduced (see Supplementary material available at *The EMBO Journal* Online for a more detailed description of the fertility defects), occasional progeny could be obtained even from homozygous parents demonstrating that Cdk4 is

Table I. The effect of *Cdk4* on the size of cells, organs and organism

Cross ^a	Genotype	Weight ^b (mg)	Wing area ^c (mm ²)	Cell size ^d (μm ²)	Cell number ^e
1	<i>Cdk4^{Δ3}/Cdk4^{Δ3}</i>	0.69	1.19	137.9	8573
2	<i>Cdk4^{Δ3}/+</i>	0.84	1.27	130.3	9687
3	<i>Cdk4^{Δ3}/Cdk4^{Δ3}; da-GAL4/+</i>	0.68	1.14	146.5	7729
	<i>Cdk4^{Δ3}/Cdk4^{Δ3}; da-GAL4/UAS-Cdk4 III.1</i>	0.94*	1.33**	137.9***	9617
4	<i>Cdk4^{Δ3}/Cdk4^{Δ3}; da-GAL4/+</i>	0.73	1.16	147.5	7811
	<i>Cdk4^{Δ3}/Cdk4^{Δ3}; da-GAL4/UAS-Cdk4^{D175N}-mycIII.7</i>	0.77	1.17	143.2	8114
5	<i>Cdk4^{Δ3}/Cdk4^{Δ3}; da-GAL4/+</i>	0.73	1.15	149.5	7641
	<i>Cdk4^{Δ3}/Cdk4^{Δ3}; da-GAL4/UAS-Cdk4^{D175N}-mycIII.4</i>	0.77	1.22	145.6	8328

^aThe crosses that resulted in progeny with the different genotypes analyzed are described in Materials and methods. As the growth of flies is very sensitive to growth conditions, comparisons between sibling progeny of a cross raised in the same bottle are most accurate. The values given in Table I are from experiments that were independent of those used for the calculation of the weight ratios (Figure 3G and H).

^bThe average weight of male flies was determined as described in Materials and methods. Standard deviations (SDs) were smaller than 0.04 mg, except for the *Cdk4^{Δ3}/Cdk4^{Δ3}; da-GAL4/+* flies in cross 3 where SD was 0.06.

^cThe area of 10 wings obtained from 10 different males was measured and averaged, except for cross 5 where only five wings were analyzed. SDs were <0.03 mm² except for cross 2 where the SD was 0.05 mm².

^dAn average cell size was determined as described in Materials and methods by analyzing the same wings that were also used for the wing area measurements. SDs were <5 μm².

^eTotal cell numbers present on one side of the wing blade were estimated by dividing the wing area by the cell size. Note that cell density is not uniform throughout the wing blade and that cell size was determined in a region that has a relatively low cell density.

*Comparison with sibling flies with *t*-test results in *P* <10⁻³.

**Comparison with sibling flies with *t*-test results in *P* <10⁻¹⁰.

***Comparison with sibling flies with *t*-test results in *P* <10⁻².

not absolutely essential for cell proliferation or development to the adult stage.

Interestingly, homozygous *Cdk4^{Δ3}* flies were found to be significantly smaller than wild-type flies (Table I; Figure 3). Size reduction affected all aspects of the flies proportionally. The weight of *Cdk4^{Δ3}* homozygotes was found to be ~20% lower than that of heterozygotes (Table I). The wing area of *Cdk4^{Δ3}* homozygotes was found to be ~10% lower than that of heterozygotes. As each cell is known to secrete one hair during wing development, we determined the hair density in a defined region of the adult wings as a measure of cell size. The results indicated that the cells in wings of *Cdk4^{Δ3}* homozygotes are slightly larger compared with heterozygotes (Table I). The values determined for the total wing size and for the cell size were used to extrapolate the total cell number present in the wing. These estimations indicated that the wings of *Cdk4^{Δ3}* homozygotes contained fewer cells than those of heterozygotes (Table I).

To demonstrate that the decrease in weight, wing size and cell numbers observed in *Cdk4^{Δ3}* homozygotes is caused by loss of Cdk4 function and not by potential second site mutations on the chromosome, we expressed *UAS-Cdk4* ubiquitously in *Cdk4^{Δ3}* homozygotes with the help of *da-GAL4*. This prevented the reduction in weight, wing size and cell numbers (Table I; Figure 3).

Analogous ubiquitous expression of mutant *Cdk4^{D175N}-myc* had essentially no effect in *Cdk4^{Δ3}* homozygotes (Table I). The D175N mutation affects an aspartate residue which is conserved in all protein kinases and known to be required for the phosphotransfer reaction (van den Heuvel and Harlow, 1993). Overexpression of cdk1 and cdk2 with analogous mutations results in dominant-negative inhibition of the corresponding endogenous cdks in mammalian cells. Mutant *Cdk4^{D175N}-myc* protein binds to Cyclin D with the same efficiency as *Cdk4-myc* (Figure 1) and thus is expected to act in a dominant-negative manner. While *Cdk4^{D175N}-myc* expression did not

reduce the weight of *Cdk4^{Δ3}* mutants, it resulted in a slight but significant weight decrease in *Cdk4⁺* flies (Figure 3G). In additional experiments, we compared directly the effects of *Cdk4^{D175N}-myc* expression on the weight of *Cdk4^{Δ3}* homo- and heterozygotes that had developed in the same bottle (Figure 3H and I). These experiments clearly confirmed that *UAS-Cdk4^{D175N}-myc* reduces fly weight when expressed in *Cdk4^{Δ3}* heterozygotes, while it had at most the opposite effect in *Cdk4^{Δ3}* homozygotes (Figure 3H and I). Moreover, this same differential effect on *Cdk4^{Δ3}* homo- and heterozygotes was also observed with a *UAS-Cdk4^{K55M}-myc* transgene (Figure 3H and I), which carried another mutation known to abolish kinase activity but not D-type cyclin binding, when introduced into mammalian cdk4 (Kato and Sherr, 1993). The finding that both *Cdk4^{D175N}-myc* and *Cdk4^{K55M}-myc* expression mimicked the *Cdk4^{Δ3}* phenotype when expressed in *Cdk4⁺* flies suggests that they act in a dominant-negative manner. The fact that they have no effect in *Cdk4^{Δ3}* flies argues strongly that Cdk4 is the only Cyclin D-dependent cdk in *Drosophila*. We emphasize that this conclusion is complicated but not invalidated by the possibility that Cdk4 might not only act as a protein kinase but also by titrating putative INK inhibitors as described for mammalian cdk4/6. We point out that the *Drosophila* genome sequence (Adams *et al.*, 2000) has not revealed INK orthologs.

To analyze the effects of loss of Cdk4 function on cell growth and cell cycle progression, we compared the behavior of homozygous *Cdk4^{Δ3}* and their wild-type sister clones induced by mitotic recombination (Figure 4). Sixty-seven hours after clone induction, the area covered by *Cdk4^{Δ3}* clones in imaginal wing discs was found to be only about two-thirds of the area covered by sister clones (Figure 4A). Analysis by flow cytometry revealed a significant difference neither in cell size nor in the cell cycle profile (Figure 4B). In addition, we did not observe pyknotic nuclei in *Cdk4^{Δ3}* mutant clones, suggesting that

Fig. 3. *Cdk4* is required for normal growth of organism and organs. (A–F) Adult male flies (A–C) and wings (D–F) are shown. The region boxed in (D) was used to determine cell densities (see Table I). Genotypes were *+Cdk4*³ (*Cdk4*⁺), *Cdk4*³/*Cdk4*³ (*Cdk4*³) and *Cdk4*³/*Cdk4*³; *da-GAL4*, *UAS-Cdk4 III.1* (*Cdk4*³; *UAS-Cdk4*). (G) Males heterozygous for a *UAS* transgene (*UAS-Cdk4 III.1*, *UAS-Cdk4*^{D175N}-*myc III.1*, *UAS-Cdk4*^{D175N}-*myc III.7* or *UAS-Cdk1 III.1*) were crossed with females homozygous for *da-GAL4* (black bars) or with control females (white bars). The *UAS* transgene therefore was only present in 50% of the progeny from these crosses. The weight of progeny with or without *UAS* transgene was determined and the weight ratio (+*UAS* transgene/–*UAS* transgene) was calculated. Black bars represent the weight ratio in flies carrying *da-GAL4* (+); white bars indicate the weight ratio in flies lacking *da-GAL4* (–). (H and I) *Cdk4*³/*CyO*; *UAS* transgene/+ males were crossed with *Cdk4*³/*CyO*; *da-GAL4/da-GAL4* females. Thus, only 50% of the progeny expressed the *UAS* transgene (*UAS-Cdk4 III.1*, *UAS-Cdk4*-*myc III.4*, *UAS-Cdk4*^{D175N}-*myc III.7*, *UAS-Cdk4*^{K55M}-*myc III.2*, *UAS-Cdk1*-*myc III.1* or *UAS-Cdk1 III.1*). The weight of progeny that either expressed the *UAS* transgene or not was determined and the weight ratio (+*UAS* transgene/–*UAS* transgene) was calculated. Weight ratios were determined for male (H) and female (I) progeny. White bars indicate the weight ratio in homozygous *Cdk4*³/*Cdk4*³ progeny (–); black bars in heterozygous *Cdk4*³/*CyO* siblings (+). The significant *P* values as determined by a *t*-test are indicated by asterisks (**P* < 0.05; ***P* < 0.01).

cell death did not play a significant role in the reduced growth of *Cdk4*³ mutant clones. These data indicate that *Cdk4*³ cells grow slowly and that their cell cycle is lengthened by a proportionate increase in the G₁, S and G₂ phases.

***Cdk4* interacts with RBF and Cyclin E–Cdk2**

In addition to D-type cyclin–cdk complexes, cyclin E–cdk2 has also been implicated in the regulation of cell cycle progression through the G₁ phase. The presence of

Cyclin E–Cdk2 might explain the relatively mild phenotype observed in flies lacking *Cdk4* function. To evaluate this notion, we studied the effects of heterozygosity for *Cyclin E* and *Cdk2* mutations in *Cdk4* mutants. While heterozygosity for mutations in *Cyclin E* is readily tolerated in *Cdk4*³ heterozygotes, it resulted in complete lethality in *Cdk4*³ homozygotes. Similarly, heterozygosity for mutations in *Cdk2* resulted in an almost complete lethality in *Cdk4*³ homozygotes, while it has no effect in *Cdk4*³ heterozygotes. In contrast, mutations in *Cyclin A*,

Fig. 4. *Cdk4* is required for normal growth of imaginal disc cells. (A) Twin spots of homozygous *Cdk4*³ clones and homozygous *Cdk4*⁺ clones were induced in a *Cdk4*³/*Cdk4*⁺ background by mitotic recombination at 48 h AED. Wing imaginal discs were fixed at 115 h AED and the size of *Cdk4*³ (black) and *Cdk4*⁺ clones (gray) was determined. The sizes measured for clone pairs are indicated by bars ordered according to the size of the *Cdk4*³/*Cdk4*⁺ clones. Values for median and average clone area are indicated. (B) *Cdk4*³ clones lacking *UAS-GFP* were induced by mitotic recombination at 48 h AED. Wing imaginal discs were dissected and dissociated for FACS analysis at 96 h AED. Green fluorescent protein (GFP) fluorescence intensity and DNA content were analyzed as well as forward scatter (FCS) as a measure of cell size. Cells were gated as indicated on the left panel and gray traces reflect *Cdk4*³ mutant cells (GFP-negative) and black traces cells with either one or two *Cdk4*⁺ copies (GFP-positive) in the panels illustrating essentially indistinguishable cell cycle profiles and cell size distribution.

Cyclin B, *Cyclin B3* and *Cdk1* had no effect on the survival of *Cdk4*³ homozygotes. These observations demonstrate that *Cdk4* mutants are particularly sensitive to reduction in Cyclin E–Cdk2 levels.

Vertebrate D-type cyclin–cdk complexes inhibit Rb function. The defects observed in *Cdk4* mutants, therefore, might reflect increased Rb function. To evaluate this notion, we studied the effects of heterozygosity for mutations in the gene encoding the *Drosophila* Rb family member (RBF) on the fertility and size of *Cdk4* mutant females. For these experiments we used two putative null alleles, *RBF*¹¹ and *RBF*¹⁴. We developed a PCR assay to monitor the presence of the *RBF*¹¹ allele (Figure 5A). *Cdk4* mutant females heterozygous for *RBF*¹¹ (*RBF*¹¹/*Cdk4*³/*Cdk4*³) were found to be significantly more fertile than sibling females without *RBF*¹¹ (*+/+*; *Cdk4*³/*Cdk4*³) (Figure 5, compare B with C). In the experiment with *RBF*¹⁴, we were unable to monitor the presence of this mutation. Based on our crossing scheme, however, one half of the *Cdk4* mutant females was expected to be heterozygous for *RBF*¹⁴ (*RBF*¹⁴/*+/+*; *Cdk4*³/*Cdk4*³), while the other half was expected to have two functional *RBF* gene copies (*+/+*; *Cdk4*³/*Cdk4*³). As in the *RBF*¹¹ experi-

Fig. 5. Reduction of *RBF* gene dose suppresses fertility defects of mutant *Cdk4* females. (A) The presence of the *RBF*¹¹ allele was monitored by a PCR assay. A 650 bp fragment is amplified exclusively when *RBF*¹¹ is present. (B and C) Sibling *Cdk4*³ mutant females, which were either *+/+* (B) or *RBF*¹¹/*+/+* (C), were crossed individually to wild-type males and the number of progeny developing to the adult stage was determined. (D) Sibling *Cdk4*³ mutant females, which were either *+/+* or *RBF*¹⁴/*+/+*, were crossed individually to wild-type males and the number of progeny developing to the adult stage was determined. The presence of *RBF*¹⁴ could not be monitored. However, ~50% of the females were found to have significantly higher numbers of adult progeny than *Cdk4*³ mutant females and are therefore presumably *RBF*¹⁴/*+/+*.

ment, we also observed an increased fertility in ~50% of the *Cdk4* mutant females in the *RBF*¹⁴ experiment (Figure 5D). These 50% females with increased fertility, therefore, presumably correspond to the *RBF*¹⁴ heterozygotes. We conclude that the fertility of *Cdk4* mutant females is increased by a reduction in the copy number of functional *RBF* genes from two to one. Similarly, heterozygosity for mutations in *RBF* was found to increase the weight of *Cdk4* mutant females (data not shown). The antagonistic activities of Cdk4 and RBF could also be demonstrated in experiments involving overexpression using the UAS-GAL4 system (Datar *et al.*, 2000).

D-type cyclin complexes are thought to be the major Rb kinase in mammalian cells (Geng *et al.*, 1999). Therefore it was of interest to compare Rb kinase activity in wild-type and *Cdk4* mutant embryos. Unfortunately, our antibodies failed to precipitate Rb kinase activity even from wild-type embryo extracts. Moreover, in contrast to vertebrate Rb, *Drosophila* RBF does not change its apparent

Fig. 6. Rb kinase in *Cdk4* mutants. Extracts from embryos with *UAS-mRb* (lane 1), *da-GAL4* and *UAS-mRb* (lane 2), *da-GAL4*, *UAS-mRb*, *UAS-Cyclin D* and *UAS-Cdk4* (lane 3), *da-GAL4*, *UAS-mRb*, *UAS-dacapo* (lane 4) and *da-GAL4*, *UAS-mRb* and *UAS-Cyclin E* (lane 5), were analyzed by immunoblotting with anti-Rb. Alternatively, mRb was immunoprecipitated from *da-GAL4*, *UAS-mRb* embryo extracts and incubated without phosphatase (lane 6) or with phosphatase either in the absence (lane 7) or presence (lane 8) of phosphatase inhibitors before immunoblotting with anti-Rb. To analyze the effect of *Cdk4* on mRb phosphorylation, we analyzed extracts from embryos collected from a cross of *Cdk4*^{Δ3}; *UAS-mRb* females with either *Cdk4*^{Δ3}; *da-GAL4* males (lane 9) or *da-GAL4* males (lane 10) by immunoblotting with anti-Rb.

electrophoretic mobility upon phosphorylation (Du et al., 1996). As also observed with mammalian Rb (Khandjian and Tremblay, 1992), we were unable to focus *Drosophila* RBF on two-dimensional gels. Thus, using *da-GAL4* we expressed mouse Rb in *Drosophila* embryos from a *UAS* transgene (*UAS-mRb*). Immunoblotting with an antibody against Rb clearly revealed two forms with different electrophoretic mobility (Figure 6A, lane 2). Phosphatase treatment converted the lower into the higher mobility form (Figure 6, lane 7). Conversely, co-expression of *UAS-Cdk4* and *UAS-Cyclin D* resulted in a relative increase in the lower mobility form (Figure 6, lane 3). These observations suggested that Cyclin D–Cdk4 might in principle function as Rb kinase. However, we failed to detect a decrease in the abundance of the low mobility form in extracts from embryos lacking both maternal and zygotic *Cdk4* function (Figure 6, lane 9). It appears therefore that kinases other than Cyclin D–Cdk4 can phosphorylate mRb. In fact, experiments involving expression of either *UAS-Cyclin E* or *UAS-dacapo* suggested that Cyclin E–Cdk2 is a major Rb kinase in *Drosophila* embryos. *UAS-Cyclin E* resulted in a strong enrichment of the lower mobility form (Figure 6, lane 5). In contrast, *UAS-dacapo* resulted in a severe reduction of the lower mobility form (Figure 6, lane 4).

Discussion

D-type cyclin–cdk complexes are thought to control specifically progression through G₁ in response to extracellular growth factors. Surprisingly, we find that

Drosophila Cdk4, which encodes the kinase partner of *Drosophila* Cyclin D, is not an absolutely essential gene. *Cdk4* mutant flies are smaller than normal and almost infertile. However, some progeny can develop into adults in the complete absence of both maternal and zygotic *Cdk4* function. Moreover, we demonstrate that imaginal disc cells lacking *Cdk4* function are characterized by a reduced cellular growth rate, an increased cell cycle duration with a proportional extension during G₁, S and G₂ and a normal cell size. Rather than promoting the progression specifically through G₁ in response to cellular growth, therefore, D-type cyclin–cdk complexes appear primarily to stimulate cellular growth in *Drosophila*. This notion is further supported by our data from overexpression analyses (Datar et al., 2000). The discussion here is focused on *Cdk4* loss-of-function mutations and their genetic interactions, while the growth-regulatory role of Cyclin D–Cdk4 is discussed in detail in Datar et al. (2000).

Progression through the cell division cycle in the absence of *Cdk4*

It is not yet clear to what extent the presence of the highly related *cdk6* gene explains the fact that *cdk4* is not required for development to the adult stage in mice (Rane et al., 1999; Tsutsui et al., 1999). In contrast, *Drosophila* does not appear to have a *Cdk6* gene (Sauer et al., 1996; Adams et al., 2000). Moreover, our experiments involving overexpression of dominant-negative *Cdk4* proteins (*Cdk4*^{D175N}, *Cdk4*^{K55M}) argue strongly against the idea that *Cdk4* null mutants are rescued by functionally redundant D-type cyclin–cdk complexes. Overexpression of these mutant kinases in *Cdk4*⁺ flies mimics a *Cdk4* loss-of-function phenotype, while it does not at all enhance the mutant phenotype in *Cdk4* null mutants.

Just like *Cdk4*, *Drosophila Cyclin D* should not be an essential gene, if Cyclin D functions exclusively in a complex with *Cdk4*. Indeed, near-saturation mutagenesis for recessive lethal mutations uncovered by a deficiency removing *Cyclin D* has not led to the identification of *Cyclin D* alleles (B.A. Edgar and A. Katzen, personal communication), supporting the idea that *cyclin D* might not be essential. In contrast to complexes of *Cdk1* and *Cdk2*, which are clearly encoded by essential genes and provide functions indispensable for progression through the cell cycle, Cyclin D–Cdk4 complexes, therefore, do not appear to be basic components of the cell cycle machinery.

Like *Drosophila*, *C.elegans* appears to have single genes for cyclin D and *cdk4* (Park and Krause, 1999). *C.elegans cdk4* has been demonstrated to be an essential gene. Mutant analysis and RNA interference experiments have indicated that cyclin D and *cdk4* are required for cell cycle progression during the larval stages. Cell proliferation during these larval stages is known to be dependent on nutrition, and thus the cyclin D–cdk4 might be required to regulate the resulting growth. However, consistent with our findings in *Drosophila*, cyclin D and *cdk4* do not appear to be necessary during embryonic proliferation in *C.elegans*. Thus these results also suggest that cell cycle progression is not necessarily dependent on the presence of D-type cyclin-dependent kinase activity.

Antagonistic activities of Cdk4 and RBF

Experiments in vertebrate cells have suggested that D-type cyclin-cdk activity is required for progression through G₁ in Rb⁺ cells and is dispensable in Rb⁻ cells (Lukas *et al.*, 1994, 1995a,b). RBF are also expressed in *Drosophila*. In addition to the previously characterized RBF gene (Du *et al.*, 1996; Du and Dyson, 1999), the genome sequence has uncovered a second family member. The genetic interactions described here indicate that Cyclin D-Cdk4 and RBF act antagonistically. We find that the fertility defect of *Cdk4* mutant females is suppressed by a reduction in the dose of *RBF*⁺ copies. Additional evidence demonstrating this antagonistic relationship is described by Datar *et al.* (2000).

Hierarchy and functional specificity of Cdk4 and Cdk2

Drosophila Cdk4 is not essential even though RBF is known to be expressed and to inhibit cell proliferation (Du *et al.*, 1996; Neufeld *et al.*, 1998; Du and Dyson, 1999; Datar *et al.*, 2000). RBF therefore might either not be sufficient for a complete inhibition of cell proliferation in *Drosophila* or it might be inactivated by a redundant pathway. In vertebrate cells, Rb is thought to be phosphorylated by both D-type-cdk complexes and cyclin E-cdk2. Recent evidence emphasized that these kinases act sequentially to inactivate separate functions of Rb. Initial phosphorylation by cyclin D-dependent kinase activity blocks the binding of histone deacetylase to Rb and disrupts Rb structure so that cyclin E-dependent kinase activity gains access to additional phosphorylation sites. The subsequent phosphorylation of these sites dissociates Rb from E2F (Harbour *et al.*, 1999). Similarly, a 'knock-in' of the *cyclin E* gene into the *cyclin D1* locus in mice results in a strong reduction of Rb hyperphosphorylation. Interestingly, this expression of cyclin E instead of cyclin D1 does not result in the characteristic cell proliferation defects associated with a cyclin D1 'knock-out', even though Rb hyperphosphorylation is not restored (Geng *et al.*, 1999). These findings suggest, therefore, that cyclin D1 complexes represent the major Rb kinase in the tissues analyzed and that they are primarily important for the induction of cyclin E expression.

Our results suggest that Cyclin D-Cdk4 and Cyclin E-Cdk2 might act as independent RBF kinases in *Drosophila*. Overexpression of either cyclin D-Cdk4 or Cyclin E resulted in an increase of hyperphosphorylated Rb. The specific Cyclin E-Cdk2 inhibitor Dacapo severely inhibited Rb hyperphosphorylation. Thus, *Drosophila* Cyclin E-Cdk2, which is known to phosphorylate RBF efficiently *in vitro*, might also phosphorylate Rb in *Cdk4* mutants, thereby explaining why a decrease in Rb phosphorylation cannot be observed in *Cdk4* mutants. For technical reasons, however, we have analyzed the phosphorylation of mouse Rb expressed in *Drosophila* embryos instead of investigating RBF phosphorylation directly. Our suggestion that Cyclin D-Cdk4 and Cyclin E-Cdk2 might act as functionally overlapping RBF kinases must therefore remain tentative, since mouse Rb might not faithfully report RBF kinase activity. Our finding that *Cdk4* mutants are extremely sensitive to a reduction of the *Cyclin E*⁺ and *Cdk2*⁺ gene dose further

stresses the intimate functional relationship between Cyclin D-Cdk4 and Cyclin E-Cdk2 but does not address their interaction hierarchy. Even though we favor the idea that Cyclin D-Cdk4 and Cyclin E-Cdk2 might act as overlapping RBF kinases in *Drosophila*, we emphasize that a number of phenotypic differences resulting from both loss- and gain-of-function mutations in the genes encoding the subunits of these different kinase complexes clearly demonstrate that they must also have distinct functions.

Cdk4 stimulates growth

Drosophila Cdk4 is clearly required for normal growth of cells and the organism. Size regulation at the level of cells, as well as at the level of organs and organism is an important but poorly understood process. A number of observations in yeast and mammalian cells have indicated that cell cycle progression is dependent on a critical cell size (for a recent review see Polymenis and Schmidt, 1999). Many cell types delay the G₁-S transition (at a point called START in yeast and restriction point in mammalian cells) until a critical size has been reached. Slow growth due to nutrient or growth factor limitations therefore results primarily in an extension of the G₁ phase. In budding yeast, the Cln cyclins, and in particular Cln3p, have been implicated in this coupling of cell growth and cell cycle progression. Cln3p is a very unstable protein which might only accumulate to an effective concentration that triggers progression through START when ribosome numbers have increased sufficiently (Polymenis and Schmidt, 1997). Experimental uncoupling of growth rates and the rates of Cln3p accumulation affects primarily cell division size without a comparable change of cell cycle duration. D-type cyclin-cdk complexes have been proposed to regulate progression through the restriction point in response to growth factors and mitogens in a comparable manner. However, proliferating *Cdk4* mutant cells in wing imaginal discs are of wild-type size and progress more slowly through the cell cycle with a proportionate extension of G₁, S and G₂ phases. This phenotype is incompatible with an exclusive role of Cdk4 in the coupling of cell size and progression through the restriction point. Cdk4 appears to regulate, rather than simply sense growth. This notion is further supported by Datar *et al.* (2000).

The observation that cells are slightly larger in adult wings of *Cdk4* mutants does not fit readily with the suggestion that Cdk4 promotes growth. We emphasize, however, that the effect on cell size was small and obvious only in differentiated adult cells and not in proliferating imaginal wing cells. Adult *Cdk4* mutant wings contain fewer cells. The slight increase in adult wing size might reflect a partial compensation for decreased cell numbers during wing differentiation late in development.

More prominent than the slight cell size increase observed in wing cells is, however, the reduced size of *Cdk4* mutant flies. A number of mutations have been shown to result in size reduction. The *Drosophila myc* homolog (Gallant *et al.*, 1996; Johnston *et al.*, 1999) and several genes of the insulin signaling pathway have been shown to be specifically required for growth to normal size (Leevers *et al.*, 1996; Böhni *et al.*, 1999; Huang *et al.*, 1999; Montagne *et al.*, 1999; Weinkove *et al.*, 1999).

Interestingly, while all these mutations result in developmental delays, we find that *Cdk4* mutants eclose on time. The size reduction observed in *Cdk4* mutants, however, is less pronounced than in most of these other mutants.

Reduced growth has also been reported to result from inactivation of mouse *cdk4* function (Rane *et al.*, 1999; Tsutsui *et al.*, 1999). Intriguingly, the phenotypic similarities observed in *cdk4* mutant mice and flies extend further. Mutant mice are also infertile particularly when female. In these females, infertility was shown to be caused by deficits in the hypothalamic pituitary axis rather than by developmental abnormalities in reproductive organs (Rane *et al.*, 1999). In addition, *cdk4* mutant mice developed diabetes within 2 months after birth due to a loss of the insulin-expressing β -islet cells (Rane *et al.*, 1999). In *Drosophila*, we note a striking similarity of the phenotypic consequences of mutations in *Cdk4* and in genes of the insulin signaling pathway. A future analysis of the relationship of *Cdk4* and insulin signaling in *Drosophila*, therefore, might reveal a striking evolutionary conservation.

Apart from emphasizing functional homology, our analysis also reveals an apparent clear difference between the regulatory role of cyclin D–Cdk4 in *Drosophila* and in mammalian cells. In mammalian cells, D-type cyclin complexes do not only function catalytically as protein kinases. They also act stoichiometrically by titrating the cdk inhibitors p21^{CIP1} and p27^{KIP1}. Thereby, they contribute to the activation of cyclin E–Cdk2 complexes. In contrast, we have been unable to detect the *Drosophila* cdk inhibitor Dacapo (the only detectable CIP/KIP family member in the known genome sequence) in Cyclin D–Cdk4 complexes. This apparent inability of *Drosophila* Cyclin D–Cdk4 to titrate Dacapo, might explain why, to some extent, mammalian D-type cyclin complexes behave in a functionally distinct manner.

In summary, our observations clearly implicate Cdk4 in the control of growth rates at the cellular and organismal level. Moreover, while our results confirm an involvement of Cdk4 in the regulation of cell proliferation, they also emphasize the importance of alternative pathways. In *Drosophila*, the developmental program of cell proliferation is surprisingly normal in the absence of Cdk4. The fact that the cyclin D–Cdk4 pathway is of paramount importance for tumorigenesis, therefore, should probably not lead to the view that it is the only pathway controlling entry into and exit from the cell cycle.

Materials and methods

Fly stocks

The P element lines *l(2)s4639* and *l(2)05428* (Spradling *et al.*, 1995) with insertions in the chromosomal region 53C containing *Cdk4* (Sauer *et al.*, 1996) were characterized by PCR using a P element primer (5'-GCA-GGTAGCACCTTATGTTATTCATCATG-3') in combination with *Cdk4*-specific primers (primer 1: 5'-GAGAACGGTGTGCCAATG-3', primer 2: 5'-GCAAGATCTAGGGGTTCTGCTGAAAG-3'). Imprecise excisions were generated from *l(2)s4639* and intragenic deletions were identified by PCR. A PCR fragment spanning the intragenic deletion in *Cdk4*³ was cloned and sequenced. Breakpoints were found at positions corresponding to base pairs 325 and 855 of the *Cdk4* cDNA (Sauer *et al.*, 1996). This intragenic deletion also results in a frame shift. PCR with primers 1 and 2 were used to detect the *Cdk4*³ allele.

To monitor the presence of *RBF*¹¹ in single *Cdk4*³ mutant females, we devised a PCR assay. *RBF*¹¹ was isolated as an imprecise excision of a

P element insertion within the 5' untranslated region of *RBF* (Du and Dyson, 1999). A molecular analysis of *RBF*¹¹ revealed the presence of residual P element sequences allowing an enzymatic amplification of a 650 bp fragment specifically from this allele with the help of the P element primer and a *RBF* primer (5'-CACATGCTATTCAC-CTCCTG-3').

Lines with *UAS* transgenes (*UAS-Cdk1-myc*, *UAS-Cdk2-myc*, *UAS-Cdk4-myc*) allowing the expression of *Cdk1*, *Cdk2* or *Cdk4* as fusion proteins with an extension of six myc epitope copies at the C-terminus were produced with the help of pUAST constructs (Brand and Perrimon, 1993). In addition, we generated lines (*UAS-Cdk4*^{D175N-myc}, *UAS-Cdk4*^{K55M-myc}) allowing the expression of mutant Cdk4 proteins. A Sculptor *in vitro* mutagenesis kit (Amersham) was used to introduce the corresponding changes. We also generated *UAS-Cdk4* lines allowing the expression of Cdk4 without a C-terminal extension.

For the production of lines with *UAS-Cyclin D* transgenes, we used a pUAST construct containing a fragment of the *Drosophila Cyclin D* cDNA clone DNB15. This and seven additional cDNA clones were isolated from an embryo cDNA library (Brown and Kafatos, 1988) using a probe generated from a partial cDNA clone (Sauer *et al.*, 1996). The cDNA DNB15 was sequenced (DDBJ/EMBL/GenBank accession No. AF260583) and found to have stop codons upstream of the open reading frame, suggesting that it contained the complete coding sequence.

For expression of mouse Rb in *Drosophila* embryos, a fragment from pECE- Δ B/X-HA (Hamel *et al.*, 1992) was inserted into a modified pUAST vector. Molecular details of the resulting *UAS-mRb* and the other transgenes described here are provided upon request. In comparison with *da-GAL4*-driven expression of *UAS-RBF*, which results in small flies with defects such as rough eyes, wing and tergite defects, analogous expression of *UAS-mRb* appeared to have little effect in *Drosophila*.

For a detailed description of the additional *Drosophila* strains used in this study see the Supplementary material available at *The EMBO Journal* Online.

Weight and size measurements

For the measurements presented in Table I, we analyzed progeny from the following crosses: *w*⁺; *Cdk4*³/*CyO* males with *w*⁺; *Cdk4*³/*CyO* females (cross 1); *w* males with *w*⁺; *Cdk4*³/*CyO* females (cross 2), *w*⁺; *Cdk4*³/*CyO*; *UAS-Cdk4* III.1/+ males with *w*⁺; *Cdk4*³/*CyO*; *da-GAL4* females (cross 3), *w*⁺; *Cdk4*³/*CyO*; *UAS-Cdk4*^{D175N-myc} III.7/+ males with *w*⁺; *Cdk4*³/*CyO*; *da-GAL4* females (cross 4), *w*⁺; *Cdk4*³/*CyO*; *UAS-Cdk4*^{D175N-myc} III.4/+ males with *w*⁺; *Cdk4*³/*CyO*; *da-GAL4* females (cross 5). Progeny were collected for 24 h, aged for 3 days after eclosion, sorted according to gender and genotype as indicated by visible markers (*Cy*, mini *w*⁺ dose), counted and stored at –70°C. Collections were repeated on four consecutive days. Subsequently, pools of known numbers of flies (in general ~30) were weighed on a Mettler AE50 balance and the average fly weight was calculated. The weight of male flies averaged from the four consecutive collections is given in Table I. The weight of female flies is strongly influenced by the weight of the ovaries. To minimize fertility effects on fly weight measurements, we analyzed male flies.

For the data shown in Figure 3G, we weighed pools of 2- to 3-day-old males. Three different measurements were averaged. For the data shown in Figure 3H and I, we weighed at least 20 flies of each genotype individually and calculated the average.

For the analysis of the size of adult wings and of cells in these wings, dissected wings were deposited on a glass slide and covered with a coverslip. To flatten the preparations, a weight was placed on top of the coverslip and nail polish was used to fix the coverslip to the slide. Microscopic images of wings were captured using a CCD camera. IPLab software was used to determine total wing area and to count wing hairs in a defined wing area of constant size.

Immunoprecipitation, immunoblotting and immunolabeling

For Cdk co-immunoprecipitation experiments, virgin females homozygous for *arm-GAL4* were crossed to males homozygous for either *UAS-Cdk1-myc* II.1, *UAS-Cdk2-myc* II.2, *UAS-Cdk4-myc* II.1 or *UAS-Cdk4*^{D175N-myc} II.1. Eggs were collected from these crosses for a 3 h period and aged for 4 h at 25°C.

To monitor Rb phosphorylation, we expressed *UAS-mRb* using *da-GAL4*. The mouse monoclonal antibody G3-245 (PharMingen) was used for immunoblot analysis. The rabbit polyclonal antibodies Rb(M153) (Santa Cruz Biotechnology Inc.) were used for immunoprecipitation from extracts prepared from 3–6 h embryos.

To monitor mRB phosphorylation in *Cdk4* mutants, we crossed *w*⁺; *Cdk4*³; *UAS-mRb* virgin females with *w*⁺; *Cdk4*³; *da-GAL4* males and

analyzed progeny lacking both maternal and zygotic *Cdk4* function 3–6 h after egg deposition (AED) by immunoblotting with anti-Rb. For control, progeny from *w**; *Cdk4*³; *UAS-mRb* females, which had been crossed to *w**; *da-GAL4* males, were analyzed in parallel. While these control embryos expressed zygotic *Cdk4* function from the paternally contributed endogenous *Cdk4* gene, they were also lacking maternal *Cdk4* function. All the different embryo collections analyzed in these experiments, therefore, were affected to the same extent by maternal effect abnormalities. Moreover, *UAS-mRb* expression occurred only in those embryos that developed beyond the stage where zygotic expression starts.

Embryos were fixed and immunolabeled as described previously (Knoblich *et al.*, 1994). Pulse labeling with BrdU was performed for 20 min, also as described previously (Knoblich *et al.*, 1994).

Information on additional antibodies and methodological details is provided in the Supplementary material available at *The EMBO Journal* Online.

Clonal analysis and flow cytometry

Mitotic clones were induced using the FLP/FRT technique (Xu and Rubin, 1993). Larvae of the genotype *y, w, Hs-FLP; FRT(42D)/FRT(42D) πMyc, Cdk4*³ were given a heat shock for 30 min at 37°C at 48 h AED. *πMyc* expression was induced by a heat shock for 1.5 h at 37°C at 112 h AED, and discs were allowed to recover for 1.5 h at 23°C before fixation and anti-myc immunolabeling.

Fluorescence activated cell sorting (FACS) was performed as described by Neufeld *et al.* (1998). For analysis of *Cdk4*³ cells, *Ub-GFPS65T#73* (generously provided by W.Bender) was recombined onto *FRT(42D)*. Mitotic clones were induced in larvae of the genotype *y, w, Hs-FLP; FRT(42D) Ub-GFPS65T#73/FRT(42D) πMyc, Cdk4*³ by administering a heat shock for 1.5 h at 37°C at 48 h AED. Discs were dissociated for >2 h at 96 h AED and analyzed in a Becton Dickinson FACS Vantage or LSR. Cell quest (Becton Dickinson) software was used for data analysis.

Supplementary material

Supplementary material to this paper is available at *The EMBO Journal* Online.

Acknowledgements

We thank J.Campos-Ortega for providing fly stocks, J.Lukas for the mouse Rb plasmid, D.Heidmann, A.Herr and E.Keidel for help during the early stages of the work. This work was supported by the Deutsche Forschungsgemeinschaft (DFG Le 987/1-2 and 1-3, DFG Le 987/2-1).

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Received March 29, 2000; revised and accepted July 19, 2000