Drosophila Minus is required for cell proliferation and influences Cyclin E turnover

Sébastien Szuplewski,[1,2] Thomas Sandmann,[2,4] Ville Hietakangas,[2,5] and Stephen M. Cohen[1,2,3,6]

[1]European Molecular Biology Laboratory, 69117 Heidelberg, Germany; [2]Temasek Life Sciences Laboratory, Singapore 117604; [3]Department of Biological Sciences, National University of Singapore, Singapore 117604

Turnover of cyclins plays a major role in oscillatory cyclin-dependent kinase (Cdk) activity and control of cell cycle progression. Here we present a novel cell cycle regulator, called *minus*, which influences Cyclin E turnover in *Drosophila*. *minus* mutants produce defects in cell proliferation, some of which are attributable to persistence of Cyclin E. Minus protein can interact physically with Cyclin E and the SCF Archipelago/Fbw7/Cdc4 ubiquitin–ligase complex. Minus does not affect dMyc, another known SCF^{Ago} substrate in *Drosophila*. We propose that Minus contributes to cell cycle regulation in part by selectively controlling turnover of Cyclin E.

Supplemental material is available at http://www.genesdev.org.

Received May 27, 2009; revised version accepted July 24, 2009.

Progression through the cell cycle requires periodic activation of cyclin-dependent kinases (Cdks) (for review, see Murray 2004). Oscillation in Cdk activity is achieved in part through cyclical synthesis and controlled degradation of cyclins, the regulatory subunits of the Cdks. Cyclin E is an evolutionarily conserved nuclear cyclin that controls G1/S transition and S-phase progression in animal cells, predominantly by activating Cdk2 (Hwang and Clurman 2005). *CycE* and *cdk2* are essential genes in *Drosophila.* Cyclin E acts as the limiting factor for G1–S-phase transition (Knoblich et al. 1994; Neufeld et al. 1998; Lane et al. 2000). Cyclin E turnover is important for cell cycle progression and is regulated by a conserved ubiquitin–ligase complex, called SCF (Welcker and Clurman 2008). The SCF complex is built on an elongated scaffold protein, Cullin-1, which recruits the substrate recognition module consisting of Skp1 and an F-box protein, as well as a ring domain-containing ubiquitin–ligase module. Substrate selectivity is mediated by the F-box subunit, in part through recognition of phosphorylated motifs on substrate proteins (Welcker and Clurman 2008). The *Drosophila* F-box protein encoded by *archipelago* (*ago*) is the ortholog of Fbw7/Cdc4. Scf–Ago promotes degradation of CycE, dMyc, and Trachealess (Moberg et al. 2001, 2004; Mortimer and Moberg 2007).

Here we report the characterization of the classical *Drosophila* mutant *minus*. Minus protein can interact physically with Cyclin E and the SCF–Ago complex. Cells lacking Minus fail to degrade CycE, resulting in persistence of CycE. *minus* mutants show defects in cell proliferation, attributable in part to excess CycE activity, reflecting that normal regulation of CycE turnover is essential for normal cell proliferation during *Drosophila* development. We propose that Minus acts as a cell cycle regulator by selectively controlling CycE turnover.

[*Keywords*: *Drosophila*; Cyclin E; SCF; cell proliferation; cell cycle]
Present addresses: [4]German Cancer Research Center, Im Neuenheimer Feld, 69120 Heidelberg, Germany; [5]Institute of Biotechnology, Viikinkaari 9, 00014 Helsinki, Finland
[6]Corresponding author.
E-MAIL cohen@tll.org.sg; FAX 65-6872-7089.
Article is online at http://www.genesdev.org/cgi/doi/10.1101/gad.1822409. Freely available online through the *Genes & Development* Open Access option.

Results and Discussion

Flies homozygous mutant for a spontaneous mutation in the *minus* gene *mi[1]* showed small body size, small bristles, and delayed completion of pupal development (Lindsley and Zimm 1992). *minus* alleles were also isolated in a screen for female sterility (Schüpbach and Wieschaus 1991), and the *minus* gene has been mapped to the cytogenetic interval 59E on the right arm of the second chromosome (FlyBase Consortium 2003). To isolate new *minus* alleles, we screened *P*-element insertions in 59E for failure to complement the *mi[1]* bristle phenotype. Flies carrying the *l(2)SH0818 P*-element insertion in *trans* to *mi[1]* showed a small bristle phenotype, milder than that produced by the combination of *mi[1]* in *trans* to a deletion (Fig. 1A–C). The stronger mutant combination was also female sterile (Supplemental Table S1). Thus, *l(2)SH0818* appears to have reduced *minus* activity. *l(2)SH0818* is semilethal, but rare homozygous survivors showed small body size (Fig. 1D) and small bristles. We confirmed that these phenotypes were due to the *P*-element insertion, as flies from which the *P*-element was precisely excised were homozygous viable and normal in size. Animals homozygous for a null allele of *minus* also showed reduced body size in larval and pupal stages (Fig. 1E,F).

The *l(2)SH0818* P-element is inserted in the 5′ untranslated region (UTR) of the annotated gene *CG5360* (Fig. 1G). Two other transposons inserted in this 5′ UTR, *EY01258* and *l(2)k06908*, also produced weak bristle phenotypes in *trans* to *mi[1]* (Supplemental Table S1), suggesting that they are weak *minus* alleles. *mi[1]* was isolated as a spontaneous mutation, which can be caused by transposon insertions. We were unable to amplify DNA spanning the second intron of *CG5360* from *mi[1]* homozygous animals by PCR, consistent with the possibility that an insertion of DNA disrupts the *CG5360* transcription unit (other parts of the gene amplified normally). We generated an additional *mi* allele by imprecise excision of the viable *P*-element insertion *EY01258*. *mi$^{\Delta EY22}$* is a deletion that removes the two first exons and part of the third exon of *CG5360* (Fig. 1G). *mi$^{\Delta EY22}$* produced phenotypes equivalent to those of a larger deletion that completely removes the gene (Supplemental Table S1), and so behaves genetically like a null allele. Homozygous *mi$^{\Delta EY22}$* mutants died mainly during early larval stages. The remaining mutants showed a developmental delay and reduced growth rate. After 5 d, the largest mutant larvae were much smaller

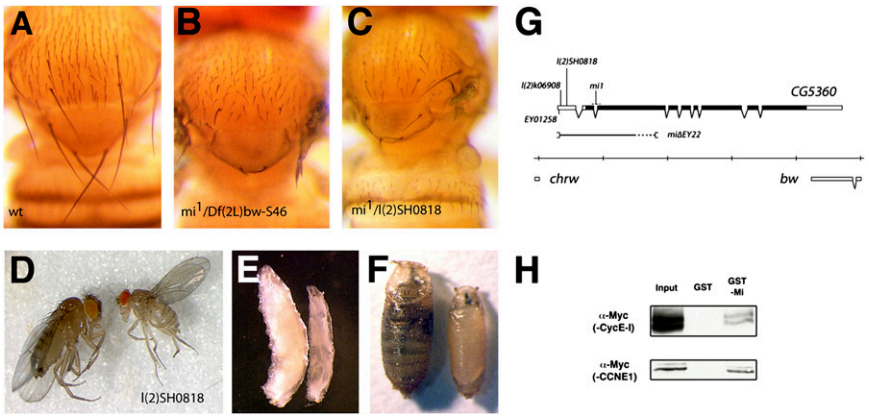

Figure 1. Genetic and molecular characterization of the *minus* gene. (*A–C*) Adult dorsal thorax preparations of wild-type (wt) control (*A*), $mi^1/Df(2L)bw\text{-}S46$ (*B*), and $mi^1/l(2)SH081$ (*C*) flies. (*D*) *l(2)SH0818*/+ control (*left*) and homozygous *l(2)SH0818* mutant females (*right*). (*E,F*) Homozygous y^1w^{1118} control (*left*) and $mi^{\Delta EY22}$ mutant larvae (*right*) after 5 and 11 d of growth in uncrowded conditions. (*G*) The *minus* locus showing transgene and transposon insertion sites and the deletion generated by imprecise excision. The ORF is shaded. (*H*) Lysates from S2 cells expressing Myc-tagged CycE-I or Myc-tagged CCNE1 were assayed for binding to GST-Minus beads or control GST beads. Proteins bound to the beads were detected on immunoblots probed with anti-Myc tag antibody. The input lane represents 8% (*top*) and 5% (*bottom*) of the total lysate.

than comparably aged control larvae (Fig. 1E). By 11 d, the surviving mutants that had pupated were also small (Fig. 1F).

Minus protein lacks domains of known function, but was identified as a CycE-interacting protein in a genome-wide yeast two-hybrid screen (Giot et al. 2003). Ten cyclin-binding sites are predicted using the Eukaryotic Linear Motif server (http://elm.eu.org; Supplemental Fig. S1). Some of the predicted cyclin-binding sites are conserved in other insects—*Anopheles gambiae*, *Tribolium castaneum*, and *Apis mellifera*—but none resides in a region of sequence conservation sufficient to permit identification of an orthologous protein outside of the insects. We confirmed the interaction between Minus and CycE in vitro using GST pull-down assays. The *Drosophila Cyclin E* gene encodes two proteins that differ in their N termini (Richardson et al. 1993). A GST-Minus fusion protein was able to bind CycE-I from lysates of S2 cells transfected to express Myc-tagged CycE-I (Fig. 1H). No binding was seen to the GST control. Similar results were obtained with Myc-tagged *Drosophila* CycE-II protein (data not shown) and with Myc-tagged human CycE isoform 1 (CCNE1) (Fig. 1H).

Interaction with CycE and its mutant phenotypes suggested that Minus might affect the cell cycle. To test this, we asked if Minus is required for mitotic cell cycle progression by FACS analysis on clones of *minus* mutant cells and by antibody staining for cell cycle markers in the wing imaginal disc. Under normal circumstances, clones of cells lacking Minus are eliminated by competition with faster-growing Minus-expressing cells (Fig. 5, below). To circumvent this problem, we provided homozygous $mi^{\Delta EY22}$ cells with a relative growth advantage by inducing the clones in a *Minute* mutant background to reduce cell competition (Morata and Ripoll 1975). Homozygous $mi^{\Delta EY22}$ mutant clones were recovered under these conditions. Labeling with an antibody to the G2/M-phase phosphorylated form of Histone H3 (Su et al. 1998) showed elevated levels of phospho-H3 (PH3) in the *minus* mutant clones compared with the surrounding $mi^{\Delta EY22}$/*Minute* heterozygous tissue (Fig. 2A), but not in control clones produced in a *Minute* mutant background (Fig. 2B). This suggests accumulation of cells in M or G2 phase in the *minus* mutant clones. The fact that this was not observed in the control indicates that it is not just a consequence of the cells having been given a growth advantage relative to the Minute/+ surrounding tissue. Comparison of control Minute/+ clones with homozygous $mi^{\Delta EY22}$ mutant clones by FACS analysis also indicated an accumulation of *minus* mutant cells in G2/M at the expense of cells in G1 (Fig. 2C, left panel). A similar shift toward G2/M was observed by comparing FACS profiles of cells isolated from wing discs from control and homozygous mutant $mi^{\Delta EY22}$ third instar larvae (Fig. 2C, center and right panels).

Homozygous *minus* mutant imaginal discs were small with elevated levels of cell death, visualized by an antibody to the activated form of Caspase 3 (Fig. 2D,E). To test if cell death contributes to the cell cycle defects in *minus* mutant clones, we made use of the *Drosophila* Inhibitor of Apoptosis Protein-1 (DIAP-1). A *UAS-DIAP-1* transgene was expressed under control of *engrailed-GAL4* with either a *UAS-GFP* transgene or a *UAS-RNAi* construct to target Minus for depletion. Expression of the *minus RNAi* transgene increased the number of cells labeling with anti-PH3 (Fig. 2F), beyond the modest increase observed in the cells expressing DIAP-1 and GFP (Fig. 2G). Thus, accumulation of cells in G/2M does not appear to reflect compensation for the elevated level of cell death in the *minus* mutant.

In *Drosophila*, most larval tissues grow using an unusual cell cycle called endoreplication, which consists of S and G phases but omits M phase (Lilly and Duronio 2005). In this way, larval cells increase their ploidy and size. To test if the growth defects reflect a defect in endoreplication, homozygous $mi^{\Delta EY22}$ mutant salivary gland cells were made using FLP/FRT-mediated recombination. Mutant cells were small, with small nuclei (Fig. 2H, arrows). There was no change in the appearance of *minus* mutant cells that expressed the baculovirus inhibitor of apoptosis, p35 (Hay et al. 1994), and GFP (Fig. 2I). Thus, the small nuclear size is unlikely to reflect cells undergoing apoptosis, and more likely reflects underreplication of DNA in the polyploid salivary gland cells. These results suggest that *minus* is required cell-autonomously to promote endoreplication. In this context, it is interesting that preventing CycE oscillation has been shown to impair endoreplication (Follette et al. 1998; Weiss et al. 1998).

Does Minus act by influencing the level of Cyclin E expression? Depleting *minus* by RNAi in salivary glands abolished the normal pattern of CycE oscillation and resulted in a uniform elevated level of anti-CycE labeling (Fig. 3A,B). Minus RNAi produced a milder version of the endoreplicative defect seen in mutant clones (cf. Figs. 3A

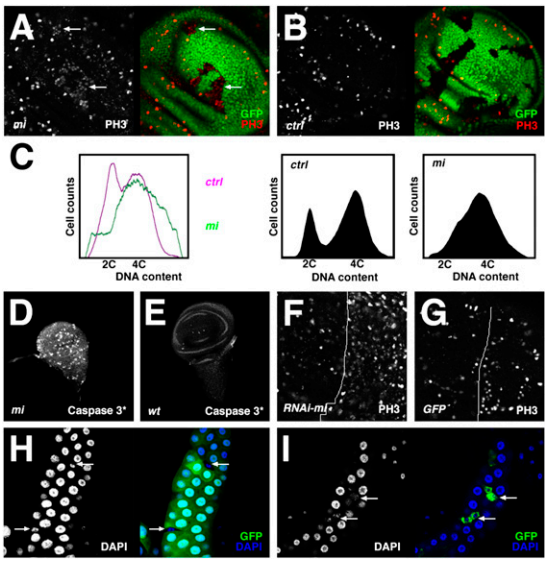

Figure 2. *minus* is required for cell proliferation and endoreplication. (*A*,*B*) Anti-PH3 of third instar wing discs containing clones of $mi^{\Delta EY22}$ mutant cells (*A*) or wild-type cells (*B*), each in a *Minute* background. Clones are labeled by the absence of GFP (arrows). (*C*) FACS analysis of cells from dissociated *Minute*/+ heterozygous wing discs containing $mi^{\Delta EY22}$ homozygous mutant clones. (*Left*) Profile of homozygous $mi^{\Delta EY22}$ mutant cells and heterozygous *Minute*/$mi^{\Delta EY22}$ "control" cells from the same discs. FACS analysis of cells from y^1w^{1118} (*ctrl*) and homozygous $mi^{\Delta EY22}$ mutant discs (*mi*). (*D*,*E*) Homozygous $mi^{\Delta EY22}$ mutant and control wing imaginal discs labeled with antibody to activated Caspase 3, to visualize apoptotic cells. (*F*,*G*) Anti-PH3 staining (PH3) of third instar wing discs expressing UAS-DIAP1 and *UAS-RNAi-mi* (*F*) or *UAS-GFP* (*G*) in the P compartment (*right* of the white line). (*H*,*I*) Salivary glands containing homozygous $mi^{\Delta EY22}$ mutant cells. Mutant cells are marked by the absence of GFP (arrows) DAPI-labeled nuclei are shown in the *left* panel. (*I*) $mi^{\Delta EY22}$ mutant cells expressing *UAS-p35* are marked by GFP (arrows).

and 2H). To test whether Minus also regulates CycE in mitotic cells, we produced clones of cells mutant for the null allele $mi^{\Delta EY22}$. Homozygous $mi^{\Delta EY22}$ mutant clones showed elevated levels of CycE protein (Fig. 3C). This was not seen in control clones produced in a *Minute* mutant background (Fig. 3D). Depleting *minus* by RNAi was also effective in S2 cells (Fig. 3E) and led to increased CycE levels (Fig. 3F). Comparable results were obtained with an independent dsRNA sequence to deplete *minus* and by depletion of Archipelago, a core component of the SCF complex that promotes CycE turnover (Fig. 3F). These observations indicate that CycE is overexpressed in cells with reduced Minus activity.

CycE levels are subject to positive autoregulatory feedback control. Elevated CycE activity, through phosphorylation of Rb, leads to activation of E2F, which in turn transcribes *CycE* (Du et al. 1996). Quantitative RT–PCR showed that depletion of *minus* led to increased *CycE* mRNA levels in S2 cells (Supplemental Fig. S2). To bypass this transcriptional feedback, we tested the effect of depleting Minus on a Myc-tagged CycE-I transgene expressed using a heterologous promoter. Depleting Minus in S2 cells increased the level of Myc-tagged CycE-I or Myc-tagged human CCNE1 (Fig. 3G) and Myc-tagged CycE-II (data not shown). We also observed elevated Myc-tagged CycE-I levels in $mi^{\Delta EY22}$ mutant clones produced in a transgenic fly expressing Myc-CycE under control of the *hsp70* promoter, which should not be

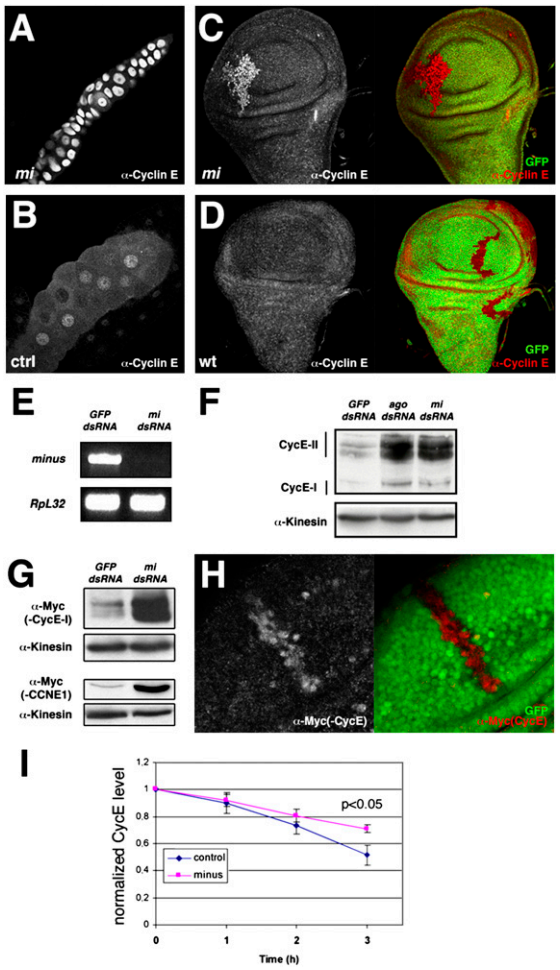

Figure 3. *minus* mutant affects CycE turnover. (*A*,*B*) Anti-CycE staining of third instar salivary glands expressing either *UAS-RNAi minus* (*mi*) (*A*) or *UAS-GFP* (ctrl) (*B*) under *ptc-GAL4* control. (*C*,*D*) Anti-CycE staining of third instar wing discs containing clones of homozygous $mi^{\Delta EY22}$ mutant cells or wild-type cells, both in a *Minute* background to give the clone a relative growth advantage. Clones are labeled by the absence of GFP. (*E*) RT–PCR to assess the efficiency of *minus* depletion in S2 cells treated with dsRNA directed to *minus* or to *GFP* as a control. The PCR primers were from a region of *minus* mRNA that did not overlap the region used for the dsRNA. *RpL32* was amplified as a control. (*F*) S2 cells treated with dsRNA to deplete *GFP*, *archipelago*, or *minus*, analyzed by immunoblotting with anti-CycE. (*Top* panel) Probed with anti-Myc antibody to detect *myc-tagged* CycE. (*Bottom* panel) Blot reprobed with anti-Kinesin as a loading control. (*G*) Extracts from S2 cells transfected to express Myc-tagged-CycE-I or Myc-tagged-Human CCNE1 treated with dsRNA to *GFP* or *minus* and analyzed by immunoblotting with anti-Myc to detect the tagged proteins. Blots reprobed with anti-Kinesin as a loading control. (*H*) Anti-Myc tag staining of a third instar wing disc heterozygous for *hsp70-Myc-CycE-I* and a *Minute* mutation, containing a large clone of $mi^{\Delta EY22}$ mutant cells, labeled by the absence of GFP. Note the elevated level of Myc-tagged CycE in the clone. (*I*) S2 cells expressing CycE-Renilla luciferase were treated with 10 μg/mL cycloheximide, and samples were collected at the indicated times. Cells had been treated 5 d previously with dsRNA to *minus* or *GFP* as a control. Error bars indicate standard deviation of three independent experiments. By 3 h of cycloheximide treatment, the difference in CycE levels was significant ($P < 0.05$) by Student's *t*-test. Note that use of an unsynchronized cell population might cause us to underestimate the magnitude of the effect.

subject to the same transcriptional regulation as *CycE* (Fig. 3H). Together, these results suggest that the direct effects of Minus on CycE levels are post-transcriptional.

To test if the increased level of CycE reflects reduced turnover, we compared the stability of CycE in control cells and in cells depleted of Minus by RNAi. To permit an accurate quantitative assessment of CycE levels, we used a CycE-Renilla luciferase fusion protein expressed in S2 cells (Fig. 3I). In control cells, the level of CycE-luciferase dropped by ~50% during 3 h of cycloheximide treatment to block new protein synthesis. In Minus-depleted cells, the level of CycE-luciferase decreased by ~30%. The data represent the average of three independent experiments ($P < 0.05$ at the 3-h time point). There was no effect of depleting Minus on Renilla luciferase protein alone (data not shown). Thus, the rate of CycE protein turnover is reduced in cells with reduced Minus activity.

The relationship between CycE and Minus is reminiscent of that between CycE and Archipelago, which promotes CycE turnover. So, we asked whether Archipelago and other components of the SCF complex physically interact with Minus. GST-Minus was able to pull down V5-tagged Archipelago, HA-tagged Skp1, and Myc-tagged Cullin1 from lysates of S2 cells (Fig. 4A). None of the proteins associated with the GST control.

We next asked if CycE, Minus, and Ago–SCF form a ternary complex. To allow quantitative measurement of CycE levels, we used the Cyclin E-luciferase fusion protein. Renilla luciferase was used as a control. In addition, the cells were cotransfected with HA-tagged Archipelago and either a GFP-Minus fusion protein or a GFP control. Cell lysates were immunoprecipitated with anti-HA to pull down the Archipelago–SCF complex and associated proteins. The precipitated complex was released from the antibody by incubation with HA peptide, and the eluate containing the resolubilized complexes was subjected to a second round of immunoprecipitation with anti-GFP to pull down GFP-Minus, or GFP in the control. After the sequential immunoprecipitation, Renilla luciferase was measured (Fig. 4B). Control cells transfected to express GFP together with the CycE-luciferase fusion protein showed a very low level of recovered luciferase activity. Luciferase activity was >40-fold higher in cells transfected to express GFP-Minus with CycE-luciferase. The data represent the average of four independent experiments, and the observed difference was statistically significant ($P < 0.05$ using either Student's *t*-test or Wilcoxon rank sum test). Because the complex was first pulled down with an antibody to tagged Archipelago, followed by an antibody to tagged Minus, recovery of CycE-luciferase depends on the existence of a complex containing all three proteins.

In addition to promoting CycE turnover, the Archipelago–SCF complex promotes turnover of dMyc and Trachealess proteins. dMyc levels were elevated in *ago* mutant clones (Fig. 4C; Moberg et al. 2004). As dMyc is required for normal cell growth (Johnston et al. 1999), we asked if Minus might also promote dMyc turnover. Clones of $mi^{\Delta EY22}$ mutant cells did not show elevated dMyc (Fig. 4D). This suggests that the Minus-containing Ago–SCF complex might be specific for CycE degradation.

We next asked to what extent elevated CycE levels contribute to the phenotypes observed in *minus* mutants. Bristle progenitors grow by endoreplication, and elevated CycE expression can reduce bristle growth (Weng et al. 2003), as seen in flies carrying a leaky *hsp70-Myc-CycE-I* transgene (Fig. 5A,B). Consistent with the idea that reduced bristle size is due to excess CycE activity in the *minus* mutant, we observed that removing one copy of the *CycE* gene suppressed this phenotype (Fig. 5C,D). Fewer bristles were rescued in the combination carrying the weaker allele $CycE^{5}$ than with the null allele $CycE^{Ar95}$. The *minus* bristle phenotype was not affected by removing one copy of the *cdk2* gene, which is not the rate-limiting element of the CycE/Cdk2 complex.

Does excess CycE also impair the growth of *minus* mutant clones in diploid tissue? Clones of cells homozygous for the null allele $mi^{\Delta EY22}$ were typically eliminated

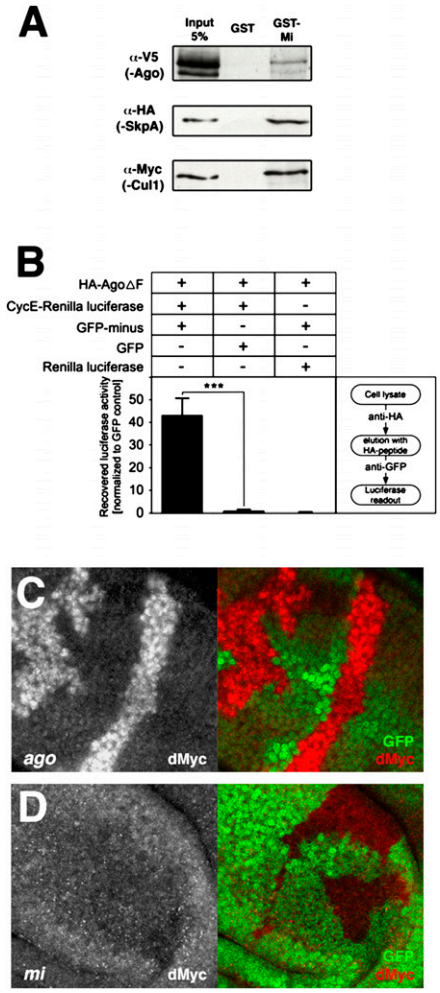

Figure 4. Minus can interact with the SCF–Archipelago complex. (*A*) Extracts from S2 cells expressing V5-tagged Archipelago, HA-tagged SkpA, or Myc-tagged Cul1 were assayed for binding to GST-Mi beads or GST control beads. Proteins bound to the beads were detected by immunoblotting with anti-V5, HA, or Myc tag antibodies as indicated. The input lane represents 5% of the total lysate. (*B*) S2 cells were transfected as indicated to express HA-tagged AgoΔF (Moberg et al. 2004), CycE-Renillla luciferase or Renilla luciferase, and GFP-Minus or GFP, as indicated. Lysates were immunoprecipitated with anti-HA, followed by release of bound complexes by incubation with HA peptide and immunoprecipitation with anti-GFP. Bound luciferase was measured. Error bars indicate standard deviation. (*C*,*D*) Anti-dMyc labeling (red) of third instar wing discs containing clones of *ago1* mutant or $mi^{\Delta EY22}$ mutant cells, as indicated. Mutant clones are labeled by the absence of GFP.

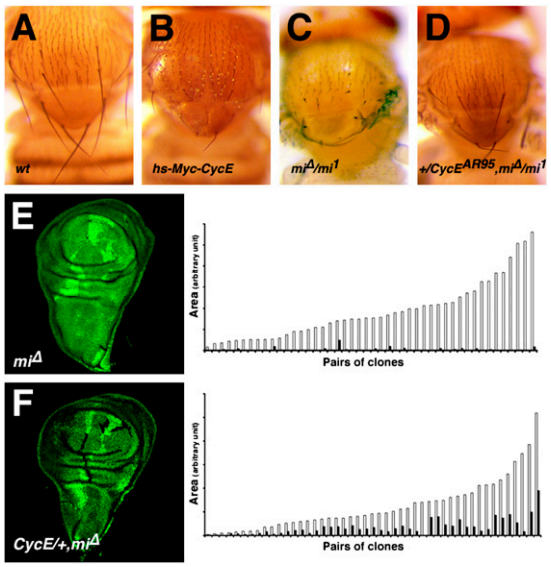

Figure 5. Some *minus* phenotypes are due to excess CycE. (*A–D*) Adult dorsal thorax preparations of wild-type (*A*), *hsp70-Myc-CycE*/+ (*B*), $mi^{\Delta EY22}/mi^{1}$ (*C*), and +/ $CycE^{Ar95}$, $mi^{\Delta EY22}/mi^{1}$ (*D*). (*E*, *left* panel): Third instar wing disc containing several twin spots (bright green) and a rare homozygous $mi^{\Delta EY22}$ mutant clone, marked by the absence of GFP. (*Right* panel) Histogram showing the area (arbitrary units) of 46 homozygous $mi^{\Delta EY22}$ mutant clones (shaded) and their twin spots. (*F*, *left* panel) Third instar wing disc heterozygote for $CycE^{AR95}$ containing several twin spots and homozygous $mi^{\Delta EY22}$ mutant clones, marked by the absence of GFP. (*Right* panel) Histogram showing the area of 47 homozygous $mi^{\Delta EY22}$ mutant clones (shaded) and their twin spots. Although smaller on average than their twins, many more mutant clones were recovered than in the discs expressing two copies of the *CycE* gene.

from the imaginal discs by competition with faster-growing Minus-expressing cells (Fig. 5E). As for the endoreplication defect, removing one copy of the *CycE* gene partially rescued survival and of growth these clones (Fig. 5F). Thus, excess CycE activity contributes to the defects in proliferation of diploid cells.

Our study provides evidence that efficient Cyclin E turnover is essential for normal cell proliferation and endoreplication during *Drosophila* development. Endoreplicative growth and more typical proliferative diploid cell cycles are impaired as a consequence of the elevated Cyclin E levels in *minus* mutants. Attempts to suppress the cell proliferation phenotype by reducing CycE activity were only partially successful, perhaps due to incomplete compensation for elevated CycE levels. It is also possible that Minus has other targets, in addition to CycE.

Minus was also identified in a screen for female sterility (Schüpbach and Wieschaus 1991). Minus' role in Cyclin E turnover suggests a possible link to the *encore* mutant. *encore* encodes a protein of unknown function that has been proposed to promote CycE degradation by localizing the SCF complex to a germline-specific cytoplasmic structure called the fusome (Ohlmeyer and Schüpbach 2003). In mammals, different Fbw7/Cdc4 isoforms can target different Myc functions in distinct subcellular compartments (Welcker and Clurman 2008). Interestingly, the *Drosophila* Fbw7/hCdc4 protein Archipelago exists in only one isoform (Moberg et al. 2001), limiting the possibility for isoform-specific subfunctions. Use of accessory proteins, such as Minus, may be another means to confer target specificity on the core Archipelago/SCF complex.

At present we cannot detect a Minus ortholog outside the insects. But, we note that Minus binds to human CCNE1 and influences its expression, much as it does with *Drosophila* CycE. Although this does not constitute evidence for the existence of a mammalian protein with a function analogous to Minus, it is compatible with this possibility. A vertebrate protein having the motifs required for Minus function but in a different number or arrangement might not be readily identified unless these short motifs were embedded in more extensive blocks of sequence similarity. In view of the importance of Cyclin E turnover in cancer (Hwang and Clurman 2005), a functional equivalent of Minus might be a good candidate for a tumor suppressor.

Materials and methods

Fly strains

The following mutant alleles were obtained from the Bloomington Stock Center: y^{1}, w^{1118}, mi^{1}, *Df(2L)bw-S46*, *EY01258*, *l(2)k06908*, *KG06805*, *da-GAL4*, $CycE^{5}$, $CycE^{Ar95}$, $cdc2c^{2}$, $cdc2c^{3}$, *UAS-p35*, *UAS-CycE*, *UAS-GFP*, and *ptc-GAL4*. *UAS-RNAi-mi* lines were from the Vienna *Drosophila* RNAi center. *l(2)SH0818* was provided by S. Hou (Oh et al. 2003), *FRT80B* ago^{1} was provided by I. Hariharan; *hs FLP; FRT42D, Ubi-GFP, M(2)53*^{1} was provided by D. Hipfner; and *hh-GAL4, UAS-DIAP1* was provided by B.J. Thompson. $mi^{\Delta EY22}$ was generated by imprecise excision of *EY01258*. Mutants were balanced with *CyO*, Kr^{GAL4}, and *UAS-GFP*, and molecular characterization was done by PCR on GFP-negative larvae. Mutant clones were induced using the FLP/FRT system. Larvae were heat-shocked for 1 h at 38°C at 48 ± 12 h in the following genotypes:

hs FLP/+ or Y; FRT42D, Ubi-GFP/FRT42D, $mi^{\Delta EY22}$;
hs FLP/+ or Y; FRT42D, Ubi-GFP/FRT42D;
hs FLP/+ or Y; FRT42D, Ubi-GFP/ $CycE^{Ar95}$, *FRT42D,* $mi^{\Delta EY22}$;
hs FLP/+ or Y; FRT42D, Ubi-GFP, M(2)53^{1}*/FRT42D,* $mi^{\Delta EY22}$;
hs FLP/+ or Y; FRT42D, Ubi-GFP, M(2)53^{1}*/FRT42D*;
hsFLP, UAS-CD8-GFP/+; FRT42D, tub-GAL80/FRT42D, $mi^{\Delta EY22}$; *tub-GAL4/UAS-p35*;
hs FLP/+ or Y; FRT42D, Ubi-GFP, M(2)53^{1}*/FRT42D,* $mi^{\Delta EY22}$; *hsp70-Myc-CycE/+*; and
hs FLP/+ or Y; FRT80B, Ubi-GFP/FRT80B, ago^{1}.

hs FLP/+ or Y; FRT42D, Ubi-GFP, M(2)53^{1}*/FRT42D,* $mi^{\Delta EY22}$; *hsp70-Myc-CycE/+* larvae were heat-shocked for 1 h at 38°C 16 h before dissection to induce *hsp70-Myc-CycE* expression. The *hsp70-Myc-CycE* transgene was derived from *pIE1-4-myc-CycE* (Moberg et al. 2001).

To produce salivary gland clones, 0- to 6-h embryos were heat-shocked for 1 h at 38°C.

Immunohistochemistry and FACS analysis

Antibody staining of imaginal discs was performed as in Hipfner and Cohen (2003). The primary antibodies used were rabbit α-CycE (C. Lehner), mouse α-CycE (H. Richardson), monoclonal mouse α-Myc P4C4-b10 (B. Edgar), and rabbit α-phospho-Histone-H3 (Cell Signaling). FACS analysis was done according to Neufeld et al. (1998).

Cell culture: dsRNA treatment, immunoprecipitation, and luciferase assays

dsRNA was prepared using MegascriptT7 (Ambion) with templates dsRNA-1_f, 5′-taatacgactcactataggTCCTTTGGGCTGTGTCGC-3′; dsRNA-1_r, 5′-taatacgactcactataggTGGGTTGGCGTTCCTTGC-3′; and

414–776: dsRNA-2_f, 5′-ctaatacgactcactatagggagCACGACAGCCACCA CGACAAC-3′; dsRNA-2_r, 5′-ctaatacgactcactatagggagTCCATCGTCTG CTCATCCACAAA-3′; nucleotides 17–633 of *EGFP2*, for *ago* as in Moberg et al. (2004). S2 cells (2×10^6) were treated with 12 μg of dsRNA per well. Two days later, cells were transfected with 4 μg of Cellfectin (Invitrogen) and 2 μg of *pMT-myc-CyE-I* or *pMT-myc-CyE-II* or *pMT-myc-CCNE1*. After 12 h, the mix was replaced with medium containing 20 μg of dsRNA. Cells were induced at 12 h with 0.7 mM $CuSO_4$ for 2 d. *pMT-myc-CyE-I* was derived from *pIE1-4-myc-CycE* (Moberg et al. 2001), and *pMT-myc-CyE-II* was made from EST *LD22682*. *pMT-myc-CCNE1* was made using *IRAUp969H0878D* from Deutsches Ressourcenzentrum für Genomforschung GmbH.

For analysis of protein levels, cells were lysed in 50 mM Tris (pH 7.5), 300 mM NaCl, 0.5% CHAPS, 25 mM NaF, and protease inhibitor cocktail (Roche). Immunoblots were probed with rabbit α-Myc (Santa Cruz Biotechnologies) or α-Cyclin E (d300; Santa Cruz Biotechnologies) and α-Kinesin (Cytoskeleton, Inc.) as a loading control.

For analysis of protein stability, cells were treated with dsRNA and transfected with *pMT-CycE-Renilla luciferase*, but not induced with $CuSO_4$. *pMT-CyE-Renilla luciferase* is a C-terminal fusion derived from *pMT-myc-CyE-I*. Cells were treated with cycloheximide (10 μg/mL) as indicated, and dual luciferase assays were according to the manufacturer's protocol (Promega).

For double immunoprecipitation, transfections were done with combinations of *pMT-GFP-mi*, *pMT-GFP-mi*, *pMT-HA-Ago-*ΔF (Moberg et al. 2004), *pMT-CyE-Renilla luciferase*, or *pMT-Renilla luciferase*. *pMT-GFP-mi* was generated by using *LD45221* and introducing EGFP2 (Clontech) at Minus residue 82. Cells were lysed in 20 mM Tris (pH 7.5), 150 mM NaCl, 0.2% NP-40, 10% glycerol, 1 mM DTT, and protease and phophatase inhibitor cocktails (Roche). Lysates were immunoprecipitated with rat monoclonal 3F10 anti-HA (Roche) bound to protein G beads (Roche). Beads were washed five times in lysis buffer and incubated in lysis buffer with 0.5 mg/mL HA peptide (Roche). Eluates were immunoprecipitated with rabbit α-GFP (Torrey Pines Biolabs) bound to protein A beads (Roche). Beads were washed four times in lysis buffer and Renilla luciferase activity was measured.

GST pull-down assays

GST-Minus (residues 82–725) was produced in *Escherichia coli BL21* and purified on glutathione-sepharose beads. GST-Minus was incubated for 2 h at 4°C with lysates of S2 cells transfected with *pMT-myc-CycE-I* or *pMT-Myc-CCNE1* or *pMT*-V5-*Ago* (Ko et al. 2002) or *pMT-HA-Skp1* (Zielke et al. 2006) or *pAct-Myc-Cullin1* (Donaldson and Duronio, unpubl.). Beads were washed four times in 50 mM Tris (pH 7.5), 100 mM NaCl, and 0.5% CHAPS, and analyzed by immunoblotting with mouse 9E10 α-Myc (Santa Cruz Biotechnologies), goat anti-V5 (Abcam), or rat 3F10 anti-HA (Roche).

Acknowledgments

We thank Ann-Mari Voie, Lidia Perez, Eva Loeser, and Andrew Riddell from the EMBL Flow Cytometry Core Facility for technical support; and members of the laboratory for support and helpful comments. S.S. was a recipient of a "La Ligue Nationale contre le Cancer" Post-Doctoral Fellowship and a Marie Curie Individual Fellowship. T.S. and V.H. were fellows of the Singapore Millennium Foundation. This work was supported by EMBL and by the Singapore Millennium Foundation via core funding to T.L.L.

References

Du W, Vidal M, Xie JE, Dyson N. 1996. RBF, a novel RB-related gene that regulates E2F activity and interacts with cyclin E in *Drosophila*. *Genes & Dev* **10:** 1206–1218.

FlyBase Consortium. 2003. The FlyBase database of the *Drosophila* genome projects and community literature. *Nucleic Acids Res* **31:** 172–175.

Follette PJ, Duronio RJ, O'Farrell PH. 1998. Fluctuations in cyclin E levels are required for multiple rounds of endocycle S phase in *Drosophila*. *Curr Biol* **8:** 235–238.

Giot L, Bader JS, Brouwer C, Chaudhuri A, Kuang B, Li Y, Hao YL, Ooi CE, Godwin B, Vitols E, et al. 2003. A protein interaction map of *Drosophila melanogaster*. *Science* **302:** 1727–1736.

Hay BA, Wolff T, Rubin GM. 1994. Expression of baculovirus P35 prevents cell death in *Drosophila*. *Development* **120:** 2121–2129.

Hipfner DR, Cohen SM. 2003. The *Drosophila* sterile-20 kinase slik controls cell proliferation and apoptosis during imaginal disc development. *PLoS Biol* **1:** E35. doi: 10.1371/journal.pbio.0000035.

Hwang HC, Clurman BE. 2005. Cyclin E in normal and neoplastic cell cycles. *Oncogene* **24:** 2776–2786.

Johnston LA, Prober DA, Edgar BA, Eisenman RN, Gallant P. 1999. *Drosophila* myc regulates cellular growth during development. *Cell* **98:** 779–790.

Knoblich JA, Sauer K, Jones L, Richardson H, Saint R, Lehner CF. 1994. Cyclin E controls S phase progression and its down-regulation during *Drosophila* embryogenesis is required for the arrest of cell proliferation. *Cell* **77:** 107–120.

Ko HW, Jiang J, Edery I. 2002. Role for Slimb in the degradation of *Drosophila* Period protein phosphorylated by Doubletime. *Nature* **420:** 673–678.

Lane ME, Elend M, Heidmann D, Herr A, Marzodko S, Herzig A, Lehner CF. 2000. A screen for modifiers of cyclin E function in *Drosophila melanogaster* identifies Cdk2 mutations, revealing the insignificance of putative phosphorylation sites in Cdk2. *Genetics* **155:** 233–244.

Lilly MA, Duronio RJ. 2005. New insights into cell cycle control from the *Drosophila* endocycle. *Oncogene* **24:** 2765–2775.

Lindsley DL, Zimm GG. 1992. *The genome of* Drosophila melanogaster. Academic Press, San Diego.

Moberg KH, Bell DW, Wahrer DC, Haber DA, Hariharan IK. 2001. Archipelago regulates Cyclin E levels in *Drosophila* and is mutated in human cancer cell lines. *Nature* **413:** 311–316.

Moberg KH, Mukherjee A, Veraksa A, Artavanis-Tsakonas S, Hariharan IK. 2004. The *Drosophila* F box protein archipelago regulates dMyc protein levels in vivo. *Curr Biol* **14:** 965–974.

Morata G, Ripoll P. 1975. Minutes: Mutants of *Drosophila* autonomously affecting cell division rate. *Dev Biol* **42:** 211–221.

Mortimer NT, Moberg KH. 2007. The *Drosophila* F-box protein Archipelago controls levels of the Trachealess transcription factor in the embryonic tracheal system. *Dev Biol* **312:** 560–571.

Murray AW. 2004. Recycling the cell cycle: Cyclins revisited. *Cell* **116:** 221–234.

Neufeld TP, de la Cruz AF, Johnston LA, Edgar BA. 1998. Coordination of growth and cell division in the *Drosophila* wing. *Cell* **93:** 1183–1193.

Oh SW, Kingsley T, Shin HH, Zheng Z, Chen HW, Chen X, Wang H, Ruan P, Moody M, Hou SX. 2003. A P-element insertion screen identified mutations in 455 novel essential genes in *Drosophila*. *Genetics* **163:** 195–201.

Ohlmeyer JT, Schüpbach T. 2003. Encore facilitates SCF-Ubiquitin-proteasome-dependent proteolysis during *Drosophila* oogenesis. *Development* **130:** 6339–6349.

Richardson HE, O'Keefe LV, Reed SI, Saint R. 1993. A *Drosophila* G1-specific cyclin E homolog exhibits different modes of expression during embryogenesis. *Development* **119:** 673–690.

Schüpbach T, Wieschaus E. 1991. Female sterile mutations on the second chromosome of *Drosophila melanogaster*. II. Mutations blocking oogenesis or altering egg morphology. *Genetics* **129:** 1119–1136.

Su TT, Sprenger F, DiGregorio PJ, Campbell SD, O'Farrell PH. 1998. Exit from mitosis in *Drosophila* syncytial embryos requires proteolysis and cyclin degradation, and is associated with localized dephosphorylation. *Genes & Dev* **12:** 1495–1503.

Weiss A, Herzig A, Jacobs H, Lehner CF. 1998. Continuous Cyclin E expression inhibits progression through endoreduplication cycles in *Drosophila*. *Curr Biol* **8:** 239–242.

Welcker M, Clurman BE. 2008. FBW7 ubiquitin ligase: A tumour suppressor at the crossroads of cell division, growth and differentiation. *Nat Rev Cancer* **8:** 83–93.

Weng L, Zhu C, Xu J, Du W. 2003. Critical role of active repression by E2F and Rb proteins in endoreplication during *Drosophila* development. *EMBO J* **22:** 3865–3875.

Zielke N, Querings S, Grosskortenhaus R, Reis T, Sprenger F. 2006. Molecular dissection of the APC/C inhibitor Rca1 shows a novel F-box-dependent function. *EMBO Rep* **7:** 1266–1272.