Encore facilitates SCF-Ubiquitin-proteasome-dependent proteolysis during *Drosophila* oogenesis

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Summary

Exit from the cell cycle requires the downregulation of Cyclin/Cdk activity. In the ovary of *Drosophila*, Encore activity is necessary in the germline to exit the division program after four mitotic divisions. We find that in *encore* mutant germaria, Cyclin A persists longer than in wild type. In addition, Cyclin E expression is not downregulated after the fourth mitosis and accumulates in a poly-ubiquitinated form. Mutations in genes coding for components of the SCF pathway such as *cul1*, *UbcD2* and *effete* enhance the extra division phenotype of *encore*. We show that Encore physically interacts with the proteasome, Cul1 and Cyclin E. The association of Cul1, phosphorylated Cyclin E and the proteasome 19S-RP subunit S1 with the fusome is affected in *encore* mutant germaria. We propose that in *encore* mutant germaria the proteolysis machinery is less efficient and, in addition, reduced association of Cul1 and S1 with the fusome may compromise Cyclin E destruction and consequently promote an extra round of mitosis.

Key words: Mitosis, Encore, Oogenesis, Cyclin E, SCF, Proteolysis, *Drosophila*

Introduction

In *Drosophila*, the development of the egg starts at the anterior tip of the ovary, in the germarium, where the germline stem cells divide to produce a cystoblast and a self-renewing stem cell. Each cystoblast undergoes four mitotic divisions with incomplete cytokinesis. The resulting 16 cells of each egg chamber are connected by intercellular bridges called ring canals (Fig. 1A). One of the cells with four ring canals develops into the oocyte and the rest give rise to nurse cells. Each of the four mitoses is oriented and synchronized by the fusome; a germline specific organelle composed of membrane and cytoskeletal proteins (Storto and King, 1989; Lin et al., 1994; Deng and Lin, 1997). After each division, the fusome grows by fusion of ER-Golgi type vesicles and extends through the ring canals in order to connect all the cells of the cysts (de Cuevas and Spradling, 1998; Leon and McKearin, 1999) (Fig. 1A). The mechanism by which the number of cyst mitoses is limited to four has not been fully elucidated. However, the studies of various mutations suggest that the fusome plays a role in regulating the timing, the synchronization, and perhaps the exit from the cell cycle in the germarium. Several genes have been implicated in the regulation of germline division. Mutations in genes coding for components of the fusome such as *hu-li tai shao* (*hts*), α and β *spectrin* (Yue and Spradling, 1992; Deng and Lin, 1997; Lin et al., 1994; de Cuevas et al., 1996), and *Dynein heavy chain* (*Dhc64*) (McGrail and Hays, 1997) result in egg chambers that contain less than 16 germline cells and often lack an oocyte. The integrity of the fusome is compromised and the resulting number of cells in these mutant egg chambers is variable and not always a factor of 2^n as in the wild-type cyst. Mutations in genes encoding proteins that associate with the fusome such as *bag of marbles* (*bam*) or genes required for proper association of Bam to the fusome such as *benign gonial cell neoplasm* (*bcgn*) result in mutant egg chambers that are tumorous and filled with proliferating cells (Lavoie et al., 1999; McKearin and Ohlstein, 1995). Mutations in the *ovarian tumor* (*otu*) gene that produce tumorous egg chambers have fragmented fusomes (King and Storto, 1988). Overexpression or loss-of-function mutations in a third group of genes such as *Cyclin A*, *Cyclin B*, *Cyclin E* and mutations in the gene encoding the E2 Ubiquitin conjugating enzyme UbcD1 lead to the production of cysts with 32 or 8 cells (Lilly et al., 2000). These genes do not affect fusome integrity and thus timing and spatial characteristics of cell division appear to be intact. The *encore* gene belongs to this group of genes, its product is necessary for exit from mitosis. Loss of Encore activity results in egg chambers containing 32 rather than 16 cells (Hawkins et al., 1996; Van Buskirk et al., 2000). Mutations in the *encore* gene produce additional phenotypes, which show differential temperature sensitivity. *encore* mutant females raised at 18°C produce egg chambers with 16 cells, but they give rise to ventralized eggs (Hawkins et al., 1997). The extra cell division phenotype is only observed when *encore* mutant females are raised at high temperatures (25-29°C). The *encore* gene encodes a 200 kDa protein with no homolog of a defined biochemical function (Van Buskirk et al., 2000). In this work we analyze the mechanism by which Encore promotes exit from the cell cycle after four germline mitoses.

Cell cycle progression is controlled by a series of cell cycle dependent kinases (Cdk). Cdk activity is carefully regulated by the levels of the Cyclin subunits, by Cdk inhibitors (CKI) and by post-translational modification of the Cdk subunit through both activating and inactivating phosphorylation (Desai et al.,

1995; Nakayama et al., 2001). Transition from G1 to S phase depends on Cdk2/Cyclin E activity, and on the timely destruction of the Cdk2/Cyclin E inhibitor p27. The *Drosophila* p27 homologue, Dacapo, is required for exit from the cell cycle in the embryo and eye imaginal disc (Lane et al., 1996; de Nooij et al., 1996). In addition, exit from the cell cycle requires destruction of the cyclins by the ubiquitin-proteasome system (UPS) (Ciechanover et al., 2000; Varshavsky et al., 2000). The addition of ubiquitin requires three different activities; the ubiquitin activating enzyme (E1), the ubiquitin conjugating enzyme (E2) and the ubiquitin ligase enzyme (E3). The ubiquitinated protein bound to E3 is presented to the proteasome, isopeptidase activities in the 19S-recognition particle (RP) of the proteasome cleave the ubiquitin tail, the protein is unfolded and finally destroyed by the proteasome 20S-core particle (CP) (Verma et al., 2002; Cope et al., 2002). There are two E3 enzyme complexes that regulate the cell cycle progression (Tyers and Jorgensen, 2000; Koepp et al., 1999). The APC/cyclosome regulates progression from G2 to M phase transition. The SCF complex regulates the G1 to S phase transition. The SCF complex is composed of Skp/Cullin/Rbx1 and F-box proteins and controls substrate ubiquitination via an interaction between the F-box component and the phosphorylated target protein. In *Drosophila* and mammalian systems, mutations in the *Cul3* and *Ago* genes cause the accumulation of Cyclin E, entry to S-phase and doubling of cell number (Moberg et al., 2001; Singer et al., 1999). Thus, proper regulation of the destruction machinery is important for maintaining normal levels of Cyclin E and assuring proper cell cycle progression. The work presented in this paper demonstrates that the *encore* gene product associates with the SCF-UPS and is required for proper exit from germline mitosis. The failure to downregulate Cyclin E after four cell divisions in conjunction with an accumulation of Cyclin A protein provide the conditions to promote an extra cell division. We show that Encore can bind to Cul1, Cyclin E-$Ub_{(n)}$ and the proteasome. We also demonstrate that Cul1 and the proteasome 19S-RP subunit S1 are associated with the fusome and these associations are very much attenuated in *encore* mutant ovaries. We propose that as a direct consequence, Cyclin E is not degraded properly, its activity is misregulated and the cyst undergoes one extra cell division.

Materials and methods

Stocks and reagents

The *encore* alleles used in this study were the EMS induced alleles enc^{Q4} and enc^{RI} and the P-element allele enc^{BB} (Hawkins et al., 1996). The *cyclin A* alleles, *l(3)183* (Lehner and O'Farrell, 1989) and *l(3)C8LRI* (Lehner et al., 1991), and stocks containing the *HS-cylin A* or *HS-cyclin E* transgenes were provided by C. Lehner (Knoblich and Lehner, 1993; Knoblich et al., 1994). The stock containing the *HS-rux* transgene was obtained form L. Zipursky (Thomas et al., 1997). H. Richardson and the Developmental Studies Hybridoma supplied the polyclonal Cyclin A antibody. Monoclonal and polyclonal antibodies against *Drosophila* Cyclin E were obtained from C. Lehner, H. Richardson, M. A. Lilly and purchased from Santa Cruz Biotechnology. V. Fillipov provided the polyclonal antibody against *Drosophila* Cul1. The S1 and LMP7 antibodies were purchased from Upstate Biotechnology. The phosphorylated Cyclin E antibody was purchased from Santa Cruz Biotechnologies. The *cyclin E* alleles (*05206* and *k05007*), and *UbcD2* (*k13206*), *effete* (*S1782* and *8*) and *cul1* [*Df(2R)CA53* and *lin19*] alleles were supplied by the Bloomington Stock Center.

Antibody staining

Ovaries of well-fed flies were dissected in ice-cold PBS and fixed with 4% paraformaldehyde in PBST for 10 minutes. Fixed ovaries were washed in PBST, blocked with 5% BSA in PBST and incubated in the primary antibody at 4°C overnight. The fluorescent secondary HRP antibodies (Vector) were used at 1 to 1000 dilution. The Cul1 and S1 antibody staining was performed with the following modifications. After dissection, the ovaries were incubated in heptane-PBST0.3% Tween) for 10 minutes, washed for 1 hour in PBST at 4°C, blocked for 2 hours at 4°C, followed by incubation with the primary antibody to 1:1000 dilution overnight at 4°C. The ovaries were then washed three times with PBST and fixed as usual at room temperature. Images of stained samples were collected with a Zeiss Confocal Microscope.

Western blots and immunoprecipitation assays

Ovary dissection was performed in ice-cold 50 mM Tris buffer containing protease inhibitors (Roche), 250 μM NEM and 10 mM MG132 and as much of the vitellarium as possible was carefully dissected away. The germarium-enriched region was then transferred to ice cold Tris buffer, homogenized, centrifuged and sample buffer was added. Super Signal Chemiluminescent kit (Pierce) was used for signal detection. For immunoprecipitation assays, the germaria-enriched extracts were precleared for 30 minutes with AG beads (Pharmacia), followed by a 1 hour incubation at room temperature with the precipitating antibody and AG beads. The beads were then washed six times and sample buffer added and run in a 7%Tris-acetate Nupage polyacrylamide gel (Invitrogen).

Proteasome activity assays

Germaria-enriched extracts were prepared in ice-cold 50 mM Tris buffer. To start the reaction, 2 mM DTT, 5 mM $MgCl_2$, 2 mM ATP and 10 μM of the fluorescent peptide succinyl-leu-leu-val-tyrosine-7-amido-methylcoumarin (Succ-LLVY-MCA) was added. The reaction was incubated at 29°C and aliquots were collected every 20 minutes and added to 200 μl of 1%SDS to stop the hydrolysis reaction. Peptide hydrolysis was monitored measuring by fluorescent emission at 440 nm.

Ubiquitination reaction

Germaria-enriched extracts were prepared in Tris buffer containing protease inhibitors. To start the ubiquitination reaction 10 mM MG132, 0.1 μg/μl Ubiquitin aldehyde, 5 μg/μl Ubiquitin, 5 mM ATPγS, 20 mM $MgCl_2$, 2 mM DTT and 2 μg of histidine-tagged p27 was added, followed by 2-8 hours incubation at 37°C. Polyubiquitinated p27 was affinity purified using nickel-agarose beads. The de-Ubiquitination reaction was performed by adding 20 mM $MgCl_2$, 2 mM DTT, 10 mM Creatine phosphate and 8 μg Creatine Kinase and 5 mM ATP to germaria-enriched ovary extract. The reaction was incubated at 37°C and aliquots were taken every 20 minutes. The addition of sample buffer stopped the reaction.

Results

Mutations in the *encore* gene cause the accumulation of Cyclin A protein in the *Drosophila* germarium

In wild-type ovaries, Cyclin A protein is expressed in a cell cycle-dependent manner (Lilly et al., 2000). The Cyclin A protein is detected in the stem cells, and in dividing cystoblasts. Its expression declines rapidly in post-mitotic cysts (Fig. 1B-D). In *encore* mutant females raised at 29°C Cyclin A protein expression lingers longer than in wild-type

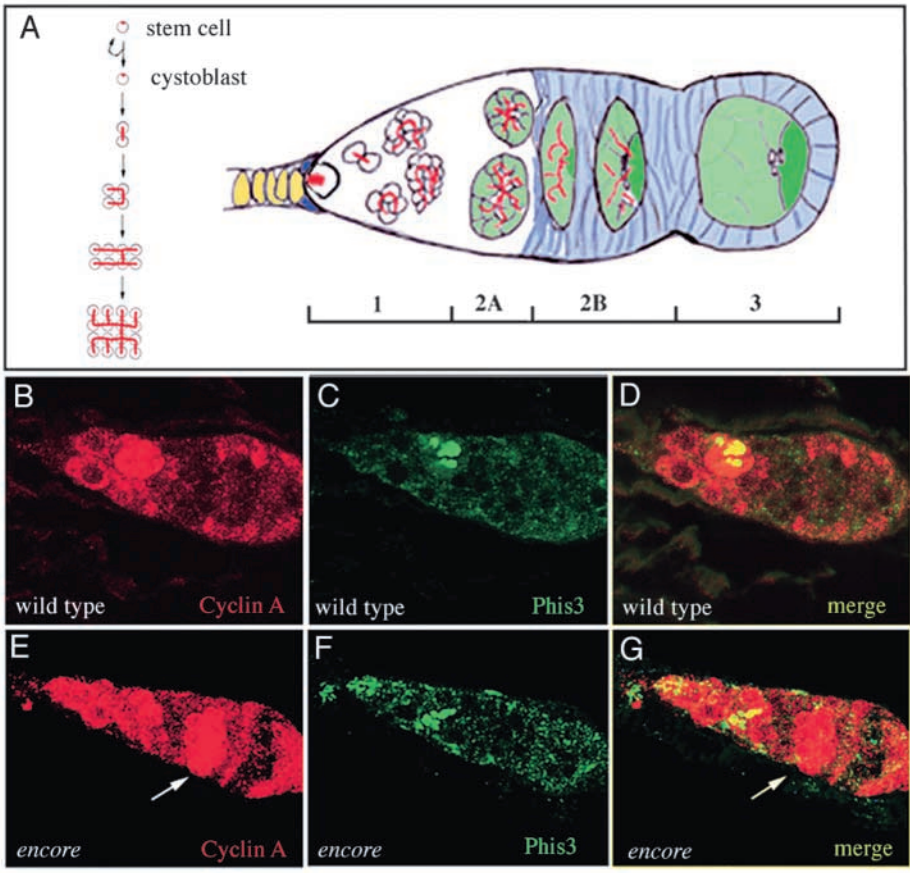

Fig. 1. Cyclin A protein expression in the germarium. (A, left) The four mitotic divisions of the cystoblast and its stereotypic relationship with each other and with the fusome (red). (A, right) The different regions of the *Drosophila* germarium. The germline stem cells, and dividing cystoblast comprise region 1. Region 2A contains post-mitotic 16 cell-cysts (green). The boundary between regions 2A and 2B is marked by the follicle cells migrating inwards to envelop the cyst and to form the egg chamber. The red lines represent the spectrosome in the germline stem cells and the fusome connecting all the cells in the dividing cystoblast and postmitotic cyst. Region 3 of the germarium is characterized by the budding of newly formed egg chambers. (B-G) Expression of Cyclin A (red) and the mitotic marker phosphohistone 3 (green) in wild-type and in *encore* mutant germaria. (B-D) In wild-type ovaries, Cyclin A is expressed in the stem cells and dividing cystoblast in region 1 of the germarium. It rapidly declines in post-mitotic cysts. (E-G) In *encore* mutant germaria, Cyclin A expression persists after the end of mitosis. The arrow shows a cyst positive for Cyclin A in a posterior area of the germarium.

germaria (Fig. 1E-G) and is detected in cysts located in a posterior area not associated with Phospho-histone3 expression. This suggests that Cyclin A protein remains present after mitoses have stopped. The persistence of Cyclin A expression in the germarium may indicate that proper Cyclin A protein turnover is defective. In the *Drosophila* embryonic cellular blastoderm Cyclin A distribution is very dynamic and accumulates in the cytoplasm during interphase, in the nucleus during prophase and is degraded during metaphase (Lehner and O'Farrell, 1989; Whitfield et al., 1990). Immunohistochemical assays also show a dynamic Cyclin A subcellular localization in the germarium, which seems to depend on the cell cycle stage. Cyclin A levels in the cytoplasm increase to fill the cyst completely. In these cysts, Cyclin A is also observed in transient association with the fusome during late prophase/metaphase of the cell cycle (Lilly et al., 2000) (Fig. 2A-C). In dividing cells, Cyclin A is degraded at metaphase and there is no detectable Cyclin A in anaphase and telophase. Cytoplasmic accumulation and nuclear localization of Cyclin A in *encore* mutant germaria is comparable with wild type. The transient association of Cyclin A with the fusome, however, is prolonged as we can observe Cyclin A in the fusome in a posterior position of the germarium (Fig. 2D-F, arrow). The accumulation of Cyclin A protein in encore mutant germaria can also be observed in western blots (Fig. 2H). Increased levels of Cyclin A protein are observed in germaria-enriched extracts from *encore* mutant ovaries compared with wild type. These data suggest that *encore* mutations at the restrictive temperature of 29°C promote the accumulation and/or prevent the timely turnover of Cyclin A protein in the germarium.

Reduction of Cyclin A protein by overexpression of the Cyclin A inhibitor Roughex suppresses the *encore* extra division phenotype

Overexpression of a *Cyclin A* transgene under the control of heat shock promoter results in an extra mitotic division in only 3% of the egg chambers. However, expression of a stable form of Cyclin A increases the number of egg chambers containing 32 rather than 16 cells to 17% (Lilly et al., 2000). In our hands, the expression of the *HS-Cyclin A* transgene causes an extra mitotic division in 4% of egg chambers. Given these results and the observed accumulation of Cyclin A protein in *encore* mutant germaria, we wanted to find out whether Cyclin A is responsible for the extra mitotic division phenotype. Reduction of *Cyclin A* gene dose by half in an *encore* mutant background does not suppress the extra division phenotype (Fig. 2G). One reason for this result could be that one copy of the *Cyclin A* gene might produce enough protein to allow an extra cell division. Another possibility is that reduction of *Cyclin A* gene dose is compensated by turning on feedback mechanisms that affect the production or stability of Cyclin A. In order to circumvent this problem we took a different approach to decreasing the amounts of Cyclin A protein by over-expressing the Cyclin A inhibitor, Roughex (Rux). The *rux* gene product binds to Cyclin A and this complex is then transported to the nuclei where it is destroyed via the UPS (Sprenger et al., 1997;

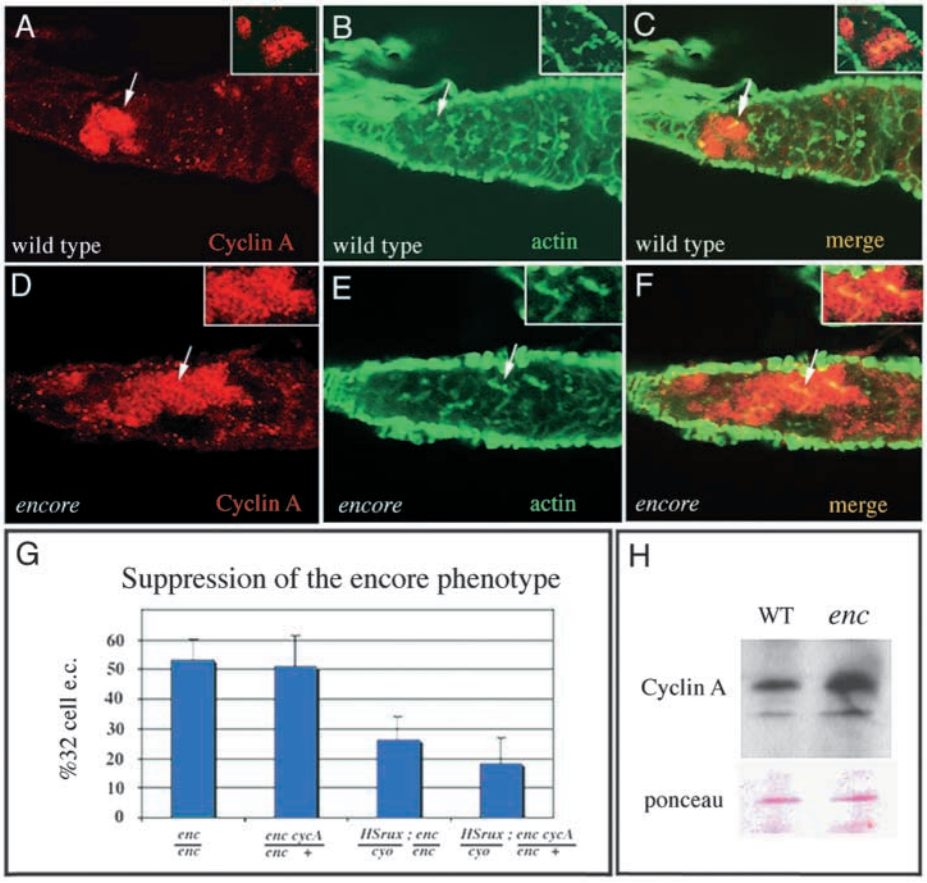

Fig. 2. Cyclin A promotes the *encore* extra division phenotype. (A-C) In wild-type germaria Cyclin A (red) associates with the fusome (green stained with phalloidin for actin) in dividing cystoblasts (arrow and inset). (D-F) Association of Cyclin A with the fusome in *encore* mutant germaria persists in posterior regions of the germarium (arrow and inset). (G) *encore* mutant females raised at 25°C produce 55% of the egg chambers with 32 cells. Reducing *Cyclin A* gene dose has no effect on the *encore* cell division phenotype. However overexpression of the *HS-rux* transgene in an *encore* mutant background reduces the 32 cells egg chamber phenotype from 55 to 25%. Additional reduction of *Cyclin A* gene dose results in 20% of the egg chambers containing 32 cells. (H) Western blot showing that the amount of Cyclin A protein in *encore* mutant extract is increased compared with the wild-type extract. Equivalent amounts of germaria-enriched extract were loaded in both lines as seen with the protein dye ponceau (pink).

Thomas et al., 1997; Foley et al., 1999; Avedisov et al., 2000). Overexpression of Rux using a *HS-rux* transgene gives rise to a reduced number of mitotic divisions. Similarly, in *Drosophila* embryonic ectoderm and imaginal discs (Thomas et al., 1997), expression of the *HS-rux* transgene at 37°C reduces the number of mitoses. The expression of the *HS-rux* transgene alone or in an *encore* heterozygous mutant background at 25°C had no effect on mitosis. Flies expressing the *HS-rux* transgene in an *encore* mutant background were raised at 25°C. In this experiment about 55% of the *encore* mutant control ovaries contained 32 cell egg chambers (Fig. 2G). The mild expression of Rux resulted in the suppression of the extra mitotic division phenotype such that only 25% of the egg chambers showed the *encore* phenotype. The extent of the *HS-rux* suppression did not vary significantly when in addition to expressing *HS-rux*, *cyclin A* gene dose was reduced by half. Thus, it seems that the extra Cyclin A protein present in the *encore* mutant germarium contributes to the promotion of an extra mitotic division.

Cyclin E protein expression is altered in *encore* mutant germaria

In hypomorphic mutations of the *Cyclin E* gene, 30% of the egg chambers have only eight cells (Lilly and Spradling, 1996). Conversely, expression of a heat inducible *cyclin E* transgene induces entry to S phase and results in an extra round of mitosis in the *Drosophila* embryo and eye imaginal disc (Knoblich et al., 1994; Richardson et al., 1995). When we express the *HS-cyclin E* transgene, a modest 6% of egg chambers produce an extra cell division. To assess Cyclin E protein expression in *encore* mutant ovaries, immunolocalization experiments were carried out on flies raised at 29°C. In wild-type germaria, Cyclin E protein is expressed in the nuclei of the germline stem cells, cystoblasts and dividing cysts (Fig. 3A). The protein levels are dramatically reduced after mitosis ends. There is no Cyclin E expression in postmitotic cysts of region 2A and it is absent in region 2B of the germarium. Cyclin E protein expression resumes in region 3 and persists throughout the rest of oogenesis. However, this second phase of Cyclin E protein expression is no longer synchronized as not all the cells in the egg chamber express Cyclin E simultaneously (Fig. 3A). This pattern of expression is in accordance with BrdU incorporation experiments (McKearin and Ohlstein, 1995) that indicate the requirement for Cyclin E during S phase of the mitotic cycle and of the endocycle. In *encore* mutant ovaries, Cyclin E protein is expressed throughout the germarium, indicating that the mechanism of downregulation of Cyclin E at stage 2A and 2B is defective (Fig. 3B). Cyclin E is degraded during S phase in the cell cycle (Follette and O'Farrell, 1997). Thus, in wild-type germaria, its expression oscillates and not all the cysts in region 1 express Cyclin E simultaneously (Fig. 3A, inset). In *encore* mutant germaria, all cysts express some Cyclin E, indicating that at each cell division Cyclin E degradation is affected (Fig. 3B, inset). The unsynchronized expression of Cyclin E in region 3 and later is comparable with wild-type ovaries. The Cyclin E protein expression in wild-type germaria suggests that cessation of mitosis in the ovary requires Cyclin E downregulation. Thus, we tested if the persistent expression of Cyclin E in *encore* mutant germaria can promote the extra

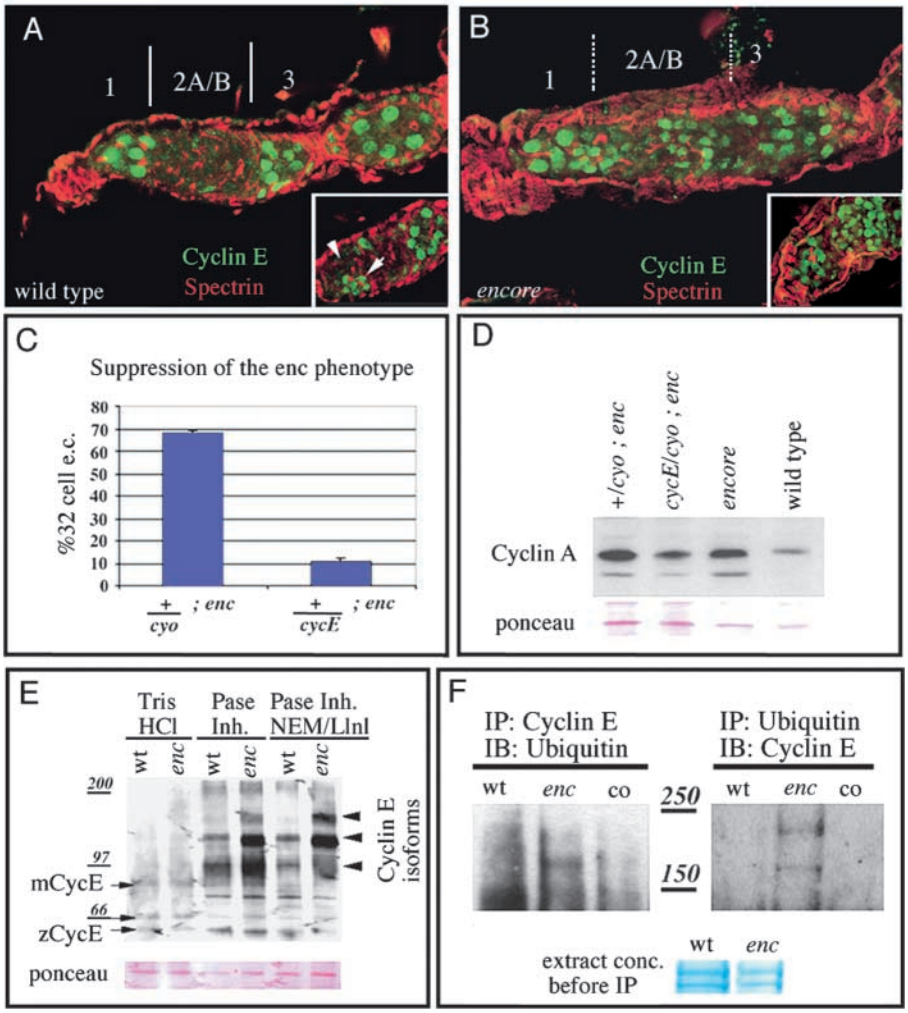

Fig. 3. Expression of Cyclin E in the germarium. (A,B) Spectrin (red) marks the spectrosome in the germline stem cell and the fusome in the developing cyst. (A) Cyclin E protein expression (green) in the wild-type ovary is observed in region 1 of the germarium and sharply decreases in regions 2A and 2B. Asynchronous expression of Cyclin E resumes in region 3 and persists in the vitellarium. In wild-type germaria, Cyclin E expression oscillates strongly (inset): some cysts show Cyclin E expression (arrow), while other cysts are depleted of Cyclin E protein (arrowhead). (B) In *encore* mutant germaria, Cyclin E expression is observed throughout the germarium. There is no downregulation in region 2A/B. In *encore* mutant germaria, all cysts express some Cyclin E protein all the time (inset). (C) Reducing *cyclin E* gene dose in an *encore* mutant background suppresses the *encore* 32-cell egg chamber phenotype from 70 to 10% in females raised at 29°C. (D-F) Western blots of germaria-enriched extracts. (D) The accumulation of Cyclin A observed in *encore* mutant females can be partly restored to more normal levels by reducing *cyclin E* gene dose. The blot was stained with the protein dye ponceau (pink) as loading control. (E) The inhibition of proteolysis (Pase Inh.) or/and 19S-RP isopeptidase activity (NEM/Llnl) reveals that in *encore* mutant extracts Cyclin E accumulates as a high molecular form protein (arrowheads). The blot was stained with the protein dye ponceau (pink) as loading control. (F) Immunoprecipitation assays using anti-*Drosophila* Cyclin E antibodies followed by IB using anti-Ubiquitin antibodies shows more Cyclin E-Ub_n in the *encore* mutant extract lane compared with wild type. The reciprocal immunoprecipitation assay shows more Cyclin E-Ub_n in the *encore* mutant lane. Extract concentration of the starting material is shown stained with Coloidal blue Coomassie. The control lane (co) represents immunoprecipitation using wild-type extracts and an unrelated antibody raised in the same animal as the immunoprecipitation test.

division phenotype. Double mutant females homozygous for *encore* and heterozygous for *Cyclin E* were raised at 29°C. We observed that indeed the *encore* extra division phenotype is suppressed from 70% to 10% in the double mutant females ovaries (Fig. 3C). The suppression of the *encore* phenotype is more pronounced when *Cyclin E* gene dose is reduced than when Cyclin A activity is reduced. It has been shown that Cyclin E overexpression can promote the accumulation of Cyclins A and B in the *Drosophila* embryonic ectoderm without affecting RNA levels (Knoblich et al., 1994; Lane et al., 1996). As S-phase progresses and Cyclin E expression increases, the CycE/Cdk2 complex promotes the destruction of the Rux protein (Thomas et al., 1997; Sprenger et al., 1997; Avedisov et al., 2000) and allows Cyclin A to accumulate in the cell. We tested whether the accumulation of Cyclin A in *encore* mutant germaria is a consequence of the abnormal expression of Cyclin E. Reduction of *cyclin E* gene dose by half in an *encore* mutant background clearly reduces the accumulation of Cyclin A protein (Fig. 3D). Given these results, we propose that the persistent expression of Cyclin E in the *encore* mutant germaria causes the accumulation of Cyclin A. Reducing Cyclin E dose brings Cyclin A expression down to more normal levels resulting in suppression of the extra mitotic division. Because reducing Cyclin A activity levels has only a partial effect in suppressing the extra division, we believe that the extra round of mitosis produced by Encore is promoted by the joint effects of accumulating Cyclin E and Cyclin A proteins.

Cyclin E protein turnover is defective in the *encore* mutant germarium

An important feature of the cell cycle is the tight regulation of the Cyclin/Cdk complexes by the rapid and timely destruction of the cyclin partner. We wanted to test whether the persistence of Cyclin E protein in *encore* mutant germaria results from improper degradation. Female flies were raised at 29°C and western blots of extracts enriched for germaria and pre-vitellogenic egg chambers were performed. These experiments revealed some differences between the expression of Cyclin E in the wild-type and *encore* mutant ovaries (Fig. 3E). In

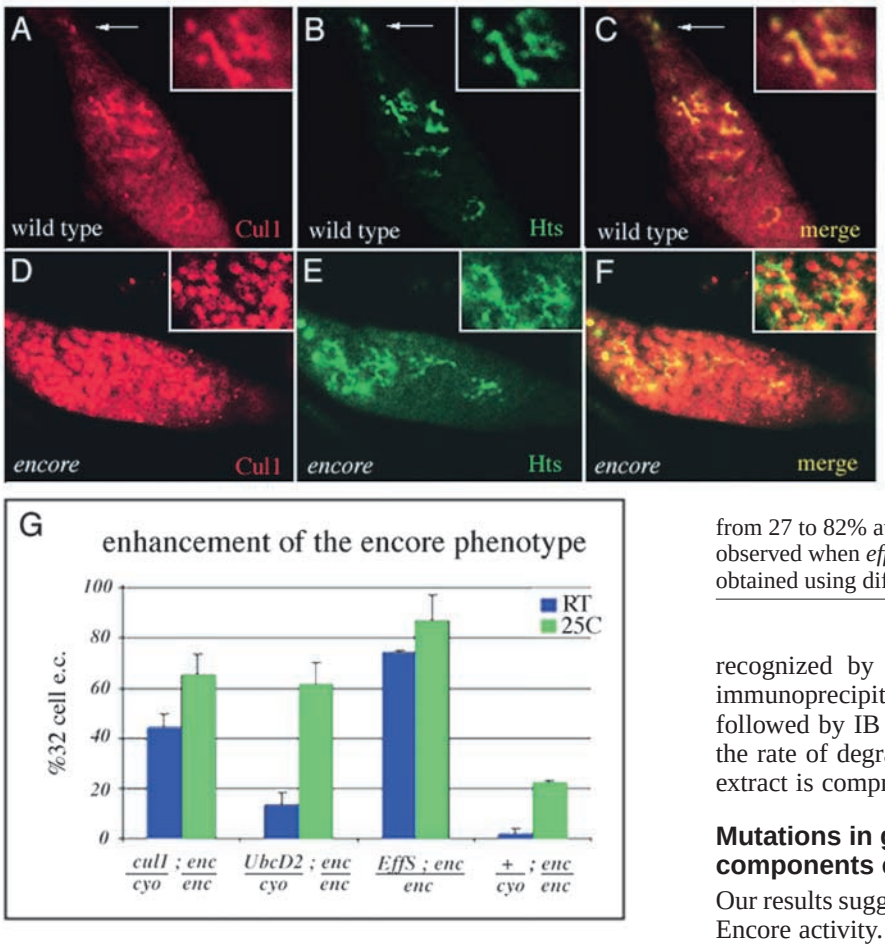

Fig. 4. Expression of Cul1 in the *Drosophila* germarium. (A-C) The Cul1 protein (red) is a nuclear protein but in the wild-type germarium mostly associates with the spectrosome (arrow) and the fusome (stained with antibodies against the Hts protein, green). (D-F) In *encore* mutant germaria, Cul1 association with the fusome is much-reduced (inset) and the Cul1 staining is prominently nuclear. Wild-type and *encore* mutant flies were raised at 29°C. (G) Genetic interactions between *encore* and *cul1*, *UbcD2* and *effete* alleles. Reduction of *cul1* gene dose in an *encore* mutant background enhances the 32-cell egg chamber phenotype from 27 to 65% at 25°C and from 3 to 44% at room temperature (RT). Enhancement of the phenotype from 27 to 60% at 25°C and from 3 to 17% at room temperature is also seen by reducing the gene dose of *UbcD2*. More dramatic enhancement from 27 to 82% at 25°C and from 3 to 75% at room temperature is observed when *effete* gene dose is reduced. Similar results were obtained using different *cul1* and *effete* alleles.

Drosophila, the *Cyclin E* transcript encodes two proteins: the zygotic and the maternal forms of Cyclin E, which are products of differential splicing (Richardson et al., 1993; Jones et al., 2000). The predicted molecular weight for the maternal Cyclin E protein is 78 kDa and for the zygotic Cyclin E is 60 kDa. Western blots using polyclonal antibodies against Cyclin E show that extracts from *encore* mutant ovaries accumulate high molecular weight forms of Cyclin E (Fig. 3E, arrowheads). Extracts made in the absence of protease inhibitors show little protein and no difference in Cyclin E expression between the *encore* mutant and wild-type extracts (arrows). The addition of protease inhibitors results in a stronger signal in both wild-type and *encore* and reveals a clear difference in Cyclin E levels between *encore* mutant and wild-type extracts. Cyclin E accumulates as high molecular weight protein in the *encore* mutant lane compared with wild-type extract lane. This observation suggests a slower rate of Cyclin E degradation in the *encore* mutant extract. The addition of isopeptidase inhibitors further protects these high molecular weight forms of Cyclin E. NEM and Llnl inhibit the action of the proteasome 19S-RP isopeptidases that de-ubiquitinate substrates. In order to confirm that the high molecular weight bands are Cyclin E-Ub_n, germaria-enriched extracts were prepared in the presence of protease inhibitors, NEM and Llnl. Immunoprecipitation assays were performed using antibodies against Cyclin E followed by immunoblot (IB) with antibodies against Ubiquitin (Fig. 3F). Cyclin E in the wild-type extract and the accumulated Cyclin E in the *encore* mutant extract are recognized by antibodies against ubiquitin. The reciprocal immunoprecipitation using antibodies against Ubiquitin followed by IB with antibodies against Cyclin E confirm that the rate of degradation of Cyclin E-Ub_n in the *encore* mutant extract is compromised.

Mutations in genes encoding SCF pathway components enhance *encore*'s mitotic phenotype

Our results suggest that proper destruction of Cyclin E requires Encore activity. In order to test this idea further, we generated double mutant flies using *encore* mutations and mutations in genes that encode for components of the SCF ubiquitination pathway. Mutations in the *cul1* and *archipelago* (*Ago*) genes result in the accumulation of Cyclin E in mammals and in the *Drosophila* eye (Wang et al., 1999; Dealy et al., 1999; Moberg et al., 2001; Koepp et al., 2001). As expected, reduction of the *cul1* gene dose in an *encore* mutant background enhances the extra division phenotype from 27 to 65% at the mildly restrictive temperature of 25°C (Fig. 4G). Moreover *cul1* mutations enhance the *encore* phenotype at room temperature from 3% to 44%. Thus, the reduction of Encore activity sensitizes the system such that even at room temperature, the proteolysis machinery can no longer ensure the destruction of Cyclin E when *cul1* gene dose is reduced. Similar results were obtained with mutations in the Ubiquitin ligase component *UbcD2* and *effete* (*UbcD1*) (Fig. 4G). Reducing *cul1* gene dose in an *encore* heterozygous background produces only 16-cell egg chambers. We propose that the reduction of Cul1 results in decreased degradation efficiency and accumulation of Cyclin E. In this situation, Encore is required to facilitate the destruction of the surplus Cyclin E. As expected for a component of the SCF-UPS, the *Drosophila* Cul1 protein is a nuclear protein and is expressed throughout oogenesis (Filippov et al., 2000). Cul1 protein expression in the germaria of wild-type females raised at room temperature or 29°C, shows strong localization to the fusome (Fig. 4A-C, inset). Unlike Cyclin A, Cul1 association with the fusome is not transient. Cul1 protein is observed in the germline stem cells in association with the spectrosome (Fig. 4A-C, arrow). During

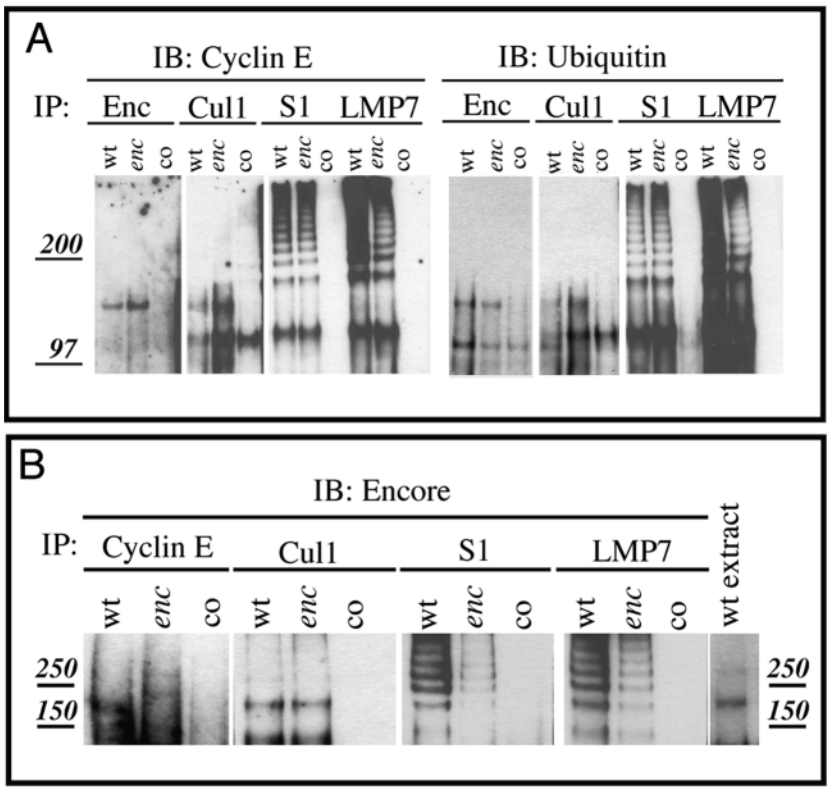

Fig. 5. Encore associates with Cyclin E, Cul1, the proteasome 19S-RP subunit S1 and the proteasome 20S-CP LMP7. (A) Immunoblot (IB) using antibodies against Cyclin E and Ubiquitin showing that Cul1 and Encore can immunoprecipitate Cyclin E-Ub_n. Immunoprecipitation with S1 and LMP7 precipitates several polyubiquitinated Cyclin E forms. The control lane (co) refers to immunoprecipitations that use unrelated antibodies raised in the same animal as the antibody used for the immunoprecipitation test. (B) IB using antibodies against Encore shows that Cyclin E, Cul1, S1 and LMP7 associate with Encore in the ovary. Notice that the amounts of immunoprecipitated protein in the Cul1 panel are comparable in wild-type and *encore* mutant extracts. The S1 and LMP7 immunoprecipitation lanes show that several forms of Encore associates with S1 and LMP7. The wild-type extract lane indicates the position of Encore in the gel. The extracts were run in 7%Tris-Acetate Nupage gel (Invitrogen).

cystoblast division and up to region three of the germarium Cul1 can be seen in association with fusome. In *encore* mutant germaria of flies raised at 29°C, the association of Cul1 with the fusome is disrupted. There is more nuclear Cul1 staining in *encore* mutant germaria compared with the wild type (Fig. 4D-F, inset). We have performed western blots using germarium-enriched extract and do not observe any difference between the levels of Cul1 in wild-type and *encore* mutant extracts (data not shown). This suggests that Encore activity is required for proper Cul1 localization to the fusome and that Cul1 association with the fusome may be important for proper Cyclin E processing. It also indicates that possibly degradation of this important cell cycle regulator occurs at the fusome.

Encore associates with components of the SCF-proteasome degradation system

At higher temperatures, the rate of the ubiquitin-proteasome dependent proteolysis decreases (Kuckelkorn et al., 2000) and polyubiquitinated proteins may require the activity of ancillary proteins for proper substrate presentation and destruction by the proteasome (Bercovich et al., 1997; Hohfeld et al., 2001; Wiederkehr et al., 2002). Our results indicate that Encore has a role in facilitating the destruction of Cyclin E and suggest a possible physical interaction between Encore, Cyclin E and components of the SCF pathway. We performed immunoprecipitation assays followed by western blots using antibodies directed against the *Drosophila* Cyclin E, Encore, Cul1 and against the mammalian proteasome 19S-RP subunit S1 and the proteasome 20S-CP subunit LMP7. We found that Encore can immunoprecipitate Cyclin E and that antibodies against Ubiquitin recognize this protein (Fig. 5A). Unexpectedly, antibodies against Encore can immunoprecipitate Cyclin E in *encore* mutant extracts. These immunoprecipitation experiments were performed using the point mutant allele enc^{Q4} and the P-element insertion allele enc^{BB}. Both *encore* mutant alleles produce protein (not shown). Encore and Cul1 antibodies can immunoprecipitate the same Cyclin E-Ub_n and there is more Cyclin E-Ub_n immunoprecipitated in the *encore* lanes compared with the wild-type lanes. Significantly, Cyclin E and Cul1 can associate with Encore (Fig. 5B). These observations suggest that Cyclin E-Ub_n, Cul1 and Encore can form a complex. By contrast, the anti-Encore antibody did not precipitate Fizzy, Cyclin A or B (data not shown). Antibodies against the S1 and LMP7 proteins immunoprecipitate several Cyclin E-Ub_n forms of the same (5A longer exposure) and higher molecular weight than the Cyclin E-Ub_n associated with Cul1 and Encore. LMP7 seems to bring down more Cyclin E-Ub_n in wild-type extracts compared with the *encore* mutant extracts. Antibodies against S1 and LMP7 can immunoprecipitate several Encore forms, suggesting that Encore may be part of a complex formed by the SCF-proteasome system and that Encore may be a substrate for UPS (Fig. 5B). The fact that Encore can be seen in the mutant and wild-type lanes suggests that the defective protein can still form a complex with SCF-proteasome components. At the moment we do not have a protein null *encore* mutation and therefore we do not know whether Encore is required for complex formation in the germarium.

The proteasome 19S subunit S1 expression is defective in *encore* mutant germaria

The localization of Cul1 to the fusome suggests that perhaps Cyclin E and other SCF-UPS substrates may be degraded at the fusome. To test whether the proteasome is also localized to the fusome, immunostaining assays were performed using antibodies against the proteasome 19S-RP subunit S1. Indeed the 19S-S1 colocalizes with the fusome in wild-type germaria (Fig. 6A-C). 19S-S1 association with the fusome is incomplete (arrowheads), as not all the fusomes are associated

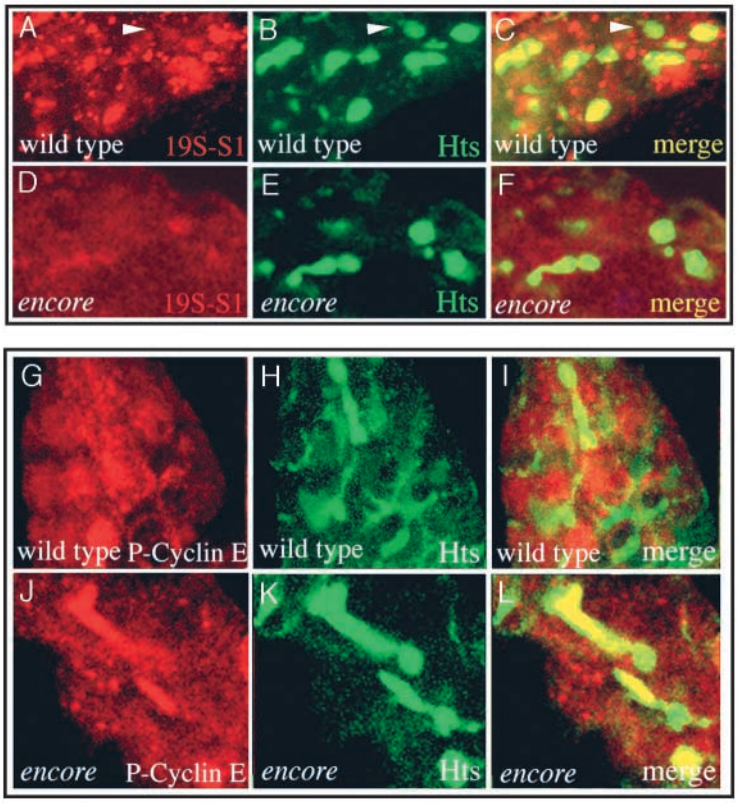

Fig. 6. Expression of the 19S-RP subunit S1 and P-Cyclin E in the *Drosophila* germarium. (A-C) 19S-S1 (red) localizes to the fusome (green, stained with Hts). Some fusomes are free of 19S-S1 staining (arrowhead). S1 is also accumulated in a granular manner in the rest of the germarium. (D-F) 19S-S1 expression in *encore* mutants is reduced; however, some S1 protein can still by observed in the mutant germarium. (G-I) In wild-type germaria, P-Cyclin E (red) localizes to the fusome (green). The expression is transient as some fusomes express some P-Cyclin E and others do not. (J-L) In *encore* mutant germaria, P-Cyclin E is more often observed localized to the fusome.

with S1. Unlike Cul1, S1 seems to be associated only with some areas of the fusome. Moreover there is also S1 accumulation in a granular appearance in the rest of the germarium. In *encore* mutant ovaries of flies raised at the restrictive temperature of 29°C, S1 expression is very much reduced (Fig. 6D-F). However some S1 protein can still be seen localized to the fusome. Thus, it seems that Cul1, 19S-S1 and presumably the rest of the 19S-RP associates with the fusome. The strong reduction of Cul1 localization to the fusome in *encore* mutant germaria could result in inefficient recruitment of the 19S-RP and the rest of the proteolytic machinery.

Phosphorylated Cyclin E association with the fusome is defective in *encore* mutant germaria

Cyclin E/Cdk2 activity is regulated by auto-phosphorylation of its regulatory subunit, Cyclin E (Clurman et al., 1996). Phosphorylation of Cyclin E results in the disassembly of the Cdk2/Cyclin E complexes, follow by ubiquitination and destruction via the SCF-UPS. Our results predict that phosphorylated Cyclin E (P-Cyclin E) would be degraded at the fusome. Using an anti-P-Cyclin E antibody we found that P-Cyclin E is expressed at the tip of the wild-type germarium in region 1 and it associates with the fusome (Fig. 6G-I). Some cysts contain very high levels of P-Cyclin E that fill the cyst completely, other cysts express intermediate levels or no P-Cyclin E. Unlike expression of Cul1, P-Cyclin E association with the fusome is not observed in all cysts suggesting some periodicity. In *encore* mutant germaria of flies raised at 29°C, P-Cyclin E is present in most of the fusomes observed in a given germarium (Fig. 6J-L). These observations suggest that in *encore* mutant germaria, P-Cyclin E localization to the fusome occurs and because the degradation process is inefficient, more P-Cyclin E accumulates at the fusome.

The Ubiquitin-proteasome pathway requires Encore activity for proper protein turnover

Our data shows that Cyclin E can be ubiquitinated in *encore* mutant germaria and that the defect resides in the destruction of Cyclin E-Ub_n. Polyubiquitinated proteins are recognized by the proteasome 19S-RP, de-ubiquitinated by resident isopeptidases and unfolded before being destroyed by the proteasome 20S-CP (Cope et al., 2002; Verma et al., 2002). In order to test whether *encore* mutations affect the activity of the proteasome 20S-CP subunit, we measured the rate of proteolysis in wild type and *encore* mutant germaria-enriched extracts. The peptidase activity was monitored by the hydrolysis of the fluorescent-labeled peptide Suc-LLVY-MCA (Glass et al., 1998). The results show that rate of proteolysis in *encore* mutant extract of females raised at 29°C or room temperature is comparable to that of wild type extracts of flies raised at 29°C (Fig. 7A). These results suggest that Encore does not affect the peptidase activity of the proteasome and that the defect may reside in substrate recognition, the formation of an inactive complex and/or in subcellular localization of the proteolysis machinery. We also tested the requirement of Encore for proper proteolysis using an exogenous mammalian protein (Fig. 7B). Commercially available histidine-tagged P27 was ubiquitinated in vitro. The necessary E1, E2 and E3 enzymes for the ubiquitination reaction were provided by germaria-enriched extracts. There was no difference in ubiquitination efficiency between wild-type and *encore* mutant extracts (Fig. 7B, left). Equal number and amount of ubiquitinated p27 bands are observed in both extracts. The P27-Ub_n was then purified using nickel-agarose beads and a de-ubiquitination reaction was performed using either wild-type or *encore* mutant germaria-enriched extract to provide the proteolysis machinery. Time zero is the P27-Ub_n from the wild-type ubiquitination reaction (Fig. 7B, left). De-ubiquitination and destruction of p27 was compromised when *encore* mutant extracts were used (Fig. 7B, right). In the wild-type situation, after 20 minutes of incubation most of the ubiquitinated p27 had disappeared. By contrast, after 1 hour, ubiquitinated p27 can still be detected in the reaction using *encore* mutant extracts.

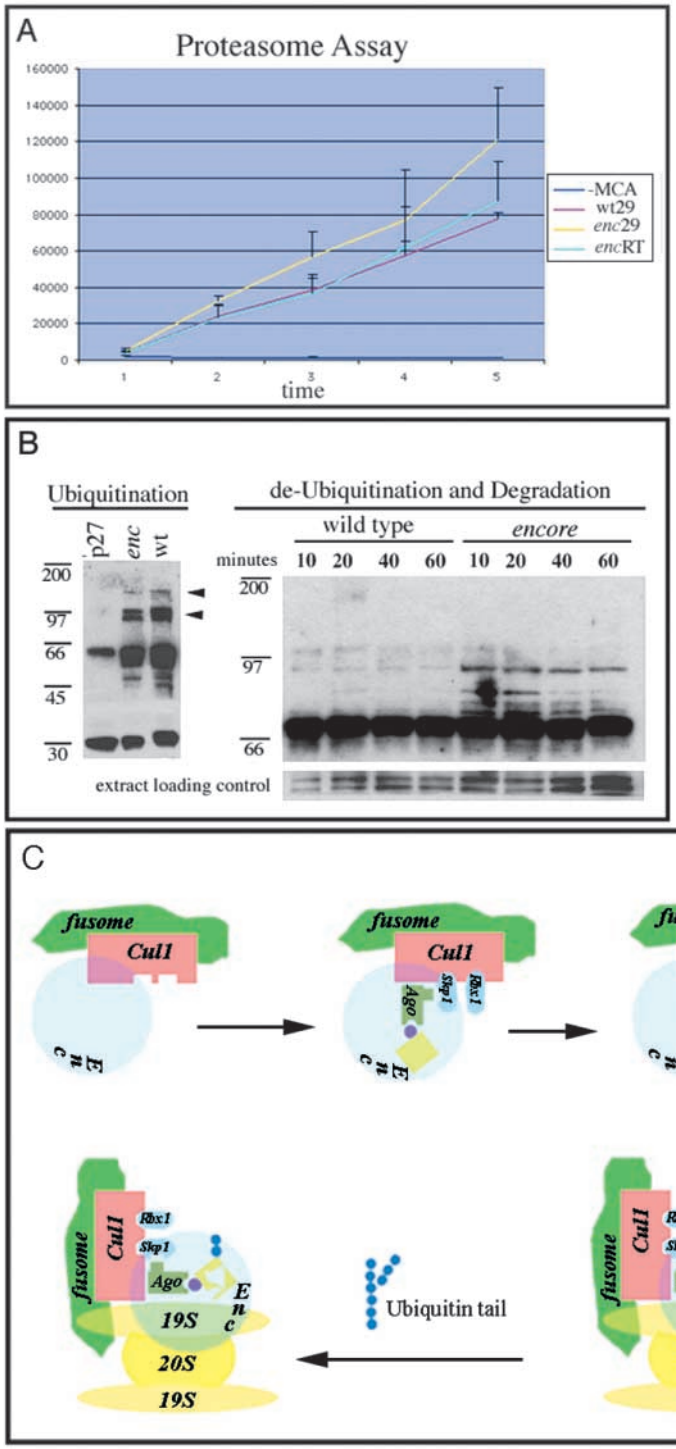

Fig. 7. Encore is required for proteolysis. (A) Proteasome assays measuring the hydrolysis of the fluorogenic peptide Suc-LLVY-MCA. The 20S-CP proteolysis activity is not significantly compromised in *encore* mutant germarium-enriched extracts at 29°C (yellow) or room temperature (light blue) compared with wild-type extracts at 29°C (pink). Control assays contain no substrate peptide (dark blue). (B) Western blot using anti p27 antibodies show that Ubiquitination reactions produce the same polyubiquitinated p27 forms (arrowheads) in wild-type and *encore* mutant extracts. The deubiquitination and degradation reaction is much slower when *encore* mutant extract was used as a source of the degradation machinery. (C) Model of Encore function. Cul1 (pink) is localized to the fusome (green) in an Encore (blue)-dependent manner (Encore may directly or indirectly modify Cul1 and thus influence its subcellular localization). Cul1 may serve as an anchor where the SCF E3 complex is assembled and a phosphorylated substrate (yellow) is recognized and ubiquitinated. The polyubiquitinated substrate is then recognized by the 19S-recognition particle. 19S-RP and presumably the 20S-core particle are recruited to the fusome where the substrate is de-ubiquitinated, unfolded and degraded by the 26S-subunit of the proteasome.

Discussion

Cyclin E proteolysis and exit of mitosis in the *Drosophila* germarium

Cell cycle transition is mainly driven by Cdk activity, which is carefully regulated by the levels of the cyclin subunits, by CKI and by Cdk post-translational modifications. Presumably, cyclin proteolysis is important for ensuring the sharp decrease in Cyclin E expression after the fourth mitosis which allows exit from the cell division program. Our genetic, biochemical and immunostaining results indicate that in *encore* mutant germaria Cyclin E proteolysis is defective which creates a surplus of Cyclin E that induces the cysts to undergo an extra cell division. Much of the accumulated Cyclin E is polyubiquitinated. Cyclin A also accumulates in *encore* mutant germaria possibly in part as a result of defective Cyclin E proteolysis. Reduction of Cyclin A activity can partially suppress the *encore* extra mitosis phenotype. We think it likely that the extra cell division is due to the combined effect of surplus Cyclin E, Cyclin A and other proteins such as Bam (Hawkins et al., 1996). *encore* mutant ovaries containing additional mutations in genes coding for the ubiquitin-proteasome components such as *Cul1*, *effete* and *UbcD2* show enhancement of the extra division phenotype. We believe that this is the result of slower destruction of Cyclin E and consequent protein accumulation. The fact that the extra division phenotype can be observed at room temperature in these double mutants suggests that Encore is involved in the proper destruction of Cyclin E and perhaps other proteins degraded by the SCF pathway. Lilly et al. (Lilly et al., 2000) showed that mutations in the E2 *UbcD1* gene produce egg chambers with an extra round of mitosis and that reducing *Cyclin A* and *Cyclin B* but not *Cyclin E* gene dose suppresses the extra division phenotype. It therefore appears that reducing *Cyclin E* dose in an *UdcD1* mutant background still leaves enough active Cyclin E to promote an extra mitosis, whereas in the *encore* mutant background the increased levels of active Cyclin E may be closer to a threshold.

The fusome is a regulator of cell division during early oogenesis

Some of the functions ascribed to the fusome are to synchronize cyst mitosis and to provide the scaffold for the transport system necessary for oocyte determination (Lin et al., 1994; McGrail and Hays, 1997; Deng and Lin, 1997). Limiting the number of cell divisions in the germarium could be achieved by regulating the association of proteins such as the cyclins and/or other cell cycle regulators with the fusome. The expression pattern of Cyclin A, Cul1, P-Cyclin E and 19S-S1 proteins in the germarium supports the idea that the fusome plays an important role in the regulation of mitosis. Indeed, Cyclin A association with the fusome is transient and occurs only during cyst division (Lilly et al., 2000). In *encore* mutant germaria, Cyclin A remains associated with the fusome after cell division has stopped. Cul1 localization to the fusome suggests that the rest of the SCF complex also associates with the fusome and that substrate ubiquitination may happen at the fusome (Fig. 7C). We have shown that the SCF component Cul1 is mainly associated with the fusome in the wild-type germaria. In *encore* mutant germaria, Cul1 localization to the fusome is very poor, leading us to propose that this may be one reason why Cyclin E is not degraded properly. This also suggests that the degradation of Cyclin E and perhaps of other proteins degraded by the SCF-UPS may occur at the fusome. The association of P-Cyclin E supports this idea. The localization of P-Cyclin E in the wild type seems to be dynamic, consistent with the idea that the phosphorylated substrate is localized to the fusome, and then rapidly degraded via the SCF-UPS. In *encore* mutant germaria, the poor localization of Cul1 may result in an inefficient assembly of SCF complexes at the fusome. P-Cyclin E is localized to the fusome, but its degradation is compromised and as a result we observed a consistent expression of P-Cyclin E at the fusome. The partial association of the proteasome 19S-RP subunit S1 to the fusome supports the idea that proteolysis may occur at the fusome. The proteasome 19S-RP would recognize the polyubiquitinated substrate and recruit the rest of the proteasome to the fusome (Fig. 7C).

The role of Encore on facilitating Cyclin E proteolysis

Our results suggest that Encore can associate with the SCF-UPS machinery and assists with the degradation of Cyclin E and perhaps other SCF substrates. As the mutant Encore protein can still interact with SCF-UPS components, the mutant protein may form complexes but these might be inactive and/or the mutant protein poisons the degradation machinery. Consistent with such a hypothesis, the *encore* extra cell division phenotype is milder in hemizygous versus homozygous females at 25°C (Hawkins et al., 1996). Encore is required for the proper localization of Cul1, P-Cyclin E, S1 and presumably the rest of the proteolysis complex to the fusome. This localization may be more crucial at 29°C, whereas at lower temperatures a less efficient degradation system may have enough time for normal cell cycle regulation. *encore* mutations do not affect the 20S-Core Particle activity as measured by the rate of degradation of a fluorogenic peptide. We do not know whether Encore retains Cul1 at the fusome or whether Encore directly or indirectly modifies Cul1 in order to promote its localization at the fusome. Cul1 is known to be modified by the addition of Nedd8 (Furukawa et al., 2000); however, Cul1 seems to be equally neddylated in *encore* and wild-type ovary extracts (data not shown).

In summary, our results suggest that the Encore protein assists with proper cell cycle progression in the *Drosophila* germarium by ensuring that Cul1 and the proteolysis machinery is localized at the mitosis coordination center, the fusome.

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