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Spatial regulation of APC^{Cdh1}-induced cyclin B1 degradation maintains G2 arrest in mouse oocytes

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SUMMARY

Within the mammalian ovary, oocytes remain arrested at G2 for several years. Then a peri-ovulatory hormonal cue triggers meiotic resumption by releasing an inhibitory phosphorylation on the kinase Cdk1. G2 arrest, however, also requires control in the concentrations of the Cdk1-binding partner cyclin B1, a process achieved by anaphase-promoting complex (APC^{Cdh1}) activity, which ubiquitylates and so targets cyclin B1 for degradation. Thus, APC^{Cdh1} activity prevents precocious meiotic entry by promoting cyclin B1 degradation. However, it remains unresolved how cyclin B1 levels are suppressed sufficiently to maintain arrest but not so low that they make oocytes hormonally insensitive. Here, we examined spatial control of this process by determining the intracellular location of the proteins involved and using nuclear-targeted cyclin B1. We found that raising nuclear cyclin B1 concentrations, an event normally observed in the minutes before nuclear envelope breakdown, was a very effective method of inducing the G2/M transition. Oocytes expressed only the α-isoform of Cdh1, which was predominantly nuclear, as were Cdc27 and Psmd11, core components of the APC and the 26S proteasome, respectively. Furthermore, APC^{Cdh1} activity appeared higher in the nucleus, as nuclear-targeted cyclin B1 was degraded at twice the rate of wild-type cyclin B1. We propose a simple spatial model of G2 arrest in which nuclear APC^{Cdh1}-proteasomal activity guards against any cyclin B1 accumulation mediated by nuclear import.

KEY WORDS: Meiosis, Mouse, Oocyte, Cdh1, Fzr1, Anaphase-promoting complex, Cyclin B1

INTRODUCTION

Oocytes from many species have a unique cell cycle arrest at dictyate prophase I, equivalent to G2 (Jones, 2008; Mehlmann, 2005). For mammalian oocytes, arrest is broken only in the hours preceding ovulation when a luteinizing hormone (LH) rise triggers nuclear envelope breakdown (NEB) and entry into the first meiotic division. G2 arrest is maintained by high intracellular concentrations of cyclic adenosine monophosphate (cAMP), which stimulate protein kinase A (PKA) (Conti et al., 1998; Eppig, 1989), and LH acts to reduce cAMP possibly by increasing phosphodiesterase activity (Norris et al., 2008; Norris et al., 2009). Oocytes are able to resume meiosis when removed from the ovary because of an inability to sustain cAMP concentrations, but pharmacological agents able to raise PKA activity, such as milrinone, a phosphodiesterase inhibitor (Tsafriri et al., 1996), can maintain oocytes in G2 arrest in culture.

PKA-mediated G2 arrest is mediated by inhibition of the kinase activity of M-phase promoting factor. This kinase, which is implicated in the G2/M transition of all cells, is a heterodimer composed of a catalytic Cdk1 and, in mammals, a regulatory cyclin B1 subunit (hereafter Cdk1/cyclin B1). Inhibition of Cdk1/cyclin B1 during G2 is through activation of Cdk1-inhibiting kinases (Myt1/Wee1) and suppression of Cdk1-activating phosphatases (Cdc25), processes also necessary for G2 arrest in oocytes (Han et al., 2005; Han and Conti, 2006; Jones, 2004; Lincoln et al., 2002). There is also spatial regulation of Cdk1/cyclin B1 because during interphase this heterodimer shuttles continuously between the nucleus and the cytoplasm through two potential mechanisms, both involving cyclin B1. Firstly, cyclin B1 can enter the nucleus by binding directly to Importin β (Moore et al., 1999; Takizawa et al., 1999), and secondly, at the G2/M transition by regulation of a cytoplasmic retention signal (CRS) located in cyclin B1 (Hagting et al., 1999; Hagting et al., 1998). Phosphorylation of the CRS leads to a rapid accumulation of Cdk1/cyclin B1 in the nucleus in the minutes before NEB (Hagting et al., 1999), allowing it to phosphorylate nuclear lamins and induce NEB (Guttinger et al., 2009). Nuclear import during interphase is counteracted by an export signal dependent on the hydrophobic residues in the CRS (Hagting et al., 1998).

G2 arrest in mammalian oocytes is dependent not only on Cdk1 regulation, but also on controlling concentrations of cyclin B1. Cdh1-mediated activation of the APC induces cyclin B1 degradation during prophase I, and such APC^{Cdh1} activity is essential to maintain G2 arrest. APC^{Cdh1}-mediated cyclin B1 loss is slow in dynamics compared with the cyclin B1 degradation observed during the mitotic division; nonetheless it is an essential activity that if reduced leads to meiotic resumption in the majority of oocytes over a period of several hours (Reis et al., 2006). Although APC^{Cdh1} activity was first observed in mouse eggs and has been corroborated by a number of groups (Marangos and Carroll, 2008; Reis et al., 2006; Schindler and Schultz, 2009), it has also been observed recently in pig oocytes (Yamamuro et al., 2008), where again it is essential in maintaining arrest. This suggests that APC^{Cdh1} may be essential in G2 arrest for a number of mammalian species. It is important to note that although G2 arrest is often perceived as being primarily due to inhibitory Cdk1 phosphorylation, APC^{Cdh1} inhibition by itself can override any cAMP-imposed arrest (Reis et al., 2006).

APC^{Cdh1} activity in oocytes is likely to be finely balanced: not too high, which would lower cyclin B1 concentrations, making oocytes refractory to LH, but not too low so as to induce premature NEB and meiotic entry. In mouse oocytes recent work has shown that this is

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achieved by protein modulators of APC^{Cdh1} activity that can act to inhibit cyclin B1 degradation, such as Emi1 and securin (Pttg1 – Mouse Genome Informatics) (Marangos and Carroll, 2008; Marangos et al., 2007), or hasten it, for example Cdc14b (Schindler and Schultz, 2009). In this study we describe a spatial aspect to APC^{Cdh1}-mediated cyclin B1 degradation that until now has been overlooked. We find that NEB in the G2 oocyte is very sensitive to concentrations of nuclear cyclin B1 but that precocious NEB is hindered by the nucleus being the predominant site of the APC^{Cdh1}-proteasomal degradation machinery.

MATERIALS AND METHODS

Reagents

All reagents were obtained from Sigma-Aldrich unless otherwise specified.

Oocyte collection and culture

Germinal vesicle oocytes were collected from 3- to 4-week-old Quackenbush Swiss mice (University of Newcastle, Australia) 44-52 hours after intraperitoneal injection of 10 IU pregnant mares' serum gonadotrophin (Intervet). For bench handling, microinjections and imaging experiments, oocytes were cultured in M2 medium following mechanical removal of granulosa cells under mineral oil. For long-term incubation, oocytes were cultured in MEM alpha media with 20% FCS (Invitrogen) in a 5% CO_2 humidified incubator at 37°C. Milrinone (1 μM or 10 μM) was used to arrest G2 oocytes, and 35 μM cycloheximide was used to inhibit protein translation.

PCR

G2 oocytes (n=1300) were collected in M2 media and washed in phosphate-buffered saline containing 1% polyvinylpyrrolidine (PBS/PVP). cDNA was prepared from the oocytes in a minimal droplet of PBS/PVP wash using the Smart PCR cDNA synthesis kit (Clontech Laboratories) according to the manufacturers' instructions. Two microliters of cDNA was used in a PCR reaction and amplified with Platinum High Fidelity Taq (Invitrogen) at 55°C annealing temperature using the following primers: Fwd, 5′-TAGCGA-ATTCATGGACCAGGACTATGAGCG-3′ and Rev, 5′-TAGTCGACC-TATCGGATCCGGGTGAAGAGGTT-3′. Gel fragments stained with ethidium bromide were excised and purified before TA ligation into pGEM T-Easy vector for sequencing, or for re-amplification.

cRNA and morpholinos

Cyclin B1 cRNA was made using a modified pRN3 vector designed to produce protein C-terminally coupled to GFP. cRNA was synthesized using T3 mMESSAGE mMACHINE (Ambion), and dissolved in nuclease-free water to a concentration of approximately 1 μg/μl before microinjection. Morpholino to Cdh1 (Gene Tools LLC) was used at a micropipette concentration of 1 mM: 5′-CCTTCGCTCATAGTCCTGGTCCATG-3′. For experiments involving cyclin-GFP fluorescence measurement, with or without morpholino microinjection, oocytes were incubated for 24 hours in MEM alpha/20% FCS to achieve knockdown (Reis et al., 2007).

Microinjections and imaging

All microinjections into oocytes were made on the heated stage of a Nikon TE300 inverted microscope. Briefly, micropipettes were inserted into cells using the negative capacitance overcompensation facility on an electrophysiological amplifier. A single 0.1-0.3% volume injection was achieved using a timed injection on a Pneumatic PicoPump (World Precision Instruments). Brightfield and epi-fluorescence images were recorded using a Princeton Interline MicroMax CCD camera. Metamorph and Metafluor software (Molecular Devices, PA, USA) were used for image capture and data analysis.

Immunoblotting

Proteins were separated on a NuPage 10% gel (Invitrogen) using the manufacturer's sample buffer and instructions. Western blotting was performed using an XCell II Blot Module (Invitrogen). Blot was incubated in 5% skimmed milk before primary antibody addition: Cdh1 (ab3242 and ab77885; Abcam, 1:100/1:300) cyclin B1 (ab72; Abcam, 1:500); Psmd11 (ab66346; Abcam, 1:200), Cdc27 (ab10538; Abcam, 1:400) and Cdc16 (ab611891; Abcam 1:300), phospho-Ser147 cyclin B1 (ab60986; Abcam, 1:500) and secondary anti-mouse or anti-rabbit IgG HRP (DAKO) incubation was followed by use of ECL detection reagents (GE Healthcare) according to the manufacturers' instructions.

Immunofluorescence microscopy

Whole ovaries were fixed overnight in 4% paraformaldehyde before washing in 75% ethanol and paraffin embedding and sectioning. Sections were deparaffinized and rehydrated followed by antigen retrieval in boiled 10 mM Tris/1 mM EDTA/0.02% Tween (pH 9). Sections were blocked in 10% normal goat serum for 1 hour at room temperature and incubated with primary antibody at 4°C overnight, before incubation with anti-mouse IgG HRP (DAKO). At least 20 follicles per condition were examined from at least five mice. DAB liquid substrate was used for detection and Hematoxylin as a counterstain. Oocytes were fixed with 4% paraformaldehyde in PBS/PVP and then transferred to the same containing 2% Triton-X100. Fixing and permeabilizing were for 30 minutes each at room temperature. Oocytes were then washed extensively in PBS+PVP before blocking in 10% goat or rabbit serum before primary antibody incubation (anti-Cdh1 mouse monoclonal, ab3242, Abcam 1:30; anti-Cdc27 mouse monoclonal, ab10538, Abcam 1:50; anti-Psmd11 rabbit polyclonal, ab66346, Abcam, 1:5150. Following further washes oocytes were incubated with conjugated goat anti-mouse and rabbit Alexa Fluor 488 (Invitrogen). These incubations were at room temperature in PBS+PVP. For confocal imaging oocytes were mounted in SlowFade (Invitrogen). Images were acquired using an Olympus FV1000 equipped with a 60×/1.2 NA UPLSAPO oil immersion objective. All confocal images are representative equatorial scans on individual oocytes. They are not background subtracted, and are presented as 8-bit (0-255 arbitrary units) images.

Statistical analysis

All one-way ANOVA statistical analysis was performed using Minitab release 14, with a 95% or 99% confidence level and Fisher's post-hoc analysis. Yates' correction was used for chi-squared analysis.

RESULTS

Oocytes express only Cdh1α

In order to examine how Cdh1 maintains prophase I arrest in mouse oocytes (Reis et al., 2006) we first had to determine which splice variants are expressed in these cells. In humans, the single copy *Cdh1* gene (Chr.19p13.3) is alternatively spliced, generating two products that may have distinct functions as a result of differing intracellular targeting: Cdh1β remains cytoplasmic but Cdh1α is nuclear because of a nuclear localization signal (NLS) (Zhou et al., 2003a; Zhou et al., 2003b). We could not find a report characterizing in detail splice variants of mouse *Cdh1*.

To examine for *Cdh1* transcripts in mouse we used a cDNA library generated from G2-arrested oocytes, and compared its profile to that of heart, which if analogous to human would express both *Cdh1*α and β. In oocytes we could detect only *Cdh1*α, although, as predicted, both transcripts were found in heart (Fig. 1A,B). Heart *Cdh1*β (GenBank accession FJ786633), was smaller than its human homolog as a result of the removal of exons 4-7. In humans only exons 7 and 8 are alternatively spliced for *Cdh1*β (Zhou et al., 2003b) (see Fig. S1 in the supplementary material).

A nuclear APC ubiquitin-proteasomal machinery

By way of confirmation for the above PCRs, immunoblotting of oocytes was performed. A single band at the predicted size of Cdh1α was seen using two independently derived antibodies (Fig. 1C; and see Fig. S2 in the supplementary material) (55 kDa; Cdh1β is ~32 kDa). These data altogether therefore suggest that G2 mouse oocytes exclusively transcribe Cdh1α.

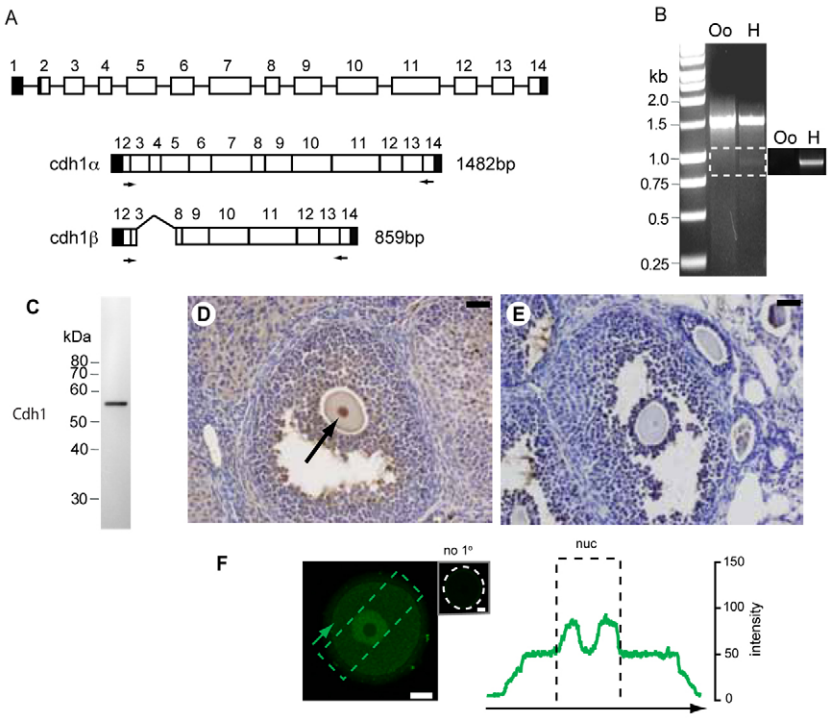

Fig. 1. Nuclear localization of Cdh1α. (**A**) (Top) The single copy murine *Cdh1* gene contains 14 exons (boxes; open reading frame white, untranslated region black). (Middle and bottom) Alternative splicing of the pre-mRNA yields Cdh1α and β. The arrows indicate the position of PCR primers used in this study. (**B**) RT-PCR on poly(A) mRNA from prophase oocytes (Oo) and heart (H), gives a prominent band at 1.5 kb in both, and a weak 0.9 kb band in heart (boxed). Re-amplification of PCR products in the 0.8-1 kb range (right) confirmed the heart-specific 0.9 kb band. Using sequencing, the 1.5 kb product was found to be *Cdh1*α, and the 0.9 kb product *Cdh1*β (see Fig. S1 in the supplementary material). (**C**) Immunoblot of prophase I oocytes using anti-Cdh1 (ab3242). Only a single protein band at 55 kDa was observed, the predicted size of Cdh1α. (**D**,**E**) Ovarian sections of young adult mice were immunostained for Cdh1 (D) or with secondary antibody only (E). (D) The arrow indicates the prominent nuclear staining in G2 oocytes. Nuclei are counterstained with Hematoxylin. (**F**) Confocal section of an oocyte immunostained for Cdh1. A boxed area across the oocyte has been drawn, and the mean 8-bit pixel intensity (0-255 arbitrary units) has been plotted against the distance traveled within that box in the direction of the arrow, as shown. The plot (representative of at least ten oocytes from two independent experiments) confirmed the enhanced levels of nuclear staining for Cdh1. Inset, secondary antibody-only control. Scale bars: 50 μm in D,E; 20 μm in F.

The nuclear localization of Cdh1α, as predicted by its NLS, and as observed in HeLa cells (Zhou et al., 2003a; Zhou et al., 2003b), was also confirmed on ovarian sections, again using these two antibodies (Fig. 1D,E; and see Fig. S2 in the supplementary material). Immunofluorescence on confocal sections of fixed oocytes collected from hormonally primed mice allowed a more detailed examination of the distribution of Cdh1 (Fig. 1F): it was absent from the nucleolus but appeared to be concentrated in the nucleus.

Interestingly, the oocyte nucleus was found to be enriched not only in Cdh1α, but also the 26S proteasome and to some extent the APC, suggesting that the nucleus might be an important site for APC^{Cdh1}-mediated protein ubiquitylation and degradation. Oocytes were probed both for Psmd11, which is a well-characterized subunit of the 26S proteasome (Coux et al., 1996; Isono et al., 2005), as well as Cdc27 and Cdc16, two essential subunits of the APC (Peters, 2006; Vodermaier et al., 2003). Using antibodies against all three we found enhanced nuclear staining relative to the bulk cytoplasm (Fig. 2A-C; corresponding immunoblots Fig. 2E-G). However, with respect to Cdc27 and Cdc16 we also saw cortical staining (Fig. 2B,C). These data together suggest that Cdh1, the APC and the 26S proteasome, although by no means exclusively nuclear, may collectively be active within the nucleus of G2 oocytes to cause degradation of APC^{Cdh1} substrates.

The staining pattern of cyclin B1 appeared to be very different from that of Cdh1, with cytoplasmic concentrations higher than nuclear concentrations, and with the antibody used showing a cortical distribution (Fig. 2D; for immunoblot see Fig. 2H). This cortical distribution was reminiscent of that observed for cyclin B in *Xenopus laevis* (Beckhelling et al., 2003); and in keeping with the cytoplasmic accumulation observed in oocytes of other species (Terasaki et al., 2003; Westendorf et al., 1989) and in adult cells (Clute and Pines, 1999). Such cytoplasmic accumulation of cyclin B1 may be due to its ability to anchor to cytoplasmic proteins (Charrasse et al., 2000; Jackman et al., 1995; Ookata et al., 1995). The nucleus was not, however, devoid of cyclin B1. Using an antibody specifically able to recognize only phospho-serine147-cyclin B1, a residue located in the CRS and phosphorylated on nuclear entry (Hagting et al., 1999), we could additionally observe some nuclear phospho-cyclin B1 (see Fig. S3 in the supplementary material). Such a finding would be consistent with continual shuttling of cyclin B1 between cytoplasm and nucleus.

Increasing nuclear cyclin B1 overrides G2 arrest

It is possible that the ubiquitin proteasomal machinery in the nucleus of G2-arrested oocytes acts as a protective mechanism to ensure prolonged G2 arrest by preventing nuclear cyclin B1 accumulation.

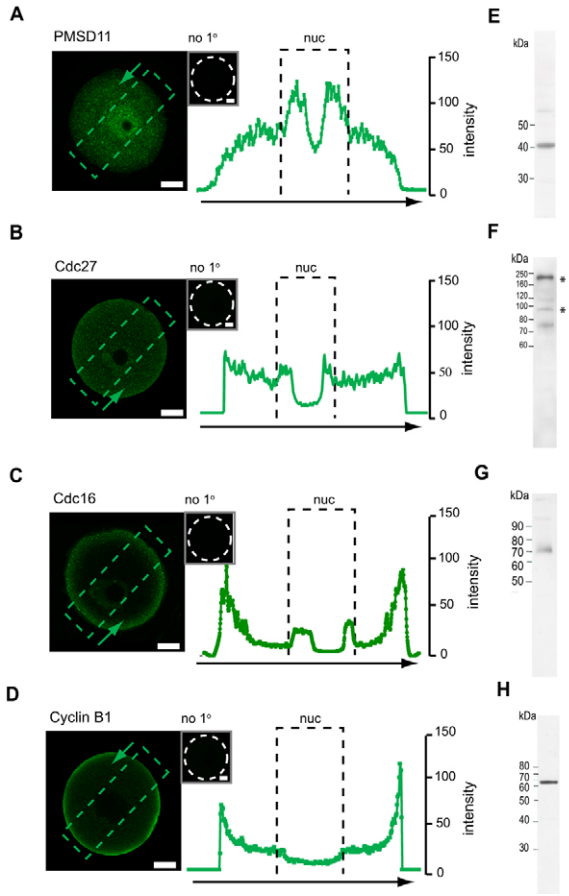

Fig. 2. APC^{Cdh1} proteasomal components in nucleus of G2 oocytes. (**A-D**) Representative immunofluorescence images on fixed and permeabilized G2 oocytes stained for Psmd11 (A), Cdc27 (B), Cdc16 (C) or cyclin B1 (D). For each image a boxed area across the oocyte is shown, and the mean 8-bit pixel intensity (0-255 arbitrary units) was plotted against the distance traveled within that box in the direction of the arrow, as shown. Insets, secondary antibody-only controls. (**E-H**) Immunoblots of prophase I oocytes were performed using the same antibodies used in A-D, to confirm specificity to (E) Psmd11, (F) Cdc27, (G) Cdc16 and (H) cyclin B1. (F) *, slow-migrating Cdc27 bands observed previously and due to protein hyperphosphorylation (Hochegger et al., 2007). Scale bars: 20 μm.

To examine more directly if raising concentrations of nuclear cyclin B1 would be an efficient mechanism to induce the G2/M transition in oocytes, we used cyclin B1 constructs that differ in their partitioning between cytoplasm and nucleus: 'cytoplasmic' (5×A) cyclin B1; and 'nuclear' (F146A) cyclin B1. 5×A cyclin B1 is hindered from accumulating in the nucleus because it has alanine substitutions of five crucial serines located in the CRS, the phosphorylation of which is known to be involved in enhancing nuclear entry during the G2/M transition (Hagting et al., 1999). By contrast, F146A cyclin B1 can more readily accumulate in the nucleus of G2 cells because CRS-mediated export from the nucleus is impeded by mutation of a crucial CRS phenylalanine to alanine (Hagting et al., 1998).

Live cell imaging following injection of copy RNA (cRNA) constructs C-terminally coupled to GFP, demonstrated the ability of the 'nuclear' F146A cyclin B1 construct to accumulate in the nucleus relative to either wild-type or 5×A cyclin B1 (Fig. 3A-D). It had a ~6-fold accumulation relative to these other constructs (Fig. 3D), suggesting that shuttling of cyclin B1 in and out of the nucleus does occur in oocytes during G2 arrest. Equally it is important to note that a substantial fraction of F146A-cyclin B1 remained in the cytoplasm, as previously observed for this construct in somatic cells (Hagting et al., 1998). This is probably because nuclear-cytoplasmic shuttling is slow, and very efficient nuclear accumulation needs CRS phosphorylation, a process normally observed at the G2/M transition (Hagting et al., 1999; Hagting et al., 1998). It is interesting to note also that 5×A cyclin B1 achieved similar nuclear accumulation to that of wild-type cyclin B1 (Fig. 3D), suggesting that nuclear import is not wholly dependent on CRS phosphorylation, a finding consistent with previous studies (Moore et al., 1999; Takizawa et al., 1999).

Nuclear-targeted F146A-cyclin B1 induced NEB at a high rate in oocytes – much higher than the other cyclin B1 constructs. Oocytes were arrested at G2 by the phosphodiesterase inhibitor milrinone, microinjected with one of the three cyclin B1 constructs and observed for NEB (Fig. 3E). Over the 11 hours of milrinone culture all non-injected oocytes remained arrested at G2 (n>90 oocytes). However, we observed NEB in oocytes for all three cyclin B1 constructs. Importantly, F146A-cyclin B1 was far more effective at inducing high rates of NEB than wild-type or 5×A cyclin B1, a finding that was conserved even when the milrinone concentration was increased 10-fold from 1 μM to 10 μM, to enhance further the PKA-drive to G2 arrest. These observations were not due to inherently greater levels of expression with F146A cyclin B1, as similar levels of expression, determined by GFP fluorescence, were achieved for all cyclin B1 constructs (Fig. 3E).

Nuclear cyclin B1 accelerates the G2/M transition

The above data suggest that nuclear accumulation of cyclin B1 in oocytes is very effective at overcoming a G2 arrest imposed by maintaining a high PKA drive. We wanted to examine this further, to establish whether nuclear cyclin B1 accumulation was an important component of spontaneous NEB, induced once oocytes were released from G2 arrest.

We found that nuclear-targeted F146A-cyclin B1 accelerated NEB in oocytes released from G2 arrest. NEB rates were monitored in oocytes following washout from milrinone-containing medium. In uninjected oocytes that were not expressing any exogenous cyclin B1 construct, measurement of cumulative NEB rates demonstrated that 50% of oocytes underwent NEB at ~110 minutes (Fig. 3F), consistent with timings reported in other mouse strains (Marangos and Carroll, 2004). Expression of any of the three cyclin B1 constructs accelerated NEB, with 50% NEB apparent by 50-60 minutes in wild-type and 5×A cyclin B1. However, greatest acceleration of NEB was observed with F146A cyclin B1, as 50% NEB rates were observed earlier than controls, just 30-40 minutes after milrinone removal.

The sudden increase in nuclear translocation of wild-type cyclin B1 ahead of NEB has already been established in both somatic cells (Hagting et al., 1998) and oocytes (Marangos and Carroll, 2004; Reis et al., 2006). We wanted to establish whether the same phenomenon occurred with our differentially targeted cyclin B1 constructs. G2-arrested oocytes were microinjected with wild-type, F146A or 5×A cyclin B1-GFP and, following milrinone washout, the translocation of cyclin B1 into the nucleus was examined relative to NEB by monitoring fluorescence and brightfield (Fig. 4A-C). A significant rise in the ratio of nuclear:cytoplasmic wild-type cyclin B1, indicative of enhanced nuclear entry, occurred 26 minutes before NEB (Fig. 4A,D). This period of nuclear entry before NEB was shorter for 5×A cyclin B1 (22 minutes; Fig. 4C,D) consistent with its nuclear entry being associated with the dissolution of the nuclear envelope. By contrast, the earliest nuclear entry was observed for F146A cyclin B1, some 34

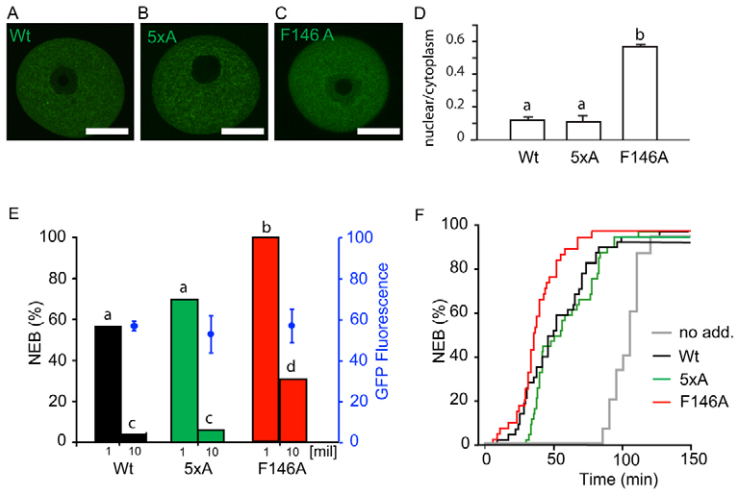

Fig. 3. Nuclear cyclin B1 overrides G2 arrest and accelerates the G2/M transition. (**A-C**) Representative equatorial sections of G2 oocytes microinjected with GFP-tagged cRNA cyclin B1: (A) wild-type, (B) 5×A and (C) F146A. (**D**) Nuclear:cytoplasmic ratios of cyclin-GFP constructs as determined by confocal image analysis of G2 oocytes (n=10 oocytes per construct). (**E**) Left axis and bars, rates of NEB in oocytes microinjected with cyclin B1 cRNA constructs and incubated in one of two concentrations of milrinone (μM), as shown (n=30-44; t=8 hours). Right axis and datapoints, level (mean ± s.e., n=31-35 per group) of GFP expression in oocytes microinjected with cRNA to cyclin B1 as indicated and incubated in 10 μM milrinone. F146A cyclin B1 induced higher levels of NEB, despite similar levels of expression to those of the other constructs. (**F**) Cumulative NEB rates recorded in G2 oocytes following milrinone wash-out (t=0 minutes). Some oocytes had been microinjected with cRNA to a cyclin B1-GFP construct as indicated 1.5 hours earlier. (D,E) Different letters indicate significant differences (P=0.01, one-way ANOVA, Fisher's post-hoc test). Scale bars: 30 μm.

minutes before NEB (Fig. 4B,D). Therefore an earlier rise in the nuclear:cytoplasmic ratio of F146A cyclin B1 is associated with precocious NEB, suggestive of a model in which NEB induction is dependent on a threshold level of nuclear cyclin B1.

Nuclear APC^{Cdh1} prevents precocious NEB

All the data presented so far are consistent with the possibility that a nuclear ubiquitin proteasomal machinery acts to block a nuclear rise in cyclin B1, which otherwise would lead to precocious NEB, the oocyte being particularly susceptible to rising concentrations of nuclear cyclin B1. If this indeed were so, then it would be important to determine the relative efficiency of APC^{Cdh1}-mediated cyclin B1 degradation in both the nucleus and the cytoplasm of the oocyte.

If the ubiquitin proteasomal machinery were more efficient in the nucleus to guard against rising cyclin B1, then one may initially conclude that F146A cyclin B1 would be degraded at a much faster rate than either wild-type or 5×A cyclin B1. However, such an assumption would be wrong, because it does not necessarily follow that the nucleus would have any greater capacity to degrade cyclin B1 just because nuclear components of the degradation machinery are present at a higher concentration. The nuclear volume of an oocyte is only ~1/60th that of the total cell (20 μm nucleus in a 80 μm diameter cell); therefore cytoplasmic cyclin B1 degradation may outweigh nuclear cyclin B1 degradation by sheer mass rather than density.

In order to compare the nuclear and cytoplasmic APC^{cdh1} activities, the cyclin B1 constructs were expressed as a C-terminal fusion with GFP in oocytes and loss of fluorescence signal recorded following translational inhibition. We have used this approach previously in oocytes using cyclin B1 to verify that such degradation was Cdh1-dependent (Reis et al., 2006). A loss in GFP signal began about 40 minutes after cycloheximide addition, consistent with a slow folding time for GFP, and the subsequent degradation profile was normalized to this point of maximum expression (Fig. 5A). Such normalization plots of degradation allowed us to calculate average rates of cyclin B1 loss for each construct (Fig. 5C). 5×A and wild-type cyclin B1 both showed similar rates of degradation. However, nuclear-targeted F146A cyclin B1 was degraded at a 2-fold higher rate. We consider this remarkable given the 15-fold larger volume of the cytoplasm to the nucleus. Furthermore, Cdh1 antisense morpholino knockdown for 24 hours reduced Cdh1 concentrations by ~60% and reduced degradation of all three constructs (Fig. 5B,C). Although the level of knockdown is less than that reported previously in a different strain of mouse (Reis et al., 2006), the present findings suggest that there is unlikely to be a different mechanism of cyclin B1 degradation for nuclear versus cytoplasmic degradation, because all three constructs were affected by Cdh1 knockdown to the same degree.

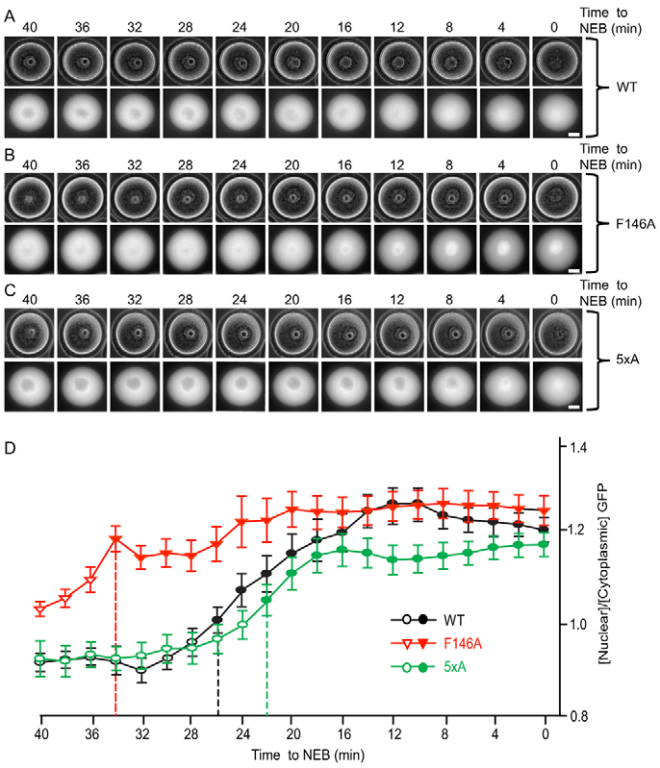

Fig. 4. Nuclear translocation of cyclin B1 before NEB. (**A-C**) Representative brightfield (top) and GFP (bottom) epifluorescence in G2 oocytes, washed out of milrinone, expressing GFP-coupled cRNA to wild-type cyclin B1 (A), F146A cyclin B1 (B) or 5×A cyclin B1 (C). Time is expressed in minutes before NEB (disappearance of the nucleolus). (**D**) Ratio of nuclear: cytoplasmic fluorescence from oocytes recorded in A-C, plotted against time before NEB. The initiation of a significant rise in this ratio (mean fluorescence ± s.e.) relative to 40 minutes before NEB is marked by the vertical dashed line, and the use of solid datapoints until the time of NEB. (P=0.01, one-way ANOVA, Fisher's post-hoc test). (A-D) n=30-31. Scale bars: 20 μm.

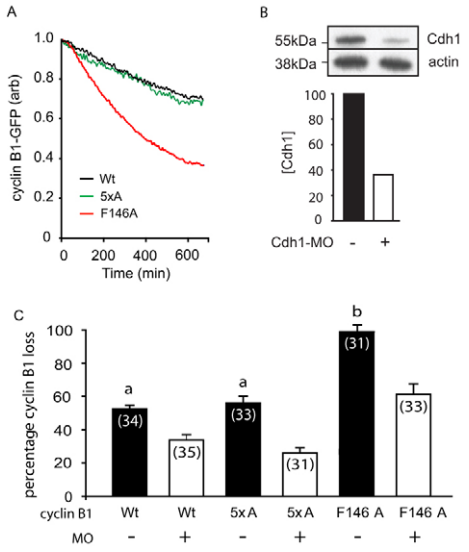

Fig. 5. Accelerated degradation of Cdh1-dependent nuclear cyclin B1. (**A**) Representative GFP fluorescence decline in oocytes expressing cRNA to wild-type, 5×A and F146A cyclin B1 following addition of cycloheximide; *t*=0 minutes, time of maximum fluorescence. (**B**) Immunoblot for Cdh1 in morpholino (MO)-injected and non-injected G2 oocytes cultured for 24 hours (*n*=45 oocytes per lane). Densitometric analysis of Cdh1 levels normalized to actin protein levels indicates that the Cdh1 morpholino reduced Cdh1 levels by 65%. (**C**) Percentage loss in cyclin B1, calculated from degradation profiles constructed as in A, for oocytes microinjected with the cyclin B1 constructs and Cdh1 MO as shown (*n*=31-35 oocytes per group). All oocytes, Cdh1 MO-injected or non-injected, were incubated for 24 hours before injection with the appropriate cyclin B1 construct. Different letters indicate significant differences. Percentages of cyclin B1 loss were also significantly different with Cdh1 MO treatment compared with non-MO-injected oocytes (*P*=0.01, one-way ANOVA, Fisher's post-hoc test). Cyclin B1 loss is expressed as a percentage relative to the F146A cyclin B1 construct.

DISCUSSION

Here we have found through immunofluorescence studies that the APC^{Cdh1} proteasomal machinery appears enhanced in the nucleus of a G2 mouse oocyte. Cyclin B1 accumulation in the nucleus, however, is normally associated with NEB and meiotic resumption, a finding consistent with the nuclear envelope and chromatin being the major sites for Cdk1 activity (Guttinger et al., 2009). Therefore, one simple hypothesis that comes from these observations is that nuclear APC^{Cdh1} protects the oocyte from precocious NEB by preventing nuclear accumulation of cyclin B1. It would have been useful to measure directly enhanced APC^{Cdh1} proteasomal activity in the nucleus. However, this is too challenging because of the problem of injecting a high-viscosity protein-based solution into the oocyte nucleus and choosing an innocuous Cdh1 substrate that would remain in the nucleus following injection. Securin would be too small and diffuse out of the nucleus (Marangos and Carroll, 2008). Instead we examined this idea using a 'nuclear-targeted' cyclin B1, in which a phenylalanine for alanine mutation is made in the CRS, such a substitution hindering nuclear export. This construct was very effective at inducing NEB in oocytes, more than the other cyclin B1 constructs tested, and importantly it was also degraded the fastest. Like all the other cyclin B1 constructs tested, this degradation was reduced by a partial knockdown achieved to Cdh1. Given that the only known difference that exists between this construct and wild-type cyclin B1 is its dwell time in the nucleus, we conclude, in agreement with the immunostaining of enhanced nuclear concentration, that the nucleus is a major site of APC^{Cdh1} activity in the oocyte.

Sensitivity of oocyte to cyclin B1 overexpression

It is interesting to note that the G2-arrested oocytes are easily triggered to undergo NEB by a rise in the concentrations of cyclin B1. A number of studies have now demonstrated that overexpressing cyclin B1 can cause G2-arrested oocytes to undergo NEB at very high rates (Ledan et al., 2001; Marangos and Carroll, 2004; Reis et al., 2006). Prophase I arrest in oocytes is unique, a very latepoint arrest in G2, with no such analogous arrest seen in somatic cells. Despite this corollary it is interesting to note that overexpressing B1 in adult somatic cells has little effect on the timing of the G2/M transition (Hagting et al., 1998; Jin et al., 1998). This is not due to differences in the levels of overexpression: a 4-fold increase in cyclin B1 achieves 60% override of a dibutyryl cAMP-mediated prophase arrest (Marangos and Carroll, 2004), but a similar 3- to 5-fold cyclin B1 overexpression in HeLa cells does not alter the timing of mitotic entry (Jin et al., 1998).

We extend the above observations by showing that oocytes are more prone to undergoing NEB when they express a cyclin B1 construct that is designed to accumulate in the nucleus. Again this is not related to dose, because our proteins are tagged with GFP, allowing comparative measurements of intracellular concentrations to be made between constructs. The sensitivity of the oocytes to raised cyclin B1 concentrations, and by inference here nuclear cyclin B1 concentrations, is in contrast to observations made in HeLa cells, in which the nuclear-targeted cyclin B1 fails to have any effect on the timing of entry into mitosis (Hagting et al., 1998). In conclusion, these differences suggest oocytes are prone to premature NEB induced by nuclear cyclin B1 accumulation in a way not observed in somatic cells, and this probably reflects their unique arrest late in G2. Such sensitivity could be accounted for simply by the absence of the inhibitory Wee1 kinase of Cdk1 from the nucleus, or the presence of activating Cdc25 phosphatase. However this seems unlikely given that Wee1B (Wee2 – Mouse Genome Informatics), the important Cdk1 inhibitory kinase in mammalian oocytes (Han et al., 2005; Shimaoka et al., 2009), possesses a nuclear localization signal (Han and Conti, 2006), and Cdc25b is observed to be cytoplasmic (Pirino et al., 2009; Zhang et al., 2008). It may be that the shuttling of cyclin B1 between cytoplasm and nucleus occurs at a greater rate or that the amplification pathways, necessary to switch on Cdk1/cyclin B1 activity, are much greater in oocytes. However, although direct comparisons of cytoplasmic-nuclear shuttling rates have yet to be made between oocyte and adult cells, it is unlikely that cyclin B1 import in an oocyte is very different from adult cells, given the observation that the nuclear-targeted cyclin B1 construct used here, in which nuclear export is inhibited, still shows a considerable presence in the cytoplasm. Therefore, in conclusion, it is not immediately obvious why the oocyte appears to be sensitive to raised cyclin B1 concentrations, but because this appears central to G2 arrest it does warrant further investigation.

APC^{Cdh1}: the nuclear guardian of G2 arrest

The APC plays a pivotal role in the control of the mitotic division. As such, any spatial investigation into the APC is often conducted with respect to the mitotic spindle and the regulation of the spindle

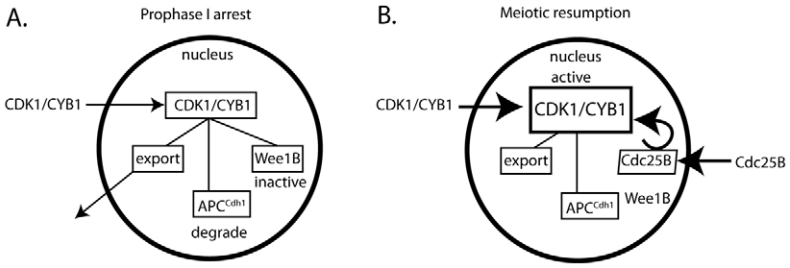

Fig. 6. Model of APC^{Cdh1} involvement in G2 arrest and meiotic resumption. (**A**) During prophase I arrest slow nuclear import of Cdk1/cyclin B1 occurs. The nucleus is the site for Cdk1 activity in the processes of NEB and chromosome condensation. In order to prevent precocious meiotic entry, we propose at least three protective mechanisms: (1) nuclear export, (2) Wee1B-mediated inhibitory Cdk1 phosphorylation; and (3) APC^{Cdh1}-mediated cyclinB1 degradation. (**B**) At meiotic resumption, an LH trigger causes translocation of Cdc25b to the nucleus and an increase in Cdk1/cyclin B1 import. We propose that neither APC^{Cdh1} activity nor nuclear export stop; they simply become overwhelmed by enhanced Cdk1/cyclin B1 entry.

assembly checkpoint, which prevents anaphase until sister chromatids are bi-orientated on the spindle (Peters, 2006). Interphase studies appear to have been limited to neurons, but here there are interesting similarities. In primary cerebellar granule neurons APC^{Cdh1} is nuclear (Konishi et al., 2004), its activity controls axonal growth and its nuclear localization is essential for this function (Stegmuller et al., 2008; Stegmuller et al., 2006). In post-mitotic neurons APC^{Cdh1} is also nuclear (Gieffers et al., 1999) and such activity is essential in preventing a build up in nuclear cyclin B1 (Almeida et al., 2005). Therefore in both neurons and oocytes a nuclear APC^{Cdh1} guards against a rise in cyclin B1 concentrations, although the outcomes when this is not achieved are very different. In oocytes, this is a physiological event triggered by LH, and it induces meiotic entry, whereas in neurons, it is pathophysiological, and the rise in nuclear Cdk1 activity triggers apoptosis (Almeida et al., 2005).

A spatial model for G2 arrest and meiotic resumption

The nucleus plays an essential part in the process of G2 arrest to eventual NEB. It does this by giving spatial segregation to the protagonists of NEB. During G2 arrest (Fig. 6A) Cdk1 activity is maintained low by several mechanisms, notably nuclear Wee1B, nuclear APC^{Cdh1}, cytoplasmic Cdc25b, and cytoplasmic cyclin B1. At meiotic resumption (Fig. 6B) there are probably at least two important events that contribute to NEB. Firstly, an LH-driven change in the phosphorylation status of Cdc25b allowing its translocation to the nucleus (Pirino et al., 2009; Solc et al., 2008; Zhang et al., 2008). Secondly, a change in shuttling activity of cyclin B1 that promotes its translocation to the nucleus. These events lead to Cdk1 activation and so NEB. We surmise that APC^{Cdh1} activity in the nucleus is not high enough to cope with the sudden influx of cyclin B1 observed just before NEB. In our model we predict that the primary function of nuclear APC^{Cdh1} is to help maintain arrest rather than actually being involved in the physiological cues of NEB. Although such an active involvement of APC^{Cdh1} in meiotic resumption cannot at this stage be discounted, we argue this is unlikely because we and others have reported that APC^{Cdh1} remains active in the hours after NEB, during prometaphase I (Homer et al., 2009; Reis et al., 2007).

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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