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Prometaphase APC^{cdh1} activity prevents non-disjunction in mammalian oocytes

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Summary

The first female meiotic division (MI) is uniquely prone to chromosome segregation errors through non-disjunction, resulting in trisomies and early pregnancy loss¹. Here, we show a fundamental difference in the control of mammalian meiosis which may underlie such susceptibility. It involved a reversal in the well-established timing of activation of the Anaphase-Promoting Complex (APC)^{2, 3} by its co-activators cdc20 and cdh1. APC^{cdh1} was active first, during prometaphase I, and was needed in order to allow homologue congression, since loss of cdh1 speeded up MI, leading to premature chromosome segregation and a non-disjunction phenotype. APC^{cdh1} targeted cdc20 for degradation but not securin and cyclin B1. These were degraded later in MI through APC^{cdc20}, making cdc20 re-synthesis essential for successful meiotic progression. The switch from APC^{cdh1} to APC^{cdc20} activity was controlled by increasing CDK1 and cdh1 loss. These findings demonstrate a fundamentally different mechanism of control for the first meiotic division in mammalian oocytes not observed in meioses of other species.

The E3 ligase activity of the Anaphase-Promoting Factor (APC) bound to its co-activator cdh1 (APC^{cdh1}) is commonly associated with late M- and early G1-phases of the cell cycle, where it contributes to M-phase exit by degradation of mitotic proteins, while simultaneously preventing precocious DNA replication^{3–6}. APC^{cdh1} activity is also observed in germinal vesicle stage (GV) mouse oocytes, equivalent to late G2, where it contributes to cyclin B1 degradation and as such is required for maintenance of GV arrest^{7, 8}.

We wanted to establish if cdh1 had any role in meiosis I (MI) after GV breakdown (GVB), independent of its role in maintaining GV arrest. Therefore, we examined the ability of oocytes to progress through MI following microinjection with a *cdh1* antisense morpholino (*cdh1*^{MO}). Culture in milrinone-containing medium for 24 h following *cdh1*^{MO} microinjection is sufficient to reduce cdh1 levels by >90% (hereafter 'cdh1 knockdown oocytes') but maintain GV arrest in the majority of oocytes, with longer term culture (48h) needed to promote GVB⁷. In cdh1 knockdown oocytes, which maintained arrest over 24 h, we found that progression through MI was accelerated following milrinone wash-out. Oocytes extruded a polar body (PB), which forms on completion of MI, 1.5 h earlier than non-injected oocytes (Fig 1a). This effect was attributed specifically to loss of cdh1, since it

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AUTHOR CONTRIBUTIONS

K.T.J. directed the work. A.R. and S.M. performed most the experiments; with H.Y.C making the initial observations on the effects of the *cdh1*^{MO}, I.N. performing some of the Westerns, and M.L. making some of the constructs. KTJ wrote the paper in consultation with A.R. and S.M.

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was not observed in mock *cdh1* depleted oocytes through addition of a 5-base-mismatch *cdh1* morpholino (5MM-*cdh1*^{MO}). The acceleration of PB could have been due to a loss in *cdh1* during either GV arrest or MI. However, it was attributed specifically to a function in MI because we could restore a normal PB timing by rescuing *cdh1* expression after GVB. Thus, in *cdh1* knockdown oocytes *cdh1* cRNA injection, which was made on release from milrinone (hereafter 'cdh1 rescue oocytes'), rescued a normal phenotype (Fig 1a).

The MI acceleration in *cdh1* knockdown oocytes led to premature segregation and non-disjunction of homologues. Following oocyte maturation, chromatin in *cdh1* knockdown oocytes often failed to fully align on a metaphase II spindle and sometimes was very dispersed suggesting segregation of homologues had not occurred properly (Fig 1b; Supplementary Fig 1). To examine this further, chromosome spreads were performed in which the hyperploidy rate was calculated, to circumvent problems associated with chromosome loss during the fixing procedure⁹. About one-third of the *cdh1* knockdown oocytes were hyperploid (Fig 1b), suggesting that non-disjunction had occurred in the majority of oocytes if chromosome segregation was stochastic between the PB and oocyte. These effects were attributed to loss of *cdh1*, since both 5MM-*cdh1*^{MO} injected oocytes and *cdh1* rescue oocytes formed metaphase II spindles and were not hyperploid (Fig 1b). Live-cell chromatin imaging during maturation of uninjected and *cdh1* knockdown oocytes confirmed the immunofluorescence and chromosome spread results. In control oocytes, chromosomes underwent condensation after GVB, congressed on a metaphase I plate, migrated to the oocyte cortex and underwent anaphase (Fig 1c; Supplementary Movie 1). Condensed chromosomes in *cdh1* knockdown oocytes however underwent anaphase without congression onto an obvious metaphase plate and so generated lagging chromosomes (Fig 1d; Supplementary Movie 2).

We wanted to establish if the acceleration of MI by *cdh1* knockdown was associated with accelerated degradation of proteins known to be degraded at this time. At the mitotic metaphase-anaphase transition both cyclin B1, the regulatory subunit of CDK1, and securin, which prevents loss of cohesin from attached sisters by inhibiting separase, are degraded simultaneously¹⁰. Loss of endogenous and fluorescent protein tagged cyclin B1 and securin during MI have been reported previously but comparison of degradation in the same oocyte has not been performed^{11, 12}. Therefore we visualised cyclin B1 and securin degradation together in an oocyte by coupling to separable fluorescent proteins, and found simultaneous degradation (Supplementary Fig S2a). When levels of *cdh1* were knocked down, the initiation of both cyclin B1 (Fig 2 a,b) and securin (Supplementary Fig S2b and Fig S2c), were brought forward by about 1.5 h, a timing which is consistent with the accelerated PB extrusion observed above. This effect was specific for *cdh1*, since it was not observed for the control 5MM-*cdh1*^{MO} and normal timing of cyclin B1 degradation was restored in *cdh1* rescue oocytes. Although *cdh1* was involved in the timing of cyclin B1 and securin degradation, it appeared not to contribute to actual degradation, since their rates of loss were unaffected by *cdh1* knockdown (Supplementary Fig S2d). This suggests that cyclin B1 and securin are degraded through the same mechanism in MI, possibly by APC^{cdc20} activity as they are in mitosis. Indeed, both cyclin B1 degradation (Fig 2c,d) and securin degradation (not shown) were blocked by addition of the spindle poison nocodazole or microinjection of cRNA to *mad2*, a component of the Spindle Assembly Checkpoint (SAC) pathway, which in mitosis is a well-established means of inhibiting APC^{cdc20} (Ref. 3). This was the case in control oocytes with unperturbed levels of *cdh1* (Fig 2c), and in *cdh1* knockdown oocytes (Fig 2d), suggesting that loss of *cdh1* does not accelerate MI by overriding any SAC imposed arrest.

The acceleration of MI and resulting non-disjunction observed following *cdh1* knockdown suggested prometaphase APC^{cdh1} may delay homologue segregation to allow full

congression on a metaphase plate. In order to demonstrate APC^{cdh1} activity directly during prometaphase I we examined for degradation of its substrate cdc20 (Ref 2, 3). Cdc20-GFP was used as a reporter for APC^{cdh1} activity but was itself biologically inactive since it lacked ability to rescue cdc20 knockdown (see Fig 4b), and we attribute this to occlusion of the C-terminal IR tail which is involved in APC binding¹³. However, cdc20-GFP proved to be a good reporter of APC^{cdh1} activity since it was degraded in oocytes shortly after GVB in a period lasting 2-4 h (Fig 3a; Supplementary Fig S3a, b). Degradation was due to cdh1, since it was blocked in cdh1 knockdown oocytes, not observed with control 5MM-*cdh1*^{MO}, and was recovered in cdh1 rescue oocytes (Fig 3a). Interestingly, with respect to these fluorescent protein chimeras, the time of initiation for cdc20 degradation appeared to occur earlier than either cyclin B1 or securin, despite all being APC^{cdh1} substrates. Therefore, to determine if such timings were an artefact of overexpressed fluorescent protein chimeras, we examined for changes in endogenous proteins levels in uninjected maturing oocytes. Importantly we observed the same relative timing of endogenous cdc20, cyclin B1 and securin degradation as that for the chimeras (Fig 3b). Maximum cdc20 degradation was seen 3-4 h after GVB, whilst at this time both cyclin B1 and securin levels were still rising, their degradation began some 1-2 h later. Cdh1 knockdown resulted in a stabilisation of endogenous cdc20 and cdh1 rescue recovered this loss (Fig 3c). Therefore with both fluorescent protein chimeras and endogenous proteins we have observed APC^{cdh1} mediated cdc20 degradation ahead of any loss in cyclin B1 and securin.

To demonstrate again that the degradation of cdc20 occurs ahead of cyclin B1, *cyclin B1-YFP* and *cdc20-Cerulean Fluorescent Protein (CeFP)* cRNA were expressed in the same oocyte and their expression monitored during oocyte maturation. Cdc20 degradation preceded cyclin B1, in a period soon after GVB (Fig 3d). There was no observable loss in cyclin B1 at this time; its degradation began as the period of cdc20 degradation ended (Fig 3d and Supplementary Fig S3c). All these findings taken together show that during MI, APC^{cdh1} has substrate preference for cdc20 over cyclin B1 and securin; and this holds for both endogenous protein and proteins expressed from cRNA. This may be explained by the manner in which these substrates are degraded, processive or distributive ubiquitination, which underlies the temporal ordering of degradation for mitotic APC^{cdh1} substrates¹⁴. In addition the binding of accessory factors to APC^{cdh1} during prometaphase I may either enhance the degradation of cdc20, or decrease that of cyclin B1 and securin at this time; a phenomenon previously observed for a number of APC^{cdh1} substrates¹⁵⁻¹⁷.

When cdh1 knockdown oocytes were co-expressing *cyclin B1-YFP* and *cdc20-CeFP* cRNA no degradation of cdc20 was seen (Fig 3d), consistent with prometaphase APC^{cdh1} activity leading to loss of cdc20. However, cyclin B1-YFP degradation proceeded at a normal rate, confirming its degradation during MI is APC^{cdh1} independent. Cdh1 knockdown however brought forward the time of cyclin B1 degradation to the point at which cdc20 would normally have been degraded (Supplementary Fig S3c). In fact regardless of the actual time of onset for cdc20 degradation, cyclin B1 degradation followed 2 h later (Supplementary Fig 3d).

Cdc20 is essential for MI in lower eukaryotes but dispensable in frogs¹⁸⁻²². Therefore, we wanted to establish the cdc20 dependency of MI in mouse. During maturation, cdc20 quickly re-accumulated after its loss, making it possible that nascent cdc20 was necessary for APC^{cdc20} mediated cyclin B1 and securin loss. Protein synthesis was needed for MI completion since cycloheximide added to oocytes at 4 h post GVB, when cdc20 levels were low, blocked PB extrusion (control, 91% PB extrusion rates, n=34; with cycloheximide 30%, n=37). We used a *cdc20* morpholino (*cdc20*^{MO}; hereafter 'cdc20 knockdown') to block cdc20 re-accumulation during MI (Fig 4a) and therefore examine directly the role played by cdc20 protein synthesis in MI. Cdc20 knockdown specifically blocked PB

extrusion (Fig 4b), since it was not observed with a 5-base mismatch morpholino (5MM-*cdc20*^{MO}) and high rates of PB extrusion could be restored by *cdc20* cRNA microinjection (Fig 4a, b; hereafter '*cdc20* rescue'). These data suggested that *cdc20* re-accumulation during MI was needed for securin and cyclin B1 degradation. To examine this directly, we measured the rate of degradation of both cyclin B1 (Fig 4c) and securin (Supplementary Fig S2e) and found them to be substantially reduced following *cdc20* knockdown. This was not due to the microinjection procedure because normal rates of cyclin B1 degradation were observed following 5MM-*cdc20*^{MO} microinjection (Fig 4c). These data show that re-accumulation of *cdc20* was needed for completion on MI and that in an unperturbed MI, APC^{cdc20} contributed to cyclin B1 and securin degradation. In *cdh1* knockdown oocytes *cdc20* was also responsible for the cyclin B1 and securin degradation. We could not knockdown *cdc20* in a *cdh1* background because *cdc20* loss was dependent on APC^{cdh1}, however large injections of *cdc20*-CeFP (4-fold greater pipette concentration than used elsewhere), acted as a dominant-negative construct to block polar body extrusion and inhibit cyclin B1 degradation in *cdh1* knockdown oocytes (Fig 4d). These data, along with the observed ability of *mad2* to block degradation (Fig 2d), strongly suggests that APC^{cdc20} is responsible for cyclin B1 and securin degradation in *cdh1* knockdown oocytes.

Although CDK1 can phosphorylate and so inhibit *cdh1* binding to the APC^{23, 24}, its rise in oocytes during prometaphase I was found to be sufficiently slow to allow APC^{cdh1} to function. $\Delta 90$ *cyclin B1* cRNA, which accelerated CDK1 activity (Fig 5a) and increased phosphorylated *cdh1* (Fig 5b, phosphorylated *cdh1* migrates more slowly²³), reduced by a half the period of APC^{cdh1} mediated *cdc20* degradation (Fig 5d,e). During oocyte maturation we also observed a loss of endogenous *cdh1* in a period between 6 to 7 h after GVB (Fig 5c). This loss in *cdh1*, which can be degraded through both APC- and SCF-dependent mechanisms^{25, 26}, suggested that the switch from APC^{cdh1} to APC^{cdc20} activity could be regulated by both CDK1 activity and *cdh1* degradation. In fact they appeared to act redundantly in switching off APC^{cdh1}, because it was only when both *cdh1* cRNA was overexpressed and CDK1 inhibited, that the period over which APC^{cdh1} was active increased (Fig 5e).

In summary we have shown that in prometaphase, APC^{cdh1} is active and has a role to play in oocyte maturation, slowing passage through MI and allowing time for homologues to congress. APC^{cdh1} activity preferentially targeted *cdc20* for degradation over cyclin B1 and securin, whose later degradation was mediated by *cdc20*. *Cdc20* resynthesis was therefore essential for completion of MI. *Cdh1* knockdown moved forward the period of APC^{cdc20} activity despite a lack of homologue congression, suggesting that the various SAC components in oocytes^{9, 27, 28}, do not act to monitor bivalent orientation; a finding suggested previously from studies in mice carrying a univalent X chromosome²⁹. The present work therefore describes a mechanism controlling the timing of mammalian meiosis which has not been observed for other cell divisions.

METHODS

Oocyte collection and culture

GV oocytes were collected from four to six week old MFI mice (Harlan, Bicester, UK) mice 44-52 h after intraperitoneal injection of 7.5 IU pregnant mares' serum gonadotrophin (Calbiochem, Nottingham, UK). For bench handling, microinjections, and imaging experiments, oocytes were cultured in medium M2 following mechanical removal of granulosa cells. For long-term incubation, oocytes were cultured in MEM (22571-020; Gibco, Paisley, UK) with 20% foetal calf serum in a 5% CO₂ humidified incubator at 37°C. Milrinone (1 μ M; Sigma-Aldrich, Poole, UK) was used to arrest GV oocytes. Chromosome spreads and H1 kinase assays on oocytes were performed as described previously^{7,30}.

cRNA and morpholinos

Cdc20, *cdh1*, *cyclin B1*, *Δ90cyclin B1* and *mad2* cRNA were made as described previously^{7,31}, using a modified pRN3 vector designed to produce protein C-terminally coupled to CFP (Cyan), CeFP (Cerulean), GFP (Green), Venus (Yellow), YFP (Yellow), or unlabelled. cRNA was synthesised using T3 mMESSAGE mMACHINE (Ambion, Huntingdon, UK), and dissolved in nuclease-free water to a concentration of approximately 1 μg μl⁻¹ before microinjection. The following morpholinos (Gene Tools LLC, OR, USA) were used at a micropipette concentration of 1mM: *cdh1*^{MO}, 5'-CCTTCGCTCATAGTCCTGGTCCATG-3'; 5MM-*cdh1*^{MO}, 5'-GTACCTGGTCCTGATACTCGCTTCC-3'; *cdc20*^{MO}, 5'-CGCTCTCGAACACGAAGTGCGCCAT-3'; and 5MM-*cdc20*^{MO}, 5'-CGGTCTCAAACACCAAGTGCGCCAT-3'. For all experiments involving *cdh1*^{MO} and 5MM-*cdh1*^{MO}, oocytes were injected with morpholino for 24 h prior to release into milrinone-free medium. This allows for >90% knockdown in *cdh1*, with around 70% of oocytes remaining GV arrested⁷. For all experiments involving *cdc20*^{MO} or 5MM-*cdc20*^{MO}, oocytes were microinjected in milrinone-arresting medium, then washed free of milrinone following completion of microinjection.

Microinjections and imaging

All microinjections into oocytes were made on the heated stage of a Nikon TE300 inverted microscope as described previously⁷. 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, Stevenage, UK). Brightfield and epi-fluorescence images were recorded using a Sony Interline MicroMax CCD camera. Confocal sections were performed on a LeicaSP2. Metamorph and Metafluor software (Universal Imaging Corp., Downingtown, PA) were used for image capture and data analysis.

Immunoblotting

Western blotting was performed as described previously⁷ using antibodies against *cdc20* (sc-8358, Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 1:200, *cdh1* (ab3242; Abcam, Cambridge, UK) at 1:200, securin (ab3305; Abcam) at 1:1000, cyclin B1 (ab72; Abcam) at 1:400, and actin (ab3280; Abcam) at 1:400. Blots were incubated in primary antibodies overnight at 4°C, using nonfat milk (5%) as a blocking agent. Anti-rabbit IgG (for polyclonals; P0448; Dako, Ely, UK), anti-mouse IgG (for monoclonals; P0447; Dako) and ECL Plus (RPN2132; GE Healthcare; Little Chalfont, UK) were used as secondary detection reagents according to the manufacturer's instructions.

Immunofluorescence microscopy

Oocytes were fixed with 3.7% paraformaldehyde in phosphate-buffered saline containing 1% polyvinylpyrrolidone (PBS+PVP) and then transferred to the same containing 2% Triton-X100. Fixing and permeabilising were for 30 mins each at room temperature. Oocytes were then washed extensively in PBS+PVP before incubation with 1:400 anti-tubulin (ab6160; Abcam). Following further washes oocytes were incubated with 1:20 TRITC-labelled rabbit anti-mouse IgG (Dako) and Hoechst 33258 to stain chromatin. These incubations were at 37°C in PBS+PVP.

Statistical analysis

All 1-way ANOVA statistical analysis was performed using Minitab release 14, with a 95% confidence level and Fisher's post-hoc analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Cdh1 loss accelerated meiosis I leading to premature homologue segregation and non-disjunction. **a**, Timecourse of PB extrusion rates in oocytes injected with *cdh1*^{MO} (n=58), 5MM-*cdh1*^{MO} (n=37), *cdh1*^{MO} and *cdh1* cRNA (n=67); or no injections (n=64). Cdh1 knockdown accelerated PB extrusion. **b**, Percentages of dispersed chromatin and hyperploidy observed in matured non-injected control oocytes, or those injected with 5MM-*cdh1*^{MO}, *cdh1*^{MO} and *cdh1* cRNA as stated. Number of oocytes examined in parentheses. **c** and **d**, Chromatin (Hoechst) and brightfield images of maturing non-injected oocytes (**c**) and those injected with *cdh1*^{MO} (**d**). Cdh1 knockdown oocytes had no discernible congression of chromosomes on a metaphase I plate and had lagging chromosomes during anaphase (arrow). (**c**) and (**d**) time stamp is h:min, scale bar is 20μm. (**c**) and (**d**) are selected frames from Supplementary Movies 1 and 2. (**a**), (**c**) and (**d**) times are relative to GVB.

Figure 2.

Premature cyclin B1 degradation following *cdh1* knockdown. **a**, Cyclin B1-YFP fluorescence during maturation of oocytes injected with *cdh1*^{MO}, 5MM-*cdh1*^{MO} and *cdh1* cRNA as stated. **b**, Calculated from (a), the timing of initiation of cyclin B1-YFP degradation after GVB (asterisk indicates $P=0.034$, significantly different from non-injected, ANOVA). The error bars represent s.d. Numbers of oocytes in parentheses, pooled for each condition from at least 2 independent experiments. **c**, Cyclin B1-YFP fluorescence in oocytes during maturation, at the arrow when indicated oocytes were injected with *mad2* cRNA or nocodazole was added to the medium. **d**, As for (c), in oocytes following *cdh1* knockdown. Mad2 and nocodazole both inhibited PB extrusion, which has been marked in control oocytes by the arrowheads. (c) and (d) are representative recordings of at least 12 oocytes.

Figure 3.

Cdc20 degradation precedes that of cyclin B1 and securin during meiosis I. **a**, Representative cdc20-GFP fluorescence recordings in oocytes microinjected with *cdh1*^{MO} (n=16); 5MM-*cdh1*^{MO} (n=23), *cdh1*^{MO} and *cdh1* cRNA (n=14); or no further injections (n=22). Note that *cdh1* knockdown prevented cdc20-GFP degradation between GVB and PB. **(b and c)**, Western blot and densitometric analysis of endogenous cdc20, cyclin B1 and securin levels (50 per lane) at the times indicated relative to GVB (0 h is GV arrest). In **(b)** oocytes were uninjected; note the early loss in cdc20 at 4h, ahead of both cyclin B1 or securin. In **(c)** oocytes were microinjected with *cdh1*^{MO} and *cdh1* cRNA as indicated; note that *cdh1* knockdown abolished cdc20 loss and brought forward cyclin B1 and securin degradation. Cdc20 degradation at 4 h was recovered by *cdh1* cRNA rescue. Actin was used as a loading control. Blots are representative of 2 further replicates. **d**, Cyclin B1-YFP and cdc20-CeFP fluorescence levels recorded simultaneously during maturation, with or without microinjection of *cdh1*^{MO} as indicated. Representative traces (top, n=40; bottom n=44) of cyclin B1-YFP and cdc20-CeFP fluorescence during maturation. In **(a)** and **(d)** the timing of GVB and PB extrusion are marked by arrowheads. **(b)** and **(c)** Uncropped Westerns for cdc20, securin and cyclin B1 are displayed in the Supplementary Information, Fig S4.

Figure 4.

Essential role of *cdc20* in meiosis I. **a**, Western blot of matured oocytes (50 per lane) following injection of *cdc20*^{MO} or *cdc20* cRNA as indicated at the GV stage. The morpholino prevented re-accumulation of *cdc20* during MI. The uncropped Western is displayed in the Supplementary Information, Fig S4. **b**, PB extrusion rates following maturation in oocytes injected with *cdc20*^{MO}, 5MM-*cdc20*^{MO}, *cdc20* cRNA, or *cdc20*-GFP cRNA as stated. *Cdc20* knockdown blocked completion of MI which was rescued by *cdc20* cRNA. **c**, Cyclin B1-Venus degradation rate, plotted with respect to time as a percentage of the maximum recorded fluorescence, in oocytes injected with *cdc20*^{MO} (n=18); 5MM-*cdc20*^{MO} (n=15); or without further microinjection (n=15), pooled for each condition from at least 2 independent experiments. **d**, Cyclin B1-Venus degradation rate plotted as for (c), in *cdh1* knockdown oocytes injected *cdc20*-CeFP cRNA (n=38) or no further injections (n=34), pooled for each condition from at least 2 independent experiments. The pipette concentration of *cdc20*-CeFP was high, four-fold that used in any other experiment. In (b), (c) and (d), asterisks imply significantly different from non-injected group, (p<0.05; b, ANOVA; c and d, t-test). In (c) and (d) the range over which PB extrusion occurred is as marked.

Figure 5.

CDK1 regulation of prometaphase APC^{cdh1}. **a**, H1 kinase assays of maturing oocytes at the times indicated, normalised with respect to time 0 h (GV arrest). Some GV oocytes were microinjected with $\Delta 90cyclin B1$ cRNA ($\Delta 90cycB1$) before maturation (n=5). $\Delta 90cyclin B1$ increased the rate of rise of H1 kinase activity following GVB. **(b and c)** *cdh1* expression in oocytes at the times indicated after GVB. In **(b)**, *cdh1*^{MO}, or cRNA for $\Delta 90cyclin B1$ and *cdh1* were microinjected as indicated. **d**, *cdc20*-GFP fluorescence changes in a control (black) and $\Delta 90cyclin B1$ cRNA injected oocytes (grey), plotted with respect to time as a percentage of the maximum recorded fluorescence before any *cdc20* loss. Time *t* is the time taken to reach the minimum recorded fluorescence from the start of *cdc20* degradation and is plotted in **(e)**. **e**, The period of *cdc20*-GFP degradation (*t*), calculated from experiments performed as in part **(d)**, in oocytes injected with cRNA for $\Delta 90cyclin B1$ and *cdh1* or treated with roscovitine (ROS). Numbers examined in parentheses with oocytes pooled for each condition from at least 2 independent experiments. In **(a)** and **(e)** error bars are s.d. and asterisks mark timepoints that are significantly different from: time 0 (**a**) or no additions (**e**) ($p < 0.05$, ANOVA).