The APC/C activator FZR1 coordinates the timing of meiotic resumption during prophase I arrest in mammalian oocytes

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SUMMARY

FZR1, an activator of the anaphase-promoting complex/cyclosome (APC/C), is recognized for its roles in the mitotic cell cycle. To examine its meiotic function in females we generated an oocyte-specific knockout of the Fzr1 gene (Fzr1−/−). The total number of fully grown oocytes enclosed in cumulus complexes was 35-40% lower in oocytes from Fzr1−/− mice and there was a commensurate rise in denuded, meiotically advanced and/or fragmented oocytes. The ability of Fzr1−/− oocytes to remain prophase I/germinal vesicle (GV) arrested in vitro was also compromised, despite the addition of the phosphodiesterase milrinone. Meiotic competency of smaller diameter oocytes was also accelerated by Fzr1 loss. Cyclin B1 levels were elevated ~5-fold in Fzr1−/− oocytes, whereas securin and CDC25B, two other APC/C FZR1 substrates, were unchanged. Cyclin B1 overexpression can mimic the effects of Fzr1 loss on GV arrest and here we show that cyclin B1 knockdown in Fzr1−/− oocytes affects the timing of meiotic resumption. Therefore, the effects of Fzr1 loss are mediated, at least in part, by raised cyclin B1. Thus, APC/C FZR1 activity is required to repress cyclin B1 levels in oocytes during prophase I arrest in the ovary, thereby maintaining meiotic quiescence until hormonal cues trigger resumption.

KEY WORDS: FZR1, Cyclin B1, Meiosis, Mouse, Oocyte

INTRODUCTION

Prophase I arrest, referred to as the germinal vesicle (GV) stage, is a conserved feature of oocytes across species (Whitaker, 1996; Mehlmann, 2005; Jones, 2008). In mice, as in all mammals, GV arrest begins shortly after meiotic recombination in fetal life. Periodic, non-hormonal recruitment of a small number of quiescent follicles into the growing pool after birth leads to follicle growth and eventual ovulation, both of which are hormone dependent. It is only near the time of ovulation in the fully grown oocytes of mature follicles that a rise in luteinizing hormone (LH) breaks this arrest, causing GV breakdown (GVB). A high level of cAMP, generated from a Gs-coupled oocyte receptor, is needed to maintain arrest in fully grown oocytes (Cho et al., 1974; Magnusson and Hillensjo, 1977; Bornslaeger et al., 1986; Mehlmann et al., 2002; Freudzon et al., 2005). This is enhanced by cGMP from the surrounding cumulus cells, which inhibits phosphodiesterase 3A (PDE3A) activity, preventing cAMP hydrolysis (Sela-Abramovich et al., 2008; Norris et al., 2009; Vaccari et al., 2009; Zhang et al., 2010). Therefore, owing to the maturation-inhibitory follicular environment, GVB is spontaneous when oocytes are released into culture, but arrest can be maintained by methods that raise cAMP. The ability to undergo meiotic resumption, referred to as meiotic competency, is not observed in the smaller oocytes from pre-antral follicles (Sorensen and Wassarman, 1976; Wickramasinghe et al., 1991). Thus, the acquisition of meiotic competency is coupled to follicle growth, and, once the oocyte is fully grown, meiotic resumption needs to be inhibited by cAMP.

GVB is similar to the G2/M transition of mitosis in being associated with a rise in CDK1 activity (a heterodimer of CDK1 and cyclin B1) (Doree and Hunt, 2002; Jones, 2004). cAMP-dependent protein kinase A (PKA) is likely to maintain arrest by keeping CDK1 in a phosphorylated and inactive state, a process that is only reversed by the peri-ovulatory LH rise, which results in an increase in CDC25B phosphatase activity (Duckworth et al., 2002; Lincol et al., 2002; Han et al., 2005; Zhang et al., 2008; Pirino et al., 2009; Oh et al., 2010). The inability of the small oocytes to switch on CDK1 activity is also likely to explain oocyte competency, given that cyclin B1 and CDK1 protein levels are reduced 10-fold in incompetent oocytes (Kanatsu-Shinohara et al., 2000) and that overexpression of both cyclin B1 and CDK1 in incompetent oocytes can induce GVB (de Vantery et al., 1997). Cyclin B1 overexpression in fully grown oocytes cultured in media designed to maintain high cAMP levels has the ability to induce GVB (Leder et al., 2001; Marangos and Carroll, 2004; Holt et al., 2010). Oocytes are therefore likely to have a mechanism to prevent cyclin B1 accumulation, and a number of recent studies in mouse have suggested that this is dependent on FZR1, an activator of the anaphase-promoting complex/cyclosome (APC/C) (Reis et al., 2006a; Marangos et al., 2007; Marangos and Carroll, 2008; Homer et al., 2009; Schindler and Schultz, 2009; Holt et al., 2010).

The mammalian FZR1 protein (also known as CDH1), encoded by Fzr1, is one of two well-established co-activators of the APC/C, which is an E3 ligase that regulates mitotic and meiotic progression through the ubiquitylation and subsequent degradation of key sets of substrates (Peters, 2006). FZR1, and another key activator, CDC20 (p55CDC/Fizzy), function at different stages of the cell cycle and confer substrate specificity upon the APC/C by recognizing substrates with either D-boxes (APC/C CDC20) or D- , KEN or CRY boxes (APC/C FZR1) (Reis et al., 2006b; Pfleger and Kirschner, 2000; Zur and Brandis, 2002). APC/C CDC20 activity is essential for the metaphase to anaphase transition through destruction of securin (Pttg1 – Mouse Genome Informatics), the

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inhibitory chaperone of the protease separase (Esp11 – Mouse Genome Informatics), which is responsible for cleavage of chromosomal cohesin (Peters, 2006; Thornton and Toczyski, 2006). APC/C(CDC20) activity also targets cyclin B1 destruction, facilitating mitotic/meiotic progression by lowering CDK1 activity. Low CDK1 activity promotes APC/C(FZR1) activation, such that APC/C(FZR1) activity is replaced by APC/C(FZR1) in late mitosis/G1. Recent somatic cell knockout studies have implicated FZR1 in G1/S phase timing, DNA replication and, consequently, genomic stability (Garcia-Higuera et al., 2008; Sigl et al., 2009), in addition to roles in the differentiation of various cell lineages including neurons (for a review, see Wasch et al., 2010). The recent identification of APC/C(FZR1) activity in vitro during prophase I of the oocyte therefore represents a temporally unique role for this APC/C co-activator.

APC/C(FZR1)-mediated cyclin B1 proteolysis appears to be under intricate control, with oocytes containing factors that can either enhance or reduce rates of cyclin B1 loss (Marangos and Schultz, 2009). It is also possible that securin is the preferred substrate of APC/C(FZR1) during GV arrest (Marangos and Carroll, 2008). As such, cyclin B1 levels in GV oocytes may be regulated by the amount of the competitive substrate securin and the extent of APC/C(FZR1)-dependent securin degradation. FZR1 has also been shown to be important in the GV arrest of porcine oocytes (Yamamuro et al., 2008). Thus, although some aspects of the mechanisms involved still need to be elucidated, it is likely that APC/C(FZR1) activity is a conserved feature of GV arrest for all mammalian oocytes.

Since GV arrest is such a fundamental process in meiosis it is essential to address the role of FZR1 using a technique that can eliminate FZR1 specifically and with high efficiency. All of the observations on FZR1 made so far have been with antisense knockdown approaches on cultured and denuded oocytes. Here, we have therefore utilized Cre-loxP technology to create an oocyte-specific knockout of the Fzr1 gene. This has enabled us to examine FZr1 function more fully during the period of GV arrest.

MATERIALS AND METHODS
Reagents
Generation of Fzr1<sup>Loxp/Loxp</sup> mice
Fzr1<sup>Loxp/Loxp</sup> mice were created according to Garcia-Higuera et al. (Garcia-Higuera et al., 2008). Female Fzr1<sup>Loxp/Loxp</sup> mice were mated with Zp3Cre<sup>+/loxp</sup> males, and male F1 offspring of the genotype Fzr1<sup>Loxp/Loxp</sup>/Zp3Cre<sup>+/-</sup> were obtained to examine the experiment. Primers used for genotyping were Cre (5`-GCG-GTCTGGAGACTAAAATCATTAT-3`) and Fzr1 (see Garcia-Higuera et al., 2008). Animals were housed in a PC2 facility under a 12-hour light/12-hour dark cycle regimen with ad libitum food and water (University of Newcastle and Australian Bioresources, Australia).

Histology
Ovaries from 5-week-old mice were fixed in Bouin’s Solution, embedded in paraffin and sectioned at 8 μm intervals prior to Hematoxylin and Eosin (H&E) staining.

Oocyte collection and culture
GV oocytes were collected from 4- to 8-week-old mice 44-52 hours after intraperitoneal injection of 10 IU PMSG (Intervet). For bench handling and microinjections, oocytes were cultured in M2 medium containing 4% BSA or, for longer incubations, in MEM alpha medium with 20% FCS in a 5% CO<sub>2</sub> humidified incubator at 37°C. The phosphodiesterase 3 inhibitor milrinone (0.6-10 μM) was used to maintain GV arrest in some experiments (Tsafiri et al., 1996). Follicle dissociation was performed by incubating ovaries in 1.5 mg/ml collagenase and 2 mg/ml DNase in M2 medium containing 4% BSA for up to 30 minutes. GVBD was scored at the disappearance of the nucleolus.

dsRNA knockdown of cyclin B1 by microinjection
A 650 bp coding region of mouse cyclin B1 and a 575 bp coding region of Fzr1 were amplified by PCR using primers incorporating T7 promoter sequences at the 5' ends: cyclin B1 Fwd 5`-TATACGACTCTATAGGGAGATGATAATCTTCCTCAAG-3` and Rev 5`-TATACGACTCTATAGGGAGATGATAATCTTCCTCAAG-3`; Fzr1 Fwd 5`-TATACGACTCTATAGGGAGATGATAATCTTCCTCAAG-3` and Rev 5`-TATACGACTCTATAGGGAGATGATAATCTTCCTCAAG-3`. The PCR products were cloned into pGEM-T Easy and sequenced for confirmation. Restriction digestion was used to obtain 1 μg of template, from which long double-stranded (ds) RNA was prepared using the MEGAscript T7 RNA Kit (Ambion) by performing transcription on the dsDNA template, and dissolved in nuclelease-free water at ~1 μg/μl before microinjection. Microinjections into oocytes were made on the heated stage of a Nikon TE3000 inverted microscope. Micropipettes were inserted into cells using the negative capacitance overcompensation facility on an electrophysiological amplifier (Madgwick et al., 2004). A single 0.1-0.3% volume injection was achieved using a timed injection on a Pneumatic PicoPump (World Precision Instruments). Following microinjection, oocytes were incubated for 48 hours in 10 μM milrinone prior to washout and imaging to assess GVBD (Biostation IM, Nikon). MetaMorph and MetaFluor software (Molecular Devices, PA, USA) were used for data analysis.

Immunoblotting
Proteins were separated on a NuPage 10% gel (Invitrogen) using the manufacturer’s sample buffer and instructions. Blotting was performed using an XCell II Blot Module (Invitrogen). Blots were incubated in 5% skimmed milk prior to primary antibody addition: FZR1 (Abcam, ab3242; 1:500), cyclin B1 (Abcam, ab72; 1:500), securin (Abcam, ab3305; 1:200), CDC25B (Santa Cruz Biotech, sc-326; 1:200), GAPDH (Sigma, G8795, 1:10,000). Secondary anti-mouse or anti-rabbit IgG HRP (DAKO) incubation was followed by signal detection using ECL reagents (GE Healthcare) according to the manufacturer’s instructions.

Statistical analysis
Statistical analysis was performed using Graphpad Prism software. ANOVA was performed with a 95% or 99% confidence level and Tukey’s post-hoc analysis. Yate’s correction was used for Fisher’s <sup>2</sup> analysis and two-way unpaired Student’s <sup>t</sup>-tests.

RESULTS
Establishment of an oocyte-specific Fzr1 knockout
To create an oocyte-specific knockout of the Fzr1 gene we bred mice harboring loxP sites in Fzr1 (Fzr1<sup>Loxp/Loxp</sup>, Fig. 1A) with Zp3-Cre<sup>-/-</sup> mice. In such a system, Cre recombinase expression follows that of ZP3, and is specifically expressed in the oocytes of activated follicles recruited into the growing pool but not in oocytes contained within quiescent primordial follicles (de Vries et al., 2000). Mice from this breeding program had one of four possible genotypes: (1) Fzr1<sup>+/loxp</sup>/Zp3-Cre<sup>-/-</sup>; (2) Fzr1<sup>+/loxp</sup>/Zp3-Cre<sup>-/-</sup>; (3) Fzr1<sup>Loxp/Loxp</sup>/Zp3-Cre<sup>-/-</sup>; and (4) Fzr1<sup>Loxp/Loxp</sup>/Zp3-Cre<sup>+/+</sup> (Fig. 1B). They were born at the expected Mendelian frequencies (see Table S1 in the supplementary material) and all mice thrived and appeared healthy. Fully grown oocytes collected from Fzr1<sup>+/loxp</sup> and Fzr1<sup>Loxp/Loxp</sup> littersmates contained levels of FZR1 protein that were no different from those taken from Fzr1<sup>+/+</sup> mice (data not shown) and are therefore referred to as controls. However, oocytes from Fzr1<sup>+/loxp</sup>/Zp3-Cre<sup>+/+</sup> mice (hereafter Fzr1<sup>+/+</sup>) showed a 30% reduction in FZR1 compared with these controls (Fig. 1C,D). Fzr1<sup>Loxp/Loxp</sup>/Zp3-Cre<sup>+/+</sup> mice (hereafter Fzr1<sup>Loxp/Loxp</sup>) were predicted to carry only the
Fzr1 deletion and this was confirmed by immunoblots in which we failed to detect any FZR1 protein in oocytes (Fig. 1C). By contrast, FZR1 was readily observed at control levels in various somatic tissues from Fzr1Δ/Δ mice, including the granulosa cells that are connected to oocytes by gap junctions (Kidder and Mhawi, 2002; Edry et al., 2006), showing that the deletion was oocyte specific (Fig. 1E). We conclude, therefore, that we have achieved a specific knockout of Fzr1 in oocytes.

**Spontaneous meiotic resumption and oocyte loss following Fzr1 knockout**

Ovaries from Fzr1Δ/Δ mice appeared histologically normal, with quiescent and growing follicles present at different stages of growth (Fig. 2A). Control, Fzr1+/Δ and Fzr1Δ/Δ mice were hormonally primed, and 48-52 hours later fully grown cumulus-enclosed oocytes (CEOs) were counted and their maturation status assessed when collected from antral follicles. The median and spread in oocyte numbers analyzed. Error bars indicate s.e.m. (Fig. 2E). The GVB rate in heterozygotes (++, P<0.01; ANOVA) was highly significant compared with controls (***, P<0.001; χ²). The GVB rate in heterozygotes was not statistically significant from that of controls (χ²).}

Fig. 1. Oocyte-specific knockout of Fzr1 by Cre-loxP recombination. (A) The mouse Fzr1 gene, showing the position of the inserted loxP sites (triangles) and the deletion caused by Cre recombinase (Fzr1Δ). For genotyping, primers spanning the intron between exons 3 and 4 were used, with the predicted PCR product sizes as indicated. 5'β′ untranslated regions (UTR), black; coding sequence (CDS), white. (B) Representative PCR products from tail-tip genomic DNA of the four genotypes produced by the breeding program: Fzr1+/loxP, Fzr1+/loxP, Zp3-Cre+/T; Fzr1+/loxP, and Fzr1+/loxP, Zp3-Cre+/T; in addition to wild-type mice. Fzr1 PCR, even-numbered lanes; Cre PCR, odd-numbered lanes. (C) Representative immunoblot for FZR1 protein in fully grown GV oocytes: pooled Fzr1+/loxP and Fzr1+/loxP (control, Ctrl); Fzr1+/loxP, Zp3-Cre+/T (+/Δ); and Fzr1+/loxP, Zp3-Cre+/T (Δ/Δ) mice. Thirty oocytes per lane, four replicates. (D) FZR1 protein levels determined by densitometric scans of immunoblots in C, normalized with respect to controls. n=4; ***, P<0.001; ANOVA. Error bars indicate s.e.m. (E) Immunobots for FZR1 protein in somatic tissues of control Fzr1+/loxP (Ctrl) and Fzr1+/loxP, Zp3-Cre+/T (Δ/Δ) mice. Br, brain; Lu, lung; Liv, liver; Sp, spleen; K, kidney; GC, ovarian granulosa cells (10 μg protein per lane).

**Fig. 2. Reduced oocyte numbers and increased GVB following Fzr1 knockout.** (A) Histological sections of ovaries from 5-week old Fzr1+/loxP and Fzr1Δ/loxP, Zp3-Cre+/T (Δ/Δ) mice. Quiescent, growing and mature follicles are evident in both ovaries. White arrowhead indicates pre-antral follicle; black arrowhead indicates antral follicle. (B) Total number of cumulus-enclosed oocytes (CEOs) collected per mouse from preovulatory follicles in hormonally primed controls Fzr1+/loxP and Fzr1+/loxP (control, Ctrl), Fzr1+/loxP, Zp3-Cre+/T (+/Δ), and Fzr1+/loxP, Zp3-Cre+/T (Δ/Δ) mice. Significantly fewer CEOs were collected from the knockout Fzr1Δ/Δ mice but not from Fzr1+/loxP heterozygotes (++, P<0.01; ANOVA). Numbers in parenthesis refer to mouse numbers analyzed. Error bars indicate s.e.m. (C) Percentage of CEOs that had undergone GVB at the time of collection in B. Significantly more Fzr1+/loxP oocytes had undergone GVB at the time of recovery compared with controls (***, P<0.001; χ²). The GVB rate in heterozygotes was not statistically significant from that of controls (χ²). (D) Percentage of abnormal oocytes that were either fragmented or had resumed meiosis collected from hormonally primed mice. Significantly more oocytes from Fzr1Δ/Δ mice were abnormal compared with controls (***, P<0.0001; χ²). Numbers in parenthesis (C,D) refer to oocyte numbers analyzed. (E) Representative brightfield image of oocytes collected from a Fzr1Δ/Δ mouse. Arrowheads indicate abnormal oocytes that have undergone meiotic resumption or fragmentation. Scale bars: 200 μm in A; 50 μm in E.
The CEOs collected from hormonally primed mice should be GV intact. As expected, almost 100% of both control and Fzr1Δ/Δ oocytes collected were GV arrested (97% and 98%, respectively; not significant, χ²). High rates of GV arrest were also observed in the oocytes collected from Fzr1Δ/Δ mice; however, here there was a significant rise to 8% in the number of oocytes that had spontaneously resumed meiosis I (P<0.01; χ²; Fig. 2C).

We predicted that the ~35% reduction in CEOs collected from Fzr1Δ/Δ mice was due to spontaneous maturation in vivo leading to follicular atresia. If so, a much higher rate of naturally denuded oocytes that are in various stages of meiotic maturation or fragmentation should be observed in the knockouts. This proved to be the case, with 23% of all fully grown oocytes collected from Fzr1Δ/Δ mice found to be denuded and in various stages of meiotic resumption or fragmentation, compared with just 4% of controls (P<0.001, χ²; Fig. 2D,E). Thus, all the above data are consistent with a model in which the loss of FZR1 causes a substantial fraction of the fully grown GV oocyte pool to undergo meiotic resumption and follicular degeneration in the ovary with an associated loss of CEO integrity.

**Fzr1 knockout causes precocious meiotic entry in small oocytes**

Small oocytes, of less than 60 μm diameter, are known to be meiotically incompetent (Sorensen and Wassarman, 1976; Wickramasinghe et al., 1991; Hirao et al., 1993). As such, when they are denuded and cultured in vitro they remain GV arrested. We questioned whether the loss of Fzr1 had the ability to cause precocious meiotic competence, given the observed increases in meiotic resumption and rates of fragmentation for fully grown oocytes collected from primed mice. We enzymatically disaggregated oocytes of various diameters from both control and Fzr1Δ/Δ ovaries and scored their ability to undergo GVB following 24 hours of culture in MEM medium without milrinone. Prior to culture, all oocytes of less than 70 μm diameter were GV arrested. After 24 hours of culture, as predicted, nearly all control oocytes below 60 μm did not undergo GVB whereas nearly half of 60-69 μm diameter oocytes did (Fig. 3A). Interestingly, a significantly greater proportion of Fzr1Δ/Δ oocytes of 50-59 μm and 60-69 μm diameter underwent GVB after culture compared with control oocytes (15% and 38% more, respectively; [Fig. 3A,B]). These data suggest that FZR1 plays a role in the timing of meiotic competency as oocytes grow in size.

Fully grown, meiotically competent and transcriptionally silent oocytes are often associated with the development of a different nuclear staining pattern termed ‘surrounded nucleolus’ (SN), as compared with that of smaller, ‘non-surrounded nucleolus’ (NSN) oocytes (Debey et al., 1993; Boumil-Baly et al., 1999; De La Fuente and Eppig, 2001; Tan et al., 2009). This change has been associated with the transcriptional silencing that occurs during the final stages of growth. We examined the chromatin configuration (NSN versus SN) of fully grown oocytes from control and Fzr1Δ/Δ ovaries. We did not find any change in the proportion of oocytes that were of the SN configuration in Fzr1Δ/Δ oocytes compared with controls, with the majority of fully grown oocytes displaying the SN configuration (Fig. 3C,D).

Thus, all of the above data taken together suggest that loss of Fzr1 promotes the ability of growing oocytes to prematurely escape from GV arrest in vivo. Despite this, fully grown GV-arrested oocytes can still be recovered from Fzr1Δ/Δ mice, and these oocytes have similar chromatin configurations to control oocytes.

**Fzr1 knockout causes spontaneous and accelerated meiotic resumption**

The ovarian environment normally prevents meiotic resumption by maintaining high levels of cAMP in the oocyte. In order to determine whether FZR1-deficient GV oocytes display any propensity for GVB in culture, control and Fzr1Δ/Δ oocytes were denuded of their cumulus cells (denuded oocytes, DOs) and cultured with one of three doses (0.6, 1 or 10 μM) of the phosphodiesterase 3 (PDE3) inhibitor milrinone for 12 hours and then GVB assessed. At the highest dose, very few oocytes (less than 3%) underwent GVB irrespective of the Fzr1 status. This suggests that high cAMP levels are sufficient to maintain GV arrest in vitro (Fig. 4A). However, clear differences in sensitivity to milrinone between control and Fzr1Δ/Δ oocytes became evident at the two lower doses. At 1 μM milrinone, GVB occurred in only 4% of control oocytes but in over 50% of Fzr1Δ/Δ oocytes (P<0.001, χ²; Fig. 4A), and at 0.6 μM, GVB increased to 90% compared with 24% in the controls (P<0.001, χ²; Fig. 4A).
ANOVA. (B) CEOs had significantly lower rates of GVB than DOs, suggesting that GVB is expressed as a cumulative percentage of the total. GVB is Student’s following 10 Fzr1 of oocytes undergoing GVB and this effect was more evident in from the oocytes, a significant reduction occurred in the percentage cumulus cells intact (CEOs) in the presence of 1 FZR1 and meiotic resumption in oocytes were assessed in control and maturation-inhibitory potential of the granulosa cells, GVB rates consistent with the idea that loss of FZR1 promotes GVB but that observed in Fig. 4. High sensitivity to meiotic resumption in cultured oocytes following FZR1 loss. (A) GVB rates in denuded oocytes (DOs) collected from control (Fzr1<sup>+/loxp</sup>, Fzr1<sup>loxp/loxp</sup>) and Fzr1<sup>ΔΔ</sup> mice. Oocytes from hormonally primed mice were cultured in media with the stated concentrations of milrinone for 12 hours and GVB rates plotted as a percentage of the total oocytes observed. The highest milrinone dose tested (10 μM) was effective at inhibiting GVB in nearly all oocytes examined, but at lower doses significantly greater rates of GVB were observed in Fzr1<sup>ΔΔ</sup> oocytes compared with controls (***, P<0.001; ANOVA). (B) GVB rates in oocytes maintained as either DOs or CEOs, collected from control (Fzr1<sup>+/loxp</sup>, Fzr1<sup>loxp/loxp</sup>) and Fzr1<sup>ΔΔ</sup> mice and cultured in 1 μM milrinone for 12 hours. Fzr1<sup>ΔΔ</sup> oocytes cultured as CEOs had significantly lower rates of GVB than DOs, suggesting that the attached granulosa cells conferred some degree of resistance to GVB in the absence of Fzr1 (***, P<0.001; ANOVA). (C) Time of GVB following 10 μM milrinone washout for control and Fzr1<sup>ΔΔ</sup> oocytes. GVB is expressed as a cumulative percentage of the total. GVB is significantly accelerated for Fzr1<sup>ΔΔ</sup> oocytes by ~21 minutes (P<0.001; Student’s t-test). Arrows indicate the time at which 50% of oocytes had undergone GVB.

The above in vivo and in vitro experiments (Figs 2 and 3) are all consistent with the idea that loss of FZR1 promotes GVB but that this can be countered, up to a point, by the maturation-inhibitory follicular environment, which in other studies has been documented about 80% of control oocytes underwent GVB within a relatively narrow window, 40-60 minutes after milrinone washout, with 45 minutes being the time at which 50% of oocytes had undergone GVB (Fig. 4C). For Fzr1<sup>ΔΔ</sup> oocytes these times were significantly shorter (mean time of GVB of 29 minutes; P<0.001, Student’s t-test).

High Cyclin B1 levels in Fzr1 knockout oocytes
Cyclin B1 and securin have both been reported to be APC/C FZR1 substrates in GV oocytes based on morpholino antisense knockdowns and overexpression studies (Reis et al., 2006a; Marangos and Carroll, 2008). We failed to detect any rise in securin levels in Fzr1<sup>ΔΔ</sup> or Fzr1<sup>ΔΔ</sup> oocytes relative to control oocytes (Fig. 5A,B). By contrast, there was a 5-fold increase in cyclin B1 levels in Fzr1<sup>ΔΔ</sup> oocytes, but not in heterozygous Fzr1<sup>ΔΔ</sup> oocytes compared with controls (Fig. 5A,C; P<0.01, ANOVA, n=3 independent blots). A rise in cyclin B1 levels was also observed in growing oocytes of 50-69 μm diameter (not shown).

We reasoned that the 5-fold increase in cyclin B1 levels is at least one of the primary reasons for the enhanced rates and earlier timing of GVB in Fzr1<sup>ΔΔ</sup>. This is because a number of studies have established that mouse oocytes show both high rates of GVB and an accelerated entry into GVB following cyclin B1 overexpression, phenocopying the effect seen here with Fzr1 knockout (Ledan et al., 2001; Marangos and Carroll, 2004; Holt et al., 2010). We therefore attempted to knockdown cyclin B1 in Fzr1<sup>ΔΔ</sup> oocytes in order to test for an effect on the timing of GVB. Oocytes were microinjected with cyclin B1 dsRNA and cultured for 48 hours in 10 μM milrinone. This achieved a 65% knockdown in cyclin B1 (see Fig. S1A in the supplementary material), which was not enough to reduce levels exactly to those of controls (see Fig. 5).

Fig. 5. High levels of cyclin B1 but not securin in Fzr1 knockout oocytes. (A) Representative immunoblot for cyclin B1, securin and GAPDH in control, Fzr1<sup>ΔΔ/ΔΔ</sup> and Fzr1<sup>ΔΔ/ΔΔ</sup> mouse oocytes. Thirty oocytes per lane. (B,C) Securin (B) and cyclin B1 (C) protein levels determined by densitometric scans of immunoblots in A, normalized to controls. n=3; **, P<0.01; ns, not significant; χ<sup>2</sup>. (D) Mean time of GVB following 10 μM milrinone washout in Fzr1<sup>ΔΔ</sup> oocytes injected with cyclin B1 dsRNA, Fzr1 dsRNA or mock injected, as well as in control oocytes. Time of GVB is significantly increased in Fzr1<sup>ΔΔ</sup> oocytes with cyclin B1 dsRNA, but not Fzr1 dsRNA, as compared with mock injected (***, P<0.001; ANOVA). Control oocytes were significantly different to all Fzr1<sup>ΔΔ</sup> conditions (***, P<0.001, ANOVA). Error bars indicate s.e.m.
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Fig. S1B in the supplementary material), but incubations beyond 2 days did not lead to any greater loss and instead started to compromisely oocyte viability (not shown). A high (10 μM) milrinone concentration was needed to maintain GV arrest in sufficient numbers for analysis. Using Fzr1 dsRNA and a sham injection as controls, we observed that the mean time of GVB in cyclin B1 dsRNA-treated Fzr1<sup>−/−</sup> oocytes was significantly delayed (P < 0.001, ANOVA), being 20 minutes later than the mean time of GVB for controls (Fig. 5D). However, it should be noted that despite a high level of knockdown, the cyclin B1 dsRNA-treated Fzr1<sup>−/−</sup> oocytes still underwent GVB at a time that was accelerated relative to control littermate oocytes (Fig. 5D). It remains possible that the still elevated cyclin B1 levels in these oocytes accounts for the difference (see Fig. S1A,B in the supplementary material), or, alternatively, that other factors affecting timing are involved in this acceleration. However, independent of this finding, we conclude that the accelerated GVB observed for Fzr1<sup>−/−</sup> oocytes can be ameliorated by cyclin B1 knockdown, consistent with the idea that the effects of FZR1 loss in oocytes are mediated, at least in part, by elevated cyclin B1.

Of two potential FZR1 substrates in the oocytes examined – cyclin B1 and securin – the levels of only one appear affected by FZR1 loss. Although a detailed screening of potential FZR1 substrates in oocytes is made difficult by the paucity of material, we decided to examine the levels of a third potential substrate CDC25B, especially as elevated levels of CDC25B may promote GVB. In mitotic cells, CDC25B is likely to be degraded by APC/C<sup>FZR1</sup> activity (Baldin et al., 1997; Kieffer et al., 2007) and in mice CDC25B activity is essential for GVB (Lincoln et al., 2002). However, we observed no difference in CDC25B protein levels in Fzr1<sup>−/−</sup> oocytes relative to controls (see Fig. S1C,D in the supplementary material). We conclude, therefore, that although CDC25B and securin may be APC/C<sup>FZR1</sup> substrates, FZR1 does not critically regulate their levels in GV oocytes. By contrast, cyclin B1 levels are regulated by FZR1, and rises in cyclin B1 contribute to the effects of FZR1 loss upon oocytes.

**DISCUSSION**

A unique and essential feature of oocytes from all mammals is that GV arrest must be strictly regulated to ensure the appropriate timing of meiotic entry following LH stimulation in the periovulatory period. Here, we have shown that loss of FZR1 results in premature GVB in the oocytes of growing and mature follicles. This represents the first in vivo report confirming the importance in GV arrest of controlling cyclin B1, the regulatory binding partner of CDK1, through FZR1-mediated protein degradation. This is distinct from the GPR3-cAMP-PKA axis that affects CDK1 phosphorylation status and is the second example of FZR1 having a non-mitotic function (Konishi et al., 2004).

**Generation of mice deficient in oocyte Fzr1**

We have deleted Fzr1 using the Zp3 promoter to drive Cre expression in order to restrict its loss to growing and mature oocytes (de Vries et al., 2000). Fzr1 is widely expressed in somatic tissues and a complete knockout is embryonic lethal due to defects in placental development (Garcia-Higuera et al., 2008; Li et al., 2008). In this study, we observed that heterozygous oocytes behaved like wild-type controls. This is most likely because these oocytes still contained ~70% of the control levels of FZR1 protein. However, Fzr1<sup>−/−</sup> mice contained no detectable levels of FZR1. Our cell-specific and temporal knockout of Fzr1 allowed us to examine FZR1 strictly during GV arrest.

**Loss of Fzr1 potentiates in vivo meiotic resumption**

CEOs from Fzr1<sup>−/−</sup> mice, as compared with controls, were 35-40% fewer in number when collected from antral follicles of hormonally primed animals, with 8% having undergone GVB. It should be noted that the in vivo resumption of meiosis was not a result of an increase in LH levels or responsiveness, as cumulus expansion, a normal event following LH action (Richards, 2005), was not detectable in oocytes upon collection. Instead, it is likely that it is due to elevated cyclin B1 in such oocytes, as discussed below.

The reduction in CEO numbers and the higher GVB rates following Fzr1 knockdown are likely to be linked, with the reduction being due to oocytes escaping arrest, resulting in follicular loss. It is well recognized that follicle growth and viability require oocyte-specific proteins, as well as continuous oocyte-granulosa cell communication that is affected by meiotic status (Dong et al., 1996; Simon et al., 1997; Edelmann et al., 1999; Rajkovic et al., 2004; Pangas and Rajkovic, 2006). Therefore, either FZR1 is important in maintaining follicle health during the growth phase by the degradation of pro-atretic factors, or precocious GVB in itself enhances follicle degeneration. The reduction is not due to a smaller pool of non-growing follicles, which was the same in control and in Fzr1<sup>−/−</sup> mice (data not shown), consistent with the lack of Cre activity at this time when driven by the Zp3 promoter (de Vries et al., 2000). It is also not due to altered follicle-stimulating hormone (FSH) responsiveness, as we observed normal follicle growth when whole follicles were cultured in vitro with FSH (not shown). Combining our observations of higher levels of GVB in CEOs and higher levels of fragmentation and GVB in the DO pool suggests that precocious GVB leads to increased rates of follicular atresia and loss of healthy CEOs in the ovaries of Fzr1 knockout mice.

**Loss of Fzr1 potentiates in vitro meiotic resumption**

The phenomenon of a maturation-inhibitory environment in the ovary has been well established and is due to maintenance of high cAMP levels in the oocyte. The oocyte receptor GPR3, which stimulates adenylyl cyclase, is the likely mechanism by which cAMP is generated, supported by PDE3 inhibition from granulosa cell cGMP that passes into the oocyte through gap junctions (Sela-Abramovich et al., 2008; Norris et al., 2009; Vaccari et al., 2009; Zhang et al., 2010). Here, we used milrinone, a PDE3 inhibitor, to block the process of spontaneous maturation when oocytes were removed from the ovary. It was clear that oocytes from Fzr1<sup>−/−</sup> mice needed much higher doses of milrinone in the culture media to maintain arrest and underwent GVB much more quickly than control oocytes when washed free of this PDE3 inhibitor. Under in vitro conditions the granulosa cells afforded partial protection against precocious GVB in knockout oocytes. This is likely to be because they contribute maturation-inhibitory factors such as cGMP to the oocyte (Norris et al., 2009; Zhang et al., 2010). We conclude, therefore, that the behavior of the Fzr1 knockout oocytes in vitro mirrors the observations that we made in vivo. In summary, Fzr1 loss makes GV oocytes much more susceptible to undergo GVB precociously; however, this can be counter-balanced by the maturation-inhibitory environment of the follicle.

**Fzr1 loss leads to premature meiotic competence**

Not all oocytes that can be recovered from ovaries are able to undergo GVB when released into culture medium. As follicles are recruited, oocytes grow in size although they remain GV arrested.
A number of studies have established that when oocytes reach a diameter of 60 µm they become competent to undergo GVB (Sorensen and Wassarman, 1976; Wickramasinghe et al., 1991; Hirao et al., 1993). Because CDK1-cyclin B1 plays a role in inducing GVB it would be expected that such inability of small oocytes to undergo GVB would be related to some aspect of CDK1 activity. We noted a lack of premature GVB in small oocytes irrespective of Fzr1 status, both within histological sections and in enzymatically isolated oocytes. Only following in vitro culture did small oocytes (50-60 µm) from Fzar1ΔA animals display a propensity to undergo GVB, in contrast to controls. This suggests that the role of FZR1 in the maintenance of GV arrest only becomes important once oocytes reach a critical size.

FZR1 downregulates cyclin B1 levels during GV arrest

cAMP-mediated PKA activity maintains GV arrest by phosphorylating, sequestering and inactivating CDC25B (Zhang et al., 2008; Pirino et al., 2009). LH triggers CDC25B translocation to the nucleus, WEE1B (WEE2 – Mouse Genome Informatics) to the cytoplasm, and consequential activation of CDK1-cyclin B1, which is also associated with nuclear translocation (Fig. 6A) (Marangos and Carroll, 2004; Han et al., 2005; Oh et al., 2010). Despite these studies establishing the importance of maintaining low CDC25B activity for GV arrest, and the fact that in mitosis CDC25B is highly likely to be degraded through an APC/CΔFZR1-dependent mechanism (Baldin et al., 1997; Kieffer et al., 2007), we did not observe any pronounced increase in CDC25B levels following Fzr1 knockout. Similar to CDC25B, following FZR1 loss levels of securin were not elevated, despite a previous report in mouse GV oocytes that APC/CΔFZR1 has substrate preference for securin over cyclin B1 (Marangos and Carroll, 2008). The present data do not necessarily contradict these latter observations, which were nonetheless largely based on exogenous, overexpressed substrates, because it might be that levels of securin in oocytes are primarily set by transcription or translation rather than degradation.

Of the three potential APC/CΔFZR1 substrates examined here, only cyclin B1, which was upregulated 5-fold in Fzar1ΔA oocytes compared with control oocytes, appeared critically regulated by FZR1. The importance of FZR1 in regulating cyclin B1 has also been observed in neurons (Almeida et al., 2005; Maestre et al., 2008). However, in these cells the outcome is different, as the raised cyclin B1 causes postmitotic G0 cells to re-enter mitotic cell cycle division, with the consequence that they undergo programmed cell death. In mouse oocytes, cyclin B1 overexpression can mimic all of the effects that we observe with Fzr1 knockout oocytes in vitro (Ledan et al., 2001; Marangos and Carroll, 2004; Reis et al., 2006a; Holt et al., 2010), and a partial cyclin B1 knockdown had effects on the timing of GVB in Fzar1ΔA oocytes. We conclude, therefore, that cyclin B1 overexpression drives the precocious meiotic resumption from prophase I arrest in oocytes following FZR1 loss. Overexpression of cyclin B1, the regulatory binding partner of CDK1, has similarly been reported to induce entry into mitosis in early Drosophila embryos (Royou et al., 2008) as well as in reconstituted somatic cell extracts (Deibler and Kirschner, 2010). Therefore, it is not a unique property of mouse oocytes that they are sensitized to undergo a G2/M-type transition by raised levels of cyclin B1. The study of precisely how raised cyclin B1 manages to activate CDK1 is made difficult by the inherently complicated feedback pathways operating at the G2/M transition that can often act in a redundant manner (Lindqvist et al., 2009). However, as revealed using somatic cell extracts, the roles played by cyclin B1 in affecting the interaction of CDK1 with its inhibitory kinase WEE1, as well as in activating CDC25 at higher concentrations, have helped to establish a molecular mechanism that would explain the ability of raised cyclin B1 to induce mitotic entry (Deibler and Kirschner, 2010).

Despite the argument in favor of the importance of cyclin B1, it remains possible that other proteins are upregulated as a result of FZR1 loss and contribute to the Fzar1ΔA phenotype. An exhaustive list of FZR1 targets in any system has not yet been established but at least 20 proteins involved in CDK1 regulation and mitotic progression have already been described, with a similar number targeted for degradation during G1 (Qiao et al., 2010). A more detailed analysis of the potential targets of APC/CΔFZR1 has to be set against the small numbers of oocytes that can be collected for analysis. There is much interest in what constitutes the physiological set of FZR1 substrates, with contrasting observations on what is stabilized following Fzr1 knockout or knockout (Floyd et al., 2008; Garcia-Higuera et al., 2008; Li et al., 2008; Sigl et al., 2009). Although these other studies have failed to detect any differences in the levels of cyclin B1 following loss of FZR1, it should be noted that they were performed on mitotically dividing cells that do have the capacity to degrade cyclin B1 through APC/CΔCDC20 activity. Although oocytes contain CDC20 at all stages of meiotic maturation (Shoji et al., 2006; Reis et al., 2007), it is unlikely to be able to regulate cyclin B1 during GV arrest because its activity requires APC/C phosphorylation, which is normally limited to M phase by Polo kinase and CDK1 (Kramer et al., 2000; Golan et al., 2002; Kraft et al., 2003). Consistent with

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**Fig. 6. A model for oocyte prophase I arrest and outcomes of oocyte-specific FZR1 loss.**

(A) Maintenance of high cAMP via a GPR3 receptor maintains inhibition of CDC25B and activation of WEE1B, thus repressing CDK1 activity, whereas APC/CΔFZR1 activity maintains low cyclin B1 levels. Upregulation of CDK1 activity or cyclin B1 levels leads to resumption of meiosis. (B) The fully grown oocyte is normally prophase VGV arrested; however, in the absence of FZR1, increased cyclin B1 levels induce premature meiotic resumption and oocyte loss.
this, APC/C^Cdc20 activity has only been observed in the equivalent meiotic period of oocytes, several hours following GVBD (Herbert et al., 2003; Reis et al., 2007).

In summary, we have established that FZR1, in concert with the well-established regulation of CDK1, plays a key role in the maintenance of oocyte GV arrest in the mouse. FZR1 maintains a low level of cyclin B1, which makes growing oocytes refractory to precocious meiotic resumption. We have thus shown that this mitotic protein plays an important role in prophase I arrest.

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Competing interests statement
The authors declare no competing financial interests.

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FZR1 and meiotic resumption in oocytes


