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Function of the A-Type Cyclins During Gametogenesis and Early Embryogenesis

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Abstract

The cyclins and their cyclin-dependent kinase partners, the Cdks, are the basic components of the machinery that regulates the passage of cells through the cell cycle. Among the cyclins, those known as the A-type cyclins are unique in that in somatic cells, they appear to function at two stages of the cell cycle, at the G1-S transition and again as the cells prepare to enter M-phase. Higher vertebrate organisms have two A-type cyclins, cyclin A1 and cyclin A2, both of which are expressed in the germ line and/or early embryo, following highly specialized patterns that suggest functions in both mitosis and meiosis. Insight into their in vivo functions has been obtained from gene targeting experiments in the mouse model. Loss of cyclin A1 results in disruption of spermatogenesis and male sterility due to cell arrest in the late diplotene stage of the meiotic cell cycle. In contrast, cyclin A2-deficiency is marked by early embryonic lethality; thus, understanding the function of cyclin A2 in the adult germ line awaits conditional mutagenesis or other approaches to knock down its expression.

17.1 Introduction to the A-Type Cyclins

Although cyclin A was the first cyclin identified and subsequently cloned in any organism (Swenson et al. 1986), our understanding of the mechanisms of regulation of expression and function of the A-type cyclins remains somewhat limited. Cyclin A was originally described as a protein that exhibited an unusual expression pattern in clam embryos (Evans et al. 1983). Subsequently, genes for cyclin A have now been found in all multicellular organisms, including humans (Pines and Hunter 1990). While only a single gene encoding cyclin A is present in the genomes of the nematode *C. elegans* and the fruitfly *Drosophila melanogaster*, we discovered that there are two distinct cyclin A genes in the mouse, one of which, *Ccna1*, is testis-specific and restricted to the germ line (Ravnik and Wolgemuth 1996; Sweeney et al. 1996). Two A-type cyclin genes have now been documented in many other organisms, including humans (Yang et al. 1997). The gene encoding the second

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mammalian A-type cyclin, cyclin A2 (*Ccna2*), is ubiquitously expressed in all proliferating cells and is upregulated in a variety of cancers (Pines and Hunter 1990; Wang et al. 1990).

Cyclin A2 is generally considered to be the critical mammalian S-phase cyclin (Hochegger et al. 2008; Pines and Hunter 1990; Yam et al. 2002), but is unique among the mammalian cyclins in that it is expressed in more than one stage of the cell cycle, specifically in both the S- and G2-phases. Cyclin A2 is induced at the beginning of S-phase (Erlandsson et al. 2000; Girard et al. 1991) and once synthesized, it binds and activates its catalytic partners, the cyclin-dependent kinases (Cdk) Cdk2 and Cdk1. The cyclin A2-Cdk complexes are the machinery that drive S-phase progression, at least in part by phosphorylating proteins that play important roles in DNA synthesis (den Elzen and Pines 2001; Girard et al. 1991; Pagano et al. 1992; Yam et al. 2002; Zindy et al. 1992; Fisher 2011). Its expression at the G2-phase further suggests a second function, involving the entry of cells into mitosis (Swenson et al. 1986). Indeed, injection of anticyclin A2 antibodies into cultured fibroblasts, or inhibition of cyclin A2 function by p21Cip1 during the G2-phase, blocked the progression of cells into mitosis (Furuno et al. 1999; Pagano et al. 1992). These and numerous other studies have led to the current model that the “core” components of the cell-cycle machinery consist of cyclins A and B and their associated Cdks and constitute the critical elements of the cell-cycle engine (Hochegger et al. 2008; Murphy et al. 1997). Perhaps not surprisingly then, targeted mutagenesis of the murine *Ccna2* gene resulted in early embryonic lethality, apparently around the peri-implantation stage (Murphy et al. 1997), as did loss of function of the cyclin B1 gene (Brandeis et al. 1998). However, as discussed in greater detail below, cyclin A2 appears to be dispensable for very early embryonic cell divisions.

17.2 Expression and Function of the A-Type Cyclins During Gametogenesis

17.2.1 Unique Features of Mammalian Gametogenesis from a Cell Cycle Perspective

Gametogenesis in higher organisms affords a unique opportunity for understanding the regulation of both the mitotic and meiotic cell cycles. Given the high level of conservation of key regulatory components across evolutionarily diverse organisms, it is likely that specific proteins will have highly conserved functions during both mitosis and meiosis in mammals, as they do in *Drosophila* and the yeasts in which they have been best characterized. However, it is clear that there are control points and checkpoints in the mitotic and meiotic cell cycles of higher eukaryotes that do not exist in simpler organisms and which further differ between the male and female germ lines (rev. in Handel et al. 1999; Wolgemuth 2002, 2003; Wolgemuth and Roberts 2010). The mitotic divisions of the gonocytes and the signals to enter and progress through meiosis are precisely controlled and exhibit sexual dimorphism with regard to their timing during development. For example, in female mammals, the oogonia commit to meiosis during embryonic development while in the male, this occurs at puberty and throughout adult life. As another example, the oocyte arrests in the diplotene stage of meiosis for a period that can last for months or years depending on the species. Progression through meiosis in the female germ cell pauses again in metaphase II awaiting fertilization to complete the second meiotic division.

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In contrast, spermatocytes progress through meiosis with very different temporal hallmarks – once spermatogonia enter preleptotene S-phase, the first and second meiotic divisions proceed without interruption, yielding haploid spermatids which then undergo the dramatic chromatin remodeling events of spermiogenesis. The mitotic divisions that precede meiosis are also rigorously regulated and vary from species to species. The spermatogonial stem cells undergo mitotic divisions wherein one product of this cell division maintains self-renewing, stem cell qualities while the other one goes on to further mitotic divisions and form spermatogonia. Spermatogonia in mammals are usually classified as type A spermatogonia which do not exhibit heterochromatin histologically or B-type spermatogonia which do. In the spermatogonial compartment of mice, the stem cell is designated as A_{single} (A_s) and the subsequent products of mitotic divisions as A_{paired} (A_{pr}) and $A_{aligned}$ (A_{al}). These cells can be identified according to their characteristic location on the basal membrane of the seminiferous tubule. The A_{al} cells, 16 in number in the mouse, then undergo six mitotic divisions, yielding the A_1, A_2, A_3, and A_4 followed by Intermediate and type B spermatogonia, which then enter the preleptotene stage of meiosis.

17.2.2 The A-Type Cyclins in the Male Germ Line

The two A-type cyclins exhibit dramatically different patterns of expression: in the mouse, *Ccna2* is ubiquitously expressed in mitotically dividing cells while expression of *Ccna1* is highly restricted, being most abundant in the testis (Sweeney et al. 1996). Within the testis, *Ccna1* is further restricted to the germ line, specifically in stage IX to XII spermatocytes (Sweeney et al. 1996). Human *CCNA1* is also expressed at highest levels in male germ cells, again in pachytene to diplotene spermatocytes and possibly in round spermatids (Liao et al. 2004). We and others have also detected very low levels of cyclin A1 expression in hematopoietic progenitor cells (Ekberg et al. 2004; Kramer et al. 1998; Yang et al. 1997), although the significance of this expression remains to be determined. In addition, although mouse *Ccna1* has also been reported to be expressed in the brain (van der Meer et al. 2004) and ovaries (Sweeney et al. 1996); (and see discussion below), these studies remain to be confirmed.

Ccna1 and *Ccna2* also exhibit a distinct, nonoverlapping pattern of expression during male germ cell development: *Ccna2* expression is downregulated early in the meiotic cell cycle before *Ccna1* is expressed (Ravnik and Wolgemuth 1999; Sweeney et al. 1996). In the adult mouse testis, *Ccna2* is expressed in spermatogonia and preleptotene spermatocytes, suggesting that it could have an S-phase function in both the mitotic cell cycle of spermatogonial germ cells, analogous to its function in somatic cells, but also in germ cells entering meiosis (Ravnik and Wolgemuth 1996, 1999). As discussed in detail below, genetic ablation of cyclin A2 function in the mouse results in early embryonic lethality, thereby precluding our use of conventional gene targeting to elucidate its function in the germ line. In contrast, the strikingly restricted expression of *Ccna1* led us to hypothesize that its primary site of function is in the male germ line, specifically at the first meiotic division.

To test this hypothesis and to begin to address possible redundancy of the two A-type cyclin genes, we generated cyclin A1-deficient mice by targeted mutagenesis of the *Ccna1* gene (Liu et al. 1998a). *Ccna1*$^{-/-}$ mice were overtly healthy and the females were fully fertile;

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however, the males were sterile. Initial histological and cytogenetic analysis revealed an absence of cells from the late diplotene stage on and no spermatids or sperm were seen. Assessment for apoptosis by TUNEL assay showed a massive wave of cell death in diplotene cells. Markers for gene expression in earlier stages of meiotic prophase did not reveal any differences when compared to wild-type animals and examination of synaptonemal complex preparations appeared indistinguishable between normal and cyclin A1-deficient spermatocytes (Liu et al. 1998a). There was a striking reduction in the activation of the maturation promoting factor (MPF) kinase at the end of meiotic prophase, although both Cdk1 and cyclin B proteins were present. The apparent relatively normal appearance of the meiotic chromosomes was supported by subsequent studies in which pachytene cyclin A1-deficient spermatocytes were artificially driven into a meiotic configuration by treatment with okadaic acid (Liu et al. 2000a). Metaphase I preparations from mutant and normal spermatocytes appeared similar, with no obvious defects in chiasmata. Cyclin A1 is therefore essential for passage into the first meiotic division in spermatocytes, a function that cannot be complemented by the concurrently expressed B-type cyclins (Chapman and Wolgemuth 1993).

Nickerson et al. were able to pinpoint the time of arrest in the cyclin A1-deficient spermatocytes to the late diplotene stage of meiosis after the resolution of chiasmata (Nickerson et al. 2007). Use of antibodies against the synaptonemal complex protein 3 (SCP3), which forms part of the lateral element of the synaptonemal complex (Lammers et al. 1994) and antibodies that recognize centrosomes, the human CREST autoimmune antisera (Earnshaw and Rothfield 1985), which are believed to recognize at least in part the CENP-A, B and C proteins (Brenner et al. 1981), enabled careful staging of chromosome spreads prepared from day 17, 21, and 28 wild-type and *Ccna1*$^{-/-}$ mice. This analysis revealed that there was normal meiotic progression until middiplotene and normal formation and resolution of chiasmata. However, instead of proceeding through diakinesis and forming metaphase I bivalents, the cyclin A1-deficient spermatocytes arrested and underwent apoptosis. Interestingly, this arrest is distinct from the meiotic arrest observed in mice deficient in the putative cyclin A1 kinase partner, Cdk2, at several levels. First, Cdk2-deficient mice exhibited meiotic defects in both the male and female germ lines (Ortega et al. 2003). Second, the arrest was observed in mid-pachytene spermatocytes and third, the pachytene chromosomes exhibited thin threads of SCP3 staining, perhaps indicating aberrant pairing (Ortega et al. 2003). In contrast, pachytene chromosomes in cyclin A1-deficient mice were normal with respect to staining of SCP3 (Nickerson et al. 2007) and were able to form apparently normal-appearing meiotic metaphase 1 bivalents upon treatment with okladaic acid to drive exit from prophase (Liu et al. 2000b).

Other interesting features of the arrested spermatocytes included a characteristic clustering of centromeric heterochromatin and the subsequent appearance of γ-H2AX foci, first at the centromere and then along the chromosomal axes (Nickerson et al. 2007). This unusual clustering preceded hallmarks of apoptosis, such as phosphorylation of Ser139 in γ-H2AX, which curiously appeared to begin at the centromeres and then spread along the length of the chromosomes. We speculated that this clustering of centromeric heterochromatin may represent mislocalization or aberrant association of centromeres and noted that it would be

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of great interest to know whether such clustering occurs in other mouse models exhibiting meiotic prophase arrest and apoptosis, such as the *Cdk2* (Ortega et al. 2003), *Spo11* (Baudat et al. 2000), *Mlh1* (Edelmann et al. 1996), and *Atm* (Xu et al. 1996) knockout mice.

Our earlier studies at the immunohistological level had revealed a clear nuclear localization of cyclin A1 in spermatocytes from midpachytene through diplotene (Ravnik and Wolgemuth 1996; Liu et al. 1998a). Subsequent analysis of meiotic chromosome preparations revealed a diffuse staining of chromatin but also specific cyclin A1 localization at foci in the pericentromeric region at late diplotene, coincident with the point of arrest in cyclin A1-deficient mice (Nickerson et al. 2007). Concomitant examination of the chromosomal distribution of Cdk2, a putative binding partner for cyclin A1 (Joshi et al. 2009; Liu et al. 1998b, 2000a), revealed a distribution of Cdk2 protein in the centromeric region at telomeres and at foci along chromosomes during pachytene to diplotene (as reported by Ashley et al. (2001)). Interestingly, despite some overlap, cyclin A1 did not completely colocalize with its putative Cdk2 partner at the centromeres. Furthermore, the distribution of Cdk2 was not altered in cyclin A1-deficient mice.

Also of interest was the observation of an apparent lack of histone H3 serine 10 phosphorylation in the cyclin A1-deficient spermatocytes (Nickerson et al. 2007). Histone H3 becomes phosphorylated at serine 10 at the pericentromeric region in late diplotene of male meiosis and this phosphorylation persists through metaphase I (Cobb et al. 1999). Examination of histological sections of testes and of chromosome spread preparations revealed that phosphorylation of H3 serine 10 was dramatically reduced in heterozygous *Ccna1*$^{+/-}$ spermatocytes and undetectable in homozygous *Ccna1*$^{-/-}$ spermatocytes that are completely devoid of cyclin A1 (Nickerson et al. 2007). The kinase that performs this phosphorylation in both mitosis and meiosis is thought to be the aurora B kinase, a component of the passenger protein complex that also includes INCENP, survivin, and borealin (Vagnarelli and Earnshaw 2004). Aurora B kinase in particular is believed to be critical for correct chromosome alignment at metaphase (Ditchfield et al. 2003). The reduction of this phosphorylation in *Ccna1*$^{+/-}$ spermatocytes and its absence in *Ccna1*$^{-/-}$ spermatocytes prompted us to examine this complex in more detail (Nickerson et al. 2007). We found the levels and distribution of survivin to be indistinguishable between *Ccna1*$^{+/-}$ and *Ccna1*$^{-/-}$ spermatocytes when compared to *Ccna1*$^{+/+}$. In contrast, while immunoblot analysis of whole testicular lysates did not indicate a significant difference in levels of aurora B protein between control and cyclin A1-deficient testicular lysates, the amount of aurora B protein associated with meiotic chromosomes was strikingly different. In particular, we observed a pronounced reduction in staining intensity of aurora B protein localizing to the pericentromeric heterochromatin in late diplotene mutant spermatocytes. This suggests that in the absence of cyclin A1 protein, aurora B does not localize properly, likely contributing to the failure to complete the first meiotic division.

A second targeted mutant allele of *Ccna1* has been generated by the insertion of a *Lac Z* reporter gene (van der Meer et al. 2004), in order to disrupt cyclin A1 production. In this case, the entire cyclin A1 coding region is still in the genome rather than having deleted required coding regions. An unusual splicing event that deleted lacZ sequences could theoretically restore functional cyclin A1 protein, although this did not occur in this case.

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The males carrying this mutation were also sterile and exhibited genetic strain-dependent differences in the fertility of the heterozygous mice as well. That is, when these *Ccna1*$^{+/-}$ mice were on amixed background of 129S6/SvEv and MF1, they were reported to have “reduced sperm production and fertility” as compared to mice carrying the wild-type *Ccna1* allele (van der Meer et al. 2004). Furthermore, mice that were heterozygous for this knockout on a pure 129S6/SvEv background were reported to be sterile due to a greatly reduced production of sperm. To the best of our knowledge, there has been no further characterization of this phenotype, andwe have never observed this reduced fertility in our *Ccna1*$^{+/-}$ mice on a mixed C57Bl/6 and 129SvEv background (Liu et al. 1998a).

Finally, as mentioned above, the lack of cyclin A1 in spermatocytes resulted in a fully penetrant induction of apoptosis. However, it was not clear whether this induction represented a primary response to a lack of cyclin A function or a secondary response to a general degeneration of the highly structured seminiferous tubules. We therefore undertook studies examining the induction of apoptosis during the first wave of spermatogenesis in cyclin A1-deficient testes (Salazar et al. 2005). The temporal appearance of cell death was observed to coincide with the G2/M cell cycle arrest that occurred in late diplotene spermatocytes, suggesting that apoptosis was in fact a primary response to the cell cycle arrest. That is, at the time when the first wave of differentiating cells should be completing the first meiotic division, significantly higher numbers (by almost twofold; $p < 0.01$) of TUNEL-positive pachytene spermatocytes were observed in the cyclin A1-deficient as compared to the control testes. It was also shown that caspase 3 was clearly involved in apoptosis occurring in the cyclin A1-deficient spermatocytes, as increases in the amount of the procaspase protein and changes in the subcellular distribution of the activated form were observed.

Apoptosis in the testis can involve both cell-intrinsic (Bcl-2 family-mediated) and/or cell-extrinsic (Fas-mediated) pathways (Beumer et al. 2000; Lee et al. 1997). Some proteins of the cell-intrinsic pathway, the Bcl-2 family of proteins, including the proapoptotic protein Bax, are essential for normal spermatogenesis. Bax-deficient testes are characterized by an accumulation of spermatogonia, consistent with a proposed failure of germ cell death during the first wave of spermatogenesis (Knudson et al. 1995). The preleptotene spermatocytes failed to undergo meiosis, presumably because of the resulting aberrant Sertoli cell to spermatocyte ratio. Immunohistochemical analysis in cyclin A1-deficient testis showed an increase of Bax-positive spermatocytes and a redistribution of localization of Bax protein from a cytoplasmic to perinuclear and nuclear localization. A positive correlation of the detection of Bax expression and TUNEL-positive cells was also observed. Thus, apoptosis that occurs in the absence of cyclin A1 at least in part involves Bax signaling.

The role (if any) of p53 in regulating cell death in the absence of cyclin A1 in vivo was addressed by producing mice that were both cyclin A1- and p53- deficient (Salazar et al. 2005). Loss of p53 gene function could not rescue the cell cycle arrest in *Ccna1*$^{-/-}$ mutant testis; however, there was a significant reduction in the apoptotic index in the doubly mutant tubules. This observation led us to suggest that cyclin A1 may have roles in regulating two signaling cascades – one leading to progression through meiosis and a second function in regulating apoptosis in spermatocytes. The fact that there were apoptotic cells in the double

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mutant indicated that cell death in response to cyclin A1-deficiency also involved a p53-independent pathway(s). The mutant mice generated by van der Meer and colleagues (van der Meer et al. 2004) were also used in a study in which double cyclin A1 and p53-deficient mice were produced (Baumer et al. 2007). It was reported that these mice had increased numbers of giant cells in the testicular tubules, although the significance of this phenomenon was not pursued. The induction of an apparent apoptotic response (TUNEL-positive) and the appearance of giant cells has been seen in various other gene knockout studies that resulted in impaired meiosis [rev. in (Salazar et al. 2003)]. It would thus appear that the induction of cell death is critical for insuring not only that the proper number of germ cells is produced but also that germ cells that have not gone through the proper reduction divisions of meiosis cannot form viable gametes.

17.2.3 The A-Type Cyclins in the Female Germ Line

Cyclin A2 has been shown to be present in protein extracts from total adult mouse ovary, (Sweeney et al. 1996) and cyclin A2 mRNA and protein were detected in fully grown oocytes (Winston et al. 2000; Fuchimoto et al. 2001). To elucidate the pattern of cyclin A2 expression throughout ovarian development, with a particular focus on the germ line, we undertook a detailed analysis of embryonic to adult ovaries using in situ hybridization, immunohistochemistry, and immunoblotting analysis (Persson et al. 2005). The progression of folliculogenesis in the adult mouse ovary can be staged according to oocyte size, morphological characteristics. and number of layers of the surrounding follicle cells (Pedersen and Peters 1968). In situ hybridization results showed that while *Ccna2* transcripts were detected in granulosa cells at all stages of follicular development, the expression was low in early stage follicles (stages 1–5), according to Pedersen and Peters (1968) and was much higher in growing follicles, particularly in the cumulus layer (cells immediately surrounding the oocyte) in stage 6–8 follicles. It should be recalled that oocytes are arrested at this time, in the diplotene stage of meiosis. However, *Ccna2* transcripts were present in oocytes at all stages of folliculogenesis, from the very early resting follicles to stage 6 and 7 follicles as well as in ovulated eggs within the oviductal ampulla. Immunohistochemistry revealed that the cellular distribution of cyclin A2 protein was similar to that observed for *Ccna2* transcripts, suggesting that cyclin A2 expression is not regulated at the level of translation. The levels of cyclin A2 expression in granulosa cells increased with follicular growth and differentiation whereas more mature oocytes contained less cyclin A2.

Examination of fetal ovaries between embryonic day (E) 13.5–18.5 by immunohistochemistry revealed that cyclin A2 protein was indeed expressed and, further, that the pattern of its distribution changed during development. At E13.5–E14.5, cyclin A2 was detected in mitotically active somatic cells, as well as oogonia and early meiotic oocytes, and was predominantly nuclear. This nuclear localization of cyclin A2 continued through E15.5, when germ cells enter meiotic prophase and are presumably leptotene or zygotene oocytes. Interstingly, at E16.5, about half of the oocytes still displayed nuclear cyclin A2 while in the remaining oocytes, it became predominantly cytoplasmic. At E18.5, when the majority of oocytes are in the pachytene to diplotene/dictyate stage, the localization was predominantly cytoplasmic with only weak nuclear staining.

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The high levels of expression and nuclear localization of cyclin A2 protein in the embryonic ovary suggested that it is active in germ cells as they undergo proliferation and enter into meiotic prophase. It is interesting to compare the striking change from a robust nuclear localization in mitotic oogonia to a predominantly cytoplasmic localization in oocytes meiotic prophase to the cyclin A2 expression in the corresponding stages in spermatocytes. The localization of cyclin A2 is always predominantly nuclear in mitotically proliferating spermatogonia and in preleptotene spermatocytes (Ravnik and Wolgemuth 1999). Cyclin A2 is then undetectable in leptotene, zygotene, pachytene, and diplotene stages of meiotic prophase (or later stages), but in late meiotic prophase, the novel cyclin A1 is expressed (Sweeney et al. 1996). We have speculated that germ cells need to exclude or prevent A-type cyclins from being active during the stages in which the cells are undergoing the "business" of meiosis, so as to prevent premature entry into a meiotic division while in the process of pairing, recombination, repair, etc. (Liao et al. 2005). We further suggest that spermatocytes could achieve this by degrading cyclin A2 and then activating cyclin A1, whereas the oocyte could sequester cyclin A2 to the cytoplasm. A differential sub-cellular distribution of cyclin A1 has also been observed in specific circumstances. While cyclin A1 is normally nuclear in both mouse (Liu et al. 1998a) and human (Liao et al. 2004) late prophase spermatocytes, it was found to be predominantly cytoplasmic in leukemic cells in a transgenic mouse model (Liao et al. 2001) and leukemic cells from patients (Ekberg et al. 2004).

One final note with regard to the two potential cyclin-dependent kinase partners of cyclin A2: readily detectable expression of both Cdk1 and Cdk2, two common partners for the A-type cyclins, was observed in granulosa cells and oocytes at all stages of folliculogenesis (Persson et al. 2005). Cdk1 was predominantly cytoplasmic, whereas Cdk2 was both cytoplasmic and nuclear in oocytes.

And what about a function for cyclin A1 in mouse oocytes? The presence of cyclin A1 protein had been reported in ovulated mouse oocytes in one study (Sweeney et al. 1996) and in another, both mRNA and protein were purported to be present in germinal vesicle-intact oocytes and to decline during meiotic maturation (Fuchimoto et al. 2001). The significance of these observations is unclear, given that the cyclin A1-deficient female mice are fully fertile (Liu et al. 1998a; van der Meer et al. 2004), and we have not detected *Ccna1* mRNA by in situ hybridization or immunohistochemistry in sectioned ovarian tissue (Ravnik S, Persson CL, Wolgemuth DJ, unpublished observations). To resolve the question of cyclin A1 expression in a definitive manner, immunoblot analysis was performed on extracts from total ovaries and oocytes from cyclin A1-deficient mice (and controls) using anticyclin A1 antibodies (Persson et al. 2005). The results revealed the presence of a weakly cross-reacting band, close in size to *bona fide* cyclin A1, in extracts from ovary, isolated oocytes, 1-cell, and 2-cell embryos. This same band was also detected in the testis of cyclin A1-deficient mice, ruling out the possibility its being *bona fide* cyclin A1. Further, in the aforementioned targeted mutagenesis experiment of van der Meer and colleagues (van der Meer et al. 2004), there was no beta-galactosidase expression in the adult ovary. This further suggests that the *Ccna1* gene was not being transcribed, an observation consistent with our conclusions that neither *Ccna1* mRNA nor cyclin A1 protein are expressed in oocytes. Expression of beta-

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galactosidase was reported to be expressed in a few regions of the adult mouse brain in this same study; however, no studies elucidating the physiological relevance of this putative expression have been reported.

Finally, since cyclin A1 is expressed during meiotic prophase in the male germ line, we examined histological sections of embryonic ovaries using anti-cyclin A1 antibodies (Persson et al. 2005). Adult testis sections were included on the same slides as positive controls. No specific staining for cyclin A1 protein was found in embryonic ovaries from E13.5 through 18.5, the latter stages of which clearly contain pachytene and diplotene oocytes. We therefore conclude that, consistent with the results of our targeted mutagenesis experiments (Liu et al. 1998a), cyclin A1 is neither expressed during oogenesis nor required for oocyte function, at least in the mouse model.

17.3 The Early Mouse Embryo and the A-Type Cyclins

Several studies have examined expression of the A-type cyclins in the early embryo, to determine both the contribution, if any, of maternal stocks of mRNA and protein to early embryonic mitotic divisions, as well as to determine when the embryonic cyclin A2 gene is activated. Levels of cyclin A2 protein were reported to decrease with the progression of the oocyte through germinal vesicle breakdown and meiotic maturation (Fuchimoto et al. 2001). However, both maternally produced cyclin A2 mRNA and protein persist through the first mitotic division following fertilization and the activation of transcription of the embryonic genome at the two-cell stage (Winston et al. 2000). They are then reduced to undetectable levels in embryos between the two- to four-cell divisions. Using the model in which *LacZ* was inserted into the endogenous *Ccna2* gene (Murphy et al. 1997), clear evidence for *Ccna2* activation was observed in blastocysts (Winston et al. 2000). In this same study, cyclin A2 protein was also detected by immunofluorescence in individual blastomeres of 8-cell embryos.

The significance of this expression is not clear, however, in light of the very dramatic phenotype seen in cyclin A2-deficient embryos: early embryos divide perfectly well up to the blastocyst stage, but undergo a demise at the time of implantation (Murphy et al. 1997; Winston et al. 2000). The same early embryonic lethality was observed in recent conditional knockout strategies of the cyclin A2 gene (Kalaszczynska et al. 2009). So clearly, cells of the early embryo can undergo mitotic cell division in the complete absence of cyclin A2. These embryos can implant as assessed in vivo and in vitro by blastocyst outgrowth assays but die shortly thereafter (Winston et al. 2000). In addition, there are no obvious differences in their ability to undergo DNA replication, as assayed by BrdU incorporation at the blastocyst stage.

17.4 Regulation of Expression of the A-Type Cyclins in the Germ Line

As noted above, the two A-type cyclins exhibit strikingly different patterns of expression from one another that also differ between the male and female germ line cells. We therefore hypothesized that there will be specific regulatory elements unique to each A-type cyclin that are critical for their distinct regulation of expression. The essential role of *Ccna1* in male germ cell development and the concurrent expression of cyclin A1 mRNA and protein

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suggested the importance of understanding mechanisms controlling transcription of the *Ccna1* gene. The lack of cell lines derived from male germ cells made it necessary to analyze the *Ccna1* promoter in these cells in vivo in transgenic mice (Lele and Wolgemuth 2004). Serial deletions in the *Ccna1* upstream sequence allowed us to define two functional segments of the promoter.

Analysis of transgenes carrying the genomic fragment of mouse *Ccna1* spanning −1.3 kb to +0.8 kb (designated 1.3cyA1lacZ) of the putative transcriptional start site, using a combination of X-gal staining and in situ hybridization, revealed expression specifically in spermatocytes at stages IX to XII of the cycle of the seminiferous epithelium (Lele and Wolgemuth 2004). No expression was observed in spermatogonia or earlier meiotic stages, a pattern that is similar to the narrow window of expression during spermatogenesis seen for the endogenous mouse gene. In contrast, 1.3 kb of human *CCNA1* promoter directs expression of EGFP in a much less restricted pattern in male germ cells (Muller-Tidow et al. 2003), reflecting differences in the mouse and human promoters despite their sharing highly conserved regions.

Within this fragment of the mouse gene are consensus sequences for two sets of paired CDE/CHR elements (Lele and Wolgemuth 2004). These elements were first discovered in the proximal promoter of the *CCNA2, CDK1*, and *CDC25C* genes, which are expressed in S- and G_2-phases of the mitotic cell cycle, and are believed to control the timing of expression of these genes during the cell cycle (Zwicker et al. 1995). In vivo footprinting of the *Ccna2* proximal promoter revealed that the bipartite element was occupied at stages when the gene was not transcribed. Mutation of the CDE/CHR element in the context of *CCNA2* or *CDK1* promoter/reporter genes caused derepression of the reporters in G_1-phase. These elements have now been shown to be involved in controlling the timing of expression of other cell cycle-regulated genes, including the genes for cyclin B2 (Lange-zu Dohna et al. 2000), rabkinesin6 (Fontijn et al. 2001), polo-like kinase (Uchiumi et al. 1997), p130 (Fajas et al. 2000), m-survivin (Otaki et al. 2000), and aurora A (Tanaka et al. 2002). Also, the CDE/CHR element appears to downregulate expression of the human *CDK1* gene in response to TPA-induced differentiation of U937 cells (Sugarman et al. 1995) and in response to p53-dependent DNA damage (Badie et al. 2000).

We further observed that the promoters of the genes for mouse and rat cyclin A1 are unique from the human in that they contain two sets of CDE/CHR elements, but like the human gene, have unpaired CDE and CHR consensus sequences (Lele and Wolgemuth 2004). There have been no previous reports of unpaired CDE elements, but the promoters of human *CCNB2* (Wasner et al. 2003) and mouse C*dc25C* (Haugwitz et al. 2002) are regulated by CHR elements that are not paired with functional CDE elements. Factors that bind CDE/CHR (Liu et al. 1997) or CHR (Kishore et al. 2002; Philips et al. 1999) have been detected in various cultured cell lines, but have not been identified. Although there are many studies examining the regulation of expression of the human and mouse cyclin A2 genes in various cell lines (rev. in Blanchard 2000), to the best of our knowledge, nothing is known with regard to the regulation of their expression in the male and female germ lines.

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17.5 Insight from Other Model Organisms

17.5.1 An Evolutionary Perspective

The processes of gametogenesis, especially in the female germ line, result in oocytes with strikingly different morphologies among various species (e.g., a small, transparent mouse egg vs. a large, opaque frog egg!). Yet the overall goal to produce haploid cells involves common strategies. It has been suggested previously that several aspects of premeiotic germ cell development are in fact widely conserved (Pepling et al. 1999). The genes responsible for many of these processes may also be conserved across species, and thus functions identified for a gene in one species at the very least provide a framework within which to address function in other more complex models. The same is true for early embryogenesis – the syncitial early *Drosophila* embryo may seem a world apart from the totally cellularized early mouse embryo – yet as far as cell cycle control is concerned, many of the same regulators and machinery are not only present but are critical for normal development.

17.5.2 Drosophila

The *Drosophila* genome contains a single A-type cyclin first identified by Lehner and O'Farrell (1989) who further showed that its expression from the zygotic genome was essential for cell division after the maternal stores of cyclin A were exhausted. Both cyclin A and B proteins are distributed evenly throughout the embryo, but cyclin A becomes more concentrated in the cortex region at the blastoderm stage (Maldonado-Codina and Glover 1992). In the early stages of zygotic divisions (cycles 4–6), cyclin A localization is strongly nuclear during interphase with only low levels detected in the cytoplasm (Stiffler et al. 1999). Several cycles later, its subcellular distribution is predominantly cytoplasmic during interphase, although there is some weak punctuate localization in the nucleus as well (Maldonado-Codina and Glover 1992). As the cell prepares to divide, cyclin A associates with the condensing chromosomes and subsequently segregates into the daughter nuclei during anaphase. After the separation of the two daughter nuclei, cyclin A is not degraded but rather returns to the cytoplasm. It should be recalled that the *Drosophila* embryo at this stage is syncitial, and interestingly there is little degradation of the cyclins until the embryo is cellularized (Maldonado-Codina and Glover 1992).

As far as cyclin A's function in the *Drosophila* germ line, it is clearly expressed in a subset of cells in both the stem cells and growing cystocytes of the germaria of adult females (as are cyclin B and cyclin E) (Lilly et al. 2000). Cyclin A was surprisingly found at the site of the fusome, a structure that is rich in vesicles, passes through ring canals, and physically connects all the cystocytes within a single cyst (de Cuevas et al. 1997; de Cuevas and Spradling 1998; Kishore et al. 2002). This association exhibits a periodicity and occurs during the late S to G2 stage of the cell cycle. Overexpression of cyclin A in the cystocytes results in an extra round of mitotic divisions and cysts with 32 rather than 16 cells (Lilly et al. 2000). Association of cyclin A with this structure is also present in male cystocyte mitotic divisions.

In early stages of meiotic prophase in developing fly oocytes, cyclin A protein levels have been reported to be at the posttranslational level by deadenylation of its mRNA (Morris et

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al. 2005) and by repression of translation by the Bruno protein during the arrest at the end of meiotic prophase (Sugimura and Lilly 2006). As meiosis resumes during meiotic maturation, cyclin A mRNA is repolyadenylated and the Bruno repressor is lost (Vardy et al. 2009). The cyclin A protein that is now made is also phosphorylated at multiple sites, likely involving autophosphorylation. Translation of cyclin A mRNA is also promoted by the PAN GU (PNG) kinase at this stage and at earlier stages of oogenesis. After the completion of meiosis, PNG kinase appears to promote further polyadenylation of the cyclin A mRNA. Cyclin A is degraded by the anaphase promoting complex/cyclosome (APC/C) at the end of the first meiotic division (Pesin and Orr-Weaver 2007). Such tight regulation of cyclin A levels is certainly consistent with our hypothesis outlined above regarding the importance of strict regulation of the A-type cyclins in mammalian gametogenesis, particularly during meiosis, and the likely occurrence of this regulation at various levels.

17.5.3 Xenopus

The *Xenopus* oocyte has been a major source of obtaining extracts for many of the pioneering studies identifying factors involved in cell cycle function and regulation (for example, Strausfeld et al. 1996). It has further been suggested that the early *Xenopus* embryo represents “a unique developmental context” in which to investigate the role of the A-type cyclins, particularly their function in both cellular proliferation and cell death (Carter et al. 2006). After fertilization, a rapid series of cell divisions characterizes proliferation in the early frog embryo, until the developmental stage known as the midblastula transition (MBT) is achieved. These early cell divisions consist of rapidly alternating S- and M-phases, with essentially no G-phases, and are driven by cyclin E/Cdk2 complexes which control S-phase, and by a combination of cyclin A1/Cdk1 and cyclin B/Cdk1 which regulate the entry into M-phase (Hartley et al. 1996, 1997; Kimelman et al. 1987; Newport and Kirschner 1982; reviewed by Gotoh et al. 2011). Interestingly, in *Xenopus* egg extracts, both cyclin A1 and cyclin E have been shown to have S-phase promoting activity (Strausfeld et al. 1996). The regulation of their synthesis and turnover is quite distinct at these stages. Cyclin A1 and cyclin B are translated from maternally stored mRNAs and are degraded at each division and synthesized anew (Hartley et al. 1996). In contrast, cyclin E protein is maternally derived and is not turned over, but rather remains constant until the MBT (Hartley et al. 1997). The presence of large stores of cyclin A1 in the frog oocyte is in striking contrasts to its complete absence in mouse oocytes (discussed above). In fact, this was initially confusing because at the time of discovery of two A-type cyclins in *Xenopus*, only a single cyclin A had been identified in the mouse and human genomes (discussed in Sweeney et al. 1996).

At the MBT, the cell cycle and its machinery undergo distinctive remodeling. While cyclin A1 is the major A-type cyclin during the first 12 mitotic cell cycles, at the MBT, the zygotic genome encoded cyclin A2 gene is activated (Howe et al. 1995). This cyclin A2 is clearly related to murine and human cyclin A2 (Howe et al. 1995; Strausfeld et al. 1996; Sweeney et al. 1996), and as noted above, it is the only A-type cyclin present in mammalian oocytes. In *Xenopus*, cyclin A1 appears to form a complex with Cdk1, but not Cdk2, while cyclin A2 interacts with both Cdks (Minshull et al. 1990; Strausfeld et al. 1996). In mammals, cyclin A1 can interact with both Cdk1 and Cdk2 in vitro (Joshi et al. 2009) and in vivo (Liu et al.

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2000a). When *Xenopus* embryos are exposed to ionizing radiation prior to the MBT, however, cyclin A1 protein was shown to persist beyond the MBT and further to complex with both Cdk1 and Cdk2 (Anderson et al. 1997). This may suggest that, depending upon the physiological context, cyclin A1 in *X. laevis* can complex with both of these Cdks, as it does in the mammalian system.

17.6 Unanswered Questions and Future Directions

17.6.1 What Are the Critical Interacting Proteins and Substrates of Cyclin A1 and A2 in the Germ Line and Early Embryo?

There have been a number of studies identifying proteins that interact with cyclin A2/Cdk2 complexes, particularly in cultured cell lines (Suryadinata et al. 2011). Much less is known about the interacting proteins for cyclin A2 in germ cells and almost nothing is known about interacting proteins for cyclin A1, due to the very limited tissue distribution of its expression. Although several approaches have been used to identify proteins that interact with cyclin A1 and cyclin A1-Cdk complexes, almost all studies have been limited to identifying partners and substrates in the myelocytic leukemia cell lines where human cyclin A1 is known to be upregulated (Yang and Kornbluth 1999; Yang et al. 1997). For example, using a yeast triple hybrid strategy, several known proteins including Ku70 as well as several less-well characterized proteins including INCA1, KARCA1, and PROCA1, were pulled out in the screen and confirmed by GST pulldown experiments (Diederichs et al. 2004). The Ku70 DNA repair protein was of particular interest, as it was subsequently reported that cells lacking cyclin A1 were deficient in the repair of double-strand breaks (Muller-Tidow et al. 2004). A possible physiological relevance to the function of cyclin A1 interacting proteins in spermatogenesis was provided by the observation that INCA (inhibitor of Cdk interacting with cyclin A1) interacts with a novel testis protein, RSB-66 (Chen et al. 2008). However, RSB-66 was reported to be most abundant in round spermatids, where cyclin A1 is expressed at low levels, if at all. Clearly an area in much need of investigation is the identification of physiologically relevant interacting proteins and potential substrates for cyclin A1-Cdk (and cyclin A2-Cdk) complexes in vivo.

17.6.2 Could the A-Type Cyclins Have Cdk Independent Functions in the Germ Line?

The observations that (a) both cyclin A1 and Cdk2-deficient mice exhibited defects in spermatogenesis but with distinctive patterns, (b) cyclin A1 and Cdk2 proteins do not completely colocalize in wild-type spermatocytes, and (c) Cdk2 localization did not change in prophase spermatocytes that lack cyclin A1, raises the possibility that cyclin A1 may interact with proteins other than its predicted Cdk partner. There is increasing evidence for Cdk-independent functions for cyclins: in addition to activating Cdks, cyclins can interact with other proteins, and thus, have very different functions. It has actually been recognized for some time now that the D-type cyclins can serve as coactivators or corepressors of tissue-specific transcription factors (Zwijsen et al. 1997). Studies from the lab of Piotr Sicinski further showed that E-type cyclins also have kinase-independent functions (Geng et al. 2007), which is of particular interest since both the E-type cyclins, like the A-type cyclins, partner with Cdk1 and Cdk2.

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17.6.3 Does Cyclin A2 Play an Important Role in the Germ Line?

The early embryonic lethality exhibited by cyclin A2-deficient mice has precluded our understanding of its potential functions in the male and female germ line (and in any other adult tissue and lineages for that matter). It was therefore of great interest that mice carrying floxed alleles of the *Ccna2* gene were recently generated and, in combination with the *Ccna1*$^{-/-}$ strain, used to explore the function of the A-type cyclins in fibroblasts, embryonic stem cells, and in the hematopoietic lineage (Kalaszczynska et al. 2009). Mouse embryonic fibroblasts (MEFs) lacking both cyclin A1 and cyclin A2 were surprisingly capable of apparently normal cellular proliferation, due at least in part to the upregulation of cyclin E1. In this same study, a tour de force set of experiments involving complex breeding schemes, tetraploid blastocyst complementation rescue, and deletion of floxed *Ccna2* alleles generated MEFs that were devoid of cyclin A1 and A2 as well as cyclin E1 and E2. These MEFs were incapable of proliferation and DNA synthesis was virtually abolished, suggesting that either cyclin A2 or an E-type cyclin is necessary for S-phase.

The next question: what about the requirement for cyclin A2 in other cell types? In contrast to the ability of MEFs to upregulate cyclin E1 and compensate for loss of the A-type cyclins, both hematopoietic and embryonic stem cells could not proliferate in the absence of cyclin A2 (Kalaszczynska et al. 2009). It will be of great interest to use transgenic strains of mice that express Cre recombinase early in the male germ line, such as the Ngn3-Cre (Yoshida et al. 2004) or Stra8-Cre (Sadate-Ngatchou et al. 2008) mice, or GDF-9-iCre mice in the case of the female germ line (Lan et al. 2004), to elucidate the requirement for cyclin A2 function in these cells. These studies are already underway in our laboratory in collaboration with the Sicinski lab.

17.6.4 Is There a Role for Cyclin A1 in Human Infertility?

The specificity of the cyclin A1-deficient phenotype has led us and others to consider loss of function of the human *CCNA1* gene as both being involved in cases of infertility in otherwise healthy men and conversely serving as a potential target for male contraception. One such study proposed to use the expression of cyclin A1 as a new molecular diagnostic marker, noting that cyclin A1 expression was absent in the majority of cases of Sertoli cell-only syndrome and only very low levels were detected in specimens with spermatogonia only or where spermatogenesis arrest occurred at the level of primary spermatocytes (Schrader et al. 2002). This is, of course, what one would predict for any late male meiotic prophase-specific marker. A second study was undertaken to screen for mutations in the human *CCNA1* gene in 347 infertile men from a western Chinese cohort that might be causal for their infertility (Zhoucun et al. 2009) While 4 point mutations were identified in the exon-screening, none resulted in changes in amino acids, and not surprisingly, no association with impaired spermatogenesis could be detected. The authors suggested that mutations in the cyclin A1 gene are not likely to represent a frequent cause of male idiopathic infertility.

Several years ago, we undertook an analogous screen of DNAs from infertile men of predominantly European origin (Mandon-Pepin et al. 2002). We identified a mutation in exon 6 in one of the alleles of human *CCNA1* in an infertile patient (Wolgemuth DJ et al.,

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unpublished observations) which was not seen in any of the other 100 nor in DNAs from 35 ethnically matched fertile men. The change would putatively cause an alteration in the corresponding amino acid from arginine to glutamine. The changed amino acid has two interesting features that suggest functional importance. In cyclin A2 from frog, human, and mouse, and in cyclin A1 from frog, it is lysine, whereas in human and mouse cyclin A1, it is arginine. Both of these amino acids are highly basic and represent putatively conservative functional substitutions. In the infertile patient, it is glutamine, a nonconservative change. The crystal structure of the cyclin A2 protein has been solved, alone, in association with one of its catalytic partners, Cdk2, and also in the presence of an inhibitor of the cyclin A2/Cdk2 complex, p27 (Noble et al. 1997; Russo et al. 1996). In cyclin A2, the corresponding mutated amino acid would be present in an alpha helix that is involved in protein–protein interactions. We suggest that there is simply too little information available to assess the significance of mutations in human cyclin A1 in cases of idiopathic male sterility.

17.7 Conclusions

In summary, the cyclin A proteins of higher eukaryotes are unique at several levels. They function at two critical stages of the cell cycle, both during S-phase and at G2-M. Depending upon the physiological context, they can complex with both Cdk1 and Cdk2, although the full physiological context of this association remains to be determined. Unlike the other cyclin families, both cyclin A1 and cyclin A2 have been shown to be essential genes: loss of cyclin A2 in the mouse model is early embryonic lethal and cyclin A2 is absolutely required for spermatocytes to undergo the first meiotic division. A critical role for cyclin A2 has also been demonstrated in two stem cell systems (hematopoietic stem cells and embryonic stem cells) – will germ cell stem cells be added to this list?

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