Cell cycle regulation of
Oikopleura dioica

A study of the cyclin CDK-complement

Jan Inge Øvrebø

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Abstract

Regulation of the eukaryotic cell cycle is a fundamental biological process which controls proliferation of all eukaryote cells. Progression through the cell cycle is highly dependent on its core regulators; Cyclins and associated Cyclin-dependent kinases (CDKs), which orchestrate a coordinated series of events through growth in the first gap phase (G1), initiation of DNA synthesis (S), the second gap phase (G2) and mitosis (M). Variations of the cell cycle include the canonical mitotic cell cycle, giving rise to identical sister cells, meiosis, giving rise to haploid gametes, and various endoreduplicative cycles, which increase ploidy of cells through repetitive S-phases without intervening cytokinesis. Although limited to a very few specialized cell types in vertebrates, endoreduplication is widespread amongst invertebrates. The marine urochordate *Oikopleura dioica*, deploys somatic endocycling as a main developmental strategy, which facilitates rapid growth during a very short life cycle. *O. dioica* females also take advantage of the elevated transcriptional capacity of endocycling nurse nuclei within the coenocyst; a single cell compartment shared by hundreds of nurse and meiotic nuclei. Being a large transparent ovary, the coenocyst provides a unique model to study both endocycling and meiosis within a shared cytoplasm. The urochordates also belong to the closest sister group to vertebrates, which places knowledge about the *O. dioica* cell cycle in an interesting evolutionary context.

By searching the fully sequenced genome of *O. dioica* we annotated the Cyclin-CDK complement of *O. dioica*, which revealed amplified Cyclin D and Cyclin B complements. We also identified a surprising amplification of CDK1, an important M-phase regulator, which is highly conserved from yeast to vertebrates. Interestingly, the majority of somatic cells grow through endocycling during *O. dioica* development, which should favor conditions with low CDK1 activity. This observation therefore raised the question; why does an organism that develops mainly through a mechanism favoring reduced CDK1 activity have several paralogs of this particular cell cycle regulator? In order to dissect possible explanations, we analyzed expression of odCDK1 paralogs throughout *O. dioica* development revealing diverse expression throughout mitotic and endocycling proliferation, in addition to male- and female-

specific expression during gametogenesis. We also assessed functions amongst the odCDK1 paralogs, which displayed variations within the highly conserved PSTAIRE motif. Because the PSTAIRE motif is decisive in Cyclin interaction and thus indirectly affects substrate specificity, functional variation amongst odCDK1 paralogs might occur. Targeted knockdown of odCDK1 expression by injection of double stranded RNA (dsRNA) revealed non-redundant and essential functions for two odCDK1 paralogs in producing viable oocytes, representing the first known case in metazoan models where CDK1 paralogs have sub-functionalized in the control of meiosis.

Abbreviations

APC/C	Anaphase promoting complex/cyclosome
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
CAK	CDK activating kinase
cAMP	cyclic adenosine 3', 5'-monophosphate
CDC	Cell division cycle
CDK	Cyclin dependent kinase
CDT11	Chromatin licensing and DNA replication factor 1
CDH1	CDC20 homolog 1
CKI	CDK inhibitor
cmRNA	Capped messenger RNA
dsRNA	Double stranded RNA
FZR	fizzy related
G0	Quiescent phase
G1	First gap phase
G2	Second gap phase
M	Mitotic phase
MAPK	Mitogen-activated protein kinase
MCM	Minichromosome maintenance protein
mRNA	Messenger RNA
MPF	Mitosis/Maturation promoting factor
MTOC	Microtubule organizing center
MYPT	Myosin phosphatase targeting protein
NEB	Nuclear envelope breakdown
NPC	Nuclear pore complex
OC	Organizing center
ORC	Origin recognition complex
PKA	protein kinase A
PLK1	Polo-like kinase 1

Pre-RC   Pre-replication complex

PCNA   Proliferating cell nuclear antigen

Rb   Retinoblastoma protein

S   DNA synthesis phase

TALEN   Transcription activator-like effector nuclease

TEM   Transmission electron microscopy

TGC   Trophoblast giant cells

TS   Trophoblast stem cells

1. Introduction

1.1 The eukaryotic cell cycle

One of the central concepts of biology is the replication/multiplication of the basic unit of life; the cell. The core process of cell proliferation is controlled by the cell cycle machinery. The canonical eukaryotic mitotic cell cycle passes through four phases: the first gap phase (G1), synthesis phase (S), second gap phase (G2) and mitosis (M) (Figure 1). After a cell has completed these four phases it can either repeat the cycle or enter a quiescent state (G0), which is commonly found in terminally differentiated cells. In G1 the cell accumulates nutrients, grows, makes organelles and produces proteins in order to reach the minimal required size for cell cycle entry. From G1, the cell can enter several fates; such as quiescence, apoptosis and senescence in response to unresolved DNA damage, or enter S-phase in response to growth hormones and nutrition. In S-phase the cell initiates replication of its genome resulting in duplication of chromatids, making the cell transiently tetraploid. After completion of S-phase the cell enters G2, where it resumes growth in preparation for M-phase. The duration of G2 varies amongst cell types and is often absent during embryogenesis of several species. As the cell enters the first sub-phase of M-phase; the prophase, chromatin starts to condense and nuclear envelope breakdown (NEB) initiates, and mitosis continues by mitotic spindle assembly and alignment of chromatin at the metaphase plate. Chromatid segregation into two identical sister cells during anaphase is triggered by activity of the anaphase-promoting complex/cyclosome (APC/C) which culminates in a decline in CDK1 activity and cleavage of cohesion complexes; the protein ring structures that keep newly replicated sister chromatids paired from S-phase onwards. This results in segregation of sister chromatids towards opposite poles during telophase, reassembly of the nuclear envelopes and the division of the cytoplasm during cytokinesis, giving rise to two identical sister cells (Morgan, 2007). Another important aspect of the cell cycle is the cycle of the centrosomes, the microtubule organizing centers (MTOC) of the mitotic spindles, which duplicate and separate in parallel with chromatin.

The well-known mitotic cell cycle is, however, one of several alternative variations of the cell cycle. Additional variants include meiosis where two rounds of
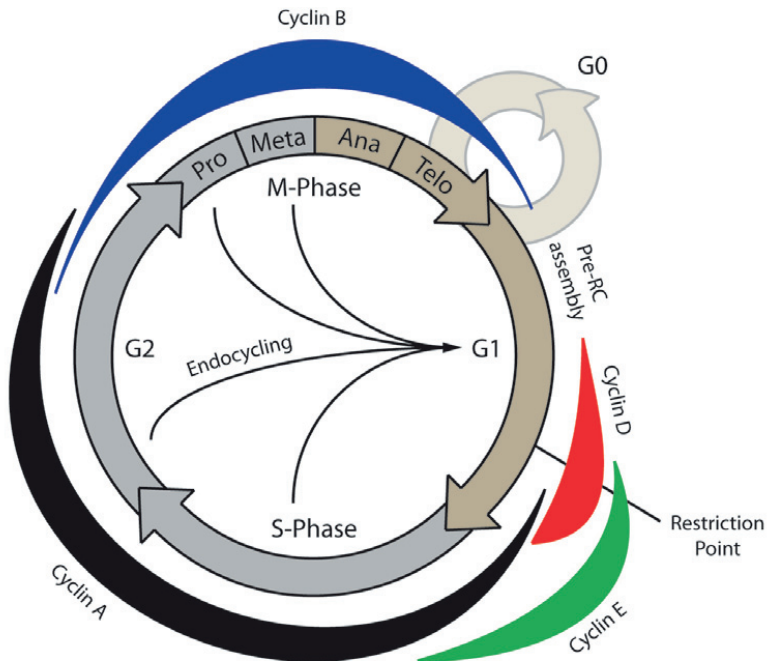
Figure 1: The eukaryotic cell cycle. The canonical cell cycle is divided into four major phases; The first gap phase (G1), DNA synthesis (S-phase), the second gap phase (G2), and mitotic phase (M-phase). The cell cycle is controlled by periodic rise of Cyclins, whose role is to activate Cyclin dependent kinases (CDKs) and provide substrate specificity. Cyclin D (red curve) rises in late G1 in response to growth signals, which in turn stimulate expression of Cyclin E and drive the cycle through the restriction point, marking commitment to complete a full unidirectional cycle. Cyclin E stimulates its own transcription, which also results in increased expression of Cyclin A. Cyclin A activates CDK2 and triggers the onset of DNA replication. Cyclin A-CDK2 maintains progression through S-phase, whereas Cyclin E and D are targeted for proteasomal degradation by active SCF complex (Grey arrows). By the end of G2, Cyclin A activates CDK1 directing the cycle towards M-phase. Accumulation of Cyclin B forms the mitosis promoting factor (MPF) together with CDK1, which triggers chromatin separation in anaphase by activation of the Anaphase promoting complex (APC). Active APC (brown arrow) targets mitotic Cyclins for proteasomal degradation, thereby creating a window of low CDK activity, allowing reassembly of the pre-replication complexes (Pre-RC). Endocycling cells bypass M-phase by re-entering gap phase following S-phase (central arrows). Modified from (Edgar and Orr-Weaver, 2001; Morgan, 2007).

cell division occur without an intervening S-phase, reducing the original cell ploidy, giving rise to haploid gametes. Another cell cycle variant is endoreplication, a strategy to increase cellular genomic copies (ploidy) through repetitive rounds of DNA replication without intervening cytokinesis (Edgar and Orr-Weaver, 2001; Zielke et al., 2013). In mammals endocycling is limited to a few cell types, including trophoblast giant cells (TGCs) and hepatocytes, but there are several organisms where endocycling is a more widespread mechanism. In *Drosophila* endocycling is found in the salivary gland and in the nurse nuclei, which are polyploid nuclei with high transcriptional activity supporting oocyte development. In the marine urochordate *Oikopleura dioica*, most somatic cells switch to endocycling shortly after hatching, and analogous to *Drosophila*, *O. dioica* also possesses nurse nuclei, undergoing endocycling, supporting oogenesis (Ganot et al., 2007a; Ganot and Thompson, 2002).

1.2 Cyclins and CDKs

Regulation of the cell cycle is enormously complex with a vast number of interacting molecules, but can be generalized to be controlled by oscillations of kinase activity, which is also the case in the prokaryotic cell cycle. The Cyclin dependent kinases (CDKs) are considered the basic regulators of the eukaryotic cell cycle because they activate critical components of the cell cycle engine (Morgan, 1997). CDKs constitute a family of protein kinases capable of phosphorylating serine and threonine residues of target proteins. Amongst the mammalian CDKs, four are directly involved in the cell cycle; CDK1, CDK2, CDK4 and CDK6. Activation of CDKs is achieved through association with their activating Cyclin subunit, which induces conformational changes to reveal the catalytic site (Jeffrey et al., 1995) and to modulate the substrate specificity of CDKs (Loog and Morgan, 2005; Roberts, 1999). Each Cyclin binds to specific CDK partners so that the levels of the different Cyclins control which CDKs are active. CDK4 and CDK6 are activated by Cyclin D during G1 phase (Sherr, 1993, 1995) in response to growth factors that trigger a kinase cascade activating transcription of early and late response genes including; Cyclin D, Cyclin E, CDK2, CDK4 and CDK6. During late G1, CDK4 and CDK6 induce expression of Cyclin E through activation of the E2F transcription factors. Cyclin E-dependent activation of

CDK2 further induces E2F activity, stimulating Cyclin A accumulation and S phase entry (Kato et al., 1993). Cyclin A activates CDK2 and triggers onset and maintenance of S-phase until G2, when Cyclin A activates CDK1 and initiates the path to M-phase entry. Finally, Cyclin B controls CDK1 activation, forming the complex known as the mitosis promoting factor (MPF), leading to initiation of and progression through M-phase (Labbe et al., 1989).

1.2.1 Cyclin-CDK structure and activation

Activation of CDKs is highly dependent on Cyclin binding and structural changes involving altered accessibility to the conserved catalytic site (Echalier et al., 2010). CDKs are composed of an N-terminal lobe mainly comprising beta-sheets and a single alpha-helix, known as the PSTAIRE helix (Figure 2), while the C-terminal lobe is mainly arranged by alpha-helices (De Bondt et al., 1993; Schulze-Gahmen et al., 1996). Within the cleft, created by the two lobes, lies the catalytic site containing the ATP binding site, orienting phosphate groups outwards from the cleft. When inactive, the catalytic site is blocked through steric hindrance by the activation loop, preventing physical access to substrates. Disruption of the activation loop and access to the catalytic site is mediated through two important mechanisms. Firstly, activation loop phosphorylation of Thr160 (pThr160), in human CDK2, causes removal of the activation loop from the catalytic site through interaction of pThr160 with a cationic binding pocket on CDK, thus CDK activation requires phosphorylation by a CDK activating kinase (CAK) (Jeffrey et al., 1995). Secondly, full CDK activation requires binding of a Cyclin partner that is characterized by two domains each containing five alpha helices termed Cyclin folds, also known as Cyclin boxes. The conserved N-terminal Cyclin box possesses an MRAIL amino acid sequence and a hydrophobic patch, which contributes to substrate specificity. Cyclin binding pushes the PSTAIRE helix towards CDK, which allows the Glu51 within the PSTAIRE to interact with and change conformation of the catalytic site, adjusting ATP into an optimal position for catalytic activity.
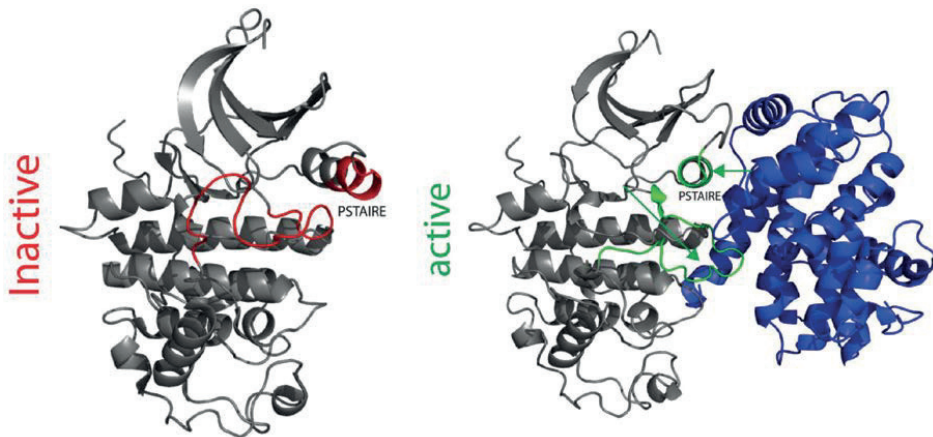
Figure 2. The Cyclin-CDK complex. The catalytic site of inactive CDKs is blocked through steric hindrance by the T-loop (red loop). CDKs are activated through Cyclin (blue molecule) interaction by repositioning the PSTAIRE helix (green helix), causing conformational changes including liberating the catalytic site by repositioning the T-loop (green loop).

1.2.2 The PSTAIRE motif

The "PSTAIRE" protein sequence of the PSTAIRE helix is highly conserved amongst CDK1 and CDK2 homologs, and has been invariable from the yeast CDK1 homolog, CDC28/CDC2, to human CDK1 and CDK2. The PSTAIRE sequence is however not retained amongst the Cyclin D interacting CDK4/6 homologs (Figure 3), although the PxxxxRE consensus is conserved in all CDKs. The proline (P) residue has been reported to maintain helix structure, and is important for Cyclin binding (Child et al., 2010), whereas Arginine (R) and Glutamate (E) are important for CDK activation by adjusting ATP position within the catalytic site. In metazoans the G2/M transition is typically regulated by PSTAIRE CDKs, which can also rescue mutants of the yeast CDK1 homologue CDC28 (Sherr, 1993), whereas the PI/LSTV/IRE CDKs are involved in G1/S transition. Amongst the plant CDK1 paralogs however only CDKA

possesses a perfect PSTAIRE motif, but is normally involved in G1/S transition through interaction with Cyclin D, whereas CDKB1, which possesses a PPTALRE motif is involved in G2/M transition through interaction with A- and B-type Cyclins (Nowack et al., 2012; Van Leene et al., 2010). Although the PSTAIRE CDKs have been conserved from yeast to plants and metazoans, they have apparently sub-functionalized towards regulation of different sub-phases of the cell cycle.

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hsCDK1     …GQVVAMKKIRLESEEEGVPSTAIREISLLKELRHPNIVSL…
hsCDK2     …GEVVALKKIRLDTETEGVPSTAIREISLLKELNHPNIVKL…
hsCDK4     …VALKSVRVPNGGGGGGGLPISTVREVALLRRLEAFEHPNV…
hsCDK6     …GRFVALKRVRVQTGEEGMPLSTIREVAVLRHLETFEHPNV…
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Figure 3. CDK PSTAIRE motifs. The Cyclin interacting PSTAIRE motif is conserved in vertebrate CDK1 and CDK2, whereas CDK4/6 share a conserved Proline, Arginine and Glutamate in the 1st, 6th and 7th positions of the motif, respectively. Conserved residues are in bold.

1.3 The mitotic cell cycle

1.3.1 The G1 – S transition

Essential for cell proliferation is replication of chromosomes in order to generate two identical sets of the genome to be distributed between the two daughter cells following mitotic cell division. An important consideration regarding entry into, and maintenance of, S-phase is to prevent unresolved DNA damage and incomplete or over-replication of the genome. Persistence of unresolved DNA problems is the leading cause of genomic instability, which may ultimately cause oncogenic transformation (Holland and Cleveland, 2009). Cancer cells accumulate mutations providing growth advantages above the native cell population, which may prove fatal to the organism as a whole. In order to prevent such developments there are several checkpoints that monitor genome integrity, which arrest the cell cycle in response to DNA damage until the damage is resolved, or if not; induce apoptosis or senescence. Another important mechanism in maintenance of genomic integrity is to ensure that the genome is completely replicated once, and only once, for each mitotic cell cycle.
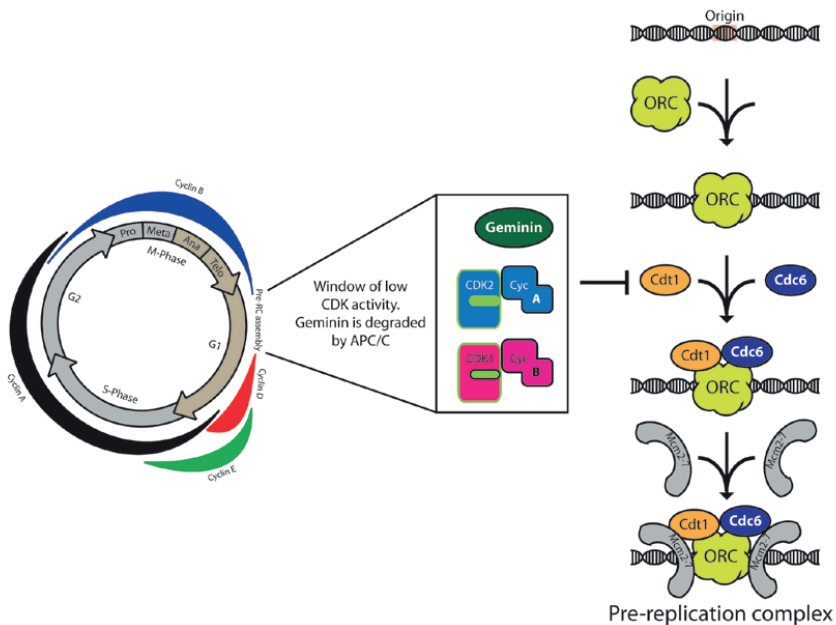
Figure 4. Assembly of the pre replication complex (Pre-RC). Replication start sites, called origins, are recognized by a multi-protein complex known as the origin recognition complex (ORC). Two other factors, CDC6 and CDT1, are recruited by ORC during a window of low CDK activity and low Geminin levels, which when present, and active, would inhibit CDT1 recruitment. Recruitment of two helicases composed of MCM2-7 finalize pre-RC assembly.

This mainly involves the DNA replication complexes which may only be assembled during G1 phase and activated once during S-phase. Regulation of the replication complex and the transition from G1 to S-phase are regulated primarily by CDK4/6 and CDK2.

The pre-replication complex (Pre-RC) is assembled during G1 phase and consists of an origin recognition complex (ORC), composed of ORC1-6, which recognizes origins of replication on DNA (Figure 4). ORC further recruits CDT1 and CDC6, which in turn recruits the DNA helicase MCM2-7, another protein complex composed of 6 subunits, assembled both downstream and upstream of the origin of replication (Bell and Dutta, 2002; Bell and Stillman, 1992; Bowers et al., 2004; Randell et al., 2006). Untimely assembly of pre-RC is prevented through inhibition of

CDT1 by Geminin and CDK-dependent phosphorylation of CDT1, which prevents recruitment by ORC, and thus low CDK activity and degradation of Geminin are required for pre-RC assembly and licensing of replication (Li and Blow, 2004; McGarry and Kirschner, 1998). Since CDK activity is also required to trigger and activate replication once pre-RCs are assembled, CDKs ensure that DNA replication is activated while preventing premature re-initiation (Bell and Dutta, 2002; Symeonidou et al., 2012).

In order to reach minimum size required for cell cycle entry the mitotic cell cycle does not progress continuously as the cell requires time to grow following cell division. Entry into S-phase therefore depends on nutritional cues and growth signals that activate the MAPK pathway responsible for Myc dependent expression of Cyclin D (Adhikary and Eilers, 2005; Bouchard et al., 1999; Hermeking et al., 2000), which in turn binds and activates CDK4/6 (Figure 5). Cyclin D however binds only weakly to CDK4/6 and thus requires assistance from the Cip/Kip family of CDK inhibitors (CKIs), p27 and p21, while facilitating assembly of an active Cyclin-p21/p27-CDK4/6 complex, as p27/p21 simultaneously inhibit CDK2 activity (Blain, 2008; Cheng et al., 1999; LaBaer et al., 1997; Sherr and Roberts, 1999). Eventually, accumulation of Cyclin D will titrate away enough p27/p21 from CDK2 to allow activation of the latter and progression of the G1/S transition. Nuclear CDK4/6 phosphorylates the Retinoblastoma protein (Rb), a proto-typical tumor suppressor and inhibitor of the E2F1-3 transcription factors, which further leads to E2F-dependent transcriptional activation (Dyson, 1998; Lees et al., 1993; Rubin et al., 2005; Weinberg, 1995) of several cell cycle regulators such as Cyclin E, Cyclin A and Cyclin B (Blais and Dynlacht, 2004; Cam and Dynlacht, 2003), and also E2F7-8, which antagonize E2F1-3 dependent transcription (de Bruin et al., 2003). Translation and accumulation of Cyclin E leads to activation of CDK2, which again amplifies its own activation through further phosphorylation of Rb, in addition to phosphorylation of p27/p21 (Akamatsu et al., 1998; Ohtani et al., 1995). This positive feedback loop creates a rapid elevation of cell cycle regulators, including Cyclin A, causing a high level of Cyclin A-CDK2 activity, which triggers firing of pre-RC and onset of S-phase.
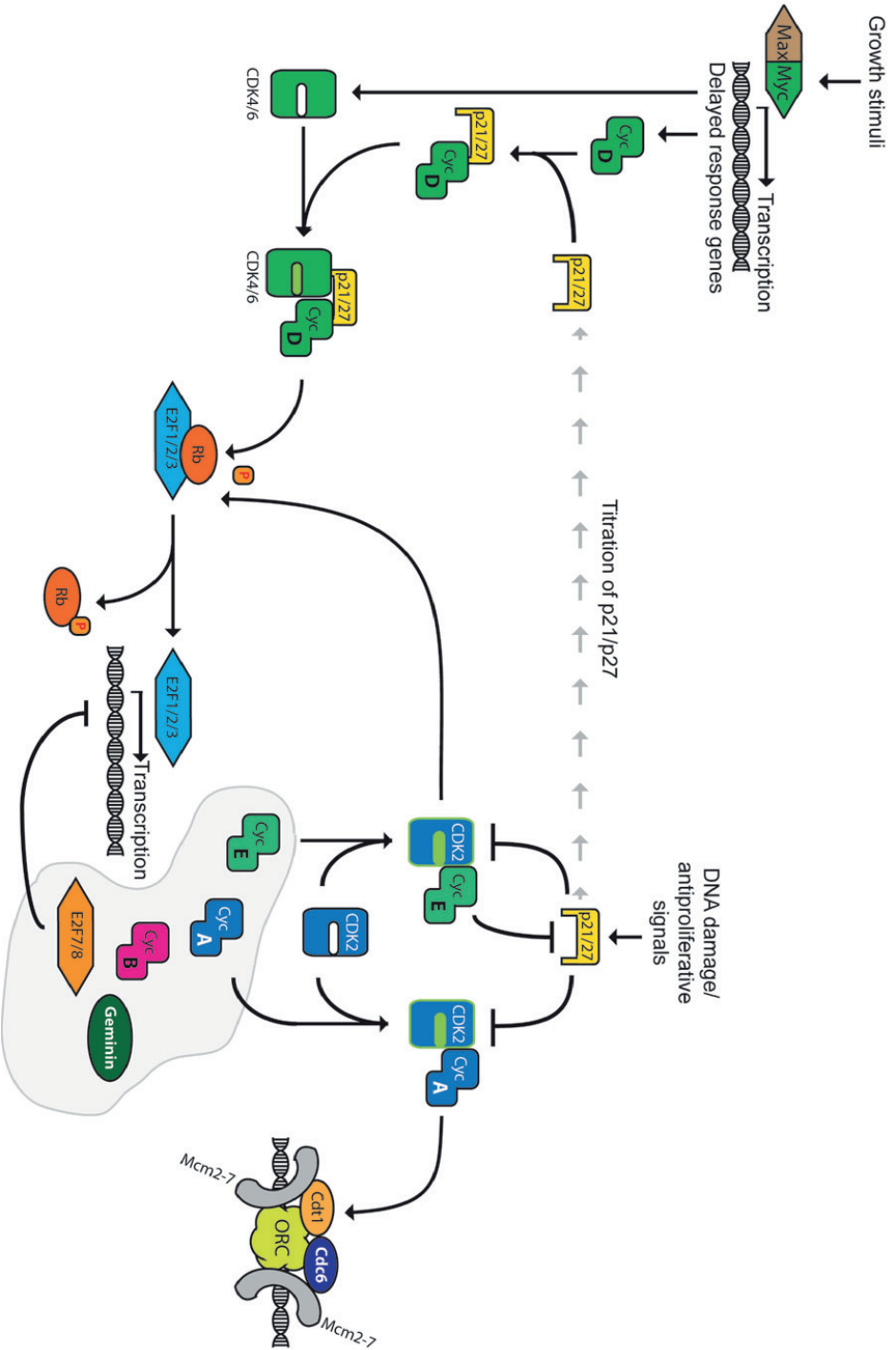
Figure 5: The G1-S transition. CDK4/6 control cell cycle entry from G1 to S-phase and are inhibited by the INK4 family of CDK inhibitors (CKIs), in response to anti-proliferative signals. Once the cell is stimulated to undergo cell division, growth signals activate transcription of Cyclin D. Cyclin D alone binds weakly to CDK4/6, but the CKI p27 stimulates binding between CDK4/6 and Cyclin D and activates CDK4/6 rather than inhibiting the complex. Cyclin D-CDK4/6 in turn phosphorylates the Retinoblastoma protein (Rb) and release inhibition of the transcription factors E2F1/2/3. E2Fs activate transcription of several Cyclins including Cyclin E and Cyclin A. Cyclin E forms an active complex with CDK2, which further phosphorylates Rb, creating a positive feedback loop which also causes an increased level of Cyclin A. Increased Cyclin A levels lead to Cyclin A-dependent activation of CDK2, which phosphorylates CDT1 of pre-replication complexes (Pre-RC) causing activation of DNA replication and the onset of S-phase.

1.3.2 The G2 – M transition

Complete and faithful replication of the genome prior to mitotic cell division is essential to ensure production of two identical copies of the genome. Premature entry into M-phase in the presence of DNA damage, or uneven duplication of sister chromatids would lead to genomic instability and could culminate in cancer (Holland and Cleveland, 2009). Regulation of M-phase entry must therefore ensure that DNA replication is complete and that DNA damage is resolved before proceeding.

When S-phase is complete, newly duplicated sister-chromatids will remain associated through sister chromatid cohesion, which helps to ensure symmetric bi-polar separation of sister chromatids during cell division (Hopfner, 2003; Nasmyth, 2002). The responsible CDK for M-phase entry is CDK1, whose main Cyclin partners are Cyclin A and Cyclin B. Cyclin B and CDK1 remain cytoplasmic during interphase and CDK1 activity is held in check through inhibitory phosphorylation, on Thr14 and Tyr15, by the protein kinases Wee1 and Myt1 (Figure 6) (Boutros et al., 2007; Gavet and Pines, 2010). When entering M-phase, Cyclin B-CDK1 localization focuses on the centrosomes where CDK1 becomes activated through de-phosphorylation of Thr14 and Tyr15 by the protein phosphatase CDC25. CDK1 further amplifies its own activity through a positive feedback loop, by activating phosphorylation of CDC25, and
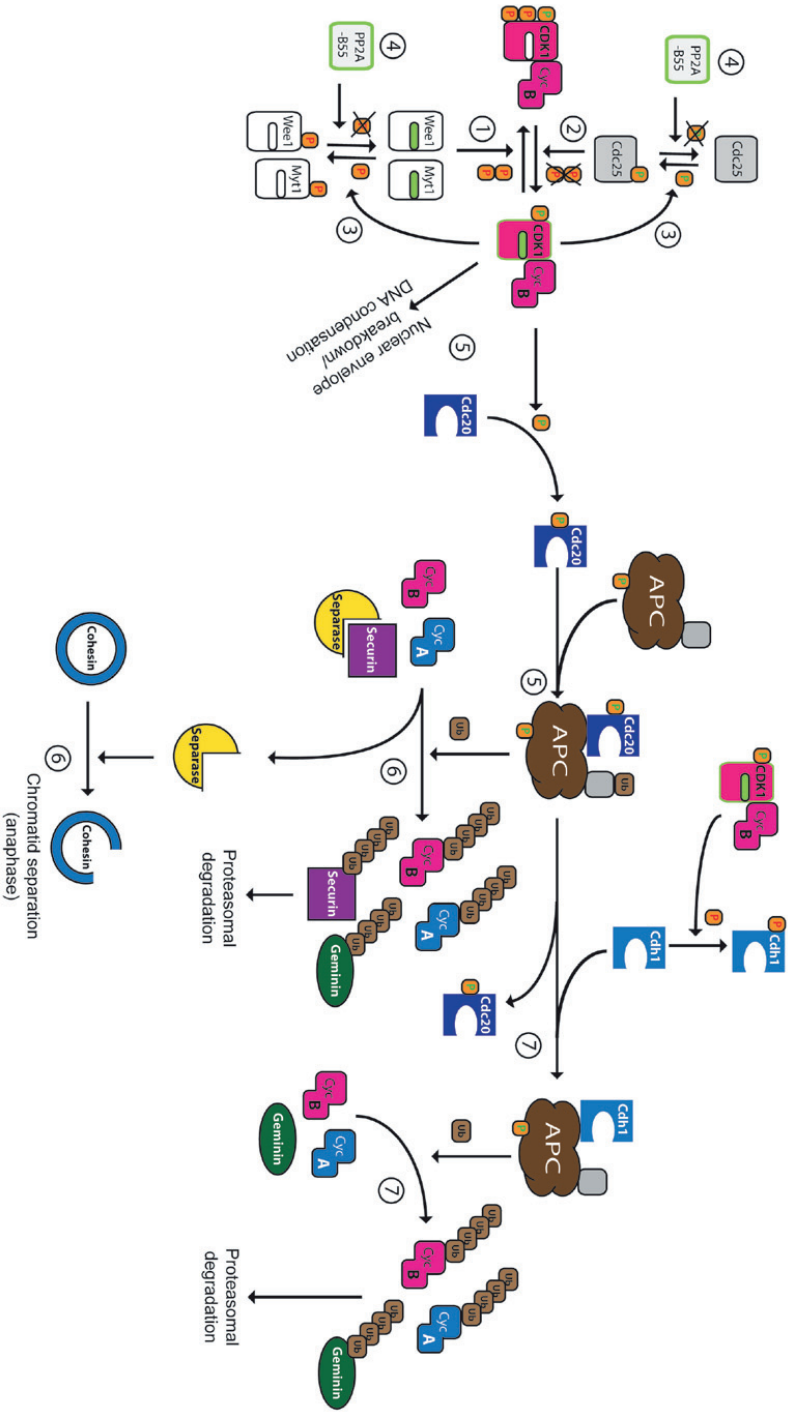
Figure 6: The G2-M transition. CDK1 is targeted by inhibitory phosphorylation by Wee1 and Myt1 ①. When the cell is ready to enter mitosis, the protein phosphatase CDC25 removes Wee1/Myt1 dependent inhibitory phosphorylations thus activating Cyclin B-CDK1 ②. Cyclin B-CDK1 creates a positive feedback loop by phosphorylation-dependent activation and inactivation of CDC25 and Wee1/Myt1 respectively ③. Cyclin B-CDK1 also prevents de-phosphorylation of its own substrates by inactivation of PP2A ④. Cyclin B-CDK1 phosphorylates several targets required for M-phase progression, which include nuclear lamins, causing nuclear envelope breakdown, and CDC20, a component of the anaphase promoting complex/cyclosome (APC/C) ⑤. APC/C^{CDC20} is an ubiquitin ligase that targets Securin, an inhibitor of Cohesin cleavage, and Geminin, an inhibitor of pre-RC assembly, for proteasomal degradation. These events trigger separation of sister chromatid and onset of anaphase, as well as degradation of mitotic Cyclins, which resets the cell cycle by creating low CDK activity ⑥. Low CDK activity is later maintained by APC/C^{Cdh1}, which allows reassembly of pre-RC and licensing for another entry into S-phase ⑦.

inhibitory phosphorylation of Wee1/Myt1 (Lindqvist et al., 2005; Mailand et al., 2002). Activation of CDK1 leads to rapid increase of CDK1 activity in an all or none mechanism (bi-stable switch), which can be inhibited by DNA damage through the ATM/ATR pathway (Zhou and Elledge, 2000), and thus CDK1 activation marks passage of the M-phase entry checkpoint.

As Cyclin B-CDK1 complexes become active, they translocate to the nuclei (Gavet and Pines, 2010) in late prophase where they promote NEB (Gong et al., 2007), through phosphorylation of Lamins amongst others, and they are also responsible for completion of chromatin condensation (Abe et al., 2011; Kimura et al., 2001). Another important target for CDK1 is the anaphase promoting complex/cyclosome (APC/C), a multi-protein Ubiquitin ligase complex that requires an activating subunit, CDC20, which also contributes to substrate recognition. Upon activation by CDK1, APC/C can ubiquitinylate and target several proteins, possessing destruction box motifs, for proteasomal degradation, including Cyclin A, Cyclin B, Geminin and Securin (Hershko, 1999). Securin destruction relieves Separase inhibition and leads to Cohesin cleavage and onset of anaphase, whereas Cyclin destruction ensures low CDK activity, which combined with Geminin destruction, allows reassembly of the pre-RC (Vodermaier, 2004). As CDK activity drops, CDC20 is replaced by CDH1 as the

APC/C activating subunit, which shares several targets with CDC20, but CDH1 does not target Securin and is therefore not able to induce chromatin separation (Morgan, 2007). APC/C^{CDH1} maintains low CDK activity through M-phase until G1, in order to allow pre-RC assembly. APC/C^{CDH1} is also involved in maintenance of prophase arrest and the MI-MII transition in the meiotic cell cycle (Homer, 2013).

1.4 Oogenesis and the meiotic cell cycle

Sexual reproduction requires fusion of two haploid gametes, a single sperm cell from the male and an oocyte from the female, merging two sets of chromosomes in order to generate a diploid zygote containing genomic information from both parents. In addition sexual reproduction allows exchange of genomic information between homologous chromosomes through homologous recombination, an important source of genetic variation (Cole et al., 2012). Differentiation and maturation of germ cells thus rely on meiosis and ploidy reduction in order to produce haploid gametes. Germline cells of insects and vertebrates initially proliferate synchronously through mitosis in order to produce a cluster of cells, interconnected through ring canals, known as germline cysts. In vertebrate females, the cyst phase exists only in juvenile individuals, whereas the cyst phase persists through most of meiosis amongst insects and appendicularians (Ganot et al., 2007a; Pepling et al., 1999). Initiation of the meiotic program starts with entry into pre-meiotic S-phase in order to replicate the genome, where sister chromatids are tightly joined by Cohesin rings before entering meiosis. During the initial steps of meiotic prophase I, homologous chromosomes will find each other and be paired together during zygotene (Figure 7.) (Klutstein and Cooper, 2014; Scherthan, 2001). Zygotene is characterized by clustering of telomeres at the nuclear membrane towards the centrosome, a conformation defined as the chromosomal bouquet. Synapsis between homologous chromosomes is further strengthened through assembly of protein scaffolds known as synaptonemal complexes, followed by resolution of the chromosomal bouquet during pachytene. The synaptonemal complexes is then disassembled in diplotene, which also marks the completion of homologous recombination, characterized by overlapping regions of condensed chromosomes known as chiasma (Morgan, 2007; Scherthan, 2001).
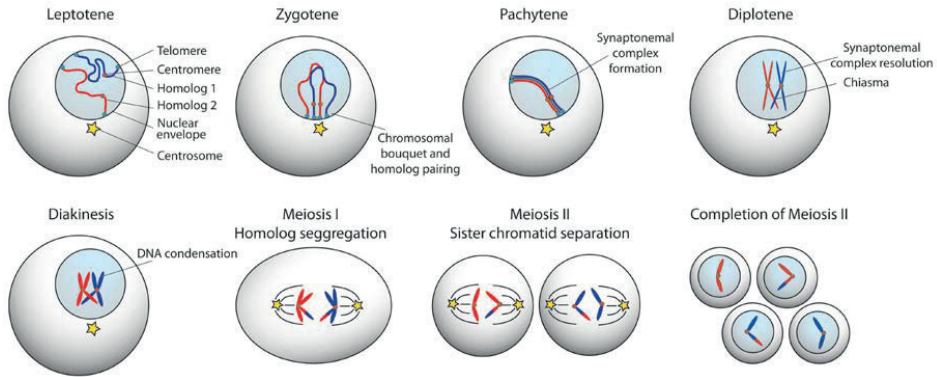
Figure 7. Meiosis. As germ cells enter the initial phase of meiotic prophase, leptotene, newly replicated chromosomes will be anchored to the nuclear membrane via the telomeres. During zygotene homologous chromosomes will pair together as the telomeres aggregate at the nuclear envelope towards the centrosome, an arrangement known as the chromosomal bouquet. Synapsis between homologous chromosomes will form as the synaptonemal complexes assemble, creating a protein scaffold supporting homolog pairing as well as homologous recombination. The synaptonemal complexes disassemble after completion of homologous recombination and are absent during diplotene. The chromosomes will also start to condense during diplotene and chiasma at sites of recombination will be visible by light microscopy. DNA condensation completes during diakinesis, marking the final step before nuclear envelope breakdown (also called germinal vesicle breakdown in oocytes of several species) and metaphase entry. The first meiotic division ensures equal separation of homologous chromosome pairs, whereas the second meiotic division ensures equal separation of sister chromatids, as in mitotic cell division. Two consecutive meiotic cell divisions ultimately give rise to four haploid germ cells. Figure is modified from (Scherthan, 2001) and (Morgan, 2007).

Chromosome condensation is completed at diakinesis, which is the final step of prophase I.

Most animal oocytes will enter a prolonged prophase I arrest at diplotene or diakinesis (Figure 8), which largely depends on maintaining low activity of CDK1-Cyclin B activity (Sagata, 1996; Von Stetina and Orr-Weaver, 2011). Mammalian germline cells enter meiosis during fetal development and enter Prophase I arrest around birth, which is maintained until puberty. Oocytes are then released from prophase I arrest during ovulation cycles. In mammals low CDK1 activity is maintained by high levels of cyclic adenosine 3', 5'-monophosphate (cAMP), a second messenger, causing activation of Protein kinase A (PKA) that in turn activates the

Wee1 kinase, which inhibits CDK1 through inhibitory phosphorylation (Han et al., 2005; Lincoln et al., 2002; Pirino et al., 2009; Von Stetina and Orr-Weaver, 2011). Another mechanism is APC/C^{CDH1} dependent proteasomal degradation of cyclin B, preventing activation of CDK1 (Reis et al., 2006).

Resumption from prophase I arrest requires CDK1 activation followed by nuclear translocation (Sagata, 1996; Von Stetina et al., 2008), and is stimulated through hormones such as progesterone in frog, or methyl adenine in starfish. Studies of *Xenopus* oocytes have shown that hormone dependent CDK1 activation occurs through several mechanisms, including down regulation of cAMP production, inhibition of Myt by the MAP kinase pathway, and Cyclin B production (Haccard and Jessus, 2006; Kishimoto, 2003). Polo-like kinase 1 (PLK1) is also found to be activated downstream of CDK1 activation in starfish and *Xenopus* oocytes, where it activates CDC25 to further increase CDK1 activity (Karaiskou et al., 1999; Okano-Uchida et al., 2003). Active CDK1 resumes the meiotic cycle from prophase I arrest and induces NEB, chromosome condensation, spindle assembly and entry into Metaphase I (Jones, 2004).

Some species, including Ascidians and Molluscs, will enter a second meiotic arrest at metaphase I whereas most vertebrate oocytes will arrest in metaphase II and remain arrested until fertilization (Whitaker, 1996). Metaphase I can be distinguished by the condensed chromosomes and visible chiasma by light microscopy. Chromosome separation in meiotic anaphase differs from mitosis in that sister chromatin cohesion is retained around centromeric regions and centrosomes are co-oriented towards the same spindle pole, guided by a Monopolin complex (Corbett et al., 2010; Marston and Amon, 2004), thus separating homologous chromosomes to opposite poles, rather than sister chromatids. Oocytes also divide asymmetrically to extrude a small diploid polar body on the surface of the oocyte. In contrast to mitotic cell cycles, meiosis requires entry into a second meiotic M-phase, without an intervening S-phase, in order to create haploid gametes. Mitotic Cyclins are degraded following anaphase, thus CDK1 reactivation requires Cyclin B re-synthesis (Hochegger et al., 2001). However, an important difference in the meiotic cell cycle is that anaphase I is followed by only partial Cyclin degradation through restrained
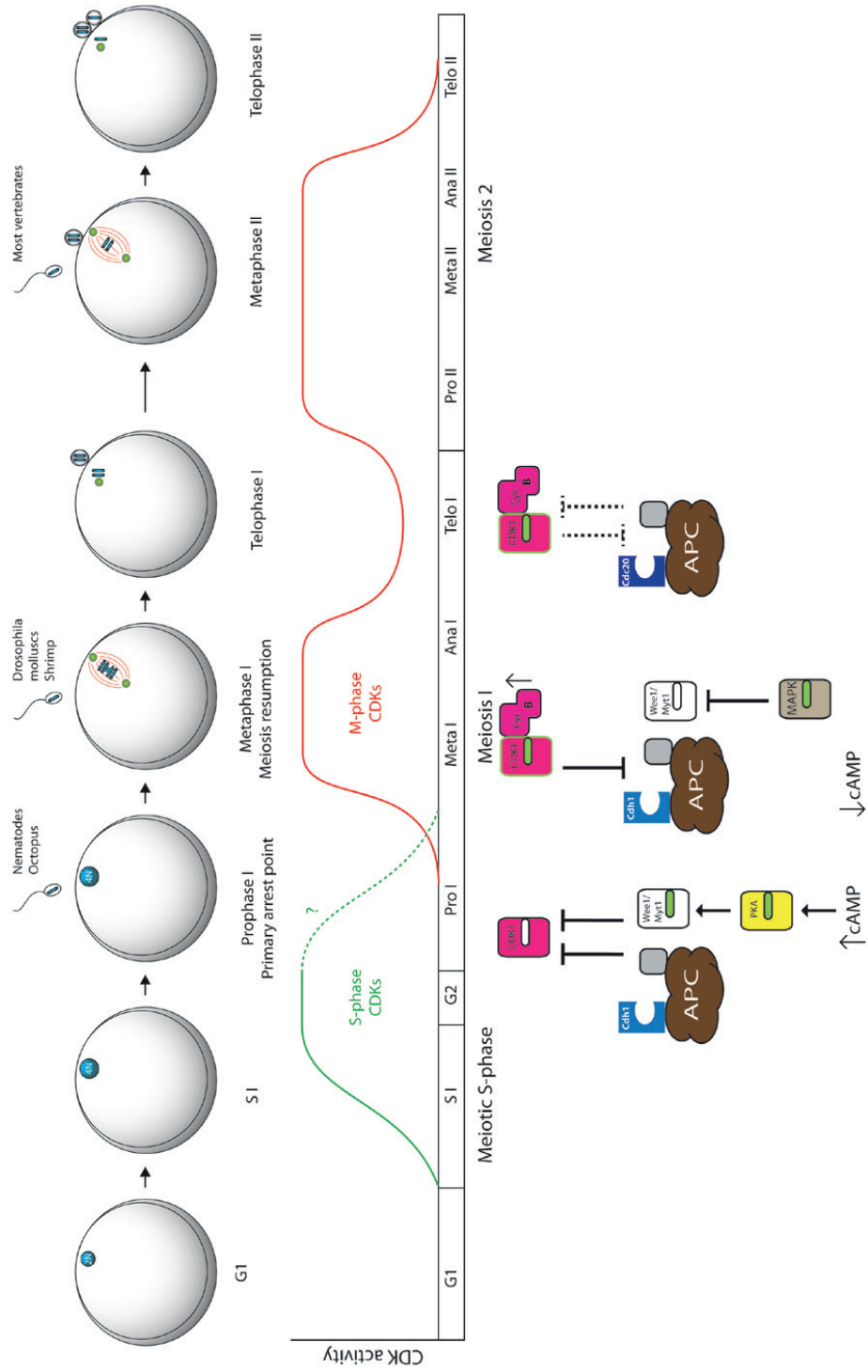
Figure 8. The meiotic cycle: oogenesis. Mitotic germline cells commit to meiosis when entering the pre-meiotic S-phase, which is controlled by G1/S Cyclin-CDKs. After completing S-phase the cell enters the meiotic M-phase followed by a prolonged primary arrest in meiotic prophase I, a feature common to most Metazoan oocytes. This is maintained by low CDK1 activity. An increase in CDK1 activity triggers meiosis resumption and nuclear envelope breakdown leading to chromosome separation in anaphase. As with meiosis, anaphase is followed by degradation of M-phase Cyclins, causing low CDK activity, though in meiosis CDK activity is only partially inactivated. Partial inactivation of CDK1 allows the cell to enter meiotic M-phase II, immediately after telophase and extrusion of the first polar body. In most mammals, germline cells enter a secondary arrest in metaphase II, which is maintained by high CDK1 activity. Completion of meiosis II is triggered by fertilization, promoting anaphase and extrusion of a second polar body, creating a haploid nucleus which becomes diploid when fusing with the male sperm nucleus.

APC/C activity (Iwabuchi et al., 2000; Taieb et al., 2001). CDK1 activity can thus be kept low enough to allow meiotic spindle disassembly and nuclear envelope re-formation, but high enough to inhibit Wee1 and prevent pre-RC assembly (Nakajo et al., 2000), allowing immediate entry into second meiotic M-phase without intervening S-phase (Marston and Amon, 2004). Chromatid separation in meiosis II resembles mitotic anaphase, and cleavage of cohesin on centromeres allow sister chromatid separation creating a second, haploid, polar body and a haploid pro-nucleus.

1.5 Endocycling

Another widespread variant of the cell cycle referred to as endoreduplication or endocycling is quite different from mitosis and meiosis in that such cells no longer complete cytokinesis, and in many cases even lack M-phases all together. Instead of duplication, these cells continue to grow by increasing their ploidy, through repetitive cycles of S-phases, above the diploid state of mitotic cells. Because cells entering endocycles becomes polyploid and cease cell division, endocyling normally occur only in terminally differentiated cells. Endocycling is ubiquitous amongst eukaryotes (De Clercq and Inze, 2006; Yin et al., 2010), but is most widespread in plants and polyploid cells as such as it may contribute up to half of the earth's biomass (Sugimoto-Shirasu and Roberts, 2003; Whitman et al., 1998; Zielke et al., 2011). Amongst metazoans, endocycling cells are abundant in insects, especially during rapid

growth of larvae, where endocycles have been well described in *Drosophila* salivary glands and nurse nuclei of adult female ovaries (Zielke et al., 2013). Endocycling cells also exist in mammals, where they are found in megakaryocytes, hepatocytes and TGCs of the placenta (Hu and Cross, 2010). Altered features of polyploid cells compared to diploid cells include the ability to maintain a larger cytoplasmic volume due to increased transcription from the amplified genomic content and also increased metabolic activity (Calvi and Spradling, 1999). Mitosis is a rather energy demanding process, considering the structural reorganizations and massive surge of phosphorylation activity associated with M-phase (Ma and Poon, 2011). Endocycling cells, however, can maintain continuous transcriptional activity while maintaining continuous growth, in contrast to mitotic cells whose transcription halts during mitosis, endocycling provides a more efficient strategy facilitating rapid cellular growth (Edgar and Orr-Weaver, 2001; Zielke et al., 2013). In addition, due to multiple gene copies, polyploid cells are considered to be less susceptible to genetic instability due to a dampened effect of mutations (Comai, 2005; Lee et al., 2009), as well as being more tolerant to genotoxic stress (Mehrotra et al., 2008). On the other hand, once committed to endocycles, cells would normally never revert to mitosis as the mitotic apparatus would be unable to properly separate the polyploid genome. When this does happen, cells can move towards the path of aneuploidy, genomic instability, tumourigenesis and cancer development (Storchova and Pellman, 2004).

1.5.1 Endocycle entry

Because endocycles do not include an M-phase they are composed of repetitive S-phases, doubling the genomic content for each cycle, separated by gap-phases allowing time for growth and pre-RC assembly (Edgar and Orr-Weaver, 2001; Edgar et al., 2014). In order to switch from a mitotic cell cycle to an endocycle, the cell needs to establish two fundamental alterations. First, the cell needs to restrict M-phase entry, which primarily involves down regulation of CDK1 activity in endocycling cells studied so far. In *Drosophila* follicle cells CDK1 deregulation occurs through transcriptional repression of String/CDC25 and an inhibitor of Fizzy related (FZR)/CDH1 named Cut, which prevents removal of CDK1 inhibitory

phosphorylation and causes destruction of mitotic Cyclins respectively, both controlled by the Notch signaling pathway (Deng et al., 2001). The endocycle switch in *Drosophila* also involves transcriptional and/or post-transcriptional (cell type dependent) down regulation of mitotic Cyclins (Maqbool et al., 2010; Zielke et al., 2008). In mammalian trophoblast stem cells (TS), endocycle transition is prevented by mitogenic activation of CHK1 (Ullah et al., 2011), a kinase that is also involved in the ATM/ATR- dependent DNA damage response. CHK1 maintains low levels of p57 and p21 through phosphorylation, which targets them for poly-ubiquitinylation and proteasomal degradation. Loss of mitogenic signal will therefore act to stabilize p57 and p21 through inactivation of CHK1 (Ullah et al., 2011). Increased levels of p57/p21 will then stimulate the transition from mitosis to endocycling by inhibiting CDK1 activity, causing G2 arrest followed by endocycle onset (Ullah et al., 2011). In plants, endocycle entry is positively regulated by CKIs: the p57^{KIP} related protein named KRP (KIP related protein) and plant specific CKIs named Siamese (SIM) and Siamese-related (SMR), which inhibit the activity of M-phase CDK activity (Churchman et al., 2006; Roeder et al., 2010; Walker et al., 2000). On the other hand, plant endocycling entry is negatively regulated by DEL1, an atypical E2F, which represses transcription of CCS52, a CDH1/FZR homolog (Lammens et al., 2008), thus down-regulation of DEL1 causes an up-regulation of CCS52, which inactivates CDK1 through proteasomal degradation of mitotic Cyclins.

Though not completing a normal mitosis, endocycling cells still need to reset the cell cycle following S-phase in order to allow reassembly of pre-RC and entry into another S-phase. This usually requires degradation of Geminin and low CDK activity, which are two requirements of pre-RC assembly (Bell and Dutta, 2002). Obtaining low CDK activity and destruction of Geminin are achieved by proteasomal degradation by APC/C$^{CDC20/CDH1}$, and this is utilized to reset the mitotic cell cycle following M-phase. Because mitotic CDK activity is specifically targeted for down-regulation when switching to endocycles in plants, insects and mammals, Fzy/CDC20, whose activity depends on mitotic kinase activity, will no longer be able to activate APC/C. However, CCS52/FZR/CDH1, activated by low mitotic kinase activity, will remain fully capable of activating the APC/C during the endocycle switch (Listovsky et al., 2000; Morgan,

2007; Takahashi et al., 2013). In both *Drosophila* and plants, CCS52/FZR activity is up-regulated through known mechanisms during endocycle entry, as mentioned above, and CDH1 has also been demonstrated to be important for endocycle entry in TGCs. Even though upstream regulation of endocycle entry varies between species, and even cell types of the same species, the overall mechanism appears to involve short-circuiting of the mitotic cell cycle through down regulation of mitotic CDK activity, while promoting APC/C activity in order to keep mitotic Cyclin levels low.

1.5.2 Maintaining endocycles

Maintenance of endocycling requires oscillations of CDK activity in order to trigger S-phase, when CDK activity is high, and relicense DNA replication, when CDK activity is low. Because entry into endocycling establishes restriction on M-phase entry through stable down regulation of mitotic CDK activity, down regulation of G1-S CDK activity thus requires a mechanism independent of CDK1 and CDC20. As discussed in the previous section, CKIs facilitate entry into endocycles where they will maintain endocycles through synchronous oscillations of CKIs and APC/C$^{CDH1/Fzr}$ activity, inverted relative to oscillations of CDK activity, which have been demonstrated in plants, insects and mammals. *Drosophila*, however, has a single CIP/KIP –type CKI named Dacapo, whose expression is promoted by CDK2-Cyclin E activity, following S-phase entry, in ovarian nurse cells. This mechanism creates out of phase oscillations between CDK2-Cyclin E activity and expression of Dacapo, allowing windows of low CDK activity and pre-RC assembly followed by S-phase entry. Dacapo is however dispensable in maintaining endocycling in salivary glands, ovarian follicle cells and innervated bristle cells, but alternative negative feedback loops are likely involved in those cells (Edgar et al., 2014). One such negative feedback loop, deployed in mouse TGCs, involves transcriptional activation followed by transcriptional repression during the G1/S transition. The transcriptional activators, E2F1-3, promote expression of G1/S Cyclins, which in turn further elevates E2F1-3 activity and thus G1/S Cyclin expression in a positive feedback loop, as explained previously. Simultaneously E2F1-3 also indirectly ensures transcriptional repression of the very same targets by promoting expression of their own antagonists, the atypical

E2Fs E2F7 and E2F8. As the E2F7/8 levels increase, they will then gradually replace E2F1-3 and thus silence the expression of G1/S Cyclins. Although they are important in endocycling TGCs, the importance of atypical E2Fs play out differently in plants and insects. As mentioned in the previous section, the plant atypical E2F ortholog, DEL1, is down regulated during endocycling because it represses the expression of CDH1, whereas in mammalian endocycling cells; atypical E2Fs acts through down regulation of Cyclin expression. *Drosophila*, however, lacks atypical repressor E2Fs all together but instead utilizes another interesting mechanism which maintains cyclic degradation of the activator E2F that is linked to DNA synthesis. As the endocycling cell initiates DNA replication the proliferating cell nuclear antigen (PCNA), a DNA clamp linking the DNA polymerase to the DNA strand, activates an ubiquitin ligase, CRL4-CDT2, which targets several proteins, including E2F1 and components of pre-RC, for proteasomal degradation by recognition of a PIP degron motif (Zielke et al., 2011). Active CRL4-CDT2 thus enforces down regulation of Cyclin E in response to DNA synthesis, creating a window of low CDK activity and pre-RC assembly.

Comparing regulation of endocycling between plants, insects and mammals, even amongst different cell types of the same organism, reveals interesting differences regarding endocycling, which suggests that endocycles have likely appeared multiple times in the course of evolution and that there are several paths to modulate the cell cycle towards endocycles. The marine urochordate, *Oikopleura dioica*, has deployed somatic endocycles as a dominant developmental strategy, supporting rapid growth from early development through adulthood. A spectacular bilateral-symmetric pattern of polyploid cells of the *O. dioica* epithelium suggests an intricate regulation of cell size and ploidy which is poorly understood, but is likely to involve regulation of gap-phase length during endocycles (Ganot and Thompson, 2002). The coenocyst, the *O. dioica* ovary, also consists of multiple endocycling nurse nuclei neighboring an equal number of meiotic nuclei, all sharing a single gigantic cell compartment. This environment exemplifies a situation where two quite different variants of the cell cycle presumably share the same proteins through a common cytoplasm, which should present various challenges regarding how to tackle incompatible cell cycle regulators.

O. dioica therefore serves as an interesting model organism to conduct cell cycle research, which may provide useful insight especially into endocycling and meiosis.

1.6 The urochordate *Oikopleura dioica*

The appendicularian, *O. dioica,* is a marine urochordate, a member of the closest extant group to the vertebrates (Delsuc et al., 2006). It is found pan-globally within the marine environment and is among the most abundant species of zooplankton and an important contributor to the marine ecosystem (Fenaux and Gorsky). Appendicularians, or larvaceans, retain a larva-like pelagic state throughout their life cycle, in contrast to their sister class ascidians and thaliaceans, which have a free swimming larval stage, common to all urochordates, but become sessile at the adult stage. The name "*Oikopleura dioica"* derives from the Greek word "Oikos" meaning "house" because *O. dioica* resides within a gelatinous house that aids in feeding. The house collect and concentrate algae and food particles from the surroundings, facilitated by water flow through particle concentration filters, controlled by beating of the tail (Fenaux, 1985). These filter-feeding houses are produced continuously by the epithelial cells, collectively referred to as the oikoplastic epithelium, and are exchanged about once every 4th h. This frequent shedding of houses is a major contributor to marine snow, which drives vertical flux of carbon, important for the marine, as well as the global, carbon cycle. The name "*dioica*" derives from the fact that *O. dioica* is the only dioecious species of *Oikopleura*, meaning they have separate individual sexes. *O. dioica* is emerging as an intriguing model organism for evolutionary studies due to its compact and rapidly evolving genome (Denoeud et al., 2010; Seo et al., 2001).

1.6.1 Life cycle of *O. dioica*

The life cycle of *O. dioica* is very short, for a chordate, and lasts from 6 to 10 days, depending on temperature (Bouquet et al., 2009; Nishida, 2008). Early embryonic development starts with the first cell division, about 35 min post fertilization, followed by rapid cell divisions leading to hatching of a free swimming larva as soon as 4 h post fertilization (Fujii et al., 2008; Nishida, 2008) (Figure 9). The early larvae develop
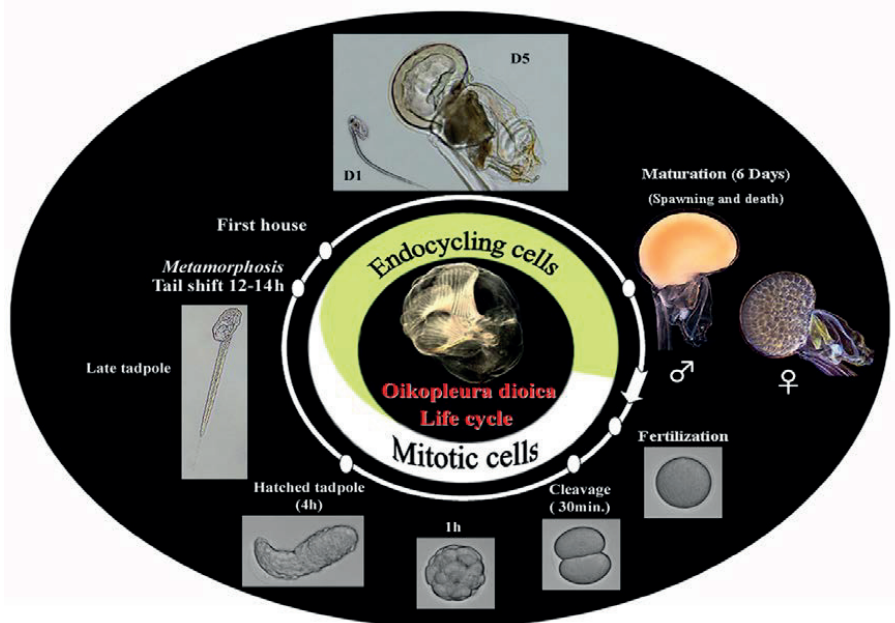
Figure 9: Life cycle of *Oikopleura dioica*. The life cycle of *Oikopleura dioica* is rapid and lasts for 6 days when cultured at 15°C. The first mitotic division occurs 35 min after fertilization and swimming larvae is hatched as soon as 4 h post fertilization. After 12 h post-fertilization *O. dioica* undergoes metamorphosis and from this point most somatic cells are committed to endocycles. Somatic endocycles are responsible for rapid growth, increasing cellular volume rather than cellular count, for the remainder of the life cycle. From day 4 the most significant growth is observed in the gonads, which will compose a larger mass than the rest of the animal by day 6. Reproduction occurs at day 6 through spawning of oocytes and sperm through rupturing of the female gonad epithelium and release of sperm through the spermiduct respectively. The figure was designed and created by Eric Thompson and Alexandra Moosmann.

through mitotic cell divisions, while an increasing number of cells, the earliest being the field of Eisen cells, exit mitosis and enter somatic endocycles (Ganot and Thompson, 2002). After 18 h most cells have entered somatic endocycles, with cells lining the gastro-intestinal tract and germline being the main exceptions. At this stage, the animal enters a metamorphic event known as tail shift, where the tail shifts 120° from posterior towards anterior orientation. Following tail shift, *O. dioica* possesses three distinct body parts: a trunk, a gonad and a tail. The trunk contains the animal's vital organs such as the gastro-intestinal tract, anterior ganglion and the oikoplastic epithelium. The gonad is located posterior to the trunk and composes the largest part

of the animal at maturity, while the tail contains the notochord and the caudal ganglion. As *O. dioica* complete metamorphosis, most somatic cells switch to endocycles, which facilitate rapid growth best exemplified by the oikoplastic epithelium, which maintains a perfect bilateral symmetry of differently sized cells with ploidies ranging from 30-1300C (C = haploid equivalents) at maturity (Ganot and Thompson, 2002). Metamorphosis is also followed by inflation of the first filter-feeding house, created by the oikoplastic epithelium (Spada et al., 2001), allowing feeding. The house is fully replaced every 4th h (Fenaux, 1985). After tail shift, *O. dioica* increases trunk size from 200 μm at day 1 to 1000 μm at day 6 (Troedsson et al., 2007), mostly due to increases in cell volume of endocycling cells. The most dramatic growth, however, is observed in the gonads, whose growth depends on nutrient availability, contributing to more than half the volume of the mature animal.

1.6.2 *O. dioica* oogenesis

The onset of germline development of the ovary occurs through syncytial mitotic divisions contained within a germline cyst (Pepling et al., 1999). *O. dioica* oogenesis may be broken into five phases, which starts at day 3 as meiosis commences. During the first phase (P1) of oogenesis, germline nuclei undergo fate selection by either committing to asynchronous endocycles, establishing polyploid nurse nuclei supporting oogenesis through high transcriptional activity, or they enter meiosis committed to seed pro-oocytes. The distribution of meiotic and asynchronously endocycling nuclei is 1:1, an arrangement defined as a coenocyst describing a heterogeneous population of nuclei sharing a common cytoplasm (Ganot et al., 2007a; Ganot et al., 2007b). Nurse nuclei start to endocycle in P2, while the meiotic nuclei enter zygotene, characterized by the chromosomal bouquet. In P3, the nuclei are organized by an actin scaffold, which partially encloses meiotic pro-oocytes in compartments. Similar to *Drosophila* egg chambers, which contain 15 nurse nuclei and 1 meiotic oocyte; all nuclei are connected to the same cytoplasm through structures known as ring canals. P3, which starts at late day 3, lasts until day 5, during which meiotic nuclei remain arrested in prophase I. The meiotic nuclei are anchored, via patches rich in nuclear pore complexes, to actin, marking the future animal pole of

Table 1: Timing of events during oogenesis in *Oikopleura dioica*.

Days at 15°C	Phase	Events in oogenesis*
D0.5-D3		- Proliferation of germ nuclei in syncytium
D3	P1	- Fate differentiation of germ nuclei
		- Onset of meiosis
D3.5	P2	- Meiotic chromosomal bouquet (zygotene)
		- Meiotic nuclear NPC cluster formation
		- Nurse nuclei endocycle
D3.5-D5.5	P3	- Prophase I arrest
		- Rapid growth of coenocyst
		- Pro-oocytes with ring canals (future vegetal pole)
		- Meiotic nuclei anchored via NPC to actin (future animal pole)
		- Meiotic nuclei exhibit H3S10P
		- Nurse nuclei endocycle
D5.5-D6	P4	- Oocyte growth by cytoplasm transfer through ring canals
		- Resumption of meiosis
		- Unselected meiotic nuclei associate with nurse nuclei
		- Meiotic chromatin adopts π-configuration, NPC clusters partially dissociate
		- Nurse nuclei endocycle
		- Extensive invaginations form in nurse nuclear envelopes (late P4)
D6-D6.5	P5	- Meiotic nuclei in oocytes enter metaphase I arrest
		- Nuclei external to oocytes undergo apoptosis
D6.5		- Spawning

Modified from (Ganot et al., 2007b). * NPC – Nuclear pore complex.

the pro-oocyte. Meiotic chromatin also becomes phosphorylated at histone 3 serine 10 (H3-pS10) during P3 (Ganot et al., 2008), an epigenetic modification known to occur during diplotene/diakinesis in other species. In later P3, meiotic chromatin becomes phosphorylated at H3S28 as well, marking meiotic/mitotic chromatin entering pro/meta phase. Also during P3 the ovary grows rapidly, supported by high transcriptional activity of the growing nurse nuclei. At mid-day 5, pro-oocytes undergo a second round of selection, at P4; determining which pro-oocytes are to reach maturity. An important selective factor is food resources, as rich sources of nutrients may improve fecundity three fold compared to a poor diet (Bouquet et al., 2009). This

also allows opportunistic population growth of *O. dioica* during periods of algal blooms (Troedsson et al., 2002). Selection coincides with chromatin condensation into a characteristic π-configuration and activation of the MAPK pathway, a well-established transducer of growth and nutrition signals. In addition meiotic nuclei, selected to seed mature oocytes, become enriched in H3-pS28 and grow rapidly by cytoplasmic transfer through the ring canals, whereas H3-S28 phosphorylation diminishes in non-selected nuclei, which then associate with nurse nuclei (Ganot et al., 2008). When oogenesis reaches its last phase (P5), growing oocytes reach metaphase I arrest, while nurse nuclei and non-selected nuclei become apoptotic. The fully grown oocytes will ultimately be released through rupture of the gonad epithelium, at which point the animal dies. *O. dioica* oogenesis is summarized in Table 1.

1.7 A perspective on cell cycle evolution

O. dioica responds effectively to available nutrients (Troedsson et al., 2002) and its rapid growth, short generation time, and efficient modulation of reproductive output, allow rapid population growth in response to rich food sources occurring during algal blooms. *O. dioica* also represents one of the most abundant species of animal plankton along with copepods, and belong to the closest evolutionary sister group to vertebrates (Delsuc et al., 2006) (Figure 10). Studying cellular regulatory networks in the light of evolution provides useful insight into conserved and specialized regulatory modules amongst species (Doonan and Kitsios, 2009). Common evolutionary mechanisms include gene expansion, through gene duplication, and gene contraction, through gene deletions or detrimental mutations. Duplicated genes allow mutations within a duplicated gene, while retaining original function within the other. This may cause loss of function mutations of a duplicated gene (non-functionalization), but may also cause advantageous mutations, giving rise to new functions (neo-functionalization) (Li et al., 2005). Another possibility is sub-functionalization, where duplicated genes divide original function amongst paralogs, leading to divided specialization of original functions. As species evolve, other genes may in turn become redundant, allowing deleterious mutations of redundant genes, and thus "simplify" regulatory pathways. *O. dioica* for instance display both constrictions, as seen with *O. dioica* notochord genes
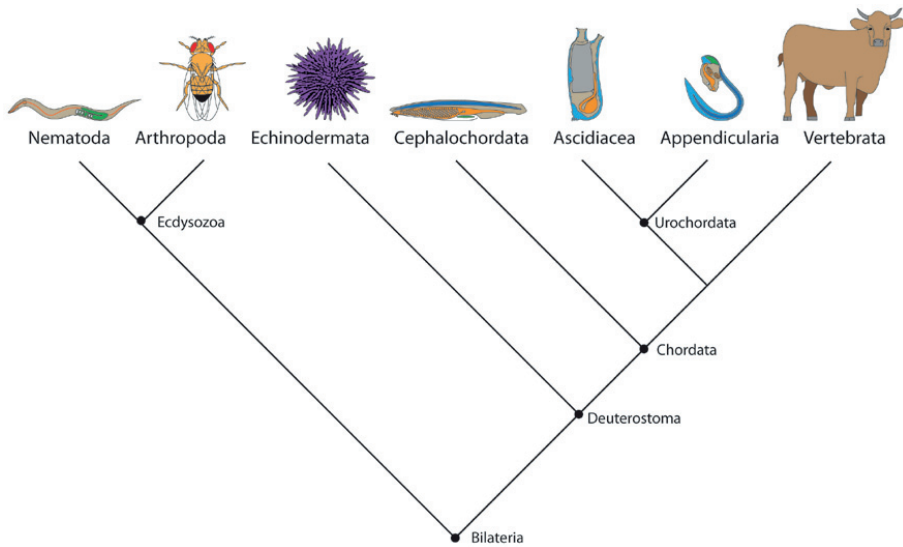
Figure 10: Bilaterian evolution. Cephalochordata have traditionally been considered the closest sister group to vertebrates. In the last ten years phylogenetic analysis of genomic data has revealed that the urochordates are closer to vertebrates than cephalochordates in the history of evolution (Delsuc et al., 2006).

which are halved compared to *C. intestinalis* (Kugler et al., 2011), and expansions, among *O. dioica* homeobox genes (Edvardsen et al., 2005), of the genome. Denoeud *et. al.* demonstrated that the highly conserved animal genome architecture is shattered in *O. dioica*, which illuminates a higher degree of plasticity of genomes than previously thought (Denoeud et al., 2010).

Regulation of the eukaryotic cell cycle retains similar cell cycle modules in yeast, plant and metazoans (Doonan and Kitsios, 2009), though there is an obvious diversification amongst the Cyclin and CDK complements. Budding yeast for instance possess a single CDK1 ortholog, CDC28, controlling the entire cell cycle, through interaction with nine Cyclins; Cln 3 (G1), Cln1-2 (G1/S), Clb5-6 (S) and Clb 1-4 (G2/M) (Nurse and Bissett, 1981; Piggott et al., 1982) (Figure 11). *Arabidopsis thaliana* (Plant) however requires three CDK1 orthologs namely CDKA1, which regulates G1/S phase together with five D-type Cyclins and one group of A-type Cyclins (CycD2-6 and CycA3), and CDKB1 and CDKB2, which regulate G2/M together with groups of A and B-type Cyclins (Cyc A2 and CycB1-3) (Van Leene et al., 2010). The plant Cyclin and CDK complement is quite different from the metazoan

cell cycle in that it possesses an amplified complement of D type Cyclins and lacks Cyclin E all together. Another remarkable difference is that the plant CDK1 homologs interact with separate cyclin partners, without overlap, which may partly be explained by their different PSTAIRE motif (conserved only in CDKA1), whereas metazoan CDK1 and CDK2 share several Cyclin partners. We see that additional CDKs perform specialized functions in cell cycle regulation from yeast to vertebrates, where three CDKs are dedicated in control of interphase and one is responsible for M-phase entry. This classical eukaryotic cell cycle model has however been challenged by CDK knockout studies in mice, revealing that there is a high level of functional redundancy amongst cell cycle CDKs (Malumbres and Barbacid, 2009). For instance CDK1, in the absence of CDK4/6 and CDK2, is sufficient to maintain early embryogenesis in mice, whereas individual knockout of CDK4/6 and CDK2 display cell type specific defects, exemplified by CDK2 which is required for meiosis (Adhikari et al., 2012; Berthet et al., 2003; Santamaria et al., 2007). This suggests that regulation of the basic eukaryotic cell cycle is in principle largely conserved from yeast. The amplification of cyclins and CDKs in higher multicellular eukaryotes may thus reflect a more complex composition of specialized cell types, which possibly require additional specialized regulatory cell cycle modules.

The invertebrate Cyclin and CDK complements are simple in comparison with the more complex Cyclin and CDK complements of the vertebrates. Are relatively simple complements retained in the rapidly evolving *O. dioica*, which belongs to the closest sister group to the vertebrates, or have they evolved greater complexity? Has a rapid evolution and shattered genome architecture also introduced alterations within the core cell cycle machinery with respect to a growth strategy favoring endocycling? We know that the homeobox genes of *O. dioica* have been amplified (Edvardsen et al., 2005), which suggests higher plasticity of developmental gene expression. A majority of the duplicated homeobox genes are broadly expressed in the oikoplastic epithelium, where they have likely neo-functionalized to control patterning and expression of this functionally important organ (Denoeud et al., 2010; Hosp et al., 2012; Spada et al., 2001). Thus we set out to determine the core cell cycle regulatory complement of *O.*

dioica and to begin to explore the functional significance of some of the variant machinery we uncovered.
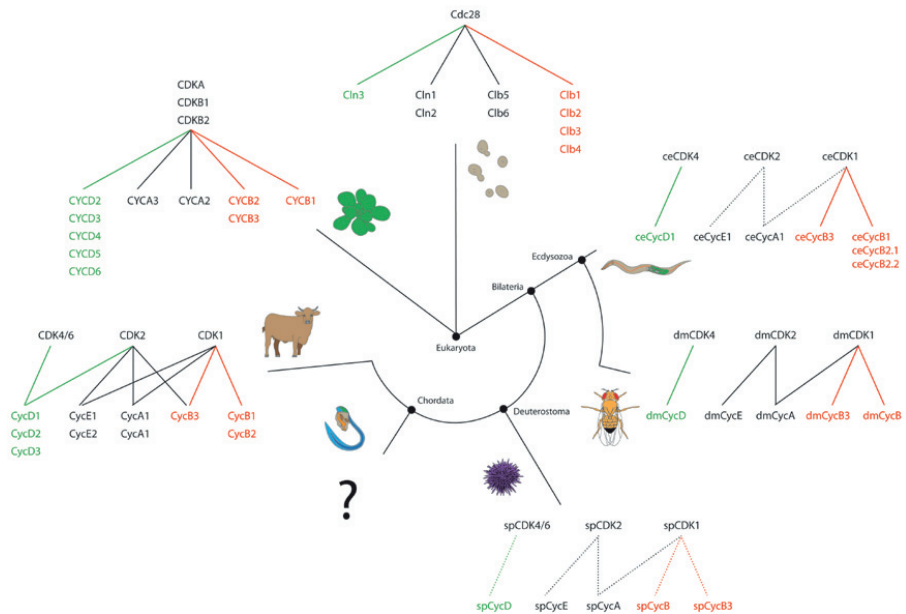
Figure 11: Evolution of the Cyclin-CDK complement. The yeast cell cycle depends on a single CDK1 ortholog (CDC28) (Mendenhall and Hodge, 1998), whereas plants (Van Leene et al., 2010) and Metazoans (Campsteijn et al., 2012; Malumbres and Barbacid, 2005; Meyer et al., 2000; Sigrist and Lehner, 1997; Sodergren et al., 2006; van den Heuvel, 2005) subdivide regulation of cell cycle entry (Green lines), G1/S/G2 (Black lines) and G2/M transition (Red lines) between several CDKs. In the chordate lineage there is also an amplification of the Cyclin complement.

2. Aims of study

O. dioica displays several unusual aspects of the cell cycle for a chordate, involving extensive use of endocycling required for both somatic and female germline development. Rapid evolution and unusual genome architecture is reflected in the *O. dioica* genomic content, as exemplified by rapidly evolving Lamins and the amplified homeobox genes, and we were therefore curious to explore to what extent highly conserved cell cycle machinery had been conserved or modified in this rapidly evolving chordate. Although the cell cycle Cyclin and CDK complement have expanded from yeast to metazoan, followed by further amplifications in vertebrates, the core function of CDK1 has been retained. Our first aim was to explore and annotate the cyclin and CDK complements of *O. dioica*, by searching for and aligning the Cyclin-box motifs, conserved in Cyclins, and kinase domains, conserved in CDKs. We also wanted to explore retention of other regulatory elements, such as the highly conserved PSTAIRE motif of CDKs, which could provide clues regarding conserved function. As a supporting study of the Cyclin and CDK annotation we also wanted to establish a developmental expression profile of Cyclins and CDKs, which could be linked to their involvement in mitosis (embryogenesis), endocycling (post-metamorphosis) and meiosis (late development). Additional support planned for this work in order to evaluate cell cycle phase specific involvement of Cyclin-CDKs, included spatio-temporal localization of Cyclins and CDKs. Following identification of the Cyclin and CDK complements we wanted to explore conserved functions with respect to cell cycle control, through dsRNA knockdown approaches, especially with respect to coordinated regulation of endocycling and meiosis within the shared cytoplasm of the coenocyst. Because the coenocyst is unique compared to germlines of other metazoans we would also expect to expand this study to include additional exploration of the architecture and process of oogenesis within the coenocyst, in order to better comprehend the regulation of this unusual cell cycle environment.

3. List of papers

Paper I

Campsteijn, C., J. I. Ovrebo, B. O. Karlsen and E. M. Thompson (2012).

"Expansion of cyclin D and CDK1 paralogs in *Oikopleura dioica*, a chordate employing diverse cell cycle variants." <u>Mol Biol Evol</u> **29**(2): 487-502.

Paper II

Jan Inge Øvrebø[1,2] , Coen Campsteijn[3,4], John Kourtesis[2], Martina Raasholm[2], Harald Hausen[2], Eric Thompson[1,2]

"Functional specialization of chordate CDK1 paralogs during oogenic meiosis"

Manuscript for submission

4. Summary of results

4.1 Expansion of Cyclin D and CDK1 Paralogs in *Oikopleura dioica*, a Chordate Employing Diverse Cell Cycle Variants (Paper I)

This work characterizes the complete Cyclin and CDK complement of *O. dioica* using the assembled genome by Genoscope. We characterized a Cyclin and CDK complement similar to that of other invertebrates, though some interesting expansions were identified. The B-type Cyclin complement contained 5 genes, which by comparison with *Ciona intestinalis* and *Drosophila,* each possessing two Cyclin Bs, represents an expansion even outnumbering the three Cyclin B genes in vertebrates. *O. dioica* B-type Cyclins were expressed during early mitotic development as well as in late development, which is dominated by mitotic and meiotic germline development. The apparent lack of expression during mid-development, consisting primarily of somatic endocycles, was consistent with Cyclin B being a G2/M Cyclin. Sex specificity of B-type Cyclins was also observed in gonads of late animals, which may be related to specialized functions related to male and female gametogenesis. Also the *O. dioica* Cyclin D complement is expanded to 4 genes in contrast to a single Cyclin D gene in invertebrates and three in vertebrates. The D-type Cyclins displayed an overlapping expression profile with some being preferentially expressed in early mitotic development, whereas others were higher expressed during mid-development. The abundance of D-type Cyclins could be involved in a rheostat like function in careful regulation of cell size in somatic endocycling cells. Even more surprisingly the highly conserved G2/M CDK, CDK1, was expanded to five paralogs, making *O. dioica* the only known metazoan to possess more than one CDK1 gene. Another peculiar observation was that none of the five CDK1 paralogs possessed a perfect PSTAIRE motif, a motif which is highly conserved and invariant in metazoan CDK1s. Because there are changes within the PSTAIRE motif as well as non-conservative variations at the Cyclin interaction interface amongst the odCDK1 paralogs, variations in Cyclin binding preference amongst them may exist. As with Cyclin B, odCDK1 paralogs also displayed sex specificity, suggesting specialization towards gametogenesis. odCDK1a is most identical to human CDK1 amongst the five

odCDK1s and is expressed through development, peaking at early mitotic development and late development. CDK1b and c have a similar expression profile, being expressed though development with highest expression from early to mid-development and late male specific expression. CDK1d and e are expressed exclusively at early and late development and are up regulated in females during oogenesis.

4.2 Summary of results (Paper II)

This work dissects odCDK1 function in *O. dioica* meiosis within the coenocyst. We observed enrichment of odCDK1 paralogs, as well as other meiotic cell cycle regulators and meiotic kinase activity (MPM-2), within cytoplasmic organelles juxtaposed to meiotic nuclei. These structures were similar to MTOCs, including presence of gamma-tubulin, and were defined as organizing centers (OCs). OCs contained odCDK1 paralogs during P3 (pre-selection) females, but odCDK1 paralogs translocated from the OCs to non-selected nuclei at P4 (post-selection). Selected nuclei condensed into π-configuration but did not contain odCDK1 and did also become enriched in nuclear Lamin1, an indication of an intact nuclear envelope, indicating these nuclei are in diakinesis of prophase I and have not resumed meiosis. Nuclear Lamin1 and lack of nuclear odCDK1 paralogs were observed until spawning and thus prophase I arrest in diakinesis lasts from day 5 until spawning. To establish whether odCDK1 paralogs are functionally redundant or possesses specialized functions, we performed RNAi experiments by injecting dsRNA directly into day 4 gonads. RNAi of odCDK1e did not give rise to any observable phenotypes, whereas RNAi of odCDK1d caused release of sterile oocytes failing to resume meiosis, consistent with canonical CDK1 activity. In addition, odCDK1a RNAi caused release of small and sterile, oocytes, suggesting problems with cytoplasmic transfer in odCDK1a depleted ovaries. This work reveals novel sub-functionalization amongst two odCDK1 paralogs.

5. General discussion

The cell cycle control system safeguards mitotic cell division ensuring reliable inheritance of undamaged genomic material, which is essential in maintenance of cell fates and healthy cell proliferation. In addition to providing robust passage through the cell cycle checkpoints, the cell cycle may also be carefully modulated, tailored to the functional role of the cell, which may involve major restructuring of the cell cycle in order to switch from mitotic to endocycles or meiotic cell cycles. The mitotic cell cycle has been scrutinized most extensively, within a diverse range of organisms, and is thus the best understood. Obtaining a complete picture of the mitotic cell cycle has many implications in cancer biology and has revealed several cell cycle checkpoints that are defective in cancer cells. What is known about regulation of the mitotic cell cycle is often, and rightfully so, transferred to regulation of meiosis, as many mitotic cell cycle regulators are also involved in meiosis. There are, however, some important differences to consider, as meiosis consists of two cell division events without an intervening S-phase in order to decrease cell ploidy. Thus, there are mechanisms allowing re-entry into M-phase following the first cell division.

Endocyling, which has been studied to a lesser extent, contrasts meiosis by maintaining repetitive S-phases and lacking M-phase all together in order to increase ploidy. One of the reasons why there is less knowledge about endocycling might be that it is less frequent in mammals, which are most relevant for medical research. Endoreplication is, however, a ubiquitous mechanism deployed, to various extents, in most eukaryotes, including the giant trophoblast cells of the mammalian placenta. Endocycling cells share several features with cancer cells including DNA re-replication, leading to genomic instability (Dutta, 2007), and insensitivity to apoptotic signals (Mehrotra et al., 2008). In our study we explored cell cycle regulation of the marine urochordate *O. dioica*. There are several advantages associated with *O. dioica* as a model organism as it deploys mitosis, endocycling and meiosis each predominating at three different developmental stages, making comparison of

developmental expression with cell cycle variants convenient. As for studying meiosis, knockdown experiments can easily be performed by microinjection directly into the exposed gonads, which also allows manipulation of meiosis during prophase I of meiosis. Gonad injection is also an efficient strategy to distribute synthetic transcripts in pre-spawned oocytes, allowing manipulation of entire oocyte clutches, providing a convenient system to study embryonic mitosis. Viewed from an evolutionary perspective *O. dioica* stands out as a member of the closest sister group to vertebrates (Delsuc et al., 2006), as well as being one of the most rapidly evolving metazoans. The *O. dioica* Cyclin and CDK complement may therefore illuminate new aspects of cell cycle evolution within the chordate lineage. An important first step in order to understand the *O. dioica* cell cycle was to uncover core cell cycle regulators, namely the Cyclins and CDKs, within the fully sequenced *O. dioica* genome (Denoeud et al., 2010; Seo et al., 2001).

5.1 Specialized function amongst the amplified CDK1 paralogs in *O. dioica*. Analysis of the *O. dioica* genome revealed a cell cycle Cyclin-CDK complement amplified above that of other invertebrates, and even vertebrates (Figure 12), but the rationale behind this amplification remains unknown. One obvious feature of *O. dioica* is its extensive use of endocycling, both somatic and during germline development. Similar to growth of *O. dioica* by endocycling, *C. elegans* also deploys endoreplication for somatic growth (Lozano et al., 2006) and in *Drosophila* endocycling is observed in several tissues, including nurse nuclei of the ovary (Hammond and Laird, 1985), but do so with a simpler Cyclin-CDK complement. Perhaps the most surprising amplification we observed amongst the odCDK complement was the discovery of five odCDK1 paralogs (Campsteijn et al., 2012), which was quite unexpected for an organism where endocycling predominates, as CDK1 is a well-known G2/M regulator in metazoans. Interestingly, CDK1 is also the most conserved CDK, which single-handedly controls the entire cell cycle in yeast. Metazoans on the other hand have evolved several cell cycle CDKs. Phylogenetic analyses indicate that the CDK4/6 subfamily emerged with the appearance of eumetazoans, while CDK2 diverged from CDK1 by gene duplication in Metazoans
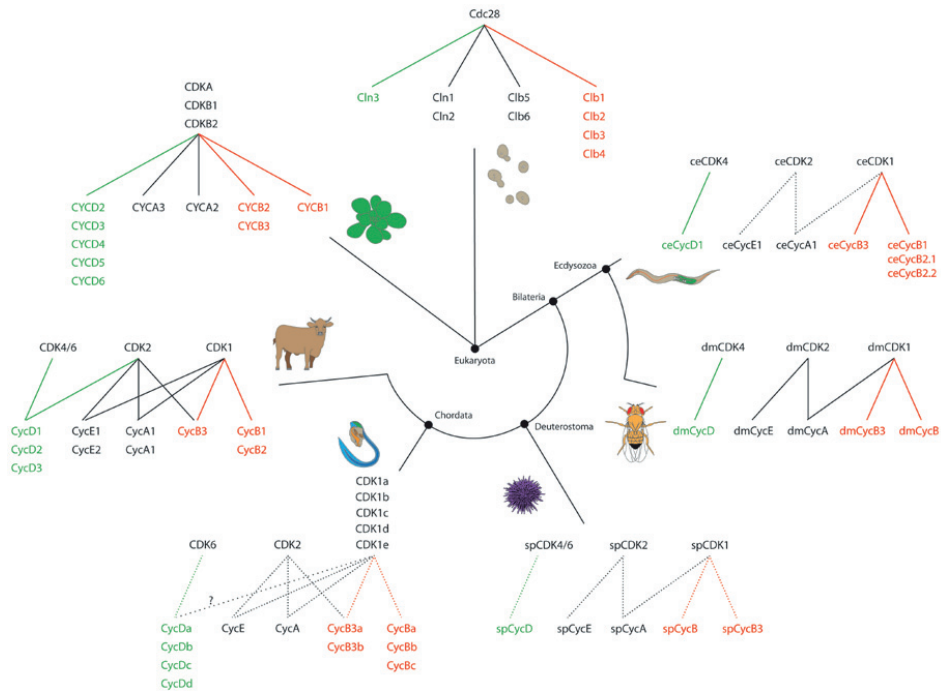
Figure 12: Expansion of the Cyclin and CDK complement of *O. dioica*. Genome search and phylogenetic analysis revealed that *O. dioica* possesses an amplified complement of D and B type Cyclins, expanded beyond the vertebrate Cyclin D and Cyclin B complement (Campsteijn et al., 2012). The CDK complement possesses five paralogs of CDK1, making *O. dioica* the only known metazoan to possess multiple CDK1 paralogs.

(Cao et al., 2014). CDK1 has retained crucial function in controlling M-phase in all metazoans studied so far. *O. dioica* represents the only known model organism where gene duplication of CDK1 has occurred in the metazoan lineage. In order to explore odCDK1 function in *O. dioica*, we performed microinjection of odCDK1-eGFP fusion cmRNAs into one-cell embryos to reveal spatio-temporal localization (Figure A1a). Immunofluorescence revealed that there were three odCDK1 paralogs; odCDK1a, odCDK1b and odCDK1c, which displayed nuclear localization during early prophase in mitotically developing tadpoles, whereas odCDK1d and odCDK1e remained cytoplasmic throughout the cell cycle. This suggests that three odCDK1 paralogs might share redundant function in mitotic regulation, whereas the latter two are committed to a meiosis related function (Paper II). Preliminary knockdown experiments of odCDK1a alone, however, revealed severe developmental defects in

early tadpoles, suggesting that even amongst the three odCDK1 paralogs involved in mitosis, there is some degree of non-redundant function concerning embryonic development (Figure A1b). We also performed co-knockdown of odCDK1b and odCDK1c, which did not reveal any evident phenotypes. Taken together this suggests that odCDK1a may act as the primary regulator of mitosis, consistent with canonical CDK1 regulation, whereas odCDK1b and odCDK1c are functionally redundant to odCDK1a, or non-essential, in regulation of mitosis. As odCDK1b and odCDK1c are preferentially expressed in male testes in late development, they may play a more important function during male germline development, analogous to the specialized function of odCDK1d and odCDK1e in the female ovary (PaperII). Our findings suggests that, amongst the odCDK1 paralogs, odCDK1a might be sufficient to maintain mitotic development, but that specific odCDK1 paralogs are required during germline development, as observed with odCDK1a and odCDK1d in the ovary (Paper II). Being functionally different may involve variations in Cyclin interactions, providing substrate specificity, and thus exploring these interactions would provide important information for further studies.

5.2 Amplified CDK1 paralogs in *O. dioica* display variations in the Cyclin interaction motif.

The CDK1 PSTAIRE motif is highly conserved from yeast (CDC2/CDC28) to vertebrate CDK1 and CDK2, and is decisive in Cyclin interactions. Plants however do possess three CDK1 paralogs whose function has sub-functionalized towards regulation of different cell cycle phases (Van Leene et al., 2010). The highly conserved PSTAIRE motif is conserved in plant CDKA, whose function is regulation of G1/S transition, analogous to Metazoan CDK4/6 and CDK2, through interaction with D and A-type Cyclins. The CDK1 paralogs; CDKB1 and CDKB2, regulate G2/M through non-overlapping Cyclin A and B interaction, but possess altered PSTAIRE motifs. Even though CDKA and CDKBs share a sequence similarity of about 83% (Hirayama et al., 1991), only CDKA is capable of substituting CDC2 function in yeast (Porceddu et al., 1999). The fact that the PSTAIRE motif, which is decisive in Cyclin interaction (Child et al., 2010), varies between the three plant CDK1 homologs might

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hsCDK1/2          PSTAIRE
odCDK1a           PSTSIRE
odCDK1b           PPTSVRE
odCDK1c           PATSIRE
odCDK1d           PPTSLRE
odCDK1e           PATSVRE
hsCDK4            PISTVRE
hsCDK6            PLSTIRE
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Figure 13. The odCDK1 PSTAIRE motifs. Vertebrate CDK4/6 regulates the G1/S transition through interaction with D-type Cyclins and possesses a $PI/LS_3T_4V/IRE$ motif. The plant CDK1 homolog, CDKB, also forms active complexes with D-type Cyclins with its $PP_2T/ALRE$ motif. odCDK1 paralogs shares an A_4 to S_4 substitution which mimics the CDK4/6 $PI/LS_3T_4V/IRE$ motif. odCDK1b and d also possesses a S_2 to P_2 substitution which mimics the plant CDKB $PP_2T/ALRE$ motif.

explain the non-overlapping Cyclin interactions, and thus their different functions. Interestingly, none of the odCDK1 paralogs possess a perfectly conserved motif, which is invariant amongst other known metazoan CDK1 homologs. Observed substitutions amongst odCDK1 paralogs includes S_2 to A_2 in odCDK1c (PA_2TSIRE) and odCDK1e (PA_2TSVRE), as well as S_2 to P_2 in odCDK1b (PP_2TSVRE) and odCDK1d (PP_2TSLRE), the latter two creating similar motifs observed in plant CDKB1 (PPTA/TLRE), which regulates M-phase through interactions with Cyclin B3 (Van Leene et al., 2010). The closest homolog to hsCDK1, odCDK1a, possessed a single A_4 to S_4 substitution ($PSTS_4IRE$), shared by the five CDK1 paralogs, mimicking the S/T sequence of the CDK4 PISTVRE motif (Figure 13), which is known to bind Cyclin D. If the different PSTAIRE motifs in odCDK1 paralogs provide largely non-overlapping Cyclin interactions, as seen in plant, the different affinity to Cyclins might contribute to the specialized functions observed for odCDK1a and odCDK1d. The amplified complement of D-type Cyclins and the odCDK1 paralog PSTAIRE motifs that mimic CDK4/6 raises the possibility of interaction between odCDK1 paralogs with the amplified Cyclin D complement.

5.3 Cyclin Ds and odCDK1 - a possible partnership?

We identified an amplified complement containing four D-type Cyclins, a fourfold amplification compared with other invertebrates which also exceeds the three D-type

Cyclins of human (Campsteijn et al., 2012). The expansion of G1-S Cyclins in *O. dioica* may reflect predominant endocycling development (Ganot and Thompson, 2002). However, similar duplications are not observed in *C. elegans*, which also grows by somatic endoreplication (van den Heuvel, 2005). Because the oikoplastic epithelium is comprised of endocycling cells which vary greatly in both size and ploidy while at the same time displaying a perfect bilateral symmetry (Ganot and Thompson, 2002; Spada et al., 2001), it is reasonable to hypothesize that *O. dioica* has evolved a sophisticated way of regulating endocycle frequency. Endocycles are composed of G and S-phases and as the duration of the S-phase is mostly constant; the frequency of S-phase entry is primarily regulated by the duration of G (Ganot and Thompson, 2002). An important role for D-type Cyclins are to relay nutrition and growth signals (Adhikary and Eilers, 2005), which in turn activate CDK4 and phosphorylate pRb, leading to released activity of E2F and entry into S-phase. The amplified Cyclin D complement's role in responding to nutrition would also reflect *O. dioica'*s observed ability to respond to algal blooms through opportunistic growth (Troedsson et al., 2002). It is therefore tempting to speculate that the amplified complement of D-type Cyclins has evolved in order to function as an endocycle rheostat, regulating G length.

As discussed in chapter 5.2, the divergent odCDK1 PSTAIRE motifs displays characteristics suggesting possible interactions with D-type Cyclins, which could imply that odCyclin Ds can function as activators of odCDK1, analogous to the plant CDK1 homolog CDKA (Doonan and Kitsios, 2009; Van Leene et al., 2010), providing substrate specificity towards G1-S function. If this is the case, odCDK6 may still retain its canonical G-phase function, while bound to a subset of D-type cyclins, whereas odCDK1 may provide an additional level of G-phase regulation, or even replace odCDK6 function, in specialized cells. For instance, most invertebrates possess a single CDK4/6 family member, whereas vertebrates require both CDK4 and CDK6 in order to produce a healthy animal; where CDK4 ablation cause reduced body size, diabetes and sterility (Rane et al., 1999; Tsutsui et al., 1999), and CDK6 knockout cause hypoplasia and defective hematopoiesis in mice (Malumbres et al., 2004). Since CDK activity is highly dependent on the respective cyclin partner,

additional G-phase CDKs may as well arise from CDK1 paralogs with altered cyclin interaction motifs, as from an existing CDK4/6 family member.

One of the *O. dioica* D-type Cyclins, odCyclin Dd, displayed sequence similarities with both D and E-type Cyclins, and immunofluorescence revealed cyclic nuclear localization in G1-S phase of both mitotic and endocycling cell cycle, thus based on our observations we concluded that odCyclin Dd acts as a hybrid between Cyclin D and E (Campsteijn et al., 2012). Surprisingly we also discovered a possible function related to the meiotic cell cycle in the ovary, where odCyclin Dd was found to translocate within non-selected nuclei similar to odCDK1 paralogs (Figure A3-1). Based on the fact that we have concluded a G1-S function for odCyclin Dd (Campsteijn et al., 2012); we assume odCyclin Dd is not involved in meiosis. Therefore we still have no clear ideas to why CDKs and Cyclins accumulate in nuclei destined for an apoptotic fate, but we have an impression that non-selected nuclei somehow function as "garbage disposals", clearing the cytoplasm exterior to growing oocytes of obsolete cell cycle regulators.

5.4 Multiple Cyclin Bs: What are they used for?

The canonical function of CDK1 is regulation of M-phase through interaction with mitotic Cyclins. The B-type cyclins were also amplified within the *O. dioica* Cyclin complement (Campsteijn et al., 2012) to five B-type Cyclins in comparison with invertebrates possessing two-three Cyclin B genes. Because B-type Cyclins are known to regulate the G2/M transition whereas most of the *O. dioica* cells are endocycling, lacking M-phase all together, it raises the question of why *O. dioica* requires that many. The odCyclin B expression profiles also reveal that they are in fact scarcely expressed during developmental stages composed primarily of endocycling, as would be expected of B-type Cyclins. odCyclin Bs are, however, expressed pre-metamorphosis, while cells are still mitotically dividing, and in late development during which germline differentiation becomes prevalent. In addition, comparison of expression relative to testes and ovaries, revealed sex specific expression levels, where odCyclin Ba/b and odCyclin B3aα were expressed in ovaries, and odCyclin Bc, odCyclin B3b and odCyclin B3aβ were expressed in testes. Interestingly, B1 and B3

Cyclins display functional redundancies during regulation of mitosis in *Drosophila*. Although, both are required in production of fertile oocytes, only Cyclin B3 is dispensable for male fertility (Jacobs et al., 1998). Cyclin B3 regulates the meta/ana-phase transition, which has been demonstrated in *C. elegans* where elevated expression of Cyclin B3 is sufficient to make the meta/ana-phase transition in the absence of important spindle assembly checkpoint components (Mdf-1/MAD1) (Tarailo-Graovac et al., 2010). This could partially explain why the male enriched odCyclin B3b is expressed earlier (at day 5) than the female enriched odCyclin B3a (at day 6), as meiosis of spermatogenesis is completed at day 6, whereas the meta/ana-phase transition has not yet occurred in pre-spawned oocytes. Functional specialization of Cyclins in meiosis control is also reminiscent of the amplified B-type Cyclins of *C. elegans*, where RNAi experiments demonstrate overlapping functions among B-type Cyclins: Cyclin B1 is required for chromosome segregation and Cyclin B3 is required for sister chromatid separation during meiosis (van der Voet et al., 2009). Because co-knockdown of Cyclin B1, Cyclin B3 and Cyclin B2.1/B2.2 is required to produce as severe effect as CDK1 knockdown alone, at least three B-type cyclins are likely to perform specific functions during meiosis in *C. elegans*. During embryogenesis, only Cyclin B1 and Cyclin B3 are required for successful mitosis, whereas during adult development Cyclin B3 is no longer detected. B-type Cyclins therefore display both overlapping and specialized function in regulation of meiosis/mitosis, as well as some functional redundancy during mitosis (van der Voet et al., 2009). Similar to *O. dioica*, *C. elegans* grow primarily through endoreplication (Lozano et al., 2006), so why this amplification of M-phase cyclins? A possible explanation may be found in the sex-specific expression patterns of B-type Cyclins, which has been observed in *O. dioica* (Campsteijn et al., 2012), allowing sex-specific specialization of duplicated Cyclins, integrated in tissue/sex-specific expression networks. Sex and tissue-specific specialization may also be beneficial in hermaphroditic species, such as *C. elegans*, as well as *O. dioica*, which is dioecious but is likely to have evolved from a hermaphroditic ancestor. This explanation, however, is not necessarily compatible with another hermaphroditic tunicate, *Ciona intestinalis*, which possess a single Cyclin B1 and B3. Nevertheless odCDK1a and odCDK1d do have different functions during

oogenesis, and *O. dioic*a B-type Cyclins may also exhibit functional specialization during meiosis.

5.5 Cell cycle regulation within the coenocyst

While studying odCDK1 functions in the coenocyst we established that *O. dioica* oocytes undergo primary prophase arrest at diakinesis around day 5, persisting until spawning (Paper II). We then wanted to explore cell cycle regulation within the coenocyst where there is co-existence of both endocycling and meiotic nuclei within a shared cytoplasm, which could offer some challenges regarding control of the cell cycle. In order to prevent reentry into the mitotic cell cycle, endocycles require a kinase environment poor in CDK1 activity and cycling activity of G1/S CDK activity to be able to re-enter S-phase (Ullah et al., 2009). Meiosis, however, requires CDK1 activity in order to resume meiosis from prophase I arrest and low Cyclin E-CDK2 activity in order to prevent exit from the meiotic cycle (Hong et al., 2003; Von Stetina and Orr-Weaver, 2011). Because the timing of meiotic and endocycling entry occurs in parallel within the *O. dioica* coenocyst, there is a possibility that canonical CDK1 activity is not required in the ovary after this point, until the oocytes resume meiosis from prophase I arrest close to the spawning of oocytes. This idea is also supported by the fact that expression of odCDK1d, which now has been shown to be important for meiosis resumption (Paper II), peaks at day 6, just prior to spawning and meiosis resumption. odCDK1a, however, is expressed throughout development, and may conduct kinase functions which are not involved in meiosis resumption within the coenocyst. As we have observed functional specialization of odCDK1 paralogs in control of oogenesis, we may speculate that *O. dioica* has adapted its Cyclin-CDK complement in order to manage a heterogeneous cell cycle environment. Similar genomic Cyclin-CDK amplification is not observed in *Drosophila,* however, whose egg chambers contain 15 endocycling nurse cells and a single meiotic oocyte nucleus, interconnected to a common cytoplasm through ring canals. *Drosophila* egg chambers contains a polarized system for mRNA transport, which may facilitate separation of meiotic and endocycling regulators (Kumano, 2012). An obvious difference between the two species, however, is that *O. dioica* maintain thousands of pro-oocytes sharing

cytoplasm with an equal number of asynchronously endocycling nuclei, a situation where a more localized regulation of the cell cycle would make sense. Further the final selection of oocytes occurs after entering prophase I arrest in response to available nutrients, which could be linked to the amplified Cyclin D complement. In this study we revealed that acentriolar MTOC like organizing centers (OC) could play a vital role in separation of meiotic and endocycling regulators in a manner which has not been previously described. Aggregation of CDKs could both contribute to a localized control of meiotic events and act as a storage node for cell cycle kinases required in maturation of the oocytes. Canonical centriolar MTOCs play an important function in mitosis during separation of sister chromatids, and act as an activation center for Cyclin B-CDK1 before translocation into the nucleus (Jackman et al., 2003), thus creating localized accumulation of CDK1 activity close to the nuclei. In *Drosophila* ovaries, which also possess a meiotic nucleus and endocycling nurse nuclei connected to a shared cytoplasm via ring canals, centriolar MTOCs from the nurse nuclei migrate into the developing oocyte (Mahowald and Strassheim, 1970), which is not surprising in the sense that endocycling nuclei do no longer require MTOCs due to lack of mitotic division. It may also be possible that MTOC removal plays an additional function by moving a central mitotic organizing center away from nuclei and thus restricts CDK1 activity. A similar loss of MTOCs related to endocycling nurse nuclei are also observed in the *O. dioica* coenocyst where we observed a 1:1 ratio of OCs juxtaposed to meiotic nuclei (Paper II). Interestingly, non-selected nuclei also lost association with their MTOC following oocyte selection, suggesting that they no longer require a meiotic control point as they are committed to undergo an apoptotic fate.

Direct interaction between OCs and meiotic nuclei were supported by TEM (Paper II), where we observed physical contact and NPCs bridging the OC/meiotic nuclei, as well as in immunofluorescence where NPC clusters were observed in the OC/meiotic nuclei interface. Interestingly, NPC rich regions of the nuclear envelope have likewise been observed at sites attached to the actin scaffold (Ganot et al., 2007b), and could thus be related to OC/meiotic nuclei in a similar manner, although OCs were not observed to contain actin.

In order to ensure that only one pair of centrioles, provided by sperm, is present following fertilization, oocytes of most vertebrates eliminate centrioles prior to the first meiotic division (Mikeladze-Dvali et al., 2012; Szollosi et al., 1972). Acentriolar MTOCs are still able to focus microtubules in order to separate chromatin during meiosis I and meiosis II, though do not form a bipolar spindle similar to centrioles but rather create short microtubule assemblies which are thought to facilitate asymmetric cell division creating small polar bodies (Luksza et al., 2013). There are still many questions remaining regarding the *O. dioica* OCs, including microtubule organization activity and RNA localization. At some stages there was even observed an envelope of Lamin surrounding the OCs of P4 and P5 ovaries and additional small Lamin vesicles surrounding the OCs (Figure A3), suggesting that the OCs are involved in vesicle transport. Because of difficulties of live imaging we are still unable to fully understand the dynamics of possible vesicle transport towards the OCs, and thus this feature of the OC remains an intriguing mystery.

5.6 Meiotic regulation - Functions of odCDK1a and odCDK1d

We wanted to study specific functions of odCDK1 paralogs within the coenocyst and functional studies revealed that there could be a link between odCDK1 paralogs and control of the ring canal. Knockdown of odCDK1a by dsRNA injection consistently produced small infertile oocytes spawned from successfully injected females (Paper II). This result is consistent with similar effects obtained by knocking down Myosin phosphatase (DMYPT) and Flw, a PP1 homolog, in *Drosophila*, causing over-constriction of the ring canals preventing influx of cytoplasm, which in turn lead to restricted growth of the oocyte (Ong et al., 2010; Yamamoto et al., 2013). Although, CDK1 has not yet been directly linked to regulation of the ring canal, but as MYPT is a confirmed target of CDK1 (Yamashiro et al., 2008), we suggest that CDK1 depletion could cause a similar effect, resulting in the small oocytes produced in odCDK1a silenced *O. dioica* ovaries. Consistent with our assumption is the observed effect of localized CDK1 inhibition in *Drosophila* oocytes, which causes premature constriction of the contractile ring, which is also regulated by MYPT (Menant and Karess, 2012). The composition of the ring canal and the actomyosin contractile ring is similar in that

ring canals are formed by incomplete cytokinesis (Haglund et al., 2011), and regulatory mechanisms between the two may share similarities. Measurements of ring canals in adult ovaries however, following odCDK1a knockdown and CDK1 inhibitor treatment, have so far not produced conclusive results to support this hypothesis (Figure A4). Therefore it's possible that odCDK1a controls cytoplasmic influx into growing oocytes through another, yet unidentified, mechanism. This mechanism appears not to involve microtubule transport or the MAPK pathway, as treatment with
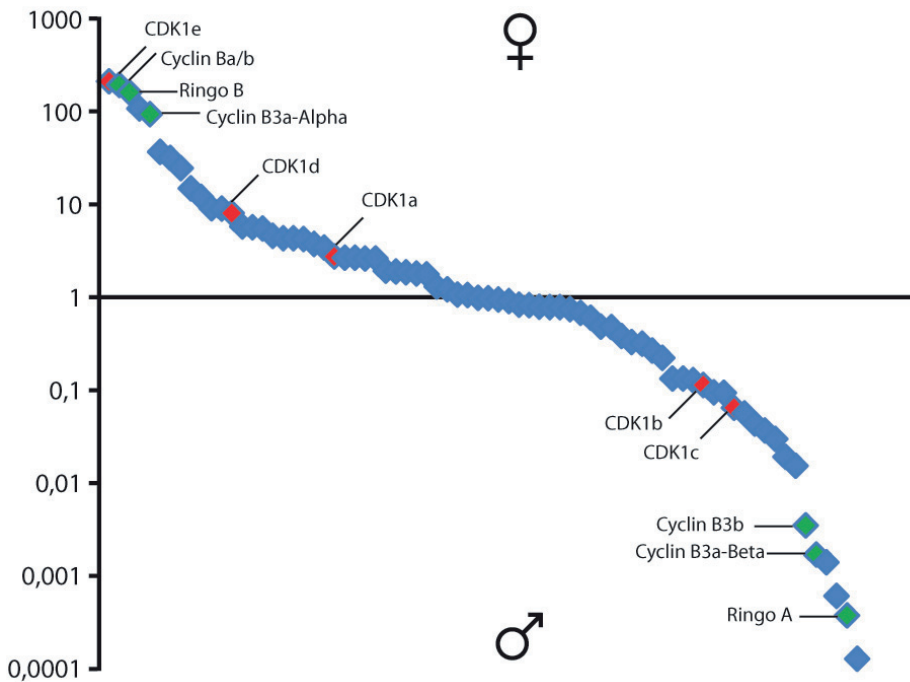
Figure 14. Relative expression of cell cycle regulators between male and female gonads.
Relative expression ratio of transcripts between day 6 male and female gonads plotted on a log scale. Values above 1 represent transcripts enriched in ovaries whereas values below 1 represent testes enriched transcripts. Figure modified from (Campsteijn et al., 2012).

colchicine or U0126 did not seem to restrict oocyte growth (Ganot et al., 2007b; Ganot et al., 2008). Consistent with canonical CDK1 function, our results suggests that odCDK1a is essential in maturation of oocytes (Paper II). In addition we found that

another odCDK1 paralog, odCDK1d, was required for successful maturation. The effect, however, appeared more specific as spawned oocytes seemed perfectly normal, but failed to resume prophase I arrest following spawning. The inability of the two paralogs to substitute the function of the other implies that they are different regarding substrate specificity, which is provided by their Cyclin partners. Interestingly, the idea of different preference of Cyclin partners is supported by the different PSTAIRE motifs observed amongst odCDK1 paralogs (Campsteijn et al., 2012). Amongst the
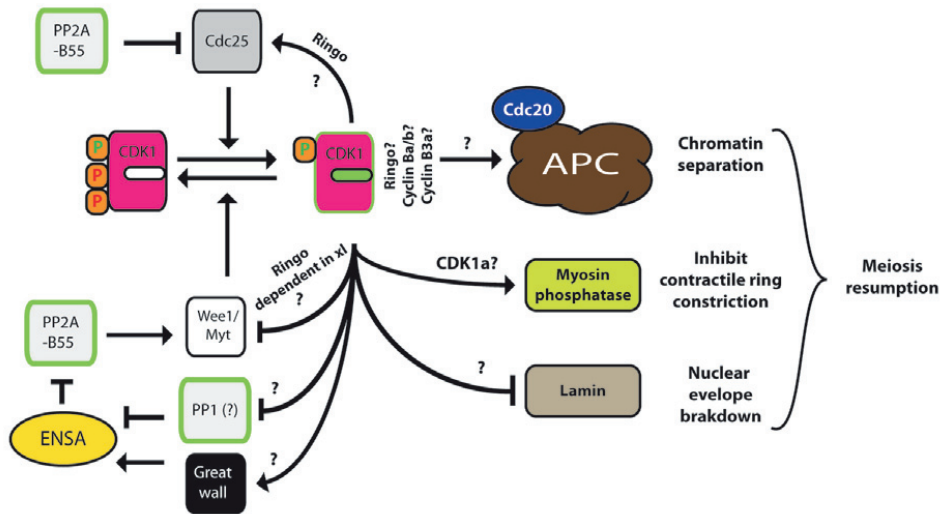
Figure 15. CDK1 target substrates required for meiosis resumption.
CDK1 creates a positive feedback loop through activation of CDC25, inhibition of Wee1/Myt1 and activation of Greatwall. CDK1 also trigger meiosis progression by initiating nuclear envelope breakdown and chromatin separation. CDK1 also inhibits constriction of the contractile ring until CDK1 is deactivated following APC activation. OdCDK1a depletion in *O. dioica* ovaries causes spawning of small oocytes, suggesting odCDK1a might control growth by activating myosin phosphatase (though see Figure A4). Specific substrates and cyclin partners of odCDK1 paralogs are thus far unknown.

five B-type Cyclins discovered in *O. dioica*, all are expressed during gonad development, of which odCyclin Ba/b and odCyclin B3a-alpha is expressed specifically in females (Campsteijn et al., 2012). Further, it has been shown that Ringo proteins are important CDK1 activators during meiosis resumption (Chauhan et al.,

2012), and we have found both a male and a female specific Ringo (Ringo A and Ringo B respectively) in *O. dioica* (Figure 14). We therefore argue that odCyclin Ba/b, odCyclin B3a-alpha and odRingo B are likely candidates to trigger meiosis resumption in complex with odCDK1a and/or odCDK1d. The different substrates that need to be phosphorylated in order to resume meiosis from prophase I arrest include; inhibitory phosphorylation of Wee1/Myt1 and activation of CDC25, NEB through phosphorylation of Lamins and finally activation of APC/C through CDC20 phosphorylation (Figure 15). A similar distribution of CDK1 function may apply in the testes, where odCDK1b, odCDK1c and possibly odCDK1a interact with the male specific CDK activators including odCyclin B3b, odCyclin B3a-beta and odRingo A. We have, however, not discovered a function of odCDK1e, which did not display any phenotypes upon knockdown; suggesting that odCDK1e shares a redundant function with odCDK1a and odCDK1d, or that odCDK1e is in the process of evolving into a pseudogene. Another possibility is that odCDK1e performs a subtle function which we are unable to observe, or that RNAi is insufficient to ablate odCDK1e function due to high expression.

5.7 *O. dioica* and evolution of the cell cycle.

Regulation of the "essential" cell cycle has been well conserved from yeast to multicellular organisms, although specialized Cyclins and CDKs appear to have evolved in order to maintain the cell cycle of specialized cell types in multicellular eukaryotes (Malumbres and Barbacid, 2009). CDK1 has retained a crucial function in regulating M-phase entry and is thus essential in mitotic proliferation, whereas additional CDKs have evolved to maintain progression through interphase, especially in specialized cells. *O. dioica* represents the only studied example where additional CDK1 paralogs have sub-functionalized control of M-phase. We have already demonstrated that odCDK1d is essential in meiosis resumption of oocytes, although we have not thus far clearly determined the mechanistic function of odCDK1a during meiosis, other than it is required for successful production of fertile oocytes of the correct size. However, the limited expression of odCDK1d suggests that its sole function is to control meiosis resumption, and possibly early development. Because

mitotic proliferation occurs throughout the life cycle in the intestinal tract and *O. dioica* testes, as well as in the ovary until day 3, we may safely assume that mitosis is controlled by one or several of the three odCDK1 paralogs being expressed throughout development: odCDK1a, odCDK1b and odCDK1c.

Vertebrates have also amplified their Cyclin complement, which similar to interphase CDKs display redundant control of the essential cell cycle. As with CDKs, knockout of individual Cyclins mostly allow proper progression through early development in mice, but cause various anomalies within tissues composed of specialized cell types. Vertebrates are composed of a large number of specialized cells (about 400 different cell types in humans (Vickaryous and Hall, 2006)) and the amplified Cyclin complement observed in vertebrates is thus likely to facilitate cell cycle control of a greater variety of cell types. As duplicated genes rapidly diverge in expression (Gu et al., 2002), duplicated Cyclins and CDKs can be incorporated into specialized developmental expression networks where they are free to evolve novel functions within different tissues. It is therefore possible that organisms of higher cellular complexity, and greater complexity of expression networks, might retain a greater number of duplicated genes. *O. dioica,* however, has a less complex composition of cell types than vertebrates, but still possesses a more complex complement of D-type Cyclins and CDK1 paralogs. Complex tissue-specific control of endocycling is however obvious in the oikoplastic epithelium, which displays a perfect bilateral symmetry of differently shaped cells with a great variety of cell sizes and ploidies (Spada et al., 2001). Different Cyclin D paralogs may therefore have been incorporated into different tissue pattern specific expression modules where they control cell size through regulation of G-phase lengths (Ganot and Thompson, 2002). odCDK1d may be part of an ovary specific expression network, where odCDK1d has evolved to conduct a meiosis specific function, whereas odCDK1e, which is apparently non-essential in oogenesis. In addition we have yet not thoroughly explored odCDK1 paralog function during mitosis. The male enriched odCDK1b and/or odCDK1c are possibly involved in spermatogenesis as suggested by male enriched expression. Another interesting view is that new genes have been proposed to originate from newly duplicated genes highly expressed in the testes (Kaessmann, 2010). Due to

high selective pressure on competing sperm and the open chromatin state of meiotic chromatin, which allows promiscuous expression of newly duplicated genes without established regulatory elements, testis is the most rapidly evolving animal tissue (Assis and Bachtrog, 2013). Over time testes expressed genes may evolve towards specialized functions within other tissues, a hypothesis referred to as "Out of testes" (Assis and Bachtrog, 2013).

5.8 Endocycling and cancer.

Basic research in simple model organisms including yeast, sea urchin and *Drosophila* has provided invaluable knowledge about the cell cycle, which has been implemented in medical research. Currently, most of the knowledge about cell division revolves around the mitotic cell cycle, but there is yet much to be learned, for instance how alterations of the cell cycle may lead to polyploidy. Mammalian livers are known to become increasingly polyploid with age, triggered by telomere damage and the DNA damage response (Lazzerini Denchi et al., 2006), where endoreplication may offer a less disruptive solution to DNA damage, opposed to apoptosis, without renouncing tissue repair and metabolic activity (Fox and Duronio, 2013). Interestingly developmentally programmed endoreplicating cells are also naturally resistant to apoptosis (Mehrotra et al., 2008) in addition to dampened effect of genomic instability due to the gene copy redundancy present in polyploid cells (Comai, 2005). Although endoreplication offers higher tolerance to genomic stress, with low risk of oncogenic transformation in liver (Diril et al., 2012; McClendon et al., 2011), polyploidization is also associated with increased genomic instability and elevated risk of tumourigenesis in other tissue cells, especially when they revert back to the mitotic cell cycle (Storchova and Pellman, 2004; Fox and Duronio, 2013). An estimation of the polyploid fraction of glioblastoma lines revealed that about 5% were polyploid (Donovan et al., 2014). Error prone cell division of tetra or polyploid cancer precursor cells is a potent source of aneuploidy, found in 90% percent of solid tumours, and is responsible for rapid evolution of cell diversity within cancers (Coward and Harding, 2014; Weaver and Cleveland, 2008). The allelic variation that arises with further polyploidization and aneuploidization is a major contributor to the genetic

heterogenicity in tumours, a major challenge regarding anti-cancer treatment (Coward and Harding, 2014).

Unscheduled re-replication of DNA occurs occasionally in proliferating cells when they fail to divide, but is allowed another entry into S-phase (Storchova and Pellman, 2004). As this form of ploidy increase is not controlled by an "endoreplication-machinery", it is rather referred to as an abortive cell cycle. Such abortive cell cycles will more often than not cause cell cycle arrest and apoptosis, though "slippage" through the cell cycle checkpoint may occur (Minn et al., 1996). Abortive cell cycles are a common initiator of cancer cell transformation, as it may often cause uneven chromosome segregation during cell division, rendering the cell chromosomally unstable and prone to chromosome loss favoring tumourigenesis (Storchova and Pellman, 2004). Induced endoreplication is also known to occur in response to genotoxic stress, including telomere shortening, and regeneration, where tissue repair is facilitated by increased cell mass in preference to proliferation of damaged cells (Fox and Duronio, 2013). Although endocycling cells are generally considered to be terminally differentiated, they are also known to revert back to the mitotic cell cycle (Fox et al., 2010). This would normally cause problems during chromatid separation due to a "polyploid" complement of centrosomes. Multiple centrosomes will either cluster into two spindle poles, but do not create proportionally bigger spindles, or may nucleate multiple spindle poles, both inevitably causing asymmetric distribution of chromatids producing aneuploid daughter cells (Holland and Cleveland, 2009). Such aneuploid cells are thus susceptible to tumourigenesis due to chromosomal instability (Fox and Duronio, 2013). Another prevailing feature of cancer cells involves uncontrolled re-entry into the cell cycle, allowing cell cycle entry independently of growth signals. Mutations in the Cyclin D phospho degron motif may render Cyclin D insensitive to ubiquitinylation and proteasomal degradation (Barbash et al., 2009). Such stable Cyclin D mutants can cause over activation of CDK4/6 and thus also E2F1-3 (Malumbres and Barbacid, 2001), as is found in several human cancers (Benzeno et al., 2006; Moreno-Bueno et al., 2003). Interestingly, *O. dioica* express an odCyclin Db spliced variant which lacks a phospho degron motif, implying resistance to proteasomal degradation, similar to a Cyclin D variant observed in human

tumors (Lu et al., 2003). On the other hand we have so far been unable to identify genes coding for p53, the guardian of the genome. It is possible that the short life cycle of *O. dioica* and the polyploid cells resistance to genomic instability relives the pressure to maintain intricate safety mechanisms avoiding tumor development. *O. dioica* as a model organism therefore provides an excellent opportunity to increase our knowledge on polyploidy, as it's a dominating aspect of the *O. dioica* life cycle.

5.9 Future perspectives

We have now established fundamental knowledge of the *O. dioica* Cyclin and CDK complements, providing a solid foundation for further research on endocycling, mitosis and meiosis in *O. dioica*. However, some improvements of *O. dioica* as a model organism still remain, such as establishing a protocol for stable transgenics, which would open up a wide range of experiments to be utilized in *O. dioica* cell cycle research. Several transgenic approaches have so far been tried in *O. dioica* including transposable elements and transcription activator-like effector nuclease (TALEN) approach, though none have so far proven effective. Another obstacle of *O. dioica*, which could be solved by transgenic lines, is technical limitations of live imaging, which could shed light on Cyclin-CDK1 dynamics in regulation of mitosis as well as OC dynamics within the coenocyst.

   O. dioica provides an excellent model to study oogenesis, as the adult female animals are large and easily accessible. This allows manipulation of thousands of pro-oocytes sharing the same cytoplasm, and may easily become an attractive model to study oogenesis. Female fertility depends on successful progression through meiosis I and meiosis II, a process which becomes more error prone with increasing maternal age (Jones and Lane, 2013), and increased knowledge about oogenesis and meiosis may thus be applied to medical research concerning infertility in women. Previous reports have demonstrated that CDK1 is the only essential CDK maintaining meiosis (Adhikari et al., 2012), but we have now demonstrated a situation where meiosis depends on two CDK1 paralogs in *O. dioica* (Paper II). Because CDK1 phosphorylates a wide range of substrates, this unique subdivision of function serves an excellent opportunity to narrow down more specific functions of CDK1 during meiosis.

Having established some important functions of the odCDK1 paralogs during meiosis, our next goal is to further explore their function in regulation of the mitotic cell cycle through knockdown studies in addition to assigning the functions for each odCDK1 paralog. Knockdown experiments can be expanded by targeting Cyclins, focusing on the B-type Cyclins, both during mitotic development and oogenesis in order to couple CDK1 function with Cyclin interaction. In support of knockdown studies we need evidence of Cyclin-CDK interactions and therefore our group is currently working with co-immunoprecipitation experiments. In addition to exploring interaction between B-type Cyclins and odCDK1 paralogs, it would be interesting to see whether there is an actual interaction between Cyclin D and odCDK1. This interaction could reveal novel communication between growth signaling and CDK1 activity, especially if we could link Cyclin D with reproductive output and nutritional response. Further it would be interesting to examine spatio-temporal localization of Cyclins, but as we are at present limited by difficulties obtaining Cyclin antibodies, we would aim to utilize injection of eGFP-Cyclin cmRNA to explore localization within mitotic cells and the coenocyst. Additionally, in order to assess whether odCDK1 OC localization within the coenocyst is mediated through Cyclin binding; we have constructed an eGFP-odCDK1a construct containing mutations within the PSTAIRE motif. By identifying the mitotic cell cycle components in *O. dioica* embryogenesis, we may better comprehend the programmed transition into endocycling.

O. dioica possesses most of the beneficial traits that are desirable in a model organism and this work pioneers cell cycle research in this urochordate. We have annotated and revealed an expanded Cyclin-CDK complement where a unique expansion of CDK1 paralogs raises a number of interesting questions, whose answers may illuminate processes of cell cycle evolution. This work may have answered some questions already, as we have demonstrated further sub-functionalization of CDK1 within the metazoan lineage. We have also developed a powerful tool for studying oogenesis, the coenocyst, which allow manipulation and study of thousands of pro-oocytes as well as endocycling nurse nuclei. Ultimately, our goal in studying *O. dioica* is to provide valuable knowledge about the cell cycle in a chordate model organism. This knowledge could be applied to obtain a better understanding of the mechanisms

behind human diseases; including reproductive dysfunction as well as cancer development, and finally, to shed further light on the evolutionary potentials of the core cell cycle regulators.

6. Appendix

Appendix 1:

Mitiple regulation by multiple odCDK1 paralogs in *Oikopleura dioica*

Jan Inge Øvrebø, Marine Gueydan, Eric M. Thompson

A1-1. Introduction

From yeast to human, the crucial function of CDK1 controlling mitotic M-phase has been highly conserved through evolution (Malumbres and Barbacid, 2009). Yeast possesses a single cell cycle CDK, the CDK1 homolog Cdc2/Cdc28, which singlehandedly maintains progression through the cell cycle. Metazoans on the other hand have evolved several cell cycle CDKs. Phylogenetic analysis indicates that the CDK4/6 subfamily emerged with the appearance of eumetazoans, while CDK2 diverged from CDK1 by gene duplication in metazoans (Cao et al., 2014), although CDK1 has retained crucial function in controlling M-phase in all metazoans studied so far. *O. dioica* represents the only known organism where gene duplication of CDK1 has reoccurred in the metazoan lineage, representing five odCDK1 paralogs (Campsteijn et al., 2012), raising the possibility of redundant control of M-phase. Due to variations within the highly conserved cyclin interaction motif, the PSTAIRE motif, there is also reason to expect functional diversification amongst the odCDK1 paralogs. In order to explore odCDK1 paralog functions in the mitotic cell cycle, we wanted to observe spatio-temporal localization of odCDK1 paralogs within mitotically proliferating cells of *O. dioica* tadpoles, as well as explore odCDK1 paralogs functions by RNA knockdown.

A1-2. Materials and Methods

A1-2.1 Animal culture and collection.

O. dioica were maintained in culture at 15 °C (Bouquet et al., 2009). D4-D6 animals were placed in filtered sea water, chased out of their house and anesthetized in cold

ethyl 3-aminobenzoate methanesulfonate salt (MS-222, 0.125 mg/ml; Sigma), before collection.

A1-2.2 *In vitro* fertilization (IVF)

Sperm solution was prepared by transferring 3-4 mature males to a small petri dish containing artificial seawater. Following spawning of at least one male, sperm solution was placed on ice and its quality was assessed on Nikon Eclipse E400 microscope. Mature females were transferred to artificial sea water in 6-well plates coated with 0,1 % gelatin. Oocytes were fertilized by careful addition of 100 µl sperm solution. Development was documented using a Spot RT-KE camera mounted on a Nikon Eclipse TE2000-S inverted microscope.

A1-2.3 Micro injection

Injection solution was prepared by mixing 400 ng/µl capped mRNA (cmRNA) and 100 ng/µl Alexa Fluor® 568 fluorescent dye (Molecular probes), adding 400 ng/µl double stranded RNA (dsRNA) for RNAi silencing, in RNase free water. Anesthetized D5 animals and 1-cell embryos were transferred to small petri dish coated with 3 % agarose in filtered seawater. Gonads and 1-cell embryos were injected using a Nikon Eclipse TE2000-S inverted microscope equipped with Narishige micromanipulators and transjector 5246 micro injector (Eppendorf). Animals were positioned with holding pipettes crafted from borosilicate capillaries with an outer diameter of 1 mm and inner diameter of 0,5 mm (Sutter instruments). Injection needles were fashioned by pulling Quartz capillaries (Sutter instruments) with outer diameter of 1 mm and inner diameter of 0,7 mm on a Flaming/Brown P87 Micropipette puller (Sutter instruments). Injected D5 animals were transferred to watch glasses containing filtered seawater for recovery until transferred to 3 L beaker and cultured according to their stage (Bouquet et al., 2009). Animals screened for positive eGFP expression were raised until spawning for IVF and immunofluorescence analysis.

A1-2.4 CDK1-eGFP constructs

Utilizing cDNA, odCDK1s (paralog a, b, c, d and e) protein coding sequences and 3'UTRs were amplified separately by polymerase chain reaction (PCR), using primers represented in Table A1-1A. PCR products were cloned into TOPO 2.1 vectors (Life technologies) which were used to transform One Shot® TOP10 Chemically Competent *E. coli* bacteria (Life Technologies) by heat shock, followed by growth in miniprep culture. Miniprep plasmids were purified using QIAprep spin mini prep kit (QIAGEN). CDK1 eGFP fusion constructs for cmRNA expression were built using a T7 promoter driven eGFP construct with ampicillin resistance, inserting CDK1a/b/c/d/e using restriction sites EcoRV (NotI for CDK1b) and BamHI. 3'UTRs were inserted using restriction sites BsrGI and XmaI. Finished constructs were amplified and purified by maxi prep (QIAGEN). Final vector maps are represented in supplementary figure 1.

A1-2.5 Capped mRNA synthesis

Oikopleura CDK1-eGFP fusion constructs including endogenous 3'UTRs (supplementary figure 1) were linearized using XmaI (odCDK1a, b, c and d) or AfeI (odCDK1e), and purified by phenol-chloroform extraction followed by ethanol precipitation, and were used as template for cmRNA synthesis. cmRNA was synthesized using mMessage mMachine (Ambion) followed by addition of 3' polyadenine tails with the poly(A) tailing kit (Ambion) according to supplied protocol, and purified by Lithium chloride precipitation.

A1-2.6 dsRNA synthesis

DNA templates for dsRNA synthesis were prepared by PCR using gene specific forward and reverse primers with T7 overhang (Table A1-1B), and purified by phenol-chloroform extraction. Sense and antisense RNA were synthesized in a single reaction following recommended protocol for dsRNA synthesis using T7 RiboMAX™ Express RNAi system (Promega).

A1-2.7 Immunofluorescence sample processing

Samples were processed for immunofluorescence following procedure described in (Campsteijn et al., 2012). Images were processed in Image J.

A1-2.8 Antibodies

As primary antibodies mitotic phospho protein MPM-2 (Millipore), histone H3-pS28 (Abcam) and GFP (ams Biotechnology) were used at 1:100 dilution. Secondary antibodies anti-rabbit Alexa488 and anti-rat Alexa568 (Molecular probes) and anti-mouse Alexa568 (Life Technologies) were used at 1:500 final dilutions.

A1-2.9 Quantitative reverse transcription-PCR

RNA was isolated from 20-30 tadpoles following recommended protocol using RNAqueous®-Micro Kit (Ambion). Collected tadpoles were first washed with 100 µl PBS, then PBS was replaced by 100 µl of lysis solution and the samples were snap-frozen in liquid nitrogen and stored at -80 °C until further processing. Total RNA (200 ng) was reverse transcribed using M-MLV reverse transcriptase (Life Technologies) following recommended protocol using oligo-dT primers (Promega). Quantitative reverse transcription–polymerase chain reactions (qRT-PCR) of 20 µl were assembled using cDNA equivalent to 2 ng total RNA, 500 nM gene specific forward and reverse primers (Campsteijn et al., 2012) and 10 µl iQ™ SYBR® Green Supermix (Biorad). qRT-PCR reactions were run on a CFX-96 (Bio-Rad). Relative expressions were normalized to RPL23 and EF-1β mRNA levels in all qRT-PCRs.

A1-3. Results

A1-3.1 CDK1d and CDK1e do not display canonical CDK1 localization during mitosis.

We wanted to explore spatio-temporal localizations of the five odCDK1 paralogs identified in *O. dioica*. odCDK1a represents the closest homolog to hsCDK1 (Campsteijn et al., 2012) possesses the most conserved PSTAIRE motif (PSTSIRE), and is expressed throughout development and in about equal proportions between males and females. odCDK1b and odCDK1c are also expressed throughout

development but are preferentially expressed in males compared to females (Campsteijn et al., 2012). In contrast, odCDK1d and odCDK1e expression is limited to oocytes and early embryonic development, dropping after 1h post fertilization before expression is increased during gametogenesis from day 5 onwards. odCDK1d and odCDK1e are also preferentially expressed in females, indicating a function related to oogenesis. In order to observe whether the odCDK1 paralogs are involved in mitosis we injected odCDK1 paralog-eGFP cmRNA in 1-cell embryos. It has been shown in various systems that CDK1 targets several substrates within the nuclei during prophase and thus relies on nuclear translocation. We would therefore expect canonical mitotic odCDK1s to translocate to the nucleus during the G2-M transition (Gavet and Pines, 2010). Immunofluorescence analysis revealed nuclear accumulation of odCDK1a, odCDK1b and odCDK1c during prophase, before dispersing before anaphase, consistent with canonical CDK1 behavior (figures A1-1A). odCDK1d and odCDK1e however appeared cytoplasmic throughout cell cycle stages, with diffusion surrounding chromatin following nuclear envelope breakdown (figure A1-1A). Taken together, these data suggest that odCDK1a, odCDK1b and odCDK1c dynamics reflect canonical CDK1 and argue for a similar role in *Oikopleura*, whereas odCDK1d and odCDK1e localization patterns argue against a canonical role linked to chromatin translocation for these kinases during mitosis.

A1-3.2 odCDK1a is possibly indispensable for proper mitotic development.

Spatio-temporal localization indicates that odCDK1a, odCDK1b and odCDK1c are actively involved in mitotic entry. We now wanted to test whether they are all required for mitotic proliferation or if their functions are redundant, by performing dsRNA mediated RNA silencing. Because we could expect stockpiling of odCDK1 paralogs in spawned embryos, suggested by expressed transcripts detected by qRT-PCR in spawned oocytes (Campsteijn et al., 2012), and in order to limit accumulation of expressed protein in spawned oocytes, we chose to inject day 5 females. Females successfully injected with dsRNA targeting odCDK1b and odCDK1c, either individually or together, spawned oocytes which developed normally following IVF, suggesting either non-essential function or non-detectable defects in mitotic control

(data not shown). Females successfully injected with dsRNA targeting odCDK1a on the other hand, displayed failure to develop normally (Figure A1-1B), which indicate an essential mitotic function for odCDK1a. Because we were unable to verify successful knockdown by qRT-PCR, we consider these results as very preliminary.

A1-4. Discussion

Among the five odCDK1 paralogs, odCDK1a, odCDK1b and odCDK1c display canonical CDK1 spatio-temporal localization during mitotic cell proliferation. In addition our results give hints towards a central role of odCDK1a maintaining proper mitotic development. odCDK1a is also the closest homolog to CDK1 of other species and possesses a PSTSIRE motif with closer resemblance to the PSTAIRE motif than that of the other four odCDK1 paralogs. It is therefore possible that although *O. dioica* has an expanded complement of CDK1, a single CDK1 is apparently still responsible for M-phase progression, analogous to the initial duplication of CDK1 giving rise to CDK2 in the metazoan lineage. We know that odCDK1a and odCDK1d possess functions important for successful oocyte production (Paper II), but we still know little about the function of odCDK1b, odCDK1c and odCDK1e. We acknowledge the requirement for experimental adjustments for knockdown experiments during early development. Because there may be significant changes in expression during the course of development, a more accurate timing of collection may be crucial in order to obtain reliable qRT-PCR data. It may also be difficult to compare expression of necrotic/apoptotic embryos with that of normal tadpoles collected at the same time of development, and collection of unfertilized oocytes for qRT-PCR prior to fertilization of remaining oocytes may prove a better approach for further studies. Because successful knockdown in embryos has still not been confirmed by qRT-PCR, we cannot exclude that odCDK1b and odCDK1c may still be important mitotic regulators. Since odCDK1b and odCDK1c are also transcriptionally enriched in males versus females, another possibility is that they maintain syncytial mitotic divisions in the male germline, which starts post metamorphosis lasting until meiotic entry at maturation at day 6.
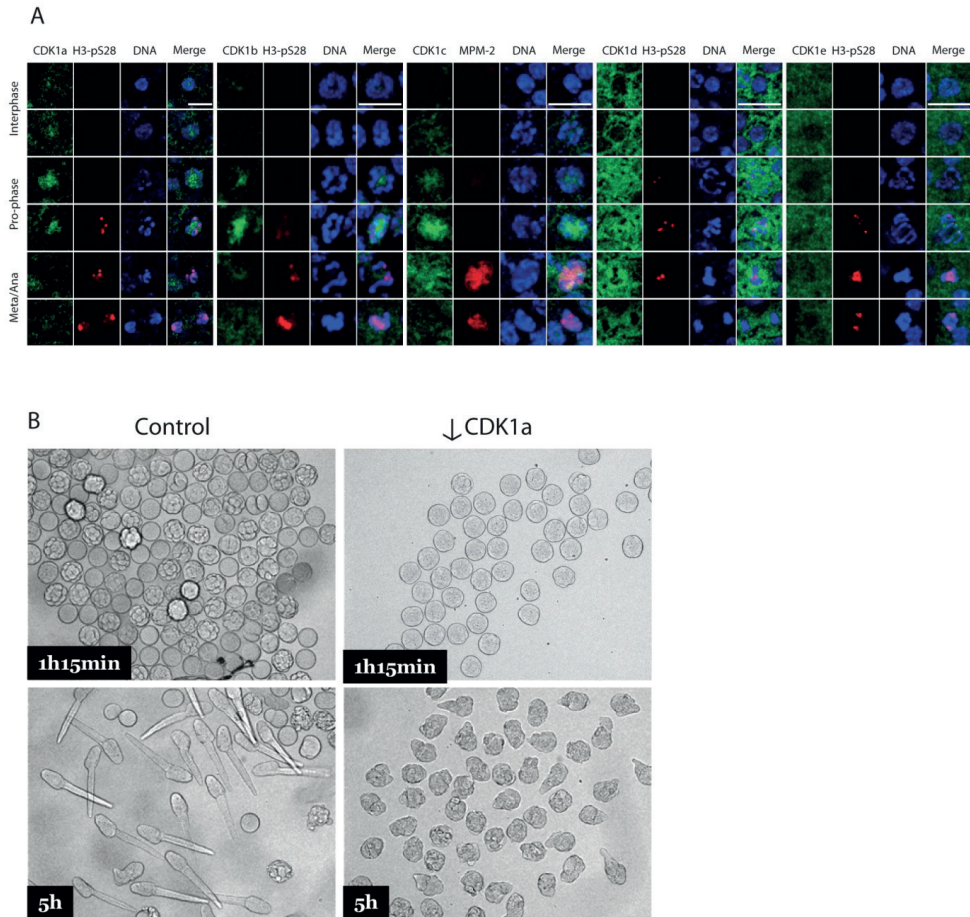
Figure A1-1. Regulation of the mitotic cell cycle by odCDK1 paralogs. (A) Localization of odCDK1 paralogs in mitotic development. Immunofluorescence using anti-eGFP antibody targeting odCDK1-eGFP fusion constructs reveal cycling nuclear localization of odCDK1a, odCDK1b and CDK1c, in mitotic cells. Background reduced to illustrate nuclear localization only. odCDK1d and odCDK1e appear cytoplasmic, but do not translocate during any of these mitotic cell cycle stages. Cytoplasmic staining illustrates the absence of nuclear translocation. (B) Injection of dsRNA targeting odCDK1a caused abnormal development of *Oikopleura dioica* embryos. Day 5 animals were injected with 400 ng/µl dsRNA targeting odCDK1a and raised until spawning and were then fertilized. Delayed development was already observed 1 h post fertilization and displayed fatal developmental defects 5 h post fertilization. Data in panel B represents preliminary results produced by Marine Gueydan.

Table A1-1

A: Vector construction primers

Primer name	Target	Sequence (5'-3')
JIP34 (forward)	CDK1a - ORF	GATATCTAATACGACTCACTATAGGGAGAGCCACCATGACGTCCAATATCGCTAATTAC
JIP35 (reverse)	CDK1a- ORF	CCATGGATCCGCTGCCGTGAATATTCGTGACATTCTTCATTATTTTCG
JIP36 (forward)	CDK1a – 3'UTR	TGTACAAGTAAGATTTTTGAAATTTAACTATTACAAATTGAAATTCAAACAAGTTC
JIP37 (reverse)	CDK1a – 3'UTR	CCCGGGAAAAATAAAATTGTCTTTGGCAGGATGACCC
JIP42 (forward)	CDK1b - ORF	TGGCCATAATACGACTCACTATAGGGAGAGCCACCATGGACGAAGAAGCCAAAATAGC
JIP43 (reverse)	CDK1b - ORF	TCATGACGGATCCGCTGCCCATGTCCATTTCGTTGAGAGAGG
JIP44 (forward)	CDK1ab – 3'UTR	TGTACAAGTAACTCGCCGTTCACGCAACAC
JIP45 (reverse)	CDK1b – 3'UTR	CCCGGGTTTTGAATGTATGGGGCAGGTATGTG
JIP50 (forward)	CDK1c - ORF	GATATCTAATACGACTCACTATAGGGAGAGCCACCATGAGAGAAAACGTAATATTGATCGTCCACC
JIP51 (reverse)	CDK1c- ORF	CCATGGATCCGCTGCCTGAGGCTATGTTTTCTTTCCACTC
JIP52 (forward)	CDK1c – 3'UTR	TGTACAAGTAACTGCCCTTTTACAGAATAACCCAAATACTG
JIP53 (reverse)	CDK1c – 3'UTR	CCCGGGCAATGAAGATATATTTTATTGTTTTTCTGCTGTCGGTAG
JIP38 (forward)	CDK1d- ORF	GATATCTAATACGACTCACTATAGGGAGAGCCACCATGATCAAAGCAAAGTCAGGAACTC
JIP54 (reverse)	CDK1d- ORF	CCATGGATCCGCTGCCGTTGACAAGCTGAAGACCTCTAACAG
JIP55 (forward)	CDK1d – 3'UTR	TGTACAAGTAAAAAAATTAAGGCCATCACTGTGCATTCTC
JIP41 (reverse)	CDK1d – 3'UTR	CCCGGGAAATGATGGTTTTAGAAAACCGTCGCG
JIP112 (forward)	CDK1e- ORF	GATATCTAATACGACTCACTATAGGGCTGCAGCCACCATGCGGCCTTTTAATCCGAG
JIP47 (reverse)	CDK1e- ORF	CCATGGATCCGCTGCCGTTGACGAGTTGTAGACCTCTG
JIP48 (forward)	CDK1e – 3'UTR	TGTACAAGTAAAAAATCACGATCACCATTCGACAC
JIP49 (reverse)	CDK1e – 3'UTR	CCCGGGATATTAATTTTGTGTTTATTTATGCCTAATATGTGATGTCAGTGATAGAAG

B: dsRNA target primers

Primer name	Target	Sequence (5'-3')
JIP120 (Forward)	CDK1a	TAATACGACTCACTATAGGGATGACGAAGGCGTACCAAGCACTA
JIP121 (Reverse)	CDK1a	TAATACGACTCACTATAGGGAGTCCTTGAAATTCTCGACGCCCT
JIP124 (Forward)	CDK1b	TAATACGACTCACTATAGGGTTTCGCCTTGGAGACGAGGAAGAA
JIP125 (Reverse)	CDK1b	TAATACGACTCACTATAGGGTGTCCGAGAAGAATTTCGGGAGCA
JIP128 (Forward)	CDK1c	TAATACGACTCACTATAGGGTTCTCGGAACACCGACCGAAGAAA
JIP129 (Reverse)	CDK1c	TAATACGACTCACTATAGGGAGAGGAAGATTCAAGATCGCCGCA
JIP132 (forward)	CDK1d	TAATACGACTCACTATAGGGAAGATATTCCGCATCCTCGGCACT
JIP133 (reverse)	CDK1d	TAATACGACTCACTATAGGGACAGCGTTTGCTTTCGAGTTCTCC
JIP136 (forward)	CDK1e	TAATACGACTCACTATAGGGTACGCATGTCCGGTTGATTGTTGG
JIP137 (reverse)	CDK1e	TAATACGACTCACTATAGGGAGCAGGGACAGCTGGATATTCCTT

Appendix 2:

No observed effect on ring canal (RC) constriction upon odCDK1a knockdown

Jan Inge Øvrebø, Eric M. Thompson

A2-1. Introduction

During mitosis assembly and constriction of the contractile ring depends on decreased cellular CDK1 activity through an unknown mechanism (Morgan, 1997). This requirement has been nicely demonstrated by localized injection of CDK1 inhibitors into unfertilized *Drosophila* oocytes, which stimulates assembly and constriction of the contractile ring at the site of injection (Menant and Karess, 2012). The RC within *Drosophila* ovarioles are formed by incomplete cytokinesis by contractile rings (Ong and Tan, 2010). Limited growth of *Drosophila* oocytes has been demonstrated by over-constriction of RCs by inhibiting Myosin phosphatase (MYPT) (Ong et al., 2010), which requires protein phosphatase 1 (PP1β) as a catalyzing subunit (Yamamoto et al., 2013). Reduced size of released oocytes was also observed in *O. dioica* upon odCDK1a knockdown (Paper II) and we speculated that odCDK1a could be involved in over-constriction of the *O. dioica* RCs through a similar mechanism. We therefore investigated whether reduced size upon odCDK1a knockdown could be explained by over constriction of RCs. In order to relate odCDK1a with regulation of RC constriction, we performed knockdown of odCDK1a by injecting dsRNA, followed by immunofluorescence and measurements of RC diameters.

A2-2. Materials and Methods

A2-2.1 Animal culture and collection

O. dioica were maintained in culture at 15 °C (Bouquet et al., 2009). D4-D6 animals were placed in filtered sea water, chased out of their house and anesthetized in cold ethyl 3-aminobenzoate methanesulfonate salt (MS-222, 0.125 mg/ml; Sigma), before collection.

A2-2.3 odCDK1a knockdown by dsRNA injection

Microinjection and preparation of injection solution, dsRNA and cmRNA were performed as in (Paper II).

A2-2.7 Immunofluorescence sample processing

Samples were processed for immunofluorescence following procedure described in (Paper II). For actin detection 0,6 units/ml Alexa488 conjugated phalloidin (Molecular Probes) were added to the secondary antibody incubation step. Images were processed in Image J.

A2-3. Results

A2-3.1 RCs did not constrict upon odCDK1a dsRNA injection.

We performed odCDK1a dsRNA injection in day 4 gonads followed by screening for positive expression of H2B-eGFP as a positive injection control. Immature animals were collected and processed for immunofluorescence at day 6 where they were stained for actin in order to measure RCs. We were unable to observe significant reduction of RC diameters in females expressing H2B-eGFP, compared to RCs of control animals, measured to about 3µm in diameter.

A2-4. Discussion

Our results indicate that RCs do not constrict upon odCDK1a knockdown and can thus not explain why reduced odCDK1a causes oocyte size reduction. Technical limitations to our approach include difficulties in confirming successful knockdown by qRT-PCR

as the same animal cannot be used for immunofluorescence. However, we have previously experienced positive H2B-eGFP expression as reliable indicator of successful knockdown. It may yet be possible that oocyte growth is facilitated by expansion of RC diameters at a later developmental time point and that odCDK1a knockdown limits this mechanism, although measured diameters of wild type coenocysts were quite similar in different developmental time points. If the opening and constriction of the RCs within the coenocyst is a more dynamic process in live animals, for instance transient opening of RCs, it may be difficult to obtain representative results by immunofluorescence in fixed animals. It is also possible that the RCs relax/constricts to 3 µm as a rapid response to stress during fixation of live animals. We can therefore not entirely exclude the possibility of odCDK1a involvement in RC constriction. Another possibility of odCDK1a control of oocyte growth, may involve modulation of the actin cytoskeleton. CDK1 is also known to activate Rho-family GTPases (Evangelista et al., 1997; Nern and Arkowitz, 2000; Shimada et al., 2000), which polarize the actin cytoskeleton and is required for delivery of vesicles to growing cell membranes. Inhibition of CDK1 in budding yeast demonstrated a possible role during post-golgi traffic to growing membranes (McCusker et al., 2012); therefore this may be a possible requirement in *O. dioica* oocyte growth as well.
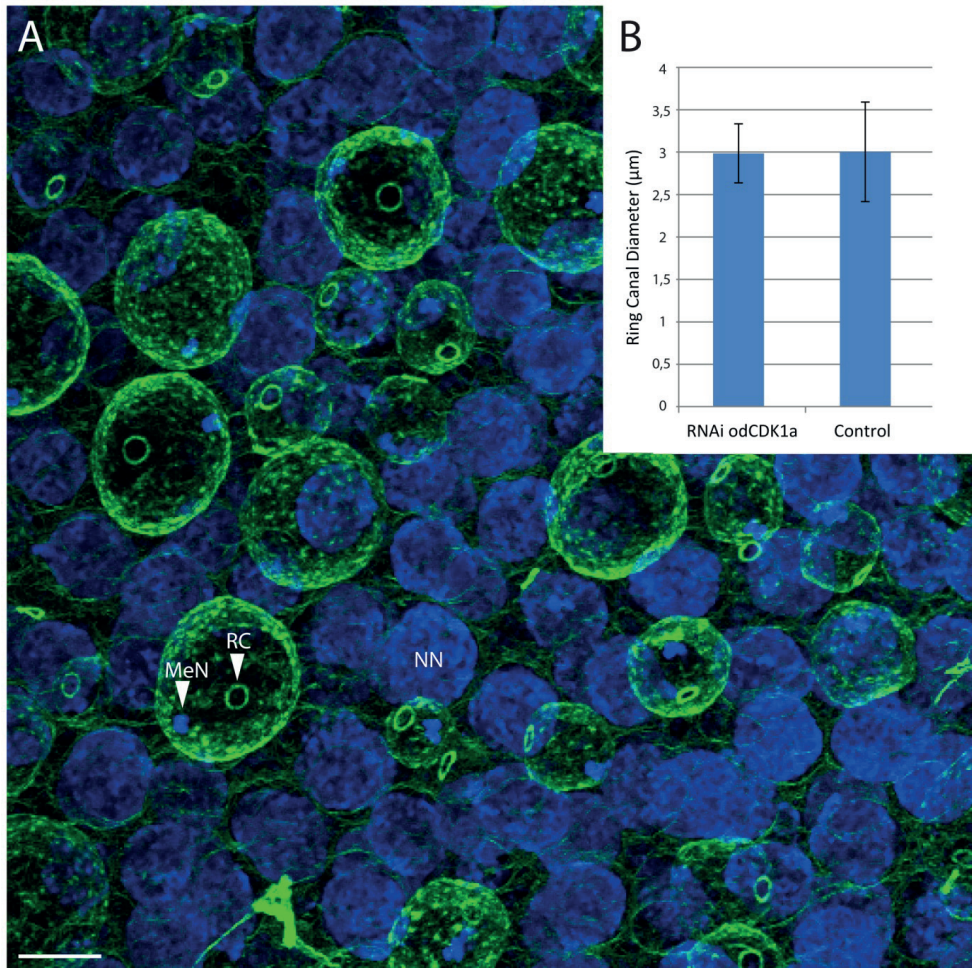
Figure A2-1. Pro-oocytes and their ring canals (RC).
A) Superimposed stack of coenocyst injected with 400 ng/ul odCDK1a-dsRNA stained with phalloidin 488 actin stain (green) and ToProII DNA stain (blue). Single meiotic nuclei (MeN) resided within growing pro-oocytes, whereas nurse nuclei (NN) resided exterior to pro-oocytes. B) RC diameter was not significantly reduced (Students T-test = P>0,05) compared to controls (3∓0,5 µm) in response to odCDK1a knockdown. 30 ring canals were measured in 3 females (90 total) for both control and RNAi treated samples. Scale bar = 15 µm.
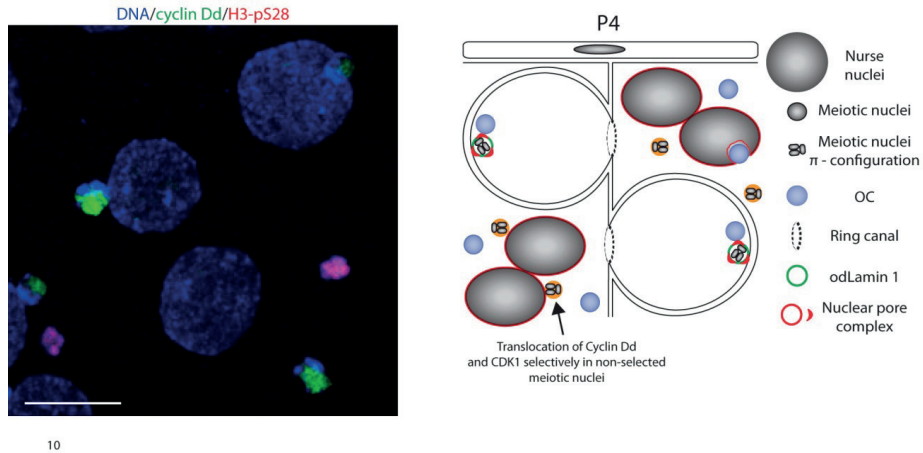
Appendix 3:

Supplementary figures

Figure A3-1. odCyclin Dd in non-selected nuclei. odCyclin Dd translocated selectively to non-selected nuclei, distinguished by negative H3-pS28 staining, following oocyte selection, consistent with behavior of odCDK1 paralogs (Paper II). odCyclin Dd were also observed in endocycling nurse nuclei, consistent with previous observations in endocycling epithelial cells (Campsteijn et al., 2012). As a G1 Cyclin, odCyclin Dd were not expected to perform any particular function during regulation of meiosis and therefore the rationale behind this translocation remains an unsolved mystery. Because the non-selected meiotic nuclei are destined to undergo apoptosis, we speculate that non-selected nuclei may be involved in a mechanism clearing the cytoplasm exterior to growing oocytes for unnecessary cell cycle regulators.
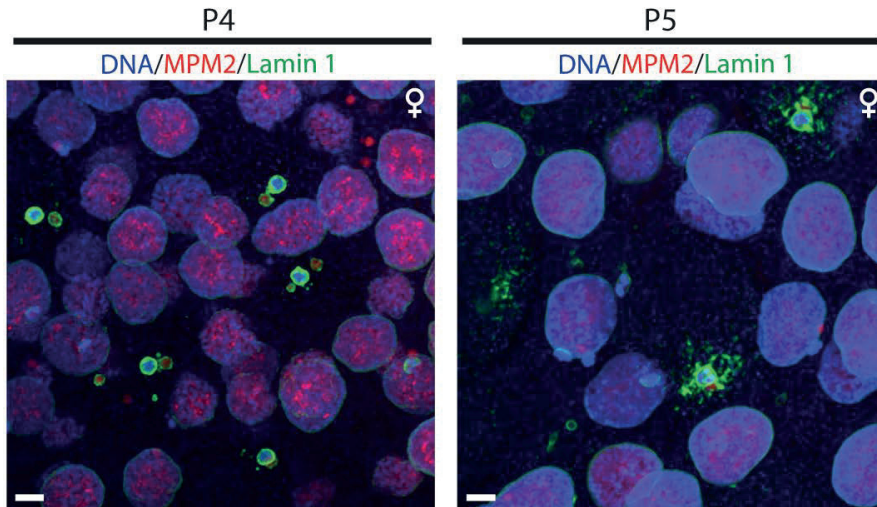
Figure A3-2. Lamin envelopment of organizing centers (OC).
OCs were fully enveloped in Lamin1 during P4 with additional small Lamin vesicles observed in close proximity. Lamin1 was retained on OCs belonging to selected meiotic nuclei in P5, although Lamin1 structures appeared fragmented. Lamin1 was not observed in OCs associated with non-selected meiotic nuclei. Never before have MTOC like structures surrounded by membrane structures been described in the literature and could therefore be a novel structure utilized by *Oikopleura*. The small Lamin vesicles could probably be involved in vesicle transport of unknown cellular components and/or regulators, orchestrated by the OCs. Detailed studies of OC and vesicle dynamics would require fluorescence live imaging, a technique not yet available for adult *O. dioica*. Scale bar = 5 μm.

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