Lifespan extension in a semelparous chordate *Oikopleura dioica* via developmental growth arrest:

Roles of Target of Rapamycin (TOR) signaling and D cyclins

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by

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Abbreviations

AMPK	Adenosine Monophosphate-Activated Protein kinase
AP	Activator Protein
ARD	Adult Reproductive Diapause
CDK	Cyclin Dependent Kinase
CKI	Cyclin Dependent Kinase Inhibitor
CmRNA	Capped messenger RNA
CREB	Cyclic AMP response element-binding protein
CRM	Chromosome Region Maintenance 1
D2SV	Cyclin D2 Splice Variant
DAF	Dauer Formation
DR	Dietary Restriction
DTC	Distal Tip Cell
4EBP1	Eukaryotic translation initiation factor 4E-binding protein 1
ERK	Extracellular signal-Regulated Kinases
FOXO	Forkhead box O
G0	Quiescent phase
G1 phase	First Gap phase
G2 phase	Second Gap phase
GA phase	Growth Arrest
GHR	Growth Hormone Receptor
GLD	Germline developmental Defective
GSC	Germline Stem Cell
GSK-3β	Glycogen synthase kinase-3β
IGF	Insulin Growth Factor
InR	Insulin Receptor
ITC	Inner Trinucleate Cell
JNK	c-Jun N-terminal kinase
JH	Juvenile Hormone
LKB	Liver Kinase B1
M-phase	Mitotic phase

MAPK	Mitogen-Activated Protein Kinases
MSK	Mitogen- and Stress-activated protein Kinase
NLS	Nuclear Localization Sequence
PCNA	Proliferating Cell Nuclear Antigen
PI3K	Phosphatidylinositide 3-kinases
PIP	PCNA Interacting Protien
PTEN	Phosphatase and Tensin homologue
Rb	Retinoblastoma
ROS	Reactive Oxygen Species
S phase	DNA Synthesis phase
S6K	S6 Kinase
TORC	Target Of Rapamycin Complex
TSC	Tuberous Sclerosis Complex

Abstract

All living organisms experience ageing during the course of their lifespan. The science of ageing has emerged into an enthralling area of research. Our knowledge of the mechanisms of ageing and reproduction has been mainly derived from iteroparous model organisms such as *C. elegans* and *D. melanogaster*. The mechanisms governing lifespan are linked to signaling from the reproductive tissues. In this study, we examined to what extent these mechanisms are evolutionarily conserved in a semelparous model organism *Oikopleura dioica.* This marine chordate is a dioecious species that belongs to the sister group of vertebrates. Rapid growth occurs by increasing the size of somatic endocycling cells. The pre-meiotic gonad consists of mitotic germline nuclei which proliferate asynchronously in a common cytoplasm (syncytium). We identify a reversible developmental growth arrest (GA) in *O. dioica* in response to high-density, nutrient-limited conditions, which extends its lifespan up to three-fold. Iteroparous models, sacrifice germ cells that have already entered meiosis, and maintain a reduced number of active germline stem cells (GSCs) under starvation. In contrast, the post-meiotic germline of *O. dioica* does not maintain GSCs and GA only occurs prior to meiotic entry. Nutrient limitation encountered after the meiotic entry led to production of reduced numbers of progeny. During GA, nutrient-dependent Target of Rapamycin (TOR) signaling activity was reduced whereas MAPK stress signaling ERK1/2 and p38 and their common downstream effector MSK1 pathways were activated. Chemical inhibition of TOR signaling alone was sufficient to prevent meiotic entry and germline differentiation. Under GA conditions, both ERK1/2 and p38 pathways were activated and shown to be critical for survival. However, chemical inhibition of TOR signaling only activated p38-MSK1, but not the ERK1/2 pathway. TOR signaling differentially regulated mitotic and endocycling cell cycles during GA. Somatic endocycles immediately ceased upon entry into GA, whereas mitotic germline nuclei and intestinal cells gradually arrested over time. This was mirrored on release from GA, mitotic germline cell cycle resumed first, followed by mitotic intestinal cell cycles, and finally, somatic endocycles. Unlike *C. elegans* and *Drosophila* GSCs, *O. dioica*

germline nuclei have a distinct G1 phase and D-type cyclins play a major role in cell cycle regulation. TOR signaling differentially regulated endocycling and mitotic germ nuclei by altering the expression of D-type cyclins, E2Fs and Cyclin-dependent Kinase Inhibitor a (CKIa). Under GA/TOR inhibition, levels of Cyclin Dd and E2F1 were reduced immediately in arrested endocycling cells, whereas they declined more gradually in proliferating germ nuclei. Simultaneously, increased expression of negative cell cycle regulators, CKIa and E2F7, was observed during somatic endocycling cell arrest. CKIa-mediated cell cycle arrest in proliferating germline nuclei was delayed through cytoplasmic sequestration of CKIa by increased levels of the Cyclin Dbβ splice variant (lacking phosphodegron and Retinoblastoma-binding motifs) in the syncytial cytoplasm. Overall in this investigation, we interpret that the single event of meiotic entry is a definitive signal that lifespan extension can no longer occur in *O. dioica*. Moreover, we demonstrated that TOR signaling integrates environmental cues such as nutrient limitation, to differentially regulate cell cycle variants and promote lifespan extension, in response to adverse environmental conditions.

1. Introduction

Ageing is an inevitable process, experienced by everyone. It occurs as a consequence of accumulation of defective biomolecules, cells and tissues over the course of a lifetime. This process often decreases an organism's capacity to maintain homeostasis under stressful conditions, leading to a greater vulnerability to many age related diseases (cancer, cardiovascular and neurodegenerative diseases) and eventually results in death. Ageing is a gradual loss of function of multiple systems in the body, accompanied by decreasing fertility and increasing risk of death overtime in an organism. Different species can have dramatically different lifespan and even within a single species lifespan shows variation. The rate of ageing can be influenced by environmental factors such as nutrients and various types of stress. Natural selection favours animals that are most likely to become reproductively successful by developing better survival strategies to escape from unfavorable environmental conditions such as starvation (Kirkwood & Rose, 1991). The process of ageing has evolved as a strategy to control population size in nature (Travis, 2004). Nonetheless, organisms too have evolved various strategy to increase their fitness, survival and effectively contribute to the next generation. One of the mechanisms involves adopting different reproductive modes such as Semelparity and Iteroparity (Ranta *et al.*, 2000). A species is considered semelparous if it has a single reproductive episode before death and iteroparous if it has multiple reproductive cycles over the course of its lifetime . In semelparity, the organisms channel major resources towards maximizing reproduction in one reproductive event, producing as many gametes/offspring as possible at the expense of their life. The mechanisms through which environmental factors influence reproduction and lifespan are mainly studied in iteroparous model organisms. Ageing studies on emerging semelparous model organisms such as *Oikopleura dioica,* which invests a major proportion of its energy budget in reproduction (Troedsson *et al.*, 2002), might help us to better understand the mechanistic link between lifespan and reproduction as well as the environmental cues governing them. In the long run these studies could contribute to improve health and delay ageing in individuals.

1.1 Link between reproduction and lifespan

The relationship between lifespan and reproduction is an intriguing question that has been studied using different model organisms. However, the investigations of the link between lifespan extension and reproduction during unfavorable conditions have been carried out in iteroparous invertebrate model organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster*. Many hypotheses have been proposed to understand the association between reproduction and lifespan extension. The most well-known and widely accepted hypothesis is the "Trade off" hypothesis, which suggests a relationship between reproduction and lifespan extension of an organism (Adler *et al.*, 2013). According to the Trade off hypothesis, reproduction and lifespan are inversely related to each other and thought to be an important process seen during nutrient-limited conditions (Partridge *et al.*, 2005). During scarcity of food there is a reduction in fecundity and delay in maturity (Kirkwood & Rose, 1991). The limited resources are reallocated to enhance the chances of survival during these unfavorable nutrient-limited conditions rather than investing in reproduction since production of gametes is an energy intensive process (Partridge *et al.*, 2005). Thus, during such a food crisis, a reduction or absence of reproduction ensures survival of an organism until the environment reaches favorable conditions. This strategy effectively increases lifespan with modulate reproductive success and at the same time increases the chances of survival of the progeny. This phenomenon of lifespan extension under nutrient scarcity possibly functions as an adaptive strategy to increase the evolutionary fitness of the organism.

1.1.1 Nutrient-dependent developmental arrest

Invertebrate model organisms such as *C. elegans* and *Drosophila* have been extensively used in studies of reproductive growth arrest and lifespan as a proxy model system to understand the process of ageing in humans. In *C. elegans,* exposure to nutrient limitation induces developmental and reproductive arrest at different stages of its life cycle **(Figure 1).** During the life cycle of *C. elegans,* adults lay embryos which progress through different embryonic stages (gastrulation, comma stage, two and three-fold embryos) before hatching in the L1 larval form (Pedersen, 2013). The larvae develop through L2, L3, and L4

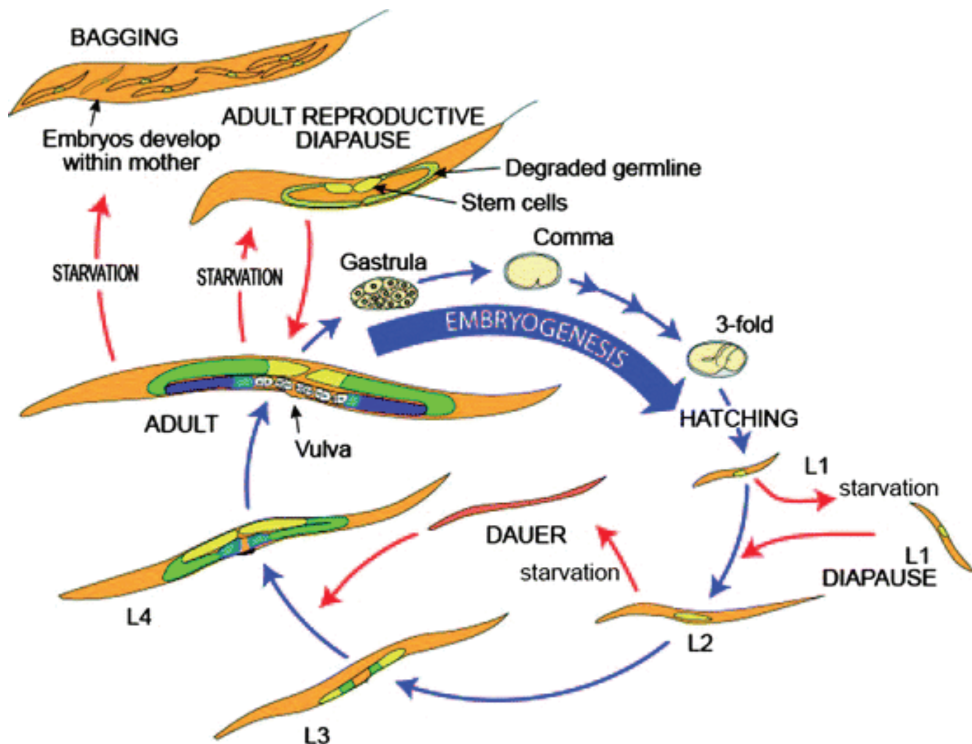

Figure 1. Lifespan extension through growth arrest in *C. elegans.* Adult *C. elegans* lay embryos which undergo gastrulation, comma and 3-fold stage during embryogenesis to form L1 larvae. L1 larvae molt into L2, L3, and L4 stages to give rise to an adult under favorable growth conditions. Worms can enter reproductive arrest and slow down metabolic activity during different stages of the lifecycle, consequently increasing lifespan during nutrient limitation. When L1 larvae hatch in the absence of nutrients, they enter "L1 Diapause" and halt further development until they find food. Similarly, when L2 larvae encounter nutrient limitation, they enter an alternative reversible developmental arrest called "Dauer" in which germ stem cell proliferation is arrested. Another reproductive diapause called Adult Reproductive Diapause (ARD) occurs in post meiotic stage L4 or in adults, when they encounter food scarcity. In ARD, reproduction is poised by degrading most of the germline, but an arrested state of germ stem cells is maintained. Upon re-feeding, germ cell proliferation, meiosis and oogenesis are resumed. Bagging is another strategy in adults to overcome nutrient-limited environments. Here the adult worm stops laying embryos which instead, continue to develop within the mother. At hatching, these embryos consume the mother from inside out. The mother does not survive but this ensures that the embryos have enough food to reach larval diapause. (Adapted from Nanette & Tim, 2013).

larval stages through molting before they reach the adult stage. Interestingly, when food becomes limiting, worms at different stages of larval development enter diapause where reproductive development is completely blocked and their cellular metabolism is slowed

down (Fielenbach & Antebi, 2008). If L1 larvae hatch in the absence of food, they enter "L1 diapause" and retain the L1 morphology, blocking further developmental progress (Baugh, 2013). When nutrients are available, arrested L1 larvae reinitiate their development. Similarly, if L2 larvae encounter environments with less or no food or overcrowding, they enter an alternate developmental form called "dauer larvae" (Fielenbach & Antebi, 2008). In the dauer larvae, the development of the reproductive system ceases. They exhibit a modified morphology where a specialized hard cuticle is formed, the mouth is closed by an internal plug, and the pharynx is constricted such that pumping is blocked (You *et al.,* 2010). The dauer larvae can persist for months and are resistant to stress and desiccation. Even though growth and development is arrested in the dauer form, the larvae actively search for food. When dauer larvae encounter food they re-enter the normal developmental program at the L3 stage and proceed to adulthood. Another form of diapause occurs in *C. elegans* when L4 or adults encounter starvation, which is called Adult Reproductive Diapause (ARD) (Angelo & Van Gilst, 2009). ARD halts reproduction by causing apoptotic death of the entire germline with the exception of a small population of cells called the Germline Stem Cells (GSCs) in the mitotic zones that are apparently arrested in proliferation. This ARD allows sexually mature adults to delay reproductive onset up to fifteen fold and expand their lifespan up to three fold. Upon return to favorable conditions, worms in ARD resume cell cycling in the reserved GSCs and become fully fertile adults. Similarly, "Bagging" is another strategy adopted by adult *C. elegans* to overcome starvation during nutrient-limited conditions wherein the adult nematodes stop laying embryos but instead allow the embryos to continue their development within the mother (Chen & Caswell-Chen, 2004, Chen & Caswell-Chen, 2003). Upon larval hatching, which now occurs inside the mother, these embryos consume the mother from the inside out. The mother does not survive but her demise ensures that the developing progeny have enough food to reach larval development.

Similar to *C. elegans, Drosophila* also prolongs its lifespan by inducing "reproductive diapause" in response to harsh environmental conditions such as low nutrient availability or low temperatures (Tatar & Yin, 2001). Lifespan studies in *Drosophila* have also shown that the germline responds to starvation similarly to ARD of *C. elegans* (Tatar & Yin, 2001). During starvation, programmed cell death in the germline of *Drosophila*

initiates at two different anatomical locations. One is in the region 2a of the Germanium where cystoblasts divide to form germline cysts, which enter meiosis. The other is in the stage 8 egg chambers at the onset of vitellogenesis. As in ARD of *C. elegans*, a small population of GSCs is maintained in the *Drosophila* ovary during starvation (Drummond-Barbosa *et al*., 2009), suggesting that upon nutrient deprivation, maintenance of GSCs is essential during growth arrest and lifespan extension in iteroparous organisms.

1.1.2 Effect of gonadal signals on lifespan

Many studies have demonstrated that the removal of the reproductive system by surgical means or genetic ablation significantly extends lifespan in several organisms **(Figure 2)**. For example, ovariectomy in *Romalea microptera* (Grass hoppers) (Drewry *et al*., 2011) and gonadectomy in *Oncorhynchus tshawytscha* (Pacific Salmon) increases their lifespans up to 3-5 fold (Robertson, 1961). Similar results have been reported in mammals such as humans and mice. In humans, castrated men have been reported to live longer than non-castrated fertile men (Min *et al.*, 2012). Similarly, lifespan increased up to 60% in overiectomized mice compared to non-ovariectomized wild type mice (Cargill *et al.*, 2003). Additionally, transplantation of ovaries from young mice into old mice resulted in lifespan extension in the old mice (Mason *et al.*, 2009). These results suggest that there exists a direct effect of signals from the gonad on lifespan extension, a phenomenon that is conserved across invertebrates to vertebrates.

Mechanisms that abrogate reproduction have a dramatic effect on lifespan in *C. elegans* and *Drosophila* **(Figure 2a and 2b)**. In *C. elegans,* laser ablation of GSC precursors Z2 and Z3 in the larvae, gives rise to adults without a germline, which have an increased lifespan (Arantes-Oliveira *et al.*, 2002). However, removal of somatic gonad precursor cells (Z1 and Z4) along with GSCs did not increase the lifespan in *C. elegans.* This finding suggests that lifespan extension in *C. elegans* does not occur merely by rendering the organism sterile but might occur as a result of antagonistic signals produced by the germline and the somatic gonads (Arantes-Oliveira *et al.*, 2002, Antebi, 2013). In addition to *C. elegans,* another nematode species, *Prisrionchus pacificus,* which diverged from *C. elegans* 100 million years ago, also showed a similar effect (Arantes-Oliveira *et al.*, 2002). In *Drosophila,* genetic manipulations involving the over-expression of germline

differentiation factor Bag of marbles (Bam) also increases lifespan (Flatt *et al.*, 2008). These examples suggest that the association between germline and lifespan has been selected and is maintained during the course of evolution.

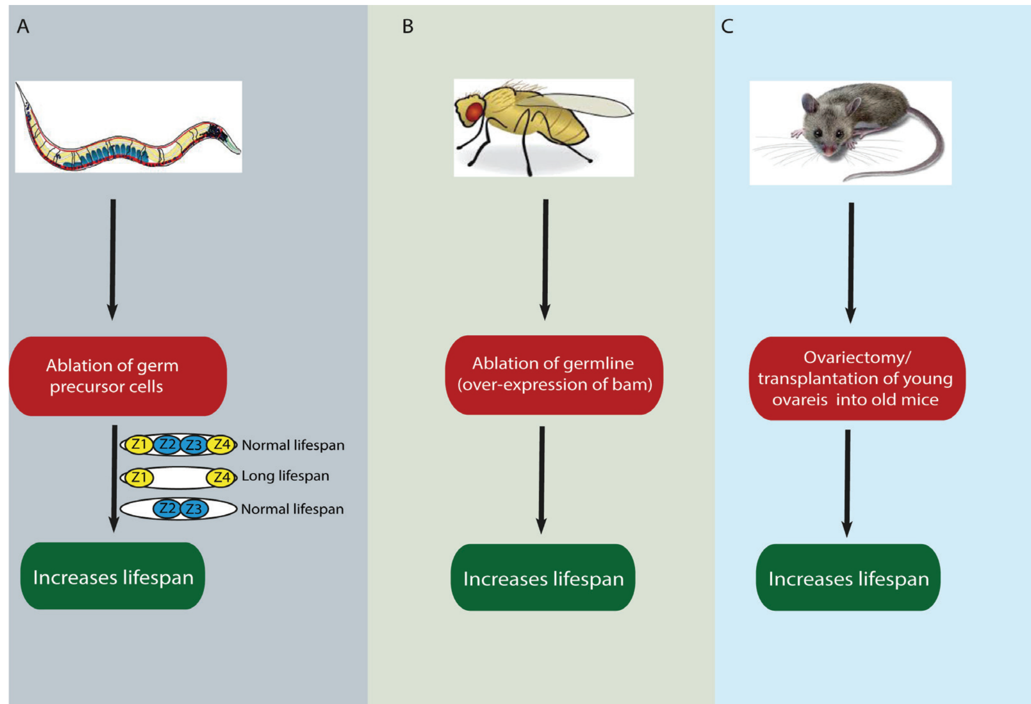

Figure 2. Links between reproductive signaling and lifespan. A) The reproductive system in *C. elegans* is derived from four germ precursor cells (Z1 to Z4). The Z1 and Z4 cells develop into the somatic gonad, whereas Z2 and Z3 give rise to the germline. Laser ablation of Z2 and Z3 cells results in loss of the germline, increasing the lifespan of the adult. However, laser ablation of all four germ cells abrogates this effect and results in normal lifespan. B) Lifespan in *Drosophila* is extended when bag of marbles **(**bam**),** a germline differentiation factor is over-expressed. C) In mice, removal of ovaries or transplantation of young ovaries into the old mice increases lifespan.

1.2. Environmental cues influence lifespan

Nutrient sensing is fundamental to the growth, development and ageing of an organism. Mutations in nutrient sensing genes or stress responsive genes impact lifespan (Kallmann, 1956, Kenyon, 2010). Under conditions of abundant nutrients or absence of environmental stress, these genes promote rapid growth and reproduction. However during

adverse environmental conditions, organisms undergo global physiological changes promoting cell protection to help increase survival (Kenyon, 2010). The Dietary Restriction (DR) effect is one of the well-known phenomena causing lifespan extension in various model organisms **(Figure 3)** including *C. elegans, Drosophila* and *Mus musculus* (Kenyon, 2010). The mechanism through which DR regulates lifespan is not well understood. Most widely accepted hypothesis suggests that DR promotes lifespan extension by reducing the uptake of nutrients and thereby reducing metabolic rate (Roth & Polotsky, 2012). This hypothesis has been supported in a number of organisms including mammals where studies involving calorie restriction resulted in lifespan extension by decreasing the rate of metabolic activity, thus reducing the production of Reactive Oxygen Spices (ROS) and free radicals, known to induce DNA damage and accelerate ageing (Roth & Polotsky, 2012). DR also promotes lifespan extension by regulating conserved nutrient and stress signaling pathways that are essential in coordinating the organismal and cellular response to nutrient limitation (Tissenbaum, 2012). These pathways promote developmental arrest in response to nutrient limitation. The most studied signaling pathways influencing lifespan include Insulin/Insulin-like Growth Factor (IGF) signaling, Target Of Rapamycin (TOR) signaling, AMP-activated protein kinase (AMPK) signaling and Mitogen-Activated Protein Kinase (MAPKs) signaling.

1.2.1 Insulin/Insulin-like Growth Factor (IGF) signaling

The Insulin/Insulin-like Growth Factor (IGF) signaling pathway was the first conserved pathway known to increase lifespan in worms, flies and mammals (Kenyon, 2010, Bartke, 2008b, Bartke, 2008a). Several genetic and functional studies on Insulin signaling components show that inhibition of Insulin-dependent regulation results in lifespan extension. The most exciting finding in ageing research was the identification of mutations in single genes encoding Insulin-like receptor *daf-2* (dauer formation-2) in *C. elegans* (Kenyon *et al.*, 1993). Mutations in *daf-2* produce dauer-like growth arrested larvae that live at least twice as long. As seen in *C. elegans daf-2* mutants, mutants of Insulin receptor *InR* or Insulin receptor substrate *chico* induce reproductive diapause by reducing Juvenile hormone (JH) levels in *Drosophila* (Tatar & Yin, 2001, Clancy *et al.*, 2002). Inhibition of Insulin/IGF-like signaling results in extended lifetime due to altered gene

expression, activation and nuclear translocation of FOXO (Forkhead box protein O) transcription factors (FOXOs; DAF-16 in *C. elegans*), which in turn activate stress resistant genes **(Figure 3).** FOXOs also modify the expression profiles of anti-oxidants and genes regulating the cell cycle, reproduction and development (Halaschek-Wiener *et al.*, 2005, Hansen *et al.*, 2005, Lee *et al.*, 2003).

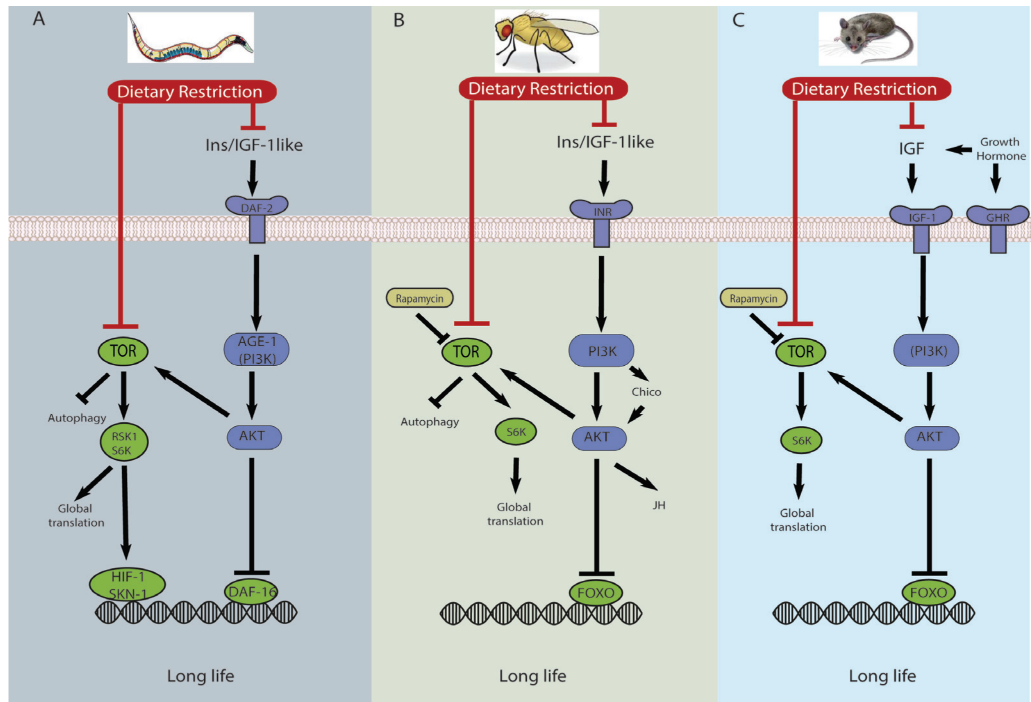

Figure 3. Conserved nutrient signaling cascades regulate lifespan. Genetic and phenotypic analyses in worms (A), flies (B) and mice (C) show that dietary restriction (DR) increases lifespan by down-regulating Insulin/IGF-like signaling and TOR signaling pathways. The role of TOR and its downstream effector S6K in influencing the ageing process by regulating global translation is conserved in these three model organisms. DAF-16/FOXO transcription factors which activate the cellular protective response are activated in the absence of IGF-1/AKT-dependent signaling whereas HIF1 and SKN1 are inactivated in the absence of TOR/S6K. This increases lifespan. In these model organisms, TOR inhibition results in lifespan extension through reduction of ribosomal S6 kinase (S6K) activity and through autophagy in worms and flies. In flies, negative effectors of TOR such as 4EBP1 are known to increase lifespan. TOR inhibition by Rapamycin extends lifespan in *Drosophila* and mice. In flies, inhibition of IGF signaling blocks juvenile hormone (JH) and increases lifespan by promoting growth arrest. In mice, longevity effects of DR appear to involve reduced activity but not absence of GHR/IGF-1 signaling since DR does not extend lifespan further in GHR deficient mice (Modified from Fontana *et al.*, 2010).

Studies in mice have also shown that mutations in Insulin receptor IGF-1 as well as altered IGF signaling prolong lifespan (Bartke, 2008b, Kappeler *et al.*, 2008, Selman *et al.*, 2008). Additionally, mice deficient in Growth Hormone Binding Receptor (GH-BR), which is known to be activated by IGF-1, showed increased lifespan (Bartke, 2005). Interestingly, FOXOs are also activated upon blocking of Insulin signaling in mice, similar to invertebrates and increase lifespan (Greer & Brunet, 2005). Thus, the effects of Insulin/IGF-like signaling and FOXOs on lifespan, appear to be conserved across species.

1.2.2. Target Of Rapamycin (TOR) signaling

TOR signaling, a major conserved signaling pathway, which acts downstream of Insulin and amino acid signaling, is known to increase lifespan (**Figure 3**). TOR is a serine/threonine kinase, which forms two different complexes called TORC1 (TOR complex1) and TORC2 (TOR complex2). The complexes have different functions and both are essential for viability (Loewith *et al.*, 2002). TORC1 complex consists of TOR, Raptor (Regulatory-associated protein of mTOR) and mLSt8 whereas the TORC2 complex substitutes Rictor (Rapamycin-insensitive companion of mTOR) for Raptor. TOR kinases transduce permissive signals for cell growth and cell division in response to the availability of nutrients, growth factors, energy levels and cellular stress. When nutrients are plentiful, TORC1 signaling is activated mainly by IGF signaling to regulate protein synthesis. TOR signaling mediates protein synthesis by regulating two main downstream effectors, ribosomal S6 Kinase (S6K1) and eukaryotic initiation factor 4E (eIF4E) binding protein (4EBP1) **(Figure 4)**. On the other hand, TORC2 acts downstream of IGF or stress response signaling, to promote cell survival. It is also responsible for regulating actin organization in the cytoskeleton. TORC1 but not TORC2, is sensitive to the drug rapamycin produced by *Streptomyces hygroscopicus* (Vezina *et al.*, 1975). Rapamycin treatment has been shown to increase the lifespan in *Drosophila* and mouse (Bjedov *et al.*, 2010, Harrison *et al.*, 2009). Due to the availability of specific TORC1 inhibitors, TORC1 has been better studied than TORC2. TORC1 is activated by AKT (also known as Protein Kinase B, PKB) in response to Insulin and other growth factors (Avruch *et al.*, 2006). Activation of TORC1 by AKT is mediated by inhibition of the Tuberous Sclerosis Complex 1/2 (TSC1/2), which inhibits

TORC1 activator Ras homolog enriched in brain (Rheb) (Inoki *et al.*, 2003). TOR signaling pathways have been the most studied pathways that are directly linked to DR mediating lifespan extension. Genetic and rapamycin-mediated pharmacological inhibition of TOR signaling mimics DR phenotypes in yeast, *C. elegans* and *Drosophila* resulting in increased lifespan (Bjedov *et al.*, 2010, Hansen *et al.*, 2007, Kaeberlein *et al.*, 2005, Kapahi *et al.*, 2004)**.** *TOR* and *daf-15* (Ortholog of raptor) mutants arrest at the L3 larval stage with a phenotype similar to arrested dauer larvae (Long *et al.*, 2002). TOR inhibition seems to activate a pathway distinct from Insulin/IGF pathways since it extends lifespan independent of DAF-16/FOXO (Hansen *et al.*, 2007, Jia *et al.*, 2004, Vellai *et al.*, 2003, Fontana *et al.*, 2010). It has also been proposed that TOR might act downstream of DAF-16, resulting in lifespan extension in *C. elegans* (Vellai *et al.*, 2003). Therefore it is possible, that antagonistic functions of TOR and DAF-16/FOXO regulate growth and ageing (Robida-Stubbs *et al.*, 2012).

TOR signaling affects lifespan and ageing by regulating mRNA translation, autophagy, stress response pathways and metabolic changes **(Figure 4)**. Activation of TOR promotes mRNA translation through phosphorylation of S6K1 and 4EBP1 to regulate cell growth and number (Wullschleger *et al.*, 2006, Thomas, 2002, Pan *et al.*, 2007). Activation of S6K1 results in phosphorylation of ribosomal protein S6, accompanied by up-regulation of a class of mRNAs containing a 5’ TOP motif (Terminal oligopyrimidine tract) present in their 5’ UTR. TOP motif functions as a *cis-regulatory* element for the control of mRNA translation mediated by nutrient-dependent TOR signaling (Meyuhas, 2000). These mRNAs then stimulate the production of ribosomal protein and ribosome biosynthesis (Jastrzebski *et al.*, 2007). Another TOR effector, 4EBP1, present in an inactive hypo-phosphorylated form, acts as a translational repressor by binding eIF4E. Activation of TOR phosphorylates 4EBP1 and releases eI4E, which stimulates translation initiation in the 5’cap of mRNAs. Thus when the level of nutrients and TOR activity are low, the rate of translation drops. This phenomenon has an impact on the lifespan of an organism since inhibition of S6 kinase (S6K) has been shown to extend lifespan in yeast, worms, flies and mice (Hansen *et al.*, 2007, Kaeberlein *et al.*, 2005, Kapahi *et al.*, 2004, Syntichaki *et al.*, 2007, Selman, 2011, Crittenden *et al.*, 2002). Similarly, inhibition of translation by 4EBP1 extends lifespan in

yeast, flies and mice (Hansen *et al.*, 2007, Kaeberlein *et al.*, 2005, Kapahi *et al.*, 2004, Syntichaki *et al.*, 2007). The same effect was shown by inhibition of TOR signaling using

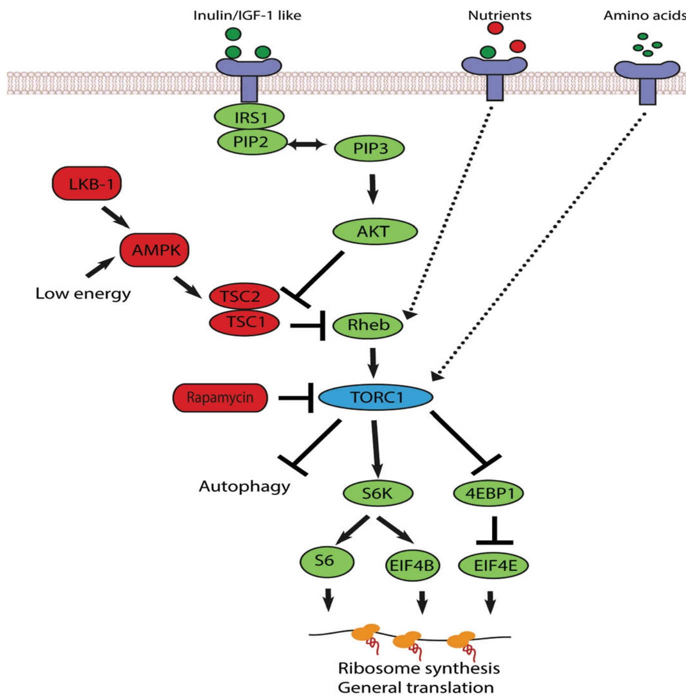

Figure 4. Nutrient-dependent upstream regulation of TOR signaling. Target of rapamycin complex (TORC1) activity is influenced by a number of positive (green) and negative (red) upstream regulators. TORC1 is activated by amino acids, nutrients, and Insulin signaling. TOR activation is mediated by PI3K and AKT (a serine/threonine protein kinase), which inhibit the TSC1/2 complex, thereby relieving TSC1/2-mediated repression of the Rheb) and allowing activation of TORC1. In contrast, low cellular energy levels (conveyed by AMP) activate AMP kinase, which represses mTORC1 both through direct phosphorylation of TSC2 and through regulatory associated protein of TOR (raptor) inhibition. The TSC1/2 complex thus acts to integrate intracellular cues and extracellular conditions. Under unfavorable cellular conditions, the TSC1/2 complex represses the activation of Rheb to inhibit TORC1. TOR-dependent phosphorylation of the 4E-BP disrupts the interaction of 4E-BP with eIF4E, which leaves eIF4A free to promote the binding of ribosomes to the translation start site. TORC1 also activates S6K, which activates the translational initiation factor eIF4B and the S6 ribosomal protein by direct phosphorylation. TORC1 signaling can inhibit autophagy as well.

rapamycin (Harrison *et al.*, 2009) or deletion of S6K1 (Selman *et al.*, 2009) in mouse. It can be envisaged that reduced TOR activity might result in decreased global mRNA translation and ribosomal synthesis, which might prevent growth and cause lifespan extension.

Reduction of TOR signaling can also influence lifespan indirectly by acting on the salvage autophagy pathway (Hands *et al.*, 2009). Accordingly, when nutrients are abundant, activation of TOR signaling prevents autophagy, whereas under nutrient-limitation, reduced TOR signaling leads to autophagy. Studies using mutants of the insulin receptor in *C. elegans* and *Drosophila*, which have reduced TOR activity, showed that reduction in TOR activity induces autophagy and lifespan extension (Kenyon, 2010).

In addition to the functions described above, several studies have reported that TOR signaling regulates germline differentiation in yeast, *C. elegans* and *Drosophila.* In *C. elegans*, RSKS-1/S6K, a downstream effector of TOR signaling acts on the germline autonomously to influence the expansion of the larval germline progenitor pool by both promoting cell cycle progression and inhibiting differentiation (Korta *et al.*, 2012). Likewise, a negative regulator of TOR signaling TSC1/2, prevents GSC differentiation in *Drosophila* (Sun *et al.*, 2010). Interestingly, TOR inhibition is proposed to suppress the translation of meiotic onset specific genes in *Saccharomyces cerevisiae* and prevents gamete differentiation (Zheng & Schreiber, 1997). However, it is not clear to what extent the pharmacological inhibition of TOR signaling affects the meiotic entry in GSCs, to influence lifespan in multicellular organisms.

1.2.3. AMP-activated kinase (AMPK) signaling

Adenosine Mono Phosphate activated Kinase (AMPK) signaling has been widely studied in many model organisms and is reported to regulate lifespan by integrating signals from nutrient and energy dependent pathways (Salminen & Kaarniranta, 2012). Under nutrient-limitation, the levels of AMP rise (high AMP/ATP ratio) activating AMP kinase. Lifespan extension has been shown by over-expression of AMP kinase of *C. elegans* (Apfeld *et al.*, 2005) and by treatment with the anti-diabetic drug Metformin, which activates AMP kinase signaling in mouse (Anisimov *et al.*, 2008). In *C. elegans,* AMPK signaling also activates lifespan extension factor, DAF-16, resulting in its translocation to the nucleus to promote longevity (Greer *et al.*, 2007). Alternatively, IGF signaling activated in response to nutrients (High ATP, low AMP) prevents AMPK signaling and decreases lifespan (Salminen & Kaarniranta, 2012). Furthermore, energy-dependent AMPK signaling activated in response to low ATP levels, represses TOR signaling, to increase lifespan under

nutrient-limited conditions in *Drosophila* (Stenesen *et al.*, 2013, Kimura *et al.*, 2003). Studies in *C. elegans* have also reported that AMPK inactivates TOR signaling and increases lifespan (Apfeld *et al.*, 2005). On the contrary, when AMPK activity is compromised during starvation in *C. elegans*, animals have reduced survival and fail to maintain mitotic quiescence in GSCs during L1 arrest. They develop into sterile adults upon nutrient restoration, which suggests that AMPK signaling regulates lifespan and fertility. These studies indicate that stress due to nutrient deprivation induces activation of AMPK which might in turn activate DAF-16 to suppress TOR signaling thereby increasing lifespan in multicellular organisms.

1.2.4. Survival MAPK signaling

Genetic and environmental factors influence lifespan extension through activation of adaptive and defensive anti-stress responses (Gems & Partridge, 2008, Kaeberlein *et al.*, 2007). Under starvation or upon inhibition of nutrient-dependent signaling, resistance to environmental stress is induced by activation of survival signaling which concomitantly causes lifespan extension. Activation of the evolutionary conserved signaling Mitogen Activated Protien Kinases (MAPK) is associated with increasing survival under various environmental stress conditions. The survival MAPK signaling cascades consist of three major components: MAPK, MAPK Kinase (MAPKK) and MAPKK Kinase (MAPKKK). MAPK activation leads to activation of trancription factors resulting in changes in gene expression profiles controlling cell proliferation, differentiation, survival and apoptosis. Three families of MAPKs have been identified, namely, Extra cellular signal-Regulated Kinase (ERKs), stress activated protein kinases/ C-Jun N-terminal Kinases (JNKs) and p38 MAPKs.

a. MAPK ERK signaling pathway: ERK is a major MAPK signaling pathway activated in response to various extracellulalar stimuli including presence of nutrients and growth factors. On the other hand, there are a few reports which demonstrate that upon nutrient depletion, ERK signaling is activated and influences survival and lifespan. MAPK ERK is activated in calorie restricted rats and mice to increase survival and lifespan (Ikeyama *et al.*, 2002). Likewise, activation of ERK signaling, increases survival in human cells (Tresini *et al.*, 2007). Similarly, activation of ERK signaling enhances survival during starvation

induced L1 growth arrest in *C. elegans* (You *et al.*, 2006, Kang *et al.*, 2007). Conversely, loss of ERK signaling reduces survival and lifespan in *C. elegans* (Okuyama *et al.,*2010).

b. MAPK JNK pathway: MAPK JNK signaling is mainly activated in response to stress, enhancing the survival and lifespan under unfavorable conditions (Mamay *et al.*, 2003, Hirosumi *et al.*, 2002, Kajimoto & Kaneto, 2004). In *C. elegans* and *Drosophila* , JNK signaling induces nuclear localization of DAF-16/FOXO, and extends lifespan when insulin signaling is inhibited (Oh *et al.*, 2005, Wang *et al.*, 2005, Essers *et al.*, 2004).

c. MAPK p38 pathway: MAPK p38 signaling is predominantly activated by environmental stress to increase survival and lifespan in *C. elegans* and *Drosophila* (Craig *et al.*, 2004, Inoue H 2001, Koga M, 2000). In germline of *C. elegans,* MAPK p38 is activated and causes lifespan extension in *daf-2* mutants probably by inducing the expression of stress-resistant genes (Troemel *et al.*, 2006, Alper *et al.*, 2010). These findings suggest that the activation of MAPK signaling plays an important role in survival and extending lifespan under nutrient-limited stress or reduced insulin signaling activity.

Cross talk between MAPK and TOR signaling: MAPK and TOR signaling pathways have been reported to cross talk in various organisms (**Figure 5**). Direct inhibition of TOR signaling activates survival MAPK pathways in mammalian cells (Anjum & Blenis, 2008, Cully *et al.*, 2010, Liu *et al.*, 2002). Inhibition of the TOR pathway activates MAPK to promote survival and cell cycle progression in unicellular yeast also (Petersen & Nurse, 2007). Additionally, mammalian cancer cell lines overcome chemical TOR inhibition by activating MAPK ERK1/2 signaling (Leung-Pineda *et al.*, 2004, Carracedo *et al.*, 2008). TOR inhibition also activates MAPK p38 signaling in megakaryocytes (Martinet *et al.*, 2012) and glioblastoma cells (Cloninger *et al.*, 2011) to increase survival. However, activation of MAPK p38 under rapamycin-mediated inhibition of TOR signaling has not been reported in multicellular organisms.

Mitogen and Stress Induced Kinase 1 (MSK1) is a common downstream effector of both MAPK ERK and p38 survival pathways (van der Heide *et al.*, 2011). MSK1 plays a major role in stress-induced epigenetic changes and regulation of transcription under stress and increases the survival during adverse environmental conditions (Mifsud *et al.*, 2011). MAPK ERK and p38 pathways activate TOR signaling to regulate mRNA translation in mammalian cells (Anjum & Blenis, 2008, Cully *et al.*, 2010, Liu *et al.*, 2002). It has been

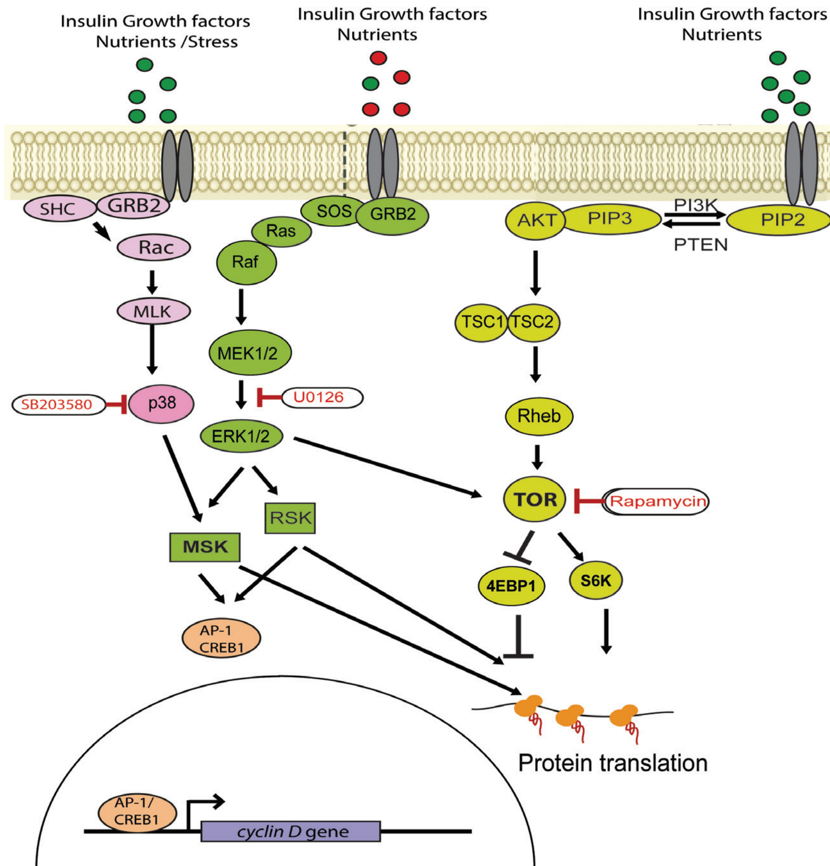

Figure 5. Cross-talk between TOR and MAPK signaling. The Mitogen-Activated Protein Kinase (MAPK) ERK1/2 pathway, induced by mitogens and growth factors, activates TOR signaling and TOR effector ribosomal S6 kinase (S6K). Mitogen Stress activated protein Kinases (MSKs) are activated by mitogens, growth factors and stress through the ERK and p38 pathways. Both the MEK inhibitor U0126 and the p38 inhibitor SB203580 have been used to delineate the function of MSKs. MSKs and RSKs (90 KDa Ribosomal S6 Kinase) are required for the stress-induced phosphorylation of the transcription factors CREB and activating transcription factor-1 (AP1), which activate D-type cyclins. Activated RSKs and MSKs positively regulate TOR-independent translation. Activated RSKs phosphorylate TSC2 and inactivate its tumour-suppressor function, thereby promoting TOR signaling and translation of TOR-dependent targets. MSK1 phosphorylates 4EBP1 whereas RSK phosphorylates Ribosomal S6 in response to MAPK-pathway activation, *via* TOR-independent pathway.

reported that MSK1 activates 4EBP1, a downstream effector of TOR and positively regulates translation in mammalian cells (Liu *et al* 2010). Thus, activation of MAPK

signaling under nutrient-stress or TOR inhibition increases survival possibly by regulating global translation and by activating stress resistant genes.

1.3. Gonadal organization and GSCs regulation in iteroparous model organisms

Iteroparous model organisms have been routinely investigated to establish the link between reproductive axis and lifespan. As described earlier, several reports have demonstrated that signals from reproductive tissues and GSCs influence lifespan. As known for adult stem cells, proliferation and differentiation of GSCs is regulated by concerted effects of environmental cues and intrinsic programs (Li & Xie, 2005, Morrison & Spradling, 2008). The *in vivo* effects of intrinsic and extrinsic factors on cell cycle regulation in GSCs of invertebrate models *Drosophila* and *C. elegans* have been established (Li & Xie, 2005). However, the nature of this regulation in vertebrate models is not yet clear.

1.3.1. *Caenorhabditis elegans*

Caenorhabditis elegans is a hermaphrodite organism with an iteroparous mode of reproduction. The reproductive tract in *C. elegans* is comprised of two U shaped gonadal tubes which fuse at their proximal ends to form a common uterus **(Figure 6a)**. The distal end of the gonadal tubes contains the Distal Tip Cell (DTC). The DTC of the gonad serves as the stem cell niche and maintains a group of approximately 200 asynchronous mitotically dividing nuclei in a syncytium (Spradling A *et al.*, 2001). By convention, GSCs in *C. elegans* refers to germ nuclei surrounded by cytoplasm which is connected to the central core of germline cytoplasm called the "rachis" in a syncytial environment (Hirsh *et al.*, 1976, Cinquin *et al.*, 2010). Short-range signals from the DTC regulate GSC specification and proliferation. These mitotic nuclei gradually migrate away from the DTC zone, forming a transition zone proximal to the DTC. As these nuclei move further proximally from the transition zone, they enter early meiotic prophase at the proximal end of the gonadal tubes. Laser ablation of the DTC causes all GSCs in the mitotic zone to arrest proliferation and enter meiosis. Thus, the DTC is essential for mitotic proliferation of GSCs (Kimble & White, 1981). Signals from the DTC regulate self-renewal and differentiation of GSCs mainly through mRNA translational control of cell fate determinants **(Figure 6c)**. The DTC

produces Lag2/Delta ligands, which bind GLP-1/Notch receptors on the adjacent mitotic nuclei to activate GLP-1 signaling. GLP-1 signaling in these nuclei activates Pumilio-like

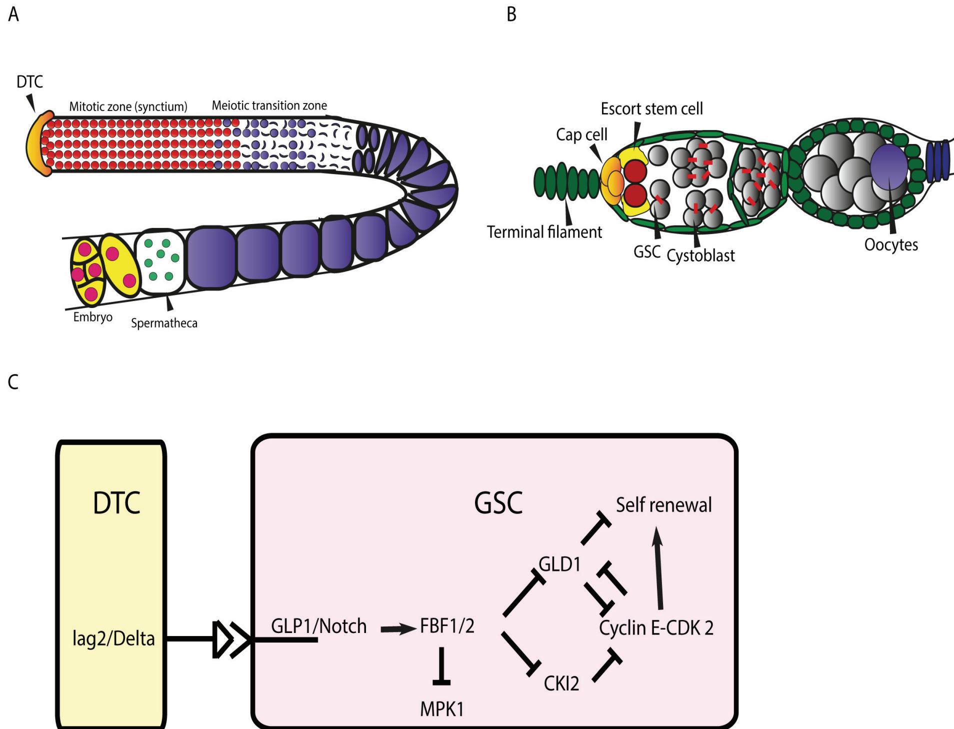

Figure 6. Organization of the ovary in iteroparous model organisms. A) Schematic representation of *C. elegans* germ nuclei in the gonadal tube. The germ nuclei are generated by mitotic divisions in response to signals from the Distal Tip Cell (DTC) at the distal end of each gonadal tube. As the nuclei move away from the DTC, they initiate meiosis in the transition zone and arrest at the pachytene stage of meiosis I. Around the time the nuclei reach the bend in the gonadal tube (past the transition zone), oogenesis is initiated. The germ nuclei exit pachytene and enter diplotene, arresting for a second time in diakinesis, the final stage of meiotic prophase. Germ nuclei become fully cellularized oocytes, following which they grow dramatically in size. The oocytes proceed in a single file through the gonadal tube, with the most mature oocyte present directly adjacent to the spermatheca. B) Schematic representation of the *Drosophila* germarium, the anterior most structure of each ovariole. Germ stem cells (GSCs) are located near the terminal filament (TF) and cap cells. In response to signals from the cap cells, GSCs asymmetrically divide to renew GSCs and to produce cystoblasts. The cystoblasts will subsequently divide synchronously four times to produce a cyst of 16 cystocytes. One cystocyte is then selected to form the oocyte. C) Simplified signaling mechanisms of the *C. elegans* GSC niche. The DTC functions as a niche to maintain GSCs, allowing germ cells beyond the signaling of the niche to differentiate. DTC-expressing LAG-2/Delta, can activate GLP1/ Notch signaling, maintain functions of the RNA binding proteins FBF1 and FBF2, and can repress functions of

differentiation-promoting GLD genes to control GSC self-renewal. The FBF proteins suppress CKI-2 which inhibits the Cyclin E-CDK2 and promotes GSC proliferation.

translational repressors FBF-1, FBF-2 and MAPK phosphatase lip-1 (Lamont *et al.*, 2004, Lee *et al.*, 2006), to suppress intrinsic germ cell differentiation. This is achieved by repressing expression of differentiation-inducing genes such as *gld-1, 2* and *3* (Crittenden *et al.*, 2002). Thus, in iteroparous *C. elegans*, the DTC zone is instrumental in specifying GSC fate to the adjacent mitotic nuclei and regulating GSC proliferation by repressing differentiation factors and maintaining the expression of stemness genes.

1.3.2 *Drosophila melanogaster*

The germline in iteroparous *Drosophila* females is comprised of two to three GSCs located at the tip of the ovariole in a structure called the germarium (Shim *et al.*, 2013) (**Figure 6b**). Three different types of somatic cells, namely the Terminal filament, Cap cells and Escort cells surround the GSCs in the ovariole. Terminal filament, cap and escort cells constitute the niche, which signals GSCs to undergo self-renewal or differentiation (Hanna & Hennebold, 2014, Xie & Spradling, 2000). Cap cells are anchored to GSCs through E-cadherin mediated cell adhesion. The number of GSCs correlates with the number of cap cells. Cap cells produce BMP-like Dpp and Notch signals that are responsible for maintaining the stemness of GSCs by suppressing the expression of differentiation markers. Signals secreted from the cap cells are short range and these signals cannot influence cells more than one cell diameter away (Shim *et al.*, 2013). Normally, a GSC divides mitotically to produce two daughter cells. Two or three GSCs are found in each germarium and form a direct connection with the cap cells, from which they receive supportive signals. The anterior region of each GSC has a single fusome/spectrosome which ensures asymmetical division of GSCs. GSCs continuously self-renew by asymmetric division, in which the daughter cell that directly adheres to the cap cells becomes another GSC. The other daughter cell moves one cell away from the cap cells and eventually gives rise to a cystoblast. Cystoblasts subsequently divide synchronously four times with incomplete cytokinesis to produce interconnected 16-cell cysts, where one of the interconnected cells will differentiate into a growing oocyte. The balance between self-renewal and differentiation is maintained by the local micro-environmental niche signaling.

1.4 Nutrient-dependent signaling regulates cell cycle progression and influences lifespan

Nutrient-dependent or growth factor mediated extracellular signaling is a key determinant of growth and development in multicellular organisms. It increases both cell number and size. Inhibition of nutrient-dependent signaling induces growth arrest by preventing cell cycle progression in both somatic and reproductive tissues. Insulin/IGF, TOR and MAPK signaling pathways are a few nutrient-dependent pathways that directly regulate proliferation and differentiation in both somatic and reproductive tissues by altering the expression of various genes.

1.4.1 The cell cycle

Development in multicellular organisms from a single-celled zygote to an adult requires multiple rounds of cell division (cell proliferation) and differentiation. During each cell division, cells complete a series of orchestrated events called the cell cycle **(Figure 7a)**. The cell cycle starts with a gap phase called G1 that integrates the cell cycle with extracellular growth and developmental stimuli. Based on external signals received during G1, cells either temporarily (reversibly) or permanently (irreversibly) exit the cell cycle and enter a quiescent or arrested phase known as G0 arrest (Sherr, 1995). Once the cell commits to proliferation in G1 based on external stimuli, it enters the next phase called S phase (DNA synthesis phase). Accurate duplication of the genome occurs during S phase followed by a second gap phase called G2. The segregation of duplicated complete sets of chromosomes to each of the daughter cells occurs during the M phase (mitotic phase). During the course of growth and development of an organism, variations of this typical **mitotic cell cycle** (G1-S-G2-M) are used to fulfill specific requirements. These include rapid **embryonic cell cycles** that consist exclusively of S and M-phases, while lacking G1 and G2 phases. Another widespread variation of the cell cycle is called **endo-reduplication.** Endo-reduplication (also called endoreplication or polytenization) is the replication of the DNA content in the absence of cytokinesis, which results in increased DNA content and polyploidy. It is a cell cycle variant in which mitosis is either partially or completely avoided.

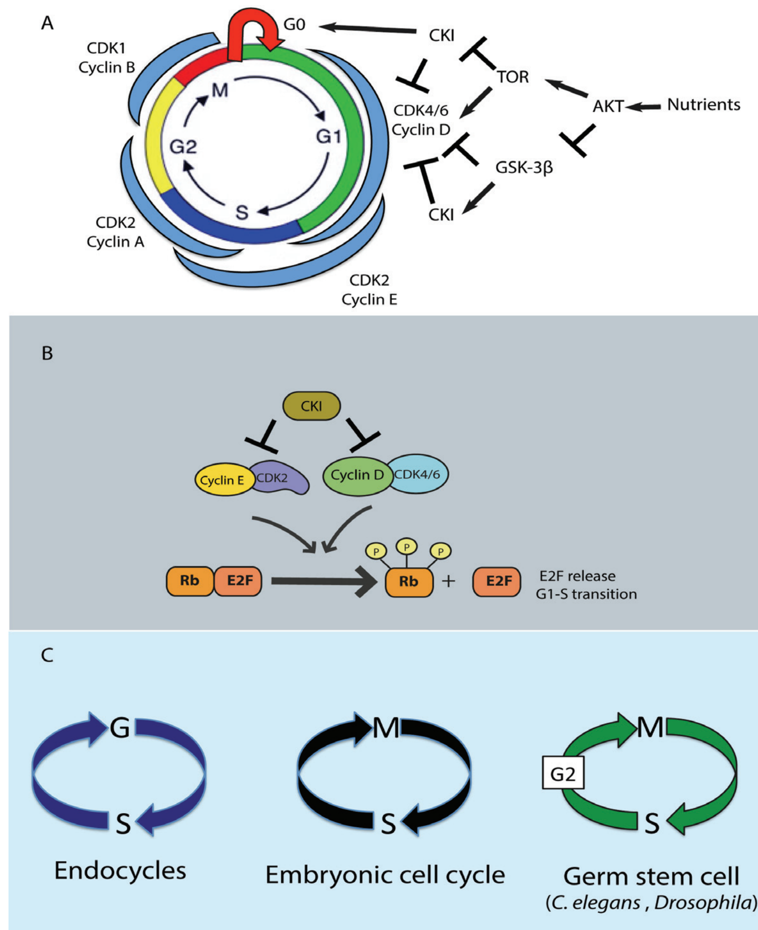

Figure 7. Nutrient signaling promotes the cell cycle. A) Schematic representation of mitotic cell cycle regulation. Different phases of the mitotic cell cycle are regulated by specific CDK-Cyclin complexes. Nutrient signaling activates TOR and initiates cell cycle progression mainly in G1 phase through increasing Cyclin D expression as well as inhibiting the negative regulatory CDK inhibitors (CKI) which are generally involved in Cyclin D degradation. Similarly, nutrient signaling negatively regulates GSK 3β, which activates CKI. Starvation can arrest the cell cycle in a reversible G0 state. B) Transition from G1 to S phase of the cell cycle is in part driven by Retinoblastoma (Rb) phosphorylation that leads to the release of E2F transcription factors and activation of genes involved in cell cycle progression. The G1 regulators Cyclin D-CDK 4/6 complexes and CyclinE-CDK2 complexes catalyse Rb phosphorylation. Cyclin-dependent kinase inhibitors (CKI) can inhibit CDK activity which causes cell cycle arrest. C) Schematic representation of cell cycle variants in which specific cell cycle phases are omitted/truncated. Endoreduplicative cell cycles either bypass mitosis completely or partially, leading to a polyploid DNA content in a cell. Embryonic cell cycles lack both G1 and G2 phases and proliferation is rapid. Another variant of the cell cycle, the Germ stem cell (GSC) cell cycle in *C. elegans* and *Drosophila* progresses through a very short or absent G1 phase followed by S, G2 and M phases.

Depending on whether mitotic events are allowed to occur and the configuration of the genetic material, endo-reduplication can be defined as endocycling and endomitosis. During endocycling, the cell largely avoids mitosis and the duplicated chromatids remain physically associated to each other. Repeated rounds of endocycling can lead to the formation of polytene chromosomes in which sister chromatids are arranged as tightly packed parallel arrays. Endomitosis, on the other hand, allows the cells to undergo aspects of mitosis but fails to initiate telophase. As a result, duplicated chromosomes produced by endomitosis exist as discrete units in a single polyploid nucleus or may be packaged into separate nuclei, depending on the phase during which mitosis was aborted. (Edgar & Orr-Weaver, 2001). Another cell cycle variant reported in GSCs of *C. elegans* and *Drosophila,* consists of G2, M and S phases but has a very short or absent G1 phase **(Figure 7c)**.

Cell cycle progression is governed by specific combinations of two major evolutionary conserved protein families known as Cyclin Dependent Kinases (CDKs) and their specific cofactors called Cyclins. They form a core regulatory complex which controls the transition between the phases and checkpoints during the cell cycle (Malumbres & Barbacid, 2009). During the cell cycle, the expression of different CDKs remains fairly constant whereas the level of cell cycle phase specific Cyclins oscillates. Oscillation of the Cyclins controls the activation of specific Cyclin-CDK complexes which ultimately drive the cell through different phases of the cell cycle (Vermeulen *et al.*, 2003). For instance, CDK1, the first human CDK to be identified, associates with either Cyclin A or B. The Cyclin B-CDK1 complex forms the M phase promoting factor (MPF), which drives the transition between G2 phase and M phase, as well as early M phase. Another mammalian CDK, CDK2, can form a complex with Cyclin E or A. The Cyclin E-CDK2 complex regulates G1 to S phase transition whereas the Cyclin A-CDK2 complex regulates the S phase. Other CDKs, CDK4 and CDK6 interact with either Cyclins D1, D2, or D3 (Malumbres & Barbacid, 2009) and promote cell cycle progression during G1 phase in response to environmental cues. The functional specificity of Cyclin D-CDK4/6 complexes is determined by the bound D-type cyclins and not by the bound CDK (Malumbres & Barbacid, 2005). The activity of Cyclin-CDK complexes is negatively regulated by direct interactions with proteins referred to as Cyclin dependent Kinase Inhibitors (CKIs). CKIs are divided into two major families: the INK4 (inhibitor of CDK4) family, which

specifically inhibits the Cyclin D-CDK4/6 complexes and the Cip/Kip (Kinase inhibitor protein) family, which inhibits most of the Cyclin-CDK complexes (Sherr & Roberts, 1999).

1.4.2 Extracellular cues activate the G1 regulator Cyclin D-CDK4/6 complex

The ultimate function of nutrient and mitogenic extracellular signaling, along with intrinsic factors is to trigger the activation of CDKs, which regulate the early events of cell cycle progression. Extracellular signaling initiates the cell cycle by activating G1 phase regulators such as D-type cyclins and CDK4/6 complexes. The failure to activate Cyclin D-CDK4/6 complexes causes a cell cycle arrest in G0 phase (Sherr, 1995). The level of Cyclin D expression begins to rise during early G1 phase, continues to accumulate until late G1 phase and gradually falls. A decline in the expression of D-type cyclins during late G1 phase marks the onset of S phase and the G1-S transition. In mammalian mitotic cell cycles, the regulatory subunits CDK and D-type cyclins are the rate-limiting factors, which control the progress of G1 phase. The Cyclin D-CDK4/6 and Cyclin E-CDK2 complexes regulate the cell cycle catalytically by phosphorylating Retinoblastoma (Rb), consequently activating the E2F transcription factor that promotes the G1-S transition (Sheaff *et al.*, 1997). Non-phosphorylated Rb sequesters E2F and prevents cell cycle progression **(Figure 7b)**. Once the cell crosses the G1 restriction point, it is committed to progression through the entire cell cycle through cyclical expression of Cyclins E, A, and B. In mammalian cells, depending upon the cell lineage, different D-type cyclins (D1, D2 and D3) are induced by a mitogenic signal during G1 phase. However, invertebrate models such as *C. elegans* and *Drosophila* have a single D-type cyclin that regulates the G1-S transition (Park & Krause, 1999, Datar *et al.*, 2000). D-type cyclins also play many non-catalytic roles to regulate the cell cycle. Cyclin D1 acts as a transcription modulator and regulates the activity of several transcription factors and histone deacetylases in humans (Coqueret, 2002). D-type cyclins also activate the Cyclin E-CDK2 complex indirectly by sequestering CKI, a negative regulator of G1 phase (Sherr & Roberts, 1999). CKI inhibits the Cyclin E-CDK2 complex and causes cell cycle arrest during G1-S transition **(Figure 8)** (Sherr & Roberts, 1999). D-type cyclins play a pivotal role in regulating the G1 phase of the cell cycle either

catalytically or non-catalytically, hence they are primarily regulated by various growth regulating signaling pathways to control proliferation in response to extrinsic cues.

Nutrient-dependent signaling such as MAPK, TOR and Insulin/IGF signaling regulate Cyclin D levels through transcriptional, translational and stability controls. MAPK signaling stimulates transcription of Cyclin D by activating transcription factors such as Activation Protein 1 (AP-1) or Cyclic AMP Responsive Element Binding protein 1 (CREB1). Similarly, TOR signaling positively regulates the cell cycle by inducing the translation of *cyclin D* mRNA (Wang & Proud, 2009). Insulin/IGF signaling promotes the cell cycle by regulating the stability of Cyclin D expression. Cyclin D-CDK4 complexes in quiescent cells are inhibited by Glycogen Synthase Kinase-3β (GSK-3β) mediated phosphorylation of Cyclin D at T286, which causes nuclear export and cytoplasmic destruction of the Cyclin D-CDK4 complex (Takahashi-Yanaga & Sasaguri, 2008). Nutrient-dependent signaling negatively regulates GSK-3β activation, thereby promoting the stability of Cyclin D. In the absence of Insulin/IGF signaling, GSK-3β is activated and degrades the Cyclin D-CDK4 complex causing cell cycle arrest in G0. GSK-3β also phosphorylates and inhibits AP1, a transcription factor that promotes *cyclin D* expression. Thus, D-type cyclins function as a node for major growth regulating signaling pathways that promote cell proliferation in response to nutrient availability.

Another major class of G1 regulators the Cip/Kip (p21and p27) family of CKI is negatively regulated by nutrient-dependent signaling. CKIs inhibit the activity of Cyclin-CDK complexes thereby preventing cell cycle progression. Most notably CKIs inhibit the Cyclin D-CDK4/6 and Cyclin E-CDK2 complexes to cause cell cycle arrest during unfavorable conditions in several organisms including *C. elegans, Drosophila* and mammals (Meyer *et al.*, 2000, Hong *et al.*, 1998, Sherr & Roberts, 1999). In *C. elegans,* CKI-1 interacts with the Cyclin D-CDK4 and Cyclin E-CDK2 complexes and inhibits their activity during starvation. Not much is known about another CKI found in *C. elegans*, CKI-2, in regulating the function of the Cyclin D-CDK4 and Cyclin E-CDK2 complexes (Boxem & van den Heuvel, 2001). Although CKI Dacapo has been reported to bind and inhibit the activity of the Cyclin E-CDK2 complexes in *Drosophila*, it does not bind the Cyclin D–CDK4 complex or regulate its activity (Meyer *et al.*, 2000). Similarly, p21 and p27 in mammals, bind and inhibit the activity of Cyclin D-CDK4 and Cyclin E-CDK2 complexes

during unfavorable conditions (Sherr & Roberts, 1999). However, it has been reported that p27 also acts as an assembly factor for forming active nuclear Cyclin D-CDK4/6 complexes and promotes G1 progression in mammalian cells during favorable conditions (Larrea *et al.*, 2008).

In *C. elegans,* activation of CKI-1 blocks the G1-S transition in L1 diapause and dauer larvae (Hong *et al.*, 1998). During starvation, absence of nutrient-dependent signaling activates DAF-16 which results in transcription of *CKI-1* and causes cell cycle arrest in *C. elegans* (Baugh & Sternberg, 2006). Similarly, FOXO also positively regulates the transcription of p27 in mammalian cells during starvation (Dijkers *et al.*, 2000). It has been shown that nutrient-dependent signaling influences CKI activity by regulating its cellular

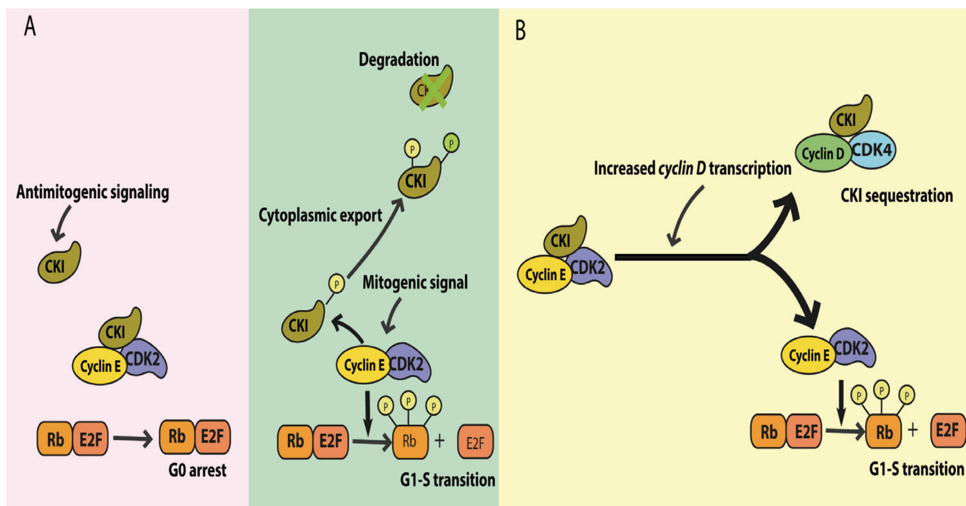

Figure 8. Regulation of CDK inhibitors during cell cycle entry. Schematic representation of regulation of Cip/Kip family CKI. A) G1-S transition promoting Cyclin E-CDK2 activity is inhibited by CKI causing reversible G0 arrest. Upon mitogenic stimulation, phosphorylation of CKI at multiple sites, results in export from the nucleus and destruction by cytoplasmic proteasomal complexes. B) After mitogenic stimulation, increasing numbers of Cyclin D-CDK4 complexes associate with CKI thereby reducing the amount of CKI available for inhibitory binding of Cyclin E-CDK2. This promotes S phase entry.

localization. Serum and glucocorticoid-regulated kinase 1 (SGK1), a downstream effector of TOR signaling phosphorylates p27, which promotes the cytoplasmic retention of p27 promoting the G1-S transition (Hong *et al.*, 2008). D-type cyclins also have been reported to

sequester CKI, p21 and p27 either in cytoplasm or nucleus to promote G1-S transition in mammalian cells under nutrient-deprived conditions **(Figure 8)** (Kim *et al.*, 2009, Meyyappan *et al.*, 1998, Narbonne & Roy, 2006a, Perez-Roger *et al.*, 1999, Bouchard *et al.*, 1999). Thus, sequestration of up-regulated CKI by activation of D-type cyclins possibly helps to overcome the block of G1-S transition under nutrient deprivation.

1.4.3 Nutrient signaling differentially regulates mitotic and endoreduplicative cell cycle variants

Organisms grow either by increasing cell number through mitotic cell division, or by increasing cell size through endoreduplication or by both. In *C. elegans* and *Drosophila* larvae, overall growth occurs mainly through endoreduplication (Edgar & Orr-Weaver, 2001, Lozano *et al.*, 2006). However, endoreduplication-associated growth in these organisms is usually confined to specialized cell types that perform specific biological functions and is not a universal mechanism that controls organism size. Endoreduplication usually occurs in tissues that develop rapidly and have a very high metabolic activity (Lee *et al.*, 2009). This endoreduplicative mode provides an efficient strategy for growth as well as resistance to genotoxic stress. The same cell cycle regulators that control G1 and S phase of mitotic cell cycles regulate the progression of endocycles. However, nutrient-dependent signaling regulates endocycling and the mitotic cell cycle in different ways **(Figure 9)** (Britton & Edgar, 1998). In endocycling cells of *Drosophila* larvae, withdrawal of nutrients causes an immediate cell cycle arrest whereas re-feeding immediately initiates the cell cycle in quiescent endocycling cells (Britton & Edgar, 1998). These findings suggest that endocycling progression is highly responsive to the availability of nutrients. In contrast, in mitotic cells, nutrient withdrawal does not cause an immediate cell cycle arrest but reduces cell cycle progression gradually. Thus nutrient-dependent signaling is essential for cell cycle progression in somatic as well as endocycling cells but might have a different mechanism of action operating in these two cell types.

Signals from amino acids alone can activate quiescent endocycling cells and this response is mediated at the cellular level by the TOR pathway in *Drosophila* larvae (Britton *et al.*, 2002, Britton & Edgar, 1998, Brogiolo *et al.*, 2001). Reduction in Insulin/IGF-like signaling inhibits endocycles and results in small cells with reduced ploidy whereas

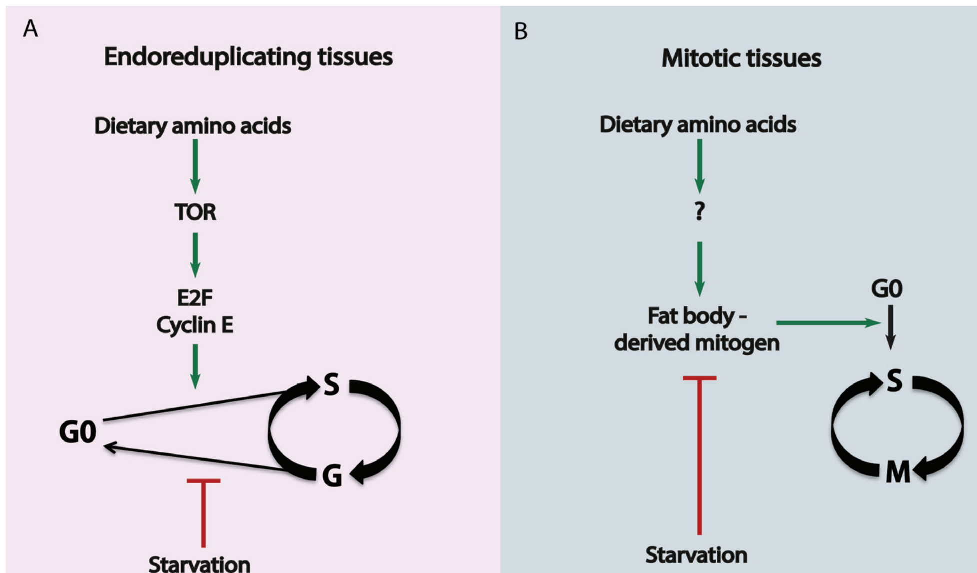

Figure 9. Nutrient signaling differentially regulates mitotic and endocycling cells. A) Endocycling cells respond immediately to starvation by undergoing cell cycle arrest and upon re-feeding they re-enter the cell cycle. Nutrients and amino acid-mediated TOR signaling stimulate endocycling cells to enter S-phase. The requirement for dietary amino acids/TOR activation can be bypassed by ectopic expression of G1-S transition factors Cyclin E or E2F. TOR signaling is continuously required for these tissues to remain in the cycling state. B) In mitotic cells, starvation gradually arrests cell cycle progression. Nutritional input is required only at the time of cell cycle activation. Inhibition of TOR does not affect the proliferation of mitotic cells. Nutrient signaling may regulate the production or secretion of fat body-derived mitogenic factors that in turn regulate mitotic cell cycle progression. (Modified from Britton & Edgar, 1998).

activation of Insulin/IGF-like signaling induces endocycle progression and results in larger than normal cells with increased ploidy (Hafen & Stocker, 2003). In *Drosophila* and *C. elegans*, loss of TOR function also reduces cytoplasmic volume in endocycling tissues whereas mitotic cells grow normally, suggesting that TOR activation differentially regulates mitotic and endocycling cells (Jia *et al.*, 2004, Long *et al.*, 2002, Oldham *et al.*, 2000). Further, investigations involving upstream regulators of TOR such as TSC1/2 or Rheb in *Drosophila* imply that TOR activation stimulates growth and endocycle progression, whereas its inhibition has the opposite effect (Saucedo *et al.*, 2003, Stocker *et al.*, 2003, Zhang *et al.*, 2003). Moreover, activation of Insulin/IGF-like signaling or TOR activity can bypass starvation arrest and initiate S phase progression (Britton *et al.*, 2002). These

findings suggest that Insulin/TOR signaling is inhibited during nutrient-limited conditions resulting in arrest of endocycles.

Nutrient-dependent oscillation of the G1-S regulator Cyclin E, is the driving force for endocycle progression (Lilly & Spradling, 1996, Royzman *et al.*, 1997, Weng *et al.*, 2003, Zielke *et al.*, 2011, Delsuc *et al.*, 2008). Nutrient withdrawal in *Drosophila* larvae induces cell cycle arrest in both endocycling and mitotic cells. Ectopic expression of Cyclin E stimulates S phase entry in these arrested endocycling cells, but not in the arrested mitotic cells (Britton & Edgar, 1998) suggesting that expression of Cyclin E differentially regulates endocycling and mitotic cells. Activation of TOR or ectopic expression of Cyclin E stimulates S-phase entry of arrested endocycling cells, thus it can be anticipated that the progression of endocycles occurs in response to TOR-mediated *cyclin E* translation (Britton & Edgar, 1998). In addition to Cyclin E, other G1 regulators such as Cyclin D-CDK4 complexes also regulate cell growth in *Drosophila.* Over-expression of Cyclin D and CDK4 stimulates growth in endocycling cells resulting in an increase in final ploidy in *Drosophila* (Meyer *et al.*, 2000, Datar *et al.*, 2000). In contrast to the mammalian cell cycle, *Drosophila* Cyclin D functions primarily in promoting growth and does not have a direct role in promoting cell cycle progression (Datar *et al.*, 2000). Moreover the mechanism by which Cyclin D-CDK4 complexes control growth is not known but it does not appear to involve either the regulation of Rb/E2F or the Insulin/IGF pathways in *Drosophila* (Lee *et al.*, 2009).

E2F transcription factors constitute another family of major cell cycle regulators known to activate transcription of several genes whose products are essential for entering S phase and initiating DNA replication (Black & Azizkhan-Clifford, 1999). Hypo-phosphorylated Rb binds E2F1 and prevents its function. Release of E2F1 is mediated by phosphorylation of Rb at multiple serine and threonine residues by Cyclin-CDK complexes. E2Fs are also known to act as growth sensors in mitotic (Real S, 2011) and endocycling cells (Zielke *et al.,* 2011). In mammals, the E2F family consists of nine proteins, which can be broadly classified into the typical and atypical E2Fs (Chen *et al.*, 2009). The typical activator E2Fs (E2F1, E2F2, E2F3a) and typical repressor E2Fs (E2F3b, E2F4, E2F5 and E2F6) differ from the atypical E2Fs (E2F7 and E2F8) in many ways. Activator E2Fs dimerize with DP (Dimerization protein) to bind target promoters whereas atypical repressor

E2Fs bind target promoters independent of DP proteins. Classical activator and repressor E2Fs possess only one DNA-binding domain but atypical E2F possess two DNA binding domains. Recent studies in *Drosophila* revealed that TOR signaling regulates the level of E2F1 to control endocycling in salivary glands (Zielke *et al.,* 2011). E2F1 levels peak during G phase and decrease during S phase whereas stabilized E2F1 levels arrest endocycling in salivary glands. Similar to G1-S regulators such as Cyclin E, ectopic expression of E2F1 promotes S phase entry in arrested endocycling cells but not in arrested mitotic cells (Britton & Edgar, 1998, Zielke *et al.,* 2011). Thus, TOR signaling differentially regulates endocycling and mitotic cells mainly by altering the translation of G1 regulators.

1.4.4 The cell cycle in GSCs of invertebrates

In invertebrate model organisms such as *C. elegans* and *Drosophila*, regulation of the core cell cycle machinery in somatic cells differs from the regulation of GSCs (Noatynska *et al.*, 2013). Unlike somatic cells, the cell cycle in these invertebrate GSCs, either lacks the G1 phase or has a very short G1 phase (Hsu *et al.*, 2008, Fox *et al.*, 2011). Other stem cell types such as embryonic stem cells in mammals also have a short G1 phase (Singh & Dalton, 2009, Becker *et al.*, 2006). Decreasing G1 phase length has been proposed as a strategy adapted by GSCs and other stem cell types, to enhance self-renewal. A decreased G1 phase length limits their sensitivity to signaling which induces differentiation, in GSCs (Lange & Calegari, 2010, Singh & Dalton, 2009, Orford & Scadden, 2008, Becker *et al.*, 2006). However, it has been reported that prolonging G1 phase in mouse embryonic stem cells did not promote differentiation (Li *et al.*, 2012).

The G1 phase regulator Cyclin D plays a major role in self-renewal and differentiation in mouse spermatogonial stem cells, which have a well-defined G1 phase (Beumer *et al.*, 2000). However, due to the absent/short G1 phase in GSCs of *C. elegans* and *Drosophila*, the role of Cyclin D and CDK4 in G1 progression has not been reported. Nonetheless, in *Drosophila* and *C. elegans* it has been reported that Cyclin E-CDK2 activity possibly bypasses the need for upstream G1 phase regulation by Cyclin D-CDK4 complexes in GSC proliferation (Wang & Kalderon, 2009, Fox *et al.*, 2011). In *C. elegans*, Cyclin E is expressed throughout the GSC cell cycle. In the mitotic zone of the gonadal tube, Cyclin E-CDK2 complexes promote self-renewal and stemness in GSCs by negatively regulating the

stability of GLD-1 (Germline development Defective 1), a differentiation factor (Jeong *et al.*, 2011). Moreover, the expression of differentiation factor *gld-1* mRNA is also inhibited by RNA binding proteins FBF1 and FBF2 **(Figure 6C)**. In the transition zone, in the absence of FBF1 and FBF2, GLD-1 inhibits *cyclin E* expression promoting GSC differentiation (Jeong *et al.*, 2011). Similarly, in *Drosophila,* niche signaling induces Cyclin E expression throughout the cell cycle of GSCs and maintains stemness (Wang & Kalderon, 2009, Ables & Drummond-Barbosa, 2013).

1.4.5 Nutrient signaling regulates GSC proliferation and lifespan extension

As we described earlier in section 1.1.2, signaling from reproductive tissues is directly linked with lifespan in iteroparous models. Self-renewal and differentiation of GSCs is regulated by external stimuli such as nutrients, hormones or physical injuries in addition to local niche signaling (Ables *et al.*, 2012). In *Drosophila* and *C. elegans*, GSC proliferation and differentiation is regulated to modulate the lifespan of the organism under limited resources (Shim *et al.*, 2002, Drummond-Barbosa & Spradling, 2001). GSCs carry information that needs to be transferred from one generation to the next. Therefore, maintaining the genetic integrity of this information is critical to prevent deleterious effects. Thus, it can be anticipated that during unfavorable environmental conditions, GSCs become quiescent in order to minimize the risk of acquiring deleterious mutations.

Insulin-like signaling has extensive roles in GSC proliferation in *Drosophila* and *C. elegans* (Ables *et al.*, 2012, Narbonne & Roy, 2006b). Functional impairment of Insulin/IGF signaling in *daf-2, daf-16* and dauer mutants in *C. elegans* have very few mitotically dividing GSCs when compared to wild type (Michaelson *et al.*, 2010, Narbonne & Roy, 2006b). Similarly, during starvation, or absence of Insulin mediated PI3K signaling, expression of FOXOs prevents GSC proliferation (Hsu *et al.*, 2008). Nutrient-dependent Insulin/IGF signaling regulates GSC proliferation independent of signals from the niche environment (Drummond-Barbosa & Spradling, 2001, Narbonne & Roy, 2006a). In *Drosophila* loss of the Insulin receptor substrate *chico* results in sterile adults (Bohni *et al.*, 1999). Similarly, loss of *inR* in GSCs suppresses GSC proliferation, cyst growth and yolk deposition. Thus, Insulin/IGF signaling regulates GSC proliferation in iteroparous *C. elegans* and *Drosophila*. Nuclear localization of DAF-16 in the germline and the ablation of

the germline extends lifespan in *C. elegans* (Berman & Kenyon, 2006). Moreover, DAF-16 was also localized to the nucleus of the somatic intestinal tissue in long-lived *C. elegans* wherein the germline was ablated (Berman & Kenyon, 2006). DAF-18 (Phosphatase and tensin homolog (PTEN) orthologue), a suppressor of AKT signaling, is required for nuclear translocation of DAF-16 to regulate gene expression and increase lifespan (Lin *et al.*, 2001). These findings suggest that the germline mediated reproductive signaling influences longevity in a DAF-16 dependent manner. *daf-2,* insulin signaling mutants have also been reported to exhibit lifespan extension similar to that associated with removal of the germline. Interestingly, double mutants of *daf-2* and *daf-18* do not alter lifespan, probably due to failure of nuclear translocation of DAF-16 (Mihaylova *et al.*, 1999). This suggests that IGF signaling and reproductive signaling coordinate to regulate the nuclear translocation of DAF-16 to increase lifespan. Consistent with the effect of reproductive signaling on lifespan extension, mutants of the *glp-1* notch like ligand, also show increased lifespan. Moreover, the lifespan was reduced by an additional mutation of *daf-16* in the *glp-1* mutants (Korta *et al.*, 2012). Thus, DAF-16 is responsible for mediating lifespan extension in *glp-1* mutants.

TOR, a downstream effector of Insulin/IGF signaling also regulates GSC proliferation and differentiation. In *Drosophila,* TOR signaling regulates the G2 phase of the GSC cell cycle and is required for proper rates of GSC proliferation (Drummond-Barbosa & Spradling, 2001). Hence, *TOR* mutants in *Drosophila* females frequently lose GSCs from their niche. These *TOR* mutants exhibit a prolonged G2 phase, a phenotype that is not rescued by proliferation inducing FOXO mutations. This suggests the existence of a parallel and independent control of GSC proliferation (LaFever *et al.*, 2010). On the contrary, removal of TSC1/2, negative regulators of TOR, or hyper-activation of TOR, causes a dramatic loss of GSCs (LaFever *et al.*, 2010, Sun *et al.*, 2010). This dramatic loss of GSCs was rescued by the TOR inhibitor rapamycin treatment in *Drosophila* (Sun *et al.*, 2010). This study also concluded that TOR signaling is required for GSC differentiation. In *C. elegans* LKB1 (Liver Kinase B1) and AMPK, upstream negative regulators of TOR signaling, play a vital role in arresting GSC proliferation during starvation (Narbonne & Roy, 2006a). Consistently, loss of downstream effectors of TOR signaling such as S6K or eIF4E decreases proliferation of GSCs more severely than mutants of *TOR* or *Rapto*r in *C.*

elegans (Korta *et al.*, 2012). These findings suggest that Insulin and TOR signaling regulate the proliferation and differentiation of GSCs, a mechanism that also directly regulates growth arrest and longevity in iteroparous models.

1.4.6 Atypical nutrient-dependent G2-M cell cycle arrest in GSCs

In mammalian somatic cells, nutrient-dependent signaling affects the cell cycle machinery that is required for regulating the G1-S and G2-M transitions (Wang & Proud, 2009). In dauer larvae of *C. elegans*, somatic cells are arrested in G1 phase whereas the GSCs are arrested in the G2 phase of the cell cycle (Narbonne & Roy, 2006a, Fukuyama *et al.*, 2006). Similarly in *Drosophila* GSCs, diet-dependent cell cycle arrest occurs in the G2 phase, and is mediated by activation of FOXOs (Hsu *et al.*, 2008) and Dacapo (Yu *et al.*, 2009). However the knockdown of *cyclin E* and *CDK2* in GSCs causes cell cycle arrest during a G1-like state. This suggests that Cyclin E expression is mainly regulated by niche signaling but not by Insulin/IGF signaling (Hsu & Drummond-Barbosa, 2009, Hsu & Drummond-Barbosa, 2011, Hsu et al., 2008). This potentially explains why G2 and not G1 phase is the major point of regulation by nutrient-dependent pathways in *Drosophila* GSCs. In *C. elegans,* RNA depletion of *CKI-1*, induces hyper-proliferation of GSCs during dauer formation (Hong *et al.*, 1998). These studies also suggest that GSCs do not arrest during G1 but rather arrest at the G2-M checkpoint in starved larvae or larvae in which Insulin/IGF signaling is compromised. Additionally, in *cul-2* mutants (component of the SCF-degradation complex) of *C. elegans*, CKI-1 protein levels accumulate and cause cell cycle arrest during the G2-M transition in GSCs (Feng *et al.*, 1999). Suppression of CKI-2 activity, another CKI in *C. elegans*, via the RNA binding protein FBF-2, is critical for GSC proliferation (Kalchhauser *et al.*, 2011). Together these data indicate that nutrient-dependent signaling regulates reversible cell cycle arrest in GSCs at the G2-M checkpoints in *C. elegans* and in *Drosophila.*

1.5 The semelparous urochordate model *Oikopleura dioica*

To date *in vivo* understanding of the effect of germline reproductive signaling on lifespan extension has been derived from iteroparous invertebrate model organisms such as

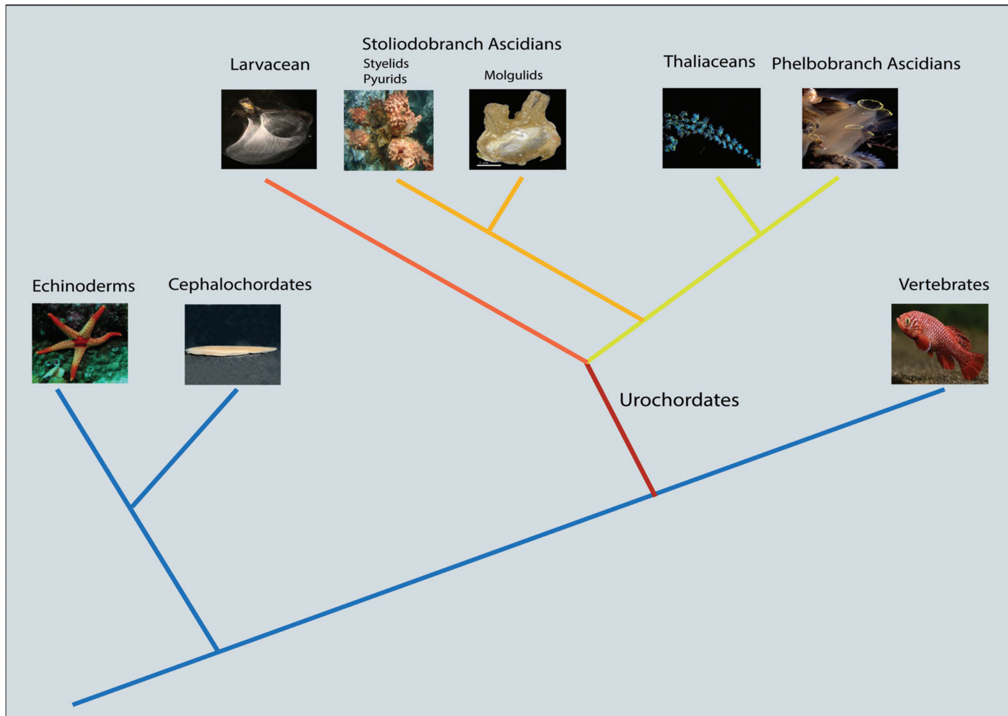

Figure 10. Phylogeny of Larvaceans. According to the new chordate phylogeny based on whole genome sequencing and ribosomal RNA analysis (Delsuc *et al.*, 2008), tunicates (Urochordates) form the closest sister group of vertebrates. *O. dioica* belongs to the class Larvacea of the subphylum urochordata. (Modified from a drawing by Billie Swalla, University of Washington).

The appendicularian *O. dioica* is a coastal marine zooplankton with a pan-global distribution (Gorsky & Fenaux, 1998). Appendicularians are also called larvaceans because they retain their tail throughout the life cycle. *O. dioica* has a compact 72 Mb sequenced genome containing more than 18000 predicted genes and is ranked among the smallest metazoan genomes (Seo *et al.*, 2001, Denoeud *et al.*, 2010). It has a short life cycle and can be cultured year-round under laboratory conditions. It has many conserved signaling pathways allowing extrapolation of findings in this system to other organisms. Additionally, the occurrence of a semelparous mode of reproduction in *O. dioica* makes it an excellent *C. elegans* and *Drosophila* and vertebrate *Mus musculus.* Due to the complexity of germline

organization in mouse, it is very difficult to decipher *in vivo* germline regulatory mechanisms (Ninga *et al.*, 2012). This investigation introduces an emerging semelparous model organism, *Oikopleura dioica,* which has a simple reproductive anatomy for studying nutrient dependency and survival signaling. *O. dioica* belongs to class appendicularia (or larvacea) of the subphylum urochordata in the phylum chordata **(Figure 10).** Urochordata is phylogenetically the closest sister group of vertebrates (Delsuc *et al.*, 2008), and thus might provide evolutionary insight towards understanding the phenomenon of lifespan extension in vertebrates. model organism for *in vivo* studies to understand the molecular regulation of growth arrest and lifespan extension and to compare these mechanisms to those found in iteroparous model organisms.

1.5.1 Feeding behavior and ecological roles

In marine food webs, they occupy an important trophic position permitting rapid energy transfer from micro-scale phytoplankton primary producers to macro-scale zooplanktivorous predators. Among appendicularians, *O. dioica* is the only dioecious species. It is found in all major oceans and is an opportunistic species with the ability to expand its population rapidly in size in response to algal blooms. They are distributed as patchy populations and attain densities up to 53,000 individuals per m^3 (Uye & Ichino, 1995). The feeding behavior of *O. dioica* differs from current model organisms. *O. dioica* are effective filter feeders living in cellulose based gelatinous houses (Greek: Oiko means house) which are secreted by specialized somatic epithelial cells called Oikoplastic cells (Sagane *et al.*, 2010, Hosp *et al.*, 2012). Oikoplastic cells stop dividing before the onset of house production, but continue to increase cell size by endocycling (Ganot & Thompson, 2002). These filter-feeding houses are continuously discarded and replaced every 3-4 h. It is hypothesized that this repetitive process of house making is a mechanism to avoid clogging and ensure maximum filtration and feeding (Tiselius *et al.*, 2003). Moreover, repetitive house production allows *O. dioica* to build a house as per its growth enabling adequate feeding. The discarded houses contribute to marine snow and vertical carbon flux in the ecosystem (Robison *et al.*, 2005).

1.5.2 Life cycle

Conditions and standard densities for *Oikopleura dioica* culture have been optimized in the laboratory. *O. dioica* completes its life cycle within 6 days at 15°C **(Figure 11)** (Bouqet *et al.*, 2009). Upon fertilization, the first cellular division occurs after 30 min. Within 4 h the embryo reaches the tailbud stage, the notochord begins to develop and it resembles a

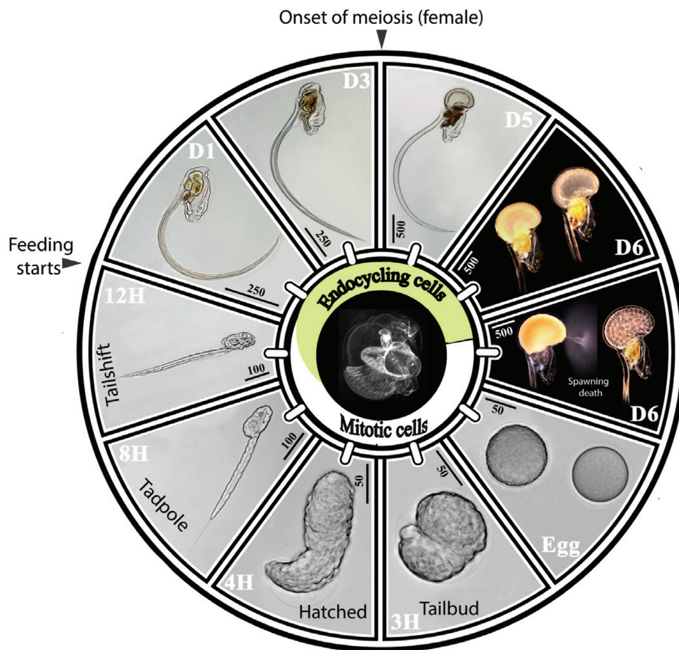

Figure 11. Short life cycle of the chordate *Oikopleura dioica*. The *Oikopleura dioica* life cycle is completed in 6 days at 15°C. After fertilization, the embryo starts mitotic divisions within 30 min. By 3 h post-fertilization, the tailbud is visible. The tadpole hatches at 4 h post-fertilization concurrent with the main onset of organogenesis, and at this stage, the majority of cells are proliferating mitotically. During the early tadpole stages (8 h post fertilisation) cells from the somatic oikoplastic epithelium progressively transit from mitotic proliferation to endocycling. Approximately 12 h after fertilisation, the embryo undergoes tailshift metamorphosis followed by opening of the mouth. 12-14 h post-fertilization, pre-house secretion is visible on the oikoplastic epithelium covering the trunk. This is followed by inflation of the first house and initiation of filter-feeding. From day 1 to day 5 (D1–D5) the animal grows rapidly through extensive use of endocycling in somatic tissues. Germline nuclei proliferate until day 3 and the gonad differentiates

from day 4. In early day 6 (D6-upper panel), sexual differentiation is readily apparent with an immature male (at left) and immature female (at right). At later day 6 (D6-lower panel) mature males (left) release sperm through the spermiduct located on the anterior dorsal surface of the testes and mature females (right) release the oocytes through rupture of the ovary (Ganot *et al.*, 2006). Scale bar in µm. (Adapted and modified from Bouqet *et al.*, 2009)**.**

tadpole, which is bent ventrally. At 8 h post fertilization (pf) development of mouth, digestive track and vacuolation in the notochord occurs. Prior to 12 h pf, the oikoplastic epithelium in the trunk starts secreting pre-houses. At 12 h pf the mouth opens, the vacuoles of the notochord fuse and the germline becomes apparent at the trunk-tail junction after metamorphosis. Under laboratory culture conditions at standard densities, day 1 to day 5 animals grow rapidly through somatic endocycling. During the pre-meiotic stages (up to day 3) the germline expands by mitotic nuclear proliferation. The onset of meiosis in females and males occurs around late day 3 and later than day 5, respectively. Around day 6, sexual differentiation is visible with the naked eye and adult *O. dioica* release their oocytes and sperm into seawater to complete the life cycle.

1.5.3. Somatic growth occurs through endocycling

Somatic growth of *Oikopleura dioica* is mainly determined by cell size rather than cell number by endocycles (Ganot & Thompson, 2002). Intestinal gut epithelial cells are among the few somatic cell types in adults that retain mitotic proliferation. Prior to tailshift, a fixed number of mitotic somatic oikoplastic cells (epithelial) asynchronously transit into endocycling cells. *O. dioica* rapidly grows about 10-fold in size within one week **(Figure 12)**. In adults, the somatic monolayer of endocycling epithelial cells are bilaterally symmetrical and have a specific cellular pattern reflected in different fields with ploidy levels ranging from 34C to 1300C at maturity (Ganot & Thompson, 2002). The potential relationship between rapid growth, lifespan and regulation of endocycling is intriguing because the endocycling epithelium itself produces the filter feeding house, which allows the animals to increase their feeding rates as the life cycle progresses. Thereby the organism can grow rapidly to complete its life cycle within a short period.

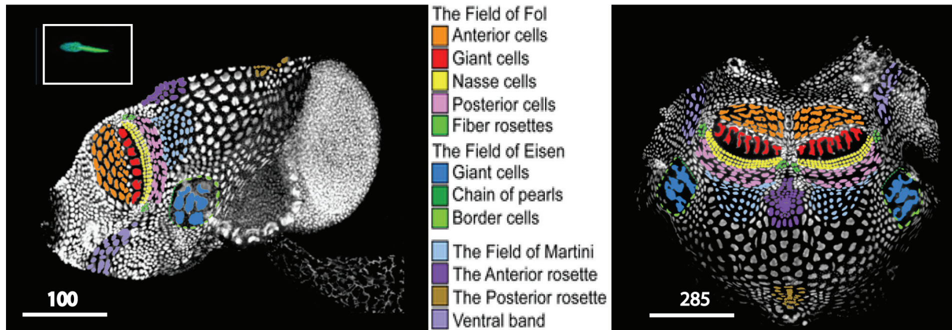

Figure 12. Rapid growth in *Oikopleura dioica via* somatic endocycling. A) *Oikopleura dioica* grows rapidly through somatic endocycling. Lateral view of a day 4 whole mount of *O. dioica,* mouth to the left and gonad at the right. Inset depicts 4h post fertilization tadpole. B) Dorsal view of the somatic epithelium in day 6 *O. dioica*, which has been slit ventrally and spread on a slide, anterior at the top. Nuclei are stained with Hoechst and colours depict specific cellular fields of the epithelium according to the legends in the centre of the figures. Scale bars in µm. (Adapted from Hosp *et al.*, 2012).

1.5.4. Reproductive anatomy and mode of reproduction

The species name "*dioica*" is derived from the fact that *O. dioica* is the only dioecious species in the genus *Oikopleura*. It is a semelparous organism with only one reproductive cycle throughout its lifespan. During embryogenesis, the germline is specified (the details of which have not been elucidated completely) and forms a transparent gonad at the trunk-tail junction after metamorphosis. The gonad in *O. dioica* is a single giant germline cyst. Its size increases throughout the life cycle. It is surrounded by a single layer of follicle cells enveloped by a monolayer epithelium **(Figure 13)**. During the early stages of the life cycle, germline consists of homogenous undifferentiated endo-mitotic nuclei which proliferate in the syncytium (Ganot *et al.*, 2007a, Ganot *et al.*, 2007b). At mid day 3 of the life cycle in females, half of the mitotic germline nuclei in the gonad enter meiosis while the other half enter endocycles. The female germline now becomes heterogenous with respect to nuclear type: there are equal numbers of smaller meiotic nuclei in prophase-I and asynchronously endocycling larger nurse nuclei, all present in a common cytoplasm which is described as coenocyst (Ganot *et al.*, 2007a, Ganot *et al.*, 2007b). The mechanism underlying this asymmetric division into meiotic and endocycling nuclei is not yet elucidated in *O. dioica*.

Following selection of a set of meiotic nuclei for oocyte formation, the surrounding cytoplasm is transferred into the oocytes through the ring canals (Ganot *et al.*, 2006).

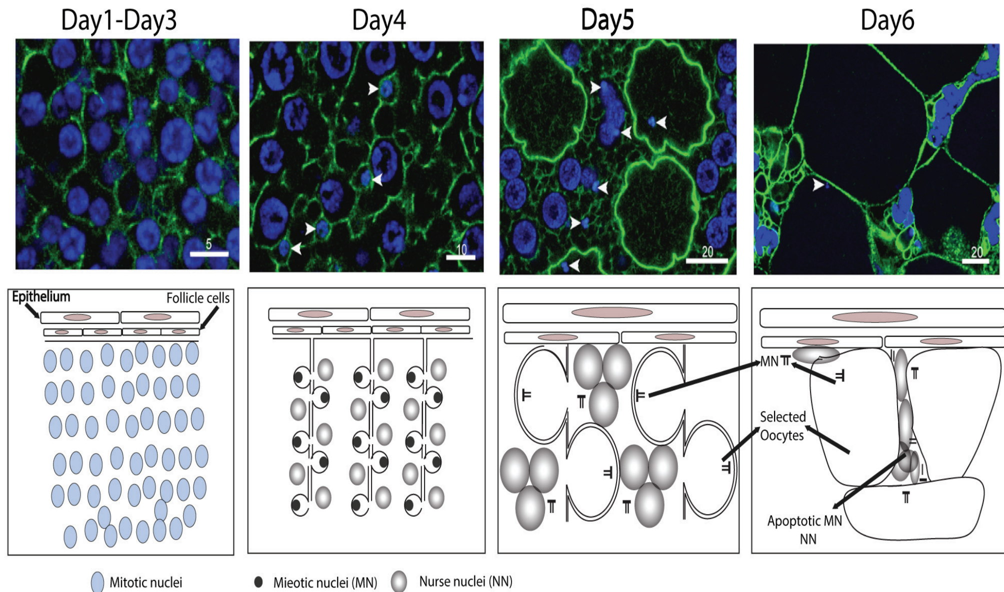

Figure 13. Organization of the ovary in *Oikopleura dioica*. The gonad of *O. dioica* is a single cell germline surrounded by a monolayer of follicle cells enclosed in a monolayer epithelium. In pre-meiotic stages (day 1-day 3) thousands of germline nuclei proliferate in the syncytium, where the nuclei share a common cytoplasm. In the ovary, around mid day 3, the onset of meiosis occurs and results in two different fates for the nuclei in the common "coenocyst" cytoplasm: half enter meiosis (MN) while the other half become endocycling nurse nuclei (NN). An internal F-actin network defines pseudo-compartments (pro-oocytes) surrounding each meiotic nucleus open to the general cytoplasm through a ring canal (F-actin green; nucleus blue in upper panel). At day 5, all meiotic nuclei proceed to a π-conformation of condensed chromatin entering prophase I. In parallel, a subset of pro-oocytes grows rapidly by transfer of cytoplasm through the ring canals. Only a selected number of meiotic nuclei within maturing oocytes will complete meiosis. This number is dependent on the availability of nutrients. At day 6, nuclei external to oocytes (Nurse nuclei and mitotic nuclei) undergo apoptosis, leaving a gonad filled with metaphase I arrested oocytes. Oocytes are released synchronously through rupture of the gonad epithelium. Meiotic nuclei are indicated by arrows. Scale bars in μm. Lower panel are schematics of the organisation of the ovary corresponding to immunofluorescent images in the upper panel (Modified from Ganot *et al.*, 2008).

This causes an increase in the size of the selected meiotic nuclei and eventually gives rise to a mature oocyte arrested in metaphase I. The number of oocytes produced depends on the amount of cytoplasm present within the oocytes, which directly depends on the availability of nutrients (Troedsson *et al.*, 2002). Thus, *O. dioica* can adjust its population size based on algal bloom density. The higher the algal bloom density the higher the fecundity and the larger the *O. dioica* population. At day 6, the unselected meiotic nuclei and the nurse nuclei undergo apoptosis (Ganot *et al.*, 2008). In contrast to female meiosis, meiotic onset and spermatogenesis in male *O. dioica* has not yet been the subject of detailed investigation. The reproductive organization and regulation of GSCs in *O. dioica* differs from that observed in iteroparous organisms like *C. elegans* and *Drosophila.* In these iteroparous organisms, polarity in ovary is maintained, and the signal from niche cell regulates GSCs proliferation and differentiation. However, in semelparous *O. dioica* , so far there is no evidence of presence of niche cells in the germline or a polarity based germline organization. The three somatic polyploid nuclei called *"*Inner Trinucleate Cells" (ITC) might constitute a signaling hub for the germline (Ganot *et al.*, 2006). Nonetheless, there is no clear evidence on regulation of germ stem nuclei governed by ITC in *O. dioica*. Moreover, *O. dioica* does not maintain a pool of GSCs unlike *C. elegans* and *Drosophila,* post the single meiotic entry.

2. Aims of the study

The ageing process is linked to signals from germline, such that the rate of ageing can be adjusted to reproductive status, promoting co-evolution of these two processes. Mechanistic insights into this link have been mainly derived from studies in nutrient-dependent growth arrest of iteroparous model systems such as nematodes and insects. However, the link between nutrients, ageing and reproductive status has not been studied in semelparous model organisms. We identify reversible growth arrest (GA) in response to crowded, nutrient-deprived conditions in *Oikopleura dioica,* a semelparous model within the sister group to vertebrates. In this study, we explore GA in *O. dioica* to understand to what extent lifespan extension mechanisms might be evolutionary conserved.

The principal focus of our work is to address the link between germline status (pre/post meiotic) and lifespan extension in *O. dioica* during nutrient-restricted GA. In order to understand the link between germline status (pre/post meiotic) and lifespan extension, we investigate very specific questions. To begin with, we asked whether nutritional TOR signaling is involved in regulating GA and lifespan? Growth arrest in other model organisms is associated with activation of stress signaling pathways to increase their survival. Hence we also investigate whether the essential stress signaling pathways are activated in *O. dioica* during GA. Further, the present study also aims at deciphering the role of TOR signaling and cell cycle regulators in regulating the mitotic and endocycling cell cycle variants during GA.

Germline stem cells (GSCs) in invertebrate models such as *C. elegans* and *Drosophila* possess a very short or absent G1-phase, and the role of Cyclin D in regulating their GSC cell cycle is unclear. However, vertebrate spermatogonial stem cells have a distinct G1 phase and Cyclin D1, a G1 specific Cyclin, plays an important role in GSC renewal. Nonetheless, the activity of Cyclin Dependent kinase Inhibitor (CKI), negative regulator of the cell cycle, is suppressed during GSC proliferation. Increase in CKI activity causes cell cycle arrest in GSCs of these vertebrate and invertebrate models upon environmental stress. Semelparous *O. dioica* occupies a key phylogenetic position near the invertebrate-vertebrate transition; hence we asked whether there exists a G1-like phase in the GSC cell cycle of *O. dioica*. Moreover, previous reports of transcript profile analysis in

the gonads, revealed that the G1 cyclins were expressed during gametogenesis. In this study, we attempt to elucidate the possible role of the D-type cyclin, in regulating the GSC proliferation in *O. dioica.* Further, we also examine whether the D-type cyclin splice variants regulate the CKI activity during GSC proliferation under standard as well as nutrient-deprived conditions in *O. dioica*.

3. List of papers

Paper I

Gunasekaran Subramaniam, Coen Campsteijn and Eric M. Thompson

Lifespan extension in a semelparous chordate occurs via developmental growth arrest just prior to meiotic entry.

PLoS one, 2014, 9(4): e93787. doi: 10.1371/journal.pone.0093787.

Paper II

Gunasekaran Subramaniam, Coen Campsteijn and Eric M. Thompson

Co-expressed Cyclin D variants cooperate to regulate proliferation of germline nuclei in a syncytium.

Cell Cycle, 2015 (In press). doi: 10.1080/15384101.2015.1041690

4. Summary of results

4.1 Lifespan extension in a semelparous chordate *Oikopleura dioica* occurs *via* developmental growth arrest just prior to meiotic entry (Paper 1)

Under standard laboratory culture densities *Oikopleura dioica* grows rapidly and completes its life cycle within 6 days at 15°C (Bouqet *et al.*, 2009). At higher culture densities, *O. dioica* undergoes growth arrest (GA) retaining the morphology of day 3 pre-meiotic animals without further growth. Under these high culture densities, GA in *O. dioica* increases its lifespan more than 3-fold. During GA, somatic endocycling cells arrest from day 3 onwards. Mitotic germline nuclei and somatic intestinal mitotic cells do not arrest immediately but gradually slow down, attaining full arrest at day 12 in dense cultures. We also observed that upon release of GA *O. dioica* into standard density, cell cycles re-initiated first in the mitotic germline and intestinal cells but later in somatic endocycling cells. Additionally, we demonstrated that increased food under dense conditions reduced GA and increased sexual maturation in a dose-dependent manner. Nutrient-sensitive TOR signaling activity was reduced in GA animals. The differential response of somatic endocycles and mitotic cycles during GA, was also observed under the rapamycin analogue CCI-779, mediated TOR signaling inhibition or in the absence of food. These findings indicate that density-dependent GA in *O. dioica* involves inhibition of nutrient-mediated TOR signaling and that this signaling differentially impacts cell cycle progression in endocycles versus mitotic cycles, as seen in *Drosophila* and *C. elegans* (Britton & Edgar, 1998, Jia *et al.*, 2004, Long *et al.*, 2002). The germline nuclei in GA animals and TOR-inhibited animals did not enter meiosis, when the pre-meiotic stages of *O. dioica* were exposed to dense culture conditions or TOR inhibition. However, exposure to high-density or TOR inhibition during post-meiotic stages did not induce GA and gametes were produced. This observation indicates that inhibition of TOR signaling prevents germline differentiation, entry into meiosis and gamete production under nutrient-limited conditions

experienced prior to meiotic entry. When GA animals were diluted to standard culture densities, they rapidly resumed normal cell cycle progression, and completed their life cycle with no effects on fecundity or F_1 hatching success. On the other hand, animals introduced to dense, nutrient-limited conditions after meiotic entry, matured and spawned as usual at day 6 but exhibited significantly reduced fecundity. Similar rates of hatching success were observed in this F1 progeny, as compared to animals maintained under standard culture densities throughout the life cycle.

Survival MAPK pathways ERK1/2 and p38 and their downstream effector MSK1 were activated in the germline during GA. Inhibition of these MAPK pathways reduced the survival of animals during GA under dense conditions. Pharmacological inhibition of TOR signaling activated p38 and MSK1 but not ERK. Additionally, phospho-ERK1/2 appeared as foci in nuclei of the mitotic germline under dense conditions whereas phospho-p38 appeared as foci in the germline cytoplasm under both dense conditions or during pharmacological inhibition of TOR. Thus, upon inhibition of TOR signaling alone, activation of p38 but not ERK1/2 was critical to survival.

TOR signaling initiates cell cycle progression in mitotic and endocycling cells by regulating G1-S cell cycle regulators, hence we examined the effect of perturbation of TOR signaling on G1-S regulators. In *O. dioica,* inhibition of TOR signaling led to the rapid arrest of somatic endocycles by acting on G1 regulators Cyclin D, E2F and CKI. *O. dioica* expresses multiple D-type cyclins (Da, Db, Dc and Dd), three Cyclin dependent kinase inhibitors (CKIa, CKIb, CKIc), and a single activator E2F1 (typical E2F) and repressor E2F7 (atypical). One of the D-type cyclins, Cyclin Dd exhibited maximal levels in G1 phase with residual Cyclin Dd observed in early S phase in both somatic endocycling cells (Campsteijn *et al.*, 2012) and mitotic germ stem nuclei, suggesting that Cyclin Dd is a major D-type G1 cyclin in both tissues. Levels of another G1 regulator, E2F1, also rose in G phase and dropped in S phase of the mitotic germline. However, E2F1 appeared to be expressed constitutively during both G and S phase in somatic endocycling cells of *O. dioica*. This suggests that oscillation of levels of E2F1 is important in regulating cell cycle progression in mitotic germline nuclei but less so in somatic endocycling cells in *O. dioica*.

TOR signaling regulates the translation of *cyclin Dd* and *E2F1* mRNAs in somatic endocycling cells. Thus, inhibition of TOR signaling during GA, down-regulates Cyclin Dd

and E2F1 levels, resulting in concomitant arrest of somatic endocycles. Similarly, mRNA levels of negative regulators of the G1-S transition, *CKIa* and *E2F7,* increased significantly during GA or inhibition of TOR signaling. CKIa showed enhanced nuclear localization during endocycle arrest in somatic cells. Similarly, very prominent accumulation of E2F7 was observed in the nucleolus of arrested somatic endocycling cells during GA when TOR signaling activity was reduced.

4.2 Co-expressed Cyclin D variants regulate proliferation of germ stem nuclei in a syncytium (Paper II)

In the pre-meiotic stage of *Oikopleura dioica,* mitotic germ nuclei proliferate asynchronously in a syncytium as seen in *C. elegans*. Unlike GSCs in *C. elegans* and *Drosophila, O. dioica* GSCs have a distinct G1 phase. Strikingly, *C. elegans and Drosophila* have only one D-type cyclin (Park & Krause, 1999, Datar *et al.*, 2000) and *O. dioica* has multiple D-type cyclins which might be differentially involved in regulating variant cell cycles in different tissues (Campsteijn *et al.*, 2012). Comparative analyses of human and *O. dioica* Cyclin D domain structure and organization were performed (SMART- http://smart.embl-heidelberg.de and ELM-http://elm.eu.org). This revealed that unlike human Cyclin D, *O. dioica* D-type cyclins contain only the N terminal cyclin box and do not retain the C-terminal cyclin box. Interaction motif prediction revealed that Cyclin Dd considerably differs from other Cyclin D splice variants. It has two Retinoblastoma class (Rb) binding motifs (LXCXE) and one putative phosphodegron motif (present in a few splice variants). GSK-3β phosphorylates Cyclin Dd at the phosphodegron motif, following which Cyclin Dd is exported to the cytoplasm by Chromosomal maintenance 1 or Exportin 1(CRM) for degradation (Alt *et al.*, 2000). Cyclin Dd also contains three predicted Nuclear Localization Sequences (NLS) as well as one nuclear export CRM binding motif. These features are consistent with a possible nuclear role in regulating the cell cycle in different tissues. Another D-type cyclin, Cyclin Db, produces four splice variants-Cyclin Dbα, Dbβ, Dbγ and Dbδ (Campsteijn *et al.*, 2012). Cyclin Dbγ is a full length splice variant. Alternative splicing removes either two exons containing the N-terminal retinoblastoma binding motif (Cyclin Dbα and β) or C-terminal putative phosphodegron motif (Cyclin Dbβ and δ), producing three other splice variants, all of

which retain the cyclin motif**.** Lack of the exon expressing the putative phosphodegron motif at the C terminus produces the short Cyclin Dbβ and δ splice variants, each having an altered unique sequence of 34 amino acids at the C-terminus. Surprisingly, even though Cyclin Dbδ and β have a distinct C terminal sequence, they still retain the C terminal CRM1 cytoplasmic export consensus sequence. However, all Cyclin Db splice variants lack a predicted NLS.

Cyclin Dd levels oscillate and peak in G1-phase before declining in early S-phase in mitotic germline nuclei. This suggests that Cyclin Dd might regulate the G1-S transition in germ nuclei cell cycle in *O. dioica*. Cyclin Dd, typically present in the nucleus, was gradually reduced in arrested germline nuclei and endocycling cells in nutrient-restricted GA animals causing cell cycle arrest. In contrast, Cyclin Dbβ/δ was typically present as cytoplasmic foci and localized adjacent to nuclei during GA and standard culture conditions**.** We observed that these Cyclin Dbβ/δ foci were not found adjacent to nuclei in G1 or M phase, but were found mainly adjacent to nuclei in S phase. Thus there exists a negative correlation between the occurrence of Cyclin Dbβ/δ cytoplasmic foci and G1- or M-phase germline nuclei. We also observed that a high number of early S-phase nuclei have cytoplasmic Cyclin Dbβ/δ foci adjacent to them when compared to late or mid S phase nuclei**.** Thus, these specifically localized Cyclin Dbβ/δ cytoplasmic foci might play a role in G1-S transition of germline nuclear proliferation in *O. dioica*. Unlike Cyclin Dd, *cyclin Dbβ* transcript and protein levels were up-regulated during GA and under TOR inhibition whereas the Cyclin Dbβ were down regulated during MAPK p38 inhibition. Similar to Cyclin Dbβ/δ, CKIa also appeared as cytoplasmic foci in germline cytoplasm in both standard and GA culture conditions of *O. dioica*. Cyclin Dbβ foci colocalized with CKIa in the germline cytoplasm. An increase in the number of CKIa-Cyclin Dbβ foci in the germline cytoplasm suggests that the up-regulated Cyclin Dbβ sequesters up-regulated CKIa in germline cytoplasm, thus permitting cell cycle progression under GA. During GA/TOR inhibition, it can be hypothesized that activated MAPK p38 signaling increases the levels of Cyclin Dbβ in the germline of animals during GA or when TOR signaling is inhibited. This would promote sequestration of CKIa and potentially explain the delayed cell cycle arrest observed in the germline as compared to somatic cells.

To summarize the key findings in this study, nutrient-limited, high-density cultures or direct TOR inhibition caused nutrient-dependent growth arrest (GA) during pre-meiotic stages in *O. dioica*. This GA promoted lifespan extension in pre-meiotic stages but not in post-meiotic stages. TOR signaling was shown to be instrumental in mediating GA as confirmed by rapamycin-mediated TOR inhibition that mimicked the GA phenotype. During GA, endocycling cells arrested immediately (day 3) in response to nutrient limitation/high-density or TOR inhibition whereas germline nuclei exhibited a delayed and more gradual arrest. In endocycling cells, during GA/TOR inhibition, there was reduced TOR activity resulting in reduced Cyclin Dd and E2F1 levels, concurrent with increased CKIa and E2F7 expression, thus causing cell cycle arrest. Despite having low Cyclin Dd levels during GA/TOR inhibition, germ stem nuclei continued to proliferate, due to p38 mediated up-regulation of Cyclin Dbβ cytoplasmic foci which sequestered up-regulated CKIa during GA. Eventually the cell cycle did arrest in germline nuclei probably due to the persistent down-regulation of Cyclin Dd.

5. General discussion

Nutrients acquired by organisms are shared between major metabolic processes such as somatic growth, repair and reproduction. Hence it becomes difficult to study the effect of nutrients on lifespan extension (Kasumovic & Andrade, 2006). Moreover, the availability of nutrients affects reproductive trade-offs between somatic growth and reproduction (Hunt *et al.*, 2004, De Loof, 2011). Generally, nutrient limitation or DR prolongs adult lifespan in a wide range of taxa (Fontana *et al.*, 2010). Prolonged lifespan under DR is associated with transient reduction in reproductive performance, possibly allowing adaptive maximization of future reproduction in organisms when food availability is periodic (Partridge *et al.*, 2005). Evolution of fitness and lifespan depends on how often organisms reproduce (semelparous or iteroparous) in their life cycle (Ranta *et al.*, 2000). In iteroparous organisms, the effects of diet and reproductive effort on lifespan are difficult to delineate for several reasons. Diet-restricted animals may increase their food intake depending upon the nutritional demands of reproduction (Maklakov *et al.*, 2008). Organisms can shift their nutrient allocation patterns based upon the environmental context (Kasumovic & Andrade, 2006). Additionally, differences in the timing and magnitude of reproductive effort vary in a non-linear fashion between animals on different diets, making it difficult to determine if effects on reproduction are solely due to diet (Hunt *et al.*, 2004). As a result, examination of diet-mediated effects on lifespan and mechanisms regulating lifespan, may be most easily examined in semelparous animals that reproduce only once in a lifetime (Stearns, 1992). For such animals, simpler predictions about optimal life history and allocation patterns are possible, wherein fitness is determined in a single reproductive episode (Kasumovic *et al.*, 2009)

Iteroparous model organisms such as mouse, *Drosophila* and *C. elegans* have been primarily used for research on developmental growth arrest (GA), ageing and lifespan extension. These model organisms have been extensively investigated to identify the association between GA, lifespan extension and the reproductive state in organisms. However, this study uses a semelparous chordate *O. dioica* as a model organism to

investigate the link between lifespan and the reproductive state in organisms. Besides having a different mode of reproduction, *O. dioica* also differs from these iteroparous model organisms in the organization of ovary and the mechanism of GSC regulation. The routine iteroparous model organisms display a polarity in organization of the ovary and have a niche cell which regulates the GSC proliferation. Unlike these model organisms, *O. dioica* does not show polarity in the organization of the ovary and does not have a niche cell to regulate GSC proliferation. Moreover, post-meiotic entry, *O. dioica* does not maintain a population of GSCs in proximity to the ITC or elsewhere in the ovary (Ganot *et al.*, 2007b, Ganot *et al.*, 2007a), in contrast to the iteroparous model organisms. We examined to what extent semelparity and germline organization in *O. dioica* affects the known signaling pathways involved in regulating GA, lifespan and the reproductive state under nutrient-restricted conditions.

5.1 *O. dioica* germline nuclei proliferate asynchronously in a syncytium

During the first half of the life cycle, the *Oikopleura dioica* germline continuously proliferates mitotically in a common cytoplasm (syncytium). During the pre-meiotic stage in *O. dioica* life cycle, the males and females cannot be morphologically distinguished. At the mid-life cycle (day 3), all the germline nuclei in the ovary enter meiosis. No reserve GSC pool is maintained in the coenocystic ovary. Due to the presence of a state of syncytial proliferation and absence of differentiated nuclei, the pre-meiotic stages of *O. dioica* serves as a simple model for studying the effect of environmental factors on GSC proliferation. In the pre-meiotic stage, the germline nuclei are present in a common cytoplasm. Ideally, the presence of a common cytoplasm during the pre-meiotic stage should allow an even distribution of cell cycle regulators, which would eventually result in synchronization of the cell cycle in the germline nuclei. However, in the current study, we have reported that despite being in a common cytoplasm, the germline nuclei proliferate asynchronously. The asynchronous proliferation of germline nuclei in the syncytium can be explained by several probable mechanisms that might be operating in the germline nuclei. In eukaryotes, active Cyclin-CDK complexes induce mitotic cell cycle progression (Nurse, 1994). Failure of diffusion of active Cyclin-CDK complexes between adjacent germline nuclei might create a discrete unique cytoplasmic zone around each nucleus, which

regulates cell cycle progression in the individual nuclei. Prevalence of nuclear autonomy wherein each nucleus enters the cell cycle independent of other nuclei, could also possibly explain the asynchronous nature of germ stem nuclei proliferation. RNA binding factors such as GLD-1, FBF1, FBF2 have been shown to regulate proliferation and differentiation of GSCs in syncytium of *C. elegans.* However, no such RNA binding factor has been reported in germline *O. dioica.* It remains to be elucidated if novel RNA binding factor-like proteins might function to regulate germline nuclei proliferation and differentiation in *O. dioica*. Alternatively, it is quite possible that mRNA from periodically transcribed genes gets translated in a local cytoplasm and is redirected back to the transcriptional mother nucleus, imparting cell cycle phase specific identity. Another possible mechanism imparting asynchrony to germline nuclei proliferation would be translation of the cell cycle proteins within individual nuclei. This would prevent post translational diffusion of translated protein into the common cytoplasm (Iborra *et al.*, 2001). Finally, the nuclear pore complex itself could be remodelled in a cell cycle dependent manner such that only certain proteins are allowed to enter nuclei in a particular cell cycle phase (Makhnevych *et al.*, 2003, De Souza *et al.*, 2004). However, it remains unclear as to what benefit the organism derives from an asynchronous proliferation in syncytial germline nuclei. It can be envisaged that this may prevent sudden doubling of the nucleo-cytoplasmic ratio. Further, a common cytoplasm facilitates diffusion of differentiation factors in the entire gonad in this semelparous chordate and also helps in movement of RNA and proteins to oocytes/gametes, where they can be packaged as maternal products (Ganot *et al.*, 2007a).

5.2 A distinct G1 phase in *O. dioica* germline nuclei

In invertebrate model organisms like *C. elegans* and *Drosophila,* self-renewal of GSCs is characterized by a cell cycle which lacks a significant G1-like phase (Hsu *et al.*, 2008, Fox *et al.*, 2011), with no obvious role of the G1 regulators Cyclin D and CDK4/6. It has been proposed that a reduced G1 phase may limit sensitivity to differentiation signaling (Lange & Calegari, 2010, Singh & Dalton, 2009, Orford & Scadden, 2008). On the contrary, prolonging G1 phase in embryonic stem cells did not promote differentiation (Li *et al.*, 2012). We have reported here that invertebrate, chordate *O. dioica* germ stem nuclei exhibit a significant G1-like phase. This is similar to mouse spermatogonial stem cells,

which have a distinct G1 phase with D-type cyclins playing a role in self-renewal (Beumer *et al.*, 2000), hinting at an evolutionary impact on G phase and Cyclin D regulation in GSC cell cycle. We have also observed the expression of another G1 regulator, E2F1, restricted to G1 phase of germline nuclei of *O. dioica*. A greater number of G2 phase nuclei were observed compared to G1 phase nuclei suggesting that the length of G1 phase is shorter than G2 phase. Taken together, these findings suggest that unlike GSCs in *C. elegans* and *Drosophila*, there is a distinct G1-like phase present in cell cycle of germ nuclei in *O. dioica*.

D-type cyclins are major regulators of the G1 phase. They generally accumulate in the nucleus during G1 phase and disappear from the nuclei of cells undergoing DNA synthesis. Earlier reports have shown that *O. dioica* has CDK6 (does not have CDK4) and several D-type cyclin paralogs: Cyclin Da, Db, Dc and Dd (Campsteijn *et al.*, 2012). Additionally, alternative splicing of Cyclin Db produces four different isoforms (α, β, γ and δ). Previously, we have reported that Cyclin Dd acts as a major D-type cyclin with expression levels reaching a peak in G1, however its expression is not restricted to G1 alone and residual expression is observed in early S phase (Campsteijn *et al.*, 2012). Similar Cyclin Dd expression was also observed in mitotic germline nuclei suggesting that Cyclin Dd acts as a major D-type cyclin, regulating the G1-S transition in both somatic endocycling cells and germline nuclei. It is possible that Cyclin D expression/degradation in *O. dioica* is regulated by mechanisms distinct from the Cyclin D expression/degradation mechanisms identified in organisms expressing Cyclin D exclusively in the G1 phase (Baldin *et al.*, 1993).

The Cyclin Db splice variants β and δ, which have a unique C terminus, occur as cytoplasmic foci preferentially adjacent to early S phase (residual expression of Cyclin Dd present) nuclei. This suggests that possibly co-expression of Cyclin Dd and Cyclin Dbβ/δ cooperate in regulating the G1-S transition proliferation of germline nuclei in a syncytium. It has been reported that the improper or partial folding of the unique amino acid sequence in the C terminus of human and mouse Cyclin D2 splice variant, Cyclin D2SV is responsible for cytoplasmic micro aggregation of the protein (Denicourt *et al.*, 2008, Wafa *et al.*, 2013). Thus, perhaps the existence of Cyclin Dbβ/δ as cytoplasmic foci might be due to the partial folding of the unique amino acid sequence in the C-terminus and the

absence of a predicted NLS. Although, these cytoplasmic Cyclin Dbβ foci lack Rb binding motifs, they might have Rb independent regulatory functions such as sequestration of cell cycle regulators.

Structural and functional comparison of mammalian Cyclin D splice variants with *O. dioica* Cyclin Dbβ provided additional insight on the role of Cyclin Dbβ in regulating the cell cycle in *O. dioica*. In mammals, the Cyclin D1 (D1b) and Cyclin D2 (D2SV) splice variants lack the phosphodegron and CRM1 motifs but have a unique C-terminus (Solomon *et al.*, 2003, Sun *et al.*, 2009). The human splice variant Cyclin D1b is constitutively expressed throughout the cell cycle of cancer cells (Solomon *et al.*, 2003) whereas the mouse splice variant D2SV is present as cytoplasmic foci restricted to G1 phase (Sun *et al.*, 2009). *O. dioica* Cyclin Db splice variants β and δ lack the predicted phosphodegron but retain the CRM1 (nuclear exportin1) motif in their unique C-terminus end. Additionally, the absence of the Rb binding motif in Cyclin Dbβ suggests a Rb-independent role and that it might act as a dominant negative in regulating cell cycle progression. Previously we have shown that the *cyclin Db* is expressed in reproductive tissues and that its expression peaked prior to meiotic entry in females, falling thereafter (Campsteijn *et al.*, 2012). These findings support the hypothesis that Cyclin Dbβ/δ might be involved in mitotic germline proliferation in a syncytium.

5.3 Nutrient limitation causes growth arrest immediately prior to meiotic entry in *O. dioica*

The phenomena of developmental arrest and prolongation of lifespan have been studied in detail in some invertebrates, however it has not been investigated in vertebrate model systems. Developmental stage specific GA and lifespan extension has been reported in *C. elegans* and *Drosophila*. In *C. elegans,* nutrient limitation in pre-meiotic stages produces L1 and L2 dauer arrest (Lee I 2012, Randall C. Cassada, 1975). Similar to the dauer larvae, *Drosophila* enter diapause during the larval stage (Tatar *et al.*, 2001). Our pioneer study demonstrates that *O. dioica,* a sister chordate to vertebrates, undergoes reversible GA and increases its lifespan in response to nutrient-limited, high-density cultures. *O. dioica* cannot survive under complete starvation but can survive with minimum nutrition in high-density cultures. High nutrient availability increases fecundity but they

cannot survive with too much food since it clogs houses (Troedsson *et al.*, 2002). In this study we demonstrated that *O. dioica* adopts two alternative mechanisms to survive under nutrient-deprived conditions. This depends on the developmental stage (pre or post-meiotic) during which it is exposed to adverse environmental conditions. High-density cultures of *O. dioica* up to day 3 induce GA and prolong lifespan up to 3- fold. Occurrence of this GA coincides with meiotic onset in females (late day 3) suggesting that GA occurs just prior to meiotic entry. These findings cannot be extended to males since meiotic onset in males occurs at a later stage than in females. We have not yet investigated which precise stage of germline progression this day 3 arrest corresponds to in males. GA animals produce normal viable progeny when returned to favorable conditions. However, if *O. dioica* are exposed to high density, nutrient-limited cultures during the post-meiotic stage, they do not undergo GA but instead produce a smaller number of oocytes. This finding also suggests that the number of nuclei selected as well as the volume of cytoplasm in the coenocystic ovary, decreased under nutrient limitation, leading to reduced number of oocytes during oogenesis **(Figure 14).** Such animals complete their life cycle in the normal time frame. The strategy of producing a small number of progeny serves as a means to generate a lower population density in the next generation. Nutrient limitation during adult post-meiotic stages in iteroparous invertebrates like *C. elegans* and *Drosophila* results in reproductive arrest but these organisms maintain a germ stem pool until the onset of favorable conditions (Angelo & Van Gilst, 2009, Drummond-Barbosa, 2001). Conversely, the post-meiotic high-density cultures of *O. dioica* retain the differentiated gonad but do not maintain any distinct GSC pool suggesting the existence of a distinct adaptive mechanism in semelparous model organisms.

5.4 Reduced TOR signaling causes growth arrest by blocking the meiotic entry in *O. dioica*

TOR signaling acts as a sensor of nutritional and Insulin cues, thus playing a central role in regulating growth, reproduction and lifespan in various organisms. We demonstrated that TOR signaling was reduced in GA animals emphasizing its role in regulating growth and lifespan in *O. dioica*. Our findings are in concert with those in other organisms where reduced TOR signaling has been associated with GA and increased

lifespan. Inactivation of *CeTOR* or TOR signaling component *daf-15* (raptor) induces larval arrest and increases lifespan in *C. elegans* (Jia *et al.*, 2004, Long X, 2002). Similarly in *Drosophila*, *TOR* homozygous mutants (strong *TOR* mutant) produce arrested larvae (Zhang H, 2000) and *TOR* mutants (weak *TOR* mutant) in females frequently lose their GSCs (Oldham *et al.*, 2000). Several genetic studies revealed that meiotic onset of GSC is regulated by TOR signaling (Fukuyama M *et al.*, 2012, Sun *et al.*, 2010), but it has never been reported that direct inhibition of TOR by rapamycin affects GSC differentiation. This study benefits from the simple reproductive anatomy and single episode of meiotic onset in semelparous *O. dioica* to understand the role of germline differentiation and longevity under direct pharmacological inhibition of TOR signaling.

We have identified the *TOR* ortholog in *O. dioica* **(Figure A1)** and shown that rapamycin-mediated TOR inhibition prevented meiotic onset and asymmetric germline differentiation. In *C. elegans,* the response to starvation and energy stress involves the activation of AMP Kinase (AMPK). AMPK activates TSC1/2, which represses TOR signaling and maintains GSC quiescence and survival during L1 arrest (Fukuyama M *et al.*, 2012). TSC1/2 also prevents germ cell differentiation by repressing TOR signaling in *Drosophila* (Sun *et al.*, 2010). Under nutrient-limited conditions, *O. dioica* might activate low energy dependent AMPK leading to TSC1/2 like protein mediated TOR inhibition to suppress germline cell cycle and differentiation or shut down the translation of meiotic onset specific genes as proposed in *Saccharomyces* (Zheng & Schreiber, 1997) and *C. elegans* (Jia *et al.*, 2004). Thus, our findings implicate nutrition and nutrition-dependent TOR signaling as a key determinant of growth, germ stem nuclei differentiation and lifespan in *O. dioica.* However, it is unclear whether TOR signaling acts directly or mediates its action through Insulin/IGF-like signaling. The possible roles of Insulin signaling in regulating *O. dioica* lifespan should be investigated.

5.5 TOR signaling differentially regulates endocycling and mitotic tissues during growth arrest in *O. dioica*

Nutrients differentially regulate endocycling and mitotic cells in *Drosophila* (Britton & Edgar, 1998). Loss of TOR function reduces cytoplasmic volume in endocycling tissues whereas mitotic cells grow normally in *Drosophila* and *C. elegans* (Oldham, 2000, Long

X, 2002, Jia *et al.*, 2004). These studies demonstrated that, starvation or inhibition of TOR signaling, arrests the endocycling cells immediately but does not arrest the mitotic cells immediately. However, which cell types re-enter the cell cycle in response to re-feeding/TOR activation was not elucidated in these model organisms. The body plan of post-metamorphic *O. dioica* is made up of endocycling (somatic epithelial) and mitotic (germline and somatic intestinal) cell types. The simple anatomy of this chordate helps in studying the nutrient-dependent, differential regulation of endocycling and mitotic cell cycles. We showed that TOR localized to the cytoplasm of both somatic endocycling cells and mitotic germline **(Figure A2)** but nutrient limitation or direct inhibition of TOR signaling in *O. dioica* instantly shuts down somatic endocycling but not the mitotic cell cycle (germ nuclei and intestinal cells). The latter gradually slowed down and ceased to cycle over time. Upon release of GA animals into standard density, cell cycling was restored in mitotic tissues within 4 h but resumed after 5 h in endocycling cells. We hypothesize that energy stress and AMPK activation might gradually reduce TOR activity and cause a gradual arrest of germline proliferation during GA in *O. dioica.* Thus there exists a differential regulation of endocycles and mitotic cycles under nutrient-limiting conditions, probably to ensure selective allocation of nutritional resources towards reproduction as opposed to investing it on growth. Mitotic cells might also adopt a different homeostasis mechanism as a means to preclude stress related to nutrient deficiency, in order that the animal survives and reproduces (McLeod *et al.*, 2010). G1 phases of mitotic and endocycling cells are differentially regulated by TOR signaling but the exact mechanism remains poorly understood. In this study, we have gained insight into molecular mechanisms underlying the differential regulation of mitotic and endocycling cells by TOR signaling.

5.5.1 Cyclin Dd and CKIa mediate the effects of TOR signaling in endocycling and mitotic cells

Nutrient-dependent TOR signaling differentially regulates the endocycling and mitotic cells. These cell cycles utilize similar G1-S transition machinery, which includes Cyclin D, CKI and E2Fs. In mammals, TOR signaling positively regulates TOP-dependent *cyclin D* translation (Coqueret, 2002). Similarly, nutrient-dependent Insulin/IGF-like

signaling also positively regulates Cyclin D stability. Absence of IGF signaling, activates GSK-3β, causing Cyclin D degradation in mammals (Takahashi-Yanaga & Sasaguri, 2008). In *C. elegans* and *Drosophila,* starvation or ablation of Insulin/IGF signaling arrests somatic cells in G1 phase whereas the GSCs are arrested in G2 phase. In these invertebrate models, G1 is nearly absent and Cyclin E is expressed throughout the cell cycle, plays an important role in GSC renewal (Hsu *et al.*, 2008, Fox *et al.*, 2011). However, the role of Cyclin D-CDK4 complexes regulating GSCs proliferation is unclear (Narbonne & Roy, 2006b, Hsu *et al.*, 2008). Moreover, TOR signaling promotes S phase entry in *Drosophila* and mutants of *cyclin E* exhibit cell cycle arrest in G1 of *Drosophila* GSCs. These findings imply that TOR may regulate the G1-S transition by controlling *cyclin E* translation in GSCs of these invertebrates (Hsu *et al.*, 2008, Hsu & Drummond-Barbosa, 2009). Likewise, nutrient-dependent TOR signaling positively regulates translation of the *Cyclin E* promotes endocycling in *Drosophila* (Britton & Edgar, 1998). Cyclin D is also expressed during the endocycle of *Drosophila*, but functions primarily in promoting growth and does not seem to have a direct role in promoting cell cycle progression (Datar *et al*., 2000). In contrast with *Drosophila,* Cyclin Dd acts as a major D-type cyclin regulating the G1-S transition of both endocycling and mitotic cells in *O. dioica* (Campsteijn *et al.*, 2012). In this study, we demonstrated that TOR signaling promotes the G1-S transition by regulating the translation of *cyclin Dd* mRNA in *O. dioica* **(Figure 13 and Figure14).** The transcript levels of *cyclin Dd* were unaffected in GA animals where TOR signaling was reduced, though Cyclin Dd protein levels were lower in GA *O. dioica.* Under TOR inhibition Cyclin Dd levels were reduced immediately in arrested endocycling cells but persisted in the proliferating germline nuclei. During GA, Cyclin Dd levels were gradually reduced in germline nuclei that eventually arrested in G1 phase of their cell cycle. In *C. elegans,* low energy levels, under nutrient-deprived growth conditions, activate TOR negative regulators AMPK and LKB1, reducing GSC division rates (Narbonne & Roy, 2006a). Thus, somatic endocycle progression is regulated by TOR signaling *via cyclin Dd* translation. In mitotic germ nuclei, cell cycle progression is possibly regulated by the expression of Cyclin Dd. It is possible that during GA, the *cyclin Dd* translation is gradually decreased due to the reduction of TOR activity and diminishing energy levels.

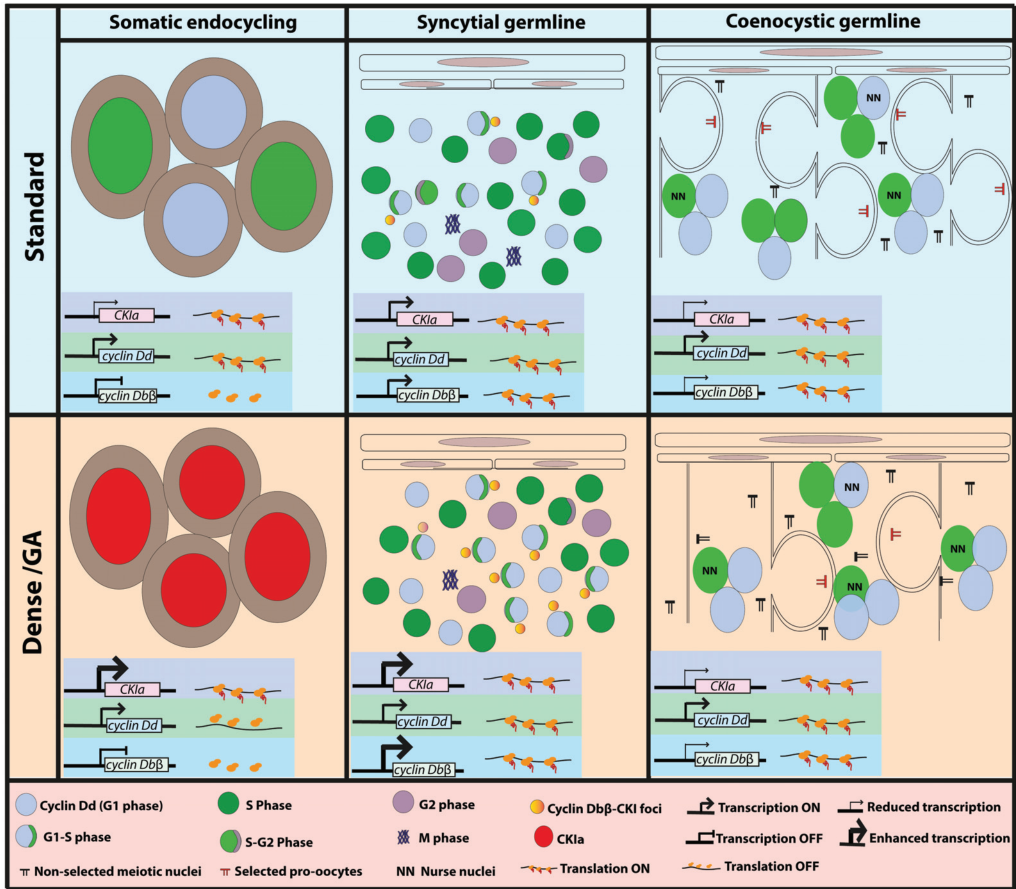

Figure 14. Nutrient-dependent differential regulation of cell cycle variants in *O dioica.* Somatic endocycling cells: In somatic endocycling cells, the transcription and translation of *cyclin Dd* occurs and regulates the G phase. However, Cyclin Dbβ and CKIa proteins are not detected under standard culture conditions. Under GA, translation of *cyclin Dd* is reduced with no change in the transcription of *cyclin Dd* mRNA. Both mRNA and protein levels of CKIa increase in endocycling cells during GA and the protein is concentrated in the nucleus. **Syncytial germline**: Cyclin Dbβ and CKIa proteins are detected in the mitotic germline. Both the proteins co-localize in the cytoplasm adjacent to early S phase nuclei to promote G1-S transition. During GA, *cyclin Dd* mRNA translation is not affected immediately but the Cyclin Dd protein level reduces gradually. *CKIa* and *cyclin Dbβ* transcription is up-regulated. Cyclin Dbβ and CKIa cytoplasmic foci increase in number during GA. **Coenocystic germline:** Under standard and dense culture conditions, Cyclin Dd protein is present in the G phase whereas the Cyclin Dbβ and CKIa proteins are not detected in the endocycling nurse nuclei. Under dense culture conditions at post-meiotic stages, the cytoplasmic volume of the coenocystic germline as well as the number of selected meiotic nuclei is reduced.

Another major class of G1 regulators, Cip/Kip family Cyclin dependent Kinase Inhibitors, (CKIs) is negatively regulated by nutrient-dependent TOR signaling. CKI has been reported to inhibit G1-S transition and cause cell cycle arrest. CKI causes G0 cell cycle arrest under TOR inhibition or starvation by inhibiting Cyclin E-CDK2, Cyclin D-CDK4/6 complexes or PCNA (Proliferating cell nuclear antigen) in the nucleus. In the *O. dioica* genome, we identified three different CKIs from the Cip/Kip family , *CKIa, CKIb and CKIc* **(Figure A3),** but did not find any CKI from the INK family**.** The role of CKIa in endocycles and the mitotic germline in *O. dioica,* under nutrient-deprived conditions has been characterized in this study. We also demonstrated that during TOR inhibition, both the transcript and protein levels of CKIa were increased. Moreover, CKIa localized to the nucleus of somatic endocycling cells under rapamycin mediated inhibition of TOR as well as in GA in *O. dioica* (**Figure 14**). In *O. dioica,* CKIa expression was undetectable in somatic endocycling cells under standard culture conditions. In *Drosophila,* CKI Dacapo is dispensable for endoreplication in salivary glands and ovarian follicles, but is expressed in an oscillatory manner in endocycling ovarian nurse cells (Edgar *et al.*, 2014). Taken together, under nutrient limitation or TOR inhibition CKIa is expressed in a non-oscillatory manner in somatic endocycling cells and causes arrest in these cells of *O. dioica* **(Figure 15).** Probably the increased nuclear localization of CKIa causes cell cycle arrest in somatic endocycling cells by inhibiting the activity of Cyclin-CDK complexes and PCNA under GA or TOR inhibition.

In the mitotic germline, CKIa occurs as cytoplasmic foci located preferentially adjacent to early S phase nuclei. The CKIa foci were not observed in the nuclei and the number of cytoplasmic CKIa foci increased under nutrient-deprived GA. Generally, CKI activity is suppressed either at the level of transcription or by modulating the stability of the protein for promoting self-renewal of GSCs in other model organisms (Hong *et al.*, 1998, Kalchhauser *et al.*, 2011, Beumer *et al.*, 1999, Beumer *et al.*, 1997). Moreover an increase in CKI activity causes cell cycle arrest in GSCs under starvation in *C. elegans* and *Drosophila* (Narbonne & Roy 2006b). Additionally, TOR signaling negatively controls the stability of p27, a CKI, in mammalian cells (Leung-Pineda *et al.*, 2004, Hong *et al.*, 1998). Taken together these findings suggest that CKIa is possibly rendered inactive by cytoplasmic sequestration in the germline under nutrient-deprived conditions. As a result,

the G1-S transition is not blocked by CKIa and the proliferation of germline nuclei continues in the germline. We need to further elucidate the possible mechanisms and roles of CKIa and other CKIs in regulating proliferation of somatic endocycling and germline nuclei in *O. dioica*. DAF-16, a FOXO transcription factor promotes the transcription of *CKI-1* which induces cell cycle arrest in L1 arrested *C. elegans* (Baugh & Sternberg, 2006). FOXOs also regulate the transcription of *p27* in mammalian cells (Dijkers *et al.*, 2000). Consistent with these findings, our study showed that the *FOXO* ortholog of DAF-16, was up-regulated during GA in *O. dioica* **(Figure A4)**. The presence of FOXO binding consensus sequences in the *CKIa* promoter, suggests that the up-regulation of CKIa transcripts might be mediated by activation of FOXO during nutrient-deprivation.

There is evolutionary diversity in the interaction and localization of Cyclin D-CDK4/6-(CKI) complexes. The Cyclin D and CDK4/6 proteins do not possess an obvious Nuclear Localization Sequence (NLS) in mammals (Bockstaele *et al.*, 2006a, 2006b). However, Cip/Kip proteins possess a well-characterized bipartite NLS signal in their C-terminus and this protein family required for the nuclear localization of Cyclin D-CDK4 (Bockstaele *et al.*, 2006b, LaBaer *et al.*, 1997, Bockstaele *et al.*, 2006a). However, *C. elegans* and *Drosophila* CKI do not act as nuclear localization factors for Cyclin D–CDK4 complexes. In *C. elegans*, the presence of the NLS in Cyclin D may drive the transport of Cyclin D-CDK4 complexes into the nucleus to promote G1-S transition in somatic cells **(Table A1).** In contrast, Dacapo, a *Drosophila* CKI, does not bind to or inhibit the Cyclin D–CDK4 complexes (Meyer *et al.*, 2000), and the NLS in CDK4 might drive Cyclin D-CDK4 complexes into the nucleus. In *O. dioica,* Cycln Dd and CKIa have a predicted NLS whereas CDK6 does not have any NLS. Moreover, we detected Cyclin Dd but not CKIa in the G1 phase of somatic and germline nuclei. This suggests that CKIa is not required for nuclear localization of Cyclin Dd-CDK6 complexes during G1 phase in *O. dioica.*

5.5.2 Differential regulation of E2Fs in mitotic and endocycling cells during growth arrest

Another cell cycle regulator, the E2F1 transcription factor, is regulated by TOR signaling in *Drosophila* (Zielke *et al.*, 2011). We asked whether E2Fs were regulated by TOR signaling in mitotic and endocycling cells of *O. dioica* under GA. In mammals, nine

activator and repressor E2Fs have been described (van den Heuvel & Dyson, 2008). Understanding the functional and structural redundancy among these E2F family members is challenging, complicating their functional interpretation, which poses as a major obstacle in *in vivo* E2F studies. Hence, organisms with fewer E2Fs are preferable when studying E2F functions. Unlike mammals, *Drosophila* has a single typical activator and typical repressor E2F, but does not have atypical E2Fs (van den Heuvel & Dyson, 2008). We identified a single typical activator *E2F1* and atypical repressor *E2F7* in the *O. dioica* genome **(Figure A5)**. Due to the presence of a single typical and atypical E2F, *O. dioica* serves as an excellent model to understand the antagonistic functions of typical activator E2F1 and atypical repressor E2F7 during cell cycle regulation.

In the mammalian mitotic cell cycle, the activity of E2F1 is controlled in a cell cycle phase dependent manner. E2F1 is expressed during G1 phase and is bound to E2F1 target promoters through the DNA-binding subunit DP1 (Flemington *et al.*, 1993). These E2F-DP1 complexes activate transcription and promote G1-S transition. During G1 phase, Rb binds E2F1 and prevents the formation of E2F1-DP1 complexes. Upon activation in response to mitogenic cues, Cyclin D/E-CDK complexes phosphorylate Rb. Phosphorylated Rb dissociates from E2F1, allowing E2F1 to activate transcription from target genes and promote S phase entry. During S-phase exit, E2F1 and DP1 subunits are phosphorylated by Cyclin A-CDK2 complexes, resulting in the release of bound E2F1-DP1 complexes from target promoters. The released E2F1 is destroyed by SCF-mediated ubiquitination and degradation in G2-M phase (Flemington *et al.*, 1993, Helin *et al.*, 1993). Similarly, E2F1 expression is detected only in G1 phase of mitotic imaginal disc cells in *Drosophila* (Asano et al., 1996). However, in *Drosophila* salivary gland endocycling cells, E2F1 is expressed in G phase but degrades during S-phase (Zieke *et al.*, 2011). This proteolytic destruction of *Drosophila* E2F1 during S phase depends on the E2F1 PIP (PCNA interacting protein) box motif. The E2F1 PIP motif binds PCNA and mediates an interaction with CRL4 ubiquitin ligase (Shibutani *et al.*, 2008). Unlike E2F1 dynamics in the *Drosophila* salivary gland, *O. dioica* E2F1 is constitutively present in G-and S-phases of somatic endocycling cells but is detected only in G1 phase of germline mitotic nuclei under standard culture conditions. Moreover, *O. dioica* E2F1 lacks the PIP box degradation motif. Taken together these findings suggest that distinct E2F1 regulatory mechanisms are

involved in controlling endocycles in *Drosophila* and *O. dioica*. The absence of the PIP box in E2F1, and apparent absence of Cyclin A-CDK2 complexes in regulating E2F1 degradation in endocycling cells (Campsteijn *et al.,* 2012), suggest that E2F1 might be differently regulated in *O. dioica*. Possibly, Cyclin D/E-CDK complexes promote G-S transition, by phosphorylating Rb on target promoters bound by Rb-E2F complexes (Flemington *et al.*, 1993, Helin *et al.*, 1993) in endocycling cells of *O. dioica.*

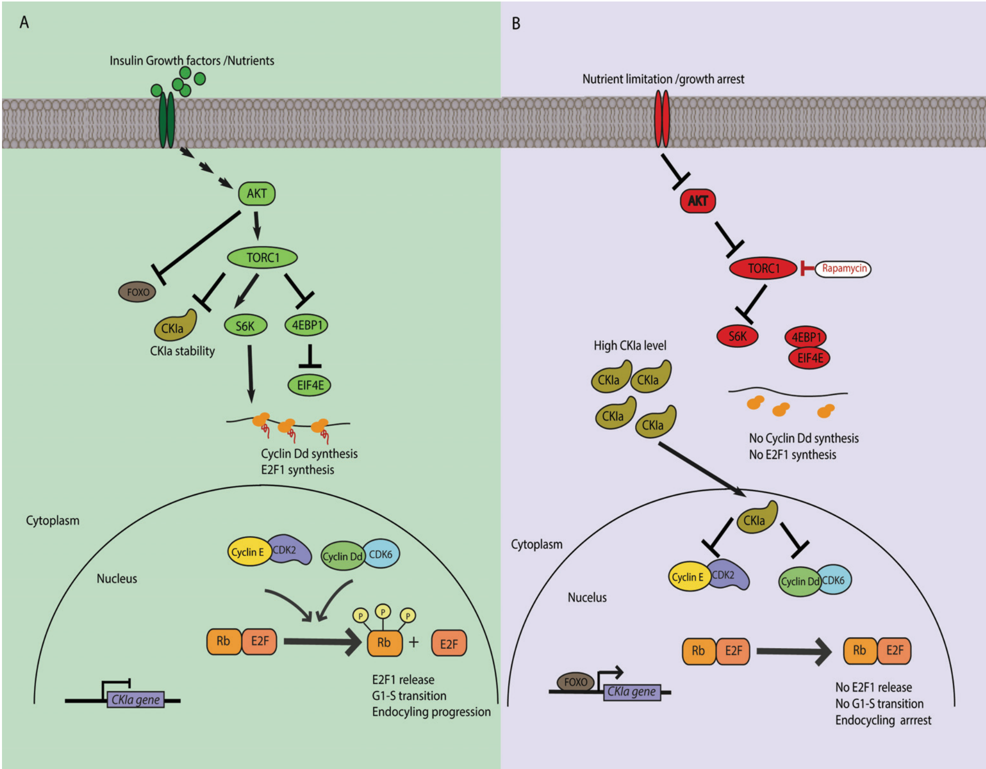

Figure 15. TOR mediates somatic endocycle progression. A. Under standard growth conditions, Insulin and TOR signaling control the G-S transition by positively regulating the translation of Cyclin Dd, E2F1 and negatively regulating the translation of CKIa. Cyclin–CDK complexes promote G-S transition through release of E2F1 from Rb. B. During growth arrest or direct inhibition of TOR by rapamycin, endocycling cells arrest immediately due to reduction of Cyclin Dd and E2F1 levels concomitant with increased levels of CKIa. CKIa is translocated to the nucleus, possibly to inhibit CyclinD-CDK6 and CycinE-CDK2 complexes**.** Under nutrient-limited conditions, CKIa up-regulation might be mediated by the activation of FOXO transcription factors during growth arrest (Unpublished data from Danks *et al*.).

Starvation in *Drosophila* larvae induces cell cycle arrest in endocycling and mitotic cells. This arrest was overcome by over-expression of E2F1 in endocycling cells but not in mitotic cells (Britton *et al* 1998). Moreover nutrient-dependent TOR signaling positively regulates translation of *E2F1* and promotes endocycling in *Drosophila* (Zielke *et al.*, 2011b, Britton & Edgar, 1998). We also demonstrated the importance of E2F1 for somatic endocycle progression in *O. dioica* during GA/ TOR inhibition **(Figure 15)**. Under nutrient-deprived growth conditions, *E2F1* mRNA levels were not affected but protein levels became almost undetectable and somatic endocycles ceased. However, expression of E2F1 protein did not change in the mitotic germ nuclei under nutrient limitation. Thus, the translation of E2F1 was responsive to nutrient availability in a TOR-dependent manner in endocycling cells, but not to the same extent in mitotic cells.

The atypical Rb independent transcription repressor E2F7 acts as an antagonist for E2F1 functions and prevents the G1-S phase transition in mammalian endocycling trophoblast giant cells (Chen H. Z *et al.*, 2012). Similarly, E2F7 promotes the G2-S transition in mitotic cells by repressing E2F1 targets (Westendorp *et al.*, 2012). In *O. dioica* cultured under standard conditions, E2F7 localized to the nucleolus of G and early S phase nuclei but not to the mid-S phase somatic endocycling cells **(Figure A6).** This suggests a possible role in inhibiting the G-S phase transition. During GA, both *E2F7* transcripts and protein levels increased and the protein accumulated in arrested endocycling cells **(Figure A6).** There is ambiguity in the literature regarding the role of different atypical E2Fs in regulating endocycles. Atypical E2Fe in *Arabidopsis* is required to prevent endocycles (Lammens T, 2008) whereas atypical E2Fs in mammals promote endocycles (Chen H. Z *et al.*, 2012). Additionally, atypical E2Fs play a major role in cell survival, regulation of stress, hypoxia, and apoptosis (Li J, 2008). In *O. dioica*, E2F7 accumulates in the nucleolus of arrested somatic endocycling cells during GA suggesting that E2F7 has a role in blocking somatic endocycle progression in *O. dioica* under nutrient limitation. Recent reports have revealed that cellular stress induces nucleolar accumulation of E2F1 causing repression of rRNA transcription, and ribosomal biosynthesis, leading to cell cycle arrest in human cells (Jin *et al.*, 2014). Accumulation of nucleolar E2F7 under nutrient limitation may also be a mechanism to promote survival of

O. dioica under GA. The precise role(s) of E2F7 in cell cycle regulation of endocycling and mitotic tissues and survival need to be investigated further in *O. dioica.*

5.6 Activation of survival signaling MAPK in the germline during growth arrest

MAPK cascades are evolutionarily conserved signaling pathways activated under various environmental stresses to promote survival and extend lifespan (Troemel *et al.*, 2006). Activation of ERK1/2 and p38 signaling has been shown to play a major role in increasing longevity in *C. elegans* (Troemel *et al.*, 2006, Okuyama *et al.*, 2010). Furthermore, activation of MAPK p38 in germline was also shown to extend lifespan in *C. elegans* (Alper S, 2010), whereas p38-dependent MSK1 activity plays a pro-survival role in mammalian cells (van der Heide *et al.*, 2011). Our studies showed that inhibition of MAPK ERK1/2 and p38 during dense cultures of *O. dioica* resulted in decreased survival, consistent with the role of these MAPK pathways during stress to improve survival. Moreover, we also demonstrated an increase in the active forms of the MAPK ERK1/2 and p38 as well as activation of their common downstream effector MSK1 in the mitotic germline under GA. These findings further substantiate the role of MAPK pathways in increasing survival and lifespan during nutrient-deprived GA. Although GA was characterized by reduced TOR signaling activity, the rapamycin-mediated inhibition of TOR signaling, activated p38-MSK1 signaling but not ERK signaling in the germline. This suggests that absence of TOR signaling in GA animals directly activates p38-MSK1 signaling in the mitotic germline whereas activation of ERK1/2 is independent of inhibition of TOR signaling. Moreover, MAPK signaling positively regulates the mitotic cell cycle (Wilkinson & Millar, 2000), suggesting that the activation of MAPK ERK1/2 and p38 signaling in the germline might be involved in maintaining the mitotic cell cycle in the germline under GA. Additionally, MAPK ERK1/2 and p38 signaling positively regulate TOR signaling and stimulate protein synthesis (Cully *et al.*, 2010, Miyazaki M *et al.*, 2011) suggesting that the MAPK p38 pathway might be activated to compensate for the reduction of TOR signaling in the mitotic germline during GA in *O. dioica.* In light of the above findings, we conclude that the activation of ERK1/2, p38 and MSK1

in the germline promotes survival and extends lifespan in *O. dioica* during nutrient limitation.

5.7 MAPK p38 signaling promotes *cyclin Dbβ* transcription during growth arrest

In human fibroblasts, activation of MAPK p38 signaling either arrests or promotes the cell cycle depending on the nature of the upstream inducer (Faust *et al.*, 2012). Chemical stress induces stronger and longer activation of p38, inhibits the transcription of *cyclin D1* and causes cell cycle arrest. On the other hand, mitogenic stimuli induce a weak and transient activation of p38, which stimulates *cyclin D1* transcription and promotes cell cycle progression. It is also known that MAPK p38 signaling activates the transcription factor CREB, which stimulates *cyclin D* transcription (Deak *et al.*, 1998, Iordanov *et al.*, 1997, Wiggin *et al.*, 2002). Hence, we investigated whether the activated MAPK p38-MSK1 promotes the transcription of D-type cyclins in *O. dioica* under nutrient deprivation in the mitotic germline. We observed that the transcripts of *cyclin Dbβ* splice variant as well as the number of cytoplasmic Cyclin Dbβ foci increased in the germline during GA. However, the precise splicing mechanism of Cyclin Dbβ under GA needs to be elucidated. The up-regulation of Cyclin Dbβ in the proliferating germline under GA, suggests that Cyclin Dbβ might positively regulate the germ nuclei proliferation under nutrient deprivation. This contrasts with studies showing that up-regulation of Cyclin D2 and splice variant D2SV was observed in cell cycle-arrested mammalian cells during starvation (Wafa *et al.*, 2013, Meyyappan *et al.*, 1998, Ahmed *et al.*, 2008). We also have demonstrated that activation of MAPK p38 signaling promotes *cyclin Dbβ* transcription. The presence of CREB-binding consensus sequences in the *cyclin Db* promoter suggests that p38-MSK1 possibly induces *cyclin Dbβ* expression through phosphorylation of the CREB protein in the mitotic germline, though this remains to be directly verified. Thus p38-mediated increase of Cyclin Dbβ in germline cytoplasm, may be one of the mechanisms responsible for promoting cell cycle in germline nuclei of *O. dioica* under GA.

5.8 Cyclin Dbβ sequesters CKIa and promotes germline nuclei proliferation during growth arrest

In mammalian cells, upregulation of Cyclin D forms Cyclin D-CDK4 complexes which sequester CKI in the nucleus thereby reducing freely available CKI. As a result, the Cyclin E-CDK2 complexes are not inhibited by CKI resulting in the G1-S transition (Sherr 1999). Sequestration of CKI has also been reported in the cytoplasm in order to promote cell cycle progression in cancer cells (Kim et al., 2009, Zhou *et al.*, 2001, Serres *et al.*, 2011, Wu *et al.*, 2006). Mutation of CDK activating kinase (CAK) residue T156 in human Cyclin D1 results in the formation of an inactive complex with CDK4 which sequesters p27 in the cytoplasm of NIH/3T3 cells (Diehl & Sherr, 1997). Similarly, a K112 mutant of Cyclin D1 forms an inactive complex with CDK6 which sequesters p27 in mammalian fibroblasts (Landis *et al.*, 2006). Inactive complexes of human and mouse Cyclin D2 splice variant D2SV (does not retain T156) with CDK4 are also localized exclusively to the cytoplasm and regulate cell cycle progression by sequestering p27 along with other cell cycle regulators (Cyclin D, Cyclin B, and CDK4) (Denicourt *et al.*, 2008, Sun *et al.*, 2009, Wafa *et al.*, 2013). It has further been shown that inhibition of TOR signaling in mammalian cells, increases the cytoplasmic sequestration of p27, promotes the G1-S transition in mitotic cell cycles and increases survival (Short *et al.*, 2008, Hong *et al.*, 2008). Similarly, reports in *C. elegans* show that Cyclin D–CDK4 complexes and CKI-1 inhibit each other in somatic cells (Boxem & van den Heuvel, 2001). This suggests that Cyclin D-CDK4 complexes may contribute to CKI-1 inactivation, possibly through CKI-1 sequestration. However in *Drosophila,* Dacapo does not bind the Cyclin D-CDK4 complex (Meyer *et al.*, 2000), and hence may not use CKI sequestration as a mechanism to drive G1-S progression.

In *O. dioica,* increased CKIa protein levels were observed in arrested endocycling somatic cells. However germline nuclei did not arrest despite increased levels of CKIa, during GA. We demonstrated that Cyclin Dbβ interacts with CDK6 and CKIa, suggesting that the Cyclin Dbβ-CDK6 complex binds CKIa. Furthermore, we also observed that cytoplasmic foci of Cyclin Dbβ and CKIa colocalize in the mitotic germline cytoplasm. The number of these cytoplasmic Cyclin Dbβ-CKIa foci increased in proliferating germline under nutrient-dependent GA. Cyclin Dbβ is thought to act as a dominant

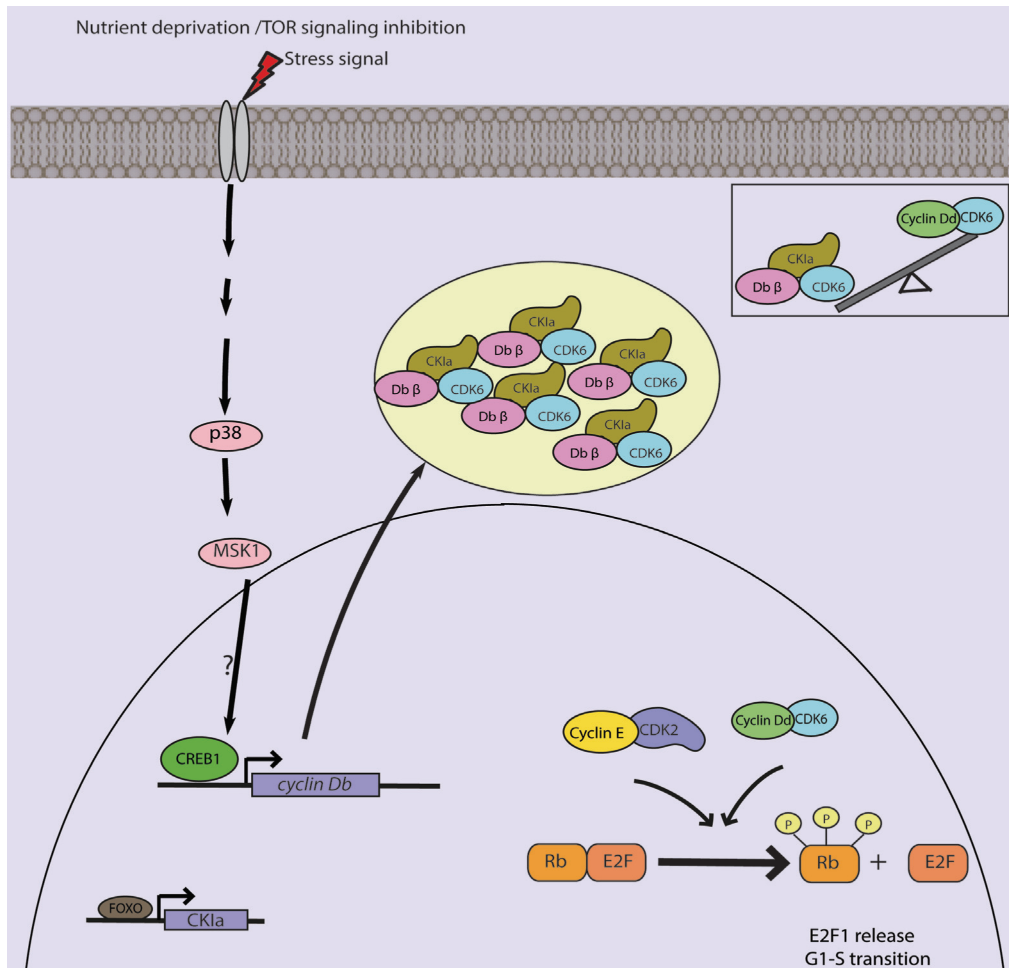

Figure 16. In the germline, MAPK p38 mediated Cyclin Dbβ stimulation overcomes CKI-mediated cell cycle arrest during TOR inhibition. During growth arrest or inhibition of TOR signaling, mitotic germ nuclei initially overcome CKIa-mediated cell cycle arrest and continue to proliferate. Proliferative rates gradually slow and full arrest occurs after several days. CKIa up-regulation might be mediated by the activation of FOXO transcription factors during nutrient deprivation (Unpublished data from Danks *et al.*,). However during nutrient limitation / TOR inhibition, stress-induced MAPK p38-MSK1 signaling is activated, promoting the transcription of *cyclin Dbβ* probably through the activation of CREB1. Up-regulated Cyclin Dbβ forms a complex with CDK6 and sequesters increased CKIa in cytoplasm to overcome CKIa-mediated cell cycle arrest. It is possible that a shift in the balance between Cyclin Dbβ-CDK6 and Cyclin Dd-CDK6 complexes causes a gradual arrest in the germline nuclei.

negative of Cyclin D, since its predicted domain structure lacks the Rb binding and phosphodegron regions. However, we observed that Cyclin Dbβ sequesters CKIa in the germline cytoplasm and promotes G1- S transition of germ nuclei. Contrary to its role as dominant negative mutant of Cyclin D, Cyclin Dbβ positively regulates the proliferation by sequestering CKIa in the syncytial germline. We hypothesize that, in the mitotic germline, activated MAPK p38 increases the levels of Cyclin Dbβ. Increased levels of Cyclin Dbβ result in sequestration of up-regulated CKIa to promote proliferation during the initial period of GA **(Figure 16).** This mechanism of suppressing CKI activity by cytoplasmic sequestration is different from other mechanisms used by GSCs of invertebrate model organisms to suppress CKI activity. GSCs of invertebrate model systems suppress CKI activity by either regulating CKIa translation or CKIa stability (Narbonne & Roy 2006b). Our findings also suggest that there is a balance between Cyclin Dd and Cyclin Dbβ expression for promoting G1-S transition in germline nuclei of *O. dioica* under GA. Such an interpretation is further supported by the differential response of somatic endocycling cells (Cyclin Dd present; Cyclin Dbβ absent) and the germline (Cyclin Dd present; Cyclin Dbβ present) to nutrient-restricted conditions **(Figure 14).** It may be possible that CDK6 is also sequestered gradually with increased Cyclin Dbβ levels under nutrient -limited condition. This results in an increase in the ratio of cytoplasmic Cyclin Dbβ-CDK6 versus Cyclin Dd-CDK6, causing a gradual arrest in cell cycle progression in mitotic germline during later stages of GA under nutrient-deprived conditions.

In summary, semelparous *O. dioica* was subjected to high-density culture which caused GA just prior to meiotic entry and extended the lifespan up to threefold. GA during nutrient deprivation was characterized by reduced TOR signaling. Under these conditions, the MAPKs, ERK and p38, were activated in the germline probably for survival of *O. dioica.* This model organism was used to study the molecular mechanisms downstream of TOR signaling that differentially regulate endocycling and mitotic cells. TOR signaling regulated the G1-S transition by stimulating the translation of Cyclin Dd, and E2F1 while repressing CKIa levels. When TOR was inhibited, endocycling cells stopped cycling due to decreased translation of *cyclin Dd* and *E2F1,* and increased expression of CKIa and E2F7, resulting in GA. However, in mitotic tissues, inhibition of

TOR signaling activated stress-induced MAPK p38 signaling, which promoted transcription of the *cyclin Dbβ* splice variant. Co-expressed Cyclin Dd and Cyclin Dbβ cooperate to promote S phase in germline nuclei of syncytium. Increased Cyclin Dbβ co-localized with the up-regulated CKIa, thus permitting the G1-S transition in mitotic germline through sequestration of this negative regulator**.** Possibly, energy-dependent reduction of TOR signaling in the germline, gradually down-regulates *cyclin Dd* translation and increases the ratio of cytoplasmic Cyclin Dbβ-CDK6 versus Cyclin Dd-CDK6 complexes. This correlated with the gradual cell cycle arrest of syncytial germline nuclei.

5.9 Conclusion

In this study we have used the semelparous reproductive model organism to gain an understanding of the link between germline status and lifespan extension. We have interpreted, in a significantly different manner, the link between reproductive status and lifespan extension in semelparous model organism in comparison with iteroparous model organisms in response to nutrient-limited stress. In iteroparous reproductive models *C. elegans* and *Drosophila*, meiotic entry occurs several times and post meiotic cells are destroyed but a pool of active GSCs is maintained during paused lifespan in response to nutrient limitation. The pool of maintained GSCs is reactivated when nutrient conditions improve in these iteroparous models. However, in the semelparous model, lifespan extension occurs only before meiotic entry under nutrient deprivation and GSCs are not maintained after meiotic entry. Nutrient limitation encountered after meiotic entry leads to production of reduced number of progeny. This single meiotic entry is a definitive signal that lifespan extension can no longer occur after onset meiosis in this semelparous model.

In contrast to *C. elegans* and *Drosophila* GSCs, *O. dioica* germ stem nuclei have a distinct G1 phase and co-expressed Cyclin D variants cooperate to regulates G1-S transition in syncytium. Moreover, CKIa sequestration by Cyclin Dbβ is a mechanism used by GSCs in *O. dioica* for suppressing CKI activity, which is distinct from GSCs in these invertebrates. Understanding the molecular regulation of syncytial germ stem nuclei proliferation sheds light on the mechanisms involved in the adaptation of adult stem cells to nutritional limitation. Deregulation of nutrient signaling, occurrence of endomitosis and

polyploidy are hallmarks associated with various types of human cancers (Showkat *et al.*, 2014, Erenpreisa *et al.*, 2005a, Erenpreisa *et al.*, 2005b). Additionally, an increase in Cyclin D1, Cyclin D1b and Cyclin D mediated cytoplasmic sequestration of CKI has also been reported in several human cancers (Knudsen *et al.*, 2006, Kim et al., 2009, Zhou *et al.*, 2001, Serres *et al.*, 2011, Wu *et al.*, 2006)). Similarly, we also reported that MAPK p38 increases Cyclin Dbβ, which sequesters CKIa in the cytoplasm of endomitotic germline under inhibition of nutrient signaling in *O. dioica.* Thus, the TOR/MAPK signaling mechanisms investigated in this study of *O. dioica,* unveil a striking relevance to cancer development in humans. A thorough understanding of these TOR/MAPK signaling mechanisms governing ageing and GSC proliferation in syncytium, in the future will provide insight for regenerative medicine and developing therapeutic approaches for treating cancers. Nonetheless, a sound understanding of ageing mechanisms, in future, will also allow us to tweak these mechanisms to benefit mankind.

5.10 Future perspectives

In this study, we have established a link between reproduction and lifespan extension in the semelparous chordate model *O. dioica*. We have also gained an understanding of the regulation of germline status and organismal lifespan through TOR signaling in *O. dioica.* However, it remains unclear as to how Insulin signaling (TOR dependent/independent) influences lifespan in *O. dioica*. Future goals will be to identify the molecular mechanisms governing meiotic onset and germline differentiation and to determine how Insulin and TOR signaling influences these mechanisms. It would also be interesting to determine whether there exists cross-talk between nutrient and stress signaling during germline nuclei proliferation and differentiation. Exploring the role of FOXOs in cell cycle regulation, reproduction and lifespan during GA in *O. dioica*, will help determine whether FOXOs are the upstream regulators of TOR signaling during nutrient deprivation in *O. dioica*. In addition to this, investigating the potential signaling roles of the ITC in germline would improve our understanding of germline proliferation and differentiation in *O. dioica*. Furthermore, deciphering the role of endocrine signaling and various other nutrient signaling pathways on germline status and lifespan of *O. dioica,* will tell us whether endocrine signaling (sex hormones) or other nutrient signaling

pathways are also involved in establishing the link between germline status and lifespan in *O. dioica.*

We have successfully established methods to express recombinant fusion proteins (Capped mRNA with reporter) and knockdown proteins using double stranded DNA in embryos, somatic cells and the germline of *O. dioica.* It would be interesting to study the effects of TOR knockdown on cell cycle regulators and effectors of TOR signaling in somatic endocycling cells and GSCs. This would provide additional insight into the differential regulation of these cell cycle variants by TOR signaling. In this study we demonstrated that cell cycle progression in mitotic tissues starts prior to endocycles in somatic tissues upon release from GA. Understanding the mechanisms which regulate the cell cycle and identifying the various signaling pathways involved in these two tissues upon reactivation, will provide a better picture of the role of the nutrient signaling in regulating these cell cycle variants

In contrast to the *C. elegans* and *Drosophila* GSC cell cycles, *O. dioica* germ nuclei have a distinct G1-phase and we have begun to understand nutrient-dependent regulation of D-type cyclins, E2Fs and CKI in GSCs. Functional characterization of Cyclin E-CDK2 complexes and regulation of Cyclin E-CDK2 complexes by nutritional signaling in *O. dioica* will provide more information on the regulation of GSCs proliferation. Studying the effects on other cell cycle regulators such as D-type cyclins, CKIb and CKIc, in response to various external stimuli such as nutrient depletion in endocycling as well as in mitotic germ nuclei will improve our understanding of the regulation of these cell cycle variants. A previous study from our lab reported that CDK1 is amplified in *O. dioica.* These amplified odCDK1 paralogs have a modified PSTAIRE motif (Campsteijn *et al.,* 2012). The PSTAIRE motif is generally present on CDK1 and it mediates the interaction with Cyclin B in all organisms (Lim and Kaldis, 2013). It is in our interest to elucidate the possible interaction between amplified CDK1 paralogs and D-type cyclins in regulating the cell cycle variants. In addition, immune-precipitation studies, yeast and mammalian two hybrid systems would help to identify the interacting partners of various CDKs, Cyclins and their splice variants in germline and endocycling cells. These interaction studies might help to obtain an improved picture of the mechanistic regulation of the cell cycle in these cell types. Further, generating stable

transgenics which knockdown or over-express TOR signaling components will improve our understanding of the role of TOR signaling components in regulating the cell cycle, germline status and lifespan in *O. dioica*.

Chromatin immunoprecipitation (ChIP) in *O. dioica* has been established in the lab and we have begun to characterize epigenetic changes over the compacted regulatory regions and open reading frames of *O. dioica* genes. It would be of interest to study the genome wide epigenetic histone H3 modifications (repressed and activated chromatin) occurring in GA, rapamycin treated and GA released animals, which might be critical in regulating the expression of the cell cycle regulators. If a method to mechanically separate somatic endocycling cells from the germ stem nuclei was practicable, then ChIP with isolated cell types could reveal cell-type specific regulation of these marks on target cell cycle promoters, thus explaining the differential regulation of these cell types epigenetically. ChIP-seq with E2F1 and E2F7 will determine the occupancy of these proteins on promoters of cell cycle regulators, although a cell type-specific ChIP-seq would be more informative on the differential occupancy of E2F1 and E2F7 in endocycling and mitotic cells. Additionally, EMSA can be performed (Electrophoretic mobility shift assay) to confirm the predicted occupancy of FOXO on *CKIa* promoter and occupancy of CREB on *cyclin Db* promoter during GA.

Genome wide transcript studies provide a global and mechanistic view of regulation of development. We established “OikoBase”, a tiling array-based genome browser resource for *O. dioica,* which provides an unbiased whole genome transcription data set from different developmental stages, including GA and GA released states (Danks *et al.,* 2013). Analysis of OikoBase has helped identify certain regions of *O. dioica* genome which are unique to GA. These genomic regions are specifically expressed during GA in *O. dioica,* which otherwise remain dormant throughout its lifespan. Elaborate investigation of the genes in these genomic regions might provide novel molecular mechanisms in ageing research (Unpublished data from Gemma Danks, Prof. Eric Thompson’s lab). Additionally, we are in the process of generating a ribosomal profiling sequencing library of TOR responsive TOP mRNAs (Danks *et al*., 2014). This library might help further elucidate the mechanisms of TOR mediated regulation of cell cycle, growth, and lifespan. Further, transcriptomic interrogations during GA and TOR

inhibition (both tiling array, RNA seq data), characterizing the GA specific transcripts and their translational regulation, will help us understand the environmental cues that regulate meiotic onset and cell cycle variants to influence lifespan in *O. dioica.* Ultimately our goal in studying *O. dioica* is to provide valuable information on lifespan extension and molecular regulation of cell cycle variants.

6. Appendix

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OdTOR     1 HYSDELVVCARRFYLHIKKTFEENVRTSKEWREFREKLQEE--------ILILLVDNR--
CeTOR     1 NRDANQRQAARELSRYVRSELKDE--PNTFSDAFLNAIDGRTDIASQSAIYNCMKSNSNI
MmTOR     1 NEETR-AKAAKELQHYVTMELREM--SQEESTRFYDQLNHH--------IFE-LVSSSDA
DmTOR     1 NRNVQ-NKATQDLLFYVKTELREM--SQEELAQFFDEFDHH--------IFT-MVNATDI

OdTOR    51 HGRQGSDFSATNLKKIVNPGGSADSKFAGAICAYICFKISHEIFQEHIIISNRLSSLLME
CeTOR    59 DQKRAGIYLIVCLAET--HSGNVIR-----YANYL---------LKMLNNGNGLDEDTVK
MmTOR    49 NERKGGILAIASLIGV--EGGNSTR--IGRFANYL---------RNLLPSS---DPVVME
DmTOR    49 NEKKGGALAMKCLINC--EGSLTARKGISPYLNRL---------RDLLLIN---DVSVME

OdTOR   111 AHDNNKNRTFQRKDILISELMMLVYSTCMSGMAIWPATSKFDELLFVEDQVKRYHACLFY
CeTOR   103 MASKALAFLIATCKSYAAELVDRCLDHCHEWLGQNVPHSQQPKNQQEIDQIRRLAASHLS
MmTOR    93 MASKAIGRLAMAGDTFTAEYVEFEVKRALEWLG------------ADRNEGRRHAAVLVL
DmTOR    95 IAARSLVKLANMPTSKGADSFDFDIKKAFEVLR------------GERQEYRRHSAVFIL

OdTOR   171 FHMASKHPIDFYRNHFRSAMRRNGRKTDKIVAKLIRIVCESVNSEHNEMRVAAFSALKAI
CeTOR   163 RELALATPTAFFLR----------------VNLFFKYIFNAVRDKNPAVRIAGIDALHVV
MmTOR   141 RELAISVPTFFFQQ----------------VQPFFDNIFVAVWDPKQAIREGAVAALRAC
DmTOR   143 RELAIALPTYFYQH----------------ILTFFEVIFNAIFDPKPAIRESAGEALRAA

OdTOR   231 LNLVASRE-TALSEGRNWPKDLVNSIIEGLK---NTES--------DRI-GPILCCYLLV
CeTOR   207 LTIVSQRE---AKNKTEWFKKCFDEALEGQP---NPSQKDDL----DRWHAVALILNELL
MmTOR   185 LILTTQRE-PKEMQKPQWYRHTFEEAEKGFD---ETLAKEKGMNRDDRIHGALLILNELV
DmTOR   187 LIVTAQRESTKQSSEPQWYRICYDEANGSFNADLGSSKDQKGVTRDDRIHGGLVVFNELF

OdTOR   278 DISAAKNN-YTNSLKTCLKKLRIAQTWNPCAQKN--KTE-KFFDAVFKYDLVEE------
CeTOR   257 RISDQRFE-----LIRCESSQFIKQKFLKEDEEE--GVEWLVLT----------------
MmTOR   241 RISSMEGERLREEMEEITQQQLVHDKY--CKDLMGFGTKPRHITPFTSFQAVQPQQPNAL
DmTOR   247 RCANATWERRYTSLKTLFPKTQ-HNKFLEASSSSSMGSQLNTLVPRLKVPFIDK------

OdTOR   328 -------ALQNPMGG----------TRFTNLQPNSSTAERLLDENVETLSRLV-----LS
CeTOR   294 -------------------------KQQTIVE--SVTARKLVLERFPKILDCVRQIIPLA
MmTOR   299 VGLLGYSSPQGLMGFGTSPSP----AKSTLVE--SRCCRDLMEEKFDQVCQWV-----LK
DmTOR   300 -----LGSTQTHLGEGEHHKGVAKFASHNVLE--SAYAQEILQEHYTSICDNV-----LE

OdTOR   366 QRSM----KNSINIHPYLLPLLPKLCAFNPKKFE--PFFVKTIEYCLQEIAPSGNQKTTT
CeTOR   327 NKTSSTKQQSSIYLNTVLMQLLPRICAFPQC---DRTFQTISFDTSFTILQ----RNA--
MmTOR   348 CRSS-----KNSLIQMTILNLLPRLAAFRPSAFTDTQYLQDTMNHVLSCVK-KEKERT--
DmTOR   348 QRTS-----KSPYVQQALLQILPRLAAFNRAVFVE-KYLQTCVSHLMQILRGKEKDRT--

OdTOR   420 RRNQGTALLSMGMIIHLICESDSELADPTTRPTTVLEEQIDTKVIPKLKSVLKEAFISFQ
CeTOR   378 -----VAAPAIGMMM----------LSNPDVHATHI------EKTISFISAAI-------K
MmTOR   400 -----AAFQALGLLS----------VAVRSEFKVYL------PRVLDIIRAALPPKDFAHK
DmTOR   400 -----VAYITIGYMA----------VAVQSAIEVHL------SSIMTSVKVALPSKDLTSK

OdTOR   480 MSRGGVDCDPTLFQCIELISISLTDLHKKLWFKPQNKEFLNMIVAFGLIEIKDSWIRCVQ
CeTOR   411 KTT-NSDVLDNYFTFLFLFVDAYHEQ-----VTTQIKAIIPQLMDITL---SRSLANVLK
MmTOR   440 RQK-TVQVDATVFTCISMLARAMGPG-----IQQDIKELLEPMLAVGL---SPALTAVLY
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OdTOR  540 SLNSKIS--RDQVSHGMEQVLLKNLFNE--RLPSQIMNGYSDDHSCLSSLSVLIPFKNGE
CeTOR  462 MIMMRIPKLRLNVQDGVMASVYQTLTGS--LIP----------PKSEPIGRPASPKAILQ
MmTOR  491 DLSRQIPQLKKDIQDGLLKMLSLVLMHK--PLR----------HPGMPKGLAHQLASPGL
DmTOR  489 ELSENVPQLKSAITEGLIGILSQVLMNKAAILP----------YTALPTIAI-------D

OdTOR  596 DFDFGRVSVRKRIASLKALEMFEFTDFSRYKKLIYRWKEYFNPKEKIQVKIAAAKAVTRV
CeTOR  510 KAETDPKELQRIVLAVDVLGEFYFSRGA-LQRIMQYVADYYLTADNVEIRLAAVSSCCEM
MmTOR  539 TTLPEASDVASITLALRTLGSFEFEGHS-LTQFVRHCADHFLNSEHKEIRMEAARTCSRL
DmTOR  532 GSLMQNGDGATTVLALKTLGTFNFEEQN-MLDFVQRCADYFIVHEQQEIRLEAVQTCTRL

OdTOR  656 LRLFI--IHSSTCGPNLVGETTISNIIRKLLQIADTIIPYKQESQSTQLKLAIYRSLKP-
CeTOR  569 VVPFVGVYKKVTSDKRNSLLQTIYGVLRAVCSVIVN-------DQDVRVRMQVISCFGQM
MmTOR  598 LTPSIHLISGHAHVVSQTAVQVVADVLSKLLVVGIT-------DPDPDIRYCVLASLDE-
DmTOR  591 LKLAV--QSSESMENSKTLSDTVSHVIERLLMVAIT-------DMDCNVRIRILRSLDE-

OdTOR  713 --EFDAYLIHQHNLERIMNAIHQPYPIKEENFTDRLEVQEAVMEIIGRLNAMNAAETMPH
CeTOR  622 PRPFLAHLAQPEMLEVQFMALHD----------EKLEMQQACVTLLGRLAELNPALVLPR
MmTOR  650 --RFDAHLAQAENLQALFVALND----------QVFEIRELAICTVGRLSSMNPAFVMPF
DmTOR  641 --TFDGKLAQPESLNSLFITLHD----------EIFEIRELAMVTIGRLSSINPAYVMPK

OdTOR  771 IRMMINDLWTQLQMP-----DQASARLLEVLFRHANQAQLSLFVDPTIRQIEPLLSYEYV
CeTOR  672 LRLMLLETLSQMQQSGQARLEQHSAKMIAQLAKQSPKF-MRPYVGSLMIAMIPKLR--ND
MmTOR  698 LRKMLIQILTELEHSGIGRIKEQSARMLGHLVSNAPRL-IRPYMEPILKALILKLKDPDP
DmTOR  689 LRTTMIELITDLKYSGMSRNKEQSAKMLDHLVISTPRL-ISSYMNPILKALVPKLH--EP

OdTOR  826 QSYPPRMTCTLIDCLSAISAVNGPRLE-KETLNRFFSLIYRLLVETTEPTVKVSALRCLS
CeTOR  729 QKY-AEVTAQVLNAVSEIAVIGG--AEIVKNLKPLFEKLTHMINDSSSLHKREAALRAIG
MmTOR  757 DPN-PGVINNVLATIGELAQVSG--LEMRKWVDELFIIIMDMLQDSSLLAKRQVALWTLG
DmTOR  746 ESN-PGVILNVLRTIGDLAEVNGGSDEMELWADDLLSILLEMLGDAGSPDKRGVALWTLG

OdTOR  885 SLIEHHNYVISPYFKHRKLMDDMLALVTADKKTMQHEINEEALRMIGRLGALDPYQYKEI
CeTOR  786 GICRSTAYVVDPYRDYPSLLDDLLRIL---KTVMSNTMRREAIKTLGILGAIDPYTHKVF
MmTOR  814 QLVASTGYVVEPYRKYPTLLEVLLNFL---KTEQNQGTRREAIRVLGLLGALDPYKHKVN
DmTOR  805 QLISATGRVVTPYHKYPVLIDILINFL---KTEQRRSIRRETIRVLGLLGAMDPYKHKMN

OdTOR  945 TKKRRSN-----------SQNEKHDGFTSIN--EMLVNDSRCPDEKF---IIRVAVELIK
CeTOR  843 TGSVQSSTAISTALSLPISETDSKDPRQDII---HWFNYEKCTLEEFYPAITIANLMLMM
MmTOR  871 IGMIDQS---RDASAVSLSESKSSQDSSDYSTSEMLVNMGNLPLDEFYPAVSMVALMRIF
DmTOR  862 KGLIDSQ---KDNVLIAYSDGKV-DESQDISTAELLVNMGNA-LDEYYPAVAIAALMRIL

OdTOR  989 AKLSHGGDVTAPLHSLDNLIQHAGTNILPHLGKVMPEL---HRIAQDHKAKKEIKSNILG
CeTOR  900 QDED-SQSYAEIAQAIVTIFRSLGDMAPLYTEQVIPRLIEVCRRATESSNRANLREFFLQ
MmTOR  928 RDQSLSHHHTMVVQAITFIFKSLGLKCVQFLPQVMPTFLNVIRVCDG-----AIREFLFQ
DmTOR  917 RDPTLSTRHTSVVQAVTFIFQSLGIKCVPYLAQVLPNLLDNVRTADN-----NLREFLFQ

OdTOR 1046 LFEKLLQKMRRRVLPFMDDIKETMETQKDISYAPLRKKLAQLIYQAAYNLQYEFKDYMSW
CeTOR  959 QLANFVAIIRKHAAPYMPAIFTIIADAWKED-ISVKMVVIEVLTDMGTAIGNDFSKYTGE
MmTOR  983 QLGMLVSFVKSHIRPYMDEIVTLMREFWVMN-TSIQSTIILLIEQIVVALGGEFKLYLPQ
DmTOR  972 QLAILVAFVKLHIISYMGDIFKLIKEFWTIN-TPLQNTLINLIEQIAVALGCEFRDYLAE
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OdTOR 1106 AVRYILELFEKLKSSTDPQDEEATIYLVRAIGKFGY-IEAHYSVLLRPLATMLQSPNSSD
CeTOR 1018 LIPYLLTVLQ----TDKTKERVLTVKVMESIQKLTHCIVQHLHLVLPPLLIILDDFSLKL
MmTOR 1042 LIPHMLRVFM----HDNSQGRIVSIKLLAAIQLFGANLDDYLHLLLPPIVKLFDAPEVPL
DmTOR 1031 LIPQILRVLQ----HDNSKDRMVTRRLLQALQKFGSTLGYYLPLILPPIVKLFDSPYVPQ

OdTOR 1165 QLRGEILVVLEARIVRTAMPDYFMILSEAILVALAYEVSADKHTVIKNHGPHSSKPTKDY
CeTOR 1074 SIRNTALSTVLHMTQQVDVSAYAPRMMQSWHHNIS-TAEM-RDKLLLLLIEIIKQLGKFF
MmTOR 1098 PSRKAALETVDRLTESLDFTDYASRIIHPIVRTLDQSPEL-RSTAMDTLSSLVFQLGKKY
DmTOR 1087 QVSMVALETINNLACQLDFTDFSSRIIHPLVRVLDAEPEL-RDQAMTTLRSLAKQLGKKY

OdTOR 1225 DLRETAVNCLRYMRSGMRHFNLWE----EEY---------------DDYFQIRTGNLWKP
CeTOR 1132 DIFKRGVD------QKLRDYNLDKSVHYEQYRKLAQRAQMSRDVLTSSVFAGSNGNIQYS
MmTOR 1157 QIFIPMVN------KVLVRHRINH----QRY------------DVLICRIVKGYTLAD---
DmTOR 1146 LVFVPMVQ------RTLNKHRIVD----PEY------------EELLSKIKSCSTLADSYG

OdTOR 1266 SEDPEMVFQDNAFKNDFSQDQSMIKEGEDE----HDT-----------------------
CeTOR 1186 STQAGMRGQANNVYANNDLHERLMNGSIDSGASRQDNRDDYYRYGVEEKKEVPKVAPTTA
MmTOR 1193 EEEDPLIYQHRMLRSS--QGDALASGPVETGPMKKLH-----------------------
DmTOR 1185 AGESELR--PSRFKNN----EPFVTDRNSNNK--NLQ-----------------------

OdTOR 1299 ---------------AHYNWLKEEFEVDCQTPED-FKDLQVRISKCFLSHSKSAVIRGLR
CeTOR 1246 RPTSELVTVQITKQRLNKDALMPQWKNENLTSKDEWLQWLMKIRIGFLTYGSSPSLRAAS
MmTOR 1228 ---------------VSTINLQKAWGAARRVSKDDWLEWLRRLSLELLKDSSSPSLRSCW
DmTOR 1214 ---------------VTTNELRTAWQVTRRVSKDDWVEWLKRLSIGLLKESPSHALRACR

OdTOR 1343 GMDRLYNVPVNLIIFNCSFYAMWAEPKPVRELQMDGSESTRIPEDPIKPYRDHITKGLLK
CeTOR 1306 SLGDQHP-HLARDLFPAAFMSVWTELDS--------------------DVQNDLTSCLLR
MmTOR 1273 ALAQAYN-PMARDLFNAAFVSCWSELNE--------------------DQQDELIRSIEL
DmTOR 1259 SLAQEYD-TLLRDLFNAAFISCWTELSP--------------------DLKNELTQSLIQ

OdTOR 1403 IIDGKNTDPPRGILSFFLDLAHFLELSDIDKERLDQPGGSLKAFGGLNGIGKLADCAERT
CeTOR 1345 AI-STGIPE---LIQTILNLAEFMDHSEKGPLPISHD--------------VLGRWAEQT
MmTOR 1312 ALTSQDIAE---VTQTLLNLAEFMEHSDKGPLPLRDD----------NGIVLLGERAAKC
DmTOR 1298 ALQVTDMPE---ITQTILNLAEFMEHCDRDPIPIETK--------------LLGTRAMAC

OdTOR 1463 DAYAKALHYREMEFLYKISEKDNR-----EKERKIAESLIKSFQKLGEQEAAEGVLEFAK
CeTOR 1387 KAFAKACRYKEMSVLKKSGSMQTTFTRKVKLEPNDCQSLITYANKLNVQEEAAGVVRYAE
MmTOR 1359 RAYAKALHYKELEFQKGP-------------TPAILESLISINNKLQQPEAASGVLEYAM
DmTOR 1341 RAYAKALRYKEEEFLLRE-------------DSQVFESLILINNKLQQREAAEGLLTYR

OdTOR 1518 SN--DITVNAEWFEKLNRWEEAKKRYA----DGNDNP---------------------YD
CeTOR 1447 RNEMNFQMRGRWYEKLNEWEKALGAYELEEKKKSSCPNLQVYDEKDHLMTPEEAATAEEA
MmTOR 1406 KHFGELEIQATWYEKLHEWEDALVAYDKKMDTNKEDP---------------------EL
DmTOR 1388 NAANELNVQGRWYEKLHNWDEALEHYERNLKTDSSDL---------------------EA

OdTOR 1551 QLGYMRCLEALGEWDELCAKV-YEF-------------DEEIEDQKIESVKLGTTAAWHL
CeTOR 1507 RMHEMRCLEALGRWDELNSKS-VVWADQRGNRNDSVRDEINKKQLDHKMAVIAARGAWAV
MmTOR 1445 MLGRMRCLEALGEWGQLHQQCCEKW-------------TLVNDETQAKMARMAAAAAWGL
DmTOR 1427 RLGHMRCLEALGDWSELSNVTKHEW-------------ENFGTEAKSRAGPLAAVAAWGL
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OdTOR 1597 GNWDEFQRLHSIIPVNSFDGAFYRAVHSVQHSRYDEARKWTMEARKDLETSLT-MQDKNY
CeTOR 1566 DNWERMADYVSVISENTQDGAMLRAVVAVHNDENTKAMGLIEKVREMIDSELTAMANESY
MmTOR 1492 GQWDSMEEYTCMIPRDTHDGAFYRAVLALHQDLFSLAQQCIDKARDLLDAELTAMAGESY
DmTOR 1474 QDWEAMREYVRCIPEDTQDGSYYRAVLAVHHDDFETAQRLIDETRDLLDTELTSMAGESY

OdTOR 1656 SRVYGTVFNAQLCSELEEVIEHRTANRQRQIAIQEMWESRLCGTSSDDGQRVDGVQLVIE
CeTOR 1626 ERAYIPMVSVQQMAELEEAIEYKTRPERRPR-IALLWSRRL-----------QGCRRNVE
MmTOR 1552 SRAYGAMVSCHMLSELEEVIQYKLVPERREI-IRQIWWERL-----------QGCQRIVE
DmTOR 1534 ERAYGAMVCVQMLAELEEVIQYKLIPERREP-LKTMWWKRL-----------QGGQRLVE

OdTOR 1716 EWQKILLLRSLVKERNTAAFPRDDTEEHFNIRSMLKFMSLAAKSGRTEIARQTIKKILPN
CeTOR 1674 QWQRLIMLRGLVLS------PQEM------HPLRVKFSSMCRKQGKNSMSRAVLRELL--
MmTOR 1600 DWQKILMVRSLVVS------PHED------MRTWLKYASLCGKSGRLALAHKTLVLLL--
DmTOR 1582 DWRRIIQVHSLVVK------PHED------IHTWLKYASLCRKSGSLHLSHKTLVMLL--

OdTOR 1776 IDNWNYVEMLSYPGLIENAQGRQVSLKISPPKIRALYKYVKHVAIPHLQVTPKSEEKGIQ
CeTOR 1720 -------------SLPANSDLVRAKAPFDKPLL--VLALAKQLY------QDDHKDEAIR
MmTOR 1646 -------------GVDPSRQLDH-PLPTAHPQV--TYAYMKNMW------KSARKIDAFQ
DmTOR 1628 -------------GTDPKLNPNQ-PLPCNQPQV--TYAYTKYMA------ANNQLQEAYE

OdTOR 1836 IITRLCDDLKPLKNRLKDLRDRER----MSDDERIVIAI---------------------
CeTOR 1759 ALEDLANHWNKRINPIPKATGRELIPPSTKEPARICAKVLLKLGEWTELKSKTSNNMQVG
MmTOR 1684 HMQHFVQTMQQQAQHAIATEDQQH----KQELHKLMARCFLKLGEW--------------
DmTOR 1666 QLTHFVSTYSQELSCLPPEALKQQ-------DQRLMARCYLRMATW--------------

OdTOR 1871 -----------TYCRQAEMRRNTVPQPNQIPHLTKKVGEIKDLYMTAIDALNKKDIQSRE
CeTOR 1819 ELSFVRQQVSPQYRTKESRTPETIAFENTINYYQ---------------------QATQ
MmTOR 1726 -----------QLNLQ-GINESTI--PKVLQYYS---------------------AATE
DmTOR 1705 -----------QNKLQDSIRPDAI--QGALECFE---------------------KATS

OdTOR 1920 FVKRRYKAVHSYANFALDSVRRIREGDAQTVRKLQHPRENSSQ-----------------
CeTOR 1857 YDPGWHKVWHKLASTHFYAVCRERPHPTTVISPPQQPQQPKKMHIPPVTRATSPPPPAQK
MmTOR 1750 HDRSWYKAWHAWAVMNFEAVLHYK-HQNQARDEKKKLRHASGANITNATTAATTAASAAA
DmTOR 1730 YDPNWYKAWHLWAYMNFKVVQAQK----SALDKQQPPGASMGM-----------------

OdTOR 1963 -----------------DDQEKAAELFKQI------------------------------
CeTOR 1917 SPQPAPFHSITEPLSVIIDYPVPPPLGSLVGLPPMPAYLSSNSSLPPQHHHVSPLSNDSP
MmTOR 1809 ATSTEGSNSESEAESN-ENSPTPSPLQKKV------------------------------
DmTOR 1769 ------------------------TMGSGL------------------------------

OdTOR 1976 -RSPIYKEIANFSVDALKYFYESVKLSQKSVLQDMLRILNIMAEINKDLEDPETSEFMHA
CeTOR 1977 SNSAENKLYLKHAAHAVRCFAKALMCSPGSRLEDTLRLMQLWFDHGDD-KDQDVYF----
MmTOR 1838 -TEDLSKTLLLYTVPAVQGFFRSISLSRGNNLQDTLRVLTLWFDYGH---WPDVNE----
DmTOR 1775 -DSDL-MIIQRYAVPAVQGFFRSISLIKGNSLQDTLRLLTLWFDYGN---HAEVYE----

OdTOR 2035 SEGRLLETIRTEPLSCDPERWLKVVPQLIAIMDMKKV----DNIPEILAHIGEKHAQGIL
CeTOR 2032 ---ALTETIFDLPVTT----WLEAIPQLMARLDCPDDQKSVQLVLRVLCEIARHRPQAVI
MmTOR 1890 ---ALVEGVKAIQIDT----WLQVIPQLIARIDTPRPLVG-RLIHQLLTDIGRYHPQALI
DmTOR 1826 ---ALLSGMKLIEINT----WLQVIPQLIARIDTHRQLVG-QLIHQLLMDIGKNHPQALV
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OdTOR 2091 WSLIYAQKNPTQENERQSDLAKSIINRIRDHSKNLVKESEMISTELVSVAMLWIDQWNEA
CeTOR 2085 YALTVASRSKDV---HRSKNAGTVLEKMMEYHSKLVREASLVTEELVRCAILWHEQWHDA
MmTOR 1942 YPLTVASKSTTT---ARHNAANKILKNMCEHSNTLVQQAMMVSEELIRVAILWHEMWHEG
DmTOR 1878 YPLTVASKSASL---ARRNAAFKILDSMRKHSPTLVEQAVMCSEELIRVAILWHEQWHEG

OdTOR 2151 LEDILQKFYCQQNNQELDRRQKIEYIKRRMRGLHEMVLNTQQTRNLSEVEFKSTFHENIT
CeTOR 2142 LDDASRVYFHRRLQDN-----NVQAMFDALRNMNDL-MQKGAPTTMKEHSFQQTYSSDLK
MmTOR 1999 LEEASRLYFGER---------NVKGMFEVLEPLHAM-MERG-PQTLKETSFNQAYGRDLM
DmTOR 1935 LEEASRLYFGDR---------NVKGMFEILEPLHAM-LERG-PQTLKETSFSQAYGRELT

OdTOR 2211 SADRHLRRLINGDETESDLEDLMRKYQEIKRKIKDKASGMTKLDLKKVSTKLLEVKDMEL
CeTOR 2196 EAGRYVQAFESSGNVK-DLNQAWEIYCSVFKKLRDQLATLNSLDLVYVSPNLVSAKDLEL
MmTOR 2048 EAQEWCRKYMKSGNVK-DLTQAWDLYYHVFRRISKQLPQLTSLELQYVSPKLLMCRDLEL
DmTOR 1984 EAYEWSQRYKTSAVVM-DLDRAWDIYYHVFQKISRQLPQLTSLELPYVSPKLMTCKDLEL

OdTOR 2271 AIPGTYQPGMDLVRIHSVASEIEILKSKQKPRKIFINGSDGQRHQFLLKGKEDLRLDERV
CeTOR 2255 VVPGTYDPSAPIVSIQSFSSKMNVITSKQRPRKMVIRGSNGLDYQFLLKGHEDPRQDERV
MmTOR 2107 AVPGTYDPNQPIIRIQSIAPSLQVITSKQRPRKLTLMGSNGHEFVFLLKGHEDLRQDERV
DmTOR 2043 AVPGSYNPGQELIRISIIKTNLQVITSKQRPRKLCIRGSNGKDYMYLLKGHEDLRQDERV

OdTOR 2331 MQLFDLMNSLIKMNNNLRQKDIEVIKYSIIPMTNENGLQSGLVRWIDNSETINELLKWVR
CeTOR 2315 MQLFGLVNTLLANNSETCRRNLTIQRYSIVALSKD----SGLIGWVPNCDTLHTLVKEYR
MmTOR 2167 MQLFGLVNTLLANDPTSLRKNLSIQRYAVIPLSTN----SGLIGWVPHCDTLHALIRDYR
DmTOR 2103 MQLFSLVNTLLLDDPDTFRRNLAIQRYAVIPLSTN----SGLIGWVPHCDTLHTLIRDYR

OdTOR 2391 QKRLNVTPHPEKEHEKERLPQDSAIQNCSILQKVEMFHYVQKVTEDRGYDLARILWLQSA
CeTOR 2371 EKKAKI---PLSIEHKTLQKLSLETEHLTLMQKLQLFESALSVTQ--GEDLRHVLWLKSP
MmTOR 2223 EKK-KI---LLNIEHRIMLRMAPDYDHLTLMQKVEVFEHAVNNTA--GDDLAKLLWLKSP
DmTOR 2159 DKK-KV---PLNQEHRTMLNFAPDYDHLTLMQKVEVFEHALGQTQ--GDDLAKLLWLKSP

OdTOR 2451 SADIWFERRTNFVCSLALMSMVGYILGLGDRHVSNILIITTSGKVAHIDFGDSFENAMKR
CeTOR 2426 SSEVWFDRRTNYTRSVACMSMVGYILGLGDRHPSNLMLDRLTGKVVHIDFGDCFEVAMLR
MmTOR 2277 SSEVWFDRRTNYTRSLAVMSMVGYILGLGDRHPSNLMLDRLSGKILHIDFGDCFEVAMTR
DmTOR 2213 SSELWFERRNNYTRSLAVMSMVGYILGLGDRHPSNLMLDRMSGKILHIDFGDCFEVAMTR

OdTOR 2511 EHVPEKVPFRLTRMLVNAMEVTGVDGTFKKICLESIDMCRSNKESILAVLEAFINDPVV-
CeTOR 2486 EKFPERVPFRLTRMLINAMEVTGLDGVYNYTAERVLKMLRTNQESLLAVLEAFVYDPVIN
MmTOR 2337 EKFPEKIPFRLTRMLTNAMEVTGLDGNYRTTCHTVMEVLREHKDSVMAVLEAFVYDPLLN
DmTOR 2273 EKFPEKIPFRLTRMLIKAMEVTGIEGTYRRTCESVMLVLRRNKDSLMAVLEAFVYDPLLN

OdTOR 2570 --------------------------------------AESQHRRVQKHGQISVEA----
CeTOR 2546 WRLVEGMKKD------PKTRKDTGG-------------------RQNMAGAVLPSS----
MmTOR 2397 WRLMDTNTKGNKR---SRTRTDSYSAGQSVEILDGVELGEPAHKK---AGTTVPESIHSF
DmTOR 2333 WRLLDVDKKGNDAVAGAGAPGGRGGSGMQDSLSNSVEDSLPMAKSKPYDPTLQQGGLHNN

OdTOR 2588 ------------DDAKVKIQRVTAKLEGKDFNEVDENT--NTLSVKDQVERLIYEARNIE
CeTOR 2577 ------------STTDSIMETIKRKLDGTEFVHTDGSTPPEPLQVTEQLAMLTEQATSPL
MmTOR 2451 IGDGLVKPEALNKKAIQIINRVRDKLTGRDFSHDD------TLDVPTQVELLIKQATSHE
DmTOR 2393 VAD------ETNSKASQVIKRVKCKLTGTDFQTEK------SVNEQSQVELLIQQATNNE

OdTOR 2634 NLCQHYTGWCIYW
CeTOR 2625 NLCQSYIGWCPFW
MmTOR 2505 NLCQCYIGWCPFW
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Figure A1. Multiple sequence alignment of TOR. *O. dioica* TOR was aligned with TOR from other species using MUSCLE. Dark shading indicates identical conserved amino acid positions and grey shading represents similar amino acids. Predicted rapamycin binding sites are indicated in a red box. *DmTOR* - *Drosophila melanogaster* TOR, *Mm*TOR - *Mus musculus* TOR, *Od*TOR - *Oikopleura dioica* TOR, *Ce* TOR - *Caenorhabditis elegans* TOR.

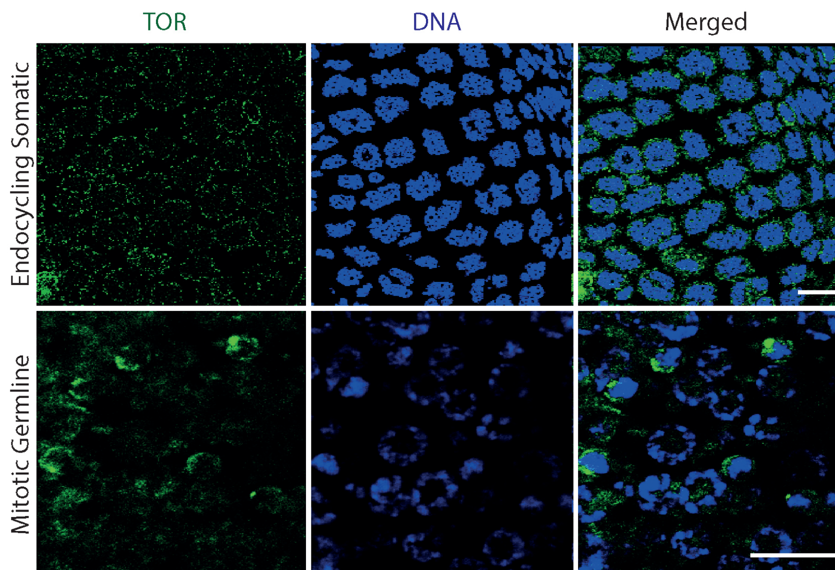


Figure A2. TOR in *Oikopleura dioica* somatic and germline cytoplasm. Immunostaining of TOR (anti TOR-VEADDAKVKIQRVTAKLEGKDF) shows that TOR is localized in the cytoplasm of both the germline and in endocycling epithelial cells. Scale bars indicate 10 µm.

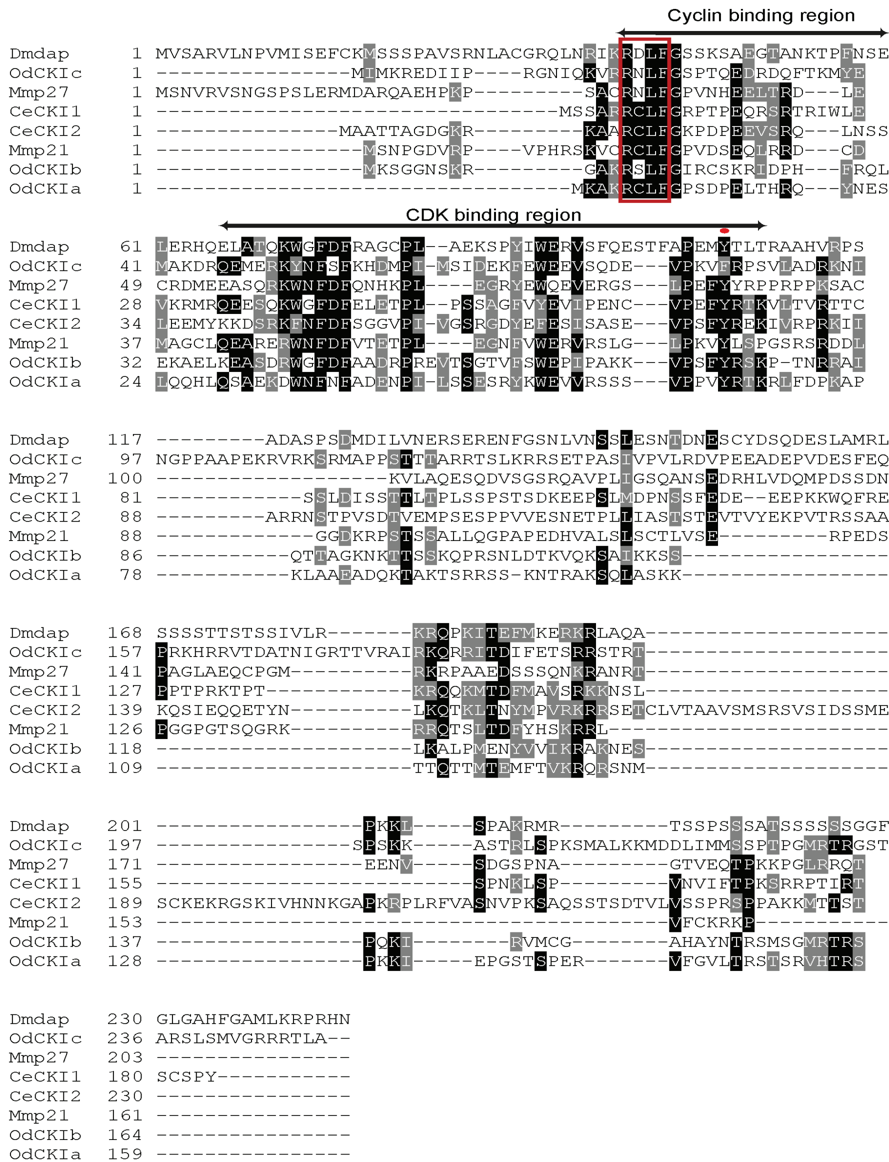
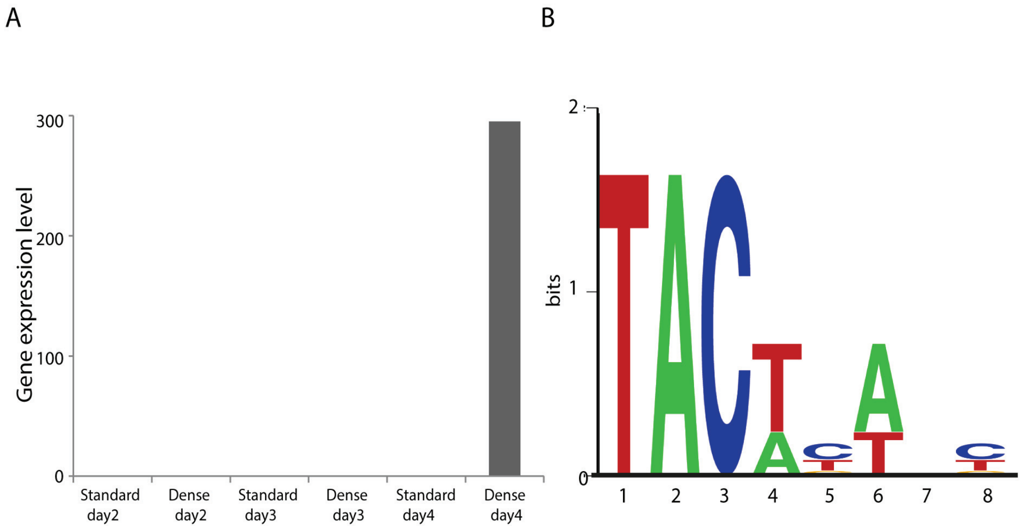


Figure A3. Multiple sequence alignment of Cip/Kip sequences with *O. dioica* CKIs. *O. dioica* CKIs (CKIa, CKIb and CKIc) were aligned with members of the Cip/Kip family from other species using MUSCLE. Dark shading indicates identical conserved amino acid positions and grey shading represents similar amino acids. Cyclin and Cyclin-dependent kinase (CDK) binding regions are indicated by double-headed arrows. Cyclin binding residues RXLF (red box) and the tyrosine residue (black circle), a phosphorylation site critical in the formation of non-inhibitory, active complexes with Cyclin D-CDK4/6, are indicated. *Dm*dap - *Drosophila melanogaster* Dacapo, *Mm*p27 - *Mus musculus* p27, *Mm*p21 - *Mus musculus* p21, *Od*CKI - *Oikopleura dioica* CKI, *Ce*CKI - *Caenorhabditis elegans* CKI.



C

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CTGCTCGATTGTTACAAGTTCGTGCTTCAAAATCGAATGCTCAGTTCTGGAAGACTTGGAAAAGGCTCCTTCCAA
GAGGTCGCCGACGAAATGAATCGGCGATGCGGGCTCGACGGCGACGGAACTTACGAGCGAGAGCAACTTGCAGCC
AAACTTCAAAACTTGAAGACGCAATTTCGGAATAGAAAACAAGCTGTTGACGACGGCCGTGAGAGTGGCAGTCTT
TGGAAGTGGTATCAGACGATGGACGACTTATCGAATGCTGAGTTTAACCTTCGCGAGTCATTCGATAGCGCGCCG
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ACCGAAGAGTCAGAGTCAGAAGATACGGATCGATGTAGCGCTAGTGTTACTCATCAGTTAAACAATATGAGATCA
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GTTTTGTTGCTTTAAAAAAGACCTCTATCCAATCATTTAAAATCATCTCTTTACTTTACCGAAGATTTCCGTTGT
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TTCAATTCATATTTTCACAGCTCTTCTTTTAGAAGATG
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Figure A4. Analysis of FOXO binding sites in the *O. dioica* CKIa promoter. A) The *FOXO* ortholog (GSOIDG00003334001) expression level was plotted using the Oikobase (http://oikoarrays.biology.uiowa.edu/Oiko/expression_matrix.html) expression matrix. Expression of FOXO was observed in day 4 dense but not in day 4 standard conditions. B) Promoter sequence of CKIa extracted from the *Oikopleura dioica* genome (Denoued *et al.,* 2010). Predicted FOXO binding sites in the promoter were identified using ConSite (http://consite.genereg.net). The predicted FOXO binding site sequence logo was created using http://weblogo.berkeley.edu/logo.cgi. The overall height of the stack in the sequence logo indicates the sequence conservation at that position while the height of the symbol within the stack indicates the relative frequency of nucleic acid at that position. C) Predicted FOXO binding consensus sequences are highlighted in yellow in the CKIa promoter sequence.

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OdE2F7     1 -----------------------------MKTDSPHSGLRLLSEA--------------I
CeEFL3     1 -------------------MTTIFGSSNAMFLNIEANKENIPPGTEFKKESFVLP-----
AtE2Fe     1 ------------------------------------------------------------
MmE2F7     1 MEVNCLTLKDLISPRQTRLDFAIEDAENAQKENIFVDRSRMTPKTPMKNEPIDLSKQRIF
MmE2F8     1 ----------------------MENQKENLFSEPHKRGLMKSPLHPSSKANMVLAE---I

OdE2F7    18 SPHIRELA-------KKDEEQLIAESLARETVIKEAP-VKERTGKRKLFEAELLSEGSSL
CeEFL3    37 EPRVNRTT-------DPDHENLLPSP-----VPRPSPAMSQLSDASFLSQDGGAVNTS-A
AtE2Fe     1 MSDLSPE---------------------RFKLAVTSP---------------SSIPES-S
MmE2F7    61 TPDRNPITPVKPVDRQPQVEPWTPTANLKMLISAASPDIRDREKKKELFRPIENKEDAFV
MmE2F8    36 QPDLGPLTTPTKPKEVSQGEPWTPTANLKMLISAVSPEIRSRDQKRGLSDNRSALPEA-R

                                                 DNA binding  domain
OdE2F7    70 GSNQDEKEC--------------LTRSLDKIVERFLERFSDKENEQ------FTLMDLEF
CeEFL3    84 DEADDDLEV-----------TSRKEKSLGLLCQRFLIAINEETVGSSTREVHLETVARKM
AtE2Fe    24 SALQLHHSY------------SRKQKSLGLLCTNFLALYNREGIEM----VGLDDAASKL
MmE2F7   121 NSLQLDVAGDGAVDEYEKQRPSRKQKSLGLLCQKFLARYPSYPLSTEKTTISLDEVAVSL
MmE2F8    95 DCLHEHLSG----DEFEKSQPSRKEKSLGLLCHKFLARYPKYPNPAVNNDICLDEVAEEL

OdE2F7   110 TAVKRRIYDVLNVLEGVGYIQKWQKKNSYLWTSKATMEEKIMKIRRSANVDSQNQDLVAT
CeEFL3   133 NVEKRRIYDIVNVMEALDAMQK-TNKSYYQWQGLESLPKLMFDLQNEA-VEEGLPERVLR
AtE2Fe    68 GVERRRIYDIVNVLESVGVLTR-RAKNQYTWKGFSAIPGALKELQEEG-VKDTFHRFYVN
MmE2F7   181 GVERRRIYDIVNVLESLHLVSR-VAKNQYGWHGRHSLPKTLRTLQRLG-EEQKYEEQMAC
MmE2F8   151 NVERRRIYDIVNVLESLHMVSR-LAKNRYTWHGRHNLTKTLGTLKSVG-EENKYAEQIMM

OdE2F7   170 IAHSIVA--------KDETFDDLTSHSDNSHLESDSSILNISELSNSSLDS--SSFSANS
CeEFL3   191 VEQAMCSFTELSSPRGKQGFKDIVGSFVSCTSTPTTPSTSFDSVTVKMEPA--GLLEKRS
AtE2Fe   126 ENV--------------KGSDDE----------DDDEESSQPHSSSQTDSSKPGSLPQSS
MmE2F7   239 LQQ--------------KELDLMGYRF---------GERRKDGSPDPRDPHLLDFSEADY
MmE2F8   209 IKR--------------KEYEQE-FDFIKSCGIEDHVIKSHTGQNGHSDMCFVELPGVEF

                                       DNA binding  domain
OdE2F7   220 SSCSKSSSMTKNLSSLTVRFISLFFRISPVNWTLTLDEAAERLV-ADSGELDQTATSKSR
CeEFL3   249 RVDTRDRQGRNSLAQLCRRFLMVLLSNPKNIRKVSLDVASTVLI-KDPETEGFEPPSRSR
AtE2Fe   162 DPSKIDNRREKSLGLLTQNFIKLFICSEA-IRIISLDDAAKLLL----GDAHNTSIMRTK
MmE2F7   276 PSSSANSRKDKSLRIMSQKFVMLFLVSKT--KIVTLDVAAKILI-EESQDTPDHSKFKTK
MmE2F8   254 RAASVNSRKDKSLRVMSQKFVMLFLVSTP--QIVSLEIAAKILIGEDHVEDLDKSKYKTK

OdE2F7   279 IASIKRRLYDITNVFLALGLIEKVQVTYGTGVHLQRKSAYHWIGVSVSGDKTGSNSTSKT
CeEFL3   308 C----RRLYDIANVLVALGLIKKVHYLFGTK----KIPLFVYCGPE--PDQTGSFDVFQS
AtE2Fe   217 V----RRLYDIANVLSSMNLIEKTHTLDS------RKPAFKWLGYNGEPTFTLSSDLLQ-
MmE2F7   333 V----RRLYDIANVLTSLALIKKVHVTEERG----RKPAFKWIGPV---DFSS-------
MmE2F8   312 I----RRLYDIANVLSSLDLIKKVHVTEERG----RKPAFKWTGPEISPNNSGSSPIMPL

OdE2F7   339 VLEDVTHLQQKNSIDRQCQTS---PSLLRSL--IADIHAELDEARAHDSISLSVNCPTCL
CeEFL3   358 VERLLS-------SSQNVPQT---PIIKAQTDKIVQQIAGFGK-RTLSEQNLTKPSGNT-
AtE2Fe   266 ---------------------------------LESRKRAFGTDITNVNVKRSKSSSSS-
MmE2F7   375 ---------------------------------IGKEMRSFDKD----------------
MmE2F8   364 PASLEAEQSAKENCAKNLFSTRGKPSFTRHP-SLIKLVKSIENDRRKISSAPSSPVKSNK
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OdE2F7   394 NQLHRHMPALCPHCRAHLPAATNNALSESSNSMLLTKQKLMFSPLPSSPLPSDLSRRQMT
CeEFL3   406 -----------------PKIAKIKSAAVRSSPMYMENNLFMFAEVVTAEAAAEKMRYDAF
AtE2Fe   292 -------------------------QENATERRLKMKKHSTPESSYNKSFDVHESRHG--
MmE2F7   386 --------------------------------------------LWYIPFPSSTCRQQ--
MmE2F8   423 AESSQNSPPVPNKMAQLAAICKMQLEEQSSEPRKKVKVNLARSGHYKPLAPLDPTVNTEL

OdE2F7   454 STPLAGVSKIASKFCKISSNLSSGTFAANRKRKS--SAPVFSTRMPPNFAARPAM-----
CeEFL3   449 AQLSRTILGSVASINSLNAPLTSSQHPMTSRLPP---LPMA----PLQLPKLPEI-----
AtE2Fe   325 ------------------------------------------------------------
MmE2F7   400 ------------------------------------------------------------
MmE2F8   483 ELLTPSLIQPLGVVPLIPSPLSSAVPVILPQAPSGPSYAIYLQPAQAQMLTPPPGLSPTV

OdE2F7   507 ------------------------------------------------------------
CeEFL3   497 ------------------------------------------------------------
AtE2Fe   325 ------------------------------------------------------------
MmE2F7   400 ------------------------------------------------------------
MmE2F8   543 CPTQPSNATGSKDPTDAPAEKTATDAATTGSLQPAPERHGAKHRSKETTGDRGTKRMITA

OdE2F7   507 -----------------------PQNTFMTAFVKK-----------------PLASKTLN
CeEFL3   497 -----------------------PKPQAQKQFTFP-----------------ATSRPRFN
AtE2Fe   325 ---------------------------SRGGYHFG-----------------PFAPGTGT
MmE2F7   400 ------------------------------NWPFP-------------------------
MmE2F8   603 EDSGPSSVKKPKEDLKALENVPTPTPLFPSGYLIPLTQCSSLGPDSVLSNTENSGTPSPN

OdE2F7   527 F---------ASPIAPNEIERQRKPSRN---GANVHFLLP--------------------
CeEFL3   517 FPDYTPVQSTIRPLVSQMTFPQESSQLHNLDVKPKHLMSN--------------------
AtE2Fe   341 YPTAGLEDNSRRAFDVENLDSDYRPSYQ---NQVLKDLFS--------------------
MmE2F7   405 ----------VLPV-----------------TRNLRLMTS--------------------
MmE2F8   663 HRIYGSPIAGVIPVASSELTAVNFPPFH---VTPLKLMVSPTSMAAVPVGNSPALNSGHP

OdE2F7   555 --------------------------------------------KMEIHLPASPKRVKDD
CeEFL3   557 -------------------------------------------------ILGESKKFKNN
AtE2Fe   378 -------------------------------------------------------HYMDA
MmE2F7   418 ------------------------------------------------------------
MmE2F8   720 APAQNPSSAIVNFTLQHLGLISPGVQMSASPGPAGTVPVSPRVEADNLSSRQRRATNHD

OdE2F7   571 SGLGPSPSLFPSNVANQSVKNSK-------------------------------------
CeEFL3   568 QNTFEHTTSSAFQVVKKGETRPKKVFGEIQNLQ---------------------------
AtE2Fe   383 WKTWFSEVTQENPLPNTSQHR---------------------------------------
MmE2F7   418 --LLEQ------------------------------------------------------
MmE2F8   780 SPVLGQSQLNGQPVAGTGAQQPVPVTPKGSQLVAENFFRTPGGPTKPTSSPYTDFDGANK

OdE2F7       ---------------------
CeEFL3       ---------------------
AtE2Fe       ---------------------
MmE2F7       ---------------------
MmE2F8   840 TSFGTLFVPQRKLEVSTEDIH
```

Figure A5. Multiple sequence alignment of atypical E2F sequences with *O. dioica* atypical E2F7. *O. dioica* E2F7 was aligned with members of atypical E2Fs from other species using MUSCLE. Dark shading indicates identical conserved amino acid positions and grey shading represents similar amino acids. Predicted DNA binding regions are indicated by red boxes. *Mm*E2F7 - *Mus musculus* E2F7, *Mm*E2F8 - *Mus musculus* E2F8, *Od*E2F7 - *Oikopleura dioica* E2F7, *Ce*EFL3 - *Caenorhabditis elegans* EFL3, AtE2Fe - *Arabidopsis thaliana* E2Fe.

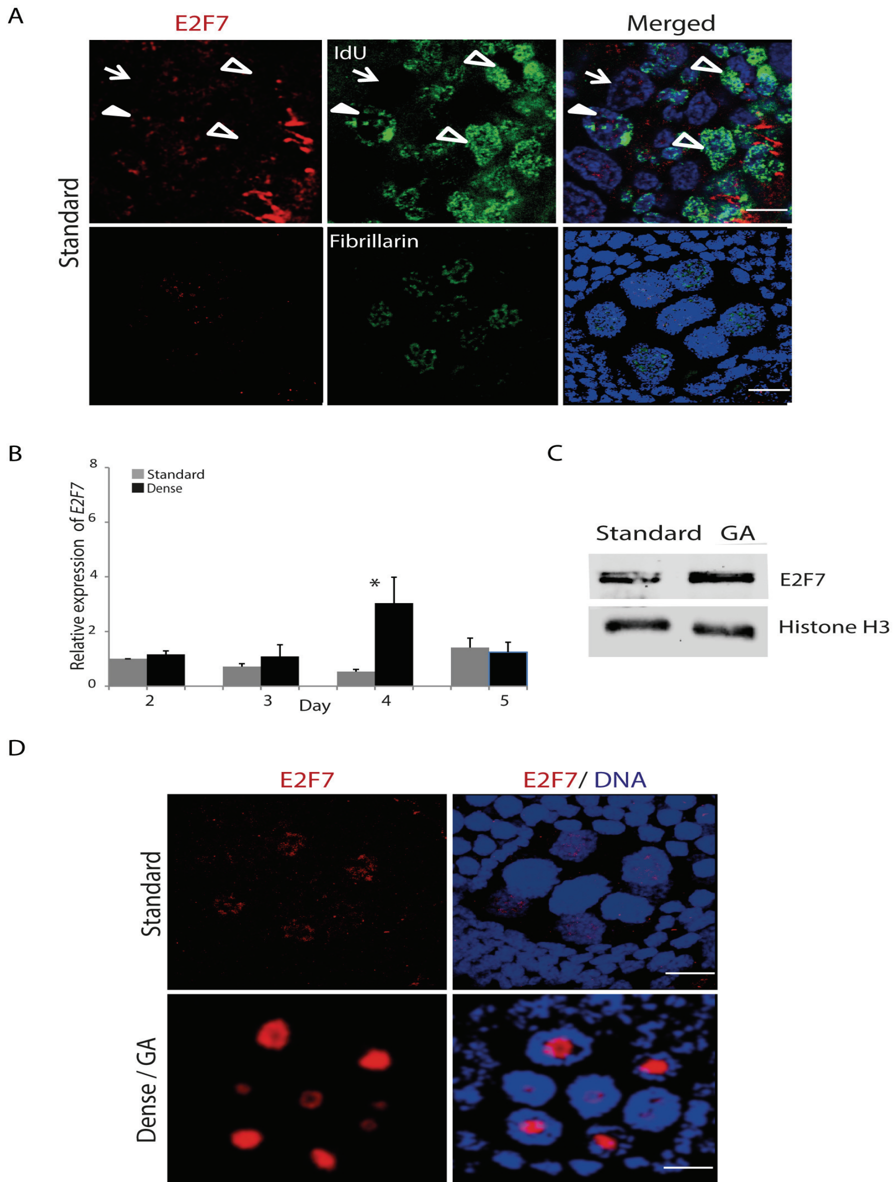

Figure A6. Nutrient signaling regulates the expression of atypical transcription repressor E2F7 in somatic endocycling cells of *O. dioica*. A) At standard culture conditions, E2F7 was present in the nucleolus of G phase (empty arrow head) and early S phase (filled arrow head) but not during mid S phase (arrows) of somatic endocycling cells. Immunostained E2F7 colocalized with the nucleolar marker fibrillarin. B) Quantitative Real time PCR was performed using RNA isolated from *O. dioica* cultured under standard and dense conditions at days 2, 3, 4 and 5. The expression of *E2F7* was not affected in day 2, day 3 and day 5 animals grown under dense conditions but increased specifically in day 4 animals at high culture densities. The relative mRNA expression values and standard error, were derived from three different

populations. mRNA expression was normalized to transcription of the house keeping gene *EF1β*. C) Western blot analysis of whole cell lysates from animals cultured at standard (Standard) or high densities that induced growth arrest (GA). In the latter case, E2F7 levels were increased D) Immunostaining of E2F7 showed accumulation in the nucleolus of arrested endocycling cells of GA *O. dioica*. Scale bars indicate 10 μm. Error bars indicate standard error.

Table A1. NLS predicted from Eukaryotic linear Motif (http://elm.eu.org)

Organisms	Protein	Predicted NLS
Human (Larrea *et al*.,2008, Bockstaele *et al*., 2006a, b)	Cyclin D1 CDK4/6 p27 p21	No No Yes Yes
Drosophila	Cyclin D CDK4 Dacapo	No Yes Yes
C. elegans	Cyclin D CDK4 CKI-1	Yes No Yes
O. dioca	Cycln Dd Cyclin Db CDK6 CKIa	Yes No No Yes

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