The APC/C Inhibitor XErp1/Emi2 Is Essential for *Xenopus* Early Embryonic Divisions

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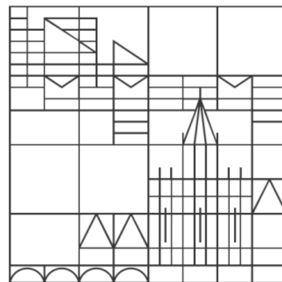

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1 Summary

In many vertebrates, mature eggs await fertilization arrested at metaphase of meiosis II. Recently, XErp1/Emi2 was identified as the cytostatic factor mediating the metaphase-II arrest in mature *Xenopus* eggs. XErp1 prevents anaphase onset by directly inhibiting the ubiquitin-ligase Anaphase promoting complex/cyclosome (APC/C). Upon fertilization, XErp1 is targeted for degradation resulting in APC/C activation and the consequent exit from meiosis.

In canonical cell cycles, Emi1 and the spindle-assembly-checkpoint control mitotic progression by inhibiting the APC/C. On the contrary, early embryonic divisions lack these APC/C-inhibitory components, which raises the question of how these cycles are regulated. In the work presented here it is shown that XErp1 quickly reaccumulates in the early embryonic divisions of *Xenopus* and that it is essential as mitotic APC/C inhibitor regulating the timely destruction of APC/C substrates.

Loss of XErp1 by injection of antisense morpholio oligos (MOs) in one-cell embryos is lethal. Co-injection of XErp1 mRNA not targeted by the MOs completely rescues the phenotype and the injected embryos develop into healthy tadpoles, which demonstrates that the phenotype is specific for XErp1. Furthermore, in contrast to exit from meiosis II, protein levels of XErp1 remain constant during the early embryonic divisions, but XErp1 is phosphorylated in a cell cycle dependent manner. Cyclin dependent kinase 1 (Cdk1/Cyclin B) and the Protein Phosphatase 2A (PP2A) together with cyclic AMP dependent Protein Kinase A (PKA) act as regulators of XErp1. Phosphorylation of XErp1 by Cdk1 inhibits its APC/C inhibitory function resulting in APC/C activation. PP2A antagonizes the Cdk1 mediated inactivation of XErp1 and promotes its binding to the APC/C. PKA phosphorylates XErp1 at sites critical for PP2A recruitment. Thus, Cdk1 and PP2A/PKA are at the core of early mitotic cell cycles by antagonistically controlling XErp1-activity, which results in oscillating APC/C-activity driving the rapid cleavage divisions.

Zusammenfassung

In den meisten Wirbeltieren arretieren reife Eizellen vor der Fertilisation in Metaphase von Meiose II. In reifen *Xenopus* Eizellen ist XErp1/Emi2 für den Meiose II Arrest verantwortlich und konnte als sogenannter „cytostatische Faktor" identifiziert werden. XErp1 verhindert den Eintritt in Anaphase indem es den Anaphase-Promoting-Complex/Cyclosome (APC/C), eine Ubiquitinligase, direkt inhibiert. Fertilisation führt zum Abbau von XErp1, was in der Aktivierung des APC/C, dem Abbau von wichtigen APC/C-Substraten wie Cyclin B und Securin sowie im Verlassen des Meiose II Arrests mündet.

In normalen Zellzyklen regulieren Emi1 und der Spindle-Assemblierungs-Checkpoint die Passage durch Mitose, indem sie den APC/C inhibieren. In embryonischen Zellteilungen von *Xenopus* sind diese Mechanismen abwesend, was die Frage aufwirft, wie die Teilungen kontrolliert werden können. In der hier vorgestellten Arbeit wird gezeigt, dass XErp1 nach Fertilisation wieder akkumuliert und als essentieller mitotischer Regulator des APC/C fungiert, der den zeitlich richtigen Abbau von APC/C Substraten kontrolliert.

In Abwesenheit von XErp1, herbeigeführt durch Injektion von Morpholino Oligos (MOs), sind die Embryonen nicht überlebensfähig. Dieser Phänotyp kann durch Expression von Wildtyp XErp1, jedoch nicht von einer Mutante mit einem Defekt in der APC/C Inhibierung, aufgehoben werden. Dies verdeutlicht, dass die APC/C-inhibierende Funktion von XErp1 für die frühen embryonischen Teilungen essentiell ist und das folgerichtig in Abwesenheit von XErp1 APC/C Substrate destabilisiert werden. Desweiteren wird aufgezeigt, dass XErp1 während der embryonischen Teilungen zellzyklusabhängig modifiziert wird. Cyclin abhängige Kinase 1 (Cdk1/Cyclin B) phosphoryliert XErp1 und verhindert die Binding an den APC/C. Protein Phosphatase 2A (PP2A) wirkt Cdk1 entgegen und aktiviert XErp1. Für die Bindung von PP2A an XErp1 ist wiederum die Phosphorylierung an weiteren Aminosäuren durch cyclische AMP abhängige Protein Kinase A (PKA) nötig.

Damit bilden Cdk1 sowie PP2A/PKA den Grundstein für embryonische Zellteilungen in *Xenopus*. Sie regulieren XErp1 antagonistisch und stellen so eine oszillierende APC/C Aktivität sicher, die die Zellteilungen vorantreibt.

2 Introduction

The generation of offspring is one of the fundamental processes of life. In single cell organisms, cell division results in the formation of offspring, while in multi cellular organisms it is needed for growth. Here, distribution of the genetic material from parents to their progenies starts with the fertilization of an oocyte by sperm. The newly generated embryo divides and provides all its cells with the same genetic material in each division. This process is controlled by the cell cycle machinery.

2.1 The eukaryotic cell cycle

The cell cycle can be divided into four separate phases (**Figure 1**): In G1 (“G” stands for gap-phase), the cell prepares for DNA replication. When environmental conditions are not favorable for division cells in most multi cellular organisms can exit G1 and enter a prolonged non-dividing stage called G0. When the decision for DNA replication is made, cells enter the synthesis- or S-Phase, where the genome is once (and only once) duplicated. In G2 it is elaborated if DNA replication was successful and possible errors are corrected. The cell is not allowed to enter M-Phase (the processes of mitosis and cytokinesis) until all DNA damage is repaired and if this does not happen, apoptosis is started to eliminate the cell. Failure to do so might lead to unequal distribution of the genetic material and can result to severe diseases like cancer (Ganem & Pellman, 2012; Suijkerbuijk & Kops, 2008; Vitre & Cleveland, 2012).

Mitosis leads to the formation of two genetically identical daughter cells and can be subdivided into multiple steps (**Figure 1**). In prophase the chromosomes condense to a degree that RNA transcription is not possible anymore (Gottesfeld & Forbes, 1997; Hernandez-Verdun, 2011). The two sister chromatids are tightly held together at the arm- and centromere-region by a ring shaped protein complex called cohesion that entraps the DNA (Haering et al, 2008). During Prometaphase the nuclear membrane is disassembled and the spindle poles, in most cells organized by the centrosomes (Keating & Borisy, 1999;

Paoletti & Bornens, 1997; Varmark, 2004), start to nucleate microtubules. These attach at the kinetochores, a highly ordered protein structure at the centromeres of sister chromatids. Current models implicate different ways how the initial contact between microtubules and the kinetochores are made. On one hand microtubules itself might “search and capture” kinetochores (Gundersen, 2002; Schuyler & Pellman, 2001), but recent data implicate also the contribution of different motor proteins and microtubule plus-end associated factors as necessary components to establish kinetochore-mircotubule interactions (Mimori-Kiyosue & Tsukita, 2003; O'Connell & Khodjakov, 2007; Odde, 2005; Wu et al, 2006).

During metaphase the chromosomes are moved to the middle of the cell due to pushing and pulling forces, where they are organized in a so called metaphase plate. When all chromosomes are attached to the mitotic spindle and correctly aligned on the metaphase plate, anaphase is initiated. The sister chromatids are finally split at their centromeres and pulled to opposite spindle poles. With this step, chromosome separation is completed, but the two DNA masses are still in the same cytoplasm. In telophase the spindle poles move even further apart from each other and the DNA already starts to decondense again. The nuclear membrane is reassembled and between the two DNA masses, where the metaphase plate was formally located, a contractile ring is formed, which leads to abscission of the two newly formed daughters during cytokinesis.

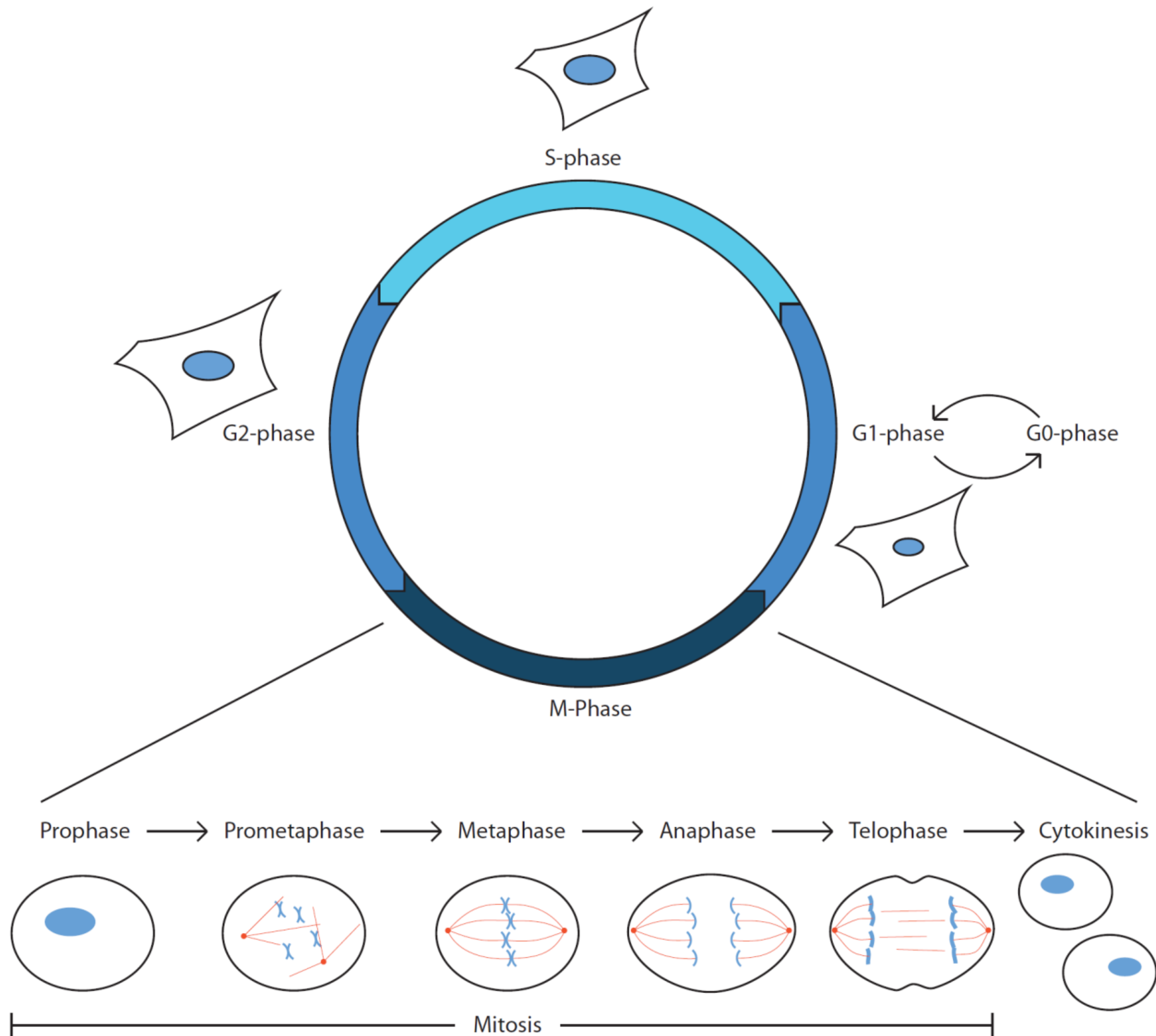

Figure 1: The eukaryotic cell cycle
The cell cycle of eukaryotes can be divided in four different phases: G1 (with the option to exit the cell cycle to G0), S-Phase, G2 and M-Phase. Mitosis itself also consists of five phases: Prophase, Prometaphase, Metaphase, Anaphase and Telophase. After cytokinesis a new round of the cell cycle starts separately in both daughter cells. Chromosomes/DNA are shown in blue, microtubules and spindle poles in red. During Mitosis the cell shape changes to a round form and the cell looses contact with the surrounding tissue. Modified from David O. Morgan “The Cell Cycle”.

2.1.1 Cdk1/Cyclin B drive the cell cycle

The above described processes of mitotic progression are mainly regulated by one protein complex, the Cyclin-dependent protein kinase 1 (Cdk1) together with its activator Cyclin B (CycB).

When cytoplasm of mature *Rana pipiens* eggs was injected into prophase arrested oocytes of the same frog species these started to perform meiotic maturation (Masui & Markert,

1971) and this activity was named accordingly maturation promoting factor (MPF). Cdk1/Cyclin B was identified as the MPF (Dunphy et al, 1988) and as we know by now, it is also responsible for normal mitotic cell divisions (Gautier et al, 1989). However, recent data suggest that MPF not only consists of Cdk1/Cyclin B but also another kinase called greatwall (Gwl). Injection of purified Cdk1/Cyclin B complexes into *Xenopus* oocytes are not able to induce meiotic progression and need active Gwl to start meiosis (Hara et al, 2012). It therefore seems that MPF is the combined activity of two kinases that complement each other: Cdk1/Cyclin B phosphorylates mitotic/meiotic substrates and at the same time Gwl inactivates the counteracting phosphatase (see below) to promote M-Phase entry. Besides this, Masui and Makert also identified another activity, the so called cytostatic factor (CSF), which will be discussed later. The activity of Cdk1/Cyclin B can be regulated by different means (**Figure 2**).

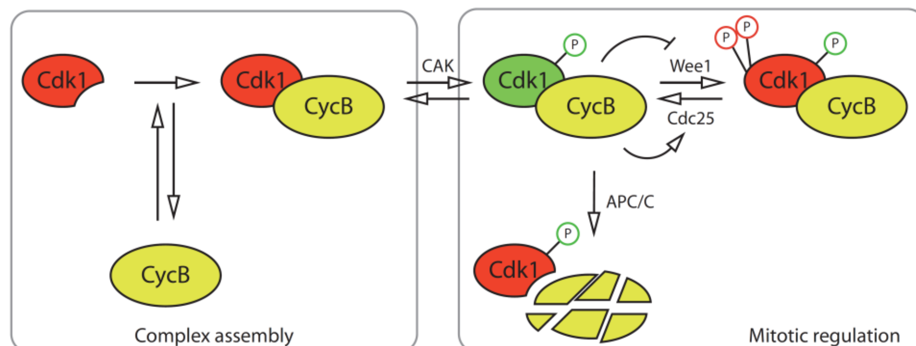

Figure 2: Cdk1 regulatory mechanisms
Green colouring indicates active Cdk1, while red color means Cdk1 inactivation. Cyclin B association with Cdk1 is the fundamental basis for its kinase activity, but to be fully active the complex needs to be phosphorylated in its T-loop at T161 by a Cdk Activating Kinase (CAK). The active complex can be inactivated by different means. Before entry into mitosis, Cdk1 is phosphorylated at T14/Y15 by the inhibitory kinases Wee and Myt1. When the cell enters mitosis the Cdk1 auto-amplification loops starts and Cdc25 dephosphorylates and activates Cdk1. With anaphase onset, Cyclin B becomes ubiquitinated by the APC/C and is degraded by the proteasome, Cdk1 is turned off. Adapded from (Hormanseder et al, 2013)

The most important one is the association between the kinase subunit Cdk1 and the activating subunit Cyclin B (complex assembly in **Figure 2**). When this complex is phosphorylated on Thr-161 by a Cdk Activating kinase (CAK), it becomes active (Krek &

Nigg, 1992), but this phosphorylation seems to be unregulated and therefore does not contribute to regulated Cdk1 activity as it is needed for entry into and exit from M-Phase (Fisher & Morgan, 1994; Tassan et al, 1994).

Cyclin B starts to be translated from S-phase on and its levels rise until mitosis, but Cdk1 activity remains low during G2 and turned on in a switch-like manner with entry into M-phase (Mitotic regulation in **Figure 2**). This is achieved by other Cdk1-regulatory mechanisms as for example inhibitory phosphorylations. During G2 phase two kinases, named Wee1 and Myt1, phosphorylate Cdk1 at Thr-14 and Tyr-15 to inactivate the Cdk1/Cyclin B complex (Mueller et al, 1995a; Mueller et al, 1995b; Parker & Piwnica-Worms, 1992). Additionally, they also respond to DNA damage and avoid mitotic onset, when errors during DNA replication in S-phase arose to give the cell sufficient time for repair (Chow & Poon, 2012; Feilotter et al, 1992). Thus the Cdk1/Cyclin B complex is assembled but inactive until the cell is ready to enter mitosis. At mitotic entry, Cdk1 is dephosphorylated at the inhibitory sites by the Cdc25 phosphatase family (**Figure 2**). Cdc25 itself is activated first by another kinase, the polo like kinase 1 (Plk1) (Abrieu et al, 1998; Qian et al, 1998; Toyoshima-Morimoto et al, 2002), then activates Cdk1 partially and is subsequently fully activated by Cdk1/Cyclin B itself (Millar et al, 1991; Moreno et al, 1989). On the other hand, Cdk1/Cyclin B can inactivate Wee1/Myt1 by phosphorylation (Okamoto & Sagata, 2007) and thus, Plk1 in conjunction with Cdk1 is able to start the auto-amplification loop of Cdk1 activation, which promotes mitotic entry. Recent data show, that additionally to Cdk1 activation, inactivation of counteracting phosphatases, namely Protein phosphatase 2 A (PP2A), are necessary. Presumably Cdk1/Cyclin B itself activates the greatwall kinase, which subsequently phosphorylates the two small proteins Ensa and Arpp19. Upon binding of Ensa/Arpp19 to PP2A-B55δ the phosphatase is inhibited (Gharbi-Ayachi et al, 2010; Mochida et al, 2010), which creates a so called bistable system (Pomerening et al, 2003) and ensures a switch-like transition from non-phosphorylated Cdk1 substrates to hyperphosphorylated ones.

2.1.2 The complex nomenclature of PP2A

PP2A is one of the major phosphatases in the cell and counteracts Cdk1/Cyclin B activity by removing phosphates on substrates (Mochida et al, 2009) and therefore regulating cell cycle progression. PP2A is a heterotrimeric complex consisting of a scaffold subunit (called A-subunit), a catalytic part (called C-subunit) and regulatory B-subunits. There are two isoforms of the A- and C-subunit each and many different types of B-subunits that mediate specificity towards PP2A substrates. The B-subunits are clustered into four groups (Sents et al, 2013):
1) B-subunits, consisting of B55 isoforms α to δ,
2) B'-subunits, containing B'56 isotypes α to ε members, where δ is not present in *Xenopus*,
3) B''-subunits, also called PR48 or PR72, with at least three members from α to γ,
4) B'''-subunits, also called PR93 or striatins (Moreno et al, 2000), with up to four isoforms.
This large variety theoretically gives rise to nearly 100 different holoenzymes. However, not every combination probably exists in the cell at the same time, since the regulation of PP2A assembly seems to be very complex. For example, the association of the A- and the C-subunit are already tightly controlled on translational and post-translational level (Hombauer et al, 2007; Sents et al, 2013). Additionally, association of B-subunits with the A-C-dimer is dependent on methylation (Bryant et al, 1999; Wu et al, 2000) and the C-subunit can be phosphorylated, which seems to inactivate it (Chen et al, 1992; Schmitz et al, 2010). How this influences the cell cycle is less understood and requires more investigations.

2.1.3 The APC/C regulates cell cycle progression

When the cell exits metaphase and anaphase is initiated, Cyclin B is ubiquitylated by the ubiquitin ligase Anaphase promoting complex/cyclosome (APC/C) and subjected to proteasomal degradation. By this event, Cdk1 is suddenly turned off and the mitotic state is left. Besides Cyclin B also securin is recognized by the APC/C. Securin is a small chaperone of the protease separase that cleaves the cohesin proteins at the centromeres of sister kinetochores and initiates their separation (Hagting et al, 2002; Stemmann et al, 2001;

Waizenegger et al, 2002; Zhang et al, 2008; Zur & Brandeis, 2001). Thus, the APC/C coordinates both, switching off Cdk1/Cyclin B activity and chromosome separation, leading to the entry into a new round of the cell cycle.

The APC/C itself is a multi subunit E3 ubiquitin ligase (Herzog et al, 2009) and, like Cdk1/Cyclin B, its activity is controlled by different means. Substrates of the APC/C including Cyclin B and Securin contain one or two common recognition motifs, called the destruction-box (D-Box) and the KEN-motif. KEN stands for the three aminoacids Lysine (K), Glutamate (E) and Asparagine (N). It is not yet entirely clear whether substrates bind directly and autonomously to the APC/C or if they are brought there by the APC/C coactivators Cdc20 and Cdh1 (Matyskiela & Morgan, 2009), which are necessary for APC/C function. Both coactivators are WD40-domain containing proteins (Yu, 2007) that bind and activate the APC/C specifically during M-Phase (Cdc20) and early G1 (Cdh1). By Cdk1 dependent phosphorylation, the APC/C can be activated (Kraft et al, 2003), which ensures high APC/C activity only at the transition from metaphase to anaphase. But at the same time the coactivator Cdc20 is inactivated by Cdk1 phosphorylation, introducing a delay and restricting the APC/C activation window to a very short time (Labit et al, 2012).

2.1.4 APC/C inhibitors

Besides this balanced regulation between Cdk1 and the APC/C, additional factors influence APC/C activity in mitosis.

For example, the APC/C should only be activated when all chromosomes are correctly attached to the mitotic spindle and this is ensured by the so called spindle assembly checkpoint (SAC). It consists of the proteins Mad2, Bub3 and BubR1 that form a complex with the APC/C coactivator Cdc20 and thereby inhibit the APC/C as the mitotic checkpoint complex (MCC) (Musacchio & Salmon, 2007). Interestingly, BubR1 contains an additional bipartite KEN-motif and binds to the APC/C in a pseudosubstrate like manner (Burton & Solomon, 2007; Elowe et al, 2010; Malureanu et al, 2009). Crystal structural data suggests

that the MCC displaces Cdc20/Cdh1 from their normal position on APC10 (Chao et al, 2012; Herzog et al, 2009) and additionally the bipartite KEN-motif of BubR1 blocks the substrate binding pocket and thereby inhibits substrate recruitment. Once all kinetochores are attached, the MCC is resolved and the APC/C can become active to destroy its targets.

During S-phase and G2 the APC/C is kept inactive by binding to another protein, the early mitotic inhibitor 1 (Emi1). Similar to BubR1 it contains a D-Box (another APC/C binding motif) and binds directly to the APC/C as pseudosubstrate inhibitor (Miller et al, 2006) but does not sequester Cdc20 away from it. Depletion of Emi1 results in overduplication of the genome due to decreased levels of the APC/C substrate geminin, an inhibitor of DNA replication (Di Fiore & Pines, 2007; Di Fiore & Pines, 2008; Machida & Dutta, 2007). Structural data of Emi1 are not available but besides the D-Box also a Zinc-binding-region and the last two aminoacids Arginine (R) and Leucine (L) are necessary for APC/C inhibition. The budding yeast protein Acm1 also inhibits the APC/C during G1 and S-phase and uses, similarly to BubR1 and Emi1, a pseudosubstrate inhibitory mechanism (Burton et al, 2011; Choi et al, 2008; Martinez et al, 2006). Besides APC/C inhibition in normal mitosis, most organisms also developed strategies to restrict APC/C activity in other cell cycle types. *Drosophila* Rca1 for example regulates the APC/C during embryogenesis by modulating APC/C activity towards Cyclin A (Zielke et al, 2006). And fission yeast restrict the APC/C activity during meiosis by the action of Mes1p (Kimata et al, 2011; Kimata et al, 2008), which is surprisingly first a substrate of the APC/C at the meiosis I to meiosis II transition but later in meiosis II an inhibitor. Knowing this, it is not unexpected that also vertebrates have developed a mechanism to regulate APC/C activity during meiosis.

2.2 XErp1 arrests oocytes at Meiosis II

As shortly pointed out above, Masui and Makert not only postulated the MPF, but also a so called cytostatic factor (CSF) (Masui & Markert, 1971). When they injected cytoplasm of mature *Rana pipiens* eggs into dividing frog embryos, these arrested in mitosis. Since this

cell cycle arresting activity was not present before and after the eggs entered meiosis, they formulated three criteria that a potential CSF has to fulfill:

1) It has to accumulate during meiotic progression and reach its highest activity at the end of meiosis II.

2) When injected into dividing embryos, it has to cause a cleavage arrest and

3) it has to disappear with fertilization.

2.2.1 Identification of XErp1 as APC/C inhibitor in the meiosis II arrest

The CSF arrest is characterized by high Cdk1/Cyclin B activity and chromosomes aligned to the meiotic spindle, comparable to a normal Mitosis. During this period, the APC/C is inactive to allow high Cdk1 activity and should only become activated when fertilization takes place. For over three decades, the molecular mechanism of the CSF resisted its identification. The first protein implicated in CSF arrest was the proto oncogene cMos, because it accumulates during Meiosis and its depletion releases the arrest. Moreover, it is degraded at fertilization and when injected into dividing embryos it causes a cell cycle arrest (Sagata et al, 1988; Sagata et al, 1989). Thereby it meets all three criteria defined by Masui and Makert. Further biochemical analysis revealed that the downstream kinases of the cMos pathway, MEK, MAPK and the ribosomal S6 kinase (p90Rsk) are involved in CSF arrest too, and that p90Rsk is the only one needed, since its sole injection in embryos causes a cleavage arrest (Abrieu et al, 1996; Bhatt & Ferrell, 1999; Gross et al, 1999; Haccard et al, 1993).

Since a cell cycle arrest can be caused by APC/C inhibition, the SAC proteins drew attention to themselves, because Bub1 can be phosphorylated and activated by p90Rsk (Schwab et al, 2001). Therefore it was speculated that the SAC not only causes a cell cycle arrest during normal mitosis, when chromosomes are not properly aligned to the mitotic spindle, but is also responsible for the CSF arrest. However, in mice oocytes the SAC proteins are dispensable for CSF arrest (Tsurumi et al, 2004) and also in *Xenopus* it remains unclear how SAC inactivation could be coupled to fertilization. Moreover, also without any DNA present, a

prerequisite for the SAC, *Xenopus* egg extract is capable of maintaining the CSF arrest for a very long time. For this reasons the contribution of SAC proteins to CSF arrest are still under discussion.

The search for the CSF was concluded by the identification of the *Xenopus* Emi1 related protein (XErp1), the meiotic homologue of Emi1. It was shown that it can directly bind and thereby inhibit the APC/C and also induces a cell cycle arrest when injected into dividing embryos (Schmidt et al, 2005). Moreover, it accumulates during meiotic progression and is inactivated at fertilization (Rauh et al, 2005). With this it fulfills all three criteria formulated by Masui and Makert. The function of the cMos pathway and its downstream kinases during CSF arrest could be linked to XErp1 by the finding that p90Rsk phosphorylation on XErp1 is essential for its function as APC/C inhibitor (Inoue et al, 2007; Nishiyama et al, 2007a). With this, the CSF is not a single protein, but an ordered cascade of different kinases finally activating an APC/C inhibitor. Without cMos and its downstream members, XErp1 would not be a functional APC/C inhibitor and likewise cMos would have no cytostatic effect without XErp1.

2.2.2 Mechanism of APC/C inhibition of XErp1

It is not yet fully understood how XErp1 inhibits the APC/C on a molecular basis, but three important regions were indentified. The C-terminal part of XErp1 contains a D-Box, a Zinc-binding-region and the last two amino acids Arginine (R) and Leucine (L), the so called RL-tail (Ohe et al, 2009; Schmidt et al, 2005; Tang et al, 2010). All three motifs are necessary for APC/C binding and inhibition, even with different contributions. The current idea is that XErp1 binds to the APC/C *via* its RL-tail and then inhibits it over the ZBR domain and blocks substrate recruitment with its D-Box. Surprisingly, XErp1 seems not to function as a pseudosubstrate inhibitor as one would expect from similarity to BubR1 or Emi1, but rather composes a catalytic mechanism that interferes with the transfer of ubiquitin from the APC/C to its substrates. Furthermore, the concentration of XErp1 compared to the APC/C is

substoichiometric so that a one-to-one inhibitory mechanism would not be possible (Tang et al, 2010). However, it is not known how much of the APC/C complexes are really in an active state during CSF arrest and therefore need to be inhibited by XErp1. It was also speculated that XErp1 itself might work as an (auto-)ubiquitin ligase, because it was found that XErp1 can ubiquitylate itself. It remains to be evaluated if the observed ubiquitylation was indeed autoubiquitylation as suggested (Tang et al, 2010) or if it was maybe catalyzed by minimal amounts of copurified APC/C. In support of this idea, recent data showed that the APC/C in conjunction with its E2 enzyme UbcX can ubiquitylate XErp1, but this ubiquitylation does not lead to proteasomal degradation and instead has regulatory functions (Hormanseder et al, 2011).

XErp1 also carries a so called F-Box domain in its C-terminus. The F-Box domain was first identified in Cyclin F as an interaction domain for the S-phase kinase associated protein 1 (Skp1) (Bai et al, 1996), which is necessary for the interaction with the E3 ubiquitin ligase complex SCF (Skp1 cullin F-Box-protein). This might implicate an APC/C independent function of XErp1. Indeed, interaction between Skp1 and XErp1 could be shown (unpublished data in (Schmidt et al, 2005)), but so far there are no observations about a functional role for the F-Box of XErp1.

2.2.3 Control of XErp1 activity during CSF arrest

XErp1 is not only target of the cMos-MAPK-pathway, which activates it, but is also negatively regulated by Cdk1. There are two Cdk1-site clusters in XErp1, one in the more N-terminal part and a second one in the C-terminal part (**Figure 3** A). When phosphorylated at the C-terminal sites, XErp1 loses its ability to bind and with this to inhibit the APC/C (Wu et al, 2007b). The negative charge might thereby directly interfere with APC/C binding, since it was recently shown that Cdc20 also needs to be in a dephosphorylated state, before it can bind to the APC/C (Labit et al, 2012). The N-terminal Cdk1 cluster contributes to XErp1 stability (Wu et al, 2007b). When XErp1 is phosphorylated here by Cdk1, it leads to the recruitment of

Casein kinase 1 (CK1) and both kinases conjoinedly create a binding site for *Xenopus* Plk1 (Plx1) (**Figure 3** B) (Isoda et al, 2011). Subsequently, Plx1 phosphorylates Ser-33 and Ser-38 as well as Ser-284 and Ser-288, leading to $SCF^{\beta\text{-TRCP}}$ dependent ubiquitylation and destruction of XErp1. Reportedly, the aforementioned F-Box of XErp1 is not necessary for this degradation. If the Cdk1/Cyclin B phosphorylation were not counteracted, XErp1 levels would decrease during CSF arrest until the APC/C is activated and pathogenic activation of the egg would happen in the absence of fertilization. As described before, the cMos pathway *via* p90Rsk is essential for XErp1 function. The kinase activates XErp1 by phosphorylating it at Ser-335 and Thr-336 and possibly at Ser-342 and Ser-344 as well (Inoue et al, 2007; Nishiyama et al, 2007a; Wu et al, 2007a), which leads to the binding of PP2A-B'56 (Isoda et al, 2011; Wu et al, 2007a) and subsequent removal of the inhibitory phosphorylations done by Cdk1 on XErp1 (**Figure 3** A and B).

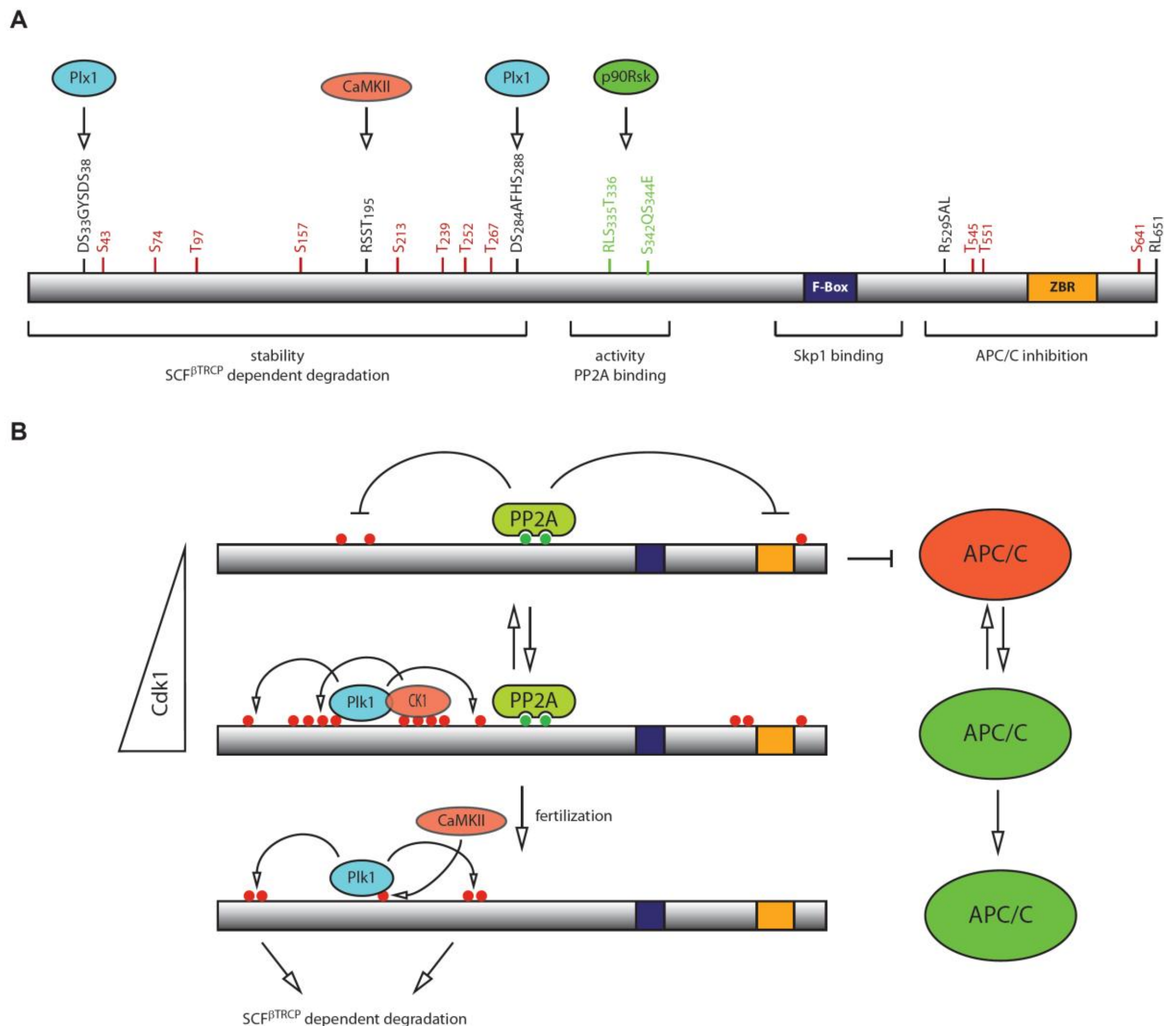

Figure 3: XErp1/Emi2 regulation

(A) The spacing of all shown sites is done according to the complete protein. Red colored sites have a negative effect on XErp1's function to inhibit the APC/C, green sites are positive. Cdk1 phosphorylates all sites shown in red of XErp1 in the N- and C-terminal parts. The N-terminal part of XErp1 is responsible for its stability. The CaMKII site is phosphorylated at fertilization leading to rapid XErp1 degradation. Phosphorylation at the p90Rsk recruits PP2A-B'56 and this activates XErp1 by removing the Cdk1 phosphorylations. The function of the F-Box in XErp1/Emi2 is not known, but it mediates a bona fide interaction with Skp1. In the C-terminal part the three motifs for APC/C binding and inhibition are located, which are the D-Box (RxxL), the ZBR-domain and the RL-tail. APC/C interaction is lost when XErp1 is phosphorylated by Cdk1 at the C-terminal sites.

(B) During CSF arrest XErp1 is dynamically regulated. PP2A is bound to XErp1 upon phosphorylation by p90Rsk, when Cdk1 activity raises it prevails over PP2A and XErp1 is transiently inactivated. The APC/C becomes active and degrades Cyclin B until Cdk1 activity is low that PP2A can take over again. At fertilization, XErp1 is sequentially phosphorylated by CaMKII and Plx1, leading to its degradation by the SCF.

Adapded from (Hormanseder et al, 2013)

Both counteracting pathways, phosphorylation and dephosphorylation of XErp1, create a self-regulatory system. When Cdk1 activity rises over a certain threshold, XErp1 becomes phosphorylated, destabilized and loses its binding to the APC/C, which is no longer inhibited. This eventually leads to its activation and a little of Cyclin B degradation until PP2A-B'56 prevails over Cdk1 phosphorylation and XErp1 is able to inhibit the APC/C again. With this fine balance, a high Cdk1 activity in Meiosis is ensured and at the same time a very responsive APC/C is formed.

2.2.4 XErp1 is degraded at fertilization

In *Xenopus*, a transient calcium influx runs over the egg at fertilization. This activates calmodulin, a calcium sensitive signaling protein, which in turn transmits the fertilization sign to two independent branches. One leads to the activation of the phosphatase PP2B/calcineurin, which is important for the dephosphorylation of proteins at fertilization (Mochida & Hunt, 2007; Nishiyama et al, 2007b). Another, triggered by Ca^{2+} and calmodulin, is the activation of calmodulin dependent kinase II (CaMKII) (Dupont, 1998; Lorca et al, 1994). This kinase phosphorylates XErp1 at Thr-195 to create a PDB-dependent binding site for Plx1, which subsequently phosphorylates Ser-33 and Ser-38, leading to recognition of XErp1 by the $SCF^{\beta\text{-TRCP}}$ and proteasome dependent destruction (Rauh et al, 2005) (**Figure 3**). In contrast to the fine balanced regulation between the Cdk1 and PP2A during the CSF arrest, this event is not reversible once fertilization is completed and the APC/C is fully activated. With this cascade, APC/C activation is perfectly coupled to fertilization, since the APC/C inhibitory function and the Ca^{2+} responsiveness are united on one protein. Only through fertilization with the occurring calcium signal, the APC/C can become active.

2.3 The prolonged first cell cycle after fertilization

In all metazoa the first cell cycle after fertilization is characterized by its unusual length compared to the following divisions. In *Xenopus* for example, the first cell cycle takes about 90 minutes, whereas the subsequent eleven fast cleavage cycles only last for about 30 minutes (Hara et al, 1980; Newport & Kirschner, 1982a) (**Figure 4**). The prolonged first division has to fulfill different functions, since only with fertilization meiosis is completed, characterized by the extrusion of the second polar body. Besides this, the male pronucleus moves through the egg and the DNA of both pronuclei decondenses to initiate DNA replication (Ferreira & Carmo-Fonseca, 1997; Luthardt & Donahue, 1973). To ensure the proper time window for all these events, the cell cycle regulation is modified and one of the most important features are inhibitory phosphorylations of Cdk1 at Thr-14 and Tyr-15 (**Figure 2**), which are not detectable in the following rapid divisions (Ferrell et al, 1991) (**Figure 4**). Also Cdc25 is inactivated during the first cell cycle (Isoda et al, 2009). Both effects, inhibitory phosphorylations on Cdk1 and inactivation of Cdc25, are directly dependent on remaining cMos that persists for about 30 min post fertilization until it is degraded (Murakami et al, 1999; Murakami & Vande Woude, 1998; Walter et al, 2000). Surprisingly, XErp1 starts to be resynthesized during the first cell cycle and persists till Mid-blastula transition (MBT) (Liu et al, 2006; Nishiyama et al, 2007a), when the cell cycle length increases again.

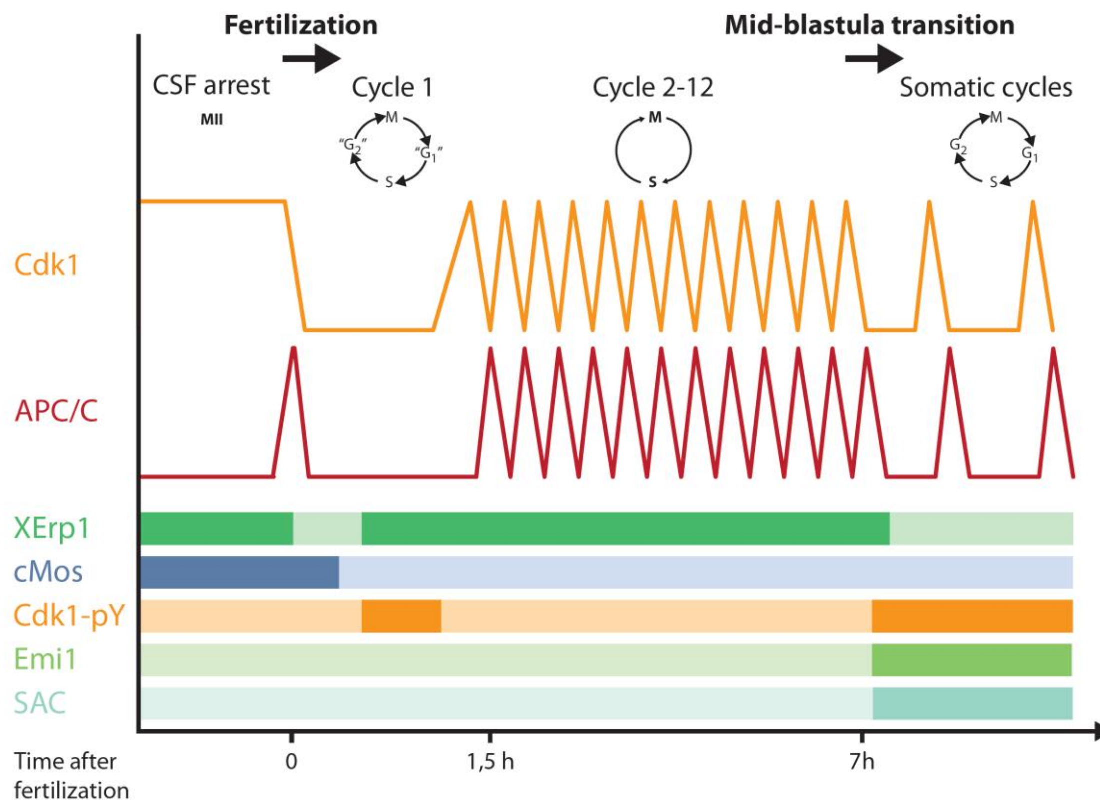

Figure 4: Schematic representation of cell cycle regulation during early development.
During CSF arrest, Cdk1 activity is high due to the presence of XErp1, which inhibits the APC/C. cMos is needed to keep XErp1 active and the SAC might be involved in APC/C inhibition. At fertilization, XErp1 is immediately degraded but re-synthesized during the first cell cycle and persists until Mid-blastulatrasition (MBT). The APC/C turns on and ubiquitylates CyclinB leading to Cdk1 inactivation. Mos degradation is delayed compared to XErp1 and contributes to the length of the first cell cycle, additionally Cdk1 is phosphorylated on Thr-14/Tyr-15 (indicated by pY). The SAC does not play a known role during this cell cycle phase. The following 11 cell cycles are very short and are regulated by oscillating Cdk1 and APC/C activity. XErp1 is expressed a second time during development. At MBT XErp1 disappears and is replaced by Emi1, the somatic cell cycle control is turned on. The SAC becomes active and Cdk1 can be regulated by inhibitory phosphorylations. Adapded from (Hormanseder et al, 2013)

2.4 Adaption of rapid cleavage divisions

A hallmark of embryonic development of most metazoan is that they perform rapid cleavage cycles without substantial growth to quickly increase their cell number. In *Xenopus* the first prolonged cell cycle is followed by 11 rapid cleavage divisions, in *Drosophila* by 12 and in the sea urchin by 10. Mammals lack the cleavage division, which probably reflects their development in a protected environment like the uterus of the mother. The cell cycle of the rapid divisions is modified in a way that it lacks both gap-phases and only consists of

alternating phases of DNA replication (S-phase) and -separation (Mitosis) (**Figure 4**). All factors needed for the first developmental steps are stored in the egg as maternal mRNAs, since transcription does not take place (Kimelman et al, 1987; Newport & Kirschner, 1982b). Additionally, cell cycle checkpoints, like the mentioned SAC and DNA damage control, are not functional and only introduced at MBT (Newport & Kirschner, 1982a) (**Figure 4**). The consequence of this is that errors occurring during the rapid cleavage cycles are not sensed until MBT, where it is first tried to repair them and if this is not possible, apoptosis of the whole embryo is induced (Anderson et al, 1997; Carter & Sible, 2003; Hensey & Gautier, 1997; Hensey & Gautier, 1999; Sible et al, 1997). The cell cycle itself is controlled by Cdk1/Cyclin B activity like in normal divisions and Cyclin B is degraded in every anaphase. Surprisingly, it is not known how the APC/C is controlled during this time, since the SAC and also Emi1 are not present. As shortly pointed out above, XErp1 reaccumulates during the first cell cycle and persists until MBT, however, studies using *Xenopus* egg extract did not identify a role for it during this period (Liu et al, 2006).

2.5 Aim of this work

XErp1 was initially identified as APC/C inhibitor during meiotic progression. The work presented here was designed to reveal a possible function of XErp1 during the early cleavage cycles of *Xenopus laevis*. Here it accumulates for a second time after it was degraded at fertilization.

A combination of phenotypical observations, micromanipulations and biochemical assays was used to investigate the function and regulation of XErp1 during early *Xenopus* development.

This work will give important insights into the progression and regulation of embryonic cell divisions and will help to improve the understanding of the molecular basis during the first phases of the life of an organism.

3 Results

3.1 XErp1 is required for early embryonic divisions

With regard to published data (Liu et al, 2006; Nishiyama et al, 2007a), it was first analyzed if XErp1 indeed reaccumulates during early embryo development. For this, oocytes were fertilized *in vitro*, cultivated at 20 °C and samples were taken at the indicated time points post fertilization (hpf) (**Figure 5**). XErp1 is present in the unfertilized egg (CSF) and disappears after fertilization. During the first cell cycle it reaccumulates and its levels remain constant during the following rapid cleavage cycles (2-9 hpf). It is degraded again at MBT (stage 8) as indicated by the swift change from maternal cyclin A1 to zygotic cyclin A2 and the disappearance of cyclin E1. To compare protein levels of XErp1, all samples were treated with calf intestinal phosphatase (CIP) to remove possible phosphorylations that might lead to a wrong estimation of protein levels.

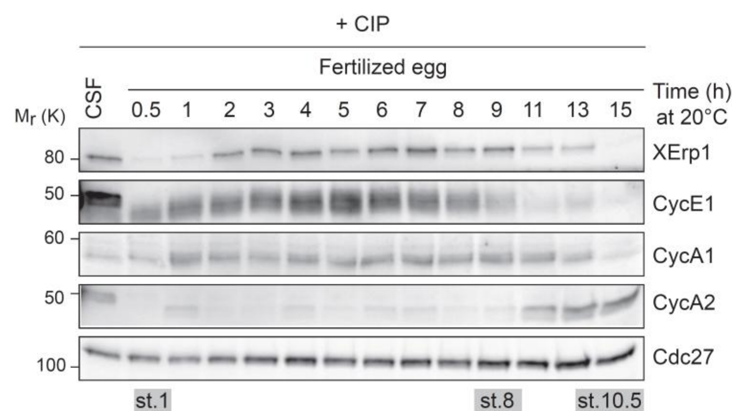

Figure 5: XErp1 reaccumulates during early embryonic divisions
Oocytes were fertilized *in vitro* and incubated at 20 °C. At the indicated time points, samples were taken and analyzed by immunoblotting for XErp1, Cyclin E1, Cyclin A1 and Cyclin A2. Cdc27 serves as loading control. Embryo staging according to Nieuwkoop and Faber is indicated, stage 8 is Mid-blastula transition (MBT), where XErp1 disappears.

Next it was analyzed if this second expression of XErp1 after CSF arrest is functionally relevant for embryonic development. One-cell embryos were injected with morpholino oligos (MOs) targeting XErp1 and development was analyzed. Six hours post fertilization samples for immunoblot analysis were removed and tested for XErp1, which can be detected in

control (Ctrl-) MO injected embryos but not in embryos depleted of XErp1 (**Figure 6** C). Closure of the blastopore as a marker for successful early development was scored 24 hpf (**Figure 6** A, B). The majority of embryos depleted of XErp1 could not close their blastopore (**Figure 6** A, B) and later died by apoptosis (**Figure 7** A, B), while Ctrl-MO injected embryos developed normally.

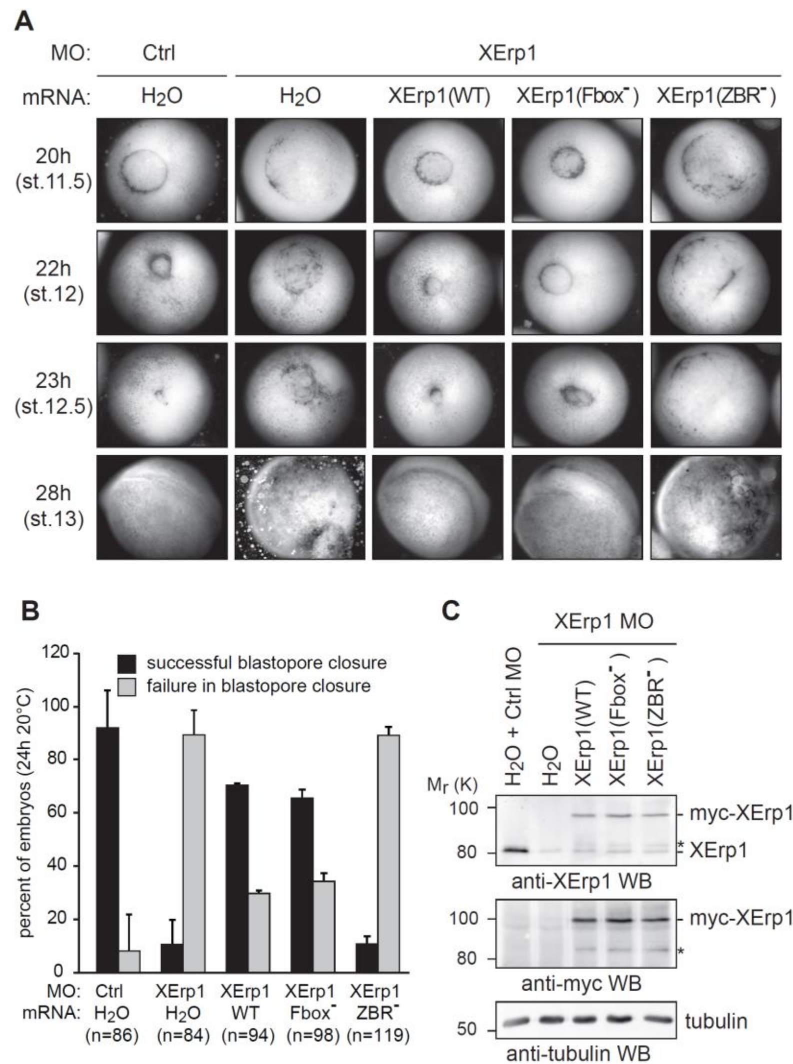

Figure 6: XErp1' APC/C inhibitory function is required for early embryonic development

(A) One-cell embryos were injected with the indicated MO and/or mRNAs and imaged under an inverted microscope from 20 to 28 hpf. Embryo staging according to Nieuwkoop and Faber is indicated. XErp1 depletion leads to a failure in blastopore closure, which can be rescued by expression of Myc-XErp1^{WT}.

(B) Embryos were treated as in (A) and phenotypes were quantified 24 hpf.

(C) Embryos were injected as in (A), samples for immunoblot were removed 6 hpf and blotted as indicated. Asterisk mark unspecific band.

To test if the observed phenotype is specific for XErp1 depletion, mRNA encoding for Myc-tagged XErp1^{WT} was coinjected with the MOs. All mRNAs of XErp1 used in this study are resistant to the MOs due to silent single point mutations and carry the XErp1-3'UTR, which ensures expression comparable to endogenous levels (**Figure 6** C) (Ohe et al, 2007). Expression of Myc-tagged XErp1^{WT} rescues the XErp1 depletion phenotype as shown by a successful blastopore closure (**Figure 6** A, B).

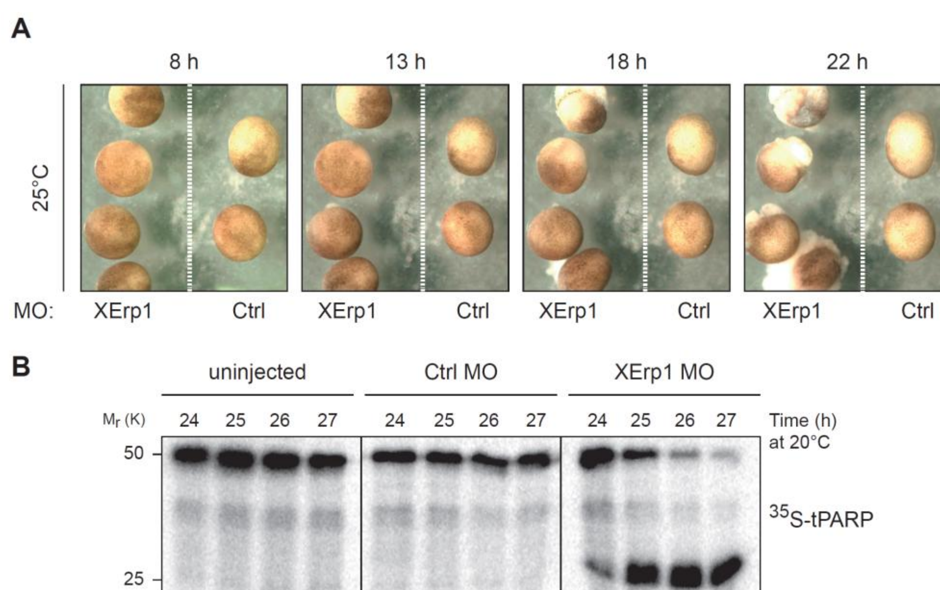

Figure 7: XErp1 depletion results in embryonic lethality
(A) One-cell embryos were injected with morpholino oligs (MO) targeting XErp1 or control oligos (Ctrl) and imaged at the indicated time points. Stage 8 is 8 hpf, stage 12.5 22 hpf.
(B) Embryos were injected as in (A) and samples were taken at indicated time points. Lysate was prepared and an N-terminal fragment of ^{35}S-labeled human PARP1 (tPARP) was added to observe caspase dependent cleavage products at 25 kDa by autoradiographie, which indicates apoptosis.

From studies of XErp1 in the meiotic system different domains of XErp1 are known that are important for various functions. To address the question which one of these could be also important during early embryonic development, mutants of XErp1 were expressed in embryos injected with the MOs. XErp1 mutated in the F-Box domain (Myc-XErp1$^{F\text{-}Box\text{-}}$, L450A) normally rescues the XErp1 depletion phenotype (**Figure 6**), suggesting that this domain is not needed for XErp1 function during early embryonic development. Strikingly, two mutants of XErp1 compromised in APC/C inhibition, either mutated in the ZBR domain (Myc-XErp1$^{ZBR\text{-}}$, C583A) or in the D-Box (Myc-XErp1$^{\Delta Box\text{-}}$ R529A), showed a failure in blastopore

closure like XErp1 depletion alone did (**Figure 6** A, B and **Figure 8**). This indicated that XErp1 might be essential as an APC/C inhibitor during early *Xenopus* development.

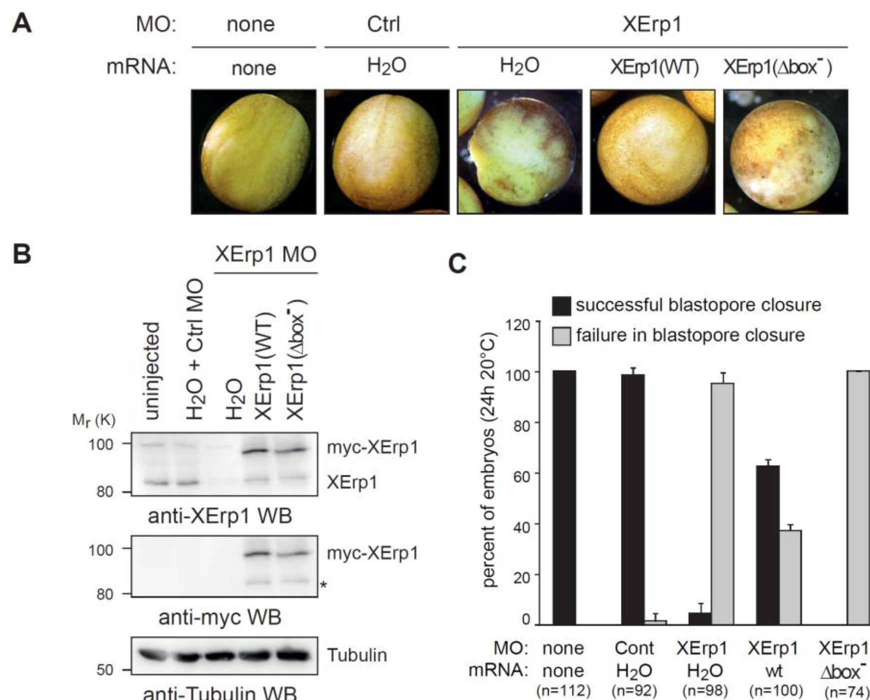

Figure 8: XErp1$^{\Delta Box-}$ cannot rescue XErp1 depletion

(A) One-cell embryos were injected with the indicated MO and/or mRNAs and imaged 24 hpf, stage 12.5 according to Nieuwkoop and Faber. Embryos depleted of XErp1 show an apoptotic phenotype.

(B) Embryos were injected as in (A), samples for immunoblot were removed at 6 hpf and blotted as indicated. XErp1 could be depleted successfully and expression of the Myc-constructs is shown. Asterisk marks unspecific band.

(C) Embryos were treated as in (A) and phenotypes of blastopore closure were quantified 24 hpf.

3.2 XErp1 works as an APC/C inhibitor

To investigate in more detail if XErp1 is needed as an APC/C inhibitor during the rapid cleavage cycles, an embryonic extract system was explored to study the effects of XErp1 depletion. If XErp1 inhibits the APC/C, its absence should lead to a hyperactive APC/C and consequently to decreased levels of its substrates. For preparation of the extract, eggs were fertilized and cultivated until 4-cell stage, where they were collected and crushed by centrifugation. The cytosolic fraction was isolated and used for further experiments. The advantage of this extract is that it is an open, synchronous system that is easily accessible

for manipulations. XErp1 was removed from the embryonic extract by antibody depletion and radioactively labeled securin (^{35}S-Sec) was added to monitor APC/C activity. After XErp1 depletion, securin was degraded while in Ctrl-depleted extract it remained stable (**Figure 9** A). Addition of an IVT of Myc-XErp1^{WT} after XErp1 depletion stabilized securin again (**Figure 9** B, C), indicating that the APC/C becomes uncontrollablly active if XErp1 is absent.

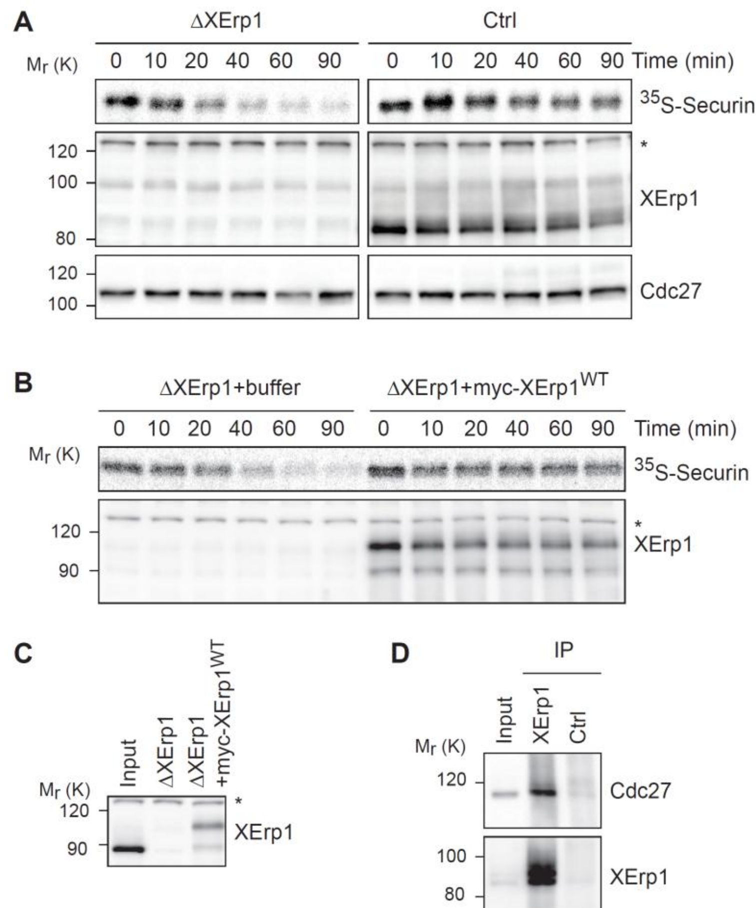

Figure 9: XErp1 depletion in embryo extract results in destabilization of APC/C substrates

(A) Embryo extract was prepared from 4-cell embryos and XErp1 was removed by antibodies or extract was treated with Ctrl-antibodies. ^{35}S-Securin was added to monitor APC/C activity and samples were analyzed by immunoblot and autoradiographie. When XErp1 is absent, Securin becomes unstable. Cdc27 serves as loading control. Asterisk marks an unspecific band.

(B) Extract was prepared as in (A) and XErp1 was depleted by antibodies. IVT of Myc-XErp1^{WT} or buffer was added and samples were analyzed by immunoblot and autoradiographie. Addition of XErp1-IVT restores Securin stability.

(C) Extracts from (B) were immunoblotted for XErp1 to compare the amount of added IVT Myc-XErp1^{WT} with endogenous protein.

(D) XErp1 immunoprecipitates from 4-cell embryos were analyzed for XErp1 and Cdc27. An interaction between XErp1 and Cdc27 is visible.

To see if this also applies for living embryos, levels of APC/C substrates after XErp1 depletion by MO injection were analyzed. In accordance with the earlier results, XErp1 depletion decreased geminin and cyclin B protein levels compared to control-MO injected embryos (**Figure 10** A). Additionally, the cell cycle length increased with decreasing XErp1 levels (**Figure 10** B, C) which could be explained by the observed low levels of cyclin B. Under this condition, Cdk1 activity needs longer to reach the necessary degree for mitosis and consequently the cell cycle time increases.

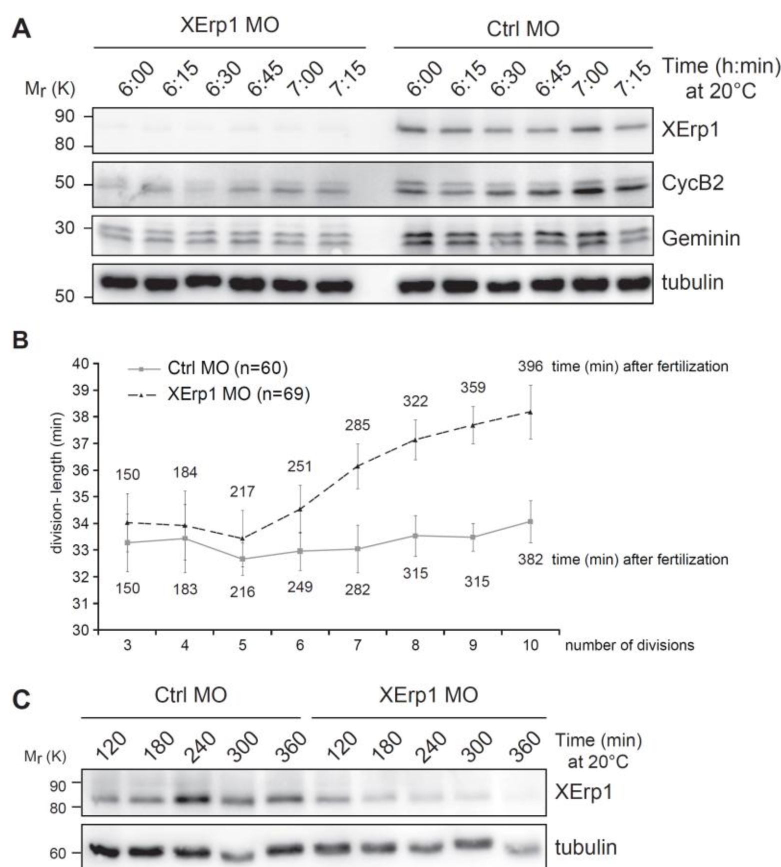

Figure 10: XErp1 depletion *in vivo* destabilizes APC/C substrates and increases cell cycle length

(A) One-cell embryos were injected with the indicated MOs and incubated for 6 h at 20 °C. At the indicated time points, samples were taken and analyzed by immunoblot for XErp1, Geminin and Cyclin B2. After XErp1 depletion, Geminin and Cyclin B levels are decreased. Tubulin serves as loading control.

(B) One-cell embryos were injected with XErp1- or Ctrl-MO and filmed under a microscope. The length of each division was counted and blotted against the division number. The length of each division after fertilization is indicated. XErp1 depletion lengthens the cell cycle.

(C) Embryos were injected as in (B), samples were taken at the indicated time points and blotted for XErp1 and tubulin as loading control.

If XErp1 inhibits the APC/C directly, as shown for meiosis (Schmidt et al, 2005), it should be associated with it. Indeed, co-immunoprecipitation experiments (co-IPs) of XErp1 and Cdc27, an APC/C core subunit, using the embryonic extract verified an association between XErp1 and Cdc27 (**Figure 9** D). These experiments conclusively show that XErp1 is needed as an APC/C inhibitor during early embryonic development and that its absence leads to failures in development.

3.3 Phosphorylation controls the activity of XErp1

After meiosis, XErp1 is degraded to allow APC/C activation, but when samples from embryos were analyzed, XErp1 levels showed no decrease within each cell cycle when cyclin B was destroyed (**Figure 11** A), leading to the question how the APC/C can become active if XErp1 is constantly present. During meiosis a cybernetic system of Cdk1 phosphorylation and PP2A mediated dephosphorylation of XErp1 ensures its APC/C inhibitory activity (Wu et al, 2007a; Wu et al, 2007b). When embryonic samples were not treated with CIP to preserve phosphorylations, a cell cycle dependent electrophoretic mobility shift of XErp1 could be observed (**Figure 11** B) that coincides with phosphorylation of greatwall kinase (Gwl), a known Cdk1 substrate in mitosis (Yu et al, 2006). This leads to the hypothesis that XErp1 might be regulated by phosphorylation and dephosphorylation *via* Cdk1 and PP2A during the rapid cleavage cycles to modulate its activity.

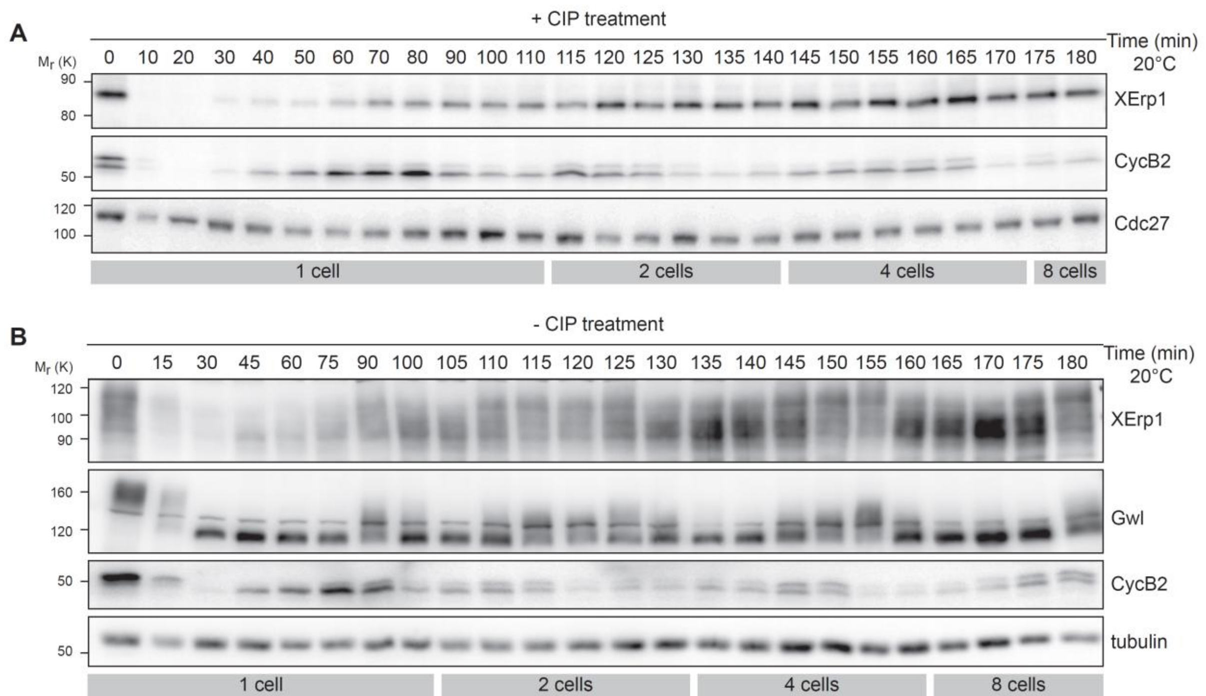

Figure 11: XErp1 is phosphorylated in a cell cycle dependent manner
(A) Oocytes were fertilized and samples were taken at indicated time points. After CIP treatment, samples were analyzed for XErp1 and Cyclin B2. XErp1 levels do not change over time. Cdc27 serves as loading control and indicated cell numbers where observed by eye.
(B) Oocytes were fertilized like in (A) and samples were taken at indicated time points, but not treated with CIP. Immunoblotting for XErp1 and greatwall kinase (Gwl) showed cell cycle dependent phosphorylations. Tubulin serves as loading control. Indicated cell numbers where observed by eye.

3.3.1 Cdk1 inactivates XErp1

In meiosis, Cdk1 phosphorylation of XErp1 negatively influences its stability and, more importantly, its ability to inhibit the APC/C (Wu et al, 2007b). If a similar mechanism exists during the early embryonic cell cycles, mutation of the six major Cdk1 phosphorylation sites on XErp1 should circumvent Cdk1 mediated inactivation and XErp1 should be able to constantly inhibit the APC/C. When this mutant of XErp1 (Myc-XErp1^{6A}, S213A, T239A, T252A, T267A, T545A and T551A) was expressed in MO injected embryos, it caused a severe cell cycle arrest (**Figure 12** A, B) with accumulation of cyclin B (**Figure 12** D) consistent with constitutive APC/C inhibition. This effect was not observed in embryos expressing Myc-XErp1^{WT} and, more importantly, also not in embryos expressing the Cdk1 mutant of XErp1 combined with a mutation in the APC/C inhibitory domain (Myc-

XErp1$^{6A,\Delta ZBR}$), which shows that the cell cycle arrest is mediated *via* APC/C inhibition (**Figure 12** A, B).

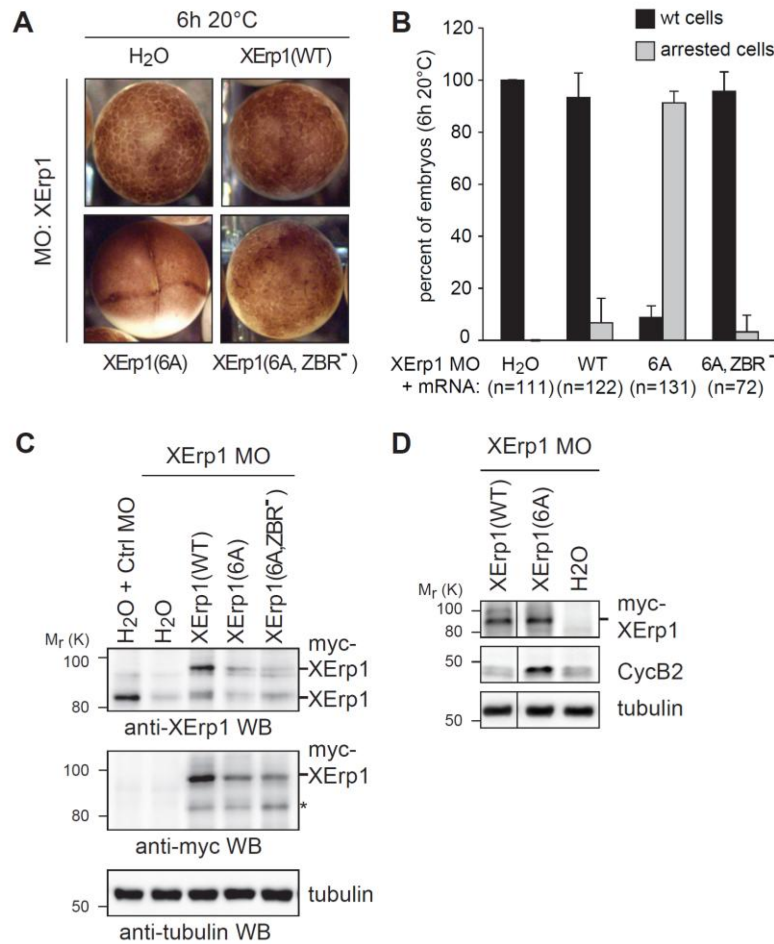

Figure 12: Mutation of six Cdk1 sites in XErp1 results in a cell cycle arrest and stabilization of APC/C substrates

(A) One-cell embryos were injected with the indicated MOs and mRNAs. 6 hpf images were taken and showed a cell cycle arrest when XErp1 could not be phosphorylated by Cdk1 anymore.

(B) Phenotypes from embryos in (A) were quantified 6 hpf. The cell cycle arrest was only visible in Myc-XErp1^{6A}.

(C) Samples from embryos in (A) were taken 6 hpf and analyzed by immunoblot for XErp1. Expression of Myc-XErp1^{WT} was stronger compared to the mutants but still showed no cell cycle arrest. Asterisk marks unspecific band.

(D) One-cell embryos were injected with the indicated constructs. 4 hpf samples were taken and immunoblotted for XErp1, Cyclin B and tubulin as loading control. Cyclin B levels are increased if XErp1 cannot be phosphorylated by Cdk1 and causes a cell cycle arrest.

If the idea of Cdk1 mediated inactivation of XErp1 is correct, artificial activation of Cdk1 should cause APC/C activation due to XErp1 hyperphosphorylation. Through addition of a stable cyclin B, missing 90 aminoacids at its N-terminus (CycB$^{\Delta 90}$), to embryonic extract,

Cdk1 was turned on. This experiment shows that XErp1 was indeed retarded in its electophoretic mobility, indicating phosphorylation, and at the same time APC/C substrates ^{35}S-Sec and endogenous cyclin B were destabilized (**Figure 13** A). Importantly, when Cdc27 was immunoprecipitated under this conditions and the association of XErp1 was tested by immunoblotting, it could only be detected in the buffer control, but not in samples where Cdk1 was activated (**Figure 13** B). To ensure that protein levels are not changed due to phosphorylations, the samples were treated with CIP. All these data are consistent with the suggestion that Cdk1 inactivates XErp1 by dissociating it from and thereby activating the APC/C.

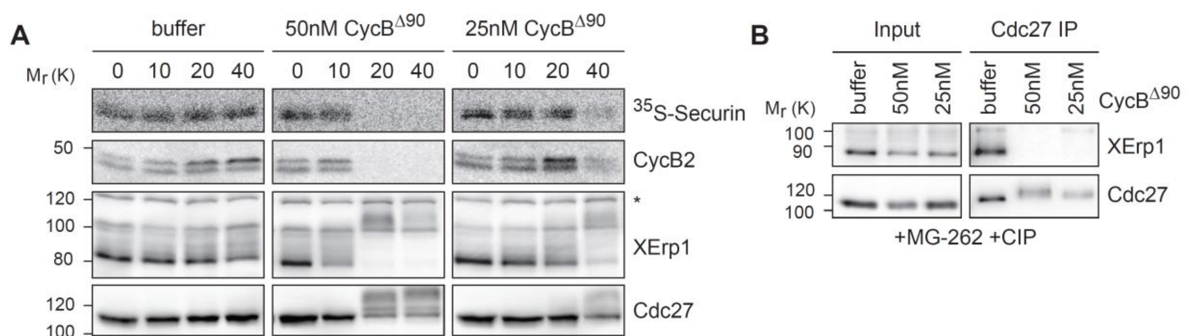

Figure 13: Cdk1 activation results in XErp1 hyper-phosphorylation and its dissociation from the APC/C
(A) Embryonic extract was treated with buffer or the indicated amounts of Cyclin B$^{\Delta 90}$ to activate Cdk1. Samples were taken and analyzed by immunoblotting and autoradiographie. Addition of CycB$^{\Delta 90}$ leads to an upshift of XErp1 and APC/C activation. Asterisk marks unspecific band.
(B) Embryo extract was treated with buffer or Cyclin B$^{\Delta 90}$ like in (A) and Cdc27 was immunoprecipitated in the presence of proteasome inhibitor MG-262 to avoid XErp1 degradation. Samples were treated with CIP to compare protein levels and co-purified XErp1 was detected by immunoblotting.

3.3.2 XErp1 is activated by PP2A mediated dephosphorylation

If Cdk1 is able to inactivate XErp1 as shown before, raising Cdk1 activity by increasing cyclin B levels due to cell cycle dependent translation would immediately inactivate XErp1. This would lead to APC/C activation and consequently cyclin B degradation. Obviously, this does not happen, because in this situation cell cycle progression would not be achieved. Therefore a Cdk1 counteracting activity, namely a phosphatase, might be necessary to keep XErp1 active. From meiosis it is known that PP2A can bind to and activate XErp1 by

dephosphorylation (Isoda et al, 2011; Wu et al, 2007b). If this also applies for embryonic cell cycles, inhibition of PP2A should lead to XErp1 inactivation. Under these conditions Cdk1 phosphorylation, even if not artificially activated, should prevail over PP2A mediated dephosphorylation. When okadaic acid (OA), an inhibitor of PP2A, was added to the embryonic extract, XErp1 showed its characteristic upshift due to phosphorylation and APC/C substrates were destabilized (**Figure 14** A, upper panel), implying that the APC/C became active as shown before. Likewise, when Cdc27 was immunoprecipitated and probed for associated XErp1, XErp1 was present in the DMSO solvent control (**Figure 14** B) and only to a lesser degree in OA treated samples. Notably, when embryo extract or living embryos were treated for a prolonged time with OA (more than 2 h), XErp1 was hardly detectable by immunoblotting, implying that PP2A might also control XErp1 stability in the long term (compare **Figure 14** A, lower panel, with C). To verify that the observed effects, Cdk1 mediated inactivation and PP2A mediated activation of XErp1, are not an artifact of the embryonic extract system, living embryos were either injected with $CycB^{\Delta 90}$ or treated with OA or the appropriate controls, respectively. After immunoprecipitation of Cdc27, samples were treated with CIP to compare the total protein-amount of co-purified XErp1 with Cdc27. Under both conditions XErp1 was not associated with the APC/C anymore, where in controls it was (**Figure 14** D), showing that also *in vivo* phosphorylation controls XErp1 activity by influencing its ability to bind to the APC/C.

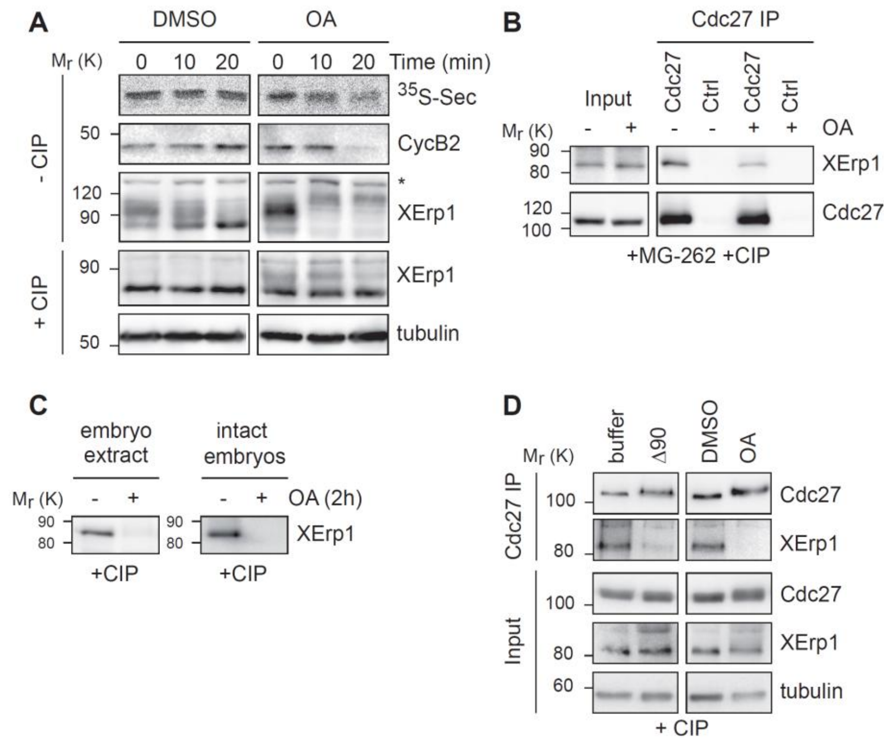

Figure 14: Inhibition PP2A leads to XErp1 hyperphosphorylation and dissociation from the APC/C

(A) Embryo extract was treated with 1 µM OA or DMSO as control and samples were taken at the indicated times. APC/C activation and XErp1 phosphorylation was analyzed by immunoblotting and autoradiographie. Addition of OA leads to an upshift of XErp1 and destabilization of Cyclin B and Securin. Total protein levels of XErp1 do not change. Asterisk marks unspecific band.

(B) DMSO or 1 µM OA was added to embryo extracts and Cdc27 was immunoprecipitated in the presence of proteasome inhibitor MG-262 to avoid XErp1 degradation. Association of XErp1 to Cdc27 was tested by immunoblotting after samples were treated with CIP.

(C) Embryo extract or embryos were treated for prolonged time periods with 1 µM OA and samples were immunoblotted for XErp1. Prolonged inhibition of PP2A leads to destabilization of XErp1.

(D) Embryos were injected with 50 nM Cyclin $B^{\Delta 90}$ or treated with 2 µM OA and incubated for 30 min. Cdc27 was immunoprecipitated and analyzed by blotting for XErp1. Total protein levels of XErp1 do not change and it is not associated with Cdc27 anymore.

3.3.3 Identification of PP2A-B'56 as XErp1 activator

The PP2A core enzyme has a rather low and unspecific activity. This basal activity is enhanced and specified by association with regulatory B-subunits. Recently, B'56α, -β and -ε were identified as XErp1 specific B-subunits (Isoda et al, 2011). To test for binding of different B-subunits, embryo extract was incubated with mRNA encoding for Flag-tagged B'56ε, B55δ or H_2O as control. The B-subunits were purified over the Flag-tag and probed for bound XErp1. Neither B55δ, which plays an important role in the Greatwall-Ensa/Arpp19

pathway, nor the H_2O control showed significant association with XErp1, while with B’56ε a weak interaction could be repeatedly detected (**Figure 15** A). The domain of XErp1 that associates with PP2A is known (Isoda et al, 2011; Wu et al, 2007b) and was therefore tested in embryonic extract. Extracts were incubated with mRNA encoding for different isoforms of Flag-tagged B’56-subunits and a bacterially purified glutathione-S-transferase tagged XErp1 fragment (GST-XErp1$^{301\text{-}400,\ WT}$). After reisolation of the GST-XErp1$^{301\text{-}400,\ WT}$ fragment, associated B-subunits were analyzed by immunoblotting against the Flag-tag. Specifically, B’56α, -β and -ε could be shown to bind to XErp1 in embryo extracts (**Figure 15** B). As control, a XErp1 fragment in which the PP2A binding sites were mutated (GST-XErp1$^{301\text{-}400,\ 4A}$: S335A, T336A, S342A, S344A) was used and with this no co-purification with any isoform could be achieved (**Figure 15** B).

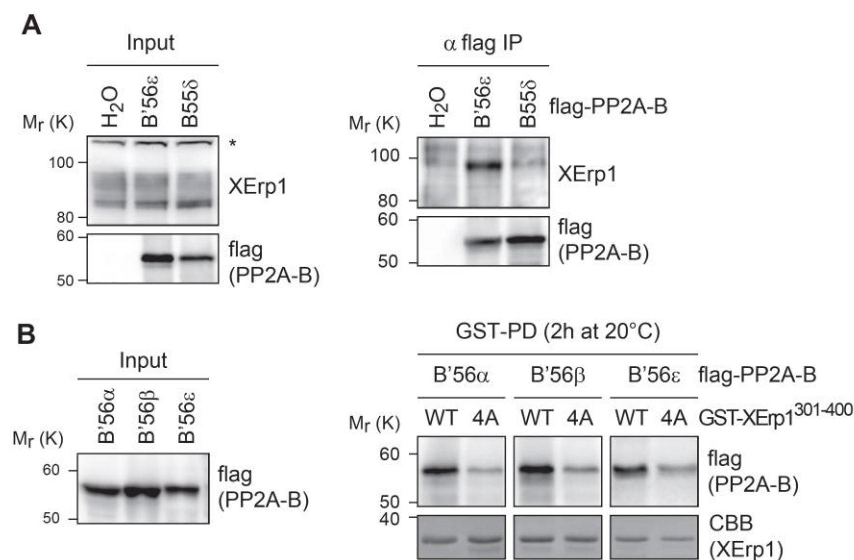

Figure 15: XErp1 interacts with PP2A-B’56
(A) mRNA encoding Flag-tagged PP2A B-subunits or H_2O (control) was incubated in embryo extract and immunoprecipitated using antibodies to Flag. Precipitates were analyzed for XErp1 and Flag epitope. A specific association could only be observed with B’56ε. Asterisk marks unspecific band.
(B) Extracts were incubated with different Flag-B′56 mRNAs and GST-tagged XErp1$^{301\text{-}400,\ WT}$ or XErp1$^{301\text{-}400,\ 4A}$. After reisolation of GST-tagged proteins, interacted B′56 subunits were analyzed by anti Flag immunoblot. Only the WT XErp1 fragment was able to co-purify Flag-tagged B-subunits. CBB, Coomassie Brilliant Blue.

3.3.4 PKA phosphorylation is required for XErp1 activity

Previous studies established that XErp1 needs to be phosphorylated at a target sequence to allow PP2A binding (Nishiyama et al, 2007a; Wu et al, 2007a). Reportedly, during meiosis this phosphorylation is done by the downstream kinase of the cMos-MAPK pathway, p90Rsk (Inoue et al, 2007). After fertilization, cMos is degraded and, in contrast to XErp1, is not reexpressed during the cleavage cycles, raising the question of how PP2A could bind to XErp1 if no kinase would phosphorylate it. That phosphorylation might indeed be necessary for PP2A binding is implied by the fact, that GST-XErp1$^{301-400,\ 4A}$, which can not be phosphorylated, does not bind PP2A (**Figure 15** B). Therefore it was tested whether XErp1 is phosphorylated in embryos. Embryos were snap frozen in the 4-cell stage and used for a kinase assay where incooperation of radioactive phosphate was monitored. A maltose-binding-protein tagged XErp1 fragment (MBP-XErp1$^{319-375}$, MBP-BD) containing the phosphorylation sites was used as substrate and could be efficiently phosphorylated in this assay (**Figure 16**). Different combinations of mutated sites were used as control and these showed reduced incooperation of radioactive phosphate. No radioactivity could be detected with the 4A mutant, which implies that the kinase responsible for the observed phosphorylation uses the same sites as reported for p90Rsk in meiosis.

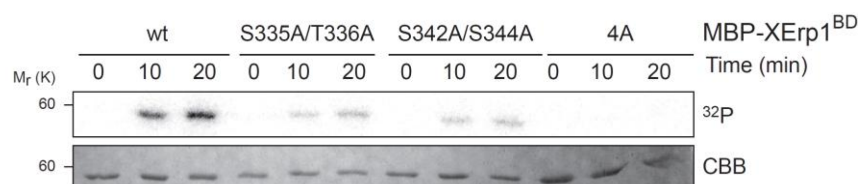

Figure 16: XErp1 is phosphorylated in embryo extract
MBP-tagged XErp1 fragments (MBP- XErp1$^{319-375}$, MBP-BD) were incubated in embryo extract supplemented with radioactive γ^{32}P-ATP and incooperation of radioactivity over time was analyzed by autoradiographie. The WT-fragment gets phosphorylated over time, while the mutant fragments show less or no incooperation of radioactivity. CBB, Coomassie Brilliant Blue.

Since it might be possible that other kinases related to cMos, like b-Raf, activate the MAPK pathway in embryos (Yue et al, 2006), it was tested if inhibition of the downstream kinase MEK influences XErp1 phosphorylation. Under these conditions XErp1 was conventionally

phosphorylated and also depletion of other components of the MAPK pathway did not disturb early embryonic development (data not shown). To further investigate whether the MAPK pathway is active during the early cleavage cycles, the activity of MAPK was tested by immunoblotting against phosphorylated Tyr-204. This phosphorylation indicates active MAPK but revealed no activity over background level after meiosis was released (**Figure 17**).

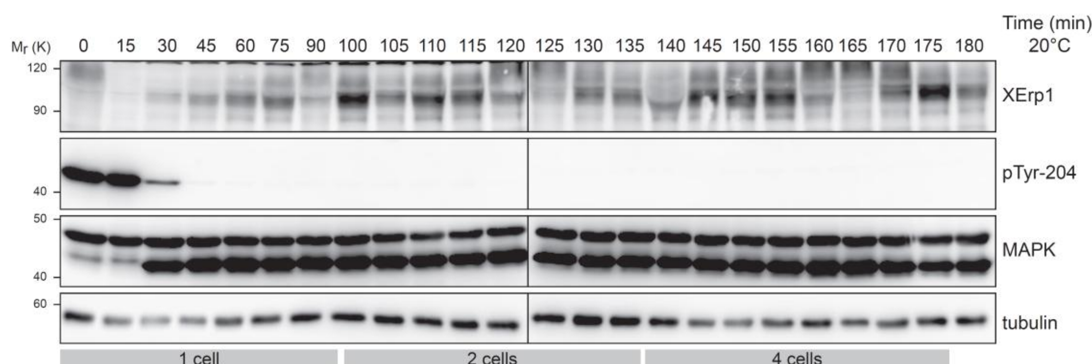

Figure 17: MAPK is not active during *Xenopus* rapid cleavage cycles
Oocytes were fertilized and samples were taken at indicated time points, but not treated with CIP. Immunoblotting for MAPK confirms that it is expressed but not active due to missing phosphorylation at Tyr-204. The MAPK antibody detects p42- and p44-MAPK, the pTyr-204 only p42-MAPK. Phosphoshift of XErp1 indicates cell cycle progression and tubulin serves as loading control. Note that the samples were distributed on two separate gels, indicated by the black line in the middle. Cell numbers were observed by eye.

A consensus sequence search for possible kinases capable of phosphorylating the mentioned sites revealed that cyclic AMP dependent protein kinase A (PKA) might be a good candidate (**Figure 18** A) (Smith et al, 1999). Depletion of PKA from embryo lysate by antibodies and subsequent kinase assays indeed showed that phosphorylation of MBP-BD^{WT} was reduced in the PKA depleted sample but not in control samples (**Figure 18** B, C). This effect was specific for PKA since addition of recombinant active PKA restored incooperation of radioactivity. Additionally, also full-length MBP-tagged XErp1^{WT} but not MBP-XErp1^{4A} could *in vitro* be phosphorylated by PKA (**Figure 18** D).

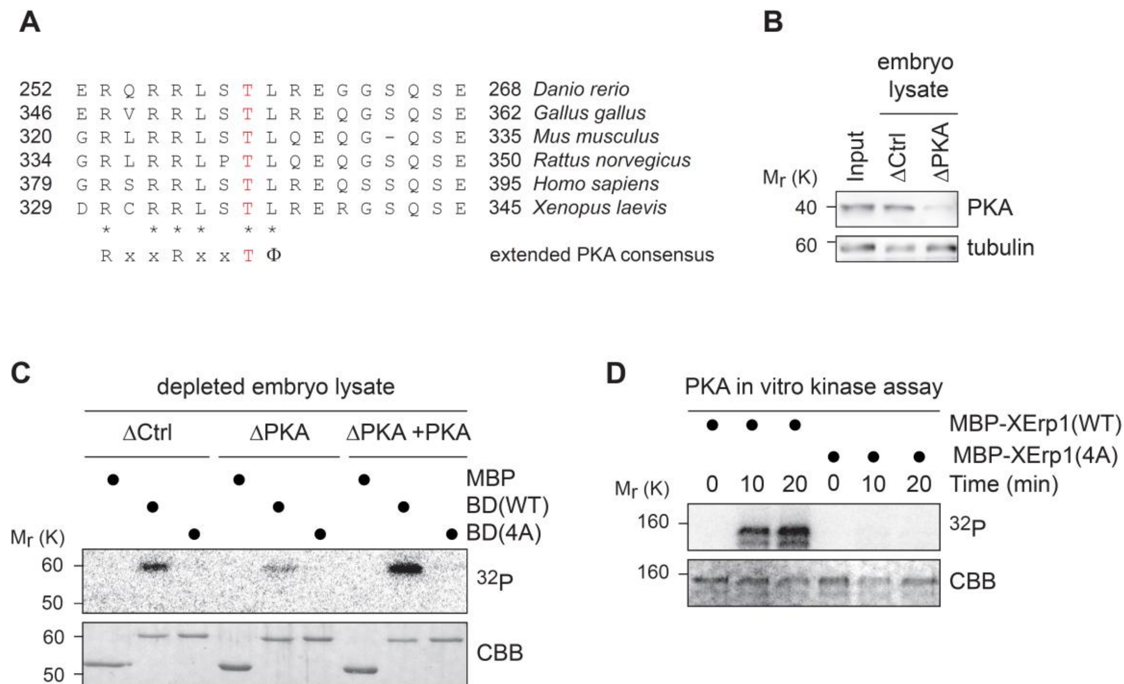

Figure 18: PKA phosphorylates XErp1

(A) Alignment of the extended protein kinase A (PKA) phosphorylation consensus motif against Erp1 proteins shows a high conservation. Φ stands for any hydrophobic aminoacid.

(B) Embryo lysates depleted of PKA (ΔPKA) or treated with control antibodies (ΔCtrl) were immunoblotted for PKA and tubulin as loading control.

(C) Embryo lysates from (B) were supplemented with γ^{32}P-ATP and phosphorylation of MBP-XErp1^{BD} was analyzed by autoradiography. Depletion of PKA diminishes incooperation of radioactivity into WT XErp1 fragment. Recombinant PKA was added to ΔPKA lysate to restore pohosphorylation.

(D) *In vitro* PKA phosphorylation assay using γ^{32}P-ATP and MBP-tagged full-length WT or PKA phosphomutant (4A) XErp1. ^{32}P incorporation was analyzed by autoradiographie and shows an increase over time using WT XErp1.

Inhibition of PKA *in vivo* by either injection of PKI protein (a small potent inhibitor of PKA) or incubation of embryos with H89 (an ATP competitive small molecule inhibitor of PKA) led to a failure in blastopore closure as seen for XErp1 depletion (**Figure 19** A, C). PKA was under both conditions efficiently inhibited as shown by immunoblotting with a pan specific antibody that detects all proteins phosphorylated at the PKA consensus motif (**Figure 19** B, phosphoRxxS/T antibody). Interestingly, when PKA was inhibited in embryos for 6 h XErp1 protein levels dropped, which would be consistent with the idea that long term inhibition of PP2A (-binding) destabilizes XErp1 (compare **Figure 14** C and **Figure 19** B).

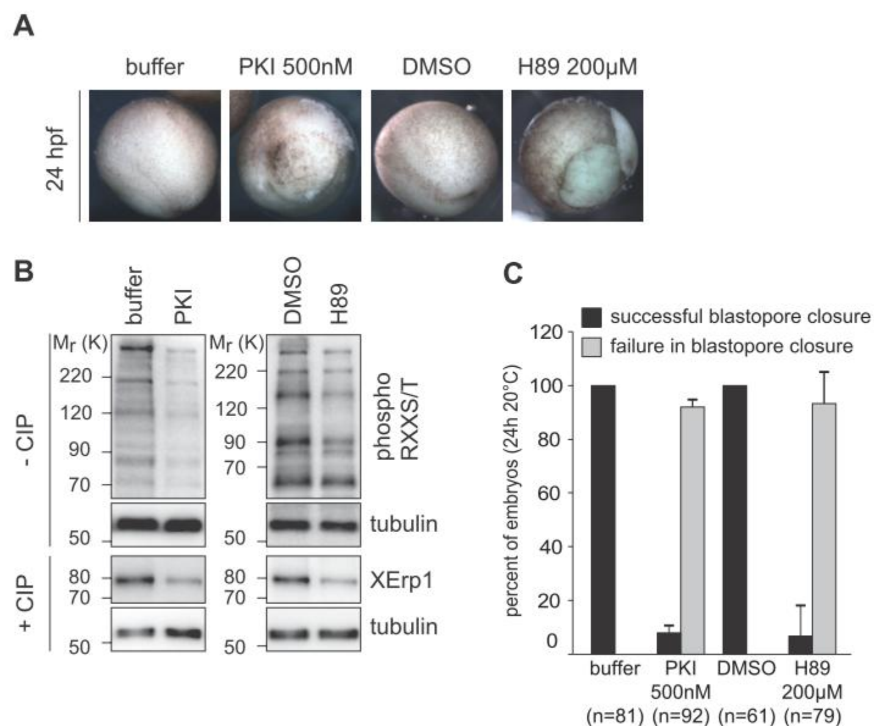

Figure 19: Inhibition PKA *in vivo* results in failure in blastopore closure

(A) Embryos at one-cell stage were either injected (PKI) or incubated (H89) with the indicated PKA inhibitors, cultivated for 24 h at 20 °C and photographed. PKA inhibition leads to an apoptotic phenotype.

(B) Embryos were treated as in (A); samples were taken 6 hpf, CIP-treated when indicated, and immunoblotted for XErp1 and the phosphorylated PKA consensus motif (RXXpS/pT, where X stands for any amino acid and pS/pT for phosphorylated serine or threonine) to monitor PKA activity. PKA inhibition leads to a decreased signal of the phosphospecific antibody and to lower XErp1 protein levels.

(C) The phenotype in blastopore closure from embryos treated as in (A) was scored 24 hpf.

Motivated by this finding, a link between PKA phosphorylation and PP2A binding should exist. To test this possibility, MBP-BD was *in vitro* phosphorylated by PKA and incubated for a short time in embryo extract. This short incubation was necessary to avoid phosphorylation of the non-phosphorylated MBP-BD^{WT} by endogenous PKA. Indeed, only MBP-BD^{WT} when pre-phosphorylated with PKA co-purified the catalytic subunit of PP2A (**Figure 20** A). Neither non-phosphorylated MBP-BD^{WT} nor MBP-BD^{4A} (under both conditions) associated with PP2A. This data show that PKA phosphorylation of XErp1 can lead to PP2A association.

If the presented model is correct, expression of Myc-XErp1^{4A} in embryos depleted of endogenous XErp1 should fail to close their blastopore since PKA can't phosphorylate this

mutant and consequently PP2A cannot bind anymore, which inactivates XErp1. In fact, expression of Myc-XErp1^{4A} could not rescue the XErp1 depletion phenotype while Myc-XErp1^{WT} efficiently did (**Figure 20** B, C, D). These experiments allow the conclusion that XErp1 is kept in an active state *via* PKA mediated recruitment of PP2A-B'56.

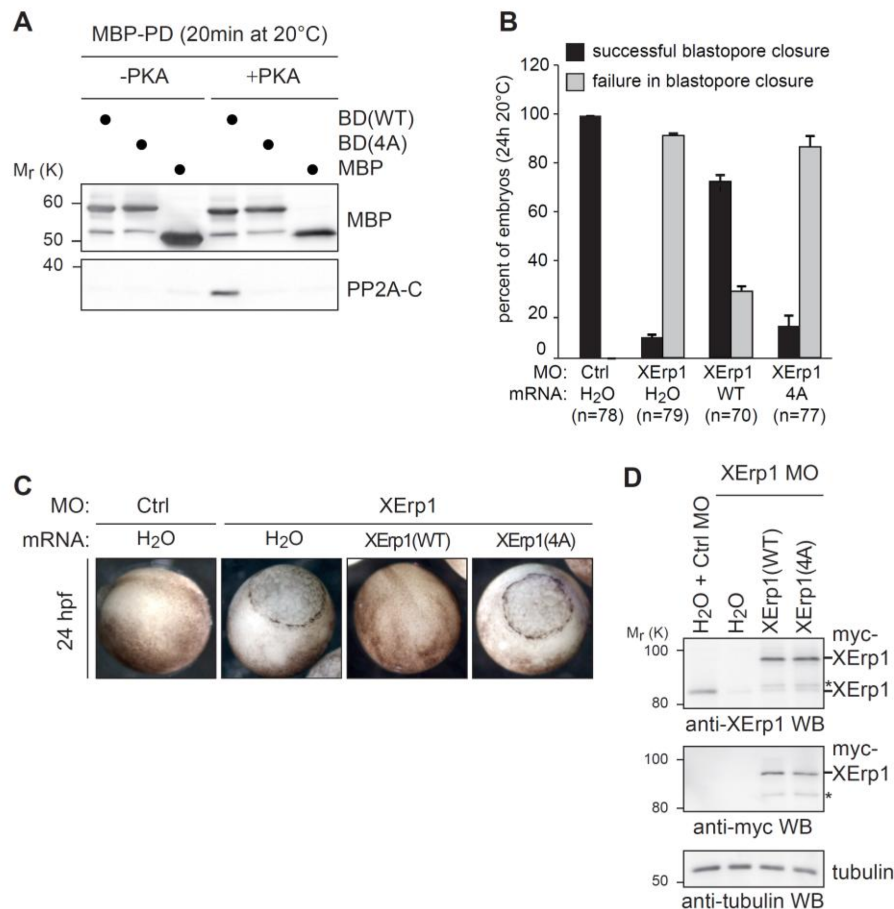

Figure 20: Inhibition of PP2A recruitment to XErp1 leads to embryonic lethality

(A) MBP-XErp1^{BD} was phosphorylated by PKA *in vitro* and incubated for 20 min in embryo extract. After repurification of MBP-XErp1^{BD}, the associated PP2A catalytic subunit (PP2A-C) was analyzed by immunoblot. Only MBP-XErp1$^{BD, WT}$ prephosphorylated by PKA associated with PP2A.

(B) One-cell embryos were injected with the indicated MOs and mRNAs and cultivated for 24 h. The phenotypes in blastopore closure were quantified.

(C) Embryos treated as in (B) were photographed 24 hpf. Expression of Myc-XErp1^{4A}, which is unable to be phosphorylated by PKA and to bind PP2A, leads to an apoptotic phenotype.

(D) Samples from embryos in (B) were removed 6 hpf and analyzed by immunoblot for XErp1 and tubulin as loading control. Asterisk marks unspecific band.

3.3.5 Global PKA activity is not regulated during early cleavage cycles

From the data presented above, one problem could still arise. When PP2A constantly counteracts the activity of Cdk1 against XErp1, XErp1 can never be inactivated. Eventually, the embryo would end up in a situation comparable to the CSF state, where Cdk1 and PP2A activity against XErp1 are equal and the cell cycle would not proceed. A solution could be that PP2A recruitment to XErp1 is regulated by cell cycle dependent changes in PKA activity. For this reason global PKA activity in the embryo was analyzed by performing kinase assays using Kemptide, a standard PKA substrate peptide. The activity of PKA was measured during one-cell cycle and compared to Cdk1 activity, which showed the expected drop after metaphase (**Figure 21**, H1). In contrast to this, PKA activity remained constant over time (**Figure 21**, Kemptide). Additionally, kinase activity against MBP-BD^{WT} was measured in the same assay and also showed no changes during the cell cycle (**Figure 21**, XErp1), which is consistent with the aforepresented data, that PKA can phosphorylate XErp1 (**Figure 18**).

So far it remains unclear how PP2A-B'56 activity against XErp1 is regulated during early embryonic development and this point needs further investigations.

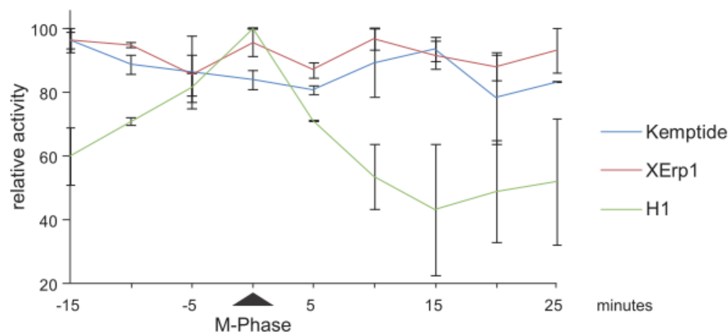

Figure 21: Global PKA activity does not change during embryonic cell cycles
Kinase activity against H1 (Cdk1), Kemptide (PKA) and MBP-XErp1^{BD} containing the PKA consensus motif was measured for embryos from two independent fertilizations by scintillation counting. Cdk1/Cyclin B activity droped after Mitosis, while PKA activity remained constantly high.

4 Discussion

4.1 Summary of the findings presented in this study

The experiments and data presented in this work extent the current understanding of the function of XErp1 and the regulation of the early embryonic cleavage cycles in *Xenopus*. Until now, it was solely known that XErp1 functions as APC/C inhibitor during oocyte maturation and that it is responsible for the CSF arrest. In this thesis, the significance of XErp1 as APC/C inhibitor could be expanded beyond meiosis and important insights into the regulation of the cell cycle machinery after fertilization were given.

Depletion of XErp1 leads to embryonic lethality. Micromanipulation of embryos and biochemical assays verified its function as APC/C inhibitor in mitosis and immunoprecipitations validated the interaction with the APC/C. Moreover, in depth analysis of the regulation of XErp1 revealed a tight link between its activity, Cdk1, PP2A and the APC/C, which are all interconnected. Cdk1 activity inactivates XErp1, which was shown by microinjections and *in vitro* experiments. PP2A counteracts Cdk1 activity by keeping XErp1 in an active state. Inhibition of PP2A binding to XErp1 results in the same phenotype, i.e. embryonic lethality, like XErp1 depletion did, which was demonstrated by the expression of a mutant unable to interact with PP2A. The important role of PKA in this system is to recruit PP2A to XErp1, as revealed by kinase- and pulldownassays.

All these findings integrate XErp1 as one of the major players in the development of early embryos and to the current knowledge XErp1 is the only known APC/C inhibitor present during this phase of development. The following chapter will relate the function of XErp1 to the context of other publications, explain a current working model and show future research directions by pointing out still open questions.

4.2 XErp1 depletion leads to APC/C misregulation

4.2.1 Domains of XErp1 necessary for APC/C inhibition

Studies of XErp1 function in meiosis have pointed out that three elements located in the C-terminus of the protein, the D-Box, the ZBR-domain and the RL-tail, are needed for APC/C inhibition (Ohe et al, 2010; Schmidt et al, 2005; Tang et al, 2010). In the study presented here using *Xenopus* embryos two of these elements (ZBR and D-Box, **Figure 6** and **Figure 8**, respectively) were investigated with regard to their importance for XErp1 function as APC/C inhibitor in mitosis. XErp1 proteins with mutations in these regions were unable to rescue the XErp1 depletion phenotype and the embryos died after MBT, like the XErp1 depleted embryos did. With this it is clear that at least these two elements are necessary for the APC/C inhibitory function of XErp1 in the rapid cleavage cycles. The role of the RL-tail was not tested in the meantime, but it is very likely that it is also needed for APC/C inhibition in the embryos as it is in meiosis.

4.2.2 Molecular consequences of XErp1 depletion

XErp1 depleted embryos die by apoptosis as shown in **Figure 7**. By observation of the embryos it is easily visible that they are first delayed in blastopore closure and then start to induce apoptosis. But this phenotype is quite common and is in general a result from errors during the rapid cleavage cycles. For example, errors in DNA replication induced by treatment with aphidicolin, X-Ray irradiation of embryos or inhibition of protein synthesis all result in similar phenotypes accompanied by apoptosis (Anderson et al, 1997; Carter & Sible, 2003; Finkielstein et al, 2001; Greenwood et al, 2001; Hensey & Gautier, 1997; Sible et al, 1997). To reveal which kind of errors arise after XErp1 depletion, the molecular consequences of the absence of XErp1 were investigated.

Since the APC/C inhibitory domains of XErp1 are needed for its function in the early embryos, depletion of XErp1 may lead to hyperactivation of the APC/C, which caused in the end the apoptotic phenotype. By immunodepletion of XErp1 from embryonic extract it could

be shown that the APC/C indeed becomes activated and this leads to the degradation of endogenous Cyclin B (**Figure 9**). Likewise, in living embryos Cyclin B levels are drastically lower after XErp1 depletion compared to control depleted embryos (**Figure 10**). A measurable consequence of APC/C hyperactivation is the lengthening of the rapid cleavage cycles (**Figure 10**). This might be explained by the fact that Cyclin B needs much longer to reach a level sufficient to induce entry into mitosis. It is therefore possible that interphase of the embryonic cell cycle is longer and entry into mitosis is delayed. Indeed, studies have shown that an artificial increase in Cyclin B levels fastens the cell cycle by promoting earlier entry into mitosis (Pomerening et al, 2005) and it is therefore tempting to speculate that a decrease slows down cell cycle progression. The individual contributions of every cell cycle phase to the total cleavage time were not investigated during this study. It is therefore possible that the actual timing of mitosis might be unaffected by XErp1 depletion or even speeded up, because the APC/C can become activated much easier without XErp1 present.

One would expect that a hyperactive APC/C ceases cell cycle progression at one point, because Cyclin B cannot accumulate anymore. The embryo possibly tries to compensate for the missing XErp1 and hyperactive APC/C by increasing the synthesis rate of Cyclin B and with this is still able to continue cycling. Recent mathematical modelling of the early embryonic cell cycles that were mainly based on the data presented here, support the finding that XErp1 depletion leads to an increase in cell cycle length due to hyperactivated APC/C (Vinod et al, 2013). The oscillations of Cdk1 activity are not as pronounced and “spike-like” anymore when the data are simulated in a way that mimics XErp1 depletion.

Besides Cyclin B other APC/C substrates are also affected by XErp1 depletion as shown by a decrease in geminin protein levels (**Figure 10**). Geminin is a suppressor of DNA replication. After Mitosis it is degraded by the APC/C and thereby allows association of the pre-replication complex (pre-RC) with replication origins to perform DNA duplication (Maiorano et al, 2004; McGarry & Kirschner, 1998; Sivaprasad et al, 2007). In S-phase, when the APC/C is inactive, it accumulates again and blocks a second round of DNA replication by inhibition of re-licensing of origins by the pre-RC. When geminin levels are

decreased after XErp1 depletion this mechanism might not work perfectly anymore. Recent data have indeed pointed out that depletion of geminin itself in *Xenopus* embryos by injection of morphonlino oligos leads to re-replication of chromosomal DNA and in the end to embryonic lethality (McGarry, 2002). Additionally, since the cell cycle of XErp1 depleted embryos is lengthened as shown before, there is possibly enough time that re-replication of DNA could take place.

Another effect of XErp1 depletion that is related to APC/C hyperactivation might be chromosome missegregation. Securin is a substrate of the APC/C that keeps the protease separase inactive until anaphase, when the APC/C is activated (Hagting et al, 2002; Stemmann et al, 2001; Waizenegger et al, 2002; Zhang et al, 2008; Zur & Brandeis, 2001). In normal cells the SAC controls APC/C activation and ensures proper sisterchromatid segregation, but the SAC is not functional in the early embryos and only activated at MBT (Kimelman et al, 1987; Newport & Kirschner, 1982a). For this reason, errors in chromosome segregation are not sensed until MBT. When securin is unstable in the absence of XErp1, like shown for Cyclin B and geminin, separase might be activated prematurely and lead in the end to wrong segregation of the chromosomes. Due to lack of a working securin antibody, protein levels could not be checked directly. But addition of ^{35}S-labelled securin (^{35}S-Sec) to embryonic extract revealed that securin behaves exactly like Cyclin B in terms of APC/C dependent degradation (**Figure 9**) and the above described scenario of chromosome segregation errors is therefore very likely.

All data presented in this paragraph implicate that the apoptotic phenotype after XErp1 depletion is probably not caused by one specific defect. Since there are more validated and even more predicted APC/C substrates (Liu et al, 2012), like the centrosomal proteins CENP-F and Ndc80, the mitotic kinases Aurora A and B or the kinesin(-like) proteins CENP-E and Kif4A, XErp1 depletion might lead to pleiotropic effects that are all related to APC/C hyperactivation. This results in different individual defects like unusually slow cell cycle progression, errors in DNA replication and probably also chromosome missegregation that all contribute partially to the XErp1 depletion phenotype.

4.2.3 The F-Box of XErp1 is dispensable for its function in early embryos

Besides the APC/C inhibitory domains of XErp1 that are clearly needed for its function during the early embryonic cell cycles, the work presented here also tried to elucidate a potential role for the F-Box domain of XErp1. Expression of an F-Box mutant of XErp1, which is unable to interact with Skp1 (Schmidt et al, 2005), showed no lethal phenotype in embryos depleted of endogenous XErp1 (**Figure 6**). Like embryos expressing WT XErp1 the F-Box mutant rescued the XErp1 depletion and with this approach a function of the F-Box could not be determined. However, there are other proteins, so called orphan F-Box proteins, that, similar to XErp1, contain an F-Box, which, however, does not seem to be essential for the function of the protein.

Another possible role for the F-Box of XErp1 might be that it helps in protein folding. Skp1 is known to interact with F-Box containing proteins and to stabilize their conformation, even if they are not part of a characterized SCF-complex (Yoshida et al, 2011). The mitotic homologue of XErp1, Emi1, also contains an F-Box domain and was indeed shown to be stabilized by co-expression of Skp1 (Moshe et al, 2011). For this reason, the role of the F-Box of XErp1 could be to control protein levels by limiting the folding of XErp1. However, the data presented here might already argue against this hypothesis, since the F-Box mutant of XErp1 was not to be found more unstable or less expressed in embryos compared to WT XErp1 (**Figure 6**). Therefore, a possible function of the XErp1 F-Box needs still to be explored.

4.3 Regulation of XErp1 activity

4.3.1 Cdk1 inactivates XErp1

It is remarkable how conserved the regulation of XErp1 between CSF arrest and mitosis is and that the same elements work together in the two different developmental stages. XErp1 can be inactivated by Cdk1/Cyclin B phosphorylation in meiosis II and in the early embryonic cell cycles. Interestingly, the same phosphosites are involved in this regulation. During CSF

arrest Cdk1 phosphorylates a cluster of serine and threonine residues in the N-terminal region to regulate XErp1 stability and this could also be shown for the embryos. Long term inhibition of PP2A, the counteracting phosphatase (that will be discussed in the next section), leads to destabilization of XErp1 (**Figure 14**). Under this condition Cdk1 phosphorylates XErp1 constantly and results in a decreased stability of XErp1 like it was afore shown during the CSF arrest (Wu et al, 2007b). Besides the N-terminal phosphosites, C-terminal sites are phosphorylated by Cdk1/Cyclin B as well to regulate XErp1's APC/C binding and inhibitory activity in early embryos. In agreement with this, coimmunoprecipitations of Cdc27 and XErp1 revealed an interaction that is lost upon Cdk1 activation (**Figure 13** B and **Figure 14** D). In CSF arrest it was shown that mutation of these phosphosites to alanines converted XErp1 to a constitutive APC/C inhibitor (Wu et al, 2007b). Expression of the same mutant form of XErp1 in embryos (XErp1-6A) blocks cell cycle progression, visible by big cells that do not divide anymore (**Figure 12** A and B), and results in high levels of Cyclin B indicative of an inactive APC/C (**Figure 12** D). Importantly, combining these mutations with changes in the ZBR domain that is necessary for APC/C inhibition cells do not arrest, showing that the observed effect is APC/C dependent (**Figure 12** A). These data lead to the conclusion that Cdk1/Cyclin B regulate XErp1 negatively by direct phosphorylation.

4.3.2 PP2A and PKA cooperate to activate XErp1

During meiosis II arrest, Cdk1/Cyclin B activity against XErp1 is counteracted by PP2A-B'56 and it could be shown that the same isoforms of B-subunits that are known to interact with XErp1 in meiosis (Isoda et al, 2011) bind to it in the embryonic cell cycles. Specifically, the B'56α, –β and –ε subunits could be verified to directly interact with XErp1 (**Figure 15** A and B) and using the embryonic extract it was possible to prove for the first time an *in vivo* interaction with the B'56ε isoform (**Figure 15** C and D). Inactivation of PP2A by OA treatment of embryonic extract results in the loss of XErp1 binding to the APC/C and consequently APC/C activation (**Figure 14** A and B). This indicates that PP2A removes the inhibitory

phosphorylations done by Cdk1 on XErp1. Moreover, expression of a mutant of XErp1 in embryos that is unable to interact with PP2A is not able to rescue the lethal XErp1 depletion phenotype (**Figure 20**) demonstrating that the activation of XErp1 *via* PP2A is clearly needed for its function in early embryos.

The kinase that recruits PP2A-B'56 to XErp1 is different in CSF arrest compared to the early embryos. In the CSF state, the cMos-MAPK pathway leads to PP2A binding *via* its downstream kinase p90Rsk, whereas in embryos this is achieved by PKA. The data presented in **Figure 17** implicate that the MAPK is inactivated after fertilization and also interfering with MAPK pathway function showed no developmental defect in the early stages (data not shown). To achieve PP2A binding to XErp1 in embryos another kinase is therefore needed and it could be shown that PKA activates XErp1 during the rapid cleavage cycles. In agreement with this, PKA inhibition leads to embryonic lethality (**Figure 19**) and a mutant of XErp1 unable to be phosphorylated by PKA does not recruit PP2A (**Figure 20** A).

4.4 Working model

The data presented above can be included into the following working model (**Figure 22**):

1) During interphase the APC/C has to be inactive ("off"), which is achieved by inhibition *via* XErp1.

2) Cyclin B is allowed to accumulate due to the inhibited APC/C. This in theory could lead to increasing Cdk1 activation, which would result in rapid XErp1 inactivation by inhibitory phosphorylations. To ensure a stable APC/C inhibition, Cdk1 phosphorylation is counteracted by the activity of PP2A-B'56, which binds to XErp1 by PKA mediated phosphorylation.

3) At a certain point in the cell cycle Cyclin B levels are high and activate Cdk1 over a threshold level that tips the balance in favor for Cdk1 and leads to XErp1 inactivation. At this point mitosis starts and PP2A is not able to remove the inhibitory phosphorylations anymore.

4) At anaphase the APC/C is activated (“on”) and degrades Cyclin B, starting a new round of the cell cycle. XErp1 is again activated by the action of PP2A.

In contrast to meiosis, where the forces of Cdk1/Cyclin B and PP2A-B’56 against XErp1 are always more or less equal, this situation is never achieved in the early embryos. If both activities would constantly counteract each other to the same degree, the cells would not be able to cycle. For this reason it is important that one part is always more active than the other one. In interphase PP2A-B’56 activity is higher as Cdk1 activity and in mitosis it is *vice versa*.

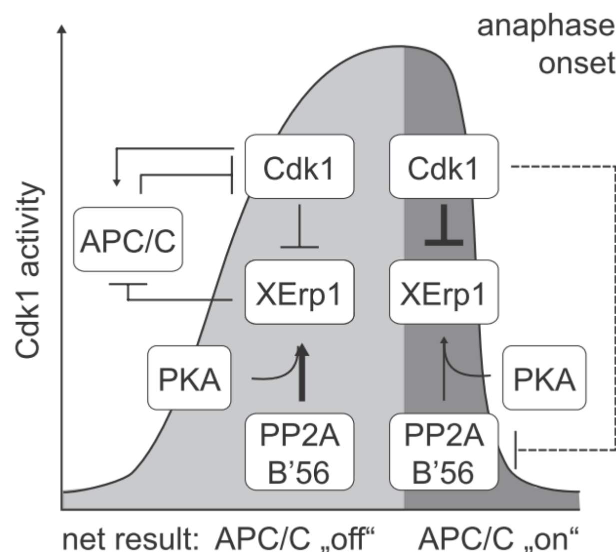

Figure 22: Current working model
During interphase, Cdk1 activity against XErp1 rises but is counteracted by PP2A-B’56. PKA promotes the association of PP2A-B’56 with XErp1 and the APC/C is “off”. When Cdk1 activity prevails over PP2A, mitosis is induced by the inactivation of XErp1 and the activation of the APC/C (“on”), which degrades Cyclin B and initiates the next round of the cell cycle.

4.5 Is *Xenopus* cycling egg extract the method of choice as model system for embryonic cell cycles?

For the here described work it was crucial to combine different approaches and to develop a new embryonic extract that was not used before. So far, data from literature suggested that XErp1 is only needed for the meiosis II arrest but dispensable for early embryonic cell cycles (Liu et al, 2006). As presented, this is not the case as XErp1 is indeed essential for the rapid cleavage cycles. How can this difference be explained?

Here, living *Xenopus* embryos were used and their *in vivo* development was observed. In the published paper so called *Xenopus* cycling egg extract was utilized. This special form of extract is prepared from CSF arrested eggs that are activated by a calcium ionophore and were thought to mimic embryonic cell cycles since their description (Murray & Kirschner, 1989). The cycling egg extract is capable of performing multiple rounds of cell cycles with raising Cdk1 activity until a mitotic state is reached, where then the APC/C is activated. But there are some differences between the cycling egg extract and living embryos and the most pronounced one is that in the extract inhibitory Cdk1 phosphorylations are detectable (Ferrell et al, 2009; Krasinska et al, 2011; Pomerening et al, 2005). In living embryos, during the fast cleavage cycles, these inhibitory phosphorylations are mostly absent (Ferrell et al, 1991; Hartley et al, 1996) and only evident during the first cell cycle (**Figure 4**). This means, the Cdk1 autoamplification loop works only in the first cell cycle but not in the following eleven divisions. Interestingly, the autoamplification loop was shown to function in the cycling egg extract (Abrieu et al, 1998). Additionally, one-cell cycle of the extract lasts about twice as long as a cleavage division in living embryos. For this reasons it is possible that a cycling egg extract represents a consecutive repetition of the first cell cycle instead of real embryonic cleavage divisions. This in turn might explain the differences observed for the function of XErp1 using cycling egg extract and living embryos.

To overcome this difficulties and to have access to an easily manipulatable and open system, an embryonic extract system was developed during this study. Since it is directly derived from 4-cell embryos all the protein contents are mitotic and not interspersed with remaining meiotic proteins, like in cycling egg extract. In combination with studies from living embryos, this new extract was crucial for the work and might reveal more interesting findings about early embryonic cell cycle regulation in the future that are not possible with cycling egg extracts.

4.6 Regulation of PP2A activity against XErp1

4.6.1 Is PKA activity regulated in a cell cycle dependent manner?

From the presented working model (**Figure 22**) an obvious question arises. How is PP2A-B'56 activity against XErp1 regulated? After initial APC/C activation in anaphase Cyclin B levels start to drop and Cdk1 is inactivated. In this situation PP2A would overwhelm Cdk1/Cyclin B and this would in turn immediately lead to XErp1 reactivation and APC/C inhibition, resulting in incomplete Cyclin B degradation. The cell cycle would cease and end up in a situation like during CSF arrest, where PP2A and Cdk1 always counteract each other and only allow so much Cyclin B degradation until the balance is equal again.

The presented model assumes that this does not happen and a solution to the situation could be that the binding of PP2A-B'56 to XErp1 is regulated in a cell cycle dependent manner *via* changes in PKA activity. Indeed, reports show that PKA activity drops in anaphase (Grieco et al, 1994), which would fit to the presented idea of regulated PP2A binding *via* different PKA activity levels. This would allow specific interaction of PP2A and XErp1 during interphase, but not at mitotic exit, when XErp1 needs to stay inactive until all Cyclin B is degraded. Unfortunately, as shown in **Figure 21** global PKA activity does not change during the early embryonic cell cycles. The difference between published results (Grieco et al, 1994) and the data shown here might again result from the different methods used. As pointed out afore, *Xenopus* cycling egg extract might not sufficiently reassemble early embryonic cell cycles and in the published paper cycling extracts were used to determine PKA activity. Here, exactly one cell cycle of living embryos was analyzed and revealed that global PKA activity does not change. These data do not exclude that PKA activity specifically against XErp1 could be regulated in a cell cycle dependent manner, but during this study no evidence for this hypothesis was found.

4.6.2 PP2A regulation *via* inhibitory proteins

As explained afore, global PKA activity does not change in the early embryos, but the model (**Figure 22**) suggests that PP2A-B'56 should be inactive against XErp1 during the metaphase to anaphase transition.

Another elegant possibility how PP2A-B'56 could be regulated stems from the observation, that Cdk1 can indirectly inactivate one specific type of PP2A holoenzymes. Recently it was shown that Cdk1 can activate another kinase, called Greatwall (Gwl) (Yu et al, 2006) that subsequently phosphorylates the two small proteins Ensa and Arpp19. These small and heat stable proteins are inhibitors of PP2A-B55δ, the major Cdk1 counteracting phosphatase (Mochida et al, 2009), when phosphorylated by Gwl (Gharbi-Ayachi et al, 2010; Mochida et al, 2010). During interphase, when Cdk1 is not or minimally active Ensa and Arpp19 are dephosphorylated and thereby inactive due to missing Gwl activity. PP2A-B55δ can counteract most phosphorylations done by Cdk1. When mitosis starts, Cdk1 reaches its highest activity and phosphorylates Gwl, which becomes activated and can transmit the inhibitory signal *via* Ensa/Arpp19 to PP2A-B55δ. By this, Cdk1 inactivates its own enemy. It is tempting to speculate that a similar mechanism might exist to inactivate PP2A-B'56. When Cdk1 activity is high it might, either directly by phosphorylation or indirectly *via* some Ensa/Arpp19-like protein, inactivate PP2A-B'56. In this situation XErp1 cannot be protected from Cdk1 activity anymore. On the highest level of Cdk1 in mitosis the APC/C would then become active and could degrade Cyclin B until Cdk1 is completely turned off.

4.7 XErp1 reactivation after anaphase

4.7.1 Phosphatases regulate mitotic exit

The data clearly show that Cdk1 inactivates XErp1 *via* phosphorylation (**Figure 12** and **Figure 13**) and the presented idea of a PP2A-B'56 inhibitor also regulated by Cdk1 is intriguing, but this raises another question. When Cdk1 inactivates both proteins and

normally PP2A-B’56 would keep XErp1 active, how can XErp1 and also PP2A-B’56 become active again after mitosis?

It is known that phosphatases are important for mitotic and meiotic exit throughout evolution. In yeast the phosphatase Cdc14 is absolutely required after anaphase to dephosphorylate mitotic Cdk1/Cyclin B targets and its deletion leads to a mitotic arrest (Holt et al, 2008; Sanchez-Diaz et al, 2012; Visintin et al, 1998). For exit from metaphase II of meiosis in *Xenopus* the phosphatase PP2B/calcineurin is crucial (Mochida & Hunt, 2007; Nishiyama et al, 2007b). Fertilization triggers a calcium wave and the activation of the signalling molecule calmodulin, which binds to and activates calcineurin. An important calcineurin substrate is the APC/C co-activator Cdc20 because its dephosphorylation is needed for APC/C activity at meiotic exit (Mochida & Hunt, 2007) and, consequently, chemical inhibition of calcineurin blocks the calcium induced release due to sustained Cdc20 phosphorylation. At anaphase during normal cell cycles of metazoan cells, Protein Phosphatase 1 (PP1) gets activated (Wu et al, 2009). Already during metaphase it localizes to kinetochores to stabilize their interactions with microtubules (Kim et al, 2010; Liu et al, 2010) and during anaphase it remains at the chromosomes to dephosphorylate mitotic substrates (Trinkle-Mulcahy et al, 2006; Vagnarelli et al, 2011). Interestingly, PP1 itself is also regulated by Cdk1/Cyclin B *via* inhibitory phosphorylations at Thr-320 (Dohadwala et al, 1994; Wu et al, 2009) and can start intrinsic auto-dephosphorylation, when Cdk1 levels drop at anaphase. Even if the function of PP1 is not as crucial as the one of Cdc14 is in yeast, the conserved principle of phosphatase activation at anaphase onset seems to be clear.

4.7.2 Another phosphatase might activate XErp1 after mitosis

Nothing is known about phosphatase activation during anaphase of the rapid cleavage cycles, but it is tempting to speculate that also here they might play an important role. It is unlikely that calcineurin is required like it is for meiotic exit since the calcium signal appears

only with fertilization. Instead, PP1 might be a good candidate for the dephosphorylation of XErp1 and/or PP2A-B'56.

There are two possibilities how it could work. The first one is that PP1 directly acts on XErp1 and dephosphorylates it at mitotic exit. Since Cdk1 activity is already low at this point in the cell cycle PP1 could auto-dephosphorylate and activate itself. Most PP1 interacting proteins and also some substrates convey a conserved RVxF motif (standing for the aminoacids arginine, valine, any aminoacid and phenylalanine). Some variations in this motif are allowed but, unfortunately, no similar sequences can be found in XErp1. This does not exclude that XErp1 might be a direct substrate of PP1 at mitotic exit, but makes this first possibility a bit more unlikely.

The second mechanism could include a stepwise activation of XErp1 after mitotic exit. PP1 might, after initial auto-dephosphorylation, first activate PP2A and this then removes the inhibitory phosphates on XErp1 as it does in interphase. If an inhibitor of PP2A-B'56 exists, that, as afore mentioned, might also be controlled by Cdk1, PP1 would only need to dephosphorylate it to mediate PP2A-B'56 reactivation.

At the moment there are no data to support one or the other idea. Also a mix between both mechanisms is thinkable. First PP1 could activate PP2A-B'56 and both phosphatases then work together to rapidly dephosphorylate XErp1 so that it can inhibit the APC/C again. Maybe one can image PP1 as some kind of reset button that after mitosis removes directly or indirectly all mitotic phosphorylations done by Cdk1.

4.8 A revised model of early embryonic cell cycles

4.8.1 APC/C activation is supported by Cdk1/Cyclin B

Besides XErp1 as APC/C inhibitor other mechanisms might be important in regulating the early embryonic cleavage cycles. Mathematical modelling based on the data presented here has revealed that robust cycling is only possible when phosphorylation of the APC/C itself and its coactivator Cdc20 are taken into account (Vinod et al, 2013). The APC/C is a target of

many mitotic kinases and also Cdk1/Cyclin B phosphorylate and thereby activate it (Kraft et al, 2003). During the here presented study, phosphorylation of the APC/C core subunit Cdc27 was observed in a cell cycle dependent manner (data not shown) providing initial evidence that during early embryonic cleavage cycles this indeed might be important as suggested by the modelling approach.

4.8.2 Cdc20 is inactivated by Cdk1/Cyclin B

Cdc20 is phosphorylated by different kinases, mostly related to the spindle assembly checkpoint (Chung & Chen, 2003; Tang et al, 2004). But since the SAC is not active during the rapid cleavage cycles it seems unlikely that these phosphorylations play a major role in regulating its activity at this time. Additionally, Cdk1/Cyclin B mediated inhibitory phosphorylation of Cdc20 was observed and, at a first glance, seemed to be counterintuitive (Yudkovsky et al, 2000). Why should Cdk1/Cyclin B simultaneously activate the core APC/C but inactivate its coactivator? Published data suggest that this mechanism restricts the activity of the APC/C to a very small time window. Only when Cdk1 activity is highest, the APC/C is fully activated and in the same moment a phosphatase gets active to remove the inhibitory phosphates on Cdc20 (Labit et al, 2012). The authors argue that it might be a B’56 containing holoenzyme since PP2A-B55δ is inactive during the metaphase-mnaphase transition by the action of the Gwl-Ensa/Arpp19 pathway. From the model presented above PP2A-B’56α, -β and -ε might also be inactive due to Cdk1 mediated phosphorylation, otherwise XErp1 could not inhibit the APC/C.

4.8.3 A complex kinase and phosphatase network regulates the early embryonic cell cycles

Interestingly, another B’ isoform, B’56γ, was already implicated in DNA damage response (Li et al, 2007; Shouse et al, 2008) and, more importantly, in cell proliferation (Chen et al, 2004). When B’56γ was down regulated, cells divided faster and uncontrolled. So it might not be

surprising to imagine a potential role during the early cleavage cycles, especially when the DNA damage checkpoint is inactive and B’56γ is not needed to function in it. Other publications suggest an inhibitory link from MAPK to PP2A-B’56γ (Letourneux et al, 2006), but, as shown afore, MAPK is also not active during the embryonic cell cycles (**Figure 17**). Interestingly, B’56γ is present in the early embryos, because expression profiles showed its abundance (Baek & Seeling, 2007). Putting these data together it is a very appealing possibility that B’56γ might play a role during the cleavage cycles. An interesting function would be to dephosphorylate Cdc20 and thereby to activate the APC/C at the right time.

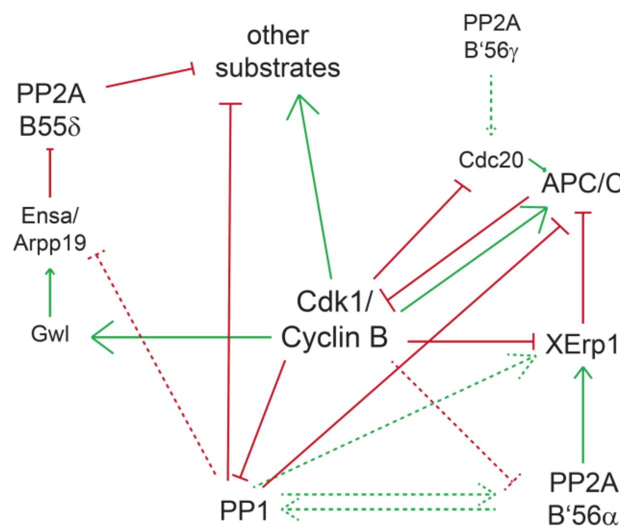

Figure 23: A possible interaction diagram of proteins regulating the rapid cleavage cycles
At the heart of cell cycle regulation is Cdk1/Cyclin B is located. Its activity is directly counteracted by the APC/C (which degrades Cyclin B) and indirectly by the two phosphates PP1 and PP2A-B55δ, removing substrate phosphorylations. The APC/C is regulated by XErp1 and through association with its coactivator Cdc20. Both proteins themselves are again subjected to regulation *via* Cdk1/Cyclin B and (possibly other) PP2A holoenzymes. Red colouring indicates inhibitory relationships, green positive interactions and dashed lines show speculative connections.

During this work only the relationships between XErp1, the APC/C, Cdk1/Cyclin B and PP2A-B’56 were analyzed. From literature it is known that PP1 is inactivated by Cdk1/Cyclin B (Berndt, 1998) but can auto-activate itself. This leads to the inactivation of the APC/C at mitotic exit (Wu et al, 2009), which was before phosphorylated and activated by Cdk1 (Kraft et al, 2003). At the same time Cdc20 is inhibited by Cdk1 mediated phosphorylation (Labit et al, 2012). Cdk1/Cyclin B also activates Gwl (Yu et al, 2006) and thereby indirectly influences

PP2A-B55δ negatively (Gharbi-Ayachi et al, 2010; Mochida et al, 2010). Speculations lead to the idea that PP1 might work as some kind of reset button after anaphase that eliminates all mitotic phosphorylations. Depending on the initial effect of Cdk1/Cyclin B phosphorylation this might either activate the target protein or inactivate it. Future research is needed to understand the regulatory network in rapid cleavage cycles, but the work presented here is crucial in revealing first connections between cell cycle proteins necessary for early development.

The most striking question is still, as already pointed out before, how all these phosphorylations are reversed when mitosis is left. PP1 was implicated as the major phosphatase during anaphase (Wu et al, 2009) and future research should narrow down the functions of phosphatases during the early cleavage cycles in general but in particular also of PP1.

One could connect all these pieces into an interaction diagram (**Figure 23**) showing the complex regulation of the, at first glance so simple appearing, rapid cleavage cycles of *Xenopus* embryos.

5 Contributions

The work presented here was done in close collaboration with Eva Hörmanseder. E.H. did experiments shown in **Figure 10** B, **Figure 12**, **Figure 14** A B C, **Figure 15** and **Figure 20** A.

6 Publications

The main part of this work was published in:

Tischer T*, Hormanseder E*, Mayer TU (2012) The APC/C Inhibitor XErp1/Emi2 Is Essential for Xenopus Early Embryonic Divisions. *Science* **338:** 520-4

Parts of the introduction are modified from an early version of:

Hormanseder E*, Tischer T*, Mayer TU (2013) Modulation of cell cycle control during oocyte-to-embryo transitions. *EMBO Journal*, July 26 Advanced Online Publication

Other publications:

Hacker SM, Pagliarini D, Tischer T, Hardt N, Schneider D, Mex M, Mayer TU, Scheffner M, Marx A (2013) Novel Fluorogenic ATP Analogues for Online Monitoring of ATP Consumption: Observing Ubiquitin Activation in Real-Time, *Angewandte Chemie International Edition*, DOI: 10.1002/anie.201304723

Hormanseder E*, Tischer T*, Heubes S, Stemmann O, Mayer TU (2011) Non-proteolytic ubiquitylation counteracts the APC/C-inhibitory function of XErp1. *EMBO reports* **12:** 436-443

*equal contribution

7 Material and Methods

All reagents and chemicals used were obtained from commercial suppliers if not differently stated. Buffers and solutions were prepared with de-ionized water from a Milli-Q system (Millipore). All reactions were stopped with 3x Laemmli sample buffer (180 mM Tris, 30 % Glycerol, 10 % SDS, pH 6.8) and boiled for 10 min at 95 °C if not otherwise stated.

7.1 Antibodies

Table 1: Antibodies used in this study (alphabetical order)

Antibody	source	species	incubation	dilution
Cdc27	homemade	rabbit	1 h, RT or o/n, 4 °C	1:500
cMyc	homemade	mouse	1 h, RT	1:10
Cyclin A1	abcam 13337	mouse	o/n, 4 °C	1:250
Cyclin A2	homemade	rabbit	o/n, 4 °C	1:1000
Cyclin B2	MBL K0189-3	mouse	o/n, 4 °C	1:500
Cyclin E1	T.Hunt	rabbit	1 h, RT	1:1000
(X)Erp1	homemade	rabbit	2 h, RT or o/n, 4 °C	1:750
Geminin	T.McGarry	rabbit	1 h, RT	1:1000
Greatwall	T.Lorca	rabbit	1 h, RT or o/n, 4 °C	1:1000
MAPK	sc-154	rabbit	o/n, 4 °C	1:200
MAPK-pY204	cell signaling 9106	mouse	1 h, RT	1:2000
MBP	NEB E8032S	mouse	1 h, RT	1:5000
PKA	sc-903	rabbit	o/n, 4 °C	1:200
PKA	abcam 26322	rabbit	2 h, RT	1:1000
Phospho RxxS/T	Cell Signaling 9621	rabbit	o/n, 4 °C	1:1000
PP2A-C	upstate 05-421	mouse	o/n, 4 °C	1:1000
Tubulin	Sigma T6199	mouse	1 h, RT	1:3000

7.2 *Xenopus* embryos and extracts

All procedures and incubations were done at 20 °C if not otherwise stated.

Testis was removed from a male *Xenopus laevis* frog by surgery. The evening before, the frog was injected with 75 U Human chorionic gonadotropin (hCG, Sigma or Intervet) and kept separately over night. The frog was anesthetized by a bath in Tricaine (MS222, Sigma) and killed. The testis were removed and stored in MMR (5 mM Na-HEPES, 0.1 mM EDTA, 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, pH 7.8) supplemented with 5 mg gentamycine (Sigma).

Oocytes were fertilized *in vitro* by collection of freshly laid eggs. For this female *Xenopus laevis* frogs were injected the evening before the experiment with 500 U hCG and kept separated. In the morning the frogs were transferred to a fresh basin with MMR and after one hour the eggs were collected. After a quick rinse with MMR they were transferred to a petri dish and most of the buffer was removed. Testis and eggs were mixed by removing small bits of the testis by a scissor and incubated for 10 min. The MMR was diluted about 10-fold with water and incubation was continued for 20 min. The jelly coat was removed by a cysteine solution (2 % L-cysteine (Sigma) in 0.1x MMR, pH 7.8 with NaOH) for 7 min and embryos were washed four times with 0.1x MMR.

Staging of embryos was carried out according to Nieuwkoop & Faber (Nieuwkoop & Faber, 1967). Embryos were photographed under a Stemi-2000-C equipped with a 18.2 Color Mosaic Camera (Zeiss/Visitron) or under an Axiovert-200M (Zeiss). At one-cell stage, embryos were injected with the indicated solutions using a Nanoliter-2000 Micromanipulator (WPI). For XErp1 depletion, 64 ng of a mixture of two antisense morpholino oligos (MOs; Gene tools) targeting both XErp1 gene copies (Ohe et al, 2007) or as control sense MOs were injected. Where indicated, 250 pg mRNA were co-injected coding for wild type or mutant XErp1 including the XErp1 3'UTR and an amino-terminal 6x Myc-tag. Embryos were lysed in NEB buffer 3 (New England Biolabs) containing *complete* protease inhibitors (Roche), cleared by centrifugation for 10 min at 4 °C, 20.000 g and treated with Calf intestinal

phosphatase (CIP, New England Biolabs). Where phosphorylations were preserved, 40 mM beta-glycerophosphate and no CIP were added.

Embryo extracts were prepared from four cell embryos or embryos 4 hours after fertilization. Embryos were collected, compacted by a brief spin in a table top centrifuge and the supernatant was removed. Embryos were crushed by centrifugation at 12.000 g for 10 min at 4 °C. The cytosolic fraction was isolated by piercing the eppi-tube with a needle and then used for further experiments.

7.3 Gel electrophoresis and immunoblotting

Gel electrophoresis and immunoblotting were performed with the Biorad MiniGel system according to standard procedures (Burnette, 1981). The detection of the protein of interest was performed in two steps: First a specific antibody against the protein (see **Table 1**) and second a horseradish peroxidise (HRP) coupled antibody (Dianova) against the species of the first antibody was used. For detection, enhanced chemo luminescence (ECL) was performed (100 mM Tris, 1.25 mM luminol, 225 µM coumaric acid, 0.015% H_2O_2, pH 8.5) with a LAS3000 (FujiFilm).

7.4 Antibody depletions and immunoprecipitations

PKA was depleted from diluted embryo lysate. Two embryos were lysed in 20 µL kinase buffer (50 mM Tris, 10 mM $MgCl_2$, pH 7.2) and incubated three times for 30 min with each 10 µg anti PKA antibody (Sigma) coupled to magnetic protein G beads (Invitrogen) on ice.

XErp1 was depleted from 100 µL 4-cell embryo extract with 3x 30 min each 10 µg antibody coupled to magnetic beads on ice. XErp1-IPs were performed with 2 µg antibody coupled to magnetic beads for 1 h on ice and subsequently washed 3 times with CSF-xB (100 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 50 mM sucrose, 5 mM EGTA, 10 mM HEPES, pH 7.7). Beads were boiled in Laemmli.

Cdc27 IPs from 50 µL 4-cell embryo extract diluted with 50 µL CSF-xB were performed with 2 µg antibody coupled to agarose protein G beads (Thermo Fisher) for 1 h at RT. For IPs from embryos, 50 embryos were lyzed by centrifugation, the cytoplasmatic fraction was isolated and used for IP with 2 µg antibody for 1 h at RT. Precipitates were washed 3 times with CSF-xB and beads were boiled in Laemmli.

Flag-tagged PP2A B-subunits were immunoprecipitated after mRNA expression for 2 h (2 µg/50 µL extract) with Flag antibody on magnetic beads like described before.

7.5 Pulldown assays

For MBP-XErp1^{BD} pull down assays, 5 µg recombinant protein was coupled to amylose beads (New England Biolabs) and when indicated pre-phosphorylated with PKA as described below. Protein beads were washed with CSF-xB buffer, residual buffer was removed and the beads were incubated with undiluted four-cell embryo extract (100 embryos per condition) for 20 min at 20 °C. The beads were washed 3 times (PBS, 200 mM NaCl, 0.1 % Triton-X 100), boiled in Laemmli and subjected to immunoblotting.

GST pulldown assays were performed as following: Flag-tagged B-subunits were expressed from mRNA in extract and purified GST-tagged proteins were added at the same time after coupling to glutathione-beads. After an 2 h incubation the beads were isolated and washed 3 times with CSF-xB.

7.6 mRNA production and IVT

For mRNA production, the Ambion mMessage mMachine T7 Ultra Kit was used, but modified accordingly. Plasmid DNA was purified with the Qiagen Midi prep Kit and 10 µg of DNA were digested to linearize at 37 °C over night in a 50 µL reaction. For XErp1 plasmids containing the 3'UTR a KpnI/NdeI double digest or a SphI digest were performed to cut directly behind the 3'UTR. PP2A-B-subunit plasmids were digested with AscI. Linear DNA was precipitated by addition of 5.5 µL EDTA, 3.3 µL Ammoniumacetate and 140 µL 100% Ethanol and

incubation over night at -20 °C or 2 h at -80 °C. After centrifugation at 4 °C for 45 min with 20.000 g DNA was recovered in 20 µL water. The mRNA reaction was set up according to the manufactors instructions but incubated for 3 h at 30 °C. Subsequently the DNA was digested with TurboDNAse at 37 °C for 20 min. XErp1 mRNA containing the 3'UTR was filled up to 100 µL with water and 60 µL Lithium Chloride (LiCl) were added. B-subunit mRNA was poly-A tailed following the instructions in the manual and also precipitated with LiCl at -20 °C over night. After centrifugation at 20.000 g 4 °C for 30 min the pellet was washed with 1 mL 70% Ethanol and centrifugation was repeated. The pellet was recovered in 20 µL to 50 µL water.

Expression of the mRNA was always analyzed by addition of 2 µg mRNA to 50 µL CSF extract and probing by immunoblotting.

Coupled *in vitro* transcription and translation (IVTs) were performed with the Promega wheat germ extract expression system according to the manuals instructions. Radioactive labelled proteins were detected with a FLA-5000 (FujiFilm).

7.7 Protein purification

MBP-XErp1^{BD} was purified over its C-terminal 6xHis-tag. Proteins were expressed in 1 L *E.coli* JM109 RIL for 4 h at 37 °C. Cells were collected by 20 min centrifugation at 4 °C, 6000 g, washed once with PBS (137 mM NaCl, 2.7 mM KCl, 10.2 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.5), centrifuged again at 4 °C, 3000 g for 10 min and frozen in liquid nitrogen to store them. After thawing, the pellet was resolved in IMAC5 (**Table 2**) and lysed with an Emulsiflex C5 homogenizer (Avestin). The lysate was cleared by centrifugation at 20.000 g, 4 °C for 20 min and the supernatant was added to 1 mL slurry of washed and equilibrated Ni-NTA agarose beads (Qiagen). After incubation for 3 h at 4 °C the beads were transferred to a Biorad column and washed 3 times with IMAC 20 and eluted with IMAC500 (**Table 2**) in fractions about half the bead volume (~250 µL).

GST proteins were expressed and purified similarly as described but incubated with Gluthation S6 sepharose (GE Healthcare). Additionally, the buffers listed in **Table 3** were used.

All purified proteins were dialyzed over night in 80 mM HEPES, 150 mM KCl, 1 mM DTT, 10 % Glycerol, pH 7.7 and aliquoted before freezing in liquid nitrogen and storing at -80 °C.

Table 2: IMAC buffers used for His-purification

IMAC5	**500 mL**
20 mM Tris-HCl pH 8.0	1.21 g
300 mM NaCl	8.76 g
5 mM Imidazol	0.17 g
1 tablet Roche *complete* protease inhibitors / 50 mL	
IMAC20:	**500 mL**
20 mM Tris-HCl pH 8.0	1.21 g
300 mM NaCl	8.76 g
20 mM Imidazol	0.68 g
0.1 % TX100	5 mL (10 %)
IMAC200:	**500 mL**
20 mM Tris HCl pH 8.0	1.21 g
300 mM NaCl	8.76 g
200 mM Imidazol	6.81 g

Table 3: Buffers used for GST-purification

Buffer A	**1000 mL**
100 mM Tris-HCl pH 7.3	12.11 g
150 mM NaCl	8.77 g
Lysisbuffer	**250 mL**
Buffer A	250 mL
0.1 % Triton	2.5 mL (10 %)
5 mM DTT	193 mg
1 tablet Roche *complete* protease inhibitors / 50 mL	
Washbuffer	**50 mL**
Buffer A	50 mL
1 mM NaATP	27.6 mg
1 mM $MgCl_2$	50 µL (1 M)
5 mM DTT	38.6 mg
0.1 % Triton	400 µL (10 %)
Elutionbuffer	**250 mL**
100 mM Tris HCl pH 8.0	3.03 g
500 mM NaCl	7.31 g
20 mM Glutathione (reduced)	1.54 g

7.8 Kinase assays

For PKA kinase assays from embryos, two embryos were lysed in 20 µL kinase buffer (see 7.4) and 1 µL was used as source of kinase. 1.25 µg recombinant MBP-Proteins coupled to amylose beads were used as substrates and incubated at 30 °C for the indicated times or 30 min. For PKA add-back experiments 3 U of active PKA (Calbiochem) was added to the depleted extracts.

PKA assays using 6 U of recombinant active PKA were carried out in Tris Buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 200 µM ATP, 200 µCi/µmol γ-labeled-^{32}P-ATP) for the indicated times at 30 °C with 2 µg of protein. Pre-phosphorylation reactions for MBP pulldown assays were carried out with an excess of PKA (100 U/µg protein, 5 µg protein

used in total) for 1 h at 30 °C and the reactions were stopped by washing the beads in ice cold reaction buffer.

The estimation of global PKA activity during the cell cycle (**Figure 21**) was performed with a scintillation counter. One frozen embryo (but not stored at -80 °C) was lysed in fresh prepared 40 µL buffer (80 mM beta-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT, Roche *complete* protease inhibitors (Ferby et al, 1999)) and centrifuged for 5 min at 20.000 g, 4 °C. 2 µL of the supernatant were taken per 30 µL reaction and four reactions from each embryo were performed in parallel. All contained 0.2 mM MgATP pH 7.0 and 6 µCi ^{32}P-γ-ATP. The first reaction for Cdk1 activity was supplemented with 0.33 mg Histon H1, the second one for PKA activity contained 100 µM Kemptide (Calbiochem) and the third reaction for kinase activity against XErp1 contained 0.6 mg MBP-XErp1^{BD}. The fourth reaction was the background reaction, which was not supplemented with any additional protein. All reactions were incubated for 10 min at 30 °C and stopped by transferring them on a p81 filter (Whatman), which was immediately dropped into 7 % H_3PO_4. Two washes with 7 % H_3PO_4 were performed and the filters were dried with a fan. The filters were put in a scintillation tube supplemented with 5 mL scintillation solution for measurement of the incooperated radioactivity with a Beckman LS 6500.

7.9 PARP cleavage assay

Human PARP1 was a gift from A. Bürkle and the first 338 aa (tPARP) were subcloned in an vector suitable for IVT. Embryos were injected in one-cell stage and incubated. Three embryos were lysed in 15 µL caspase buffer (80 mM beta-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 10 mM DTT) and the supernatant after centrifugation (10.000 g, 4 °C, 10 min) was transferred to a new reaction tube. 1 µL of ^{35}S-tPARP was added to the supernatant and incubated for 20 min at 25 °C.

7.10 Data analysis

All data were collected from at least three independent experiments. n indicates the number of embryos counted if not otherwise stated. Data were processed using Microsoft Excel and Adobe Photoshop and Illustator. Statistical significance was determined by a two tailed student's t-test, even if not explicitly stated in the figures. Error bars represent SD.

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9 Abbreviations

aa	amino acid(s)
AMP	adenosine 5'-monophosphate
APC/C	Anaphase promoting complex/cyclosome
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
cAMP	cyclic adenosine 5'-monophosphate
CBB	Coomassie Brilliant Blue
Cdk1	Cyclin dependent kinase 1
CSF	cytostatic factor
C-terminal	carboxyterminal
C-terminus	carboxy terminus
Cyc	Cyclin
D-box	Destruction box
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylendiamine tetraacetic acid
EGTA	ethylen glycol tetraacetic acid
Emi1	early mitotic inhibitor 1
Fig.	figure
g	gram or centrifugal force
GST	glutathione-S-transferase
h	hour
hpf	hours post fertilization
HEPES	4-(2-hydroxyethyl)-1piperazineethansulfonic acid

HRP	horseradish peroxidase
IgG	immunoglobulin G
IP	immunoprecipitation
IPTG	isopropyl-β-D-thiogalactopyranoside
IVT	coupled *in vitro* transcription-translation
k	kilo
kb	kilo base pairs
kDa	kilo dalton
L	liter
m	milli
μ	micro
M	molar
MBT	Mid-blastula transition
MBP	Maltose Binding Protein
min	minutes
mRNA	messenger RNA
n	nano
N-terminal	aminoterminal
N-terminus	amino terminus
OA	okadaic acid
OD	optical density
o/n	over night
ORF	open reading frame
p	pico
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PKA	cAMP dependent protein kinase A
PP1	Protein Phosphatase 1

PP2A	Protein Phosphatase 2 A
Rca1	Regulator of Cyclin A1
SAC	spindle assembly checkpoint
SDS	sodium dodecylsulfate
sec	seconds
^{35}S-Securin	^{35}S(ulfur)-Methionin labeled securin
Tris	tris(hydroxymethyl)aminomethane
U	units
v/v	volume per volume
w/v	weight per volume
WT	wild type
XErp1	*Xenopus* Emi1 related protein
ZBR	Zinc binding region

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