

**Functional characterization of a
novel *Xenopus* polo-like kinase
interacting protein**

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Table of contents

Table of contents	1
1 Introduction	2
1.1 The cell division cycle	2
1.2 Principles of cell cycle regulation	6
1.3 Vertebrate oocyte biology	9
1.4 The molecular basis of CSF arrest	12
1.5 Objective of the project and experimental approach	18
2 Results	21
2.1 Characterization of an anti-XErp1 antibody	21
2.2 Expression and behaviour of XErp1 protein	22
2.3 Functional analysis of XErp1	25
2.4 Regulation of XErp1 by Plx1	34
2.5 <i>In vivo</i> analysis of XErp1 function	43
3 Discussion	45
3.1 XErp1 behaviour in maturing oocytes and CSF extracts	45
3.2 XErp1's contribution to CSF arrest	47
3.3 Mechanism of XErp1 function	50
3.4 Link between XErp1 and Plx1	51
3.5 Relationship between XErp1 and Emi1	53
3.6 Conclusion	55
4 Materials and Methods	56
4.1 Chemicals and buffers	56
4.2 Molecular Biology	56
4.3 Protein Biochemistry	58
4.4 Cell biology	62
Literature	68
Summary	78
Zusammenfassung	80
Abbreviations	82
Danksagungen, curriculum vitae, publications	84

1 Introduction

Cell division is one of the most fundamental processes in biology. In unicellular and multicellular organisms the generation of new cells from a parent cell serves reproductive purposes (sexual or asexual). In addition, in multicellular eukaryotes cell division forms the basis for ontogenesis and the maintenance of the adult organism's cellular structure.

The scope of this work is the functional characterization of a novel protein involved in the sexual reproduction of vertebrates. In particular, the protein functions in a pathway coordinating cell cycle progression of female germ cells with their fertilization.

1.1 The cell division cycle

The formation of daughter cells from a parent cell proceeds through a set of successive events that can be described as the cell division cycle (Alberts et al., 2002). The somatic cell division cycle is classically divided into different phases during which the cell grows, duplicates its genetic material, distributes it equally and forms physically separate daughter cells (see *Figure 1.1*). The phase during which the cell replicates its DNA is

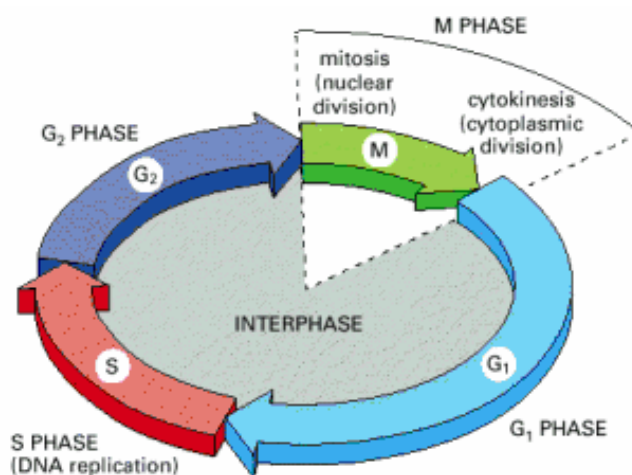


Figure 1.1: A basic eukaryotic cell division cycle (Alberts et al., 2002). The cell division cycle can be divided into S-phase where the DNA is replicated and M-phase where the replicated and condensed chromosomes are segregated. Both are interrupted by gap-phases G₁ and G₂ that can be more or less pronounced. Together, the gap-phases and the intervening S-phase are called interphase. The transition from interphase to M-phase where the cell divides is associated with very pronounced morphological and biochemical changes.

called S-phase (for synthesis-phase), while the phase during which the replicated DNA is distributed to the daughter cells is called M-phase (for mitosis-phase). The final step of M-phase called cytokinesis constitutes the actual physical separation of the cytoplasm to yield the resulting two daughter cells. The S- and M-phases are usually separated by so called gap-phases (G_1 and G_2) of variable length during which cells grow and prepare for M-phase, respectively.

In order to contribute to a functional tissue and perform their physiological role in this context, cells might exit the cell cycle from G_1 into a phase called G_0 to differentiate. Embryonic cell cycles (the first cell divisions of the developing metazoan organism) on the other hand often lack prominent gap-phases altogether. They consist solely of rapidly alternating S- and M-phases (Gilbert, 1997).

Eukaryotic cells have two different types of cell division, termed mitosis and meiosis, which have different purposes. Particularly, in the case of meiosis this is reflected in a variation in the sequence of events of the basic cell division cycle depicted in *Figure 1.1* as will be described below.

1.1.1 Mitosis

A mitotic cell division leads to the formation of two genetically identical daughter cells. Mitosis is therefore involved in the asexual reproduction of single celled organisms; furthermore it is the foundation of the development and function of tissues in higher organisms.

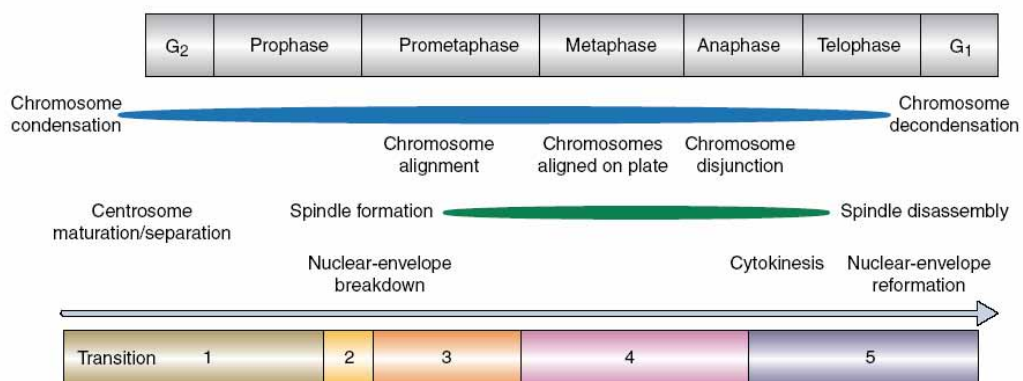


Figure 1.2: Staging of a mitotic nuclear division (Pines and Rieder, 2001). Mitosis can be staged phenomenologically (top) or based on more detailed knowledge of cellular processes (bottom).

Mitosis has historically been divided into certain stages that are discernable by light microscopy in tissue preparations or cultured cells (see *Figure 1.2* top). After DNA replication cells begin to condense their chromatin leading to the formation of visible chromosomes during **prophase**. Chromosomes are aligned in the future cell division plane during **prometaphase** by a microtubule structure called the mitotic spindle. During the process of alignment the chromatids of a chromosome are attached to opposite poles of the mitotic spindle via a protein structure at their centromeres called the kinetochore. This process is referred to as bipolar attachment. After all chromosomes have been aligned in **metaphase**, spindle forces move the chromatids to opposite poles of the cell **anaphase**. The nuclear envelope reforms and the chromatin decondenses in **telophase**, which is also the time when cytokinesis takes place.

Based on advances in understanding the cell division cycle at the molecular level, an alternative staging of mitotic progression has been proposed (Pines and Rieder, 2001). It relies on biochemical changes that constitute important transitions throughout mitotic progression (see *Figure 1.2* bottom). In this work, however, the more common classic terminology will be used.

1.1.2 Meiosis

Meiosis is a specialized cell division that leads to the production of germ cells (also called gametes) from somatic precursor cells (Marston and Amon, 2004; Petronczki et al., 2003). Meiosis proceeds through two consecutive nuclear divisions, meiosis I and meiosis II, without an intervening S-phase, theoretically resulting in four genetically non-equivalent cells that carry only one genetic complement (i.e. they are haploid). In meiosis I homologous chromosomes are separated, whereas meiosis II segregates the chromatids leading to a reduction of the genetic complement. Fertilization, the fusion of the haploid paternal and maternal gametes, restores the diploid state leading to the formation of a zygote from which a new organism can develop by mitotic division of its somatic cells (see *Figure 1.3*).

Reduction of the genetic complement leading to gamete formation is not the only peculiarity of meiosis. An equally important feature of meiosis and sexual reproduction in general is recombination of maternal and paternal traits, which is thought to be evolutionarily extremely advantageous for the respective species (Hoekstra, 2005). Recombination of maternal and paternal genes happens through two different mechanisms. Upon entry into meiosis I after pre-meiotic S-phase homologous chromosomes pair before they are segregated in anaphase I. First, the segregation process is inherently random in the sense that maternal and paternal homologues have an equal chance to be pulled to one or the other pole. Second, the cell actively introduces double-strand breaks into the DNA after replication, which are often repaired by a mechanism leading to strand exchange (crossing-over).

Even though the phases of a meiotic nuclear division look similar to those of mitosis (and are in fact called prophase I, metaphase I, anaphase I, metaphase II and anaphase II), reduction of the genetic complement and recombination of the genetic material requires important changes to the process of chromosome segregation especially in meiosis I.

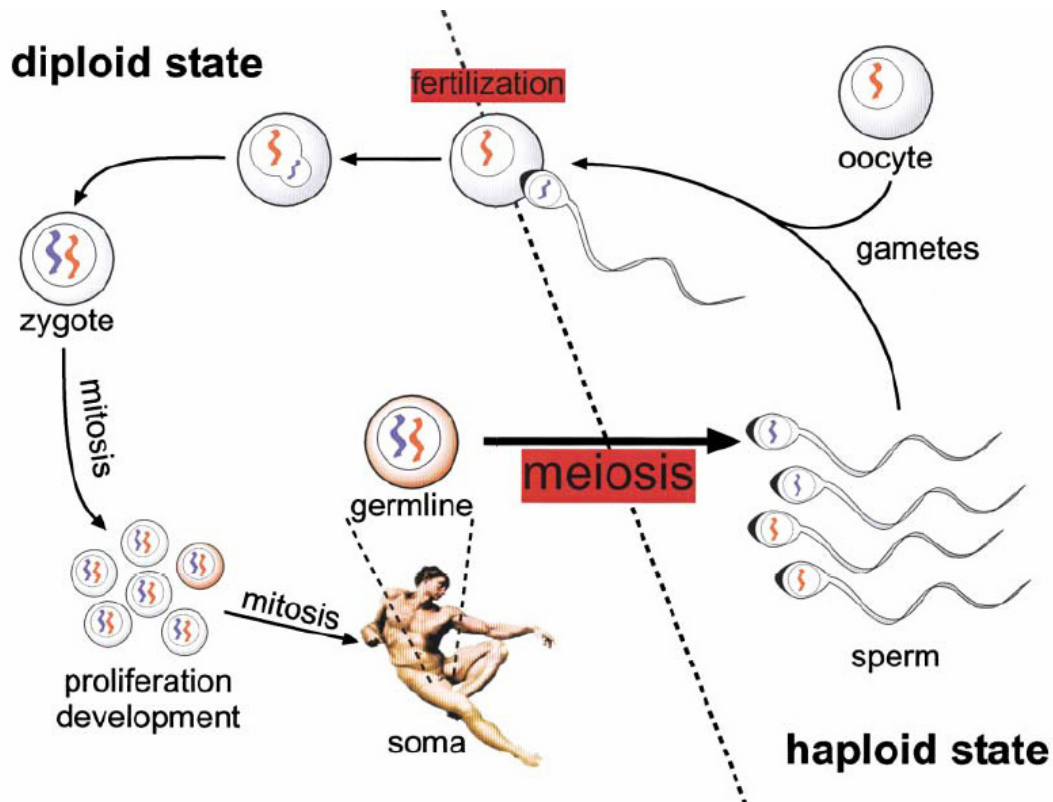


Figure 1.3: Life cycle of a typical diploid organism (Petronczki et al., 2003). The organism's body is made up of diploid cells which divide mitotically. Sexual reproduction is initiated by producing haploid germ cells that fuse to lead to the formation of zygote from which a new organism develops.

Linkage of homologous chromosomes has to be established by formation of chiasmata that result from crossing-over. Orderly segregation of homologues in meiosis I requires the suppression of bipolar attachment of sister centromeres as well as the protection of centromeric cohesion during anaphase I. Reduction of the genetic complement requires the suppression of DNA synthesis between meiosis I and meiosis II.

1.2 Principles of cell cycle regulation

Progression through the process of cell division, mitotic or meiotic, is chiefly regulated by post-translational modification of cell cycle-regulatory proteins. The two best-understood modifications are phosphorylation by protein kinases and multi-ubiquitylation. Both mechanisms often act together on the same protein to influence its activity (Nigg, 2001). Phosphorylation/de-phosphorylation is a rapidly reversible way of modulating protein activity by inducing structural change or altering the physico-chemical properties of the protein to create or block binding sites for other proteins. Multi-ubiquitylation targets proteins for proteolysis by the 26 S proteasome. This makes major cell cycle transitions, which are usually associated with degradation of key regulatory proteins, virtually irreversible ultimately resulting in directionality of the cell cycle (Reed, 2003).

1.2.1 Phosphorylation and cell cycle regulatory kinases

The most prominent example of cell cycle regulatory protein kinases is the family of cyclin-dependent kinases (Cdks) (Murray, 2004). The catalytically active form of these enzymes consists of a small Cdk-subunit containing the catalytic domain associated with one of various cyclin-subunits, which act as activators of kinase activity. Different combinations of Cdk/cyclin complexes promote specific events in different phases of the cell division cycle. The activity of the different Cdk/cyclin complexes is, besides other mechanisms, tightly regulated through availability of the corresponding cyclin. M-phase progression for example is mainly driven by Cdk1 in complex with cyclin B. Cyclin B protein accumulates at the beginning of mitosis and is degraded upon exit from mitosis.

Together with Cdks other families of protein kinases regulate different aspects of cell cycle progression, especially in M-phase. These include the Aurora and Polo-like kinases (Barr et al., 2004; Nigg, 2001). Members of these kinase families have numerous functions related to spindle function and chromosome segregation throughout M-phase progression. Particularly, the polo-like kinase 1 (Plk1) and its involvement in promoting metaphase to

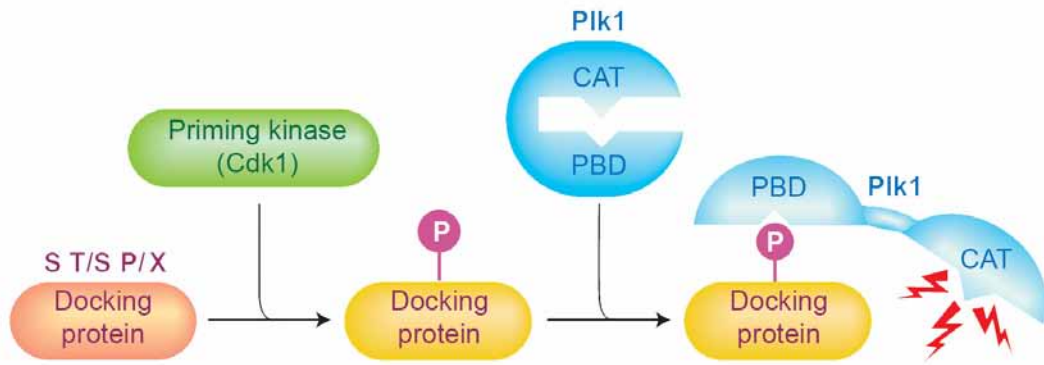


Figure 1.4: Model of Plk1 targeting and activation (Sillje and Nigg, 2003). Plk1 is auto-inhibited by its PBD until it binds to a docking site that has been phosphorylated by a priming kinase. The kinase can then phosphorylate either the docking protein itself or other proteins in the vicinity.

anaphase transition is of high importance for this work. The polo-like kinase family has been identified through a mutation in the *Drosophila* polo gene (Llamazares et al., 1991; Sunkel and Glover, 1988). The mammalian genome contains four genes coding for Plks of which the Plk1 protein is the best characterized.

Plk1 has an amino-terminal kinase domain and two carboxy-terminal sequence motifs called polo-boxes which together form the so called polo-box-domain (PBD). This domain was found to be a phospho-peptide-binding domain that preferentially targets the kinase to proteins that contain PBD “docking sites” (see **Figure 1.4**). By peptide library screening the consensus sequence for optimal binding was determined to be S/T-Sp/Tp-P (where Sp/Tp stands for phosphorylated serine or threonine residues) (Elia et al., 2003a). The phosphorylation at the docking site is carried out by a so called priming kinase. The sequence of the optimal docking site suggests that mainly proline directed kinases like Cdk1 act as priming kinases for Plk1. There are, however, studies that show that other kinases including Plk1 itself can create docking sites (Neef et al., 2003; Rauh et al., 2005). Furthermore, binding of Plk1 to its docking site is thought to activate the kinase by releasing the kinase domain from auto-inhibitory binding to the PBD (Jang et al., 2002).

1.2.2 Ubiquitylation and proteolysis in cell cycle control

Ubiquitin is a small protein that has been named after its ubiquitous presence in virtually all eukaryotic cells (Hershko and Ciechanover, 1998; Hershko et al., 2000). It serves as a modifier that is attached post-translationally to lysine residues of other proteins via an iso-peptide bond. Ubiquitin can form chains if internal lysine residues in ubiquitin itself are used for isopeptide bond formation with further ubiquitin molecules. These multi-

ubiquitin chains serve as signals to recruit the modified proteins to the 26 S proteasome, a high molecular weight protease complex that hydrolyses its substrates into small peptides.

Ubiquitin chain formation on the target is brought about by a cascade of enzymes that sequentially act to transfer ubiquitin to the target protein (see **Figure 1.5**). Free ubiquitin is first covalently attached to an “ubiquitin-activating enzyme” E1 via a thioester bond in an ATP-dependent manner. It is then transferred to an “ubiquitin-conjugating enzyme” E2. The E2-bound ubiquitin is then attached to a lysine residue in the target protein with the help of an “ubiquitin-ligase” E3 which is thought to position the E2-ubiquitin and the substrate for efficient ubiquitin transfer.

E3 enzymes are heavily regulated throughout the cell cycle in terms of their activity and/or substrate specificity. In cell cycle control two types of E3 ligases, have well established functions. They both belong to the family of cullin-based ubiquitin ligases, because they contain subunits that share homology with a group of proteins called the cullins. These E3 enzymes are the anaphase-promoting complex/cyclosome (APC/C) and the Skp1-Cullin-F-box (SCF) complexes (Peters, 1998; Vodermaier, 2004).

The APC/C is a multi-protein complex consisting of at least twelve subunits. It associates with one of its two activators, Cdc20 (also called xFzy in *Xenopus*) or Cdh1 (also called xFzr in *Xenopus*), to direct ubiquitylation of cell cycle-regulatory proteins. There is evidence that the activators as well as core APC/C subunits are involved in substrate specificity and recruitment (Burton et al., 2005; Carroll et al., 2005; Kraft et al., 2005; Passmore et al., 2003). APC/C substrates usually contain sequence-motifs which are essential signals for recognition by APC/C. The first signal to be identified was pictorially labelled “destruction”-box (d-box) (Glotzer et al., 1991), however more signals have been and keep on being identified. In M-phase APC/C^{Cdc20} targets cyclin B and an inhibitor of sister-chromatid separation called securin for degradation (Zou et al., 1999). This leads to

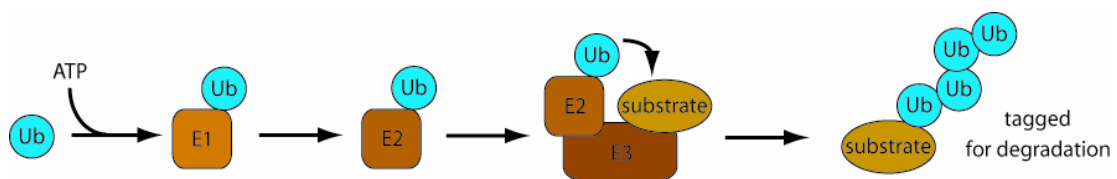


Figure 1.5: Simplified scheme of the biochemistry of ubiquitin modification of proteins. Posttranslational modification of protein with a ubiquitin chain takes place through a cascade of enzymes generally called E1 to E3. The ubiquitin chain serves as a signal for degradation of the substrate proteins by the 26 S proteasome, a cellular protease complex.

decreased Cdk1 activity and loss of centromeric cohesion on sister-chromatids, respectively. These two events are important for the ordered transition from metaphase to anaphase.

SCF complexes are composed of an elongated cullin-backbone with the Skp1 protein bound to one side and the small Rbx1 subunit on the other (Zheng et al., 2002). Skp1 is associated with one of many so-called F-box proteins via its own F-box. The variable F-box protein associated with Skp1 is thought to act as the substrate specificity factor for the complex. Binding of the respective substrate to the F-box protein requires phosphorylation on sequence motifs, that are thus called phospho-degrons (Ang and Harper, 2005). The archaetypal example for such a process is the yeast Cdk-inhibitor Sic1p (Feldman et al., 1997). One of the best characterized examples in vertebrate systems is the SCF complex containing the F-box protein β -transducin repeat containing protein (β -TRCP). The paradigms of SCF ^{β -TRCP} function are its involvement in the degradation of the NF- κ B inhibitor I κ B α and the wingless-signalling pathway component β -catenin (Winston et al., 1999; Yaron et al., 1998). However, a number of other proteins some of which are critical cell cycle regulators have been identified as targets of this E3-ligase (Ang and Harper, 2005).

1.3 Vertebrate oocyte biology

Metazoans produce two different kinds of gametes – sperm and oocyte. The motile sperm carries the paternal genetic information, whereas the oocyte contains the maternal complement. The oocyte is a specialized, often rather large cell, that accumulates lots of biological molecules (RNA, proteins) that are sufficient to support the early embryo's metabolism and development through a series of rapid cell divisions after fertilization in the absence of extra nutrition (Gilbert, 1997). Oocytes of the African clawed frog *Xenopus laevis*, which served as a model system for this work, are in fact over 1 mm in diameter.

1.3.1 Oocyte maturation

Oocytes arise from precursor stem cells in the oogonia of the respective species through an asymmetric meiotic division that produces one functional oocyte and, depending on whether the first polar body divides again or not, two or three small cells termed “polar bodies”. Throughout oogenesis, vertebrate oocytes are arrested in meiotic prophase and terminate their growth-phase as so called “immature oocytes” (Gilbert, 1997) (see **Figure**

1.6 top). In the frog stimulation of these oocytes with progesterone secreted from the surrounding follicle cells induces resumption of meiosis, a process that is synonymously called oocyte maturation (Ferrell, 1999; Kishimoto, 2003). Reentry into the meiotic cell cycle from prophase I arrest is triggered essentially by activation of a mitogen-activated protein kinase (MAPK) pathway with the germ-cell specific MAPK-kinase-kinase (MAPKKK) c-Mos at its top. This leads to activation of the Cdk1/cyclin B complex that drives progression through meiosis (see also 1.2.1).

1.3.2 CSF-arrest

Oocyte maturation proceeds until a second cell cycle arrest in metaphase of meiosis II. In this state the eggs are laid and await fertilization. Originating from a seminal publication by Masui and Markert in 1971 this arrest of the now “mature” oocyte is called cytostatic factor (CSF) arrest (Masui and Markert, 1971; Tunquist and Maller, 2003). In this work cytoplasmic injection experiments with frog eggs led to the identification of two major biochemical activities that control cell cycle progression of the oocyte (see *Figure*

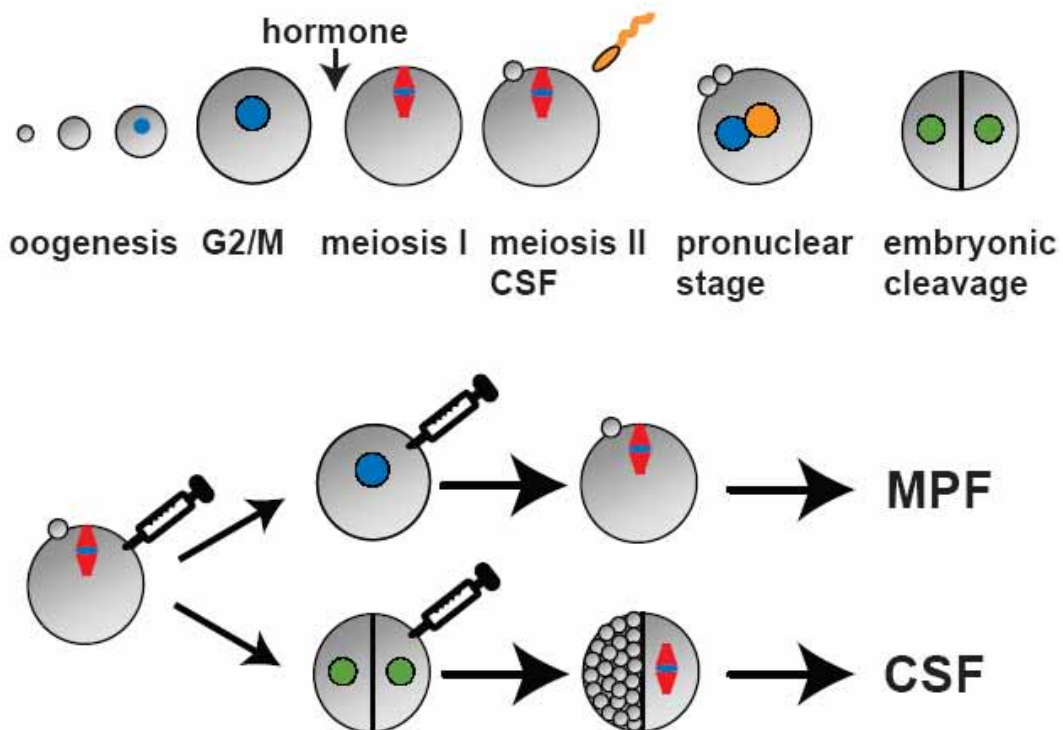


Figure 1.6: Overview of oocyte biology and the identification of MPF and CSF (Schmidt et al., 2006). After oogenesis oocytes are arrested at the G2/M transition (prophase I). In frogs progesterone triggers meiosis resumption. Release from CSF arrest by fertilization leads to the onset of embryonic development (top). Cytoplasmic injection experiments with frog eggs have led to the identification of MPF and CSF (bottom).

1.6 bottom).

When small amounts of cytoplasm from a mature oocyte were transferred into an immature oocyte, this cell entered meiosis and progressed to metaphase of meiosis II in the absence of progesterone stimulation. This led Masui and Markert to propose that the cytoplasm of mature oocytes contains an activity they termed maturation-promoting factor (MPF). Purified MPF was subsequently found to contain Cdk1, the vertebrate homolog of the fission yeast *cdc2* protein (Gautier et al., 1988). It became clear that MPF equals the evolutionary conserved complex of Cdk1/cyclin B that drives M-phase progression in all eukaryotic cells (Nurse, 2002). In a second set of experiments cytoplasm of mature oocytes was transferred into one cell of a mitotically dividing two-cell embryo. Following injection, the injected cell stopped dividing with the spindle apparatus in a metaphase configuration similar to the metaphase of meiosis II arrest. It was concluded that the mature cytoplasm also contains an activity able to arrest cells in metaphase. This activity was called CSF (see above).

In summary the mature oocyte is arrested by an activity called CSF in metaphase of meiosis II with high MPF activity awaiting fertilization. The biological function of this second arrest is thought to be the prevention of development in the absence of fertilization, which is called parthenogenesis (see below).

1.3.3 Fertilization

The fusion of male and female gametes resulting in the subsequent mixing of paternal and maternal genetic material is called fertilization. The fusion event triggers signalling pathways, which release the oocyte from CSF arrest and allow for progression beyond metaphase II, a process that is also called egg activation (Jones, 2005). Egg activation synchronizes the cell cycle phases of sperm and egg. The egg can complete meiosis II and after the female pronucleus has formed it can fuse with the male pronucleus to yield the diploid zygote. The principal event that allows cell cycle progression is the release of calcium from intracellular stores. Calcium release happens in a wave-like fashion that can last from roughly ten minutes in frog eggs to several hours in mammalian eggs (Halet et al., 2003). It has been found that activation of Calmodulin-dependent kinase II (CaMKII) is sufficient to trigger CSF release in *Xenopus* eggs (Lorca et al., 1993), but the relevant targets of this kinase have remained obscure.

1.4 The molecular basis of CSF arrest

As opposed to MPF which has been purified in the late 1980s, CSF has ever since it was proposed resisted detailed biochemical characterization. CSF has in fact never been assumed to be a single protein or protein complex. However, the experiments conducted on CSF together with its properties as an inhibitor of metaphase to anaphase transition have predicted criteria that molecules involved in CSF activity would have to fulfil. These molecules would have to a) accumulate and/or become active during oocyte maturation, b) be active at metaphase of meiosis II c) be inactivated upon fertilization/activation of the egg. These criteria have led the field to test candidate proteins potentially involved in CSF arrest.

1.4.1 The classical CSF pathways

The first candidate protein implicated in CSF activity in *Xenopus* eggs was the cellular counterpart of the oncogenic protein kinase *mos* (Sagata et al., 1989). *Mos* is a MAPKKK that is uniquely induced upon resumption of meiosis after prophase I arrest (see also 1.3.1). It was shown that *mos* is present in unfertilized (CSF arrested) eggs, but not in fertilized ones. Injection of *mos* RNA into two-cell embryos resulted in cleavage arrest of the injected half dependent on the amount of RNA injected, whereas cytosol from CSF arrested eggs depleted of *mos* protein lost its CSF activity (Sagata et al., 1989).

Subsequently, it was found that *mos* is upstream of a cascade of kinases that successively activate each other to bring about CSF arrest. It was known that phosphorylation of MAPKK by *mos* can activate this kinase *in vitro* (Posada et al., 1993). Consistently, Haccard *et al.* found that a constitutively active MAPKK (also called MEK) that activates MAPK can induce metaphase arrest in cleaving embryos in the absence of *mos*, indicating that it has CSF activity (Haccard et al., 1993). This led to the idea that *mos* induced CSF arrest works through the MEK/MAPK module. MAPK in turn was shown to be able to activate proteins of the ribosomal S6 kinase (p90^{RSK}) family in different contexts (Frodin and Gammeltoft, 1999). The p90^{RSK} kinase is active in unfertilized eggs and therefore a good candidate of yet another downstream factor in *mos*-induced CSF arrest. Two independent studies showed that p90^{RSK} is required for the establishment of a metaphase arrest in *Xenopus* cycling extracts and that injection of constitutively active kinase into cleaving embryos causes metaphase arrest (Bhatt and Ferrell, 1999; Gross et al., 1999). However, p90^{RSK} was found to be dispensable once CSF arrest is established as

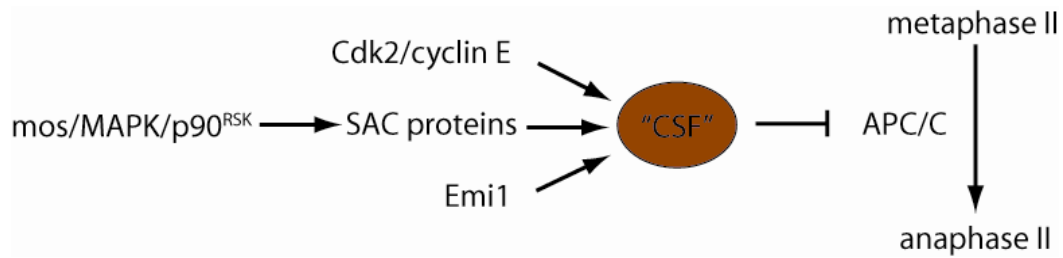


Figure 1.7: Pathways shown to be involved in CSF-mediated APC/C inhibition in meiosis II. At the moment, the picture that emerges from the literature is that CSF activity is the sum of distinct, parallel pathways that all inhibit cyclin degradation (and therefore MPF inactivation) by the APC/C. For some of these pathways/proteins the molecular mode of action has begun to be explored (SAC proteins, Emi1), whereas for Cdk2 a mechanistic explanation has not emerged, yet. Notably, mutual dependencies of these seemingly parallel pathways have not been extensively tested.

immuno-depletion of the protein from CSF arrested egg extracts does not lead to CSF release (Bhatt and Ferrell, 1999).

At about the time *mos* was found to mediate CSF activity through the MAPK-pathway an independent activity required for CSF arrest was found. Using anti-sense oligo-nucleotides Gabrielli *et al.* found that Cdk2 is required for CSF arrest in frog eggs (Gabrielli *et al.*, 1993). Also, gain-of-function experiments with a Cdk2 that cannot be inactivated showed that a metaphase arrest can be induced in *Xenopus* cycling extracts (Tunquist *et al.*, 2002). These results were, however, challenged by a study showing that injection of oocytes with the Cdk2 inhibitor p21^{Cip} does not perturb metaphase arrest in oocytes (Furuno *et al.*, 1997).

1.4.2 CSF arrest and the APC/C

Most of the early work on CSF described in section 1.4.1 has been conducted before the discovery of the APC/C (King *et al.*, 1995; Sudakin *et al.*, 1995) or even before ubiquitin/proteasome-dependent proteolysis of cell cycle regulators was well established (Glotzer *et al.*, 1991) (see also 1.2.2). CSF arrest is a state, where transition from metaphase to anaphase is blocked as exemplified by high MPF (Cdk1/Cyclin B) activity and stable cohesion between sister-chromatids. Thus, the arrest is ultimately due to inactivity of the APC/C and CSF release is triggered by APC/C activation (see **Figure 1.7**). The APC/C activator Cdc20 was found to be absolutely required for APC/C activation at the metaphase II to anaphase II transition and therefore for CSF release (Lorca *et al.*, 1998). *Xenopus* eggs have been assumed to express only Cdc20, which is also called xFzy in the frog. This has only very recently been questioned (Papin *et al.*, 2004). Here, expression of the *Xenopus* Cdh1 homolog in oocytes was reported, but its functions might

be independent of APC/C-dependent proteolysis. Other meiosis specific APC/C activators, like the yeast Ama1 protein or the Drosophila cortex protein (Chu et al., 2001; Cooper et al., 2000), have not been found in *Xenopus*, yet. Thus, currently CSF release can be considered to be solely due to APC/C^{Cdc20} activation.

1.4.3 Spindle-assembly checkpoint proteins as CSF components downstream of the MAPK-pathway

In mitotic cells the spindle-assembly checkpoint (SAC) is a pathway that prevents anaphase onset as long as not all chromosomes are attached to the mitotic spindle in a bipolar fashion and aligned at the metaphase plate (Musacchio and Hardwick, 2002; Yu, 2002). It does so by creating a diffusible signal that prevents APC/C activation. Although the exact nature of this signal is still being explored, the components of this highly conserved pathway are well known. They have been identified in genetic screens undertaken in yeast as members of the budding uninhibited by benzimidazole (Bub) and mitotic arrest deficient (Mad) families (Hoyt et al., 1991; Li and Murray, 1991).

The *Xenopus* homolog of protein kinase Bub1 was shown to be a substrate p90^{RSK} in oocytes. Phosphorylation of Bub1 by p90^{RSK} was able to activate its kinase activity *in vitro* suggesting that Bub1 could act as a mediator of p90^{RSK} activities in oocytes (Schwab et al., 2001) (see **Figure 1.7**). Depletion of Bub1 from *Xenopus* cycling extracts prevents the establishment of a mos-dependent metaphase arrest indicating that it is downstream of the mos/MAPK/p90^{RSK} pathway of CSF arrest (Tunquist et al., 2002).

Similarly, two other components of SAC have been shown to be involved in CSF mediated APC/C inhibition. The Mad1 and Mad2 proteins are thought to be at the very bottom of the SAC pathway. Mad1 is thought to act as a template for Mad2 converting it into a conformation competent to inhibit APC/C^{Cdc20} directly by sequestration of Cdc20 (Musacchio and Hardwick, 2002). Injection of Mad1 or Mad2 protein causes metaphase arrest in blastomeres of dividing *Xenopus* embryos (Tunquist et al., 2003). Conversely, depletion of both Mad1 and Mad2 prevents the establishment of a mos-dependent metaphase arrest in *Xenopus* cycling extracts. Surprisingly however, only the depletion of Mad1 and not Mad2 from CSF extracts leads to calcium-independent CSF release indicating that only Mad1 is required for CSF maintenance. This implies that the generation of the signal that brings about APC/C inhibition in the case of CSF arrest must mechanistically differ from that of the SAC.

In summary, the work presented in section 1.4.1 and in this section suggests that CSF arrest may use the MAPK-kinase pathway to specifically modify the behaviour of the SAC resulting in stable APC/C inhibition. In addition, Cdk2 activity might be required independently to establish this arrest (for an overview see **Figure 1.7**).

1.4.4 The APC/C inhibitor Emi1 as a putative CSF component

Recently, the F-box protein early mitotic inhibitor 1 (Emi1) has been proposed as yet another independent element contributing to CSF arrest (Reimann et al., 2001a; Reimann and Jackson, 2002). The protein has been shown to be able to directly inhibit APC/C ubiquitylation activity *in vitro*. This inhibition was found to be mediated by an inhibitory interaction with the APC/C activator Cdc20, but by a mechanism different from the SAC protein Mad2 (Reimann et al., 2001b). Overexpression of Emi1 in CSF extracts prevents calcium-induced CSF release, whereas depletion of the protein from CSF arrested egg extract was reported to induce calcium-independent CSF release indicating that Emi1 is required to maintain CSF arrest. Interestingly, the ability of Emi1 to block CSF release was found to be independent of MAPK activity suggesting that Emi1 might act in parallel to this “classical” pathway involved in CSF activity (see **Figure 1.7**).

In addition to its role as a CSF component other functions for Emi1 have been proposed. In fact, initially the protein was characterized mainly as a regulator of the mitotic, early embryonic cell cycles in *Xenopus* (Reimann et al., 2001a). Here, Emi1-mediated APC/C^{Cdc20}-inhibition was thought to allow re-accumulation of mitotic cyclins to facilitate entry into M-phase. The human Emi1 homolog was shown to have a function promoting S-phase in tissue culture cells. In this case, inhibition of APC/C^{Cdh1} by Emi1 that is expressed in an E2F-dependent manner allows accumulation of cyclin A (Hsu et al., 2002). This study also reported that Emi1 is degraded in prophase, a process that was subsequently shown to require Plk1 and the SCF^{β-TRCP}-complex (Hansen et al., 2004; Margottin-Goguet et al., 2003; Moshe et al., 2004).

Doubts about a role for Emi1 as a true CSF component arose not only because the timing of Emi1 destruction in mitosis seemed to be incompatible with a function in metaphase (there was no obvious explanation how Emi1 could escape premature degradation in meiosis). Also, it was reported that Emi1 protein is undetectable in CSF arrested *Xenopus* eggs and that its mis-expression interferes with oocyte maturation altogether (Ohsumi et al., 2004). These results conflict not only with Emi1’s function as a

mediator of CSF activity, but also with reports describing a role for Emi1 in oocyte maturation (Tung and Jackson, 2005). Thus, a possible role of Emi1 in CSF arrest is currently heavily debated, also because of the work presented here (Zachariae, 2005).

1.4.5 Activation of APC/C activity by kinases

It has been realized quite early after its identification that tight regulation of the APC/C's activity could be ensured by phosphorylation of APC/C subunits (Lahav-Baratz et al., 1995). In fact, a significant fraction of the vertebrate APC/C subunits, including the activators, are found phosphorylated specifically in M-phase (Kraft et al., 2003). The kinases that seem to create these sites are Cdk1/CyclinB and to a lesser extent Plk1. The roles for Cdk1/CyclinB in APC/C activation are well established, whereas the situation for Plk1 is less clear. It has been variously shown that Cdk1 phosphorylation of the APC/C is required for its activation by Cdc20 *in vitro* and *in vivo* (Kraft et al., 2003; Kramer et al., 1998; Kramer et al., 2000; Rudner et al., 2000).

For Plk1 conflicting results exist on whether phosphorylation of APC/C subunits is sufficient, required or even dispensable for APC/C activity. It has been shown for purified mammalian APC/C that Plk1, pre-phosphorylated by Cdk1, can activate its ubiquitin-ligase activity *in vitro* (Kotani et al., 1998). Golan *et al.* showed that both Cdk1 and Plk1 can contribute to APC/C activation *in vitro* by phosphorylating its subunits and that their effects are additive (Golan et al., 2002). However, reinvestigation of the relative contribution of Plk1 and Cdk1 to *in vitro* APC/C activation could only confirm a role for Cdk1 phosphorylation (Kraft et al., 2003).

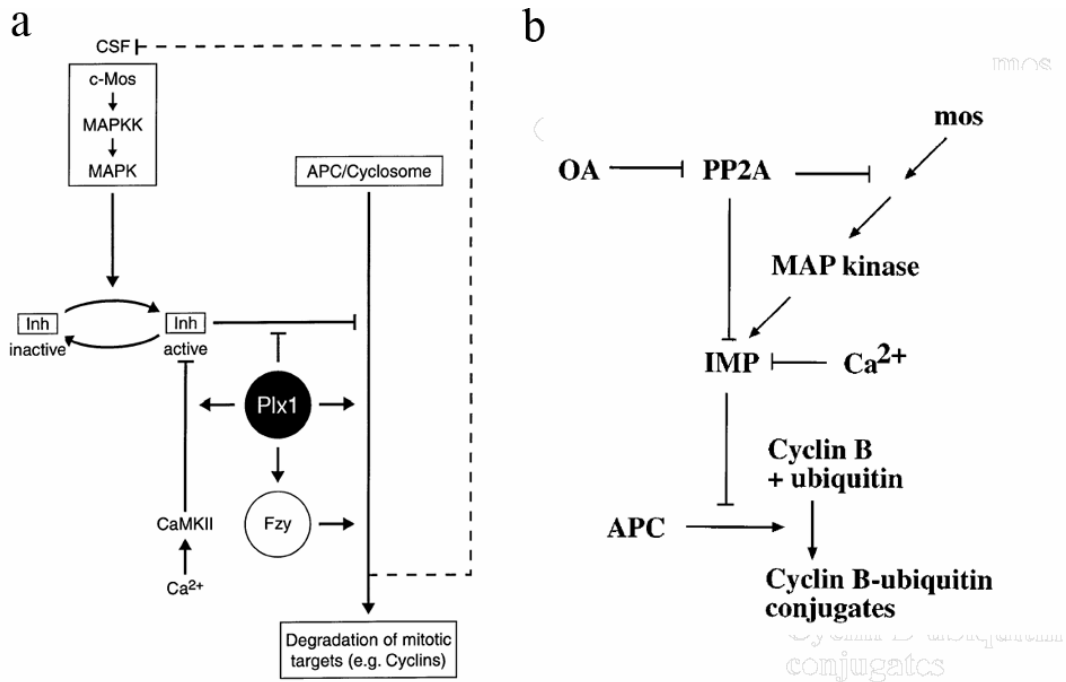


Figure 1.8: Models of APC/C regulation upon calcium-induced CSF release (Descombes and Nigg, 1998; Vorlaufer and Peters, 1998). a) Plx1 could be required for APC/C activation through many alternative pathways, one of them being the inactivation of a putative APC/C inhibitor. b) An inhibitor of cyclin-proteolysis has also been proposed by Vorlaufer and Peters based on its phosphatase-sensitivity.

Despite the uncertainties about direct APC/C activation by Plk1 phosphorylation *in vitro* a role for Plk1 in promoting anaphase onset and APC/C activity is clearly established. Particularly, in *Xenopus* it was clearly shown that interfering with the activity of Plx1 (the *Xenopus* homolog of Plk1) prevents APC/C activation and consequently destruction of APC/C substrates (Descombes and Nigg, 1998). Therefore, Plx1 activity is essential for CSF release in CSF-arrested *Xenopus* egg extracts. Also, Plx1 was found to be required to maintain APC/C activity once it has been turned on by counteracting an unknown phosphatase (Brassac et al., 2000).

These studies did not address the exact molecular mechanism by which Plx1 activates the APC/C, but Descombes and Nigg have proposed a model including all likely possibilities of how Plx1 could be involved in APC/C activation (Descombes and Nigg, 1998) (see **Figure 1.8a**). The model included the classic interpretations of Plk action on the APC/C, namely direct phosphorylation of the APC/C or its activator Cdc20/Fizzy. Furthermore, involvement of Plx1 in transduction of the calcium signal that leads to CSF release was envisioned. As a third alternative Plx1 was proposed to negatively regulate a putative APC/C inhibitor. A similar inhibitor has been proposed by Vorlaufer and Peters

independently of Plx1 function based on their studies of putative APC/C regulatory phosphatases (Vorlaufer and Peters, 1998) (see *Figure 1.8b*).

1.5 Objective of the project and experimental approach

The general aim of this work was to perform a basic functional characterization of *Xenopus* Emi-related protein 1 (XErp1, also called Emi2 and formerly designated Pxp17). The *Xenopus* egg extract system proved to be extremely useful as a tool to study XErp1 function and also part of its regulation. Both aspects of the project, the objective as well as experimental approach, will be explained in the following sections.

1.5.1 Objective of the project

The F-box protein XErp1 has been identified in a yeast two-hybrid screen of a *Xenopus* oocyte library with kinase-dead Plx1 as a bait aimed at identifying effectors of Plx1 function (Duncan and Nigg, see (Schmidt et al., 2005)). The protein is evolutionarily conserved in vertebrates and its carboxy-terminal domain containing the F-box and a zinc-binding region (ZBR) is very similar to Emi1 (see *Figure 1.9*). As a Plx1-interacting protein it is a potential effector and substrate of Plx1. Thus, in addition to a basic characterization of the protein, exploring the functional significance of its interaction with Plx1, particularly in the context of CSF regulation, was a major goal of the project. The well established function of Plx1 in activation of the APC/C in *Xenopus* egg extract guided

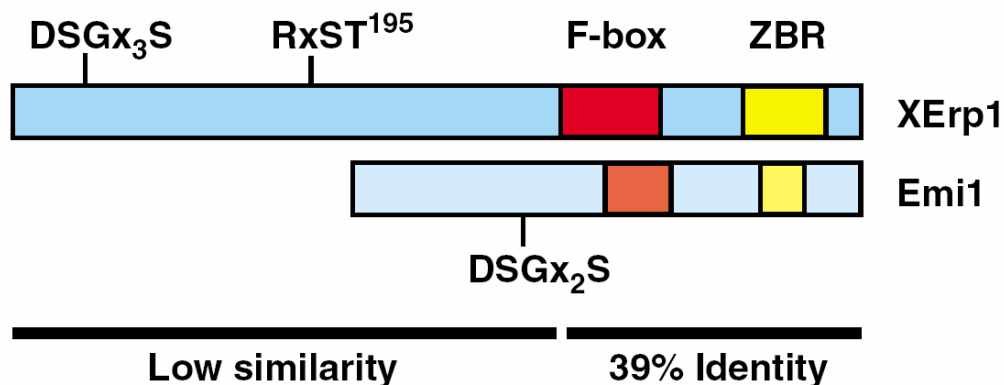


Figure 1.9: Schematic representation of XErp1 and Xenopus Emi1 (Schmidt et al., 2006). XErp1 is an F-box protein harbouring a carboxy-terminal zinc-binding region (ZBR). The amino-terminus contains two important sequence motifs that are involved in regulating the stability of XErp1 (partly described in this work). The carboxy-terminus shows significant sequence identity to the already described Emi1 protein.

the experiments as well as the choice of model system.

1.5.2 The *Xenopus* egg extract system

Cytoplasmic extracts from *Xenopus laevis* eggs are a widely accepted *in vitro* system to study cell cycle-related processes (for an overview see **Figure 1.10**). The system has been developed and has been successful because it uniquely combines ease of manipulation with a high degree of preservation of *in vivo* processes. This work uses cytoplasmic extracts from CSF-arrested *Xenopus* eggs (or short CSF extracts). These

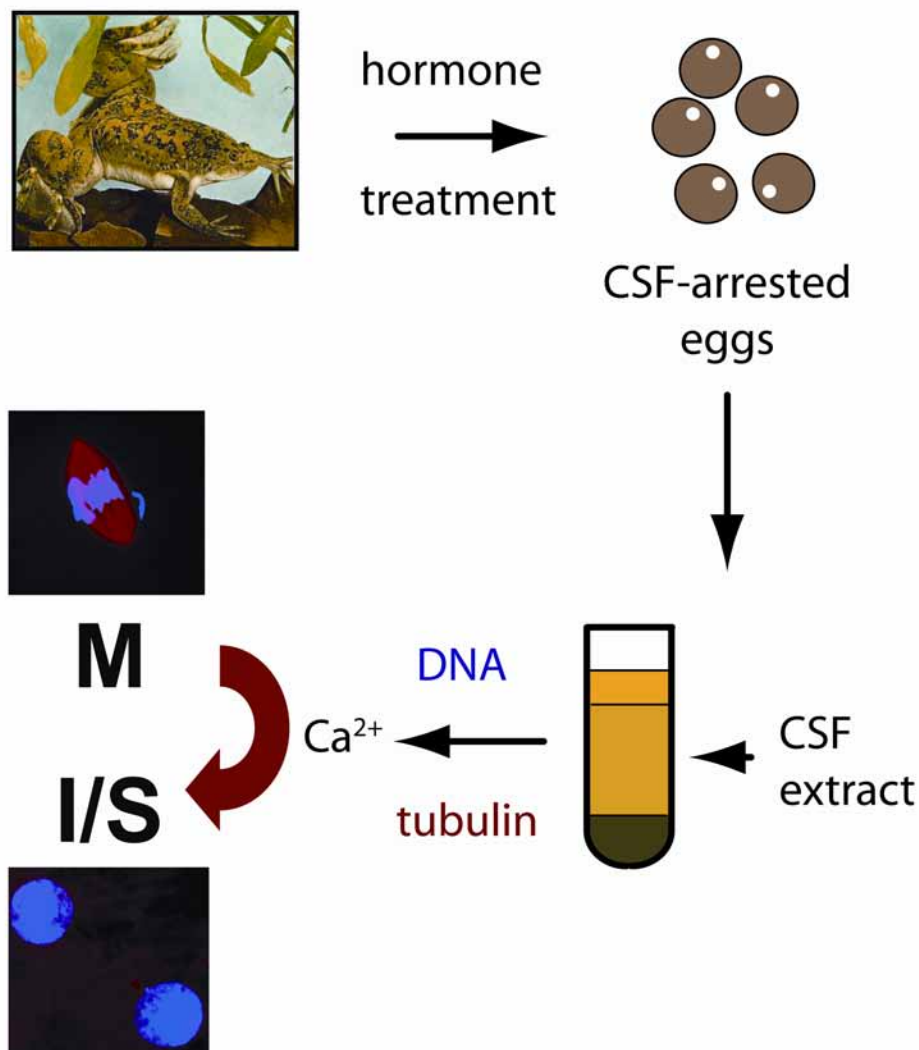


Figure 1.10: Schematic of the *Xenopus* egg extract system. *Xenopus laevis* females are injected with gonadotropin to induce ovulation. The laid eggs, which are CSF arrested, are collected and lysed by centrifugation to yield the cytoplasmic extract. After addition of sperm nuclei (DNA) and fluorescently labelled tubulin the configuration of the chromatin and the microtubules are easily observable and serve as indicators of the cell cycle phase of the extract. CSF release into interphase/S-phase is triggered by the addition of calcium ions.

extracts, as opposed to extracts from activated eggs which are called cycling extracts, remain CSF arrested after preparation, but can be induced to undergo CSF release by the addition of CaCl_2 (see Materials and Methods for a detailed description).

The extracts faithfully reproduce many complex biochemical processes *in vitro* like chromosome condensation of exogenously added sperm nuclei, assembly of bipolar spindle structures and reformation of fully functional nuclei and DNA synthesis upon release from CSF arrest. Importantly, at the biochemical level events like Cdc20-dependent APC/C activation and consequently timely degradation of cell cycle regulatory proteins are observed in this system. In addition, a very valuable feature particularly for cell cycle studies is the fact that the extract can be assumed to behave fully synchronous. The processes mentioned above are easily observed by sampling the extract either for microscopic inspection or biochemical analysis.

2 Results

Following a basic characterization of XErp1 expression and behaviour in oocytes and their extracts, its function and regulation have been examined. Also, initial steps have been taken to elucidate the functional relationship to its interaction partner Plx1 and its functional significance *in vivo*. The results of these experiments will be presented in the following sections.

2.1 Characterization of an anti-XErp1 antibody

In order to analyze XErp1 function and regulation, a polyclonal antibody (XErp1-NT) was affinity-purified by Thomas Mayer from serum raised by Peter Duncan against an amino-terminal fragment of XErp1. To examine its specificity, this antibody was characterized by Western blotting. The antibody recognizes a prominent band running at

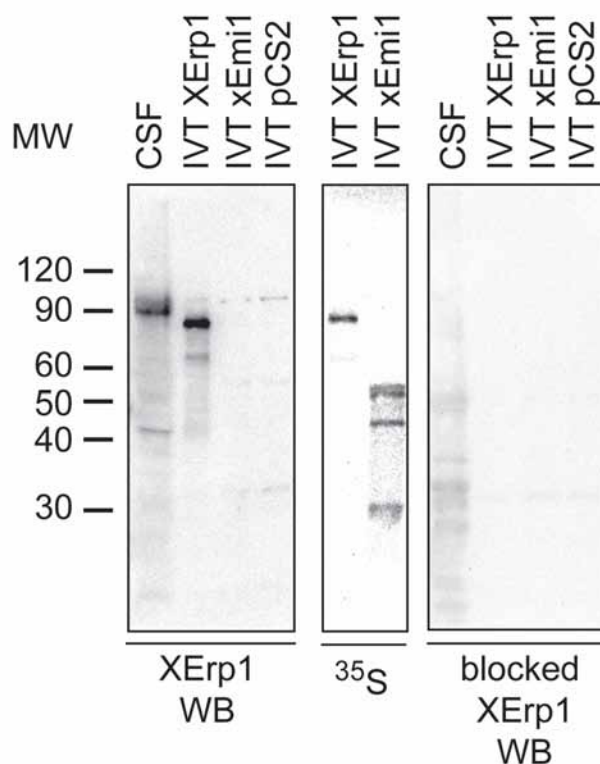


Figure 2.1: Anti-XErp1 antibody characterization. CSF extract (corresponding to 50 μ g protein/lane), IVTted XErp1, Emi1 and unprogrammed wheat germ extract were processed for Western blotting with 2 μ g of affinity-purified anti-XErp1 antibody (left panel). Expression levels of the 35 S-Met-labelled IVT products were examined by autoradiography (middle panel). For specificity analysis the antibody was blocked with a tenfold molar excess of the XErp1 fragment the antibody was made against (right panel).

an apparent molecular weight of about 90 kDa in extract from CSF-arrested *Xenopus* eggs (see **Figure 2.1** left panel). The calculated molecular weight of XErp1 is 72 kDa, which is significantly less than what is observed for the protein recognized in CSF extract. This difference is possibly due to the physico-chemical properties of the protein or post-translational modifications or a combination of both (see following sections).

The antibody also recognizes *in vitro* translated XErp1 which has a slightly higher electrophoretic mobility (for an explanation see sections below). Importantly, the antibody does not recognize *in vitro* translated *Xenopus* Emi1 or other proteins in the wheat germ extract used for translation. To test whether the observed signals derive from antibodies that bind XErp1 protein, the antibody was pre-incubated with a tenfold molar excess of the XErp1 fragment the antibody was raised against prior to Western blotting. After this treatment the signals for the prominent band in CSF extract as well as for IVTed XErp1 vanished (see **Figure 2.1** right panel). Together these data indicate that this antibody specifically recognizes XErp1 and is a useful tool to study its function and regulation.

2.2 Expression and behaviour of XErp1 protein

The *Xenopus* polo-like kinase Plx1 has been shown to be required for activation of the APC/C and destruction of its targets upon CSF release in *Xenopus* egg extract (Descombes and Nigg, 1998). The mechanism of Plx1-mediated APC/C activation was not addressed in this study. However, a yeast two-hybrid screen conducted to identify possible regulators or mediators of Plx1 action on the APC/C has identified XErp1 (Duncan and Nigg, see introduction). The identification of XErp1 as an interacting protein of a cell cycle regulatory kinase implicated in APC/C regulation upon CSF release suggested that XErp1 might be a cell cycle regulatory protein implicated in this process. Cell cycle regulatory proteins often undergo cell cycle phase-dependent post-translational modifications and changes in protein level to regulate their activity. To investigate the fate of XErp1 during oocyte maturation up until CSF arrest, oocyte lysates have been analysed for XErp1 abundance and modifications.

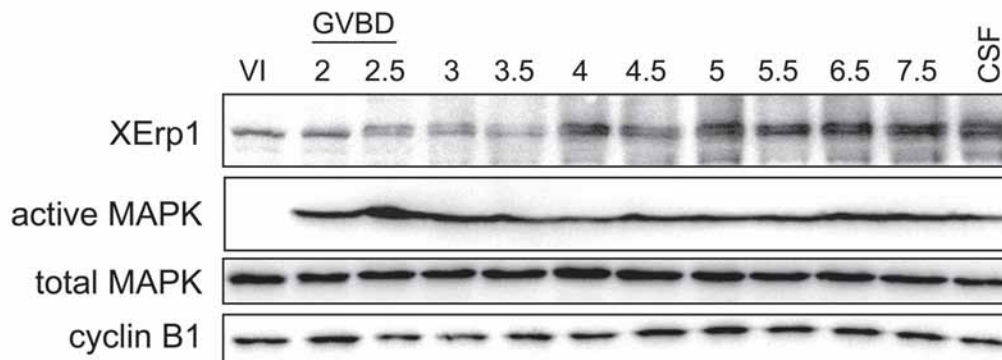


Figure 2.2: Analysis of XErp1 levels during oocyte maturation. Lysates from *Xenopus* oocytes before (VI) and after progesterone treatment (time is given in hours after treatment) were processed for Western Blot analysis with antibodies against XErp1, phosphorylated Erk1/2 (active MAPK), Erk1/2 (MAPK) and cyclin B1. The bar “GVBD” indicates the approximate time at which the GVBD occurred in most oocytes.

2.2.1 XErp1 protein during oocyte maturation

For analysis of XErp1 protein levels during oocyte maturation, immature (stage VI) oocytes were isolated from female *Xenopus laevis* frogs (with the kind help of Ingmar Schön). The oocytes were treated with progesterone to induce oocyte maturation. In order to synchronize the oocytes, they were grouped according to the time they underwent germinal vesicle breakdown (GVBD) as a sign of meiotic entry. This process is analogous to nuclear envelope breakdown in mitotic prophase and is clearly visible by the appearance of a white spot on the animal pole of the oocyte.

Samples of the synchronized oocytes were taken at different time points and processed for Western blot analysis of their lysates (see **Figure 2.2**). Initiation of oocyte maturation after progesterone treatment is apparent by the signal for active MAPK after progesterone treatment. The slight drop in cyclin B1 levels at 2.5 and 3 hours indicates the transition from meiosis I to meiosis II where about half of the cyclin is degraded (Iwabuchi et al., 2000). Western blot analysis shows that XErp1 is already present in stage VI oocytes. XErp1 protein levels or modification status do not change immediately after progesterone treatment. However, after GVBD an electrophoretic shift is visible and later on at about 4 hours an increase in protein levels up until CSF arrest. Electrophoretic shifts are often caused by post-translational modifications like phosphorylation.

2.2.2 Behaviour of XErp1 protein upon CSF release in extract

The basic expression analysis in *Xenopus* oocytes indicated that XErp1 is a cell cycle regulated protein. It is present throughout meiosis until CSF arrest and undergoes cell cycle specific changes in abundance and modification status. Therefore, *Xenopus* egg extract seemed to be a well suited system to further investigate XErp1 function and regulation. Thus, CSF extract was prepared and analysed for XErp1 protein levels before and at different times after treatment with CaCl_2 which mimics fertilization and induces release from CSF arrest (see **Figure 2.3**).

Supporting the observations made in maturing oocytes, XErp1 is present in CSF extract in a high mobility form. This electrophoretic shift has been shown to result from protein phosphorylation (N.R. Rauh, see (Schmidt et al., 2005)). Upon calcium addition, XErp1 levels drop dramatically before the protein reappears in a lower mobility form beginning from forty minutes after CSF release. The reappearance of the protein requires protein synthesis since it was not observed when the extract was treated with calcium and the protein synthesis inhibitor cycloheximide (CHX) (see **Figure 2.3** right panel). Inspection of DNA morphology of extract samples confirmed that the extract has been CSF arrested before calcium addition and released into interphase after addition of calcium (**Figure 2.3** bottom).

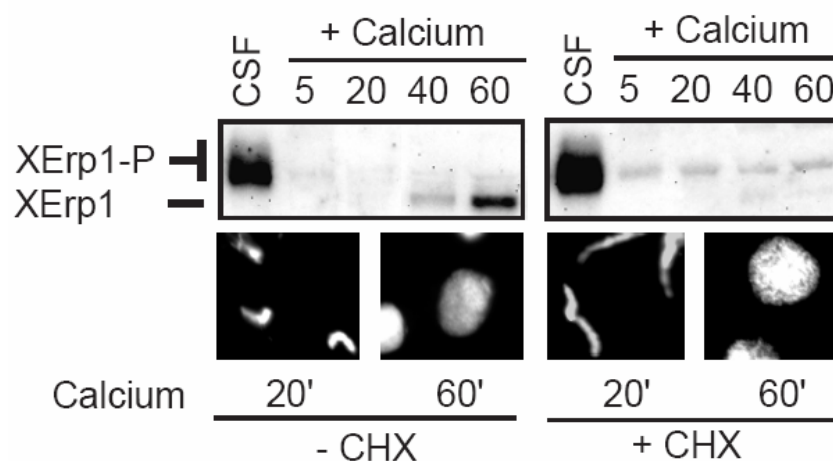


Figure 2.3: Behaviour of XErp1 protein upon calcium addition to CSF extracts. CSF extracts were released into interphase with CaCl_2 in the presence or absence of the translation inhibitor cycloheximide (CHX). Samples were taken at different times and Western blotted for XErp1 protein. Release of the extract was monitored by analysing sperm DNA morphology.

2.3 Functional analysis of XErp1

Analyses of XErp1 protein levels and modifications in whole *Xenopus* oocytes and egg extracts indicated that its abundance and phosphorylation status is very tightly coupled to CSF release, indicating a possible role for XErp1 in regulating CSF activity. To investigate whether XErp1 has a role in regulating CSF activity, XErp1 loss-of-function as well as gain-of-function studies in CSF-arrested *Xenopus* egg extracts were employed.

2.3.1 Inhibition of XErp1 function in CSF extracts

To test a putative role of XErp1 in the regulation of CSF arrest, experiments to inhibit its function in CSF arrested egg extracts were performed. To this end different antibodies were immobilized on magnetic beads and added to CSF extracts. The fate of these extracts was observed over time by microscopic inspection of added sperm DNA morphology and by measuring H1 kinase activity as an indicator of Cdk1 activity. Extracts treated with antibodies specific for XErp1 released from CSF arrest in the absence of calcium addition as shown by decondensed sperm DNA (see **Figure 2.4** left panel, 2).

Release from CSF arrest is triggered by APC/C activation resulting in cyclin B destruction and a consequent drop of Cdk1 activity. A rapid drop in histone H1 kinase activity, which corresponds to Cdk1 activity, could be observed in the extract treated with

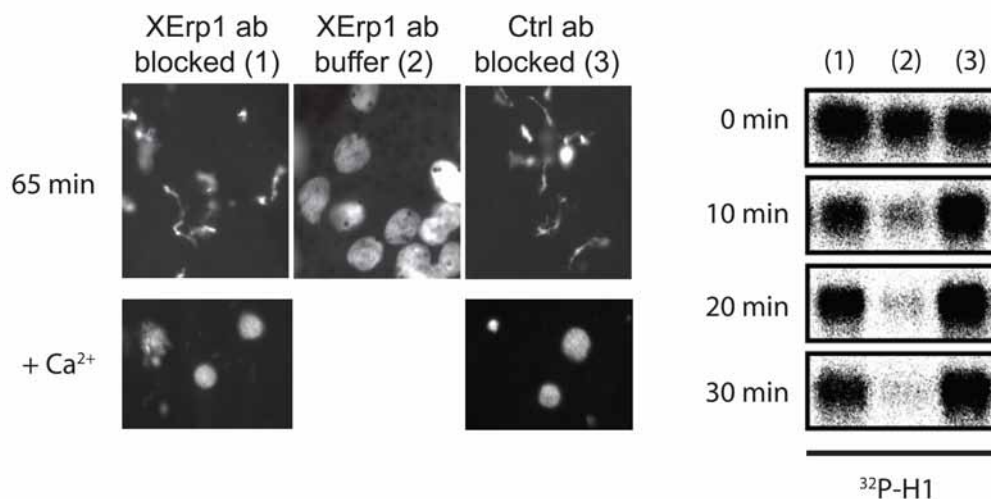


Figure 2.4: Inhibition of XErp1 function causes CSF release. CSF extracts were incubated with the indicated antibodies on ice, warmed to 20°C and the cell cycle phase of the extracts was monitored 65 minutes after warming by microscopic inspection of DNA morphology. The unreleased control extracts were treated with calcium and examined for DNA decondensation (left panel). Cdk1 activity in the extracts was measured by assaying histone H1 kinase activity after warming the extracts in the presence of blocked XErp1 antibody (1), XErp1 antibody (2) or blocked control antibody (3) (right panel).

XErp1 specific antibodies (see **Figure 2.4** right panel, 2). This did not happen in extracts treated with an anti-XErp1 antibody that had been blocked with an excess of antigen before addition or an equally treated control antibody (see **Figure 2.4**, 1 and 3). To confirm that the control extracts were capable of release from CSF arrest, they were treated with CaCl_2 after the experiment. Both extracts showed formation of nuclei after calcium treatment indicating that they were able to activate APC/C and release from CSF arrest.

To confirm these results, the experiments were repeated with different affinity-purifications from serum that has been raised against full-length XErp1 protein (#32.1 to 3). These experiments lead to the same results as described above (XErp1-NT antibody). In all cases XErp1 specific antibodies lead to calcium-independent CSF release, whereas unspecific control antibodies did not perturb CSF arrest as shown by sperm DNA

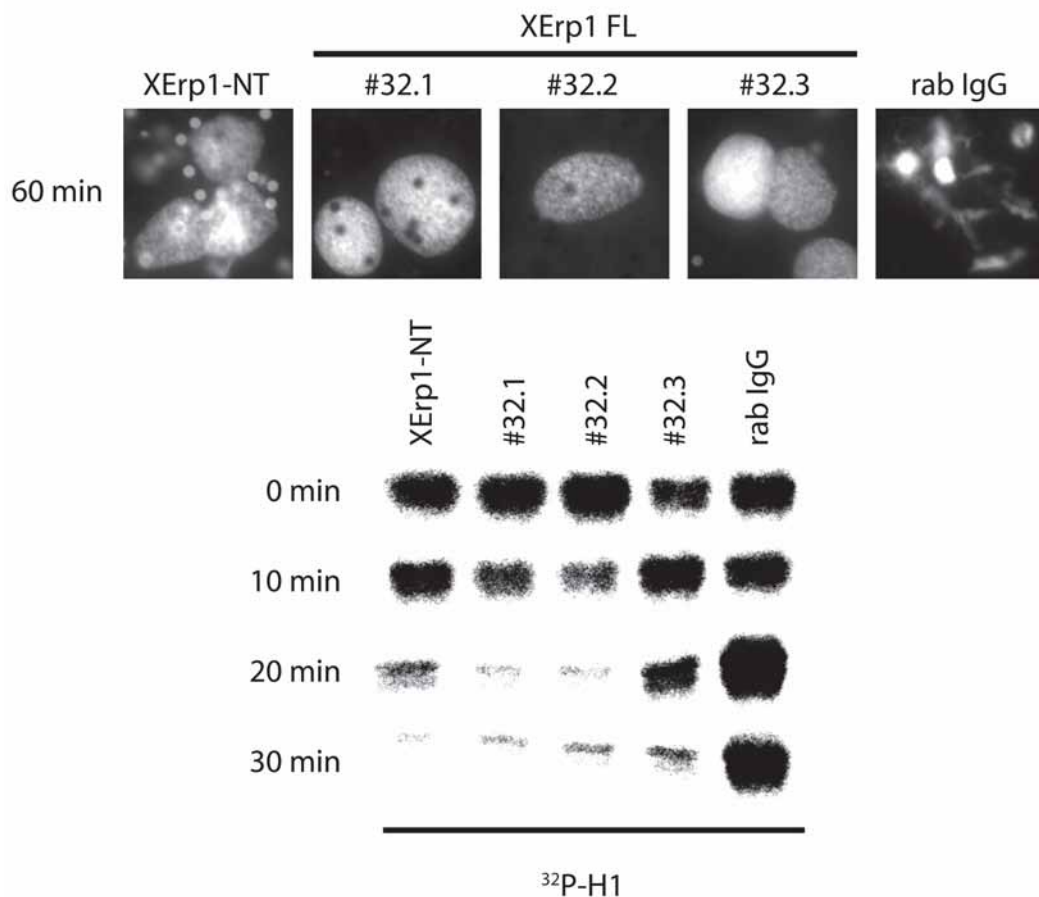


Figure 2.5: Inhibition of XErp1 function with different antibody preparations. CSF extracts were incubated with antibody used for the previous experiments (XErp1-NT) and different affinity purifications from serum raised against full-length XErp1 (#32.1 to 3). The extracts were warmed to 20°C and DNA morphology was examined after 60 minutes (top panel). Histone H1 kinase activity (bottom panel) was examined to monitor the cell cycle phase of the extracts throughout the experiment in samples taken at the indicated times after warming the extract.

morphology (see **Figure 2.5**, top panel) and H1 kinase activity of extract samples (see **Figure 2.5**, bottom panel). Together, these results indicated that inhibition of XErp1 function interferes with the maintenance of CSF arrest in *Xenopus* egg extracts.

In order to make sure that the observed effect is solely due to inhibition of XErp1 function, rescue experiments have been performed. Here, endogenous XErp1 was immunodepleted from CSF extract and replaced with different *in vitro* translated proteins to examine their ability to complement loss of the endogenous protein. After warming to 20°C, the extract was examined for integrity of CSF arrest. Since the inactivation of XErp1 triggers the irreversible calcium-independent release from CSF arrest (see above), the rescue proteins had to be added before immunodepletion of the endogenous XErp1 was started. Thus, the antibody raised against an amino-terminal fragment of XErp1 was used for depletion and carboxy-terminal fragments encompassing the two functional domains and the full-length as one negative control were used for rescue.

As expected immunodepletion with an unspecific rabbit antibody (ctrl ab) did not remove XErp1 from the extract and did not perturb CSF arrest (see **Figure 2.6a** and b, 1 and 6). However, the XErp1 specific antibody significantly depleted the protein from the extract and led to calcium-independent CSF release in the absence of rescue protein as evident from decondensed DNA morphology and stable IVT securin (see **Figure 2.6a**, 5 and 10). When myc-tagged full-length XErp1 was added to the extract as a rescue construct, DNA morphology and instability of securin indicated that CSF release had taken place. In this case no rescue could be observed, because myc-tagged full-length XErp1 was recognized by the antibody and depleted along with the endogenous XErp1 (see **Figure 2.6b**, myc WB and XErp1 WB). However, when a carboxy-terminal fragment of XErp1 containing the ZBR and the F-box was added before depletion, CSF arrest was maintained as evident from condensed DNA, intact spindle structures and stable securin, indicating that it was sufficient to rescue depletion of endogenous XErp1. (see **Figure 2.6a**, 3 and 8; **Figure 2.6b**). The ability of this fragment to rescue CSF arrest and therefore APC/C inhibition critically depended on the integrity of the ZBR, since a fragment bearing a mutation in a conserved residue within the ZBR domain (C583A) could not rescue the effects of XErp1 depletion (see **Figure 2.6a**, 4 and 9).

The Western Blot for XErp1 shows the endogenous protein as well as the full-length rescue construct (marked with an asterisk). Comparison of these two bands and the signals detected for the myc-tagged rescue constructs shows that they have been used at concentrations that approximately match the endogenous level of XErp1.

Taken together loss-of-function analyses of XErp1 indicate that it is required to maintain CSF arrest and consequently APC/C inhibition in *Xenopus* egg extract. This

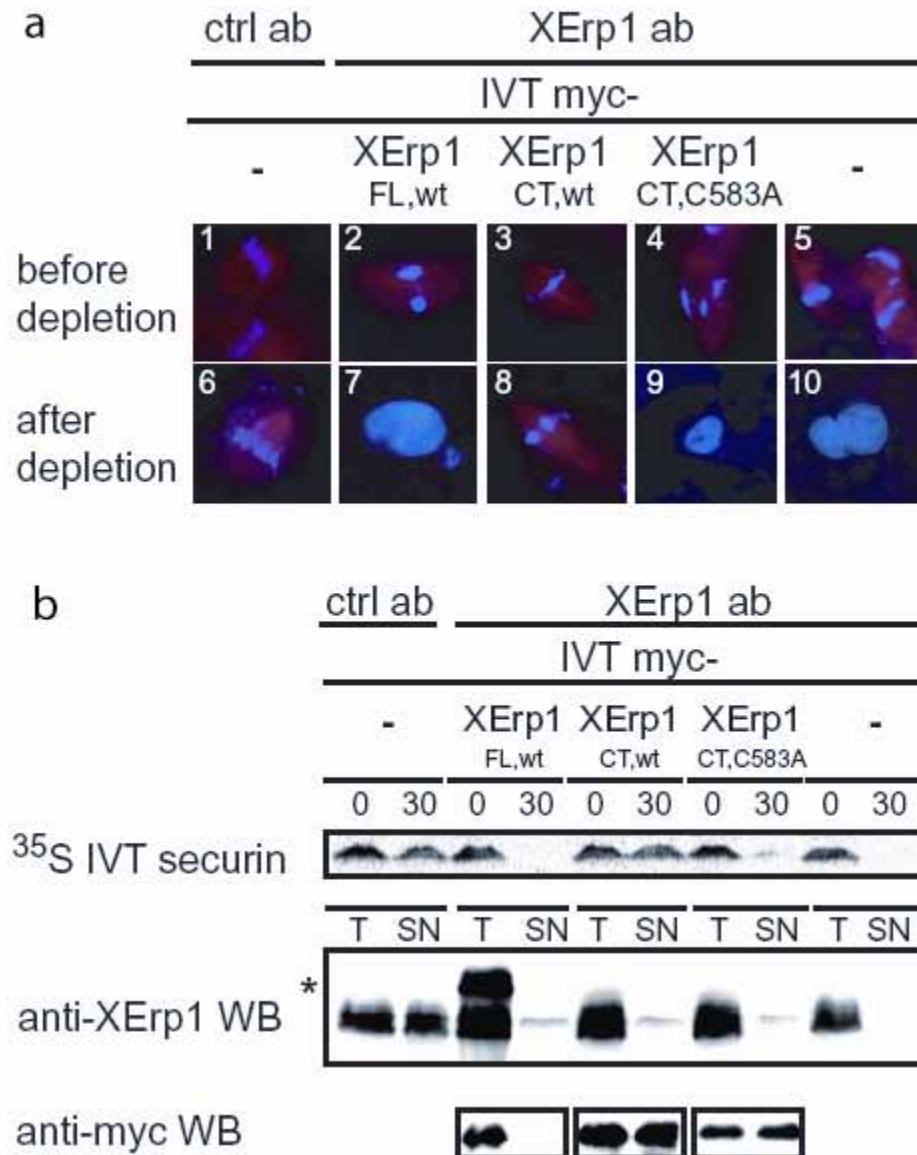


Figure 2.6: Rescue of XErp1 depletion from CSF extracts. (a) DNA and spindle morphology of the differently treated extracts have been examined before and after depletion of XErp1. (b) XErp1 depletion and the fate of the rescue constructs were analysed by Western Blotting of extract samples against endogenous XErp1 and the myc-epitope tag (T is before and SN after depletion). APC/C activity was monitored by examining the stability of radioactively labelled securin that was added to the extract after depletion. The extract was sampled before (0) and 30 minutes after depletion and warming to 20°C (30).

function is mediated by the carboxy-terminal domain with an intact zinc-binding region, since this part of the protein is sufficient to rescue the effects of XErp1 depletion.

2.3.2 Overexpression of XErp1

To further support a role for XErp1 in preventing CSF release and therefore APC/C activation, the effects of XErp1 overexpression were investigated. To this end different

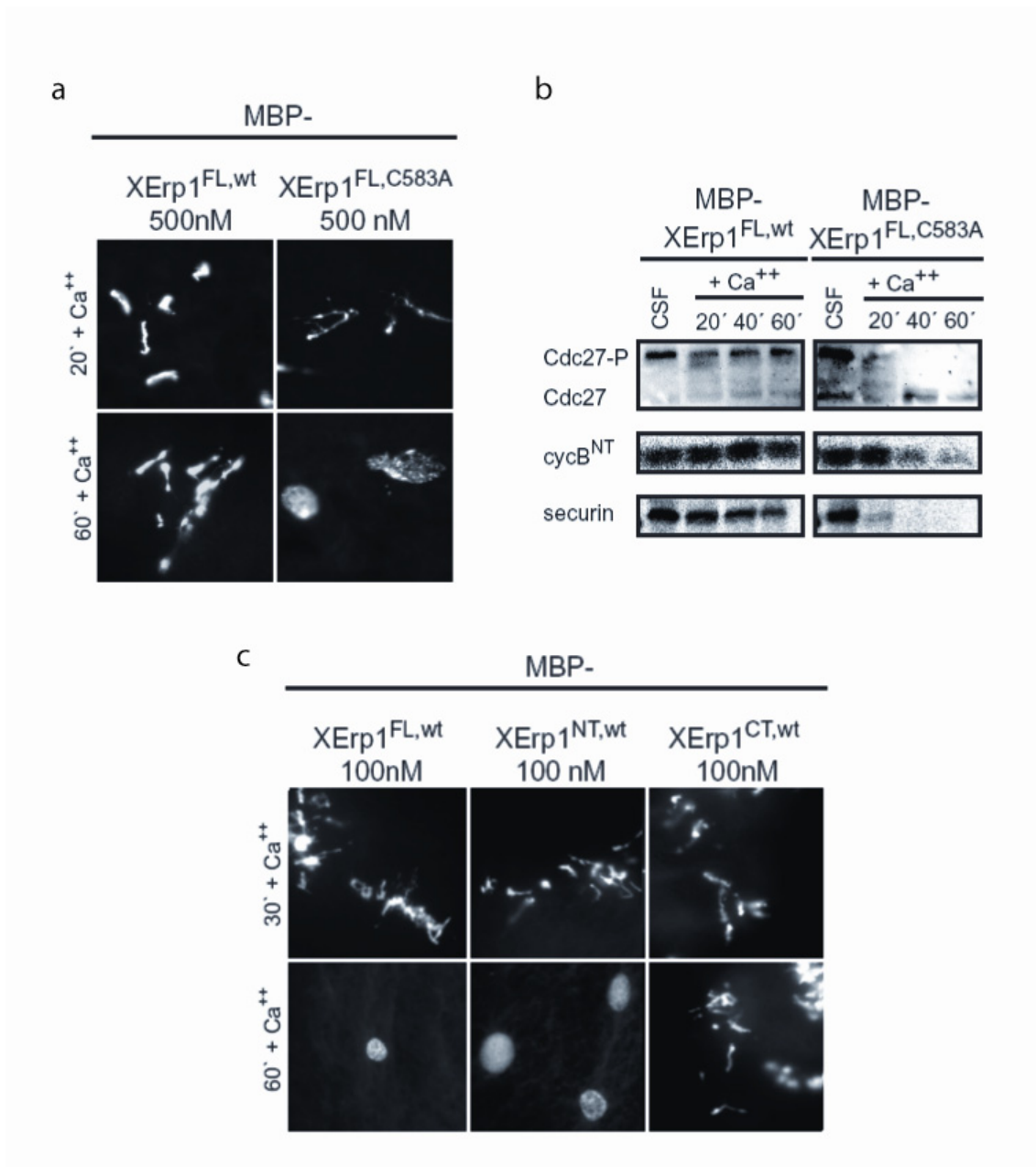


Figure 2.7: Effects of XErp1 overexpression on CSF release. Recombinant MBP-XErp1 proteins were purified from bacteria, added to CSF extracts and their effect on CSF release and APC/C activation was examined. (a) DNA morphology was analysed by fixing extract samples 20 and 60 minutes after calcium addition. (b) Cdc27 gel mobility and stability of cyclin B amino-terminus and securin were monitored as markers of M-phase exit and APC/C activation. (c) Different fragments of XErp1 were examined for their effect on CSF at low concentrations.

recombinant full-length MBP-XErp1 fusion proteins were purified from bacteria and added to CSF extracts at defined concentrations to examine their effect on calcium-dependent CSF release. Wildtype MBP-XErp1^{FL,wt} was added to the extract to a final concentration of 500 nM (corresponding to 5-10 fold over endogenous levels as determined by semi-quantitative Western Blotting, data not shown) and its effect on CSF release was examined after addition of calcium. As shown by condensed DNA until 60 minutes after calcium addition, overexpression of MBP-XErp1 completely prevented calcium-dependent CSF release (see **Figure 2.7a**, left). As expected from the loss-of-function/rescue analysis, a mutant protein lacking a conserved cysteine residue in the ZBR (MBP-XErp1^{FL,C583A}) was unable to prevent CSF release as evident from decondensed chromatin 60 minutes after calcium addition (see **Figure 2.7a**, right).

To corroborate these findings, biochemical markers of CSF release and APC/C activation were examined in the same experiment (see **Figure 2.7b**). The APC/C subunit Cdc27 is phosphorylated in M-phase by Cdk1 causing a pronounced mobility shift of the protein (King et al., 1995; Patra and Dunphy, 1998). Upon M-phase exit inactivation of Cdk1 by cyclin B degradation and removal of Cdk1 phosphorylation by opposing phosphatases abolishes this mobility shift. To examine this mobility shift as a marker for the cell cycle phase of the extract, samples were analysed by Western Blotting for Cdc27. In samples from CSF arrested egg extracts a low mobility form of Cdc27 was clearly visible. This band was not shifted down after calcium addition in the samples treated with wildtype MBP-XErp1 which also showed condensed chromatin after 60 minutes. In samples treated with mutant MBP-XErp1, Cdc27 was shifted down in the presence of calcium indicating that exit from M-phase has taken place. To investigate APC/C activation and M-phase exit more directly we examined the stability of the APC/C substrates cyclin B and securin. IVTted and radio-labelled securin and amino-terminus of cyclin B (cycB^{NT}) were stable in extracts treated with wildtype MBP-XErp1, but not in extracts containing the mutant MBP-XErp1 indicating that the APC/C could not be activated properly in the presence of wildtype MBP-XErp1.

To further clarify the role of the functional domains/motifs of XErp1 in preventing APC/C activation different fragments of MBP-XErp1 were examined for their effect on CSF release at different concentrations (not shown). At a final concentration of 100 nM only a carboxy-terminal fragment of XErp1 (aa 374-651, see also **Figure 2.7c**) was still able to prevent calcium-induced chromatin decondensation and therefore CSF release.

Equimolar amounts of full-length MBP-XErp1 or MBP-XErp1 amino-terminus did not prevent CSF release under these conditions. This indicated that the carboxy-terminal fragment alone was more effective than the full-length protein or the amino-terminus (see *Figure 2.7c*). This argues for possible negative effects of the amino-terminal part of the protein on the activity of full-length XErp1.

2.3.3 MAPK-independency of XErp1 overexpression effects

The results of the experiments addressing a possible function of XErp1 in CSF arrest suggested that XErp1 is in fact an indispensable component of CSF activity. The MAPK pathway has been shown to play a pivotal role in CSF arrest by preventing APC/C-mediated M-phase exit (Abrieu et al., 1996). Thus, the requirement of this pathway for XErp1-induced prevention of CSF release and APC/C activation was tested. MAPK (specifically Erk1/2) is activated through phosphorylation of two neighbouring residues (corresponding to T183 and Y185 in mammalian Erk1/2) by a MAPK-kinase. Small-molecule inhibitors have been developed that efficiently prevent MAPK activation by

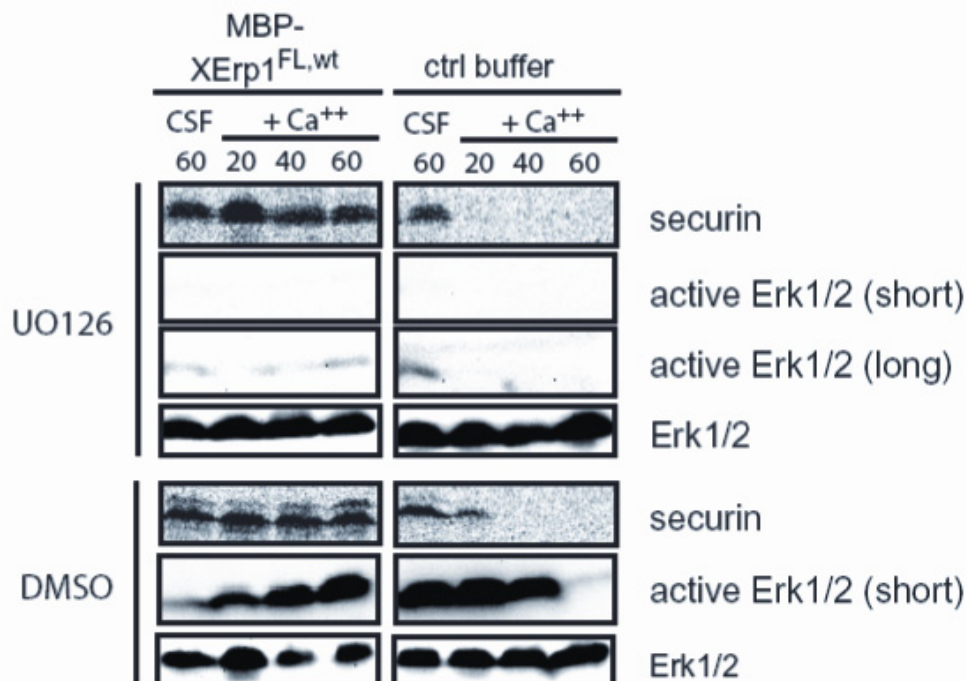


Figure 2.8: Effect of MAPK of XErp1 function. MBP-XErp1 or control buffer were incubated in CSF extract in the presence or absence of the MAPK pathway inhibitor UO126. CSF release after calcium addition was followed by analysing securin stability. The status of MAPK activity was monitored by comparing signals for total Erk1/2 to signals for phosphorylated (active) Erk1/2.

inhibiting the upstream MAPK-kinase Mek. One of these molecules called UO126 was used to inhibit MAPK activity in CSF extracts and to examine MBP-XErp1's ability to suppress APC/C activation in the absence of MAPK activity.

To this end extracts were treated with UO126 or DMSO as a solvent control and incubated with full-length, wildtype MBP-XErp1 or a control buffer. In the buffer control the APC/C substrate securin was readily degraded after calcium addition. This happened in the presence or absence of an active MAPK pathway as judged from staining with an antibody specific for the region around T183/Y185 in Erk1/2 (see **Figure 2.8**, right panel). However, when excess MBP-XErp1^{FL,wt} was added to extracts treated with UO126 or DMSO, APC/C activation was prevented as shown by stable securin in both cases. Thus, suppression of MAPK kinase activity with UO126 did not prevent excess XErp1 from inhibiting APC/C activation (see **Figure 2.8**, left panel). This indicates that full activity of the MAPK pathway might not be strictly required for XErp1's function in CSF arrest.

2.3.4 Mechanism of XErp1 function

The results from loss-of-function as well as gain-of-function experiments strongly support a role for XErp1 in preventing APC/C activation during CSF arrest. In particular the carboxy-terminus seems to be sufficient for this function, whereas the amino-terminus might exert a negative regulatory function. An intriguing possibility to test was a direct inhibition of APC/C's ubiquitin ligase activity by XErp1. In order to investigate whether inhibition of the APC/C by XErp1 is direct, an *in vitro* ubiquitylation assay was established and the effect of different XErp1 fragments on the reaction was monitored.

The assay uses purified components of the ubiquitin pathway (E1, E2 and ubiquitin) including immuno-purified APC/C plus its activator Cdc20 as an E3 to modify an amino-terminal fragment of cyclin B (cycB^{NT}) as a model substrate. The APC/C was immuno-purified from M-phase extract and incubated with or without Cdc20 and the respective recombinant MBP-XErp1 fragments. After washing, the immobilized APC/C was mixed with the other assay components including the substrate. Ubiquitin modification is visible by the appearance of low-mobility bands over time, which represent multiple conjugates of the cyclin B amino-terminus with ubiquitin.

When immuno-purified APC/C was not incubated with exogenous Cdc20, it showed only basal activity towards the cyclin B amino-terminus, probably because the purification procedure removes most of the bound activator Cdc20 which is required for efficient substrate ubiquitylation (see **Figure 2.9**). As expected, pre-incubation of the APC/C with recombinant Cdc20 greatly increased its activity towards the substrate as shown by the formation high molecular weight conjugates and rapid loss of the band representing unconjugated substrate (Kramer et al., 1998; Kramer et al., 2000). However, if the pre-incubation step was carried out in the presence of the XErp1 carboxy-terminus, which rescued XErp1 depletion and was most efficient in preventing calcium-triggered CSF release, ubiquitin conjugation to the cyclin B amino-terminus was largely suppressed in a concentration-dependent manner (see **Figure 2.9**). High molecular weight ubiquitin conjugates did not form as efficiently and considerable amounts of substrate remained unconjugated. In contrast, up to ten-fold higher concentrations of the amino-terminus did

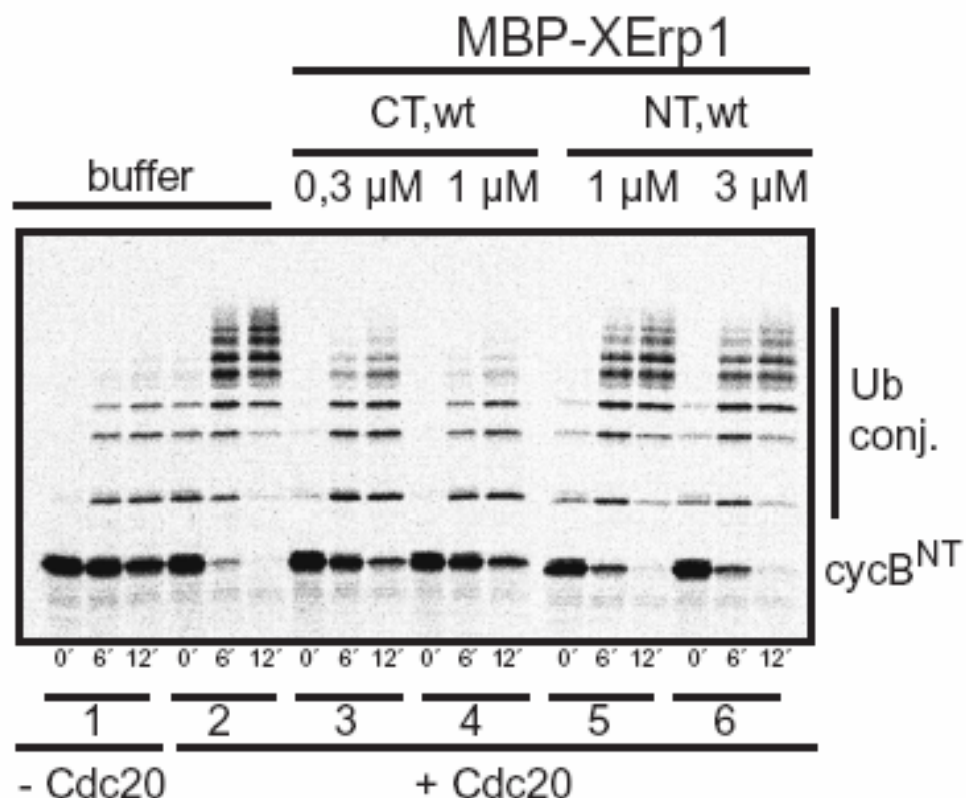


Figure 2.9: Effect of XErp1 fragments on APC/C activity *in vitro*. To analyze XErp1's effect on APC/C activity *in vitro*, immuno-purified APC/C and Cdc20 were incubated with recombinant MBP-XErp1 fragments. The APC/C was subsequently assayed for activity towards its model substrate amino-terminus of cyclin B. Samples of the reaction were taken at 0, 6 and 12 minutes, boiled in sample buffer and examined for cyclin B ubiquitylation by PAGE and autoradiography.

not have a visible effect on APC/C activity towards its model substrate.

These results corroborate the finding that the carboxy-terminus of XErp1 is required and could be sufficient to ensure APC/C inhibition in CSF arrested egg extracts. They furthermore suggest that the mechanism by which XErp1 exerts its function is by direct inhibition of APC/C^{Cdc20}-dependent ubiquitylation of M-phase substrates.

2.4 Regulation of XErp1 by Plx1

XErp1 was identified as an interacting protein of Plx1, a kinase that has been shown to be involved in APC/C regulation. The results on XErp1 function show that the protein directly affects APC/C activity. This opens the possibility that XErp1 might be a mediator of Plx1's function in activating the APC/C. Thus, the link between XErp1 and Plx1 in regulating APC/C activity was investigated.

2.4.1 Phosphorylation of XErp1 by Plx1

A straight-forward link between a kinase and any given protein could be a direct kinase/substrate relationship. This possibility is easily testable *in vitro* by incubating recombinant kinase and the candidate protein(s) in the presence of radioactively labelled ATP. To test whether XErp1 could serve as a substrate for Plx1, *in vitro* kinase assays were performed on full-length MBP-XErp1 and the two fragments that have also been used for the analysis of APC/C activity *in vitro* (see 2.3.4). Incorporation of radioactively labelled phosphate shows that the MBP-tagged full-length as well as the amino-terminus of XErp1 are *in vitro* substrates of Plx1. In contrast, the MBP-tagged carboxy-terminal part of the protein does not show significant phosphate incorporation (see **Figure 2.10a**).

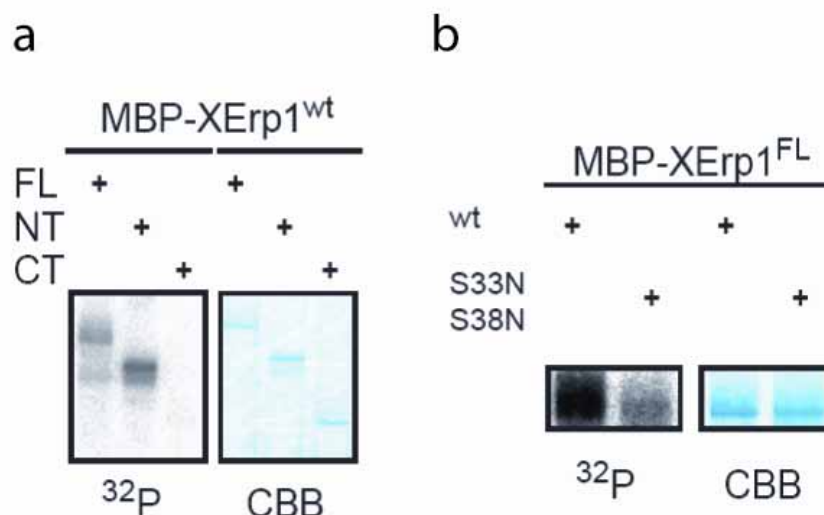


Figure 2.10: In vitro phosphorylation of amino-terminal XErp1 by Plx1. Recombinant MBP-tagged XErp1 fusion proteins were incubated with ^{32}P -labelled ATP and recombinant Plx1. (a) Incorporation of labelled phosphate into full-length XErp1 and the amino- (NT) or carboxy-terminal (CT) fragments as shown by autoradiography (^{32}P) and Coomassie staining (CBB) to control for approximately equal amounts in the assay. (b) Comparison of full-length wildtype XErp1 with a mutant in the phospho-degron (S33N, S38N) as in (a).

The overexpression experiments had already indicated a possible role of the amino-terminus in negatively regulating XErp1 function (see 2.3.2, **Figure 2.7**) and Plx1 was postulated to negatively regulate an inhibitor of the APC/C (see introduction). Since the amino-terminus was phosphorylated by Plx1, this part of the protein was scanned for possible Plx1 phosphorylation sites that could be implicated in negative regulation of XErp1 activity. Indeed, the amino-terminus contained sequence motif that is known to target proteins for degradation dependent upon phosphorylation of serine residues within this motif (phospho-degron, DSGXXXS, where X can be any amino acid). This motif is described to be recognized by an SCF complex containing β -TRCP as a targeting subunit (SCF ^{β -TRCP}) (Fuchs et al., 2004). The motif does not fit the consensus generally assumed to be optimal for Plk phosphorylation (D/E-X-S/T- Φ , where Φ is a hydrophobic amino acid) (Barr et al., 2004), but the sequence around this motif contains several acidic residues and might thus be considered favourable for Plk phosphorylation. Also, Plx1 has been shown to target similar motifs in other proteins (Watanabe et al., 2004). When phosphate incorporation into full-length MBP-XErp1 was compared to a mutant where the critical residues S33 and S38 have been changed to a non-phosphorylatable residue, it was almost completely abolished (see **Figure 2.10b**). This indicated that these two serine residues represent the main sites of Plx1 phosphorylation on XErp1 *in vitro*.

2.4.2 Relevance of the XErp1 phospho-degron

The results of the *in vitro* kinase assays have identified a potential phospho-degron in the amino-terminal part of XErp1 as a putative target site for negative regulation of XErp1 by Plx1. To check whether the identified motif is implicated in phosphorylation-dependent degradation of XErp1, experiments were conducted to examine the behaviour of XErp1 variants lacking this potential phospho-degron or made non-phosphorylatable at the critical positions. Low amounts of ³⁵S-Met-labelled IVTted proteins were incubated in CSF-arrested egg extract in the presence or absence of calcium and their stability was monitored by autoradiography of extract samples after PAGE. To exclude that the addition of *in vitro* translated XErp1 proteins had an effect on M-phase exit similar to the addition of excess recombinant protein, the cell cycle phase before and after calcium addition was monitored. In analogy to previous experiments, this was done by microscopy of added

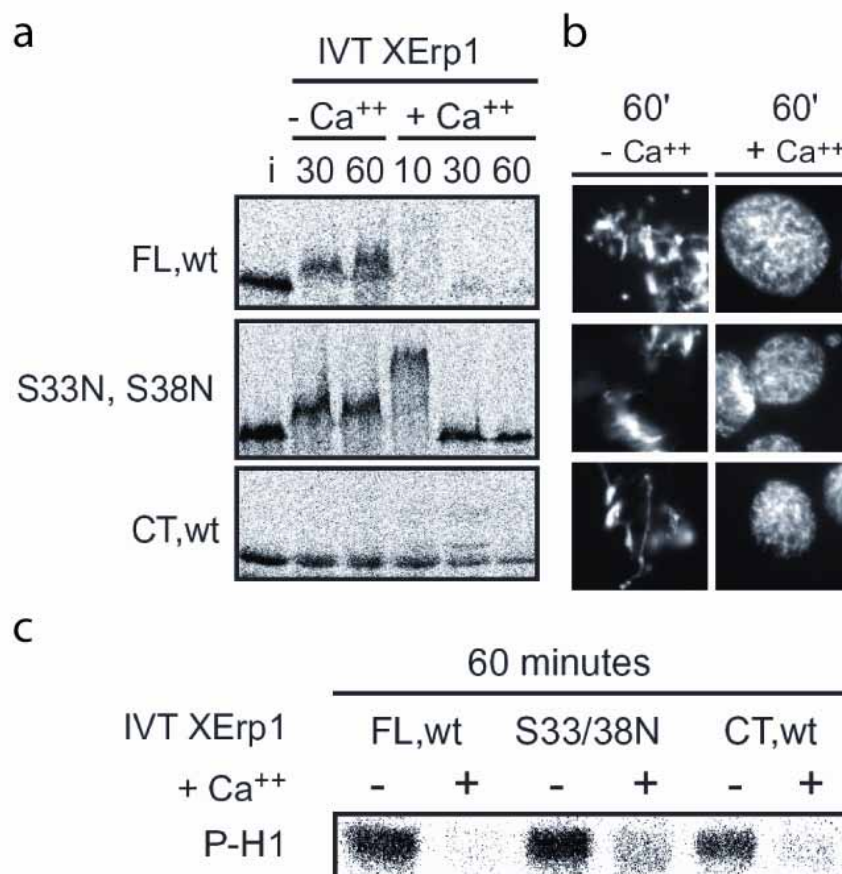


Figure 2.11: Role of the phospho-degron for XErp1 stability. (a) Radioactively labelled IVT XErp1 proteins were incubated in CSF extract in the presence or absence of calcium to examine their stability. (b) and (c) Analysis of CSF release by microscopic inspection of DNA morphology and assaying H1 kinase activity confirmed that IVT addition had no effect on M-phase exit.

sperm nuclei and assaying H1 kinase activity of extract samples.

When wildtype XErp1 IVT was added to ice cold CSF extract that was subsequently warmed to 20°C, it experienced a mobility shift similar to that seen in endogenous XErp1 in M-phase. In the absence of calcium this upshifted form remained stable throughout the course of the experiment. However, in samples treated with calcium the labelled protein was completely degraded within ten minutes (see **Figure 2.11a**). Thus, wildtype IVT XErp1 like the endogenous protein is stable in CSF extract and degraded in a calcium-dependent manner. IVT XErp1 carrying a mutation in the two critical serine residues of the DSGXXS-motif (XErp1 S33N, S38N) also showed an upshift upon incubation in CSF extract and remained stable in the absence of calcium. In contrast to the wildtype protein however this XErp1 mutant did not get degraded in the presence of calcium. Instead, shortly after calcium addition it experienced a pronounced, transient mobility shift (henceforth called hypershift). At later time points the stabilized protein regains the increased gel mobility of unmodified IVT that is similar to endogenous protein in interphase extract.

When the carboxy-terminal fragment of XErp1 was added to CSF extract it showed no sign of post-translational modifications and remained stable. No significant degradation after calcium addition was observed with this fragment. Also, the hypershift could not be observed upon CSF release. This is consistent with the idea that the phospho-degron motif in the amino-terminus is required for efficient XErp1 destruction upon CSF release. It also shows that the modifications of XErp1 seen in CSF extract and associated with CSF release depend on the presence of amino acids 1-373 (for a detailed analysis see N. Rauh, PhD thesis).

In all cases the addition of the IVT proteins did not have a visible effect on CSF release as shown by the formation of nuclei and the pronounced drop in H1 kinase activity after calcium addition (see **Figure 2.11b** and c). Taken together these results indicate that XErp1's phospho-degron, which is the main site of Plx1 phosphorylation *in vitro* (see 2.4.1), is required for its timely degradation in extract.

2.4.3 Interplay between XErp1 and Plx1

The evidence obtained so far indicated that APC/C activation upon CSF release in *Xenopus* egg extract could work through Plx1-dependent destabilization of the APC/C-

inhibitor XErp1. To investigate this potential link between XErp1 and Plx1 in more detail Plx1 loss-of-function studies have been carried out. These were focussed on the fate of XErp1 and its role in Plx1-mediated APC/C activation under these conditions. Plx1 loss-of-function has been successfully achieved in *Xenopus* egg extract by either depleting endogenous Plx1 or by adding excess recombinant, kinase-dead Plx1 (Plx1 N172A) to the extract (Descombes and Nigg, 1998). Kinase-dead Plx1 is assumed to act in a dominant-negative fashion by recruiting Plx1 substrates into non-productive complexes. It has been shown that the PBD of Plx1 is required for dominant-negative effects of Plx1 on CSF release (Liu et al., 2004). This is consistent with the recently introduced model of PBD-mediated targeting of Plks to substrates or structures in the cell and suggests that APC/C activation by Plx1 employs such a mechanism (Sillje and Nigg, 2003).

To simplify the dominant-negative approach, it was checked whether the PBD of Plx1 is not only required, but whether its overexpression is also sufficient to create a Plx1

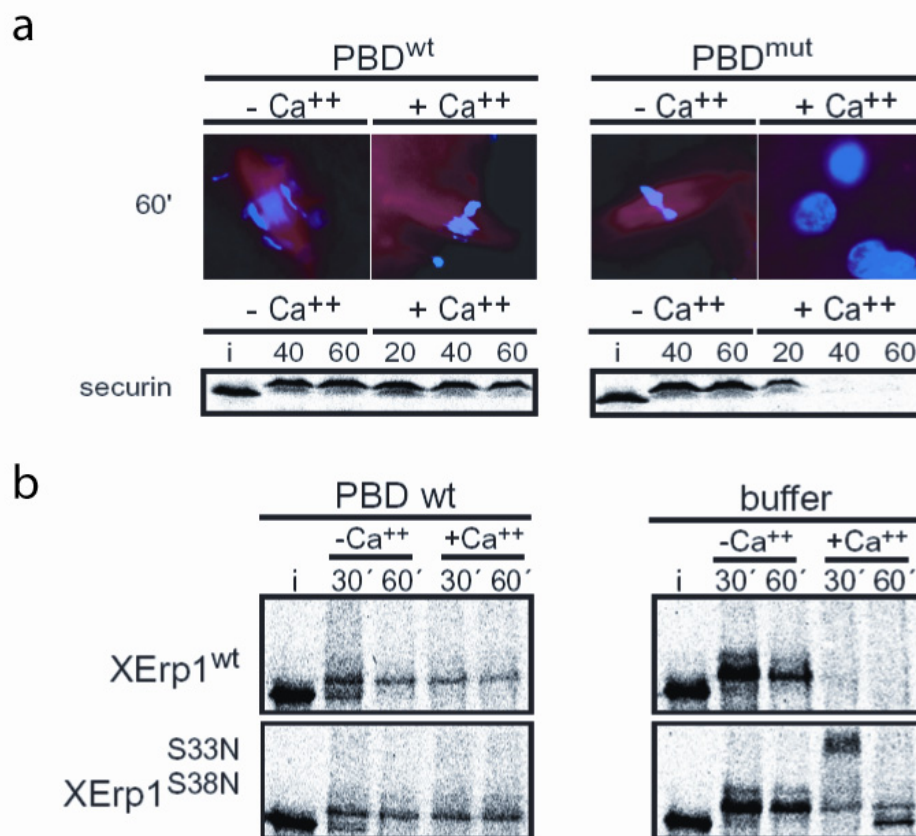


Figure 2.12: Effects of dominant-negative MBP-PBD on CSF release and XErp1 stability. (a) MBP-tagged PBD or a mutant incapable of phospho-peptide binding where added to CSF extracts in the presence or absence of calcium. (b) The effect of dominant-negative PBD on XErp1 stability was tested by monitoring IVT XErp1 stability.

loss-of-function situation in *Xenopus* egg extracts. To this end MBP-tagged PBD was purified from bacteria and added to CSF extracts to examine its effect on calcium-induced CSF release. Similar to kinase-dead Plx1, MBP-PBD prevented CSF release and destruction of the APC/C-target securin after addition of calcium (see **Figure 2.12a**, left panel). As for kinase-dead Plx1 this effect of the PBD required the ability to bind phosphopeptides, because an MBP-PBD in which three residues critical for phosphate-binding were mutated (W408F, H532A, K534A; see (Elia et al., 2003b)) did not show a visible effect on CSF release as observed by spindle disassembly, chromatin decondensation and securin degradation (see **Figure 2.12a**, right panel). Thus, these results revealed that overexpression of wildtype MBP-PBD could be used to study XErp1's fate and function under Plx1 loss-of-function conditions.

If XErp1 was a target of Plx1-dependent destabilization upon CSF release, then it should be stable when CSF release is prevented by dominant-negative interference with Plx1 function. To examine the fate of XErp1 in the presence of the dominant-negative MBP-PBD we incubated IVT XErp1 in CSF extract supplemented with MBP-PBD or control buffer (see **Figure 2.12b**). Upon induction of CSF release wildtype XErp1 was degraded in the control case. As expected, this degradation depended on the phosphodegron, because a mutant in this motif was not degraded in the control. In contrast, when calcium was added to extract containing the dominant-negative PBD, wildtype XErp1 was not degraded, indicating that its degradation requires Plx1-activity. Again, a mutant in the phosphodegron did not get degraded under these conditions similar to the wildtype. Interestingly, the transient hypershift, that was well visible in this mutant in the control, was suppressed when MBP-PBD was present, suggesting that requires functional Plx1.

The working hypothesis that has emerged thus far suggests that Plx1-mediated inactivation of the APC/C inhibitor XErp1 leads to APC/C activation and therefore CSF release. This suggests that dominant-negative effects of the PBD might be largely mediated by preventing XErp1 inactivation through sequestration. To establish whether XErp1 is an important target of Plx1 in APC/C activation and CSF release, it was investigated if XErp1 inactivation is sufficient to overcome dominant-negative effects of the PBD on these processes. To examine this, the MBP-PBD was used to interfere with Plx1 activity as before; in addition, anti-XErp1 antibody was used to inhibit XErp1 function at the same time as in 2.3.1.

Consistent with the previous results, addition of MBP-PBD to CSF extract prevented CSF release in the presence of calcium and unspecific rabbit antibody as evident from condensed sperm DNA one hour after calcium addition and a moderate drop in H1 kinase activity by only about 40 % (see **Figure 2.13**, 3). However, when XErp1-specific antibody was added under these conditions, H1 kinase activity rapidly declined to interphase levels and sperm DNA decondensed to form nuclei, indicating that XErp1 inactivation is

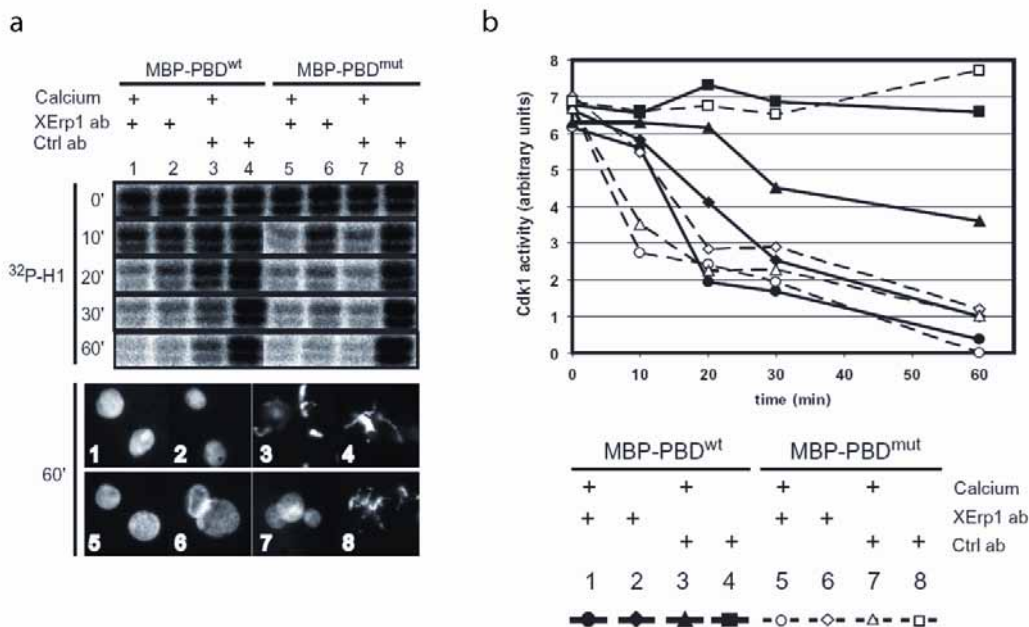


Figure 2.13: XErp1 as a target of Plx1-mediated APC/C activation. (a) XErp1 function was inhibited in the presence of dominant negative acting MBP-PBD or a mutant unable to bind phospho-peptides and in the presence or absence of calcium. CSF release and APC/C activation was monitored by H1 kinase assay and microscopy of sperm DNA. (b) The results of the H1 kinase assay were quantified by densitometric analysis of band intensities.

sufficient to overcome a block in calcium-induced CSF release induced by Plx1 loss-of-function (see **Figure 2.13**, 1). Thus, MBP-PDB cannot prevent calcium-induced CSF release in the absence of active XErp1. The fact that inactivation of the Plx1 substrate XErp1 is sufficient to rescue an MBP-PDB-induced block in CSF release shows that XErp1 is an important target of Plx1 in mediating this process.

Moreover, CSF release and drop in H1 kinase activity was also observed in the absence of calcium when XErp1 function was inhibited in the presence of MBP-PDB (see **Figure 2.13**, 2). This shows that the PDB cannot prevent CSF release induced solely by XErp1 inactivation which would be consistent with a model in which Plx1 acts upstream of its effector XErp1 in activating the APC/C. Again, the effects seen with MBP-PDB were dependent on phospho-peptide binding as a mutant MBP-PDB could not prevent CSF release in the presence of control antibody and calcium (see **Figure 2.13**, 5-8).

Taken together, the experiments carried out to elucidate the functional relationship between XErp1 and Plx1 support a model in which Plx1-mediated inactivation of XErp1 is an important trigger for APC/C activation and subsequent CSF release.

2.4.4 Regulation of XErp1 degradation by calcium

Besides Plx1 activity, the calcium signal is a second obvious requirement for CSF release and therefore possibly XErp1 degradation. In fact, the calcium signal seems to determine the timing of XErp1 degradation in extracts. Also, the working model proposed by Descombes and Nigg predicts, that if the Plx1-regulated APC/C inhibitor exists, it would be inactivated by the calcium signal. Work by Lorca *et al.* has shown that calmodulin-dependent kinase II (CaMKII) is the sole mediator of this calcium signal in *Xenopus* egg extract (Lorca *et al.*, 1993). Therefore, experiments were conducted to examine whether CaMKII activity is required for XErp1 degradation in extract using a CaMKII inhibitory peptide. Because the calcium signal, XErp1 degradation and CSF release are very tightly linked, the experiments had to be done under conditions where CaMKII activation and cell cycle progression are uncoupled. Anaphase arrested extract that does not have CaMKII activity is such a system. Extract can be arrested in anaphase with non-destructible cyclin B (Holloway *et al.*, 1993; Stemmann *et al.*, 2001). CaMKII activity can be retriggered by addition of calcium in the absence of cell cycle progression (Rauh *et al.*, 2005). It is important to be able to trigger CaMKII activity because available

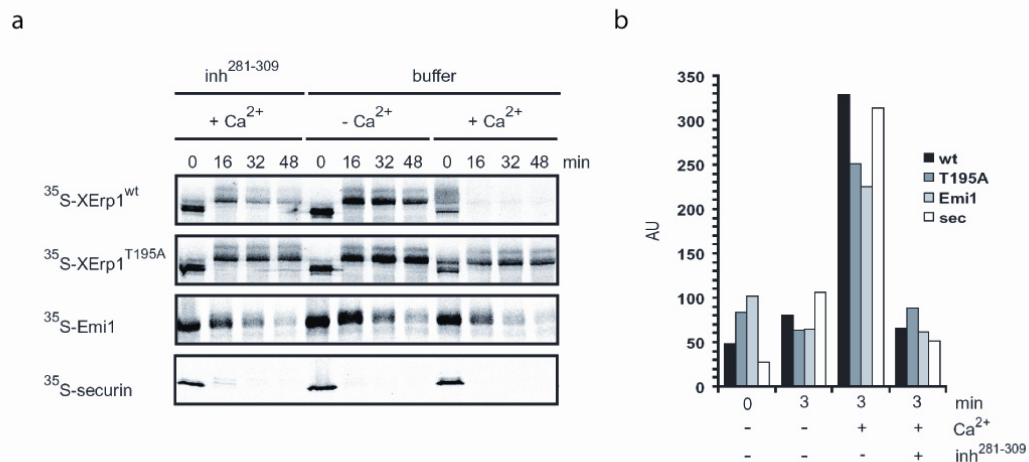


Figure 2.14: CaMKII-dependency of XErp1 degradation in extract. (a) Radioactively labelled IVT XErp1 wildtype or a mutant incapable of efficient Plx1 binding (T195A) and IVT Emi1 were incubated in extract without CaMKII activity (middle). Calcium was added to the extracts in the presence (left) or absence (right) of an inhibitory peptide. Anaphase state of the extract was confirmed by monitoring securin stability under all conditions. (b) CaMKII activity was measured in all samples by assaying extract samples for CaMKII activity towards a specific peptide-substrate before (0) and 3 minutes after calcium addition (3).

CaMKII inhibitors, be it small molecules or peptides, prevent only CaMKII activation and not activity.

When wildtype XErp1 was incubated in anaphase-arrested egg extract that lacks CaMKII activity it experienced the M-phase specific upshift like in CSF extract, but remained stable over the course of the experiment (see **Figure 2.14a**, middle). In contrast, wildtype XErp1 was very rapidly degraded in extract in which CaMKII activity was retriggered by the addition of calcium (see **Figure 2.14a**, right). This degradation could be prevented with a peptide corresponding to the calmodulin-binding region in CaMKII that prevents efficient activation of the kinase (see **Figure 2.14a**, left). It was shown that CaMKII can phosphorylate XErp1 directly and that phosphorylation on threonine 195 leads to enhanced Plx1 recruitment which triggers XErp1 degradation (Rauh et al., 2005). Consequently, a mutant in this critical residue was resistant to calcium-induced degradation. Interestingly, the previously proposed CSF component Emi1 was unstable in anaphase extract in the presence or absence of CaMKII activity suggesting that its stability might not be regulated by fertilization and the subsequent calcium-signal.

A hallmark of anaphase is APC/C activity and consequently instability of its substrates. Rapid degradation of the APC/C substrate securin under all conditions showed that the manipulations did not affect anaphase arrest. CaMKII activity in this experiment

was monitored by assaying extract samples for their phosphorylation of a CaMKII-specific peptide (see **Figure 2.14b**). Together these results show that CaMKII activity is required for XErp1 inactivation probably via enhanced Plx1 recruitment.

2.5 *In vivo* analysis of XErp1 function

Extracts from CSF arrested *Xenopus* eggs have mainly been used throughout this work to study XErp1 function and regulation. The available evidence suggests that XErp1 plays an important role in maintaining CSF activity in these extracts and that its degradation after Plx1 phosphorylation on the phospho-degron sequence allows for APC/C activation and CSF release. CSF activity has originally been identified by cytoplasmic injection experiments into two-cell embryos. The observed cleavage arrest was used an indicator of potential CSF activity also for single proteins of the CSF pathways. Therefore, as a first step towards an *in vivo* analysis of XErp1 cytoplasmic injection experiments were

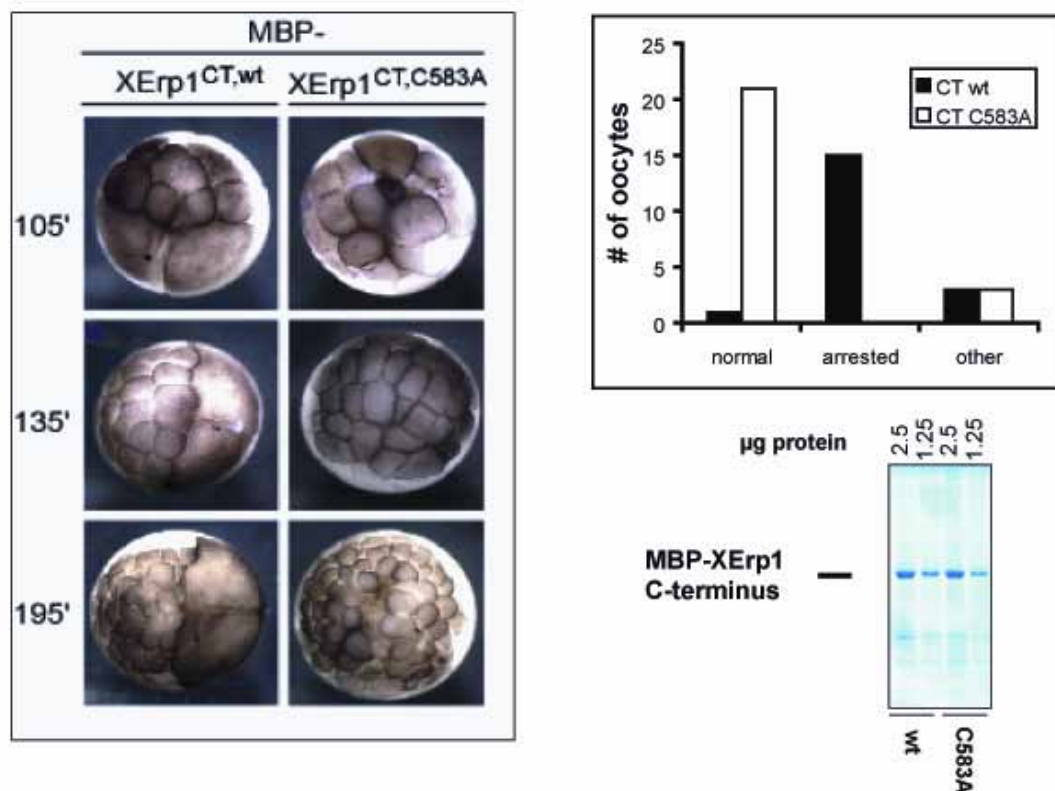


Figure 2.15: Injection of XErp1 into *Xenopus* embryos. The carboxy-terminus of XErp1 or a mutant lacking a functional ZBR were injected into one cell of two-cell embryos and their development followed after injection (examples, left panel). All injected embryos were scored for their phenotype after the observation period (right panel, top). The purity of the injected protein is shown on a Coomassie stained gel (right panel, bottom).

carried out using recombinant XErp1.

To this end the carboxy-terminal fragment, that was sufficient to rescue CSF release after XErp1 depletion from CSF extract, was injected into one cell of dividing two-cell embryos obtained by *in vitro* fertilization. As a control, embryos were injected with a carboxy-terminal fragment mutated in a critical residue of the ZBR, that was unable to rescue the effects of XErp1 depletion from CSF extracts. Development of the embryos was followed for about three hours after injection (see **Figure 2.15**, left panel). Of 19 embryos injected with the wildtype protein the injected half appeared arrested in 15 cases, whereas the other half seemed unaffected. In one case the embryo looked normal and in three cases the embryos were not reliably scorable due to signs of deterioration. Of the 24 embryos injected with the control protein carrying a point mutation in the ZBR, 21 had a normal appearance. No blastomere-arrest could be observed in any of the control-injected embryos (see **Figure 2.15**, right/top). Again, three embryos could not be unambiguously assigned due to beginning degradation. These results clearly indicate that misexpression of XErp1 interferes with embryonic cell cycle progression *in vivo* similar to what has been observed for other proteins implicated in CSF activity.

3 Discussion

This work has led to a basic characterization of XErp1 protein expression, function and regulation. The protein is expressed in immature oocytes and throughout oocyte maturation, where it experiences cell cycle-dependent changes in abundance and post-translational modification status. Experiments using the *Xenopus* egg extract system show that XErp1 is present in CSF-arrested *Xenopus* egg extract and is rapidly degraded after addition of calcium which mimics fertilization. Loss-of-function as well as gain-of-function studies demonstrate that XErp1 activity is essential for CSF arrest and that its misregulation interferes with APC/C activation and therefore CSF release. *In vitro* experiments using immuno-purified APC/C show that this is due to direct inhibition of APC/C substrate ubiquitylation by XErp1. Moreover, oocyte injection experiments show that XErp1 misexpression *in vivo* leads to cell division arrest.

Investigation of a potential link to its interacting protein Plx1 shows that a phospho-degron motif in XErp1's amino-terminus, which is required for XErp1 degradation upon CSF release in extract, is the main Plx1-phosphorylation site on XErp1. Experiments combining XErp1/Plx1 loss-of-function demonstrate that XErp1 is a critical target for Plx1 in APC/C activation and CSF release. XErp1 degradation in extract is shown to depend on CaMKII activity, the sole mediator of the fertilization signal. As opposed to XErp1, the closely related Emil protein does not appear to be regulated by CaMKII in terms of its degradation.

In this chapter the results of this work will be discussed with respect to their contribution to the understanding of CSF arrest and a possible mechanism of polo-like kinase-mediated APC/C activation. The data collected here will also be put into context with studies, that have been published on this topic throughout the course of this work and shortly thereafter. Moreover, emphasis will be put on observations that were not followed up on in this work, but pose interesting questions for future work on the subject.

3.1 XErp1 behaviour in maturing oocytes and CSF extracts

Analysis of XErp1 function has shown that it is an important component of CSF activity in *Xenopus* egg extracts. According to the current view in the field, CSF

components have to fulfil basic criteria. These derive from properties that CSF activity shows: the component should (1) become active during oocyte maturation (2) be present and active in metaphase of meiosis II (3) be inactivated upon fertilization or parthenogenetic activation of the egg (see also introduction). In this section the results obtained for XErp1's behaviour during oocyte maturation and in CSF extracts will be discussed based on the criteria outlined above. The question of how XErp1 becomes active in the first place is of particular interest in this context.

In CSF extracts XErp1 exists in a form with reduced gel mobility that was shown to result from protein phosphorylation (N. Rauh, see (Schmidt et al., 2005)). In the analysis of whole oocytes undergoing meiotic maturation a similar electrophoretic shift was observed around the meiosis I to II transition that persists until CSF arrest. It is tempting to assume that both shifts correspond to the same modification. Protein phosphorylation is often used by the cell to modulate protein activity, thus it seems reasonable to postulate that XErp1 activity is modulated by phosphorylation throughout oocyte maturation. Since the functional investigation has shown that XErp1 is active as an APC/C inhibitor in the CSF state (consistent with the second criterion) and the modification occurs before CSF arrest, it also seems legitimate to hypothesize that if this phosphorylation modifies XErp1 activity, it would probably have a positive effect (i.e. be an activating phosphorylation). This would be consistent with the first criterion, stating that a CSF component would have to become active during oocyte maturation.

From a simplified point of view, progression through the meiotic cell cycle with two consecutive nuclear divisions is, like the mitotic cell cycle, regulated by alternating phases of Cdk1 and APC/C activity. However, XErp1 is present throughout meiosis which would exclude APC/C activation during the whole process if XErp1 was always active (i.e. also in meiosis I). This is clearly not what has been observed, lending further support to the hypothesis, that XErp1 activity is turned on or relieved from repression at a later stage of oocyte maturation before it fulfils its function at metaphase of meiosis II. The timing of the putative activating phosphorylation well after GVBD would fit with activation in meiosis II. The upshift is clearly delayed with respect to activation of the MAPK pathway, making it seem unlikely that it is MAPK itself that phosphorylates XErp1. This would fit well with the results showing that MAPK activity is not strictly required for XErp1 activity at least under overexpression conditions.

For Emi1, a protein closely related to XErp1, it has been suggested that downstream mediators of the MAPK-pathway influence its activity (Paronetto et al., 2004). In particular, phosphorylation of mouse Emi1 by p90^{RSK} was published to enhance its binding to the APC/C activator Cdc20. This is thought to promote its potential as an APC/C inhibitor to ensure maintenance of CSF arrest in mouse oocytes. However, these results have been questioned by a recent publication showing that mice lacking all three p90^{RSK} isoforms do not show defects in CSF arrest (Dumont et al., 2005). Moreover, a study showing that mouse XErp1 (which is here referred to as Emi2) is required for CSF arrest does not find such a role for Emi1 (Shoji et al., 2006). Also, the study that initially proposed a role for Emi1 in CSF arrest in *Xenopus* shows that Emi1 like XErp1 does not require full MAPK activity to prevent CSF release when overexpressed (Reimann and Jackson, 2002).

In summary, XErp1 is clearly modified during oocyte maturation and this modification is probably an activating phosphorylation. The kinase that could carry out this phosphorylation is currently unknown. Possible candidates include the kinases already implicated in CSF-arrest establishment such as Cdk2 or downstream mediators of MAPK activity. The data shown in this work do not support a role for the MAPK-pathway or its downstream mediators, but do not completely exclude it either (see also below).

3.2 XErp1's contribution to CSF arrest

Since its discovery as a biochemical activity in 1971 many proteins have been implicated in CSF arrest. Some of these are thought to act in linear pathways (mos/MAPK/p90^{RSK}/Bub1), others have been identified as independent components of CSF activity or their relationship to the already known pathways has not been studied (Emi1 and Cdk2). The loss-of-function studies conducted on XErp1 clearly show that it is essential for CSF arrest maintenance in *Xenopus* egg extracts (see **Figure 3.1a**).

As mentioned before, the available evidence collected suggests that this function might be independent of a fully active MAPK pathway. However, there is work showing that under certain circumstances low levels of MAPK activity might be sufficient to ensure CSF arrest maintenance (Yamamoto et al., 2005). This study also discusses the possibility that in experiments in which small molecule MAPKK inhibitors are used, these might not be potent enough to lower MAPK activity under the critical threshold that would interfere with CSF arrest maintenance. This would question the conclusion reached in this work as

well as those reached for Emi1 concerning their functional independence of the MAPK-pathway (Reimann and Jackson, 2002; Schmidt et al., 2005). On the other hand, the data shown in the study by Yamamoto *et al.* clearly contradict the current view that the MAPK-pathway is required only for CSF arrest establishment but not maintenance based on several studies addressing this question (for a review see (Tunquist and Maller, 2003)). Thus, the question of whether MAPK activity or its downstream mediators can be placed in one pathway with XErp1 (and/or Emi1) or are completely separate entities cannot be answered, yet.

The MAPK-pathway and Cdk2, the two classical CSF components, are thought to act in an additive fashion (Tunquist et al., 2002). Whereas a possible link between the MAPK-pathway and the two novel seemingly independent elements of CSF activity Emi1 and XErp1 has at least been addressed experimentally, no data exist on a possible link between these proteins and Cdk2. In *Xenopus* it has been shown that a complex of Cdk2 and cyclin E functions during the meiotic cell cycle (Rempel et al., 1995). The kinase activity of this complex increases markedly around entry into meiosis II. This would fit well with the observed modification of XErp1 after GVBD. The mechanism of Cdk2-mediated CSF arrest is presently unclear (Gabielli et al., 1993; Tunquist et al., 2002). Thus, the hypothesis that Cdk2 targets XErp1 to enhance its APC/C inhibitory activity is valid and worth testing. This would put XErp1 downstream of Cdk2/cyclin E.

Very recent work on *Xenopus* oocyte maturation shows that there are redundant pathways for MPF activation after progesterone exposure (Haccard and Jessus, 2006). It is thought that both of these pathways are normally active, but none of them is exclusively required for oocyte maturation to take place. One pathway acts by directly activating Cdk1 through the synthesis of new B-type cyclins, the other acts by inducing the synthesis of mos. As we have seen before, mos activates the MAPK-pathway and its downstream component p90^{RSK}. This kinase is able to inhibit Cdk1-inhibitory kinases and thereby leads to activation of MPF (Palmer et al., 1998). The fact that new cyclin synthesis in the absence of MAPK-pathway activity would be sufficient to observe seemingly normal oocyte maturation opens the possibility that the effects seen on CSF arrest after MAPK inactivation are an indirect consequence of processes that usually happen after MAPK activation in meiosis I. It is tempting to speculate that one such process could also be Cdk2/cyclin E activation in meiosis II. This would put the MAPK-pathway upstream of Cdk2/cyclin E which might in turn be upstream of XErp1. Such a view would unify most

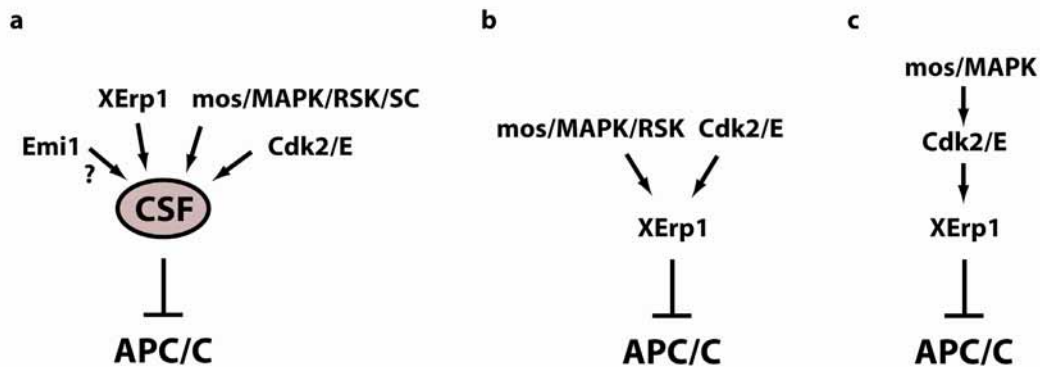


Figure 3.1: Alternative models of how XErp1 could contribute to CSF. (a) XErp1 could be an independent element among the other already identified components of CSF. This would support a view where CSF is the sum of several independent pathways inhibiting the APC/C. (b and c) Data produced in this work suggest that XErp1 might get activated through phosphorylation. Also, there is serious doubt about the role of Emi1 in CSF arrest. The mechanism of SAC-mediated CSF arrest is unclear. Thus, XErp1 could be the only APC/C inhibitor important for CSF onto which already described pathways converge.

activities that have so far been shown to be critical for CSF activity into one common pathway (see **Figure 3.1c**). However, until now no data exist to support this speculation. Alternatively, the two pathways could converge on XErp1 independently, so that phosphorylation of XErp1 by downstream mediators of the MAPK-pathway and Cdk2 are both necessary for full XErp1 activity (see **Figure 3.1b**).

As a last point the injection experiments should be taken into account here. Clearly, the injection of the XErp1 functional carboxy-terminus leads to a cell cycle arrest similar to that observed for other factors implicated in CSF activity, suggesting that XErp1 acts as a CSF also *in vivo*. However, in this experiment the exact arrest point is not defined. It has not been analysed whether the XErp1 injected blastocysts arrest with high Cdk1 activity and metaphase-like spindles. Thus, in principle other mechanisms than APC/C inhibition before anaphase could have led to a cleavage arrest anywhere in the cell cycle. Although this would be surprising, this is an issue that needs more detailed analysis.

Taken together, this work has shown that XErp1 is an essential component of CSF arrest in *Xenopus* egg extract. Recent work on mouse oocytes has shown that the mouse homolog also fulfils this function *in vivo* and it is likely that this is also true for XErp1 in intact *Xenopus* oocytes (Shoji et al., 2006). How XErp1 function relates to the other pathways that have been implicated remains largely unclear.

3.3 Mechanism of XErp1 function

The experiments carried out in this work show that XErp1 is able to significantly stabilize APC/C substrates in extract and that it can inhibit APC/C^{Cdc20}-dependent ubiquitylation *in vitro*. These activities require the presence of the ZBR. Similar results have been obtained for XErp1's close relative Emi1 (Reimann et al., 2001a). The mechanism of Emi1-mediated APC/C inhibition has been studied in some detail (Reimann et al., 2001b). Emi1 is thought to bind the substrate binding region of the APC/C activator Cdc20 thereby preventing the recruitment of substrates to APC/C^{Cdc20}-complexes. How XErp1 exactly acts to inhibit APC/C^{Cdc20} has not been investigated in this work. However, the sequence similarity of XErp1's carboxy-terminus to Emi1 suggests that the mechanism by which XErp1 and Emi1 inhibit the APC/C might be largely the same.

A close relative of Cdc20 is the other well characterized APC/C activator Cdh1. In mitotic cells Cdh1 functions in late mitosis and G1-phase to target late mitotic proteins for degradation and to prevent premature reaccumulation of cyclins, respectively. Emi1 has been shown to be able to inhibit APC/C^{Cdh1}-dependent ubiquitylation and stabilize cyclin A (Reimann et al., 2001b). In dividing Hela cells accumulation of Emi1 and the resulting stabilisation of cyclin A was shown to promote S-phase progression (Hsu et al., 2002). However, this result is called into question by the very recent analysis of embryos from Emi1 knock-out mice, which show normal S-phase progression (Lee et al., 2006). Nevertheless, abnormal cyclin A levels and a clear requirement for Emi1 in mitotic cell division is observed.

Whether XErp1 is also able to inhibit APC/C^{Cdh1} has not been tested in this work. Again, the level of conservation between XErp1 and Emi1 make it seem possible that this is the case. However, the functional significance of XErp1 inhibiting Cdh1 would remain elusive, since the Emi1 knock-out embryos show that functional redundancy between XErp1 and Emi1 at least in terms of mitotic progression might not exist (Shoji et al., 2006). An involvement of Cdh1 in meiotic processes in *Xenopus* has recently been published (see introduction), but no obvious functional link to CSF activity exists (Papin et al., 2004). Thus, if XErp1 is also able to functionally inhibit APC/C^{Cdh1} in a cellular context this is likely to be unrelated to its function in CSF arrest which was subject of this work.

3.4 Link between XErp1 and Plx1

The question of how Plx1 might contribute to APC/C activation has long been tried to answer by looking at the effects of direct phosphorylation of APC/C subunits by Plx1 (Golan et al., 2002; Kotani et al., 1998; Kraft et al., 2003). Results from this work together with studies that have been carried out in parallel on Emi1 now show that Plx1-mediated inactivation of APC/C inhibitory proteins might answer the question at least in part (see *Figure 3.2*).

XErp1 was initially identified as a Plx1 binding protein in a yeast two-hybrid screen using kinase-dead Plx1 as a bait (Duncan and Nigg, see (Schmidt et al., 2005)). Typically kinase/substrate interactions are thought to be fairly transient which would lower the chances of detecting such an interaction in a yeast two-hybrid approach. However, the kinase-dead form of Plx1, which can bind substrates but not phosphorylate them, was used

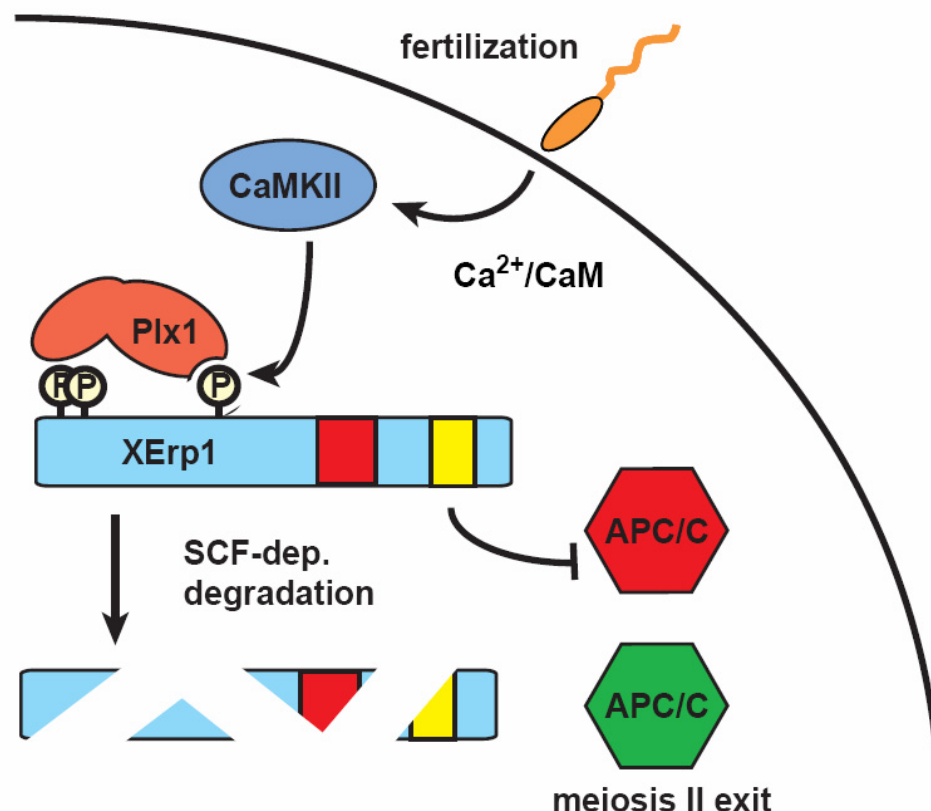


Figure 3.2: Link between XErp1 function in CSF arrest and Plx1 function in APC/C activation. In the CSF state the APC/C is kept inactive by XErp1. After fertilization, calcium release leads to enhanced binding of Plx1 to XErp1 (Rauh et al., 2005). Plx1-mediated phosphorylation of the phospho-degron sequence leads to destruction of XErp1 which derepresses the APC/C and allows for meiosis II exit. It is seems likely that the destruction is carried step is SCF^{B-TRCP}-dependent.

for the screen. This might have led to prolonged trapping of substrates in a complex with the kinase facilitating the identification of XErp1. In this work the physical interactions of XErp1 and Plx1 were not further characterized. Experiments examining the interaction of XErp1 and Plx1 have been conducted in the context of a study investigating the role of the calcium signal for XErp1 degradation (N. Rauh, see (Rauh et al., 2005)).

Proving, that a certain kinase directly phosphorylates a substrate *in vivo* or even in an extract is virtually impossible, because the system is much too complex and intermitting players can never be ruled out. Thus, only correlating *in vivo* data with *in vitro* evidence leads to a sufficient level of confidence that a protein is a direct substrate of a kinase in a physiological setting. However, *in vitro* phosphorylation reactions are often flawed by promiscuity of the kinase concerning the substrate itself and sites that are phosphorylated within the substrate. XErp1 has a fairly high serine/threonine content of about 14 % which would predict the protein to be prone to unspecific phosphorylation. In the case of XErp1 the results of the *in vitro* phosphorylation assays clearly show that XErp1 can be a substrate for Plx1. Importantly, it is only the amino-terminus which is phosphorylated, whereas the carboxy-terminus is not phosphorylated at all. Since the phosphorylatable residues are approximately evenly distributed throughout the sequence of XErp1, this shows that the *in vitro* reaction cannot be completely unspecific. Moreover, removal of the putative target serines of the phospho-degron sequence from XErp1 led to a significant decrease in phosphate incorporation into the full-length protein providing evidence that these two residues are preferential sites of Plx1-mediated phosphorylation.

Evidence that this phosphorylation happens in the extract comes from experiments examining the behaviour of mutants in these two sites in the extract and from Plx1 loss-of-function experiments. These experiments clearly show that changing the respective residues to non-phosphorylatable ones completely stabilizes the protein after CSF release has been triggered. Also, the protein is not degraded when dominant-negative MBP-PBD is present before calcium is added to the extract. This experiment has however the caveat, that XErp1 degradation, Plx1 activity and CSF release are tightly coupled. Thus, it cannot be distinguished here whether XErp1 stability is a direct consequence of Plx1 loss-of-function or of inhibited CSF release. Notably, the fact that XErp1 is stabilized under such conditions is consistent with the model that Plx1-dependent phosphorylation and degradation of XErp1 is required for CSF release. Experiments to show that XErp1 is stabilized by Plx1 loss-of-function independently of cell cycle progression in anaphase

arrested extracts have been carried out in the context of the project investigating the CaMKII requirement for XErp1 degradation (Rauh et al., 2005).

A similar link to Plk1 has been found for the XErp1-related Emi1 protein. Initially, it was shown that Plk1 phosphorylation greatly enhances its recognition and ubiquitylation by SCF^{β-TRCP}-complexes *in vitro* (Moshe et al., 2004). It has also been shown that Emi1 destruction, which happens in prophase in mitotic cell cycles, depends on Plk1 phosphorylation and subsequent recognition by SCF^{β-TRCP} (Hansen et al., 2004; Margottin-Goguet et al., 2003). Emi1 contains a phospho-degron sequence similar to that found in XErp1 (DSGXXS instead of DSGXXXS) which is required for its degradation. Notably, for XErp1 it has not been formally demonstrated, that it is in fact the SCF^{β-TRCP}-complex which targets the protein for degradation, even though this seems extremely likely.

A clear link between XErp1 and Plx1 function has been established by the experiments combining XErp1 and Plx1 loss-of-function. The result that inactivation of the Plx1-substrate XErp1 is sufficient to overcome an PBD-induced block in CSF release demonstrates that XErp1 is an important target of Plx1-dependent APC/C activation pathways. However, slight differences in the kinetics of H1 kinase activity loss upon CSF release leave room for a more complex interpretation of the results involving unknown players. It is apparent that the calcium-induced CSF release is always faster than the one induced by XErp1 inactivation. This could be due to incomplete or less efficient inactivation of XErp1 by the inhibitory antibody as compared to degradation. Assuming that the antibody inactivates XErp1 as efficiently as calcium, this observation could be taken as evidence that calcium activates other pathways than the Plx1/XErp1-mediated pathway of APC/C activation. Also, calcium-induced CSF release in the absence of XErp1 is slower when wildtype PBD is present as opposed to the mutant. This could indicate that Plx1 has other targets than XErp1 that facilitate APC/C activity upon CSF release. Thus APC/C activation by Plx1 could indeed be chiefly caused by XErp1 inactivation, but supported by phosphorylation of APC/C subunits or other proteins that influence APC/C activity.

3.5 Relationship between XErp1 and Emi1

In this work XErp1 protein was characterized as a Plx1-regulated APC/C inhibitory protein required for CSF arrest in *Xenopus* egg extracts. Some aspects of XErp1 function and regulation have also been ascribed to the closely related Emi1 protein before or during

the course of this work. This immediately poses the question of whether the cellular functions of these two proteins are separable, redundant or partially overlapping. As this work was focussed on a basic characterization of XErp1 independently of Emi1, almost no comparative data have been acquired to elucidate the relationship of XErp1 and Emi1 (except for the data on CaMKII-mediated degradation in anaphase). Yet, the literature available on Emi1 and the work of others as well as this work on XErp1 allow for some discussion of this issue.

The available data on XErp1 and *Xenopus* Emi1 have until very recently suggested that they function in the same processes (namely CSF arrest, APC/C inhibition) and are regulated in the same way (Plk1/Plx1-dependent destruction by SCF ^{β -TRCP}). However, doubts about whether Emi1 could function in CSF arrest were raised by several observations. First, an Emi1 induced block to cell cycle progression stabilizes cyclin A and B, whereas a *mos*-induced cell cycle arrest stabilizes only cyclin B. Notably, it has not been tested whether XErp1 induced cell cycle arrest in extracts stabilizes cyclin A in addition to cyclin B. Second, the timing of Emi1 degradation in somatic cells already in prophase seemed incompatible with a function of Emi1 in metaphase of meiosis II, unless one assumes meiosis specific regulation of the degradation process. However, no evidence existed for such a mechanism.

More severely, Ohsumi *et al.* raised an apparently sensitive antibody to Emi1 and found the protein to be absent from maturing and CSF arrested oocytes (Ohsumi *et al.*, 2004). They also found that exogenous Emi1 is unstable in CSF extracts, an observation also made by Jackson and colleagues (Ohsumi *et al.*, 2004; Tung *et al.*, 2005). Yet, data from the Jackson laboratory support the view that endogenous Emi1 is present at detectable levels in CSF-arrested oocytes (Tung *et al.*, 2005). An explanation for the differences between the exogenously added protein and the endogenous Emi1 in terms of their stability does not exist so far. Thus, the question of Emi1's presence in CSF-arrested oocytes is not definitively answered at the moment.

Apart from the discussion about Emi1's presence or absence from CSF arrested eggs, an important issue is the functional redundancy between XErp1 and Emi1, provided Emi1 would be present. This issue has not been addressed satisfactorily due to the lack of specific reagents. It has been realized that some antibodies that have been used to study Emi1 function also recognize XErp1 (Tung *et al.*, 2005; Tung and Jackson, 2005). Thus, especially the data obtained from "Emi1" loss-of-function experiments likely reflect the

effect of combined inactivation of Emi1 and XErp1. Since this work has shown that specific inactivation of XErp1 with an antibody raised against a non-conserved region is sufficient to ablate CSF activity, the role for Emi1 in this context remains to be clarified. This would have to include selective inhibition of Emi1 function in CSF extracts as an important piece of data to resolve this issue.

An important hint as to whether XErp1 and Emi1 both function in CSF arrest might be the experiment investigating the CaMKII requirement for XErp1 and Emi1 degradation in anaphase extract. Whereas XErp1 degradation in anaphase is strictly CaMKII-dependent, Emi1 is constitutively degraded seemingly unaffected by the presence or absence of CaMKII activity. This would lead to the conclusion that XErp1 but not Emi1 stability is regulated by fertilization. However, the observations made on Emi1 in this experiment are difficult to interpret, since two groups have already observed independently that exogenous IVT Emi1 might behave different from the endogenous protein showing that it is unstable even in the CSF state (Ohsumi et al., 2004; Tung et al., 2005).

Taken together, recent publications and the experiments presented in this work do not conclusively resolve the problem of XErp1 vs. Emi1 in CSF arrest. However, evidence accumulates that argues for a view in which XErp1 is the main player in regulating the meiosis-specific cell cycle arrest termed CSF, whereas Emi1 is more important for mitotic cell division.

3.6 Conclusion

This work was aimed at characterizing XErp1 function and regulation. It was based on models which predict the existence of putative APC/C inhibitors that regulate APC/C activity and CSF release. As a result of the basic characterization of XErp1 and further functional analyses, this protein seems to have many properties that these models predict. The functional characterization of XErp1 has contributed to the understanding of APC/C activation by polo-like kinases and to the understanding of CSF arrest of vertebrate oocytes. Open questions remain mainly concerning a possible activation of XErp1 during oocyte maturation. Also, a possible functional relevance of the F-box in XErp1, an aspect that has not been covered in this work, will be of interest for future research.

4 Materials and Methods

4.1 Chemicals and buffers

All chemicals that have been used for this study were at least of purity grade p.a. (*pro analysi*). Buffers and solutions were prepared with deionized water from a Milli-Q system (Millipore GmbH, Germany) which will be referred to as H₂O. Buffers and solutions were either autoclaved or sterile filtered before use.

4.2 Molecular Biology

Standard molecular biology techniques have been used to generate, clone and subclone DNA encoding the genes of interest or fragments thereof. Plasmid DNA was usually purified from the *E. coli* strain TG1 using the Qiagen Mini kit according to the manufacturers instructions. DNA fragments were isolated from agarose gels using the Qiagen gel elution kit. Restriction digests were carried out as recommended by the enzyme supplier (New England Biolabs). Polymerase chain reaction (PCR) was carried out using Pfu Turbo polymerase (Stratagene). Ligation reactions were done overnight at 18 °C with T4 ligase (Roche).

4.2.1 Cloning and Mutagenesis of XErp1, Emil and Plx1 fragments

Full-length XErp1 was initially cloned by Peter Duncan and was then subcloned from the identified two-hybrid plasmid into a modified pCS2 vector introducing restriction sites for the FseI and AscI restriction enzymes. Primers with the sequences 5'-ATTATGGCCGGCCAGAGATGGCAAATCTCTTAGAG-3' and 5'-ATTATGGCGCGCCGGAAGACTAGCTTCAAAGTCTC-3' were used to amplify the coding sequence. Site-directed mutagenesis to yield the C583A mutation in the ZBR was carried out by Peter Duncan using the QuikChange kit (Stratagene) according to the manufacturer's instructions. The amino-terminal and carboxy-terminal fragments of XErp1 comprise the amino acids 1–424 and 374–651, respectively. Wild-type or mutant fragments were subcloned from plasmids containing the desired full-length XErp1 as a template following the strategy described above. For the carboxy terminal fragment primers 5'-ATTATGGCCGGCCAGCTCAGATGAAAGCAGAGTCC-3' and 5'-

ATTATGGCGCGCCGGAAGACTAGCTTCAAAGTCTC-3' were used. The amino-terminal fragment was subcloned into a modified pMAL vector by Thomas Mayer.

The polo-box domain construct (PBD^{wt}) comprising amino acids 358–598 of Plx1 was subcloned from full-length Plx1 in a pCS2-Myc plasmid using primers 5'-ATTATGGCCGGCCGGAGTTCACGGAGCCTGC-3' and 5'-ATTATGGCGCGCCCTATGCCGAGGCCTTTAC-3'. Full-length Emi1 was cloned from a *Xenopus* oocyte cDNA library using primers 5'-ATTATGGCCGGCCCAATATGATGTGCGGATTTGCAAGTAACC-3' and 5'-ATTATGGCGCGCCCTATAACCTCCGTAAATTCTGTTTGC-3' into a modified pCS2 vector.

4.2.2 List of used plasmids

The plasmids that have been used in this for protein expression or coupled *in vitro* transcription/translation are listed below referring to the TUM number in the Mayer laboratory plasmid collection:

Table 1: List of plasmids used in this work. TUM numbers reflect the position in the Mayer laboratory plasmid collection which contains further information on these constructs.

TUM	gene	insert	vector
101	XErp1	WT, fl	pCS2-FA
94	XErp1	WT, fl	pCS2-Myc-FA
155	XErp1	WT, fl	pMAL-Tev-FA
156	XErp1	C583A, fl	pMAL-Tev-FA
437	XErp1	WT, 374-651	pCS2-FA
201	XErp1	WT, 374-651	pCS2-Myc-FA
271	XErp1	WT, 374-651	pMAL-Tev-FA
389	XErp1	C583A, 374-651	pCS2-Myc-FA
697	XErp1	C583A, 374-651	pMAL-Tev-FA

269	XErp1	WT, 1-424	pMAL-Tev-FA
380	XErp1	S33N/S38N, fl	pMAL-Tev-FA
381	XErp1	S33N/S38N, fl	pCS2-FA
535	XErp1	T195A, fl	pCS2-FA
174	Emi1	WT, fl	pCS2-FA
341	Plx1	WT, 358-598 (PBD)	pMAL-Tev-FA
423	Plx1	W408F/H532A/K534H, 358-598 (PBD)	pMAL-Tev-FA
237	cyclin B	WT, NT	pCS2-FA
163	securin	WT, fl	pCS2-FA

4.3 Protein Biochemistry

Recombinant proteins for functional analysis in *Xenopus* egg extract or for *in vitro* assays have been produced by expression in bacteria or SF9 cells and subsequent affinity-tag purification or by coupled *in vitro* transcription/translation in either wheat-germ extract or reticulocyte lysate. Standard protocols have been followed to analyse proteins and extracts by SDS-PAGE and Western blotting.

4.3.1 Protein expression and purification from bacteria

Bacteria of the *E. coli* strain BL21 were transformed with a suitable plasmid for expression of Maltose-binding protein (MBP)-tagged fusion proteins (pMal-Tev-FA, a modified form of pMal (New England Biolabs) encoding Tev-protease cleavage sites and unique FseI and AscI restriction sites in the MCS). Transformed clones were picked after overnight culture on LB-agar plates containing the selective antibiotics ampicillin (amp) and chloramphenicol (chl). A liquid overnight culture was used to inoculate 2 to 8 litres of LB^{amp, chl} containing 0,2 % Glucose and 0,3 M saccharose. The culture was grown until

OD⁶⁰⁰ reached 0,6 and was then induced for protein expression by addition IPTG to a final concentration of 0,3 mM. Expression was allowed for 3 hours at a temperature of 30 °C.

After expression the cultures were spun down and the bacterial pellet resolved in column buffer (20 mM HEPES 7.7, 200 mM NaCl, 1mM EDTA, 1mM DTT). The bacteria were lysed by high pressure treatment in a french press apparatus. The lysate was then cleared by centrifugation in a Beckmann Ti45 rotor at 27000 rpm for 35 minutes. For affinity purification the cleared lysate was incubated with amylose beads (New England Biolabs) for at least 1 hour at 4 °C. The beads were the batch-washed with column buffer without DTT three times and allowed to settle onto a column. The fusion proteins were eluted from the column in fractions with elution buffer (column buffer without DTT containing 20 mM Maltose). The fraction size was adjusted to approximately equal the bed volume of the column.

Fractions were analysed by PAGE and the ones containing protein of sufficient concentration and purity were dialysed against a storage buffer (20 mM HEPES 7.7, 50 mM KCl, 40 % Glycerol) and stored in aliquots at -80 °C until further use.

4.3.2 Protein expression and purification from SF9 cells

Protein expression and purification from SF9 cells was carried out with the help of Thomas Mayer and Jenny Bormann. SF9 insect cells were kept at 27 °C in TC-100 medium (Gibco or PAN) supplemented with 10 % fetal calf serum (FCS) and antibiotics (1000 units/ml penicillin G and 1 mg/ml streptomycin). For protein expression SF9 cells were seeded on 15 cm culture dishes at of 2×10^7 cells per dish. Cells were infected one hour later with the desired baculo-virus by addition of fresh culture medium containing the desired recombinant baculo-virus supernatant. After incubation for 48 hours the cells were collected by washing them off the dish with culture medium (for purification of recombinant Plx1 kinase, cells were cultured in medium containing 100 nM okadaic acid three hours prior to harvesting). Then, the collected solution was centrifuged at 400 x g for 10 minutes. The supernatant was aspirated and the pellet flash frozen in liquid nitrogen and stored until purification.

For purification the pellet was resuspended in lysis buffer (different, depending on the expressed protein) and either dounced in a glass homogenizer or lysed in a french press apparatus. The lysate was cleared by centrifugation and bound to Ni-NTA beads (Qiagen)

at least 1 hour at 4 °C. After binding beads were batch-washed in lysis buffer and loaded onto a column. Proteins were eluted from the column in lysis buffer containing 200 – 250 mM imidazole. Purity of the eluted fractions was accessed by PAGE and the desired fractions were flash frozen in liquid nitrogen and stored at -80 degrees until further use.

4.3.3 Coupled *in vitro* transcription/translation (IVT)

IVT reactions to make ³⁵S-Met-labelled proteins were carried out using the TNT kit (Promega) in either wheat-germ extract or reticulocyte lysate according to the manufacturer's instructions. The reactions were programmed with 1 µg of the desired variant of pCS2 plasmid DNA and allowed to go on for approximately two hours at 30 °C. To make non-radioactive IVT products -Met amino acid mix was complemented with -Leu mix instead of ³⁵S-Met.

4.3.4 Production of antibodies

The antibody against XErp1 (XErp1^{NT}) that was mainly used for this work was produced by Peter Duncan as described in Schmidt *et al.*. Others antibodies against XErp1 were produced from recombinant full-length XErp1 protein. To this end full-length XErp1 was purified from bacteria as described in 4.3.1. Sufficiently concentrated protein fractions were then incubated with TEV protease to remove the amino-terminal MBP-tag. Since full-length XErp1 is insoluble without the MBP-tag, the precipitate was boiled in sample buffer and subjected to preparative PAGE. The gel was negatively stained in 0,2 M imidazole, 0,1 % SDS and developed with 0,2 M ZnSO₄. After washing in H₂O the desired band containing the full-length XErp1 protein without tag was cut out. The gel pieces containing the protein were eluted in 0,5 x running buffer in a gel elution apparatus (BIO-RAD). Different elutions were collected and concentrated by ultrafiltration using the Amicon system (Millipore). The antigen was send to Elevage Scientific des Dombes (ESD, France) for injection into rabbits.

4.3.5 Affinity purification of antibodies

Antibodies were affinity purified from the desired serum over an HiTrap NHS-activated sepharose column (1 ml column volume, Amersham Biosciences) coupled to the antigen. The columns are shipped filled with isopropanol. To remove the isopropanol and prepare the column for coupling the antigen, the column was washed with 2 times 3 ml ice cold 1 mM HCl. After washing the solution containing the antigen (1 mg/ml in 20 mM

HEPES pH 8.3, 500 mM NaCl, 1 mM EDTA) was directly applied to the column and incubated for one hour at room temperature. After coupling the unbound fraction was removed by washing the column following the procedure:

Inject 6 ml of Buffer A.

Inject 6 ml of Buffer B.

Inject 6 ml of Buffer A.

Leave the column for 15-30 min in room temp. or approx. 4 hours in +4°C.

Inject 6ml of Buffer B.

Inject 6 ml of Buffer A.

Inject 6 ml of Buffer B.

Finally, inject around 5 ml TBS.

Buffer A is 0,5 M ethanolamine, 0,5 M NaCl, pH 8,3

Buffer B is 0,1 M acetate, 0,5 M NaCl, pH 4

After the column has been coupled to the antigen serum was allowed to circulate over the column to bind the antibodies. To this end the column was washed with 3ml TBS, then with 3 ml elution buffer (0,15 M NaCl, 0,2 M Glycin pH 2,3) and again with 5 ml TBS. 2-4 mls of serum were mixed 1:1 with PBS and the solution was centrifuged for 10 min at 4500 xg at 4 °C. The supernatant was applied to the column by pumping it over the column repeatedly over night.

Before elution of the antibodies the column was again washed with 5ml TBS, 3ml wash buffer (20 mM Tris pH 7,5, 500mM NaCl, 0,2 % Triton X 100) and 3 ml TBS. The antibodies were eluted in 500 µl fractions with elution buffer. The tubes for fraction collection were prefilled with 90 µl Tris pH 8.5 to neutralize the pH. The pH was checked for neutrality and further adjusted if necessary. Initially, the fraction were tested by spotting 1µl on nitrocellulose membrane and Ponceau staining. Protein containing fractions

were examined by SDS-PAGE. The desired fractions were dialysed against PBS and stored at 4°C.

4.4 Cell biology

All cell biological experiments to characterize XErp1 function were carried out using the *Xenopus* egg extract system or *Xenopus* eggs. The protocols for extract preparation and analysis described below are largely based on the standard procedures described by Murray and Desai and colleagues (Desai et al., 1999; Murray, 1991). The buffers and solutions used for the experiments were exactly as described by Desai *et al.*. These include: Marc's modified Ringer medium (MMR), CSF extraction buffer (CSF-XB), dejellying solution, energy mix, phospho-creatine kinase, ubiquitin, cytochalasin B.

4.4.1 Preparation of CSF arrested *Xenopus* egg extract

Female *Xenopus laevis* frogs were induced to ovulate by injection of 800 units human chorionic gonadotropin (Sigma) into the dorsal lymph sack approximately 1 day before egg collection. Frogs were put into tanks filled with 1x MMR for ovulation. Laid eggs of sufficient quality were collected and washed with 1x MMR. Subsequently they were treated with dejellying solution for up to ten minutes and then washed into CSF-XB. At this step activated and lysed eggs were removed as far as possible before transferring the eggs into centrifugation tubes pre-filled with 1 ml CSF-XB containing 0,1 mg/ml cytochalasin B. The eggs were then compacted by a two-step centrifugation for 1 minute at 1000 rpm and another minute at 2000 rpm in a Sorvall HB-6 rotor. Excess buffer was removed from the tube after centrifugation leaving only the compacted, but intact eggs behind.

In a final centrifugation step the eggs were then lysed by centrifugation for 10 minutes at 10000 rpm at 4 °C leading to a characteristic separation and layering of the egg components in the tube. The middle layer contained the cytoplasmic extract which was isolated by puncturing the tube at the bottom of this layer and sucking it out with a 1 ml syringe. After isolation the extract was supplemented with energy mix (1:50), phospho-creatine kinase (1:1000), ubiquitin (1:250) and cytochalasin B (1:1000) and stored on ice until further use. Routinely, an aliquot of the extract was tested for integrity of the CSF arrest and the ability to release from CSF arrest after addition of 0,6 mM CaCl₂ by examining the morphology of added *Xenopus* sperm nuclei (500 to 1500 nuclei/μl extract).

To this end 1 μ l of extract was pipetted into a 3 μ l drop of fixing solution containing DAPI (4',6-diamidino-2-phenylindole) on a glass slide and squashed under a coverslip. For some experiments examining spindle morphology low amounts of rhodamine-labelled tubulin was added to the extracts.

4.4.2 Experiments examining protein stability in extract

To examine the stability of different XErp1 variants or cell cycle markers, CSF extract was supplemented with up to 1/10 vol. of an IVT reaction containing 35 S-Met-labelled protein of interest on ice. The extract was then warmed to 20 °C and reactions containing the same IVT were split into two aliquots one of which was treated with 0,6 mM CaCl_2 to induce CSF release. Samples were taken at different times, boiled in sample buffer usually in a 1:10 dilution and flash frozen in liquid nitrogen for further analysis.

The cell cycle state of the extract at the different time points was controlled by microscopic inspection of DNA morphology and/or assaying extract aliquots for H1 kinase activity. The experiments were analysed by running up to 1 μ l of extract/lane on an SDS-PAGE gel and subsequent autoradiography of the dried gel.

4.4.3 Recombinant protein addition to CSF extract

To investigate their effect on *in vitro* cell cycle progression recombinant proteins were added to CSF-arrested egg extract. Aliquots of these proteins were thawed and centrifuged for 35 minutes at 100000 g at 4 °C. The concentration of the supernatant was determined using either a standard Bradford assay (BIO-RAD) or by comparing Coomassie stained gel bands to a BSA standard. Proteins were added to the extract in the nano-molar concentration range with the final volume of addition not exceeding 1/8 of the extract volume. Proteins were usually allowed to incubate in the extract for at least 10 minutes before addition of calcium or other manipulations. Here experiments were analysed by inspection of DNA morphology, assaying H1 kinase activity or analysis of cell cycle markers by SDS-PAGE/Western blotting.

4.4.4 Antibody addition and depletion of proteins

For antibody addition as well as for XErp1 depletion experiments the desired amount of polyclonal anti-XErp1 antibody or a control antibody was coupled to Protein G Dynabeads (Dyna) in PBS buffer containing 0.1% Triton X-100 (PBSTx) at 4°C. Beads

were then washed three times in PBSTx by retrieving the beads on a magnet. The washed beads were mixed with the extract and incubated on ice under occasional mixing. Depending on the experiment the antibody beads were left in the extract throughout the course of the experiment or retrieved after incubation for at least 1 hour on ice.

4.4.5 Injection of *Xenopus* embryos

To obtain CSF-arrested oocytes for injection with recombinant protein, frogs were induced to ovulate by injection of 800 units human chorionic gonadotropin (Sigma). On the next day the already ovulated eggs were discarded and the frogs were gently squeezed to induce laying of fresh eggs into 1 x MMR in a glass petri dish. Eggs from different frogs were handled separately.

To inject protein into one cell of a two-cell embryo the CSF arrested eggs have to be fertilized *in vitro*. Fertilization is carried out using the testes dissected out of a male frog after injection with 25 units pregnant mare serum gonadotropin (PMSG) 8 days and another 50 units human chorionic gonadotropin 3 days before dissection. To fertilize the eggs most of the 1 x MMR was removed from the petri dish containing the eggs, the testis was macerated from the tip and the sperm vigorously mixed with the eggs by moving the testis through the dish. Subsequently, the dish was filled with 0,1 x MMR and allowed to sit at room temperature for 20 minutes. Successful fertilization and therefore activation of the eggs is visible from a contraction of the pigmented animal pole of the embryo. To prepare the embryos for injection, they were dejellied by replacing the 0,1 x MMR with dejelling solution (2 % cysteine in H₂O, pH 7,8 with NaOH). Dejelling was complete after approximately five minutes. Embryos were washed six times in 0,1 x MMR after the dejelling step and arranged on a rubber grid with their pigmented animal poles facing up ready to inject.

Injections were carried out using home-pulled needles from borosilicate glass capillaries (0,58 mm inner diameter, 1 mm outer diameter, Hilgenberg). After pulling the needles are closed at the tip and had to be broken off to give an orifice suitable for injection. Whether needles are suitable for injection is determined by the ratio of injection volume and injection pulse time at a given pressure. Needles were calibrated by counting the number of injection pulses needed to clear the needle from 1 µl of H₂O. Needles that inject between 10 and 20 pl liquid over a pulse of approximately 300 ms can be considered

suitable for injection. To reduce errors from the calibration procedure, one experiment was carried out using the same needle.

For the actual injection a Harvard apparatus PLI-100 connected to a compressed nitrogen bottle was used with the following settings:

injection pressure (p_{inject}) was 10 psi

clearance pressure (p_{clear}) was 70 psi

balance pressure (p_{balance}) was around 1 psi

Here, the injection pressure is the pressure with which the actual injection carried out. The clearance pressure is used to clean the needle and equals the maximal pressure the apparatus can produce. The balance pressure is constantly applied to the needle and prevents clogging of the needle by the internal pressure of the oocyte after the needle has entered the cell. For each oocyte 20 pl of a 800 ng/ μl protein solution (wildtype protein or mutant control) was injected into one blastomere of a two-cell embryo. The other blastomere was left uninjected. The embryos were cultivated at 18°C for the indicated time and their phenotype was documented using a CCD camera mounted on the stereomicroscope used for injection.

4.4.6 H1 kinase assays

To measure Cdk1 activity in the extract, the activity of extract samples towards the Cdk1 model substrate histone H1 was examined in *in vitro* kinase assays using ^{32}P -labelled ATP. To this end 1 to 2 μl aliquots of extract were flash frozen in liquid nitrogen throughout the course of the experiment. For the assay the frozen extract was incubated with kinase assay mix containing 8 μg histone H1, 14 μM ATP, and 6.5 μCi ^{32}P -ATP in H1 buffer (20 mM β -glycerophosphate, 3mM MgCl_2 , 4mM EGTA, 0.025% NP40). Reactions were carried out at room temperature and stopped by boiling samples in SDS-sample buffer.

4.4.7 *In vitro* ubiquitylation assays

To assay the effect of XErp1 protein on APC/C^{Cdc20} activity, *in vitro* ubiquitylation assays on APC/C immuno-purified from M-phase *Xenopus* egg extract were performed. The protocol described here is largely adapted from procedures described by Kramer and colleagues (Kramer et al., 1998; Kramer et al., 2000).

To purify M-phase APC/C, CSF extract was released into interphase by the addition of 0,6 mM CaCl₂ and driven back into M-phase by the addition of non-destructible cyclin B (approximate final concentration 5µg/ml, a kind gift from Olaf Stemmann). Monoclonal anti-Cdc27 antibody (purchased from Sigma) was coupled to Protein G dynabeads (Dyna) in PBSTx buffer and washed twice with PBSTx and three times with CSF-XB. To immuno-purify the APC/C, Cdc27 beads and M-phase extract were mixed and incubated on ice for at least 1 hour. Cdc27 beads were then retrieved from the extract and washed twice in buffer QA⁺⁺ (10 mM Tris pH 7,5, 500 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 0,5 % NP-40) and three times in buffer QA (10 mM Tris pH 7,5, 100 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂) to yield APC/C beads.

APC/C beads were preincubated with exogenous histidin-tagged Cdc20 (or control buffer) in 30 µl QA to activate the APC/C. To analyze XErp1's effect on APC/C activity, recombinant protein that has been centrifuged and assayed for protein concentration as described in 4.4.3 was added to the reaction along with Cdc20. Preincubation was allowed for 30 minutes at room temperature before the beads were washed once in QA and three times in CSF-XB. To start the assay the activated APC/C beads were resuspended in 30 µl reaction mix containing yeast E1 enzyme (Boston Biochemicals), E2 enzyme UbcX, ubiquitin (Sigma), an ATP regeneration system and a radiolabelled IVT of the amino-terminus of cyclin B. The assay was carried out at room temperature and stopped by boiling samples in sample buffer after 0, 6 and 12 minutes.

4.4.8 Plx1 kinase assays

For kinase assays substrates were centrifuged and assayed for protein concentration as described in 4.4.3. The reaction was started by mixing the substrate proteins with a mix containing the kinase, 1mM ATP, 2 µCi ³²P-labelled ATP in assay buffer (20 mM HEPES pH 7,7, 10 mM MgCl₂, 1 mM EGTA, 1mM DTT, 5 mM NaF). Reactions were carried out at room temperature for 1 hour. The reaction was stopped by boiling a sample in sample

buffer. Samples were analysed by SDS-PAGE and subsequent autoradiography of the vacuum-dried gel.

4.4.9 CaMKII activity assays

To measure CaMKII activity in extract, samples were assayed for their ability to transfer ^{32}P -labelled phosphate to a peptide derived from the CaMKII autophosphorylation sequence (AC-2, KKALRRQETVDAL, New England Biolabs). To this end 2 μl extract samples were flash frozen in liquid nitrogen and stored at -80°C until the assay. For the assay the frozen extract pellets were resuspended in CaMKII assay buffer (70 mM HEPES pH 7.7, 0,2 mM Mg-ATP, 200 mM EGTA) containing 1 μCi ^{32}P -labelled ATP and 250 μM AC-2 (or H_2O as a background control) in a total volume of 20 μl . The reaction was incubated at room temperature for 8 minutes until 9 μl of each reaction were spotted onto P81 membrane (phosphocellulose, Whatman Inc.). The spots were allowed to dry and then the membrane was washed extensively in H_2O . After washing the membrane was analysed by phosphoimaging and densitometric analysis of the spots. For initial establishment of the assay and some experiments the membrane pieces containing one spot each were analysed by liquid scintillation counting.

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Summary

Cell division is one of the most fundamental processes in biology. Meiosis is a specialized form of cell division resulting in cells capable of sexual reproduction. In higher animals male reproductive cells are called sperm cells, the female ones are called oocytes. The life cycle of a typical sexually reproducing organism includes a point where sperm and oocyte fuse to yield a diploid zygote, which develops into a new individual. This process is termed fertilization.

The oocytes of most vertebrates are arrested at metaphase of meiosis II before fertilization. This is thought to prevent development in the absence of fertilization (parthenogenesis). In 1971 a biochemical activity was discovered that mediates this cell cycle arrest and was hence termed cytostatic factor (CSF). CSF inhibits a protein complex called the anaphase-promoting complex/cyclosome (APC/C). The APC/C is a ligase that covalently attaches the small protein ubiquitin to cell cycle regulatory proteins in order to target them for proteolytic degradation. This results in ordered cell cycle progression. After fertilization of CSF-arrested oocytes calcium triggers the APC/C-dependent destruction of anaphase inhibitors like cyclin B or securin. This allows for progression beyond metaphase of meiosis II, pronuclear fusion and the subsequent onset of embryonic development. Studies in *Xenopus* egg extract have shown that the protein kinase Plx1 of the polo-like kinase family is also required for this process.

This work has begun to characterize the protein XErp1, which has been found as interacting partner of Plx1. Function and regulation of XErp1 have been investigated in *Xenopus* egg extracts and oocytes. Inhibition of XErp1 in CSF-arrested *Xenopus* egg extracts has shown that the protein is required to maintain CSF arrest. Consistently, overexpression of XErp1 leads to an inability of the extract to activate the APC/C after a calcium stimulus which prevents metaphase to anaphase transition. These results suggested that XErp1 might be a direct inhibitor of the APC/C. Indeed, a carboxy-terminal domain of XErp1 was found to be able to directly inhibit the ubiquitin ligase activity of immuno-purified APC/C.

Experiments examining the regulation of XErp1 have shown that the protein is degraded rapidly after calcium stimulation. This degradation could be shown to be dependent on a phosphorylated degradation signal in the amino-terminus of XErp1. Furthermore, it was shown that Plx1 phosphorylates the two serine residues 33 and 38 within this motif *in vitro*. Together these results lead to a mechanistic model of APC/C activation associated with release from CSF arrest. According to this model, Plx1-dependent phosphorylation of XErp1 leads to its destruction and therefore derepression of the APC/C.

Zusammenfassung

Zellteilung ist einer der fundamentalsten biologischen Prozesse. Die Meiose ist eine spezielle Art der Zellteilung, die der Produktion von zur geschlechtlichen Fortpflanzung befähigten Zellen dient. Bei höheren Tieren bezeichnet man die männlichen Geschlechtszellen als Spermien und die weiblichen als Eizellen. Im Lebenszyklus eines typischen höheren Organismus verschmelzen Spermium und Eizelle und ergeben eine Zygote, die den Ausgangspunkt für die Entwicklung eines neuen Individuums bildet. Diesen Prozess nennt man Befruchtung.

Die Eizellen der meisten Wirbeltierspezies sind vor der Befruchtung in der Metaphase II des meiotischen Teilungszykluses blockiert, um eine Entwicklung ohne vorherige Befruchtung (Parthenogenese) zu verhindern. Dieser Arrest wird von einer 1971 entdeckten biochemischen Aktivität verursacht, die man als zytostatischen Faktor (engl. cytostatic factor, CSF) bezeichnet hat. Der zytostatische Faktor inhibiert einen Proteinkomplex, den man „anaphase-promoting complex/cyclosome“ (APC/C) nennt. Der APC/C ist eine Ligase die das kleine Protein Ubiquitin kettenförmig an andere Zellzyklus-regulierende Proteine koppelt und diese damit für den proteolytischen Abbau markiert. Der durch ein Kalziumsignal induzierte, APC/C-abhängige Abbau von Anaphase Inhibitoren wie Zyklin B und Securin ist nötig, damit die Zelle den Metaphase-Anaphase Übergang der Meiose II nach Befruchtung vollziehen kann. Desweiteren, haben Untersuchungen in Eiextrakten des Krallenfrosches *Xenopus laevis* gezeigt, dass die Proteinkinase Plx1 für die Aktivierung des APC/C am Metaphase-Anaphase Übergang nötig ist.

Diese Arbeit befasst sich mit der Charakterisierung des Proteins XErp1, dass als Bindungspartner von Plx1 identifiziert wurde. Die Untersuchungen zur Funktion und Regulation von XErp1 wurden in *Xenopus* Eiern und deren Extrakten durchgeführt. Durch Inhibition der XErp1 Funktion in CSF-blockierten Eiextrakten konnte gezeigt werden, dass XErp1 essentiell für die Aufrechterhaltung des zytostatischen Faktor Arrestes ist. Im Einklang mit diesen Daten führt die Überexpression von XErp1 zu einer Situation, in der das Kalziumsignal den APC/C nicht mehr aktivieren kann und dem zu

Folge der Metaphase-Anaphase Übergang verhindert wird. Diese Ergebnisse deuten an, dass XErp1 ein Inhibitor des APC/C sein könnte. Tatsächlich wurde gefunden, dass eine carboxy-terminale Domäne von XErp1 in der Lage ist, die Ubiquitylierungsaktivität von immun-gereinigtem APC/C *in vitro* direkt zu inhibieren.

Experimente zum Verhalten und zur Regulation von XErp1 haben gezeigt, dass XErp1 selbst unmittelbar nach dem Kalziumsignal abgebaut wird. Dieser Abbau hängt von einem Sequenzmotiv im amino-Terminus von XErp1 ab, das durch Phosphorylierung aktiviert wird. Es konnte im Folgenden gezeigt werden, dass Plx1 die Phosphorylierung an den Serinresten 33 und 38 in diesem Sequenzmotiv *in vitro* ausführen kann. Zusammen führen diese Ergebnisse zu einem mechanistischen Modell der APC/C Aktivierung, welche zur Auflösung des zytostatischen Faktor Arrestes führt. Hiernach führt die Phosphorylierung des APC/C Inhibitors XErp1 durch Plx1 nach Befruchtung zu dessen Abbau und damit zur Aktivierung des APC/C.

Abbreviations

APC/C	anaphase promoting complex/cyclosome
CaMKII	calcium/calmodulin-dependent kinase II
Cdk1	cyclin-dependent kinase 1
CSF	cytostatic factor
DNA	desoxyribonucleic acid
DTT	dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
Emi1	early mitotic inhibitor 1
GVBD	germinal vesicle breakdown
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethane sulfonic acid
i.e.	<i>id est</i> (that is)
IVT	coupled <i>in vitro</i> transcription/translation
M	molar
min	minutes
MPF	maturation-promoting factor
PAGE	polyacrylamid gel electrophoresis
PBD	polo-box domain
PBS	phosphate-buffered saline
PCR	polymerase chain reaction

Plk1	polo-like kinase 1
Plx1	<i>Xenopus</i> polo-like kinase 1
rpm	rounds per minute
SCF	Skp1 cullin F-box
WT	wildtype
<i>Xenopus</i>	<i>Xenopus laevis</i>
XErp1	<i>Xenopus</i> Emi-related protein 1
ZBR	zinc-binding region

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