# Regulation of DNA replication in *Xenopus*

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.
O Presidente do Júri,

Porto, ______/______/_________

MSc

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# Resumo

A replicação do ADN é um mecanismo essencial para a célula, pois permite a manutenção do material genético. Este mecanismo é extremamente complexo devido ao elevado número de moléculas envolvidas e como tal necessita de ser cuidadosamente regulado. A iniciação da replicação do ADN ocorre em locais específicos do genoma conhecidos como origens da replicação. Experiências anteriormente realizadas pelo grupo demonstraram que a adição de dNTPs exógenos aumenta o número de origens ativadas e consequentemente a extensão da replicação do ADN. Durante o meu estágio recorrendo a Western Blot, DNA combing e espectrometria de massa pretendeu-se determinar o efeito dos dNTPs nas proteínas envolvidas no mecanismo de replicação do ADN. Os resultados demonstraram que os dNTPs afetam a replicação ao nível da formação do complexo de pré-iniciação.

O controlo do ciclo celular previne a acumulação de erros no ADN. A via ATR/Chk1 participa neste controlo, sendo capaz de reconhecer os locais onde ocorreu formação de ADN de cadeia simples normalmente derivados do bloqueio dos garfos de replicação. Sabendo que esta via condiciona a iniciação das origens de replicação o objetivo do meu estágio seria determinar de que forma Chk1 condiciona a ativação das origens tanto na presença como na ausência de stress. Recorrendo à técnica de DNA combing e usando um inibidor de Chk1 (AZD 7762) foi demonstrado que Chk1 é capaz de inibir a ativação de grupos de origens que são normalmente ativadas tardiamente durante a fase S.

# Abstract

The DNA replication it is an important mechanism for the cell as it allows the maintenance of the genetic material. This extremely complex mechanism due to the high number of molecules involved needs to be carefully regulated. The initiation of DNA replication begins at specific sites known as replication origins which are activated at different times during S phase. Experiments conducted in the laboratory have shown previously that dNTPs addition leads to an increase in the number of activated origins as well as an increase in the replication extent in the *Xenopus in vitro* system. During my stage I tried to verify the effect of dNTPs on the recruitment onto the chromatin of proteins involved in DNA replication mechanism. Using Western Blot, DNA combing and mass spectrometry I was able to verify that the dNTPs affect the replication at the level of pre-initiation complex formation.

The cell cycle control prevents the accumulation of DNA lesions. The ATR / Chk1 pathway participates in this control, being able to recognize DNA lesions resulting in single-stranded DNA. This pathway determines the spatial-temporal origins firing. My objective was to determine the role of Chk1 in the presence and absence of replication stress. Using the DNA combing technique and a Chk1 inhibitor (AZD 7762) I showed that Chk1 inhibits origin firing in non-activated late replication clusters, however it is unable to inhibit the origin firing near already activated origins (early clusters).

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# Abbreviations List

**ACS** : ARS consensus sequence
**ADP**: adenosine diphosphate
**ARS:** autonomously replicating sequence
**ATM:** ataxia-telangiectasia mutated
**ATP:** adenosine triphosphate
**ATR:** ataxia-telangiectasia and RAD3 related
**ATRIP:** ATR interacting protein

**bp :** base pair
**BrdU :** bromodeoxyuridine
**BSA :** bovine serum albumin

**Cdc:** cell division control
**CDK :** cyclin dependent kinase,
**Cdt1 :** Cdc10-dependent transcript 1
**Chk :** checkpoint kinase
**CIB:** chromatine isolation buffer
**CMG :** Cdc45-MCM-GINS
**CpG :** cytosine-phosphate-guanine

**dATP:** deoxyadenosine triphosphate
**Dbf4:** dumbbell former protein 4
**Dbp11:** DNA polymerase B possible subunit 11
**dCTP:** deoxycytidine triphosphate
**DDK:** Dbf4 dependent kinase
**DNA** : deoxyribonucleic acid
**Drf1:** Dbf4-related factor 1
**dGTP:** deoxyguanosine triphosphate

**dNDP:** deoxyribonucleoside diphosphate
**dNTP:** deoxyribonucleoside triphosphate
**dTMP:** deoxythymidine monophosphate
**DTT:** Dithiothreitol
**dTTP:** deoxythymidine triphosphate
**dUMP:** deoxyuridine monophophate

**EB :** extraction buffer
**eCG :** equine chorionic gonadotropin
**EM:** energy mix
**ETED:** eye-to-eye distance

**G:** guanine
**G4:** G quadruplex
**GINS:** go ichi ni san for «five one two three » representing the four subunits: Sld5, Psf1, Psf2 et Psf3

**HAT:** histone acetyltransferase
**HBO1:** HAT bound to Orc1
**hCG:** human chorionic gonadotropin
**HeLa:** cervical cancer cell line (Henrietta Lacks)
**HSB:** high salt buffer

**kDa:** kilodalton
**Kb:** kilobase

**LSS:** low speed supernatant

**Mb:** megabase

**MBT :** midblastula transition
**MCM :** mini-chromosome maintenance
**MES :** 2-N-morpholino-ethane-sulfonic acid
**mRNA**: messenger RNA

**N/C :** ratio nucleo-cytosol
**NDPK:** nucleoside diphosphate kinase

**OGRE:** origin G-rich repeat element
**ORC :** origin recognition complex

**PBS:** phosphate buffered salin
**PCNA:** proliferating cell nuclear antigen
**PK:** proteinase K
**Plk1:** Polo-like kinase 1
**PMSG :** pregnant mare serum gonadotropin
**Pre-IC :** pre-initiation complex
**Pre-LC :** pre-loading complex
**Pre-RC:** pre-replicative complex
**Psf:** partner of Sld five
**PVDF:** polyvinylidene fluoride

**RAD :** radiation sensitive
**Rif1 :** Rap1-interacting factor 1
**RNA:** ribonucleic acid
**RPA :** replication protein A
**rpm :** rotation per minute
**RRM1/2 ou R1/2 :** ribonucleotide reductase family member ½
**RT :** room temperature

**SCF :** Skp1–Cullin1–F–box protein
**SDS:** sodium dodecyl sulfate
**Sld:** synthetically lethal with dbp11
**SNS:** small nascents strands

**TMPK:** thymidylate phosphate kinase
**TopBP1:** Topoisomerase II Binding Protein as called Dbp11/Cut5
**Treslin:** TopBP1-interacting replication-stimulating protein as called Ticrr/Sld3

**9-1-1 :** Rad9-Hus1-Rad1

# Introduction

# 1. The cell cycle

The cell cycle includes different stages which allow one original DNA molecule to divide into two molecules genetically identical. For this to happen the original DNA molecule needs to duplicate its genetic content, in a process called DNA replication.

The cell cycle (fig.1) is constituted by 4 different stages: G1, S, G2 and M. The stages G1 and G2 (G for gap) are important for preparing and controlling the progress to S (synthesis) and M (mitosis) stages. During the G1 phase the cell evaluates the environmental conditions as well as the intra and extracellular signals to decide whether it should commit to the S phase. In situations where the cells are not prepared to initiate S phase they enter into the quiescence stage (G0), in this stage the cells are neither replicating nor preparing to replicate.

One time per cell cycle, the genetic material replicates in an identical and integral manner. During the cell cycle also occurs the centrosome duplication and the sister chromatid cohesion allowing an equitable repartition of the genetic content during mitosis. The G2 phase prepares the cell to mitosis by verifying if the proper conditions to an efficient chromosomes segregation are reunited. Then the cell enters in mitosis which is the end of a cell cycle. The M phase takes less than one hour in mammalians cells and is constituted by two divisional stages, the nuclear division and the cytoplasmic division also known as cytokinesis. The two cells resulted from the mitosis undergo a new cell cycle that will result in four genetically identical cells. This process continues letting the cell to divide exponentially. Thus, departing from a unique egg cell is possible to obtain a complete multicellular organism.

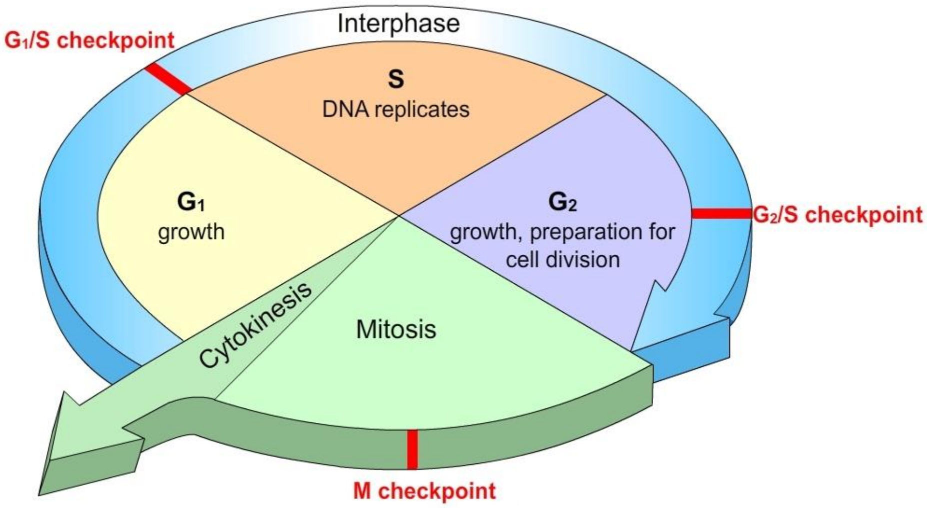

**Fig.1- The cell cycle**. It is divided into 4 phases (G1, S, G2 and M). In the S phase the DNA is replicated and in the M phase it occurs nuclear division. There are checkpoints in each of those phases to assure the correct cell cycle progress. Figure adapted from Ricochet Science

# 2. DNA replication

## 2.1 Historical discoveries

In 1953, James Watson and Francis Crick have described the DNA as a double helix structure (fig.2) and suggested a mechanism for the copy of genetic material (Watson and Crick, 1953).

In 1957, Herbert Taylor, Philip Woods and Walter Hughes showed the semi-conservative behavior of the replication (fig.2), using tritiated thymidine to study chromosomes replication (Taylor et al., 1957). Matthew Meselson and Franklin Stahl confirmed this discovery using an ultracentrifugation technique (Meselson and Stahl, 1958). Also, the *Escherichia coli* DNA molecules labelling with a nitrogen heavy isotope showed a differentiation between first and second generation and confirmed that each molecule contains a strand from the parental DNA and a newly synthetized strand (daughter strand).

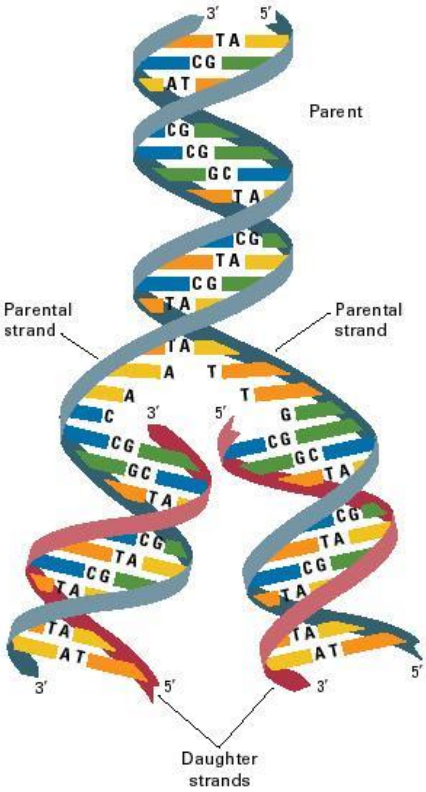

**Fig.2- DNA structure.** DNA has double helix structure and semi-conservative replication. The two molecules formed contain a parental and daughter strand. Figure adapted from Navrachana University

## 2.2 Replicon model

François Jacob and Sydney Brenner in 1963 proposed a model for the replicon (fig.3). They suggested that the replication was initiated by the interaction between two elements, the initiator, a protein factor acting in trans to the DNA sequence and the replicator, a genetic element located cis on the DNA sequence. This interaction leads to the unwinding of the double stranded DNA chain, to the recruitment of protein factors and to the synthesis of the complementary DNA strand.

The replicon is defined as a unit over the replicated chromatin from a unique initiation event. For example, the prokaryotes are constituted by a unique circular DNA molecule and they are submitted to a single initiation. However, in eukaryotes the genome is larger and has several DNA molecules called chromosomes. It is impossible for the DNA to be completely replicated with a unique initiation event due to time restrictions. Therefore numerous replicators are presented across the genome in eukaryotes. Although the initiation sites can co-localize with replicators, not all the replicators are going to initiate DNA replication in each cell cycle (Aladjem, 2007).

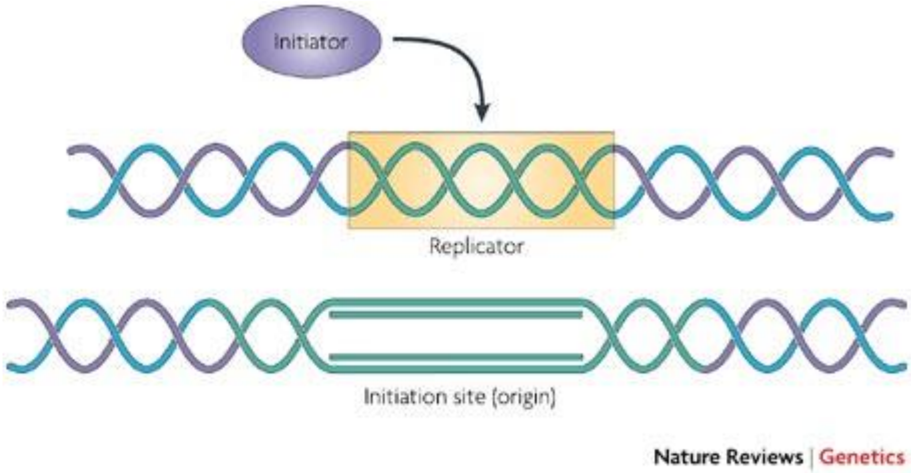

**Fig.3- Replicon Model.** The replicator in *trans* and initiator in *cis* interact to initiate DNA replication. More concretely, the double helix is unwound at the origins and the template strand is complemented by a daughter strand. Figure adapted from Aladjem et al. 2007.

## 2.3 Replication steps-an overview

The replication can be summarized in three main steps: initiation, elongation and termination (fig.4).

In the initiation step, the initiator protein interacts with the replicator, more concretely with the origin of replication, and recruits other proteins. The DNA double helix begins unwinding to allow the incorporation of the replisome, a large protein complex positioned in a symmetric manner at the replication forks. The forks together with the polymerase action assure the elongation of the newly synthetized DNA strands by nucleotide incorporation in the 5’→3’ sense (fig.4). The DNA synthesis occurs complementary and antiparallel to the parental strands. The DNA termination occurs when the two replication forks fuse.

The replication is bidirectional and polarized causing an asymmetry at the replication fork. There is the formation of two replication forks that move in opposite directions and the new DNA strands synthesis by the polymerases is done in the 5’→3’ sense. Because DNA replication is realized in the same sense for both of the strands, in one of the strands the replication is a continuous process (leading strand), the DNA is synthesized in the same direction as the replication fork. The other strand is synthesized in short separated fragments and in opposite direction to the replication fork (lagging strand). The short fragments formed are called Okazaki fragments and they are removed when the strands join each other (fig.4).

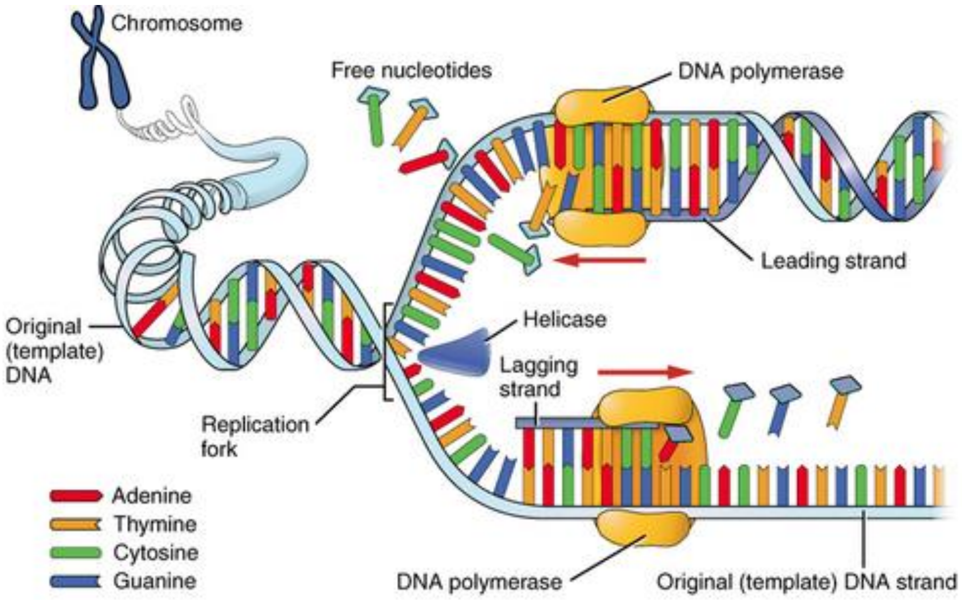

**Fig.4- Replication steps.** The DNA is replicated in the 5'-3' sense based on the complementary of the template strand. In one of the replication forks the strand complementary to the 3'-5' parental strand is continuous, in contrast in the other fork the strand complementary to the 5'-3' parental strand is discontinuous. Figure adapted from University of Michigan webscope.

# 3. Molecular Mechanisms of DNA replication

Both sequential assembly and reorganization of complex arrays of proteins are crucial for the coordinated execution of initiation, elongation and termination processes of DNA replication. The progression of replication fork is strictly controlled to ensure whole genome replication. Defects in replication forks or proteins assembly during this process may lead to genomic instability, carcinogenesis and “chromosome instability syndromes”.

The replication factors seem to be conserved across all the species, indicating that the basic mechanism of DNA replication is evolutionally preserved. However, the recognition and firing of DNA replication is more elaborated in eukaryotes in comparison with prokaryotes and there are even differences between lower and higher eukaryotes.

In eukaryotes, the DNA replication starts at specific regions of the chromosomes called replication origins. The consensus sequence required for a replication origin is usually differs between eukaryotes. In *Saccharomyces cerevisiae*, the replication origin sites corresponds to a sequence of about 200 pb called ARS (Autonomously Replicating Sequence). The ARS contains a conserved and essential 11bp sequence called ACS (ARS- consensus sequence) or element A and several less important B elements with 10-15 pb. (Palzkill et al. 1988, Marahrens et al. 1992, Theis et al. 1997).

In other species, the sequences required for the initiation of DNA replication are more complex or in some cases not totally identified. In the fission yeast namely *Schizosacharomyces pombe*, the sequence that directs DNA replication initiation spreads over 800-1000 bp (Clyne et al. 1995, Dubey et al. 1994, Dubey et al. 1996). This sequence is constituted by 20-50 bp AT-rich sequences that seem to be important in origin function, however they do not exhibit the same strong sequence similarity observed in the S.cerevisiae ACS (Chuang et al. 1999). In *Drosophila melanogaster*, the AT-rich sequence also appear to be related with DNA replication initiation sites. In metazoans no specific consensus sequence for DNA initiation has been identified yet. Recently identified GC rich elements seem to be important in replication initiation (Cadoret et al. 2008, Cayrou et al. 2011)

Eukaryotic origins direct the assembly of a multiprotein mechanism that leads to the formation of two replication forks at each origin. This mechanism begins with the formation of the pre-replicative complex (pre-RC) at origins of replication during G1. The pre-RC formation involves the coordinated assembly of some replication factors including ORC, Cdc6, Cdt1 and MCM complex.

## 3.1 The pre-replicative complex regulation

### 3.1.1 The Origin Recognition Complex

The origin recognition complex (ORC) is a complex constituted by six-subunits and acts as the initiator at the replication origins because it is able to select the sites where initiation of replication can start. Firstly identified in *S. cerevisiae* bound to the conserved ACS (Bell et al. 1992), subsequent studies showed that this complex is also crucial for the initiation in other eukaryotes. In *Xenopus* egg extracts an analogous complex is reported as necessary for initiation of replication (Romanowski et al. 1996, Rowles et al. 1996, Carpenter et al. 1996). Similarly, in *Drosophila melanogaster* recessive lethal mutations of ORC subunits showed decrease of replication (Pflumm et al. 2001, Pinto et al. 1999), the same was demonstrated when DNA replication was reconstituted in Drosophila extracts immunodepleted for ORC with the addition of recombinant ORC (Chesnokov et al. 1999).

As mentioned previously, ORC binds to the DNA to form the pre-RC. In S. cerevisiae, ORC interacts specifically with A and B1 elements of yeast origins contained in approximately 30 bp (Rao et al. 1995, Rowley et al. 1995) more precisely ORC interacts with A-rich strand of this region (Lee et al. 1997). Recently, genome-wide studies showed that however no consensus sequence have been found yet, AT-rich intergenic regions serve as potential origins in fission yeast (Segurado et al. 2003, Hayashi et al. 2003, Feng et al. 2006, Dai et al. 2005). In Drosophila, although the consensus sequences for origins and ORC binding have not yet been identified it was shown that the ORC-binding sites are overlapping with RNA polymerase II-binding sites, suggesting that transcription factors may be involved in the selective binding of ORC (MacAlpine et al. 2004).

In metazoans, genome-wide experiments demonstrated the abundance of origins in the CpG island promoter, actually half of the origins are localized within or near CpG islands (Cadoret et al. 2008, Brockdorff et al. 2009). Moreover, origin G-rich repeat element (OGRE) is present in 80-90% of replication origins and can form G quadruplexes, a four-stranded DNA structure formed by guanines (Cayrou et al. 2011, 2012).

### 3.1.2 The Cdc6 protein

Cdc6 was identified in a screen for *S. cerevisiae* mutants with changes in the cell division cycle (Hartwell et al. 1973). Cdc6 belongs to a large family of ATPases and is related to ORC1 and in a more limited extent to ORC4, ORC5 and MCM 2-7 proteins (Neuwald et al. 1999). In the last decade the crucial role of Cdc6 in pre-RC assembly was demonstrated at a step after ORC and before Mcm2-7 proteins. Cdc6 requires ORC to associate to the chromatin and is in turn required for Mcm2-7 chromatin association (Coleman et al. 1996). In genetic studies in *S.cerevisiae*, Cdc6 was showed to be required for MCM association with the origin but not for ORC (Aparicio et al.1997).

### 3.1.3 The Cdt1 protein

CDT1 gene was identified in *S.pombe*, it is regulated by the Cdc10 transcription factor (Hofmann et al.1994). Mutations in CDT1 resulted in DNA replication blocking and defects in the S-phase checkpoint suggesting Cdt1 as a key factor in pre-RC assembly (Hofmann et al.1994). In fact Cdt1 has been shown to associate with the C-terminus of Cdc6 to promote the consequent association of MCM onto the chromatin (Nishitani et al.2000). To complement, in *Drosophila melanogaster* Cdt1 co-localizes with ORC at DNA replication sites and ORC is needed for this Cdt1 localization (Whittaker et al.2000).

### 3.1.4 The MCM 2-7 Protein Complex

The MCM complex genes were first identified in genetic screens for proteins involved cell cycle progression and chromosome missegregation (Chong et al. 1996, Dutta et al. 1997). All eukaryotes appear to have six MCM protein analogs (MCM2-7) and each MCM protein has a unique and important function. This is supported by the lethality associated with the deletion of any individual MCM gene in *S.cerevisiae* and *S.pombe* (Dutta et al. 1997, Kelly et al. 2000).

The assembly of MCM onto the chromatin requires the coordinated action of ORC, Cdc6 and Cdt1. Surprisingly, once the MCM-complex is loaded onto the chromatin, both ORC and Cdc6 can be removed from the chromatin without affecting DNA replication, suggesting that the main purpose of the pre-RC is loading the MCM-complex (Rowles et al. 1999, Hua et al. 1998, Maiorano et al. 2000).

MCM proteins have a role in both initiation and elongation steps of DNA synthesis since it has been reported that the MCM proteins act as a DNA helicase. Studies in *S.pombe* MCM proteins identified a DNA helicase activity dependent on Mcm4, 6, 7 (Lee et al. 2000).

EM studies in human and *S.pombe* support a hexameric in doughnutlike structure for the MCM complex (Adachi et al. 1997, Sato et al. 2000).

### 3.1.5 Assembly of the pre-replicative complex

ORC is recruited to the origin site, then both Cdc6 and Cdt1 associate with ORC bound to the chromatin in an independent manner (Nishitani et al.2000, Maiorano et al. 2000) to initiate the assembly of the pre-RC. These three factors are required for the stable association of the MCM proteins with the origin during G1.In *Xenopus* egg extracts, the association of Cdc6 with chromatin but not ORC appears to be transient (Hua et al. 1998). Similar studies of Cdc6 *S.cerevisiae* association with the origins showed that MCM remains bound at late origins even after Cdc6 is degraded (Tanaka et al. 1998).

### 3.1.6 Selection of Replication Initiation Sites of DNA Synthesis

The cells are equipped with excess of pre-RCs on the genome and only a fraction is utilized for the initiation (Edwards et al.2002, Hyrien et al. 2003). Selection of initiation sites is regulated by various factors and is achieved at the activation step as well as at the pre-RC assembly step.

#### 3.1.6.1 Distal elements

The distal DNA elements which are involved in the transcription process may also influence the initiation sites. At the DHFR locus, the replication initiation needs the presence of a transcriptional promoter in the 5’ of the initiation site. The human β-globin locus depends of the LCR (locus control region) to be replicated. This LCR region which regulates the genetic expression is located 20kb apart from the β-globin locus (Aladjem et al. 2007). At the moment, the mechanism by which these distant elements affect initiation events is unknown.

#### 3.1.6.2 Chromatin Structures

The chromatin structure is an important factor in origin selection. Studies in *Xenopus* egg extracts and human cells show that HBO1, a histone acetyltransferase is required for the loading of MCM onto the chromatin (Iizuka et al. 2006). HBO1 interacts with Cdt1 and enhances Cdt1-dependent re-replication (Miotto et al. 2008), it also interacts with ORC and MCM2 suggesting that the pre-RC formation can be affected by histone acetylation and deacetylation (Burke et al. 2001). In *Xenopus* egg extracts the initiation is random, however by introducing a transcriptional complex at a specific site, there is a correlation with the local hyperacetylation of the histones facilitating the pre-RC assembly and/or its activation (Danis et al. 2004). Thus, chromatin structures have a role in origin selection but not in origin firing (Prioleau et al. 2003).

### 3.1.7 Prevention of Re-Replication

Replication licensing should occur only one time per cell cycle to an accurate genome duplication. Eukaryotic cells have developed a mechanism to prevent re-initiation of replication from the same origins during a single cell cycle that relies on CDK action. CDK regulates the eukaryotic DNA replication, when in elevated levels in one hand it promotes origin activation in cells entering into S phase and in the other hand it is required to prevent origins re-initiation during S, G2 and M phases of the cell cycle (Piatti et al. 1996).

The pre-RC formation inhibitor (geminin) discovered in metazoans showed that not all mechanisms that prevent pre-RC formation are mediated by CDK elevated activity. It was discovered that human geminin associates with Cdt1 and consequently inhibits pre-RC assembly, also it was showed in *Xenopus* egg extracts that excess of Cdt1 can reverse the geminin inhibitory action (Wohlschlegel et al. 2000).

Another mechanism that represses re-replication is the ATR/Chk1 pathway that will be addressed in more detail later in this report.

## 3.2 Initiation of DNA replication

In eukaryotic cells the DNA replication initiation is a precisely regulated process which requires ordered assembly of multiple protein complexes at origin replication sites (fig.5). The initiation replication process is divided into two steps. In the first step during early G1 phase, ORC, Cdc6, Cdt1 and MCM2-7 proteins are assembled onto the chromatin in a sequential manner, generating the pre-RC in a process known as origin licensing (fig.5). In the second step of replication initiation, the pre-RC and the helicase complex are activated. At the G1/S transition phase, two kinases, DDK (Dbf4 dependent kinase) and CDK (cyclin dependent kinase) phosphorylate other essential replication proteins, stimulating their loading onto the chromatin and consequent DNA helicase activation as well as chain elongation by DNA polymerases (Masai et al. 2002, Sheu et al. 2006, Masai et al. 2006, Yabuuchi et al. 2006, Krasinska et al. 2008, Francis et al. 2009). ORC bounds to the origin DNA sites, then Cdc6 in a complex with ATP binds to the ORC on DNA. The MCM2-7 and Cdt1 probably in a complex, join the ORC-Cdc6 complex. ATP bound to Cdc6 is now hydrolyzed and dissociates from the complex. Cdt1 also dissociates from the complex triggering a stable binding of the MCM complex with the DNA.

## 3.3 Elongation of DNA replication

After pre-RC formation the activation of DNA helicase requires additional factors to form the replication fork complex (replisome) such as GINS, Cdc45, MCM10, Treslin, TopBP1 and ReQL4 (fig.5).

In budding yeast, Sld2 and Sld3 (RecQL4 and Treslin orthologous respectively) are required in DNA replication initiation in a CDK dependent manner (Masumoto et al. 2002, Tanaka et al.2007, Zegerman et al.2007).

In budding yeast, Sld3 interacts with Cdc45 and both proteins associate with the origins. Sld3 action is necessary for the MCM complex-Cdc45 interaction (Kamimura et al. 2001). TopBP1, Treslin, Cdc45 and GINS associate origins in a dependent manner to initiate DNA replication (Takayama et al. 2003). Both Cdc45 and GINS remain associated with the MCM complex across the S phase, however Treslin is no longer required for DNA synthesis after forks replication activation (Kanemaki et al. 2006).

In fission yeast, Cdc7 yeast orthologous is necessary for Sld3 (Treslin) loading onto the chromatin. Moreover, in *Xenopus* extracts Cdc7 action is required before CDK step for DNA replication activation (Jares et al. 2000, Walter et al. 2000). Besides Sld3 (Treslin) also Sld2 (RecQ4L homologs) is a target of CDK phosphorylation (Sangrithi et al. 2005, Kumagai et al. 2010, Sansam at al. 2010).

### 3.3.1 Activation of helicase

In the S phase, the MCM complex moves away from replication origins as part of the replication fork (Aparicio et al. 1997, Labib et al. 2000, Pacek et al. 2004, Pacek et al. 2006). Some studies showed a helicase activity by the MCM 4-6-7 complex suggesting that MCM might be the replicative helicase, however the isolated MCM2-7 complex showed no signs of DNA helicase activity. Actually, the replicative helicase is constituted not only by the MCM complex but also by GINS and Cdc45 in a complex known as CMG (Cdc45, MCM,GINS) (Moyer et al. 2006, Aparicio et al. 1997, Pacek et al. 2004, Pacek et al. 2006).

Activation of the MCM helicase in the pre-RC is dependent of two conserved kinases, CDK and Cdc7-Dbf4 (Masai et al. 2002, Sclafani et al. 2000, Masai et al. 2005). It has shown that Cdc7 phosphorylates MCM2-7 complex facilitating the Cdc45 loading onto the chromatin (Sheu et al. 2006, Masai et al. 2006).

Some studies showed that Cdc7 phosphorylates N-terminal tail of MCM2, MCM4, MCM6 proteins and this phosphorylation is stimulated by prior CDK phosphorylation (Masai et al. 2000).

### 3.3.2 MCM10 Protein

Mcm10 allele characterization showed its importance in efficient DNA replication initiation (Merchant et al. 1997). In addition, Mcm10 seems to have a role in replication fork progression in contrast to other initiation factors like Cd6 and ORC that are dispensable (Kawasaki et al. 2000). Some publications confirmed a role of MCM10 in initiation of replication (Homesley et al. 2000) as well as its location in origins and interactions with ORC (Kawasaki et al. 2000). Studies in *Xenopus laevis* showed that MCM2-7 bounded to the chromatin is a pre-requisition for MCM10 loading onto the chromatin. Furthermore, MCM10 depletion blocks Cdc45 loading, suggesting a role of MCM10 between the pre-RC and elongation step (Wohlschlegel et al. 2002).

### 3.3.3 GINS

GINS (Go, Ichi, Nii, and San; five, one, two, and three in Japanese) is a heterotetrameric complex (Kamada et al. 2007, Boskovic et al. 2007, Choi et al.2007, Chang et al. 2007) which was originally discovered through genetic screening for lethal mutations and it is essential for DNA replication in budding yeast as well as in *Xenopus* egg extracts. GINS allows Cdc45 to associate with the MCM complex in a stable manner (Kanemaki et al. 2006, Labib et al. 2007). The exact structure of GINS is still unclear. GINS complex may be important in coordinating MCM helicase progression and priming events on the replication fork (Kamada et. 2007).

### 3.3.4 Cdc45

Cdc45 was originally identified in a screen for cold-sensitive mutants in cell cycle progression (Moir et al.1982). Results using *Xenopus* egg extracts, showed an interaction between Cdc45 and polymerase α and consequent loading of pol α on chromatin. Similar results in *S.cerevisiae* were obtained, demonstrating that in Cdc45 mutants the DNA polymerases α and ε are unable to assembly at origins of replication (Aparicio et al. 1999, Zou et al. 2000). The same interactions were shown in vitro studies in humans (Kukimoto et al. 1999). Cdc45 loads before the DNA unwinding step and the DNA polymerase association as reported in xenopus egg extracts (Mimura et al. 2000, Walter et al. 2000). Cdc45 does not interact exclusively with polymerase α, it also associates with ORC, DNA pol ε, RPA and the MCM proteins (Kukimoto et al. 1999, Kumimura et al. 2001, Saha et al. 1998, Uchiyama et al. 2001).

Once Cdc45 is loaded onto the chromatin, it is incorporated into the replication fork and colocalizes with polymerases (Aparicio et al. 1999, Zou et al. 2000). Studies of association between Cdc45 and chromatin showed a strong dependence on CDK activity (Walter et al. 2000, Zou et al. 1998). In addition, Cdc45 origin association correlates with the time of replication initiation (Zou et al. 2000, Aparicio et al. 1999) suggesting Cdc45 loading as a requirement beyond CDK activation. It is also clear that the level of Cdc45 association is increased by high levels of CDK activity.

### 3.3.5 TopBP1

DPB11 (homologous of TopBP1) gene was first identified as a multicopy suppressor of mutations in DNA pol ε (Araki et al. 1995). Co-immunoprecipitation assays support an interaction between Dpb11 and DNA pol ε (Masumoto et al. 2000). Dpb11 associates with replication origins in cooperation with DNA pol ε, suggesting that Dpb11 is required during S phase in DNA pol ε recruitment (Araki et al. 1995, McFarlane et al. 1997). Moreover, TopBP1 is needed for Cdc45 loading into the chromatin (Hashimoto and Takisawa, 2003, Van Hatten et al. 2002) and during checkpoint activation it is a direct activator of ATR-ATRIP complex (Kumagai et al. 2006, Mordes et al. 2008).

### 3.3.6 Treslin

Treslin is a large protein (220kD) about three times bigger than Sld3 orthologous. Due to this, Treslin may have additional properties in comparison with yeast Sld3 that allows it to meet the demands of higher eukaryotes. Treslin interacts with an effector kinase in checkpoint control mechanism (Chk1) and contributes in the negative regulatory mechanism that suppresses initiation (Guo et al. 2015). Treslin is also required for the initiation of replication and forms a complex with Cdc45 that associates with origins of replication (Kamimura et al. 2001).

### 3.3.7 RecQL4

Sld2 (RecQL4 orthologous) is required for the initiation of DNA replication. However, RecQ4 seems to have a different role from Sld2 since it is not required for Cdc45 and GINS loading into the chromatin instead it contributes in the RPA and polymerase α interaction with the chromatin (Sangrithi et al. 2005, Matsuno et al. 2006). In *Xenopus* egg extracts, RecQ4 depletion compromises both DNA unwinding and Polα binding to the origins (Sanuki et al. 2015). Thus, RecQL4 has an important role in pre-IC conversion into active replisomes.

### 3.3.8 Assembly of the DNA polymerases

The objective of the DNA replication initiation is the assembly of the DNA polymerases to the origins. The progression of the replication forks across the DNA allow the synthesis of daughter strands. The DNA is unwind by the MCM replication helicase associated with the Mcm10, GINS and Cdc45. Mcm10 is important in the stabilization of the polymerase α. After the DNA unwinding the RPA (replication protein A) protein interacts specifically with the single stranded DNA, The primase, a subunit of the polymerase α, synthetizes small RNA primers (between 8 and 12 nucleotides) in the template strand. This enzyme is only able to synthetize DNA departing from the 3' extremity. It extends the small RNA primers to some nucleotides and it is posteriorly replaced by DNA polymerase δ (lagging strand) or by polymerase ε (leading strand) (Kunkel and Burgers, 2008). These two polymerases advance more quickly than the polymerase α, additionally they are able to correct the synthesis due to their exonuclease activity 3’-5’. The CMG assures the polymerase ε binding to the leading strand. If polymerase δ binds to the leading strand it is replaced by the polymerase ε (Georgescu et al. 2014).

PCNA (proliferating cell nuclear antigen) is a homotrimer factor that acts as a processivity factor for DNA polymerases. PCNA, also known as clamp, is loaded into the chromatin encircling the DNA and it is going to interact with the polymerases. Simultaneously, the replication factor C (RFC) known as clamp loader recognizes the RNA-DNA primer and it is recruited into the chromatin.

The leading strand synthesis is dependent on the polymerase ε action and occurs alongside the helicase progression (Pursell et al. 2007). In contrast, the lagging strand is produced discontinuously by the polymerase δ with the formation of Okazaki fragments.

The helicase action leads to the formation of single stranded DNA which is protected by the RPA proteins. The DNA synthesis starts close to the gap area by polymerase α activity until encountering the previously introduced RNA primer. Then, the RNA primer is removed with the help of polymerase δ, Fen1 (Flap endonuclease 1) and DNA ligase I (Liu et al. 2004). Finally, additional proteins involved in the forks stability, chromatin assembly and chromatids cohesion are also recruited to the replication forks (fig.5).

## 3.4 Kinases action in the transition to replication

Two kinases (Cdc7/Dbf4 and CDKs) control the assembly and the activity of the DNA replication machinery at the origin. These two kinases phosphorylate several other replication factors particularly Cdc45 leading to its association with chromatin and the unwinding of origin in DNA replication.

### 3.4.1 Cdc7/Dbf4 (DDK)

The genes encoding Cdc7 and Dbf4 were identified in screens for mutant genes that assumed a characteristic phenotypes for cell-cycle arrest (McFarlane et al. 1997).

The similarity in the regulation between CDK and Cdc7/Dbf4 led to be referred as DDK.

In *Xenopus* egg extract, MCM is required for Cdc7 recruitment but not ORC nor Cdc6 (Jares et al. 2000, Walter et al. 2000). MCM is considered the main target of the DDK initiating DNA replication.

In addition to the MCM also Cdc45 and the catalytic subunit of polymerase α are phosphorylated by DDK (Nougarede et al. 2000).

### 3.4.2 Cyclin-Dependent Kinases (CDKs)

CDKs associate with multiple proteins involved on the pre-RC such as ORC and Cdc6 and seem to be important in CDK recruitment at the origins (Furstenthal et al. 2001).

CDK activity is essential for the DNA replication, it acts after MCM loading and before Cdc45 association onto the origins (Zou et al. 1997, Walter et al. 2000). Although Cdc6, MCM and ORC are targeted by CDKs, mutant forms of these protein cannot be phosphorylated by CDKs but the initiation of DNA replication continues, indicating that these proteins are phosphorylated in a CDK independent event (Elsasser et al. 1999, Drury et al. 2000, Jallepalli et al. 1997, Lopez-Girona et al. 1998, Nguyen et al. 2001, Vas et al. 2001, Pelizon et al. 2000).

### 3.4.3 Time of DDK and CDK action

It is known that these two kinases are of extreme importance in initiation of replication and in the association of Cdc45 with chromatin. In *S.cerevisiae*, CDK function is required for DDK function (Nougarede et al. 2000). However, in Xenopus egg extracts the opposite was verified meaning that DDK action is necessary before CDK function (Jares et al. 2000, Walter et al. 2000).

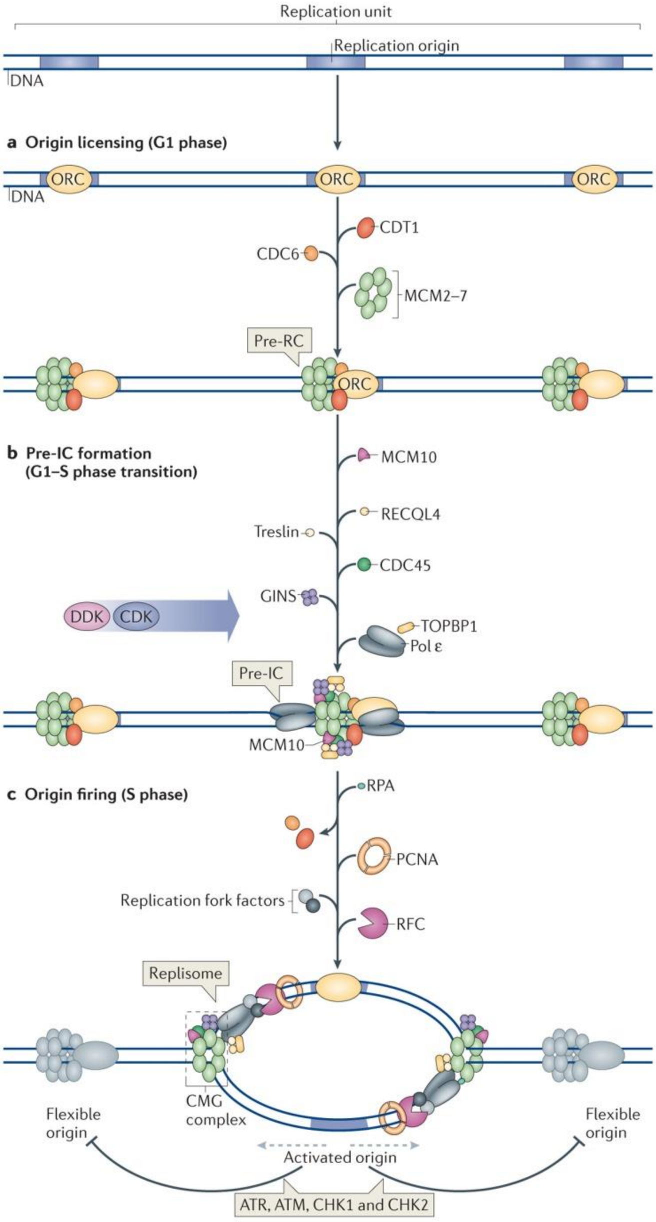

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**Fig.5**- **Activation of origins and assemble of the replisome.** Figure adapted from Fragkos M. et al. 2015.

# 4. Deoxyribonucleotides pool influences the replication program

The DNA consists in a helix double strand being each strand synthesized from four types of deoxyribonucleosides triphosphates or deoxyribonucleotides (dNTPs) such as deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate or thymidine (dTTP/TTP), deoxycytidine triphosphate (dCTP) and deoxyguanosine (dGTP). The dNTPs pool needs to be strictly regulated since an unbalanced level of dNTPs leads to errors at the replication (Kumar et al. 2010, Aird and Zhang et al. 2014).

The concentration of dNTPs is maximal at the S phase to allow the DNA replication and it is minimal in G0/G1 phases allowing reparation and mitochondrial DNA synthesis (Aye et al. 2015).

## 4.1 Deoxyribonucleotides synthesis

The nucleotides can be synthetized in two different ways, the “de novo” synthesis from glutamine and the salvage pathway. In the salvage pathway already used nucleotides are recovered. The “de novo” production is different for purines and the pyrimidines nucleotides.

### 4.1.1 The ribonucleotide reductase (RNR)

The ribonucleotide reductase catalyzes the limiting step from the dNTPs “de novo” synthesis. The enzyme is able to reduce the ribonucleotide diphosphate (NDP) 2’ carbon to produce the dNDP (deoxyribonucleoside diphosphate) corresponding to dADP, dGDP, dCDP, dTDP. Then, the NDPK phosphorylates dNDPs producing the deoxyribonucleosides triphosphate (dNTPs).

There is no rTDP precursor to produce dTTP (deoxythymidine triphosphate). The dTTP synthesis needs the dUMP (deoxyuridine) conversion into dTMP (deoxythymidine monophosphate) by the thymidylate synthetize. Then, there is the phosphorylation by TMPK (thymidylate phosphate kinase) and NDPK to obtain dTTP (Aye et al. 2015).

The RNR is a tetrameric enzyme composed by two large catalytic subunits (RRM1) and two small regulator subunits (RRM2). RRM1 contains the catalytic sites and it is expressed across all the cell cycle. The RRM2 is present mainly at the S phase to produce the dNTPs during DNA replication. In *Xenopus* egg extracts and early embryos the RNR is present but inhibition of this enzyme does not inhibit DNA replication which led to the conclusion that the RNR activity is not necessary during early stages and that the dNTP concentration is sufficient (Newport et al. 1989).

## 4.2 The effect of dNTP pool on the DNA replication

In mammalians, the dNTPs concentration affects the number of origins activated and the fork speed. In cell cultures, the fork speed increases significantly across the S phase when an augmentation of dNTPs is observable due to high regulation of RRM2. In HeLa cells the addition of exogenous dNTPs accelerates fork speed in the beginning of the S phase, however it has no effect in end of S phase (Malinsky et al. 2001).

Using BrdU to label DNA fibers, it was shown that in culture cell mediums the increase in dNTPs precursors leads to fork speed acceleration. In contrast in the presence of a low nucleotides concentration, a fork speed deceleration and an increase in dormant origins activation was observed (Anglana et al. 2003). Actually, the single addition of adenine and uridine in cell cultures is enough to transform a slow replication in a rapid one with decrease in the number of activated origins and increase in fork speed (Courbet et al. 2008). Moreover, another study showed similar results, when the pyrimidine bases synthesis was altered the fork speed decreased and the dormant origins activation augmented in compensation. The addition of dTTP and dCTP allows to revert this effect (Gay et al. 2010).

In budding yeast, it has been shown that changes at dNTPs levels affect replication dynamics. Upregulation of RNR activity increases elongation indicating dNTPs concentration as a limiting factor. In contrast, when adding hydroxyurea (HU) the RNR activity is inhibited inducing a transition to a slow replication. The upregulation of RNR seems to delay this transition since it increases fork progression (Poli et al. 2012).

In *Xenopus* an early study demonstrated that the addition of dNTP delayed the midblastula transition. It was therefore proposed the dNTP pool becomes limiting only after 12 cell divisions at the midblastula transition (Landström et al., 1975). But other studies did not detect any effect of dNTP addition on early development (Vastag et al. 2011, Collart et al. 2013). However, experiments with dNTPs addition have not been performed in the *Xenopus in vitro* system.

# 5. Cell cycle checkpoints

Cell cycle checkpoints are of extreme importance to counteract the internal and external threats posed to the DNA. Checkpoints inhibit cell cycle progression before entry into the S phase (G1/S checkpoint), throughout S phase (S-phase checkpoint), before mitotic entry (G2/M checkpoint) or before entry into anaphase (mitotic spindle checkpoint) (Jackson et al. 2009). S-phase checkpoint is essential since it controls the progression of DNA replication. In the presence of DNA damage, DNA replication is stopped in order to allow DNA repair or even trigger apoptosis in cases of aberrant genomes (Roos et al. 2013). Thus, checkpoint signaling maintains genome integrity and inhibits the arising of genomic instability associated diseases.

## 5.1 Checkpoint action

During replication some cells undergo aberrant replication fork structures responsible for genomic instability (Zeman et al. 2014). Some sources of replication stress are highlighted below. Nicks, gaps and ssDNA are related with replication stress and they can be a symptom or a source of stress. The most common source of replication stress are DNA lesions, some of the most common DNA lesions agents are UV light, chemical mutagens and byproducts of cellular metabolism (Shi et al. 2004, Brooks et al. 2005). Secondary DNA structures such as hairpins or triplexes are formed by trinucleotide repeats and are able to block replication fork progression leading to stress (McMurray et al. 2010, Kim et al. 2013). The replication process requires some components (nucleotide, replication machinery and histones) that in limited quantity slow fork speed and induce replication stress (Poli et al. 2012, Bester et al. 2011, Anglana et al. 2003, Aguilera et al, 2013). An improper control of replication initiation can also lead to replication stress since too many origins activated will deplete the endogenous nucleotide pool and slow fork speed (Sørensen et al. 2012, Beck et al.

2012) and few origins activated contributes to loss of genetic material due to deficient replication (Shima et al. 2007, Debatisse et al. 2012).

In the presence of DNA lesions, DNA polymerases are stalled while the helicases continue to unwind the parental DNA (Byun et al. 2005, Walter et al. 2000), this favors the formation of single stranded DNA (ssDNA). In addition, cells subjected to DNA damaging agents like hydroxyurea (HU), aphidicolin (APH) and ultraviolet radiaton (UV) also promote accumulation of ssDNA (Lupardus et al. 2002, Zhao et al. 2000, Sogo et al. 2002). ssDNA are recognized and bounded with RPA (replication protein A), this protein protects the ssDNA and contributes to checkpoint activation (Oakley et al. 2010, Zou et al. 2003). Although it has been reported that the amount of RPA-ssDNA influence the checkpoint activation (Shechter et al. 2004), this is not enough for checkpoint activation, in addition 5' single stranded/double stranded DNA junctions are needed (MacDougall et al. 2007, Zeman et al. 2014, Byun et al. 2005). RPA-ssDNA at stalled forks activate ATR (ataxia-telangiectasia mutated and Rad3-related), preceded by Chk1 activation.

## 5.2 ATR-ATRIP activation

### 5.2.1 RPA-ssDNA

ATR is a kinase, member of the phosphoinositide 3-kinase-related kinase (PIKK) family. ATR forms a stable heterodimeric complex with ATRIP (ATR-interacting protein) (Cimprich et al. 2008, Cortez et al. 2001). ATR-ATRIP complex is considered as a sensor of genotoxic stress because it is recruited by RPA-ssDNA to sites of replication stress (Zou et al. 2003). Other ssDNA- binding proteins may also lead to checkpoint activation by Chk1 phosphorylation (Bolderson et al. 2014).

### 5.2.2 TopBP1

TopBP1 seems to be of extreme importance in the activation of the ATR-ATRIP complex (Zhou et al. 2013). In vitro studies showed that ATR-ATRIP complex activation requires TopBP1 action, however it is independent of DNA (Choi et al. 2010).

### 5.2.3 Rad17/911 complex

The PCNA-related Rad9-Hus1-Rad1 heterotrimer also known as 9-1-1 and its loading protein Rad17/RFC enhance Chk1 phosphorylation dependent of ATR (Kobayashi et al. 2004, Weiss et al. 2002).  Rad17 loads 9-1-1 on site of fork stalling (Majka et al. 2006), then this complex interacts with ATR-ATRIP in an interaction mediated by TopBP1 (Lee et al. 2007, Mordes et al. 2008). The recruitment of TopBP1 to ssDNA sites is still unclear, in vivo studies showed that Rad17/9-1-1 complex recruits TopBP1 (Lee at al. 2010, Delacroix et al. 2007), however others showed that TopBP1 localized to sites of DNA damage leads to the 9-1-1 loading onto stalled replication forks (Yan et al. 2009).

## 5.3 ATR-mediated Chk1 phosphorylation

In response to stalled replication forks, after ATR activation the major step is the phosphorylation of the checkpoint kinase Chk1. Other factors act upstream Chk1 activation, such as Claspin (Dai et al. 2010).

### 5.3.1 Claspin

In cases of replication stress phosphorylated Claspin associates with Chk1 (Chini et al. 2006, Kumagai et al. 2003). The formation of this complex enhances Chk1 activation since ATR affinity is higher for this complex than for unbound Chk1 (Lindsey-Boltz et al. 2009). Studies in Claspin deficiency demonstrated a decrease in Chk1 phosphorylation (Errico et al. 2007, Kemp et al. 2010, Kumagai et al. 2000) as well as checkpoint deregulation (Chini et al. 2003, Chou et al. 2006).

### 5.3.2 Chk1

Chk1 is phosphorylated by ATR within the C-terminal in the serines 317 and 345 (Walker et al. 2009, Zhao et al. 2001). The role of each phosphorylation in Chk1 activation and checkpoint action remains unclear. Serine 345 phosphorylation seems to be necessary for cell survival and checkpoint action, in the case of the serine 317 phosphorylation, it seems to be important for an efficient ATR- dependent Serine 345 phosphorylation (Niida et al. 2007, Wilsker et al. 2008). Chk1 appears to have multiple roles in cell-regulation and it is considered an essential protein for cell viability (Liu et al. 2000).

To summarize ATR-ATRIP and Rad17/RFC are recruited in parallel to RPA-ssDNA and fork stalling sites respectively. Then, Rad17/RFC loads 9-1-1 onto the fork stalling sites. TopBP1 also recruited to the sites, interacts with ATR-ATRIP and promotes ATR activation (fig.6). To conclude activated ATR phosphorylates Chk1 found in complex with Claspin.

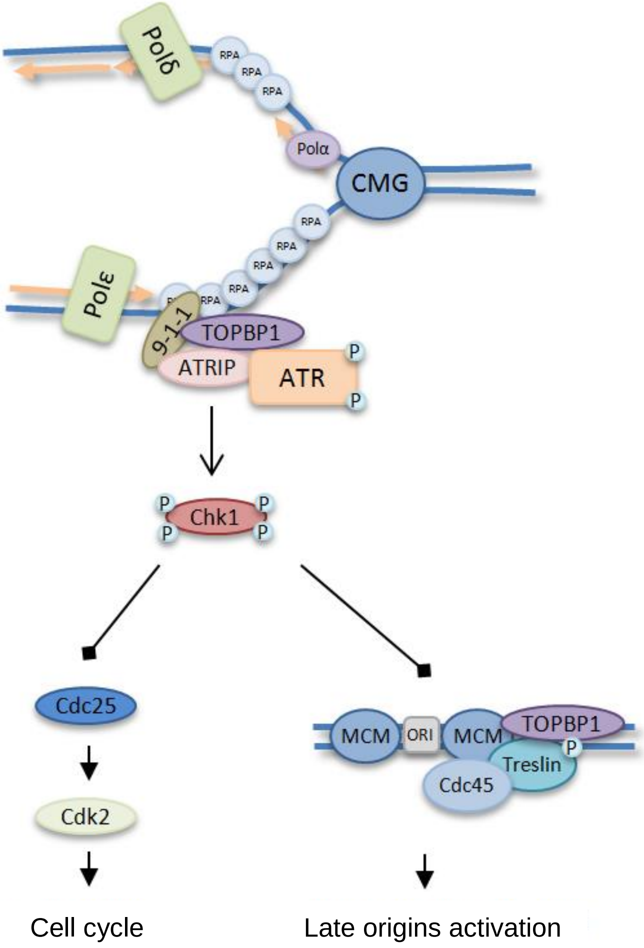


Fig.6 – ATR/Chk1 pathway activation. ATR/Chk1 pathway upon Chk1 activation is responsible for the cell cycle blocking due to Cdc25 and Cdk2 inhibition. Also it inhibits the late origins activation via Cdc45 inhibition. Figure adapted from Wu et al., 2014.

## 5.4 Chk1 action during DNA replication

It is known that several replication origins are organized into clusters meaning that adjacent origins fire simultaneously. So these clusters are activated in a spatial-temporal dependent manner and they are classified as early or late replication clusters depending on the moment of activation (Zink et al. 1999). In addition, there is the existence of another kind of origins, the dormant origins, present near the clusters and normally inactive, these origins are extremely important to maintain genome replication in case of replication stress (Blow et al. 2011, Ge et al. 2007, Ibarra et al. 2008, Woodward et al. 2006).

At every S-M transition Chk1 becomes active and localizes to the nucleus in a manner independent of DNA damaging agents (Sorensen et al. 2003, Kaneko et al. 1999).

During replication stress Chk1 is able to control origin firing by decreasing the number of active forks and it also stabilizes the replication fork so that after replication stress resolution the restart of replication is possible. As cited before there are a lot of causes to the replication stress HU, UV radiation, APH are the most commonly used in experiments, in addition with Chk1 specific inhibitors like AZD-7762 and UCN-01.

UCN-01 is a Chk1 competitive inhibitor that prevents $G_2$/M checkpoint action induced by DNA-damaging agents. UCN-01 binds to Chk1 in the ATP- binding pocket more concretely a hydroxyl group in the lactam moiety interacts with ATP—binding pocket, explaining its selectivity towards Chk1 over cyclin-dependent kinases (Zhao et al. 2002).

AZD7762 is an ATP-competitive inhibitor of Chk1 and it shows an high selectivity for checkpoint kinases in comparison with the other serine/threonine and tyrosine kinases. Thus, AZD has a different selectivity profile from UCN-01, revealed as a more potent inhibitor (Takahashi et al. 1989, Kawakami et al. 1996, Reinhardt et al. 2007).

In mammalian cells and in the presence of aphidicolin (APH), it has been shown that Chk1 inhibits the transition from early S phase to late S phase and also the activation of new replication clusters (Feijoo et al. 2001). Moreover, it has been showed that Chk1 dependent replication checkpoint effect acts mainly at late S phase clusters since early clusters activation is needed for checkpoint activation (Zegerman et al. 2009).

The exact mechanism that explains how Chk1 acts predominantly by inhibiting the firing of new replication clusters and not inside already activated clusters is unknown.

### 5.4.1 Chk1 role in replication origins firing

Some studies showed that the S phase checkpoint has an effect in the initiation before Cdc45 loading preventing helicase and polymerase actions (Karnani et al. 2011, Liu et al. 2006). These results are in agreement with another publication where a decrease in Cdc45 bound to the chromatin in a Chk1 dependent manner was observable (Falck et al. 2002).

The Chk1 related mechanism responsible for the prevention of Cdc45 loading onto the chromatin seems to involve different levels of regulation such as the Cdk2 dependent pathway, the Cdc7 regulated manner and the TopBP1/Treslin interaction mechanism.

### 5.4.2 Regulation of Cdc45 loading by Cdk2

Cdk2, activated by the upstream phosphatase Cdc25A, promotes the Cdc45 loading, in cases of unstressed cells (Scorah et al. 2009, Sorensen et al. 2004, Zhao et al.2002).

In contrast, in the presence of UV irradiation the Cdc45 loading is inhibited in a Chk1 dependent manner but independent of Cdk2 inactivation and Cdc25A degradation (Liu et al. 2006, Heffernan et al. 2007). So the differences observable between unstressed cells and UV irradiation conditions indicate an alternative pathway independent of Cdc25A and Cdk2.

### 5.4.3 Cdc45 loading regulated by Cdc7

Cdc7 kinase regulated by Dbf4 seems to participate in the pathway independent of Cdk2 and is responsible for the Cdc45 loading onto the chromatin (Heffernan et al.

2007). It has been shown that Chk1 regulates some aspects of Cdc7 activation (Yamada et al. 2013) which leads to conclude that the Dbf4-Cdc7 kinase complex is important in origin firing and is regulated by the ATR-Chk1 pathway.

### 5.4.4 Interaction TopBP1/Treslin controlling Cdc45 loading

The interaction between TopBP1 and Treslin as mentioned before promotes the formation and activation of the helicase complex. Chk1 seems to be able to disrupt the TopBP1/Treslin interaction and inhibits Cdc45 loading onto the chromatin (Boos et al. 2011).

Guo et al. 2015 showed that Chk1 is able to phosphorylate Treslin which may alter Treslin conformation or affect its interactions with other proteins involved in the replication mechanism. Chk1 action does not compromise the Treslin-TopBP1 interaction instead it alters the helicase-activating properties of this complex.

Although Cdk2 pathway explains the effect of Chk1 in cells subject to replication stress, this mechanism is unable to clarify the Chk1 action under basal conditions suggesting the existence of another mechanism. The regulation of Treslin by Chk1 may be part of that mechanism.

In conclusion, Treslin replication initiating function is negatively regulated by Chk1.

# 6. The projects

During my stay in this laboratory I had the opportunity to participate in two different projects.

In the first project I aimed to determine the effect of dNTPs addition on the DNA replication mechanism using western blotting and mass spectrometry analysis.

As previously mentioned the dNTPs effects on the DNA replication have already been reported in other species namely bacteria, yeast and mammalian cells. Thus, the lab proposed to determine the dNTP effect on DNA replication in our model of study, *Xenopus laevis*.

The lab performed experiences with high concentration of spermatozoids (4000 sperm nuclei per µl) incubated in egg extracts in the presence and absence of additional 50µM of each dNTP. This concentration doubled the endogenous concentration known for dATP and cCTP. DNA combing experiments revealed that addition of dNTP increased the replication content (Fig.7)

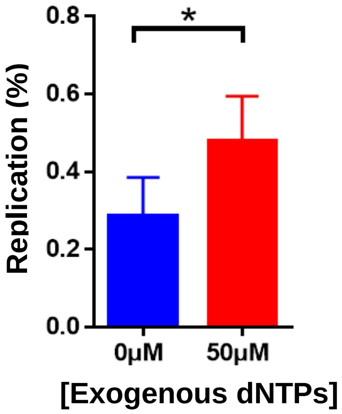


**Fig.7- Effect of dNTP addition in the replication extent.** Average replication percentages of 5 experiences (2-6) at 4000 sperm nuclei/µl from results obtained between 60 and 75min (t test, p=0,021).

In addition to the percentage of DNA replication other parameters related to replication were studied namely, fork density (number of fork/100kb), distance inter-origins and the size of the replication eyes.

The fork density is calculated by ratio between the number of activated forks and total analyzed DNA. The average of fork density for all the experiences realized showed an increase of 1, 75 folds in the presence of exogenous dNTPs (Fig.8).

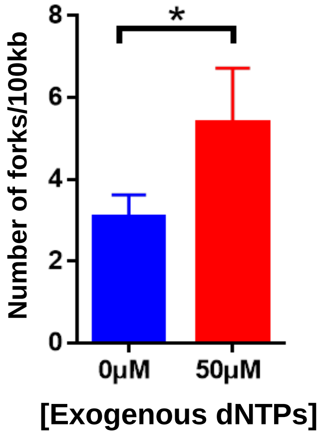


**Fig.8 – Effect of dNTP addition in the fork density.** Average fork density (number of forks/100kb) from 4 experiences (2, 4, 5 and 6) at 4000 sperm nuclei/µl, between 55 and 70min (t test, p=0, 0185).

The eye length is a parameter that informs the about the fork speed. And it also indicates the fusion of neighbors origins already activated.

For some experiences (Fig.9), an increase in eye length was observable when adding dNTPs. This suggests that the exogenous dNTPs also increases the fork speed in addition to the number of activated origins.

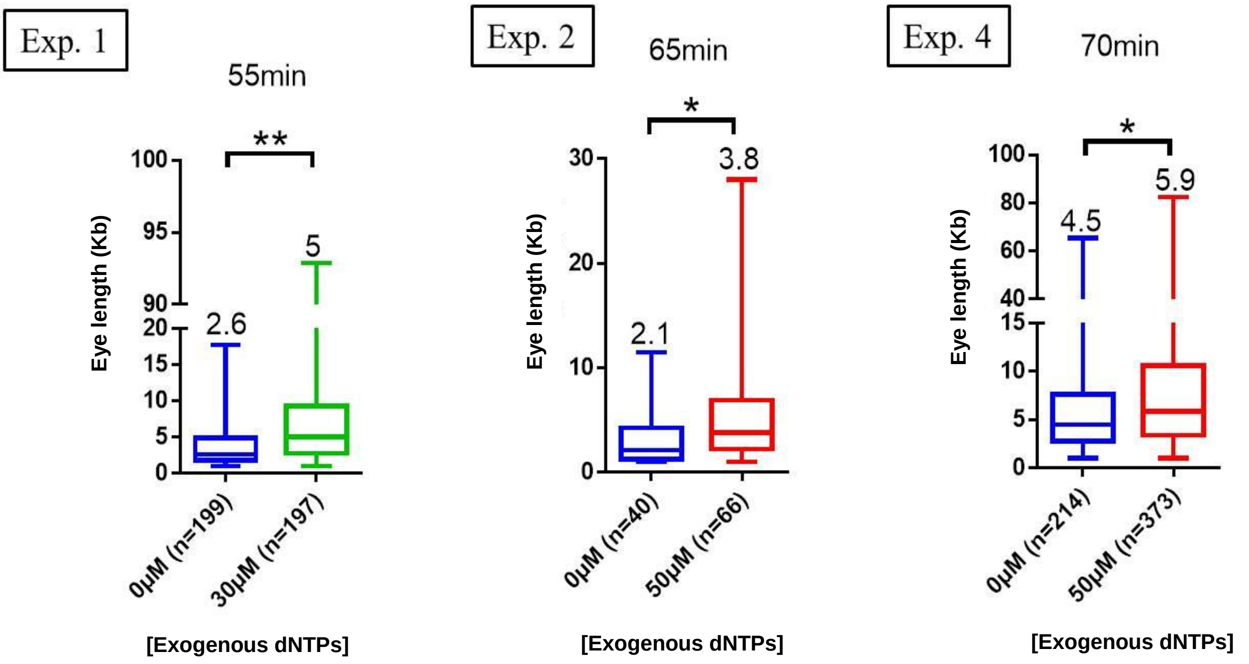


**Fig.9- Effect of dNTP addition in the eye length**. Box plot of eye length obtained from 1, 2 and 4 experiences (kb) at 4000 sperm nuclei/µl in of dNTPs concentration (0µM vs 30 or 50µM). The values present in graphics represent the median. The n corresponds to the number of replicated eyes. (t test : * p value < 0,01).

My first project consisted in using western blotting of chromatin fractions isolated during S phase to analyze whether known replication factors involved in the initiation or elongation are recruited differently onto the chromatin when exogenous dNTPs are added to the system.

In addition, using mass spectrometry (MS) analysis of all the proteins recruited into the chromatin was performed in order to quantify changes in all known chromatin bound proteins in the absence and presence of dNTPs. This approach offer us a more global view of all protein interacting with the chromatin not only the ones belonging to the replication mechinary but also proteins involved in other biological process such as chromatin remodelers or DNA cohesion proteins. About 50 proteins are directly involved in the DNA replication process, many more indirectly and in addition there might exist also so for unknown replication proteins.

In my second project, I proposed to reveal the effect of AZD 7762, a checkpoint kinase 1 inhibitor, on DNA replication.

The host lab has shown that the Chk1 inhibitor UCN-01 inhibits the activation of late origins clusters. Moreover, Chk1 is unable to inhibit origin activation of already activated clusters. The same results were obtained when Chk1 was immunodepleted. A more recent specific Chk1 inhibitor needed to be tested (AZD 7762).

Using DNA combing experiments I aimed to verify whether AZD 7762 has similar effects to the ones obtained for UCN-01 on the DNA replication in the *Xenopus in vitro* system.

# *7. Xenopus in vitro* system

During these projects I used the *Xenopus in vitro* system. *In vitro* systems use molecular and biochemical techniques to revise protein function, protein-protein and protein-DNA interactions that offer an important insight into several aspects of checkpoint activation signaling and activation of DNA replication mechanism.

The *Xenopus laevis* egg extract is able to recapitulate chromatin formation, DNA replication, mitotic spindle assembly, nuclear assembly and chromosome segregation when *Xenopus* sperm nuclei or plasmid DNA are incubated with these extracts for a determined period of time.

The initial observations about early embryonic cell cycle events were reported from the study of *Xenopus* egg cell division (Gerhart et al. 1985, Gerhart et al. 1984, Evans et al. 1983). Posteriorly, the need for a more flexible system to study biochemical aspects allowed the development of an egg extract cell free system (Blow et al. 1987, Blow et al. 1986, Hutchison et al. 1987, Lohka et al. 1983, Desai et al. 1999, Stearns et al. 1994, Miake et al. 1985, Almouzni et al. 1988). Although, the protocols have been improved in the way the extract is prepared over the years, the fundamental process remained the same.

This system allows multiple passages through the cell cycle, making the egg extracts a powerful tool for DNA replication and cell cycle progression studies (Blow et al. 1987, Blow et al. 1986, and Gautier et al. 1990). It mostly recapitulates rapid embryonic cell cycle events in the absence of transcription so it is an approximation to the somatic cell cycle process.

*Xenopus in vitro* system has some advantages and only a few disadvantages in biological processes studies. The main advantages are the short S phase (30-60min) in comparison to several hours required in differentiated cell cultures and the high protein concentration and enrichment of cell cycle factors present in limited concentration in other systems. Moreover, *Xenopus* and the other mammals are evolutionary close facilitating the characterization and isolation of proteins only abundant in complex vertebrate organisms.

On one hand, the absence of transcription, a poorly understood biological process, in egg extracts avoids the study in transcription involved biological events. On the other hand this fact provides the opportunity to establish in an unambiguous manner the functions of proteins involved in cell events like DNA replication and checkpoint activation. For example and since it is possible to deplete or add proteins to the extract the consequences of that addiction/depletion is directly related with the specific protein and not to indirect transcriptional effects.

## 7.1 DNA replication studies using *Xenopus in vitro* system

In *Xenopus* early development the S phase is really short, it has a duration of only 15-20 minutes. The medium fork speed is 0,5kb/min and considering the two replication forks departing from the same origin it is not possible to replicate more than 20kb in 20 minutes. In order to replicate the total DNA (6, 2 x $10^9$pb) it is necessary that the initiation sites are not more than 20kb apart and activated at the same time.

The *Xenopus in vitro* system based on the incubation of egg extracts with sperm nuclei is able to mimic the S phase in *Xenopus* early development. In this system the S phase takes between 40 to 60 minutes. *In vitro* studies revealed that the origins are randomly distributed across the genome (Hyrien et al,. 1995). DNA combing experiments showed that the origins are spaced between 8 and 15 kb from each other and their activation is asynchronous (Marheineke and Hyrien, 2001 and Blow et al. 2001). Kathrin Marheineke group compared the origins activation between two consecutives S phases. The results obtained show that some domains are preferentially activated in the beginning of the S phase suggesting a temporal regulation (Goldar et al., 2008).

The ATR-Chk1 checkpoint is involved in the spatial-temporal origin firing. In *Xenopus laevis* the replication origins are spaced 10kb between each other and are activated in clusters, a group of origins. These clusters may be activated at different moments of the S phase, according with the firing moment they are classified as early origins, late origins or dormant origins. ATR is involved in this spatial-temporal program since activated ATR decreases the number of activated origins deaccelerating the S phase (Marheineke and Hyrien, 2004)

To conclude in *Xenopus* the origins activation is a stochastic process and they are randomly distributed without specific sequences.

# Material and Methods

# 1. Model of study: *Xenopus laevis*

*Xenopus laevis* (fig.10) also known as African clawed frog is a species of aquatic frog that belongs to the Pipidae family.

**Fig.10- *Xenopus laevis*, model of study**

# 2. *In vitro* system using *Xenopus*

The main technique used in the laboratory is the *in vitro* system using *Xenopus* (Blow and Laskey, 1986). In this technique the cytoplasmic fraction is recovered to study the cell cycle activity present in the eggs.

## 2.1 Xenopus egg extracts

In order to mimic the S phase in vitro, the *Xenopus* eggs are incubated in a solution containing calcium that will activate the eggs. After successive washes using this time a solution without calcium and with the eggs in the interphase, they are able to simulate important biological events *in vitro*.

## 2.2 Preparation of cytoplasmic extracts in interphase

The frogs are injected with 50 units of eCG (horse chorionic gonadotropin) and 72h later they are subjected to another injection this time with 800 units of hCG (human chorionic gonadotrophine) stimulating the ovulation. To a proper ovulation the frogs are left overnight at 18ºC in a medium rich in NaCl (100mM).

Then, the eggs are washed with water three times and left resting for about 5 minutes allowing the swelling of the membrane surrounding the eggs. A solution containing 2% of cysteine in BARTH+Calcium (Nacl 88mM, KCl 2mM, MgCl2.6H2O 1mM, CaCl2 0,5 mM, Tris pH 7,6 15 mM) is used to degrade the protection shell surrounding the eggs. The eggs are submitted to rinsing three times with BARTH+ and activated by adding calcic ionosphere (0,25 µl/ml) to allow the calcium entrance present in the BARTH+ solution. The eggs are then washed two times with BARTH- (Nacl 88mM, KCl 2mM, MgCl2.6H2O 1mM, Tris pH 7,6 15 mM ) and two times with EB solution (HEPES pH 7,4 50mM, KCl 50mM, MgCl2 5mM, dithiothreitol 1mM). The activation step changed the cell cycle status of eggs from meiose to S phase.

The eggs are submitted to a first centrifugation at low velocity (2000 rpm, 4ºC) during one minute to pack the eggs. A second centrifugation at 14 000 rpm during 12 minutes leads to the eggs explosion. Three layers are obtained (fig.11), the inferior layer that contains the pigments and vitellus, the middle layer as known as LSS (low speed supernatant) corresponding to the cytoplasm more concretely to soluble proteins, mRNA, mitochondria and precursor of the nuclear envelope, the top layer has accumulated the lipid fraction of the eggs. The middle layer corresponding to the LSS is extracted using a syringe and the proteases inhibitors (1µg/ml of leupeptin, aprotinin and pepstatin A) are added before another centrifugation of 14 000 rpm during 12 minutes. This second centrifugation allows to eliminate pigments and lipids that can contaminate the cytoplasmic layer during extraction and affect the extract quality. A third or even a fourth centrifugation are performed depending on the quality and quantity of extract available. The extracts can be frozen in the form of beads using liquid nitrogen and adding 2% of glycerol and then stocked at -196ºC during several months.

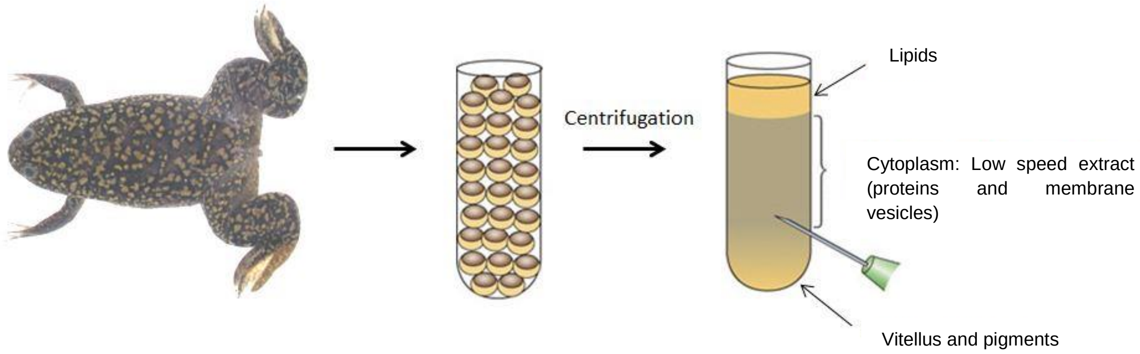


**Fig.11- Preparation of LSS extracts (Low Speed Supernatant) from Xenopus eggs.** The xenopus eggs are washed, activated, packed and centrifuged at low speed. The middle layer corresponding to the cytoplasm is extracted to be used in the experiments

## 2.3 Preparation of xenopus demembranated sperm

The males are euthanized in tricaine 0, 3 % pH7 and dissected for testicles extraction. The maximum possible of tissues and blood vessels are removed and the testicles are washed three times in HSB 1X solution (NaCl 0,1M, KCl 2mM, $MgSO_4$ 1mM, $Na_2HPO_4$ 0,5mM, Tris 15mM) and two times in SuNaSp 1X solution (sucrose 250mM, NaCl 75mM, spermidine 0,5 mM, spermine 0,2mM, dithiothreitol, leupeptin 5µg/ml, Pefabloc 100 µM).

Then, the testicles are nicely chopped in order to extract the maximum of sperm nuclei possible. After being included in SuNaSp 1X, the solution is filtered with a nylon filter of 100 microns. The sperm nuclei are centrifuged 10 minutes at 3000 rpm and this process is repeated one more time. The resulting pellet is re-suspended in TE pH7, 6 solution (Tris HCl ph7,6, 10mM, EDTA 1mM) and centrifuged during 5 minutes at 3000 rpm. One last time the pellet is re-suspended in SuNaSp 1X and centrifuged during 5 minutes. The pellet obtained is re-suspended in 2ml of SuNaSp 1X containing lysolecithin (10mg/ml) which is going to increase nuclear membranes permeability. This reaction is stopped by adding 10ml of SuNaSp 1X + 3% BSA and centrifuged at 3000 rpm during 5 minutes. To finalize the final pellet is diluted in a small volume (500 µl) of SuNaSp 1X + 0,3 % BSA + 30% glycerol. The spermatozoids concentration within the final solution can be determined by using Thoma counting chamber and diluted to obtain a stock concentration of $10^5$ and $4x10^5$ spermatozoids/µl. The stock solutions are then frozen in liquid nitrogen and stocked at -80ºC.

## 2.4 Replication in xenopus egg extracts

The cytoplasmic extracts obtained from the xenopus eggs are maintained in interphase due to the addition of cycloheximide, a protein synthesis inhibitor, which is going to inhibit the translation of the cyclin B. The stock solutions prepared containing *Xenopus* sperm nuclei are incubated with the extract at 23ºC in the presence of EM (energy mix, 7,5 mM creatine phosphate, ATP 1mM, EGTA pH 7,7 0,1 mM, $MgCl_2$ 1mM), cycloheximide (250µg/ml) and 20µM of modified nucleotides like biotin-dUTP or rhodamine-dUTP. The type of modified nucleotides and the concentration of sperm change according with the realized experiments.

A basic experiment is normally realized each time the replication in *Xenopus* egg extracts is performed (fig.12).

The extract is incubated with Tetramethyl-rhodamine-5-dUTP and sperm nuclei. The reaction is stopped with 500µl of PBS 1X (Phosphate-buffered saline 1X) and fixed by adding 500µl of paraformaldehyde 8%. The samples are transferred to a cushion of PBS 20% Sucrose covering one polylysine treated coverslip. After a 5 minutes centrifugation at 2000rpm the nuclei are fixed into the coverslip surface. The supernatant is removed and the coverslip is washed with PBS 1X SDS 0,02%, Triton 0,1%. Then, the coverslip is incubated with (1/1000) Hoechst 33342 (a blue fluorescent dye used to stain DNA) diluted in the solution previously used (PBS 1X SDS 0,02%, Triton 0,1%) during 20 minutes. After washing with PBS 1X SDS 0,02%, Triton 0,1% to remove the excess of fluorescent dye it is possible to prepare the coverslip with the microscope slides and a drop of VECTASHIELD to visualize the samples under fluorescence microscope. This relatively quick test allows to verify whether the extracts incubated with sperm nuclei have replicated and to estimate the percentage of replication.

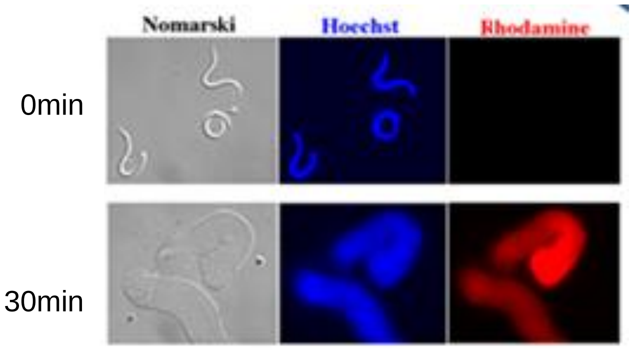


**Fig.12- DNA replication *in vitro*.** The sperm nuclei incubated with the Xenopus egg extracts will initiate the replication 30 min after incubation. Then, the samples can be observed by fluorescence microscopy due to incubation with Rhodamin-UTP and Hoechst staining

# 3. DNA combing

DNA combing allows the stretching of DNA molecules and visualizing them in an individual manner by using immunofluorescence staining. The stretching is performed by the withdrawal of a glass coverslip from a reservoir containing purified DNA in suspension (fig.13). The presence of an air-solution meniscus is enough to stretch the DNA in a regular way onto the coverslip. This technique was first used by Huberman and Riggs allowing DNA fibers visualization stained with tritiated thymidine (Huberman and Riggs, 1966). The technique used nowadays is being used since 1990 (Bensimon et al. 1995), it consists in immersing a glass silanized coverslip into a solution of MES (acide 2-N-Morpholino-ethane-sulfonic) containing DNA and then removing the coverslip in a regular way with the help of a mechanic system. This technique has as advantages the reproducibility and the same degree of stretch.

DNA combing is particularly important in the study of DNA replication, since it allows to visualize the replicon, the positon of the origins and speed of the replication forks.

DNA combing is a technique that fixes the DNA molecules irreversibly, orderly and in a reproducible manner (1µm=2kb). However, the efficiency of this technique depends on two factors: the pH of the MES solution where the DNA is suspended and the quality of silanized coverslips.

DNA molecules in suspension interact spontaneously with the hydrophobic surface of the coverslip and the air-solution meniscus allows the stretching of the fibers across the glass surface. There is a strong interaction between the hydrophobic surface of the coverslip and the hydrophobic domains of bases of the extremities exposed to the denaturation.

As mentioned before the pH of the MES solution is crucial for the fibers fixation to the coverslip (Allemand et al. 1997), a pH too acidic leads to fibers strongly fixated but in a non-specific manner so the fibers are not properly stretched across the coverslip. A pH too basic does not allow fiber fixation. To conclude, only an optimal pH results in a specific (by the extremities) and strong fixation. In the experimental conditions used in our lab the pH optimum was around 5, 9.

The other factor that determines fibers fixation is a correct silanization of the coverslips. The coverslips are subjected to a treatment to turn them hydrophobic. The coverslips are rigorously cleaned first with acetone and then left for about 20 minutes in a 50% methanol solution in a globet into an ultrasonic cleaner. The same procedure is followed this time with chloroform. Afterwards the coverslips are left drying individually and exposed to the Plasma Cleaner during 5 minutes each side in order to clean the coverslip surface. After that the coverslips are dried at 110ºC during at least 1 hour before being placed inside heptane containing 0,1 % of silane in a desiccator overnight. Now that the silanisation was realized, the coverslips are submitted another time to ultrasound cleaning primarily with heptane pure and posteriorly with water and chloroform. With the conclusion of this procedure the coverslips obtained have the proper conditions to stretch the DNA fibers properly.

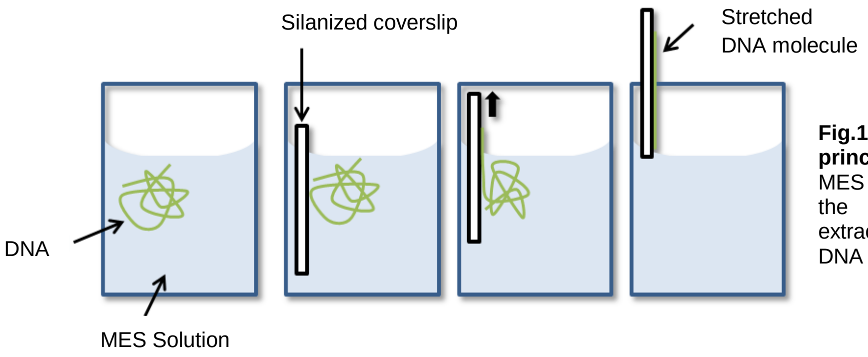


**Fig.13 – DNA Combing principle.** The soluble DNA in MES at a specific pH will bind to the silanized coverslip. The extraction of the coverslip leads to DNA stretching.

## 3.1 DNA combing protocol

When performing DNA combing the modified nucleotide used is biotin-16-dUTP. The extracts are incubated with a certain concentration of sperm that varies according with the type of experiment. The reaction is then stopped by adding 500µl PBS 1X and centrifuged during 5 minutes at 3000rpm. The resulting pellet is re-suspended in PBS 1X and re-centrifuged. After removing the supernatant the samples are re-suspended in 40 µl of LMP (low melting point) 1% agarose. Next, the agarose blocks are submitted to digestion by proteinase K (1mg/ml) at 65ºC during approximately 48 hours. This reaction is stopped with the incubation of Pefabloc (10µg/ml) at 37ºC. To conclude, the agarose blocs are melted first by submitting them at 68ºC during 20 minutes and then by adding β-agarase at 42ºC overnight.

### 3.1.1 DNA stretching

The purified DNA is labeled with YOYO (0,3µM), a DNA intercalant agent absorbing at 491nm and emitting at 509nm. The samples are diluted in 1,2ml of MES solution and the coverslips are mechanically dived in the solution and slowly removed in order to stretch the fibers. The YOYO allows to verify the correct stretching and fibers density. If these conditions are fulfilled the DNA is treated, denatured and incubated with specific antibodies.

Then, the coverslips are dried during several hours at 65ºC to assure an irreversible fixation of the molecules to the silane and to prepare them to the labelling. The coverslips are submitted to a dehydration by successive incubations of ethanol 70%, 90%, 100% during 3 minutes. The next step, the denaturation is realized in a solution of 50% formamide-2X SSC at 72ºC during three minutes. The YOYO labelling is removed across the denaturation. There is a second phase of dehydration with frozen ethanol at 70%, 90%, and 100%.

Before immunofluorescent labelling the samples are blocked with the blocking solution 1,5% (1,5% Blocking Reagent, PBS 1X, 0,05% Tween, pH 7,3) overnight at 4ºC.

### 3.1.2 Immunofluorescent labelling

The coverslips are now labelled to visualize the incorporated biotin and the totality of DNA. The biotin labelling is realized by a succession of five incubations at 37ºC alternating between Streptavidin Alexa Fluor 594 (40µg/ml) (one hour incubation for the first and 30 minutes for the following incubations) and biotinyled anti-Streptavidin antibodies (10µg/ml, 30 minutes of incubation) inside 1,5% blocking solution (fig.14). The presence of three Streptavidins allows a signal amplification.

Then, the coverslips are blocked during one hour with 2% BSA solution (2% BSA, PBS1X, 0,05% Tween) and the totality of DNA is labelled with human anti-DNA antibody in 2% BSA solution followed by two other antibodies, Alexa Fluor 488 anti-mouse (45 minutes of incubation) and anti-rabbit (30 minutes of incubation) in a 1,5% blocking solution.

Streptavidin absorbs at 590nm and emits at 617nm in contrast the anti-mouse antibody absorbs at 495nm and emits at 519nm, since there are differences between the two antibodies two different signals are observed in the fluorescence microscope.

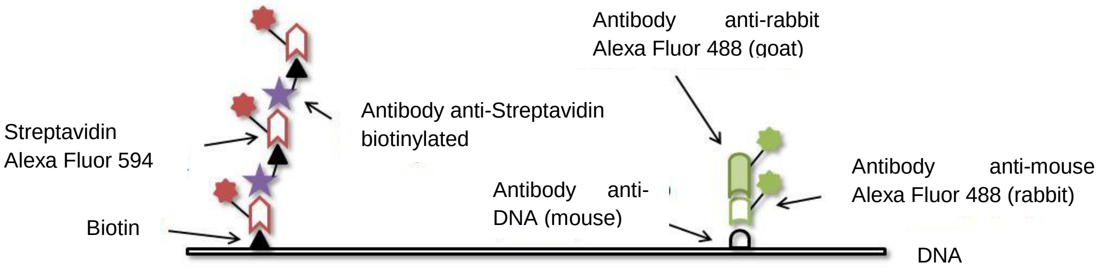


**Fig.14- Immunofluorescence labelling of the DNA contained in the coverslip**. The biotin incorporated during replication is labeled with Streptavidin Alexa Fluor 594 and the antibody anti-Streptavidin. The total DNA is labeled by anti-DNA and posteriorly by two Alexa Fluor antibodies.

The observation of the labelled fibers (fig.15) is done using a fluorescence microscope, in this case the inverted microscope Zeiss observer Z1, with the object-glass X100. The images are acquired by the Evolve (photometrics technology) camera associated to the AxioVision V4.8.2.0 software. The analysis was made with the help of ImageJ software. The measures obtained in pixels are converted in Kb and compiled with a small program (Macro) in Microsoft Excel. The maximal resolution of this technique is 1kb using a 100X objective. The different parameters obtained with this analysis are showed in table 1.

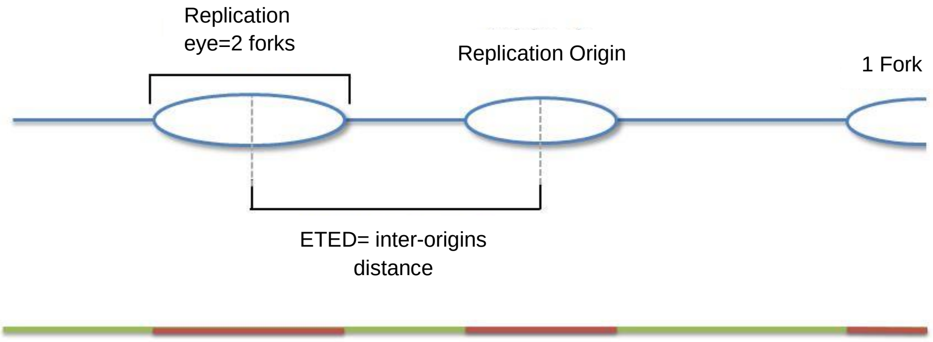


**Fig.15- DNA combing parameters.** Schematic representation of some observable parameters by DNA combing and microscopic appearance of DNA fiber, the total DNA is observed in green at the microscope and the replicated DNA in red.

**Table 1- List of parameters obtained from the analysis of DNA combing experiments.**

| Parameters | Definitions |
|---|---|
| **DNA total (Kb)** | Size of total DNA detected (green staining) |
| **DNA replicated (Kb)** | Size of total replicated DNA (red staining) |

| | |
|---|---|
| **Replication extent (%)** | Ratio replicated DNA/ total DNA |
| **Average size of all fibers (Kb)** | Average size of all fibers |
| **Average eye length (Kb)** | Average size of all replicated eyes |
| **Distance inter-origins (ETED, Kb)** | Distance between two replication eyes |
| **Number of replication forks** | Number of complete eyes (corresponding to 2 forks) more number of eye at the fibers extremities (corresponding to 1 fork) |
| **Number of origins** | Number of forks/2 |
| **Fork density (N/100Kb)** | Relation number of forks/ total DNA |
| **Average size of replication eye** | Quotient-replicated DNA/ number of origins |
| **ETED overall (Kb)** | Ratio total DNA/ number of origins |

# 4. Purification of the chromatin proteins and analysis by Western Blot

The egg extract (50µl) is incubated with the sperm nuclei (2000 nuclei per µl) in presence of cycloheximide (250µg/ml), EM (7,5 mM creatine phosphate, ATP 1mM, EGTA pH7,7 0,1mM, $MgCl_2$ 1mM) cytochalasin B (10µg/ml) and dNTPs (50µM). The controls without dNTPs are also prepared, changing the dNTPs for water. After incubation at 23ºC during different times according to the experiment conditions, the reaction is stopped with 750µl of chromatin isolation buffer (CIB) 0,1 % Triton (HEPES-NaOH pH7,5 50mM, NaCl 100mM, $MgCl_2$ 2,5mM, DTT 500µM, Spermidine 500µM, Spermine 200µM, Leupeptin 5µg/ml, Pepstatin A 5µg/ml, Aprotinin 5µg/ml, PhosSTOP 0,5X, Triton 0,1%) and the content is transferred to another tube containing a cushion of CIB 20% Sucrose (200µl). The tubes are centrifuged at 6800 rpm, 4ºC during 5 minutes. The resulting supernatant is removed, leaving only a small volume and the pellet, and the interphase is going to be washed three times with 150µl of CIB 0,1% Triton to retrieve the chromatin remaining in the extremities of the tube. The tubes are now subjected to a stronger centrifugation (13000rpm, 4ºC) during 5 minutes. Then, almost all the volume of the tubes is removed leaving about 20µl, posteriorly 20µl of Laemmli 2X is added before denaturation at 95ºC during 5 minutes.

Next, the DTT (Dithiothreitol 100mM) and the Benzonase nuclease (75u) are added to reduce proteins and digest the DNA respectively. Then, the samples are charged on a 10% or 8% acrylamide gel (depending on the molecular weight of the protein of interest). Once the samples are charged in the gel, it is submitted to a SDS-PAGE gel electrophoresis that will separate the proteins by size (fig.16).

Afterwards, the gel content is transferred to a Polyvinylidene fluoride (PVDF) membrane activated by methanol; the time of transfer is about 2 hours (fig.16). When the transfer process has finished the membrane is blocked around 2 hours with Tris-buffered saline (TBS-T) 2% milk, the proteins present in the TBS-T 2% milk bind with membrane in all the places where the chromatin proteins are not attached (fig.16). Thus, when the antibody is added it will only interact with the specific protein of interest and prevents non-specific binding, reducing the background signal.

Posteriorly to the blocking, the membrane is incubated normally overnight with the primary antibody (fig.16), which is going to interact with the protein of interest. The antibodies used were: anti-Cdc45, anti-Treslin, anti-MCM4, anti-MCM7, anti-Cdc7, anti-Drf1, anti-H3, anti-Orc1, anti-Orc2, anti-TopBP1, anti-ReQ4, anti-polymerase β, anti-polymerase ε (table 2).

**Table 2- List of primary antibodies used with respective molecular weight, antibody dilution, secondary antibody affinity and origin of the antibody.**

| Protein | Molecular Weight | Antibody Dilution | Origin | Secondary antibody affinity |
|---|---|---|---|---|
| ***Cdc45*** | 60kDa | 1/1000 (in TBS-T 5% milk) | Santa-Cruz Biotechnology | rabbit polyclonal |
| ***Cdc7*** | 58kDa | 1/1000 (in TBS-T 5% milk) | Received from J. Walter | rabbit polyclonal |
| ***Drf1*** | 112kDa | 1/1000 (in TBS-T 5% milk) | Received from J. Walter | rabbit polyclonal |
| ***Histone H3*** | 18kDa | 1/1000 (in TBS-T 5% milk) | Bethyl Laboratories | rabbit polyclonal |
| ***MCM4*** | 100kDa | 1/2000 (in TBS-T 5% milk) | Bethyl Laboratories | rabbit polyclonal |
| ***Orc1*** | 100kDa | 1/1000 (in TBS-T 5% milk) | Received from R. Laskey | rabbit polyclonal |
| ***Orc2*** | 66kDa | 1/1000 (in TBS-T 5% milk) | Received from R. Laskey | rabbit polyclonal |
| ***Polymerase δ*** | 124kDa | 1/1000 (in TBS-T 5% milk) | Received from J.C. Cadoret | mouse polyclonal |
| ***Polymerase ε*** | 55kDa | 1/1000 (in TBS-T 5% milk) | Received from J.C. Cadoret | mouse polyclonal |
| ***RecQ4*** | 133kDa | 1/1000 (in TBS-T 5% milk) | Thermoscientific | rabbit polyclonal |
| ***TopBp1*** | 200 kDa | 1/1000 (in TBS-T 5% milk) | Bethyl Laboratories | rabbit polyclonal |
| ***Treslin*** | 210kDa | 1/1000 (in TBS-T 5% milk) | Bethyl Laboratories | rabbit polyclonal |

The excess of primary antibody is removed with TBS-T and the membrane is incubated with a secondary antibody during 1 hour at room temperature with a dilution factor of 1/2000 TBS-T 5% milk (fig.16). The secondary antibody linked to the horseradish peroxidase binds to the primary antibody and using the Super Signal West Pico Chemiluminescence kit (Pierce) produces a chemiluminescent signal. The chemiluminescence is a property of chemical reactions which emit light as a byproduct. Using peroxidase as the enzyme label, the luminescent substance luminol will be oxidized by hydrogen peroxide creating an excited state product (3-aminophthalate). As this excited state product decays back to the ground state a light at 425 nm is emitted which is registered with the Las-3000 imaging system (Fuji).

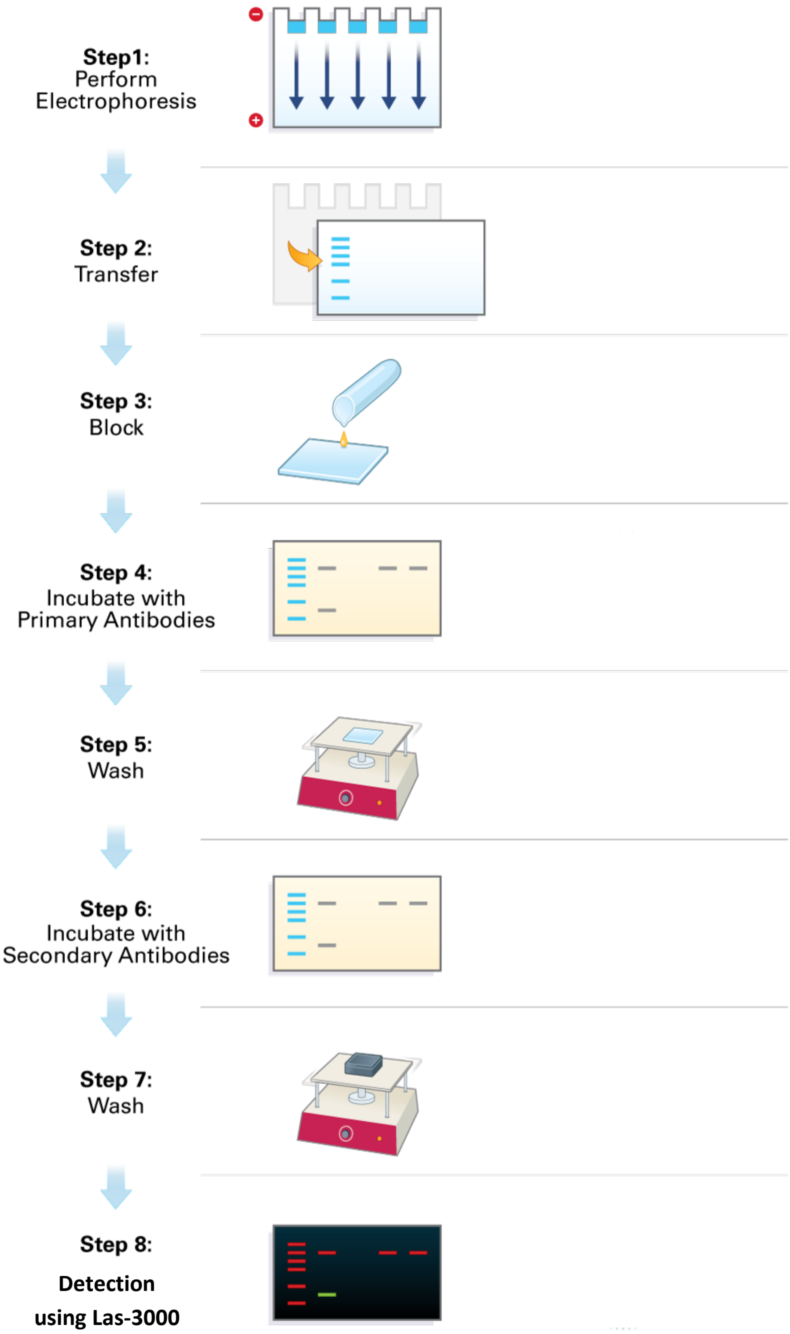


**Fig.16- Western blot steps.** Representation of Western Blot steps followed after chromatin purification. Figure adapted from Molecular Devices.

## 4.1 Membrane stripping

Stripping is the term used to describe the removal of primary and secondary antibodies from a Western blot membrane. This method is useful when we want to study more than one antibodies in the same membrane. For example in the quantitative western blot analysis performed it was necessary to incubate in the same membrane at least one protein of interest and a loading control. The stripping buffer (200mM glycine, 0, 1 % SDS, 1% Tween) is adjusted to a 2,2 pH and incubated with the membrane at 80ºC during 20 min. Then the stripping buffer is removed using PBS 1X during 20 min and TBS-T during 10 min. The next steps are the same followed as for a normal western blot protocol consisting in blocking with TBS-T 2% milk during 2 hours, followed by primary antibody and secondary antibody incubations.

# 5. λ-phosphatase action in chromatin proteins

The lambda phosphatase is a $Mn^{2+}$ dependent protein phosphatase with activity towards phosphorylated tyrosine, serine and threonine residues. This enzyme has a dephosphorylated activity and it was used to study MCM4 and Treslin proteins. Both MCM4 and Treslin are proteins subjected to phosphorylation using λ-phosphatase it is possible to verify whether these phosphorylation is observable in western blot analysis. The λ-phosphatase (100U/µl) is incubated with chromatin fractions prepared according with the chromatin purification protocol mentioned previously in the "Purification of the chromatin proteins and analysis by Western Blot" section. Moreover, the reaction solution is prepared with additional Lambda Phosphatase Buffer 10X (10-fold dilution), $MnCl_2$ solution with a 10-fold dilution and incubated at 30ºC during 20 minutes. Four samples were prepared following the conditions showed below in the table 3. After λ-phosphatase reaction the samples were prepared for western analysis.

**Table 3**- **Specificities of the samples prepared to λ-phosphatase reaction.** Four different tubes were prepared a control, containing only water and extract, a negative containing not only supernatant and water but also $MgCl_2$ and buffer solution needed to a proper enzyme action. And a sample containing the enzyme and another with the enzyme more inhibitor.

| | **Control** | **Negative** | **Enzyme** | **Enzyme+ Inhibitor** |
|---|---|---|---|---|
| **Temperature** | 4ºC | 30ºC | 30ºC | 30ºC |
| **Chromatin fraction (µl)** | 10 | 10 | 10 | 10 |
| **$H_2O$ (µl)** | 10 | 6 | 5 | 2 |
| **$MgCl_2$ (µl)** | | 2 | 2 | 2 |
| **Buffer solution 10X (µl)** | | 2 | 2 | 2 |
| **Enzyme (λ-phosphatase) (µl)** | | | 1 | 1 |
| **Inhibitor (PhosSTOP) (µl)** | | | | 3 |

# 6. CHROMASS: Chromatin Isolation and Sample Preparation for Mass Spectrometry

The xenopus egg extract (20µl) is incubated with the sperm nuclei (4000 nuclei per µl) in the presence of cycloheximide (250µg/ml), EM (7,5 mM creatine phosphate, ATP 1mM, EGTA pH7,7 0,1mM, $MgCl_2$ 1mM) cytochalasine B (10µg/ml) and dNTPs (50µM). The controls without dNTPs are also prepared, changing the dNTPs for water. The reaction is stopped with 130µl of ELB (10 mM HEPES–KOH at pH7.7, 50 mM KCl, 2.5 mM MgCl2) containing 0,2% Triton and transferred to another tube containing a cushion of ELB 500mM Sucrose (180µl). The tubes are centrifuged at 8000rpm, during 50 seconds; after the supernatant removed the pellet is washed with 200µl of ELB 250mM Sucrose and centrifuged during 50 seconds at 8000rpm. This step is repeated once more before removing the supernatant and leaving only 10µl. The additional steps needed to be followed for the samples destined to mass spectrometry analysis, were performed by ImaGiF platform. At the same time, samples for western blot were prepared by adding Laemmli 4X containing β-mercaptoethanol and denaturated at 95ºC during 5 minutes. Samples were analyzed by the platform using NanoLC/MS/MS spectroscopy and nanoESI-Triple TOF 4600 (AB Sciex)

# Results

# 1. Analysis of a possible modification in the replication factors recruitment onto the chromatin after dNTP addition

The aim of this project was to identify the molecular mechanism by which dNTP increase DNA replication extent. I first performed western blot analysis of various DNA replication factors during DNA replication. The egg extracts were incubated with sperm nuclei in a concentration of 1000-4000 nuclei per µl in presence or absence of exogenous dNTPs (50µM). Afterwards the reaction is stopped at different moments of the S phase and chromatin purifications are performed to study the proteins recruited onto the chromatin. Non-published results of the lab using DNA combing showed that the number of origins was increased upon dNTP addition and that fork speed was increased. In order to further investigate this result by western blot analysis I first tested the amount of Cdc45 recruited onto the chromatin. Cdc45 is involved in the pre-IC formation which leads to DNA replication initiation. In addition, Cdc45 is part of the CMG helicase complex which travels with the replication fork and unwinds the DNA double strand. According with the DNA combing results showing an increase in the number of activated origins in the presence of exogenous dNTPs, we expected an increase in the amount of CMG complexes bound to the chromatin implying an increase in the Cdc45 recruitment onto the chromatin.

Several different and independent experiments were performed. Since the purification of the chromatin is not always equal for each time point, Cdc45 signal obtained from western blot analysis was normalized either with histone 3 (H3) or Orc1 or Orc2 which are constant during S phase. Although two blots with positive results, showing an increase of Cdc45, were obtained (Exp. E and F) other western blot analysis performed showed a less consistent Cdc45 increase (Exp. A, B, C and D). The discrepancies obtained from the different analysis can either be due to the chromatin isolation protocol that is not very reproducible or the dNTPs effect that is variable. In order to control the effect of dNTP addition DNA combing experiments were performed in parallel with western blotting (experiment F), however these experiments are very time consuming.

For the experiment A, the sperm nuclei (2000 nuclei/µl) were incubated with egg extracts during 40, 50, 60 and 70 min and chromatin was purified as described in the material and method section. Chromatin bound proteins were analyzed by western blotting using anti-Cdc45 antibody and anti-H3 antibody (histone 3) as a loading control allowing a signal normalization when the loading is uneven. The bands intensity is quantified with ImageJ and normalized with the H3 signal. Although the Cdc45 recruitment increases in the presence of 50µM dNTPs in the first time (40min), for the remaining times a significant decrease was observed (Fig.17). These decreases can be due to a weak signal intensity in some H3 bands preventing a proper signal normalization.

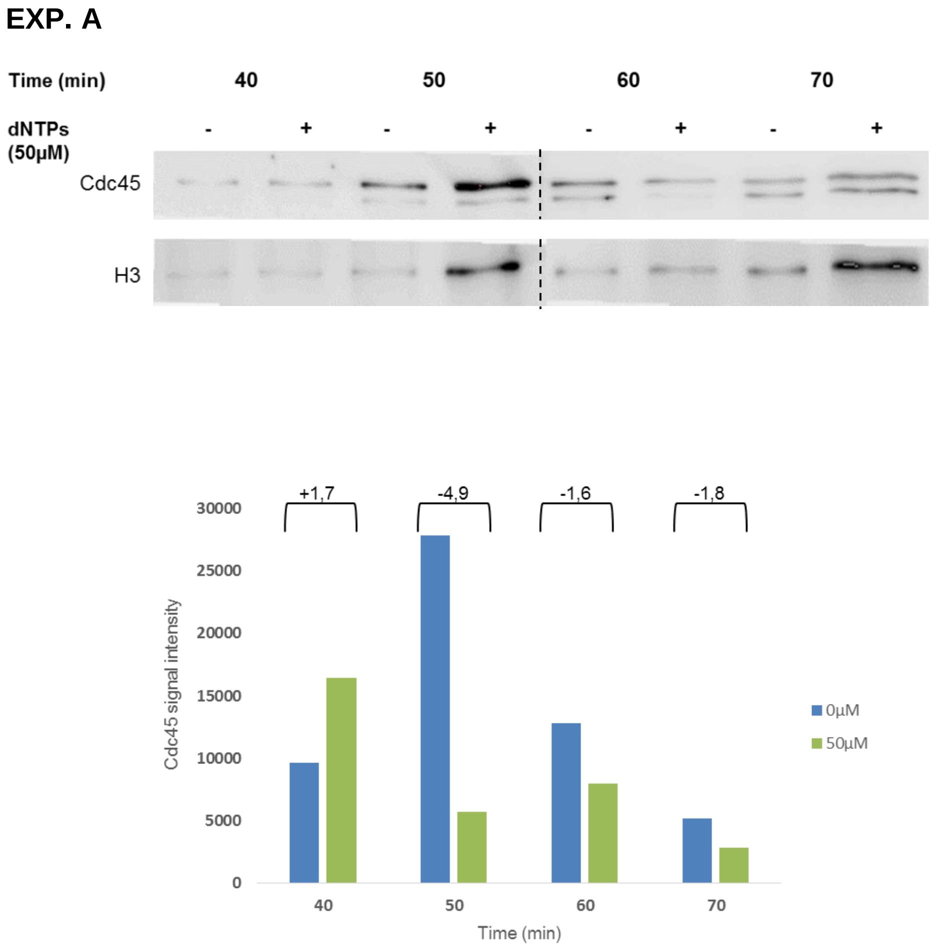


**Fig.17- Results from experiment A.** Western Blot from Cdc45 and H3 as a loading control. Graphic representation of the bands intensity for the Cdc45 present in the blot.

The experiment B (Fig. 18) obtained from egg extracts incubation with sperm nuclei (1000 nuclei/µl) during 30, 40, 50 and 60min showed for the Cdc45, a decrease in its recruitment when adding exogenous dNTPs for 30, 40 and 50 min and a slight increase in the last time (60min). Cdc45 signal measured from the bands intensity was normalized with H3 as loading control. During the experiment the egg extract revealed to be of poor quality leading too slow replication without the proper conditions (Rhodamine dUTP incorporation control, not shown). We reduced the nuclei concentration to 1000n/µl in order to obtain more optimal replication kinetics in the next experiment.

**EXP. B**

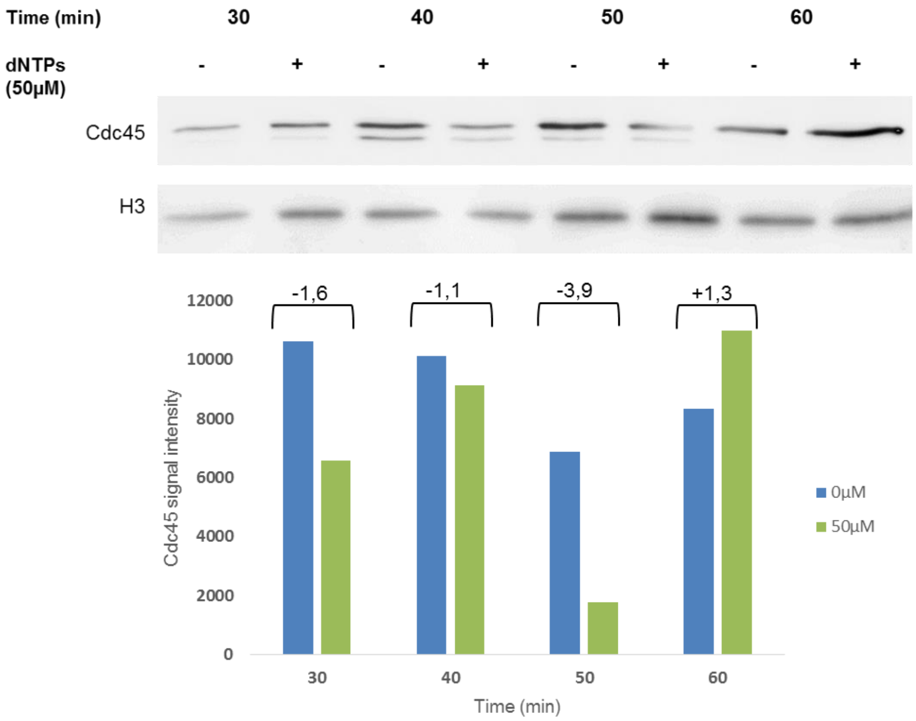


**Fig.18- Results from experiment B.** Western Blot from Cdc45 and H3 as a loading control. Graphic representation of the bands intensity for the Cdc45 present in the blot.

In experiment C (Fig. 19) egg extracts react with sperm nuclei (1000 nuclei/µl) at different times (30, 40, 50 and 60min). The western blot was incubated with anti-Cdc45, anti-Cdc7, anti-MCM4 and anti-H3. Only the bands corresponding to the Cdc45 were quantified since the Cdc7 and MCM4 bands were too faint for a proper quantification. Cdc45 quantification shows an increase at 30 min in the presence of 50µM of dNTPs, however for the remaining times (40, 50 and 60min) a decrease is observable. The increase at 30min may be explained by the weak signal of the H3 band corresponding to the $30^+$ which compromises a proper Cdc45 signal normalization.

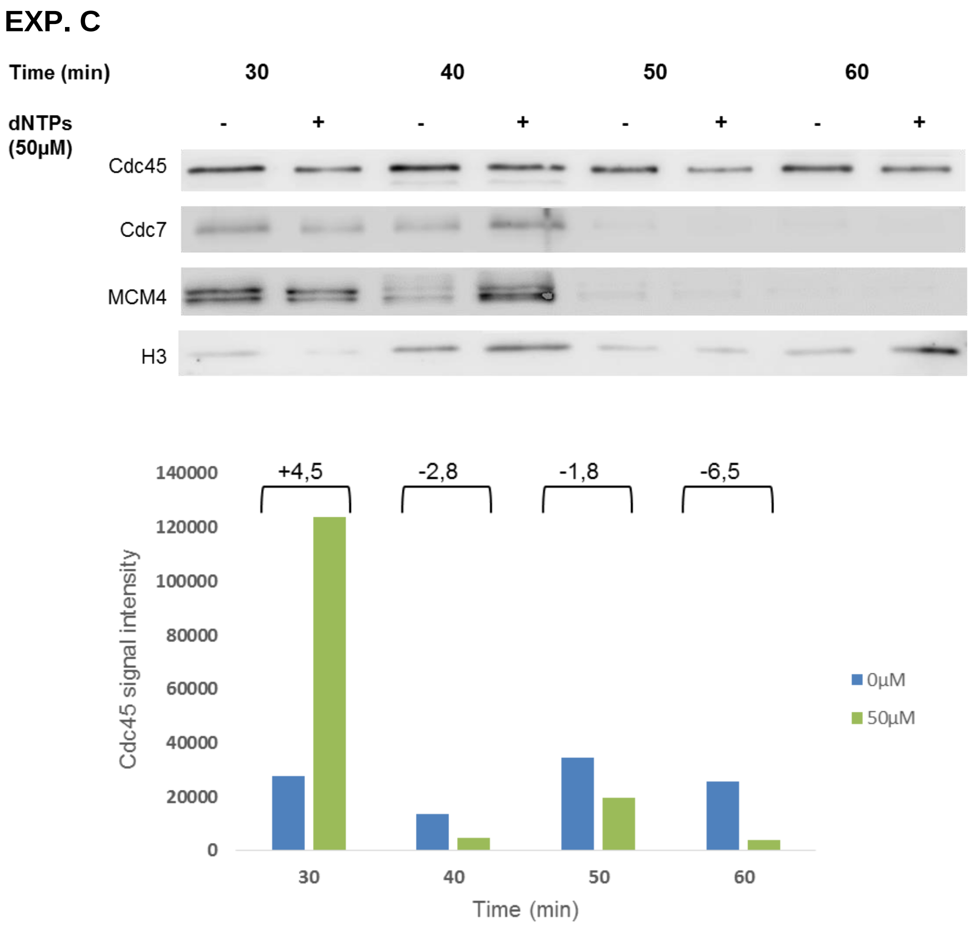


**Fig.19- Results from experiment C.** Western Blot from Cdc45, Cdc7, MCM4 and H3 as a loading control. Graphic representation of the bands intensity was only represented Cdc45 due to poor signal for Cdc7 and MCM4 western blots

To obtain the results shown in the Fig.20 egg extracts were incubated with sperm nuclei (1000 nuclei/µl) at 25, 35, 45 and 55min. The Western blot from the experiment D (Fig.20) incubated with anti-Cdc45 suggests that at 25min there is no significant difference between the control and the sample with added dNTPs. In the presence of 50µM dNTPs, an increase of Cdc45 recruitment into the chromatin at 35 and 55min is observable. In contrast, the considerable decline in the Cdc45 recruitment at 45min may be explained for the $45^{+}$ band saturation of H3 conditioning a suitable signal intensity normalization.

## EXP. D

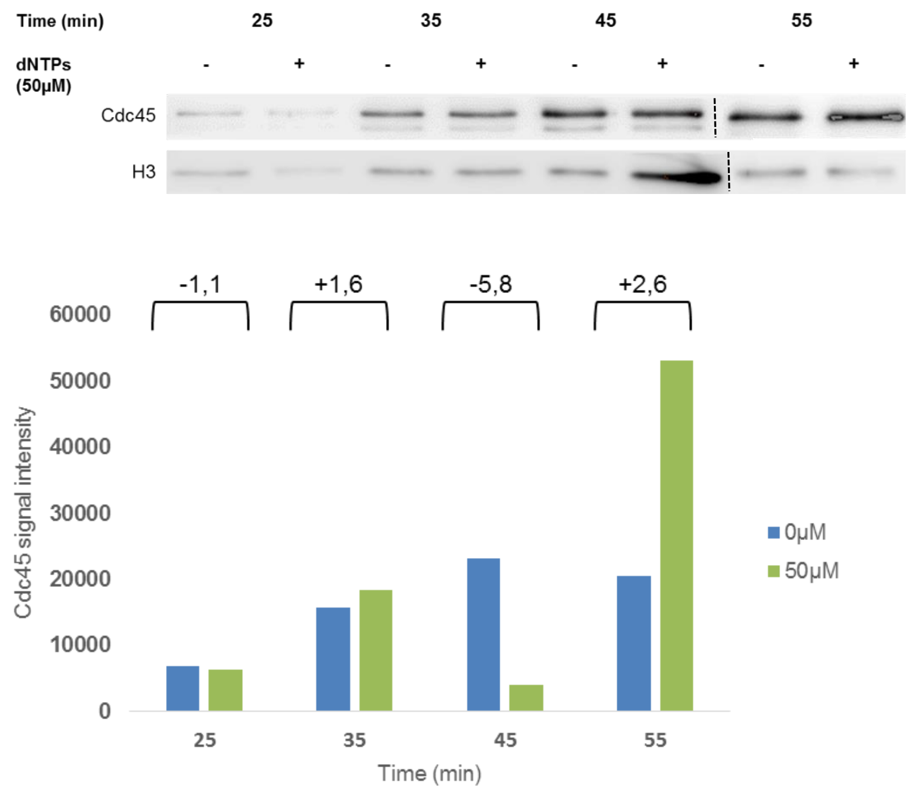


**Fig.20- Results from experiment D.** Western Blot for Cdc45 and H3. Graphic representation of the bands intensity for the Cdc45 using H3 to signal normalization.

The experiment E resulting from egg extracts incubation with sperm nuclei (2000 nuclei/µl) showed an increase in Cdc45 recruitment in the presence of exogenous dNTPs (Fig.21). However this increase is only significant at 50min (2, 8 fold) since at 40 min there is only an increase of 1, 5 fold and for 60 min a 1, 1 fold. Since the western-blot results for the Cdc45 recruitment seemed variable, in the next experiment we controlled the dNTP effect by a parallel DNA combing experiment.

**EXP. E**

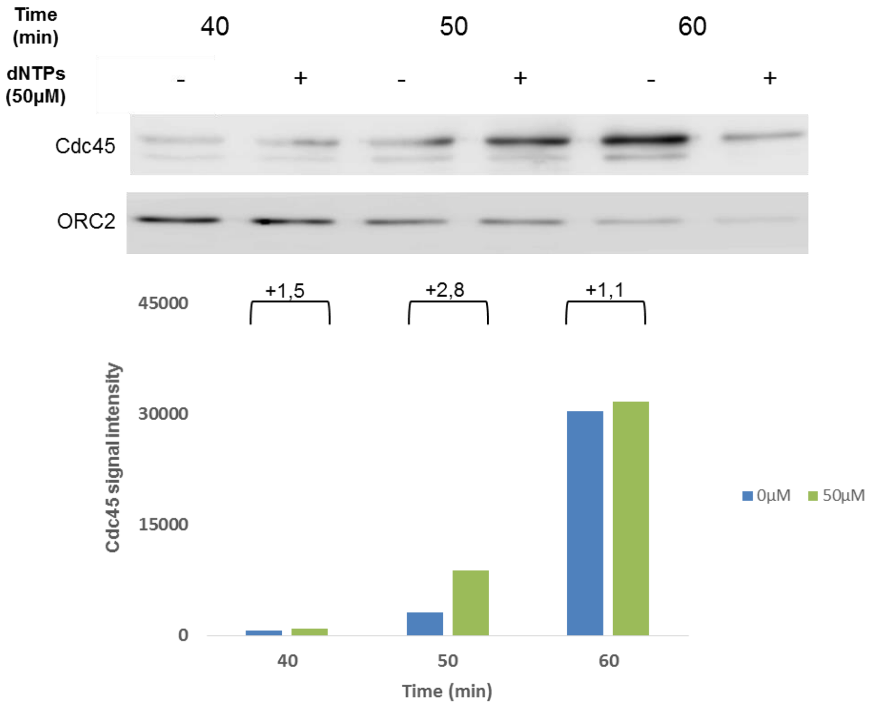
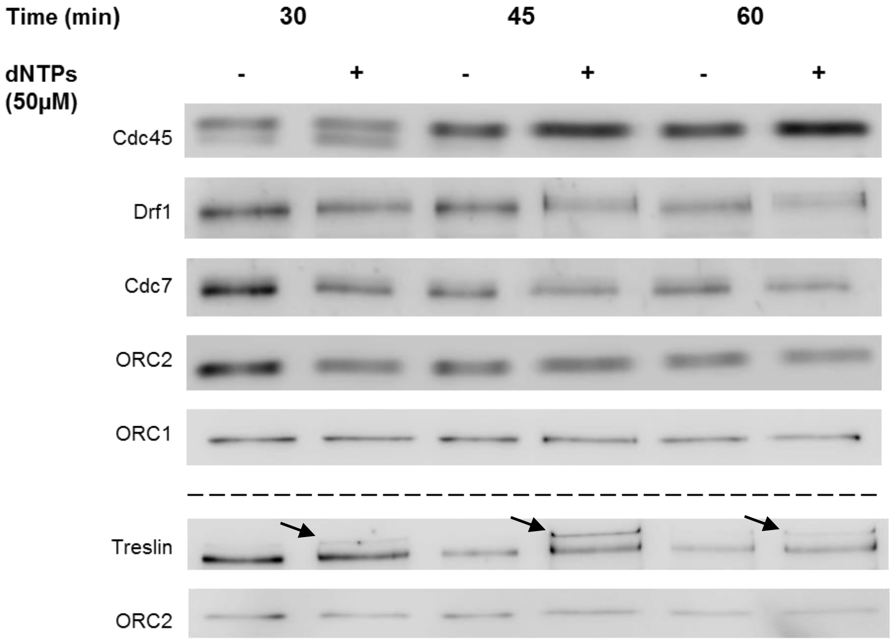
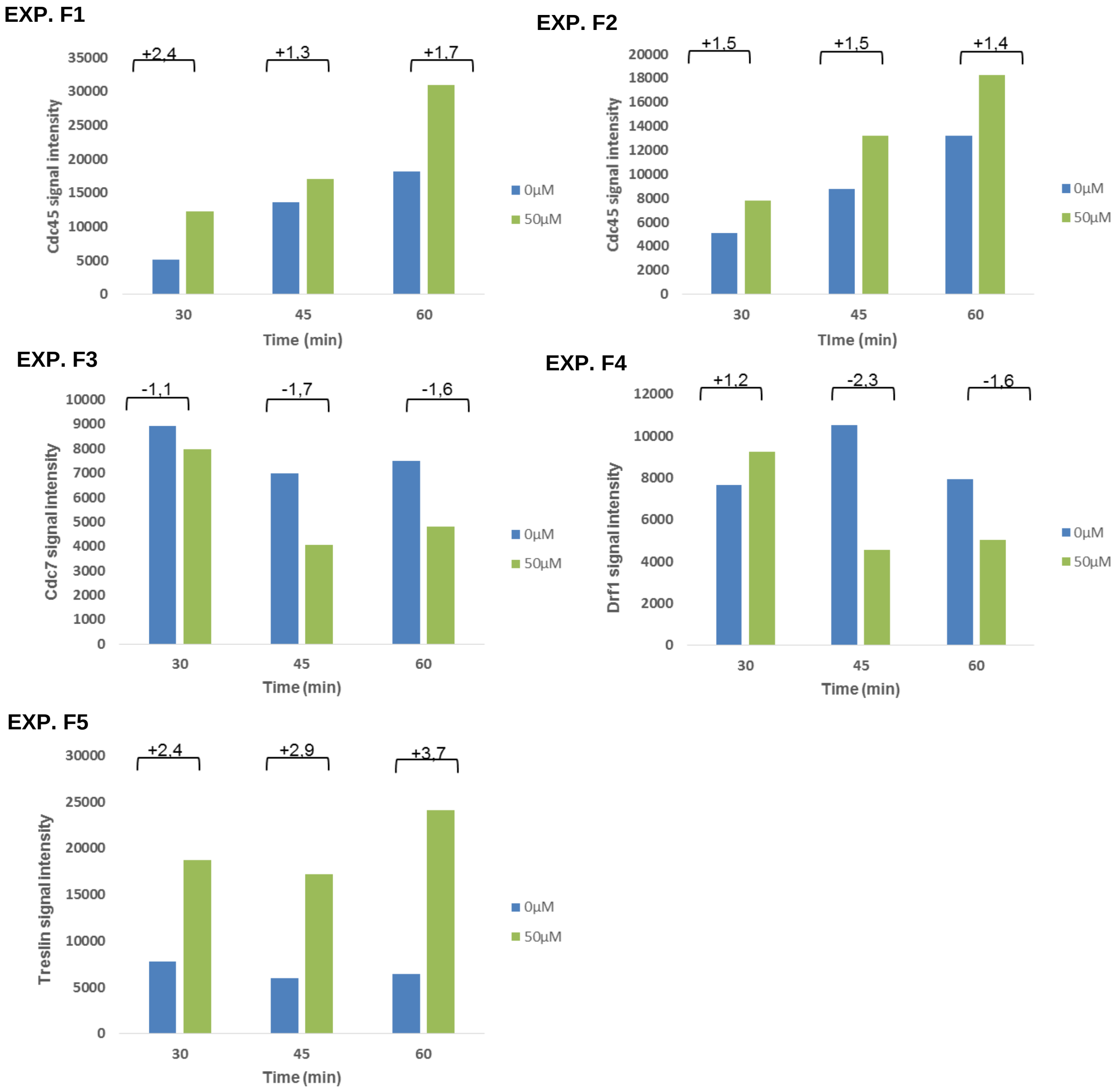


**Fig.21- Results from experiment E.** Western Blot from Cdc45 and ORC2 as a loading control. Graphic representation of the bands intensity for the Cdc45 present in the blot.

For the experience F, egg extracts were incubated with sperm nuclei (4000 nuclei/µl), showing an increase of Cdc45 recruitment at 30, 45 and 60 (Fig 22). In this experiment we increased the nuclei concentration in order to obtain a more prominent effect of dNTP addition, visible by western blotting. The egg extract quality in this case corresponded to the normal standards and the replication dynamics were not too slow.

## EXP. F


Time (min)
30
45
60
dNTPs (50μM)
-
+
-
+
-
+
Cdc45
Drf1
Cdc7
ORC2
ORC1
Treslin
ORC2


EXP. F1

EXP. F2

EXP. F3

EXP. F4

EXP. F5

**Fig.22- Results from experiment F.** Western Blot results obtained from the egg extract incubation with sperm nuclei (4000 nuclei/µl) for Cdc45, Cdc7, Drf1 and Treslin in the presence or absence of dNTPs. ORC1 and ORC2 were used as loading controls. For Exp. F1, F3, F4 and F5 the band intensity was normalized with ORC2. For the Exp. F2 the ORC1 was used to normalize the signal. The black arrow present in the western blot results for the Treslin represent the Treslin superior bands not always observable.

**EXP. F**

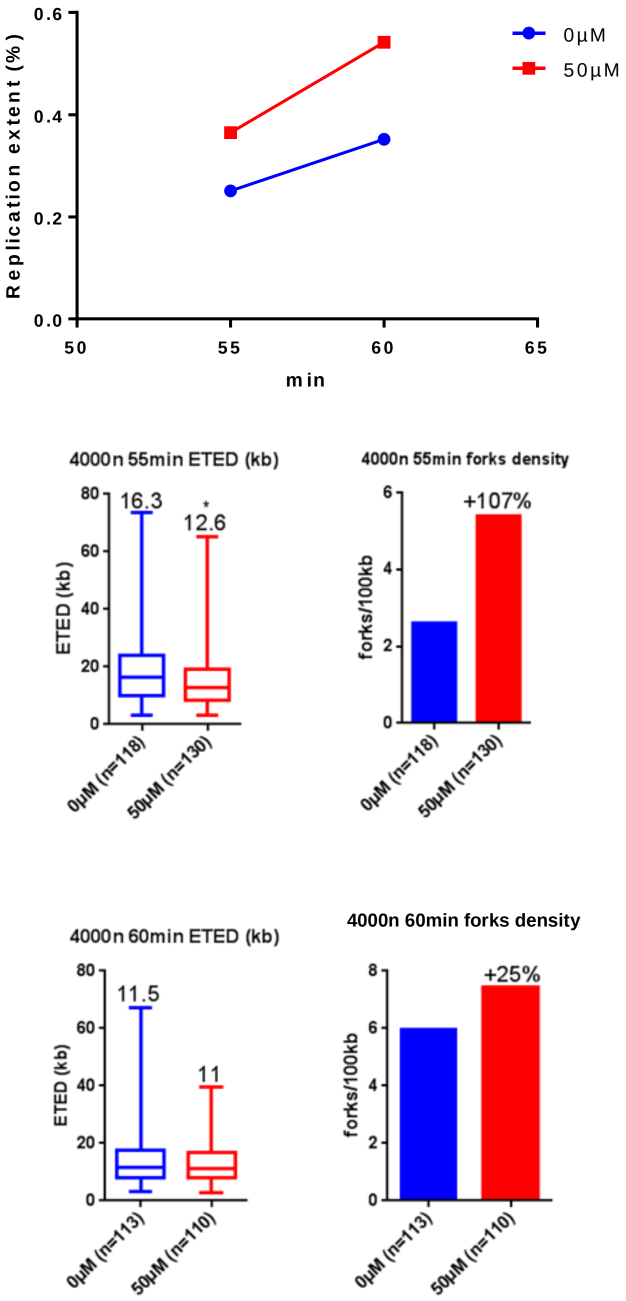


**Fig.23- DNA combing results from experiment F.** Replication extent, fork density and ETED obtained by DNA combing at 55 and 60min from the same extract as the extract used for western blot analysis of the Exp. F

Moreover the DNA combing results demonstrated an increase in replication extent with 50µM of dNTPs as well as well an increase of fork density and in the number of activated origins (Fig.23). Taken together these results confirm that the exogenous dNTPs lead to an increase in the Cdc45 recruitment and to an increase in the number of activated origins. The increase in the Cdc45 suggests a role of dNTPs during the pre-initiation complex formation. DDK and CDK are two important kinases involved in the activation of the pre-IC complex. These two kinases phosphorylate several pre-IC factors stimulating their recruitment into the chromatin and they are also responsible for the CMG helicase activation due to MCM phosphorylation. If western blot analysis showed an increase in the recruitment of these kinases in the presence of exogenous dNTPs, then the increase in the number of activated origins could be explained by an increase in the activity of these kinases leading to an increase in the CMG helicase activation.

However, the results obtained from the DDK Cdc7 and its subunit Drf1 showed a decrease in their recruitment in the presence of 50µM dNTPs. For the Cdc7 there is a constant decrease in this protein recruitment, about 1, 1 times for 30min, 1, 7 times for 45min and 1, 6 times for 60min. These results suggest that Cdc7 and Drf1 are not involved in the mechanism unleashed by the dNTPs addition because there is a decrease in their recruitment instead of an increase. However these preliminary results need to be confirmed.

Studies in *Xenopus laevis* demonstrated that contrarily to the Cdc45 that stays attached to the chromatin, Cdc7 is released at the end of S phase (Jares and Blow, 2000). Since the dNTPs addition leads to replication acceleration, verified by an increase in Cdc45, is possible that Cdc7 begins to detach from the chromatin sooner than in normal conditions.

Other preliminary experiments in the lab showed that the activity of Cdk2 in nuclear extracts is not increased upon addition of dNTP. However, further experiments must be performed to confirm these results.

I tested other proteins involved in the pre-IC formation to verify whether only Cdc45 was involved or other pre-IC factors were involved.

Treslin is involved in the pre-IC formation and it is recruited to the chromatin by MCM complex and TopBP1 action. Moreover, it is activated by the phosphorylation of either Cdk2 that stimulates Treslin action or inhibited by Chk1 kinase.

Analysis for Treslin recruitment were performed for the experience F with a different membrane but with the same samples. The Treslin western blots show two different bands and both were quantified demonstrating the increase in Treslin recruitment in the presence of dNTPs (µM). There is a 2, 4 fold increase at 30min, 2, 9 fold at 45min and 3, 7 fold at 60min. The abundance of the superior band (represented by a black arrow) seems to be more prominent in the cases where exogenous dNTPs are added. Since this band migrates more slowly than the other we asked whether this band could represent a phosphorylated band of Treslin resulting either from Cdk2 or Chk1 action. In order to test this hypothesis new chromatin fractions of a different experiment were digested with λ-phosphatase and analyzed by western blot using as antibodies anti-Treslin. In addition, anti-MCM4 was used as a control to confirm the proper λ-phosphatase action.

The λ-phosphatase experiments consisting in the digestion of chromatin with this enzyme did not detect any secondary bands as in the previous experiment. Treatment with the enzyme resulted in a small decrease in mobility showing that Treslin as well as MCM4 is phosphorylated. Thus, it is not possible to determine whether the superior band is a Treslin phosphorylated form or not, since this band does not seem to appear in a reproducible manner.

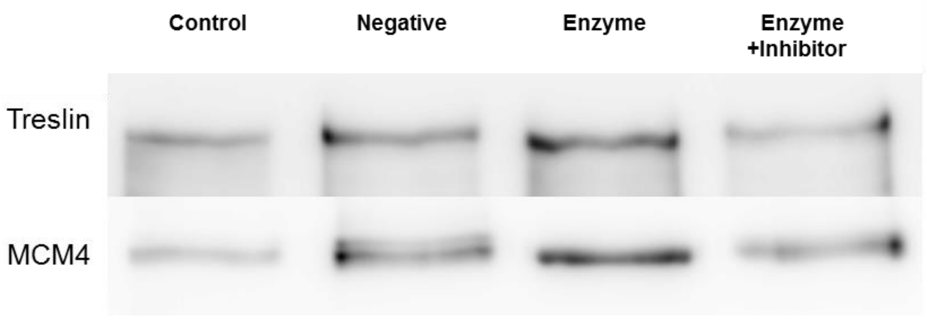


**Fig.24- λ-phosphatase experiments.** Western blots for Treslin and MCM4 resulting from λ-phosphatase experiments of chromatin fractions at 90min with 2000 sperm nuclei/µl.

The TopBP1 and RecQL4 as Cdc45 and Treslin are proteins involved in the pre-initiation complex formation. Thus, the study of these proteins by western blot was tried in order to confirm the results obtained for Treslin and Cdc45. However, the commercial antibodies against human homologues that we tested did not detected any specific bands corresponding to TopBP1 or RecQL4 (data not shown).

In addition, the recruitment of polymerases ε and δ was also tested to find a possible mechanism for the increase of the fork speed observed. However, no specific bands were obtained for neither of the commercial antibodies acquired for these proteins (Fig.25,26).

Several conditions were tested for each of the antibodies, for example two different incubation times of the primary antibody were experienced two hour or incubation overnight. In addition, two different kits (Femto kit and Pico kit) were used to enhance the chemiluminescent signal originated from the horseradish peroxidase enzyme contained in the secondary antibody, increasing the detection sensitivity.

For example, the fig. 25 shows the western blot results obtained for the polymerase δ after the incubation of egg extracts with nuclei (2000 sperm nuclei/μl) during 60 minutes and from the egg extract. The egg extract without nuclei was used to function as a control, it must contain all the proteins present in the extract and it may also indicate possible experimental errors in the chromatin purification protocol when a protein that normally is found on the chromatin appears in the extracts but not in the chromatin purified sample. The polymerase δ has a predicted molecular weight of 124 kDa, however for the western blot results the only band obtained was at approximately 100 kDa and only for the 60min sample suggesting that the observable band results from an unspecific interaction and probably it does not correspond to the polymerase δ. Moreover, the results achieved with the pico kit were extremely faint (fig.25 (a)) which led to the use of a different kit (femto kit), much more sensitive, still the results were similar (fig.25 (b)).

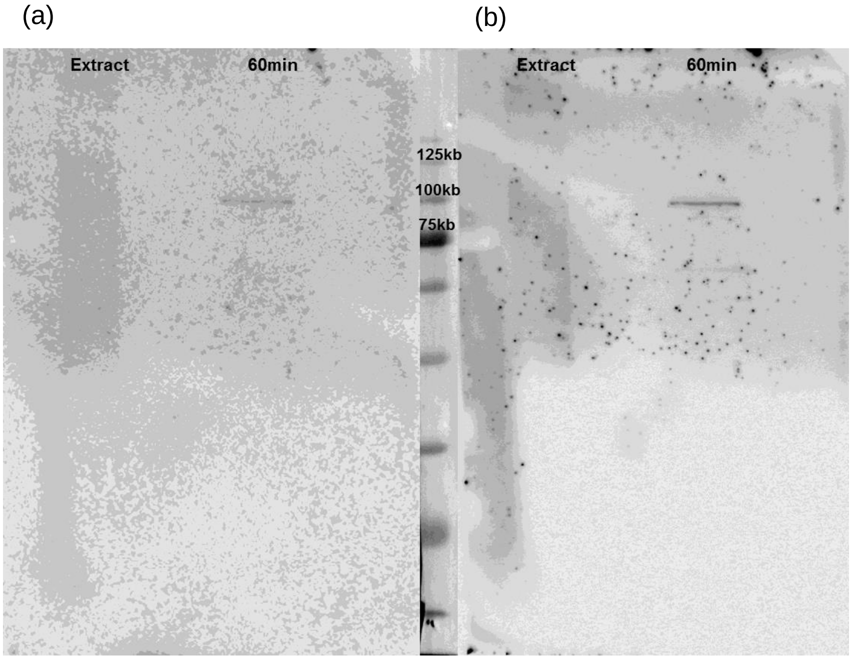


**Fig.25- Western blots obtained for Polymerase δ.** Results obtained using two different kits, the pico kit (a) and the femto kit (b). The samples analyzed were the extract and the incubation of egg extracts with sperm nuclei (2000 sperm nuclei/μl) during 60min.

The fig. 26 shows the western blots for the Polymerase ε using as samples the extract and the chromatin samples resulting from incubation of egg extracts with sperm nuclei (2000 sperm nuclei/µl) during 30, 45 and 60 in the presence or absence of dNTPs. Although the Polymerase ε predicted molecular weight is about 55kDa, the western blot analysis with anti-mouse as the secondary antibody are characterized by an abundance of bands with various molecular weights suggesting unspecific interactions. Despite the several attempts I was unable to achieve clear, convincing results with these antibodies indicating that possibly they do not reveal affinity for *Xenopus laevis* homologous since the antibodies used were against human proteins and not specific for *Xenopus.*

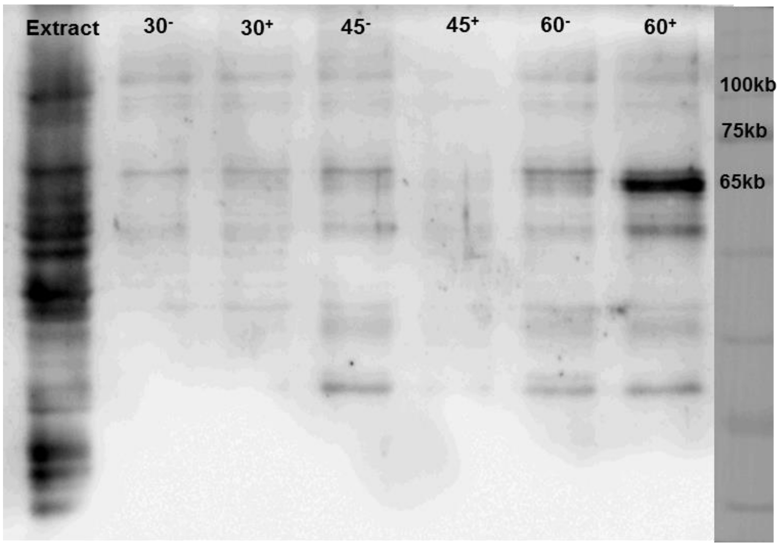


**Fig.26- Western blot results for Polymerase ε.** It was used anti-mouse as secondary antibodies. The samples analyzed were the extract and the incubation of egg extracts with sperm nuclei (2000 sperm nuclei/µl) during 30, 45 and 60 in the presence or absence of dNTPs.

# 2. Mass spectrometry analysis of the chromatin proteins in the presence and absence of exogenous dNTPs

Using *Xenopus* egg extract system for cell-free DNA replication with quantitative mass spectrometry a new method was developed by Räschle et al. 2015, called CHROMASS.

The same method was adapted and optimized to our experiments to try to determine possible variations in chromatin proteins when exogenous dNTPs are added.

Sperm nuclei were incubated with egg extracts in the presence or absence of dNTPs (50µM). The reaction was stopped at 70min and a chromatin purification was performed, to isolate the proteins bound to the chromatin. Then, the samples were analyzed by mass spectrometry.

A total of 787 proteins were identified (Fig.27). However, only a subfraction corresponded to nuclear chromatin bound proteins that were analyzed with a MS program (Scaffold). Additionally, proteins of other cellular processes were detected suggesting that the chromatin isolation was contaminated with cytosolic proteins (Fig.28).

Finally, from the 800 proteins that were analyzed by hand, 66 chromatin bound proteins potentially involved in the DNA replication process were identified and organized according with function in DNA replication or other process (table 3).

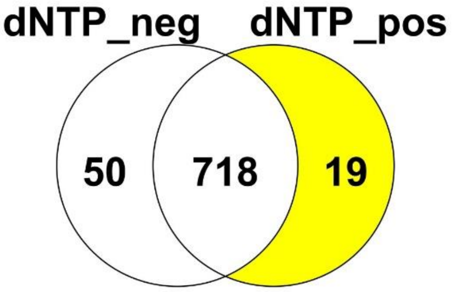


**Fig.27- Number of detected proteins by mass spectrometry.** Schematic representation of the number of proteins that are present in both samples (without dNTPs and 50µM of dNTPs) and only in one of the samples

The results showed that by using this technique the majority of the proteins involved in the replication were detected (Table 3). A total of 66 proteins were recognized, these proteins interact in different processes namely in the pre-RC initiation (14 proteins), pre-IC formation (5 proteins), regulation of replication (8 proteins), elongation process (15 proteins), cell cycle checkpoint (5), repair mechanism (4) and chromatin remodeling (13 proteins).

From the pre-Replicative complex almost all the proteins from the ORC complex (ORC 1,2,3,4 and 5), MCM complex (MCM 2,3,4,5,6 and 7) and the Cdc6 were identified. Only ORC6 was not detected using this method, however Orc6 was previously shown to be loosely bound to the chromatin.

In the first analysis, we performed only TopBp1, Treslin involved in the pre-initiation complex as well as Cdc7 and DBF4 (Cdc7 regulatory subunit) and some important phosphatases in the replication process were identified. A second analyze using a longer run of the samples with liquid chromatography (120min) and comparing the results with a different database lead to the detection of others pre-initiation complex proteins such as Cdc45, MCM10 and Psf3 (GINS). Nevertheless, RecQL4 and the other components of GINS also involved in the pre-initiation complex were not detected.

Noteworthy, the Chk1 absence, a protein normally bounded with the chromatin and related with cell cycle checkpoint is not found in the mass spectrometry detections despite other checkpoint proteins identified for example Plx1, ATM and ATR. Chk1 is absent probably because it is less abundant on the chromatin in comparison with the remaining cell cycle proteins.

The absence of the mentioned pre-IC proteins can be due to a slow replication after a less replication competent egg extract diminishing the amount of these proteins that attach to chromatin. Or the mass spectrometry sensibility was not enough to detect these proteins. Replication control reactions using Rhodamine-dUTP incorporation showed indeed that in this reaction only about 50% of the nuclei entered in S phase.

**Table 4- List of detected proteins by mass spectrometry involved in the replication mechanism.**

| Mechanism involved | Protein name |
|---|---|
| **Pre-RC complex** | origin recognition complex, subunit 1 (Orc1) |
| | origin recognition complex subunit 2 (Orc2) |
| | origin recognition complex subunit 4 (Orc4) |
| | origin recognition complex, subunit 3 (Orc3) |
| | origin recognition complex, subunit 5 (Orc5) |
| | cell division cycle 6 (Cdc6) |
| | DNA replication licensing factor mcm3 (MCM3) |
| | DNA replication licensing factor mcm2 (MCM2) |
| | DNA replication licensing factor mcm6 (MCM6) |
| | DNA replication licensing factor mcm7-B (MCM7-B) |
| | DNA replication licensing factor mcm4-B (MCM4-B) |
| | DNA replication licensing factor mcm5-B (MCM5-B) |
| | DNA replication licensing factor mcm5-A (MCM5-A) |
| | DNA replication licensing factor mcm4-A (MCM4-A) |
| **Pre-IC complex** | DNA topoisomerase II binding protein 1 (TopBP1) |
| | Treslin |
| | mdm2-binding protein (MTBP) |
| | MiniChromosome Maintenance (MCM10) |
| | cell division cycle 45 (Cdc45) |
| | DNA replication complex GINS protein PSF3 (GINS-PSF3) |
| **Regulation phosphatases** | protein phosphatase 1A |
| | protein phosphatase 1, catalytic subunit, alpha isozyme |
| | serine/threonine-protein phosphatase PP1-beta catalytic subunit |
| | protein phosphatase 2, regulatory subunit A, beta |
| | protein phosphatase 2, catalytic subunit, alpha isozyme |
| **Regulation Kinases** | cell division cycle 7 (Cdc7) |
| | protein DBF4 homolog B |
| **Elongation** | Proliferating cell nuclear antigen-like (PCNA) |
| | replication factor C (activator 1) 2, 40kDa |
| | replication factor C (activator 1) 4, 37kDa |
| | replication factor C (activator 1) 3, 38kDa |
| | replication protein A 70 kDa DNA-binding subunit |
| | replication protein A 32 kDa subunit-A |
| | replication protein A3, 14kDa |
| **Polymerases** | DNA-directed DNA polymerase delta 1 |
| | DNA polymerase epsilon |
| | DNA polymerase delta subunit 2 |
| | DNA polymerase delta 3 |
| | DNA polymerase alpha catalytic subunit |

| | |
|---|---|
| **Okazaki** | flap endonuclease 1-A |
| | ligase III, DNA, ATP-dependent |
| | flap endonuclease 1-B |
| **Checkpoint** | serine/threonine-protein kinase PLK1 (Plx1) |
| | aurora B |
| | ATR protein |
| | ATM serine/threonine kinase |
| | TIMELESS protein |
| **Repair** | RAD50 |
| | double-strand break repair protein MRE11 |
| | RAD52 |
| | DNA damage-binding protein 1 |
| **Regulation** | 14-3-3 protein zeta a |
| | 14-3-3 protein beta/alpha-B |
| | SUMO-conjugating enzyme UBC9 |
| **Chromatin remodeler** | topoisomerase (DNA) II alpha |
| | SWI/SNF related, matrix associated, actin dependent regulator of chromatin (SMAC) |
| | FACT complex subunit (SPT16) |
| | FACT complex subunit (SSRP1) |
| | histone chaperone (asf1a-B) |
| | nucleosome assembly protein 1-like 1-B |
| | SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a (SMCA) |
| | Rif1 protein |
| | Structural maintenance of chromosomes protein 3 (SMC protein 3) |
| | structural maintenance of chromosomes protein 1A (SMC protein 1A) |
| | sister chromatid cohesion protein PDS5 homolog B-A |
| | cohesin subunit SA-1 |
| | protein HIRA |

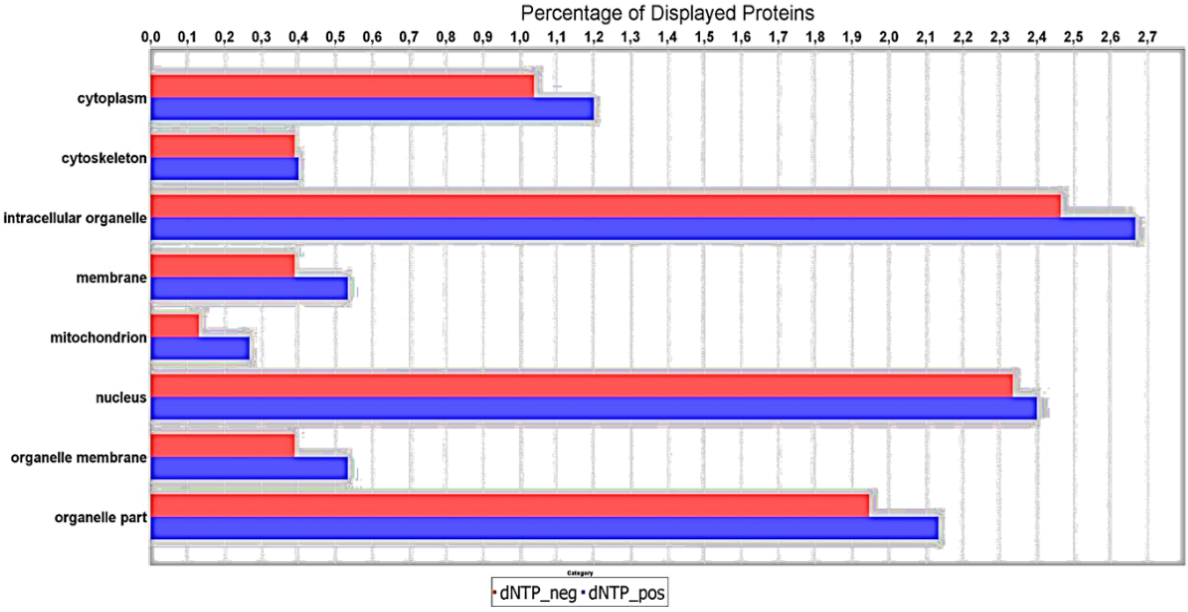


**Fig.28 – Origin of the detected proteins by mass spectrometry.** Graphic representation of the proteins nature obtained from the mass spectrometry analysis. The red represents the sample without exogenous dNTPs and the blue shows the results for the samples with added dNTPs.

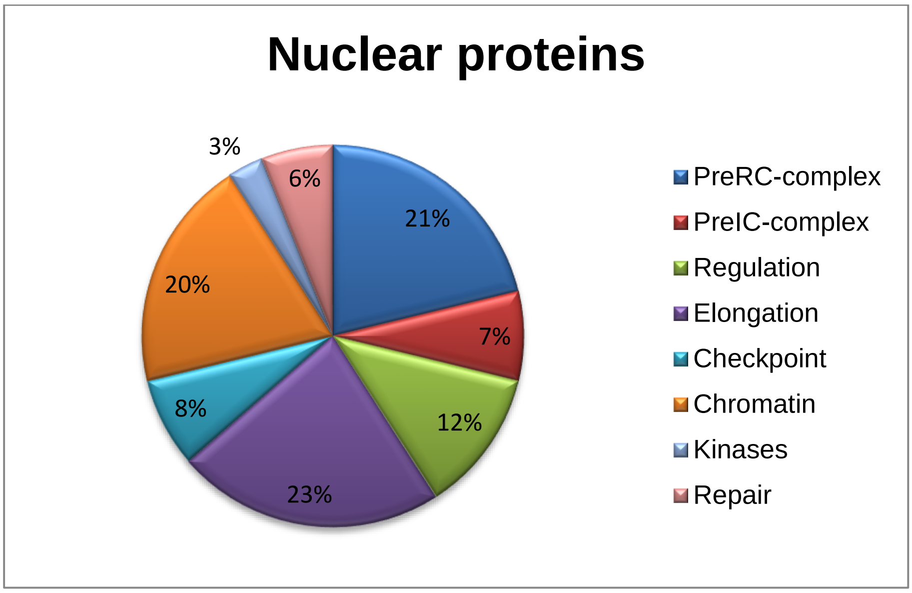


**Fig.29 – Mechanisms of the proteins detected.** Graphic that represents from the identified proteins by mass spectrometry, the percentage of proteins involved in a specific cell mechanism, such as in the pre-RC complex formation or in the cell cycle checkpoint

As shown in the figure 28, a big percentage of the proteins present in the samples are from the nucleus or intracellular organelles and only a small percentage of proteins are derived from mitochondria, cytoskeleton and membrane.

The main objective of the mass spectrometry analysis is to quantify possible changes in the chromatin associated proteins in the presence of dNTPs. The next step consists in analyzing the samples in triplicates in order to obtain a proper mass spectrometry quantification. During my master thesis I was able to obtain this qualitative analysis and the validation of the experimental protocol.

# 3. Analysis of the replication program after Chk1 inhibition by AZD-7762 in presence and absence of exogenous stress

I aim to determine the effect of AZD 7762, a checkpoint kinase 1 inhibitor, on DNA replication considering absence or presence of stress.

## 3.1 In the presence of aphidicolin

Aphidicolin is tetracyclic antibiotic and a reversible DNA polymerase inhibitor. Aphidicolin interacts specifically with DNA polymerase α and δ inhibiting DNA elongation. However initiation of replication still occurs which means that the replication forks are assembled but unable to progress. The single strand DNA originated from stalled forks induce checkpoint activation, more concretely Chk1 phosphorylation in an ATM/ATR dependent pathway. In order to inhibit Chk1 function I used AZD-7762, a competitive Chk1 inhibitor. As control I used DMSO, the AZD dilution solvent.

Sperm nuclei (2000 sperm nuclei/µl) were incubated with egg extracts in the presence of aphidicolin (7, 5µg/ml) and AZD (0, 5µM) or DMSO (0, 5µM) for 105min. At the beginning of the reaction biotin-dUTP was added to label replication eyes. Following the previously mentioned experimental procedure the DNA is stretched in fibers and immunolabeled using anti-DNA antibody to label all the DNA and fluorescent streptavidin conjugates to label the replication eyes. Then, the labelled DNA fibers are observed under the fluorescent microscope, randomly photographed, about 5 Mb of DNA analyzed with the help of an Excel software.

The fig. 30 shows some of the fibers obtained from the DNA combing experiment. It is possible to verify that the presence of AZD significantly increases the number of replication eyes (represented in red due to labelling with biotin-dUTP) whereas in the absence of AZD replication percentage is very low due to the effect of the elongation inhibitor aphidicolin. This shows that more origins are activated when Chk1 is inhibited.

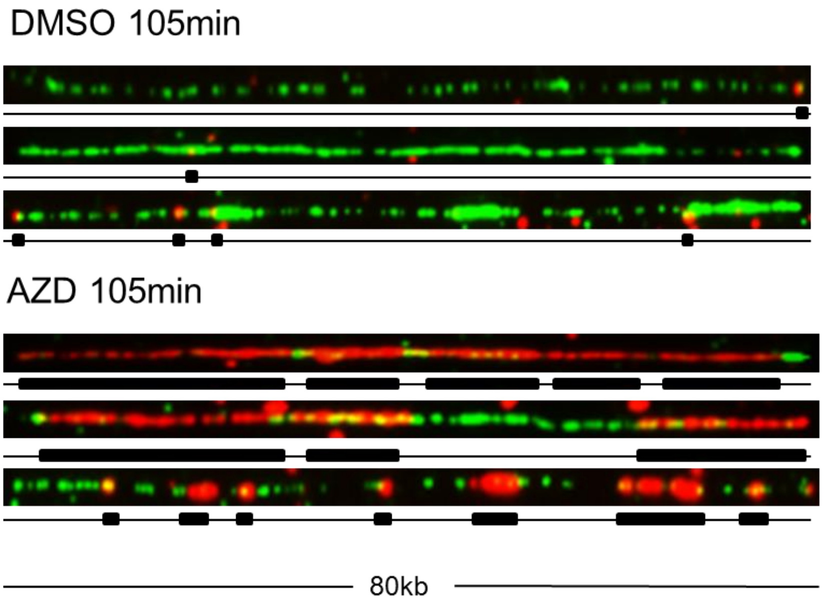


**Fig.30 – Combed DNA fibers in the presence of Aph.** Representative, in the absence (above) or in the presence (below) of AZD. The green represents whole DNA label and in red are the replication eyes labeled with biotin.

The results obtained from the fibers analysis showed a significant increase of the DNA replication extent in the presence of AZD in comparison with the control (DMSO) (Appendix 2). The addition of AZD increases DNA replication four folds (fig.31) since in the control it is about 5% and in AZD is 20% (t-test, P<0,0001). This augmentation can be due to an increase in the number of activated origins and or to an increase in fork speed. As previously described, the replication origins are organized in clusters and each cluster has several origins with different firing moments. The increase in activated origins can occur inside already activated cluster or outside, in new regions of the genome. In order to determine this, the eye-to-eye distance and the fork density were measured. The eye-to-eye distance represents the distance between origins inside the same replication cluster due to the limited length of DNA fibers. The fork density refers to the amount of activated origins across all the genome. Thus, an increase in the fork density with stable eye-to-eye distances indicates an increase in newly activated clusters (late replication clusters). In contrast, a decrease in eye-to-eye distances complemented by an augmentation of fork density reveals more activated origins inside already activated clusters.

The results for the fork density indicate a significant increase in the presence of AZD (fig.31 (b)), being six times higher in comparison with the control (it is about 1, 23 for DMSO and 4, 77 for AZD (t-test, P= 0.0183)).

Moreover, when comparing the AZD condition with the condition control from the (fig 31 (c)) a slight increase in median eye-to-eye distance is observable, AZD value (5, 40) versus control value (4, 35) (Mann-Whitney, P=0, 0112). No difference in mean eye length was detected, implying that fork speed was not affected (data not shown).

These results show that Chk1 inhibition by AZD similar to UCN leads mainly to an increase in the number of origins in newly activated clusters.

The experience was repeated twice with similar results.

In conclusion, in the presence of exogenous stress (Aph), Chk1 inhibitory action only affects origins outside already activated clusters.

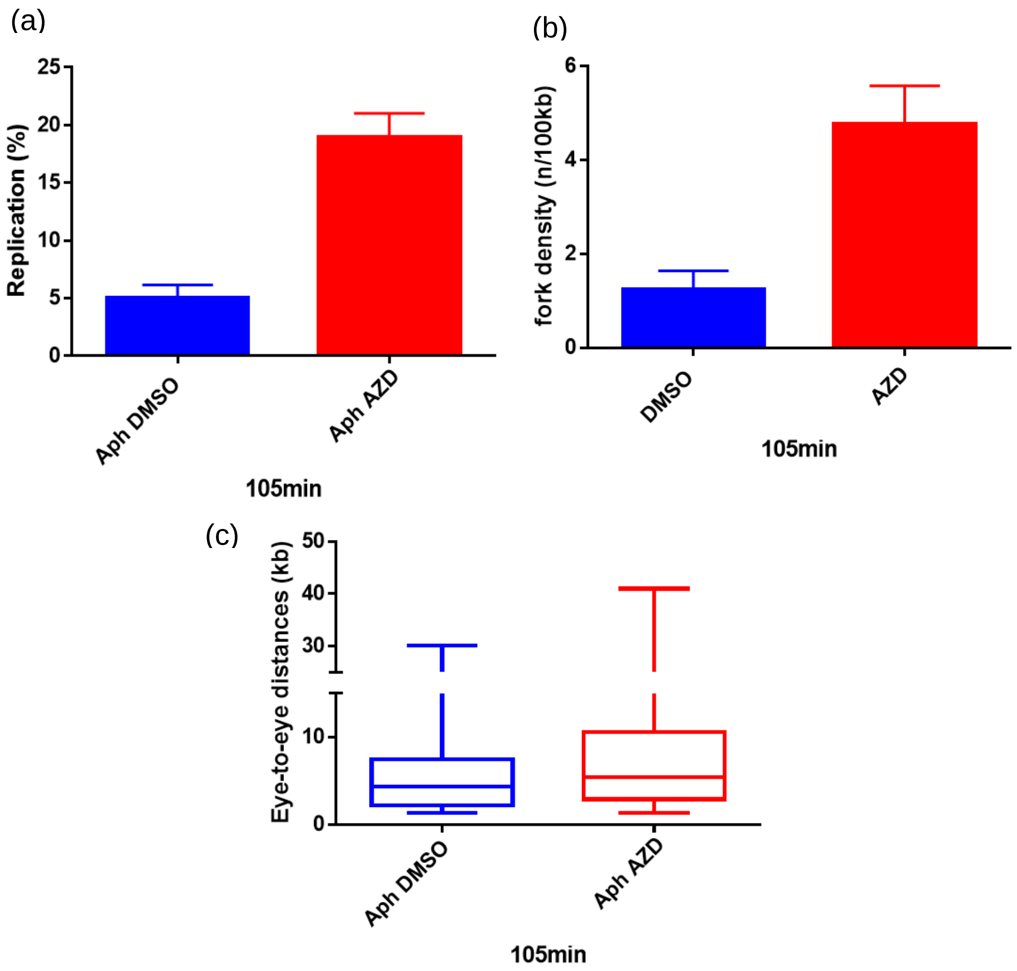


**Fig.31 – Inhibtion of Chk1 action by AZD-7762 increases DNA synthesis and fork density in the presence of aphidicolin.** (a) mean replication extent with SEM (t-test, P<0,0001), (b) fork density (t-test, P= 0.0183), (c) eye-to-eye distance (Mann-Whitney, P=0,0112)

## 3.2 In the absence of exogenous replication stress

Chk1 function is also observable in the absence of exogenous stressful conditions. During normal S phase, the replication encounters blocked forks derived endogenously from hard to replicate regions or spontaneous DNA damage.

To study origin activation, sperm nuclei (2000 nuclei/µl) were incubated with egg extracts during 45, 55 and 75min, in the absence of stress (no aphidicolin), in the presence of biotin-dUTP and AZD, Chk1 competitive inhibitor, (0, 5µM) or DMSO. I proceeded to the DNA isolation followed by DNA stretching and DNA fibers immunolabelling which are observed under the fluorescent microscope, randomly photographed and analyzed with the help of an Excel software (the quantity of analyzed DNA varied between 4,5 and 6,5kb according with experimental conditions). The fig.32 shows examples of labelled DNA fibers isolated after 55min. One can observe an increase in the replication eyes (labeled in red) after AZD addition.

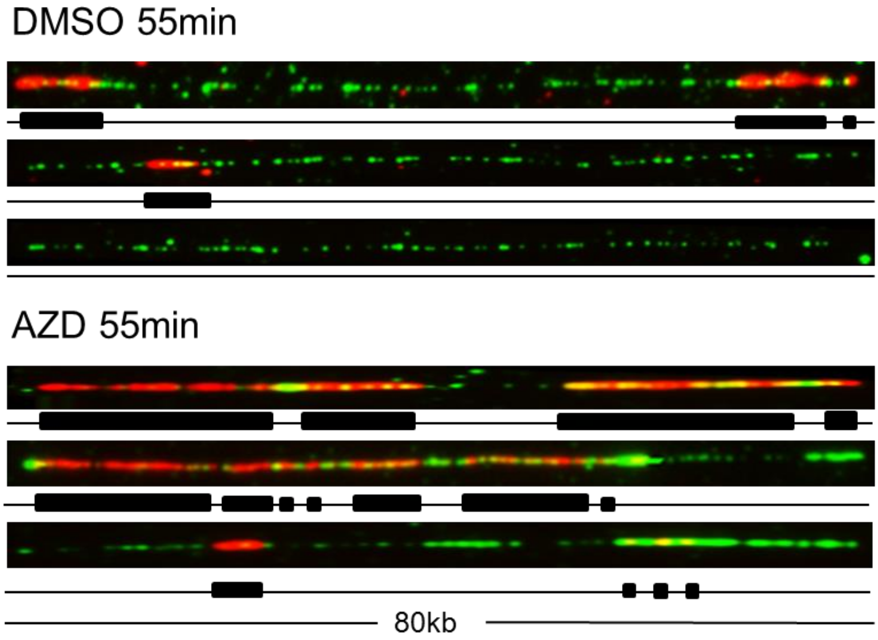


**Fig.32 – Combed DNA fibers in the absence of Aph.** Representative at 55min, in the absence (above) or in the presence (below) of AZD. The green represents whole DNA label and in red are the replication eyes labeled with biotin.

DNA replication parameters (DNA replication extent, eye-eye distance and fork density) were obtained for 45, 55 and 75min time points (Appendix 1). The results obtained for the DNA replication extent showed at 45 min (fig.33 (a)) an increase in the DNA replication percentage in the presence of AZD (40%) in comparison with the control (DMSO) which has only 10% (t-test, P<0,0001). After 55min of incubation (fig.33 (d)), a 20% of DNA replication extent was observed for the control and a significant increase in this percentage (60%) in the presence of AZD (t-test, P<0,0001). However, for the DNA replication corresponding to 75min of incubation (fig.33 (g)) the increase in DNA replication extent is less pronounced since it is 40% of DNA replication extent for the control versus 70% for the AZD condition (t-test, P<0, 0001).

This increase in DNA replication extent could be explained by an increase of fork density or/and an increase of fork speed. The fork density was three time higher after AZD addition (5,82) at 45min (fig.33 (b)) than in the control (1,65) (t-test, P= 0.021). At 55min the balance between the control and AZD conditions reveals an increase of the fork density, from 2 forks for each 100kb to approximately 5 forks for each 100kb (t-test, P= 0.0028). The fig. 33 (h) shows an increase in fork density with the AZD addition compared to the control, however this augmentation is less pronounced but still significant, 4,49 control to 6,54 AZD (t-test, P= 0.0064) This is in agreement with the DNA replication extent. The increase of fork density indicates a rise of activated origins.

These additional origins may be activated in already activated clusters, in non-activated cluster or in both. In order to determine specifically which clusters are triggered the eye-to-eye distances was analyzed. The median eye-to-eye remains unchanged when comparing the control (5,7) with the AZD condition (5,8) for the reaction after 45min of incubation and distributions were not significantly different (Mann-Whitney, P=0, 7880). For 55min the median eye-to-eye distance is unchanged between DMSO (6,8) and AZD (7,6) conditions and distributions were not significantly different (Mann-Whitney, P=0, 7849). The median eye-to-eye distance measured for the last time (75min) was slightly increased with the AZD addition (8,3) versus the control (7,4), however distributions were not significantly different (Mann-Whitney, P=0,2268). During the statistical studies realized it was always considered the probability P > 0.05 as significantly different.

Thus, the increase in fork density together with the almost unchanged eye-to-eye distances lead us to conclude that in Chk1 inhibition conditions, only a few additional origins are activated inside already activated cluster, most of the new origins are activated as later non-active clusters.

These experiments were repeated twice with identical results.

Our results are consistent with the ones obtained in the presence of aphidicolin meaning that Chk1 regulates origin activation in origin outside already activated origin clusters.

The results obtained for the Chk1 activity in the presence and absence of replication stress (aphidicolin) using AZD-7726 as a second Chk1 inhibitor, were incorporated in a recently published article (see appendix).

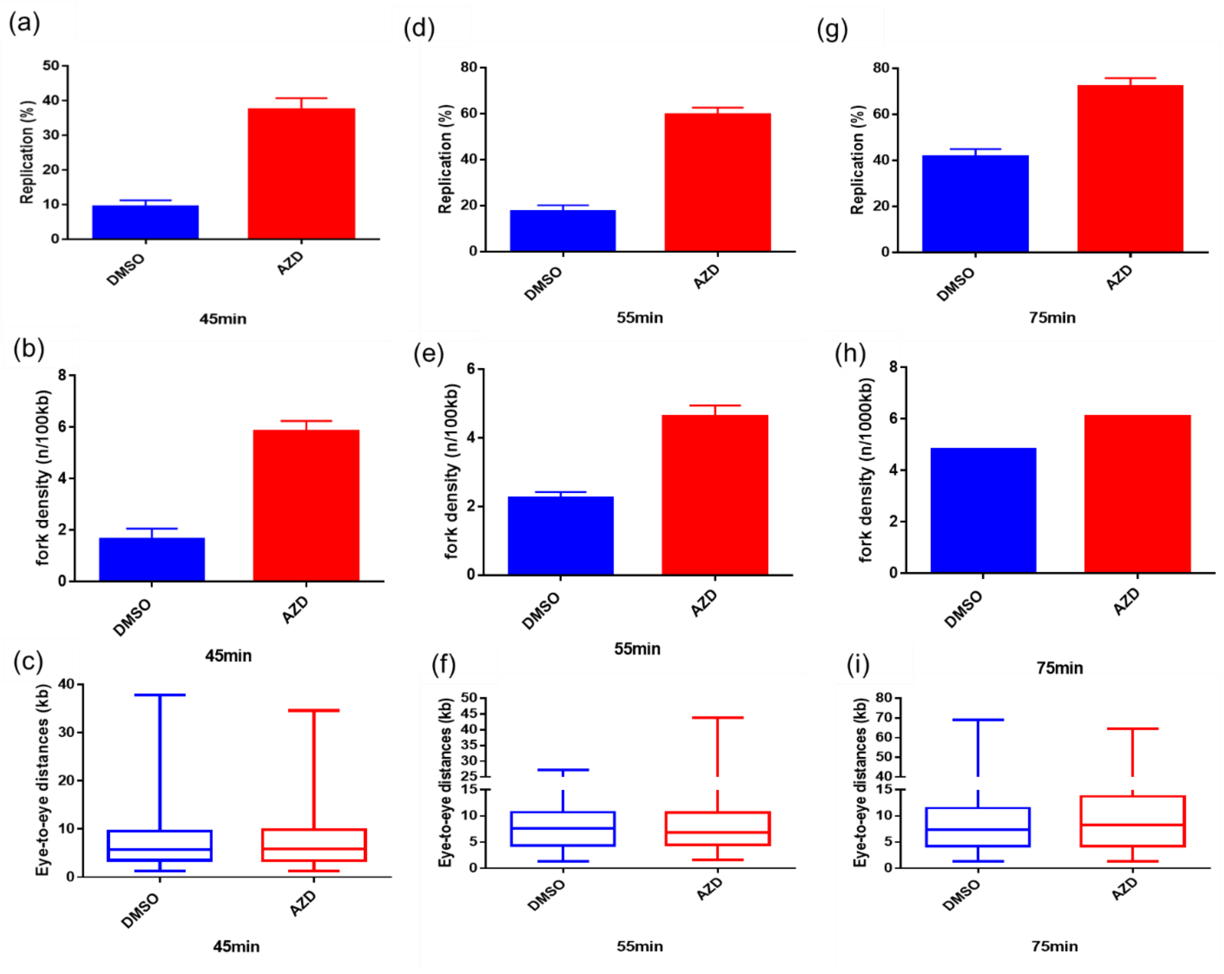


**Fig.33 – Inhibtion of Chk1 action by AZD-7762 increases DNA synthesis and fork density in the absence of stress.** Sperm nuclei were incubated with egg extracts in the presence of biotin-dUTP and AZD-7762 for 45, 55 and 75min and analyzed by DNA combing. (a) mean replication extent with SEM (t-test, P<0,0001), (b) fork density (t-test, P= 0.021), (c) eye-to-eye distance (Mann-Whitney, P=0,7880) (d) mean replication extent with SEM (t-test, P<0,0001), (e) fork density (t-test, P= 0.028), (f) eye-to-eye distance (Mann-Whitney, P=0,7849) (g) mean replication extent with SEM (t-test, P<0,0001), (h) fork density (t-test, P= 0.064), (i) eye-to-eye distance (Mann-Whitney, P=0,2268)

# Discussion

# 1. Exogenous dNTPs addition increases initiation

Studies in mammalian cells showed that a decrease in the available dNTP pool increases number of activated origins and slows the fork speed (Anglana et al. 2003). The addition of nucleotides precursors converts a slow replication in a fast one with less activated origins and higher fork speed (Courbet et al. 2008, Gay et al. 2010). Fork speed regulates the number of activated origins.

In *Xenopus* egg extracts the mechanism stimulated by dNTPs addition seems to be different from the one observed in mammalian cells. Fork speed increase is not related with a decrease of initiations since dNTP addition increases both fork speed and initiations, probably in an independent manner. Unpublished data from the lab also showed that addition of dNTPs does not decrease the activity of the replication checkpoint as shown in yeast (Poli et al. 2012).

In order to unveil the molecular mechanism behind the replication acceleration by dNTP addition in *Xenopus*, I studied the proteins involved in the replication process. Both Treslin and RecQL4 are Cdk2 targets, one of the kinases responsible for origin activation. Unfortunately, RecQL4 commercial antibody showed no affinity for xenopus samples. However Treslin and Cdc45 necessary for the CMG complex formation were more abundant on the chromatin when dNTPs were added. Although Cdc7 kinase and Drf1 regulator subunit are less recruited onto the chromatin in exogenous dNTPs conditions, we did not test a possible decrease in Cdc7/Dbf4 activity.

Altogether these results indicate that the increase in the number of origins after dNTP addition is due to an increase in the recruitment of proteins participating in the pre-IC complex (fig.34).

Recently, it was shown that Treslin phosphorylation by CDKs determines the S phase duration. This phosphorylation increases the number of initiations and accelerates the synthesis in human cells (Sansam et al. 2015). It is possible that dNTPs stimulate Treslin recruitment onto the chromatin and its phosphorylation by the CDKs leading to an increase in the number of initiations. To test this hypothesis it should be verified whether an increase in Cdk2 activity is observed when adding exogenous dNTPs. Preliminary experiments showed that this is not the case however additional experiments must be performed.

The diminution of Cdc7 and Drf1 can be due to the system acceleration that detach these protein from the chromatin sooner. Actually, it was already shown in xenopus the Cdc7 detaching from the chromatin before Cdc45 (Jares and Blow, 2000).

To verify the western blots results it should be studied whether possible changes are observable when using different loading controls to signal normalization. This experience have already been performed between ORC1 and ORC2, the same must be done with H3.

A future study using exogenous dATP instead of dNTPs could be interesting since most of the protein belonging to the pre-RC complex (ORC, MCM, and Cdc6) interact with ATP as well as with dATP. Although the DNA combing and western blot analysis show that the number of activated origins increases due to an increase in the recruitment of pre-IC proteins, it is still unclear how the increase of dNTPs concentration regulates this step. Since at the present we cannot exclude that pre-RC loading is affected upon dNTP addition, further experiments must be performed to quantify Cdc6 and MCM after dNTP addition.

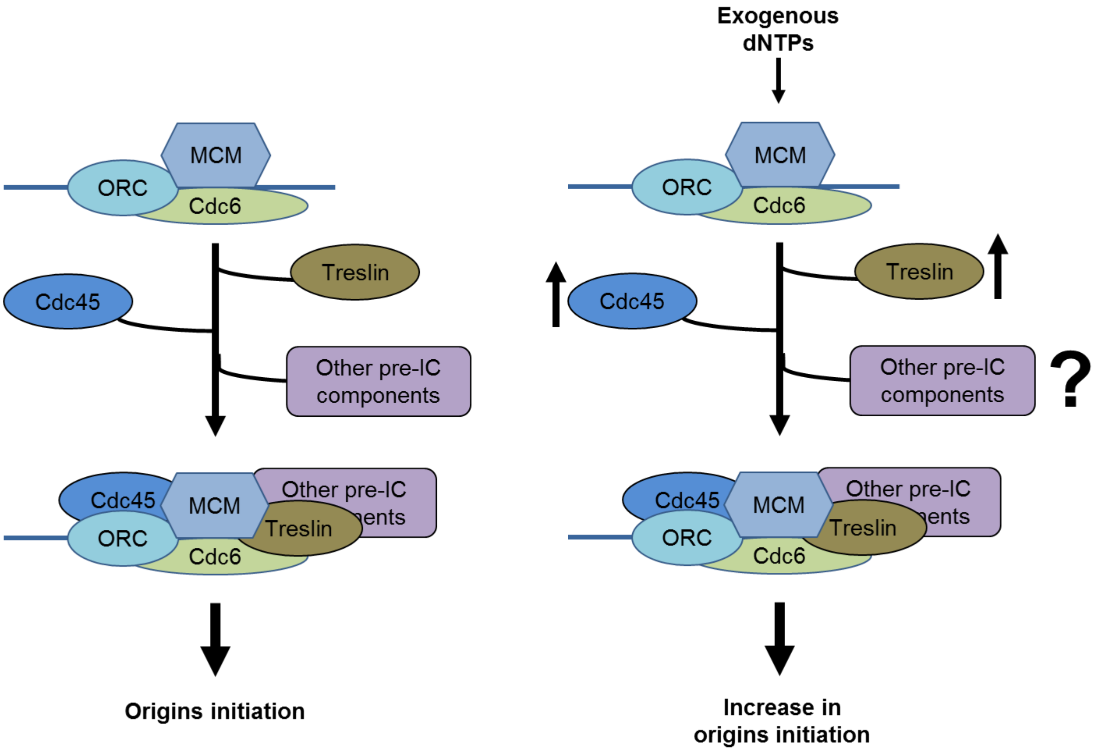


**Fig.34 – Model for the regulation of the replication mechanism in *xenopus laevis*.** It is represented the regular replication process leading to origins activation (on the left) and the altered replication process due to exogenous dNTPs addition (on the right). It is characterized the dNTPs effect at the level of the pre-IC complex more concretely an increase of Cdc45 and Treslin recruitment since for the remaining pre-IC components the effect is still unknown.

# 2. CHROMASS analyses in the presence of exogenous dNTPs

Using mass spectrometry, a more global approach was followed to study the proteins recruited onto the chromatin in the presence of exogenous dNTPs. The lab first tried to adapt the protocol used by Khoudoli et al. 2008 however only a few replication proteins were identified due to excess of mitochondrial contamination. Thus, I adapted a new protocol based on Raschle et al. 2015 and optimized it showing some promising results with a much lower percentage of mitochondrial protein contamination and the identification of the great majority of proteins involved in replication, in exception of RecQL4, GINS, Chk1, ORC6. An increase in the number of detected proteins when a second analysis with another database and longer run was performed may suggest that the protocol can be further optimized.

At the moment, a quantitative analyze in the presence or absence of dNTPs is in progress.

# 3. Chk1 inhibitory effect in the DNA replication

Studies in yeast showed that Rad53 (Chk1 homologous) inactivation leads to late origins activation (Santocanale and Diffley, 1998). In mammalian cells, Chk1 inhibition by UCN-01 increase the number of origins activated in the presence of replication stress (Feijoo et al. 2001). Our results confirmed what was shown in mammalian cells for the Chk1 function in origins activation mechanism in the presence of replication stress due to aphidicolin addition. Moreover, our results showed the same Chk1 ability in the regulation of origin activation in inactive clusters in absence of replication stress.

In absence of stress, Chk1 is still active since even in this condition the replication encounters blocked forks derived endogenously from regions hard to replicate, such as fragile sites, repeated regions or secondary structures. The Chk1 inhibition by AZD-7762 increases the origins activation in early S phase. The lab previously showed that origins are organized as cluster that became active in different moments of the S phase (Marheineke and Hyrien, 2001). A cluster corresponds to a group of replicons with a size between 50 and 100 kb, this cluster of origins is located at a replication domain a lot bigger, obligating the coordinated replication.

The results obtained in this project suggested that Chk1 inhibits origins activation in late clusters and it seems to be unable to inhibit origins in already activated clusters. This inhibition allows to slow down the global S phase progression and at the same time restarts the replication near stalled replication forks.

The inhibition of Chk1 action near already activated clusters suggests the existence of another mechanism still unknown where Chk1 is involved and inhibited. Vincenzo Costanzo team has proposed a model where Plx1 (Plk1 orthologous) is important in Chk1 action since it inhibits Chk1 function near already activated clusters. The model proposed was based on *Xenopus* studies indicating that Plx1 depletion in situations of replication stress leads in one hand to an augmentation on Chk1 activation and in the other hand to a decrease in Cdk2 activity and Cdc45 recruitment (Trenz et al. 2008). However, this study did not complement a detailed analysis of the origins and clusters activation by DNA combing.

Various mechanisms explaining the local and global regulation of origins activation were suggested, however always in the presence of exogenous replication stress (Yekezare et al. 2013). Nevertheless, it is possible that these mechanisms are also relevant in the normal S phase. The lab had previously suggested a mathematical model for the replication control in *Xenopus* (Goldar et al. 2008). In this model the replication origins are considered to be evenly spread across all the genome. And activated by a *trans* factor with an initiation probability ($P_{init}$). An environmental protein is going to bind with the origin, activating it. In the beginning of the replication, this protein splits in two and associates with the bidirectional replication forks. When the two forks encounter each other the two units of the proteins assemble and it will activate further origins. Moreover, the origins activation can be randomly controlled by the regulator protein Chk1. It inhibits the activation of a non-activated origin with a $P_{Chk1}$ probability. A correction between the number of forks and the number of initiations at a definite time was considered. Thus, this first model combined the availability, variable with time, of the protein and the origins affinity with this protein, factor dependent of the fork density. From the results obtained in this project it was possible to improve the suggested model by adding the origin inhibition resulted from Chk1 action combined with a local suppression of the Chk1 effect near activated origins (fig.35). It is not yet known whether the suppression of the Chk1 effect is controlled by the Plx1, as previously suggested, or by other factor that still remains to be discovered. This checkpoint regulation allows the cell to replicate specific clusters even though we are in the presence of forks stalling and the global replication is slowed down until disappearance of the replication stress. In order to comprehend the possible Plx1 contribution to this mechanism, DNA combing experiments with Plx1 inhibitor in the presence or absence of Chk1 inhibitor must be performed.

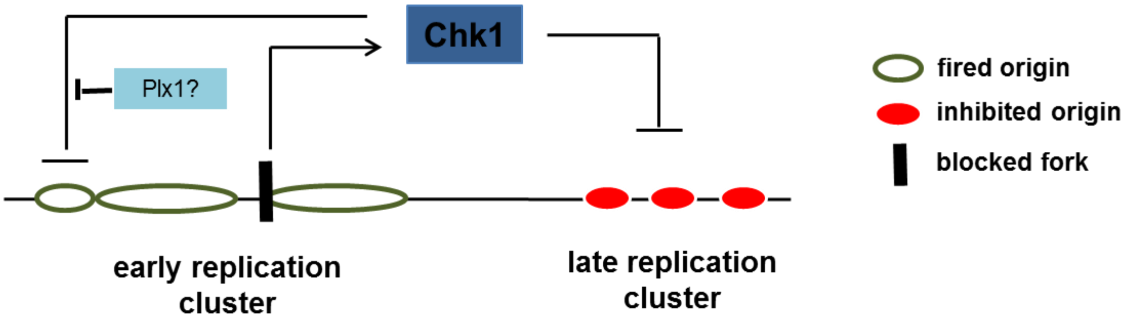


**Fig.35 – Model of the spatio-temporal origins activation in *xenopus laevis***. This model has represented the Chk1 inhibitory effect in late replication clusters and the inhibition of this effect near already activated clusters that could be due to Plx1 action or another unknown protein is involved. Adapted from Platel et al. 2015

Moreover, this numerical model shows that the probability of Chk1 action in absence of exogenous replication stress is elevated. This outcome is in agreement with the results obtained in the last article published by the lab (Platel et al. 2015), showing that a low Chk1 overexpression results in deceleration of the replication due to inhibition of origins activation in a non-perturbed S phase.

## Future Perspectives

- Perform a quantitative MS analysis to verify possible changes in the recruitment of replication proteins in the presence of dNTPs
- Acquire new antibodies to Treslin, TopBP1 and ReQL4 to confirm the results obtained for the other pre-IC proteins
- Verify the MCM complex phosphorylation status
- Understand the effect on fork speed due to exogenous dNTPs addition
- Confirm that the proteins belonging to the pre-RC remain unchanged when adding dNTPs

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# Appendix

# Appendix 1

| | 45min | | 55min | | 75min | |
|---|---|---|---|---|---|---|
| | **DMSO** | **AZD** | **DMSO** | **AZD** | **DMSO** | **AZD** |
| **Analysed DNA (kb)** | 6450,9 | 4340,5 | 4751,0 | 6241,3 | 5960,0 | 5759,0 |
| **Replicated DNA(kb)** | 585,0 | 1470,7 | 744,6 | 3649,3 | 2381,8 | 4249,6 |
| **Replication Extent** | 0,091 | 0,339 | 0,157 | 0,585 | 0,400 | 0,738 |
| **Number of analysed fibers** | 165 | 132 | 151 | 193 | 157 | 108 |
| **Number of fully replicated fibers** | 1 | 7 | 11 | 54 | 17 | 26 |
| **Number of unreplicated Fibers** | 130 | 55 | 116 | 60 | 64 | 17 |
| **Average size of all fibers (kb)** | 39,1 | 32,9 | 31,5 | 32,3 | 38,0 | 53,3 |
| **Average size of fully replicated fibers (kb)** | 25,6 | 26,0 | 22,0 | 28,2 | 24,7 | 36,2 |
| **Average size of unreplicated fibers (kb)** | 39,7 | 35,7 | 32,1 | 32,4 | 39,2 | 48,0 |
| | | | | | | |
| **Number of replication eyes** | 64 | 101 | 25 | 81 | 88 | 116 |
| **Average Eye Length (kb)** | | | | | | |
| **Local** | 5,9 | 6,6 | 7,7 | 8,7 | 9,2 | 11,2 |
| **Overall** | 7,5 | 10,2 | 16,2 | 22,2 | 16,5 | 24,1 |
| **Excluded** | 14,3 | 18,7 | 26,3 | 35,2 | 28,1 | 49,0 |
| | | | | | | |
| **Number of eye to eye distances (ETED)** | | | | | | |
| **Average ETED length (kb)** | 38 | 50 | 11 | 35 | 35 | 73 |
| **Local** | 10,7 | 8,6 | 10,7 | 8,4 | 10,4 | 11,5 |
| **Overall** | 82,2 | 30,1 | 103,3 | 37,9 | 41,4 | 32,7 |
| **Excluded** | 149,3 | 41,6 | 132,4 | 45,9 | 51,3 | 47,8 |
| | | | | | | |
| **Number of replication fork** | 157 | 288 | 92 | 329 | 288 | 352 |
| **Fork Density (forks/100 kb)** | 2,43 | 6,64 | 1,94 | 5,27 | 4,83 | 6,11 |

# Appendix 2

| | 105min | |
|---|---|---|
| | **Aph/DMSO** | **Aph/AZD** |
| **Analysed DNA (kb)** | 5716,5 | 4703,0 |
| **Replicated DNA(kb)** | 210,6 | 889,9 |
| **Replication Extent** | 0,037 | 0,189 |
| **Number of analysed fibers** | 242 | 141 |
| **Number of fully replicated fibers** | 4 | 2 |
| **Number of unreplicated Fibers** | 207 | 62 |
| **Average size of all fibers (kb)** | 23,6 | 33,4 |
| **Average size of fully replicated fibers (kb)** | 6,9 | 31,4 |
| **Average size of unreplicated fibers (kb)** | 23,6 | 32,2 |
| | | |
| **Number of replication eyes** | 38 | 149 |
| **Average Eye Length (kb)** | | |
| **Local** | 4,6 | 5,5 |
| **Overall** | 5,2 | 5,9 |
| **Excluded** | 13,6 | 43,3 |
| | | |
| **Number of eye to eye distances (ETED)** | 8 | 74 |
| **Average ETED length (kb)** | | |
| **Local** | 9,1 | 12,3 |
| **Overall** | 141,1 | 31,2 |
| **Excluded** | 173,6 | 49,6 |
| | | |
| **Number of replication fork** | 81 | 301 |
| **Fork Density (forks/100 kb)** | 1,42 | 6,40 |

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RESEARCH ARTICLE

# Tight Chk1 Levels Control Replication Cluster Activation in *Xenopus*

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## Abstract

DNA replication in higher eukaryotes initiates at thousands of origins according to a spatio-temporal program. The ATR/Chk1 dependent replication checkpoint inhibits the activation of later firing origins. In the *Xenopus in vitro* system initiations are not sequence dependent and 2-5 origins are grouped in clusters that fire at different times despite a very short S phase. We have shown that the temporal program is stochastic at the level of single origins and replication clusters. It is unclear how the replication checkpoint inhibits late origins but permits origin activation in early clusters. Here, we analyze the role of Chk1 in the replication program in sperm nuclei replicating in *Xenopus* egg extracts by a combination of experimental and modelling approaches. After Chk1 inhibition or immunodepletion, we observed an increase of the replication extent and fork density in the presence or absence of external stress. However, overexpression of Chk1 in the absence of external replication stress inhibited DNA replication by decreasing fork densities due to lower Cdk2 kinase activity. Thus, Chk1 levels need to be tightly controlled in order to properly regulate the replication program even during normal S phase. DNA combing experiments showed that Chk1 inhibits origins outside, but not inside, already active clusters. Numerical simulations of initiation frequencies in the absence and presence of Chk1 activity are consistent with a global inhibition of origins by Chk1 at the level of clusters but need to be combined with a local repression of Chk1 action close to activated origins to fit our data.

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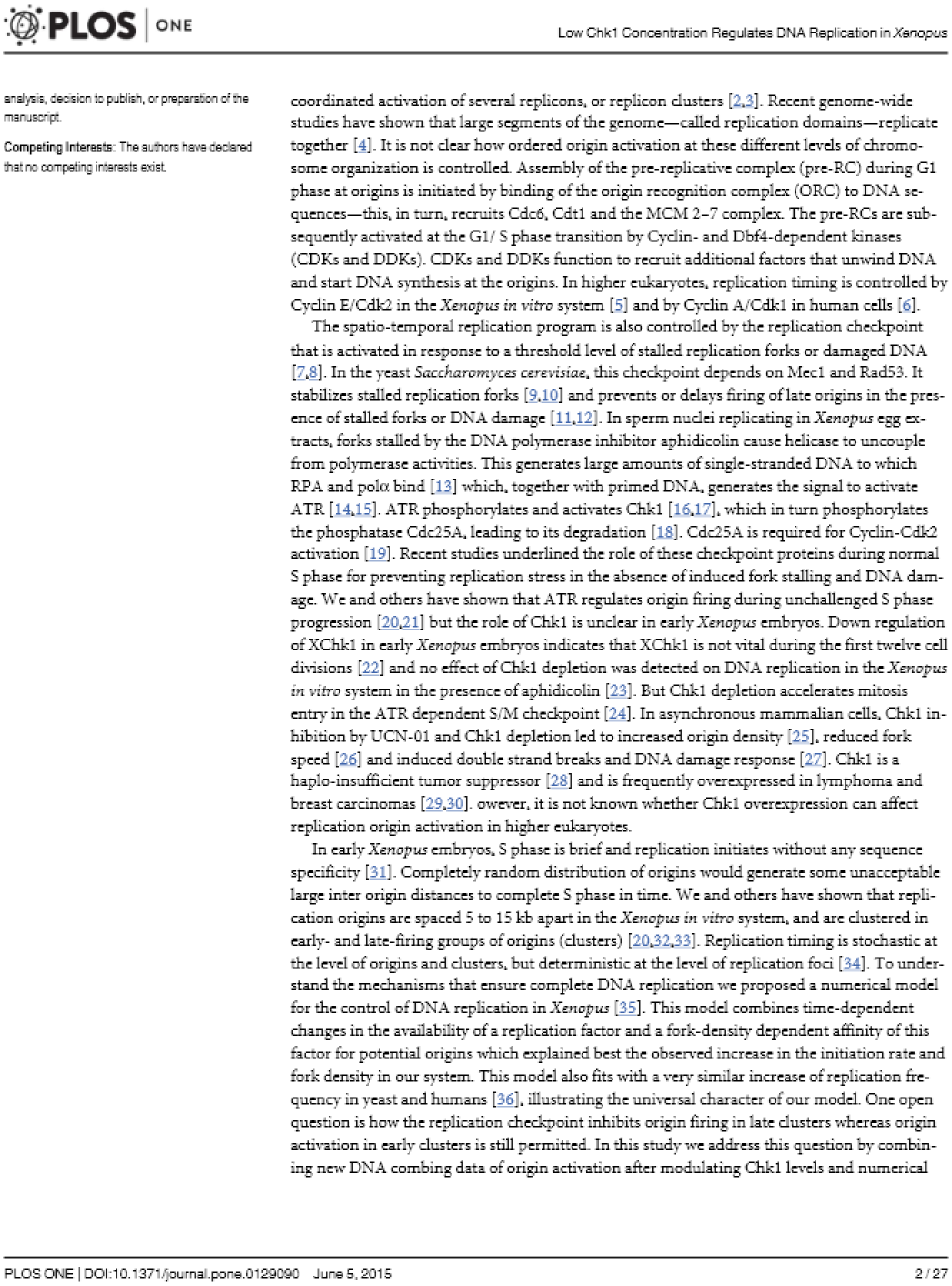
## Introduction

To maintain genome stability, eukaryotic DNA replication must be strictly controlled in space and time during S phase [1]. In higher eukaryotes, DNA replication starts from several thousand replication origins, each activated at different times during S phase. It also involves the



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coordinated activation of several replicons, or replicon clusters [2,3]. Recent genome-wide studies have shown that large segments of the genome—called replication domains—replicate together [4]. It is not clear how ordered origin activation at these different levels of chromosome organization is controlled. Assembly of the pre-replicative complex (pre-RC) during G1 phase at origins is initiated by binding of the origin recognition complex (ORC) to DNA sequences—this, in turn, recruits Cdc6, Cdt1 and the MCM 2–7 complex. The pre-RCs are subsequently activated at the G1/ S phase transition by Cyclin- and Dbf4-dependent kinases (CDKs and DDKs). CDKs and DDKs function to recruit additional factors that unwind DNA and start DNA synthesis at the origins. In higher eukaryotes, replication timing is controlled by Cyclin E/Cdk2 in the *Xenopus in vitro* system [5] and by Cyclin A/Cdk1 in human cells [6].

The spatio-temporal replication program is also controlled by the replication checkpoint that is activated in response to a threshold level of stalled replication forks or damaged DNA [7,8]. In the yeast *Saccharomyces cerevisiae*, this checkpoint depends on Mec1 and Rad53. It stabilizes stalled replication forks [9,10] and prevents or delays firing of late origins in the presence of stalled forks or DNA damage [11,12]. In sperm nuclei replicating in *Xenopus* egg extracts, forks stalled by the DNA polymerase inhibitor aphidicolin cause helicase to uncouple from polymerase activities. This generates large amounts of single-stranded DNA to which RPA and polα bind [13] which, together with primed DNA, generates the signal to activate ATR [14,15]. ATR phosphorylates and activates Chk1 [16,17], which in turn phosphorylates the phosphatase Cdc25A, leading to its degradation [18]. Cdc25A is required for Cyclin-Cdk2 activation [19]. Recent studies underlined the role of these checkpoint proteins during normal S phase for preventing replication stress in the absence of induced fork stalling and DNA damage. We and others have shown that ATR regulates origin firing during unchallenged S phase progression [20,21] but the role of Chk1 is unclear in early *Xenopus* embryos. Down regulation of XChk1 in early *Xenopus* embryos indicates that XChk1 is not vital during the first twelve cell divisions [22] and no effect of Chk1 depletion was detected on DNA replication in the *Xenopus in vitro* system in the presence of aphidicolin [23]. But Chk1 depletion accelerates mitosis entry in the ATR dependent S/M checkpoint [24]. In asynchronous mammalian cells, Chk1 inhibition by UCN-01 and Chk1 depletion led to increased origin density [25], reduced fork speed [26] and induced double strand breaks and DNA damage response [27]. Chk1 is a haplo-insufficient tumor suppressor [28] and is frequently overexpressed in lymphoma and breast carcinomas [29,30]. owever, it is not known whether Chk1 overexpression can affect replication origin activation in higher eukaryotes.

In early *Xenopus* embryos, S phase is brief and replication initiates without any sequence specificity [31]. Completely random distribution of origins would generate some unacceptable large inter origin distances to complete S phase in time. We and others have shown that replication origins are spaced 5 to 15 kb apart in the *Xenopus in vitro* system, and are clustered in early- and late-firing groups of origins (clusters) [20,32,33]. Replication timing is stochastic at the level of origins and clusters, but deterministic at the level of replication foci [34]. To understand the mechanisms that ensure complete DNA replication we proposed a numerical model for the control of DNA replication in *Xenopus* [35]. This model combines time-dependent changes in the availability of a replication factor and a fork-density dependent affinity of this factor for potential origins which explained best the observed increase in the initiation rate and fork density in our system. This model also fits with a very similar increase of replication frequency in yeast and humans [36], illustrating the universal character of our model. One open question is how the replication checkpoint inhibits origin firing in late clusters whereas origin activation in early clusters is still permitted. In this study we address this question by combining new DNA combing data of origin activation after modulating Chk1 levels and numerical

simulations in the presence and absence of Chk1 kinase activity in the synchronous *Xenopus in vitro* system.

By specific inhibition using UCN-01 and AZD-7762 or immunodepletion of Chk1 we show that Chk1 regulates the spatio-temporal replication program at the level of replication clusters and not inside active clusters—both in the presence and absence of external replication stress. We show that Chk1 inhibition results in an increase in initiations in S phase in the absence and presence of aphidicolin, consistent with studies in mammalian cells. Surprisingly, modest Chk1 overexpression by adding recombinant Chk1 inhibits DNA replication by decreasing fork density and inhibiting cluster activation showing for the first time that Chk1 levels must be tightly controlled in our system to allow correct origin activation even in the absence of external stress. The numerical simulation of initiation frequencies in the presence and absence of checkpoint activity, and subsequent fitting to our experimental data, shows that Chk1 globally inhibits replication clusters whereas Chk1 is itself inhibited close to activated origins in active early clusters. Thus, we provide for the first time a numerical model for the spatio-temporal replication program including the replication checkpoint for higher eukaryotes.

## Materials and Methods

### Reagents and antibodies

Aphidicolin and UCN-01 were purchased from Sigma-Aldrich, AZD-7762 from Selleck Chemicals, aliquoted at -20°C and used only once. Human Anti-Phospho-Serine345-Chk1 (recognizes Phospho-Ser344-XChk1) was purchased from Cell Signaling Technology, anti-human Chk1 antibody from SantaCruzBiotech, anti-Phospho (Y15) cdk2 (ab76147) from Abcam, Anti-DNA antibody (Mab3032) from Merck-Millipore, Streptavidin and AlexaFluor antibodies from Invitrogen. XOrc2 antibody was a gift from R. A. Laskey.

### Production of antibody against XChk1 and recombinant XChk1

XChk1 cDNA (gift from B. Dunphy) was cloned into a pDEST vector (Invitrogen) including an N-terminal Histag. The protein was expressed in *E.coli* C41 (DE3) (gift of B. Miroux) and purified using Ni-Sepharose (GE Healthcare) according to the manufacturer. Two specific polyclonal antibodies against the full length recombinant protein were produced by P.A.R.I.S antibodies (Compiegne, France). These antibodies worked well in western blot analysis but did not work in immunodepletions experiments. For depletion and add back experiments recombinant and active XChk1 with a N-terminal His-tag was expressed in the baculovirus expression system (BD BaculoGold), purified using Nickel-Sepharose (Amersham Bioscience) beads as described by the supplier and dialyzed over night against 50 mM Hepes pH 7.8, 10% glycerol, 1mM DTT, 300mM KCl. Its kinase activity was tested using the Cdc25 peptide substrate CHKtide (Upstate) as indicated by the supplier.

### Replication of sperm nuclei in *Xenopus* egg extracts

Replication competent extracts from unfertilized *Xenopus* eggs were prepared as described [37] and used fresh unless stated otherwise. We routinely checked for Chk1 phosphorylation before nuclei addition in order to exclude low quality extracts. Sperm nuclei (100 or 2000 nuclei/μl) were incubated in extracts in the presence of cycloheximide (250 μg/ml), energy mix (7.5 mM creatine phosphate, 1 mM ATP, 0.1 mM EGTA, pH 7.7, 1 mM $MgCl_2$) and 20μm biotin-dUTP (Roche Applied Science). Replication was allowed to continue for indicated time points. Aphidicolin was added at 7.5 μg/ml and replication continued for 90 to 120 min. UCN-01 (or solvent (DMSO) alone as control) was added at 1 μM. Caffeine (or buffer alone as control) was added where indicated, to a final concentration of 5 mM from a 100 mM solution, freshly dissolved in 10 mM Pipes-NaOH, pH 7.4. *In vitro* fertilization of *Xenopus* eggs with sperm was performed according to standard techniques [38], and developmental stages of embryos were determined according to Nieuwkoop and Faber (1994). Our institutional Animal Care and Use Committee (IACUC) namely Paris Center and South number 59 approved the study and the protocols herein (approvals number 2012–0062 and 2012–0063) following the French and the European laws on animal experimentation.

## Immunodepletions

Anti-XChk1 serum [24] or mock serum (rabbit IgG) was incubated 3h or overnight at 4° C with native protein A sepharose beads (GE Healthcare). Beads were washed with EB buffer without DTT buffer and briefly with a small volume of fresh extract to eliminate buffer and incubated twice 30 min at 4°C with egg extract (volume ratio 1:2) under agitation. Extracts were separated from beads by centrifugation for 2 min at 1000 g in compact reaction columns (USB) with cellulose filters and used for replication reactions.

## Molecular combing and detection by fluorescent antibodies

DNA was extracted and combed as described [39]. Biotin was detected with AlexaFluor594 conjugated streptavidin followed by anti-avidin biotinylated antibodies. This was repeated twice, then followed by anti-DNA antibody, AlexaFluor488 rabbit anti-mouse, and goat anti-rabbit antibodies for enhancement [40].

## Measurements and data analysis

Images of the combed DNA molecules were acquired and measured as described [39]. For each combing experiment a total of 6–12 Mb DNA was measured. The fields of view were chosen at random, unless mentioned otherwise. Measurements on each molecule were made using Image Gauge version 4.2 (Fujifilm) and compiled using macros in Microsoft Excel (2010). Replication eyes were defined as the incorporation tracks of biotin–dUTP. Replication eyes were considered to be the products of two replication forks, incorporation tracks at the extremities of DNA fibers were considered to be the products of one replication fork. Tracts of biotin-labeled DNA needed to be at least 1 kb to be considered significant and scored as eyes. When label was discontinuous, the tract of unlabeled DNA needed to be at least 1 kb to be considered a real gap. The replication extent was determined as the sum of eye lengths divided by the total DNA length. Fork density was calculated as the total DNA divided by the total number of forks. The midpoints of replication eyes were defined as the origins of replication. Eye-to-eye distances (ETED), also known as inter-origin distances, were measured between the midpoints of adjacent replication eyes. The means of fiber lengths were comparable inside each individual experiment in order to avoid biases in eye to eye distances. Incorporation tracks at the extremities of DNA fibers were not regarded as replication eyes, but were included in the determination of the replication extent, calculated as the sum of all eye lengths (EL) divided by total DNA. Box plots of ETED (with n ranging from 80–400) were made using GraphPad version 6.0 (La Jolla, CA, USA). Statistical analysis of repeated experiments have been included as means including standard error of the mean (SEM). Non parametric unpaired tests (Mann-Whitney Test) and unpaired Student's t-tests were used to determine statistical significance. A P-value less than 0.05 was considered statistically significant. When experiments were repeated with a different egg extract replication extent differs at identical time scales because different egg extracts replicate nuclei with different replication kinetics. It is therefore difficult to combine all of them and include statistics of independent kinetics experiments.

## Neutral and alkaline agarose gel electrophoresis

Sperm nuclei were incubated in fresh extracts complemented with indicated reagents and one-fiftieth volume of [$\alpha$-$^{32}$P]dATP (3000 Ci/mmol). DNA was purified, separated on 0.8% TBE-agarose or 1.1% alkaline agarose gels, and analyzed as described [33].

## Western blot analysis

For analysis of whole extract samples, replication reactions were stopped at indicated times by the addition of SDS sample buffer. For analysis of nuclei, reactions were diluted into a 20-fold volume of nuclear isolation buffer (NIBS) (50 mM Hepes, 150 mM NaCl, 2 mM $MgCl_2$, protease inhibitors, phosphatase inhibitors, 10% sucrose) and nuclei were pelleted through a NIBS buffer with 20% sucrose at 4000 g, 5 min, 4°C. The purification was repeated, then the pellet was dissolved in SDS sample buffer. For analysis of chromatin-bound proteins, reactions were diluted into a 20-fold volume of nuclear isolation buffer (NIB) (50 mM Hepes pH 7.5, 100 mM NaCl, 2 mM $MgCl_2$, 2 mM DTT, spermin 0.2 mM, spermidine 0.5 mM, protease inhibitors, phosphatase inhibitors, 0.1% TritonX100) and chromatin was recovered through a NIBS buffer, 0.1% TritonX100, 15% sucrose at 4000 g, 5 min, 4°C. Interphase was washed twice with 200 μl NIB+ TritonX-100. The pellet was centrifuged again at 10 000 g for 5 min, 4°C and was resuspended in SDS sample buffer. Proteins were subjected to SDS gel electrophoresis and transferred to PVDF membranes. Immunodetection was performed according to the manufacturer, and peroxidase activity was revealed using Super Signal West Pico or Femto Chemiluminescence Kit (Pierce).

## Cdk2 immunoprecipitation and kinase assays

Anti-Xenopus Cdk2 antibody or mock rabbit IgG were coupled to Protein A Sepharose as described above and washed in dilution buffer (50mM Hepes/KOH pH8.0, 50mM KCl, 20 mM $K_2HPO_4/KH_2PO_4$ pH8). Replication reactions (50μl) supplemented with 2000 nuclei/μl were stopped after 45 min with 5 fold dilution buffer, proteinase and phosphatase inhibitors, overlayed on 150μl dilution buffer and 30% Sucrose and centrifuged 5000 g for 5 min. The pellet was resuspended in 200μl dilution buffer supplemented with 0.2% Triton X100 to extract nuclear proteins, incubated 10 min on ice and centrifuged 14 000 rpm for 5 min. The supernatant was incubated with Cdk2 or mock coupled beads at 4°C for 2 h. Beads were washed three times in dilution buffer with Triton, once in dilution buffer without Triton and finally in EB buffer. H1 histone kinase assays in duplicates were performed with 10 μl beads, 0.1 μCi $\gamma^{32}$P-ATP, 50μM ATP and 0.5 μg H1 histone for 30 min at 30°C. Reactions were stopped with 2x Laemmli buffer, proteins were separated by SDS gel electrophoresis, gels were dried and bands were quantified on a phosphoimager Typhoon Trio (GE Healthcare).

## Numerical simulation of initiation frequency I(f)

We used a dynamic Monte Carlo method to simulate DNA replication as a one-dimensional nucleation and growth process [35,41]. The replicating genome is schematised as a one-dimensional array of $L$ elements (L = 1000000 here). Each element corresponds to 1 kb. We made the following assumptions: 1. The initiation process is governed by the stochastic encounter of a limiting factor ($N$) and a potential replication origin; 2. The number $N$ of limiting factors increases with a rate $J$ as replication progresses ($N = N_0 + Jt$, where $N_0$ is the initial number of limiting factors); 3. Replication origins are uniformly distributed along the genome and can only fire once during the simulation. Once an initiation has occurred, the limiting factor is sequestered by the two diverging replication forks; replication forks will progress with a speed v = 1 element per round of calculation. Each round of calculation corresponds to 2 min, so the measured speed v of replication forks is 0.5 kb $\text{min}^{-1}$. The encounter between a limiting factor and a replication origin will trigger firing with a probability $P(x,t)$, where $x$ is the location of replication origin and $t$ is the elapsed time from the beginning of S phase. $P(x,t)$ represents the probability of replication origins firing, which is conditional on the inhibitory action of Chk1. As helicases are integrated parts of replication forks, in our numerical simulations we assumed that the number of activated Chk1 ($N_{Chk1}$) is equal to the number of active replication forks. We checked by quantitative western blotting that the amount of chromatin bound Chk1 is roughly is sufficient for one Chk1 molecule to bind to one replication fork (data not shown). Furthermore, as the inhibitory action of Chk1 is thought to be global over the genome, we assumed that during an unchallenged S phase, Chk1 inhibits unfired potential replication origins with a probability $k_{Chk1}$. Also, we assumed that in the absence of Chk1, the encounter between a limiting factor and a replication origin will trigger firing with a constant probability $P_0$ if the latter has not been inhibited for firing by Chk1. We introduced a third, local mechanism that removes the inhibitory action of Chk1 over the potential replication origins. Assuming that this mechanism is also active during an unchallenged S phase, we considered that if a Chk1-inhibited potential replication origin is at a distance $d = 45$ kb (cluster size) of a replication fork, it would have a probability $k_{polo}$ of recovering its ability to fire. Combining our numerical simulation with a Simplex optimisation algorithm, we obtained the fit of $I(f)$ with experimental data.

## Results

### Global origin inhibition after induced fork stalling is Chk1-dependent in *Xenopus* egg extracts

We used nascent strand analysis and DNA combing to analyze origin activation of sperm nuclei replicating in the *Xenopus* egg extracts in the presence of aphidicolin and in the presence or absence of the specific Chk1 inhibitor UCN-01 as well as after Chk1 depletion. When sperm chromatin is incubated in *Xenopus* egg extract, it is assembled into normal interphase nuclei surrounded by a nuclear envelope and replicated semi-conservatively [42]. DNA replication starts synchronously after a typical 20 to 30 min lag phase, and is complete 40 to 60 min later, depending on the extract. Aphidicolin, an inhibitor of DNA polymerases α,ε and δ, inhibits elongation of DNA nascent strands, and thereby stalls replication forks, but does not prevent the initiation of DNA replication and the formation of short nascent strands. Upon addition of aphidicolin to the replication reaction, we observed a caffeine sensitive phosphorylation of Chk1 in western blot analysis from whole replication reaction samples using a specific phospho-Ser345 Chk1 antibody (Fig 1A), consistent with previous studies [21,23]. Next, we checked whether UCN-01, a specific inhibitor for Chk1 in mammalian cells [43,44], inhibits Chk1 in *Xenopus* egg extracts. Using western blot analysis with an antibody against the inhibitory Y15 phosphorylation site of Cdk2, we confirmed that the P-Cdk2 signal decreased in comparison to the aphidicolin control and that therefore the Chk1-Cdc25-Cdk2 axis was also abrogated by 1 μM UCN-01 in the *Xenopus in vitro* system in the presence of aphidicolin (Fig 1B). We analyzed the effect of UCN-01-mediated Chk1 inhibition on nascent strand synthesis by alkaline DNA electrophoresis. Sperm nuclei were incubated for 90 min in the presence of 5 or 10 μg/ml aphidicolin and [$\alpha$-$^{32}$P]-dATP with or without UCN-01. We expected that inhibition of Chk1 would increase nascent strand synthesis due to unscheduled origin firing if Chk1 inhibits origin activation in the replication checkpoint pathway. We observed that UCN-01 increased indeed the accumulation of nascent strand synthesis 2- and 5-fold at 5 and 10 μg/ml aphidicolin, respectively (Fig 1C). Thus, the inhibitory effect of Chk1 on replication increased with the dose

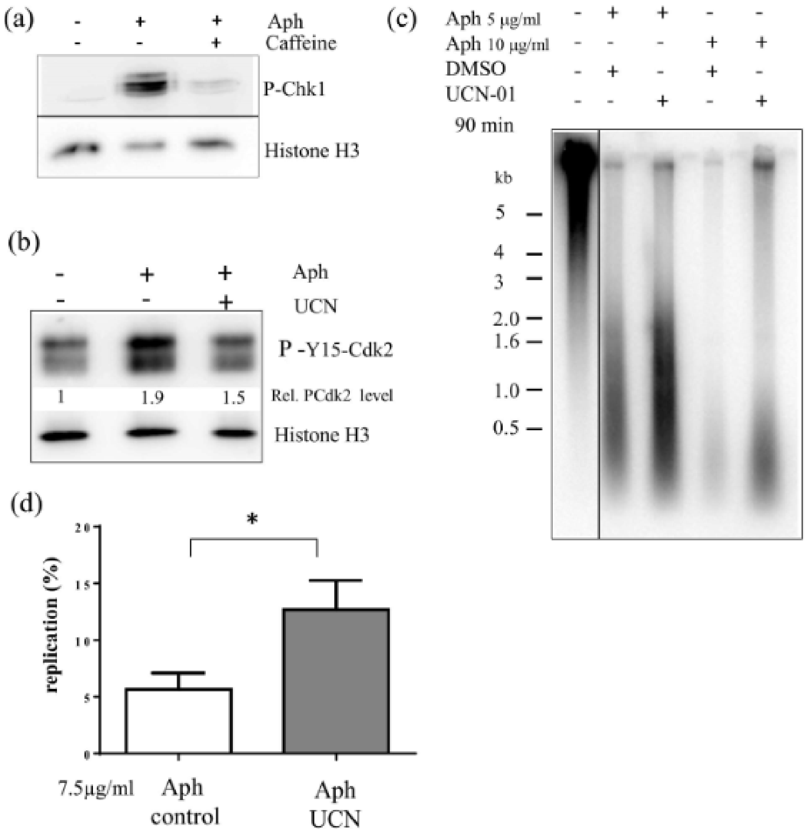

**Fig 1. Inhibition of Chk1 activity by UCN-01 increases DNA synthesis in the presence of aphidicolin.** (a) Sperm nuclei (1000n/μl) were added to egg extracts for 35 and 60 min in the presence or absence of aphidicolin and caffeine, and whole extracts were subjected to gel electrophoresis and western blot analysis using an anti-P-S345-Chk1 antibody, (b) Sperm nuclei were added to egg extracts for 90 min in the presence or absence of aphidicolin and 1μM UCN-01, western blot against whole extracts using antibody against phospho-Y15 Cdk2, loading control histone H3, (c) Sperm nuclei were added to egg extracts in the presence of [α-$^{32}$P]-dATP with or without 1μM UCN and aphidicolin (5 or 10 μg/ml) and nascent DNA strands synthesized after 90 min were analyzed by alkaline DNA electrophoresis, (d) Quantification of six independent alkaline DNA electrophoresis experiments at 7.5 μg/ml aphidicolin, mean with SEM (t-test, P = 0.0387), 100% replication is defined as the signal in the–Aph-control after 90 min, * marks significant difference ($P < 0.05$).

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of aphidicolin. Nascent strands were smaller with 10 μg/ml aphidicolin than with 5 μg/ml, owing to a stronger elongation inhibition, and were, in both cases, not affected by Chk1 inhibition. We repeated this experiment six times with very similar results but with an intermediate aphidicolin concentration (7.5 μg/ml) which allowed optimal DNA combing analysis (see below). We found a significant 2.2 fold mean increase upon UCN-01 addition (Fig 1D).

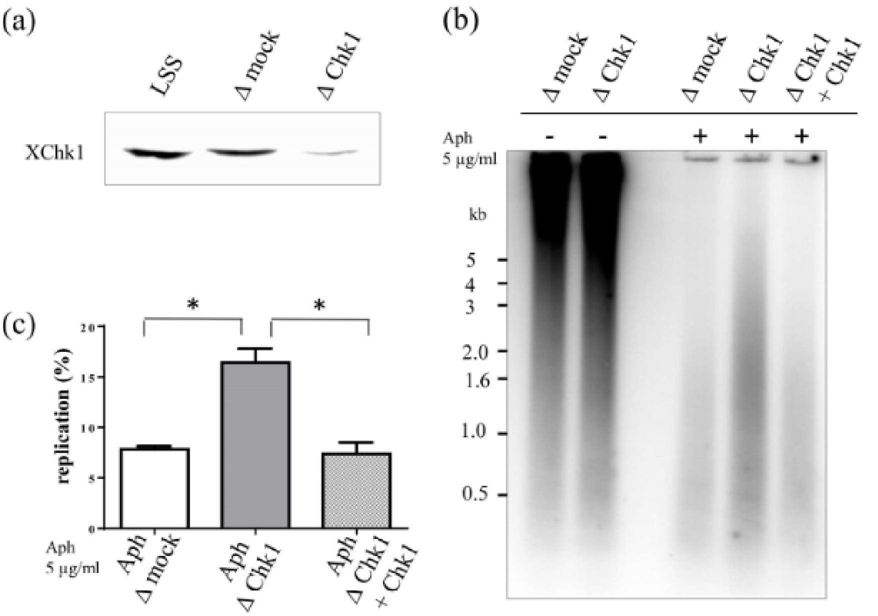

**Fig 2. Chk1 depletion increases DNA synthesis in the presence of aphidicolin.** (a) Egg extracts were mock- or Chk1-depleted and aliquots analyzed by western blotting using an anti-XChk1 antibody, (b) Sperm nuclei were incubated for 150 min in mock-depleted, Chk1-depleted egg extracts, Chk1-depleted egg extracts supplemented with recombinant 40 nM XChk1 in the presence of [α-$^{32}$P]-dATP with or without aphidicolin (5 µg/ml). Nascent DNA strands synthesized after 150 min were analyzed by alkaline gel electrophoresis, (c) Mean replication in mock depleted, Chk1 depleted, Chk1 depleted + Chk1 add back extracts of two independent experiments with SEM (t-tests: mock versus Chk1 depletion: $P = 0.026$, Chk1 depletion versus add back: $P = 0.037$), * significantly different ($P < 0.05$).

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In order to further confirm the role of Chk1 we performed immunodepletion experiments of Chk1. Using a specific *Xenopus* anti-Chk1 antibody [24] we depleted 85% of endogenous XChk1 in egg extracts (Fig 2A). Sperm nuclei were then incubated in the presence of 5 µg/ml aphidicolin and [α-$^{32}$P]-dATP in mock or XChk1 depleted extracts. The experiment was repeated once. We found that nascent strand synthesis under denaturing conditions was twofold higher in XChk1-depleted extracts in comparison to mock depleted extracts (Fig 2B and 2C), consistent with our experiments with Chk1 inhibitors. Adding back recombinant active XChk1 (40nM, [24]) to XChk1-depleted extracts decreased DNA synthesis to control levels, which demonstrated the specificity of our immunodepletion. We conclude that Chk1 is activated and regulates origin firing upon fork stalling by aphidicolin in *Xenopus* egg extracts.

We performed DNA combing experiments to understand how Chk1 regulates origin firing in the presence of replication stress. Sperm nuclei were incubated in the presence of 7.5 µg/ml aphidicolin and DMSO or UCN-01 for 90 min in egg extracts. To label replication eyes, biotin-dUTP was added at the beginning of the reaction which was stopped after 90 min. DNA was purified, combed and labeled as described in the experimental procedures. DNA fibers were

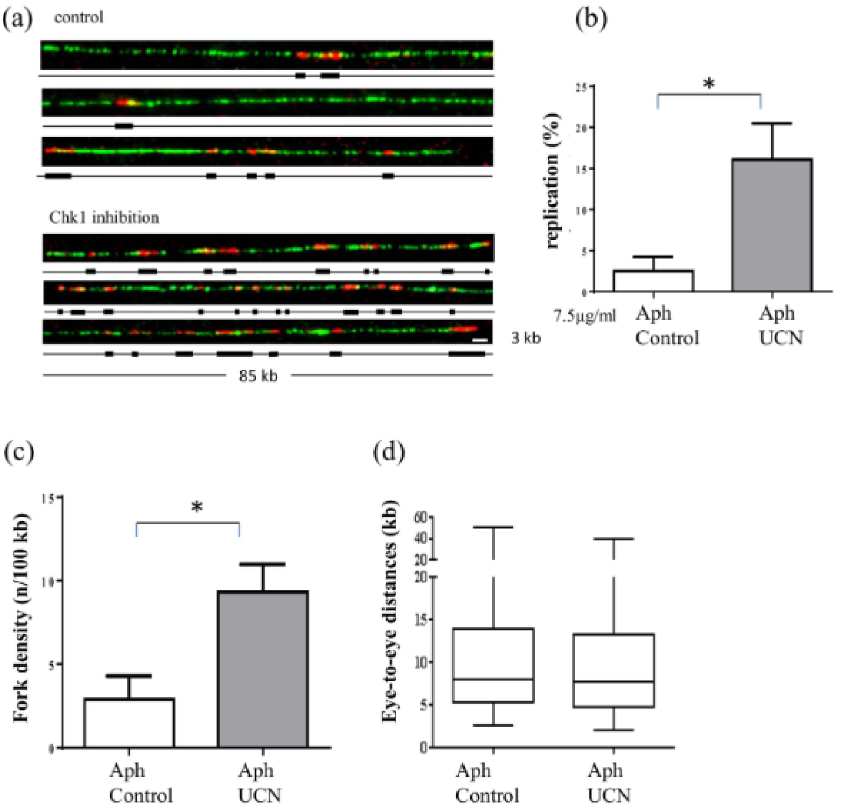

**Fig 3. Fork density increases after Chk1 inhibition in the presence of aphidicolin induced stalled forks.** Sperm nuclei (2000 nuclei/µl) were replicated in egg extracts in the presence of Biotin-dUTP, aphidicolin (7.5µg/ml) and in the presence (1µM) or absence of UCN-01 for 90 min. (a) Three representative combed DNA fibers replicated in the absence (above) or the presence of UCN-01 (below) (merge: green, whole DNA label; red, biotin labeled replication eyes), (b) Mean replication extent of four independent experiments with SEM (t-test, P = 0.027), (c) Mean fork density (number of forks/100kb) of four experiments with SEM (t-test, P = 0.018), (d) Box-plot of distances between replication eyes (kb) of representative experiment from (a), scale bar 3 kb,* significantly different (P < 0.05).

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visualized using an anti-DNA antibody, replication eyes were visualized using fluorescent streptavidin conjugates (Fig 3A) and replication extent was determined. The mean replication extent of four independent experiments is shown in Fig 3B. We found that in the presence of aphidicolin the mean replication extent was around 6-fold higher in the presence of UCN compared to the control. In *Xenopus*, 2–5 origins are grouped in replication clusters (30–50 kb) that fire asynchronously throughout S phase. The increase of replication extent can result therefore from an increase in the number of origins activated either inside or outside already activated replication clusters, or both. To determine which origins are activated, we directly measured eye-to-eye distances on individual fibers. In addition, we calculated the overall fork density (number of forks/100 kb) by dividing the total DNA length by the total number of forks. Because DNA fibers analyzed by DNA combing are in general not longer than 80–100 kb due to DNA breaks a difference exists between fork density and eye-to-eye distances, especially in early S phase. Eye-to-eye distances can only be measured on fibers containing at least two origins, whereas the calculation of fork density also includes those fibers with only one origin, or no origins and therefore include all replication clusters which have not yet been activated. Therefore local eye-to-eye distances mainly reflect the origin distances from origins inside the same replication cluster, whereas fork density is representative of the amount of active origins in the whole population of DNA fibers. Thus, an increase of the fork density with no change in eye-to-eye distances would reflect an increase in replication cluster activation. We observed a mean 2.6-fold increase in fork density in aphidicolin-treated extracts when Chk1 was inhibited (Fig 3C). However, there was no significant decrease in eye-to-eye distances when Chk1 was inhibited (Fig 3D, median 8.1 kb control versus 7.8 kb plus UCN-01, Mann-Whitney, two-tailed test, P = 0.370), which would have been expected if additional origins fired inside active clusters. No significant difference was detected in eye-to-eye distributions in another independent experiment (S1 Fig). We conclude that when replication forks are stalled by aphidicolin, a Chk1 dependent replication checkpoint is activated in the *Xenopus in vitro* system, which inhibits origins outside, but not inside, activated clusters.

## Chk1 dependent checkpoint activation at low nuclei to cytoplasm ratios

In *Xenopus* embryos, the DNA content per cell increases rapidly in the absence of transcription during the first 12 cell divisions until the mid-blastula transition (MBT). Chk1 only becomes essential after 12 cell cycles, and is transiently phosphorylated at this stage [22]. We tested whether the replication checkpoint is activated at low nuclei concentration in the *in vitro* system that mimics pre-MBT embryos. Nuclei were incubated at 100 nuclei/μl instead of 2000 nuclei/μl in egg extracts, in the absence or presence of aphidicolin. Proteins of isolated nuclei were analyzed using western blotting. The low nuclei concentration corresponded to 32 cell embryos, about 5 cell cycles after fertilization. We detected strong Chk1 phosphorylation in the presence of aphidicolin, but no signal in its absence (Fig 4A). DNA combing experiments were compared in the presence or absence of Chk1 activity in the presence of aphidicolin. The mean extent of DNA replication (Fig 4B) and the mean fork density (data not shown) in two independent experiments increased in the absence of Chk1 activity. This result shows that the replication checkpoint is activated at low nuclei to cytoplasm (N/C) ratios *in vitro*. We then tested whether Chk1 is phosphorylated in aphidicolin-treated embryos before the MBT. *In vitro* fertilization was performed, and embryos were incubated for 45 min with aphidicolin (100 μM) before nuclear isolation at stage 8 (5 h post fertilization, pre-MBT) or at stage 9 (7 h p.f, post-MBT). Western blot analysis of isolated nuclei rather than whole embryos showed that Chk1 was phosphorylated after replication stress before and after MBT (Fig 4C). We conclude that at low N/C ratios, Chk1 phosphorylation can be detected *in vitro* and *in vivo*, suggesting that Chk1 controls origin activation upon replication stress under these conditions *in vivo*.

## Chk1 inhibition increases fork density during unchallenged S phase

After having observed that only a few induced stalled forks were needed to activate the DNA replication checkpoint, we tested whether Chk1 can regulate origin activation in the absence of external stress, during an unchallenged S phase. In contrast to studies in asynchronous

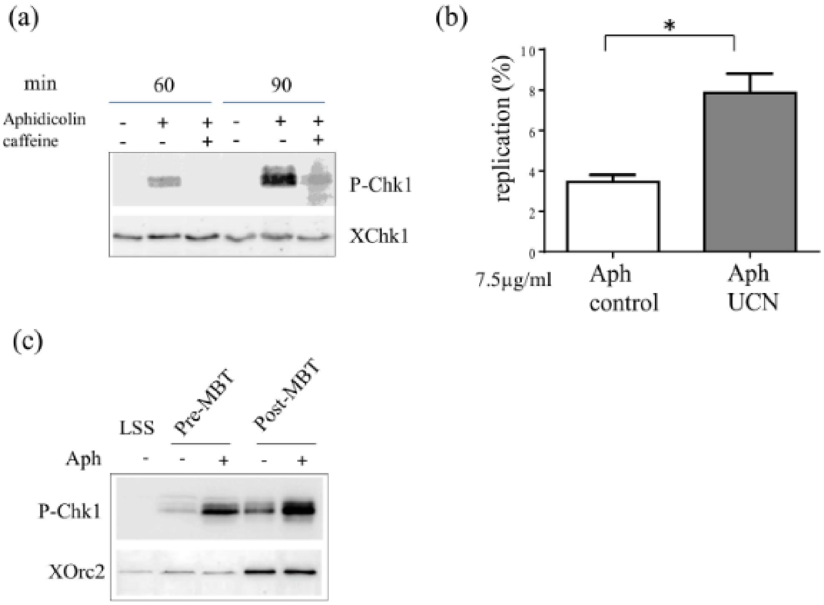

**Fig 4. Checkpoint activation upon low nuclei to cytoplasm ratios in the presence of aphidicolin.** (a) Sperm nuclei (100 nuclei/µl) were added to egg extracts in the presence of aphidicolin, nuclear extracts were prepared and subjected to gel electrophoresis and western blot analysis using an anti- P-Chk1 and XChk1 antibody, (b) For combing experiments sperm nuclei (100 nuclei/µl) were added to egg extracts in the presence of aphidicolin (7.5 µg/ml), biotin-dUTP and in the presence or absence of UCN-01 (1µM) for 90 min, DNA was isolated and combed, mean replication extent of two independent experiments with SEM (t-test, $P = 0.049$), (c) *Xenopus* embryos were incubated in aphidicolin (100 µM) for 30 min prior to harvest at stage 8 (pre-MBT) and stage 9 (post-MBT), nuclei extracts were prepared, subjected to gel electrophoresis and western blot analysis using a P-Chk1 antibody and XORC as loading control, LSS (low speed supernatant, extract),* significantly different ($P < 0.05$).

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mammalian cells, we use the synchronous *Xenopus in vitro* system that allows us to distinguish temporally distinct events during early, mid and late S phase without synchronization procedures that interfere with checkpoint activation. Sperm nuclei were incubated in egg extracts in the absence of aphidicolin and reactions were stopped at different times for western blot analysis. Chk1 phosphorylation was observed after 30 min, at the onset of replication, and was not observed in controls (extract with or without nuclei incubated on ice for 5 min) (Fig 5A). Chk1 phosphorylation increased in the presence of aphidicolin and was sensitive to the ATM/ATR inhibitor caffeine, as expected. Chk1 phosphorylation during unchallenged S phase has been shown in other studies, although under different experimental conditions [21,45]. Phosphorylated Chk1 was present mainly in nuclear and much less in chromatin bound fractions (S2 Fig), indicating that Chk1 is released from chromatin upon phosphorylation, consistent with results in human cells [46]. In order to analyze origin activation we performed two independent DNA combing experiments using two different egg extracts. Sperm nuclei were incubated in egg extracts in the presence of biotin-dUTP with or without 1 µM UCN-01. The reaction was stopped in early middle or late S phase and DNA was isolated, combed and labeled

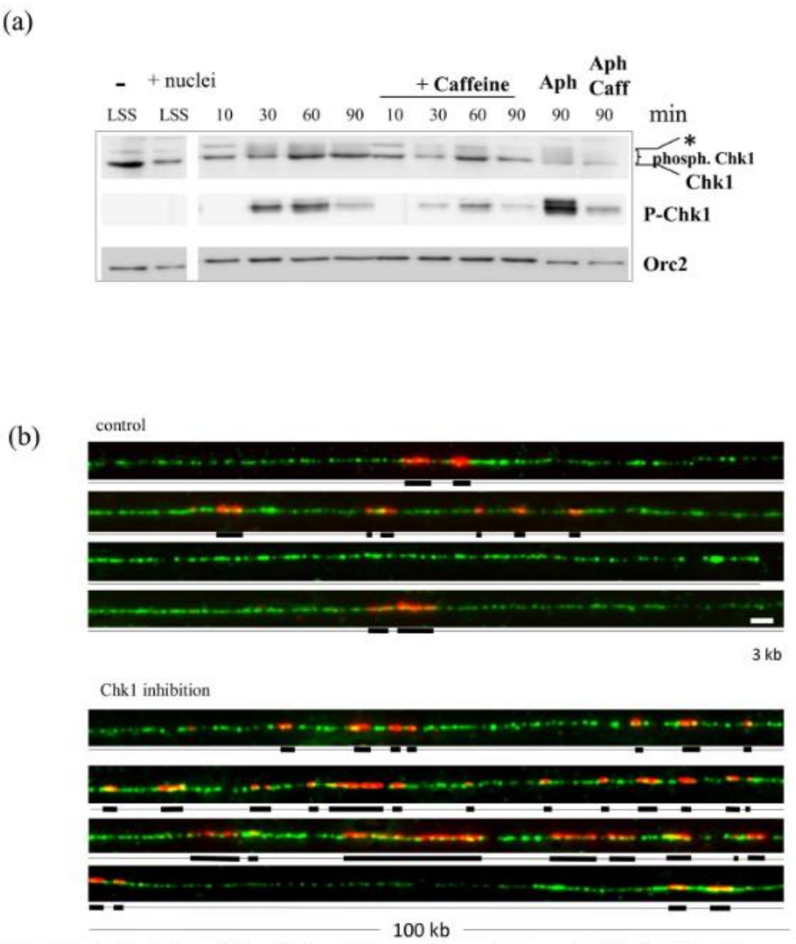

**Fig 5. Chk1 activation during unchallenged S phase.** (a) Sperm nuclei were added to egg extract for indicated times in the presence or absence of aphidicolin and caffeine, isolated nuclei were subjected to gel electrophoresis and western blot analysis using antibodies against XChk1, anti P-Chk1, XORC2, LSS, low speed supernatant, * marks a non-specific band, (b) Sperm nuclei were added to egg extracts in the presence of Biotin-dUTP for indicated times in the presence or absence of UCN-01 (1μM), Representative combed DNA fibers from early S phase (40 min), in the absence (above) or presence (below) of UCN-01 (merge: green, whole DNA label; red, biotin labeled replication eyes), scale bar 3 kb.

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(Fig 5B). In Fig 6 we show the results of the DNA combing analysis of both experiments separately (a, b) because the replication in experiment 1 was slightly slower than in experiment 2 due to the use of another egg extract. Therefore time points are not identical and not all results cannot be combined and compared directly, especially at later time points. For both experiments replication was accelerated at all time points during S phase in the absence of Chk1 function (Fig 6A, b, top panels). Fork density analysis (Fig 6A and 6B, middle) showed that it strongly increases in early S, less in middle S, and slightly decreased in late S phase in the UCN treated samples. This latter decrease is probably due to more merged eye lengths in the UCN treated sample since we observed an increase in mean eye length (data not shown). Next, we analyzed eye-to-eye distances which we expected to be smaller because fork densities were higher in the presence of UCN. The analysis was performed at the earliest time point in order to avoid replication eye mergers. The comparison of eye-to-eye distance distributions between control and UCN show that either median distances were slightly bigger for experiment 1 at 40 min upon UCN treatment (Fig 6A, bottom, Mann-Whitney test, $P = 0.0418$) or not significantly different at 35 min ($P = 0.398$) for experiment 2 (Fig 6B, bottom). Slightly larger eye-to-eye distances in exp.1 could result from more eye mergers due to a small increase in initiations inside clusters after UCN treatment despite an early S phase time point. We combined replication extent and fork density data for early S phase from four independent experiments and found a significant increase of 2.8 and 2.7, respectively (Fig 6C and 6D) after treatment with UCN-01. We conclude that only few additional origins are activated inside already activated clusters but new origins are mainly activated in later clusters upon Chk1 inhibition. These results are therefore in agreement with our aphidicolin data and show that in the absence of external stress, Chk1 also regulates origin activity mainly outside activated replication clusters during S phase. We conclude that after Chk1 inhibition, more origins are activated especially in the beginning of S phase.

In order to confirm the effect of UCN-01, we used a second, more recent Chk1 inhibitor, AZD-7762 [47] in experiments both in the presence and absence of aphidicolin. In the presence of aphidicolin we found in four independent experiments, two nascent strand analysis and two DNA combing experiments, that addition of 0.5μM AZD increased the replication extent in nascent strand (Fig 7A and 7B) and combing analysis (Fig 7C) as observed with UCN-01. This increase was due to a sevenfold higher fork density (Fig 7D) in the presence of AZD. Finally, the distribution of eye-to-eye distances was slightly larger in the presence of AZD in comparison with the control (Fig 7E), but not smaller as expected if origins were activated inside already activated clusters. Furtheron, in the absence of aphidicolin, we found in two independent DNA combing experiments a fivefold increase of replication (Fig 7F) early in S phase which was again due to an increase of fork density (Fig 7G). Distributions of eye-to-eye distances were unchanged as observed after UCN inhibition (Fig 7H). Time course experiments by alkaline DNA gel electrophoresis (S3 Fig) showed that replication extent was still higher at mid and late S phase upon AZD addition. We conclude that Chk1 inhibition by AZD-7762, very similar to UCN-01, results in the activation of replication origins outside but not inside active replication clusters.

## Chk1 overexpression inhibits late replication cluster activation

Kumagai *et al.* reported that Chk1 is present in replication competent *Xenopus* egg extracts at a relatively low concentration of 40 nM (2 ng/μl) and that Chk1 overexpression delays mitotic entry. This observation suggested that XChk1 concentration could also be already optimal for DNA replication in the *Xenopus in vitro* system and that overexpression of Chk1 would actually inhibit DNA replication in the absence of external stress. In order to test this hypothesis we

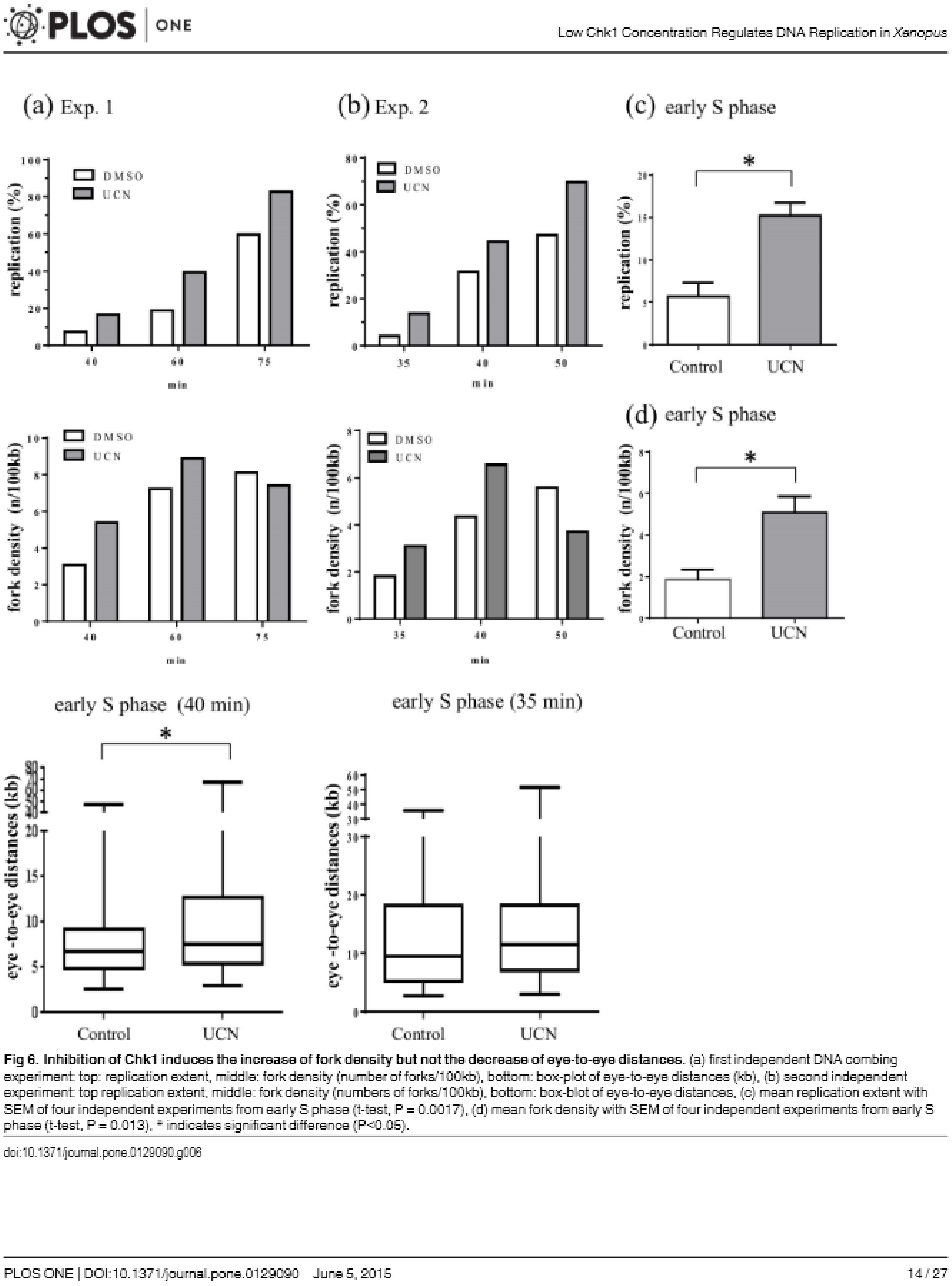

**Fig 6. Inhibition of Chk1 induces the increase of fork density but not the decrease of eye-to-eye distances.** (a) first independent DNA combing experiment: top: replication extent, middle: fork density (number of forks/100kb), bottom: box-plot of eye-to-eye distances (kb), (b) second independent experiment: top replication extent, middle: fork density (numbers of forks/100kb), bottom: box-blot of eye-to-eye distances, (c) mean replication extent with SEM of four independent experiments from early S phase (t-test, P = 0.0017), (d) mean fork density with SEM of four independent experiments from early S phase (t-test, P = 0.013), * indicates significant difference (P<0.05).

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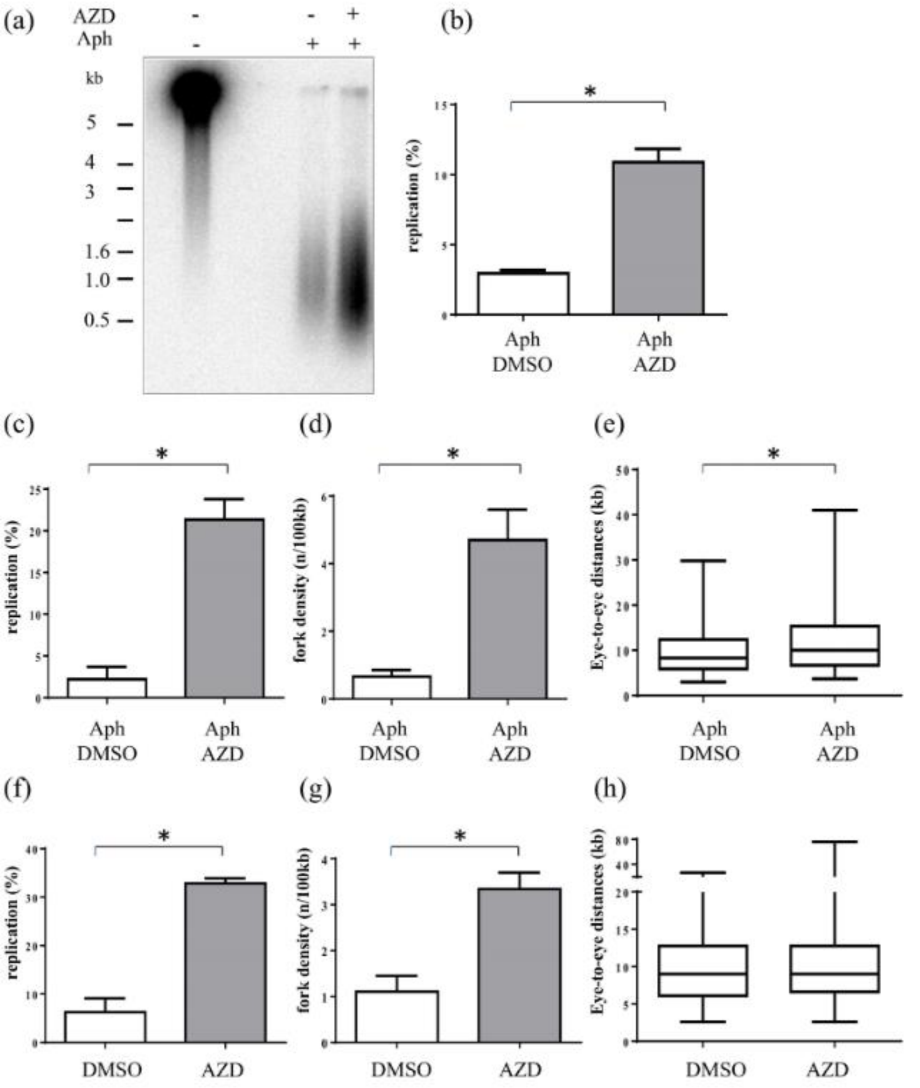
(a)
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**Fig 7. Inhibition of Chk1 activity by AZD-7762 increases DNA synthesis and fork density in the presence and absence of aphidicolin.** (a) Sperm nuclei were added to egg extracts in the presence of [α-$^{32}$P]-dATP with or without 0.5 μM AZD-7762 and aphidicolin (7.5 μg/ml) and nascent DNA strands synthesized after 90 min were analyzed by alkaline gel electrophoresis, (b) Quantification of (a) and another independent experiment, mean replication with SEM (t-test, $P = 0.013$), (c) sperm nuclei were added to egg extracts in the presence of biotin-dUTP, aphidicolin with or without AZD-7762 for 105 min and DNA combing analysis was performed, mean replication extent with SEM of two independent experiments (t-test, $P = 0.021$), (d) fork density (t-test, $P = 0.048$), (e) eye-to-eye distances (Mann-Whitney, $P = 0.045$), (f) sperm nuclei were added to egg extracts in the presence of biotin-dUTP, with or without AZD-7762 and DNA combing analysis was performed, mean replication extent with SEM of two independent experiments at early S phase (t-test, $P = 0.013$), (g) fork density (t-test, $P = 0.046$), (h) eye-to-eye distances (Mann-Whitney, $P = 0.434$), * significantly different ($P < 0.05$).

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produced active recombinant XChk1 (S4 Fig, S5 Fig and S6 Fig), added 120 nM of XChk1 to frozen egg extracts and replicate sperm nuclei in the presence of [α-$^{32}$P]-dATP. The reactions were stopped at indicated time points and DNA was purified. Quantification of DNA synthesis after DNA gel electrophoresis showed a decrease of DNA replication when XChk1 was overexpressed (Fig 8A, S7 Fig). No difference in the timely entry into S phase was detected upon Chk1 overexpression (data not shown). In order to find out how Chk1 addition inhibits DNA replication we performed DNA combing experiments. Sperm nuclei were incubated for 45 min in egg extract the presence of biotin-dUTP and in the absence or presence of 120 nM recombinant XChk1 (Fig 8B). Consistent with the quantification by gel electrophoresis, DNA combing analysis showed that XChk1 addition decreased the percentage of DNA replication in two independent experiments (Fig 8B and 8C) in mid S phase. Next, we analyzed the fork density and found that XChk1 addition decreased more than twofold the number of active forks (Fig 8D). We compared the local eye-to-eye distances in the absence and presence of XChk1 and found a small significant increase upon Chk1 overexpression in one experiment (Fig 8E, median = 8.3 kb control versus 10.1 kb Chk1 addition, Mann Whitney, P-value = 0.0002), but not for a second experiment (S8 Fig) in early S phase. No significant decrease in the median eye length was detected (data not shown) which shows that Chk1 addition did not inhibit elongation. We conclude that Chk1 overexpression in *Xenopus* inhibits DNA replication mainly by inhibition of not activated replication clusters and to a much lesser extent of single origins in already activated clusters.

Next, we wanted to test by which mechanism XChk1 overexpression inhibits replication initiation. Western analysis of chromatin isolated during S phase from replication reactions in the absence and presence of recombinant XChk1 shows that addition of recombinant XChk1 resulted in a threefold increase of chromatin bound XChk1 (Fig 9A). Absolute quantification of chromatin-bound XChk1 using recombinant XChk1 gave an estimate of 700 fg per nucleus before Chk1 addition (data not shown) which corresponds to one Chk1 molecule per replication fork if origins are spaced in average 10kb as reported [33]. Further on, we could detect both an increase of phosphorylated Chk1 and the inhibitory P-Y15-Cdk2 protein levels in nuclei upon Chk1 overexpression (Fig 9B). In order to confirm that Cdk2 activity is actually decreased we performed XCdk2 immuno-precipitations of nuclear extracts prepared from replication reactions in the absence and presence of recombinant XChk1 and tested kinase activity in Cdk2 or mock immunoprecipitations (IP) (Fig 9C) in H1 histone kinase assays (Fig 9D and 9E). The experiment was repeated twice. We found that upon Chk1 addition mean kinase activity of Cdk2-IPs decreased to 61% of the control in a specific manner. We conclude that overexpression of Chk1 in *Xenopus* egg extracts leads to replication cluster inhibition by inhibiting Cdk2 kinase activity.

## Numerical simulation of checkpoint action on origin activation

In order to better understand the role of Chk1 in the replication program we conducted numerical simulations to recapitulate origin activation including checkpoint pathways, and fitted

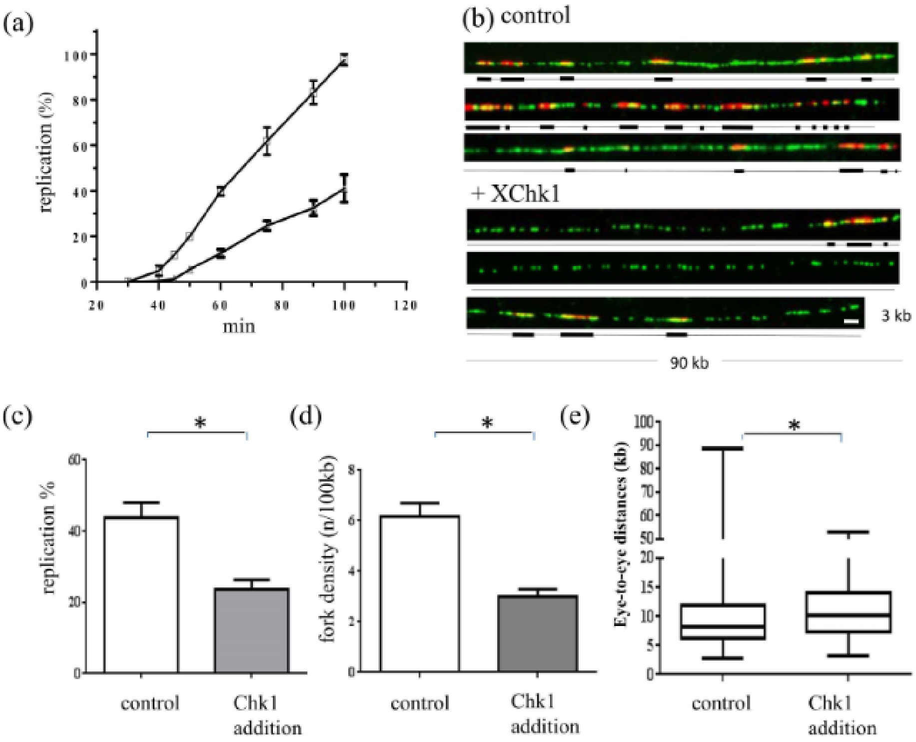

**Fig 8. Chk1 overexpression inhibits DNA replication by inhibiting replication cluster activation.** (a) Sperm nuclei were added to frozen egg extracts in the presence of [α-$^{32}$P]-dATP for indicated times in the absence (squares) or presence of 120 nM (triangles) recombinant XChk1, DNA was isolated, separated by agarose gel electrophoresis and quantified, mean with SEM of two independent experiments (t-tests: P values <0.05). For combing experiments sperm nuclei were added to egg extracts in the presence of Biotin-dUTP for 45 min in the presence or absence of recombinant XChk1 (120 nM). (b) Representative combed DNA fibers, in the absence (above) or presence (below) of supplemented XChk1 (merge: green, whole DNA label; red, biotin labeled replication eyes), (c) Mean replication extent of two independent experiments with SEM (t-test: P = 0.029), 45 min, (d) Fork density (number of forks/100kb) mean with SEM (t-test: P = 0.037), (e) Box-plot of eye-to-eye distances, 35 min (Mann Whitney, P-value = 0.0002) (kb). Scale bar 3 kb, *significantly different (P < 0.05).

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the results to the experimental DNA combing data. Small replication eyes (1 to 3 kb) from DNA combing data obtained in the presence and absence of Chk1 activity during an unchallenged S phase were used (Figs 5 and 6A), and initiation frequency (*I(f)*) (see Materials and Methods and as described previously [35]) was plotted as a function of replication extent per fiber (Fig 10A, circles). *I(f)* first increased until a plateau was reached after about 45% of replication extent. This plateau was abolished (or maybe reached later) when the checkpoint function was compromised (Fig 10B), as expected from the higher fork density observed in the absence of Chk1 activity. In order to propose a model of replication control that could

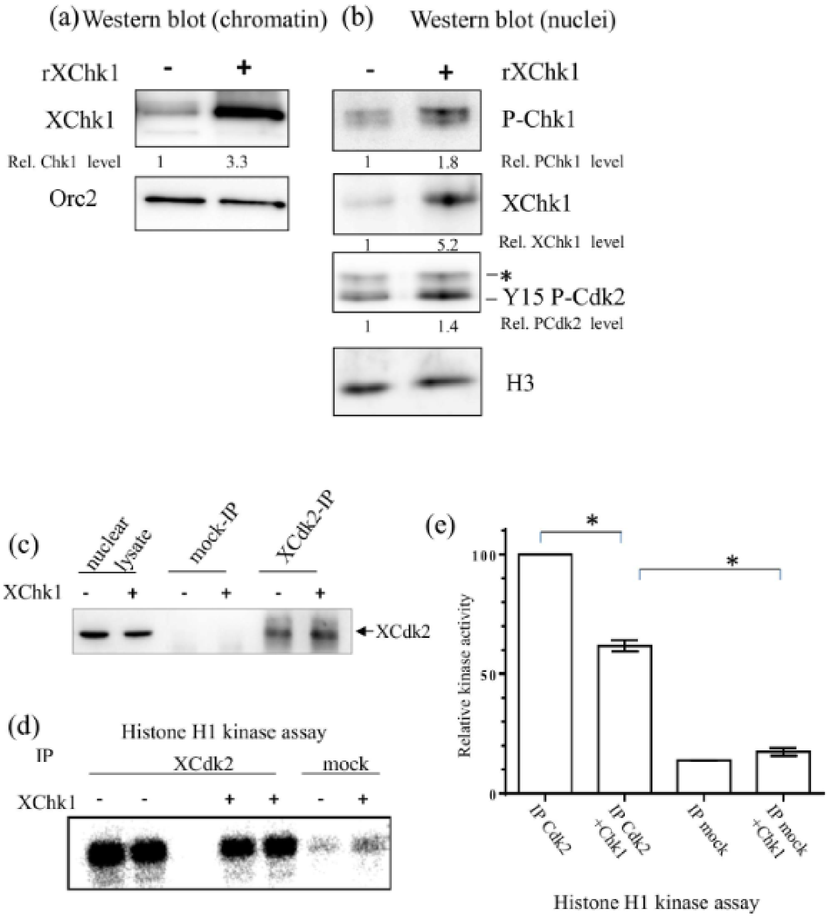

**Fig 9. Chk1 overexpression decreases Cdk2 activity.** (a) Isolated chromatin and (b) sperm nuclei from reactions in Fig 8B were subjected to gel electrophoresis and western blot analysis using indicated antibodies. Relative protein levels normalized to the loading control Orc2 or histone H3 are indicated below. (c) Western blot for Cdk2 of nuclear lysate (input), mock and Cdk2 IP, (d) Histone H1 kinase assay of cdk2 or mock immunoprecipitates (IP) of nuclear lysates in the absence or presence of recombinant Chk1, duplicates for Cdk2 IPs (e) Quantification of relative histone H1 kinase activity in (d) and two other independent experiments, means of three independent experiments with SEM (t-test: P values < 0.005), * significant difference (P < 0.05).

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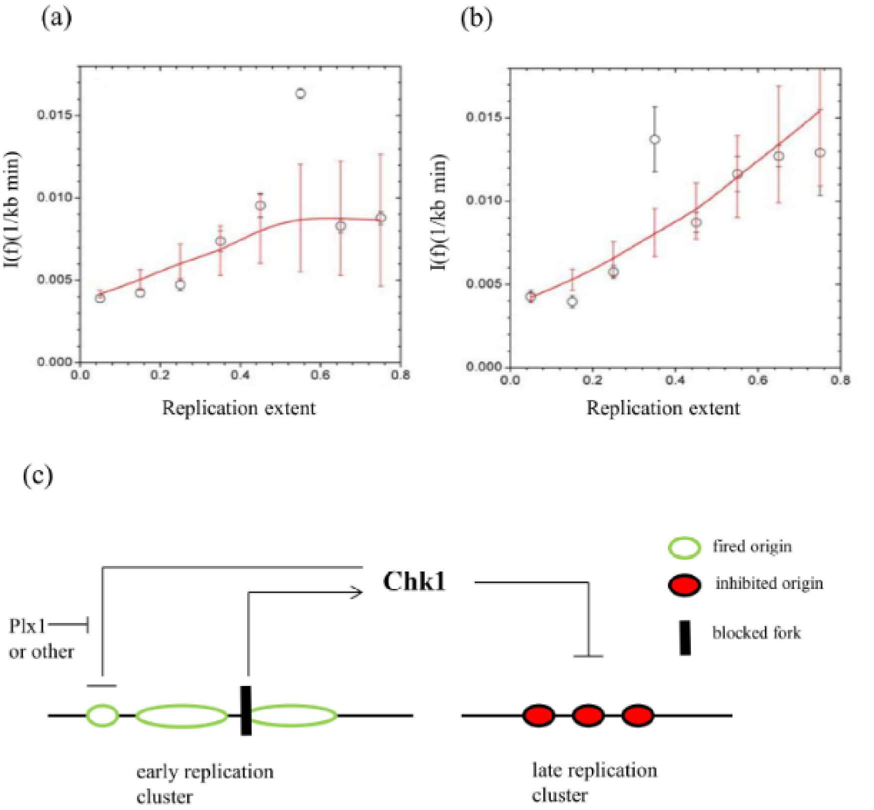

**Fig 10. Numerical simulation of initiation frequencies including Chk1 action.** Initiation frequency (*I(f)*) increases during early S phase to a higher value after Chk1 inhibition. Experimental (circles) and theoretical, fitted (line) *I(f)* values. (a) in presence of Chk1 activity, (b) in absence of Chk1 activity, see main text for more details. (c) Model of spatio-temporal regulation of origin activation in *Xenopus*, see discussion for details.

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faithfully reproduce the behavior of $I(f)$ in the presence or absence of UCN-01, we built our simulations on our previous model of DNA replication [35] (see Materials and Methods). First, we considered that an encounter between a limiting replication factor and a replication origin will trigger firing with a probability $P$. Second, we added the checkpoint action of Chk1, which inhibits potential replication origins with a temporal probability rate $k_{Chk1}$. We ran a series of simulations using only these two control variables, but were unable to extract an $I(f)$ profile similar to the one extracted from the experimental data. It was previously shown that *Xenopus* Polo-like kinase 1 (Plx1) suppresses the inhibitory action of Chk1 in the presence of aphidicolin [48]. The authors proposed a model in which Plx1 inhibits Chk1 action on replication origins in the neighbourhood of a stalled replication fork. Therefore, we introduced a third, local pathway that blocks the inhibitory action of Chk1 protein over the potential replication origins and assumed that this pathway is also active during an unchallenged S phase. We considered that if a Chk1-inhibited potential replication origin is at a distance *d* of a replication fork, it would have a probability $k_{polo}$ of recovering its ability to fire. Using this third variable, we found a better match between the *I(f)* extracted from the numerical simulation and the experimental data. We obtained the best fit of *I(f)* with experimental data in the absence of UCN-01 for a probability of inhibition of Chk1 $k_{Chk1} = 0.99$ ($P < 10^{-4}$, $\chi^2 = 1.03$) (Fig 10A, plotted line). This high probability of origin inhibition by Chk1 probably illustrates that regulating the initiation rate by the fork density during a normal, unchallenged S phase is essential. Note that this is also consistent with the observed quantity of Chk1 recruitment onto chromatin (one Chk1 molecule/fork, see above). In the presence of UCN-01, however, we obtained the best fit of *I(f)* with experimental data for a probability of inhibition of Chk1 $k_{Chk1} = 0.3$ (Fig 10B, plotted line). This observation suggests that UCN-01 does not completely inhibit Chk1. The initiation rate increases, but is limited by the overall initiation probability and the partial loss of the correlation between fork density and initiation rate. Using combing data from a second independent experiment we obtained very similar results (data not shown). We conclude that to fit our experimental DNA combing data with numerical simulations, we need a combination of two independent means of controlling origin activation: a limiting replication factor and a global checkpoint response but with local checkpoint regulation. These two controls can explain the observed initiation frequencies during S phase in *Xenopus*.

## Discussion

We investigated the role of the checkpoint kinase Chk1 in the replication checkpoint and the spatio-temporal regulation of S phase in the *Xenopus in vitro* system. First, we report that when replication stress is induced by aphidicolin, Chk1 controls chromosomal origin firing in *Xenopus*, consistent with studies in mammalian cells. Second, our experiments demonstrated that during normal, unchallenged S phase and challenged S phase, Chk1 inhibits origin firing at the level of replication clusters, but not within active clusters. Third, we provide the first evidence that modest Chk1 overexpression inhibits DNA replication by inhibiting origin firing in the absence of external replication stress in higher eukaryotes illustrating that Chk1 levels are tightly regulated during normal, unchallenged S phase in higher eukaryotes. Finally, based on fitted mathematical simulations we propose a refined model for spatio-temporal replication program in the *Xenopus* model system showing how Chk1 inhibits late clusters whereas origin firing in early clusters is prohibited by Chk1 inhibition close to activated forks.

### Regulation of replication origin and cluster activation by Chk1 in *Xenopus*

Rad53 inactivation leads to the firing of late replication origins in *S. cerevisiae* [11], and Chk1 inhibition by UCN-01 in mammalian cells to the firing of additional origins [49] in the presence of DNA damage or replication stress. Consistent with these results, we found that more replication origins fire in *Xenopus* egg extracts that are replicating nuclei treated with aphidicolin in the absence of Chk1 activity, by inhibiting with two different Chk1 inhibitors or Chk1 depletion. In the absence of induced stress our DNA combing analysis showed that, in the absence of Chk1 activity (Chk1 inhibition by UCN-01 and AZD-7762), 2–3 times more origins fire early in S phase. In an earlier study we also observed an increase of global fork density following ATR inhibition and that *Xenopus* replication origins are organized in clusters that fire at different times during S phase [20]. Combing experiments demonstrated that Chk1 inhibits origins mainly in non-activated replication clusters, but not in already active replication clusters. This differential regulation by the replication checkpoint efficiently inhibits S phase progression, but allows replication of a region with a stalled fork from neighboring origins within an already activated replication cluster.

Several replication clusters are probably present in each cytologically visible replication focus [2]. We previously showed that replication foci number increases early in S phase and decreases late in S phase in *Xenopus* [34]. We tried to investigate foci number in control and UCN-01 treated samples, but single replication foci could not be resolved under these experimental conditions. Upon Chk1 inhibition by UCN-01 or Chk1 depletion, changes in foci patterns or number were detected in chicken DT40 cells during a normal, unchallenged S phase [25] and upon replication stress in human cells [49,50], which illustrates that Chk1 also regulates replication at the level of large chromatin domains. Replication cluster activation has not been addressed in these studies and its organisation is clearly different. Further on, foci activation was studied in the presence of replication inhibitors only. How replication clusters and the larger domains are established and maintained during the cell cycle is still not clear. In *Xenopus*, it probably involves tethering replicons together with different factors such as topoisomerase II [40,51], which might restrict the access of rate limiting initiation factors to later replicating replication clusters. In yeast, forkhead transcription factors Fox2/3 might be needed to tether early origins together [52].

All S phase checkpoint pathways are functional in the *Xenopus in vitro* system, which mimics early developmental stages. However, pre-MBT *Xenopus* embryos exposed to high and prolonged concentrations of aphidicolin continued to divide despite incomplete replication [53], which illustrates the absence of the ATR/Chk1 dependent S-M checkpoint *in vivo*. Therefore it has been proposed that checkpoint activation occurs at the MBT when a critical signal threshold is reached [54]. However, the replication checkpoint is active in the *in vitro* system at a concentration of 1000 nuclei/μl, corresponding to nuclei to cytosolic ratio (N/C ratio) just before the MBT. To confirm that replication can also be activated at low N/C ratios, we reduced the nuclear concentration in the *in vitro* system 10-fold. Even at these very low N/C ratios, the replication checkpoint is activated, as we observed both Chk1 phosphorylation and an increase in fork density, though the checkpoint seems less active at low N/C ratios than at high N/C ratios. In addition, we also detected Chk1 phosphorylation in nuclei from pre-MBT embryos treated with aphidicolin for one cell cycle. These results clearly show that the replication checkpoint can be activated by low N/C ratios *in vitro* and *in vivo*, which challenges the idea that a critical concentration of stalled forks at the MBT is needed to activate ATR and Chk1. Rather than a threshold, we propose that the replication checkpoint shows a gradual response to stalled forks, which is also consistent with its activation during normal, unchallenged S phase [20,21] (our results in this study). These stalled or slowed down forks during unchallenged S phase could arise due to spontaneous DNA damage, a decrease in the optimal concentration of some replication factors or in regions which are difficult to replicate.

A former study did not detect an effect of Chk1 depletion on chromosomal DNA replication in the presence of aphidicolin [23] using an anti-human Chk1 antibody. We speculate that our use of an anti-*Xenopus* antibody or the fact that we used a higher aphidicolin concentration which, as we show, increased the effect of Chk1 inhibition could explain the discrepancy between the studies. While our study was under submission a very recent study showed that inhibition or depletion of Chk1 increases the replication extent of DNA replication during normal S phase in *Xenopus* egg extracts, which is in agreement with our results [55]. However, no combing experiments were performed to show origin and cluster activation upon Chk1 inhibition or depletion.

## Tight Chk1 levels regulate origin activation during normal S phase

In this study we provide the first evidence that modest Chk1 overexpression inhibits DNA replication by inhibiting origin firing in the absence of external replication stress in higher eukaryotes. Our experimental observations are further confirmed by our numerical model which shows that during normal S phase the probability of origin inhibition by Chk1 needs to be already high, in order to fit our experimental combing data. Therefore our results show that the Chk1 activity is negatively rate limiting for DNA replication in the *Xenopus in vitro* system because additional Chk1 inhibits DNA replication. Together with the depletion experiments our study therefore demonstrates that nuclear Chk1 activity needs to be tightly regulated by the cell for proper S phase progression. Loss of one copy of CHK1 causes spontaneous cell death even in the absence of external stress in mammalian cells which the authors interpreted as limiting endogenous Chk1 levels [28]. A recent study reported that expression of one extra-allele of Chk1 in transgenic mice protects against replication stress [56]. The viability of these cells was increased and was associated with a decrease of double strand breaks when transgenic cells were treated with hydroxyurea and aphidicolin. No effect of Chk1 overexpression on BrdU incorporation analyzed by FACS was detected. In *S. cerevisiae*, overexpression of a hyperactive allele of the RAD53, the functional CHK1 homologue, is lethal [57]. Our DNA combing experiments show that even in the absence of replication stress three-fold overexpression of Chk1 changes the spatio-temporal program by inhibiting late firing replication clusters mainly. These different effects of Chk1 overexpression could be due to differences in the experimental systems, different levels of overexpression and our more sensitive methods to quantify DNA replication. In mammalian culture cells 20–50% of cellular Chk1 is bound to chromatin [46,58]. In our system, absolute quantification of chromatin bound XChk1 during replication gave an estimate of one chromatin bound Chk1 molecule per active replication fork if one considers that origins are spaced in average 10 kb apart. Upon XChk1 overexpression the nuclear and chromatin bound fraction of Chk1 is increased which leads to the inhibition of DNA replication by downregulation of Cdk2 kinase activity probably via Cdc25A. How exactly Chk1 is recruited to chromatin and the replication fork is not well understood but Chk1 is associated with DNA polymerase alpha [14,59] and the GINS-MCM-Cdc45 helicase [60]. One interesting question is how Chk1 overexpression affects DNA replication if one considers that the amount of Chk1 found on chromatin during normal S phase matches in theory with the presence of one Chk1 molecule per replication fork. A simple explanation would be that supplying additional Chk1 increases the probability of chromatin binding of Chk1 and thereby increasing Chk1 activity on chromatin. Another explanation could be linked to the fact that there are around 20 fold more MCM complexes than ORC complexes bound to the DNA, also known as the MCM paradox [61] which has been shown to be important in dormant origin firing [62]. It is tempting to speculate that the additional Chk1 is recruited to chromatin via excess MCM complexes which then would affect the replication program.

## A model for checkpoint dependent control of DNA replication

Our finding that Chk1 mainly regulates origins distal to already activated replication clusters suggests that mechanisms exist that prevent Chk1 from inhibiting potential origins in clusters that already contain activated origins. In *Xenopus*, depletion of Polo-like kinase 1 leads to an increase in Chk1 phosphorylation and a decrease in both Cdk2 activity and Cdc45 loading in response to replication stress [48]. Costanzo and co-workers proposed a model suggesting that Plx1 plays a role in checkpoint adaptation by inhibiting checkpoint action close to stalled forks but they did not analyze replication origin and cluster activation in detail by DNA combing.

Different scenarios for local/global regulation of origin firing have been suggested for cells under replication stress or DNA damage [63], but that could also apply to a normal S phase.

In a previous study, based on DNA combing experiments with intact checkpoint pathways, we proposed a model for the control of DNA replication in *Xenopus* [35]. This model combines time-dependent changes in the availability of a replication factor and a fork-density dependent affinity of this factor for potential origins to explain the observed increase in the initiation rate and fork density in our system. But the inhibitory regulation by the replication checkpoint had not been included. Here, we refine this model by adding a Chk1-dependent inhibition of origins combined with local repression of this inhibition in the proximity of previously fired origins (Fig 10C). This local repression could be mediated by Plx1 and/or by unknown factors. The Chk1-dependent control together with a control implying a limiting replication factor best matches the experimental initiation frequencies ($I(f)$). This differential regulation of the checkpoint pathway would allow the cell to replicate a replication cluster with a stalled fork, which might not be able to resume from neighbor origins. But globally replication is slowed down until the replication stress disappears. It would be interesting to test whether these pathways could also explain initiation rates in mammalian systems.

In conclusion, our study demonstrates that both a highly active Chk1-dependent replication checkpoint and rate limiting initiation factors are needed for the sequential activation of replication clusters in *Xenopus* egg extracts, which explains the essential role of Chk1 in regulating origin firing and genome stability during S phase. Thus, this basal replication checkpoint activity is an efficient way for cells to adapt the optimal replication fork density to the concentration of replication factors during S phase.

## Supporting Information

**S1 Fig. Eye-to-eye distance distribution does not significantly change upon Chk1 inhibition by UCN in the presence of aphidicolin.** Box-plot of eye-to-eye distances (ETED), second independent experiment, control DMSO, UCN addition, 90 min Aphidicolin (Mann-Whitney Test, P = 0.3702).
(PDF)

**S2 Fig. Phospho-Chk1 is not bound to chromatin.** Sperm nuclei were added to egg extracts for the indicated times, nuclear extracts or chromatin fractions were subjected to gel electrophoresis and western blot analysis using antibodies against anti P-Chk1, XORC2.
(PDF)

**S3 Fig. Time course of replication upon AZD addition.** Sperm nuclei were added to egg extracts in the presence of [$\alpha^{32}$P]-dATP, replication was stopped at indicated times, purified DNA was subjected to gel alkaline electrophoresis and replication quantified on a phosphorimager with 90 min AZD time point as 100%, mean with SEM of two independent experiments.
(PDF)

**S4 Fig. Production of recombinant XChk1.** Recombinant XChk1 was purified from Baculovirus-infected insect cells His-tagged XChk1 after purification with Nickel-Sepharose loaded on a 10% polyacrylamide gel and Coomassie stained. Lanes: 1. Protein Marker, 2. 10 µl XChk1-6His (0.2mg/ml).
(PDF)

**S5 Fig. Production of anti-XChk1 antibody.** Anti-XChk1 antibody made against full length XChk11 recognizes recombinant XChk1 and endogenous XChk1, Lanes: 1. Recombinant

6His-XChk1, 2. S phase Xenopus egg extract,* marks non-specific band.
(PDF)

**S6 Fig. Chk1 kinase assay.** CHKtide kinase assay, recombinant Chk1 was incubated with or without a specific Chk1 substrate CHKtide in the presence of [$\gamma^{32}$P]-ATP for 30 min at 30°C, separated on 15% SDS polyacrylamide gel, dried and analyzed on a phosphoimager.
(PDF)

**S7 Fig. Effect of Chk1 overexpression on DNA replication.** Sperm nuclei were replicated in egg extract in the presence of$\alpha^{32}$P]-dATP, replication was stopped at indicated times, purified DNA was subjected to agarose electrophoresis.
(PDF)

**S8 Fig. Eye-to-eye distance distribution of second independent DNA combing experiment in absence and presence of recombinant Chk1, 45 min (Mann-Whitney, P = 0.296).**
(PDF)

**S1 File. Raw DNA combing data from Figs 3, 4, 6, 7 and 8.**
(ZIP)

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Raw DNA combing data from Fig 3, Fig 4, Fig 6, Fig 7, Fig 8 can be found in S1 File.

## Author Contributions

Conceived and designed the experiments: KM AG. Performed the experiments: KM AG MP PL JMW HN PP XG PB. Analyzed the data: KM AG MP XG PB. Wrote the paper: KM AG.

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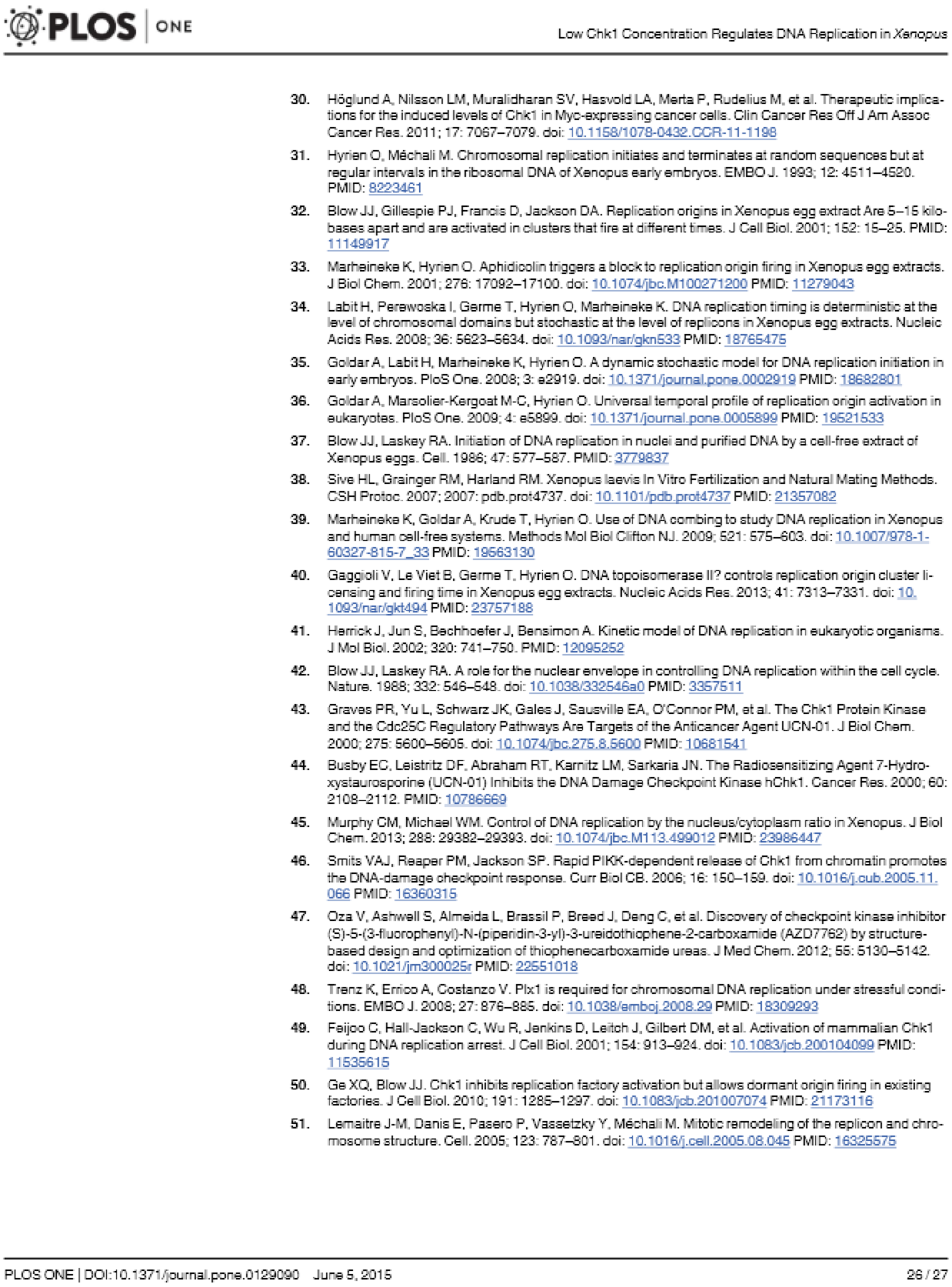

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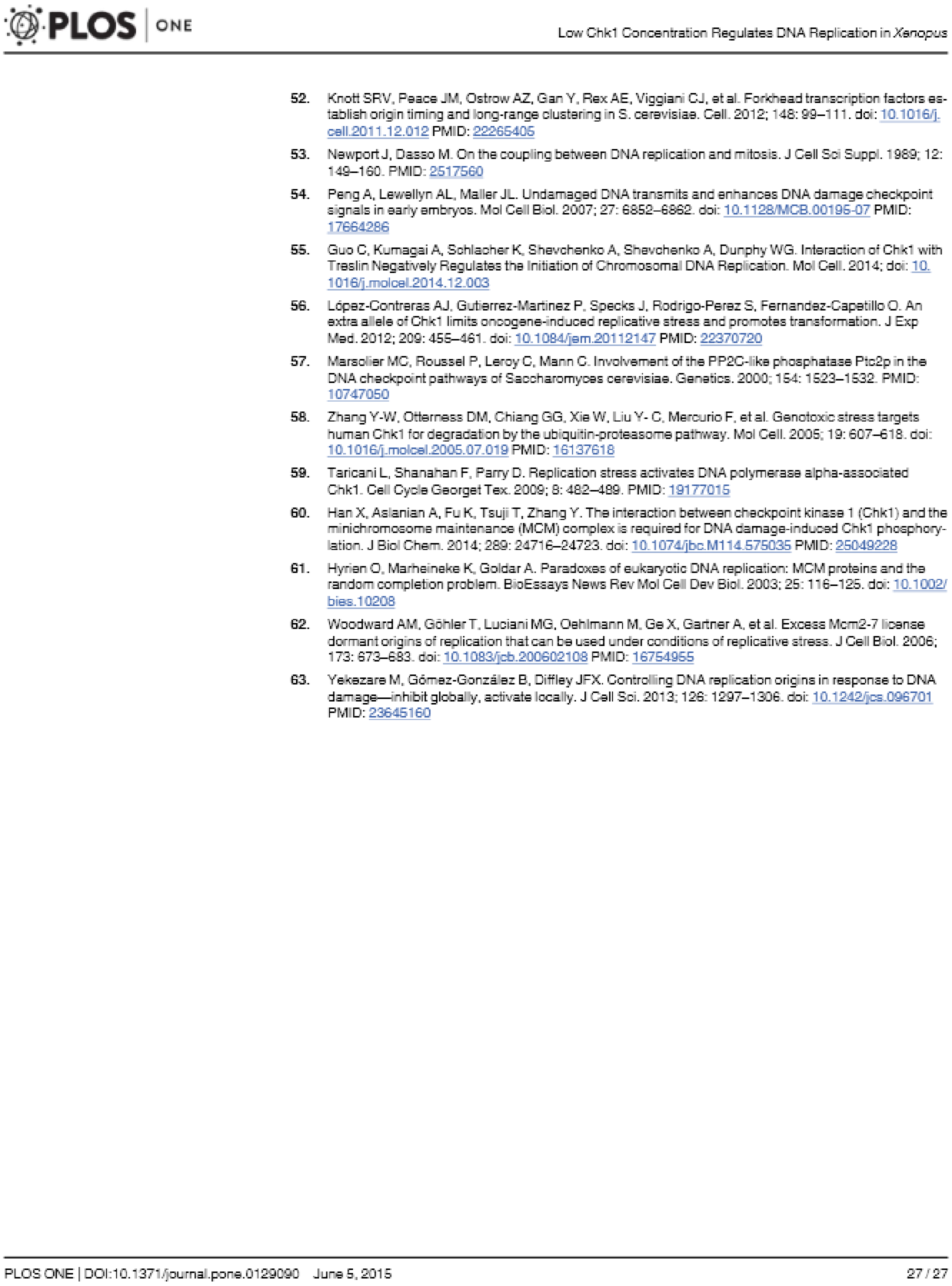

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