Dissertationes Biologicae Universitatis Tartuensis
237

MARKO LÕOKE

Studies on DNA replication initiation in *Saccharomyces cerevisiae*

DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
237

DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
237

MARKO LÕOKE

Studies on DNA replication initiation in *Saccharomyces cerevisiae*

UNIVERSITY OF TARTU
PRESS
1632

Institute of Molecular and Cell Biology, University of Tartu, Estonia

Dissertation is accepted for the commencement of the degree of Doctor of Philosophy (in Cell Biology) on 07.05.2013 by the Council of the Institute of Molecular and Cell Biology, University of Tartu

Supervisor: Arnold Kristjuhan, PhD
Institute of Molecular and Cell Biology
University of Tartu
Estonia

Opponent: John F.X. Diffley, PhD
Cancer Research UK
London Research Institute, Clare Hall Laboratories
United Kingdom

Commencement: Room No. 105, 23B Riia St. Tartu, on May 7th, 2013, at 12:00.

Publication of this thesis is granted by the Institute of Molecular and Cell Biology, University of Tartu and by the Graduate School in Biomedicine and Biotechnology created under the auspices of European Social Fund.

ISSN 1024–6479
ISBN 978–9949–32–260–2 (print)
ISBN 978–9949–32–261–9 (pdf)

University of Tartu Press
www.tyk.ee
Order No. 117

TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The current thesis is based on the following original publications.

Ref. I Lõoke M, Kristjuhan K, Kristjuhan A (2011) Extraction of genomic DNA from yeasts for PCR-based applications. BioTechniques, 50(5):325–8.

Ref. II Lõoke M, Reimand J, Sedman T, Sedman J, Järvinen L, Värv S, Peil K, Kristjuhan K, Vilo J, Kristjuhan A (2010) Relicensing of transcriptionally inactivated replication origins in budding yeast. The Journal of Biological Chemistry, 285:40004–11.

Ref. III Lõoke M, Kristjuhan K, Värv S, Kristjuhan A (2013) Chromatin-dependent and -independent regulation of DNA replication origin activation in budding yeast. EMBO reports, 14(2):191–8.

Author contribution: In all presented articles I participated in experimental design, performed experiments, analyzed data and co-wrote the manuscripts.

LIST OF ABBREVIATIONS

AAA+	ATPases Associated with diverse cellular Activities
Abf1	ARS-Binding Factor 1
ACS	ARS Consensus Sequence
ARS	Autonomously Replicating Sequence
ATP	Adenosine TriPhosphate
Cdc6, 7, 45	Cell Division Cycle (6, 7, 45)
CDK	Cyclin-Dependent Kinase
Cdt1	Chromatin licensing and DNA replication factor 1
Clb2, 5, 6	CycLin B2, 5, 6 (B-type cyclin 2, 5, 6)
CMG	Cdc45/MCM2-7/GINS complex
CUT	Cryptic Unstable Transcript
Ddc2	DNA Damage Checkpoint protein 2
DDK	Dbf4-Dependent Kinase (Cdc7-Dbf4 protein kinase)
Dpb11	DNA polymerase B 11
Fkh1, 2	ForKhead Homolog (1, 2)
Gcn5	General Control Nonderepressible 5
GINS	Complex of Sld5, Psf1, Psf2, and Psf3 (Go - five, Ichi - one, Nii - two, and San - three in Japanese)
HML	Hidden Mat Left
HMR	Hidden Mat Right
Mec1	Mitosis Entry Checkpoint 1
Mcm2-7	MiniChromosome Maintenance 2-7
Mrc1	Mediator of the Replication Checkpoint 1
NFR	Nucleosome Free Region
ORC	Origin Recognition Complex
PCNA	Proliferating Cell Nuclear Antigen
Pfh1	PiF1 Helicase homolog 1
Pre-RC	Pre-Replicative Complex
Psf1, 2, 3	Partner of Sld Five (1, 2, 3)

Rad53	RADiation sensitive 53
RNAPII	RNA Polymerase II
RPA	Replication Protein A
Rpd3	Reduced Potassium Dependency 3
SAGE	Serial Analysis of Gene Expression
Sen1	Splicing ENdonuclease 1
Sin3	Switch INdependent 3
Sir1, 2, 3, 4	Silent Information Regulator (1, 2, 3, 4)
Sld2, 3, 5, 7	Synthetic Lethality with Dpb11 (2, 3, 5, 7)
SWI4	SWItching deficient 4

INTRODUCTION

Duplication of DNA during the S phase is critical for the accurate transmission of genetic material to daughter cells. Successful progression trough the S phase requires DNA replication to be properly regulated and monitored to ensure that the entire genome is duplicated exactly once, without errors. In order to achieve this, replication has evolved into a tightly regulated process that includes the coordinated action of numerous factors that function in all phases of the cell cycle. This thesis reviews the current understanding of these processes from the formation of replicative complexes in preparation for DNA synthesis to the series of events that culminate in the S phase with their activation. I will review the constitution of the timely and coordinated replication origin activation program and describe the concept of replication licensing, which protects against genomic instability by limiting initiation events to once per cell cycle. Lastly, I will discuss, how chromatin and chromatin dependent processes, such as DNA replication and transcription, manage to successfully coexist and complete their independent tasks.

In the second part of the thesis, results of my practical work are presented. First, a novel yeast DNA extraction protocol is introduced and its potential uses and advantages are discussed. Additionally, results concerning the dynamics of replication origin licensing under transcriptional conditions are outlined. They demonstrate that DNA replication origins can be licensed multiple times *in vivo*, and this ensures the presence of maximal number of functional replication origins in the S phase. And finally the results about the role of chromatin environment in the regulation of replication origin activation are introduced. These findings indicate that the chromatin context indeed is a major influencing force of the temporal dynamics of replication origin activation, but these effects can be bypassed by specific mechanisms.

1. OVERVIEW OF LITERATURE

1.1. Replication initiation

DNA replication is initiated from specific sequences called replication origins. Eukaryotic genomes have many such sites and the synthesis of each chromosome is started from multiple origins. In budding yeast (*Saccharomyces cerevisiae*) replication origins are short DNA regions of a few hundred base pairs in length, referred to as autonomously replicating sequences (ARS) because of their ability to promote autonomous replication of plasmids (Struhl et al., 1979). Dynamic multi-protein complexes bind onto origins throughout the cell cycle and govern where and when replisomes catalyzing DNA synthesis will assemble. Empirically we can divide this process into several steps. In the first stages, replication origin sites where the multi-protein complexes will form have to be selected. Then in G1 phase of the cell cycle, the inactive replication complexes are assembled onto these sites. Finally these complexes are activated in the S phase. Here, in the literature overview, the key events that take place at origins of DNA replication through the cell cycle are outlined. Additionally, the regulatory mechanisms that control origin activation in time and space and ensure that all genomic regions are replicated exactly once within the same cell cycle are discussed.

1.1.1. Recognition of replication origin sites

The first step in budding yeast DNA replication is the selection of replication origin sites from the 12 million bp genome. All budding yeast replication origins share a highly conserved ARS consensus sequence (ACS) or A domain, which is required for the recruitment of the origin recognition complex (ORC) (Bell and Stillman, 1992; Stinchcomb et al., 1979). The ACS consensus sequence consists of an 11 bp motif (T/A)TTTAT(A/G)TTT(T/A), although a functional ACS may contain one or more mismatches to this sequence. More recently, it has been found that three additional conserved nucleotides on each side of the ACS are important, resulting in a 17 bp extended ACS (Theis and Newlon, 1997; Chang et al., 2011).

While obligatory to origin functioning, the ACS alone is not sufficient for selection of ORC binding sites. The yeast genome contains over 12,000 matches to the ACS motif, yet only 400 are functional, indicating that there are additional requirements (Nieduszynski et al., 2006). The additional sequence other than ACS needed for ORC binding is often referred to as the B1 element. Together, the ACS and B1 serve as a bipartite DNA binding site for ORC (Rao and Stillman, 1995; Rowley et al., 1995).

In addition to ORC binding sequence, every origin contains a set of flanking domains that enables it to serve as a site for DNA replication initiation (Walker et al., 1991; Marahrens and Stillman, 1992). Historically, these elements have been

referred to as B and C domains. Regardless of the name, the exact mechanism of enhancement by the flanking regions varies between replication origins and no clear consensus sequence of these elements can be brought out. Some of these well-characterized elements have been shown to be transcription factor binding sites, while others are regions of DNA unwinding (Bielinsky and Gerbi, 1998; Umek and Kowalski, 1988). The role of transcription factors here is thought to involve the recruitment of chromatin remodelers or modifiers that position nucleosomes or otherwise increase accessibility of origins to transacting factors (Li and Herskowitz, 1993; Flanagan and Peterson, 1999; Venditti et al., 1994). Similarly, it has been found that the favorable chromatin modifications can lead to better origin activation (Knott et al., 2009; Vogelauer et al., 2002).

Therefore, whether a particular sequence matching the ACS behaves as an origin may be determined by several contributing factors. These include, the ease of unwinding of DNA in the vicinity of the ACS, the presence of transcription factor binding sites that may act as nucleosome excluding elements, as well as the surrounding chromatin context that is favorable for the binding of replication factors. While no single one of these contributing properties may be essential for origin activity, together these features may determine whether a particular ACS motif can function as a replication origin.

1.1.2. Assembly of pre-replicative complexes

The first steps towards DNA replication are made long before the start of the S phase. Already in the late mitosis and early G1 phase, relevant protein complexes are formed at the replication origin sites, culminating in the formation of pre-replicative complexes (pre-RCs) (Fig. 1). These events are conserved among eukaryotic organisms, and this process results in the loading of the hetero-hexameric Mcm2-7 (MiniChromosome Maintenance 2-7) replicative DNA helicase onto origin DNA. Events needed for Mcm2-7 loading are the binding of ORC to origin DNA and the recruitment of cofactors Cdc6 (Cell Division Cycle 6) and Cdt1 (Chromatin licensing and DNA replication factor 1) to the complex. Furthermore, ATP binding and hydrolysis are essential for pre-RC formation as at least ten of the proteins that participate in pre-RC assembly are members of the AAA+ family of ATP binding proteins (Iyer et al., 2004). Analyses of mutations in the conserved ATP binding motifs of ORC, Cdc6, and all six Mcm2-7 subunits demonstrate that each of these elements is essential *in vivo* (Klemm and Bell, 2001; Schwacha and Bell, 2001; Weinreich et al., 1999). Reconstitution of pre-RC assembly from purified yeast proteins has established that ORC, Cdc6 and Cdt1 are necessary and sufficient to load Mcm2-7 onto origin DNA in a reaction requiring ATP hydrolysis (Evrin et al., 2009; Kawasaki et al., 2006; Remus et al., 2009).

Selecting replication origins involves binding of the ORC to the origin DNA. This complex marks all potential origins, providing spatial control of origin posi-

tioning, and is required for the loading of downstream replication factors. ORC consists of six subunits (Orc1–Orc6) and binds DNA only in its ATP-bound state. Specifically, ATP binding (but not hydrolysis) by the Orc1 subunit is required for origin-specific DNA binding (Bell and Stillman, 1992; Klemm et al., 1997). ORC requires five of its largest subunits (Orc1–Orc5) to recognize DNA, four of which (Orc1, 2, 4, and 5) are in close contact with the origin (Lee and Bell, 1997). Although Orc6 is not required for DNA binding, it remains essential for DNA replication and cell viability (Li and Herskowitz, 1993). ORC binding to DNA is required for the recruitment of the Cdc6 ATPase and Cdt1. Moreover, targeting of ORC to specific chromosomal locations can also be accomplished through its interaction with with Cdc6, which increases the stability of ORC on chromatin and inhibits ORC binding to nonspecific DNA (Mizushima et al., 2000; Harvey and Newport, 2003).

Both Cdc6 and Cdt1 are necessary for the subsequent association of the Mcm2–7 helicases with origin DNA. In budding yeast, Cdt1 and Mcm2–7 form a stable complex and are recruited to origin DNA as a complex. Interactions between Cdt1 and Orc6 are important for this recruitment step (Chen and Bell, 2011). ATP hydrolysis by Cdc6 stimulates the stable loading of the Mcm2–7 complex onto chromatin, which is accompanied by the dissociation of Cdt1 and Cdc6 (Randell et al., 2006). Additionally, ATP hydrolysis by the ORC complex is necessary for the reiterative loading of Mcm2–7 complexes to origins (Bowers et al., 2004). As mentioned, after helicase loading, Cdc6 and Cdt1 are released from the complex whilst ORC and Mcm2-7 are retained on DNA. *In vitro*, ORC can also be removed with high salt buffer washing, but Mcm2-7 is retained on the DNA, which is a hallmark of pre-RC assembly (Bowers et al., 2004; Donovan et al., 1997).

In vitro reconstitution of pre-RC formation using purified budding yeast proteins and electron microscopy have revealed that Mcm2–7 proteins are loaded as double hexamers at replication origins. The two hexamers are arranged in a head-to-head orientation and are connected via their N-terminal rings. Moreover, the Mcm2–7 double hexamers encircle double stranded DNA and are able to slide along it (Remus et al., 2009; Evrin et al., 2009). The ability of Mcm2-7 to slide along DNA is also interesting because it provides a mechanism for multiple double hexamers to be loaded at a single origin. This could help explain the fact that the number of Mcm2-7 hexamers loaded during G1 phase greatly exceeds the number of replication origins used in the subsequent S phase (Donovan et al., 1997; Lei et al., 1996).

Taken together, current results indicate that Mcm2-7 in the pre-RC exists as a stable head-to-head double hexamer encircling double-stranded DNA. ORC, Cdc6 and Cdt1 are required for Mcm2-7 loading, and the process also requires ATP binding and hydrolysis (Fig. 1).

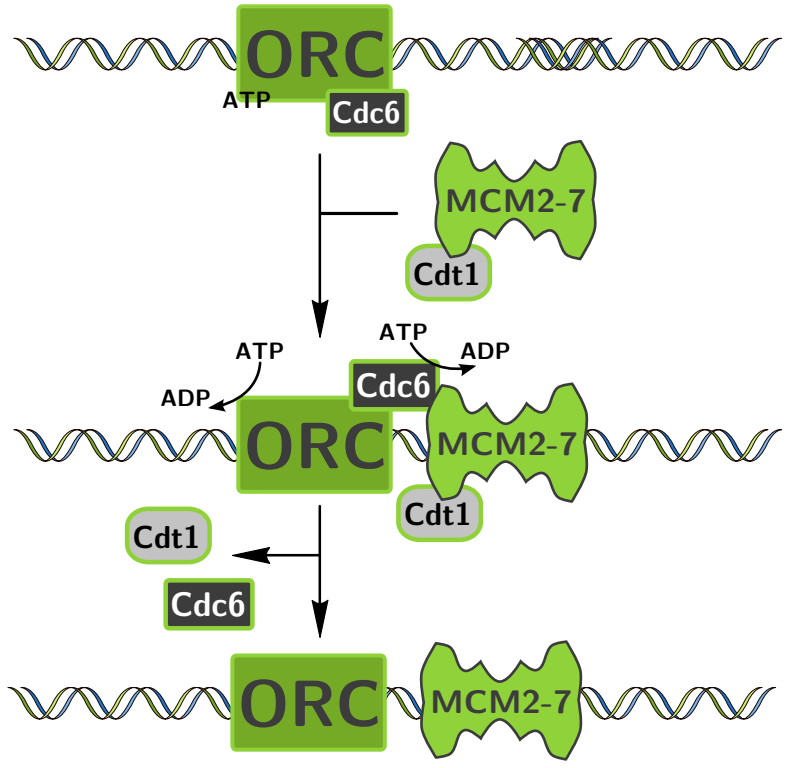

Figure 1. Assembly of pre-replicative complexes. Replication origin sites in budding yeast are marked by stable binding of ORC. Specificity of ORC binding is stabilized by ATP and Cdc6. In G1 phase of the cell cycle, ORC together with Cdc6 and Cdt1 help to load pre-RC complexes. This results in the stable binding of Mcm2-7 double hexamers. In order to achieve this, ORC - Cdc6 subcomplex has to interact with Cdt1 bound Mcm2-7. ATP hydrolysis by ORC and Cdc6 is necessary. The loaded helicase complexes are activated in the S phase where they form the core of the replication initiation process. Cdc6 and Cdt1 are not needed for Mcm2-7 activation.

1.1.3. Activation of the Mcm2-7 helicase complex

Multiple lines of evidence indicate that the Mcm2-7 complex is the engine of the replicative helicase. First, Mcm2-7 travels with replication forks, forming part of the purified replisome complex (Gambus et al., 2006; Aparicio et al., 1997). Second, inactivation of Mcm2-7 subunits during the S phase results in rapid replisome inactivation (Pacek and Walter, 2004; Labib et al., 2000). And third, purified yeast Mcm2-7 complex displays a weak helicase activity under appropriate conditions (Bochman and Schwacha, 2008). However, the key aspect of the Mcm2-7 activation is its association with other proteins that are important for the helicase to function

properly. Current evidence indicates that the active eukaryotic replicative DNA helicase contains at least three components. In addition to hetero-hexameric ATPase Mcm2-7, two cofactors are needed, Cdc45 and the GINS complex (Moyer et al., 2006; Pacek and Walter, 2004; Ilves et al., 2010). The Cdc45, Mcm2-7 and GINS assembly (collectively termed as the CMG complex) is several hundred fold more active as a DNA helicase than the Mcm2-7 alone, providing strong evidence that it is the functional form of eukaryotic DNA replicative helicase (Ilves et al., 2010). The GINS complex (Go, Ichi, Nii, and San; five, one, two, and three in Japanese) itself consists of Sld5, Psf1, Psf2, and Psf3 proteins (Psf - Partner of Sld Five; Sld - Synthetic Lethality with Dpb11), all of which are highly conserved in eukaryotic cells (Takayama et al., 2003). Just like Cdc45 and Mcm2-7, GINS has been shown to be important both for the initial helicase activation and for progression of DNA replication forks (Aparicio et al., 1997; Labib et al., 2000; Kanemaki et al., 2003; Kanemaki and Labib, 2006; Tercero et al., 2000).

The active helicase complexes are assembled only in the S phase, and this process is dependent on the activities of two kinases, CDK (cyclin-dependent kinase) and DDK (Dbf4-dependent kinase) (Zou and Stillman, 2000; Sheu and Stillman, 2006; Masai et al., 2006). Both conserved protein kinases are regulated independently of each other, but by similar mechanisms (Sclafani, 2000; Masai and Arai, 2002). The enzymes are inactive in their monomeric forms and are activated by the binding of unstable activating factors, cyclins for CDK and Dbf4 protein for DDK. Unlike higher eukaryotes, budding yeast has only one cyclin-dependent kinase, Cdk1. At the transition from G1 to the S phase, as CDK and DDK activities increase, numerous factors are recruited to convert the Mcm2-7 double hexamer into an active helicase complex (Fig. 2).

DDK has been shown to act prior to CDK in activating replication origins (Heller et al., 2011). The catalytic subunit of DDK is Cdc7, a serine/threonine kinase activated by the enzyme's regulatory subunit Dbf4. DDK is activated at the onset of the S phase when Dbf4 protein levels are elevated (Ferreira et al., 2000). The primary targets for DDK phosphorylation are the Mcm2-7 components Mcm2, Mcm4, and Mcm6 (Francis et al., 2009). It has been shown that DDK preferentially phosphorylates Mcm2–7 complexes that are stably bound to chromatin (Sheu and Stillman, 2006; Francis et al., 2009; Masai et al., 2006). Moreover, DDK is targeted to replication origins through interactions of Dbf4 with several pre-RC components, including Mcm2, Mcm4, Orc2 and Orc3 (Duncker et al., 2002; Varrin et al., 2005; Sheu and Stillman, 2006). The exact mechanism through which the phosphorylation of Mcm2–7 complex activates the Mcm2–7 helicases remains unknown, but it is likely that this post-translational modification generates a conformational change in Mcm2–7 complex, enabling it to function as a helicase. This model is supported by the finding of a specific mutation in the budding yeast Mcm5 subunit termed *mcm5-bob1*, which can bypass the requirement for Cdc7-

Dbf4 in initiation of DNA replication (Hardy et al., 1997). The *mcm5-bob1* mutant may mimic a conformational change in the Mcm2–7 complex that is normally induced by DDK phosphorylation (Fletcher et al., 2003).

In addition to the DDK induced conformational change in the Mcm2-7 complex, the helicase activation is also dependent on the recruitment of several cofactor proteins. This is directed by CDK dependent phosphorylation events. Unlike DDK, activation and substrate specificity of CDK relies on multiple activation subunits called cyclins. Activation of DNA replication is primarily dependent on the S phase cyclins Clb5 and Clb6 (B-type cyclin 5 and 6). The primary targets of CDK are Sld2 and Sld3 proteins. Sld2 is the preferred substrate for Clb5/Cdk1, but Sld3 is equally well phosphorylated by the mitotic Clb2/Cdk1 pair (Loog and Morgan, 2005). In the absence of Clb5, the short-lived Clb6 is able to substitute for it and activate early replication origins, but the S phase is prolonged due to the failure of late replication origin activation (Jackson et al., 2006; McCune et al., 2008).

Once phosphorylated, the phosphopeptides in Sld2 and Sld3 act as docking sites for Dpb11 (DNA polymerase B 11). Dpb11 is then able to bind to both Sld2 and Sld3 simultaneously and bring the two together. Both Sld2 and Sld3 in turn form complexes with other proteins. Therefore, the Dpb11 dependent scaffolding of Sld2 and Sld3 helps to form a large protein complex (Fig. 2)(Zegerman and Diffley, 2007; Tanaka et al., 2007; Masumoto et al., 2002). The current model suggests that Sld3 interacts with Sld7 and Cdc45. This complex is recruited to origins prior to their activation: in late G1 to early firing origins and during the S phase to late firing origins (Kamimura et al., 2001; Tanaka et al., 2011). Other essential proteins are then recruited to the complex via the Sld3 and Dpb11 interactions. First, the binding of Dpb11 to phosphorylated Sld2 triggers the assembly of a subcomplex composed of Dpb11, Sld2, polymerase ε and GINS. Next, when phosphorylated Sld3 associates with Dpb11, all of the components are brought together (Fig. 2) (Muramatsu et al., 2010). Upon initiation of DNA replication, Sld2, Sld3 and Dpb11 do not participate in the replisome and dissociate, whereas Cdc45, GINS and Mcm2–7 assemble into an active helicase complex (Muramatsu et al., 2010). An additional evolutionarily conserved factor, Mcm10, participates in the conversion of the pre-RC to an active replisome. It is required for the recruitment of DNA polymerase α to origins and for activation of the CMG complex (Kanke et al., 2012; Watase et al., 2012; van Deursen et al., 2012; Ricke and Bielinsky, 2004).

Dynamically, the whole process appears to occur very quickly, as Cdc45 has been shown to associate with origins at the time of replication initiation, and its binding to the origins has been utilized as a reliable marker of origin firing (Pryde et al., 2009; Zou and Stillman, 2000; Vogelauer et al., 2002; Aparicio et al., 1999, 2004; Lõoke et al., 2010).

In summary, activation of the replicative helicase is initiated by the action of two S phase kinases, DDK and CDK. DDK phosphorylates Mcm2-7 proteins resulting in the conformational change in the hexamer needed for Mcm2-7 activation. Concurrently, CDK dependent phosphorylations of Sld2 and Sld3 allows recruitment of the Dpb11 protein which acts as a scaffold to bring the CMG complex together so that DNA can be unwound.

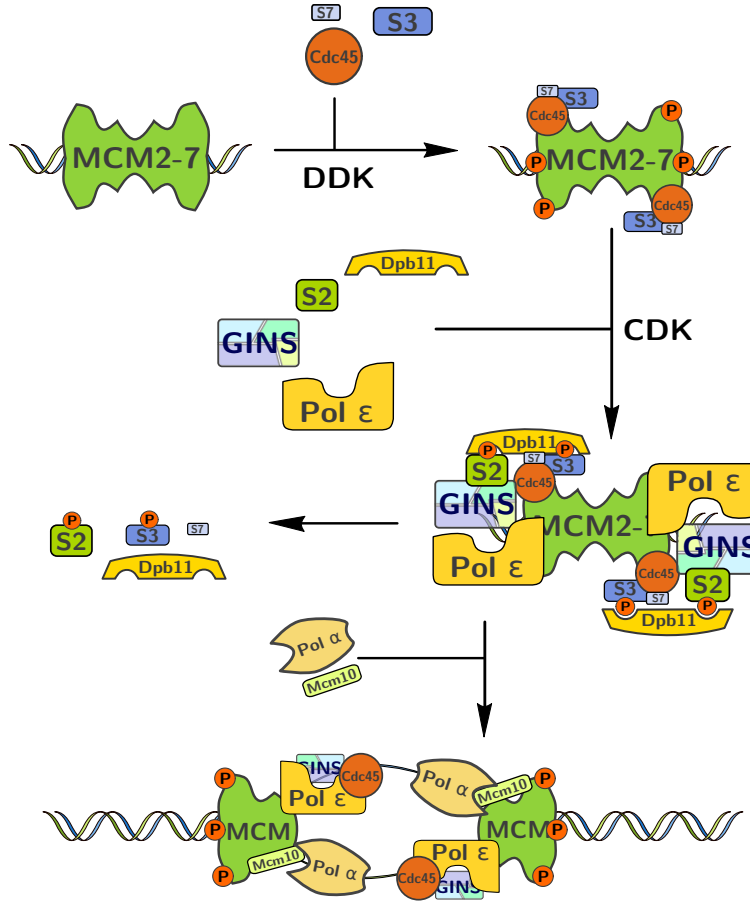

Figure 2. Activation of Mcm2-7 complexes. Abbreviations: S3 - Sld3; S2 - Sld2; S7 - Sld7. Increased activity of CDK and DDK in the S phase triggers the activation of Mcm2-7. First, subunits of Mcm2-7 are phosphorylated by DDK, which induces a conformational change in the complex, enabling the binding of Sld3, Sld7 and Cdc45. CDK dependent phosphorylation of Sld3 and Sld2 creates binding sites for Dpb11 in each protein. Dpb11 binds both of the phosphorylated proteins simultaneously and acts as a scaffold, bringing Sld2 and its binding partners Pol ε and GINS to the complex. Finally, before replication can start, the Mcm10 protein helps to recruit Pol α to the replication complex. Sld2, Sld3, Sld7 and Dpb11 are not needed for the following synthesis of DNA.

1.2. Replication elongation

At the start of replication, the previously formed CMG complex unwinds the DNA duplex and a short region of single-stranded DNA (ssDNA) is exposed. This stretch of DNA is stabilized by the ssDNA binding protein RPA (Replication Protein A), hence RPA associates with origin DNA prior to replication initiation (Tanaka and Nasmyth, 1998; Walter and Newport, 2000). After origin unwinding, Pol α/primase primes DNA synthesis and Pol ε starts the elongation of both strands. Afterwards the loading of PCNA (Proliferating Cell Nuclear Antigen) effects a switch from Pol ε to Pol δ for continuous elongation of the leading strand (Burgers, 2009).

The leading strand is replicated continuously in 5' to 3' direction, but since DNA polymerases only show unilateral processivity, the lagging strand must also be replicated in the same fashion. This is achieved by producing Okazaki fragments which are about 300 bp in length and where continuous priming and Pol ε dependent 5' to 3' elongation takes place. Later the fragments are fused together by DNA ligase (Burgers, 2009). On average, a replication fork replicates ~1-3kb/minute, but fork velocity can vary between different regions of the genome (Conti et al., 2007). Replication forks can pause for some time at certain sites, in particular during replication of long stretches of repeated sequences. This can cause fork collapse and termination of DNA replication at that site (Rothstein et al., 2000). Therefore, an integral part of genome duplication is the surveillance of DNA replication and genome integrity. An evolutionarily conserved signal transduction pathway called intra-S phase checkpoint or DNA damage checkpoint serves as such surveillance mechanism and responds to both DNA damage and replication perturbations.

Activation of this control mechanism starts with generation of long stretches of ssDNA either by the functional uncoupling of replicative helicases and polymerases during fork stalling (Byun et al., 2005; Nedelcheva et al., 2005), processing of DNA double strand breaks (Mimitou and Symington, 2008; Zhu et al., 2008), or by the nucleotide excision repair process (Giannattasio et al., 2004). The generated ssDNA binds RPA and triggers the activation of checkpoint response by Ddc2 (DNA Damage Checkpoint protein 2) dependent recruitment of Mec1 (Mitosis Entry Checkpoint 1) kinase (Rouse and Jackson, 2002; Zou and Elledge, 2003). Mec1 in turn phosphorylates Mrc1 (Mediator of the Replication Checkpoint 1), a mediator that transduces the signal to the effector kinase Rad53 (RADiation sensitive 53), which becomes phosphorylated and activated (Alcasabas et al., 2001). Once activated the S-phase checkpoint response coordinates various aspects of DNA replication, such as firing of new replication origins (Santocanale and Diffley, 1998; Shirahige et al., 1998; Santocanale et al., 1999), stabilization of DNA replication forks (Tercero and Diffley, 2001; Lopes et al., 2001), and resumption of stalled replication forks (Szyjka et al., 2008).

In the event that the stalled replication forks cannot be reactivated, it is important to have additional pre-RCs within the un-replicated region to finish DNA replication. Completion of DNA replication of the whole genome in a timely fashion is thought to be to be crucial, since some evidence suggests lack of a mechanism controlling the completion of DNA replication at the end of the S phase (Torres-Rosell et al., 2007). This demonstrates that the usage of multiple replication origins in a correct and timely fashion is essential for proper cell cycle progression and genome stability.

1.3. Avoiding re-replication

Eukaryotic cells need to duplicate vast amounts of genetic information quickly and accurately before each cell division. To meet this requirement multiple replication origins have to be used. At the same time it is also important to ensure that DNA replication is not only fast, but accurate, and no segment of the chromosome is duplicated more than once. Indeed, re-initiation from even a single origin within the same cell cycle may cause genomic instability. This is avoided by dividing the process into two non-overlapping phases. In the first phase, during late mitosis and G1, the Mcm2-7 complexes are recruited to DNA in an inactive state. This is also called replication licensing. These DNA bound Mcm2-7 complexes are then essential for initiation and elongation of replication forks during the S phase. As we will see, the spatial and temporal separation of factors needed for loading and activation of the Mcm2-7 is tightly regulated.

The existence of this elegant system was first noticed using cell fusion experiments, where replicated DNA was shown to differ from un-replicated DNA in its replication potential. G1 DNA was able to replicate when G1 cells were fused with those in the S phase, but G2 DNA needed to pass through mitosis beforehand (Rao and Johnson, 1970). Later, this finding was refined to a model according to which replication origins were "licensed" for replication during late mitosis and G1, but the license was removed as the DNA replication was started (Blow and Laskey, 1988). Dividing the process of DNA replication into two non-overlapping phases (one phase permissive for the licensing of DNA replication and the second permissive for the initiation of replication but not for licensing) can explain how cells ensure the precise duplication of chromosomal DNA in a single cell cycle. To date, a large amount of detailed experimental data has been acquired supporting the licensing model and we next will examine the essential features of this elegant system.

To prevent the possibility of replicated origins becoming re-licensed during the S phase, it is important that the ability to license new replication origins is downregulated before entry into the S phase. Therefore, the licensing components ORC, Cdc6 and Cdt1 are only needed for the loading of Mcm2-7 onto DNA in G1, but are not required for the continued association of the Mcm2-7 helicase complex with DNA, and thus their activities are down-regulated of at the end of

G1 (Donovan et al., 1997; Hua and Newport, 1998; Maiorano et al., 2000; Rowles et al., 1999). The key component needed to achieve this is again CDK, whose activity directly inhibits the assembly of pre-RCs (Dahmann et al., 1995). One of the major substrates of CDK in budding yeast is Cdc6. CDK can bind to Cdc6 and the phosphorylation of Cdc6 targets it for degradation at the G1 to the S phase transition (Elsasser et al., 1999; Mimura et al., 2004; Drury et al., 2000). Cdc6 levels are also regulated at the transcriptional level to give maximum expression in late mitosis and G1, with its transcription being under the control of the CDK-dependent transcription factor SWI4 (SWItching deficient 4) (McInerny et al., 1997; Zwerschke et al., 1994). In addition to Cdc6, ORC activities are also directly inhibited by CDK. It has been shown that CDK binds ORC via its activating subunit Clb5 and that this helps maintain ORC in an inactive state during S and G2 phases. Eliminating the Clb5–Orc6 interaction has no effect on initiation of replication but instead sensitizes cells to lethal over-replication. (Wuarin et al., 2002; Wilmes et al., 2004). Subsequently, re-replication is further inhibited by CDK dependent phosphorylation of ORC (Nguyen et al., 2001). The two remaining components of pre-RC, Ctd1 and Mcm2-7, are targeted in a different fashion. In budding yeast, CDK promotes nuclear export of both proteins during the S phase, G2 and early mitosis, thus preventing them from gaining access to chromosomal DNA (Nguyen et al., 2000; Labib et al., 1999).

All the mechanisms described above assure that DNA is not replicated more than once per cell cycle. Experiments in budding yeast have shown that in order for significant re-replication to occur, all these different CDK-dependent controls must be inactivated. Partial over-replication is possible when un-phosphorylatable mutants of Orc2 and Orc6 are combined with non-degradable Cdc6. In addition Mcm2-7 and Cdt1 have to be constitutively expressed in the nucleus (Nguyen et al., 2001). The presence of redundant control mechanisms highlights the importance of avoiding over-replication.

1.4. Chromatin environment

In essence, the yeast genome represents a rather compact organization of genes and regulatory sequences. Unlike in many other organisms, very little noncoding DNA is present. From the number of genes and the total size of the yeast genome one arrives at a gene density of about one open reading frame per 2 kb (Goffeau et al., 1996). This leaves only limited space for the intergenic regions that harbor the major regulatory elements involved in chromosome maintenance, transcription and DNA replication. Therefore, in order to develop a more accurate picture, the process of DNA replication must be considered in an *in vivo* environment, where the cell has to deal with many important tasks simultaneously. Additionally, it is also necessary to view the genome as a whole nucleo-protein complex called chromatin, to take into account how the presence of nucleosomes or other DNA

binding proteins affects replication, and also to fit other DNA dependent processes, especially transcription, into the scheme.

1.4.1. Timing and efficiency of replication origins

To date, ~400 loci of budding yeast replication origins have been identified and found to be distributed evenly throughout the genome. However, not all origins are identical (Nieduszynski et al., 2006). The discriminating features between these origins are their initiation time during the S phase and their efficiency of initiation, the latter of which is defined as the frequency at which an origin initiates DNA replication (fires) within a population of cells. In yeast, many origins are efficient, firing in virtually every dividing cell within a population. However, some replication origins are less efficient, initiating in fewer than half the dividing cells within a population. Although the exact mechanisms that determine initiation timing and efficiency remain unclear, chromatin structure clearly plays an important role.

In addition to firing efficiencies, individual replication origins also fire at characteristic and reproducible times during the S phase. For example, the yeast origins *ARS305* and *ARS607* fire shortly after cells have entered the S phase and are considered to be ''early'' origins, while the yeast origin *ARS1* fires during the first half of the S phase but several minutes after *ARS305* (Raghuraman et al., 2001). On the other hand, some replication origins such as *ARS501* or *ARS609* fire near the end of the S phase and are considered to be ''late'' origins (Friedman et al., 1996; Ferguson et al., 1991; Friedman et al., 1997). In general, most early origins are positioned near the central portions of yeast chromosomes, while late firing origins are positioned nearer to the telomeres. Therefore, the central portions of chromosomes tend to replicate before the ends (Raghuraman et al., 2001).

There is no consistent relationship between replication origin efficiency and the time at which an origin fires during the S phase: for instance some late firing origins are efficient and others are not (Friedman et al., 1997; Yamashita et al., 1997). Some late origins are inefficient because they are located near an early firing origin. The earlier origin establishes a replication fork that replicates the later origin before it has a chance to fire (Vujcic et al., 1999). As with origin efficiencies, the chromatin context appears to be the major determinant of origin firing times.

1.4.2. Chromatin context

The first evidence of the influence of chromatin context on replication origin activity came from studies showing that some ARS sequences that function properly when inserted into plasmids fail to function as chromosomal origins in their native loci. In particular, it was found that the origins in silenced mating type loci were inhibited by heterochromatin (Vujcic et al., 1999; Dubey et al., 1991). When an otherwise

additionally efficient origin *ARS305* was inserted into the mating type locus, it failed to function (Sharma et al., 2001). Therefore, the inherent capability of some sequences to function as origins can be inhibited by chromatin context.

In budding yeast, heterochromatin is formed in three main regions – telomeres, the ribosomal DNA (*rDNA*) locus and silent mating type loci (*HML* and *HMR;* Hidden Mat Left or Right). To establish silenced chromatin, SIR (Silent Information Regulator) proteins Sir1, Sir4, Sir2 and Sir3 have to be recruited to silencer sequences. This recruitment process is hierarchical where one event (the recruitment of a protein) leads to another. Although the initial assembly varies at different silenced loci, it is interesting to note that the silencing of mating type loci is partially initiated by ORC complex, which binds to silencer sequences and helps to recruit SIR proteins (Zhang et al., 2002). After their initial assembly, SIR proteins spread out from the silencer region through the binding of Sir3 and Sir4 proteins to the tails of histones H3 and H4. Sir3 and Sir4 bind more efficiently to hypoacetylated histone tails, and therefore they need the deacetylase activity of Sir2 for this interaction. The binding of Sir4 and Sir3, in turn helps to recruit additional Sir2 proteins, which facilitate further binding of Sir3 and Sir4 to adjacent nucleosomes, leading to effective silencing of the locus. This spreading of heterochromatin is regulated by chromatin modifications that inhibit SIR binding. This is often achieved by active transcription in boundary areas (Rusché et al., 2002, 2003; Carmen et al., 2002).

In accordance with this model, it has been found that disruptive mutations in Sir3 activate certain replication origins near the ends of chromosomes and cause telomeric DNA to replicate earlier (Stevenson and Gottschling, 1999). On the other hand, relocating a transcriptional silencer and artificially forming heterochromatin in certain parts of the genome can inhibit origin functioning as well (Zappulla et al., 2002). Instead of direct inhibition of replication origins by heterochromatin, it has also been found that an origin's activation time can be altered when it is shuffled between regions with different chromatin states. Taken into account that origins in subtelomeric regions are generally late firing, whereas origins in centromeric regions are early firing (Raghuraman et al., 2001; Pohl et al., 2012), it is possible to render an early origin *ARS1* to fire significantly later when relocated to subtelomeric areas near *ARS501* (Ferguson and Fangman, 1992). Likewise, the late *ARS501* can fire significantly earlier when taken out of subtelomeric DNA and moved to a plasmid (Ferguson et al., 1991). Similar positional effects on replication origin activation are confirmed in one of my publications with a larger variety of origins, as discussed in Chapter 2.3 (Lõoke et al., 2013).

In addition to origin relocation experiments, much information on temporal behavior of replication origins has been gathered by studying chromatin modifications. Similar to their regulatory role in transcription, histone modifications regulate the access of replication factors to replication origins and thus help to

determine the time of origin activation. For example, the global increase in histone acetylation has been shown to advance the timing of many replication origins. This can be achieved by inactivating the Sin3–Rpd3 histone deacetylase complex (Sin3 - Switch INdependent 3; Rpd3 - Reduced Potassium Dependency 3), known for its role as a gene-specific transcriptional repressor. Deletion of *RPD3* causes significantly earlier initiation of late-firing origins, along with increased acetylation of histones flanking these origins (Vogelauer et al., 2002; Aparicio et al., 2004; Knott et al., 2009). Consistent with the idea that Rpd3 deacetylates chromatin surrounding a large number of yeast origins, replication of the entire genome is advanced in a strain lacking *RPD3* (Aparicio et al., 2004). On the other hand, inducing histone acetylation improves origin activation. For instance, targeting of Gcn5 (General Control Nonderepressible 5) histone acetylase adjacent to a late-firing origin advances its time of initiation (Goren et al., 2008; Vogelauer et al., 2002). Importantly, mutating the acetylation sites in H3 and H4 severely impairs origin function, indicating that these acetylation events are necessary for efficient origin firing (Unnikrishnan et al., 2010).

Although local chromatin environment is a major influencer of origin timing, recent findings indicate that some origins with Forkhead transcription factor binding sites are activated early in the S phase regardless of their location in the genome (Chapter 2.3.; Lõoke et al., 2013). It has been found that Forkhead transcription factor-dependent clustering of these replication origins is required for their early firing, indicating that the spatial distribution of replication origins in the cell nucleus might also influence their activation (Knott et al., 2012). Additional evidence of the importance of spacial organization of chromatin in the nucleus comes from studies of the Ku complex. This versatile complex composed of yKu70 and yKu80 proteins, is important for the repair of double-stranded DNA breaks, and also functions at telomeres. At chromosome ends, the Ku complex is required for correct localization of telomeres to the nuclear periphery, and also contributes to recruitment of telomerase and to transcriptional repression by Sir proteins (Fisher and Zakian, 2005). It has been found that deletion of Ku complex components alters the positioning of telomeric DNA in the yeast nucleus, which in turn affects repression of telomere-proximal genes (Laroche et al., 1998). Importantly, replication origins located close to telomeres or within subtelomeric regions are activated much earlier in mutants lacking Ku function (Cosgrove et al., 2002).

The final aspect influencing replication origin activation is the positioning of nucleosomes. Generally, nucleosomes are depleted from regions of replication origins due to active positioning and maintenance of nucleosome free regions (Field et al., 2008; Eaton et al., 2010). When nucleosomes are positioned incorrectly origin functioning is disrupted. For example, histone deacetylase Sir2 has been shown to inhibit origin activation by inducing unfavorable nucleosome positioning

(Crampton et al., 2008). Several studies have found that active positioning of nucleosomes is important for origin to function properly. For example, *ARS1* activity is disrupted when the functional sequences of the origin are moved into a DNA region masked by a nucleosome (Simpson, 1990). Ordinarily, the nucleosome free region of *ARS1* is maintained by ORC and Abf1 (ARS-Binding Factor 1). Mutations in the Abf1 binding site allow nucleosome invasion into the functional region and reduce origin activity (Venditti et al., 1994). Similarly disruption of ORC-directed nucleosomal positioning interferes with *ARS1* functioning (Lipford and Bell, 2001).

Although not fully understood, chromatin environment remains the major determinant of origin timing and efficiency. It is likely that all the described mechanisms cooperatively regulate the access of replication factors to the origin DNA, since overexpression of limiting replication factors help to overcome chromatin restraints and advance the timing of late origins (Mantiero et al., 2011).

1.4.3. Transcription

A positive correlation between gene expression and timing of replication in both human and fruit flies has been reported, suggesting a connection between these nuclear functions (Woodfine et al., 2005; White et al., 2004; Schübeler et al., 2002). However, this is not found to be true in yeast, where the majority of replication origins are located in intergenic regions that are not directly involved in transcription of protein-coding genes (Wyrick et al., 2001). Nevertheless, the influence of global transcription can not be neglected in budding yeast. Several studies have reported that transcription by RNA polymerase II (RNAPII) inactivates DNA replication origins. The activity of a plasmid borne *ARS1* is inhibited by transcription induced from an adjacent promoter (Tanaka et al., 1994). Likewise, *ARS605*, located within the open reading frame of a meiosis specific gene, is active when transcription is repressed in mitosis, but becomes inactivated upon transcriptional induction of this gene during meiosis (Mori and Shirahige, 2007). My research shows that transcriptional inactivation of these origins arises from RNAPII-dependent transcriptional inhibition of pre-RC formation. All the components of the pre-RC, including ORC and Mcm2-7 complexes dissociate from origin DNA when transcribed (Chapter 2.2.; Lõoke et al., 2010).

As mentioned previously, intergenic regions of yeast genome are short. Although the majority of replication origins in budding yeast are located in intergenic loci, several studies show that transcription of non-coding regions is widespread in yeast (David et al., 2006; Neil et al., 2009; Xu et al., 2009; Davis and Ares, 2006). Thus, it is likely that many, if not all, replication origins suffer from some transcriptional stress and can potentially be inactivated by transcription. In order to cope with such conditions, yeast origins can be relicensed multiple times in G1 phase of the cell cycle. It appears that when all requirements are met, formation of pre-RC

complexes is a fairly dynamic process, and an equilibrium between licensing and transcriptional inactivation is formed (Chapter 2.2.; Lõoke et al., 2010).

In addition to transcription related perturbation of replication complex formation, conflicts between moving transcription and replication forks have also been described. Both head-to-head and head-to-tail collisions between replication and transcription are possible, although it has been found that head-on collisions inhibit replication fork movement to a greater extent (McGlynn et al., 2012; French, 1992). Replication forks which arrest due to encounters with transcription complexes can give rise to DNA damage response, mutagenesis and chromosomal deletions (Vilette et al., 1996; Srivatsan et al., 2010). To prevent this, certain features of genome organization help to avoid head-on collisions of highly transcribed genes and replication forks. For example replication of the rDNA cluster in budding yeast is regulated in a manner that ensures that replication forks move through the rDNA in the same direction as the transcribing RNA polymerase I (Brewer and Fangman, 1988). Similarly, in higher eukaryotes, transcription of highly expressed genes in close proximity to replication origins is directed away from the origins, minimizing the frequency of head-on collisions (Huvet et al., 2007). While replication origin placement serves as a good method of reducing head-on collisions, it is also clear that not all transcription related stress can be avoided in this way. Recently, two helicases, Sen1 (Splicing ENdonuclease 1) in budding yeast and Pfh1 (PiF1 Helicase homolog 1) in fission yeast, have been found to be necessary for efficient movement of replication forks through transcribed areas. Deletion of either factor results in aberrant DNA structures (Alzu et al., 2012; Sabouri et al., 2012). Therefore, in addition to passive reduction of possible fork collisions, certain factors actively help to reduce harmful effects in situations of concurrent replication and transcription.

In brief, although yeast replication origins are located in genomic regions with low transcriptional activity, usage of the same template molecule may easily lead to conflicts between these two processes. First, the widespread transcription of intergenic regions causes constant disruption and ensuing relicensing of replication origins, at least in budding yeast. Second, collisions between replication and transcription machineries can cause replication fork stalling and consequent genetic instability. The cell prevents this by first minimizing the possible hazardous head-on fork collisions by favorable genomic placement of replication origins with respect to highly transcribed genes. However, when concurrent transcription and replication can not be avoided, specific helicases have been shown to assist replication fork movement through transcribed areas.

AIMS OF THE STUDY

The overall goal of the study was to find out how replication origin functioning is influenced by surrounding chromatin environments. The experimental part consists of three linked studies, each dealing with individual objectives:

Ref. I The first objective of the study was to simplify the procedure of introducing and verifying genetic manipulations in yeast. A large fraction of this process consists of extraction of DNA from yeast colonies and validation of the altered traits by PCR. Therefore, the motivation was to create a simple and reliable DNA extraction protocol to simplify the routine work in yeast laboratories.

Ref. II The second study presented in the thesis was initially inspired by the discovery of the global bidirectionality of yeast promoters and the resulting possibility that many replication origins might be transcribed. Knowing that transcription disrupts the function of these origins, we sought to find the likely number of transcribed origins and also to dissect the dynamics and fate of transcribed origins.

Ref. III The third paper presents results that help to understand how chromatin context determines the activation time of different replication origins. The initial aim was to introduce many different replication origins into similar chromatin environments and to determine the role of surrounding landscape in origin activation. Later, when apparently chromatin-independent origins were identified, the objective was to describe this phenomenon in greater mechanistic detail.

2. RESULTS AND DISCUSSION

2.1. Extraction of yeast genomic DNA (Ref. I)

Presently, the process of DNA replication is best understood in budding yeast *Saccharomyces cerevisiae.* Therefore, it is an excellent tool to unveil the uncharacterized aspects of this process. In the current study, the focus was set on the relation between chromatin context, chromatin dependent processes and DNA replication initiation. In order to accomplish this goal, significant genetic manipulations had to be performed in yeast strains. Altogether, nearly 100 different yeast strains were created throughout the course of this study. Typically, genetic manipulation of yeast cells involves several rounds of genomic DNA extraction: initially for the gathering of template genetic material, next for the analysis of colonies with desired traits, and finally for the verification of finished strains. PCR is commonly used to gather information at each of the mentioned steps. Therefore, the need for convenient and reliable DNA extraction protocol for PCR based applications was apparent.

2.1.1. Development of the LiOAc-SDS DNA extraction protocol

The main obstacle in efficient isolation of yeast genomic DNA (gDNA) is the very inefficient disruption of the strong cell wall. Conventional methods for gDNA preparation from yeast cells utilize either enzymatic or physical degradation of the cell wall, followed generally by lysis of cells and extraction of gDNA with phenol/chloroform (Ling et al., 1995; Amberg et al., 2005). When analyzing a large number of samples, these methods become time-consuming and/or relatively expensive. At the same time, the resulting amount and purity of DNA greatly exceeds the requirements for simple PCR analysis. Alternatively, for quick genotyping, cells can also be lysed by repeated freeze-thaw cycles in a buffer containing Triton X-100 and SDS, followed by extraction of gDNA with chloroform (Harju et al., 2004). Although this method is considerably faster than conventional gDNA preparation methods, it requires transfer of the sample to a new test tube after chloroform extraction, which slows down the protocol and makes it inconvenient for simultaneous handling of a large number of samples. It has also been reported that usable gDNA can be isolated in a single tube by simple SDS treatment (Akada et al., 2000). However, the yield of gDNA from this protocol is relatively low and the results are poorly reproducible (Ref. I, Fig. 1A). In addition, a large number of cells is required for the protocol, and the buffer for subsequent PCR reactions has to be supplemented with Triton X-100 (Akada et al., 2000).

Principally, the idea was to improve the DNA yield of the SDS treatment by using it in conjunction with a cell wall disruptive agent, and then to remove traces of

SDS before PCR analysis. Initially, lithium acetate (LiOAc) and sodium hydroxide (NaOH) were used to disrupt cell walls. Lithium acetate is commonly used in yeast transformation protocols to weaken cell walls, and sodium hydroxide is used in protein extraction protocols (Thompson et al., 1998; Ito et al., 1983; Kushnirov, 2000). Both chemicals produced adequate results, but the output in PCR signals was diminished when using NaOH, possibly due to unsuitable pH (data not shown). Therefore, LiOAc-SDS lysis was chosen for further calibration and validation.

Following the initial lysis, ethanol was used for precipitation of the DNA, thereby removing the potentially inhibiting chemicals prior to PCR analysis. The protocol subjected to testing, therefore, consisted of following steps:

- Lysis of yeast cells in 100 μl LiOAc-SDS solution.
- Precipitation of the DNA by addition of 300 μl of 96-100% ethanol.
- Washing the resulting pellet with 70% ethanol to remove traces of LiOAc and SDS.
- Dissolving the pellet (which includes gDNA) in 100 μl of water
- Removing the insoluble components by centrifugation
- Using 1 μl of the extract for PCR

2.1.2. Calibration of the LiOAc-SDS DNA extraction protocol

In order to identify the critical conditions for efficient DNA extraction, a panel of calibration experiments were carried out. In all of these 100 μl aliquots of mid-log phase liquid culture were collected, DNA was extracted in 100 μl of lysis solution and analyzed by PCR. The test conditions were varied so that the tested component in each panel would be the limiting factor in the reaction. For example, short incubation time was used for testing of different temperatures; reactions were carried out at room temperature to test a range of SDS concentrations and incubation time; and long incubation time at high temperature was used for determination of optimal LiOAc concentration (Ref. I, Fig. 1C-F). The best results were obtained using 200 mM LiOAc and 1% SDS in the lysis solution, while carrying out the lysis at 70°C for $\geq$5 min. However, based on these experiments, many other suitable conditions can be selected if needed.

Having obtained the optimal conditions, the usability of the protocol was evaluated in its intended purpose - analysis of yeast colonies. A number of colonies were selected for DNA extraction, and two PCR fragments of 489 and 2383 bp in length were amplified. In all cases, robust reproducible output was detected,

confirming the usability of the method, especially when compared to the SDS extraction method described in Akada et al., 2000 (Ref. I, Fig. 1A).

In order to quantify the developed protocol, the yield and purity of the extracted gDNA was determined. For this purpose, gDNA from samples corresponding to 10^7cells was separated in 0.9% TAE-agarose gel along with serial dilutions of gDNA with known concentrations. Approximately 100 nanograms of gDNA was extracted from 10^7 cells, again representing a considerable improvement compared to Akada et al., 2000. In addition, the experiment verified the presence of large amounts of RNA, which can be removed with RNase treatment, if needed (Ref. I, Fig. 1B).

Another important parameter to consider when choosing a DNA extraction method for PCR is the maximal possible length of amplicons that can be amplified. In order to validate the suitability of the developed protocol in this aspect, PCR reactions were performed with different expected product lengths from 718 bp to 5920 bp. The LiOAc-SDS protocol produced robust results up to 3505 bp, with amplicons of 4449 bp also clearly detectable, although if longer fragments need to be amplified, more delicate DNA extraction methods should be used (Ref. I, Fig. 2A). Taking into account that the average gene density of yeast the genome is about one open reading frame per 2 kb (Goffeau et al., 1996), the achieved length of PCR fragment should be more than adequate in most situations.

Lastly, the potential wide use of the method was confirmed by the successful extraction and amplification of DNA from six different yeast species (*Kluyveromyces lactis, Hansenula polymorpha, Schizosaccharomyces pombe, Candida albicans, Pichia pastoris,* and *Saccharomyces cerevisiae*) (Ref. I, Fig. 2B). In addition to simple PCR-based genotyping, we have used LiOAc-SDS extracted and Pfu DNA polymerase amplified *S. cerevisiae* gDNA for BigDye v3.1–based sequencing and obtained DNA sequence readout of the entire 850-bp PCR fragment (data not shown). We have also used LiOAc-SDS extracted gDNA directly in real-time qPCR reactions and for quick genotyping of yeast colonies (data not shown).

In summary, the developed method is a quick and reliable solution for gDNA extraction from yeasts that is suitable for PCR amplification of DNA fragments $\leq$3500 bp. The protocol can be carried out in a single test tube in under 15 minutes, and cells from liquid media and single colonies grown on solid media can be used. The method is suitable for routine genotyping of yeasts either by simple detection of PCR products or for initial amplification of genomic DNA for subsequent sequencing - procedures that are widely used for analysis of scientific, environmental, industrial and clinical samples.

2.2. Relicensing of transcriptionally inactivated origins (Ref. II)

DNA replication origins are licensed in G1 phase of the cell cycle when the Mcm2-7 helicase complexes are loaded onto origins. These pre-replicative complexes remain inactive until replication is initiated in the S phase. In budding yeast, the majority of replication origins are located in intergenic regions that are not directly transcribed (Wyrick et al., 2001). However, it has been found that bidirectional initiation of transcription from many promoters is common, leading to transcription of intergenic regions and production of unstable cryptic transcripts (David et al., 2006; Neil et al., 2009; Xu et al., 2009; Davis and Ares, 2006; Wyers et al., 2005). The widespread transcription of noncoding DNA and the compact nature of the yeast genome raise the possibility that a large fraction of replication origins are transcribed on a regular basis, which can lead to their inactivation before cells enter the S phase.

2.2.1. Transcription of replication origins

To estimate the fraction of replication origins that are regularly transcribed, genome-wide data of yeast transcripts in the loci of replication origins were analyzed. First, the tiling array data of total transcriptome from Neil et al., 2009 were analyzed, and transcription of replication origins was compared to the average level of noncoding DNA transcription genome-wide. This analysis revealed that more than 10% of replication origins were transcribed (Ref. II, Fig. 1A). However, this approach probably underestimated the total number of transcribed replication origins because the widespread transcription of noncoding DNA in yeast cells significantly increases the average level of background signal that was calculated for this analysis. Therefore, the genome-wide locations of CUTs (Cryptic Unstable Transcripts), defined by the SAGE (Serial Analysis of Gene Expression) analysis in Neil et al., 2009 was also analyzed. This analysis showed that at least one-third of the replication origin sequences are transcribed on a regular basis as CUTs (Ref. II, Fig. 1B). However, the replication origin placement in the yeast genome still appears to favor transcriptionally silent regions, as the number of heavily transcribed origins is significantly smaller than the number of those transcribed occasionally (Ref. II, Fig. 1). As mentioned, only 10% of replication origins are transcribed more frequently than the average noncoding DNA (Ref. II, Fig. 1A).

This is in good agreement with previous findings showing that active transcription abolishes replication origin firing both in yeast and in higher eukaryotes (Mori and Shirahige, 2007; Tanaka et al., 1994; Sasaki et al., 2006). However, the fact that many replication origins are situated in transcriptionally active loci suggests that the replication machinery might benefit from transcription-coupled remodeling and modifications of the chromatin that help pre-RC components to access DNA

and facilitate the initiation of replication. The observation that replication timing and transcription are in positively correlated in higher eukaryotes surely supports this interpretation (Woodfine et al., 2005; White et al., 2004; Schübeler et al., 2002). However, consequently the question about the fate of transcribed origins is raised. Can sporadic transcription events permanently inactivate replication origins or is there a rescue mechanism?

2.2.2. Dynamics of pre-RC formation

An efficient solution to the transcription-coupled loss of functional origins would be re-establishment of pre-RCs on origins after sporadic transcription events. To investigate this possibility, different ARS elements were inserted into a galactose-inducible *GAL-VPS13* gene, 3 kb downstream from the transcription start site (Ref. II, Fig. 2A). ChIP assays were used to monitor the binding of ORC (Orc2) and Mcm2-7 (Mcm4) to the newly engineered origins and to their corresponding natural loci. When transcription was repressed, both complexes were detected at the *GAL-VPS13-ARS* loci while, in accordance with previous findings, recruitment of ORC and Mcm2-7 was severely reduced in conditions of active transcription (Ref. II, Fig. 2C and 2D). This confirms the disrupting effect of active transcription on replication origin functioning.

To further understand the temporal dynamics of transcription-related disruption of pre-RCs and to investigate the possible re-establishment of pre-RC components, the *GAL-VPS13-ARS607* locus was first induced with galactose and then repressed with glucose. As expected, both ORC and Mcm2-7 were gradually removed from chromatin as the origin was transcribed. Interestingly, both complexes quickly returned after transcriptional repression and disappearance of RNAPII (Ref. II, Fig. 3). Half of the maximal amounts of Orc2 and Mcm4 were detected within the first 10 minutes after transcriptional repression, indicating that the pre-RCs reformed in the same cell cycle and the passage through the S phase was not necessary.

Naturally, when rescuing pre-RCs from transcriptional stress, it would be important to achieve this before the inhibition of licensing in the S phase. In order to eliminate the possibility of cells entering the S phase and to illustrate the possibility of origin relicensing in G1, cells were next arrested with α-factor and the transcription-coupled dynamics of Mcm2-7 were monitored. In all surveyed origins, Mcm4 was removed from *GAL-VPS13-ARS* loci in response to transcriptional induction. When transcription was subsequently repressed, but cells were arrested in G1, Mcm4 was efficiently reloaded (Ref. II, Fig. 4B). The time needed for complete rescue of transcriptionally inactivated replication origins was between 30 and 60 minutes at the *GAL-VPS13* locus (compare Ref. II, Fig. 3B and 4B). Whether this time is sufficient to rescue origins in their natural loci remains unanswered in the course of this study. However, it is clear that the repeated formation of pre-RCs in G1 is possible.

2.2.3. Activation of relicensed origin

After establishing that transcriptionally disrupted pre-RCs can be reloaded onto origins in G1 without passing through the S phase, the next question was whether the relicensed replication origins are activated in the following S phase. To this end, the transcription at *GAL-VPS13* locus was either constantly repressed, constantly activated, or activated and then repressed to allow origin relicensing before the release of α-factor arrested cells into the S phase. As an origin activation indicator, recruitment of Cdc45 protein to origin DNA was measured through the course of the S phase (Ref. II, Fig. 5A). As expected, both *ARS609* and *GAL-VPS13-ARS609* replication origins were activated in transcriptionally repressed conditions, and the *GAL-VPS13-ARS609* origin failed to activate when transcription was constantly activated. More importantly, the relicensed *ARS609* origin in the *GAL-VPS13* locus was efficiently activated, demonstrating that there is no functional loss in origin function. Furthermore, no change in the timing or efficiency was monitored when the origin was relicensed (Ref. II, Fig. 5A).

To further confirm that Cdc45 recruitment to a relicensed origin reflects its firing, replication structures were visualized at the 60 minute time-point, when Cdc45 associated with origin DNA. A replication bubble was successfully detected at the relicensed *GAL-VPS13-ARS609* locus, indicating origin firing (Ref. II, Fig. 5C). In addition, origin firing was also detected in cells that were grown continuously in transcriptionally repressed conditions, but not in cells where transcription at the *GAL-VPS13-ARS609* locus was active (Ref. II, Fig. 5D and 5E).

Taken together, these results show that transcriptionally disrupted pre-RCs are quickly reloaded onto origins as soon as transcription is shut down, demonstrating a possible rescue mechanism for transcriptionally inactivated replication origins. Importantly, in G1-arrested cells, pre-RCs can reform without transition through the S phase, and following release from G1 block, DNA replication can be initiated from these relicensed origins. Therefore, this mechanism may allow pre-RC assembly in conditions of sporadic transcription, ensuring that a sufficient number of functional replication initiation loci will be available to carry out DNA synthesis.

But what is the sufficient number of origins? Surely, cells can afford to lose some of the licensed origins as there is an an excess number of origins in yeast and not all of them fire in a single S phase. Furthermore, even the most efficient origins do not fire in every S phase (Hyrien et al., 2003). Nevertheless, several lines of evidence suggest that replication origins are actively relicensed and that this helps to maintain the integrity of the genome. First, our finding that several efficient and active replication origins, like *ARS214*, *ARS305*, *ARS453*, and *ARS519*, are highly transcribed as CUTs, argues that the relicensing mechanism indeed is constantly used to maintain the potency of these origins (Ref. II, Suppl. Table 1). It has been argued that the surplus of licensed origins helps to safeguard genomic stability in situations where replication is disturbed and licensing is repressed

(Blow and Ge, 2009). Furthermore, considering the importance of accurate genome duplication, redundancy in DNA replication control naturally appears to be an inherent feature of the system. For example, similar redundancy can be found in mechanisms controlling re-initiation events in the S phase. The existence of multiple mechanisms that prevent re-initiation results in an error rate lower than 10^{-7}per origin per cell cycle (Diffley, 2011). Analogously, the high copy number of licensing factors compared to the number of origins may be responsible for generating redundancy in the number of licensed replication origins (Tanaka et al., 2011). This, in turn, can ensure the very low probability of failure of complete genome duplication.

Lastly, in addition to safeguarding the DNA replication process in yeast, relicensing of origins can also explain how replication origins in other eukaryotes can be positioned in transcriptionally active loci and why replication timing and transcription are in positive correlation (Woodfine et al., 2005; White et al., 2004; Schübeler et al., 2002; Hassan et al., 1994). As a good example, it has been found recently that the replication origins in *Trypanosoma brucei* are localized to the boundaries of transcription units (Tiengwe et al., 2012). In these situations, the otherwise harmful effects of accidental transcription can be balanced out by the relicensing system, while the same time replication complex formation benefits from being situated in the areas of open chromatin.

2.3. Chromatin-dependent and -independent regulation of DNA replication origin activation (Ref. III)

One characteristic feature of budding yeast replication origins is their time of activation in the S phase. This temporal behavior is well described, and individual replication origins fire at characteristic and reproducible times during the S phase. As discussed previously, chromatin context and chromosomal positioning of replication origins are the major determinants of replication timing (Chapter 1.4.2.). Nevertheless, it is also possible that replication origin sequences carry intrinsic DNA elements that mediate their time of activation and may help replication origins to overcome certain chromatin directed constraints.

2.3.1. Location-dependent origins

To find out whether epigenetic environment or internal properties of replication origins determine origin activation, different ARS sequences were relocated to a common chromosomal position and their activation time in this new location was determined. We reasoned that, if the timing of origin activation is determined solely by chromatin context, all origins should fire at the same time in their new position, whereas if the timing is derived from their intrinsic DNA sequence elements, the

origins should maintain their own original firing times regardless of their location in genome. The hallmark of origin activation used throughout the study was primarily the binding of Cdc45, although the firing times of all native replication origins we used were also verified by determining the binding of Pol2 (catalytic subunit DNA polymerase ε). Our measured activation times of native origins corresponded well with those previously reported (Ref. III, Fig. 1).

In order to dissect the sequence-derived and chromatin-dependent components of origin firing, activation times of these origins were determined when their sequences were copied to a common *VPS13* locus on chromosome XII. The VPS13 locus itself contains no other DNA replication origins within a 60-kb region and is a genuinely late-replicating part of the chromosome, thus serving as good target locus to study activation of inserted ARS sequences (Knott et al., 2009). After relocation to *VPS13,* origins‘ firing times were compared to those in their native positions. Upon relocation into the *VPS13* locus, the dynamics of Cdc45 recruitment were changed at four replication origins. In their native locations, the peak of Cdc45 binding was observed at 30 min after G1 release at *ARS605*, at 40 min at *ARS409*, at 50 min at *ARS501* and at 70 min at *ARS609*. However, when inserted into the *VPS13* locus, activation of these origins was changed, suggesting that their firing was determined primarily by the genomic location rather by than the intrinsic properties of their sequences. Moreover, as chromatin dependency would suggest, the new activation times of these ARS sequences were synchronized in *VPS13,* indicating that regardless of their original timing, different replication origins can adopt similar firing patterns when inserted into identical chromatin environment (Ref. III, Fig. 2).

These results are in good agreement with what has emerged from previous origin relocation experiments. For example, the timing of *ARS1* can be delayed upon relocation to late replicating chromatin near the end of chromosome V (Ferguson and Fangman, 1992). Similarly, loosening the chromatin derived restraints when relocating *ARS501* from a subtelomeric region to a plasmid advances its timing (Ferguson et al., 1991). The additional layer of information emerging from our study indicates that origins with very different initial activation times can adopt similar chromatin derived activation times. Therefore, it appears that chromatin context is the major determinant of origin firing time, and that changing the chromatin environment can both advance and delay origin firing. This idea is further supported by the finding that late replication origins retain their firing times when moved to a plasmid together with their surrounding sequences, but not when moved alone (Friedman et al., 1996). Additionally, changing the global chromatin state results in similar effects. For example, deletion of *RPD3* causes significantly earlier initiation of late-firing origins. The deletion of *RPD3* deacetylase increases histone acetylation and expands the range of euchromatin. (Vogelauer et al., 2002; Aparicio et al., 2004; Knott et al., 2009).

What is the mechanism that underlies the regulation of origin timing by changing chromatin structure? Such observations can be explained by the now widely accepted stochastic replication model (Bechhoefer and Rhind, 2012). The idea behind the model is that the key components needed for the activation of replication origins are limiting in cells. Therefore, the origins situated in more accessible parts of the genome have an advantage when competing for these activation factors. This rationale also suggests that overexpression of the limiting factors should advance the timing of late replication origins, and this is indeed what has been found (Mantiero et al., 2011; Tanaka et al., 2011). Furthermore, when overexpression of limiting replication factors is combined with the deletion of *RPD3,* an additional set of dormant origins is activated (Mantiero et al., 2011). This indicates that even when initiation factors are present in excess, some parts of chromatin remain inaccessible. This, in turn, means that limiting the number of initiation factors serves as an instrument to mediate origin firing times, and the process is self-regulated by the affinity of replication origins to such factors, which can be regulated epigenetically.

2.3.2. Location-independent origins

In addition to the location dependent origins, whose activation times were synchronized in the *VPS13* locus, another set of replication origins emerged from our study. Contrary to those described above, no shift in Cdc45-binding peak was detected at origins *ARS305*, *ARS607* or *ARS737* when they were inserted into the *VPS13* locus. All these origins were early firing both in their original and in the *VPS13* loci, as the binding of Cdc45 to these origins peaked at 30 min after the release from G1 arrest (Ref. III, Fig. 3). These results demonstrate that some early-firing origins can override the chromatin-derived control and retain their activation pattern in the new environment. However, this is not a common feature of all early replication origins, as *ARS605* shifted from early- to late-firing upon relocation into the *VPS13* locus (Ref. III, Fig. 2C). Therefore, a set of early-firing ARS sequences must possess specific sequence elements that ensure their early firing even when relocated to different sites in the genome.

In order to test whether this location independence is also maintained in other genomic loci, the *ARS607* sequence was inserted into various other chromosomal locations. First, *ARS607* was placed into the *DPB11* locus on chromosome X, which resides in late-replicating chromatin with no other adjacent replication origins (Knott et al., 2009). As expected, Cdc45 was recruited to the locus 30 min after the release from G1 arrest as it was in *VPS13* and in its native locus (Ref. III, Fig. 4J). Next, the *ARS607* sequence was tested in genomic regions that already contain native replication origins. Since neither *VPS13* nor *DPB11* normally contain active replication origins, it was possible that activation of *ARS607* was not properly regulated in these loci because some of the distal regulatory sequences

were missing in these genuinely origin-free regions. To test this possibility, the early origin *ARS728* located near the *CLD1* locus and the late origin *ARS609* near the *HXK1* locus were replaced with *ARS607*. The *CLD1-ARS607* locus was activated early in the S phase which was expected as both *ARS728* and *ARS607* are early-firing origins. More importantly, *ARS607* also activated early in the *HXK1* locus where it replaced the late-firing *ARS609.* While the firing time of the native *ARS609* locus was around 70 min after release from G1 arrest, the recruitment of Cdc45 was shifted 40 min forward in the *HXK1-ARS607* locus and peaked at 30 min after release from G1. Notably, the replaced *ARS609* itself did not harbor any intrinsic information for very late firing, as upon insertion into the *VPS13* locus, its activation shifted earlier and synchronized with the timing of other location-dependent origins in this locus (Ref. III, Fig. 2D). These findings confirm that *ARS607* is activated early in the S phase regardless of its location in the genome, and that it can convert late-replicating regions into early-replicating ones. In our experimental system, this origin fires 30 min after release from the G1 arrest in all tested loci. This demonstrates that early firing of *ARS607* is an intrinsic property of its sequence, enabling early initiation of DNA synthesis regardless of the surrounding genomic structures.

2.3.3. Forkhead binding ensures early activation

To identify the sequence elements required for chromatin independent activation of origins, truncated variants of the *ARS607* sequence were made (Ref. III, Fig. 4A). First, 21 nucleotides were removed in the *ARS607Δ1* mutant with no effect on its activation timing. Next, additional 21 nucleotides were removed in *ARS607Δ2,* which led to delayed activation of this origin in the *VPS13* locus (Ref. III, Fig. 4B and 4C). This deletion successfully separated the sequence needed for basic origin functioning from the sequence needed for its early activation in location-independent manner. The importance of this region becomes clear when it is analyzed in the context of recent findings from Knott et al., 2012. Namely, it was found that deletion of Forkhead transcription factors leads to genome-wide deregulation of the origin firing pattern in budding yeast, resulting in delayed activation of early origins and shifting many late origins to fire earlier. All three origins (*ARS305*, *ARS607* and *ARS737*) we found to be location-independent were delayed in the Fkh1/2 (ForKhead Homolog) double mutant.

ARS607 contains two putative binding sites for Fkh1/2 proteins, one of which is deleted in the *ARS607Δ2* mutant, whereas both sites are retained in the *ARS607Δ1*. To confirm the importance of Forkhead factors for early firing of the three origins, point mutations were introduced into the Fkh1/2 binding sites in *ARS607*, *ARS305* and *ARS737* (Ref. III, Fig. 4A). All of these origins have two Forkhead sites, and when either one of these sites was mutated, early activation of these origins was lost, suggesting that both Forkhead binding sites are necessary to ensure early firing

of these origins (Fig 4D-4H). The double mutant of *ARS305,* where both Forkhead sites were mutated, was also analyzed in Knott et al. 2012, and its activation was impaired. In accordance with this, our results showed that the Forkhead mutants of *ARS607* and *ARS737* also fire significantly later. Furthermore, the conversion of the *HXK1* locus into an early-replicating region is dependent on functional Fkh1/2 sites in *ARS607*, as the point-mutated version of *ARS607* fires late in this locus (Ref. III, Fig. 4J).

Naturally, in order to connect the delayed activation of the mutant origins with Forkhead factors, binding of the Fkh1 protein to replication origins was followed by ChIP assay. Fkh1 was detected at the early-replicating *ARS305*, *ARS737*, *ARS607* and *VPS13-ARS607* loci, but not at the *ARS409*, *ARS501*, *ARS605*, *ARS609* or at the origin-free *VPS13* locus. Importantly, when either one or both Fhk1/2 sites were disrupted in *ARS607*, *ARS305* or *ARS737*, recruitment of Fkh1 to the origins was severely reduced (Ref. III, Fig. 5A). Possibly, the binding of Forkhead proteins to replication origins must exceed a certain threshold level to ensure their early activation, and two Forkhead binding sites establish efficient recruitment of these factors through cooperative binding to the target DNA sequence as shown by *in vitro* assays (Hollenhorst et al., 2001). Altogether, these findings confirm that the sufficient binding of Fkh1 to some origins ensures their ability to activate early irrespective of their genomic location.

As the presence of two Forkhead binding sites was necessary for early firing of location-independent origins, two Forkhead binding sites were also introduced into the *ARS609* sequence in order to render it early. Unfortunately, this did not change the firing pattern of *ARS609* (Ref. III, Fig. S2A, S2B and S2C). However, binding of Fkh1 protein to this altered sequence was not observed either, indicating that the sole the presence of Fkh1/2 recognition sequences within the locus is not sufficient for efficient binding of Forkhead factors (Ref. III, Fig. S2D). It is likely that accessibility of Fkh1/2 sites is hindered by nucleosomes. This possibility is supported by the genome-wide nucleosome localization data indicating that the *ARS609* locus is covered by nucleosomes, whereas *ARS305*, *ARS607* and *ARS737* are located in nucleosome free regions (NFRs) (Jiang and Pugh, 2009). Therefore, at least one of the Fkh1/2 sites inserted into the *ARS609* locus is probably covered by a nucleosome, which may make the site inaccessible to Forkhead factors. Earlier studies have shown that localization of the *ARS1* regulatory sequences within an NFR is crucial for the function of the origin, and that ORC is a key factor for precise nucleosome positioning at the borders of origins (Simpson, 1990; Lipford and Bell, 2001; Eaton et al., 2010). Interestingly, when the *ARS607* sequence was inserted into the *HXK1* or *VPS13* loci, Forkhead binding and early activation was maintained (Ref. III, Fig. 4J). Therefore, it is be possible that in addition to Forkhead binding sites, elements necessary for maintaining NFRs are also needed for determining chromatin independency of replication origins.

In order to put the described findings into a broader context, the existence and positioning of Forkhead binding sites were analyzed in the context of temporal activation of replication origins. In particular, the values of replication indexes (Raghuraman et al., 2001; Nieduszynski et al., 2007; Yabuki et al., 2002) of origins containing different numbers of Forkhead binding sites were compared. The mean activation time of replication origins harboring zero or one Fkh1/2 site did not differ from the activation time of all origins, but origins containing two Forkhead sites separated from each other by 60–120 bp were almost exclusively early-firing. This suggests that Forkhead regulation affects a large number of origins and that, not only the presence but also the positioning of the Forkhead sites within an origin is important (Ref. III, Fig. 5B).

Taken together, these results indicate that some immediate-early replication origins contain two Fkh1/2-binding sites, one of which is found in close proximity to the ACS and another is located 60–120 bp away. It is also apparent that efficient binding of the Forkhead factors assures the position independent firing of some replication origins. In line with the previously discussed model of the temporal regulation of origin firing, the binding of Forkhead factors may give replication origins an advantage when competing for the limiting initiation factors. Some proof supporting this idea can be found in the Knott et al., 2012 study. First, it was found that the Forkhead activated origins were enriched in Cdc45 binding in G1 and that this was Fkh1/2 dependent. Additionally, binding of Cdc45 to early origins that were not regulated by Forkhead increased after Fkh1/2 deletion. In other words, when the binding of Cdc45 to Forkhead regulated origins was disrupted, the effective concentration of Cdc45 increased for other origins.

Forkhead induced binding of limiting factors could be facilitated in several ways. First, since Cdc45 recruitment to origins is dependent on DDK (Tanaka et al., 2011), Forkhead factors could directly recruit DDK to replication origins and therefore actively regulate origin firing. However, Forkhead factors are unlikely to achieve this without altering the accessibility of the chromatin environment, since it has been shown that the DDK subunit Dbf4 itself is one of the limiting initiation factors (Tanaka et al., 2011) and that even when it is overexpressed, tightly packed areas of chromatin remain inaccessible to limiting factors (Mantiero et al., 2011). Conversely, our results indicate that even the very late *HXK1* locus can be efficiently converted to early initiation via the insertion of the *ARS607* sequence, and that this is Forkhead dependent (Ref. III, Fig. 4J). Therefore, it is likely that instead of active recruitment of initiation factors, Forkhead binding alters the accessibility of chromatin near replication origins.

Currently, available evidence suggests that Forkhead binding can alter the nuclear positioning of replication origins and facilitate the clustering of early replication origins. First, enrichment of interactions between early replication origins was observed when the arrangement of DNA in nuclear space was analyzed (Duan et al.,

2010). Further analysis of this dataset revealed that the Forkhead activated origins form distinct clusters in the nucleus and that the interaction between two Forkhead activated origins (*ARS305* and *ARS607*) is lost upon Fkh1/2 deletion (Knott et al., 2012). Fkh1 dependent association between distant genomic regions has been also described in budding yeast mating type switching (Sun et al., 2002). Therefore, the molecular capacity of Fkh1 to functionally mediate genomic clustering has been demonstrated several times.

Although direct evidence that the Forkhead dependent clustering facilitates open chromatin and better DNA accessibility at replication origins is currently lacking, such mode of action would fit well with the proposed mechanism of controlling replication timing through origin access by limiting initiation factors.

CONCLUSIONS

This study was launched to find the effects of the chromatin environment on the functioning of replication origins. Problems arising from chromosomal positioning of replication origins and the effects of active transcription on origin regulation were primarily addressed. Methodologically, both approaches were based on genetically relocating a number of replication origins either to transcriptionally active loci or to different chromosomal positions. In order to easily produce and verify the yeast strains used in this study, a novel genomic DNA extraction protocol was developed. The new method is a quick and easy way to reliably extract DNA from yeast cell colonies and liquid culture for a downstream array of PCR based applications. The protocol has been adopted into the everyday work routine of our laboratory, and I hope that others will also benefit from it. Therefore, the first practical outcome of this work has the potential to impact general yeast genetics research.

When dissecting the importance of active transcription on origin activation, many replication origins in the budding yeast genome were found to be transcribed *in vivo*. Previous observations, that this kind of transcriptional disruption in replication origin loci inhibits replication complex formation, were confirmed in this study. At the same time it was found that pre-RC formation at these loci is a fairly dynamic process, and that transcriptionally removed replication complexes can quickly reassemble after transcriptional repression. Moreover, these reassembled, or relicensed origins are capable of Mcm2-7 activation and origin firing in the following S phase. Based on these results, it is likely that in *in vivo* conditions a balance is formed between accidental transcriptional disruption of replication origins and their constant relicensing in G1 phase. This balance may be responsible for assuring an adequate number of functional licensed origins when cells enter the S phase.

Lastly, the importance of the chromosomal environment on origin activation was studied. In agreement with previous findings, it was found that chromatin context is the sole determinant of activation for many origins. The activation time of these origins changes when they are inserted into different chromosomal positions. At the same time, origins containing double binding sites for Forkhead transcription factors are able to override the chromatin determined replication timing, and relatively late-replicating parts of the genome can be converted to early. Additionally, a set of exclusively early firing origins from the budding yeast genome were found to have two Forkhead binding sites with a specific 60 to 120 bp spacing possibly needed for efficient Forkhead binding at these loci. These results indicate that while replication origins are subject to a range different control mechanisms, the existence of chromatin independent replication origins may assure an efficient start of DNA synthesis in various conditions.

SUMMARY IN ESTONIAN

DNA replikatsiooni initsiatsiooni uurimine *Saccharomyces cerevisiaes*

Antud uurimustöö raames uuriti, kuidas kromatiini kontekst mõjutab replikatsiooni alguspunktide aktivatsiooni pagaripärmis. Käsitleti replikatsiooni alguspunktide kromosomaalse paiknemise ja transkriptsioonilise inaktivatsiooniga kaasnevaid probleeme. Töö esimeses osas kirjeldati käesoleva hetke arusaami replikatiivsete komplekside moodustumisest ja nende aktivatsioonist rakutsükli S faasis. Samuti iseloomustati mehhanisme, mis tagavad DNA täpse, ühekordse duplitseerumise. Töö praktilise osa paremaks mõistmiseks anti ka ülevaade replikatsiooni alguspunktide ajalisest aktivatsioonist ja juba teadaolevatest faktoritest, mis seda mõjutavad. Viimasena tutvustati kahe fundamentaalse DNA-seoselise protsessi, DNA replikatsiooni ja transkriptsiooni omavahelisi seoseid, keskendudes eelkõige sellele kuidas aktiivne transkriptsioon mõjutab DNA replikatsiooni.

Töö praktiline osa hõlmas endas metodoloogiliselt paljude replikatsiooni alguspunktide geneetilist ümberpaigutamist genoomi erinevatesse kromosomaalsetesse positsioonidesse ning ka transkriptiooniliselt aktiivsesse lookustesse. Selleks, et lihtsustada nende pärmitüvede tegemist, arendati esmalt uudne genoomse DNA ekstrahheerimise meetod, millega on võimalik kiirelt ja usaldusväärselt eraldada DNA-d kasutamaks seda erinevate PCR-il põhinevate rakenduste jaoks.

Uurides transkriptsiooni võimaliku mõju DNA replikatsiooni algatamisele leidsime, et paljud replikatsiooni alguspunktid paiknevad pärmi genoomis positsioonides, mis teeb võimalikuks nende transkriptsioonilise inaktivatsiooni. Samuti selgus, et transkriptsioon takistab funktsionaalsete replikatsiooni komplekside moodustumist. Kuna aga replikatsiooni komplekside moodustumise dünaamika oli oodatust kiirem, on võimalik nende kiire reassambleerumise transkriptsioonilise inaktivatsiooni järel. Lisaks näitasime, et sellised uuesti moodustunud replikatsiooni kompleksid on funktsionaalsed. Antud mehhanism on vajalik tagamaks piisava hulga funktsionaalseid replikatsiooni alguspunkte genoomi efektiivseks paljundamiseks.

Viimasena otsiti antud töös vastust küsimusele, kuidas kromatiini kontekst mõjutab replikatsiooni alguspunktide ajalist aktivatsiooni. Esmalt leidsime, et paljude replikatsiooni alguspunktide aktivatsiooni jaoks on nende kromosomaalne paiknemine peamine ajastust määrav faktor. Samas kirjeldasime ka mehhanismi, millega teatud replikatsiooni alguspunktid väldivad kromatiini kontekstist tulenevaid piiranguid. Sellised replikatsiooni alguspunktid omavad kahte kindla paigutusega Forkhead transkriptsiooni faktori seostumis-kohta. Forkheadi seostumine aga tagab replikatsiooni alguspunkti varajase aktivatsiooni sõltumata tema kromosomaalsest paiknemisest. Selliselt tagatakse efektiivne DNA replikatsiooni algatamine paljudes erinevates tingimustes.

LIST OF REFERENCES

Akada R, Murakane T, Nishizawa Y (2000) DNA extraction method for screening yeast clones by PCR. BioTechniques 28: 668–70, 672, 674.

Alcasabas AA, Osborn AJ, Bachant J, Hu F, Werler PJ, Bousset K, Furuya K, Diffley JF, Carr AM, Elledge SJ (2001) Mrc1 transduces signals of DNA replication stress to activate Rad53. Nature Cell Biology 3: 958–65.

Alzu A, Bermejo R, Begnis M, Lucca C, Piccini D, Carotenuto W, Saponaro M, Brambati A, Cocito A, Foiani M, Liberi G (2012) Senataxin associates with replication forks to protect fork integrity across RNA-polymerase-II-transcribed genes. Cell 151: 835–46.

Amberg DC, Burke DJ, Strathern JN (2005) Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual, 2005 Edition. Cold Spring Harbor Laboratory Press.

Aparicio JG, Viggiani CJ, Gibson DG, Aparicio OM (2004) The Rpd3-Sin3 histone deacetylase regulates replication timing and enables intra-S origin control in Saccharomyces cerevisiae. Molecular and Cellular Biology 24: 4769–80.

Aparicio OM, Stout AM, Bell SP (1999) Differential assembly of Cdc45p and DNA polymerases at early and late origins of DNA replication. Proceedings of the National Academy of Sciences of the United States of America 96: 9130–5.

Aparicio OM, Weinstein DM, Bell SP (1997) Components and dynamics of DNA replication complexes in S. cerevisiae: redistribution of MCM proteins and Cdc45p during S phase. Cell 91: 59–69.

Bechhoefer J, Rhind N (2012) Replication timing and its emergence from stochastic processes. Trends in Genetics 28: 374–81.

Bell SP, Stillman B (1992) ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex. Nature 357: 128–34.

Bielinsky AK, Gerbi SA (1998) Discrete start sites for DNA synthesis in the yeast ARS1 origin. Science 279: 95–8.

Blow JJ, Ge XQ (2009) A model for DNA replication showing how dormant origins safeguard against replication fork failure. EMBO Reports 10: 406–12.

Blow JJ, Laskey RA (1988) A role for the nuclear envelope in controlling DNA replication within the cell cycle. Nature 332: 546–8.

Bochman ML, Schwacha A (2008) The Mcm2-7 complex has in vitro helicase activity. Molecular Cell 31: 287–93.

Bowers JL, Randell JCW, Chen S, Bell SP (2004) ATP hydrolysis by ORC catalyzes reiterative Mcm2-7 assembly at a defined origin of replication. Molecular Cell 16: 967–78.

Brewer BJ, Fangman WL (1988) A replication fork barrier at the 3' end of yeast ribosomal RNA genes. Cell 55: 637–43.

Burgers PMJ (2009) Polymerase dynamics at the eukaryotic DNA replication fork. The Journal of Biological Chemistry 284: 4041–5.

Byun TS, Pacek M, Yee Mc, Walter JC, Cimprich KA (2005) Functional uncoupling of MCM helicase and DNA polymerase activities activates the ATR-dependent checkpoint. Genes & Development 19: 1040–52.

Carmen AA, Milne L, Grunstein M (2002) Acetylation of the yeast histone H4 N terminus regulates its binding to heterochromatin protein SIR3. The Journal of Biological Chemistry 277: 4778–81.

Chang F, May CD, Hoggard T, Miller J, Fox CA, Weinreich M (2011) High-resolution analysis of four efficient yeast replication origins reveals new insights into the ORC and putative MCM binding elements. Nucleic Acids Research 39: 6523–35.

Chen S, Bell SP (2011) CDK prevents Mcm2-7 helicase loading by inhibiting Cdt1 interaction with Orc6. Genes & Development 25: 363–72.

Conti C, Saccà B, Herrick J, Lalou C, Pommier Y, Bensimon A (2007) Replication fork velocities at adjacent replication origins are coordinately modified during DNA replication in human cells. Molecular Biology of the Cell 18: 3059–67.

Cosgrove AJ, Nieduszynski CA, Donaldson AD (2002) Ku complex controls the replication time of DNA in telomere regions. Genes & Development 16: 2485–90.

Crampton A, Chang F, Pappas DL, Frisch RL, Weinreich M (2008) An ARS element inhibits DNA replication through a SIR2-dependent mechanism. Molecular Cell 30: 156–66.

Dahmann C, Diffley JF, Nasmyth KA (1995) S-phase-promoting cyclin-dependent kinases prevent re-replication by inhibiting the transition of replication origins to a pre-replicative state. Current Biology 5: 1257–69.

David L, Huber W, Granovskaia M, Toedling J, Palm CJ, Bofkin L, Jones T, Davis RW, Steinmetz LM (2006) A high-resolution map of transcription in the yeast genome. Proceedings of the National Academy of Sciences of the United States of America 103: 5320–5.

Davis CA, Ares M (2006) Accumulation of unstable promoter-associated transcripts upon loss of the nuclear exosome subunit Rrp6p in Saccharomyces cerevisiae. Proceedings of the National Academy of Sciences of the United States of America 103: 3262–7.

Diffley JF (2011) Quality control in the initiation of eukaryotic DNA replication. Philosophical Transactions of the Royal Society of London, Biological Sciences 366: 3545–53.

Donovan S, Harwood J, Drury LS, Diffley JF (1997) Cdc6p-dependent loading of Mcm proteins onto pre-replicative chromatin in budding yeast. Proceedings

of the National Academy of Sciences of the United States of America 94: 5611–6.

Drury LS, Perkins G, Diffley JF (2000) The cyclin-dependent kinase Cdc28p regulates distinct modes of Cdc6p proteolysis during the budding yeast cell cycle. Current Biology 10: 231–40.

Duan Z, Andronescu M, Schutz K, McIlwain S, Kim YJ, Lee C, Shendure J, Fields S, Blau CA, Noble WS (2010) A three-dimensional model of the yeast genome. Nature 465: 363–7.

Dubey DD, Davis LR, Greenfeder SA, Ong LY, Zhu JG, Broach JR, Newlon CS, Huberman JA (1991) Evidence suggesting that the ARS elements associated with silencers of the yeast mating-type locus HML do not function as chromosomal DNA replication origins. Molecular and Cellular Biology 11: 5346–55.

Duncker BP, Shimada K, Tsai-Pflugfelder M, Pasero P, Gasser SM (2002) An N-terminal domain of Dbf4p mediates interaction with both origin recognition complex (ORC) and Rad53p and can deregulate late origin firing. Proceedings of the National Academy of Sciences of the United States of America 99: 16087–92.

Eaton ML, Galani K, Kang S, Bell SP, MacAlpine DM (2010) Conserved nucleosome positioning defines replication origins. Genes & Development 24: 748–53.

Elsasser S, Chi Y, Yang P, Campbell JL (1999) Phosphorylation controls timing of Cdc6p destruction: A biochemical analysis. Molecular Biology of the Cell 10: 3263–77.

Evrin C, Clarke P, Zech J, Lurz R, Sun J, Uhle S, Li H, Stillman B, Speck C (2009) A double-hexameric MCM2-7 complex is loaded onto origin DNA during licensing of eukaryotic DNA replication. Proceedings of the National Academy of Sciences of the United States of America 106: 20240–5.

Ferguson BM, Brewer BJ, Reynolds AE, Fangman WL (1991) A yeast origin of replication is activated late in S phase. Cell 65: 507–15.

Ferguson BM, Fangman WL (1992) A position effect on the time of replication origin activation in yeast. Cell 68: 333–9.

Ferreira MF, Santocanale C, Drury LS, Diffley JF (2000) Dbf4p, an essential S phase-promoting factor, is targeted for degradation by the anaphase-promoting complex. Molecular and Cellular Biology 20: 242–8.

Field Y, Kaplan N, Fondufe-Mittendorf Y, Moore IK, Sharon E, Lubling Y, Widom J, Segal E (2008) Distinct modes of regulation by chromatin encoded through nucleosome positioning signals. PLoS Computational Biology 4: e1000216.

Fisher TS, Zakian VA (2005) Ku: a multifunctional protein involved in telomere maintenance. DNA Repair 4: 1215–26.

Flanagan JF, Peterson CL (1999) A role for the yeast SWI/SNF complex in DNA replication. Nucleic Acids Research 27: 2022–8.

Fletcher RJ, Bishop BE, Leon RP, Sclafani Ra, Ogata CM, Chen XS (2003) The structure and function of MCM from archaeal M. thermoautotrophicum. Nature Structural Biology 10: 160–7.

Francis LI, Randell JCW, Takara TJ, Uchima L, Bell SP (2009) Incorporation into the prereplicative complex activates the Mcm2-7 helicase for Cdc7-Dbf4 phosphorylation. Genes & Development 23: 643–54.

French S (1992) Consequences of replication fork movement through transcription units in vivo. Science 258: 1362–5.

Friedman KL, Brewer BJ, Fangman WL (1997) Replication profile of Saccharomyces cerevisiae chromosome VI. Genes to Cells 2: 667–78.

Friedman KL, Diller JD, Ferguson BM, Nyland SV, Brewer BJ, Fangman WL (1996) Multiple determinants controlling activation of yeast replication origins late in S phase. Genes & Development 10: 1595–607.

Gambus A, Jones RC, Sanchez-Diaz A, Kanemaki M, van Deursen F, Edmondson RD, Labib K (2006) GINS maintains association of Cdc45 with MCM in replisome progression complexes at eukaryotic DNA replication forks. Nature Cell Biology 8: 358–66.

Giannattasio M, Lazzaro F, Longhese MP, Plevani P, Muzi-Falconi M (2004) Physical and functional interactions between nucleotide excision repair and DNA damage checkpoint. The EMBO Journal 23: 429–38.

Goffeau A, Barrell BG, Bussey H, Davis RW, Dujon B, Feldmann H, Galibert F, Hoheisel JD, Jacq C, Johnston M, Louis EJ, Mewes HW, Murakami Y, Philippsen P, Tettelin H, Oliver SG (1996) Life with 6000 genes. Science 274: 546, 563–7.

Goren A, Tabib A, Hecht M, Cedar H (2008) DNA replication timing of the human beta-globin domain is controlled by histone modification at the origin. Genes & Development 22: 1319–24.

Hardy CF, Dryga O, Seematter S, Pahl PM, Sclafani RA (1997) mcm5/cdc46-bob1 bypasses the requirement for the S phase activator Cdc7p. Proceedings of the National Academy of Sciences of the United States of America 94: 3151–5.

Harju S, Fedosyuk H, Peterson KR (2004) Rapid isolation of yeast genomic DNA: Bust n' Grab. BMC Biotechnology 4: 8.

Harvey KJ, Newport J (2003) Metazoan origin selection: origin recognition complex chromatin binding is regulated by CDC6 recruitment and ATP hydrolysis. The Journal of Biological Chemistry 278: 48524–8.

Hassan AB, Errington RJ, White NS, Jackson DA, Cook PR (1994) Replication and transcription sites are colocalized in human cells. Journal of Cell Science 107 (Pt 2: 425–34.

Heller RC, Kang S, Lam WM, Chen S, Chan CS, Bell SP (2011) Eukaryotic origin-dependent DNA replication in vitro reveals sequential action of DDK and S-CDK kinases. Cell 146: 80–91.

Hollenhorst PC, Pietz G, Fox CA (2001) Mechanisms controlling differential promoter-occupancy by the yeast forkhead proteins Fkh1p and Fkh2p: implications for regulating the cell cycle and differentiation. Genes & Development 15: 2445–56.

Hua XH, Newport J (1998) Identification of a preinitiation step in DNA replication that is independent of origin recognition complex and cdc6, but dependent on cdk2. The Journal of Cell Biology 140: 271–81.

Huvet M, Nicolay S, Touchon M, Audit B, D'Aubenton-Carafa Y, Arneodo A, Thermes C (2007) Human gene organization driven by the coordination of replication and transcription. Genome Research 17: 1278–85.

Hyrien O, Marheineke K, Goldar A (2003) Paradoxes of eukaryotic DNA replication: MCM proteins and the random completion problem. BioEssays 25: 116–25.

Ilves I, Petojevic T, Pesavento JJ, Botchan MR (2010) Activation of the MCM2-7 helicase by association with Cdc45 and GINS proteins. Molecular Cell 37: 247–58.

Ito H, Fukuda Y, Murata K, Kimura A (1983) Transformation of intact yeast cells treated with alkali cations. Journal of Bacteriology 153: 163–8.

Iyer LM, Leipe DD, Koonin EV, Aravind L (2004) Evolutionary history and higher order classification of AAA+ ATPases. Journal of Structural Biology 146: 11–31.

Jackson LP, Reed SI, Haase SB (2006) Distinct mechanisms control the stability of the related S-phase cyclins Clb5 and Clb6. Molecular and Cellular Biology 26: 2456–66.

Jiang C, Pugh BF (2009) A compiled and systematic reference map of nucleosome positions across the Saccharomyces cerevisiae genome. Genome Biology 10: R109.

Kamimura Y, Tak YS, Sugino A, Araki H (2001) Sld3, which interacts with Cdc45 (Sld4), functions for chromosomal DNA replication in Saccharomyces cerevisiae. The EMBO Journal 20: 2097–107.

Kanemaki M, Labib K (2006) Distinct roles for Sld3 and GINS during establishment and progression of eukaryotic DNA replication forks. The EMBO Journal 25: 1753–63.

Kanemaki M, Sanchez-Diaz A, Gambus A, Labib K (2003) Functional proteomic identification of DNA replication proteins by induced proteolysis in vivo. Nature 423: 720–4.

Kanke M, Kodama Y, Takahashi TS, Nakagawa T, Masukata H (2012) Mcm10 plays an essential role in origin DNA unwinding after loading of the CMG components. The EMBO Journal 31: 2182–94.

Kawasaki Y, Kim HD, Kojima A, Seki T, Sugino A (2006) Reconstitution of Saccharomyces cerevisiae prereplicative complex assembly in vitro. Genes to Cells 11: 745–56.

Klemm RD, Austin RJ, Bell SP (1997) Coordinate binding of ATP and origin DNA regulates the ATPase activity of the origin recognition complex. Cell 88: 493–502.

Klemm RD, Bell SP (2001) ATP bound to the origin recognition complex is important for preRC formation. Proceedings of the National Academy of Sciences of the United States of America 98: 8361–7.

Knott SRV, Peace JM, Ostrow AZ, Gan Y, Rex AE, Viggiani CJ, Tavaré S, Aparicio OM (2012) Forkhead transcription factors establish origin timing and long-range clustering in S. cerevisiae. Cell 148: 99–111.

Knott SRV, Viggiani CJ, Tavaré S, Aparicio OM (2009) Genome-wide replication profiles indicate an expansive role for Rpd3L in regulating replication initiation timing or efficiency, and reveal genomic loci of Rpd3 function in Saccharomyces cerevisiae. Genes & Development 23: 1077–90.

Kushnirov VV (2000) Rapid and reliable protein extraction from yeast. Yeast 16: 857–60.

Lõoke M, Kristjuhan K, Värv S, Kristjuhan A (2013) Chromatin-dependent and -independent regulation of DNA replication origin activation in budding yeast. EMBO Reports 14: 191–8.

Lõoke M, Reimand J, Sedman T, Sedman J, Järvinen L, Värv S, Peil K, Kristjuhan K, Vilo J, Kristjuhan A (2010) Relicensing of transcriptionally inactivated replication origins in budding yeast. The Journal of Biological Chemistry 285: 40004–11.

Labib K, Diffley JF, Kearsey SE (1999) G1-phase and B-type cyclins exclude the DNA-replication factor Mcm4 from the nucleus. Nature Cell Biology 1: 415–22.

Labib K, Tercero JA, Diffley JF (2000) Uninterrupted MCM2-7 function required for DNA replication fork progression. Science 288: 1643–7.

Laroche T, Martin SG, Gotta M, Gorham HC, Pryde FE, Louis EJ, Gasser SM (1998) Mutation of yeast Ku genes disrupts the subnuclear organization of telomeres. Current Biology 8: 653–6.

Lee DG, Bell SP (1997) Architecture of the yeast origin recognition complex bound to origins of DNA replication. Molecular and Cellular Biology 17: 7159–68.

Lei M, Kawasaki Y, Tye BK (1996) Physical interactions among Mcm proteins and effects of Mcm dosage on DNA replication in Saccharomyces cerevisiae. Molecular and Cellular Biology 16: 5081–90.

Li JJ, Herskowitz I (1993) Isolation of ORC6, a component of the yeast origin recognition complex by a one-hybrid system. Science 262: 1870–4.

Ling M, Merante F, Robinson BH (1995) A rapid and reliable DNA preparation method for screening a large number of yeast clones by polymerase chain reaction. Nucleic Acids Research 23: 4924–5.

Lipford JR, Bell SP (2001) Nucleosomes positioned by ORC facilitate the initiation of DNA replication. Molecular Cell 7: 21–30.

Loog M, Morgan DO (2005) Cyclin specificity in the phosphorylation of cyclin-dependent kinase substrates. Nature 434: 104–8.

Lopes M, Cotta-Ramusino C, Pellicioli A, Liberi G, Plevani P, Muzi-Falconi M, Newlon CS, Foiani M (2001) The DNA replication checkpoint response stabilizes stalled replication forks. Nature 412: 557–61.

Maiorano D, Moreau J, Méchali M (2000) XCDT1 is required for the assembly of pre-replicative complexes in Xenopus laevis. Nature 404: 622–5.

Mantiero D, Mackenzie A, Donaldson A, Zegerman P (2011) Limiting replication initiation factors execute the temporal programme of origin firing in budding yeast. The EMBO Journal 30: 4805–14.

Marahrens Y, Stillman B (1992) A yeast chromosomal origin of DNA replication defined by multiple functional elements. Science 255: 817–23.

Masai H, Arai KI (2002) Cdc7 kinase complex: a key regulator in the initiation of DNA replication. Journal of Cellular Physiology 190: 287–96.

Masai H, Taniyama C, Ogino K, Matsui E, Kakusho N, Matsumoto S, Kim JM, Ishii A, Tanaka T, Kobayashi T, Tamai K, Ohtani K, Arai KI (2006) Phosphorylation of MCM4 by Cdc7 kinase facilitates its interaction with Cdc45 on the chromatin. The Journal of Biological Chemistry 281: 39249–61.

Masumoto H, Muramatsu S, Kamimura Y, Araki H (2002) S-Cdk-dependent phosphorylation of Sld2 essential for chromosomal DNA replication in budding yeast. Nature 415: 651–5.

McCune HJ, Danielson LS, Alvino GM, Collingwood D, Delrow JJ, Fangman WL, Brewer BJ, Raghuraman MK (2008) The temporal program of chromosome replication: genomewide replication in clb5 (delta) Saccharomyces cerevisiae. Genetics 180: 1833–47.

McGlynn P, Savery NJ, Dillingham MS (2012) The conflict between DNA replication and transcription. Molecular Microbiology 85: 12–20.

McInerny CJ, Partridge JF, Mikesell GE, Creemer DP, Breeden LL (1997) A novel Mcm1-dependent element in the SWI4, CLN3, CDC6, and CDC47 promoters activates M/G1-specific transcription. Genes & Development 11: 1277–88.

Mimitou EP, Symington LS (2008) Sae2, Exo1 and Sgs1 collaborate in DNA double-strand break processing. Nature 455: 770–4.

Mimura S, Seki T, Tanaka S, Diffley JF (2004) Phosphorylation-dependent binding of mitotic cyclins to Cdc6 contributes to DNA replication control. Nature 431: 1118–23.

Mizushima T, Takahashi N, Stillman B (2000) Cdc6p modulates the structure and DNA binding activity of the origin recognition complex in vitro. Genes & Development 14: 1631–41.

Mori S, Shirahige K (2007) Perturbation of the activity of replication origin by meiosis-specific transcription. The Journal of Biological Chemistry 282: 4447–52.

Moyer SE, Lewis PW, Botchan MR (2006) Isolation of the Cdc45/Mcm2-7/GINS (CMG) complex, a candidate for the eukaryotic DNA replication fork helicase. Proceedings of the National Academy of Sciences of the United States of America 103: 10236–41.

Muramatsu S, Hirai K, Tak YS, Kamimura Y, Araki H (2010) CDK-dependent complex formation between replication proteins Dpb11, Sld2, Pol (epsilon), and GINS in budding yeast. Genes & Development 24: 602–12.

Nedelcheva MN, Roguev A, Dolapchiev LB, Shevchenko A, Taskov HB, Shevchenko A, Stewart AF, Stoynov SS (2005) Uncoupling of unwinding from DNA synthesis implies regulation of MCM helicase by Tof1/Mrc1/Csm3 checkpoint complex. Journal of Molecular Biology 347: 509–21.

Neil H, Malabat C, D'Aubenton-Carafa Y, Xu Z, Steinmetz LM, Jacquier A (2009) Widespread bidirectional promoters are the major source of cryptic transcripts in yeast. Nature 457: 1038–42.

Nguyen VQ, Co C, Irie K, Li JJ (2000) Clb/Cdc28 kinases promote nuclear export of the replication initiator proteins Mcm2-7. Current Biology 10: 195–205.

Nguyen VQ, Co C, Li JJ (2001) Cyclin-dependent kinases prevent DNA re-replication through multiple mechanisms. Nature 411: 1068–73.

Nieduszynski CA, Hiraga Si, Ak P, Benham CJ, Donaldson AD (2007) OriDB: a DNA replication origin database. Nucleic Acids Research 35: D40–6.

Nieduszynski CA, Knox Y, Donaldson AD (2006) Genome-wide identification of replication origins in yeast by comparative genomics. Genes & Development 20: 1874–9.

Pacek M, Walter JC (2004) A requirement for MCM7 and Cdc45 in chromosome unwinding during eukaryotic DNA replication. The EMBO Journal 23: 3667–76.

Pohl TJ, Brewer BJ, Raghuraman MK (2012) Functional centromeres determine the activation time of pericentric origins of DNA replication in Saccharomyces cerevisiae. PLoS Genetics 8: e1002677.

Pryde F, Jain D, Kerr A, Curley R, Mariotti FR, Vogelauer M (2009) H3 k36 methylation helps determine the timing of cdc45 association with replication origins. PloS One 4: e5882.

Raghuraman MK, Winzeler EA, Collingwood D, Hunt S, Wodicka L, Conway A, Lockhart DJ, Davis RW, Brewer BJ, Fangman WL (2001) Replication dynamics of the yeast genome. Science 294: 115–21.

Randell JCW, Bowers JL, Rodríguez HK, Bell SP (2006) Sequential ATP hydrolysis by Cdc6 and ORC directs loading of the Mcm2-7 helicase. Molecular Cell 21: 29–39.

Rao H, Stillman B (1995) The origin recognition complex interacts with a bipartite DNA binding site within yeast replicators. Proceedings of the National Academy of Sciences of the United States of America 92: 2224–8.

Rao PN, Johnson RT (1970) Mammalian cell fusion: studies on the regulation of DNA synthesis and mitosis. Nature 225: 159–64.

Remus D, Beuron F, Tolun G, Griffith JD, Morris EP, Diffley JF (2009) Concerted loading of Mcm2-7 double hexamers around DNA during DNA replication origin licensing. Cell 139: 719–30.

Ricke RM, Bielinsky AK (2004) Mcm10 regulates the stability and chromatin association of DNA polymerase-alpha. Molecular Cell 16: 173–85.

Rothstein R, Michel B, Gangloff S (2000) Replication fork pausing and recombination or "gimme a break". Genes & Development 14: 1–10.

Rouse J, Jackson SP (2002) Lcd1p recruits Mec1p to DNA lesions in vitro and in vivo. Molecular cell 9: 857–69.

Rowles a, Tada S, Blow JJ (1999) Changes in association of the Xenopus origin recognition complex with chromatin on licensing of replication origins. Journal of Cell Science 112: 2011–8.

Rowley A, Cocker JH, Harwood J, Diffley JF (1995) Initiation complex assembly at budding yeast replication origins begins with the recognition of a bipartite sequence by limiting amounts of the initiator, ORC. The EMBO Journal 14: 2631–41.

Rusché LN, Kirchmaier AL, Rine J (2002) Ordered nucleation and spreading of silenced chromatin in Saccharomyces cerevisiae. Molecular Biology of the Cell 13: 2207–22.

Rusché LN, Kirchmaier AL, Rine J (2003) The establishment, inheritance, and function of silenced chromatin in Saccharomyces cerevisiae. Annual Review of Biochemistry 72: 481–516.

Sabouri N, McDonald KR, Webb CJ, Cristea IM, Zakian VA (2012) DNA replication through hard-to-replicate sites, including both highly transcribed RNA Pol II and Pol III genes, requires the S. pombe Pfh1 helicase. Genes & Development 26: 581–93.

Santocanale C, Diffley JF (1998) A Mec1- and Rad53-dependent checkpoint controls late-firing origins of DNA replication. Nature 395: 615–8.

Santocanale C, Sharma K, Diffley JF (1999) Activation of dormant origins of DNA replication in budding yeast. Genes & Development 13: 2360–4.

Sasaki T, Ramanathan S, Okuno Y, Kumagai C, Shaikh SS, Gilbert DM (2006) The Chinese hamster dihydrofolate reductase replication origin decision point follows activation of transcription and suppresses initiation of replication within transcription units. Molecular and Cellular Biology 26: 1051–62.

Schübeler D, Scalzo D, Kooperberg C, van Steensel B, Delrow J, Groudine M (2002) Genome-wide DNA replication profile for Drosophila melanogaster: a link between transcription and replication timing. Nature Genetics 32: 438–42.

Schwacha A, Bell SP (2001) Interactions between two catalytically distinct MCM subgroups are essential for coordinated ATP hydrolysis and DNA replication. Molecular Cell 8: 1093–104.

Sclafani RA (2000) Cdc7p-Dbf4p becomes famous in the cell cycle. Journal of Cell Science 113: 2111–7.

Sharma K, Weinberger M, Huberman JA (2001) Roles for internal and flanking sequences in regulating the activity of mating-type-silencer-associated replication origins in Saccharomyces cerevisiae. Genetics 159: 35–45.

Sheu YJ, Stillman B (2006) Cdc7-Dbf4 phosphorylates MCM proteins via a docking site-mediated mechanism to promote S phase progression. Molecular Cell 24: 101–13.

Shirahige K, Hori Y, Shiraishi K, Yamashita M, Takahashi K, Obuse C, Tsurimoto T, Yoshikawa H (1998) Regulation of DNA-replication origins during cell-cycle progression. Nature 395: 618–21.

Simpson RT (1990) Nucleosome positioning can affect the function of a cis-acting DNA element in vivo. Nature 343: 387–9.

Srivatsan A, Tehranchi A, MacAlpine DM, Wang JD (2010) Co-orientation of replication and transcription preserves genome integrity. PLoS Genetics 6: e1000810.

Stevenson JB, Gottschling DE (1999) Telomeric chromatin modulates replication timing near chromosome ends. Genes & Development 13: 146–51.

Stinchcomb DT, Struhl K, Davis RW (1979) Isolation and characterisation of a yeast chromosomal replicator. Nature 282: 39–43.

Struhl K, Stinchcomb DT, Scherer S, Davis RW (1979) High-frequency transformation of yeast: autonomous replication of hybrid DNA molecules. Proceedings of the National Academy of Sciences of the United States of America 76: 1035–9.

Sun K, Coïc E, Zhou Z, Durrens P, Haber JE (2002) Saccharomyces forkhead protein Fkh1 regulates donor preference during mating-type switching through the recombination enhancer. Genes & Development 16: 2085–96.

Szyjka SJ, Aparicio JG, Viggiani CJ, Knott S, Xu W, Tavaré S, Aparicio OM (2008) Rad53 regulates replication fork restart after DNA damage in Saccharomyces cerevisiae. Genes & Development 22: 1906–20.

Takayama Y, Kamimura Y, Okawa M, Muramatsu S, Sugino A, Araki H (2003) GINS, a novel multiprotein complex required for chromosomal DNA replication in budding yeast. Genes & Development 17: 1153–65.

Tanaka S, Halter D, Livingstone-Zatchej M, Reszel B, Thoma F (1994) Transcription through the yeast origin of replication ARS1 ends at the ABFI binding site and affects extrachromosomal maintenance of minichromosomes. Nucleic Acids Research 22: 3904–10.

Tanaka S, Nakato R, Katou Y, Shirahige K, Araki H (2011) Origin association of Sld3, Sld7, and Cdc45 proteins is a key step for determination of origin-firing timing. Current Biology 21: 2055–63.

Tanaka S, Umemori T, Hirai K, Muramatsu S, Kamimura Y, Araki H (2007) CDK-dependent phosphorylation of Sld2 and Sld3 initiates DNA replication in budding yeast. Nature 445: 328–32.

Tanaka T, Nasmyth K (1998) Association of RPA with chromosomal replication origins requires an Mcm protein, and is regulated by Rad53, and cyclin- and Dbf4-dependent kinases. The EMBO Journal 17: 5182–91.

Tercero JA, Diffley JF (2001) Regulation of DNA replication fork progression through damaged DNA by the Mec1/Rad53 checkpoint. Nature 412: 553–7.

Tercero JA, Labib K, Diffley JF (2000) DNA synthesis at individual replication forks requires the essential initiation factor Cdc45p. The EMBO Journal 19: 2082–93.

Theis JF, Newlon CS (1997) The ARS309 chromosomal replicator of Saccharomyces cerevisiae depends on an exceptional ARS consensus sequence. Proceedings of the National Academy of Sciences of the United States of America 94: 10786–91.

Thompson JR, Register E, Curotto J, Kurtz M, Kelly R (1998) An improved protocol for the preparation of yeast cells for transformation by electroporation. Yeast 14: 565–71.

Tiengwe C, Marcello L, Farr H, Dickens N, Kelly S, Swiderski M, Vaughan D, Gull K, Barry JD, Bell SD, McCulloch R (2012) Genome-wide analysis reveals extensive functional interaction between DNA replication initiation and transcription in the genome of Trypanosoma brucei. Cell Reports 2: 185–97.

Torres-Rosell J, De Piccoli G, Cordon-Preciado V, Farmer S, Jarmuz A, Machin F, Pasero P, Lisby M, Haber JE, Aragón L (2007) Anaphase onset before complete DNA replication with intact checkpoint responses. Science 315: 1411–5.

Umek RM, Kowalski D (1988) The ease of DNA unwinding as a determinant of initiation at yeast replication origins. Cell 52: 559–67.

Unnikrishnan A, Gafken PR, Tsukiyama T (2010) Dynamic changes in histone acetylation regulate origins of DNA replication. Nature Structural & Molecular Biology 17: 430–7.

van Deursen F, Sengupta S, De Piccoli G, Sanchez-Diaz A, Labib K (2012) Mcm10 associates with the loaded DNA helicase at replication origins and defines a novel step in its activation. The EMBO Journal 31: 2195–206.

Varrin AE, Prasad AA, Scholz RP, Ramer MD, Duncker BP (2005) A mutation in Dbf4 motif M impairs interactions with DNA replication factors and confers increased resistance to genotoxic agents. Molecular and Cellular Biology 25: 7494–504.

Venditti P, Costanzo G, Negri R, Camilloni G (1994) ABFI contributes to the chromatin organization of Saccharomyces cerevisiae ARS1 B-domain. Biochimica et Biophysica Acta 1219: 677–89.

Vilette D, Ehrlich SD, Michel B (1996) Transcription-induced deletions in plasmid vectors: M13 DNA replication as a source of instability. Molecular & General Genetics 252: 398–403.

Vogelauer M, Rubbi L, Lucas I, Brewer BJ, Grunstein M (2002) Histone acetylation regulates the time of replication origin firing. Molecular Cell 10: 1223–33.

Vujcic M, Miller CA, Kowalski D (1999) Activation of silent replication origins at autonomously replicating sequence elements near the HML locus in budding yeast. Molecular and Cellular Biology 19: 6098–109.

Walker SS, Malik AK, Eisenberg S (1991) Analysis of the interactions of functional domains of a nuclear origin of replication from Saccharomyces cerevisiae. Nucleic Acids Research 19: 6255–62.

Walter J, Newport J (2000) Initiation of eukaryotic DNA replication: origin unwinding and sequential chromatin association of Cdc45, RPA, and DNA polymerase alpha. Molecular Cell 5: 617–27.

Watase G, Takisawa H, Kanemaki MT (2012) Mcm10 plays a role in functioning of the eukaryotic replicative DNA helicase, Cdc45-Mcm-GINS. Current Biology 22: 343–9.

Weinreich M, Liang C, Stillman B (1999) The Cdc6p nucleotide-binding motif is required for loading mcm proteins onto chromatin. Proceedings of the National Academy of Sciences of the United States of America 96: 441–6.

White EJ, Emanuelsson O, Scalzo D, Royce T, Kosak S, Oakeley EJ, Weissman S, Gerstein M, Groudine M, Snyder M, Schübeler D (2004) DNA replication-timing analysis of human chromosome 22 at high resolution and different developmental states. Proceedings of the National Academy of Sciences of the United States of America 101: 17771–6.

Wilmes GM, Archambault V, Austin RJ, Jacobson MD, Bell SP, Cross FR (2004) Interaction of the S-phase cyclin Clb5 with an "RXL" docking sequence in the initiator protein Orc6 provides an origin-localized replication control switch. Genes & Development 18: 981–91.

Woodfine K, Beare DM, Ichimura K, Debernardi S, Mungall AJ, Fiegler H, Collins VP, Carter NP, Dunham I (2005) Replication timing of human chromosome 6. Cell Cycle 4: 172–6.

Wuarin J, Buck V, Nurse P, Millar JBA (2002) Stable association of mitotic cyclin B/Cdc2 to replication origins prevents endoreduplication. Cell 111: 419–31.

Wyers F, Rougemaille M, Badis G, Rousselle JC, Dufour ME, Boulay J, Régnault B, Devaux F, Namane A, Séraphin B, Libri D, Jacquier A (2005) Cryptic pol II transcripts are degraded by a nuclear quality control pathway involving a new poly(A) polymerase. Cell 121: 725–37.

Wyrick JJ, Aparicio JG, Chen T, Barnett JD, Jennings EG, Young RA, Bell SP, Aparicio OM (2001) Genome-wide distribution of ORC and MCM proteins in S. cerevisiae: high-resolution mapping of replication origins. Science 294: 2357–60.

Xu Z, Wei W, Gagneur J, Perocchi F, Clauder-Münster S, Camblong J, Guffanti E, Stutz F, Huber W, Steinmetz LM (2009) Bidirectional promoters generate pervasive transcription in yeast. Nature 457: 1033–7.

Yabuki N, Terashima H, Kitada K (2002) Mapping of early firing origins on a replication profile of budding yeast. Genes to Cells 7: 781–9.

Yamashita M, Hori Y, Shinomiya T, Obuse C, Tsurimoto T, Yoshikawa H, Shirahige K (1997) The efficiency and timing of initiation of replication of multiple replicons of Saccharomyces cerevisiae chromosome VI. Genes to Cells 2: 655–65.

Zappulla DC, Sternglanz R, Leatherwood J (2002) Control of replication timing by a transcriptional silencer. Current Biology 12: 869–75.

Zegerman P, Diffley JF (2007) Phosphorylation of Sld2 and Sld3 by cyclin-dependent kinases promotes DNA replication in budding yeast. Nature 445: 281–5.

Zhang Z, Hayashi MK, Merkel O, Stillman B, Xu RM (2002) Structure and function of the BAH-containing domain of Orc1p in epigenetic silencing. The EMBO Journal 21: 4600–11.

Zhu Z, Chung WH, Shim EY, Lee SE, Ira G (2008) Sgs1 helicase and two nucleases Dna2 and Exo1 resect DNA double-strand break ends. Cell 134: 981–94.

Zou L, Elledge SJ (2003) Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. Science 300: 1542–8.

Zou L, Stillman B (2000) Assembly of a complex containing Cdc45p, replication protein A, and Mcm2p at replication origins controlled by S-phase cyclin-dependent kinases and Cdc7p-Dbf4p kinase. Molecular and Cellular Biology 20: 3086–96.

Zwerschke W, Rottjakob HW, Küntzel H (1994) The Saccharomyces cerevisiae CDC6 gene is transcribed at late mitosis and encodes a ATP/GTPase controlling S phase initiation. The Journal of Biological Chemistry 269: 23351–6.

ACKNOWLEDGEMENTS

First and foremost I would like to thank my supervisor Arnold Kristjuhan for all the hard work and patience put into my education as a researcher. Much assistance has been provided by Kersti Kristjuhan and all the other lab members of our chromatin research group. In addition, I would like to thank all the coauthors of the papers presented in this thesis. Kersti also pointed out that Signe's name should be mentioned in this section. So, thank you Signe! My gratitude also goes to Tiina Tamm and Nikita Avvakumov for critical reading and helpful notes on this thesis.

Last, but definitely not least, I would like to thank my parents Reet and Viktor, whose support has made the now twenty-something year circling in the educational system possible. The time spent without latex gloves has been mighty fun thanks to my friends Kuldar, Ott, Liis, Jaanis, Annely, Paula, Rebane and my girlfriend Kristel.

PUBLICATIONS

CURRICULUM VITAE

Name:	Marko Lõoke
Date of birth:	17.07.1982
Citizenship:	Estonian
Address:	Department of Cell Biology
	Institute of Molecular and Cell Biology, University of Tartu,
	Riia 23, 51010
Telephone:	+372 55572686
E-mail:	marko.looke@ut.ee

Education:

2007 - ...	University of Tartu, doctoral studies in molecular and cell biology
2007	University of Tartu, MSc in molecular and cell biology
2005	University of Tartu, BSc in gene technology
2001	Hugo Treffner Gymnasium

Working experience:

2007 - ...	University of Tartu, Specialist

List of publications:

1. Lõoke M, Kristjuhan K, Värv S, Kristjuhan A (2013) Chromatin-dependent and -independent regulation of DNA replication origin activation in budding yeast. EMBO reports, 14(2):191–8.

2. Kadaja-Saarepuu L, Lõoke M, Balikova A, Maimets T (2012) Tumor suppressor p53 down-regulates expression of human leukocyte marker CD43 in non-hematopoietic tumor cells. International Journal of Oncology 40: 567–76.

3. Lõoke M, Kristjuhan K, Kristjuhan A (2011) Extraction of genomic DNA from yeasts for PCR-based applications. BioTechniques, 50: 325–8.

4. Peil K, Värv S, Lõoke M, Kristjuhan K, Kristjuhan A (2011) Uniform distribution of elongating RNA polymerase II complexes in transcribed gene locus. The Journal of Biological Chemistry 286: 23817–22.

5. Lõoke M, Reimand J, Sedman T, Sedman J, Järvinen L, Värv S, Peil K, Kristjuhan K, Vilo J, Kristjuhan A (2010) Relicensing of transcriptionally inactivated replication origins in budding yeast. The Journal of Biological Chemistry 285: 40004–11.

6. Värv S, Kristjuhan K, Peil K, Lõoke M, Mahlakõiv T, Paapsi K, Kristjuhan A (2010) Acetylation of H3 K56 is required for RNA polymerase II transcript elongation through heterochromatin in yeast. Molecular and Cellular Biology 30: 1467–77.

7. Kadaja-Saarepuu L, Laos S, Jääger K, Viil J, Balikova A, Lõoke M, Hansson GC, Maimets T (2008) CD43 promotes cell growth and helps to evade FAS- mediated apoptosis in non-hematopoietic cancer cells lacking the tumor suppressors p53 or ARF. Oncogene 27: 1705–15.

ELULOOKIRJELDUS

Nimi:	Marko Lõoke
Sünniaeg:	17.07.1982
Kodakondsus:	Estonian
Aadress:	Rakubioloogia Õppetool
	Molekulaar- ja Rakuboloogia Instituut, Tartu Ülikool,
	Riia 23, 51010
Telefon:	+372 55572686
E-mail:	marko.looke@ut.ee

Haridus:

2007 - ...	Tartu Ülikool, doktorantuur molekulaar- ja rakubioloogias
2007	Tartu Ülikool, MSc molekulaar- ja rakubioloogias
2005	Tartu Ülikool, BSc molekulaar- ja rakubioloogias
2001	Hugo Treffneri Gümnaasium

Teenistuskäik:

2007 - ...	Tartu Ülikool, Spetsialist

Publikatsioonid:

1. Lõoke M, Kristjuhan K, Värv S, Kristjuhan A (2013) Chromatin-dependent and -independent regulation of DNA replication origin activation in budding yeast. EMBO reports, 14(2):191–8.

2. Kadaja-Saarepuu L, Lõoke M, Balikova A, Maimets T (2012) Tumor suppressor p53 down-regulates expression of human leukocyte marker CD43 in non-hematopoietic tumor cells. International Journal of Oncology 40: 567–76.

3. Lõoke M, Kristjuhan K, Kristjuhan A (2011) Extraction of genomic DNA from yeasts for PCR-based applications. BioTechniques 50: 325–8.

4. Peil K, Värv S, Lõoke M, Kristjuhan K, Kristjuhan A (2011) Uniform distribution of elongating RNA polymerase II complexes in transcribed gene locus. The Journal of Biological Chemistry 286: 23817–22.

5. Lõoke M, Reimand J, Sedman T, Sedman J, Järvinen L, Värv S, Peil K, Kristjuhan K, Vilo J, Kristjuhan A (2010) Relicensing of transcriptionally inactivated replication origins in budding yeast. The Journal of Biological Chemistry 285: 40004–11.

6. Värv S, Kristjuhan K, Peil K, Lõoke M, Mahlakõiv T, Paapsi K, Kristjuhan A (2010) Acetylation of H3 K56 is required for RNA polymerase II transcript elongation through heterochromatin in yeast. Molecular and Cellular Biology 30: 1467–77.

7. Kadaja-Saarepuu L, Laos S, Jääger K, Viil J, Balikova A, Lõoke M, Hansson GC, Maimets T (2008) CD43 promotes cell growth and helps to evade FAS- mediated apoptosis in non-hematopoietic cancer cells lacking the tumor suppressors p53 or ARF. Oncogene 27: 1705–15.

DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS

1. **Toivo Maimets**. Studies of human oncoprotein p53. Tartu, 1991, 96 p.
2. **Enn K. Seppet**. Thyroid state control over energy metabolism, ion transport and contractile functions in rat heart. Tartu, 1991, 135 p.
3. **Kristjan Zobel**. Epifüütsete makrosamblike väärtus õhu saastuse indikaatoritena Hamar-Dobani boreaalsetes mägimetsades. Tartu, 1992, 131 lk.
4. **Andres Mäe**. Conjugal mobilization of catabolic plasmids by transposable elements in helper plasmids. Tartu, 1992, 91 p.
5. **Maia Kivisaar**. Studies on phenol degradation genes of *Pseudomonas* sp. strain EST 1001. Tartu, 1992, 61 p.
6. **Allan Nurk**. Nucleotide sequences of phenol degradative genes from *Pseudomonas sp.* strain EST 1001 and their transcriptional activation in *Pseudomonas putida.* Tartu, 1992, 72 p.
7. **Ülo Tamm**. The genus *Populus* L. in Estonia: variation of the species biology and introduction. Tartu, 1993, 91 p.
8. **Jaanus Remme**. Studies on the peptidyltransferase centre of the *E.coli* ribosome. Tartu, 1993, 68 p.
9. **Ülo Langel**. Galanin and galanin antagonists. Tartu, 1993, 97 p.
10. **Arvo Käärd**. The development of an automatic online dynamic fluorescense-based pH-dependent fiber optic penicillin flowthrought biosensor for the control of the benzylpenicillin hydrolysis. Tartu, 1993, 117 p.
11. **Lilian Järvekülg**. Antigenic analysis and development of sensitive immunoassay for potato viruses. Tartu, 1993, 147 p.
12. **Jaak Palumets**. Analysis of phytomass partition in Norway spruce. Tartu, 1993, 47 p.
13. **Arne Sellin**. Variation in hydraulic architecture of *Picea abies* (L.) Karst. trees grown under different enviromental conditions. Tartu, 1994, 119 p.
13. **Mati Reeben**. Regulation of light neurofilament gene expression. Tartu, 1994, 108 p.
14. **Urmas Tartes**. Respiration rhytms in insects. Tartu, 1995, 109 p.
15. **Ülo Puurand.** The complete nucleotide sequence and infections *in vitro* transcripts from cloned cDNA of a potato A potyvirus. Tartu, 1995, 96 p.
16. **Peeter Hõrak**. Pathways of selection in avian reproduction: a functional framework and its application in the population study of the great tit (*Parus major*). Tartu, 1995, 118 p.
17. **Erkki Truve**. Studies on specific and broad spectrum virus resistance in transgenic plants. Tartu, 1996, 158 p.
18. **Illar Pata**. Cloning and characterization of human and mouse ribosomal protein S6-encoding genes. Tartu, 1996, 60 p.
19. **Ülo Niinemets**. Importance of structural features of leaves and canopy in determining species shade-tolerance in temperature deciduous woody taxa. Tartu, 1996, 150 p.

20. **Ants Kurg**. Bovine leukemia virus: molecular studies on the packaging region and DNA diagnostics in cattle. Tartu, 1996, 104 p.
21. **Ene Ustav**. E2 as the modulator of the BPV1 DNA replication. Tartu, 1996, 100 p.
22. **Aksel Soosaar**. Role of helix-loop-helix and nuclear hormone receptor transcription factors in neurogenesis. Tartu, 1996, 109 p.
23. **Maido Remm**. Human papillomavirus type 18: replication, transformation and gene expression. Tartu, 1997, 117 p.
24. **Tiiu Kull**. Population dynamics in *Cypripedium calceolus* L. Tartu, 1997, 124 p.
25. **Kalle Olli**. Evolutionary life-strategies of autotrophic planktonic microorganisms in the Baltic Sea. Tartu, 1997, 180 p.
26. **Meelis Pärtel**. Species diversity and community dynamics in calcareous grassland communities in Western Estonia. Tartu, 1997, 124 p.
27. **Malle Leht**. The Genus *Potentilla* L. in Estonia, Latvia and Lithuania: distribution, morphology and taxonomy. Tartu, 1997, 186 p.
28. **Tanel Tenson**. Ribosomes, peptides and antibiotic resistance. Tartu, 1997, 80 p.
29. **Arvo Tuvikene**. Assessment of inland water pollution using biomarker responses in fish *in vivo* and *in vitro*. Tartu, 1997, 160 p.
30. **Urmas Saarma**. Tuning ribosomal elongation cycle by mutagenesis of 23S rRNA. Tartu, 1997, 134 p.
31. **Henn Ojaveer**. Composition and dynamics of fish stocks in the gulf of Riga ecosystem. Tartu, 1997, 138 p.
32. **Lembi Lõugas**. Post-glacial development of vertebrate fauna in Estonian water bodies. Tartu, 1997, 138 p.
33. **Margus Pooga**. Cell penetrating peptide, transportan, and its predecessors, galanin-based chimeric peptides. Tartu, 1998, 110 p.
34. **Andres Saag**. Evolutionary relationships in some cetrarioid genera (Lichenized Ascomycota). Tartu, 1998, 196 p.
35. **Aivar Liiv**. Ribosomal large subunit assembly *in vivo*. Tartu, 1998, 158 p.
36. **Tatjana Oja**. Isoenzyme diversity and phylogenetic affinities among the eurasian annual bromes (*Bromus* L., Poaceae). Tartu, 1998, 92 p.
37. **Mari Moora**. The influence of arbuscular mycorrhizal (AM) symbiosis on the competition and coexistence of calcareous grassland plant species. Tartu, 1998, 78 p.
38. **Olavi Kurina**. Fungus gnats in Estonia (*Diptera: Bolitophilidae, Keroplatidae, Macroceridae, Ditomyiidae, Diadocidiidae, Mycetophilidae*). Tartu, 1998, 200 p.
39. **Andrus Tasa**. Biological leaching of shales: black shale and oil shale. Tartu, 1998, 98 p.
40. **Arnold Kristjuhan.** Studies on transcriptional activator properties of tumor suppressor protein p53. Tartu, 1998, 86 p.

41. **Sulev Ingerpuu.** Characterization of some human myeloid cell surface and nuclear differentiation antigens. Tartu, 1998, 163 p.
42. **Veljo Kisand.** Responses of planktonic bacteria to the abiotic and biotic factors in the shallow lake Võrtsjärv. Tartu, 1998, 118 p.
43. **Kadri Põldmaa.** Studies in the systematics of hypomyces and allied genera (Hypocreales, Ascomycota). Tartu, 1998, 178 p.
44. **Markus Vetemaa.** Reproduction parameters of fish as indicators in environmental monitoring. Tartu, 1998, 117 p.
45. **Heli Talvik.** Prepatent periods and species composition of different *Oesophagostomum* spp. populations in Estonia and Denmark. Tartu, 1998, 104 p.
46. **Katrin Heinsoo.** Cuticular and stomatal antechamber conductance to water vapour diffusion in *Picea abies* (L.) karst. Tartu, 1999, 133 p.
47. **Tarmo Annilo.** Studies on mammalian ribosomal protein S7. Tartu, 1998, 77 p.
48. **Indrek Ots.** Health state indicies of reproducing great tits (*Parus major*): sources of variation and connections with life-history traits. Tartu, 1999, 117 p.
49. **Juan Jose Cantero.** Plant community diversity and habitat relationships in central Argentina grasslands. Tartu, 1999, 161 p.
50. **Rein Kalamees.** Seed bank, seed rain and community regeneration in Estonian calcareous grasslands. Tartu, 1999, 107 p.
51. **Sulev Kõks.** Cholecystokinin (CCK) — induced anxiety in rats: influence of environmental stimuli and involvement of endopioid mechanisms and erotonin. Tartu, 1999, 123 p.
52. **Ebe Sild.** Impact of increasing concentrations of O_3 and CO_2 on wheat, clover and pasture. Tartu, 1999, 123 p.
53. **Ljudmilla Timofejeva.** Electron microscopical analysis of the synaptonemal complex formation in cereals. Tartu, 1999, 99 p.
54. **Andres Valkna.** Interactions of galanin receptor with ligands and G-proteins: studies with synthetic peptides. Tartu, 1999, 103 p.
55. **Taavi Virro.** Life cycles of planktonic rotifers in lake Peipsi. Tartu, 1999, 101 p.
56. **Ana Rebane.** Mammalian ribosomal protein S3a genes and intron-encoded small nucleolar RNAs U73 and U82. Tartu, 1999, 85 p.
57. **Tiina Tamm.** Cocksfoot mottle virus: the genome organisation and translational strategies. Tartu, 2000, 101 p.
58. **Reet Kurg.** Structure-function relationship of the bovine papilloma virus E2 protein. Tartu, 2000, 89 p.
59. **Toomas Kivisild.** The origins of Southern and Western Eurasian populations: an mtDNA study. Tartu, 2000, 121 p.
60. **Niilo Kaldalu.** Studies of the TOL plasmid transcription factor XylS. Tartu 2000. 88 p.

61. **Dina Lepik.** Modulation of viral DNA replication by tumor suppressor protein p53. Tartu 2000. 106 p.
62. **Kai Vellak.** Influence of different factors on the diversity of the bryophyte vegetation in forest and wooded meadow communities. Tartu 2000. 122 p.
63. **Jonne Kotta.** Impact of eutrophication and biological invasionas on the structure and functions of benthic macrofauna. Tartu 2000. 160 p.
64. **Georg Martin.** Phytobenthic communities of the Gulf of Riga and the inner sea the West-Estonian archipelago. Tartu, 2000. 139 p.
65. **Silvia Sepp.** Morphological and genetical variation of *Alchemilla L.* in Estonia. Tartu, 2000. 124 p.
66. **Jaan Liira.** On the determinants of structure and diversity in herbaceous plant communities. Tartu, 2000. 96 p.
67. **Priit Zingel.** The role of planktonic ciliates in lake ecosystems. Tartu 2001. 111 p.
68. **Tiit Teder.** Direct and indirect effects in Host-parasitoid interactions: ecological and evolutionary consequences. Tartu 2001. 122 p.
69. **Hannes Kollist.** Leaf apoplastic ascorbate as ozone scavenger and its transport across the plasma membrane. Tartu 2001. 80 p.
70. **Reet Marits.** Role of two-component regulator system PehR-PehS and extracellular protease PrtW in virulence of *Erwinia Carotovora* subsp. *Carotovora*. Tartu 2001. 112 p.
71. **Vallo Tilgar.** Effect of calcium supplementation on reproductive performance of the pied flycatcher *Ficedula hypoleuca* and the great tit *Parus major,* breeding in Nothern temperate forests. Tartu, 2002. 126 p.
72. **Rita Hõrak.** Regulation of transposition of transposon Tn*4652* in *Pseudomonas putida*. Tartu, 2002. 108 p.
73. **Liina Eek-Piirsoo.** The effect of fertilization, mowing and additional illumination on the structure of a species-rich grassland community. Tartu, 2002. 74 p.
74. **Krõõt Aasamaa.** Shoot hydraulic conductance and stomatal conductance of six temperate deciduous tree species. Tartu, 2002. 110 p.
75. **Nele Ingerpuu.** Bryophyte diversity and vascular plants. Tartu, 2002. 112 p.
76. **Neeme Tõnisson.** Mutation detection by primer extension on oligonucleotide microarrays. Tartu, 2002. 124 p.
77. **Margus Pensa.** Variation in needle retention of Scots pine in relation to leaf morphology, nitrogen conservation and tree age. Tartu, 2003. 110 p.
78. **Asko Lõhmus.** Habitat preferences and quality for birds of prey: from principles to applications. Tartu, 2003. 168 p.
79. **Viljar Jaks.** p53 — a switch in cellular circuit. Tartu, 2003. 160 p.
80. **Jaana Männik.** Characterization and genetic studies of four ATP-binding cassette (ABC) transporters. Tartu, 2003. 140 p.
81. **Marek Sammul.** Competition and coexistence of clonal plants in relation to productivity. Tartu, 2003. 159 p

82. **Ivar Ilves.** Virus-cell interactions in the replication cycle of bovine papillomavirus type 1. Tartu, 2003. 89 p.
83. **Andres Männik.** Design and characterization of a novel vector system based on the stable replicator of bovine papillomavirus type 1. Tartu, 2003. 109 p.
84. **Ivika Ostonen.** Fine root structure, dynamics and proportion in net primary production of Norway spruce forest ecosystem in relation to site conditions. Tartu, 2003. 158 p.
85. **Gudrun Veldre.** Somatic status of 12–15-year-old Tartu schoolchildren. Tartu, 2003. 199 p.
86. **Ülo Väli.** The greater spotted eagle *Aquila clanga* and the lesser spotted eagle *A. pomarina*: taxonomy, phylogeography and ecology. Tartu, 2004. 159 p.
87. **Aare Abroi.** The determinants for the native activities of the bovine papillomavirus type 1 E2 protein are separable. Tartu, 2004. 135 p.
88. **Tiina Kahre.** Cystic fibrosis in Estonia. Tartu, 2004. 116 p.
89. **Helen Orav-Kotta.** Habitat choice and feeding activity of benthic suspension feeders and mesograzers in the northern Baltic Sea. Tartu, 2004. 117 p.
90. **Maarja Öpik.** Diversity of arbuscular mycorrhizal fungi in the roots of perennial plants and their effect on plant performance. Tartu, 2004. 175 p.
91. **Kadri Tali.** Species structure of *Neotinea ustulata*. Tartu, 2004. 109 p.
92. **Kristiina Tambets.** Towards the understanding of post-glacial spread of human mitochondrial DNA haplogroups in Europe and beyond: a phylogeographic approach. Tartu, 2004. 163 p.
93. **Arvi Jõers.** Regulation of p53-dependent transcription. Tartu, 2004. 103 p.
94. **Lilian Kadaja.** Studies on modulation of the activity of tumor suppressor protein p53. Tartu, 2004. 103 p.
95. **Jaak Truu.** Oil shale industry wastewater: impact on river microbial community and possibilities for bioremediation. Tartu, 2004. 128 p.
96. **Maire Peters.** Natural horizontal transfer of the *pheBA* operon. Tartu, 2004. 105 p.
97. **Ülo Maiväli.** Studies on the structure-function relationship of the bacterial ribosome. Tartu, 2004. 130 p.
98. **Merit Otsus.** Plant community regeneration and species diversity in dry calcareous grasslands. Tartu, 2004. 103 p.
99. **Mikk Heidemaa.** Systematic studies on sawflies of the genera *Dolerus, Empria,* and *Caliroa* (Hymenoptera: Tenthredinidae). Tartu, 2004. 167 p.
100. **Ilmar Tõnno.** The impact of nitrogen and phosphorus concentration and N/P ratio on cyanobacterial dominance and N_2 fixation in some Estonian lakes. Tartu, 2004. 111 p.
101. **Lauri Saks.** Immune function, parasites, and carotenoid-based ornaments in greenfinches. Tartu, 2004. 144 p.
102. **Siiri Rootsi.** Human Y-chromosomal variation in European populations. Tartu, 2004. 142 p.

103. **Eve Vedler.** Structure of the 2,4-dichloro-phenoxyacetic acid-degradative plasmid pEST4011. Tartu, 2005. 106 p.
104. **Andres Tover.** Regulation of transcription of the phenol degradation *pheBA* operon in *Pseudomonas putida*. Tartu, 2005. 126 p.
105. **Helen Udras.** Hexose kinases and glucose transport in the yeast *Hansenula polymorpha*. Tartu, 2005. 100 p.
106. **Ave Suija.** Lichens and lichenicolous fungi in Estonia: diversity, distribution patterns, taxonomy. Tartu, 2005. 162 p.
107. **Piret Lõhmus.** Forest lichens and their substrata in Estonia. Tartu, 2005. 162 p.
108. **Inga Lips.** Abiotic factors controlling the cyanobacterial bloom occurrence in the Gulf of Finland. Tartu, 2005. 156 p.
109. **Kaasik, Krista.** Circadian clock genes in mammalian clockwork, metabolism and behaviour. Tartu, 2005. 121 p.
110. **Juhan Javoiš.** The effects of experience on host acceptance in ovipositing moths. Tartu, 2005. 112 p.
111. **Tiina Sedman.** Characterization of the yeast *Saccharomyces cerevisiae* mitochondrial DNA helicase Hmi1. Tartu, 2005. 103 p.
112. **Ruth Aguraiuja.** Hawaiian endemic fern lineage *Diellia* (Aspleniaceae): distribution, population structure and ecology. Tartu, 2005. 112 p.
113. **Riho Teras.** Regulation of transcription from the fusion promoters generated by transposition of Tn*4652* into the upstream region of *pheBA* operon in *Pseudomonas putida*. Tartu, 2005. 106 p.
114. **Mait Metspalu.** Through the course of prehistory in india: tracing the mtDNA trail. Tartu, 2005. 138 p.
115. **Elin Lõhmussaar.** The comparative patterns of linkage disequilibrium in European populations and its implication for genetic association studies. Tartu, 2006. 124 p.
116. **Priit Kupper.** Hydraulic and environmental limitations to leaf water relations in trees with respect to canopy position. Tartu, 2006. 126 p.
117. **Heili Ilves.** Stress-induced transposition of Tn*4652* in *Pseudomonas Putida.* Tartu, 2006. 120 p.
118. **Silja Kuusk.** Biochemical properties of Hmi1p, a DNA helicase from *Saccharomyces cerevisiae* mitochondria. Tartu, 2006. 126 p.
119. **Kersti Püssa.** Forest edges on medium resolution landsat thematic mapper satellite images. Tartu, 2006. 90 p.
120. **Lea Tummeleht.** Physiological condition and immune function in great tits (*Parus major* l.): Sources of variation and trade-offs in relation to growth. Tartu, 2006. 94 p.
121. **Toomas Esperk.** Larval instar as a key element of insect growth schedules. Tartu, 2006. 186 p.
122. **Harri Valdmann.** Lynx (*Lynx lynx*) and wolf (*Canis lupus*) in the Baltic region: Diets, helminth parasites and genetic variation. Tartu, 2006. 102 p.

123. **Priit Jõers.** Studies of the mitochondrial helicase Hmi1p in *Candida albicans* and *Saccharomyces cerevisia*. Tartu, 2006. 113 p.
124. **Kersti Lilleväli.** Gata3 and Gata2 in inner ear development. Tartu, 2007. 123 p.
125. **Kai Rünk.** Comparative ecology of three fern species: *Dryopteris carthusiana* (Vill.) H.P. Fuchs, *D. expansa* (C. Presl) Fraser-Jenkins & Jermy and *D. dilatata* (Hoffm.) A. Gray (Dryopteridaceae). Tartu, 2007. 143 p.
126. **Aveliina Helm.** Formation and persistence of dry grassland diversity: role of human history and landscape structure. Tartu, 2007. 89 p.
127. **Leho Tedersoo.** Ectomycorrhizal fungi: diversity and community structure in Estonia, Seychelles and Australia. Tartu, 2007. 233 p.
128. **Marko Mägi.** The habitat-related variation of reproductive performance of great tits in a deciduous-coniferous forest mosaic: looking for causes and consequences. Tartu, 2007. 135 p.
129. **Valeria Lulla.** Replication strategies and applications of Semliki Forest virus. Tartu, 2007. 109 p.
130. **Ülle Reier**. Estonian threatened vascular plant species: causes of rarity and conservation. Tartu, 2007. 79 p.
131. **Inga Jüriado**. Diversity of lichen species in Estonia: influence of regional and local factors. Tartu, 2007. 171 p.
132. **Tatjana Krama.** Mobbing behaviour in birds: costs and reciprocity based cooperation. Tartu, 2007. 112 p.
133. **Signe Saumaa.** The role of DNA mismatch repair and oxidative DNA damage defense systems in avoidance of stationary phase mutations in *Pseudomonas putida.* Tartu, 2007. 172 p.
134. **Reedik Mägi**. The linkage disequilibrium and the selection of genetic markers for association studies in european populations. Tartu, 2007. 96 p.
135. **Priit Kilgas.** Blood parameters as indicators of physiological condition and skeletal development in great tits (*Parus major*): natural variation and application in the reproductive ecology of birds. Tartu, 2007. 129 p.
136. **Anu Albert**. The role of water salinity in structuring eastern Baltic coastal fish communities. Tartu, 2007. 95 p.
137. **Kärt Padari.** Protein transduction mechanisms of transportans. Tartu, 2008. 128 p.
138. **Siiri-Lii Sandre.** Selective forces on larval colouration in a moth. Tartu, 2008. 125 p.
139. **Ülle Jõgar.** Conservation and restoration of semi-natural floodplain meadows and their rare plant species. Tartu, 2008. 99 p.
140. **Lauri Laanisto.** Macroecological approach in vegetation science: generality of ecological relationships at the global scale. Tartu, 2008. 133 p.
141. **Reidar Andreson**. Methods and software for predicting PCR failure rate in large genomes. Tartu, 2008. 105 p.
142. **Birgot Paavel.** Bio-optical properties of turbid lakes. Tartu, 2008. 175 p.

143. **Kaire Torn.** Distribution and ecology of charophytes in the Baltic Sea. Tartu, 2008, 98 p.
144. **Vladimir Vimberg.** Peptide mediated macrolide resistance. Tartu, 2008, 190 p.
145. **Daima Örd.** Studies on the stress-inducible pseudokinase TRB3, a novel inhibitor of transcription factor ATF4. Tartu, 2008, 108 p.
146. **Lauri Saag.** Taxonomic and ecologic problems in the genus *Lepraria* (*Stereocaulaceae*, lichenised *Ascomycota*). Tartu, 2008, 175 p.
147. **Ulvi Karu.** Antioxidant protection, carotenoids and coccidians in greenfinches – assessment of the costs of immune activation and mechanisms of parasite resistance in a passerine with carotenoid-based ornaments. Tartu, 2008, 124 p.
148. **Jaanus Remm.** Tree-cavities in forests: density, characteristics and occupancy by animals. Tartu, 2008, 128 p.
149. **Epp Moks.** Tapeworm parasites *Echinococcus multilocularis* and *E. granulosus* in Estonia: phylogenetic relationships and occurrence in wild carnivores and ungulates. Tartu, 2008, 82 p.
150. **Eve Eensalu.** Acclimation of stomatal structure and function in tree canopy: effect of light and CO_2 concentration. Tartu, 2008, 108 p.
151. **Janne Pullat**. Design, functionlization and application of an *in situ* synthesized oligonucleotide microarray. Tartu, 2008, 108 p.
152. **Marta Putrinš.** Responses of *Pseudomonas putida* to phenol-induced metabolic and stress signals. Tartu, 2008, 142 p.
153. **Marina Semtšenko.** Plant root behaviour: responses to neighbours and physical obstructions. Tartu, 2008, 106 p.
154. **Marge Starast.** Influence of cultivation techniques on productivity and fruit quality of some *Vaccinium* and *Rubus* taxa. Tartu, 2008, 154 p.
155. **Age Tats.** Sequence motifs influencing the efficiency of translation. Tartu, 2009, 104 p.
156. **Radi Tegova.** The role of specialized DNA polymerases in mutagenesis in *Pseudomonas putida.* Tartu, 2009, 124 p.
157. **Tsipe Aavik.** Plant species richness, composition and functional trait pattern in agricultural landscapes – the role of land use intensity and landscape structure. Tartu, 2009, 112 p.
158. **Kaja Kiiver.** Semliki forest virus based vectors and cell lines for studying the replication and interactions of alphaviruses and hepaciviruses. Tartu, 2009, 104 p.
159. **Meelis Kadaja.** Papillomavirus Replication Machinery Induces Genomic Instability in its Host Cell. Tartu, 2009, 126 p.
160. **Pille Hallast.** Human and chimpanzee Luteinizing hormone/Chorionic Gonadotropin beta (*LHB/CGB*) gene clusters: diversity and divergence of young duplicated genes. Tartu, 2009, 168 p.
161. **Ain Vellak.** Spatial and temporal aspects of plant species conservation. Tartu, 2009, 86 p.

162. **Triinu Remmel.** Body size evolution in insects with different colouration strategies: the role of predation risk. Tartu, 2009, 168 p.
163. **Jaana Salujõe.** Zooplankton as the indicator of ecological quality and fish predation in lake ecosystems. Tartu, 2009, 129 p.
164. **Ele Vahtmäe.** Mapping benthic habitat with remote sensing in optically complex coastal environments. Tartu, 2009, 109 p.
165. **Liisa Metsamaa.** Model-based assessment to improve the use of remote sensing in recognition and quantitative mapping of cyanobacteria. Tartu, 2009, 114 p.
166. **Pille Säälik.** The role of endocytosis in the protein transduction by cell-penetrating peptides. Tartu, 2009, 155 p.
167. **Lauri Peil.** Ribosome assembly factors in *Escherichia coli.* Tartu, 2009, 147 p.
168. **Lea Hallik.** Generality and specificity in light harvesting, carbon gain capacity and shade tolerance among plant functional groups. Tartu, 2009, 99 p.
169. **Mariliis Tark.** Mutagenic potential of DNA damage repair and tolerance mechanisms under starvation stress. Tartu, 2009, 191 p.
170. **Riinu Rannap.** Impacts of habitat loss and restoration on amphibian populations. Tartu, 2009, 117 p.
171. **Maarja Adojaan.** Molecular variation of HIV-1 and the use of this knowledge in vaccine development. Tartu, 2009, 95 p.
172. **Signe Altmäe.** Genomics and transcriptomics of human induced ovarian folliculogenesis. Tartu, 2010, 179 p.
173. **Triin Suvi.** Mycorrhizal fungi of native and introduced trees in the Seychelles Islands. Tartu, 2010, 107 p.
174. **Velda Lauringson.** Role of suspension feeding in a brackish-water coastal sea. Tartu, 2010, 123 p.
175. **Eero Talts.** Photosynthetic cyclic electron transport – measurement and variably proton-coupled mechanism. Tartu, 2010, 121 p.
176. **Mari Nelis.** Genetic structure of the Estonian population and genetic distance from other populations of European descent. Tartu, 2010, 97 p.
177. **Kaarel Krjutškov.** Arrayed Primer Extension-2 as a multiplex PCR-based method for nucleic acid variation analysis: method and applications. Tartu, 2010, 129 p.
178. **Egle Köster.** Morphological and genetical variation within species complexes: *Anthyllis vulneraria* s. l. and *Alchemilla vulgaris* (coll.). Tartu, 2010, 101 p.
179. **Erki Õunap.** Systematic studies on the subfamily Sterrhinae (Lepidoptera: Geometridae). Tartu, 2010, 111 p.
180. **Merike Jõesaar.** Diversity of key catabolic genes at degradation of phenol and *p*-cresol in pseudomonads. Tartu, 2010, 125 p.
181. **Kristjan Herkül.** Effects of physical disturbance and habitat-modifying species on sediment properties and benthic communities in the northern Baltic Sea. Tartu, 2010, 123 p.

182. **Arto Pulk.** Studies on bacterial ribosomes by chemical modification approaches. Tartu, 2010, 161 p.
183. **Maria Põllupüü.** Ecological relations of cladocerans in a brackish-water ecosystem. Tartu, 2010, 126 p.
184. **Toomas Silla.** Study of the segregation mechanism of the Bovine Papillomavirus Type 1. Tartu, 2010, 188 p.
185. **Gyaneshwer Chaubey.** The demographic history of India: A perspective based on genetic evidence. Tartu, 2010, 184 p.
186. **Katrin Kepp.** Genes involved in cardiovascular traits: detection of genetic variation in Estonian and Czech populations. Tartu, 2010, 164 p.
187. **Virve Sõber.** The role of biotic interactions in plant reproductive performance. Tartu, 2010, 92 p.
188. **Kersti Kangro.** The response of phytoplankton community to the changes in nutrient loading. Tartu, 2010, 144 p.
189. **Joachim M. Gerhold.** Replication and Recombination of mitochondrial DNA in Yeast. Tartu, 2010, 120 p.
190. **Helen Tammert.** Ecological role of physiological and phylogenetic diversity in aquatic bacterial communities. Tartu, 2010, 140 p.
191. **Elle Rajandu.** Factors determining plant and lichen species diversity and composition in Estonian *Calamagrostis* and *Hepatica* site type forests. Tartu, 2010, 123 p.
192. **Paula Ann Kivistik.** ColR-ColS signalling system and transposition of Tn*4652* in the adaptation of *Pseudomonas putida.* Tartu, 2010, 118 p.
193. **Siim Sõber.** Blood pressure genetics: from candidate genes to genome-wide association studies. Tartu, 2011, 120 p.
194. **Kalle Kipper.** Studies on the role of helix 69 of 23S rRNA in the factor-dependent stages of translation initiation, elongation, and termination. Tartu, 2011, 178 p.
195. **Triinu Siibak.** Effect of antibiotics on ribosome assembly is indirect. Tartu, 2011, 134 p.
196. **Tambet Tõnissoo.** Identification and molecular analysis of the role of guanine nucleotide exchange factor RIC-8 in mouse development and neural function. Tartu, 2011, 110 p.
197. **Helin Räägel.** Multiple faces of cell-penetrating peptides – their intracellular trafficking, stability and endosomal escape during protein transduction. Tartu, 2011, 161 p.
198. **Andres Jaanus.** Phytoplankton in Estonian coastal waters – variability, trends and response to environmental pressures. Tartu, 2011, 157 p.
199. **Tiit Nikopensius.** Genetic predisposition to nonsyndromic orofacial clefts. Tartu, 2011, 152 p.
200. **Signe Värv.** Studies on the mechanisms of RNA polymerase II-dependent transcription elongation. Tartu, 2011, 108 p.
201. **Kristjan Välk.** Gene expression profiling and genome-wide association studies of non-small cell lung cancer. Tartu, 2011, 98 p.

202. **Arno Põllumäe.** Spatio-temporal patterns of native and invasive zooplankton species under changing climate and eutrophication conditions. Tartu, 2011, 153 p.
203. **Egle Tammeleht.** Brown bear (*Ursus arctos*) population structure, demographic processes and variations in diet in northern Eurasia. Tartu, 2011, 143 p.
205. **Teele Jairus.** Species composition and host preference among ectomycorrhizal fungi in Australian and African ecosystems. Tartu, 2011, 106 p.
206. **Kessy Abarenkov.** PlutoF – cloud database and computing services supporting biological research. Tartu, 2011, 125 p.
207. **Marina Grigorova.** Fine-scale genetic variation of follicle-stimulating hormone beta-subunit coding gene (*FSHB*) and its association with reproductive health. Tartu, 2011, 184 p.
208. **Anu Tiitsaar.** The effects of predation risk and habitat history on butterfly communities. Tartu, 2011, 97 p.
209. **Elin Sild.** Oxidative defences in immunoecological context: validation and application of assays for nitric oxide production and oxidative burst in a wild passerine. Tartu, 2011, 105 p.
210. **Irja Saar**. The taxonomy and phylogeny of the genera *Cystoderma* and *Cystodermella* (Agaricales, Fungi). Tartu, 2012, 167 p.
211. **Pauli Saag.** Natural variation in plumage bacterial assemblages in two wild breeding passerines. Tartu, 2012, 113 p.
212. **Aleksei Lulla.** Alphaviral nonstructural protease and its polyprotein substrate: arrangements for the perfect marriage. Tartu, 2012, 143 p.
213. **Mari Järve.** Different genetic perspectives on human history in Europe and the Caucasus: the stories told by uniparental and autosomal markers. Tartu, 2012, 119 p.
214. **Ott Scheler**. The application of tmRNA as a marker molecule in bacterial diagnostics using microarray and biosensor technology. Tartu, 2012, 93 p.
215. **Anna Balikova**. Studies on the functions of tumor-associated mucin-like leukosialin (CD43) in human cancer cells. Tartu, 2012, 129 p.
216. **Triinu Kõressaar.** Improvement of PCR primer design for detection of prokaryotic species. Tartu, 2012, 83 p.
217. **Tuul Sepp.** Hematological health state indices of greenfinches: sources of individual variation and responses to immune system manipulation. Tartu, 2012, 117 p.
218. **Rya Ero.** Modifier view of the bacterial ribosome. Tartu, 2012, 146 p.
219. **Mohammad Bahram.** Biogeography of ectomycorrhizal fungi across different spatial scales. Tartu, 2012, 165 p.
220. **Annely Lorents.** Overcoming the plasma membrane barrier: uptake of amphipathic cell-penetrating peptides induces influx of calcium ions and downstream responses. Tartu, 2012, 113 p.

221. **Katrin Männik.** Exploring the genomics of cognitive impairment: whole-genome SNP genotyping experience in Estonian patients and general population. Tartu, 2012, 171 p.
222. **Marko Prous.** Taxonomy and phylogeny of the sawfly genus *Empria* (Hymenoptera, Tenthredinidae). Tartu, 2012, 192 p.
223. **Triinu Visnapuu.** Levansucrases encoded in the genome of *Pseudomonas syringae* pv. tomato DC3000: heterologous expression, biochemical characterization, mutational analysis and spectrum of polymerization products. Tartu, 2012, 160 p.
224. **Nele Tamberg.** Studies on Semliki Forest virus replication and pathogenesis. Tartu, 2012, 109 p.
225. **Tõnu Esko.** Novel applications of SNP array data in the analysis of the genetic structure of Europeans and in genetic association studies. Tartu, 2012, 149 p.
226. **Timo Arula.** Ecology of early life-history stages of herring *Clupea harengus membras* in the northeastern Baltic Sea. Tartu, 2012, 143 p.
227. **Inga Hiiesalu.** Belowground plant diversity and coexistence patterns in grassland ecosystems. Tartu, 2012, 130 p.
228. **Kadri Koorem.** The influence of abiotic and biotic factors on small-scale plant community patterns and regeneration in boreonemoral forest. Tartu, 2012, 114 p.
229. **Liis Andresen.** Regulation of virulence in plant-pathogenic pectobacteria. Tartu, 2012, 122 p.
230. **Kaupo Kohv.** The direct and indirect effects of management on boreal forest structure and field layer vegetation. Tartu, 2012, 124 p.
231. **Mart Jüssi.** Living on an edge: landlocked seals in changing climate. Tartu, 2012, 114 p.
232. **Riina Klais.** Phytoplankton trends in the Baltic Sea. Tartu, 2012, 136 p.
233. **Rauno Veeroja.** Effects of winter weather, population density and timing of reproduction on life-history traits and population dynamics of moose (*Alces alces*) in Estonia. Tartu, 2012, 92 p.
234. **Marju Keis.** Brown bear (*Ursus arctos*) phylogeography in northern Eurasia. Tartu, 2013, 142 p.
235. **Sergei Põlme.** Biogeography and ecology of *alnus*- associated ectomycorrhizal fungi – from regional to global scale. Tartu, 2013, 90 p.
236. **Liis Uusküla.** Placental gene expression in normal and complicated pregnancy. Tartu, 2013, 173 p.