Creation and characterization of a cell line expressing the C-terminal non-catalytic domain of POLE1

Bachelor thesis

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POLE1 C-terminaalset mittekatalüütilist domeeni ekspresseeriva rakuliini loomine ja iseloomustamine

Bakalaureusetöö

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Abbreviations

DNA- Deoxyribonucleic acid

MCM- Minichromosome maintenance protein complex

Cdc45- Cell division control protein 45

GINS - **G**o-**i**chi-**n**i-**s**an

CMG - CDC45, MCM, GINS

ORC- Origin recognition complex

DDK- DBF4-dependent kinase

PCNA- Proliferating cell nuclear antigen

RPA- replication protein A

ATP- Adenosine triphosphate

RNA- Ribonucleic acid

EdU- 5-ethynyl-2'-deoxyuridine

mAID- Auxin-Inducible Degron

Pre-IC - preinitiation complex

pre-RC - prereplication complex

TBST- Tris Buffered Saline with Tween

PBS- phosphate buffered saline

Introduction

DNA replication is arguably the most fundamental process required for the multiplication of all living cells. As a result of cell division, each daughter cell receives the same genetic information that was encoded in the DNA of the parent cell (Hanawalt, 2004). Replication is catalyzed by the replisome, which coordinates DNA separation into two individual strands and its synthesis (Zhou et al., 2017). DNA helicase is capable of unwinding duplex DNA to provide single-stranded DNA templates that are required in DNA replication (Petojevic et al., 2015). MCM helicase is inactive unless it is in complex with two other components: CDC45 and GINS (Crevel, 2001).

DNA polymerases are essential for all DNA replication processes. There are three polymerases that have major roles in genomic replication: polymerase alpha, delta, and epsilon.

Studies in yeast have shown that polymerase epsilon is an essential protein required for DNA replication on the leading strand: it conducts the majority of continuous leading strand synthesis, as well as plays an important role in genome stability, replisome assembly and replication fork movement (Zhou et al., 2017, 2019). DNA polymerase epsilon catalytic subunit (POLE1 in human, Pol2 in yeast) consists of two domains: the N-terminal catalytic domain and the C-terminal noncatalytic domain (Dua et al., 1999). The C-terminal half of the POLE1 subunit includes binding domains for smaller subunits of the polymerase epsilon: POLE2, POLE3 and POLE4.

The N-terminus of Pol2 (POLE1 in humans) has been shown to be dispensable for cell survival in yeast (Isoz et al., 2012). Yeast studies state that the CMG helicase and polymerase e form a stable complex that is essential for replisome assembly, activation, and replication fork movement in yeast (Zhou et al., 2017), however POLE1 is not required for CMG assembly in humans cells (Vipat et al., 2022). A study (Vipat et al., 2022) in human cells concludes that the C-terminal non-catalytic domain of POLE1 is capable of rescuing DNA synthesis and prevent replication stress caused by the absence of POLE1. There have been many studies where the role of polymerase epsilon in replication initiation has been studied in yeast, however the non-catalytic function of DNA polymerase epsilon in human cells is not known yet. DNA replication has been studied extensively in bacteria, SV40 and yeast. These systems however do not fully represent how DNA replication initiation step happens in human cells.

The main results of the current thesis are:

1. A cell line expressing the C-terminal non-catalytic domain (CTD) of POLE1, and capable of depleting the endogenous POLE1, was created.

2. POLE1 depletion in human cells resulted in replication stress, POLE2 degradation, growth arrest, and DNA synthesis block.

3. Stable expression of CTD in the new cell line did not prevent replication stress, POLE2 degradation, growth defects, or DNA synthesis block caused by POLE1 depletion.

4. Transient expression of CTD partially restored DNA synthesis in POLE1-depleted cells.

5. The localization of stably expressed CTD in the novel cell line was cytoplasmic, which explained the absence of the effects on POLE1 depleted cells.

1. Literature review

1.1 DNA replication

DNA replication is an essential process required for any cell division (Hanawalt, 2004). The genome is replicated exactly once during every cell cycle (Sclafani and Holzen, 2007). As a result of cell division, each daughter cell receives the same genetic information that was encoded in the DNA of the parent cell (Hanawalt, 2004). Replication is catalyzed by the replisome, which coordinates DNA separation into two individual strands and DNA copying. These functions must be tightly linked to prevent genomic instability, which is a major cause of cancer (Zhou et al., 2017). The replisome is composed of different polymerases, the helicase, which is responsible for the separation of the duplex DNA, and other factors. These proteins assemble together at the origins of replications, and move with the replication fork (Yao and O'Donnell, 2010).

The DNA replication is a semi-conservative copying of the nucleotide sequence into two daughter strands (Chagin et al., 2010). According to the semi-conservative replication model, two parent strands unwind and each of the strand forms a new molecule with the newly synthesized complementary daughter strand, resulting in two DNA molecules with one original strand and one new strand (Norris, 2019). DNA is always synthesized in the 5´to 3´direction (Yao and O'Donnell, 2010). One strand, the leading strand, that is synthesized in the same direction as the MCM helicase is moving and is replicated continuously, whereas the second strand, the lagging strand is synthesized in the opposite direction. It is replicated discontinuously in small 120-250 nucleotides long pieces called Okazaki fragments (Pavlov et al., 2006; Burgers and Kunkel, 2017).

1.1.1 Components of the replicative complex

The initiation of eukaryotic DNA replication requires the assembly and activation of highly specialized nucleoprotein complexes at chromosomal origins of replication (Egel, 2004). DNA replication can only happen when the helicase separates the DNA double helix (Petojevic et al., 2015) (**Figure 1**). The MCM helicase has an important role in the progression of the replisome and it activates after paring with CDC45 and GINS complex, forming CMG helicase (Crevel, 2001).

The DNA helicase core is formed by six different polypeptides MCM2-MCM7 (Petojevic et al., 2015). The protein has a ATP-independent DNA-binding activity that distinguishes single- and double-stranded DNA and strand-displacement activity that unwinds the helix when in complex with CDC45 and GINS complex (Chong et al., 2000). For the MCM to be activated, GINS complex and CDC45 need to establish a connection between the MCM2 and MCM5 subunits, creating a topologically segregated channel to the side of the MCM2-7 double-hexameric ring (Petojevic et al., 2015).

Cell division control protein 45 (CDC45) is essential in all eukaryotes as it functions with the replisome CMG helicase (Petojevic et al., 2015). CDC45 is required for the recruitment of many other components of the replicative complex, including RPA, PCNA, Pol α and Pol ε (Bell and Dutta, 2002).

In all eukaryotes the GINS complex is composed of four subunits: the Sld5, Psf1, Psf2 and Psf3, all of which are highly conserved in eukaryotic cells. Beside its role as a component of the CMG complex,

GINS complex interacts with other key proteins at the fork and it is associated with maintaining an active replisome progression (Takayama et al., 2003; Jedrychowska et al., 2019). GINS was also shown to interact with all three replicative polymerases (Pol α, Pol ε, Pol δ) in human cells and noticeably stimulated the Pol α and Pol ε activities (Bermudez et al., 2011).

In eukaryotes, there are three main DNA polymerases involved in DNA replication Pol α, Pol ε and Pol δ (Burgers and Kunkel, 2017). The Polα-primase complex is present on the lagging stand. The primase synthesizes an initial RNA primer, which is extended by Pol α. The replication is continued by polymerases ε and δ, on leading strand and lagging strand, respectively. These RNA-DNA primers made by Pol α-primase are extended by Pol δ. Polymerase δ displaces the 5′ end of the Okazaki fragment into a single-stranded flap structure (Balakrishnan and Bambara, 2013). Flap endonuclease (FEN1) recognizes this structure, binds to the base of the flap and removes the RNA primer to make a nick (Balakrishnan and Bambara, 2013). To complete the maturation process, DNA ligase seals the nick by linking the ends to form a single, unbroken molecule of DNA (Burgers and Kunkel, 2017).

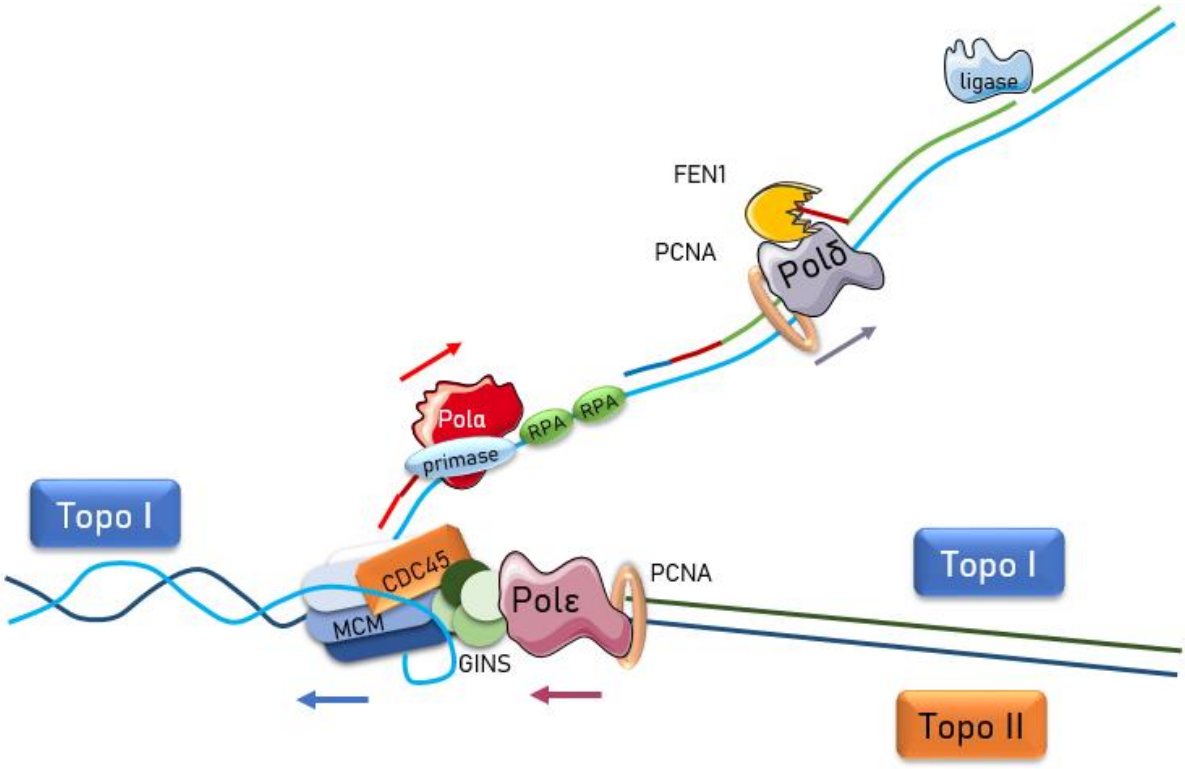


Figure 1: Replication fork components. The helicase unwinds DNA in front of the fork to create regions of singled-stranded DNA. The helicase components shown are the MCM2-7 helicase, CDC45, and associated GINS complex. The ssDNA is coated in replication protein A (RPA) to keep strands from reannealing. Replication on the leading and lagging strands is performed Pol ε and Pol δ, respectively. Pol α-primase is responsible for primer synthesis for the leading strand and each of the Okazaki fragments. In the presence of FEN1, the RNA primers are removed, and the newly synthesized fragments are ligated together by DNA ligase I. The direction of DNA synthesis and RF movement is indicated by arrows.

Replication protein A (RPA) binds to the exposed single stranded DNA of Okazaki fragments, providing sites for DNA Polα-primase complex for lagging strand synthesis (Burgers and Kunkel, 2017). PCNA is a protein connects Pol ε and Pol δ to the DNA template for rapid and processive DNA synthesis (Kelman, 1997).

Topoisomerases are enzymes that are capable of altering the topological state of DNA, such as relaxing supercoiled DNA and performing catenation and decatenation of DNA rings. There are two types of topoisomerases. Type I makes cuts in one strand of the DNA molecule, and type II cuts both strands of the DNA (Nitiss et al., 2012).

1.1.2 Replication initiation

DNA replication is initiated at thousands of specific sites in the genome called origins of replication (Bell and Dutta, 2002). Replication origins are distributed along the length of each chromosome and are activated during S phase of cell cycle. Replication origins are essential for chromosome replication and therefore are essential for life. Once a chromosome loses most or all of its origins, it can no longer replicate, and chromosome loss is lethal (Egel, 2004). Replication initiation is a two-step process: origin licensing and origin firing (Mazouzi et al., 2014).

The replication initiation process starts with origin licensing in G1 phase of the cell cycle, when inactive DNA helicase complexes are loaded onto the double stranded DNA (**Figure 2A**). In eukaryotes, a chromatin-bound origin recognition complex (ORC) is recruited to replication origins (Walter, 2000). ORC consists of subunits 1-6, and together with CDT1 and CDC6 it loads the inactive DNA helicase MCM (Hyrien, 2016). The licensing system is turned off during S phase to ensure that no origins can't fire more than once in a cell-cycle, thereby preventing re-replication (Mei and Cook, 2021).

After origin licensing, two active CMG complexes need to be assembled from MCM, GINS and CDC45, leading to the unwinding of DNA and establishment of the replication fork (Boos and Ferreira, 2019; Lewis et al., 2022) (**Figure 2B**). Treslin (TopBP1-interacting, replication-stimulating protein) and TOPBP1 (topoisomerase IIβ-binding protein 1) are the proteins which facilitate the loading of CDC45 onto chromatin (Moiseeva and Bakkenist, 2018). Studies have shown that MTBP (MDM two binding protein) forms a complex with Treslin and TOPBP1 and has a role in CMG assembly (Moiseeva and Bakkenist, 2018).

Cyclin-Dependent kinases (CDKs) which associate with respective Cyclin regulatory subunits initiate origin activation, the assembly of the CMG complex and regulate the transition through cell cycle phases (Fagundes & Teixeira, 2021; Moiseeva & Bakkenist, 2018). During G1 phase, Cyclin E binds and activates CDK2, forming the Cyclin E/CDK2 complex to promote S phase entry and directly initiating replication via phosphorylation (Fagundes and Teixeira, 2021). Cyclin A forms complexes with CDK2 and also CDK1, which are required for entry into S and M phases (Yang et al., 1999). The major function of Dbf4- CDC7 (DDK) kinase is to convert the pre-replication complex (ORC, CDC6, CDT1 and MCM2-7) into an active replicative complex via phosphorylation of MCM proteins (Walter, 2000). In late S phase Cyclin E/CDK2 activity is eliminated by complete degradation of Cyclin E (Fagundes and Teixeira, 2021).

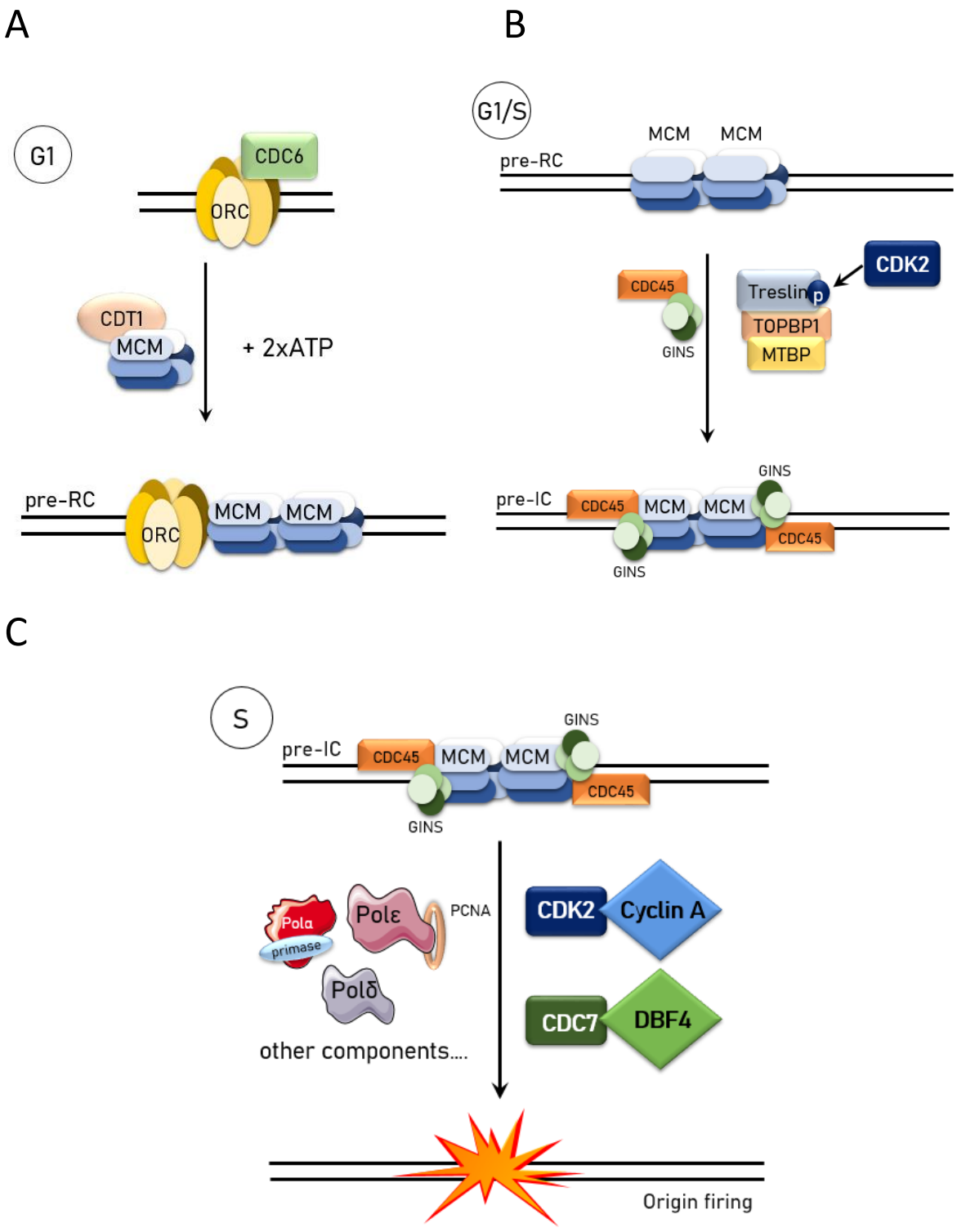


Figure 2: Steps of replication initiation **A.** Schematic of MCM loading onto chromatin at replication origins during G1 phase. CDC6 and CDT1 proteins are required for MCM loading onto ORC-marked sites. **B.** Schematic of CMG assembly in G1/S phase. **C.** Schematic of CMG activation and recruitment of DNA polymerases in S phase.

CMG activation in S-phase is necessary for origin unwinding, polymerase recruitment, and initiation of bidirectional DNA synthesis (Hyrien, 2016) (**Figure 2C**). Chromosomal DNA replication in eukaryotic cells needs three different polymerases to act sequential to create DNA: Polα-primase, Pol δ and Pol ε (Balakrishnan and Bambara, 2013). The primase synthesizes an initial RNA primer, which is 7-12 nucleotides long (Shanbhag et al., 2018). This RNA primer is extended by Pol α with approximately 20 nucleotides (Balakrishnan and Bambara, 2013). The replication is continued by Pol ε and Pol δ, on leading strand and lagging strand, respectively. In yeast Pol ε is recruited as a complex with GINS, in human cells no such complex has been described (Moiseeva and Bakkenist, 2018).

1.2 Replication stress

Replication stress is defined as slowing or stalling the progression of the replication fork (Mazouzi et al., 2014). High levels of DNA damage result in replication stress, which causes genome instability and may lead to the formation of cancer (Gaillard et al., 2015). There are many different mechanisms that cells have evolved to deal with different kinds of DNA damage (Mazouzi et al., 2014). The goal of DNA damage response is to either lead to the repair of the damage and resume normal cell cycle progression, or to initiate apoptosis (Mazouzi et al., 2014).

There are many different sources of replication stress. The most common cases of replication stress are nicks, gaps and stretches of ssDNA, unrepaired DNA lesions, misincorporation of ribonucleotides, unusual DNA structures, interference between replication and transcription, fragile sites, limited concentration of essential replication components and various human diseases (Zeman and Cimprich, 2014).

Slowed replication fork activity results in ssDNA areas, if the helicase proceeds to unwind the parental DNA, while the polymerase has stalled (Zeman and Cimprich, 2014). Single-stranded DNA accumulation happens when DNA helicase and DNA polymerase activities become uncoupled due to physical obstructions or nucleotide deficiencies that block DNA polymerase progression (Hao et al., 2015).

Ataxia telangiectasia and Rad3-related (ATR) is a protein kinase that is activated in response to replication fork stalling and DNA damage (Gaillard et al., 2015). Single-stranded DNA, bound by replication protein A (RPA), generates a signal and promotes activation of ATR (Zeman and Cimprich, 2014). RPA and ssDNA interactions serve as a base for the recruitment of ATRIP (ATR-interacting protein) and several other regulatory components, which are needed for ATR recruitment to stalled replication forks (Saldivar et al., 2017). Main purpose of ATR is to phosphorylate CHK1 at Ser-317 and Ser-345, following replication stress, which leads to the activation of CHK1 and the downstream checkpoint pathway (Gaillard et al., 2015; Hao et al., 2015).

ATR-Chk1 signaling pathway has many functions in maintaining the integrity of the genome, including cell cycle arrest, inhibition of origin firing, replication fork stabilization and promoting fork repair and restart (Saldivar et al., 2017).

1.3 History and Classification of DNA Polymerases

The first DNA polymerase (from E.coli) was discovered and characterized by A. Konberg and his team in 1955 (Lehman, 2003). Since then, there have been 5 different classes of cellular DNA polymerases

identified in prokaryotes. In eukaryotes the number of DNA polymerases is much higher than in prokaryotes: 15 are known, of which three are well studied and have major roles in genomic replication (Stillman, 2008). Overview of the functions of prokaryotic and eukaryotic DNA polymerases is presented in **Table 1**.

Function	**Polymerases in prokaryotes**	**Polymerases in eukaryotes**
Chromosomal replication	Pol III	Pol α, Pol ε, Pol δ
DNA repair	Pol I, Pol II, Pol IV	Pol β, Pol θ, Pol λ, Pol ε, Pol δ
Translesion synthesis	Pol IV, Pol V	Pol ζ, Pol η, Pol ι, Pol κ, REV1
Mitochondrial replication and repair	-	Pol γ
Somatic hypermutation (immune response)	-	Pol μ, Pol ι

Table 1. Overview of the prokaryotic and eukaryotic DNA polymerases and their functions in the cell.

DNA polymerases are divided into seven different family groups (A, B, C, D, X, Y, RT) based on functionality, structure and amino-acid sequence (Shanbhag et al., 2018). The majority of replication is conducted by B Family polymerases α, δ and ε, that share many structural and catalytic properties (Shanbhag et al., 2018) (**Table 2**).

Polymerase	Subunits (kDa)	3´-5´ exonuclease	Processivity	Primary function
Polα	166 POLA1; 70 POLA2; 49 PRIM1; 58 PRIM2	No	Low	DNA/RNA primers
Polδ	124 POLD1; 50 POLD2; 68 POLD3; 12 POLD4	Yes	High	Synthesis of the lagging-strand and DNA repair
Polε	261 POLE1; 59 POLE2; 12 POLE3; 17 POLE4	Yes	High	Synthesis of the leading-strand

Table 2. DNA polymerases and associated subunits.

DNA polymerase alpha (Pol α) initiates DNA synthesis at replication origins and on the lagging strand (Shcherbakova et al., 2003). Pol α forms a complex with primase, which synthesizes short RNA primers, extended by Pol α (Balakrishnan and Bambara, 2013). Pol α-primase complex consists of four subunits: the catalytic subunit POLA1, the regulatory subunit POLA2, and the primase subunits PRIM1 and PRIM2

(Pavlov et al., 2006). Pol α lacks 3´-5´ exonuclease proofreading activity, errors made by Pol α are mended by the mismatch repair system and Pol δ (Shanbhag et al., 2018).

DNA polymerase delta (Pol δ) is responsible for the replication on the lagging strand. Pol δ complex consists of four subunits: the catalytic subunit p125 (POLD1), p50 (POLD2), p68 (POLD3) and p12 (POLD4) (Prindle and Loeb, 2012). Additionally, Pol δ is involved in several DNA repair mechanisms and has 3´-5´exonuclease activity (Prindle and Loeb, 2012).

DNA polymerase epsilon (Pol ε) is the enzyme responsible for the leading strand synthesis (Zhou et al., 2017). It is the largest known polymerase and was identified in 1980s, first as a DNA repair factor but was soon recognized to play essential roles in replication, cell cycle control and maintaining genome stability (Korona et al., 2011; Henninger and Pursell, 2014). Both Pol ε and Pol δ carry out high fidelity DNA synthesis due to their 3'-5' exonuclease activity (Shanbhag et al., 2018). It has been shown that mutations affecting proof-reading activity can lead to genome instability (Shanbhag et al., 2018).

DNA synthesis in eukaryotes is efficient and highly accurate, primarily because of two DNA polymerase enzymes Pol ε and Pol δ (Korona et al., 2011). These polymerases can proofread their own mistakes and correct errors made by other polymerases. As mentioned before Pol δ can correct errors made by Pol α on the lagging stand and Pol ε on the leading strand (Henninger and Pursell, 2014). Additionally, Pol ε and Pol δ play essential roles in several DNA repair processes: base excision repair, nucleotide excision repair, post-replication mismatch repair and proofreading exonuclease activity (Korona et al., 2011). DNA damage can result in mutation or stop DNA replication, which can cause chromosome loss, cell death, and serious health problems, including cancer (Pavlov et al., 2006).

1.4 DNA polymerase epsilon

DNA polymerase ε was first discovered in yeast (*S. cerevisiae*). Studies in yeast systems have shown that Pol ε conducts the majority of continuous leading strand synthesis (Zhou et al., 2019). The CMG helicase and Pol ε form a stable complex, playing a critical role in replisome assembly, activation and replication fork movement (Zhou et al., 2017). Studies have shown that the reduction of Pol ε slows the movement of the DNA replication fork resulting in cells completing S phase slower (Bermudez et al., 2011).

The Pol ε consists of four subunits- POLE1, POLE2, POLE3 and POLE4 (Shanbhag et al., 2018) (**Figure 3**). The largest catalytic subunit: Pol2 in S. cerevisiae and p261/POLE1 in human cells. The second subunit: Dpb2 in S. cerevisiae and p59/POLE2 in human. The two smallest subunits: Dpb3 in S. cerevisiae and p12/POLE3 in human and Dpb4 in S. cerevisiae and p17/POLE3/CHRAC in human (Bermudez et al., 2011; Henninger and Pursell, 2014). The three subunits POLE2, POLE3 and POLE4 form a complex at the C-terminus of POLE1, which enhanced the binding of Pol ε to the DNA template (Bermudez et al., 2011).

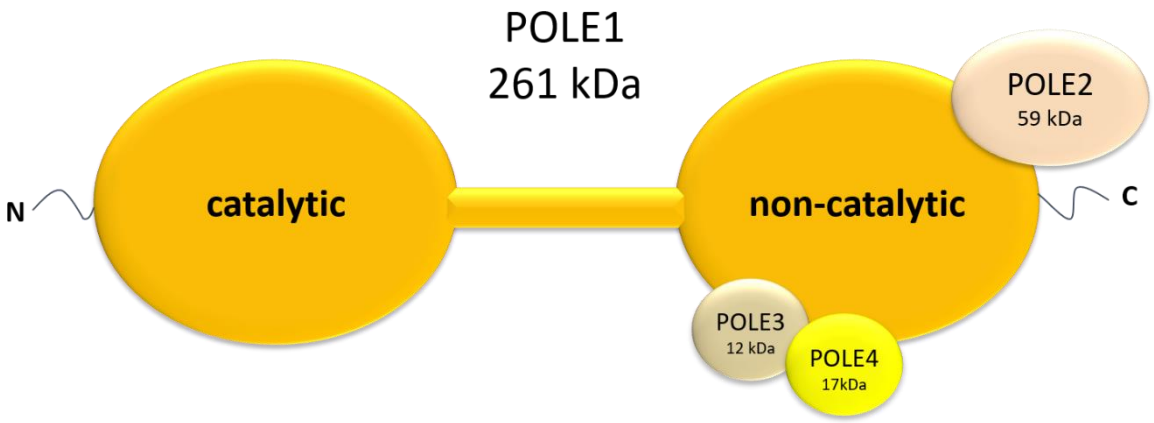


Figure 3. DNA polymerase Pol ε with four subunits.

In yeast, a study has shown that Pol ε associates with the preinitiation complex prior to Pol α and suggested that it interacts with other critical components involved in the generation of the preinitiation complex, particularly the four-subunit GINS complex. Such interactions suggest an additional role of Pol ε in helping to recruit other replication proteins prior to the initiation of replication (Bermudez et al., 2011). It is known that Pol ε also has a role in DNA repair (Kelman, 1997).

1.4.1 Polymerase epsilon catalytic subunit – POLE1

The catalytic subunit of Pol ε is encoded by the POLE gene (Henninger and Pursell, 2014). In both yeast and humans Pol ε catalytic subunit has two domains: catalytic N-terminal and non-catalytic C-terminal domain (Dua et al., 1999) (**Figure 3**). The molecular weight of the N-terminal domain is 140 kDa and C-terminal domain is ∼120 kDa (Henninger and Pursell, 2014). The non-catalytic C-terminal domain is required for replisome assembly and checkpoint activation (Burgers and Kunkel, 2017). The N-terminal domain of POLE1 is responsible for catalyzing DNA synthesis and 3'–5' exonuclease proofreading activity in human cells (Bermudez et al., 2011).

The Pol ε subunit POLE1 interacts with the CDC45, MCM complex, and Psf2 (a member of the GINS complex), according to research done in human cells (Bauerschmidt et al., 2007). Research has shown that protein-protein interactions between the C-terminal domain of POLE1 and POLE2 subunit are associated with supporting the formation of CMG helicase at replication origins (Yuan et al., 2020). The C-terminal domain of the POLE1 and POLE2 subunit serve as a connecting link between the other CMG components. While the CTD of POLE1 connects with MCM, POLE2 interacts with GINS (Goswami et al., 2018).

1.4.2 Smaller subunits – POLE2, POLE3 and POLE4

POLE2 is the second largest subunit and it is essential for viability of yeast cells (Goswami et al., 2018). It has been suggested that in eukaryotes the main role of POLE2 is to bind to the C-terminus of POLE1 and connect Pol ε to the CMG helicase via protein-protein interactions. A study has proven that by binding the POLE2 to the C-terminus of POLE1 will enhance the solubility and activity of the POLE1 subunit (Bermudez et al., 2011).

POLE3 and POLE4 form a complex that interacts with double-stranded DNA but are non-essential genes in *S. cerevisiae.* Studies in human cells have indicated that POLE3 and POLE4 binds to both parental and newly synthesized histones. The depletion of these two Pol ε subunits directly impacts chromatin maintenance, as it results in issues in the in the unwinding of the DNA by helicase and PCNA unloading (Bellelli et al., 2018a). The depletion of both POLE3 and POLE4 also causes failure of the mismatch repair system (Isoz et al., 2012).

A study in mice revealed that POLE4 is required to maintain the stability of the whole Pol ε complex suggesting an important structural role of polymerase epsilon in mammals (Bellelli et al., 2018b). A study has examined whether POLE2, POLE3 and POLE4 subunits would form complexes in the absence of POLE1. The results were surprising as the POLE3 and POLE4 revealed a stable interaction, but neither of these subunits interacted with POLE2 (Dua et al., 1999).

1.4.3 POLE depletion

1.4.3.1 Auxin-inducible (mAID) system for rapid protein depletion in mammalian cells

One effective method to study gene function is to reduce or eliminate the gene product. In this thesis we used the auxin-inducible degradations system for inducing protein depletion (**Figure 4**). This system is derived from plants and allows rapid depletion of the mAID-tagged protein by the cell's own ubiquitin-mediated protein degradation processes, together with the plant hormone auxin. osTIR1 is able to form a complex between SKP1 and CUL1 proteins in non-plant cells forming a functional E3 ubiquitin ligase. It is known that osTIR1 identifies the AID-tag, only when auxin is present (Camlin and Evans, 2019). In the presence of auxin, the mAID-tag of the protein of interest gets polyubiquitinated by E3 ligase that is recruited by F-box protein osTIR1 and the protein is therefore targeted for degradation by the proteasome (Natsume et al., 2016). The depletion of the protein is rapid and very efficient and takes approximately 30-60 minutes for the protein to be depleted (Shetty et al., 2019).

The mAID system has been widely and successfully used in many model organisms, , such as *C. elegans* (Zhang et al., 2015), *S. cerevisiae* (Shetty et al., 2019), and fruit fly (Trost et al., 2016). The mAID system can conduct rapid depletion of endogenous protein, by adding auxin in cells that express AID-tagged proteins together with osTIR1 (Natsume et al., 2016). It has been shown that the expression of osTIR1 together with mAID-tagged protein may cause a slight reduction of the target protein level even in the absence of auxin (Natsume et al., 2016). To overcome this reduction, it is recommended to express osTIR1 from an inducible promotor, such as the doxycycline-inducible promotor. This slightly increases the time required to deplete the mAID-tagged protein (as doxycycline induction takes at least 6 h), but it ensures protein stability in the absence of doxycycline and auxin,

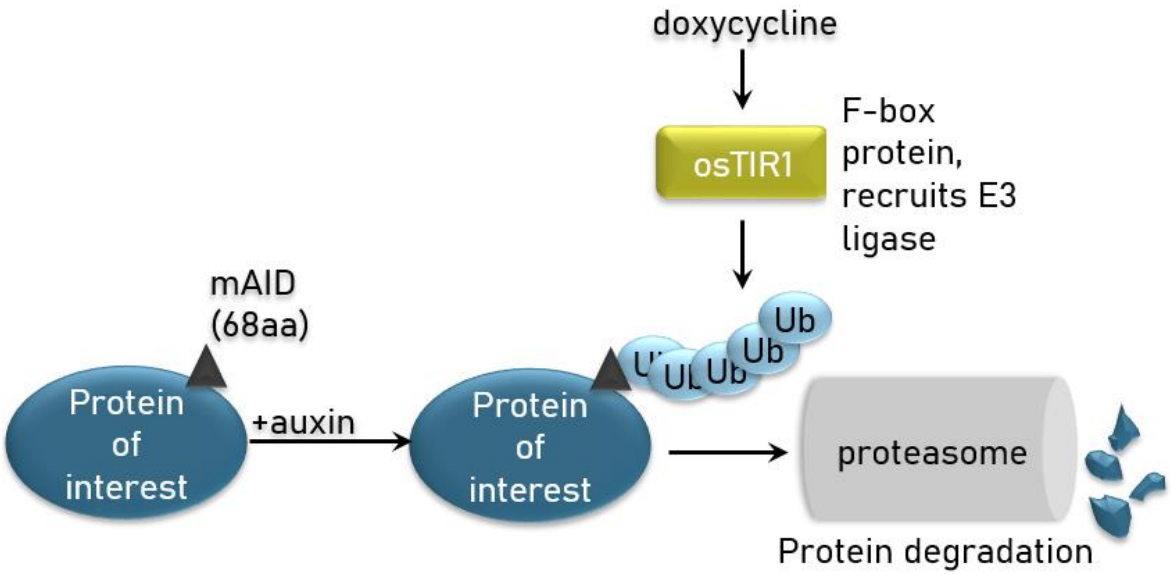


Figure 4. **Auxin-inducible degradation (mAID) system for protein degradation.** Doxycycline + auxin induced osTIR1 expression leads to quick protein degradation.

1.4.3.2 The effect of POLE1 depletion and complementation with C-terminal non-catalytic domain of POLE1 on DNA synthesis

Yeast studies have demonstrated that the deletion of the full-length Pol2 (POLE1 in humans) protein is lethal to yeast cells, however deleting the N-terminal domain which is responsible for the polymerase functions is not lethal (Dua et al., 1999; Kesti et al., 1999). The C-terminal half of Pol2 was found to be essential for yeast replication and being able to function independently of the known polymerization function (Dua et al., 1999). This indicates that the polymerase activity of Pol2 is not crucial, and that its primary role is carried out by the non-catalytic C-terminal domain. These outstanding discoveries were made more than 20 years ago, since then many studies have been conducted to clarify the essential non-catalytic role of Pol2 in DNA synthesis and the mechanism allowing cells to survive in the absence of the catalytic domain of Pol ε.

The absence of Pol2 catalytic domain results in delayed S-phase and therefore slower cell cycle progression. Studies in yeast cells have shown that the lack of the catalytic part of polymerase ε causes replication stress and checkpoint activation, which increases dNTP concentrations and mutation rates (Garbacz et al., 2019). In yeast the C-terminal domain of Pol2 participates in the formation of a complex with the second subunit of Pol ε and the CMG helicase, this is why its absence prevents the initiation and progression of DNA synthesis (Stepchenkova et al., 2021).

According to a study conducted in yeast, the C-terminal domain of Pol2 can interact with other DNA polymerases, such as Pol δ, and this interaction may cause the N-terminal domain of Pol2 to be substituted in DNA synthesis (Feng and D'Urso, 2001). Due to lack of catalytic domain of Pol ε, the Pol δ can conduct majority of replication on the lagging strand and rescue DNA synthesis on the leading

strand. This substitution is associated with slower replication fork movement and disrupted polymerase delta proofreading function caused by missing catalytic domains (Garbacz et al., 2019).

The first study (Vipat et al., 2022) where the non-catalytic function of Pol ε was described in human cells was published recently. This study stated that in the absence of the catalytic N-terminal domain the C-terminal domain of POLE1 is sufficient to assemble a full helicase and support replication in vivo however DNA synthesis is slower due to slower DNA unwinding (Vipat et al., 2022). Similar observations in yeast have already been described (Goswami et al., 2018).This study showed that the C-terminal domain of POLE1 binds to the second subunit POLE2 and in the absence of POLE1 the POLE1 CTD was sufficient to prevent POLE2 degradation. In this study it was identified that POLE1 depleted cells showed ATR activation, which indicates the accumulation of ssDNA and replications, while the expression of the CTD of POLE1 rescued it. Overall, this study provided important knowledge about human polymerase epsilon and its role in replication initiation, however the limitations associated with the instability of the used cell lines did not allow to properly assess the non-catalytic function of POLE1 in human cells and test their viability in the absence of POLE1 NTD that was previously described in yeast.

2. Aims of the thesis

The aim of this thesis was to study the possible non-catalytic role of DNA polymerase epsilon in DNA replication in human cells.

Specific tasks:

1. Create a cell line expressing the C-terminal domain of POLE1.
2. Study the effects of POLE1 depletion on DNA replication in U2OS cells.
3. Check if the expression of the non-catalytic C-terminal domain of POLE1 is sufficient to support DNA synthesis, cell growth and prevent replication stress in the absence of the endogenous protein in human cells.

3. Materials and methods

3.1 Materials

3.1.1 Cell lines

U2OS (homo sapiens bone osteosarcoma cell).

Clones 24 and 25 are U2OS-based cell lines with two modifications. First, osTIR1 was ectopically expressed in U2OS cells, puromycin was used for selection. Second, hygromycin was used for selecting CRISPR/Cas9-mediated mAID-mCherry knock-in at the C-terminus of POLE1. These cell lines were created by the supervisor.

3.1.2 Antibodies

For western blots and Immunofluorescence staining, the following antibodies were used:

Primary antibodies:

GAPDH (Santa Cruz, #sc-47724) 1:1000 dilution

POLE1 (Santa Cruz, #sc-390785) 1:500 dilution

POLE2 (Santa Cruz, #sc-398582) 1:500 dilution

Myc-Tag Mouse mAb (Cell Signaling, #2276) 1:500 or 1:1000 dilution

Chk1-pS345 (Cell Signaling, #2348S) 1:500 dilution

Monoclonal ANTI-FLAG (Sigma-Aldrich, #F3165) 1:500 dilution

Secondary antibodies:

Goat anti-Mouse IgG (Invitrogen, #A16066) 1:10 000 dilution

Goat anti-Rabbit IgG (Invitrogen, #A16104) 1:10 000 dilution

Alexa Fluor 488 goat anti-mouse IgG (Invitrogen, #A11001) 1:300 dilution

3.2 Methods

3.2.1 Cell Culture

U2OS cell line was grown in RPMI medium supplemented with 10% FBS (GIBCO) and 1% penicillin-streptomycin. The parameters for the incubator where the cells were growing were set to 37°C and CO_2 level to 5%. All the passing, treatments and seedings were done under a laminar flow hood using sterile

techniques. All solutions needed for cell growing, were pre-warmed to 37°C. The cells were grown in 100mm cell culture dishes and passed when reached 80-90% confluency.

In order to pass the cells, the old medium was vacuumed away and discarded; cells were washed with 5ml PBS, cells were detached from the plate by incubating for 5 minutes with 1ml of pre-warmed Trypsin; after incubation the cells were resuspended in fresh growth medium, diluted according to the target density (60mm cell culture dishes for following experiments or 100mm dishes for passaging purposes), the cells were evenly distributed by mild shaking and then the dishes were moved back to the incubator.

3.2.2 Transfections

Transfections were performed on 80% confluent U2OS cells that were previously seeded in 6 well plates. In one tube 2.5µg of plasmid DNA were added to 100µL of Opti-MEM (Gibco, #31985070) and mixed. In another tube 5µL of Lipofectamine 2000 (Invitrogen, #11668019) was added to 100µL of Opti-MEM and mixed. The DNA mixture was added to the lipofectamine mixture and incubated for 20 minutes at room temperature. After the incubation the mixture was added to U2OS cells for 6 hours, followed by medium change. The samples were analyzed 48 hours after transfection.

3.2.3 Generation of stable cell lines

24 hours after the transfection cells were selected with 400 µg/ml concentration of G418 (Biowest, #L0015). Every 2-3 days the growth medium was changed, and new antibiotic was added until all the cells on the non-transfected dish died. Old growth medium was removed and discarded; cells were washed with PBS; trypsinized and collected into tubes. Cells were counted using a hemocytometer and based on that diluted until reached the required concentration. Transfected U2OS cells were seeded as singe cells into a 96-well plate. The appropriate concentration was 3 cells per 1 milliliter. Using a multichannel pipette, 50µL of medium and 100µL of cell suspension was added to each well. Pre-labelled plates were left in the incubator and checked under the microscope every day. Wells in which there was one colony were marked and passaged and expanded as needed. Wells with more than one cell colony were discarded.

3.2.4 Doxycycline and Auxin treatment

The doxycycline and auxin treatment was performed on 80% confluent U2OS cells at the time of seeding into 6cm dishes: the cells were treated with 4 ug/ml of doxycycline (Sigma, #D9891-1G) and 500uM of 3-IAA (Indole-3-acetic acid, Sigma, #I2886-5G) or 2ul of DMSO (ThermoFisher, #D/4120/PB08). Cells were treated with dox/aux or DMSO for 16 hours. After the incubation, cells were collected and analyzed.

3.2.5 Cell lysis

Growth medium was removed from the samples and cells were washed with 5ml PBS. After removing the PBS, the cells were put on ice where 150µL of TGN buffer (50 mM TrisHCl pH 7.5, 150 mM NaCl, 50 mM NaF, 1% Tween-20, 0.5% NP-40, and protease inhibitors #A32953) was added. Cells were scraped with a plastic scraper and collected into 1.5ml tubes. The mix was incubated on ice for 20

minutes, followed by clearing the lysates by centrifugation at 4°C 14 000 rpm for 5 minutes. 100µL of supernatant was collected into fresh tubes, mixed with 100µL 2x Laemmli Sample Buffer (with beta- 22 mercaptoethanol) and incubated for 10 min at 96 °C. Samples were briefly centrifuged and stored at -20°C.

3.2.6 Western Blot

Proteins were separated in 10% SDS-polyacrylamide gels with 4% stacking gel that were made using SureCast Gel Handcast system and reagents, according to manufacturer' s instructions. Electrophoresis was run using an Invitrogen Mini Gel Tank. Samples were boiled on a heat block at 96°C for 10 min, centrifuged and loaded on the gel. 3µL of PageRuler™ Plus Prestained Protein Ladder (ThermoFisher #26619) was loaded as a marker. Running buffer 1x (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) was used for the gel electrophoresis. The gel electrophoresis was run at 70 volts for approximately 30 minutes or until the samples had entered the resolving gel continuing with 150 volts until the marker dye reached the bottom of the gel.

After running the gel, proteins were transferred onto a blotting membrane (0.45µm Nitrocellulose Blotting Membrane) using an Invitrogen Mini Blot appliance, using transfer buffer 1x (25 mM Tris, 192 mM glycine, pH8.3 and 10% ethanol added), blotting papers, sponges, blotting membrane and previously made gel with separated proteins. Transfer was run at 30 volts for 2 hours. The membrane was blocked for 1 hour in 5% nonfat dry milk solution in TBST 1x (150 mM NaCl, 10 mM Tris pH8.0, 0.1% Tween20).

The membranes were incubated with primary antibodies (5% non-fat dry milk solution, TBST 1x, 0.01% sodium azide) on a rocker overnight at 4°C. After the overnight incubation primary antibodies were removed, the membranes were washed with TBST 1x for 30 minutes, changing the buffer every 5 minutes. Following the wash, membranes were incubated at room temperature with secondary antibody (5% non-fat dry milk solution in TBST 1x) for 1 hour. The secondary antibody was removed, and the membranes were washed again with TBST1x for 30 minutes, changing the buffer every 5 minutes. For chemiluminescence analysis 20X LumiGLO reagent and 20X Peroxide (Cell Signaling Technology, #7003S) were used according to the manufacturer's instructions. The blot images were taken using ImageQuant LAS 4000 (GE Healthcare).

3.2.7 Detection of EdU incorporation with click chemistry reaction

EdU is incorporated into DNA during active DNA synthesis and it can be detected via copper-catalyzed alkyne-azide click reaction (Da Silva et al., 2017) (**Figure 5**). The click reaction is known for having high selectivity and low background signal (Buck et al., 2008). The reaction is catalyzed by copper and the detection happens when fluorophore-labeled azide covalently binds to the alkyne group, forming a stable triazole ring.

For EdU FACS, cells were treated with 4µL of EdU (Invitrogen, #E10187), diluted in DMSO to the final concentration of 10µM) for 30 minutes. After the incubation, the cells were washed with PBS, trypsinized and collected into prelabelled tubes. The tubes were centrifuged at 1200rpm for 5 minutes, the media was removed, and the pellets were resuspended in 250µL of PBS. Cells were fixed by adding 750µL of cold 96% ethanol continuously during low speed vortexing and kept on ice for minimum 30

minutes. The cells were centrifuged at room temperature at 2000rpm for 5 minutes. The ethanol was washed away with 5ml of PBS with 2000rpm spin, followed by rehydration by incubating with 5ml PBS at room temperature for 15 minutes.

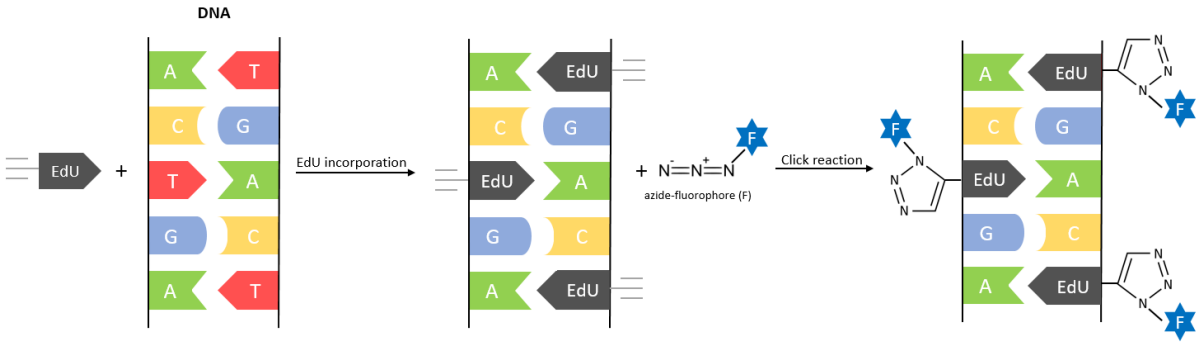


Figure 5. Principle of EdU incorporation and click reaction. EdU incorporates into replicating DNA and forms a covalent bond with an azide fluorophore in the presence of copper, consequently resulting to a fluorescently tagged replicating DNA.

The click reaction mixture was composed of PBS, 2mM of copper sulfate (100mM, #451657), 10mM of sodium ascorbate (1M, #AC352680050) and 5uM of Alexa Fluor 488 Azide (1mM, Invitrogen, #A10266). The cells were incubated with the click reaction mixture at room temperature for 30 minutes, protected from light, and then washed with 5 ml of PBS and centrifuged at 2000rpm for 5 minutes. After removing the supernatant, the cell pellets were resuspended in 300µL of PBS and transferred to special flow cytometry tubes for flow cytometry analysis.

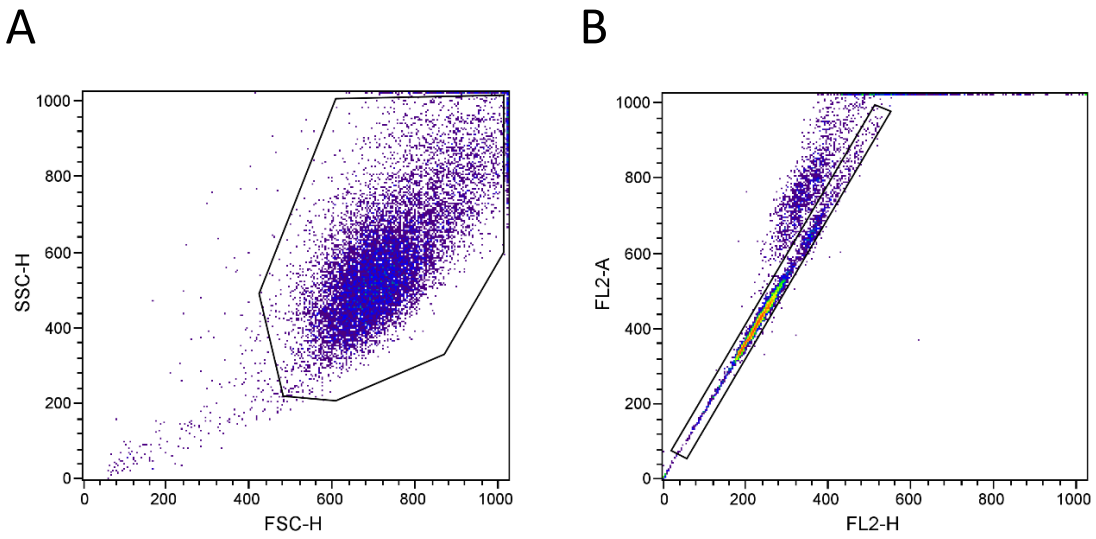


Figure 6: Flow cytometry gating strategy. A. SSC-H and FSC-H channels were used to select cells by size. The gate excludes abnormally small cells that were not usable for analysis. **B.** FL2-A and FL2-H were used to select single cells. The diagonal gate excludes cell doubles and clumps.

Flow cytometry was performed using 2002 FACSCalibur flow cytometer with CellQuest Pro software, and data were analyzed by using FCSalyzer software. 15 000 cells were collected from each sample. A gate has been applied to select cells in SSC-H and FSC-H channels based on size, to exclude cell debris (**Figure 6A**). FL2 channels FL2-A (area) versus FL2-H (height) are used to identify single cell events (**Figure 6B**). Cells were selected with a diagonal gate in FL2-H and FL2-A channels to filter out singles from doubles or cell clumps. The main purpose of the gates was to select single cells to include for further analysis and prevent inaccurate conclusions.

3.2.8 Growth curves

The cells were washed with PBS, trypsinized and collected into prelabelled tubes. To calculate the cell concentration and count the cells, a hemocytometer was used, according to the manufacturer's instructions. Cell suspensions were diluted to 350 000 cells per 1ml and seeded onto a 6cm dish. After 24, 48, and 72 hours, cells were also washed with PBS, trypsinized, collected into prelabelled tubes and counted.

3.2.9 Immunofluorescence staining

Coverslips were sterilized by dipping in 96% ethanol, dried, and placed at the bottom of a 6-well plate. For immunofluorescence staining cells were seeded onto the coverslips at 70-80% confluency. After removing the growth medium, the cells were washed with PBS, fixed with 4% PFA for 10 minutes at room temperature protected from light. After fixation, the cells were washed 2x with PBS to remove the fixation solution completely and permeabilized with 0.1% Triton X100 in a PBS solution for 20 minutes at room temperature. After permeabilization, cells were blocked by incubation with PBS/2%BSA/0.1%Triton in PBS for at least 1 hour. Primary antibody (Monoclonal ANTI-FLAG, #F3165) was diluted in PBS/0.5%BSA/0.1%Triton and added to the samples following overnight incubation at 4°C. After incubation with the primary antibody, the samples were washed 2x with PBS/0.1%Triton and PBS/0.5%BSA/0.1%Triton, each wash was with 1 minute incubation. Secondary antibody (Alexa Fluor 488, #A11001) was diluted in PBS/0.5%BSA/0.1%Triton and added to the samples following incubation for 1 hour at room temperature. Samples were washed 5x with PBS/0.5%BSA/0.1%Triton, 4x with PBS/0.1%Triton and once with PBS. Coverslips were carefully removed from the wells and dried vertically. Coverslips were flipped onto new slides with one drop of mounting medium (ProLong™ Diamond Antifade Mountant with DAPI, #P36966). Samples were cured overnight at room temperature, protected from light. Samples were stored at 4°C and analyzed using Olympus BX61 fluorescent microscope.

4. Results

4.1 Creating a cell line expressing the C-terminal non-catalytic domain of POLE1

In order to check whether DNA synthesis in POLE1-depleted human cells could be rescued by expressing the C-terminal non-catalytic domain of POLE1 (Δcat) in the absence of the full-length POLE1, we transfected clone 24 and clone 25 (U2OS-based cell lines with ectopically expressed osTIR1 and CRISPR/Cas9-mediated mAID-mCherry knock-in at the C-terminus of POLE1) with a plasmid expressing Δcat tagged with myc-FLAG and a Neomycin resistance marker, and selected transfected cells with 400 µg/ml concentration of G418 (Biowest, #L0015) until all the cells on the non-transfected dish died. The efficiency of transfection and selection was confirmed by western blot (**Figure 7**).

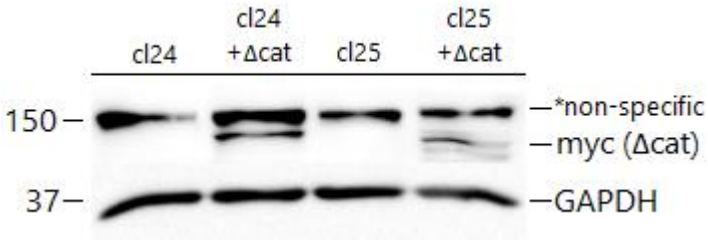


Figure 7: Clone 24 and 25 transfected with Δcat-myc-FLAG. U2OS cells were transfected with C-terminal part of POLE1 and selected with G418.

Single-cell cloning was used to create genetically identical populations of cells which is necessary for the downstream experiments. We allowed single cells from heterogeneous clone 24 and clone 25 transfections to repopulate and create homogenous populations of cells. In total, we obtained 17 clones using single-cell cloning, including eight clones from clone 24 and nine clones from clone 25. The expression of myc-tagged Δcat was tested using western blot analysis (**Figure 8**). Three clones expressed Δcat-myc-FLAG (clone 24.5; clone 25.3 and clone 25.2). One of three clones had lower Δcat-myc signal and therefore was not used further in this project.

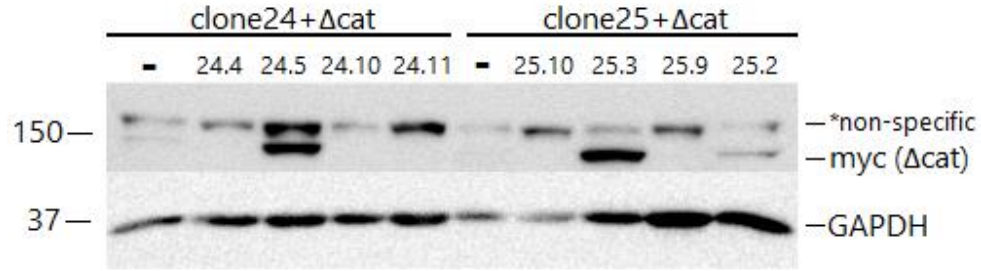


Figure 8: Δcat-myc-FLAG expression in single cell clones. Clones from single-cell cloning were lysed and western blot analysis was performed. The clones that are stably expressing myc-tagged Δcat with are marked with red rectangle.

4.2 The effect of stably expressed C-terminal domain of POLE1 on POLE2 stability and replication stress in POLE1-depleted cells

Next, we tested the effects of POLE1 depletion on clones 24 and 25 with and without Δcat expression. Using western blot analysis, we confirmed that 16-hour doxycycline and auxin (dox/aux) treatment was sufficient to fully degrade POLE1 in all four clones (**Figure 9**).

Previous data (Vipat et al., 2022) showed that the depletion of POLE1 causes POLE2 destabilization, but the C-terminal domain of POLE1 is able to retain the interaction with POLE2 and maintain its stability. As a result of dox/aux treatment, we saw a strong reduction of POLE2 in POLE1 depleted clones 24 and 25, this destabilization was also detected in clones 24.5 and 25.3 (**Figure 9**), indicating that the expression of Δcat was not capable of supporting the stability of POLE2. Based on our experiment we can conclude that POLE1 depletion caused POLE2 degradation in all four clones.

Previous studies (Vipat et al., 2022) have confirmed that POLE1 depletion causes ATR activation and replication stress. We studied if the C-terminal domain of POLE1 could prevent replication stress caused by POLE1 depletion. As a result of dox/aux treatment, we saw replication stress in clones 24 and 25 as well as in clones 24.5 and 25.3, as seen by ATR-dependent phosphorylation of CHK1 at serine-345. Since clone 24.5 and 25.3 expressed Δcat-myc-FLAG, after comparing them with clone 24 and 25 we can conclude that stable expression of Δcat did not prevent replication stress after the depletion of the endogenous POLE1(**Figure 9**).

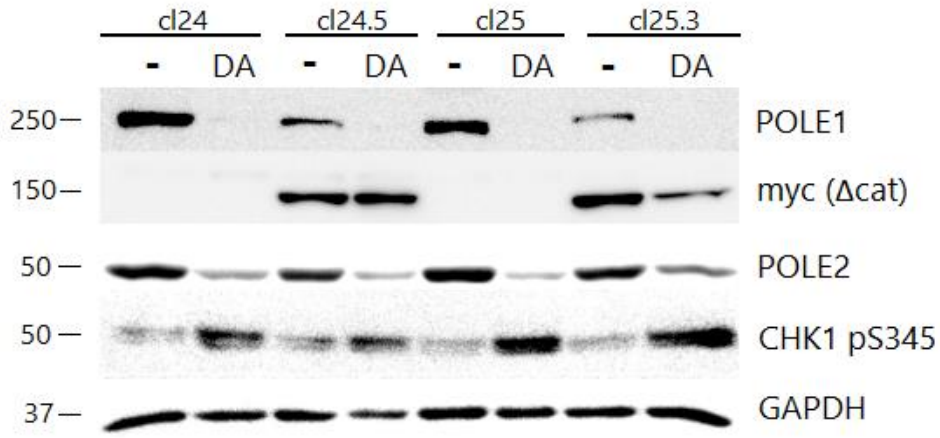


Figure 9: The effect of POLE1 depletion and its complementation with stably expressed CTD of POLE1 on POLE2 and replication stress. Clones 24, 25, 24.5, and clone 25.3 were treated for 16 hours with dox/aux or DMSO. Cell lysates were analyzed by western blot using indicated antibodies.

4.3 The effect of stably expressed C-terminal domain of POLE1 on DNA synthesis in POLE1-depleted cells

Previously published data (Vipat et al., 2022) indicated that Δcat expression can partially rescue DNA synthesis in POLE1-depleted cells. In order to test this in our newly obtained clones, we treated four clones (clone 24; clone 25; clone 24.5 and clone 25.3) with DMSO or dox/aux for 16 hours followed by a 30-minute incubation with EdU to label the ongoing DNA synthesis. After fixation, EdU was stained using click chemistry reaction, and the EdU incorporation was analyzed by flow cytometry. Our experiment confirmed that POLE1 depletion stopped DNA synthesis in clone 24 and clone 25 (**Figure 10**). However, we saw no significant difference in the percentage of EdU positive cells between dox/aux treated clones 24.5 and 25.3 expressing the C-terminal non-catalytic domain of POLE1 and clones 24 and 25, indicating that stable expression of Δcat did not rescue DNA synthesis in the absence of the endogenous POLE1 (**Figure 10**).

4.4 Growth rate analysis

Previously published data indicated that the depletion of POLE1 stopped yeast cell growth. The expression the C-terminal non-catalytic domain of Pol2 can rescue yeast cell growth, however in a slower rate in the absence of the endogenous protein (Dua et al., 1999). The viability of human cells after POLE1 complementation with CTD of POLE1 has not been studied yet. Our created cell line is the first system that would allow it to be tested in human cells. We wanted to test if Δcat expression would rescue cell growth in the absence of the full length POLE1. In order to test whether POLE1 depletion affects the cell growth, we treated four clones (clone 24; clone 25; clone 24.5 and clone 25.3) with DMSO or dox/aux for 72 hours. Cells were counted at three timepoints, every 24 hours. Based on the results (**Figure 11**) we can conclude that stable expression of Δcat did not support cell growth in the absence of the POLE1 full-length protein.

4.5 The effect of transient complementation with C-terminal non-catalytic domain of POLE1 in the absence of the full-length protein, on DNA synthesis

According to the results of this project using clones expressing Δcat-myc-FLAG (clone 24.5 and clone 24.3), the expression of the C-terminal non-catalytic domain (Δcat) of POLE1 did not prevent replication stress, rescue DNA synthesis, or cell growth in the POLE1-depleted cells. These data contradict previously published results (Vipat et al., 2022), therefore we decided to perform additional experiments to figure out what could be the reason for this inconsistency.

First, we decided to use EdU incorporation analysis to confirm that after POLE1 depletion, the C-terminal non-catalytic domain (Δcat) is enough to partially rescue DNA synthesis in human cells. In order to do that, clones 24 and 25 were transiently transfected with a plasmid expressing Δcat or an empty vector as a negative control. Cells were incubated with dox/aux for 16 hours followed by a 30-minute incubation with EdU. 48 hours after transient transfection cells were fixed and EdU was stained using click chemistry reaction. EdU incorporation was analyzed by flow cytometry.

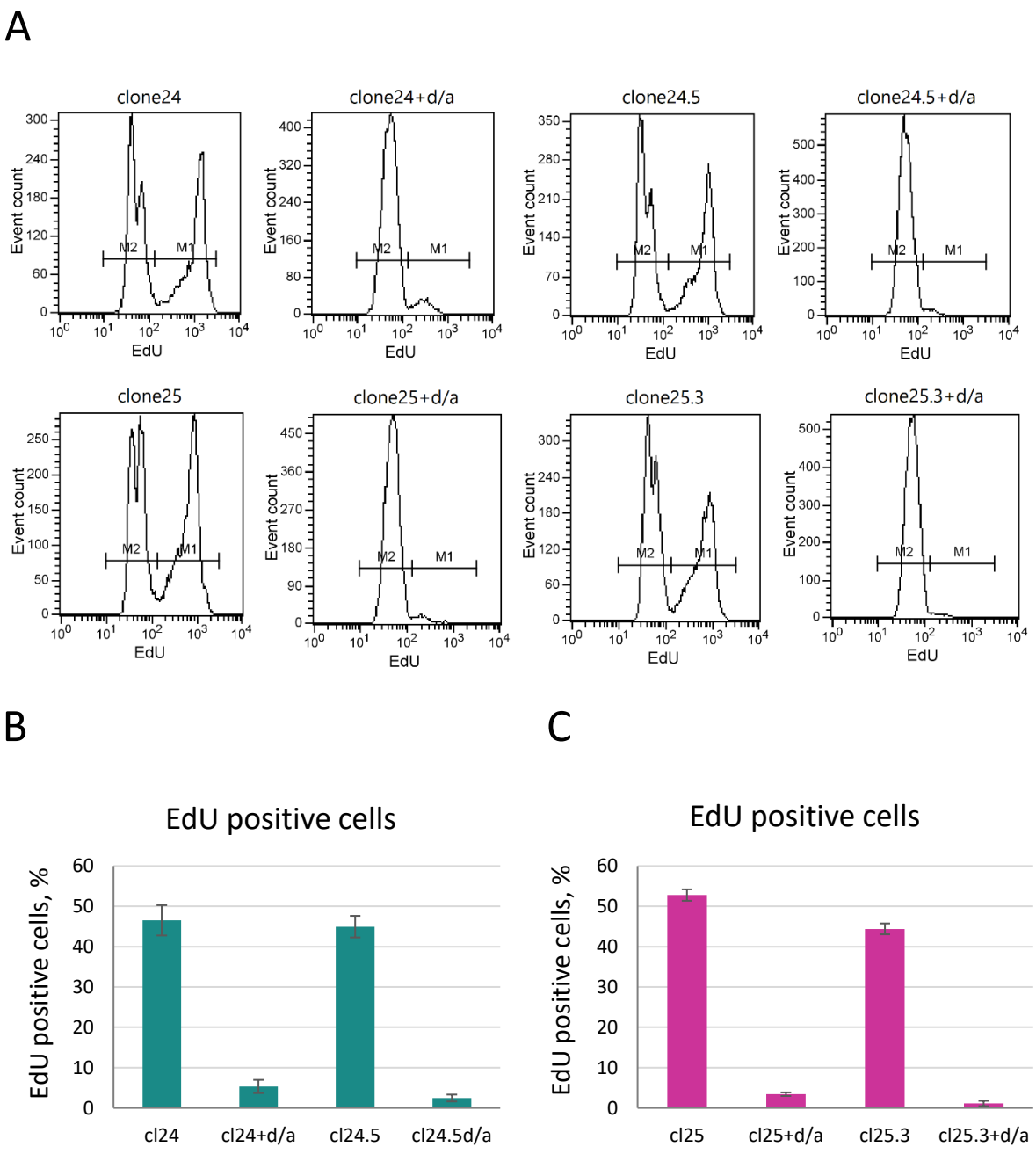
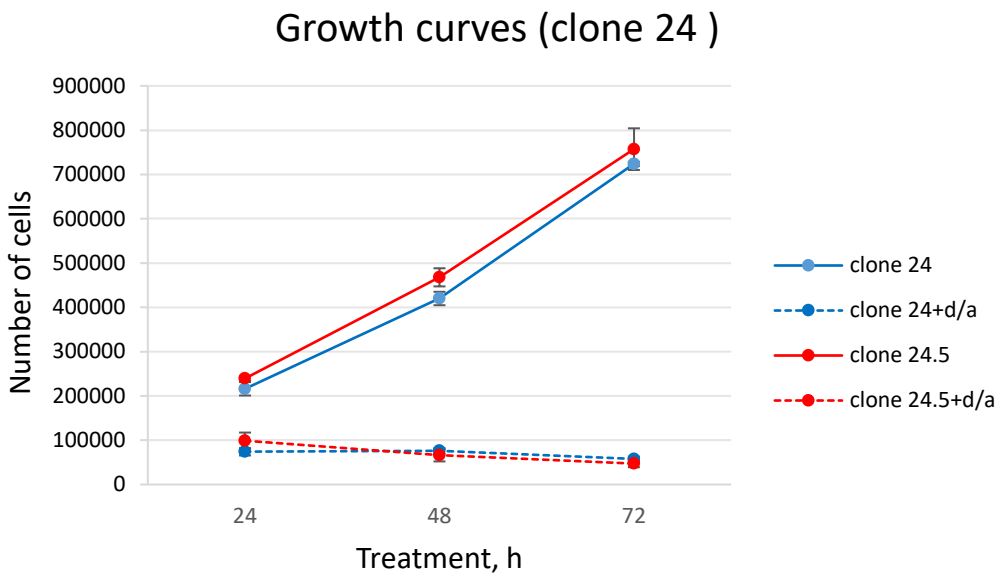


Figure 10: DNA synthesis-dependent on the stable expression of C-terminal non-catalytic domain of POLE1. Clones 24, 25, 24.5, and clone 25.3 were treated for 16 hours with dox/aux or DMSO. EdU was added for the last 30 min of treatment. **A.** Flow cytometry histograms of EdU incorporation shown with M1 and M2 gates. **B-C.** The quantifications of EdU positive cells of clones 24 and 25 in the absence of the endogenous POLE1 are shown. Quantifications are based on three independent experiments, error bars from standard deviations are shown. M1 and M2 gates were used to gate cells that represent EdU positive cells and EdU negative cells respectively.

A



B

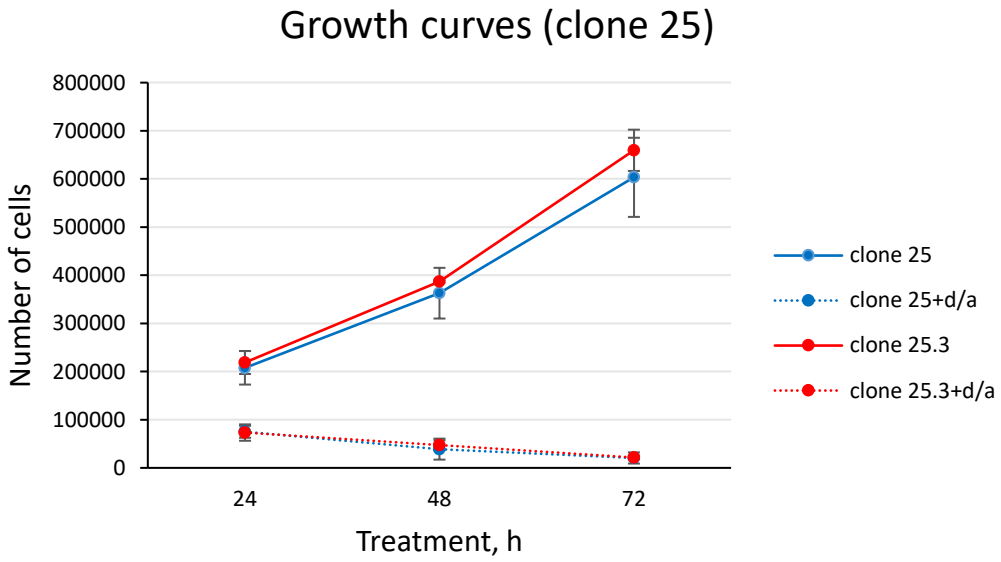


Figure 11: The effect of POLE1 depletion and its complementation with stably expressed CTD of POLE1 on cell growth. Clones 24, 25, 24.5, and clone 25.3 were treated for 16 hours with dox/aux or DMSO. The quantifications are based on three independent experiments where the cells were counted at three different timepoints. Error bars from standard deviations are shown.

Flow cytometry analysis of cells transiently expressing Δcat showed that the C-terminal non-catalytic domain of POLE1 partially rescued DNA replication in human cells in the absence of a full-length protein (**Figure 12**), indicating a non-catalytic role of DNA Pol ε in DNA replication, in agreement with the previously published results (Vipat et al., 2022).

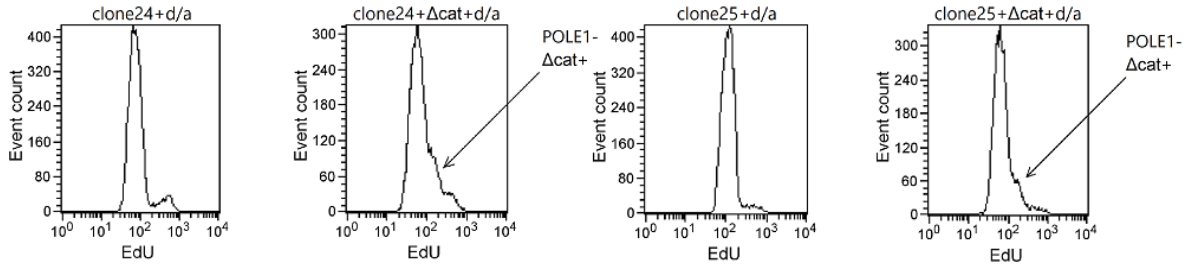


Figure 12: DNA synthesis-dependent on the transient expression of C-terminal non-catalytic domain of POLE1. Clones 24, 25, 24.5, and clone 25.3 were treated for 16 hours with dox/aux or DMSO. EdU was added for the last 30 min of treatment. Flow cytometry histograms of EdU incorporation are shown. Δcat positive cells peaks are marked.

4.6 Determining the localization of the stable expressed C-terminal non-catalytic domain of POLE1 within the cell

Second, we hypothesized that during the creation of the stable cell lines, the protein expression was mislocalized within the cell. In order to check, whether the localization of the C-terminal non-catalytic domain (Δcat) of POLE1 was in the cell nucleus in our newly obtained clones, we used immunofluorescent staining. Cells were seeded on glass coverslips, fixed and permeabilized. Anti-FLAG antibody was used as a primary antibody because in the newly obtained cell line Δcat was tagged with myc-FLAG which allows FLAG antibody to bind to it. Mounting medium with DAPI was used to mount coverslips on glass slides and stain the nuclei. Although the cells were immersed in 2% BSA to block nonspecific binding, there is still some nonspecific binding and unclear signal (**Figure 13)**.

DAPI signal shows the location of the nucleus as the stain bounds to double-stranded DNA. FLAG signal shows the location of the Alexa Fluor 488 bound to anti-FLAG antibody, and this signal does not overlap with the DAPI signal very well. The microcopy images (**Figure 13)** show that in our created clones 24.5 and 25.3 Δcat is primarily localized in the cytoplasm and not in the nucleus. Clone 25.3 has mixed population of cells as Δcat is localized in the cytoplasm in some cells and other cells - throughout the cell.

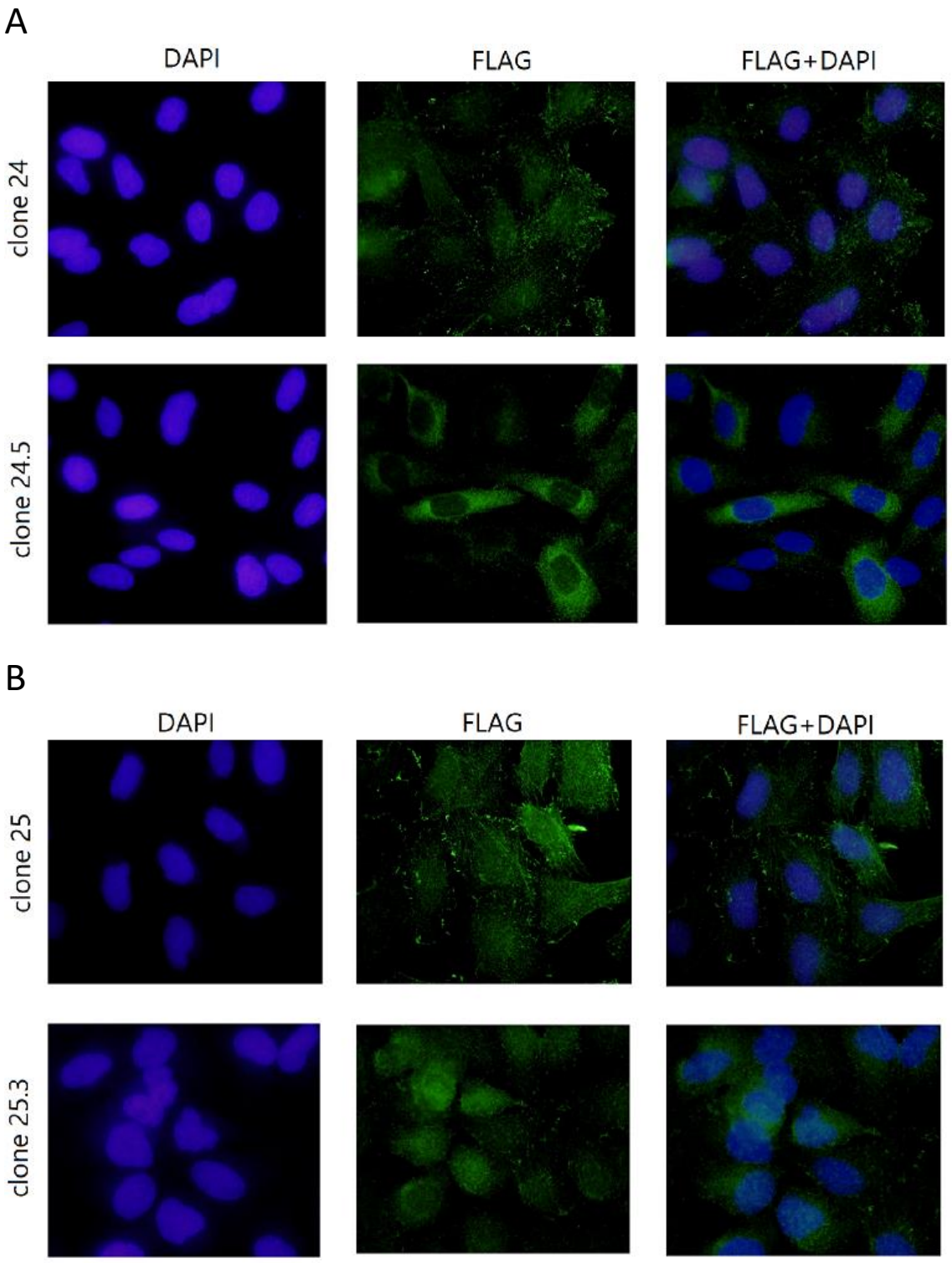


Figure 13: Localization of the overexpressed C-terminal non-catalytic domain of POLE1 within the cell in clones 24.5 and 25.3. A. Immunofluorescence staining of clone 24 and clone 24.5 with DAPI and FLAG antibody. **B.** Immunofluorescence staining of clone 25 and clone 25.3 with DAPI and FLAG antibody.

5. Discussion

POLE1 has two different domains, N-terminal catalytic domain and C-terminal non-catalytic. Yeast studies have shown that the deletion of the full-length Pol2 (POLE1 in humans) is lethal to yeast cells, however deleting only the domain which is responsible for the polymerase function is not lethal (Dua et al., 1999). This indicates that the polymerase activity of Pol2 is not crucial, and that its essential role is carried out by the non-catalytic C-terminal domain. In yeast the C-terminal domain of Pol2 participates in the formation of a complex with the second subunit of Pol ε and the CMG helicase (Stepchenkova et al., 2021). Multiple different yeast studies have stated that the catalytic subunit of polymerase ε is necessary and sufficient for cell viability. According to a recent study (Vipat et al. 2022), POLE1's C-terminal non-catalytic domain can rescue DNA synthesis and eliminate replication stress caused by POLE1's absence in human cells. The C-terminal domain of Pol2 is necessary and sufficient for yeast cell survival, although this has not yet been proven in human cells. Numerous studies have been conducted on DNA polymerase ε non-catalytic role in the initiation of replication in yeast, however, less is known about its role in human cells. The aim of this project was to further study the effects of the depletion of the endogenous POLE1 and its substitution with the non-catalytic C terminal domain of POLE1.

During this project we were able to create a cell line expressing the C-terminal non-catalytic domain (CTD) of POLE1, and capable of depleting the endogenous POLE1 after treatment with doxycycline and auxin. In this study we used auxin-inducible degron (mAID) system for POLE depletion. Introducing the plant-specific degradation pathway, controlled by auxin, into non-plant cells leads to quick protein degradation of mAID tagged proteins (Natsume et al., 2016). The belief that this method can be applied as a standard method in various organisms is supported by investigations of the AID technology applied to other model organisms, such as *C. elegans* (Zhang et al., 2015), *S. cerevisiae* (Shetty et al., 2019), and fruit fly (Trost et al., 2016).

The cell line (clone 1.6 expressing Δcat-myc-FLAG) used in the previous study (Vipat et al., 2022) developed resistance to doxycycline treatment, showing wild type level of EdU incorporation by a subpopulation of cells failing to deplete POLE1. This cell line was not able to express osTIR1 in response to doxycycline treatment due to doxycycline-inducible promoter being methylated. In the absence of osTIR1 cells are unable to degrade POLE1. The issue was caused by methylation of the dox-inducible promoter. To prevent the subclones from methylating the doxycycline inducible promotor we used recently created clone 24 and clone 25 that express osTIR1 ectopically and not as a knock-in, and therefore did not develop resistance to doxycycline. We observed that our newly created cell line expressing POLE1 CTD also effectively degraded POLE1 in response to the 16h doxycycline and auxin treatment.

One of the key properties of the C-terminal domain of POLE1 and POLE2 is to serve as a connecting link between CMG components and support its formation at replication origins. The Pol ε subunit Dpb2 interacts with the C-terminal domain of Pol2, and it is suggested that the CTD of Pol2 is needed to properly localize and regulate the activity of Dpb2 (Kesti et al., 1999). Studies in yeast have demonstrated that the absence of the Dpb2 subunit (POLE2 in humans) does not influence the catalytic activity of polymerase epsilon, but it can reduce the stability of Pol2 (POLE1 in humans) (Kesti et al., 1999). Our data confirmed that the depletion of POLE1 causes POLE2 destabilization, in agreement with

previously published data (Vipat et al., 2022), reconfirming that POLE1 is necessary for POLE2 stability in human cells. The expression of the CTD of POLE1 did not prevent POLE2 degradation in the absence of the full-length protein, which contradicted previously published data (Vipat et al., 2022), that show that expression of CTD was sufficient for POLE2 stability.

The absence of Pol2 catalytic domain results in delayed S-phase completion and therefore slower cell cycle progression in yeast. It has been shown that the expression of CTD of Pol2 can rescue yeast cell growth, however at a slower rate (Dua et al., 1999). A study also suggests that Pol δ is able to take over Pol ε role in the absence of NTD but the absence of CTD results in inviable cells (Isoz et al., 2012). The viability of human cells following POLE1 depletion and its complementation with POLE1 CTD has not been studied before. Our created cell lines would have been the first to test this complementation in human cells. Our experiment showed that stable expression of CTD did not support cell growth in the absence of the POLE1 full-length protein in human cells. This result differs from previous studies (Dua et al., 1999) in yeast cells, showing that the expression of CTD of Pol2 can restore cell proliferation in yeast cells.

Without the Pol2 catalytic domain, Pol δ synthesizes both the leading and lagging strands of DNA in yeast. DNA synthesis is slower due to the reduced speed of DNA unwinding, given that polymerase ε interacts with the CMG helicase but polymerase δ is not known to bind to CMG. Pol ε is essential for the helicase activation, which suggests that the CMG unwinds the double-helix slower as it is not fully activated in Pol ε depleted cells (Yeeles et al., 2017). Previous study (Vipat et al., 2022) on the effect of POLE1 depletion and its complementation with the CTD of POLE1 in human cells has confirmed that cells lacking POLE1 catalytic domain are able to synthesize DNA, however at a slower rate. Based on the flow cytometry analysis for measuring EdU incorporation we saw that in our cell lines, the stably expressed C-terminal non-catalytic domain of POLE1 is insufficient to rescue DNA synthesis in the absence of the full-length protein. These results contradicted previously published data (Vipat et al., 2022), showing that slow DNA synthesis can occur in cells lacking full-length POLE1, where POLE1 C-terminal non-catalytic domain was expressed.

POLE depletion is known to induce replication stress and genomic instability in mice (Bellelli et al., 2018b), which is generally caused by defective origin firing. The progression of the CMG helicase without a comparable amount of DNA synthesis is the source of replication stress in yeast cells lacking the essential protein Pol2 (Stepchenkova et al., 2021). Previous studies (Vipat et al., 2022) have confirmed that POLE1 depletion causes ATR activation and replication stress in human cells, however the expression of CTD of POLE1 rescued this, indicating that the complementation of POLE1 with CTD of POLE1 can prevent replication stress. Our data reconfirmed that POLE1 depleted cells showed ATR activation, indicating accumulation of single-stranded DNA and replication stress. However, the newly obtained clones expressing the CTD of POLE1 were not able to prevent replication stress in the absence of POLE1, which contradicted previously published data (Vipat et al., 2022).

According to the results of this project using clones stably expressing Δcat-myc-FLAG (clone 24.5 and clone 24.3), the stable expression of the CTD of POLE1 did not prevent replication stress, rescue DNA synthesis, or promote cell proliferation in POLE1-depleted cells. We considered two explanations: either our newly obtained clones were not operating normally, or the previously published findings are

not reproducible and POLE1 CTD without the full-length POLE1 has no beneficial effects on human cells. We checked both hypotheses.

First, we checked the reproducibility of the previously published experiment (Vipat et al., 2022) in human cells. We transiently transfected clones with Δcat-myc-FLAG and used EdU incorporation analysis to confirm that after POLE1 depletion, the expression of the C-terminal non-catalytic domain is enough to partially rescue DNA synthesis in human cells. The cells transiently expressing Δcat showed that the CTD of POLE1 partially rescued DNA replication in human cells in the absence of a full-length protein, indicating a non-catalytic role of DNA Pol ε in DNA replication, in agreement with the previously published results (Vipat et al., 2022).

Second, we investigated the alternative hypothesis, checking the quality of our newly created cell line. One possible explanation for the absence of the effect is the mislocalization of the stably expressed CTD within the cell. In eukaryotic cells the nucleus is the primary compartment where Pol ε functions in DNA replication. Our data showed that in our newly obtained clones the CTD of POLE1 is primarily localized in the cytoplasm and not in the nucleus, explaining the absence of the effects. A study in human cancer cells has previously shown that mislocalization of a stably expressed protein accounted for loss of its essential cell functions (Lau et al., 2006).

Since the N-terminal domain of Pol ε catalytic subunit was discovered to be dispensable for yeast cell survival, numerous studies have been carried out in yeast cells, but because the human body is more challenging and complex than yeast, all of the information we know from yeast is not inheritable to humans. Overall, our project reconfirms the effect of POLE1 depletion on DNA replication in human cells. In the future we would create new cell lines to test the effect of POLE1 depletion and complementation with C-terminal non-catalytic domain of POLE1 even further. We think that further studies of the consequences of the absence of POLE1 will help understand mutations and human cancer.

6. Abstract

DNA replication is the process in which a DNA molecule is duplicated to create two DNA molecules that are identical to each other. DNA replication ensures that when a cell divides, the genetic information is passed to each daughter cell. In eukaryotes the majority of replication is conducted by B family polymerases α, δ and ε. These polymerases perform an essential role by adding the appropriate nucleotide to the nascent strand to match the template strand.

Pol ε is responsible for the continuous synthesis of the leading strand and plays an important role in genome stability, replisome assembly and replication fork movement. It consists of four subunits: the catalytic subunit POLE1 and three smaller subunits POLE2, POLE3 and POLE4. POLE1 has two different domains, N-terminal catalytic domain and C-terminal non-catalytic domain. The N-terminal domain of POLE1 is responsible for catalyzing DNA synthesis in human cells. Based on a recent study (Vipat et al., 2022) in human cells the C-terminal non-catalytic domain of POLE1 is capable of rescuing DNA synthesis and prevents replication stress caused by the absence of POLE1. Although this has not yet been established in human cells, the C-terminal domain of the catalytic subunit of DNA Pol ε is required and sufficient for cell survival in yeast. The non-catalytic role of DNA polymerase ε in replication initiation in yeast has been extensively studied, however the non-catalytic function of polymerase ε in human cells is not known yet.

The aim of this thesis was to determine if the non-catalytic C-terminal domain of POLE1 could support DNA synthesis, cell growth, and prevent replication stress in human cells in the absence of the full-length POLE1. A cell line expressing the C-terminal non-catalytic domain (CTD) of POLE1, and capable of depleting the endogenous POLE1 after treatment with doxycycline and auxin, was created. Stable expression of CTD in the new cell line did not prevent replication stress, POLE2 degradation, growth defects, or DNA synthesis block caused by POLE1 depletion. We were able to show that transiently expressed CTD of POLE1 is able to partially rescue DNA synthesis in the absence of the full-length protein and in this situation DNA synthesis is slower. The absence of the effects of stably expressed CTD on POLE1 depletion-induced phenotypes in the new cell line were caused by the mislocalization of the protein within the cell.

Kokkuvõte

DNA replikatsioon on protsess, mille käigus DNA molekul dubleeritakse, et luua kaks teineteisega identset DNA molekuli. DNA replikatsioon tagab, et raku jagunemisel edastatakse geneetiline informatsioon igale tütarrakule. Eukarüootides viivad suurema osa replikatsioonist läbi B perekonna polümeraasid α, δ ja ε. Need polümeraasid täidavad olulist rolli, lisades sobiva nukleotiidi tekkivale ahelale, et see sobiks matriitsi ahelaga.

Polümeraas ε vastutab juhtiva ahela sünteesi eest, samuti mängib olulist rolli genoomi stabiilsuses, replisoomi kokkupanekus ja replikatsioonikahvli liikumises. See sisaldab nelja alaühikut: katalüütilist alaühikut POLE1 ja kolme väiksemat alaühikut POLE2, POLE3 ja POLE4. POLE1 alaühikul on kaks erinevat domeeni, N-terminaalne katalüütiline domeen ja C-terminaalne mittekatalüütiline domeen. Inimese rakkudes vastutab POLE1 N-terminaalne domeen DNA sünteesi katalüüsimise eest. Tuginedes hiljuti avaldatud uuringule inimese rakkudes on POLE1 C-terminaalne mittekatalüütiline domeen võimeline päästma DNA sünteesi ja ennetama POLE1 puudumisest põhjustatud replikatsioonistressi. Polümeraas epsiloni katalüütilise alaühiku C-terminaalne domeen on vajalik ja piisav rakkude ellujäämiseks pärmis, kuid inimese rakkudes ei ole veel seda kindlaks tehtud. DNA polümeraas epsiloni mittekatalüütilist rolli pärmi replikatsiooni initsiatsioonis on põhjalikult uuritud, kuid polümeraas epsiloni mittekatalüütiline funktsioon inimese rakkudes pole veel teada.

Käesoleva lõputöö eesmärk oli kindlaks teha, kas POLE1 mittekatalüütiline C-terminaalne domeen suudab toetada DNA sünteesi, rakkude kasvu ja vältida replikatsioonistressi inimese rakkudes täispika POLE1 puudumisel. Loodi rakuliin, mis ekspresseerib POLE1 C-terminaalset mittekatalüütilist domeeni (CTD) ja on võimeline endogeenset POLE1 alaühikut pärast doksütsükliini ja auksiiniga töötlemist lagundada. CTD stabiilne ekspressioon uues rakuliinis ei hoidnud ära replikatsioonistressi, POLE2 lagunemist, kasvudefekte ega POLE1 lagunemise poolt põhjustatud DNA sünteesi blokeerimist. Suutsime näidata, et POLE1 ajutiselt ekspresseeritud CTD on võimeline täispika valgu puudumisel DNA sünteesi osaliselt päästma ja sellises olukorras on DNA süntees aeglasem. Stabiilselt ekspresseeritud CTD mõju puudumine POLE1 lagundamisel uues rakuliinis oli põhjustatud valgu valest lokaliseerimisest rakus.

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References

Balakrishnan, L., and R. A. Bambara. 2013. Flap endonuclease 1. *Annual Review of Biochemistry* 82: 119–138.

Bauerschmidt, C., S. Pollok, E. Kremmer, H.-P. Nasheuer, and F. Grosse. 2007. Interactions of human Cdc45 with the Mcm2?7 complex, the GINS complex, and DNA polymerases ? and ? during S phase. *Genes to Cells* 0: 070606122915004-???

Bell, S. P., and A. Dutta. 2002. DNA replication in eukaryotic cells. *Annual Review of Biochemistry* 71: 333–374.

Bellelli, R., O. Belan, V. E. Pye, C. Clement, S. L. Maslen, J. M. Skehel, P. Cherepanov, et al. 2018a. POLE3-POLE4 Is a Histone H3-H4 Chaperone that Maintains Chromatin Integrity during DNA Replication. *Molecular Cell* 72: 112-126.e5.

Bellelli, R., V. Borel, C. Logan, J. Svendsen, D. E. Cox, E. Nye, K. Metcalfe, et al. 2018b. Polε Instability Drives Replication Stress, Abnormal Development, and Tumorigenesis. *Molecular Cell* 70: 707-721.e7.

Bermudez, V. P., A. Farina, V. Raghavan, I. Tappin, and J. Hurwitz. 2011. Studies on Human DNA Polymerase ϵ and GINS Complex and Their Role in DNA Replication. *Journal of Biological Chemistry* 286: 28963–28977.

Boos, D., and P. Ferreira. 2019. Origin Firing Regulations to Control Genome Replication Timing. *Genes* 10: 199.

Buck, S. B., J. Bradford, K. R. Gee, B. J. Agnew, S. T. Clarke, and A. Salic. 2008. Detection of S-phase cell cycle progression using 5-ethynyl-2′-deoxyuridine incorporation with click chemistry, an alternative to using 5-bromo-2′-deoxyuridine antibodies. *BioTechniques* 44: 927–929.

Burgers, P. M. J., and T. A. Kunkel. 2017. Eukaryotic DNA Replication Fork. *Annual Review of Biochemistry* 86: 417–438.

Chagin, V. O., J. H. Stear, and M. C. Cardoso. 2010. Organization of DNA Replication. *Cold Spring Harbor Perspectives in Biology* 2: a000737.

Chong, J. P., M. K. Hayashi, M. N. Simon, R. M. Xu, and B. Stillman. 2000. A double-hexamer archaeal minichromosome maintenance protein is an ATP-dependent DNA helicase. *Proceedings of the National Academy of Sciences of the United States of America* 97: 1530–1535.

Crevel, G. 2001. Nearest neighbour analysis of MCM protein complexes in Drosophila melanogaster. *Nucleic Acids Research* 29: 4834–4842.

Da Silva, M. S., P. A. M. Muñoz, H. A. Armelin, and M. C. Elias. 2017. Differences in the Detection of BrdU/EdU Incorporation Assays Alter the Calculation for G1, S, and G2 Phases of the Cell Cycle in Trypanosomatids. *Journal of Eukaryotic Microbiology* 64: 756–770.

Dua, R., D. L. Levy, and J. L. Campbell. 1999. Analysis of the Essential Functions of the C-terminal Protein/Protein Interaction Domain of Saccharomyces cerevisiae pol ε and Its Unexpected

Ability to Support Growth in the Absence of the DNA Polymerase Domain. *Journal of Biological Chemistry* 274: 22283–22288.

Egel, R. ed. . 2004. The molecular biology of Schizosaccaromyces pombe: genetics, genomics, and beyond. Springer, Berlin ; New York.

Fagundes, R., and L. K. Teixeira. 2021. Cyclin E/CDK2: DNA Replication, Replication Stress and Genomic Instability. *Frontiers in Cell and Developmental Biology* 9: 774845.

Feng, W., and G. D'Urso. 2001. *Schizosaccharomyces pombe* Cells Lacking the Amino-Terminal Catalytic Domains of DNA Polymerase Epsilon Are Viable but Require the DNA Damage Checkpoint Control. *Molecular and Cellular Biology* 21: 4495–4504.

Gaillard, H., T. García-Muse, and A. Aguilera. 2015. Replication stress and cancer. *Nature Reviews Cancer* 15: 276–289.

Goswami, P., F. Abid Ali, M. E. Douglas, J. Locke, A. Purkiss, A. Janska, P. Eickhoff, et al. 2018. Structure of DNA-CMG-Pol epsilon elucidates the roles of the non-catalytic polymerase modules in the eukaryotic replisome. *Nature Communications* 9: 5061.

Hanawalt, P. C. 2004. Density matters: the semiconservative replication of DNA. *Proceedings of the National Academy of Sciences of the United States of America* 101: 17889–17894.

Hao, J., C. de Renty, Y. Li, H. Xiao, M. G. Kemp, Z. Han, M. L. DePamphilis, and W. Zhu. 2015. And-1 coordinates with Claspin for efficient Chk1 activation in response to replication stress. *The EMBO journal* 34: 2096–2110.

Henninger, E. E., and Z. F. Pursell. 2014. DNA polymerase ε and its roles in genome stability: ROLES OF DNA Pol ε IN GENOME STABILITY. *IUBMB Life* 66: 339–351.

Hyrien, O. 2016. How MCM loading and spreading specify eukaryotic DNA replication initiation sites. *F1000Research* 5: F1000 Faculty Rev-2063.

Isoz, I., U. Persson, K. Volkov, and E. Johansson. 2012. The C-terminus of Dpb2 is required for interaction with Pol2 and for cell viability. *Nucleic Acids Research* 40: 11545–11553.

Jedrychowska, M., M. Denkiewicz-Kruk, M. Alabrudzinska, A. Skoneczna, P. Jonczyk, M. Dmowski, and I. J. Fijalkowska. 2019. Defects in the GINS complex increase the instability of repetitive sequences via a recombination-dependent mechanism. *PLoS genetics* 15: e1008494.

Kelman, Z. 1997. PCNA: structure, functions and interactions. *Oncogene* 14: 629–640.

Kesti, T., K. Flick, S. Keränen, J. E. Syväoja, and C. Wittenberg. 1999. DNA polymerase epsilon catalytic domains are dispensable for DNA replication, DNA repair, and cell viability. *Molecular Cell* 3: 679–685.

Korona, D. A., K. G. LeCompte, and Z. F. Pursell. 2011. The high fidelity and unique error signature of human DNA polymerase. *Nucleic Acids Research* 39: 1763–1773.

Lau, Q. C., E. Raja, M. Salto-Tellez, Q. Liu, K. Ito, M. Inoue, T. C. Putti, et al. 2006. RUNX3 Is Frequently Inactivated by Dual Mechanisms of Protein Mislocalization and Promoter Hypermethylation in Breast Cancer. *Cancer Research* 66: 6512–6520.

Lehman, I. R. 2003. Discovery of DNA Polymerase. *Journal of Biological Chemistry* 278: 34733–34738.

Lewis, J. S., M. H. Gross, J. Sousa, S. S. Henrikus, J. F. Greiwe, A. Nans, J. F. X. Diffley, and A. Costa. 2022. Mechanism of replication origin melting nucleated by CMG helicase assembly. *Nature* 606: 1007–1014.

Mazouzi, A., G. Velimezi, and J. I. Loizou. 2014. DNA replication stress: causes, resolution and disease. *Experimental Cell Research* 329: 85–93.

Mei, L., and J. G. Cook. 2021. Efficiency and equity in origin licensing to ensure complete DNA replication. *Biochemical Society Transactions* 49: 2133–2141.

Moiseeva, T. N., and C. J. Bakkenist. 2018. Regulation of the initiation of DNA replication in human cells. *DNA repair* 72: 99–106.

Natsume, T., T. Kiyomitsu, Y. Saga, and M. T. Kanemaki. 2016. Rapid Protein Depletion in Human Cells by Auxin-Inducible Degron Tagging with Short Homology Donors. *Cell Reports* 15: 210–218.

Nitiss, J. L., E. Soans, A. Rogojina, A. Seth, and M. Mishina. 2012. Topoisomerase assays. *Current Protocols in Pharmacology* Chapter 3: Unit 3.3.

Norris, V. 2019. Does the Semiconservative Nature of DNA Replication Facilitate Coherent Phenotypic Diversity? *Journal of Bacteriology* 201: e00119-19.

Pavlov, Y. I., P. V. Shcherbakova, and I. B. Rogozin. 2006. Roles of DNA Polymerases in Replication, Repair, and Recombination in Eukaryotes. International Review of Cytology, 41–132. Elsevier.

Petojevic, T., J. J. Pesavento, A. Costa, J. Liang, Z. Wang, J. M. Berger, and M. R. Botchan. 2015. Cdc45 (cell division cycle protein 45) guards the gate of the Eukaryote Replisome helicase stabilizing leading strand engagement. *Proceedings of the National Academy of Sciences* 112.

Prindle, M. J., and L. A. Loeb. 2012. DNA polymerase delta in DNA replication and genome maintenance. *Environmental and Molecular Mutagenesis* 53: 666–682.

Saldivar, J. C., D. Cortez, and K. A. Cimprich. 2017. The essential kinase ATR: ensuring faithful duplication of a challenging genome. *Nature Reviews Molecular Cell Biology* 18: 622–636.

Sclafani, R. A., and T. M. Holzen. 2007. Cell cycle regulation of DNA replication. *Annual Review of Genetics* 41: 237–280.

Shanbhag, V., S. Sachdev, J. A. Flores, M. J. Modak, and K. Singh. 2018. Family A and B DNA Polymerases in Cancer: Opportunities for Therapeutic Interventions. *Biology* 7: 5.

Shcherbakova, P. V., K. Bebenek, and T. A. Kunkel. 2003. Functions of Eukaryotic DNA Polymerases. *Science of Aging Knowledge Environment* 2003.

Shetty, A., N. I. Reim, and F. Winston. 2019. Auxin-Inducible Degron System for Depletion of Proteins in Saccharomyces cerevisiae. *Current Protocols in Molecular Biology* 128: e104.

Stepchenkova, E. I., A. S. Zhuk, J. Cui, E. R. Tarakhovskaya, S. R. Barbari, P. V. Shcherbakova, D. E. Polev, et al. 2021. Compensation for the absence of the catalytically active half of DNA polymerase ε in yeast by positively selected mutations in *CDC28* J. Nickoloff [ed.],. *Genetics* 218: iyab060.

Stillman, B. 2008. DNA polymerases at the replication fork in eukaryotes. *Molecular Cell* 30: 259–260.

Takayama, Y., Y. Kamimura, M. Okawa, S. Muramatsu, A. Sugino, and H. Araki. 2003. GINS, a novel multiprotein complex required for chromosomal DNA replication in budding yeast. *Genes & Development* 17: 1153–1165.

Trost, M., A. C. Blattner, and C. F. Lehner. 2016. Regulated protein depletion by the auxin-inducible degradation system in *Drosophila melanogaster*. *Fly* 10: 35–46.

Vipat, S., D. Gupta, S. Jonchhe, H. Anderspuk, E. Rothenberg, and T. N. Moiseeva. 2022. The non-catalytic role of DNA polymerase epsilon in replication initiation in human cells. *Nature Communications* 13: 7099.

Walter, J. C. 2000. Evidence for Sequential Action of cdc7 and cdk2 Protein Kinases during Initiation of DNA Replication in Xenopus Egg Extracts. *Journal of Biological Chemistry* 275: 39773–39778.

Yang, R., C. Müller, V. Huynh, Y. K. Fung, A. S. Yee, and H. P. Koeffler. 1999. Functions of cyclin A1 in the cell cycle and its interactions with transcription factor E2F-1 and the Rb family of proteins. *Molecular and Cellular Biology* 19: 2400–2407.

Yao, N. Y., and M. O'Donnell. 2010. SnapShot: The replisome. *Cell* 141: 1088, 1088.e1.

Yeeles, J. T. P., A. Janska, A. Early, and J. F. X. Diffley. 2017. How the Eukaryotic Replisome Achieves Rapid and Efficient DNA Replication. *Molecular Cell* 65: 105–116.

Zeman, M. K., and K. A. Cimprich. 2014. Causes and consequences of replication stress. *Nature Cell Biology* 16: 2–9.

Zhang, L., J. D. Ward, Z. Cheng, and A. F. Dernburg. 2015. The auxin-inducible degradation (AID) system enables versatile conditional protein depletion in *C. elegans*. *Development*: dev.129635.

Zhou, J. C., A. Janska, P. Goswami, L. Renault, F. Abid Ali, A. Kotecha, J. F. X. Diffley, and A. Costa. 2017. CMG-Pol epsilon dynamics suggests a mechanism for the establishment of leading-strand synthesis in the eukaryotic replisome. *Proceedings of the National Academy of Sciences of the United States of America* 114: 4141–4146.

Zhou, Z.-X., S. A. Lujan, A. B. Burkholder, M. A. Garbacz, and T. A. Kunkel. 2019. Roles for DNA polymerase δ in initiating and terminating leading strand DNA replication. *Nature Communications* 10: 3992.

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