Effect of TIMELESS protein overexpression on DNA replication in human cells

Bachelor thesis

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TIMELESS valgu üleekspressiooni efekt DNA replikatsioonile inimrakkudes

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Declaration

Hereby I declare that I have compiled the paper independently and all works, important standpoints and data by other authors have been properly referenced and the same paper has not been previously presented for grading.

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Contents

Abbreviations

AND-1 - Acidic nucleoplasmic DNA-binding protein

ATR – Ataxia telangiectasia and Rad3 related protein

BCA – Bicinchoninic acid assay

CDC-45 – Cell division cycle 45

CHK1 – Checkpoint kinase 1

CIdU - Chlorodeoxyuridine

CMG - CDC45-MCM-GINS

CMV - Cytomegalovirus

CRY1 – Cryptochrome circadian regulator 1

DNA - Deoxyribonucleic acid

DDX11 – DEAD/H-box helicase 11

EdU - 5-ethynyl-2'-deoxyuridin

ERα – Estrogen receptor alpha

FBS – Fetal bovine serum

GINS – Go-Ichi-Ni-San

IdU - Iododeoxyuridine

MCM - Minichromosome mainteinance protein complex

MTBP - Mdm-2-binding protein

ORC - Origin recognition complex

PBS - Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

pre-RC - Pre-replication complex

TBST – Tris-Buffered Salin with Tween

TIM – TIMELESS

TIPIN – TIMELESS-interacting protein

TOPBP1 – Topoisomerase IIβ-binding protein 1

Treslin - TOPBP1-interacting, replication-stimulating protein

Introduction

DNA replication is a very complex yet pivotal process that is necessary prior to cell division (Aze & Maiorano, 2018). This process is strictly regulated to ensure that DNA replication happens once and only once per cell cycle (Sclafani & Holzen, 2007). DNA replication follows a semi-conservative model, where each newly synthesized DNA molecule consists of two strands: one, that is derived from the original template, and another, which is newly synthesized (Vouzas & Gilbert, 2021). During DNA replication, the leading strand is synthesized continuously, while the lagging strand is formed through a series of short Okazaki fragments (Langston & O'Donnell, 2006).

DNA replication is initiated at specific sites called origins (Prioleau & MacAlpine, 2016). DNA replication is driven by the replisome. The replisome is a complex molecular machine composed of different proteins, including the CMG helicase(CDC45, MCM GINS), polymerase alpha-primase, polymerase delta and epsilon, and other non-catalytic components (Jones et al., 2021). Replisome is formed during the process of DNA replication initiation and is responsible for the replication of DNA (Hashimoto et al., 2023). In the process of origin firing, the CMG helicase unwinds the DNA strands, and the replisome moves along the DNA. The replication fork is a site where DNA double helix is separated, and the new DNA strands are being synthesized (A. Leman & Noguchi, 2013). Polymerase alpha-primase allows DNA replication by generating primers for leading-strand synthesis, and it also generates primers every few hundred nucleotides for lagging-strand synthesis. Leading strand synthesis is performed by polymerase epsilon (Vipat et al., 2022), while DNA polymerase delta handles synthesis at the lagging strand (Prindle & Loeb, 2012).

TIMELESS (TIM), TIPIN, CLASPIN and AND-1 make up a Fork Protection Complex (FPC), which is required for maintaining the integrity of the replisome (Patel & Kim, 2023). One of the FPC components – protein TIMELESS, is the main focus of this research project. TIMELESS protein is best known for its role in replication stress response and in maintaining fork stability. TIMELESS depletion is known to impact DNA replication: it leads to a defective progression of S-phase of the cell cycle, causes reduced speed of the replication fork and increased firing of replication origins, and inhibition of DNA synthesis (reviewed in Vipat & Moiseeva, 2024).

Interestingly, elevated expression of TIMELESS is often detected in cancers and confers resistance to some chemotherapy drugs. Thus, understanding the precise roles of TIM in the process of DNA replication is important. Especially valuable would be the studies on the effects of abnormal levels of TIM on DNA replication and carcinogenesis (reviewed in Vipat & Moiseeva, 2024). This project is focused on the effects of TIMELESS protein overexpression on DNA replication in human cells.

The main results of the current thesis are:

1. Overexpression of TIMELESS protein in non-cancerous RPE hTERT-1 cells does not cause any significant change in the growth rate of cells

2. Overexpression of TIMELESS protein does not cause any significant change in the rate of DNA synthesis, and does not affect the proportion of actively replicating S-phase cells in the cell population

3. Overexpression of TIMELESS protein does not significantly affect the speed of the DNA replication fork

1. Literature review

1.1. DNA replication

DNA is the molecule that carries hereditary information. DNA replication is necessary prior to every cell division, as it ensures that each of the two daughter cells receives an intact copy of the genetic material (Ekundayo & Bleichert, 2019). Overreplication of DNA is harmful, resulting in genes amplifications and other sorts of genome instability - one of hallmarks of tumorigenesis (Truong & Wu, 2011).

DNA molecule consists of two strands, each serving as a template for newly synthesized complementary strands during DNA replication. DNA replication follows a semi-conservative model, which means that each daughter DNA molecule consists of one original strand and one newly generated strand (Hanawalt, 2004). During DNA replication, the synthesis of DNA occurs in the 5' to 3' direction (Pavlov et al., 2003). The leading strand is synthesized continuously in the same direction as the moving replication fork, while the lagging strand is synthesized in the opposite direction through a series of short Okazaki fragments (Burgers & Kunkel, 2017).

1.2. DNA replication initiation and the replication fork

DNA replication is initiated at specific sites in the genome called replication origins (Ekundayo & Bleichert, 2019). For eukaryotic cells that possess large genome size, to fully duplicate their genomic material in an adequate time, tens of thousands replication origins are used to initiate DNA replication (Boos & Ferreira, 2019; Klotz-Noack et al., 2012).

Before the beginning of DNA replication, in G1 phase of the cell cycle, origins are licensed by the origin recognition complex (ORC), which binds to double-stranded DNA at origin sites (S. Li et al., 2022). Then, other important players like CDT1 and CDC6 proteins are loaded (Frigola et al., 2017). These three components: ORC, CDT1 and CDC6 support the loading of inactive helicases - heterohexameric minichromosome maintenance (MCM) complexes onto DNA, forming a pre-replication complex (pre-RC) (S. Li et al., 2022; Mei & Cook, 2021).

During the G1/S transition of the cell cycle, levels of cyclin-dependent kinases increase, and this stimulates MCM phosphorylation and facilitates the recruitment of downstream initiation proteins like CDC45 and GINS (Fei & Xu, 2018).

At the next step, CDC45 and GINS proteins load onto MCM complexes, leading to the assembly of the CMG (CDC45-MCM-GINS) helicase (reviewed in Moiseeva & Bakkenist, 2018). In human cells, other pivotal proteins like TOPBP-1, Treslin (TOPBP1-interacting, replication-stimulating protein), and MTBP (MDM2-binding protein) (Kumagai et al., 2010; Kumagai & Dunphy, 2017), are required for CDC45 loading to MCM (Kumagai et al., 2010). Another protein, DONSON, is believed to be necessary for recruitment of GINS to MCM (Kingsley et al., 2023). Activation of CMG helicase depends on MCM10, which is thought to induce origin unwinding along with promoting the subsequent recruitment of other essential proteins, and polymerase alpha-primase (Pol α-primase) (Devbhandari et al., 2017). Polymerase α-primase plays a pivotal role in DNA replication

as it initiates leading-strand synthesis by generating primers at replication origins. Furthermore, it generates primers during the discontinuous process of lagging-strand synthesis (Jones et al., 2023). DNA polymerase delta is required for lagging-strand synthesis, however, it was also observed, that it establishes the leading-strand synthesis, before the engagement of polymerase epsilon (Yeeles et al., 2017). Then, polymerase epsilon takes over the leading-strand synthesis, which completes the assembly of the full replication complex, after which two replication forks moving in the opposite directions are established (Moiseeva & Bakkenist, 2018; Pellegrini, 2023; Vipat et al., 2022).

Another key component of replisome is Fork Protection Complex (FPC) (**Figure 1**), that consists of the TIMELESS-TIPIN complex, CLASPIN and AND-1. The TIMELESS-TIPIN complex, located ahead of the moving fork, is essential for maintaining normal replication rates (A. R. Leman et al., 2010; A. R. Leman & Noguchi, 2012), while Claspin is thought to play important roles during replication stress to activate checkpoints (Baretić et al., 2020). AND-1 plays crucial roles in sister chromatid cohesion and checkpoint activation (Hao et al., 2015; Pellegrini, 2023).

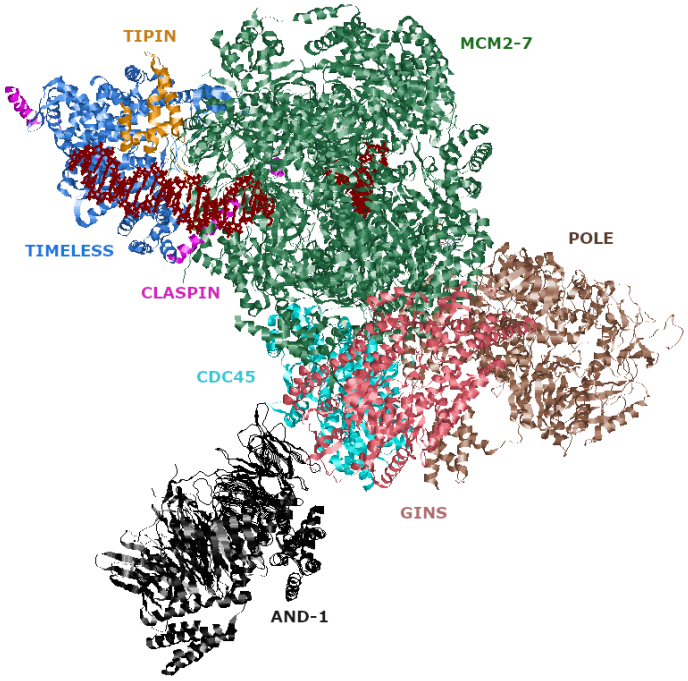


Figure 1: Structure of the human replisome. Source: PDB: 7PFO. TIMELESS, TIPIN CLASPIN and AND-1 are parts of the Fork Protection Complex, which helps maintain the integrity of the replisome. Behind the FPC is the replicative helicase CMG (CDC45-MCM2-7-GINS). On the leading strand, behind the CMG helicase, is polymerase epsilon, the leading strand DNA polymerase.

1.3. TIMELESS protein

TIMELESS (TIM) protein was first discovered in Drosophila Melanogaster and a substantial body of work in Drosophila has revealed its circadian clock related functions (Cai & Chiu, 2022). However, roles of mammalian TIM in circadian rhythm are still not well established (Kurien et al., 2019). In Drosophila, there are two known TIM paralogs: dTIM and TIMEOUT. Mammalian TIM shares

sequence homology with dTIM and TIMEOUT, but functionally it is orthologous to TIMEOUT only, which plays a role in normal DNA replication and chromosome integrity in Drosophila (Benna et al., 2010; reviewed in Vipat & Moiseeva, 2024).

Mammalian TIMELESS protein has an obligate binding partner called TIPIN (reviewed in Vipat & Moiseeva, 2024), and they form a heterodimeric complex (Gotter, 2003). Their expression levels are interdependent, depletion of one of these proteins leads to a depletion of the other protein and vice versa (A. R. Leman & Noguchi, 2012). Binding to TIPIN is required for TIM to perform most of its replication-related functions.

TIMELESS, TIPIN, CLASPIN and AND-1 make up the Fork Protection Complex (FPC), which acts as a grip to preserve the integrity of advancing replication fork (Patel & Kim, 2023). FPC is also believed to be involved in maintaining normal speed of DNA replication, and in supporting unperturbed movement of the replisome through repetitive and difficult-to-replicate regions (Escorcia & Forsburg, 2017; reviewed in Vipat & Moiseeva, 2024). For instance, TIMELESS helps moving fork to progress through G-quadruplex structures by recruiting DDX11 (DEAD/H-box helicase 11) helicase, that resolves G-quadruplex(G4) DNA (Lerner et al., 2020).

1.3.1 Functions of protein TIMELESS

Mammalian TIMELESS is a protein with diverse functions in various processes in the cell (**Figure 2**). However, mammalian TIM is best studied in the context of its roles in the DNA damage and replication stress responses (recently reviewed in Vipat & Moiseeva, 2024).

TIM/TIPIN complex is known to be essential for normal cell cycle progression as depletion of either one of these proteins leads to accumulation of cells in late S-phase of the cell cycle, indicating defects in the S-phase progression, leading to incomplete DNA replication and consequent replication stress. This shows that TIM is important for maintaining genome stability during the S-phase progression (Smith-Roe et al., 2013; Yoshizawa-Sugata & Masai, 2007).

During DNA replication stress, TIM is important, as it is needed for the activation of ATR-checkpoint response that leads to the phosphorylation of CHK1, fulfilling its checkpoint-related functions (Kemp et al., 2010). Depletion of TIM resulted in a delay in entering S phase and a decrease in the population of cells in the S phase (Xu et al., 2016).

TIMELESS is believed to mediate timely pausing of the fork at difficult-to-replicate regions and the engagement of DNA repair machinery (Patel & Kim, 2023). TIM-TIPIN complex is localized at the leading edge of the replisome and is perfectly suited for the recognition of repetitive and difficult-to-replicate regions as well as regions of DNA having suffered exogenous DNA damage. TIMELESS is believed to mediate the engagement of DNA repair machinery. It then supports fork progression through such difficult regions, that might otherwise have impeded DNA replication and eventually led to further DNA damage and loss of genomic integrity (Vipat & Moiseeva, 2024).

Other than its roles in DNA replication, TIM also binds to, and activates the estrogen receptor α (ERα), that facilitates cell division and tumor growth, inducing expression of ERα target genes (Magne Nde et al., 2018). The role of TIMELESS in circadian rhythm requires further investigation,

however, it is known that TIM interacts with circadian rhythm protein – CRY1, that is necessary for regulating DNA repair (Shafi et al., 2021). TIM facilitates the sister chromatid cohesion by letting the cohesin bind to the chromatin (reviewed in Vipat & Moiseeva, 2024).

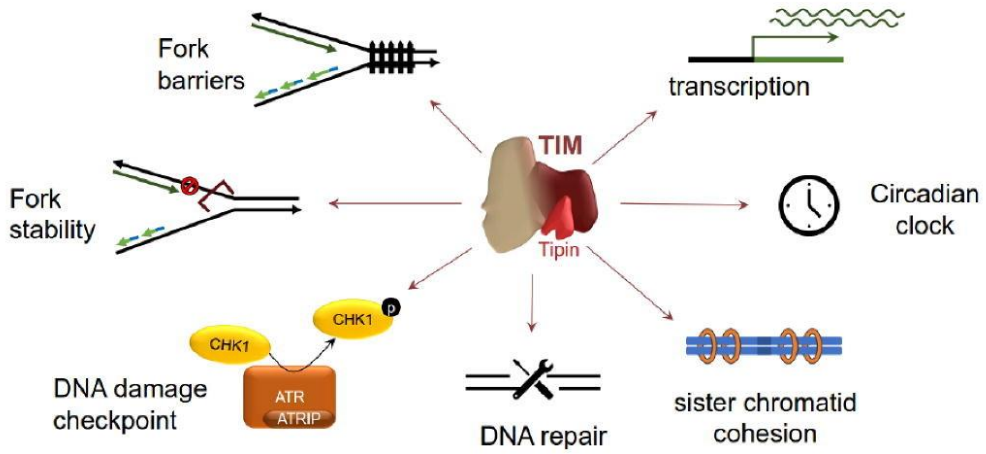


Figure 2: Various roles of TIMELESS protein in human cells. TIM is essential for normal cell cycle progression. To combat replication stress and DNA damage, TIM prevents moving replication forks from stalling during difficult-to-replicate regions. TIM-TIPIN complex helps to activate the ATR-CHK1 pathway during the replication stress. TIM establishes the association of cohesin with chromatin, facilitating sister chromatin cohesion. TIM is thought to act as a transcription factor through its interaction with ERα. TIM is thought to play some role in circadian clock function in mammals (from Vipat & Moiseeva, 2024).

1.3.2 TIMELESS in cancer

TIMELESS protein overexpression is known to have pro-tumorigenic effects and is associated with cancer (F. Li et al., 2022). It was demonstrated that TIM protein is overexpressed in lung cancer, cutaneous melanoma and breast cancer cells (Fu et al., 2012; Yoshida et al., 2013; Zhao et al., 2022). Its elevated expression in lung cancer correlates with poor survival prognosis (Yoshida et al., 2013).

TIM upregulation protects cancer cells from oncogene-induced replication stress without increasing activation of ATR-pathway (Bianco et al., 2019). TIMELESS overexpression is important for proliferation of tumor cells. It was also observed that TIMELESS knockdown caused the inhibition of the growth of tumors, but also induced apoptosis and senescence in cancer cells (Hosseini et al., 2023). Interestingly, overexpression of TIMELESS exacerbates the progression of oral squamous cancer cells by promoting their proliferation and increasing their glycolytic activity, a hallmark of cancer (Chen et al., 2024).

The overexpression of TIMELESS is associated with resistance to various genotoxic drugs (reviewed in Vipat & Moiseeva, 2024). Abnormally elevated expression of TIMELESS is related to formation of resistance to cisplatin in nasopharyngeal carcinoma (Liu et al., 2017). TIM is known to activate ERα and is implicated in supporting tumor growth in breast cancer (Mao et al., 2013). Moreover, elevated TIM expression also facilitates tamoxifen resistance in this type of cancer (Magne Nde et al., 2018).

Current data (Fu et al., 2012; Liu et al., 2017; Magne Nde et al., 2018; Mao et al., 2013; Yoshida et al., 2013; Zhao et al., 2022) clearly indicate that TIMELESS overexpression is a serious concern due to its proven association with various cancers and its role in promoting resistance to genotoxic drugs. However, the available data are still limited, leaving many questions unanswered. Beyond its pro-tumorigenic effects, what other effects does TIMELESS upregulation have? Are there other drugs to which it promotes resistance, and how can this issue be addressed? Since depletion of TIMELESS results in apoptosis, presenting a challenge for targeting this protein directly, what alternative strategies could be used to manage it in the future?

2. Aim of the thesis

The aim of this thesis was to analyze the effect of TIMELESS protein overexpression on DNA replication in human cells.

Specific tasks:

1. Generate TIMELESS overexpressing cell lines in non-cancerous RPE hTERT-1 cells, RPE hTERT-1 p53 knock-out cells, and cancerous U2OS cells
2. Study the effects of TIM overexpression on cell growth and DNA replication dynamics in these cell lines

3. Research materials and methods

3.1. Materials

3.1.1. Cell lines

U2OS (Homo sapiens osteosarcoma cells)

hTERT-RPE1 (Homo sapiens retinal pigment epithelium cells immortalized with hTERT)

RPE p53 knock-out cells are RPE hTERT-1-based cells that were generously gifted by Dr. Raphael Ceccaldi, Institut Curie

3.1.2. Antibodies

For Western Blot and DNA Fiber assay analyses, the following antibodies were used:

Primary antibodies:

α-TIMELESS (Santa Cruz, #sc-47724) 1:500 dilution

α-FLAG (Sigma Aldrich, F3165-1MG) 1:3000 dilution

α-TIPIN (Santa Cruz, #sc-135580) 1:500 dilution

α-GAPDH (Santa Cruz, #sc-47724) 1:500 dilution

α-CIdU (Abcam, #AB6326) 1:50 dilution

α-IdU (BD, #347580) 1:66 dilution

Secondary antibodies:

Goat-α-mouse (Invitrogen, #A16066) 1:10000 dilution

Goat-α-rabbit (Invitrogen, #A16104) 1:10000 dilution

Alexa fluor 488 goat-α-mouse (Invitrogen, #A-11001) 1:150 dilution

Alexa fluor 594 goat-α-mouse (Invitrogen, #A-11007) 1:150 dilution

3.1.3. Polymerase chain reaction primers

For PCR verification of TIM-expression transgene, the following primers were used:

FLAG forward primer – 5'GAT TAC AAG GAT GAC GAC GAT AAG3'

TIM_Mlu1 reverse primer – 5'CGA CGC GTG TCA TCC TCA TCA TCC TCA A3'

3.2. Methods

3.2.1. Cell culture

U2OS- and RPE-based cell lines were grown using RPMI and DMEM media respectively, supplemented with 10% FBS (GIBCO) and 1% penicillin/streptomycin. Cells were grown in a cell culture incubator at 37°C and CO_2 level of 5%.

Cells were handled under sterile conditions using a laminar flow hood. Prior to working with cells, the surface of the hood was disinfected with 70% ethanol. All necessary solutions for growing cells were pre-warmed up to 37°C.

Cells were passaged before they would reach full confluency. For passaging cells, the medium was aspirated, then cells were washed with PBS. After removing PBS, cells were incubated with trypsin for 2-5 minutes at 37°C. When cells were detached from the culture dish surface, they were resuspended in pre-warmed fresh growth medium, giving an appropriate split ratio. 1 ml of cell-containing medium was returned back to the plate followed by the addition of 9 ml fresh growth medium. Plates were rocked to suspend cells evenly and then incubated.

3.2.2. Vector digestion

Coding sequence of TIM was cloned out from the donor vector into a pCMV-based mammalian expression recipient vector containing doxycycline-inducible promoter using restriction enzyme-based cloning. TIMELESS insert was cut out from the donor vector using 1.5 µl FastDigest MluI and 1.5 µl FastDigest SfaAI restriction enzymes, along with 5 µl of 10x FastDigest buffer and 5 µg of donor vector, at 37°C overnight.

3.2.3. Gel electrophoresis and purification

For further separation, isolation, and purification of generated DNA fragments by gel electrophoresis, 1% agarose gel was prepared as follows: 0.5g of agarose powder (#BA010110) were melted in 50ml of 1x TAE buffer (Tris-Acetate-EDTA, ThermoScientific 50x). When cooled down, 2.5µl of Ethidium Bromide were added and gel was poured into a gel tray to solidify with a 15-well comb added. The gel was placed into a gel electrophoresis apparatus, 2.5µl of Gene Ruler 1 kb Plus DNA Lader (ThermoFisher Scientific) was used as a molecular weight marker and 10µl of digestion mixture was added for further separation of generated DNA fragments. The gel electrophoresis was run at 120V. Then, the bands of interest were cut under UV light, put into 1.5 ml centrifuge tubes, and weighed for further gel purification using GeneJET Gel Extraction Kit (ThermoFisher Scientific).

Binding Buffer was added to 1.5 ml centrifuge tubes, followed by a 10-minute incubation at 55°C to dissolve the gel. The mixture was transferred to GeneJET purification columns, centrifuged for 1 minute at 24000g. The flow-through was discarded and 700 µl of Wash Buffer was added, followed by a centrifugation for 1 minute at 24000g. The flow-through was discarded, and empty columns were centrifuged once again for complete removal of Wash Buffer residues. The GeneJet Purification Columns were then transferred to 1.5 ml centrifuge tubes and 30 µl of distilled water was added in the middle of the column membrane, and let to incubate for 4 minutes, following a 1-minute centrifugation at 24000g. Then, the DNA concentrations were measured using a BioSpec-nano spectrophotometer (Shimadzu Biotech).

3.2.4. Ligation

For the ligation, 100 ng of vector and 150 ng of insert were used along with 2 µl of Ligase Buffer 10x, 1.5µl of T4 ligase and water. Following a mix by pipetting, the mixture was incubated at room temperature for 2 hours.

3.2.5. Transformation

Transformation was performed using DH5α competent cells (ThermoFisher Scientific). The competent cells were thawed on ice. 50 µl of cells were added to a 1.5 ml centrifuge tube and mixed with 1.25 µl of the ligation mixture, followed by a 30-minute incubation on ice. Then, a 35-second heat shock was performed at 42°C. The tube was placed on ice for 3 minutes and 1 ml of LB solution medium was added. The tube was incubated in the shaker at 37°C for 1 hour. Transformants were selected using LB plate with 50 µg/ml kanamycin.

3.2.6. Plasmid DNA isolation and validation

For the isolation of plasmid DNA from bacteria, a miniprep kit (ThermoFisher Scientific) was used, following the manufacturer's instructions.

Following an overnight incubation, bacteria colonies were taken to sterile 15 ml tubes with 3 ml of LB and 100 µg/ml of ampicillin (ThermoFisher Scientific) that were added before. The tubes were incubated in a shaker overnight for bacteria to grow for further plasmid DNA isolation. The overnight shaken bacteria were collected by a 1-minute centrifugation at 12000g, and the supernatant was discarded. For further isolation of plasmid DNA using DNA maxiprep, glycerol stocks were prepared as follows: 20 µl of 10% glycerol were added to new 1.5 ml centrifuge tubes, where also 20 µl of bacteria culture were added, and were moved for storage at -20°C.

The collected bacteria were resuspended in 250 µl of Resuspension Solution. 250 µl of Lysis Solution was added and tubes were mixed by inverting, following the addition of 350 µl of Neutralization Solution to stop the lysis. Then, the tubes were centrifuged for 5 minutes at 12000g to pellet cell debris and chromosomal DNA, and the supernatant was transferred to GeneJet columns and centrifuged for 1 minute at 12000g. The flow-throughs were discarded and 700 µl of Wash Solution was added to the columns, followed by a 1-minute centrifugation at 12000g. The liquid was removed, and this step was repeated once more with 500 µl of Wash Solution. To remove the excess of Wash Solution, empty columns were centrifuged for 1 minute at 12000g. 30µl of distilled water were added to the middle of each tube and incubated for 4 minutes. Then the tubes were centrifuged for 1 minute at 12000g and DNA concentrations were measured using a BioSpec-nano spectrophotometer (Shimadzu Biotech).

Obtained plasmid DNA was then tested by restriction digestion using FastDigest MluI (ThermoFisher Scientific) and FastDigest SfaAI (ThermoFisher Scientific) restriction enzymes. Digested DNA was separated on an agarose gel and the sizes of the products were verified. The correct sequences of the plasmids were verified by Sanger sequencing performed at University of Tartu facility of Genomics.

3.2.7. Plasmid DNA maxiprep

For the isolation of plasmid DNA for further transfections, the GeneJET Plasmid Maxiprep kit (ThermoFisher Scientific) was used, following the manufacturer's instructions.

10 µl of glycerol stock with bacteria were diluted in 250 ml of LB with 100 mg/ml of ampicillin. The following day, the culture was transferred to 50 ml centrifuge tubes and cells were collected with a 10-minute centrifugation at 5000g. The supernatant was removed, and bacteria were resuspended in 6 ml of Resuspension Solution. Then, 6 ml of Lysis Solution was added and mixed gently by inverting up and down. Following a 3-minute incubation, 6 ml of Neutralization Solution was added. 800 µl of Endotoxin Binding Reagent was added and mixed immediately by inverting the tubes and incubated for 5 minutes at room temperature. Tubes were centrifuged for 40 minutes at 5000g to pellet cell debris and chromosomal DNA. The supernatants were moved to new 50 ml centrifuge tubes and 6 ml of 96% ethanol was added and mixed by inverting the tubes. Then, approximately 20 ml were moved to columns and centrifuged for 3 minutes at 2000g Following the disposal of the flow-through, centrifugations were repeated until all the supernatant was used. 8 ml of Wash Solution I (with isopropanol added) were added, and tubes were centrifuged for 2 minutes at 3000g. The flow-through was removed and 8 ml of Wash Solution II (with ethanol added) were added, following a 2-minute centrifugation at 3000g, and disposal of the flow-through. Then, empty columns were centrifuged for 5 minutes at 3000g once to remove residual wash solution and transferred to the new 50 ml collection tubes. Then, 700 µl of distilled water were added to the centers of the purification column membranes, incubated for 5 minutes, and centrifuged for 5 minutes at 3000g to elute plasmid DNA. DNA concentrations were measured using a BioSpec-nano spectrophotometer (Shimadzu Biotech).

3.2.8. Transfections and stable cell lines generation

TIM-expressing construct was transfected into each of the parental cell lines to generate stable cell lines with doxycycline-inducible TIM transgene. Transfections were performed on approximately 70% confluent cells in 6-well plates. 2.5 µg of plasmid DNA was transfected into the cells using Lipofectamine 2000, following the manufacturer's protocol.

24 hours after the transfection, U2OS cells were selected with 500 µg/µl and RPE hTERT-1 cells were selected with 1000 µg/µl of G-418 (BioWest) antibiotic. Every two days the growth medium was changed with the addition of fresh G-418, until all the cells in the untransfected control were dead. Single cell cloning was then performed to obtain clonal cell lines originating from one single cell, as follows. Cells were trypsinized and collected in sterile 15 ml centrifuge tubes. Using a hemocytometer, cells were counted, and serially diluted to 3 cells/ml. The cells were seeded in 96 well plates to obtain 3 cells per 10 wells, to minimize the chance of having more than one cell per well. After 8 days of incubation, each well was observed and wells, containing two or more colonies, were discarded, to avoid having cell lines originating from multiple cells. Each well that contained a single cell-derived colony was marked and colonies were sequentially transferred to bigger plates to obtain cells in 6 cm dishes.

When a single cell-derived colony was transferred to a 6 cm dish and reached 50-60% confluency, half of the cells were frozen in medium containing 10% DMSO (ThermoFisher Scientific) for stock storage, while the other half was let to grow for a next-day harvest for genomic DNA extraction followed by PCR analysis.

3.2.9. Polymerase chain reaction

We first screened all obtained clonal cell lines to verify the presence of the TIM transgene by PCR. Genomic DNA of clones was extracted from frozen pellets following the manufacturer's protocol (Omega BIO-TEK, E.Z.N.A. Tissue DNA kit, D3396-02). PCR primers that were used are described in **3.1.3. PCR primers**.

PCR master mix	1x
FLAG forward primers (10µM)	0.5µl
TIM_Mlu1 reverse primers (10µM)	0.5µl
dNTP (10mM)	0.5µl
DreamTaq Green Buffer 10x	2.5µl
DreamTaq DNA Polymerase	0.125µl
H_2O	20.4µ
Total	**24.5µl**

PCR cycle parameters
Initial denaturation – 95°C – 2 minutes
Denaturation – 95°C – 30 seconds
Annealing – 57°C – 30 seconds
Extension – 72°C – 2 minutes 30 seconds
Final extension – 72°C – 5 minutes

30 cycles were performed. PCR results were analyzed via gel electrophoresis with 1% agarose gel. GeneRuler 1 kb Plus DNA ladder (ThermoFisher Scientific) was used as a molecular weight marker.

3.2.10. Cell lysis

Cells were harvested by trypsinization in 15 ml centrifuge tubes and collected by centrifugation (5 minutes at 270g). The supernatant was aspirated without disturbing cell pellets. The cell pellets were washed once with 1 ml of cold PBS and centrifuged for 5 minutes at 720g, and then resuspended in 200 µl 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), and allowed to lyse for 20 minutes on ice. Lysates were cleared by centrifugation at 4°C 24000g for 5 minutes. Afterwards, 50 µl of lysates were mixed with 50 µl of 2x Laemmli Sample Buffer (ChemCruz, #sc-286962), vortexed to mix and boiled for 10 minutes at 96°C. Samples were stored at -20°C.

3.2.11. Western blots

For detection of specific proteins in the lysates, Western Blot analysis was performed. For Western blot analysis, proteins were separated in 8% SDS-polyacrylamide gel with 4% stacking gel, which were prepared using SureCast Gel Handcast system and reagents, according to manufacturer's instructions. Gel electrophoresis was done using the Invitrogen Mini Gel Tank. Frozen samples

were thawed, incubated in a heat block at 96°C for 10 minutes and vortexed, prior to loading onto the gel. PageRulerTM Plus Prestained Protein Ladder (ThermoFisher Scientific) was used as a molecular weight marker. Gel electrophoresis was performed using Running Buffer 1x (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) and the voltage was set at 70V for approximately 30 minutes. When samples reached resolving gel and started separating, the voltage was set to 150V until the dye reached the bottom of the gel.

After separating the proteins, they were wet transferred from the gel onto a blotting membrane (0.45µm Nitrocellulose Blotting Membrane) using the Invitrogen Mini Blot Module. Transfer was performed using Transfer Buffer 1x (25 mM Tris, 192 mM glycine, pH 8.3 and 10% ethanol added) at 30V for 2 hours. Membranes with transferred proteins were stained with Fast Green staining solution (30% ethanol, 10% acetic acid, Fast Green) to confirm uniform transfer of proteins, and then strips were cut at appropriate protein sizes for further detection of proteins of interest. Strips were then blocked for 1 hour in 5% nonfat dry milk solution in TBST (150 mM NaCl, 10 mM Tris pH 8.0, 0.1% Tween20). Afterwards, the strips were incubated in primary antibody dilutions (5% non-fat dry milk solution, TBST, 0.01% sodium azide) on a rocker overnight at 4°C. The following day, primary antibodies were removed, and the membranes were washed with TBST for 30 minutes, changing the buffer every 5 minutes. Strips were then incubated in secondary antibody dilutions (5% non-fat dry milk solution in TBST) for 1 hour at room temperature, followed by washes with TBST for 30 minutes, changing the buffer every 5 minutes. For chemiluminescent visualization of proteins 20X LumiGLO reagent A and 20X Peroxide reagent B (Cell Signaling Technology, #7003S) were used according to the manufacturer's instructions. The blots were imaged using the ImageQuant LAS 4000 machine (GE Healthcare).

3.2.12. Bicinchoninic Acid Assay (BCA)

Equal protein loading in the gels was ensured by adjusting loading volumes according to protein concentrations in each sample. Protein concentrations in the samples were measured by the bicinchoninic acid assay (BCA, cat #23227), performed according to the manufacturer's instructions. 200 µL of BCA solution was added to 96-well plate wells, 25 µL of the lysate samples were added to the wells and mixed, and incubated for 30 minutes at 37°C. Then, protein concentrations were measured with TECAN plate reader.

3.2.13. Doxycycline treatment

TIMELESS protein overexpression was induced by the introduction of doxycycline to growth medium. Cells were seeded to be 70% confluent the following day and were treated with 5 µg/ml doxycycline for 24 hours. Following a 24-hour incubation, cells were harvested and analyzed.

3.2.14. EdU FACS

Cells were seeded onto 100 mm dishes to be 70% confluent the following day and treated with doxycycline for 24 hours. 30 minutes before harvesting, 10µM of EdU (5-ethynyl-2'-deoxyuridine) was added to the cells. The cells were harvested by trypsinization and washed once with cold PBS. After removing PBS, cell pellets were fixed by slow addition of cold ethanol to a final concentration of 70% ethanol, while agitating the cells at low speed on a vortex to avoid localized

heating of the cells. The fixed cells were incubated at -20°C for 1 hour to complete the fixation process.

For staining, cells were centrifuged for 5 minutes at 270g, and the ethanol was removed. The cells were resuspended in 5 ml PBS, centrifuged for 5 minutes at 270g, and then rehydrated in the dark in 10 ml PBS for 15 minutes.

Cells were centrifuged for 5 minutes at 720g. The click reaction mix (PBS, 2mM of Cu_2SO_4 (#451657), 10mM of sodium ascorbate(#AC352680050) and 5uM of Alexa Fluor 488 Azide (Invitrogen, #A10266) was prepared in the dark. Cells resuspended in 200 µl of click staining mix and incubated for 30 minutes in the dark. Cells were then washed by adding 10 ml of PBS and centrifuged for 5 minutes at 720g. Following removal of PBS, the cells were resuspended in 300 µl of PBS for further flow cytometry analysis.

Flow cytometry analysis was performed using 2002 FACSCalibur flow cytometer with CellQuest Pro software, and data were analyzed by using FCSalyzer software. 15,000 cells were collected per sample.

3.2.15. Growth curves

In order to analyze the effect of TIM protein overexpression on growth rate of cells, a growth curve experiment was performed. 350,000 cells of each of the following cell lines: RPE, C1, C12, were seeded in 60 mm plates and treated with doxycycline along with untreated controls. Cells were harvested at 24-hour, 48-hour and 72-hour time points and counted using a hemocytometer. Once the cells were collected, the tubes were inverted 10-15 times to ensure even cell distribution throughout the medium. 10 µl of medium with cells were then taken and added into a hemocytometer. To achieve a good overall representation of the number of cells, the following approach was used: cell counting was performed using a microscope, focusing on four corner squares for cell counting. Further analyses were conducted in Excel.

The moment the cells were seeded and treated with doxycycline was considered the 0-hour time point, and all the plates were seeded to have 350,000 cells each. At each subsequent time point, the actual number of cells was counted as follows: the mean number of cells was multiplied by 10^4(multiplication factor) and by the dilution factor. The cell numbers were then normalized to the number of cells at 0 hours for better representation. The experiment was repeated three times, and the normalized mean cells numbers were used to construct growth curves. The standard deviation was calculated from the average values and represented as error bars on the graph.

3.2.16. DNA Fiber assay

To analyze the effect of TIM protein overexpression on moving fork speed, DNA Fiber assay was performed, using RPE, C1 and C12 cells. The cells were seeded into 6-well plates, to be 70% confluent the following day, and treated with doxycycline (4 µg/ml) for 24 hours. After 24 hours, the cells were treated with a 10-minute 20uM CIdU pulse. Following medium wash and addition of fresh medium, the cells were treated with a 20-minute 200uM IdU pulse. The cells were harvested

and centrifuged for 5 minutes at 270g. Following PBS wash, the cells were resuspended in 1 ml cold PBS. As nucleotide analogues and fluorophores are photosensitive, all following steps were done protecting from light.

To make the smear of DNA fibers, 2 µl of cell suspension was lysed on a glass slide by adding 6 µl of lysis buffer (200 mM Tris-HCl pH 7.4, 50 mm EDTA, 0.5% SDS in ddH2O) and pipetting up and down once. The drop was spread around with a pipette tip, making a spiral. The slide was incubated for 5 minutes at room temperature to allow for complete cell lysis. Then, the slides were tilted to 15° to allow the drop to slowly travel down under gravity, which led to the formation of a delta-like branching pattern, indicative of well-spread DNA fibers. The slides were incubated for 10 minutes in the dark to dry them completely. To prevent DNA fibers from moving, they were immobilized by fixation with 3:1 methanol-acetic acid mixture in Coplin jars for 5 minutes. The slides were dried and then washed 2 times with PBS for 5 minutes by immersing in Coplin jars.

The DNA in the smear was denatured by treating the slides with 2.5 N HCl solution for 1 hour. After that, the slides were washed by immersing in PBS 2 times for 5 minutes. Afterwards, 50µl of blocking buffer was added to each slide and covered with coverslips avoiding trapped bubbles, and smears were blocked in humid chamber for 1 hour in 37°C incubator. 50µl of primary antibody mixture (α-CIdU + α-IdU in 2.5% BSA, 0.1%-Triton-PBS) was added to the smears and covered with coverslip avoiding trapped bubbles, and incubated overnight in humid chamber at 4°C.

On the next day, the slides were washed on a rocker with PBS-0.1% Tween solution for 20 minutes, with a change of the buffer every 5 minutes. 50µl of secondary antibody mixture (goat-anti-mouse Alexa fluor 488 + goat-anti-rat Alexa fluor 594 in 2.5% BSA, 0.1 %-Triton-PBS was added to the smears, covered with coverslip, and incubated for 1 hour in 37°C incubator in the humid chamber. The slides were then washed with wash buffer (PBS-0.1% Tween 20) on a rocker for 20 minutes, changing buffer every 5 minutes. Then, the slides were further washed with PBS 2 times for 5 minutes to remove residual detergent. Finally, mounting medium (ThermoFisher Scientific) was added to the smears, and covered with coverslips avoiding trapped bubbles, and allowed to solidify for 24 hours in the dark. The following day, DNA fibers were visualized by fluorescence microscopy on Olympus BX61 fluorescent microscope at 60x magnification. Pictures were taken across the entire area of the slide containing fibers. For each unique area with fibers, two pictures were taken: one with red signal, representing incorporated CldU and another with green signal, representing incorporated IdU. Using GIMP, the pictures were superimposed. To quantify the fork speed parameter, the lengths of fiber tracks were measured using ImageJ, and analysed in GraphPad Prism. For a reliable estimation of replication fork speed, 100 fibers in total - 10 fibers per picture - were measured and analyzed.

4. Results

4.1. Generation of stable cell lines overexpressing TIMELESS protein on cancerous and non-cancerous background

To study the effect of TIMELESS protein overexpression on growth rate of cells and DNA replication dynamics, we aimed to generate TIM overexpressing cell lines on non-cancerous (RPE hTERT-1, RPE hTERT-1 p53 knock-out) and cancerous (U2OS) background. For this, we generated a plasmid construct containing FLAG-tagged TIMELESS coding sequence under a doxycycline-inducible promoter. To generate such a construct, two vectors were used: donor vector with TIMELESS coding sequence and a pCMV-based mammalian expression vector with doxycycline-inducible promoter. Both vectors were digested with the following restriction enzymes: SfaAI and MluI. Following the digestion reaction, generated DNA fragments were separated in 1% agarose gel using gel electrophoresis (**described in 3.2.3. Gel electrophoresis and purification**). Fragments of interest – FLAG-tagged TIM and recipient vector backbone with doxycycline-inducible promoter, were purified from the gel using GeneJet Extraction Kit (ThermoFisher Scientific). Then, generated DNA fragments were ligated (**described in 3.2.4. Ligation**) together such, that TIM was inserted into a purified plasmid. The ligation reaction mix was then transformed into DH5α-competent cells (**described in 3.2.5. Transformation**). Transformed cells were seeded on a plate with kanamycin antibiotic. GeneJet Plasmid Mini- and Maxiprep kits (ThermoFisher Scientific) (**described in 3.2.6 Plasmid DNA isolation and validation and 3.2.7. Plasmid DNA maxiprep**) were used for following isolation of generated plasmid from bacteria. The results of plasmid DNA isolation using miniprep kit were tested using SfaAI and MluI restriction enzymes. Generated DNA fragments were identified using 1% agarose gel and the correct sequence was verified by Sanger sequencing performed at University of Tartu facility of Genomics.

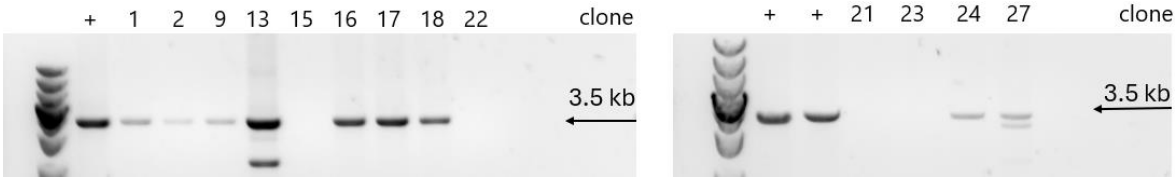


Figure 3: PCR screening of U2OS-based TIM overexpressing clones. Clones 1 and 12, generated from RPE hTERT-1 parental cell line by the supervisor, were used as positive controls ("+"). The desired band with its size is represented with an arrow. PCR-positive clones were then selected for further testing for ectopic TIM expression.

Parental cells were transfected with this construct. RPE hTERT-1 p53 KO-based cells were selected with 1000 µg/ml G-418, and U2OS-based cells were selected with 500 µg/ml G-418. Then, these cells were single cell-cloned to obtain clonal cell lines (**described in 3.2.8. Transfections and stable cell lines generation**). RPE hTERT-1-based clones were generated by the supervisor.

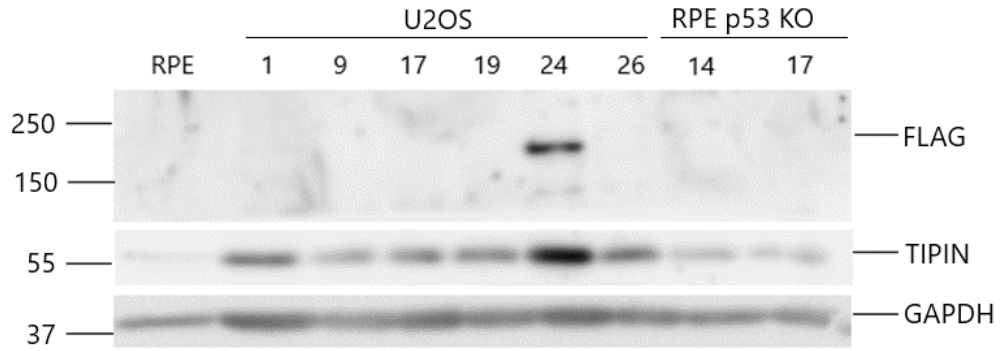


Figure 4: Western blot screening of U2OS- and RPE p53KO-based TIM overexpressing clones PCR-positive clones were treated with doxycycline for 24 hours. The following day cells were collected, and proteins were extracted for Western Blot analysis. Ectopic TIM expression was assessed with anti-FLAG antibody.

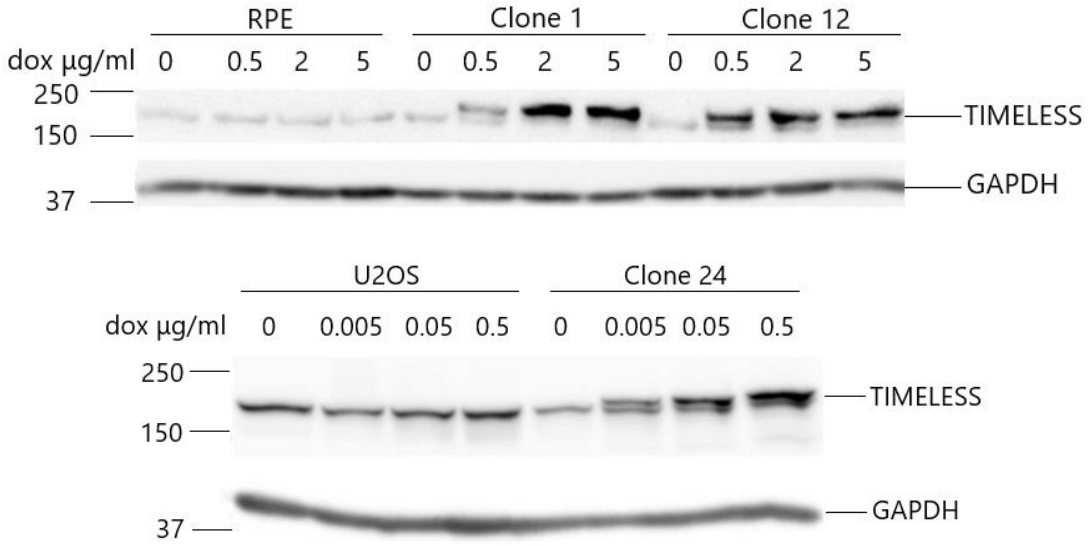
In total, 61 clones in U2OS and 46 clones in RPE hTERT-1 p53 KO cells were obtained after single cell cloning. We first screened these clones by PCR for the presence of the TIM transgene (**Figure 3**). Eight clones were positive by PCR screening. These clones were then tested by Western Blot (**Figure 4**) for their ability to overexpress TIM protein after 24-hour doxycycline treatment.

We obtained one U2OS-based clone positive for TIM overexpression - C24. Further clones will be screened as well. We proceeded with further experiments with C24.

4.2. Doxycycline titrations for induction of TIM overexpression in RPE- and U2OS-based clones

We aimed to achieve TIM overexpression levels about 3-4 times higher than normal TIM levels in the cell, as there is evidence to suggest that TIM is overexpressed in cancers up to such levels (Yoshida et al., 2013). We optimized the concentration of doxycycline needed to induce the desired TIM overexpression. RPE hTERT-1, C1 and C12, along with U2OS and C24 cells, were seeded in 60 mm plates and treated with various doxycycline concentrations. For the RPE hTERT-1-based clones, we tested the following doxycycline concentrations: 0, 0.5, 2, 5 µg/ml. In contrast, for the U2OS-based clone, the doxycycline concentrations were as follows: 0, 0.005, 0.05, 0.5 µg/ml. 24 hours later, cells were lysed, and TIM overexpression levels were quantified by Western Blots (**Figure 5**). Doxycycline concentrations: 5 µg/ml for RPE-based clones and 0.05 µg/ml for U2OS-based clones, were found to induce TIM overexpression to the desired levels.

A



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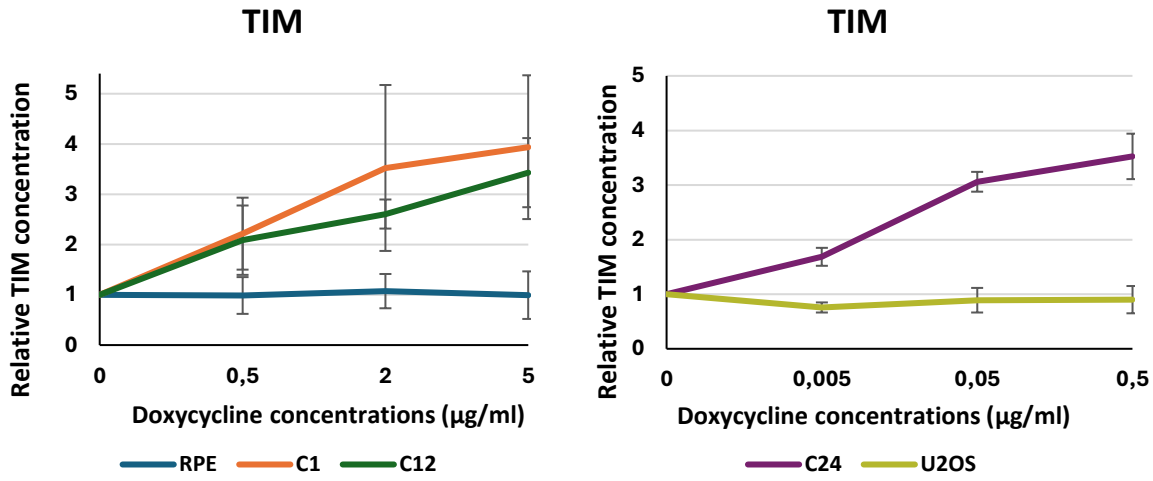


Figure 5: Optimization of doxycycline concentrations for induction of TIM overexpression in RPE- and U2OS-based clones A. RPE-based clones along with RPE control, and U2OS-based clone along with its U2OS control, were treated with various doxycycline concentrations for 24 hours and overexpression levels were quantified by Western blot. **B.** Quantifications of TIMELESS relative expression levels after a 24-hour treatment with different doxycycline concentrations. The quantifications are based on three independent experiments, means and standard deviations are shown.

4.3. The effect of TIMELESS overexpression on the growth rate of cells

In order to assess the effect of TIMELESS overexpression on the growth rate of cells, RPE-based clones were treated with 5 µg/ml of doxycycline to induce TIM overexpression, and cells were collected and counted after 24, 48 and 72 hours of treatment (**Figure 6**). Our data showed that TIM overexpression does not lead to any significant change in the growth rate of cells. Similar studies in U2OS-based TIM-overexpressing cells will be performed in the future.

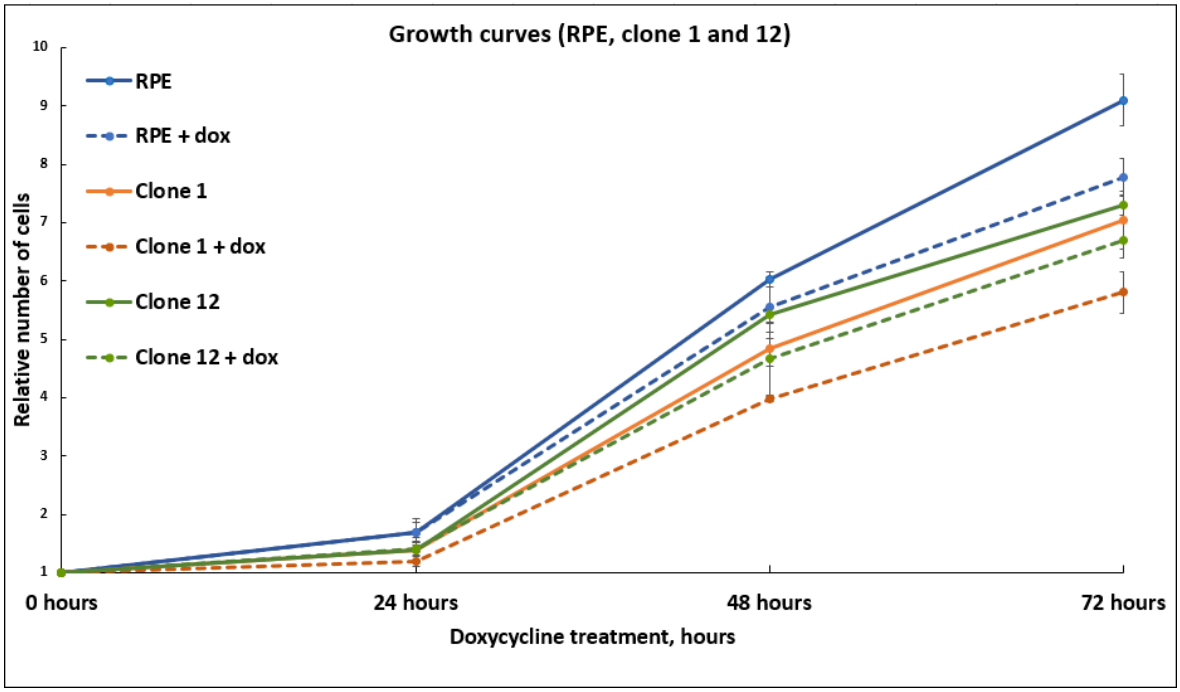


Figure 6: The effect of TIMELESS overexpression on the growth rate of cells. Indicated cell lines were treated with 5 µg/ml of doxycycline to induce TIM overexpression. Cells were collected and counted after 24, 48 and 72 hours of treatment. The quantifications are based on three independent experiments, means and standard deviations are shown.

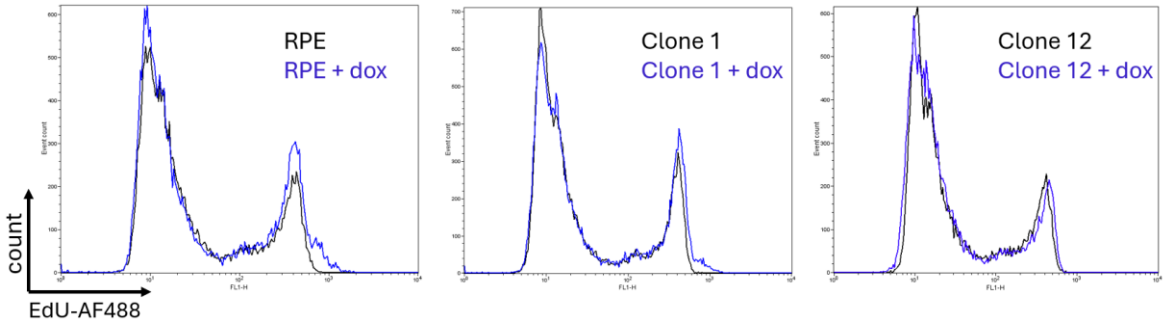
4.4. The effect of TIMELESS overexpression on the rate of DNA synthesis in cells

To assess the effect of TIM overexpression on the rate of DNA synthesis, RPE-based clones were treated with doxycycline for 24 hours, and then newly synthesized DNA was marked by treating cells with nucleotide analog EdU(5-ethynyl-2'-deoxyuridin) for 30 mins prior to harvesting them for flow cytometry analysis. EdU was then conjugated with a fluorophore AlexaFluor488, using a click reaction. Numbers of EdU-positive cells, actively synthesizing DNA, as well as levels of EdU incorporation, were analyzed by flow cytometry (**Figure 7**).

To study the effect of TIMELESS overexpression on change of cells proportion in S-phase, percentages of EdU-positive cells were compared with their doxycycline-untreated controls.

To assess the effect of TIMELESS overexpression on rate of DNA synthesis, EdU incorporation levels were compared between doxycycline-untreated and doxycycline-treated cells. For each sample, we subtracted the median background EdU signal of EdU negative cells from the median EdU signal of the EdU-positive cells. Then, we normalized the results to RPE untreated control. The results were analyzed using GraphPad Prism.

A



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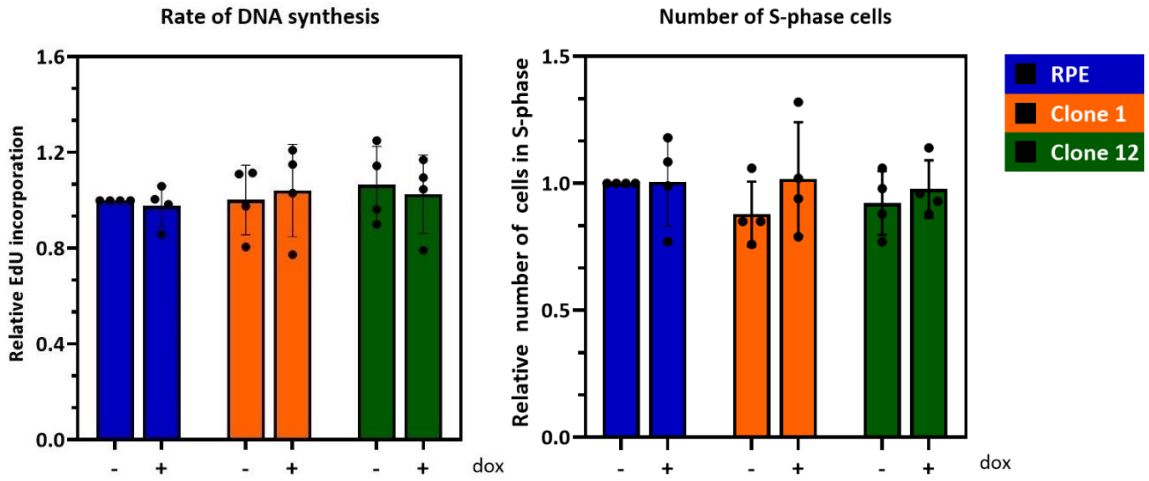

Figure 7: The effect of TIM overexpression on the rate of DNA synthesis and the proportion on RPE-based clones in S phase. The cells were treated with doxycycline for 24 hours, and then given a pulse of nucleotide analog EdU(5-ethynyl-2'-deoxyuridin) for 30 mins prior to harvesting them for flow cytometry analysis. **A.** Flow cytometry plots showing EdU incorporation histograms are shown. **B**. Relative EdU incorporation and relative number of cells in S phase were quantified. All quantifications are based on four independent experiments, means and standard deviations are shown.

We observed that TIMELESS overexpression has no significant effect on either the proportion of cells actively synthesizing DNA, or on the rate of DNA synthesis in actively replicating cells.

4.5. The effect of TIMELESS overexpression on the speed of DNA replication forks

In order to study the effect of TIMELESS overexpression on the speed of active DNA replication forks, we visualized tracks of newly synthesized DNA using DNA Fiber analysis and measured the track lengths, using ImageJ. The results were analyzed using GraphPad Prism (**Figure 8**). We observed that TIMELESS overexpression does not significantly affect the speed of DNA replication forks. However, only one repeat of this experiment has been carried out so far, and more repeats will be done in the future to obtain conclusive results.

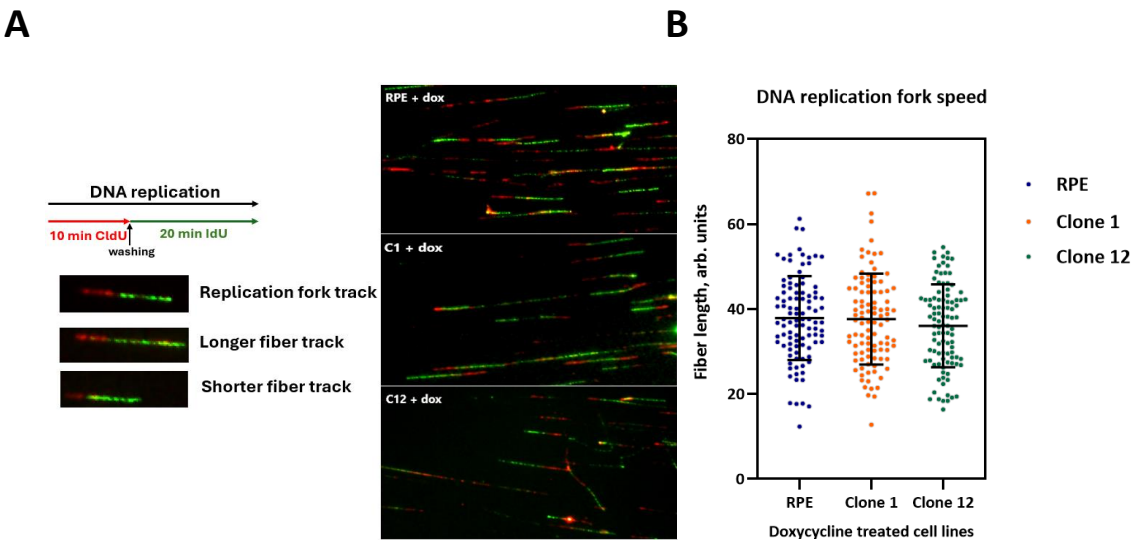

Figure 8. Assessment of DNA replication fork speed with DNA Fiber analysis. A. RPE-based clones were treated with doxycycline for 24 hours, and then pulses of nucleotide analogues CldU and IdU were given for 10 and 20 minutes respectively. Cells were lysed with lysis buffer and spread on glass slides by tilting. Tracks of newly synthesized DNA were stained with primary antibodies and fluorescently labeled secondary antibodies. DNA fibers were visualized by fluorescence microscopy on Olympus BX61 fluorescent microscope at 60x magnification, and the track lengths were measured subsequently using ImageJ software. Track length is proportionate to the speed of the replication fork. Representative pictures of obtained fibers in RPE, C1 and C12 cells are shown. **B.** DNA Fiber analysis quantifications are presented as column scatter plot. For each cell line, 100 fibers in total were measured and analyzed in GraphPad Prism. Quantifications are based on one experiment, means and standard deviations are shown.

5. Discussion

The investigation of TIMELESS overexpression is important due to its association with different cancers. Elevated levels of TIM expression not only correlate with poor survival prognosis, but also promote resistance to several chemotherapeutic drugs. TIMELESS depletion in human cells affects cell cycle parameters (like triggering G2/M arrest) and leads to a reduction in the growth rate of cells (Neilsen et al., 2019). TIM depletion caused a reduction in the proportion of actively replicating cells, the rate of active DNA synthesis, and also the speed of DNA replication forks (Bianco et al., 2019; Brewster, 2021; Mao et al., 2013). However, the effect of TIMELESS overexpression on these DNA replication-related parameters has not been studied. Thus, we aimed to study the effects of TIMELESS overexpression on the growth speed of cells and DNA replication dynamics in both cancerous and non-cancerous cells.

We started with creating a doxycycline-inducible TIMELESS-expressing construct on the background of a mammalian expression vector. With this construct, we generated a U2OS-based cell line (C24) capable of overexpressing TIM after doxycycline treatment. Two more such cell lines on the RPE-hTERT1 background (C1 and C12) were generated by the supervisor.

We hypothesized that, since TIMELESS downregulation slows cell proliferation, TIMELESS overexpression may have an opposite effect on cells. Understanding that is crucial, because if TIMELESS overexpression promotes cell proliferation, it might serve as a potential target for cancer therapies. However, growth curve analysis indicated that TIMELESS overexpression does not have any significant effect on the growth rate of cells in non-cancerous RPE hTERT-1-based cells, which was not known before.

In TIMELESS-depleted cells, the reduction in the proportion of cells undergoing DNA replication, in the rate of DNA synthesis, and the speed of DNA replication fork, was observed (Bianco et al., 2019; Brewster, 2021; Mao et al., 2013). The performed flow cytometry based EdU incorporation experiments revealed that there is no significant effect either on the proportion of cells in the S phase of the cell cycle or in the overall rate of DNA synthesis in the cells. Additionally, DNA fiber analysis showed that there was no significant change in the speed of DNA synthesis after overexpressing TIMELESS. Thus, TIMELESS overexpression does not affect the process of DNA synthesis and relatively same proportions of cells undergo DNA synthesis even after TIM upregulation. Based on the results of DNA fibers experiments, TIMELESS overexpression does not appear to affect the dynamics of fork progression during DNA replication. However, only one repeat was done, and more must be done before making any definitive conclusions. Since no such research has been done in non-cancerous cells, we cannot compare our results to those of other studies.

Non-cancerous cells were selected for this project to investigate the effect of TIMELESS overexpression on DNA replication-related parameters without the confounding factors that are present in cancer cells. We have not studied the effects of TIMELESS overexpression on the previously mentioned parameters in cancerous U2OS cells, but these studies will be done in future. Cancer cells, that are characterized by many different mutations and abnormal

proliferation, may respond to TIMELESS overexpression differently, compared to currently studied non-cancerous cells. There is a possibility that overexpressed TIMELESS can support cell proliferation because cell growth regulatory mechanisms are lost. Increased proliferative rate of cells could also enhance the rate of DNA synthesis. Cancer cells, experiencing constant DNA replication stress, may rely on TIMELESS overexpression as an adaptive mechanism for their survival, unlike normal cells, which do not require it.

Our initial study aimed to determine the effect of TIMELESS overexpression on the rate of DNA synthesis using EdU FACS analysis. The results indicated no change in the rate of DNA synthesis, and relatively same amounts of cells in S-phase after overexpressing TIMELESS. However, it is possible that a 24-hour induction of TIMELESS overexpression is insufficient to observe significant changes. In the future, this experiment is worth repeating with a longer duration of TIMELESS overexpression, up to several days, to potentially get any significantly important results.

Overall, our results imply that TIMELESS overexpression is not significant in the context of cell growth rate and DNA replication dynamics. However, more studies need to be done to confirm this conclusion. Further attention could be given to studies on genotoxic drugs. Given the limited data, it would be valuable to find out if resistance to anti-cancer drugs associated with TIMELESS overexpression, could be overcome. Additionally, more research is needed to identify significant alterations caused by TIMELESS overexpression that could be targeted in therapeutic purposes.

6. Abstract

DNA replication is a fundamental process that ensures equal distribution of genetic information between daughter cells. Because DNA replication is a central process in cell division, it needs to be performed with exceptional accuracy, as any errors in this process can have a lethal outcome. Fork Protection Complex (FPC), comprising of TIMELESS, TIPIN, CLASPIN and AND-1 proteins, helps maintain replication fork stability and support DNA replication.

Protein TIMELESS, the focus of this study, is known to be crucial for successful DNA replication. Mammalian TIMELESS is best studied in the context of DNA replication stress, DNA damage and fork stability maintenance. Interestingly, it is known that elevated expression of TIMELESS is associated with several cancers. TIMELESS presumably supports genome stability in cancer cells through stabilization of replication forks under conditions of replication stress. Further, TIM overexpression is also correlated with resistance to certain genotoxic anti-cancer drugs. However, more studies are needed to clarify TIM's pro-tumorigenic effects.

The aim of this thesis was to study the effects of TIMELESS protein overexpression on growth rate of cells and DNA replication dynamics. For this, several cell lines were created in cancerous and non-cancerous background that ectopically express TIMELESS in response to doxycycline treatment.

Our study revealed that TIMELESS overexpression does not have any significant effect on the growth rate of non-cancerous RPE hTERT-1 cells. Further, there is no significant effect on either the proportion of cells in the S-phase or on the rate of active DNA synthesis. Additionally, our data showed that TIMELESS overexpression does not significantly affect the speed of the replication fork. TIMELESS overexpression appears to have no effect on cell cycle progression and DNA replication dynamics in non-cancerous cells. But more studies are needed to confirm this data and shed light on the molecular basis of the pro-tumorigenic effects of this important protein, that could be targeted in anti-cancer therapy.

Kokkuvõte

DNA replikatsioon on fundamentaalne protsess mis tagab võrdse geneetilise informatsiooni jaotamist tütarrakkude vahel. Sellepärast, et DNA replikatsioon on kõige tähtsam protsess raku jagunemisel, see protsess vajab erandlikku täpsust, kuna igasugused probleemid DNA replikatsioonis võivad põhjustada letaalse tagajärge. DNA replikatsiooni kaitsev kahvel, mis koosneb järgmistest valkudest: TIMELESS, TIPIN, CLASPIN ja AND-1, toetab DNA replikatsiooni kahvli stabiilsust ning DNA replikatsiooni tervikuna. On teada, et valk nimega TIMELESS mängib olulist rolli edukas DNA replikatsioonis.

Imetajate TIMELESS on kõige paremini uuritud DNA replikatsiooni stressis, DNA kahjustuses ja DNA replikatsiooni kahvli stabiilsuse hoidmises. On teada, et TIMELESS valgu üleekspressioon on assotsieeritud mõnede. Arvatavasti, TIMELESS toetab genoomi stabiilsust vähirakkudes, stabiliseerides replikatsiooni kahvli replikatsiooni stressi ajal. Veel on teada, et TIMELESS valgu üleekspressioon korrileerub genotokosilistele ravimitele püsivuse tekkimisega. Aga, rohkem uuringuid on vaja teha, et selgitada TIMELESS valgu pro-tuumorigeenseid efekte.

Selle töö eesmärgiks oli uurida TIMELESS valgu üleekspressiooni efekte rakutsükli parameetritele ja DNA replikatsiooni dünaamikale. Selleks, mõned rakuliinid olid loodud mõlemal vähirakkudel ja mittevähirakkudel, mis ektoopiliselt ekspresseerivad TIMELESS valku peale mõjutamist doksütsükliiniga.

Meie uuring näitas, et TIMELESS valgu üleekspressioon ei mõjutanud oluliselt RPE hTERT-1 mittevähirakkude kasvu. Täiendavalt, olulist muutust ei avaldunud nii rakutsükli S-faasis olevate rakkude proportsioonil kui ka aktiivse DNA replikatsiooni kiirusel. Meie andmed näitavad ka, et TIMELESS'i üleekspressioon ei põhjustanud olulist muutust replikatsiooni kahvli kiirusel. Tundub, et TIMELESS'i üleekspressioon ei tekita olulisi muutusi rakutsükli progressioonis ja DNA replikatsioni dünaamikas mittevähirakkudes. Siiski on vaja täiendavaid uuringuid, et selgitada selle valgu pro-tuumorigeenseid efekte, mida võib potentsiaalselt kasutada vähiteraapias sihtmärgina.

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Lisa
rektori 07.04.2020 käskkirjale nr 1-8/17

Lihtlitsents lõputöö reprodutseerimiseks ja lõputöö üldsusele kättesaadavaks tegemiseks[1]

Mina, **Artur Morgunov**

1. Annan Tallinna Tehnikaülikoolile tasuta loa (lihtlitsentsi) enda loodud teose
Effect of TIMELESS protein overexpression on DNA replication in human cells,

mille juhendaja on **Tatiana Moiseeva**,

1.1 reprodutseerimiseks lõputöö säilitamise ja elektroonse avaldamise eesmärgil, sh Tallinna Tehnikaülikooli raamatukogu digikogusse lisamise eesmärgil kuni autoriõiguse kehtivuse tähtaja lõppemiseni;

1.2 üldsusele kättesaadavaks tegemiseks Tallinna Tehnikaülikooli veebikeskkonna kaudu, sealhulgas Tallinna Tehnikaülikooli raamatukogu digikogu kaudu kuni autoriõiguse kehtivuse tähtaja lõppemiseni.

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