

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

ANALYSIS OF THE LOCALIZATION OF DEK ISOFORM 2

HASAN AKYOL
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

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2022



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...../...../..... tarih ve/..... sayılı kararı.

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SUMMARY

DEK is a nucleic acid binding protein involved in a variety of sub-nuclear functions including gene regulation, quality control of mRNA splicing, and cell cycle regulation. Although DEK is known and studied for decades, there is no satisfying amount of data on DEK isoform 2. Here I showed that DEK isoform 2 localization throughout the cell cycle in proliferative human cell lines for the first time. It is shown that DEK isoform 2 localizes in the nucleolus but slightly remains in the nucleus during the interphase. While the cell entering the mitosis, DEK isoform 2 diffuses into the cytosol until the late mitosis. At telophase, it reassociates with daughter nuclei, and while the cells enter the interphase, it localizes into the nucleolus again. Summarily, it is shown that overexpressed DEK isoform 2 shows different localization properties than overexpressed canonical DEK isoform.

Key Words: DEK, Cell Cycle, DEK Isoform 2, Immunofluorescence.

ÖZET

DEK, gen regülasyonu, mRNA uçbirleştirmesinde kalite kontrolü ve hücre döngüsü regülasyonu dahil olmak üzere çeşitli çekirdek içi fonksiyonlarda yer alan bir nükleik asit bağlayıcı proteindir. DEK on yıllardır bilinmesine ve çalışılmasına rağmen, DEK izoformu 2 hakkında tatmin edici miktarda veri bulunmamaktadır. Burada ilk kez proliferatif insan hücre dizilerinde DEK izoform 2 lokalizasyonunu hücre döngüsü boyunca gösterdim. Interfaz sırasında, DEK izoformu 2'nin nükleolusta lokalize olduğu, ancak biraz çekirdekte kaldığı gösterilmiştir. Hücre mitozaya girerken, DEK izoformu 2 geç mitozaya kadar sitozole difüze olarak kalır. Telofazda yavru çekirdeklerle yeniden birleşir ve hücreler interfaza girerken tekrar çekirdekçikte yerleşir. Özetle, aşırı ifade edilen DEK izoformu 2'nin, yine aşırı ifade edilen kanonik DEK izoformundan farklı lokalizasyon davranışı gösterdiği gösterilmiştir.

Anahtar Kelimeler: DEK, Hücre Döngüsü, DEK izoformu 2, Immunofluoresans.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS AND ACRONYMS

<u>Abbreviations and Acronyms</u>	<u>Explanations</u>
%	: Percent
°C	: Centigrade degree
μl	: Microliter
μg	: Microgram
g	: Gram
L	: Liter
ng	: Nanogram
mg	: Milligram
ml	: Milliliter
mM	: Millimolar
M	: Molar
AML	: Acute myeloid leukemia
AP2α	: Activating enhancer binding protein 2 alpha
APC/C	: Anaphase promoting complex / cyclosome
BCA	: Bicinchoninic acid
BSA	: Bovine serum albumin
CaCl ₂	: Calcium chloride
CAKs	: Cdk activating kinases
Cdk	: Cyclin dependent kinase
cDNA	: complemeter deoxyribonucleic acid
CIP/KIP	: Cdk intibitor protein/kinase interacting protein
CK2	: Casein kinase 2
CKI	: Cyclin dependent kinase inhibitor
DAPI	: 4',6-diamidino-2-phenylindole
ddH ₂ O	: Double distilled water
DEK1	: DEK isoform 1
DEK2	: DEK isoform 2
DFC	: Dense fibrillar component
DMSO	: Dimethyl sulphoxide

DNA	: Deoxyribonucleic acid
dsDNA	: double stranded deoxyribonucleic acid
E. coli	: Escherichia coli
E2F	: Transcription factor E2F
ECL	: Electrochemiluminescence
EF	: Endotoxin free
EGFP	: Enhanced green fluorescent protein
FC	: Fibrillar center
G1	: Gap 1
G2	: Gap 2
GC	: Granular component
GFP	: Green fluorescent protein
H3.3	: Histone 3.3
HA	: Hemagglutinin
HBS	: Hepes buffered saline
HCl	: Hydrochloric acid
HEK	: Human embryonic fibroblast
HMG	: High mobility group
HRP	: Horseradish peroxidase
HS27A	: Human stroma 27a
HSP70	: Heat-shock protein 70 kilodalton
IGCs	: Intrachromosomal granular clusters
KIF15	: Kinesine interacting factor 15
M phase	: Mitotic phase
MEF	: Mouse embryonic fibroblast
mRNA	: messenger ribonucleic acid
NaOH	: Sodium hydroxide
NIKS	: Near diploid keratinocyte cells
NLS	: Nuclear localization signal
NoLS	: Nucleolar localization signal
NUP214	: Nuclear pore complex protein 214
OD	: Optical density
p300/CBP	: p300/CREB binding protein

PCAF	: p300/CBP associated factor
PCNA	: Proliferating cell nuclear antigen
pH	: Power of Hydrogen
PLK1	: Polo-like kinase 1
PML	: Premyelotic leukemia
Pol	: Polymerase
PP1	: Protein phosphatase 1
pRB	: Protein retinoblastoma
PRs	: Perichromosomal regions
rDNA	: Ribosomal deoxyribonucleic acid
RNA	: Ribonucleic acid
rRNA	: Ribosomal ribonucleic acid
S	: Synthesis phase
SAF	: Scaffold attachment factor
SAP	: SAF A/B - Acinus - PIAS
SR	: Serine-Arginine rich protein
SUMO	: Small ubiquitin like modifier
TBS	: Tris buffered saline
TE	: Tris-EDTA
U2AF35	: Splicing factor U2AF 35 kDa subunit
USP15	: Ubiquitin carboxyl-terminal hydrolase 15
UV	: Ultraviolet
X.laevis	: <i>Xenopus Laevis</i>
xUBF	: Upstream binding protein of <i>Xenopus laevis</i>

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1. INTRODUCTION

Cell cycle describes the formation, growing, and the division of the cell. It has composed of mainly two phases as interphase and the mitosis. During interphase, the cell grows, duplicates its genetic material, and prepares to division. During mitosis, chromosomes disentangle and condense, sister chromatids are pulled to two poles of the cell, and the cell divides into two new cells (Alberts et al., 2015). Some of the proteins bound to the chromatin in the interphase are regulated to dissociate from the chromatin at the beginning of the mitosis, such as DEK protein.

DEK is a chromatin-binding protein first described in acute myeloid leukemia (AML) as a fusion with NUP214 [von Lindern et al., 1992]. It codes 375 amino acids long protein which has roles in chromatin architecture, transcriptional regulation, and mRNA-splicing [Privette Vinnedge et al., 2013]. The mRNA of the DEK gene produce two known splice isoforms. The unspliced isoform is the first identified DEK isoform (DEK1), and DEK isoform 2 (DEK2) loses its amino acids 49-82 during splicing and produces 341 amino acids long protein [Yu et al., 2016]. Endogenous DEK1 is mostly bound to chromatin, and it localizes in the nucleus at interphase, and it dissociates from the chromatin at the beginning of mitosis, then re-associates with chromatin after late anaphase. When overexpressed, DEK1 remains bound to the DNA throughout the cell cycle [Matrka et al., 2015].

To our knowledge, subcellular localization of DEK2 is not investigated thoroughly in the literature. Recently we found that overexpressed DEK2 forms aggregates in the nucleus in addition to disperse nuclear location (Manuscript in preparation). Given that DEK1 mainly bounds to chromatin in this thesis we further traced location of DEK2 during the phases of cell cycle. Here it is shown that DEK2 localizes mainly in the nucleolus and slightly diffuse in the nucleus during the interphase and shows diffuse distribution during mitosis until late telophase. After telophase, DEK2 re-associates with nucleus and re-accumulates in the nucleoli. Part of this work was supported by TUBITAK project (grant number 118Z765).

2. THE CELL CYCLE

Cell cycle refers the process in which a cell duplicates itself and divide into two cells. It consists of 4 main phases as gap 1 (G1), synthesis (S), gap 2 (G2), and mitosis (M), and the phases until mitosis named interphase. The whole life of a proliferating cell passes by developing until division and interphase encapsulates this period of the cell's life. It takes approximately 23 hours depending on the type of the cell. In G1 phase, the cell grows volumetrically and matures to reach the point that it can start replicating its DNA and organelles like centrosome. This point is regulated by environmental conditions as well as subcellular events like DNA damage. If appropriate conditions exist, the S phase begins, and the DNA is started to replicate. The proper replication is checked in G2 phase. If the DNA is replicated without any obstacles, the G2/M checkpoint system permits the cell to enter the M phase. Any DNA damage or re-replication may inhibit the entry to M phase [Chen et al., 2012], [Morgan, 2007].

2.1. The Regulation of The Cell Cycle

The life circle of the cell is regulated mainly by cyclins and cyclin-dependent kinases (Cdks) (Figure 2.1). The amount of Cdks does not change during cell cycle, but their activity is regulated by the presence of distinct cyclin type at different cell cycle stages. These main regulators initiate or regulate downstream cell cycle events by phosphorylation. Cyclins and Cdks work together as complexes. The cyclin-Cdk complexes, cyclins and associated Cdks are shown in Table 2.1. The activation of Cdk complexes is completed by Cdk-activating kinases (CAKs). In humans (as well as other vertebrates and fruit flies) the CAK is Cdk7, and cyclin H accompanies Cdk7. The inhibition of Cdk complexes is regulated by phosphorylation activity of Wee1, and this inhibitory effect is eliminated by the dephosphorylation activity of Cdc25 at the same site [Enserink and Kolodner, 2010], [Harper and Elledge, 1998], [Morgan, 2007].

There are four main Cdk complexes: G1, G1/S, S, and M. G1-Cdk complex initiates the entrance to the cycle of the cell upon changes in the environment in a favorable way. The main function of G1/S-Cdks is to make the cell enter S-phase by

targeting Cdk inhibitors and APC/C, which inactivate S-Cdks. G1/S-Cdks then shut down their own activity by targeting G1/S cyclins in the S phase. During the S phase, M-Cyclin gene is activated and the amount of M-Cdk complexes increases during the G2 phase. M-Cdks activate the G2/M checkpoint and ultimately the APC/C that initiates the degradation of S- and M-Cyclins. In late mitosis, the concentration of Cdk inhibitors (CKIs) raises, which cause dephosphorylation of Cdk targets [Morgan, 2007].

Table 2.1: Cdk-cyclin complex components of animal cells.

Cyclin-Cdk complex	Cyclin	Cdk
G1-Cdk	Cyclin D	Cdk4, Cdk6
G1/S-Cdk	Cyclin E	Cdk2
S-Cdk	Cyclin A	Cdk2, Cdk1
M-Cdk	Cyclin B	Cdk1

Table 2.2: CKI proteins and their roles.

CKIs	Role
p21, p27, p57	Inhibit G1/S- and S-Cdk, activate G1-Cdk
p15, p16, p18, p19	Inhibits CDK4/6

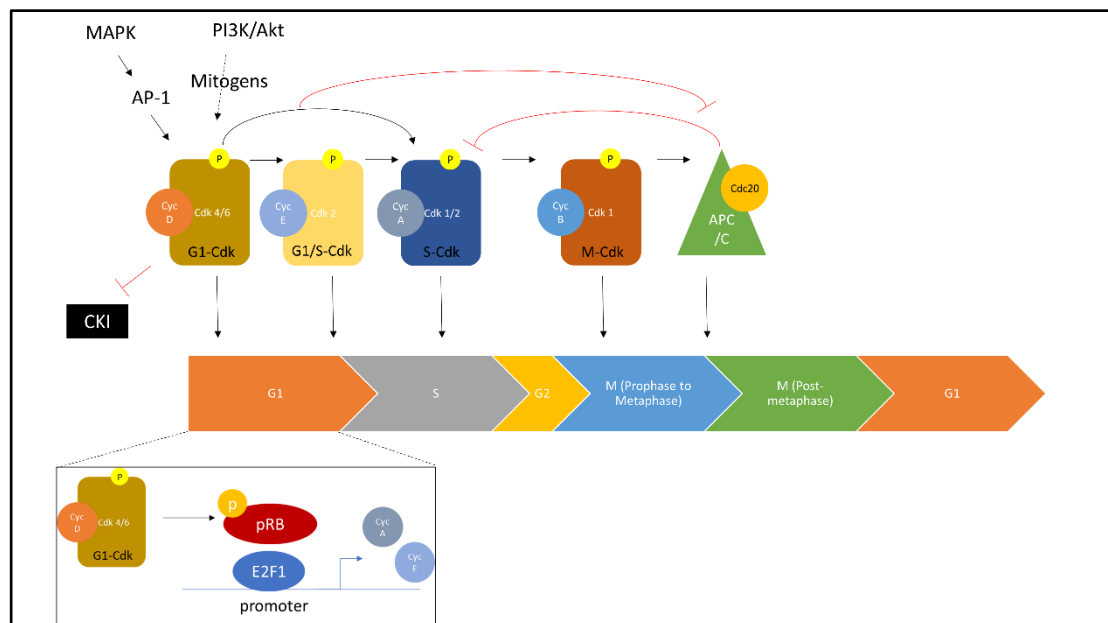


Figure 2.1: The regulation mechanism of the cell cycle.

2.2. Mitosis

When activated M-Cdk levels increase, the cell is able to pass through G2/M checkpoint and enter the M phase. The M phase consists of two main events: The division of the nucleus (karyokinesis or mitosis) and the division of cytoplasm (cytokinesis). The karyokinesis consists of several sub-phases: Prophase, prometaphase, metaphase, anaphase, and telophase [Alberts et al., 2015].

Prophase is the phase when the replicated chromosomes condense, and the mitotic spindles are formed between two centrosomes by rearranged interphase microtubules. During chromosome condensation, cohesion proteins which hold sister chromatids together during DNA replication in the S phase are removed from sister chromatids by phosphorylation of either cohesion proteins by PLK1 or histones by aurora kinase B [Morgan, 2007]. These kinases may also regulate other chromosome proteins during condensation in the prophase. In addition, transcription and translation mechanisms stop at this phase (at the beginning of the mitosis) [Lodish et al., 2007].

Prometaphase is the phase when the nuclear envelop dissociates, and the spindles bind to kinetochores, the assembling point of chromatids for microtubules. When the spindle microtubules attached to kinetochores, the chromosomes are pulled towards each spindle poles, until they reach equal distance from the poles during prometaphase. This process is called congression. When they reach the optimum distance, the metaphase starts [Lodish et al., 2007].

Metaphase is the phase when the chromosomes are placed at the halfway of the poles. During metaphase, metaphase-to-anaphase transition (or spindle checkpoint system) controls the proper assembly of microtubules to the kinetochores and makes possible bi-oriental movement of sister chromatids during anaphase [Alberts et al., 2015], [Morgan, 2007].

Anaphase starts with the degradation of cohesins by anaphase promoting complex/cyclosome (APC/C). This event occurs in early anaphase and this period is called as anaphase A. When the interaction between sister chromatids is destructed, each chromatid is pulled opposite site of the cell. To increase the distance between chromatids, the spindle poles also move away (opposite site) from each other. This period is called as anaphase B (or late anaphase). Dynein motor proteins are involved

in separation of centrosomes. Dyneins also help the fragmented nuclear envelope transportation toward spindle poles [Lodish et al., 2007], [Morgan, 2007].

Telophase is the last step of mitosis. The separated chromosomes are now at the spindle poles. The nuclear envelope begins to re-organize around each set of daughter chromosomes. Mitotic-ware (centrosomes, mitotic spindles) return their interphase states. Cytokinesis is about to start by the contraction of the contractile ring around the middle point of the cell membrane [Morgan, 2007].

2.3. Interphase

After the cytokinesis accomplished, the ongoing cycle of the cell is also completed, and a brand-new lifetime for the cell starts. As previously mentioned, during telophase, nuclear envelope rearranges, nuclear proteins return their interphase state, and gene expression and protein production start. Proliferative cells grow until intrinsic and extrinsic conditions are favorable to initiate a new cell cycle [Alberts et al., 2015].

Under favorable conditions, mitogens (extracellular proliferation factors) stimulate the cell to initiate cyclin D (G1 phase) production. Activation of upstream RAS signaling induces expression of the cyclin D. PI3K/AKT pathway leads the translation and causes lowering in the degradation of cyclin D. Produced cyclin D is then assembled with Cdk4/6 in the presence of CDK-interacting protein/kinase inhibitory protein (CIP/KIP) family members. This association let G1-Cdk complex (Cdk4/6-cyclin D) enter into the nucleus. In the nucleus, this complex causes the transcription of cyclin E (G1/S transition) and cyclin A (S phase) and the phosphorylation retinoblastoma (pRB) and activation E2F dependent transcription (together with G1/S-Cdk activity) [Tchakarska and Sola, 2020]. Until that point, Cdk inhibitors, APC/C, and suppressed expression of cyclin genes stabilize the cell in G1 phase [Morgan, 2007] (Figure 2.2).

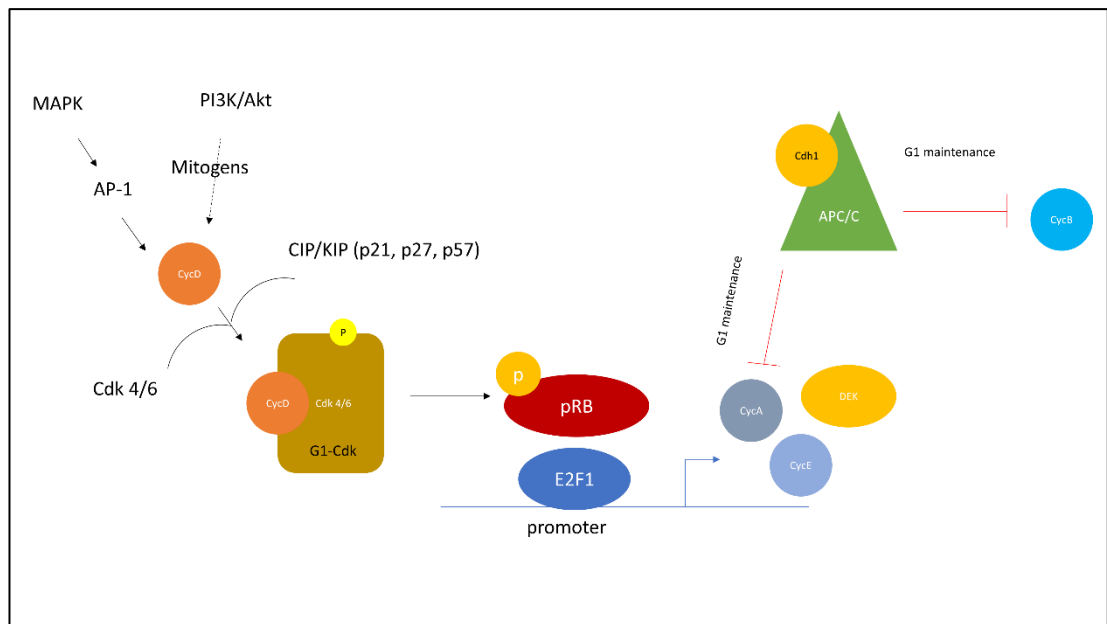


Figure 2.2: The molecular basis of the regulation of interphase.

3. THE NUCLEOLUS

The interphase nucleus contains diverse compartments called membrane-less organelles which are formed by nucleation of proteins and/or RNAs in specific locations, a phenomenon called liquid-liquid phase separation. Nuclear speckles, promyelocytic leukemia (PML) body, and the nucleolus are the examples of membrane-less nuclear bodies. The functions of nuclear bodies include, for example, mRNA processing, ribosome biogenesis, and telomere maintenance [Sawyer et al., 2018], [Staněk and Fox, 2017].

The nucleolus is a membrane-less organelle localized inside the nucleus. It emerges around the ribosomal DNA (rDNA) transcribed by RNA Polymerase I (Pol I) and the product of this transcription, ribosomal RNA (rRNA). Ribosomal genes are located on acrocentric chromosomes 13, 14, 15, 21, and 22, and each of these chromosomes has ribosomal gene groups which are called nucleolar organizer regions (NORs), because the nucleoli (plural form of nucleolus) form around these regions [Boisvert et al., 2007]. rDNA is transcribed into 47S pre-rRNA which then gives rise to 28S, 18S, and 5.8S rRNA. The gene of the fourth rRNA, 5S rRNA, is found on chromosome 1, outside of the nucleolus and transcribed by RNA Pol III rather than RNA Pol I. Nucleolar proteins contain a nucleolar localization signal (NoLS) which has no consensus but consists of mainly positively charged arginines (R) and lysines (K) [Scott et al., 2011]. This sequence directs proteins to the nucleolus. Because nucleolar localization requires charged residues, the organization of the nucleolus may arise such weak protein-protein interactions. [Iarovaia et al., 2019] The proteins that involved in the transcription of 47S rRNA form the fibrillar center (FC) of the nucleolus. rRNA modification proteins like fibrillarin localize around the newly synthesized rRNA and form the dense fibrillar center (DFC) phase of the nucleolus. Finally, the proteins and RNAs which are involved in ribosomal subunit assembly, rRNA modifications, and other functions generate the outer phase of the nucleolus, called as granular component (GC) (Figure 3.1) [Iarovaia et al., 2019], [Pederson, 2011], [Savino et al., 2001], [Yao et al., 2019].

either diffuse into the cytosol or remain associated with the chromosome at perichromosomal regions (PRs) [Boisvert et al., 2007].

Regulation of the nucleolus during the cell cycle is governed by posttranslational modifications on the nucleolar proteins. The main modification is phosphorylation of nucleolar components, and so the regulators of the mitosis in the nucleolus are M-Cdk complex and protein phosphatase 1 (PP1) (Figure 3.2). Phosphorylation of the nucleolar components in early prophase by M-Cdk cause the dissolution of GC and DFC phases. Among the substrates of M-Cdk complex, RNA Pol I transcription factors, fibrillarin, and NPM1 can be cited [Hernandez-Verdun, 2011], [Negi and Olson, 2006]. This indicates that M-Cdk complex acts on each subphase of the nucleolus. Of note, the phosphorylation of NPM1 by M-Cdk lowers the ability of NPM1 to bind RNAs. Moreover, spatial correlation between fibrillarin translocation from DFC and the nuclear envelope fragmentation indicates the possibility of a common upstream regulator [Boisvert et al., 2007]. The loss of RNAs from nucleolus is also a reason of nucleolar dissolution [Chamoussset et al., 2010; Negi and Olson, 2006]. The reason behind the RNA loss from the nucleolus is the stopped rRNA production at the prophase. Although the production of rRNA stops, the RNA Pol I machinery stays bound to rDNA and its transcriptional activity continues until some point of the mitosis. Supportively, some of the transcription machinery subunits dissociates in the middle of the mitosis indicating continuing RNA Pol I activity [Hernandez-Verdun, 2011]. The reason why transcription machinery stays bound to rDNA is to complete the ongoing transcription [Boisvert et al., 2007]. The activity of M-Cdk complex keeps the nucleolar component away until the late mitosis.

The spark of the formation of the nucleolus is the onset of the transcriptional activity of RNA Pol I during telophase. PP1 β and PP1 γ dephosphorylate M-Cdk substrates and support the formation and maintenance of nucleolar integrity [Hernandez-Verdun, 2011]. Re-formation of the nucleolus in telophase depends on re-activation of rRNA transcription and re-localization of nucleolar proteins from PNBs. Dephosphorylation of Pol I transcription factors lead the transcription of rRNA. During late anaphase, nucleolar proteins either remain localized in the PRs or start to localize in nucleolar derived foci which then give rise to PNBs [Boisvert et al., 2007]. After rRNA emerges, DFC and GC components translocate to the rRNA transcription site to generate the other parts of the nucleolus.

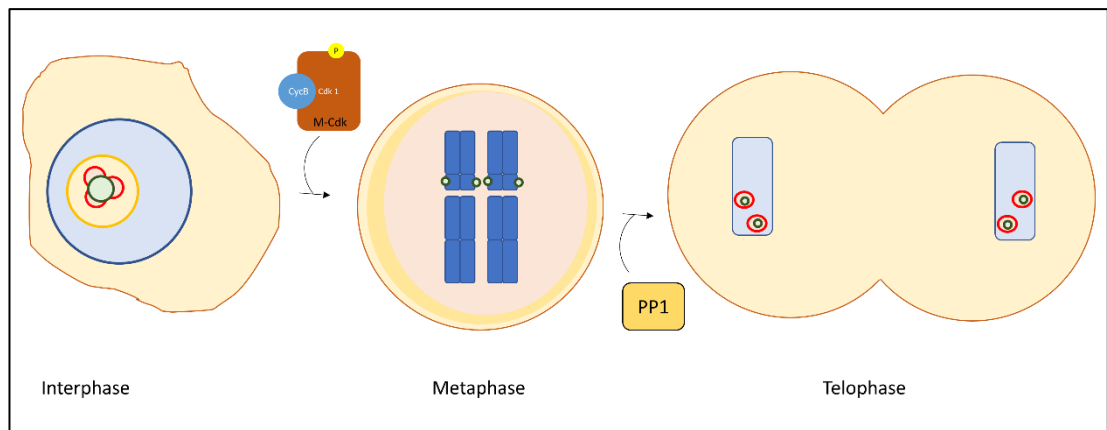


Figure 3.2. Mitotic regulation of the nucleolus.

4. DEK

DEK is a DNA/RNA-binding protein which is involved in chromatin architecture, transcriptional regulation, and mRNA-splicing [Sawatsubashi et al., 2010], [Soares, 2006], [Waldmann, 2003]. The gene of DEK was first identified in a bone marrow sample obtained from an AML patient as DEK-CAN fusion gene formed by t(6;9)(p23;q34) translocation [von Lindern et al., 1992]. It has been conserved among higher organisms since fruit flies, indicating a crucial role in humans [Sawatsubashi et al., 2010]. After expression, DEK mRNA either produces the most common isoform (DEK1) or is spliced to produce a second isoform (DEK2) lacking the amino acids 49-82 [Privette Vinnedge et al., 2013]. DEK protein has been related to the maintenance of proliferation and progression of different types of cancers, however DEK2 is less effective in these roles [Yu et al., 2016].

DEK gene is located on the short-arm of the 6th chromosome in humans and contains 11 exons whereas the 3rd exon is missing in DEK2 [Kappes et al., 2004b], [Yu et al., 2016]. Its expression is known to be regulated by different transcription factors such as E2F which is activated upon induction of the cell cycle [Carro et al., 2006] and human papilloma virus protein E7 upon infection, thus, it induces proliferation. On the other hand, although *DEK* knockout mice are viable [Smith et al., 2017], it has been associated with shown to cause Alzheimer's disease morphology in human neural cell lines and cognition in mice which is lethal [Ghisays et al., 2018], [Greene et al., 2020].

Some of the upstream and downstream transcriptional regulators of *DEK* are closely connected with the cell cycle organizing proteins. E2F family proteins E2F1 and E2F3 affect *DEK* expression positively, on the other hand E2F inhibitor pRB and CDK4 inhibitor p16 negatively affect expression of *DEK*. At the *DEK* promoter, seven putative E2F binding-site located, and one of them (TCCCGC) is conserved in both rodents and human. A luciferase reporter assay was showed that mutation in this site fully inhibits the E2F mediated *DEK* transcription [Carro et al., 2006]. In another study it was shown that silencing of DEK causes inhibition of mitotic entrance and either DEK isoforms can remove this arrest. Bioinformatical analyses showed a correlative expression between DEK and genes involved in mitotic regulation like E2F family and CDK family genes (E2F1,2,3,6,7 and 8 and CDK1,2, and 4, respectively), and also

replication marker protein PCNA. The expression of these genes is downregulated by the silencing of DEK and upregulated by overexpression of either DEK isoforms, however, the enhancing effect of DEK2 is not as powerful as DEK1 [Yu et al., 2016]. Taken together, DEK both as a gene and a protein can affect different stages of the cell cycle through its transcriptional regulator properties.

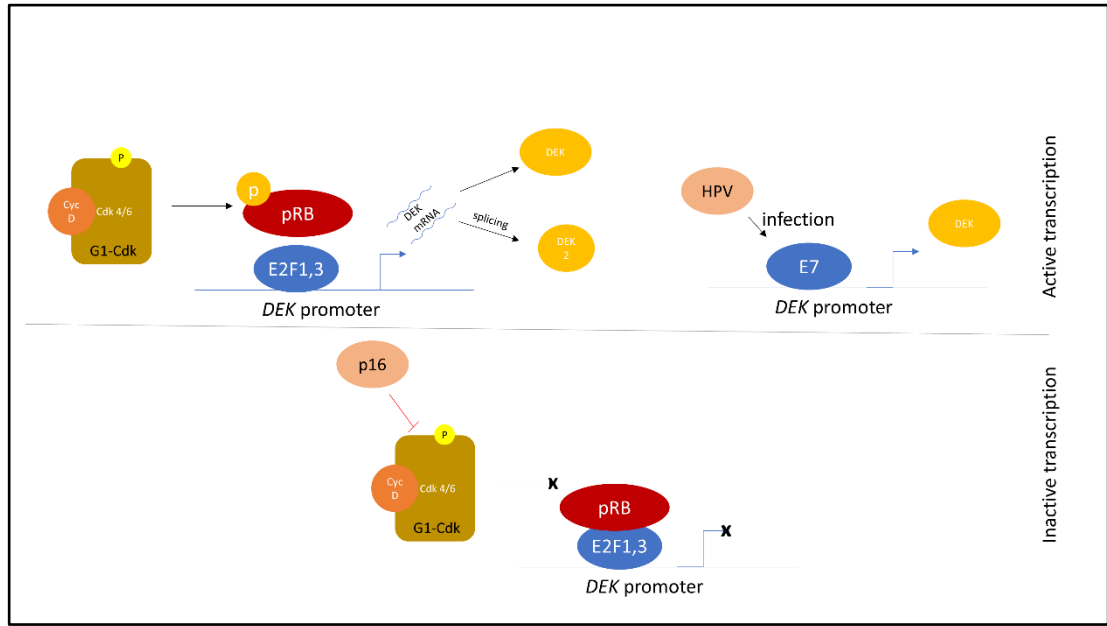


Figure 4.1: Mechanisms of *DEK* gene expression for activation or inactivation.

4.1. The Structure of DEK

DEK gene product is a 375 amino acid long protein comprises two main DNA-binding domains (87-187 and 310-375), one RNA-binding domain (187-270), and three acidic patches (29-49, 203-261, and 293-325) [Guo et al., 2021], [Kappes et al., 2004b]. One DNA-binding domain is composed of the combination of a pseudo-SAF box and a SAP box DNA-binding motifs which give DEK the aforementioned abilities [Aravind and Koonin, 2000] and is located N-terminally. The other DNA binding domain of DEK is located at the C-terminal of the protein [Devany et al., 2004]. This region is also phosphorylated by casein kinase II (CK2) causing dissociation of DEK from the DNA and multimerization [Kappes et al., 2004; Kappes, Scholten, et al., 2004]. Between two DNA-binding domains, RNA-binding domain of DEK is located

[Guo et al., 2021]. However, RNA-binding ability of DEK has not been studied broadly yet.

DEK protein comprises a variety of posttranslational modification sites or protein binding sites. DEK contains a SUMOylation site between amino acids 260-263 [Ivanauskiene et al., 2014], a citrullination site on arginine 75 [Tanikawa et al., 2018], possible PLK1 and aurora kinase phosphorylation sites [Matrka et al., 2015], in addition to CK2 phosphorylation, acetylation, and PARylation sites [Cleary et al., 2005], [Ganz et al., 2019], [Kappes et al., 2004a].

The N-terminal domain of DEK comprising the amino acids between 72 and 222 tends to bind cruciform (cloverleaf) DNA over double stranded DNA (dsDNA). It can also introduce either negative or positive supercoils. The peptide sequences comprising amino acids 68-87 and 187-208 are required to introduce positive supercoils [Böhm et al., 2005]. Of note, these sequences are outside of the pseudo-SAF/SAP box region (87-187). DEK2 is missing amino acids between 49-82 which comprises one of the regions needed for introducing positive supercoils [Privette Vinnedge et al., 2013]. However, this is not solid evidence for DEK2 introducing negative supercoils.

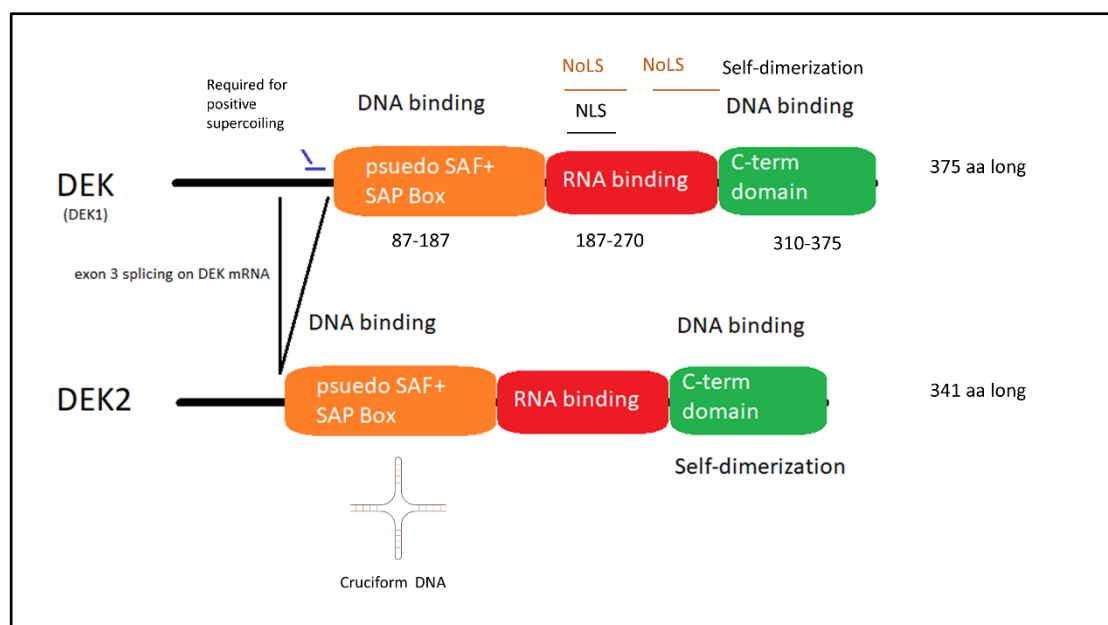


Figure 4.2. The schematic structure of DEK.

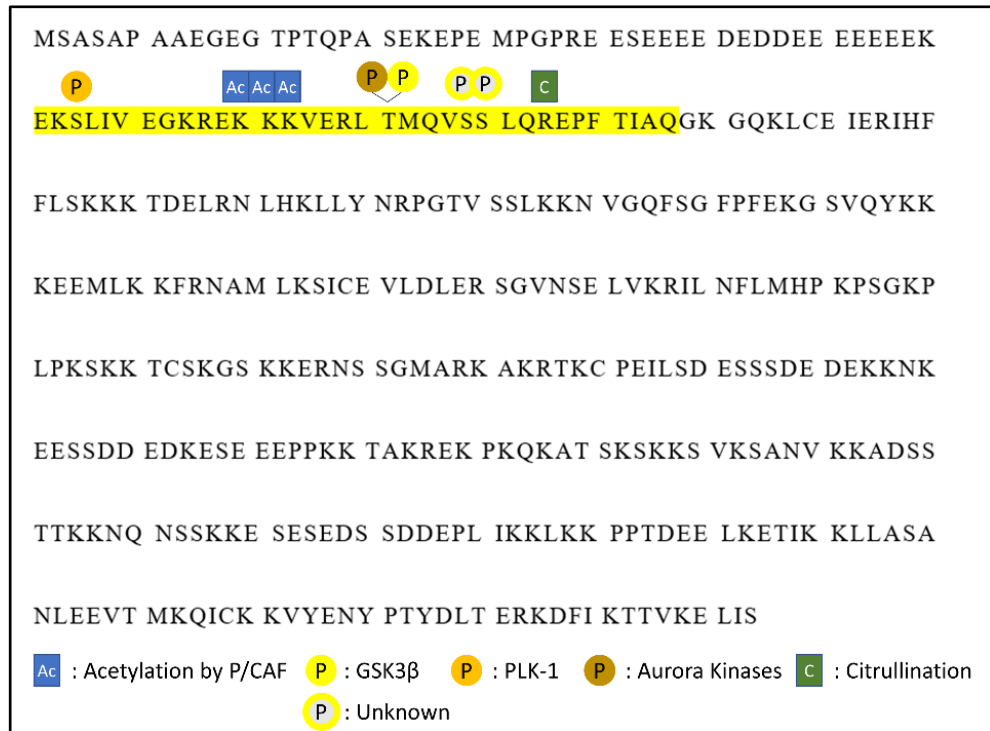


Figure 4.3: Amino acid sequence of DEK protein. Highlighted letters indicate amino acids not found in DEK2. Only posttranslational modifications not found in DEK2 are shown. P: Phosphorylation. Ac: Acetylation. C: Citrullination.

4.2. Cellular Functions of DEK

DEK protein has been associated with several nuclear functions including transcriptional regulation, mRNA splicing, and histone chaperone [Ivanauskiene et al., 2014], [Sandén et al., 2014], [Soares, 2006]. DEK is known to bind chromatin, especially transcriptionally active sites with no sequence specificity. Chromatin immunoprecipitation experiments show that DEK binds to chromatin near to transcription start sites, mostly on promoter regions [Sandén et al., 2014]. In correlation with that, the higher expression is related with the higher DEK binding. Interestingly, some of the genes whose expression is regulated by DEK are binding partners of the DEK [Smith et al., 2018] or involved in RNA splicing, chromatin architecture and cell cycle. Likewise, in DEK knockdown cells, expression levels of proteins involved in the same processes are lowered [Sandén et al., 2014]. In addition to directly binding to DNA, DEK can regulate gene expression via binding other transcription factors. DEK can bind AP-2alpha, a transcription factor involved in the

regulation of developmental genes as well as cell cycle genes, and this binding increases the DNA-binding ability of AP-2alpha [Campillos et al., 2003].

DEK protein is involved in mRNA splicing as a pre-splicing proofreader. DEK increases the 3' splicing factor protein U2AF35 to AG-rich RNA which is found on splicing sites. In the absence of DEK, U2AF can bind both AG or CG sequences, however, in the presence of DEK it prefers AG over CG [Soares, 2006]. The interaction between U2AF35 and DEK requires N-terminal amino acids of DEK with phosphorylation at S19 and S32. In addition to involving in pre-splicing events, DEK is involved in the formation of "splicing-dependent exon-exon junction complex" which is formed by proteins binding to spliced mRNA and transport the mRNA to nuclear pore complexes (NPCs) and then cytoplasm. However, DEK accompanies this complex until the NPCs [V. N. Kim and Dreyfuss, 2001].

In addition to aforementioned functions, DEK has a chaperon function. SUMOylated DEK can bind to histone 3.3 which is a histone 3 variant found in telomere regions of embryonic stem cells (ESCs). Loss of DEK causes relocation of H3.3 to heterochromatin as well as relocation of PML from telomeres, and in the presence of DEK, H3.3 is led to PML bodies at the telomere regions [Ivanauskiene et al., 2014]. Sawatsubashi and coworkers showed that DEK homolog in fruit fly also has H3.3 chaperon activity, and *in vitro* experiments showed that DEK has conserved nucleosome assembly function in the presence of phosphorylation via CK2 [Sawatsubashi et al., 2010].

4.3. Localization of DEK

DEK is a pseudo SAF/SAP-box containing DNA-binding protein having a nuclear protein signal between 205 – 221 amino acid residues (NLS) [Privette Vinnedge et al., 2013]. These indicates a possible localization into the nucleus. Supportively, immunofluorescence staining showed that DEK colocalizes with nuclear DNA [Kappes et al., 2001a]. On the DNA, DEK preferentially binds to euchromatin, and even near the transcription start site [Sandén et al., 2014]. DNase I treatment affects the localization of DEK, however RNase A treatment cause a minimal effect on the localization of DEK [Kappes et al., 2001]. Nevertheless, DEK can colocalize with splicing-associated SR-protein NM4, which is also a marker of interchromatin

granule clusters (IGCs). IGCs are proposed to splicing protein reserve areas (Alberts et al., 2015). DEK is known to be acetylated by p300/CBP-associated factor (P/CAF), and this modification leads DEK to localize in the IGCs [Cleary et al., 2005]. The reason behind this relocation of DEK to IGCs may arise from interfering its DNA-binding ability with acetylation [Cleary et al., 2005]. In addition to acetylation, phosphorylation by CK2 also interferes with DNA-binding ability of DEK and lead DEK dissociation from the chromatin [Kappes et al., 2004]. Another localization of DEK is with PML body. It has been shown that DEK has SUMO motif which is found proteins associated with PML body and that DEK and PML protein co-localize [Ivanauskiene et al., 2014]. In addition to its nuclear localization, recently it is found that DEK is associated with kinesin family member 15 (KIF15), and this interaction lead DEK to localize cytoplasm where it is deubiquitylated by ubiquitin specific protease 15 (USP15) in leiomyosarcoma cells [Ge et al., 2021].

Beyond the nuclear and cytoplasmic localization, nucleolar localization of DEK is not well understood. DEK protein has two NoLS located in the RBD [Scott et al., 2011]. High throughput nucleolar H1 interactome analysis showed that DEK is involved in the nucleolar histone H1 interactome, indicating DEK has a possible nucleolar role and localization [Szerlong et al., 2015]. Moreover, under heat stress DEK is not associated with NPM1, however during recovery from heat stress in HSP70 inhibited cells, DEK interacts with NPM1 [Frottin et al., 2019]. These data are found in the supplementary materials, so there is no direct investigation on nucleolar localization of DEK.

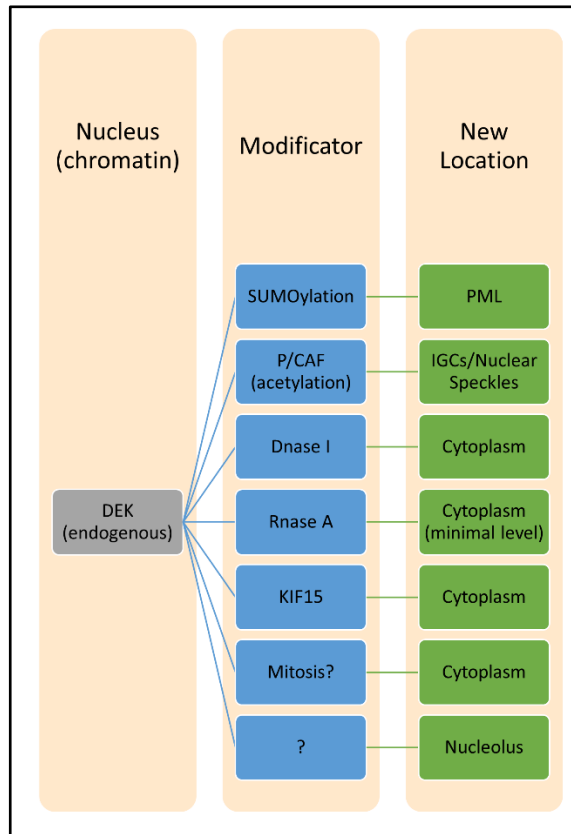


Figure 4.4: The scheme of the localization of DEK.

4.4. Mitotic Distribution of DEK

DEK is known to be associated with interphase chromosome [Kappes et al., 2001]. In the same study, immunofluorescence staining with polyclonal DEK antibodies showed that DEK remained on the DNA of HeLa cells throughout the cell cycle. However, a recent study with Near-diploid immortalized keratinocytes (NIKS) and mouse embryonic fibroblast (MEF) cells showed contrary results [Matrka et al., 2015]. Endogenous DEK in NIKS cells localize in the nucleus during the interphase but dissociates from the DNA at the beginning of the mitosis and re-associates with the DNA at telophase. DEK knockout MEF cells were transduced with either N-terminally or C-terminally GFP-tagged DEK, and thus, GFP-tagged DEK versions were overexpressed. N-terminal GFP-tag let DEK behave like endogenous DEK in NIKS. On the other hand, C-terminally GFP-tagged DEK behave like DEK in HeLa cells: It remained bound to the DNA during all cell cycle steps. The rationale behind the diverse localization behavior may arise from the DEK regulation in different cell types. DEK is somehow removed from chromatin while the cell enters the mitosis.

Supportively, the degradation mechanism of DEK is shown in LMS cell lines, and it has been known that phosphorylation is a major modification used in cell while entering the mitosis [Ge et al., 2021], [Wang et al., 2018]. Interestingly, when DEK-GFP expressing MEF cells sorted according to DEK expression level as high or low, DEK localization was outside the DNA in low DEK expressing cells and correlates with the DNA in high DEK expressing cells, indicating that colocalization of DEK with the DNA was affected by the amount of the DEK in the cell [Matrka et al., 2015]. It is thought that DEK is not removed from the DNA properly when it is overexpressed.

5. MATERIALS

5.1. Chemicals and Kits

The chemicals that were used in this study were listed in the Table 5.1, and the kits were listed in the Table 5.2.

Table 5.1: Chemicals used in this thesis.

Chemicals	Catalogue Number	Company
10X TBS	1706435	Biorad
2-Propanol	961.023.2500	Isolab
Agar Bios Special LL	4110302	Biolife
Bolt MES Buffer (20X)	B0002	Novex
Bolt Transfer Buffer (20X)	BT0006	Novex
BSA	A2153-10G	Sigma-Aldrich
DMEM	41966029	Gibco
DMSO	BP231-1	Fisher Scientific
DPBS	14190144	Gibco
Ethanol (absolute)	32221-2.5L	Sigma-Aldrich
FBS	26140-111	Gibco
Kanamycin	K4000-1G	Sigma-Aldrich
Methanol	1060092511	Merck
NaCl	7653-250G	Sigma-Aldrich
NaCl	1064041000	Merck
Nonidet P40	A1694,0250	AppliChem
Penicillin/Streptomycin	151401	Gibco
Peptone Bacteriological	4122592	Biolife
RefSafe	21141	Intron
RPMI 1640	22400-089	Gibco
Sodium Deoxycholate	D7650-10G	Sigma-Aldrich
Sodium Dodecyl Sulphate	L3771-100G	Sigma-Aldrich
Trisma Base	T1503-1KG	Sigma-Aldrich
Triton X-100	T8787-100ml	Sigma-Aldrich
Vectashield Mounting Medium	H-1200	Vector Labs
Yeast Extract	41222202	Biolife

Table 5.2: Kits used in this thesis.

Kits	Cataloge Number	Company
Nucleobond Xtra Midi EF	740420.10	MN
PCR Clean Up	740609.50	MN
jetPRIME	114-07	Polyplus
ProFection Transfection Kit	E1200	Promega
Pierce BCA Protein Assay	23227	Thermo Scientific
NucleoSpin Plasmid	740588	MN

5.2. Equipments

The equipments used in this study were listed in the Table 5.3.

Table 5.3: Equipments used in this thesis.

Equipment	Cataloge Number	Company
Azure Imager	C150	Thermo Scientific
Bolt™ Bis-Tris Welcome Pack	NW0010A	Invitrogen
Centrifuge	905607	Hitachi
Chemi Doc XRS+	721BR04545	Biorad
Confocal Microscope	LSM880	Zeiss
Heatblock	BSH1002E	Benchmark
Incubator	HF70	Heal Force
Inverted Light Microscope	Eclipse TS100	Nikon
Micropipettes	F167300	Gilson
Minicentrifuge	603.01.001	Isolab
Multi-rotator	RS-60	Biosan
NanoDrop	EC 1000 XL	Thermo Scientific
Shaker	12101150	Labnet
TProfessional TRIO Thermocycler	846-070-720	Biometra
Varioskan Plate Reader	3001-1571	Thermo Scientific
Vortex	444-1372	VWR International

5.3. Oligonucleotides

The oligonucleotides used in this study were listed in the Table 5.4.

Table 5.4: Oligonucleotides used in this thesis.

Oligo Name	Sequence
FD_mCh_DEK_F	GTAGCTAGCATGGACTACAAAGACGATGACGACAAGTC C
R-EcoRI- DEK2fuzGFP	TGCTAAGAATTCAGAAATTAGCTCTTTTACAG
XhoISiteAddedDEK RevForFusion	TAAGCACTCGAGAGAAATTAGCTCTTTTACAG
XhoISiteAddedHATa gForPrimers	TAAGCACTCGAGATGTACCCATACGATGTTCCAGATTA CGCTTCCGCCTCGGCCCTGCTG
DEKiso2R	GGAAAGCCACTGAACTGACC
MSCV_seq_F1	GAAAACCGATGAACTTAGAAATC
MSCV_seq_F2	CAGATGAATCTAGTAGTGATG
CMV_F	CGCAAATGGGCGGTAGGCGTG
EGFP_NR	CGTCGCCGTCCAGCTC

5.4. Antibodies

The antibodies used in this thesis was listed in the Table 5.5.

Table 5.5: Antibodies used in this thesis.

Antibody	Catalog Number	Company	WB	IF
Mouse anti-DEK	610948	BD	1:1000	-
Mouse anti-Myc (9B11)	2276S	CST	-	1:400
Mouse anti-NPM1 (FC-61991)	35-5200	Invitrogen	-	1:400
Rabbit anti-Alpha-Tubulin (11H10)	2125S	CST	-	1:100
Rabbit anti-Beta-tubulin (9F3)	2128P	CST	-	1:200
Mouse anti-Alpha-Tubulin (B7)	sc-5286	Santa Cruz	-	1:200
Horse anti-mouse HRP-linked IgG	7076S	CST	1:3000	-
Goat anti-mouse Alexa Fluor 555	4409S	CST	-	1:500
Goat anti-mouse Alexa Fluor 594	8890S	CST	-	1:500
Goat anti-rabbit Alexa Fluor 594	8889S	CST	-	1:500
Goat anti-mouse Alexa Fluor 647	4410S	CST	-	1:500

5.5. Nucleic Acid Solutions

5.5.1. Agarose Gel

1% agarose gel were prepared as 40 ml for small (8 well) tray (100 ml for big (15 well) tray). First, 0,4 g agarose were weighed (1 g for 100 ml), and 40 ml 1X TAE was added. It was warmed in microwave oven at 600 W for 2 minutes and 30 seconds. It was important that the solution was mixed time to time by hand during warming (outside the microwave, the lid of the bottle was closed). When the solid agarose dissolved, it was cooled down under 55°C. While it was cooling, an appropriate gel comb was placed onto the tray, and the open sides of the tray was closed by using the tray platform. All equipments were sterilized by 70% ethanol. After that 2 µl of RedSafe (Intron, 21141) nucleic acid dye was added to solution, the bottle was swirled, and the agarose solution was poured to tray. When the gel was solidified, the gel with the comb was placed into the agarose gel tank which was full of 1X TAE buffer till the max point. After the upside of the gel was covered with the buffer, the comb was removed gently.

5.6. Microbiology Solutions

5.6.1. Luria-Bertani (LB) media

Bacterial cells were grown by using LB media. 10 g of peptone, 10 g of NaCl, and 5 g of yeast extract were weighed and put into a 1 L glass measure. 700 ml of distilled water was added, and the powder was dissolved by using magnetic mixer. When it was dissolved, the volume was completed to 1 L with distilled water. Then, it was poured into a heat-proof glass bottle and autoclaved at 121 °C for 20 minutes. It was stored at +4°C and used at room temperature. Prior to use the necessary antibiotics (for example kanamycin) was added.

5.6.2. Luria-Bertani agar (LB agar) media

15 g Bacto Agar was weighed and dissolved in LB medium. Then, it was autoclaved at 121°C for 20 minutes. When it was cooled down under 55°C, desired antibiotic was added, and it was poured onto petri dishes.

5.6.3. 80% Glycerol Solution

80 ml of sterile glycerol was completed with 20 ml of ddH₂O to generate 80% glycerol. The mixture was sterilized by autoclaving.

5.6.4. 0.5 M PIPES

0,755 g PIPES was weighed and dissolved in 3 ml autoclaved water. The color of the solution was opaque white. pH of the solution was adjusted to 6,7 by adding 5 M KOH. The color of the solution turned into transparent. The volume of the solution was completed to 5 ml by adding autoclaved water. Then, the solution was filtered by 0.22 µm filter and stored at +4°C.

5.6.5. Inoue Buffer

0,544 g MnCl₂, 0,11 g CaCl₂, and 0,934 g KCl was weighed and put into a 50 ml tube. Then, the chemical powder mix was dissolved in 15 ml autoclaved water by vortexing. 1 ml 0,5 M PIPES was added to the solution. The volume of the solution was completed to 50 ml by adding autoclaved water. Finally, the solution was filtered by 0.22 µm filter and stored at +4°C.

5.6.6. Antibiotics

Kanamycin stock solution was prepared as 50 mg/ml concentration. 0,05 g Kanamycin powder was weighed, dissolved in 1 ml ddH₂O, and filtered by using 0.22 µm filter. Working concentration was 50 µg/ml, therefore 1 µl of stock kanamycin solution was added to 1 ml media.

5.7. Cell Culture Solutions

5.7.1. Completed Cell Culture Media

50 ml of fetal bovine serum (FBS) (Gibco, 26140-111) and 5 ml of penicillin/streptomycin (P/S) (Gibco, 151401) were added to 500 ml of culture media (either DMEM (Gibco, 41966029) or RPMI 1640 (22400-089)).

5.7.2. Serum Starvation (SS) Media

2 µl of FBS was added to 1 ml of serum-free 1% P/S containing cell culture media. Final FBS percentage was 0,2%.

5.8. Immunofluorescence Solutions

5.8.1. 2% BSA Solution

0,1 g of BSA was weighed and dissolved in 5 ml of 1X DPBS by vortexing well.

5.8.2. 1% BSA Solution

1 ml of 2% BSA solution was diluted in 1 ml of 1X DPBS and vortexed well.

5.8.3. 0.5% PBS-T Solution

10 µl of Triton X-100 was diluted in 2 ml of 1X DPBS, then vortexed well. It was important to cut off the very end of the pipette tip.

5.9. Western Blot Solutions

5.9.1. 1 M NaCl

2,922 g NaCl (Sigma, 7653-250G) was weighed and dissolved in 45 ml autoclaved water. When NaCl was totally dissolved, the volume was completed to 50 ml.

5.9.2. 10% Sodium Deoxycholate (SDC)

1 g SDC (Sigma, D7650-10G) was weighed and dissolved in 8 ml autoclaved water. When SDC was totally dissolved, the volume was completed to 10 ml.

5.9.3. 10% Sodium Dodecyl Sulphate (SDS)

5 g SDS (Sigma, L3771-100G) was weighed and dissolved in 40 ml autoclaved water by heating. When SDS was totally dissolved, the volume was completed to 50 ml.

5.9.4. 1 M Trisma Base (Tris)

6,057 g Trisma base (Sigma, T1503-1KG) was weighed and dissolved in 40 ml autoclaved water. pH of Tris was adjusted to 8.0 (± 0.1) by adding either 37% HCl or 10 M NaOH. The volume was completed to 50 ml.

5.9.5. RIPA Lysis Buffer

7,3 ml autoclaved water, 1,5 ml 1 M NaCl, 0,5 ml %10 SDC, 0,1 ml NP-40/Nonidet P-40 (AppliChem, cat#A1694,0250), 0,1 ml %10 SDS, and 0,5 ml 1 M Tris (pH 8.0) was mixed in a 15 ml tube and then vortexed kindly. Prior to use, 100 μ l of protease/phosphatase inhibitor solution was added to the lysis buffer.

5.9.6. 1X MES SDS Running Buffer

20 ml of 20 X MES SDS running buffer was diluted in 380 ml of autoclaved water.

5.9.7. 1X Transfer Buffer

20 ml of 20 X Bolt transfer buffer was diluted in 380 ml of autoclaved water.

6. METHODS

6.1. Cloning of DEK1 and DEK2

6.1.1. Primer Design

DEK protein has two isoforms named DEK1 and DEK2. Primers that recognize the DEK1 isoform also recognize the DEK2 isoform due to sharing the same sequence at the N-terminal and C-terminal. The primers were designed to subclone either DEK1 or DEK2 coding sequence from MSCV-IRES-GFP-DEK1 (MIG-DEK1) or MSCV-IRES-GFP-DEK2 (MIG-DEK2) plasmids, which had been cloned in a previous work in the lab. We used pEGFP-N2 or mCherry2-N1 plasmid in an attempt to generate C-terminally EGFP or mCherry2 tagged DEK isoforms (Figure 6.1). The sequences of DEK isoform transcripts were obtained from NCBI Nucleotide in FASTA format. Forward primers were designed with 3-4 bp random sequence for restriction enzyme settling, a restriction enzyme recognition site, an HA or Flag tag sequence, and a sufficient number of N-terminal DEK nucleotides, respectively. Reverse primers were planned to include enzyme settling site and cutting site, and C-terminal DEK sequence without a stop code (table 1). After being designed, primers were analyzed to adjust T_m and to check hairpin and self-dimer possibilities. Finally, designed primers were synthesized by SenteBioLab (Ankara, Turkey) (Table 5.4).

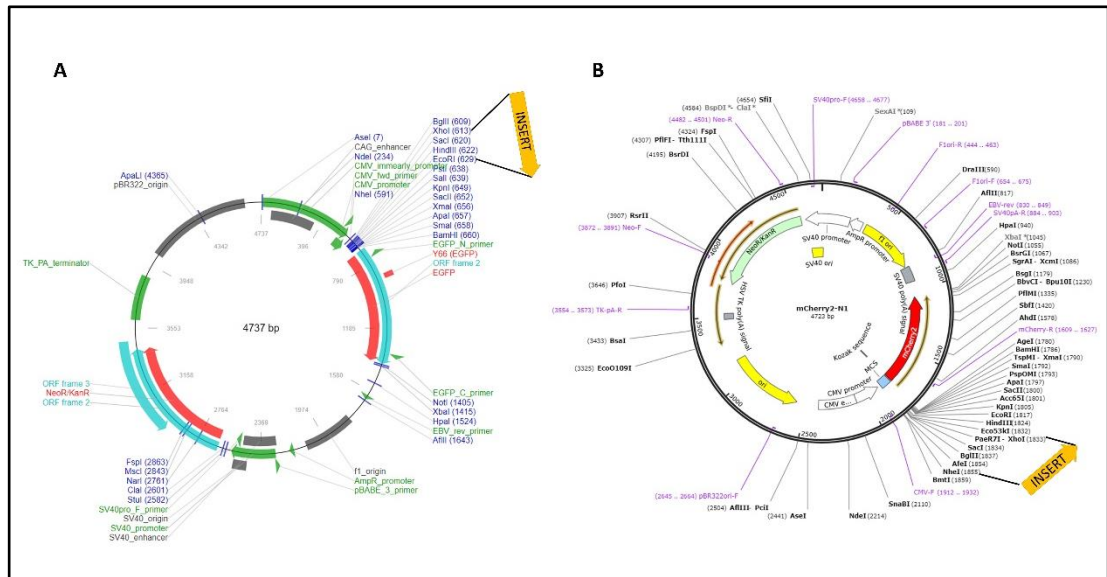


Figure 6.1: Plasmid maps of pEGFP-N2 (A) and mCherry2-N1 (B) plasmids. Black lines indicate restriction points, “INSERT” labeled yellow arrows indicate inserted sequences and their reading direction.

6.1.2. Polymerase Chain Reaction (PCR)

Polymerase chain reactions were conducted to add restriction sites to the ends of DEK1 and DEK2 coding sequences. Primers were aliquoted as 10 μ M solutions when first delivered. PCR ingredients are listed in Table 4.1 and Table 4.2, and reaction conditions are listed in Table 4.3. First, PCR ingredients except polymerase were added to a thin-walled PCR tube by the order given in Table 4.1. To generate HA-tagged DEK coding sequence, XhoISiteAddedHATagForPrimers named forward primer and R-EcoRI-DEK2fuzGFP named reverse primer (cite the Table showing the name and seq of this primers) were used. To generate Flag-tagged DEK coding sequence, FD_mCh_DEK_F named forward primer and XhoISiteAddedDEKRevForFusion named reverse primer was used (cite the Table showing the name and seq of this primers). Solution was mixed by flicking several times and spined down. DNA polymerase was added into the solution, mixed by pipetting, and spined down. When the mix was ready, the tubes was placed into PCR machine (TProfessional TRIO Thermocycler, Biometra, 846-070-720) and the reaction was performed with the conditions indicated in the Table 4.2. In the final step, 15 μ l of PCR reaction solution was mixed with 6x loading dye and loaded onto %1 agarose gel to control whether the reaction worked or not.

Table 6.1: PCR ingredients for DEK1GFP and DEK2GFP cloning.

Samples Ingredients	Non-Template Control	DEK1 (for DEK1GFP)	DEK2 (for DEK2GFP)
ddH ₂ O	41 µl	40 µl	40 µl
10X Pfu Buffer	5 µl	5 µl	5 µl
dNTPs (10 mM each)	1 µl	1 µl	1 µl
Forward Primer	1 µl	1 µl (XhoISiteAddedHATagforPrimers)	1 µl (XhoISiteAddedHATagforPrimers)
Reverse Primer	1 µl	1 µl (R-EcoRI-DEKfuzGFP)	1 µl (R-EcoRI-DEKfuzGFP)
Template DNA (20 µg)	-	1 µl (MIGR1 FD1)	1 µl (MIGR1 FD2)
PfuUltra HF	1 µl	1 µl	1 µl
Total	50 µl	50 µl	50 µl

Table 6.2: PCR ingredients for DEK2mCherry cloning.

Samples Ingredients	Non-Template Control	DEK2 (for DEK2mCherry)
ddH ₂ O	20 µl	80 µl
10X Pfu Buffer	2,5 µl	10 µl
dNTPs (10 mM each)	0,5 µl	2 µl
Forward Primer	0,5 µl	2 µl (FD2_mCh_DEK_F)
Reverse Primer	0,5 µl	2 µl (XhoISiteAddedDEKRevforFusion)
Template DNA (10 µg)	-	2 µl (MIGR1 FD2)
PfuUltra HF	0,5 µl	2 µl
Total	25 µl	100 µl

Table 6.3: PCR conditions.

	Temperature	Duration	Cycle
Main Denaturation	95°C	2 minutes	x 1
Denaturation	95°C	1 minutes	x 30
Annealing	44°C	1 minutes	
Extension	72°C	2 minutes	
Final Extension	72°C	10 minutes	x 1
Store	+4°C	∞	x 1

6.1.3. PCR Clean-Up

PCR amplified DEK cDNAs were cleaned up to be ready for cloning. MN NucleoSpin Gel and PCR Clean-up kit was used. First PCR products were mixed with NTI buffer with a 1:2 ratio. This mix was loaded into a clean-up colon, and the colon was placed into a collection tube. After centrifugation for 1 minute at 11000g, 700 µl NT3 buffer loaded into the colon and centrifuged for 1 minute at 11000g, twice. The collection was poured when it was full. To dry out the colon, it was centrifuged for 1 minute at 11000g as empty. After that, the colon was placed into a new 1,5 ml microcentrifuge tube, 30 µl Elution Buffer NE was added into the colon, incubated for 2 minutes at room temperature, and centrifuged for 2 minutes at 11000g. The concentration of the collected samples was measured with NanoDrop.

6.1.4. Restriction Digestion

Cleaned-up PCR products and the plasmid that genes will be cloned were digested before ligation by appropriate restriction digestion enzymes. The reaction mix was prepared as on the Table 4.1 for DEK1GFP and DEK2GFP cloning, and Table 4.2. for DEK2mCherry cloning. Restriction enzymes were added into the mix with the ratio of the enzyme (20 units/µl): DNA (µg) as 1:1. The reaction was performed at 37°C for 1 hour for PCR products and 1.5 hours for the plasmid. The reaction was stopped by adding 6x loading dye. The restricted samples were run in 1% agarose gel at 80 V for 1 hour.

Table 6.4: Restriction Digestion Mix Ingredients for DEK1GFP and DEK2GFP.

Mix	Vector	PCR Product	
Ingredients	pEGFP-N2	DEK1	DEK2
ddH ₂ O	78,5 µl	1,8 µl	3 µl
10X CutSmart Buffer	10 µl	5 µl	4 µl
XhoI	5 µl	1,6 µl	2 µl
EcoRI-HF	5 µl	1,6 µl	2 µl
DNA	(5 µg) 1,5 µl	(1,6 µg) 40 µl	(2 µg) 29 µl
Total	50 µl	50 µl	40 µl

Table 6.5: Restriction Digestion Mix Ingredients for DEK2mCherry.

	mCherry2-N1	PCR Product (DEK2)
ddH ₂ O	65,5 µl	3 µl
10X CutSmart Buffer	10 µl	4 µl
NheI-HF	5 µl	2 µl
XhoI	5 µl	2 µl
DNA	(5 µg) 14,5 µl	(2 µg) 29 µl
Total	100 µl	40 µl

6.1.5. Agarose Gel Clean-Up

When the run of restricted DNA was completed, the DNA having desired length was cut off and extracted from agarose gel. First, an 1,5 ml microcentrifuge tube was weighed. Second, the UV table was covered with stretch film, and the gel was placed onto the prepared UV table. Third, the UV lamp was turned on, the DNA bands were located and cut by the blunt end of a scalpel. Then the UV lamp was turned off, and the gel was placed into the microcentrifuge tube. The tube including the gel was weighed, and the weight of the gel was calculated by determining the tare of the tube. NTI buffer was added with the volume twice the weight of the gel into the tube. The tube was incubated at 50°C until the gel melted down and vortexed once in a while. The rest of the protocol was performed as in PCR Clean-Up.

6.1.6. Ligation

Restricted and cleaned-up vector and PCR products were ligated in the presence of T4 ligase. The reaction mix preparation order is given on the Table 4.6 for DEK1GFP cloning, Table 4.7 for DEK2GFP cloning, and Table 4.8. for DEK2mCherry cloning. Insert: Vector ratio was calculated by using NEBioCalculator. 150 ng of vector was ligated to inserts (DEK1 and DEK2 PCR products). The reaction mixes were prepared in a 1,5 ml microcentrifuge tube, mixed by flicking, and spined down. Centrifuge machine temperature was adjusted to 16°C and the reaction tubes were placed in the machine where they were incubated overnight not centrifuged.

Table 6.6: Ligation Mix Ingredients for DEK1GFP.

	Self Ligation	5:1	3:1
ddH ₂ O	14,7 µl	17,4 µl	11 µl
10X T4 Buffer	2 µl	2 µl	2 µl
pEGFP-N2	2,3 µl	2,3 µl	2,3 µl
DEK1	-	2,3 µl	3,7 µl
T4 Ligase	1 µl	1 µl	1 µl
Total	20 µl	20 µl	20 µl

Table 6.7: Ligation Mix Ingredients for DEK2GFP.

	Self Ligation	5:1	3:1
ddH ₂ O	14,7 µl	-	4,2 µl
10X T4 Buffer	2 µl	2 µl	2 µl
pEGFP-N2	2,3 µl	2,3 µl	2,3 µl
DEK2	-	14,7 µl	10,5 µl
T4 Ligase	1 µl	1 µl	1 µl
Total	20 µl	20 µl	20 µl

Table 6.8: Ligation Mix Ingredients for DEK2mCherry.

	Self Ligation	5:1	3:1
ddH ₂ O	16 µl	11,5 µl	13,3 µl
10X T4 Buffer	2 µl	2 µl	2 µl
mCherry2-N1	1 µl	1 µl	1 µl
DEK2	-	4,5 µl	2,7 µl
T4 Ligase	1 µl	1 µl	1 µl
Total	20 µl	20 µl	20 µl

6.1.7. Ultra-Competent Bacterial Cell Preparation

50 µl thawed incompetent DH5α cells were added into a 3 ml LB medium in a 15 ml tube. Cells were incubated for 16 hours at 37°C with 200 rpm shaking. 2 ml of this culture were added into 125 ml LB medium in a 250 ml Erlenmeyer flask. Cells were incubated at 37°C with shaking until OD600 measurement reached the value of approximately 0.55. The OD600 value was obtained by measuring 1 ml of culture at 600 nm wavelength once per hour. Bacterial culture was split into 50 ml tubes when OD600 measurement was accomplished. After splitting, cells were incubated for 10

minutes on ice. Incubated cells were centrifuged for 13 minutes at +4°C and 4000 rpm. The supernatant was removed, and each pellet was dispersed in 16 ml Inoue buffer by using an electronic pipettor. The two tubes of bacterial cells in the buffer were gathered together. Cells were centrifuged for 10 minutes at +4°C and 4000 rpm, and the supernatant was removed. The pellet was first unfasted by flicking several times and was resolved in 8 ml Inoue buffer by using a pipettor. After that, 600 µl DMSO was added into the bacterial solution. 400 µl of the final competent bacterial solution was added into each readily prepared 1,5 ml microcentrifuge tubes. The tubes were quickly frozen in liquid nitrogen and stored at -80°C.

6.1.8. Transformation

DH5α cells were transformed with DEK isoform cloned vectors to amplify the plasmids. First, 30 ng of plasmid was put into a 1,5 ml tube, and 100 µl of competent DH5α cells were added to the tube. One tube of 100 µl competent bacteria without plasmids was used as the negative control. The cells were incubated on ice for 30 minutes. After incubation, cells were incubated at 42°C for 2 minutes and then on ice for 3 minutes. This process allows the pores on the cell surface to open and let the plasmids inside of the cell. 800 µl of LB media without antibiotics was added to the tubes, and the cells were incubated at 37°C for 1 hour with shaking. After incubation at 37°C, the tubes were centrifuged at 10000 rpm for 3 minutes. The supernatant was removed, but not totally, and the pellet was resolved with the remaining supernatant and spread on kanamycin containing LB agar.

6.1.9. Colony Selection and Plasmid DNA Isolation

The colonies on the agar plates were counted and the biggest five colonies were selected. The selected colonies were taken with a disposable loop and inoculated into kanamycin containing 5 ml LB media. The cells were incubated at 37°C overnight prior to plasmid isolation.

The next day, plasmids were extracted from cells by using NucleoSpin Plasmid kit (MN, 740588). The tubes were centrifuged at 4100 rpm +4°C for 20 minutes. Supernatant was discarded and the pellet was resolved in 250 µl A1 (resolving) buffer

with pipetting. The solution was put into a new 1,5 ml microcentrifuge tube. 250 µl of A2 (lysis) buffer was added to each tube and the tubes were mixed by turning upside down 8 times. The tubes were incubated at room temperature for 5 minutes and centrifuged at 11000 g for 5 minutes post-incubation. The supernatant was loaded into colons, and it was important not to touch the pellet. The colons were centrifuged at 11000 g for 1 minute. 500 µl of Buffer AW was added to each colon and the colons were centrifuged at 11000 g for 1 minute. The collection tubes under the colons were emptied whenever they were full. 600 µl of Buffer A4 was added to each colon and the colons were centrifuged for 1 minute. The colons were dried by empty centrifugation at 11000g for 2 minutes. The colons were loaded into new 1,5 ml tubes which were the collection and storage tubes of eluted DNA. 50 µl of Elution Buffer AE was added to each colon and incubated at room temperature for 2 minutes. The tubes were centrifuged at 11000 g for 2 minutes post-incubation, and the DNA was eluted into the microcentrifuge tubes. The concentration of the eluted DNA was measured by using NanoDrop. The tubes were stored at -20°C.

6.1.10. Endotoxin-Free Plasmid DNA Isolation

Plasmids were isolated from transformed cells by using NucleoBond Xtra-EF (MN, 740420.10) plasmid DNA extraction kit. 200 ml bacterial culture which was inoculated in the previous evening, was loaded into 4 x 50 ml tubes and centrifuged at 4100 rpm +4°C for 20 minutes. Supernatant was discarded and pellet was resolved in 8 ml (2 ml for each tube) RES-EF buffer by pipetting and vortex. The resolved pellet solutions were collected in one tube. 8 ml Lysis buffer was added, and the tube was mixed by turning upside down 5 times and incubated at room temperature for 5 minutes. During incubation, a colon with plastic washers were placed into a 50 ml tube, and a colon filter was placed into the colons and equilibrated with 15 ml EQU-EF buffer. It was important that the EQU-EF buffer was loaded through the wall of the colon filter. The lysate tube was placed onto ice and neutralized with 8 ml NEU-EF buffer. The tube was mixed by turning upside down until the color of the solution turned from blue into white. After that the solution was incubated on ice for 5 minutes. The lysate was loaded into the colon through the wall of the colon filter post-incubation. The lysate was filtered by gravity. After filtration, 5 ml FIL-EF buffer was

loaded through the wall of the colon filter. After the buffer run out, the filter was removed, and 35 ml of ENDO-EF buffer was loaded into the colon. The leaked buffer in the 50 ml tube was removed whenever it reached 30 ml. After the buffer run out, 15 ml of WASH-EF buffer was loaded into the colon. After washing step, the colon was placed into a 15 ml tube, and 5 ml ELU-EF was added into the colon. After elution, the colon was discarded, 3,5 ml of isopropanol was added into the eluate and mixed with vortex. The tube was centrifuged at 4100 rpm for 30 minutes. Supernatant was removed by using a pipette. The pellet was resolved with 2 ml of 70% ethanol (70% EtOH in the kit) and centrifuged at 4100 rpm for 15 minutes. Ethanol was removed by using a pipette. The cap of the tubes was opened, and the pellet was let to dry. Dried pellet was resolved in 400 µl of TE-EF buffer and was mixed on the rotator for 10 minutes. The tube was incubated for fully dissolution of the DNA overnight at +4°C, and the next morning the solution was placed into 1,5 tube and stored at -20°C.

6.1.11. Bacterial Cell Stock

Bacterial cells were collected from agar plates by using a disposable loop (Isolab) and inoculated in 5 ml LB medium. The culture was incubated at 37°C overnight with shaking. The next morning, 625 µl of the culture was mixed with 375 µl of sterile 80% glycerol in a cryotube and stored at -80°C.

6.1.12. Sequencing

After insert-vector ligation and DNA isolation, the plasmids were sequenced by IONTEK (Kocaeli, Turkey). Samples were prepared at 100 ng/µl concentration and 100 µl of volume. Sequencing results were controlled by using FinchTV application and ClustalW.

6.2. Cell Culture

6.2.1. Cell Thawing

The cells stored at -196°C in liquid nitrogen tanks were first thawed at room temperature until the ice became half-melted. The cell solution was added into 12 ml completed cell media (DMEM or RPMI 1640 according to cell type, including 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S)) and mixed by pipetting. The solution was centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded, and the pellet was dissolved first by flicking and then by adding 12 ml of completed media. After pipetting several times, the cell-media solution was added into a 75 cm² flask and distributed evenly by rocking the flask back and forth and from side to side. The cells were incubated in a humid incubator at 37°C and 5% CO₂.

6.2.2. Cell Passaging

Confluent cells (above 70%) were passaged into new flasks to maintain their proliferation. First, the old media was discarded by vacuum, and the cells were washed with 2,5 ml 1X DPBS. The DPBS was discarded, and 1 ml 0,05% trypsin was added onto the cells. The flask was incubated at 37°C for 2 minutes until trypsin removed the extracellular attachment proteins, and then, completed media with a 1:8 (trypsin/media, v/v) ratio was added, and the cells were collected in a 15 ml tube by washing. The tube was centrifuged at 1200 rpm for 5 minutes, then the supernatant was discarded, and the pellet was solved first by flicking and later by adding the appropriate amount of completed media. A new flask was loaded with 12 ml completed media, and the cells were seeded with a ratio according to the number of days they wanted to remain in culture. The flask was placed into the incubator and incubated at 37°C 5% CO₂.

6.2.3. Cell Freezing

The cells were frozen in freezing medium that was composed of 10% DMSO and 90% FBS. After collecting and centrifugation, the pellet of cells was dissolved in 1 ml freezing medium and added into a cryotube, and the tube was placed into -80°C

in a Mr. Frosty (Nalgene) box. The day after, the cells were placed into the liquid nitrogen tank.

6.2.4. Poly-L-Lysine Coating

Prior to immunofluorescence assays, for the cells that tend to detach when solutions applied are seeded onto coverslips, slides were coated with poly-L-lysine which is polymeric form of positively charged amino acid lysine. First of all, 200 μ l of poly-L-lysine with 100 μ g/ml working concentration was prepared from 1 mg/ml poly-L-lysine stock (Sigma, P5899) by diluting in ddH₂O. The coverslips (22 mm x 22 mm) were sterilized by soaking in 70% ethanol and flame and then placed into 6-well-plate wells. 200 μ l of 100 μ g/ml poly-L-lysine was added onto each coverslip and incubated at room temperature for 15 minutes. After incubation, poly lysine solutions were collected back into a 1,5 ml tube for reusing and stored at +4°C. Coverslips were rinsed with 500 μ l 1X DPBS two times and with 500 μ l completed medium once. The liquid on coverslips was discarded and the plate was dried overnight under UV light by leaning 45° angle.

6.2.5. Transfection

HEK293T or HeLa cells were transfected by using ProFection calcium-phosphate kit (Polyplus, E1200) or jetPRIME transfection reagent following the manufacturers instructions (Polyplus, 114-07), respectively.

6.2.5.1. Calcium-Phosphate (CaPO₄)

Calcium-phosphate precipitation is a transfection method based on the precipitation of negatively charged phosphate backbone of the DNA by positively charged calcium ions derived from CaCl₂. In this method, 2x10⁵ HEK293T cells were seeded on coverslips in 6-well-plate for imaging, or 2x10⁶ HEK293T cells were seeded on a 10 cm plate. The day after, at least 3 hours prior to transfection, cell media was renewed with completed media. 1 hour before the transfection, transfection solutions were begun to prepare. For each DNA sample, two polystyrene falcon tubes were used.

2X Hepes Buffered Saline (HBS) was added to one tube (200 μ l for 6-well-plate and 500 μ l for 10 cm plate). In the other tube, DNA- CaCl_2 solution was prepared according to the order in the Table 4.9. After DNA and CaCl_2 addition, the tube was mixed by vortex and spined down. While gently vortex the HBS tube, DNA- CaCl_2 solution was added dropwise into the center of the HBS tube. It was important to drop slowly and from as higher as it could be. The final opaque solution was incubated for 30 minutes at room temperature. After that, the solution was added onto the cells dropwise, and the cells incubated overnight at 37°C 5% CO_2 in a humid incubator.

18 hours after transfection the old media was discarded, and the cells were rinsed with 1X DPBS twice (1 ml for 6-well-plate, 5 ml for 10 cm plate). After DPBS discarded, fresh completed media was added onto the cells, and the cells incubated 24 - 48 hours post-transfection until the next assay.

Table 6.9: CaPO_4 transfection DNA solution ingredients.

	6-well-plate	10 cm plate
ddH₂O	89,7-X μ l	437,5-X μ l
DNA	X μ l (2 μ g)	X μ l (20 μ g)
CaCl₂	10,3 μ l	62,5 μ l
Total	200 μ l	500 μ l

6.2.5.2. jetPRIME

HeLa cells were transfected by using jetPRIME reagent. 2×10^5 cells were seeded into coverslip containing 6-well-plate the day before transfection. On the transfection day, 2 μ g of DNA was diluted in 200 μ l jetPRIME buffer, mixed by vortex and spined down. jetPRIME reagent was added with a 1:2 ratio into the solution, mixed by vortex and spined down. The solution was incubated at room temperature for 10 minutes. After incubation, the transfection solution was added onto the cells dropwise. 4 hours post-transfection, the old media was discarded, cells were rinsed with 1X DPBS once, and fresh completed media was added. 48 hours post-transfection, the cells were analyzed.

6.2.6. Serum Starvation

Increasing the number of mitotic cells was achieved by using serum starvation to synchronize cells in the G1 phase, restarting the cell cycle in the presence of serum, and monitoring when the cells enter the mitosis.

On day-0 of the serum starvation (SS) assay, 2×10^5 cells were seeded on coverslips in a 6-well-plate with completed cell media. Next day, the old cell media was discarded, and 2 ml of serum starvation media (SS media) composed of 0,02% FBS, 1% P/S, and 98,98% cell media was added to each well. On the second day, 18 hours later serum starvation, cell media was changed with completed (10% FBS containing) media. On the third day, 20-24 hours later serum recovery, the cells were fixed for the following analysis.

6.3. Immunofluorescence

6.3.1. Fixation

Prior to immunofluorescence staining, the cells were fixed by using 4% PFA. The old medium of the cells in a 6-well-plate was discarded, the cells were rinsed with 500 μ l 1X DPBS thrice for 5 minutes each on a shaker and fixed by using 500 μ l 4% paraformaldehyde (PFA). All solutions were added through the well wall gently. After addition of 4% PFA, the cells were incubated on the shaker at room temperature for 20 minutes. PFA solution was discarded, and the cells were rinsed with 500 μ l 1X DPBS on the shaker for 5 minutes, thrice. At this point, the cells were stored at +4°C in 1X DPBS until immunofluorescence staining or mounting only, or it was continued to stain.

6.3.2. Immunofluorescence Staining

Fixed cells were permeabilized with 500 μ l 0.5% Triton X-100 (prepared in 1X DPBS) for 20 minutes on a shaker. All steps in this section were performed at room temperature. Then the cells were rinsed with 500 μ l 1X DPBS once quick and once for 5 minutes on the shaker. The cells were incubated in 500 μ l blocking solution (2% BSA in 1X DPBS) for 1 hour on the shaker. After blocking step, the solution was

discarded, and 500 μ l of primary antibody solution was added, and the cells were incubated for 1 hour on the shaker. All antibody solutions were prepared in 1% BSA in 1X DPBS. After primary antibody staining, the cells were rinsed with 500 μ l 1X DPBS thrice (1 quick, 2 times for 5 minutes) on the shaker. After washing step, 500 μ l of fluorophore conjugated secondary antibody solution was added onto cells, and the cells were incubated for 1 hours in a humid chamber on the shaker. Final washing was practiced by rinsing the cells with 500 μ l of ddH₂O five times.

6.3.3. Mounting

Stained or only fixed cells were mounted by using 12 μ l of DAPI containing Vectashield Mounting Medium (Vector lab, H-1200). First, the coverslip was airdried by using a hair drier. The mounting medium was added onto a microscope slide cleaned with 70% ethanol, and the dried coverslip was placed onto the mounting liquid on the slide. When the medium was fully covered the coverslip surface, the sides of the coverslip were sealed with nail polish. The slide was stored at +4°C for a long time.

1.3.4 Fluorescence imaging

Images were captured by using Zeiss LSM 880 Airy Scan confocal microscope. 63x oil objective were used. Zoom was 1.0.

1.3.5 Cell counting

Image J software was recruited to count cells from images. To obtain total cell number, DAPI staining image were used. To obtain total GFP+ cell number, GFP images were used. First, images were opened on Image J, image types were changed to 8-bit (Image > Type > 8-bit). Then, black-white threshold was adjusted to determine the shape of nuclei (Image > Adjust > Threshold). The threshold was adjusted manually according to cell density and image quality, to determine approximately true number of cells. If generated nuclei pictures contained holes, they were filled (Process > Binary > Fill holes). To distinguish two adjacent nuclei from each other, watershed was applied (Process > Binary > Watershed). Finally, the generated nuclei pictures

were counted (Analyze > Analyze particles). On the analyze particles tool, the particle diameter was specified as 25 pixels. Circularity was kept between 0 and 1. Nuclei on edges were included. Results were displayed. After Image J counting, mitotic cells counted manually on the original images.

6.4. Western Blot

2×10^6 HEK293T cells were seeded on a 10 cm plate, and the next day the cells were transfected by using calcium-phosphate method. This protocol includes the assays 48 hours after the transfection. The steps of Western blot are explained below.

6.4.1. Cell Lysis

The old cell media was discarded, and the cells were washed with 5 ml 1X DPBS. 1 ml of 0.05% trypsin was added onto bare cells, and the cells were incubated at 37°C for 2 minutes. After incubation, 8 ml of completed DMEM was added, the cells were collected and centrifuged at 1200 rpm for 5 minutes. The supernatant was discarded, the pellet dissolved first by flicking and then by adding 5 ml ice-cold 1X DPBS. The cells were centrifuged at 1200 rpm +4°C for 5 minutes. The supernatant was removed, and the pellet was dissolved in 200 μ l of RIPA lysis buffer. The lysate was placed from the centrifuge tube into a 1,5 ml tube. For this purpose, the pointy end of the tip was cut by scissors to enlarge the hole. The lysate was incubated on ice for 30 minutes. After incubation, the lysate was sonicated for a total of 30 seconds (15 seconds sonication, 30 seconds wait, 15 seconds sonication again). Sonicated lysate was centrifuged at 13000 rpm +4°C for 10 minutes. During centrifugation, the protein content went out of the cells into the supernatant. Therefore, post-centrifugation, the supernatant (~200 μ l) was sucked up by using a pipette and put into a new 1,5 ml tube. The protein sample was stored as aliquots in -80°C. 6 μ l of protein was placed aside for the BCA assay.

6.4.2. BCA Assay

In this protocol, the Pierce BCA Protein Assay kit (Thermo Scientific, 23227) was used, and the manual of the manufacturer was followed. First of all, the samples that were placed aside as 6 μ l aliquots were diluted with RIPA buffer, and the volume was completed up to 60 μ l. Then, the number of wells (96-well plate) used was calculated for two replicates, including the BCA standards. 200 μ l of the reagent mix was added to each well. Therefore, by multiplying the number of wells by 200, total reagent mix amount was calculated, and reagent A and reagent B were mixed in a 50:1 ratio. After distributing the reagent, 25 μ l of protein (either BCA standard or sample protein) was added to the reagent containing wells. The plate was incubated at 37°C for 30 minutes on a shaker. Then, the plate was placed into the plate reader, shook for 15 seconds in the device, and the absorbance was read at 592 nm.

6.4.3. SDS-PAGE

Protein running and transfer were accomplished by using the Bolt™ Bis-Tris Welcome Pack (Thermofisher, NW0010A) system.

Sample(s) were prepared at 20 μ l of a volume containing 20 μ g of protein, and to avoid pipetting issues half sample was more prepared for each gel. The sample mix was shown in Table 4.10. After the mix was prepared, it was boiled at 70°C for 10 minutes. The sample was cooled down at room temperature prior to loading. The running tank was filled with 1X MES SDS running buffer up to 400 ml, the gel was placed, the comb was removed, and the wells were washed by using a syringe. Finally, 3 μ l of the protein ladder and 20 μ l of the sample(s) were loaded into the wells, and the proteins were run at 80V for 10 minutes first, and then, at 100V for at least 90 minutes, until the dye got out of the gel.

Table 6.10: SDS-PAGE mix ingredients for each sample.

Ingredients	Volume
ddH ₂ O	30-X μ l
Protein (20 μ g)	X μ l
10X Sample Reducing Agent	3 μ l
4X LDS Sample Buffer	7,5 μ l
Total	30 μ l

6.4.4. Transfer

The transfer was conducted by using the transfer equipment of the previously mentioned system. Firstly, general preparation was accomplished as follows. PVDF membrane was cut with an 8 cm width and a 6 cm height, activated in absolute methanol for 1 minute, and then placed into 1X transfer buffer. When the running was completed, the running buffer was loaded into a glass bottle to reuse and stored at +4°C. The gel cassette was rinsed with distilled water. The glasses of the gel cassette were separated by using a gel knife. The bare gel was rinsed with distilled water. The well and bottom parts of the gel were cut by the gel knife. A filter paper wet by 1X transfer buffer was put on the gel, the gel was turned upside down and was detached by the gel knife starting from a corner. When the gel was completely separated from the glass, it was put on a sponge wet by 1X transfer buffer. The activated membrane was placed onto the gel once without creating bubbles. Wet filter paper and sponge were placed onto the membrane, the last bubbles were removed by using the roll. The sandwich was placed into the transfer cassette in the order of cathode-sponge-filter paper-gel-membrane-filter paper-sponge-anode. The transfer was performed at 20V for 1 hour.

6.4.5. Immunoblotting

After the transfer was completed, the membrane was blotted by using mouse α -DEK antibody (BD, 610948) and HRP-conjugated anti-mouse antibody (CST, 7076S). The transfer buffer was loaded into a bottle and stored at +4°C for reusing. The sandwich was opened, and the membrane was held remaining towards the upside as the side that was faced with the gel. The up-left corner of the membrane was cut to mark the front face of the membrane. A 50 ml tube was filled with 5 ml TBS-T. The

membrane was placed into the tube as the front face of the membrane towards the inside of the tube by using forceps and washed for 5 minutes on the rotator. After that, the membrane was blocked in 5 ml 5% BSA solution at room temperature for 1 hour. Following the blocking, the membrane was blotted by using α -DEK antibody which was diluted in 3 ml 5% BSA with a 1:1000 ratio at +4°C overnight on rotator. Post-primer blotting the membrane was washed with 5 ml TBS-T for 5 minutes at room temperature on rotator thrice. Then, the membrane was incubated in 3 ml of HRP-conjugated anti-mouse antibody diluted in 1% BSA solution with 1:3000 ratio for 1 hour at room temperature on the rotator. After the incubation completed, the membrane was washed with 5 ml TBS-T for 5 minutes on rotator thrice. At this point, the membrane was stored at +4°C in 50 ml of TBS until imaging.

6.4.6. Imaging

The protein bands on the membrane were imaged by using 800 μ l of ECL (Advansta, WesternBright ECL, K-12045-D20). The mix was prepared as gathering 400 μ l WesternBright peroxide and 400 μ l WesternBright ECL. The mix was added onto the membrane and spreaded well. Then the ECL mix was discarded, the membrane was incubated for 2 minutes in dark at room temperature. The images were obtained every 0.5 seconds from 1 second to 30 seconds by using ChemiDoc (Thermo,).

7. RESULTS

7.1. DEK-GFP Fusion Constructions

DEK1GFP and DEK2GFP fusions were generated by cloning DEK1 and DEK2 cDNA into pEGFP-N2 vector. DEK2mCherry fusion was generated by cloning DEK2 cDNA into mCherry2-N1 vector.

As explained in method section, PCR was conducted to add tag sequences and restriction sites and to amplify DEK1 and DEK2 cDNAs. HA tag and XhoI-EcoRI restriction sites were added to DEK1 and DEK2 to introduce them into pEGFP-N2 plasmid. Flag tag and NheI-XhoI restriction sites were added to only DEK2 to clone it into mCherry2-N1 plasmid. The length of HA-DEK1 PCR product was 1176 bp, HA-DEK2 was 1074 bp, and Flag-DEK2 was 1068 bp. PCR products were run in 1% agarose gel and visualized by Azure (Name of company) at 305 nm. As expected, the bands were located between 1200 bp and 1000 bp ladder bands (Figure 7.1).

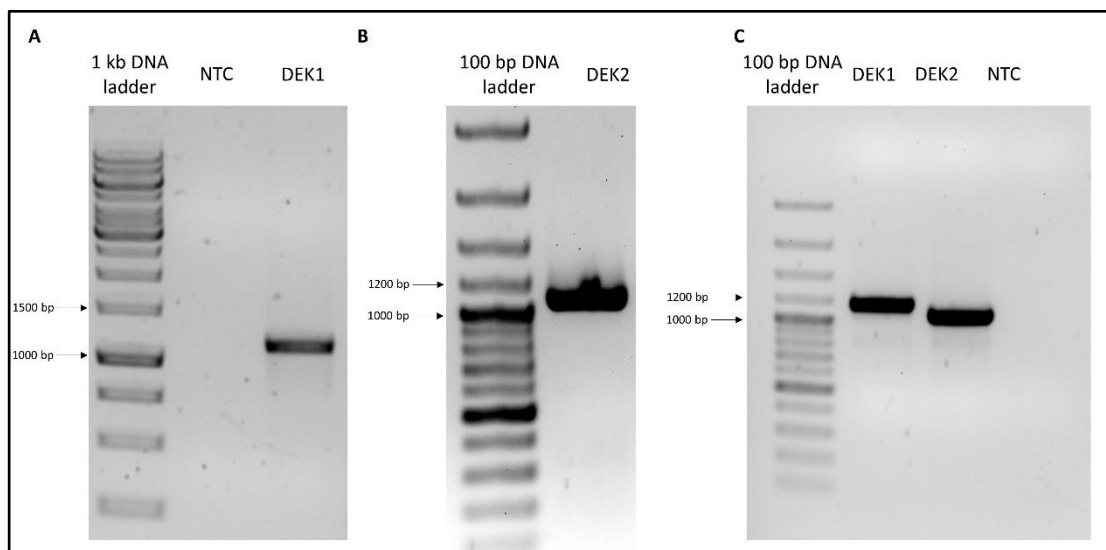


Figure 7.1: PCR results of DEK1 (A) and DEK2 (B) for adding XhoI-EcoRI sites and DEK2 (C) for adding NheI-XhoI sites. PCR products were run in 1% agarose gel.

After clean-up, PCR products and the vectors were digested by restriction endonucleases (HA-DEK1 and HA-DEK2 were lost 14 bp, and Flag-DEK2 was lost 11 bp) prior to ligation.. Digested product samples were run in 1% agarose gel. pEGFP-N2 vector was located between 5000 bp and 4000 bp marker bands, and digested PCR products were again located between 1200 bp and 1000 bp marker bands (Figure 7.2).

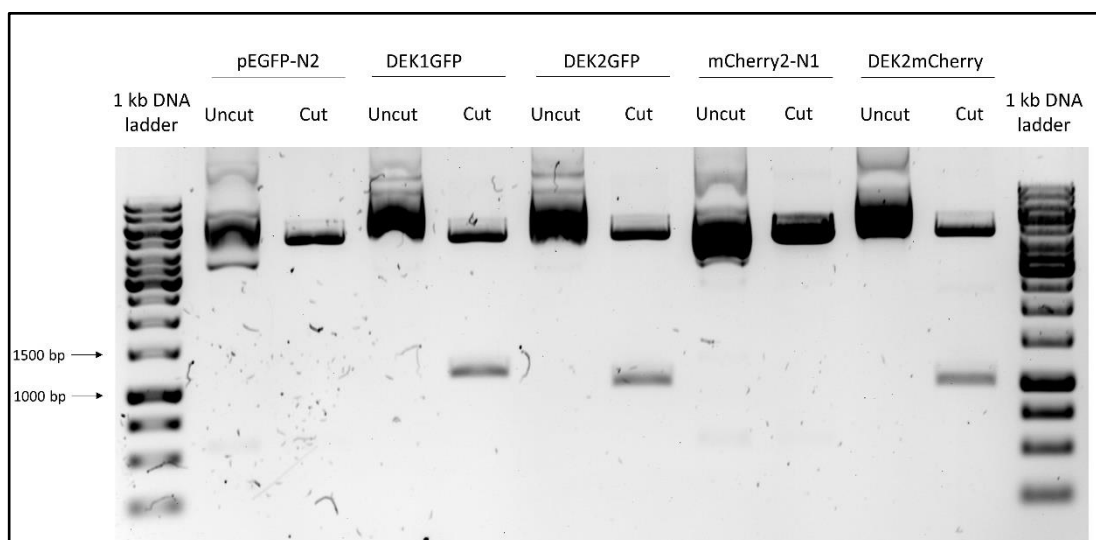


Figure 7.2: Digestion of ligation products and empty vectors.

All products had sticky ends at both 5' and 3' termini, thereby the self-ligation of the vector itself was avoided. The 5' end and 3' end had different sticky sequences so that the introduction of inserts in 3'-5' direction was deflected. Digested PCR products were used directly in the ligation reaction, but vectors were isolated from agarose gel. Ligation was performed in the presence of T4 ligase at 16°C overnight.

Ligated products were then transformed into DH5α bacterial cells. Self-ligation control petri dishes were empty. All colonies were enumerated. Relatively the biggest 5 bacterial colonies were selected from either 3:1 or 5:1 plates, inoculated to 5 ml LB with required antibiotic, and plasmids were isolated by NucleoSpin Plasmid kit (MN, 740588). The concentrations of the isolated plasmids were in Table 7.1.

All five DEK1GFP grew very well, however 2 of 5 DEK2GFP colonies did not grow, and only one DEK2mCherry colony grew. The plasmids having the biggest concentration and optimal A260/280 values were selected for the following experiments. The constructs were then digested to control whether the inserts were

able to be introduced into the vectors. As shown in figure 7.2, the ligation was successful. The three plasmids were then transformed again, the biggest three colonies were selected, and plasmids were isolated by using Nucleobond Xtra Midi EF kit (MN, 740420.10). Endotoxin-free plasmid yield was shown in Table 7.2. The plasmids that yielded the most concentration were selected for subsequent verification.

Table 7.1: Plasmid concentrations after NucleoSpin Plasmid DNA isolation. Selected plasmids were marked with an asterisk (*).

Plasmid	Colony Number	Concentration	A260/280
DEK1GFP	5:1-1 (1)*	40 ng/μl	0.8
	5:1-1 (2)*	67 ng/μl	1.342
	5:1-2 (1)*	84,3 ng/μl	1.686
	5:1-2 (2)	19,7 ng/μl	0.394
	3:1-2 (1)	15 ng/μl	0.3
DEK2GFP	5:1-1 (1)*	240,9 ng/μl	1.79
	5:1-1 (8)	60,7 ng/μl	1.71
	5:1-1 (10)*	146,8 ng/μl	1.70
DEK2mCherry	3:1-1 (1)*	219,1 ng/μl	1.83

Table 7.2: Plasmid concentrations after NucleoBond Xtra Midi EF DNA isolation.

Plasmid	Colony Number	Concentration	A260/280
DEK1GFP	5:1-1 (1)	176,6 ng/μl	1.83
	5:1-2 (1)	247,5 ng/μl	1.80
	5:1-1 (2)	135,1 ng/μl	1.82
DEK2GFP	5:1-1 (1)	641,9 ng/μl	1.80
	5:1-1 (10)	130,8 ng/μl	1.79
DEK2mCherry	3:1-1 (1)	433,5 ng/μl	1.85

After this step, DEK1GFP 5:1-1 (1) was named as DEK1GFP-C1, DEK1GFP 5:1-2 (1) as DEK1GFP-C2, DEK2GFP 5:1-1 (1) as DEK2GFP-C1, DEK2GFP 5:1-1 (10) as DEK2GFP-C10, and DEK2mCherry 3:1-1 (1) as DEK2mCherry.

7.2. Verification of Constructs

Once the insertions were completed successfully, the quality of the constructs were analyzed by both sequencing and then Western blotting. The sequencing results were analyzed by FincTV application. DEK1GFP-C1, DEK1GFP-C2, DEK2GFP-C1, DEK2GFP-C10, and DEK2mCherry were sequenced. There were no mutations in the DEK1GFP-C1, DEK1GFP-C2, DEK2GFP-C1, and DEK2mCherry constructs. On the

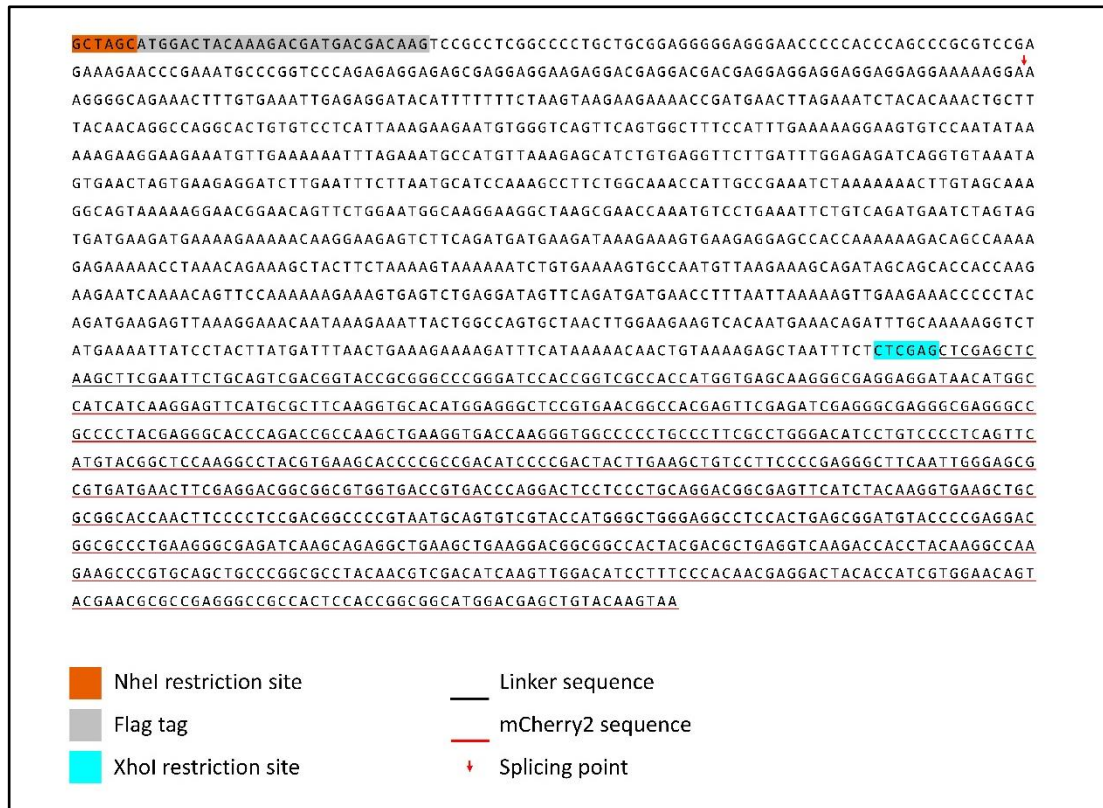


Figure 7.5: Complete nucleotide sequence of DEK2mCherry construct. Unmodified nucleotides indicate DEK1 sequence.

SDS-PAGE and Western blot were applied to investigate the protein product of each construct. Total protein was isolated from HEK293T cells either untransfected or transfected with each construct vector or empty vector. The endogenous DEK runs around the 55 kilodaltons (kD) marker band, probably due to posttranslational modifications. DEK1 is known to have 48 kD molecular weight (mW) and DEK2 has 4 kD less than DEK1. Besides, EGFP has a 26.9 kD mW. Therefore, the expected mW of DEK1GFP was 75 kD, DEK2GFP was 71 kD, and DEK2mCherry was 71 kD (mCherry2 and EGFP have the same mW). As expected, all fusion proteins were placed between 70 kD and 100 kD bands (Fig. 7.6). DEK2GFP was located slightly below the DEK1GFP band. However, DEK2mCherry construct was produced more than one protein product. Hence, DEK2mCherry was not used in subsequent experiments.

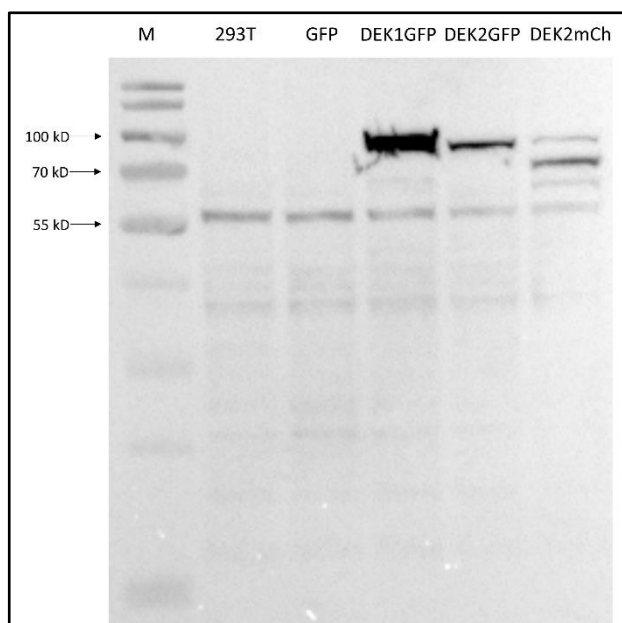


Figure 7.6: Western blot analysis of DEK overexpression. M: Marker; 293T: Untransfected HEK293T cell lysate; GFP: pEGFP-N2 (empty vector) transfected HEK293T cell lysate; DEK1GFP: DEK1GFP transfected HEK293T cell lysate; DEK2GFP: DEK2GFP transfected HEK293T cell lysate; DEK2mCh: DEK2mCherry transfected HEK293T cell lysate.

7.3. DEK Localization in the Interphase

Verified DEKGFP fusion constructs or empty vector were transfected to HeLa and HEK293T cells by using jetPRIME reagent or CaPO₄ method, respectively. DEK1GFP was introduced into only HEK293T cells, but DEK2GFP was introduced into both HeLa and HEK293T cells. Using confocal microscope, we showed that similar to endogenous DEK1, DEK1GFP locates diffusely of HEK293T cells, whereas DEK2GFP exhibited slightly diffuse localization as well as strong aggregation in the nucleus of both HeLa and HEK293T cells during interphase (Figure 7.7a and b). The nuclear localization was confirmed by a DNA binding dye “DAPI”, and the nuclear aggregates was confirmed as nucleolus by immunofluorescence staining with anti-nucleophosmin (NPM1) antibody which labels nucleolus. Moreover, double immunofluorescence staining with anti-fibrillarin and anti-NPM1 antibodies revealed that DEK2 densely co-localizes with fibrillarin and with NPM1, marking the DFC and GC components of nucleolus, respectively (Figure 7.7c). In addition to nucleolar localization, some of the DEK2GFP expressing HEK293T cells showed a punctuated staining in the nucleus resembling nuclear bodies (Figure 7.7d).

Subcellular localization of these GFP fused DEK proteins shown in this thesis were consistent with our previous studies showing similar localization of myc-epitope tagged DEK1 (MD1) or DEK2 (MD2) in human stromal cell line (Figure 7.7e).

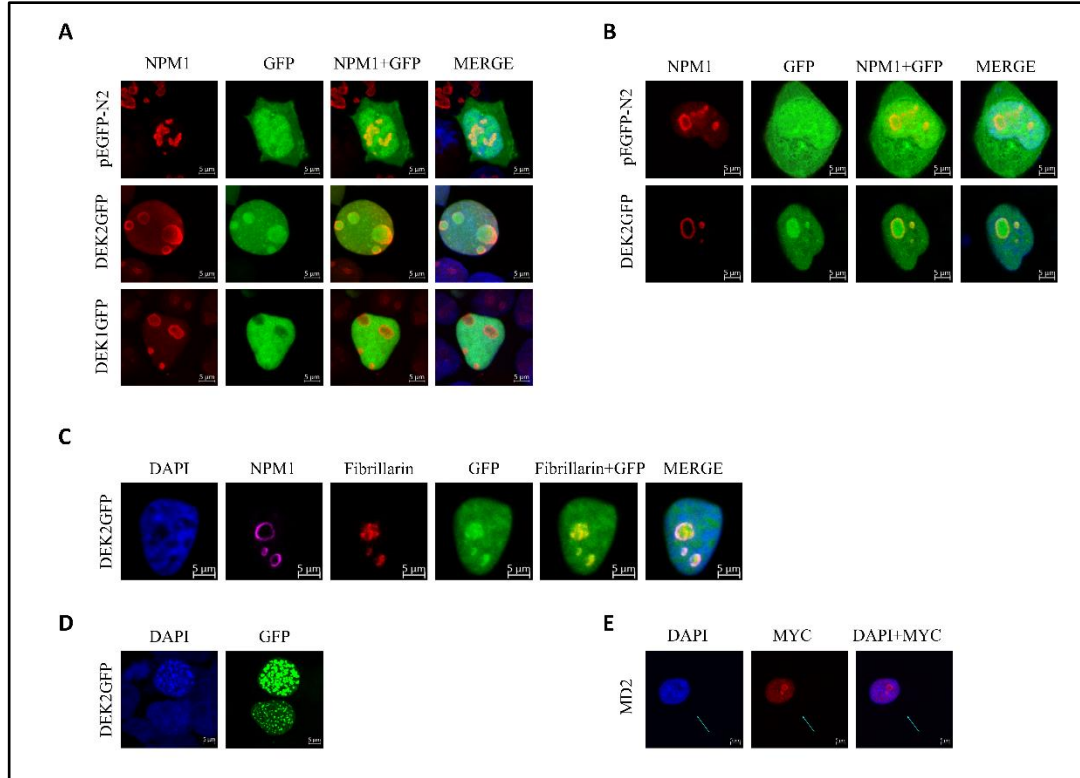


Figure 7.7: Localization of DEK protein isoforms during interphase. (A) HEK293T cells transfected with pEGFP-N2, DEK1GFP, or DEK2GFP (green) and stained with mouse α -NPM1 antibody and mouse Alexa Fluor 555 (red). Colocalization indicated with a yellowish color. (B) HeLa cells transfected with pEGFP-N2 or DEK2GFP (green) and stained with mouse α -NPM1 antibody and α -mouse Alexa Fluor 555 (red). Colocalization indicated with a yellowish color. (C) HeLa cells transfected with DEK2GFP (green) and stained with both mouse α -NPM1 (magenta) and α -mouse Alexa Fluor 647 and rabbit α -Fibrillarin (red) & α -rabbit Alexa Fluor 594. Colocalization of DEK2 and Fibrillarin indicated with yellow. (D) DEK2GFP shows another localization in HEK293T cells. (E) DEK2 localization in MD2 transduced HS27A cells stained with rabbit α -Myc (red) & α -rabbit Alexa Fluor 594. In all cells, DNA was stained with DAPI which indicates the nucleus (blue). Images captured by Zeiss LSM 880 AiryScan confocal microscope with 63x oil objective and 1.0 zoom.

7.4. DEK Localization During Cell Cycle

The cell cycle is mostly consisted of interphase (~23 hours) and mitosis (~1 hour) [Morgan, 2007]. Cells can be manipulated to capture mitotic phase by using different methods. Different chemicals can be used to stop the cell cycle at a desired phase, for example nocodazole for G2/M transition point or thymidine for S phase [Matrka et al., 2015]. In this study we first used unsynchronized cells but obtained very low levels of mitotic cells (Figure 7.8). Therefore, we aimed to increase the number of mitotic cells. Serum starvation was recruited to synchronize (arrest) the cells at G1 phase. After addition of medium containing the 10% serum, the cells synchronously entered the cell cycle, giving the opportunity to catch the cells at mitosis at the same time. According to Chen et al. (2012), 18 hours of serum starvation is enough to synchronize cells in G1 phase, and different cell types enter mitotic phase at different time after the restart of the cell cycle. Therefore, it was needed to optimize fixation time after serum recovery for each cell type. HS27A cells were fixed at each hour from 20 to 24 hours after serum recovery, and it was found that 23 hours after serum recovery (23h) was the optimal time for capturing mitotic cells (Figure 7.9). Although HEK293T cells are very widely used cells, there is no data about their mitotic time after serum starvation in the literature. Thus, a serum starvation assay was conducted for HEK293T cells too. The time range for capturing the mitotic phase was designated from 22 hours to 24 hours after serum recovery together with the fixation of the cells every half an hour because these cells have been known to rapidly proliferate. It was found that 23,5 hours after serum recovery was optimal time to capture mitotic HEK293T cells. All cells were grown on coverslips (poly-L-lysine coating was used for coverslips of HEK293T), fixed with 4% PFA, permeabilized with 0.5% PBS-T, blocked in 2% BSA, stained with rabbit α -Tubulin and anti-rabbit Alexa Fluor 647 (related with Figure 7.10 and 7.11, not shown) or Alexa Fluor 555 (Figure 7.9), and mounted with DAPI containing medium.

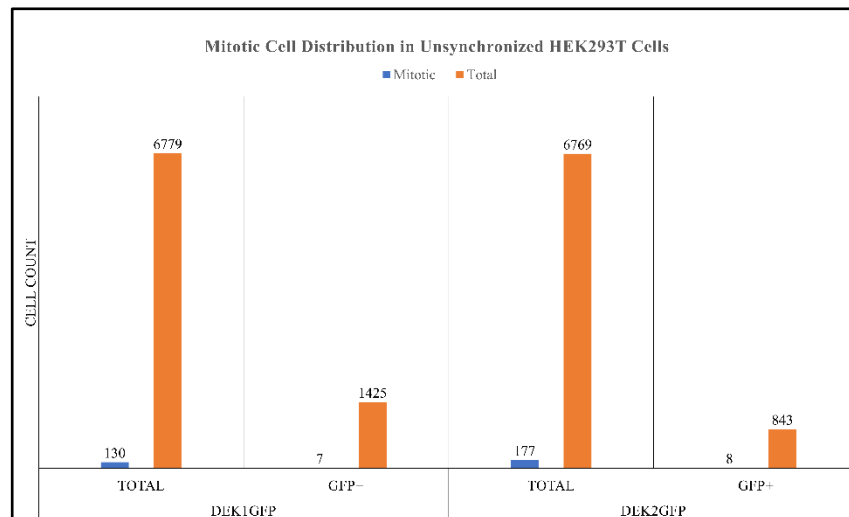


Figure 7.8: Mitotic cell counts of unsynchronized HEK293T cells expressing either DEK1GFP or DEK2GFP.

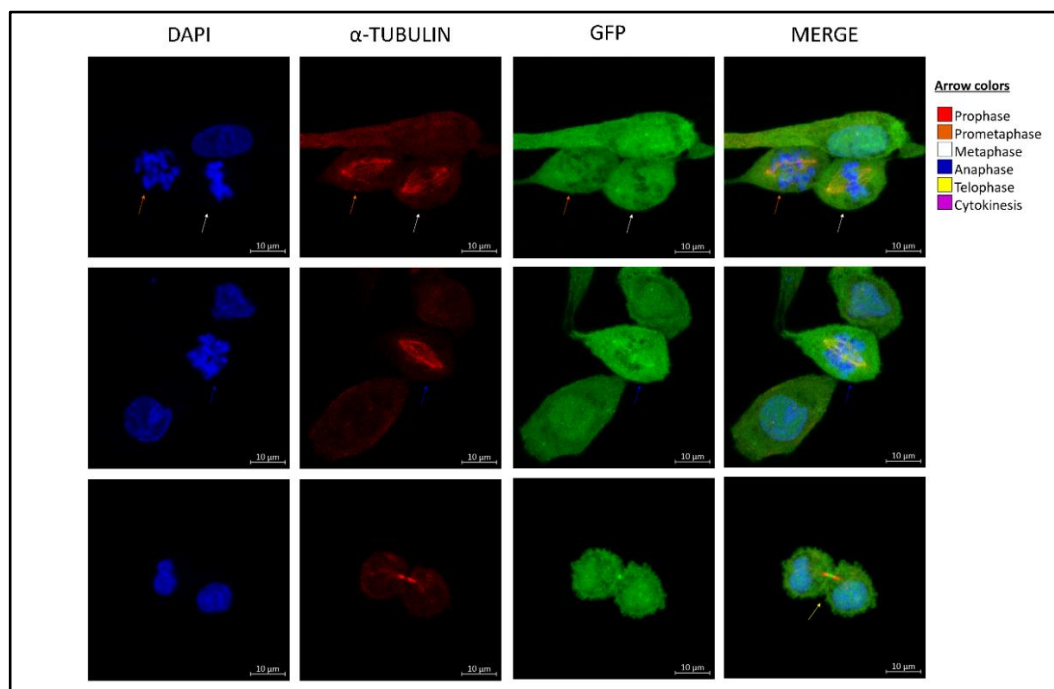


Figure 7.9: Synchronized and cell cycle restarted cells. HS27A GFP cells stained with rabbit α -Tubulin (11H10) antibody and Alexa fluor 555 anti-rabbit antibody. Blue color indicates DAPI, red color indicates α -Tubulin, and green indicates free EGFP. Images captured by Zeiss LSM 880 AiryScan confocal microscope with 63x oil objective and 1.0 zoom.

After this optimization assays, we first analyzed the DEK isoform 1 (DEK1) localization throughout the cell cycle, which has been studied before in the literature. It was shown before that when overexpressed, DEK1 remained in-contact with the chromosomes in all phases of the cell cycle [Matrka et al., 2015]. Our findings were consistent with the literature showing that either overexpression of DEK1GFP in HEK293T or in HS27A-MD1 cells, show a containing with chromosomes during mitosis (Figure 7.10). Mitotic phases were discriminated by DAPI staining (Figures 7.10 and 7.11). Total 119 mitotic MD1 cells and 152 DEK1GFP cells were counted, and mitotic phase distributions of MD1 are shown in Figure 7.10c. DAPI and MD1 co-localization percentage is shown in Figure 7.10e: More than 40% of the cells show DAPI and MD1 colocalization for each cell cycle phase. At the beginning of the mitosis, the colocalization is decreased probably because of aforementioned regulations to dissociate DEK from the DNA, however while the nuclear envelope reorganizes, the transcription begins, and the degradation of the proteins decreases, DEK re-accumulates on the DNA [Morgan, 2007]. Similar results are obtained from DEK1GFP expressing HEK293T cells (Figure 7.10b, d, f) At least 50% of the cells show DAPI and DEK1GFP colocalization in all mitosis phases. Collectively, our results showed that overexpressed DEK1 stays attached to chromosomes during mitosis in both cell lines, as judged by colocalization with DAPI.

The aim of this thesis was to investigate whether DEK2 follows a similar pattern with DEK1 during mitosis. Therefore, DEK2 localization during mitosis in HS27A-MD2 or DEK2GFP/HEK293T cells was also investigated. We found that in HS27A-MD2 cells DEK2 was not associated with the chromosomes during the mitosis and only in telophase we found there was a colocalization with DAPI (9 out of 10 telophase cells showed DAPI+MD2 co-localization) (Figure 7.11a). Total count of mitotic cells are 180 for MD2 and 171 for DEK2GFP and number of cells in each mitotic phase were shown in Figure 7.11c,d. In DEK2GFP-HEK293T cells we did not find any chromosomal localization of DEK2GFP at any stage of mitosis (Figure 7.11f). In summary, opposite to DEK1, our GFP fused DEK2 or Myc-tagged DEK2 proteins do not co-localize with chromosomes during mitosis.

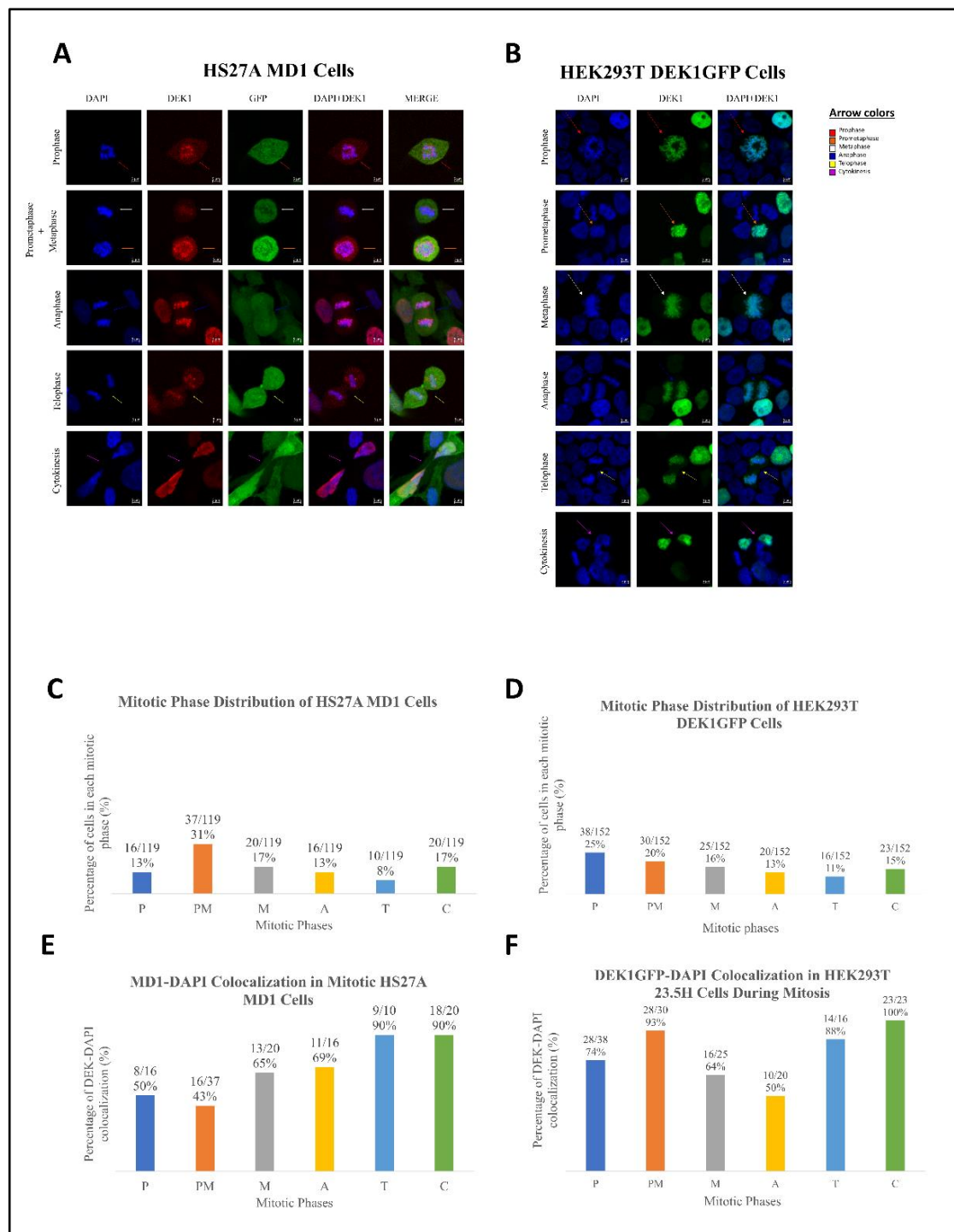


Figure 7.10: DEK isoform 1 localization during the cell cycle. (A) HS27A cells expressing MycDEK1 (MD1) fixed with 4% PFA and stained with mouse anti-Myc & anti-mouse Alexa fluor 555 antibodies as well as DAPI. (B) HEK293T cells expressing DEK1GFP fixed with 4% PFA and stained with only DAPI. (C) Mitotic phase distributions of counted HS27A MD1 cells are shown. (D) Mitotic phase distributions of counted HEK293T DEK1GFP cells are shown. (E),(F) DEK-DAPI colocalized cell counts in each mitotic phase. Images were captured by Zeiss LSM 880 Airy Scan confocal microscope with 63x oil objective and 1.0 zoom.

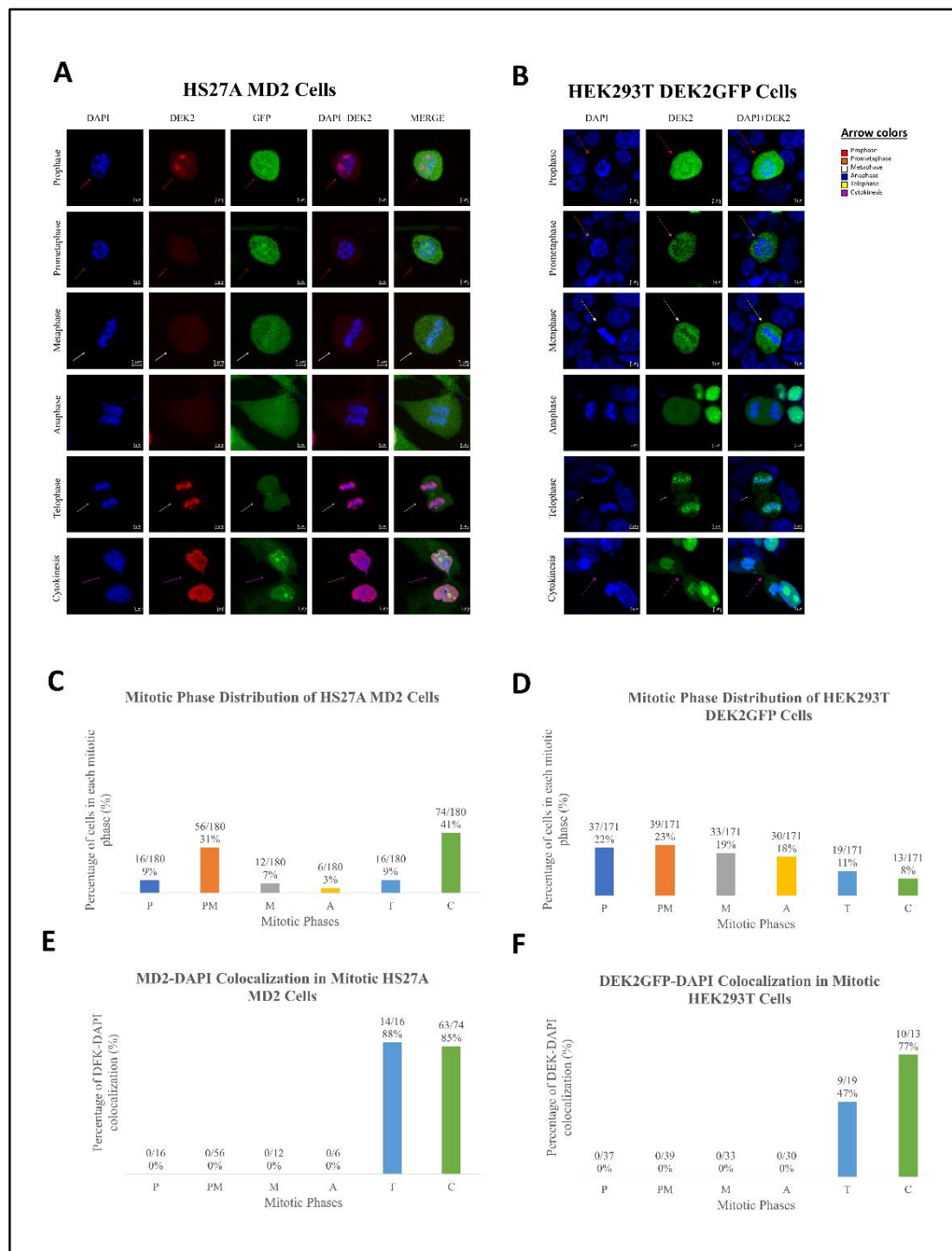


Figure 7.11: DEK isoform 2 localization during the cell cycle. (A) HS27A cells expressing MycDEK2 (MD2) fixed with 4% PFA and stained with mouse anti-Myc & anti-mouse Alexa fluor 555 antibodies as well as DAPI. (B) HEK293T cells expressing DEK2GFP fixed with 4% PFA and stained with only DAPI. (C) Mitotic phase distributions of counted HS27A MD2 cells are shown. (D) Mitotic phase distributions of counted HEK293T DEK2GFP cells are shown. (E),(F) DEK-DAPI colocalized cell counts in each mitotic phase. Images were captured by Zeiss LSM 880 Airy Scan confocal microscope with 63x oil objective and 1.0 zoom.

8. DISCUSSION

DEK is a well-known nucleic acid binding protein function in mainly cell proliferation and correlatively cancer progression [Privette Vinnedge et al., 2013]. It is expressed under E2F transcription factor which is activated while the cell enter mitosis and dysregulated in certain cancer types [Carro et al., 2006]. It has been reported that DEK involved in certain nuclear functions including gene expression control, quality control of mRNA-splicing, and promotion of cell cycle via upregulating CDK1-4 [Privette Vinnedge et al., 2013], [Soares, 2006], [Yu et al., 2016].

Despite the broad knowledge on DEK, there is not much data on DEK isoform 2 (DEK2). To date, the only data on DEK2 is about its comparison with DEK1 on the effect on cell proliferation [Privette Vinnedge et al., 2013], [Yu et al., 2016]. In this thesis, the localization behavior of DEK2 was investigated throughout the cell cycle by either immunofluorescence staining against Myc-tag or directly observing EGFP signal which was fused to DEK proteins at C-terminal. Myc-tagged DEK1 and DEK2 variants were generated in previous studies in our laboratory. In this study, DEK1 and DEK2 were sub-cloned into pEGFP-N2 vector to generate C-terminally EGFP-tagged variants, because N-terminal GFP was shown to decrease the effect of DEK overexpression [Matrka et al., 2015]. We found that although overexpressed DEK1 show co-localization with chromosomes during mitosis, DEK2 does not co-localize with chromosomes (Figure 7.8). At telophase, DEK2 relocates in the nascent nucleus, and during cytokinesis DEK2 begins to appear as nuclear aggregates, probably in the nucleolus. Moreover, the mitotic localization of DEK1 was shown in different cell lines from the literature [Kappes et al., 2001], [Matrka et al., 2015] and similar results were obtained with our work (Figure 7.7) indicating similar upstream regulation events in different human cell lines.

We also generated C-terminally mCherry2-tagged DEK2 variant to study both DEK1 and DEK2 localization at the same time during mitosis however This construct was not used because Western blot analysis showed that DEK2mCherry fusion produced three different protein variants. The reason behind this might be protein cleavage or mRNA splicing.

Using immunofluorescence staining we previously found that myc-tagged ectopic DEK2 shows nucleolar location in about 90% of HS27A human bone marrow stroma cell lines (Emrah Özçelik and Ahmet Kalaycı, unpublished results). To confirm this finding, in this thesis we generated DEK2GFP and DEK1GFPs fusion proteins and followed their nuclear location directly by using confocal microscope. The distinct localization of DEK2 during the interphase and mitosis are intriguing findings. The spliced-out part in DEK2 contains one of the two regions needed for introducing positive supercoils, a citrullination site, and the missing peptide is disordered, but has not a known effect on DNA-binding ability of DEK. In addition, DEK has several steric zippers, and one of them (which one; you should state aa residues here) is lost in DEK2. Steric zippers are hexamer amino acidic disordered sequences that form beta sheet aggregates when come together. These structures may indicate a way of stabilization of protein-protein interactions [H. J. Kim et al., 2013], and they can be one of the reason behind the different localization property of DEK2.

The nucleolus consists of three submerging phases. The core phase (FC) is responsible for rRNA transcription, the mid phase (DFC) is responsible for rRNA modification, and the outer phase (GC) is responsible for several tasks including rRNA processing and ribosome production [Feric et al., 2016]. Further investigation of the sub-nucleolar localization of DEK2 revealed that DEK2 heavily co-localizes with DFC marker fibrillarin, but still found diffuse (in accordance with DFC) in the GC colocalized with NPM1. (Figure 7.5c) These findings indicate a role for DEK2 in each sub-nucleolar phase. DEK has roles in transcriptional regulation and mRNA splicing related with events in the nucleolus [Sandén et al., 2014], [Soares, 2006]. DEK is also known to bind one of the non-canonical DNA structures, cruciform DNA, via its pseudo SAP/SAP domain [Böhm et al., 2005]. It has been shown that cruciform DNAs are found near to transcription start sites [Pandya et al., 2021], DEK is localized on the chromosomes near to transcription start sites [Sandén et al., 2014], and that DEK binds to transcribed region of *TOP1* gene [Hu et al., 2007]. A member of high-mobility group protein (HMG) Hmo1 was found to bind cruciform DNA structures near to transcription start sites and stabilizing the DNA in negatively supercoiled formation. Unlike DEK, Hmo1 does not possess the ability to produce supercoils, and like DEK, upstream binding protein of *Xenopus laevis* (xUBF), a HMG protein, can produce positive supercoils near to transcription start site [Kamau et al., 2004], [Putnam et al., 1994]. DEK1 has 2 extension regions near to pseudo SAP/SAP box domain, and these

regions are required for introducing positive supercoils [Waldmann et al., 2002]. The missing part in DEK2 contains one of these regions, thus DEK2 may lose the ability to produce positive supercoils. However, the positive effect on gene activation of DEK2 is lower than DEK1 [Yu et al., 2016]. Consequently, these arise the possibility that DEK2 may directly or indirectly contribute to one or all of the ribosome production steps.

The localization of DEK during mitosis and cytokinesis was previously described [Kappes et al., 2001], [Matrka et al., 2015]. Endogenous DEK1 was found to bind the DNA during the cell cycle in HeLa cells [Kappes et al., 2001] but not until telophase in NIKS (Near-diploid immortalized keratinocytes) cells [Matrka et al., 2015]. These contradictory results may emerge from the expression levels of endogenous DEK1 in different cell lines. Serum starvation method was used to increase the number of mitotic cells. When cells enter mitosis, they begin to round up and become easily detach [Alberts et al., 2015]. Mitotic HEK293T cells were much more prone to detach, and this caused the loss of mitotic cells during washing steps. Therefore, the number of mitotic cells were less than expected, but it was enough to generate meaningful data. Here we showed for the first time that on the contrary to DEK1, DEK isoform 2 does not co-localize with chromosomes during mitosis. The exclusion of DEK2 from chromosomes may arise from post-translational modifications. It was shown that DEK1 is phosphorylated by different type of kinases including CK2 and acetylated by p300/PCAF, and these modifications interfere with the DNA binding ability of DEK [Cleary et al., 2005], [Kappes et al., 2004a]. However, there is no data on DEK modifications while the cell entering the mitosis. Matrka and coworkers suggested that DEK has Plk1 and aurora kinase binding motifs [Matrka et al., 2015] which are regulators of mitosis in addition to M-Cdk complex [Morgan, 2007]. After the cell passes through metaphase/anaphase gate, the sister chromatids begin to pull apart and the nuclear envelope begin to re-organize [Morgan, 2007]. DEK2 begin to localize in the newly formed daughter nuclei (Figure 7.10). If DEK2 protein level is so high, it may produce small coacervates in HEK293T cells (Figure 7.10, DEK2GFP/Telophase). In addition to mitotic coacervates, DEK2 also localizes as coacervates during the interphase in HEK293T cells, as colocalized with DAPI staining indicating possible residence of double-stranded nucleic acids (Figure 7.7d).

In summary, our results show that during interphase, DEK2 localizes mainly into the nucleolus, and during the mitosis, it is mostly diffuse in the cell. Our findings raise many questions about regulation of DEK2 localization during interphase and mitosis. For instance, the levels of 47S rRNA gene transcription and the production of 28S, 18S, and 5.8S would be analyzed by quantitative PCR (qPCR) to investigate whether DEK2 involved in the transcription or splicing of rRNAs. Plk1 and aurora kinase binding site mutations might be introduced to determine the upstream regulators of DEK2 at the beginning of the mitosis. Supercoiling assay may be recruited to show what type of supercoiling DEK can introduce to the DNA.

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REFERENCES

- Alberts B., Johnson A., Lewis J., Raff M., Roberts K., Walter P., (2015), "Molecular biology of the cell", 6th Edition, Garland Science.
- Aravind L., Koonin E. V., (2000), "SAP - a putative DNA-binding motif involved in chromosomal organization", *Trends in Biochemical Sciences*, 25 (3), 112–114.
- Böhm F., Kappes F., Scholten I., Richter N., Matsuo H., Knippers R., Waldmann T., (2005), "The SAF-box domain of chromatin protein DEK", *Nucleic Acids Research*.
- Boisvert F. M., Van Koningsbruggen S., Navascués J., Lamond A. I., (2007), "The multifunctional nucleolus", *Nature Reviews Molecular Cell Biology*, 8 (7), 574–585.
- Campillos M., García M. A., Valdivieso F., Vázquez J., (2003), "Transcriptional activation by AP-2 α is modulated by the oncogene DEK", *Nucleic Acids Research*, 31 (5), 1571.
- Caragine C. M., Haley S. C., Zidovska A., (2019), "Nucleolar dynamics and interactions with nucleoplasm in living cells", *ELife*, 8, 1–21.
- Carro M. S., Spiga F. M., Quarto M., Di Ninni V., Volorio S., Alcalay M., Müller H., (2006), "DEK expression is controlled by E2F and deregulated in diverse tumor types", *Cell Cycle*, 5 (11), 1202–1207.
- Chamoussat D., De Wever V., Moorhead G. B., Chen Y., Boisvert F. M., Lamond A. I., Trinkle-Mulcahy L., (2010), "RRP1B targets PP1 to mammalian cell nucleoli and is associated with pre-60S ribosomal subunits", *Molecular Biology of the Cell*, 21 (23), 4212–4226.
- Chen M., Huang J., Yang X., Liu B., Zhang W., Huang L., Deng F., Ma J., Bai Y., Lu R., Huang B., Gao Q., Zhuo Y., Ge J., (2012), "Serum starvation induced cell cycle synchronization facilitates human somatic cells reprogramming", *Plos One*, 7 (4).
- Cleary J., Sitwala K. V., Khodadoust M. S., Kwok R. P. S., Mor-Vaknin N., Cebrat M., Cole P. A., Markovitz D. M., (2005), "p300/CBP-associated factor drives DEK into interchromatin granule clusters", *Journal of Biological Chemistry*, 280 (36), 31760–31767.
- Devany M., Kotharu N. P., Matsuo H., (2004), "Solution NMR structure of the C-terminal domain of the human protein DEK", *Protein Science*, 13 (8), 2252–2259.
- Enserink J. M., Kolodner R. D., (2010), "An overview of Cdk1-controlled targets and processes", *Cell Division*, 5 (1), 1–41.
- Feric M., Vaidya N., Harmon T. S., Mitrea D. M., Zhu L., Richardson T. M., Kriwacki R. W., Pappu R. V., Brangwynne C. P., (2016), "Coexisting Liquid Phases Underlie Nucleolar Subcompartments", *Cell*, 165 (7), 1686–1697.

Frottin F., Schueder F., Tiwary S., Gupta R., Körner R., Schlichthaerle T., Cox J., Jungmann R., Hartl F. U., Hipp M. S., (2019), "The nucleolus functions as a phase-separated protein quality control compartment", *Science*, 365 (6451), 342–347.

Ganz M., Vogel C., Czada C., Jörke V., Gwosch E. C., Kleiner R., Pierzynska-Mach A., Zancacchi F. C., Diaspro A., Kappes F., Bürkle A., Ferrando-May E., (2019), "The oncoprotein DEK affects the outcome of PARP1/2 inhibition during mild replication stress", *Plos One*, 14 (8).

Ge W., Chen Y., Guo Y., Zhao D., Mu L., Zhang K., Zhuo W., (2021), "KIF15 upregulation promotes leiomyosarcoma cell growth via promoting USP15-mediated DEK deubiquitylation", *Biochemical and Biophysical Research Communications*, 570, 117–124.

Ghisays V., Nguyen E. T., Streicher J., Pease N. A., Fitzgerald M., Estrada C. M., Franco-Villanueva A., Privette Vinnedge L., Solomon M. B., (2018), "Neuroanatomical Distribution of DEK Protein in Corticolimbic Circuits Associated with Learning and Memory in Adult Male and Female Mice", *Neuroscience*, 371, 254–267.

Greene A. N., Parks L. G., Solomon M. B., Privette Vinnedge L. M., (2020), "Loss of DEK Expression Induces Alzheimer's Disease Phenotypes in Differentiated SH-SY5Y Cells", *Frontiers in Molecular Neuroscience*, 13, 1–10.

Guo H., Prell M., Königs H., Xu N., Waldmann T., Hermans-Sachweh B., Ferrando-May E., Lüscher B., Kappes F., (2021), "1Bacterial Growth Inhibition Screen (BGIS) identifies a loss-of-function mutant of the DEK oncogene, indicating DNA modulating activities of DEK in chromatin", *FEBS Letters*, 595 (10), 1438–1453.

Harper J. W., Elledge S. J., (1998), "The role of Cdk7 in CAK function, a retro-retrospective", *Genes and Development*, 12(3), 285–289.

Hernandez-Verdun D., (2011), "Assembly and disassembly of the nucleolus during the cell cycle", *Nucleus*, 2 (3), 189–194.

Hu H. gang, Scholten I., Gruss C., Knippers R., (2007), "The distribution of the DEK protein in mammalian chromatin", *Biochemical and Biophysical Research Communications*, 358 (4), 1008–1014.

Iarovaia O. V., Minina E. P., Sheval E. V., Onichtchouk D., Dokudovskaya S., Razin S. V., Vassetzky Y. S., (2019), "Nucleolus: A Central Hub for Nuclear Functions", *Trends in Cell Biology*, 29(8), 647–659.

Ivanauskiene K., Delbarre E., McGhie J. D., Kuntziger T., Wong L. H., Collas P., (2014), "The PML-associated protein DEK regulates the balance of H3.3 loading on chromatin and is important for telomere integrity", *Genome Research*, 24 (10), 1584–1594.

Kamau E., Bauerle K. T., Grove A., (2004), "The *Saccharomyces cerevisiae* high

mobility group box protein HMO1 contains two functional DNA binding domains", *The Journal of Biological Chemistry*, 279 (53), 55234–55240.

Kappes F., Burger K., Baack M., Fackelmayer F. O., Gruss C., (2001), "Subcellular Localization of the Human Proto-oncogene Protein DEK", *Journal of Biological Chemistry*, 276(28), 26317–26323.

Kappes F., Damoc C., Knippers R., Przybylski M., Pinna L. A., Gruss C., (2004a), "Phosphorylation by Protein Kinase CK2 Changes the DNA Binding Properties of the Human Chromatin Protein DEK", *Molecular and Cellular Biology*, 24 (13), 6011–6020.

Kappes F., Scholten I., Richter N., Gruss C., Waldmann T., (2004b), "Functional Domains of the Ubiquitous Chromatin Protein DEK", *Molecular and Cellular Biology*, 24 (13), 6000–6010.

Kim H. J., Kim N. C., Wang Y.-D., Scarborough E. A., Moore J., Diaz Z., MacLea K. S., Freibaum B., Li S., Molliex A., Kanagaraj A. P., Carter R., Boylan K. B., Wojtas A. M., Rademakers R., Pinkus J. L., Greenberg S. A., Trojanowski J. Q., Traynor B. J., Taylor J. P., (2013), "Mutations in prion-like domains in hnRNPA2B1 and hnRNPA1 cause multisystem proteinopathy and ALS", *Nature*, 495 (7442), 467–473.

Kim V. N., Dreyfuss G., (2001), "Nuclear mRNA binding proteins couple pre-mRNA splicing and post-splicing events", *Molecules and Cells*, 12 (1), 1–10.

Lodish H., Berk A., Kaiser C. A., Krieger M., Scott M., Bretscher A., Ploegh H., Matsudaira P., (2007), "Molecular Cell Biology", 6th Edition, W.H. Freeman and Company.

López D. J., de Blas A., Hurtado M., García-Alija M., Mentxaka J., de la Arada I., Urbaneja M. A., Alonso-Mariño M., Bañuelos S., (2020), "Nucleophosmin interaction with APE1: Insights into DNA repair regulation", *DNA Repair*, 88, 102809.

Matrka M. C., Hennigan R. F., Kappes F., DeLay M. L., Lambert P. F., Aronow B. J., Wells S. I., (2015), "DEK over-expression promotes mitotic defects and micronucleus formation", *Cell Cycle*, 14 (24), 3939–3953.

Morgan D. O., (2007), "Primers in Biology The Cell Cycle Primers in Biology : Protein Structure and Function", New Science Press.

Negi S. S., Olson M. O. J., (2006), "Effects of interphase and mitotic phosphorylation on the mobility and location of nucleolar protein B23", *Journal of Cell Science*, 119(Pt 17), 3676–3685.

Pandya N., Bhagwat S. R., Kumar A., (2021), "Regulatory role of Non-canonical DNA Polymorphisms in human genome and their relevance in Cancer", *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*, 1876 (2), 188594.

Pederson T., (2011), "The Nucleolus", *Cold Spring Harbor Perspectives in Biology*, 3 (3), a000638–a000638.

Privette Vinnedge L. M., Kappes F., Nassar N., Wells S. I., (2013), "Stacking the DEK: From chromatin topology to cancer stem cells", *Cell Cycle*, 12 (1), 51–66.

Putnam C. D., Copenhaver G. P., Denton M. L., Pikaard C. S., (1994), "The RNA polymerase I transactivator upstream binding factor requires its dimerization domain and high-mobility-group (HMG) box 1 to bend, wrap, and positively supercoil enhancer DNA", *Molecular and Cellular Biology*, 14 (10), 6476.

Sandén C., Järvstråt L., Lennartsson A., Brattås P., Nilsson B., Gullberg U., (2014), "The DEK oncoprotein binds to highly and ubiquitously expressed genes with a dual role in their transcriptional regulation", *Molecular Cancer*, 13 (1), 215.

Savino T. M., Gébrane-Younès J., De Mey J., Sibarita J. B., Hernandez-Verdun D., (2001), "Nucleolar Assembly of the Rna Processing Machinery in Living Cells", *The Journal of Cell Biology*, 153 (5), 1097.

Sawatsubashi S., Murata T., Lim J., Fujiki R., Ito S., Suzuki E., Tanabe M., Zhao Y., Kimura S., Fujiyama S., Ueda T., Umetsu D., Ito T., Takeyama K. I., Kato S., (2010), "A histone chaperone, DEK, transcriptionally coactivates a nuclear receptor", *Genes and Development*, 24 (2), 159–170.

Sawyer I. A., Sturgill D., Dundr M., (2018), "Membraneless nuclear organelles and the search for phases within phases", *Wiley Interdisciplinary Reviews: RNA*, 10 (2), 1–20.

Scott M. S., Troshin P. V., Barton G. J., (2011), "NoD: A Nucleolar localization sequence detector for eukaryotic and viral proteins", *BMC Bioinformatics*, 12.

Smith E. A., Gole B., Willis N. A., Soria R., Starnes L. M., Krumpelbeck E. F., Jegga A. G., Ali A. M., Guo H., Meetei A. R., Andreassen P. R., Kappes F., Vinnedge L. M. P., Daniel J. A., Scully R., Wiesmüller L., Wells S. I., (2017), "DEK is required for homologous recombination repair of DNA breaks", *Scientific Reports*, 7, 1–12.

Smith E. A., Krumpelbeck E. F., Jegga A. G., Prell M., Matrk M. M., Kappes F., Greis K. D., Ali A. M., Meetei A. R., Wells S. I., (2018), "The nuclear DEK interactome supports multi-functionality", *Proteins: Structure, Function, and Bioinformatics*, 86 (1), 88–97.

Soares L. M. M., (2006), "Intron Removal Requires Proofreading of U2AF/3' Splice Site Recognition by DEK", *Science*, 312 (5782), 1961–1965.

Staněk D., Fox A., (2017), "Nuclear bodies: news insights into structure and function, *Current Opinion in Cell Biology*", 46, 94–101.

Szerlong H. J., Herman J. A., Krause C. M., Deluca J. G., Skoultchi A., Winger Q. A., Prenni J. E., Hansen J. C., (2015), "Proteomic Characterization of the Nucleolar Linker Histone H1 Interaction Network", *Journal of Molecular Biology*, 427 (11), 2056–2071.

Tanikawa C., Ueda K., Suzuki A., Iida A., Nakamura R., Atsuta N., Tohnai G., Sobue

G., Saichi N., Momozawa Y., Kamatani Y., Kubo M., Yamamoto K., Nakamura Y., Matsuda K., (2018), "Citruination of RGG Motifs in FET Proteins by PAD4 Regulates Protein Aggregation and ALS Susceptibility", *Cell Reports*, 22 (6), 1473–1483.

Tchakarska G., Sola B., (2020), "The double dealing of cyclin D1", *Cell Cycle*, 19 (2), 163–178.

von Lindern M., Fornerod M., van Baal S., Jaegle M., de Wit T., Buijs A., Grosveld G., (1992), "The translocation (6;9), associated with a specific subtype of acute myeloid leukemia, results in the fusion of two genes, *dek* and *can*, and the expression of a chimeric, leukemia-specific *dek-can* mRNA", *Molecular and Cellular Biology*, 12 (4), 1687–1697.

Waldmann T., (2003), "Structure-specific binding of the proto-oncogene protein DEK to DNA", *Nucleic Acids Research*, 31 (23), 7003–7010.

Waldmann T., Eckerich C., Baack M., Gruss C., (2002), "The ubiquitous chromatin protein DEK alters the structure of DNA by introducing positive supercoils", *The Journal of Biological Chemistry*, 277 (28), 24988–24994.

Wang Z., Ma J., Miyoshi C., Li Y., Sato M., Ogawa Y., Lou T., Ma C., Gao X., Lee C., Fujiyama T., Yang X., Zhou S., Hotta-Hirashima N., Klewe-Nebenius D., Ikkyu A., Kakizaki M., Kanno S., Cao L., iu Q., (2018), "Quantitative phosphoproteomic analysis of the molecular substrates of sleep need", *Nature*, 558 (7710), 435–439.

Yao R.-W., Xu G., Wang Y., Shan L., Luan P.-F., Wang Y., Wu M., Yang L.-Z., Xing Y.-H., Yang L., Chen L.-L., (2019), "Nascent Pre-rRNA Sorting via Phase Separation Drives the Assembly of Dense Fibrillar Components in the Human Nucleolus", *Molecular Cell*, 1–17.

Yu L., Huang X., Zhang W., Zhao H., Wu G., Lv F., Shi L., Teng Y., (2016), "Critical role of DEK and its regulation in tumorigenesis and metastasis of hepatocellular carcinoma", *Oncotarget*, 7 (18), 26844–26855.

BIOGRAPHY

In the high school, I studied database programming in high school along with formal training. I decided to be a biologist during the last year of high school, then enrolled in Molecular Biology and Genetics program at Gebze Technical University (GTU). After graduated with BSc honors degree, I continued graduate school first with integrated PhD at GTU but then decided to switch to Master's program and continue PhD in elsewhere. At the end of my Master's, I joined Michael Jantsch lab with ERASMUS to learn the basics of RNA biology and epitranscriptomics. I now wonder what will his wibbly wobbly, timey wimey, spacey wazey curriculum bring to him.