Cell Cycle Regulation in Cancer:

A noncoding perspective

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Cover illustration: Confocal imaging of human lung adenocarcinoma cells nuclei (blue) immunostained for γH2A.X (red) and pCHK2 (green).

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"مصر ليست دولة تاريخية....مصر جاءت أولا ثم جاء التاريخ"

نجيب محفوظ

To the heroes who lost their lives defending dignity and freedom;

To Gamal Abdel Nasser and Shimaa al-Sabbagh

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ABSTRACT

The cell cycle progression is tightly regulated to ensure error-free cell replication. The complexity of the transcriptional machinery aids to function in a spatiotemporal pattern across different phases and genomic loci. However, the cell cycle regulation has always been associated with a "protein-centric" view that implicates an intricate network of closely related proteins and transcription factors. This view neglects the fact that only 2−2.3% of the human genome codes for proteins. On the other hand, more than 70% of the human genome undergoes pervasive transcription of, most likely, regulatory non-coding RNA (ncRNA) counterparts. Thus, the interrogation of the intimate functional relationship of ncRNAs to cell cycle progression and tumor homeostasis in different cancer types is indispensable. To this end, in the first study of the current thesis, we optimized a nascent RNA capture assay coupled with high throughput sequencing that enables high-resolution mapping of ongoing RNA transcriptional events. The study revealed the temporal separation between DNA replication and RNA transcription, where replication timing has an inverse correlation with transcription.

Given that the DNA replication is the most critical process during cellular division, the regulatory elements governing the S phase progression would be of great importance for cell survival. Thus, in the second study, we utilized our optimized nascent RNA capture assay to identify the long noncoding RNAs (lncRNAs), which are enriched in different compartments of the S phase in HeLa cells. Then, we analyzed the expression patterns of the identified lncRNAs across the cancer genome atlas (TCGA) datasets and determined their clinical relevance in different types of cancer. We uncoupled the function of an uncharacterized lncRNA, termed as *SCAT7*, which harbored oncogenic properties that promote cell cycle progression. Transcriptome-wide analysis of cells depleted of *SCAT7* demonstrated its role in activating FGF/FGFR signaling and the downstream PI3K-AKT pathway

in different cancer models, including lung adenocarcinoma (LUAD) and renal cell carcinoma. The *SCAT7*-mediated activation of PI3K/AKT signaling depends on the lncRNA interaction with a protein complex comprising hnRNPK and YBX1 proteins. Therefore, the therapeutic targeting of *SCAT7* in mouse xenografts and PDX models reduced tumors progression significantly.

In the third study, we uncoupled the DNA replication-related functions of *SCAT7*. Using a combination of immuno-precipitation, immuno-fluorescence, and DNA combing assays, we report that *SCAT7* physically interacts and regulates the topoisomerase I (TOP1) turnover via protein ubiquitination. The depletion of *SCAT7* induces accumulation of TOP1 that creates replication stress and double-stranded breaks. However, *SCAT7* abrogation also interferes with DNA homology-directed repair and inhibits the phosphorylation of ATM protein. Subsequently, the TOP1-induced DNA damage persists, causing further replication stress and cellular death. We also uncover the potential implication of *SCAT7* silencing in circumventing cisplatin resistance in LUAD cells.

In the last study, we identified *LY6K-AS* lncRNA, which has elevated expression levels in LUAD tissues compared to healthy counterparts. *LY6K-AS* acts as an independent prognostic biomarker of survival for LUAD patients. The silencing of *LY6K-AS* induces chromosomal abnormalities and interferes with the mitotic progression of LUAD cells. Mechanistically, it interacts with 14-3-3 proteins to modulate the transcriptional programs of several factors involved in spindle assembly checkpoint. The silencing of *LY6K-AS* in cisplatin-resistant and crizotinib-resistant cells reduces their proliferation significantly. *In vivo* experiments indicated that *LY6K-AS* is a potential therapeutic target against naive and chemoresistant tumors. Collectively, the presented studies in the current thesis establish novel functions for lncRNAs in regulating cell cycle progression in different cancer models.

Keywords: Long Noncoding RNA, lncRNA, Cell Cycle, S phase, Mitosis, Checkpoints, Cancer, SCAT7, LY6K-AS

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SAMMANFATTNING PÅ SVENSKA

Cellcykeln är en noggrant reglerad process, som säkerställer korrekt kopiering av en cell till två. Det transkriptionella maskineriet bidrar till denna reglering genom att slå på geners uttryck i vid rätt plats och tid, under cellcykelns olika faser och vid olika genomiska loci. Enligt den gängse modellen styrs cellcyklen av ett intrikat nätverk av transkriptionsfaktorer och andra proteiner. Detta synsätt tar inte hänsyn till att endast 2 – 2,3% av det humana genomet kodar för proteiner, samtidigt som 70% av genomet transkriberas och i många fall sannolikt ger upphov till icke-kodande RNA molekyler (ncRNA) med regulatorisk funktion. Det är därför av stor vikt att vi undersöker den funktionella betydelsen av regulatoriska, ncRNA-molekyler för cellcykelprogression och tumörutveckling vid olika typer av cancer. Med detta mål i sikte genomförde vi den första studien i denna avhandling, med avsikt att optimera analysmetoder som gör det möjligt att utnyttja tekniker för djup sekvensering (Next Generation Sequencing), för att få en högupplösande bild av pågående transkription vid olika platser i det eukaryota genomet. Denna studie visade att DNA replikation och RNA transkription var separerade i tiden, och att det råder ett omvänt förhållande vad gäller tidpunkten för replikation och transkription.

Med tanke på att DNA-replikation är den mest grundläggande processen under celldelning, förefaller det sannolikt att de regulatoriska element som styr progressionen av S-fas är nödvändiga för cellers överlevnad. I vår andra studie utnyttjade vi därför de tekniker vi utvecklat i den första studien för att identifiera långa, icke-kodande RNA-molekyler (lncRNA) som anrikas under S-fas i HeLa-celler. Vi analyserade sedan hur dessa lncRNA uttrycktes i olika cancertyper samt deras kliniska relevans genom att analysera tillgängliga data från ”the Cancer Genome Atlas”. Vi valde att inrikta våra studier på funktionen hos en specifik lncRNA-molekyl, *SCAT7*, som uppvisade oncogena egenskaper och stimulerarde cellcykelprogression. Analys av effekter på global transkription efter nedreglering av *SCAT7*-uttryck visade att *SCAT7* aktverar FGF/FGFR och den nedströms belägna PI3K/AKT-signaleringsvägen i olika typer av cancer, inklusive carcinom i lunga och njure. *SCAT7*-medierad aktivering av PI3K/AKT-signalering beror på en interaktion mellan detta lncRNA och ett proteinkomplex bestående av hnRNPK och YBX1 proteiner. Experimentell nedreglering av *SCAT7* ledde till en signifikant minskining av tumörtillväxt i xenograftmodeller, både med cell-linjer och och patientderiverat tumörmaterial.

I den tredje studien granskade vi betydelsen av *SCAT7* för DNA-replikation. Genom att använda en kombination av immunoprecipitering, immunofluorescens och en teknik kallad *DNA combing* kunde vi visa att *SCAT7* interagerar fysiskt med topoisomeras 1 (TOP1) och kan reglera stabiliteten hos detta protein via ubiquitylering. Minskade mängder av *SCAT7* orsakar en ackumulation av TOP1, vilket leder till replikationsstress och dubbelsträngsbrott. Vidare stör lägre nivåer av *SCAT7,* homologi-beroende DNA-reparation och förhindrar fosforylering av ATM-proteinet. Detta leder i sin tur till att TOP1-inducerade skador inte åtgärdas på ett adekvat vis, vilket orsakar ytterligare replikationsstress och celldöd. Vi kunde också visa att nedreglering av *SCAT7* kan användas för motverka resistens mot cisplatin i celler från lungadenocarcinom.

I vår sista studie, visade vi att *LY6K-AS* lncRNA föreligger i högre nivåer i lungadenocarcinom än vad som ses i normal lungvävnad. Vi visade också att *LY6K-AS* kan fungera som en oberoende, prognostisk biomarkör för överlevnad hos patienter med denna sjukdom. När vi tystar uttrycket av *LY6K-AS* i celler från lungadenocarcinom leder detta till kromosomala avvikelser och störd progression genom mitos. *LY6K-AS* utövar sin funktion genom att interagera med 14-3-3 proteiner och modulerar därigenom transkriptionsmönster för flera faktorer inblandade i kontrollstationer (checkpoints) för korrekt formering av den mitotiska kärnspolen. Genom att tysta uttrycket av *LY6K-AS* kunde vi signifikant minska cisplatin- resp. crizotibnibresistenta cellers proliferation. Vidare etablerade experiment *in vivo LY6K-AS* som en potentiell måtlavla för utvecklingen av terapier mot såväl naiva som kemoresistenta tumörer. Tillsammans visar de studier som presenteras här på en ny funktion för lncRNA-molekyler, som regulatorer för cellcykelprogression vid olika former av cancer.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Meryet-Figuiere M, Alaei-Mahabadi B, **Ali MM**, Mitra M, Subhash S, Pandey GK, Larsson E, and Kanduri C. "Temporal separation of replication and transcription during S-phase progression" 2014, Cell Cycle, 13: 3241-8.

II. **Ali MM***, Akhade VS*, Kosalai ST*, Subhash S*, Statello L, Meryet-Figuiere M, Abrahamsson J, Mondal T and Kanduri C. "PAN-cancer analysis of S-phase enriched lncRNAs identifies oncogenic drivers and biomarkers" 2018, Nature Communications, 9: 883. * Authors contributed equally

III. Statello L, **Ali MM**, Reischl S, Kosalai ST, Akhade VS, and Kanduri C. "*SCAT7* lncRNA regulates TOP1 turnover and DNA homology-directed repair in lung cancer" (Manuscript)

IV. **Ali MM**, Mahale S, Marco M, Kosalai ST, Mishra K, Statello L, Umpathy G, Hallberg B, and Kanduri C. "*LY6K-AS* lncRNA regulates mitotic progression and chemoresistance in lung adenocarcinoma cells" (Manuscript)

CONTENT

ABBREVIATIONS

lncRNAs	Long noncoding RNAs
CDKs	Cyclin-dependent kinases
CKIs	Cyclin-dependent kinase inhibitors
CAK	Cyclin-dependent activating kinase
APC/C	Anaphase-promoting complex/cyclosome
Rb	Retinoblastoma
CIP/KIP	CDK-interacting protein/kinase inhibitory protein
INK4	Inhibitors of CDK4
SAC	Spindle assembly checkpoint
DDR	DNA damage response
HR	Homologous repair
NHRJ	Non-homologous end joining
ATM	Ataxia-telangiectasia mutated
ATR	ATM- and Rad3-Related
RTK	Receptor tyrosine kinase
CHEK	Checkpoint kinase
TOP1	Topoisomerase 1
MAPK	Mitogen-activated protein kinase
ERK	Extracellular signal-regulated kinase
EGFR	Epidermal growth factor receptor

FGF/FGFR	Fibroblast growth factor/fibroblast growth factor receptor
PI3K	Phosphatidylinositol 3-kinase
PKB	Protein kinase B
mTOR	Mammalian target of rapamycin
PIP_3	Phosphatidylinositol 3,4,5-triphosphate
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PTEN	Phosphatase and tensin homolog
GSK	Glycogen synthase kinase
TCGA	The cancer genome atlas
NSCLC	Non-small cell lung cancer
LUAD	Lung adenocarcinoma
ccRCC	Clear cell renal cell carcinoma
SCAT	S-phase cancer-associated transcript
IP	Immunoprecipitation
RIP	RNA immunoprecipitation
ChIP	Chromatin immunoprecipitation
ChOP	Chromatin oligo-affinity precipitation
IF	Immunofluorescence
FISH	Fluorescent *in situ* hybridization
LNA/ASOs	Locked nucleic acid/Antisense oligos
CPT	Camptothecin

1 INTRODUCTION

Cancer is a complex and highly dynamic disease, which does not imply the action of individual malignant cells. Instead, the multidimensional complexity of the disease originates from the heterogeneous interactions of different cell types, constituting a favorable microenvironment for neoplasia [1]. The onset of carcinogenic transformation and subsequent tumorigenesis require the progressive evolution of healthy cells to a pre-malignant state. This transitioning state further develops into a life-threating neoplasm that may acquire self-autonomy away from the supporting host [2]. The transformation onset may stem out from an intrinsic predisposition, as the case in some familial forms of cancer, or as a consequence of external stimuli. However, the neoplastic evolution entails indispensable traits that permit rapid clonal expansion and oncogenic sustainability. This clonal evolution is somehow analogous to the Darwinian evolution, where the sequentially acquired alterations provide a growth advantage to the next wave of accumulating malignancies [3]. Therefore, from a conceptual perspective, Hanahan and Weinberg defined the hallmarks of cancer, which enable continuous tumor growth and metastatic dissemination [4]. The logical framework encompassing the hallmarks of cancer presumed initially six manifesting traits, including the self-sufficiency in growth signals, insensitivity to growth inhibition signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis [4]. The 20-year-old framework anticipated and argued optimistically for the emerging simplicity of cancer research in the following years. This unrealistic optimism was overwhelmed with the simple idea that virtually all mammalian cells possess common mechanisms to regulate cell proliferation, differentiation, and death. However, a decade later, Hanahan and Weinberg acknowledged the shortage in their outlook, revisiting the fundamental hallmarks and adding further traits. Thus, the so-called next generation of cancer hallmarks includes the reprogramming of energy metabolism and evading immune destruction. In addition to the core and emerging hallmarks, genomic instability and tumor-promoting inflammation are consequential characteristics that facilitate neoplasia [5]. In my personal opinion, which is supported by various research articles, the decade-long advances in DNA sequencing technologies aided Hanahan and Weinberg to underlie the updated definition of cancer hallmarks. For instance, a remarkable breakthrough came in 2002, reporting the association between malignant melanoma and an activating single substitution mutation in BRAF (V599E) gene [6]. Shortly following the identification of this point mutation, a study exploited this point mutation to abrogate the transformation

phenotype of melanoma cells and provide further evidence on the versatility of targeted therapeutics in cancer milieu [7]. Another example is the discovery of the mechanism underlying the acquired resistance to the EGFR inhibitor gefitinib in non-small cell lung cancer (NSCLC) [8]. The first investigation, which was reported in 2005, relied on the feasibility of DNA sequencing technology to specify the T790M substitution mutation as the primary resistance mechanism toward EGFR inhibitors. With many other seminal discoveries, it became evident that different cell types of the same organism regulate their proliferation, differentiation, and death programs with unanticipated complexity. Of note, the conceptual advances in understanding the complexity of cancer, thanks to the emerging hallmarks, led to the definition of distinguishing features of the core hallmarks. For example, insights into the process of metastasis identified four essential pillars, comprising motility and invasion, modulating the secondary site or the local microenvironment, plasticity, and colonization of secondary sites [2]. In a similar context, the inauguration of the "omics" era led the way, not only to restructure the priorities in cancer research but also to introduce new crucial players, such as long noncoding RNAs (lncRNAs) [9].

The relationship between the genomic content and organismal complexity has been one of the most debated concepts since the dawn of the central dogma of molecular biology [10]. A humble sentiment would view the cellular DNA content (C-value) as a direct measurement of the organismal complexity. Unfortunately, the C-value is far from being an informative measurement. For instance, the salamander has an extraordinary huge genome size, which is nearly 10–25 times larger than other vertebrates. Also, the lungfish species have a C-value larger than primates [10]. In both cases, the genomes exhibit a remarkable degree of polyploidy, which can not seem plausible to serve the purpose of phylogenetic complexity. This inconsistency is known as the C-value paradox. However, a further look would consider the number of genes as a relevant manifestation of the organismal complexity. This measurement (G-value) appreciates only the number of protein-coding genes and the associated regulatory elements. Nevertheless, similar to the C-value paradox, the G-value does not fit the expected evolutionary association. In a direct comparison between the human genome and the rice genome, it is surprising to know that the estimated numbers are 20,000 and 37,000 protein-coding genes, respectively [11, 12]. Thus, associating the G-value with the evolution hierarchy results in another G-value paradox [10]. Strikingly, the expansion in noncoding sequences showed a positive correlation with the biological complexity in eukaryotes. Also, the introns' size and distribution are not random. Instead, the increase in intronic sequences correlates with higher

expression in the nervous system [10]. Therefore, the noncoding genome composition may be a direct indication of the organismal complexity.

Concordant with this evolutionary relevance, the rapid advances in DNA sequencing technologies introduced other dimensions to the noncoding sequences expansion. These dimensions have more functional and regulatory perspectives, which are also under selective evolutionary pressure. One of the first indications on the relevance of the noncoding genome arose from the striking observation that more than 70% of the human genome is pervasively transcribed [13]. The estimated total number of protein-coding genes within the human genome can not justify this unexpected firing of transcriptional events [12]. Therefore, the notion of noncoding transcripts started to gain attention as a gateway to understanding more about the cellular dynamics. Among the several classes of noncoding RNAs, the class of lncRNAs has emerged as one of the most prominent players in various physiological and pathological contexts. In a simple term, lncRNAs are endogenous transcripts longer than 200 nucleotides in length that lack significant open reading frames [9]. Over the past few years, tens of studies have laid the foundations that comprehended our understanding of the lncRNAs functional relevance. However, owing to the poor sequence conservation and functional heterogeneity among lncRNAs, experimental investigations are necessary to conclude the context-dependent relevance of each lncRNA. Thus, the main focus of the current thesis is to draw a functional connection between lncRNAs and cancer. Although there are many promising areas to venture, we thought to exploit the cell cycle vulnerabilities to get functional insights into the connection between the noncoding transcriptome and cancer hallmarks.

The first section of the introduction highlights the main constituents and the basic concept of cell cycle regulation. However, it does not include the detailed mechanisms of DNA replication and repair. The second part of the introduction discusses the general features of lncRNAs and their mode of actions. The last section of the introduction describes several examples of cell cycle-related lncRNAs, which are also relevant to different types of cancer.

1.1 Cell cycle regulation

Eukaryotic cell cycle progression is one of the most evolutionary conserved processes that require high spatial and temporal coordination to ensure the generation of defects-free progeny. The cellular division program comprises confined well-regulated stages that, from a broad perspective, promote the duplication of parental genetic material and segregate it into daughter cells. More specifically, the eukaryotic cell partitions its division process into distinct phases, where each phase comprises a set of molecular events subjected to both negative and positive regulation. These active phases are gap1 phase (G1), synthesis phase (S), gap2 phase (G2), mitosis and cytokinesis phase (M). The active duplication of the parental DNA occurs within the S phase, while the chromosomal segregation takes place in the M phase. It is worth noting that G1 and G2 are not inert phases; instead, they are periods of active metabolism and molecular events [14]. These phases promote biomass expansion, signal integration, and organization of the replicated genome. However, among various reasons, eukaryotic cells can enter an inactive phase of quiescence known as G0 due to the absence of growth signals or terminal differentiation [15]. The accurate transition between successive cell cycle phases involves dynamic oscillation in transcriptional, translational, and post-translational programs. Indeed, the cellular reprogramming relies mainly on the activity of different molecular drivers, known as cyclins and cyclin-dependent kinases (CDKs). The latter molecules are serine/threonine kinases, which are usually inactive, though they present in molar excess at particular time points. The association between CDKs and their respective cyclins promotes active configuration of the complex, and in turn, phosphorylates critical molecules to permit cell cycle transition [16]. In addition to the transcriptional and translational restraints imposed on CDKs availability, the activity of CDKs inhibitors (CKIs) confers another layer of regulation [16]. For further scrutiny, mitotically-committed cells deploy an integrated surveillance-based mechanism at each stage, known as checkpoints. These protective measures comprise intricate signaling networks that scrutinize cell size, DNA damage, DNA replication, DNA post-replication, and mitotic spindles assembly [17-20]. Intriguingly, the existence of cell size homeostasis checkpoint in mammalian cells remains controversial [21, 22]. The consequences of checkpoints activation depend on the type, severity, as well as the timing of incident defects [23, 24]. The safeguard system dictates the cellular fate into completion, repair, permanent senescence, or eventual programmable death [23]. The destined cells rely, in most of the cases, on the dynamics of the primary tumor suppressor gene p53. As such, it can induce a transient response of cell cycle arrest and repair, or destine the cells into terminal fates

[25, 26]. Although the exact mechanism that determines the selection between recovery and permanent arrest is doubtful, earlier studies suggested that p53 protein dynamics predominantly govern the decision. In this context, sustained p53 signaling leads to terminal senescence, while intermittent signaling favors damage recovery [27, 28].

As mentioned earlier, cell cycle regulation is very complex and exhibits a multi-layered network of interactions. The next sections will highlight the principal factors involved in cell division with simplified illustrations.

1.1.1 Cyclins

The terminology of cyclin originated from the phenomenal synthesis and degradation of these proteins in each cell cycle. Cyclins contain heterogeneous protein members with less conserved sequence homology and molecular weights ranging from 35–90 kDa. These members harbor a characteristic cyclin box and carboxy-terminal box. The later box is essential for proper protein folding, while the former box mediates binding and allosteric activation of the respective cyclin-dependent kinase [29]. In the human genome, there are approximately 30 genes that encode cyclins, whereas phylogenetic analysis classified these proteins into 16 subfamilies. However, in mammals, the cell cycle-related cyclins comprise four subfamilies or types, known as A, B, D, and E. The D-type cyclins are conserved only in eumetazoans [30]. The B-type cyclins are conserved in amoeba, fungi, and animals, exhibiting a cytoplasmic localization, while the other types are predominantly nuclear proteins. Broadly, depending on their temporal dynamics, cell cycle-related cyclins are categorized into four classes. The first class mediates cell cycle entry into the G1 phase in response to different stimuli. Other classes of cyclins include G1/S cyclins, S phase cyclins, and M phase cyclins. The presence of multiple cyclin molecules in yeast and mammalian cells suggested functional redundancy and compensatory actions. The generation of various knock out models elucidated, to a certain extent, the compensation and importance of different cyclins in homeostasis and development [31].

1.1.1.1 A-type cyclins

In mammalian cells, two types of cyclin A exist. Cyclin A1 present exclusively in germ cells while cyclin A2 present in all types of cells [32]. The cyclin A1 knockout mice are phenotypically viable and develop normally. On the other hand, cyclin A2 knock out is embryonically lethal shortly after blastocyst implantation. The cyclin A1 type may compensate

cyclin A2 prior to implantation, but not at the following stages. In cultured cells, cyclin A2 is indispensable to the S phase and G2/M transition.

1.1.1.2 B-type cyclins

The B-type cyclins consist of three members; B1, B2, and B3 cyclins, which are essential for the mitotic division. Both B1 and B2 cyclins are predominantly expressed in the majority of the cells, whereas B3 cyclin is limited to meiotically-dividing cells [33]. The B1 cyclin has a higher level of expression than B2 cyclin. Several studies suggested non-redundant functions of B1 and B2 cyclins due to their unique subcellular localization [34]. The cyclin B1 co-localizes to the microtubules and relocates to the nucleus during mitosis. The B2-type, in contrast, is associated with the Golgi apparatus and does not translocate to the nucleus during mitosis. Instead, it distributes evenly throughout the cell [35-38]. It became clear that the interaction between cyclin B1 and CDK1 promotes nuclear lamina disintegration, chromosomal condensation, and mitotic spindle assembly. The CDK1-cyclin B2, however, is essential to Golgi apparatus disassembly during mitosis [39, 40]. A previous study reported that the CDK1-cyclin B2 complex localizes to the centriolar satellite [41], whereas other studies indicated that the proper control of cyclin B2 is essential for centrosome separation [42]. In *Xenopus* oocytes, the bipolar spindle formation relies on the appropriate localization of cyclin B2 [43]. The cyclin B1-deficient mice suffer from embryonic lethality, while cyclin B2 knockout mice develop typically [44]. These observations suggest that B1-type cyclin is indispensable for embryonic development and can compensate cyclin B2, though they are different in cultured cells [39, 45].

1.1.1.3 D-type cyclins

Mammalian cells express three D-type cyclins, namely D1, D2, and D3. In human cells, CCND1, CCND2, and CCND3 genes encode D1, D2, and D3 cyclins, respectively. These types of cyclins are relatively unstable and exhibit a lineage-specific manner of expression in response to external mitogenic stimuli [46-49]. For instance, the NF-κB factor is a crucial regulator of D cyclins transcriptional activity owing to its enhanced occupancy over the promoters of CCND1 [50]. The temporal expression of D cyclins dictates the fate of proliferating cells, where the withdrawal of growth factors depletes cyclin D expression, regardless of the cell cycle phase [51]. Cyclin D1 is virtually expressed in all proliferating cells, while D2 and D3 cyclins are associated with B lymphocytes and T lymphocytes, respectively [52, 53]. Among several growth factors stimuli, the transcriptional activation

of D cyclins relies mainly on the RAS/RAF/MEK/ERK signaling pathway [31, 54, 55]. At the translational level, the PI3K-AKT-mTOR/SK1 signaling cascade promotes the expression of cyclin D proteins. The autophosphorylation of D cyclins mediates their stability and nuclear localization [56], whereas GSK-3β negatively regulates the stability of the protein through ubiquitination and proteasomal-mediated degradation [57]. Once the cell commits to divide and exits the G0 phase, the elevated D-type cyclins bind with CDK4/6 to form holoenzymes, which mediate cell cycle progression. The association with either CDK4 or CDK6 exhibits a cell-specific manner. The assembly of cyclin D-CDK4/6 complex requires a sustained RAF/MEK/ERK signaling to drive the G1 beyond a restriction point, where the mitogen induction is no longer required [31]. It is worth noting that the genomic locus of cyclin D1 (CCND1 gene) is one of the most frequently amplified hotspots among all types of tumors [56].

Triple knockout mice lacking D-type cyclins suffer from defected hematopoietic cells and myocardial cells, leading to ultimate death at late gestation [58]. Nevertheless, the loss of individual D cyclins does not interfere with viability and leads to cell-specific impairments. For instance, cyclin D1-deficient mice are viable but experience a reduction in body size accompanied by neurological impairment, defects in mammary glands development, and resistance to breast cancer [59-61]. Cyclin D2 knockout mice exhibit an impairment in B-lymphocyte proliferation, post-natal pancreatic β-cell proliferation, and neurological defects [52, 62]. On the other hand, cyclin D3-defected mice are viable and demonstrate defected T-cell maturation, resistance to T-cell lymphoma, and B-cell development [53, 63]. Further studies indicated that specific double knockout of D cyclins are lethal either during embryogenesis or post-natal [64]. Thus, D-type cyclins are indispensable for proper development, at least in a cell-specific manner.

1.1.1.4 E-type cyclins

Similar to A-type cyclins, E cyclins contain two members with substantial sequence homology; E1 and E2 [65, 66]. The E cyclins share the same expression pattern in proliferating cells during normal mouse embryogenesis [67]. However, in adult tissues, E1 and E2 cyclins demonstrate some differences in their expression patterns. The two types show identical expression in all adult tissues except in spleen, which has E1 only, whereas skeletal muscles and heart mostly express E2 cyclin. Of note, E2 cyclin mRNA demonstrates elevated expression levels in polyploid hepatocytes with higher DNA content [68]. Nevertheless, both cyclins are involved in G1/S phase progression through binding and activation of CDK2 before S

phase entry [69, 70]. Subsequently, cyclin E-CDK2 complex phosphorylates downstream substrates to mediate DNA replication [71], centrosome duplication [72], histone genes' transcription, and DNA repair [73, 74]. A recent study established a functional relationship between cyclin E1 and sex chromosomes synapses, while E2 cyclin is crucial for homologous pairing and telomere integrity during mouse spermatogenesis [75].

Apart from the role in cell cycle regulation, E cyclins also have a kinase-independent function in hepatocellular carcinoma (HCC). This proposed function is mostly due to the frequent integration of hepatitis B/C virus (HBV/HCV) into cyclin E1 genomic locus [76]. The stable integration leads to constitutive expression of cyclin E and tumorigenesis, regardless of cyclin E-CDK2 interaction [77]. Considering the functional redundancy between E-type cyclins, double knockout mice die during the early stages of embryogenesis due to endoreplication inhibition of placental giant cells [78]. Interestingly, cyclin E-deficient cells exhibit persistent quiescence in the G0 phase, where the deficient cells fail to integrate MCM proteins into DNA replication origins [79]. On the other hand, a single knockout of E cyclins does not compromise on viability and development [78]. These observations suggest that E1 and E2 cyclins are interchangeable and redundant. However, in a contradicting study, cyclin E2-null mice expressed cyclin E1 at higher levels following a partial hepatectomy, which led to enhanced liver regeneration. Meanwhile, cyclin E1-deficient mice demonstrated a delay in the G1/S phase associated with defected endoreplication of hepatocytes following the hepatectomy [80]. Therefore, E cyclins may have a non-redundant function in the S phase and endoreplication, at least during liver regeneration.

1.1.2 CDKs and CKIs

1.1.2.1 CDKs

Initial biochemical screening in mutants of different yeast species identified cell division cycle proteins, abbreviated as Cdc2 and Cdc28, as essential components for cell cycle progression [81]. The ability of human homologs to complement and drive cell division of mutant yeasts defected in Cdc2 led to the identification of human Cdc2 counterpart [82]. Later on, with the discovery of different Cdc proteins [83], the scientific community introduced a new nomenclature to these proteins, and hence it became cyclin-dependent kinases [84]. Current genomic approaches estimated that the human genome encodes 20 different members of CDKs, starting from CDK1 to CDK20 [84].

Of note, CDKs are highly divergent in terms of evolution and specialization. However, all CDKs harbor a characteristic catalytic core consists of an ATP-binding pocket, active T-loop motif, and PSTAIRE-like cyclin binding domain. The later domain binds to respective cyclin, which promotes the T-loop displacement and hence exposes the substrate-binding domain to mediate the phosphorylation reaction [85]. The phosphorylation of most CDKs can either possess an activating or inhibitory outcome depending on the phosphorylated residue. For instance, the phosphorylation of threonine 161 residue by cyclin-dependent activating kinase1 (CAK1, also known as cyclin H-CDK7) promotes substrate binding and stability of CDK complex. On the contrary, the kinase inhibitors WEE1 and MYT1 provoke CDK inactivation by phosphorylating the threonine 14 residue and tyrosine 15 residue, respectively [86]. However, the CDC25 phosphatases can render CDKs active by dephosphorylating these residues [87].

In yeast cells, the CDKs homologs fall into two major categories based on their temporal and functional relevance. The first category does not bind to a specific cyclin; instead, it interacts with many cyclins. The second group has a cycling-specific binding. Though the former group is associated with cell cycle functions and oscillation, the latter group, on the other hand, regulates the transcriptional activity of other genes [86]. However, despite the widespread acceptance of the same concept in human cells, recent studies challenged the postulation of the cell cycle-specific functions attributed to the first group of CDKs [88]. For instance, the cyclin D1-CDK4 complex phosphorylates the run-related transcription factor 2 (RUNX2) and mediates its degradation, which in turn inhibits osteoblasts differentiation [89]. In a similar line, CDK1 and CDK2 phosphorylate the enhancer of zeste homolog 2 (EZH2) at the threonine 350 residue to enhance the protein recruitment at target genes promoters [90]. This recruitment mediates epigenetic silencing of target genes through the deposition and maintenance of the repressive histone chromatin mark H3K27me3. Therefore, CDK1 and CDK2 aid in global epigenetic-derived transcriptional reprogramming. It is also not surprising to deduce kinase-independent functions of cell cycle-related CDKs in transcriptional regulation. For example, CDK6 interacts physically with the RUNX1 transcription factor and diminishes its transcriptional activity, causing myeloid differentiation blockade [91]. Intriguingly, CDK6 induces transcriptional activation of its repressor $p16^{INK4A}$ to antagonize the uncontrolled carcinogenesis [92]. Thus, despite all controversies, the impact of cyclins and CDKs discovery awarded the 2001 Nobel Prize in Physiology or Medicine to the three distinguished scientists Tim Hunt, Paul Nurse, and Leland Hartwell.

1.1.2.2 CDK1

The landmark discovery of CDK1 homolog (known as Cdc2 or Cdc28) in budding yeasts contributed to an unprecedented understanding of the eukaryotic cell cycle regulation [93]. In the model organism *Xenopus laevis*, Cdk1, and Cdk2 are the main drivers of the cell cycle, despite the active expression of other CDKs [94]. Similarly, in yeast cells, Cdk1 alone is sufficient for steady cell cycle progression through an association with various stage-specific cyclins [95]. Also, the crosstalk between Cdk1 and other CDKs coordinates different regulatory processes [96]. The sole capability of CDK1 to drive cell cycle progression did not demonstrate the same reliability in mammalian cells. However, a landmark study conducted in 2007 challenged that notion and indicated that CDK1 alone promotes mammalian cell division and compensates for the diminished activities of other interphase CDKs [97]. CDK1 has a preferential binding to B-type cyclins, where it binds to B1 and B2, but not B3 cyclin. The kinase-dependent activity of cyclin B-CDK1 complex triggers the post-translational modification of more than 70 distinct proteins and a considerable number of putative proteins [84].

Interestingly, the subcellular localization of B-type cyclins dictates the substrate specificity and downstream mitotic events mediated by cyclin B-CDK1 complexes [36]. In this context, the nuclear localization of cyclin B1 is necessary to direct its CDK1 heterodimeric complex to induce chromosomes condensation, nuclear lamina disintegration, and microtubules reorganization. As mentioned before, however, the cytoplasmic localization of cyclin B2-CDK1 is limited to the Golgi apparatus. So, as expected, different localization-associated motifs lie within B-type cyclins and not within CDK1 itself to allow shuttling between cytoplasm and nucleus. Of note, Gavet and pines performed an elegant study using the Forster resonance energy transfer (FRET) microscopy to elucidate the events driven by cyclin B1-CDK1 in a temporal resolution [98]. The complex mediates its activity before the nuclear lamina disintegration and accumulates over time to reach a maximal level in almost 30 minutes. Remarkably, varying levels of cyclin B1-CDK1 trigger different mitotic events. In HeLa cells, initial activation of the complex showed a high correlation and induced cell rounding in prophase, which refers to cytoskeleton reorganization associated with mitosis. Lower levels of cyclin B-CDK1 triggers its nuclear shuttling and can also activate the anaphase progression. On the contrary, higher levels of the complex are crucial to initiate nuclear envelop breakdown and centrioles disassembly. Therefore, the inhibition of CDK1 results in cell cycle arrest at the G2/M phase.

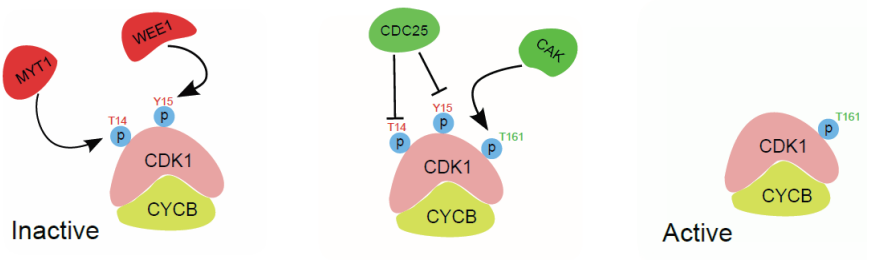

Figure 1. The regulatory phosphorylation statuses of cyclin B-CDK1 complex

The remarkable observation of the Cdk1 ability to compensate for the loss of Cdk2 by the interaction with cyclin E and permitting G1/S transition raised another question [99]. In a reversible situation, is Cdk2 able to compensate for the loss of Cdk1? To address this hypothesis, Satyanarayana and colleagues substituted both copies of mouse Cdk1 with Cdk2 in the same genomic locus [100]. This substitution caused an early embryonic lethality indicating the pre-eminence of Cdk1 in proliferation and early development. In addition to cyclins B and E, CDK1 also interacts with cyclin A at the end of the S phase to phosphorylate different proteins, such as MCMs, p53, and BRCA2 [84]. Thus, CDK1 may have overlapping functions with CDK2 considering their association with A-type cyclins. A proposed model suggests that CDK1 interacts with cyclin E and shuttles immediately to the nucleus to induce G1/S transition. Later on, CDK1 associates with B cyclins to initiate the M phase [39]. Notably, ATP-competitive potent inhibitors of CDK2 tend to inhibit CDK1 as well, causing higher toxicity. The advances in X-ray crystallography revealed a subtle but profound difference between the conformational energy of cyclin-free CDK1 and CDK2 [96]. Thus, one would speculate that the binding specificity dictates the non-redundant functions of both CDKs.

Apart from the association with cyclins, a recent report exhibited that CDK1 kinase-dependent activity is required to inactivate the spindle assembly checkpoint and activate the anaphase-promoting complex/cyclosome (APC/C). In this context, CDK1 phosphorylates DIAPH1 protein to maintain the metaphase cortical tension [101]. Intriguingly, another recent study demonstrated a new role of CDK1 in yielding nascent DNA synthesis and

DNA replication fork progression, whereas its inhibition alters DNA integrity [102]. Taken together, CDK1 may have a diverse interaction network that contributes directly or indirectly in cell cycle progression.

1.1.2.3 CDK2

The S phase entry relies mainly on the activity of CDK2 in association with cyclin E [69, 103]. The CDK1 localization is strictly nuclear regardless of the cell cycle stage. Unlike CDK1, which is phosphorylated upon binding with cyclins, CDK2 acquires phosphorylation before the association with cyclin E [104]. The bound CDK2 complex gets activated by CDC25 phosphatases, which remove the inhibitory phosphorylation at tyrosine 15 residue. The cyclin E-CDK2 complex reaches a maximal activity in G1-S cells, whereas quiescent cells are almost devoid of any activity [105]. The passage through the G1 restriction point is crucial for the accumulation and downstream activity of CDK2 and cyclin E [106]. The complex facilitates the loading of CDC45 protein, a member of replicative helicase, in the early S phase to initiate DNA synthesis. This loading of CDC45 is a rate-limiting step to progress initially through the S phase and fire dormant replication origins in case of DNA damage [107]. The ablation of cyclin E-CDK2 results in cell cycle arrest at the G1 phase. However, replicating cells are intolerant to the higher activity of the CDK2 complex, which leads to exhaustive origins firing and replication stress [104].

In addition to the function mentioned above, cyclin E-CDK2-mediated kinase activity regulates several downstream target proteins. For instance, it inactivates the retinoblastoma protein (RB) to promote the release of E2F transcription factors. Also, the complex primes the degradation of CDKN1B protein ($p27^{Kip1}$), which is a negative regulator of G1 progression [73]. Nevertheless, cyclin E-CDK2 mediates the phosphorylation and subsequent activation of the acetyltransferases coactivator proteins p300/CBP [108]. Later in the S phase, cyclin E dissociates from CDK2, and cyclin A replaces it to drive the S/G2 transition [84]. Concomitantly, cyclin A-CDK2 permits phosphorylation-mediated activation of the B-MYB transcription factor. This factor, in turn, induces the transcription of cell cycle-related genes, such as topoisomerase II α and HSP70 [109].

In contrast to the widely adopted notion that views the elevated level of cyclin E as an indicator of active CDK2, recent observations challenged this notion [104]. In unperturbed cells, CDK2 phosphorylates the serine 384 residue of cyclin E upon complex formation. This phosphorylation renders cyclin E susceptible to further inhibitory phosphorylation by GSK-3β at

threonine 380 residue. The latter event, cooperatively with other phosphorylation, triggers binding to ubiquitin ligases and degradation [110]. However, higher levels of available cyclin E may reflect inhibition of GSK-3β itself rather than an elevated activity of CDK2. Also, the ablation of CDK2 leads to the nuclear localization of CDK1 earlier in the cell cycle [111]. In this case, it is possible that CDK1 binds to cyclin E and translocates prematurely to the nucleus in order to take over the function of CDK2. However, a functionally-active CDK2 complex is indispensable for the proper repair of damaged DNA, whereas CDK1 can not compensate CDK2 [111]. Of note, Cdk2-null mice experience senescence upon sustainable exposure to the oncogenic MYC signaling. On the other hand, the expression of wildtype Cdk2 circumvents the induced senescence and is essential for MYC phosphorylation [112, 113].

1.1.2.4 CDK4/6

Among other CDKs, CDK4 and CDK6 are, perhaps, the most extensively studied proteins due to their oncogenic hyperactivity or amplification in the majority of tumors [56, 114]. CDK4 and CDK6 share almost 71% amino acids identity, and as mentioned earlier, D-type cyclins form the main catalytic heterodimers of CDK4/6 complex [39]. Both CDKs demonstrate distinct subcellular localization in specific cell types. For instance, mouse astrocytes harbor exclusive nuclear distribution of Cdk4, whereas Cdk6 is predominantly cytoplasmic [115]. The mitogenic-induced transcriptional accumulation of D cyclins has to avert the inhibitory effect imposed on CDK4/6 to permit efficient binding. Following the association with D cyclins, CDK4 and CDK6 undergo T-loop phosphorylation mediated by CAK1 at threonine 172 and 177 residues, respectively [116]. Though, there is evidence against the CAK1-mediated activation of CDK4/6, as the activation may involve another proline-directed kinase [117]. Nevertheless, historically, activated cyclin D-CDK4/6 have been shown to drive cell proliferation and G1 phase entry through downstream phosphorylation of a limited number of targets. For example, CDK4/6 holoenzymes implement monophosphorylation at multiple sites of the tumor suppressor retinoblastoma (RB), yielding it partially inactive [118]. The phosphorylated RB receives further phosphorylation mediated by cyclin E-CDK2 to allow cell cycle progression at the late G1 phase. Of note, CDK4 and CDK6 possess a preferential selection of the RB phosphorylation site. CDK4 modifies the threonine 821 residue, while CDK6 acts on threonine 826 residue [119]. Thus, it is believed that CDK4 demonstrates a higher kinase efficiency towards RB. The CDK4/6 complexes also inactivate other related pocket proteins; p107 and p130 [120]. An additional example of cyclin D-CDK4/6 targets is the Forkhead Box M1

(FOXM1) transcription factor, which is crucial to surpass cellular senescence and promote G1/S transition [121]. Further targets include SMAD3, which has anti-proliferative functions and regulated by the upstream tumor growth factor-β (TGF β). The CDK4-mediated phosphorylation of SMAD3 induces the transcription of proliferation-associated genes [122].

In a surprising changing paradigm observation, Cdk4 and Cdk6 were explicitly shown to be dispensable for proliferation and cell cycle entry from quiescence [123]. The double knockout mice exhibited normal embryonic organogenesis and proliferation despite dying at the late embryonic stage or post-natally. These mutant mice displayed severe anemia, which was the primary cause of death. Intriguingly, the CDK4/6-null mouse fibroblasts usually proliferate in response to stimulatory growth signals and even acquire immortality [123]. On the contrary, Cdk4-deficient mice are viable but suffer from a reduction in various organs size as well as the total body size. Also, proliferating mouse fibroblasts lacking Cdk4 display a delay in the S phase entry from quiescence. As the case in cyclin D-deficient mice, Cdk4 knockout affects pancreatic β cells resulting in insulin-deficient diabetic mice [124]. However, a recent study demonstrated that insulin, which plays a mitogenic role in proliferating cells, increases the activity of cyclin D1-CDK4 complex. Subsequently, the complex maintains transcriptional silencing of gluconeogenesis genes in a cell cycle-independent fashion [125]. Interestingly, Cdk6-null mice exhibit severe thymic atrophy owing to the impaired proliferation and development of thymocytes [126]. Collectively, CDK4 and CDK6 may act in a distinct spatio-temporal fashion depending on tissue type, localization, and expression timing.

1.1.2.5 CKIs

In addition to the upstream inhibitory effects of WEE1 and MYT1, cyclin-dependent kinase inhibitors (CKIs) represent the major regulatory brakes on CDKs activities. Currently, CKIs comprise two families of closely related proteins. The first one is the CDK-interacting protein/kinase inhibitory protein (CIP/KIP) family, while the second is the inhibitors of CDK4 (INK4) family. The CIP/KIP family consists, so far, of three members; $p21^{Cip1}$ (encoded by CDKN1A), $p27^{Kip1}$ (encoded by CDKN1B), $p57^{Kip2}$ (encoded by CDKN1C) [127-129]. Despite the overwhelming association with inhibitory functions, depending on their phosphorylation status, the CIP/KIP proteins can also activate their corresponding CDKs [130, 131]. Unphosphorylated $p21^{Cip1}$ and $p27^{Kip1}$ binds directly to cyclin D-CDK4/6 and block their kinase activity. Moreover, these unphosphorylated CIP/KIP proteins accumulate upon withdrawal of growth factors to inhibit cell cycle entry [56].

Phosphorylated CIP/KIP proteins are indispensable for proper association and activation of Cyclin-CDK complexes, especially for CDK4/6 complexes [132-134]. In this context, $p27^{Kip1}$ dissociates from cyclin E-CDK2 and binds to CDK4/6 complex, which in turn activates the CDK2 complex and permits S phase progression. Meanwhile, CIP/KIP proteins can also inhibit cell cycle progression in CDK2-deficient cells, casting more doubts on the mode of action of these proteins [135]. Notably, $p21^{Cip1}$-null mice develop typically without spontaneous tumors unless they experience genotoxic-induced DNA damage [136]. Also, cells devoid of $p21^{Cip1}$ are more susceptible to Ras-induced transformation [137].

As deduced from the nomenclature, the INK4 family specifically binds to monomeric CDK4 and CDK6 to hinder their association with D cyclins. This family includes various protein members; $p16^{INK4a}$ (encoded by CDKN2A), $p15^{INK4b}$ (encoded by CDKN2B), $p18^{INK4c}$ (encoded by CDKN2C), $p19^{INK4d}$ (encoded by CDKN2D) [138-140]. The INK4 proteins inhibit CDK4/6 monomers in response to growth inhibition signal and DNA damage. In turn, INK4 proteins direct either cell cycle arrest or apoptosis [141]. The genomic locus encoding $p16^{INK4a}$ and $p15^{INK4b}$ is frequently deleted in a wide array of tumors [142]. Somatic alterations associated with deletion or point inactivating mutations of $p16^{INK4a}$ are common also among many cancer [141]. Interestingly, the elevated level of $p16^{INK4a}$ expression is a hallmark of oncogene-induced cellular senescence, which promotes premature aging to circumvent the oncogenic transformation [143].

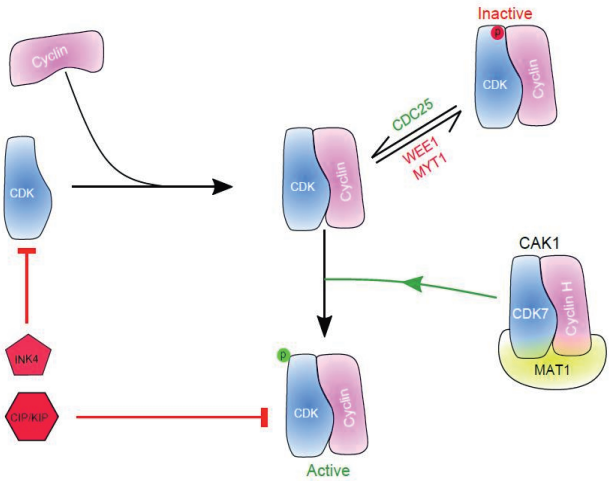

Figure 2. A simplified illustration of the general regulation of cyclin-CDK complexes

1.1.3 E2F factors and Retinoblastoma proteins

1.1.3.1 E2F transcription factors

The first E2F factor was initially discovered more than 30 years ago as a DNA-binding protein that associates and activates adenovirus E2 promoter [144, 145]. The E2F consensus binding motif, TTTCGCG, present twice within the adenovirus E2 promoter. Further studies indicated that the same consensus sequence present within promoters of various growth-responsive elements, such as c-MYC, cyclin A, cyclin D, CDK1, and DNA polymerase α [146]. Currently, the mammalian E2F family consists of eight members; E2F1−E2F8 [147]. Among the eight members, E2F1−E2F3 represent the activator members, while E2F4−E2F8 are associated with repressive functions. The E2F1-E2F6 members are typical E2F factors comprising one DNA-binding domain. These factors form heterodimers with the dimerization partner (DP1/DP2) proteins to pursue their functions. Also, they bind to different members or retinoblastoma pocket proteins [148]. In contrast, E2F7 and E2F8 exhibit two DNA-binding domains, and they neither dimerize with DP proteins nor bind to pocket proteins. Thus, E2F7 and E2F8 are the atypical class of E2Fs [149]. The tremendous progress in the research area concerned with cell growth response established E2F members as crucial regulators of transcriptional programs associated with cell cycle progression [150, 151].

The activator E2F1−E2F3 factors demonstrate temporal variation in their levels across different cell cycle phases. These factors start to accumulate in the G1 phase, reach a peak level upon the S phase entry, and decline in G2. The intricate balance of E2F1−E2F3 levels determines the cell fate, as their constitutive expression after the S phase induces apoptotic response [152]. However, as expected, they are almost diminished in quiescent cells. Mouse knockout experiments demonstrated the indispensable role of activator E2Fs in proliferation and development. The ablation of E2F1−E2F3 severely restricts cell proliferation. Intriguingly, the loss of either E2F1 or E2F2 in mouse fibroblasts does not interfere with cell proliferation, while E2F3 deficiency restricts proliferation by 50%. The E2F1 and E2F2 double knockout mice usually develop till adulthood, whereas E2F1 and E2F3 double mutant mice die during early embryonic development. The same outcome also applies to E2F2 and E2F3 mutant mice, indicating the central role of E2F3 for proper development [150]. The transactivation-mediated functions of E2F1−E2F3 in cell cycle regulation are mostly attributed to the association with pocket proteins.

Considering the repressive E2F factors, the association of E2F4 and E2F5 with retinoblastoma pocket proteins prompts a quiescent state at the G0 phase. The formation of these repressive complexes counteracts the action of activator E2Fs through transcriptional repression of target genes. Of note, E2F4 and E2F6 present throughout the whole cell cycle phases; however, their subcellular localization is the determinant factor [153]. During G0 and early G1 phases, these factors are predominantly nuclear in complex with pocket proteins. Further progression in the G1 phase provokes the dissociation of these repressive complexes, followed by cytoplasmic redistribution of E2F factors [154]. Nevertheless, the genome-wide analysis, using chromatin immunoprecipitation followed by sequencing (ChIP-seq), unraveled a versatile function of E2F4 in transcriptional modulation of target genes. Indeed, E2F4 also acts as a transcriptional activator of genes involved in cell cycle regulation, DNA repair, and apoptosis [155]. Therefore, E2F4 may have overlapping functions depending on the cell identity and cell cycle phase.

The expression of other factors, E2F6-E2F8, follows a cyclic pattern, where it peaks at S/G2 phase and declines during G2/M phase transition and progression [156, 157]. The repressive action of these factors is crucial at late DNA replication, especially in stress conditions, and seems to be independent of retinoblastoma proteins [158]. The repressive role of E2F6 is redundant with E2F4, with the latter being able to rescue E2F6 loss. In a similar line, the singular loss of either E2F7 or E2F8 does not interfere with the mouse development, reflecting functional redundancy imposed by E2F4 and E2F6. However, a double mutant is embryonic lethal due to extensive apoptosis and improper vascularization [159].

1.1.3.2 Retinoblastoma proteins (RB)

Historically, the retinoblastoma family of proteins was identified in retinal cells neoplasm due to loss-of-function mutation in a candidate 4.7 kb fragment at chromosome 13q [160]. The first cloned fragment was denoted as the RB1 gene. Later investigations revealed the frequent alterations associated with RB1 gene loss in various types of tumors. Thus, it became clear that RB1 functions as a tumor suppressor gene, and particularly in cell cycle regulation [161, 162]. The retinoblastoma family contains three proteins members, namely RB (RB1/p105), RBL1 (p107), and RBL2 (p130). Altogether, the three members are known as pocket proteins. This terminology reflects the presence of a highly homologous pocket structure that mediates the interaction with other proteins and transcription factors [84]. The pocket structure contains a spacer between two conserved domains;

A and B. E2F factors, as well as other proteins harboring the LXCXE motif, interact with these conserved domains.

The RB proteins regulate cell cycle progression distinctively in E2F-dependent or independent manners. The first mode of action relies on the physical association between RB proteins and E2F members. The other mode of action involves direct or indirect binding with more than 200 proteins, including histone modifiers and DNA repair proteins [162]. Considering the E2F-dependent regulation, RB/p105 interacts with the transcriptional activators E2F1−E2F3 to hinder their accessibility to the promoters of downstream target genes. Similarly, RB/p107 and p130 proteins interact with the E2F4 and E2F5 repressors. Regarding E2F-independent regulation, RB proteins bind to histone deacetylases, such as HDAC1, to maintain epigenetic silencing of cell cycle-related genes. Broadly speaking, histone acetyltransferases introduce acetyl marks at the promoters of actively transcribed genes, whereas HDAC activity counteracts this hyper-acetylation. The RB-HDAC1 complex epigenetically represses the transcription of cyclin E, as well as other E2F-responsive genes, restricting cell proliferation [163]. The SUV39H1 methyltransferase interacts with RB to establish the repressive chromatin mark at histone 3 lysine 9 at the promoters of E2F targets [164]. Another prominent aspect of RB-mediated regulation, albeit it is indirectly related to cell cycle progression, is the interaction with DNA repair proteins. For instance, RB/p105, p107, and p130 directly interact with the Ku70/Ku80 heterodimer complex in response to DNA damage. The latter heterodimer is a crucial member of the non-homologous end joining (NHEJ) DNA repair pathway. The Ku70/Ku80 complex recognizes the sites of DNA double-stranded breaks (DSBs) and recruits other members of repair machinery [165]. However, the role of RB proteins in recruiting Ku70/Ku80 remains elusive [162]. Though, a recent study revealed that the homologous recombination (HR) pathway, which acts to repair DNA in the S phase, recruits RB directly at sites of DSBs [166]. In a combination of E2F-dependent and independent regulation, RB proteins induce cellular senescence by positive regulation of senescence-associated heterochromatin formation (SAHF) [167, 168]. The establishment of SAHF requires HDAC activity, while the transcriptional reprogramming urges transcriptional repression of E2F targets [169]. The RB pocket proteins contribute to other non-canonical functions [162], which are summarized in figure 3, though they are beyond our current focus.

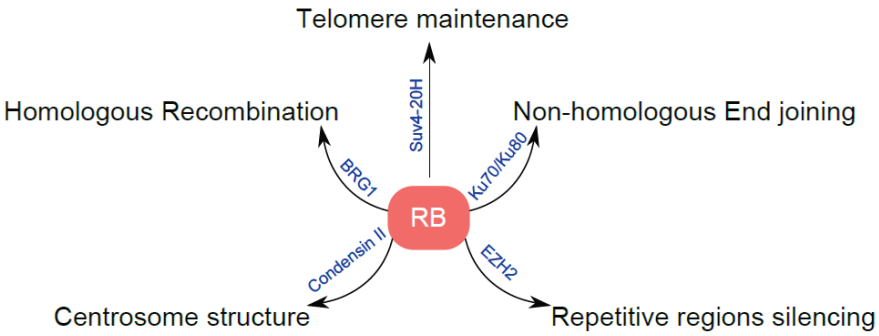

Figure 3. A summary of RB non-canonical functions (Redrawn from Velez-Cruz and Johnson; Int J Mol Sci; 2017 [162])

The widely accepted model of RB regulation utilizes a balance between three oscillating states; unphosphorylated, hypo-phosphorylated, and hyper-phosphorylated RB proteins. The RB/p107 protein has multiple phosphorylation sites (16 sites in case of RB1), but cyclin D-CDK4/6 complexes do not recognize all of them [84]. In response to mitogens at the early G1 phase, cyclin D-CDK4/6 complexes-mediated kinase activity targets RB/p105 and RBL2/p130 to inactivate RB proteins partially [170]. Subsequently, E2F1−E2F3 factors dissociate from RB/p105, at least partially. Further cell cycle progression into the late G1 phase permits the accumulation of cyclin E-CDK2 complexe, which phosphorylates all RB proteins; p105, p107, and p130. The late G1 phase experiences a complete release and translocation of E2F4 and E2F5 into the cytoplasm. However, a recent study challenged this model and provided evidence for the existence of various mono-phosphorylated RB isoforms exclusively in the early G1 phase [118]. In this scenario, cyclin D-CDK4/6 complexes prompt 14 independent mono-phosphorylated RB isoforms. Following the passage through the G1 phase restriction point, the active cyclin E-CDK2 complex promotes hyperphosphorylation of RB isoforms. Intriguingly, the cyclin D-CDK4/6-mediated mono-phosphorylation of RB isoforms is functionally active and essential to provoke DNA damage response as a consequence of genotoxic stress. In contrast; however, the unphosphorylated status of RB is required for differentiation and cell cycle exit. The unphosphorylated state depends on the activity of protein phosphatase 1 (PP1), which occurs within the anaphase stage till G1-phase [171]. Nevertheless, other studies indicated that the high prevalence of active CDKs could yield permanent inactivation of RB proteins through priming the serine 567 residue and exposing the protein to

proteolytic degradation [172]. Although this proteolytic activity may help in counteracting the RB inhibitory effects, it can also trigger apoptosis and anti-proliferative measures to circumvent the undesirable proliferation [172]. Of note, the transcription factor ICBP90 negatively modulates the transcriptional activity of RB1 through occupying putative binding sites at the gene promoter. Thus, the overexpression of ICBP90 reduces the mRNA level of RB1, which occurs in several cancer types [173]. The proper expression of Rb1 is also crucial for development [174]. Homozygous deletion of Rb1 is embryonic lethal in mice due to excessive p53-dependent apoptotic events in the nervous system leading to defected neurogenesis [175, 176]. Mouse models devoid of both Rb1 and p53 develop aggressive tumors [174]. Interestingly, mice carrying a heterozygous deletion of Rb1 do not develop spontaneous tumors, whereas an additional loss of Rb/p107 causes retinal dysplasia. Thus, Rb/p107 can rescue the partial loss of Rb1 *in vivo* [177].

Collectively, as presented in the previous sections, the mammalian cell cycle regulation involves tens or even hundreds of cellular proteins and factors. It is very challenging to provide an integrated model of all different regulators. However, to a certain extent, one would provide a simplified model of these interconnected factors by considering only cyclins, CDKs, CKIs, RB, and E2F factors. A streamlined model depicting the general cell cycle regulatory events is presented on the next page. Also, The following sections discuss the surveillance-based mechanisms that ensure the error-free progression of the cell cycle.

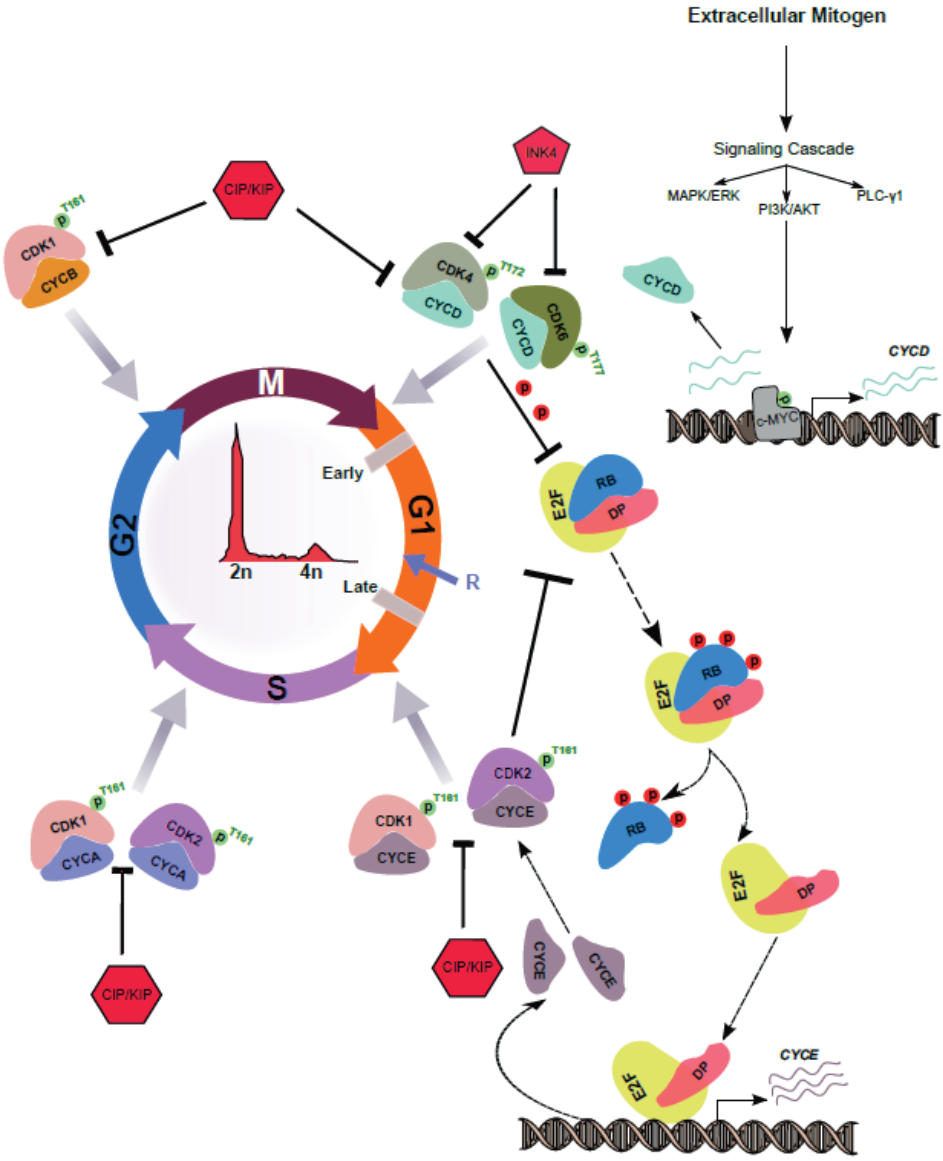

Figure 4. A comprehensive schematic illustration of mammalian cell cycle regulation. *The green phosphorylation represents an activating event, and the red phosphorylation means an inhibitory event*

1.1.4 Cell cycle checkpoints

Almost 30 years ago, Hartwell and Weinert proposed that the replicating cells implicate a sequential cell cycle dependencies to ascertain the generation of error-free progeny [178]. The ancient evolution of intricate sensory-transducing-effector circuitries assents the cell to surveil the sequence, integrity, and fidelity of the cell division process [16]. These surveillance circuitries evolved into well-defined checkpoints that operate at distinctive phases. The faultless fulfillment of checkpoints requisites underlies the faithful cell cycle progression. Thus, proficient eukaryotic cell harbors four critical checkpoints. The first one assesses the G1/S phase entry, while the second checkpoint is active in the S phase. Following the entry to the G2 phase, the third checkpoint assures the DNA integrity and assembles the DNA damage response elements to approve the G2/M transition. Lastly, the spindle assembly checkpoint (SAC) is the master regulatory self-assessment point in the M phase. The unfaithful achievement of checkpoints requirements perturbs cell cycle progression and triggers arrest, leading to various subsequent outcomes. Therefore, these checkpoints are valuable therapeutic targets for neoplasia exploitation [23].

1.1.4.1 G1/S Checkpoint

The exit from a quiescent state and the onset of the G1 phase occur in response to growth factors stimulation, as mentioned earlier. These stimuli drive the cell through a restriction point known as “R” checkpoint. Beyond this point, the mitogens are no longer required to enter into the DNA replication stage. Therefore, the R point discriminates between two distinct compartments of the G1 phase [179]. The first compartment is the G1-pm, which refers to the post-mitotic events that continue from the previous cycle to the R point. The second compartment is the G1-ps, which defines the pre S phase entry that does not respond to mitogen withdrawal. The rapid biosynthesis of D cyclins and their association with CDK4/6 lead to the release of E2F factors and the accumulation of cyclin E-CDK2 complexes. The active cyclin D-CDK4/6 complexes interact with $p21^{Cip}$ and $p27^{Kip}$ proteins and titrate them away from cyclin E-CDK2 complexes. This stoichiometric titration is necessary for the accumulation and functional activity of cyclin E-CDK2 complexes before the S phase transition [31]. In the case of unfavorable conditions, the INK4 proteins inhibit the activity of CDK4 and CDK6 monomers, whereas the unbound D cyclins are ubiquitinated and targeted for degradation [141, 180]. Thus, the loss of cyclin D-CDK4/6 triggers the inhibitory actions of CIP/KIP proteins. The ectopic

expression of INK4 proteins enforces RB-dependent cell cycle arrest at the G1 phase [181].

The exposure to genotoxic stress induces rapid DNA damage response (DDR), which is an intricate signaling cascade of upstream sensors, transducers, and downstream effectors. During the G1 phase, initial double-stranded breaks (DSBs) mediate the phosphorylation of the sensory Ataxia Telangiectasia Mutated (ATM) kinase. The phosphorylated ATM protein activates the transducer checkpoint kinase 2 (CHEK2) via phosphorylation [182]. The latter kinase primes the CDC25A phosphatase, leading to its ubiquitination and subsequent degradation. Thus, the depletion of CDC25A results in the inactivation of cyclin E-CDK2 and cyclin A-CDK2 complexes, causing a blockade of S phase entry [183]. Moreover, the p53 activity represents a cornerstone in DDR during G1 phase progression. The activated ATM mediates p53 phosphorylation, which releases it from its associations with the negative regulator MDM2 [184, 185]. This release and stabilization of p53 induce transcriptional activation of $p21^{Cip1}$, which in turn binds to and inhibits cyclin E-CDK2 and cyclin A-CDK2 complexes [127]. Subsequently, the cell alters its progression into the DNA synthesis phase and arrests at the G1 phase either temporarily or permanently.

1.1.4.2 Intra-S checkpoint

The progression through S phase requires a coordinated effort to allow replication origin firing and replication fork elongation until the completion of DNA synthesis. In unperturbed cells, the assembly of a multiprotein complex, known as replisome, at the replication fork demarcates the DNA replication process. The eukaryotic replisome consists of the following components: 1) the minichromosome maintenance (MCM) helicase complex that unwinds the duplex DNA strands. 2) The clamp proliferating cell nuclear antigen (PCNA) protein, which forms homotrimer and encircles the parental DNA to tether the catalytic subunit of DNA polymerase and slide bi-directionally. 3) The RFC clamp loader that loads, opens and closes the PCNA sliding clamp using hydrolysis-derived ATPs. 4) The pol α-primase which creates the RNA-DNA hybrids. 5) The replication protein A (RPA) that removed the DNA secondary structure. 6) The DNA polymerases pol ε and pol δ, which replicate the leading and lagging strands, respectively [186, 187]. Other proteins also present at the replisomes such as CDC45, GINS, and CTF4; however, their exact functions yet to be elucidated clearly.

When the replication fork encounters a blockade or deprivation of nucleotides pool, it stabilizes the replisome with other factors. This

stabilization allows the replication fork to re-initiate the DNA synthesis upon the physiological relief. The inability to circumvent the blockade promotes replication fork stalling. The prolonged fork stalling results in DNA gabs and single-stranded breaks, which escalate to DSBs. Of note, among the stabilization factors, the heterotrimeric Csm3-Tof1-Mrc1 checkpoint mediator complex responds to stalled forks and activates the intra S checkpoint [188]. Thus, DSBs induced by stalled forks or genotoxic stress during the DNA replication activate the internal surveillance machinery in the S phase [23]. Subsequently, the Ataxia Telangiectasia and Rad3-related (ATR) kinase is phosphorylated and activates the downstream checkpoint kinase 1 (CHEK1) protein [189]. Similar to CHEK2 protein, the activated CHEK1 primes CDC25A and destines it for proteasomal degradation. In turn, the cell halts its progression and arrest in the S phase. Notably, the ATR-dependent activation of CHEK1 in the intra S checkpoint is independent of ATM signaling, despite their functional redundancy in other phases [190-192]. Intriguingly, ATR seems to respond to a wide variety of DNA-damaging agents and stress, whereas ATM is more specific to DNA DSBs [193]. Therefore, recent investigations suggest non-redundant functions of both ATM and ATR [194].

1.1.4.3 G2/M checkpoint

The presence of either unrepaired DNA lesions or improperly replicated genome alerts the G2/M checkpoint to prevent the mitotic onset. As explained earlier, the cyclin B-CDK1 complex represents the primary driving force towards the M phase. The alleviation of the inhibitory phosphorylation status, maintained by WEE1 and MYT1, requires a counteracting activity of CDC25 phosphatases [195, 196]. However, upon the activation of the G2/M checkpoint, the phosphorylated ATR/ATM proteins activate CHEK1 and CHEK2, respectively. The latter proteins mediate CDC25A degradation and sequestration of CDC25C into the cytoplasm through the binding of 14-3-3σ protein [197, 198]. Moreover, the activated CHEK1 and CHECK2 proteins phosphorylate and inhibit the polo-like kinase 1 (PLK1) protein [199]. In unperturbed cells, PLK1-mediated kinase activity is crucial to activating CDC25C phosphatase [200]. The rapid activation of PLK1 in the late G2 phase commits the cells towards mitosis, regulates centrosome maturation, phosphorylation of Aurora proteins, chromosomes condensation, and cytokinesis [201]. Hence, inhibiting the PLK1 activity restricts the mitotic entry upon checkpoint activation. Interestingly, the MYT1 kinase is a rate-limiting factor in checkpoint recovery upon DNA damage [202]. The ablation of MYT1 activity enhances the mitotic entry without proper alleviation of DNA damage. Of note, the p53-mediated transcriptional regulation is crucial

for maintaining an active G2/M checkpoint. Similar to G1/S checkpoint, challenging the replicating cells with DNA-damaging stress activates p53, which induces the transcription of $p21^{Cip1}$ [203]. Also, it upregulates the growth arrest and DNA-damage inducible 45 (GAAD45) protein and 14-3-3σ protein [204, 205]. As expected, $p21^{Cip1}$ binds and inactivates cyclin B-CDK1 complex, while GAAD45 dissociates CDK1 from cyclin B. The 14-3-3σ protein also sequesters the cyclin B-CDK1 into the cytoplasm [206]. Collectively, these successive events lead to G2/M cell cycle arrest.

1.1.4.4 Spindle assembly checkpoint (SAC) and mitotic catastrophe

Once the cell is committed to entering the M phase, it proceeds through four stages; prophase, metaphase, anaphase, and telophase, followed by cytokinesis. Following the nuclear envelop breakdown in prophase, the chromosomes reach maximum condensation and align across the equatorial plane of the cell. The metaphase chromosomes consist of sister chromatids held together by cohesion, and they attach to microtubules spindles through their kinetochores. The proper attachment underlies the faithful chromosomal segregation in anaphase, and hence the dividing cells deploy the SAC machinery to ensure the segregation fidelity [19]. The core SAC consists of MAD2, BUB1/BUBR1/BUB3 proteins, and the regulatory subunit CDC20 [207]. In favorable conditions, CDC20 binds and activates the anaphase-promoting complex/cyclosome (APC/C) ubiquitin ligase. The activated APC/C triggers the onset of chromosomes segregation through its E3 ubiquitin ligase activity that targets cyclin B and securin protein. The degradation of these proteins authorizes the release of the separase protein that resolves the sister chromatids cohesion and facilitates congressional movement [19]. Importantly, the cyclic alteration in the cyclin B-CDK1 level is crucial for APC/C-mediated activation of chromosomal disjunction. The declining levels of cyclin B-CDK1 dictate the activation of APC/C. However, the imbalance of these levels leads to the improper onset of anaphase and premature exit of mitosis [101]. In this scenario, cells harboring defected SAC signaling will undergo chromosomal instability and aneuploidy, which may result in oncogenic attributes [208]. Therefore, cells with functional SAC signaling prevent the onset of the anaphase stage upon misalignment or improper attachment of the microtubules to the kinetochores. As such, unattached kinetochores generate inhibitory signals that activate SAC to sequester the CDC20 subunit and halt the cell cycle [209].

The unfaithful chromosomal segregation, DNA replication stress, or high levels of aberrant aneuploidy coupled with checkpoint deficiency may lead to mitotic catastrophe [210, 211]. This phenomenon is an onco-suppressive

mechanism that obstructs cell proliferation and survival of mitotically-defected cells [212]. Cells with ongoing mitotic catastrophe exhibit unique morphological manifestations, including gigantic multi-nucleated cells associated with macronuclei or micronuclei. The emergence of macronuclei and micronuclei is an indication of chromosomal missegregation, and persistence of lagging chromosomes, respectively [212]. However, mitotic catastrophe dictates the defected cells to three alternate fates depending, partly, on cyclin B levels. In this regard, mitotic catastrophe destines the defected cells to death, known as mitotic death, when the cyclin B is abundant without mitotic exit. On the contrary, following cyclin B decline, the mitotic slippage promotes the exit of defected cells without the execution of death. Thus, the mitotic catastrophe may engage the apoptotic machinery in the subsequent G1 phase to mediate cell death. Alternatively, cells may undergo permanent senescence [212, 213].

Although the exact sensory mechanism that initiates mitotic catastrophe is partly unclear, several studies suggest the involvement of p53. As supporting evidence, p53-deficient cells undergo necrosis-mediated death upon the accumulation of mitotic defects [214]. Similarly, the caspase-2 precursor (CASP2) protein may also take part in signal transduction to execute mitotic death through BCL-2 proteins [215]. Mice models deficient in Casp2 accumulate aneuploidy cells during aging, whereas CASP2-deficient cells are more susceptible to aneuploidy-derived oncogenesis [216-218].

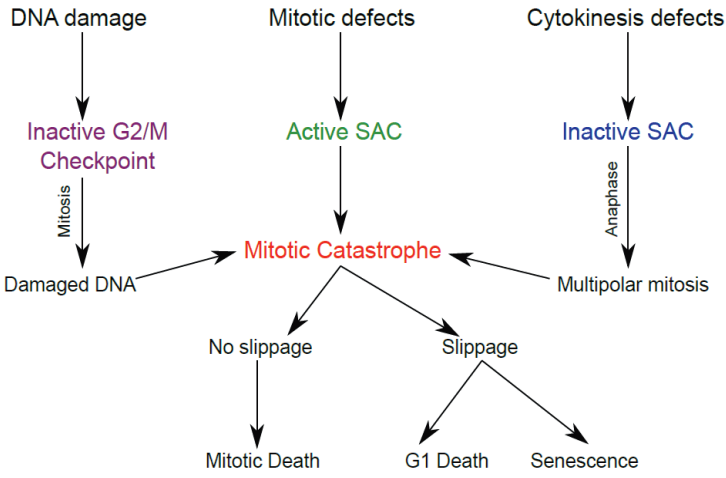

Figure 5. Summary of mitotic catastrophe events in response to various defects (Redrawn from Mc Gee; Mediators Inflamm; 2015 [213])

1.1.5 Upstream regulatory signaling pathways in cell cycle

The successive cell cycling requires sustained proliferative signaling to drive the G0/G1 transition and rewire the cellular transcriptional programs. Over the past decades, a growing body of evidence indicated the utmost importance of particular pathways underlying the oncogenic transformation and subsequent carcinogenesis. Among these pathways, the MAPK/ERK and PI3K/AKT signaling cascades are of prime importance. Therefore, the following sections will focus on these two pathways in terms of cell cycle regulation and oncogenicity.

1.1.5.1 RAS/RAF/MEK/MAPK signaling cascade

The mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway, also known as RAS/RAF/MEK/ERK, is a signaling cascade that links extracellular milieu to intracellular response [219]. The MAPK/ERK pathway stimulates cell cycle entry and progression to overcome quiescence. It is also involved in cell differentiation, migration, senescence, tissue repair, and malignant drug resistance [220, 221]. At the heart of the cascade lies the RAS family of proteins. The RAS members belong to a small GTPase class of proteins. Human cells contain three RAS members; HRAS, KRAS, and NRAS. The constitutive activation of RAS proteins is a frequent oncogenic driver in various tumors, including lung adenocarcinoma and pancreatic cancer [219]. Similarly, RAF is another central serine/threonine kinase in the pathway. There are three related RAF proteins, A-RAF, B-RAF, and C-RAF, which is also known as RAF-1. RAF mutations are associated with several types of cancer, especially melanoma and thyroid carcinoma [6, 222].

The signaling cascade starts when an extracellular ligand binds to a receptor tyrosine kinase (RTK), such as epidermal growth factor receptor (EGFR) and fibroblast growth factor receptor (FGFR) [223, 224]. Also, other receptors, such as G-protein coupled receptors (GPCR) and cytokines receptors, can mediate the Ras activation. The ligand-binding induces receptor dimerization, which exposes the cytoplasmic tyrosine kinase domain aiding to the receptor auto-phosphorylation. The activated receptor recruits the adaptor protein Grb2-ShC, which recognizes the sequence homology 2 (SH2) domains on the receptor. The Grb2-bound complex further recruits guanine exchange factors (GEF), such as the son of sevenless 1 (SOS-1), to the cell membrane complex. In turn, the SOS-1 promotes the GDP to GTP exchange of RAS protein at the cell membrane. The activated Ras represents a crucial element

to mediate the Raf kinase activity, which sequentially phosphorylates MAPK protein, which is also known as MEK protein. Subsequently, the active MAPK phosphorylates the downstream ERK1/2 proteins [219]. The phosphorylated ERK1/2 proteins simultaneously activate several cytoplasmic proteins and also translocate to the nucleus. In the cytoplasm, ERK1/2 kinase activity mediates the activation and dimerization of c-Fos and c-Jun to form the activator protein 1 (AP-1). The AP-1 complex translocates to the nucleus and induces the transcription of different genes, and also it suppresses various anti-proliferative genes [225]. Active ERK1/2 phosphorylate p90RSK kinase, which leads to downstream activation of the CREB transcription factor. In the nucleolar compartment, the translocated ERK1/2 activates various transcription factors, including c-MYC. On the other hand, the p90RSK-dependent phosphorylation of SOS-1 creates a docking site of 14-3-3 proteins to bind and alleviate the GEF activity, and in turn, negatively regulate the MAPK cascade [226]. Additionally, the RAS GTPase activating proteins (RAS GAPs) inhibits the activated RAS by hydrolyzing the RAS-GTP interaction, rendering the inactivated RAS-GDP form [227].

The majority of ERK1/2 targets are involved in vital cellular processes that instruct cell cycle progression, proliferation, apoptosis, and homeostasis [228, 229]. For instance, Active ERK1/2 induces G1 phase onset and G1/S transition through phosphorylated c-MYC, which directly binds to cyclin D1 promoter and initiates its transcription [230]. Concordantly, ERK1/2 positively regulate the transcription of the *c-FOS* factor. Although c-Fos suppresses the transcription of cyclin D, it induces the transcription of Fra-1 protein, which later on induces the cyclin D transcription [231]. Notably, cyclin D-1 deficient-mice are less responsive to oncogenic transformation induced by Ras [232]. Activated ERK proteins, in contrast, permit the translocation of CDK2 into the nucleus and subsequent activating phosphorylation by CAK as well as de-phosphorylation by CDC25 to promote cyclin E-CDK2 assembly [228]. ERK kinase activity determines the proper nuclear localization of cyclin B1 during mitosis [233]. In a similar context, ERK1 proteins activate RSK kinase, which introduces inhibitory phosphorylation to MYT1 kinase, thus maintains the activity of cyclin B-CDK1 complex [234]. However, surprisingly, MAPK induction results in a delay of G2/M transition by introducing inhibitory phosphorylation to CDC25B phosphatase. The imbalance of CDC25B delays the mitotic entry and induces cell cycle arrest. So, it is possible to conclude that the stress conditions or DNA damage promote extracellular signals that fire MAPK/ERK cascade. In turn, the MAPK would halt the cell cycle progression until the stress is resolved [235].

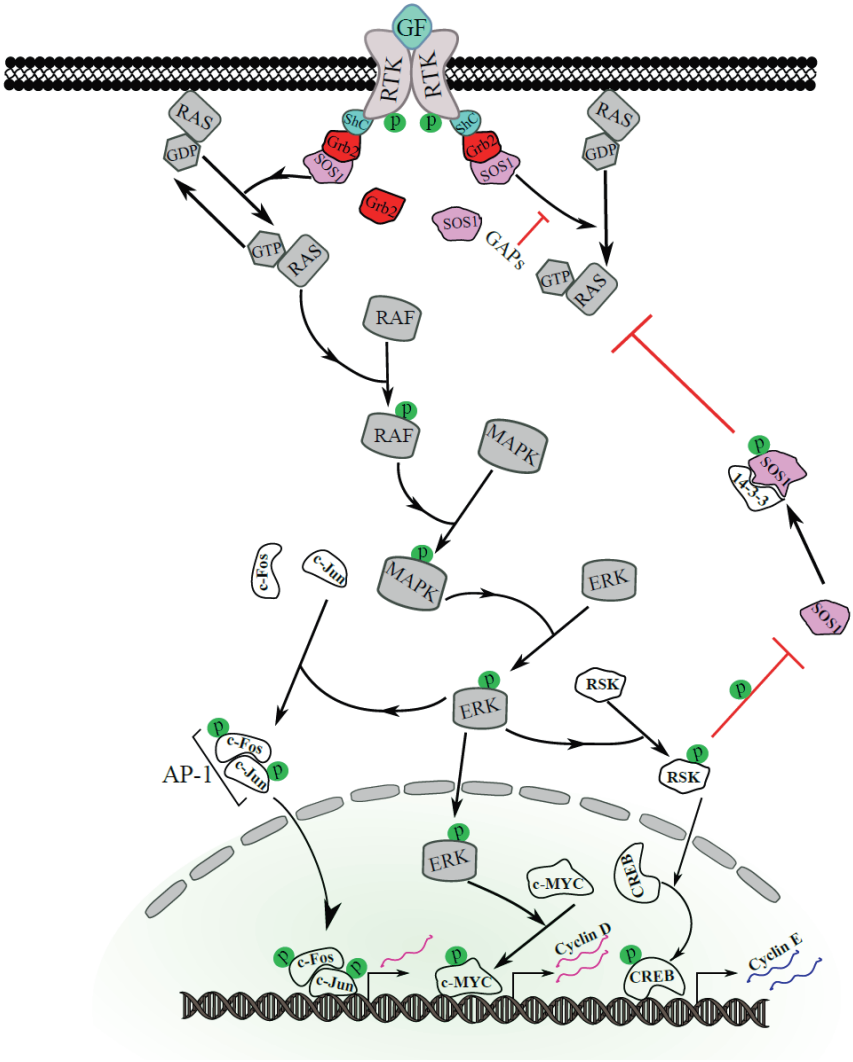

Figure 6. A schematic representation of the RAS/RAF/MEK/MAPK pathway

1.1.5.2 PI3K/AKT/mTOR signaling pathway

Phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) is one of the most extensively studied pathways; owing to its diverse implications on oncogenic transformation, cell cycle regulation, inflammation, and insulin resistance [236, 237]. The PI3K members are heterodimeric lipid kinases that are induced in response to phosphorylation of phosphatidylinositol (PtdIns) lipids in the plasma membrane [238]. The kinases consist of regulatory and catalytic subunits, and hence the PI3K proteins have eight isoforms categorized into three classes that differ in their structure and lipid substrates [239]. These three classes of PI3Ks share a common PI3K core structure, which comprises a C2 domain, helical domain, and a catalytic domain (**Figure 7**). Nevertheless, the class I PI3K has the most important role, among other classes, in carcinogenesis and aberrant cell cycle regulation [238]. The regulatory subunit is known as p85, which is encoded by seven genes, while p110 represents the catalytic subunit. This catalytic subunit of class I PI3K also comprises four different isoforms (p110α, p110β, p110γ, and p110δ) [237]. Under normal physiological conditions, the individual catalytic p110 (α, β, δ) subunit binds to the regulatory p85 subunit, which in turn stabilizes the heterodimer and inhibits PI3K-mediated activity. Similarly, p110γ binds to either p87 or p101 regulatory subunits [239]. The Src homology (SH2) domain of regulatory subunits promotes the recruitment and interaction with phosphorylated tyrosine residues of activated upstream inducers in a controlled manner. This interaction mediates the recruitment of the PI3K heterodimer complex to the plasma membrane and induces conformational changes that relieve the inhibitory status [240]. Not surprisingly, the catalytic isoform p110α (also known as PI3KCA) is frequently mutated in cancer. On the other hand, mice bearing an inactivating mutation in the PI3KCA gene, which can no longer mediate the p110 interaction with Ras, die at the perinatal stage owing to the developmental defects in the lymphatic vasculature. However, cells derived from these mice, as well as the few surviving mice, are resistant to RAS-induced oncogenic transformation [241]. Therefore, collective observations indicate that p85 truncations alongside with PI3KCA activation lead to sustained signaling and oncogenic transformation [242, 243].

The other principal constituent of the PI3K-derived signaling is the protein kinase B, which was first described in the late 1980s [244]. The human genome contains three isoforms of AKT, known as AKT1 (PKBα), AKT2 (PKBβ), and AKT3 (PKBγ). The AKT proteins have standard structural features characterized by a pleckstrin homology (PH) domain, a catalytic kinase domain, and a regulatory domain (**Figure 7**). The maximal activation

of AKT1 requires concomitant phosphorylation of threonine residue 308 and serine residue 473. The AKT2 isoform requires similar phosphorylation on threonine and serine residue 309, and 474, respectively, while AKT3 is activated through threonine 305 and serine 472 residues [237]. Further regulatory post-transcriptional modifications alter the activity, stability, localization, or substrate affinity of AKT in isoform and cell type-specific manner [237]. For instance, cyclin A/CDK2 activity underlies the phosphorylation of serine 477 and threonine 497 residues in a cell cycle-regulated pattern. Of note, this concomitant dual phosphorylation is crucial for apoptosis inhibition in mouse embryonic stem cells [245]. Another intriguing modification is the acetylation of lysine 14 residue that restricts the AKT preferential localization to the plasma membrane and thereby affects AKT-mediated signaling [246].

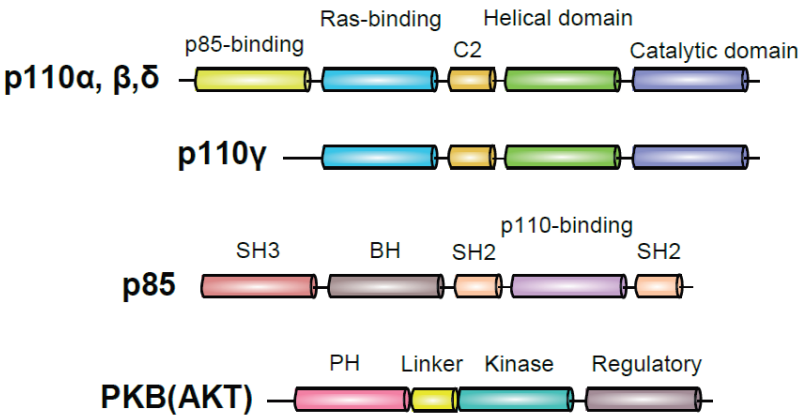

Figure 7. A schematic representations of different protein domains constituting PI3K subunits and AKT (Redrawn from Jung K. et al., Cancers Head Neck; 2018 [247] and Vanhaesebroeck B. et al., Nat Rev Mol Cell Biol; 2010 [239])

The canonical activation of PI3K/AKT cascade occurs immediately in response to extracellular stimuli, such as growth factors, hormones, and cytokines. As mentioned in the previous section, such stimuli activate individual or a combination of RTK, GPCR, and small GTPases [237, 248]. These stimulated receptors/molecules bind directly or indirectly to PI3Ks through scaffolding adaptors such as IRS1. In turn, such binding mediates the PI3Ks recruitment to the plasma membrane leading to allosteric activation

and exposure of respective kinase domains. Thereby, concerning class I PI3K, the phospholipid substrate phosphatidylinositol 4,5-bisphosphate (also known as PIP_2, or $PI4,5P_2$) is readily phosphorylated into phosphatidylinositol 3,4,5-triphosphate (PIP_3). Class II PI3K, on the other hand, preferentially phosphorylates phosphatidylinositol 4-phosphate (PI4P) substrate into $PI3,4P_2$ [249]. Subsequently, the inactive AKT translocates to the plasma membrane and gets recruited to the phosphorylated PIP3 and/or PI3, $4P_2$ sites through the PH domain of AKT. The recruitment of AKT promotes a conformational change that abolishes the inhibitory constraint of the PH domain and releases the kinase domain. Simultaneously, the phosphatidylinositol-dependent kinase 1 (PDK1) is recruited to PI3K phosphorylated substrates at the plasma membrane. The PDK1-mediated kinase activity drives the phosphorylation of AKT at threonine 308 residue, which lies within the T-loop of the AKT kinase domain [250]. Besides, the mechanistic target of rapamycin complex 2 (mTORC2) phosphorylates AKT at serine 473 residue for further stabilization and activation [251]. On the contrary, the negative regulation of the PI3K/AKT cascade integrates multiple factors targeting essential events in the cascade. Most importantly, the tumor suppressor phosphatase and tensin homolog (PTEN) protein counteracts PI3K functions where it dephosphorylates PIP_3 into PIP_2 [252, 253]. The loss of PTEN tumor suppressor functions is frequently observed in a wide array of tumors through locus deletion, inactivating mutation, transcriptional repression, or protein instability [254, 255]. Thus, PTEN inactivation leads to the accumulation of PI3K-mediated phospholipid products and sustained proliferative signals [256]. Another negative regulator of the PI3K-mediated phospholipids is the tumor suppressor protein phosphatase INPP4B. This phosphatase mediates the conversion of $PI3,4P_2$ into PI3P, most likely at the endosomal membranes [257]. The loss of INPP4B is associated with the oncogenesis process and defines aggressive basal-like breast carcinomas [258, 259]. Nevertheless, the PI3K/AKT proliferative signal can also be terminated by dephosphorylating AKT at different sites. The protein phosphatase 2A (PP2A) counteracts the PDK1-mediated phosphorylation of AKT by dephosphorylating the threonine 308 residue, leading to AKT kinase inactivation [260, 261]. Similarly, the PH domain leucine-rich repeat protein phosphatases (PHLPP1 and PHLPP2) dephosphorylate the serine 473 residue of AKT in an antagonistic manner to mTORC2 [237, 262].

Interestingly, recent studies indicated two contradicting models that dictate the localization and downstream signaling of the activated AKT proteins. The first model suggests the immediate release of the fully activated AKT from its anchoring PIP3/PI3, $4P_2$ at the plasma membrane into the cytoplasm

leading to downstream phosphorylation of AKT substrates. This model relies on the short lifetime of the activated AKT at the plasma membrane, as well as its high intracellular prevalence that leads to the phosphorylation of authentic cytosolic proteins [237]. The second model, however, restricts the kinase-mediated activity of AKT to the PIP3/PI3, $4P_2$-containing cellular membranes [263]. The model demonstrates that PH domain-dependent binding to PI3K phosphorylated lipid products results in allosteric activation of AKT, and thereby ensure substrate-specific phosphorylation at the plasma membrane. Although the two models propose counteracting mechanisms for the downstream signal transduction, a convergent mode of action may exist in a cell-specific and temporal manner.

The fully-activated AKT proteins have a repertoire consisting of tens of downstream targets. However, these substrates possess minimal consensus motif required for AKT recognition. Though, in some cases, authentic AKT substrates harbor other modified recognition motifs. The heterogeneity of AKT responsive targets, as well as their implications in a wide array of biological processes, raise numerous issues about the nature of AKT *bona fide* substrates. Thus, it is very legitimate to ask what defines the real targets of AKT *in vivo* in normal and pathological contexts [237, 264]. In a general context, regardless of the authenticity of recognition motifs, the AKT direct substrates contribute to a hitherto of biological functions. To date, tens of studies implicated AKT-catalyzed phosphorylation in modulating cellular proliferation, survival, metabolism, angiogenesis, and growth processes [264]. Among several AKT targets, the glycogen synthase kinase-3 (GSK-3) [265], the Forkhead box O (FoxO) transcription factor [266], and mTORC1 [267] are of prime importance.

The GSK-3 is a serine/threonine-protein kinase that has two closely related isoforms in mammals; GSK-3α and GSK-3β, which possess several isoform-specific functions [268]. The GSK-3 activity peaks in the absence of external growth factors, whereas it decreases dramatically following insulin uptake [265]. As evident by the kinase nomenclature, GSK-3 introduces inhibitory phosphorylation to its glycogen synthase substrate. Thus, GSK-3 alters glycogen biosynthesis and glucose transport through insulin receptor substrate (IRS) phosphorylation [269, 270]. The GSK-3 also primes crucial factors, involved in survival and proliferation, and destines them to proteasomal-mediated degradation. For instance, GSK-3 targets the MCL-1, a protein member of the prosurvival Bcl-2 family, which plays an anti-apoptotic role and inhibits multiple caspases [271]. Similarly, GSK-3-catalyzed phosphorylation alters the c-MYC-dependent transcriptional programs by destabilizing the c-MYC protein [272]. Moreover, GSK-3

inhibits the hypoxia-inducing factor 1α (HIF-1α) and thereby alters cell growth and oxygen sensation [273]. Nevertheless, activated AKT counteracts the GSK-3-mediated inhibitory effects by phosphorylating GSK-3α and GSK-3β at serine 21, and 9 residues, respectively [265]. The latter event obstructs the phosphate-binding pocket of GSK-3 and hinders the substrate accessibility.

The FoxO transcriptions factors regulate the transcriptional activity of several genes involved in apoptosis, cell cycle regulation, and metabolism [266]. FoxO factors induce *BIM* and *PUMA* transcription to promote apoptosis, either dependent or independent from p53 [274, 275]. The PI3K/AKT active signaling perturbs FoxO-dependent transcriptional activation. In response to the phosphorylation mediated by AKT, FoxO factors acquire recognition motifs for 14-3-3 proteins. The latter binding proteins sequester FoxO into the cytosolic compartment, titrating them away from the promoters of their target [276].

The PI3K/AKT-dependent regulation of cell growth and metabolism mainly relies on the selective activation of the mTORC1 complex and its downstream signaling. Extensive studies implicated mTORC1 in regulating pivotal processes such as autophagy and metabolites biosynthesis, whereas aberrant mTORC1 activity contributes to cancer and aging [277]. In order to maintain an active form, mTORC1 binds a RAS-related GTPase molecule known as RHEB. The GTP-bound RHEB activates the mTORC1 complex, whereas the conversion into GDP-bound RHEB causes an inhibitory action [277]. The tuberous sclerosis complex (TSC), composed of TSC1, TSC2, and TBC1D7, negatively regulates the mTORC1 complex. The TSC2 protein converts RHEB-bound GTP to GDP, rendering the whole mTORC1 inactive. The spatial distribution of mTORC1 imposes another layer of upstream regulation, depending on amino acids availability. In this context, a nutrient-deficient microenvironment alters a GTPase protein known as RAG, which localizes to the cytoplasmic side of lysosomes. RAG interacts with the mTORC1 complex in its GTP-bound form and recruits the complex to lysosomes where internal RHEB molecules reside [278]. The induction of growth signaling releases the TSC2-mediated inhibition on the lysosomes-localized RHEB. In turn, the activated RHEB-GTP binds to the lysosomes-recruited mTORC1 and permits downstream processes. Therefore, as expected, growth signaling-induced activation of PI3K/AKT stimulates mTORC1 by altering TSC status. The activated AKT phosphorylates TSC2 and thus reverses the inhibition on RHEB and maintains its GTP-bound state, leading to mTORC1 activation [267, 279].

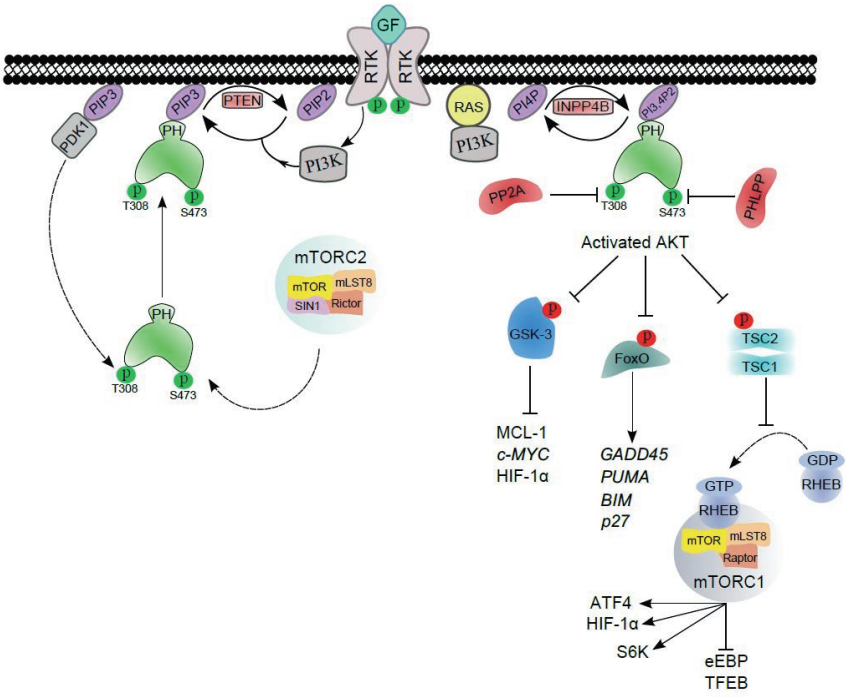

Figure 8. A simplified overview of PI3K/AKT signaling cascade

The PI3K/AKT signaling cascade contributes to cell cycle modulation either directly or indirectly through regulating downstream targets [236]. Considering the indirect regulation, GSK-3 and FoxO proteins are immensely involved in controlling cell cycle progression. For instance, the GSK-3 proteins directly target cyclin D1 through priming phosphorylation at threonine 286 residue that triggers a rapid cytoplasmic translocation and ubiquitin-mediated degradation [57]. As mentioned earlier, the $p21^{Cip1}$ protein is crucial for cyclin D-CDK4/6 complex formation [132, 133]. The GSK-3-mediated kinase activity primes $p21^{Cip1}$ protein inhibitory phosphorylation at the threonine 57 residue, resulting in a higher degradation rate. However, AKT activity counteracts $p21^{Cip1}$ degradation by inhibiting GSK-3 proteins [280]. Moreover, an AKT-dependent phosphorylation event at the serine 146 residue of $p21^{Cip1}$ increases the protein stability and promotes its association with the CDK4/6 complex [281]. Therefore, the AKT-mediated inhibition of GSK-3s permits the entry into the G1 phase, stressing the necessity of

extracellular stimuli in driving cell cycle progression prior to the restriction point. Similarly, the connection between FoxO and cell cycle regulation is firmly-established through the positive regulation of $p27^{Kip1}$ and retinoblastoma p130 that confers cell cycle exit and quiescence [282]. The elevated activity of FoxO members also transactivates INK4 family members, which restricts the G1 phase progression, leading to cell cycle arrest [283]. Nevertheless, activated AKT also nurtures the cell cycle continuance beyond the R point where it specifically phosphorylates $p27^{Kip1}$ and $p21^{Cip1}$ to allow S-phase entry and DNA synthesis, respectively [236]. In this context, AKT phosphorylates $p27^{Kip1}$ at threonine 157 residue, which retains the protein in the cytosolic compartment and hinders its association with cyclin A/E-CDK2 complexes. Hence, the cell can progress through the late G1 phase and enters the S phase. In a parallel context, $p21^{Cip1}$ binds to PCNA and inhibits its association with the DNA polymerase δ (Pol δ) [284]. Thereby, ahead of the DNA replication process, AKT-dependent kinase activity stimulates DNA synthesis through $p21^{Cip1}$ phosphorylation at threonine 145 residue [285]. The latter modification facilitates PCNA release and subsequent binding with the Pol δ holoenzyme [286].

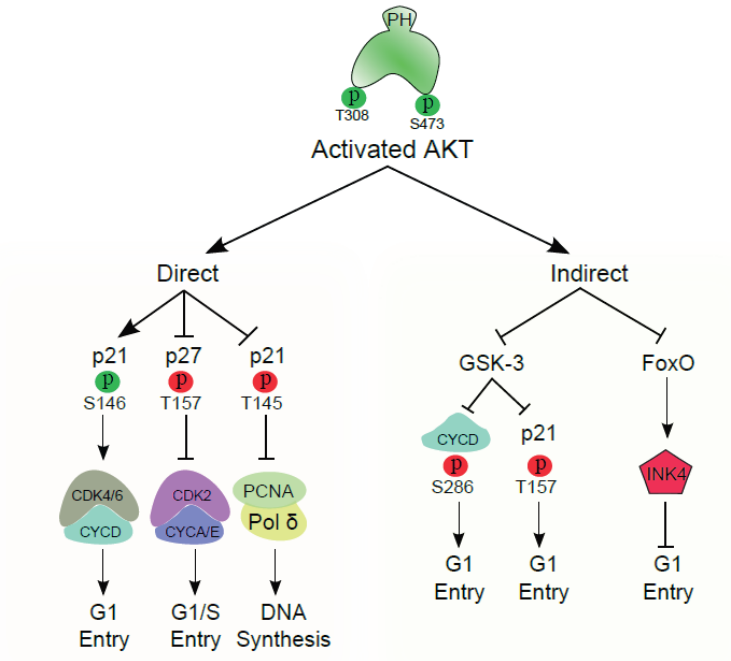

Figure 9. The connection between PI3K/AKT signaling and cell cycle regulation

1.2 long noncoding RNAs

The relevance of the noncoding portion of the genome to various cellular processes has been a matter of debate for decades, especially with the completion of the human genome project [287]. The complete sequence of the human euchromatic regions estimated the presence of 20,000-25,000 protein-coding genes [12]. From a classical perspective, this repertoire of genes would be sufficient for human cell functions. However, the concurrent genomic mapping of mouse transcriptome comprehended our understanding unprecedently into the extensive and pervasive transcription of the mammalian genomes [288]. In this regard, the functional annotation of the mammalian genome (FANTOM) initiative performed comprehensive sequencing analyses of full-length cDNA, 5'-and 3'-end sequencing of the cloned cDNAs combined with cap-analysis gene expression (CAGE). In addition to other sequencing analyses, the FANTOM project identified the 5' and 3' boundaries of 181,047 transcripts, including 5154 previously unknown protein-coding genes. Importantly, the project identified 3652 noncoding transcripts and confirmed that 3021 noncoding transcripts were previously annotated as truncated protein-coding sequences. So, the FANTOM project, in 2005, produced the most extensively curated catalog of mammalian ncRNAs. Similar collaborative efforts from the encyclopedia of DNA elements (ENCODE) project identified and mapped all protein-coding genes within 487 loci covering almost 1% of the human genome. The manual curation combined with experimental validations inaugurated the GENCODE consortium, which provided evidence on the noncoding capacity of 46 validated loci [289]. The unprecedented growth in the number of identified noncoding transcripts cast more doubts on the functionality of these transcripts. Therefore, in 2009, Guttman and colleagues introduced a new approach to identify the transcriptionally-independent long noncoding RNAs (lncRNAs), utilizing the active chromatin signature in mouse cells [290]. To this end, the approach implied the global identification of chromatin boundaries marked with H3K4me3 and H3K36me3 (K4–K36) at the promoters and gene body of transcribed regions, respectively. This approach relies on the fact that RNA polymerase II-mediated transcription takes place within these K4–K36 chromatin boundaries [291]. Subsequent integrative analysis identified new 1675 K4–K36 domains that do not overlap with previously annotated regions. Out of the identified domains, 1586 were novel uncharacterized domains correspond to large intervening noncoding RNAs (lincRNAs). Simultaneously, Khalil et al. expanded the human lincRNAs catalog, utilizing co-immunoprecipitation assays, and analyzing transcripts associated with several chromatin remodelers [292]. These analyses yielded a substantial catalog of ≈3300 lincRNAs. Concomitantly, experimental

validations demonstrated the ability of lincRNA to mediate epigenetic modulation of specific genomic loci through the association with chromatin remodelers. In 2011, the human lincRNAs landscape escalated to reach more than 8000 putative transcripts identified by combined RNA sequencing of 24 tissues and cell types [293]. Out of these putative transcripts, 4662 correspond strictly to the lincRNA category. This expanded catalog highlighted the main features of human lincRNAs, such as the high tissue specificity associated with lincRNAs expression. It also argued for the co-expression patterns of lincRNAs and the neighboring genes, showing that the association is not higher than any randomly expected value. Shortly in 2012, the GENCODE consortium reported the most comprehensive catalog of human lncRNAs, comprising 14,880 transcripts of 9277 manually annotated genes [294]. Among several features analyzed in the GENCODE catalog, lncRNAs showed a remarkable positive correlation with the antisense coding genes. It was also clear that lncRNAs expression follows tissue-specific patterns, confirming other reports on lincRNAs. Currently, the most updated version of GENCODE annotation (GRCh38.p13; v32; 2019) comprises 60,609 genes, out of which 17,910 correspond to lncRNA genes, and 19,965 are protein-coding genes. The complete overview of the human GENCODE annotation, as of December 2019, is available at https://www.gencodegenes.org/human/stats.html

The following sections will briefly discuss the general features of lncRNAs with an emphasis on their different modes of action. Also, I will elaborate on the connection between lncRNAs and cell cycle regulation, highlighting the most relevant examples with a discussion on the growing concerns at the moment.

1.2.1 General features of lncRNAs

As defined by the terminology, lncRNAs are transcripts larger than 200 base-pairs devoid of any protein-coding capacity. These transcripts are independent products of RNA polymerase II and have similar 5'-cap structure. However, not all lncRNAs possess the 3' adenosine tails. Based on primary sequence analysis, GENCODE lncRNAs exhibit less open reading frame (ORF) quality than expected of protein-coding and random sequence [289]. The initial computational analysis concluded that lncRNAs encode short ORFs with poor start codons that fail to translate into peptides. It is possibly due to the initiation of the nonsense-mediated decay pathway [295]. Nevertheless, further investigations indicated that a small subset of lncRNAs corresponds to short translated peptides, mostly less than 100 amino acids [296, 297]. In a related context, the association between lncRNAs and

ribosomes raised intriguing concerns. However, the most relevant answer attributed this association to the ability of lncRNAs to interfere with the polysomes assembly at a particular protein-coding target, inhibiting its translation [298]. Recently, the advances in ribosome profiling coupled with RNA sequencing and mass spectrometry techniques documented the translation of short peptides from putative lncRNAs [299, 300]. Importantly, a growing body of evidence supports the role of ncRNAs in generating short peptides through novel back splicing, which gives rise to circular RNAs. These species of newly-classified RNAs show aberrant expression patterns in various pathological contexts [301]. However, based on individual observations, these short peptides seem to have more implications in organogenesis and differentiation. These observations, in turn, raise more alerting concerns over the definition of noncoding transcripts.

The analysis of human body map (HBM) datasets indicated that lncRNAs have less median expression levels than mRNAs in all tissues, except for testis [294]. Intriguingly, the lower expression levels may point out to the high turnover rates of lncRNAs in comparison to mRNAs. To test this hypothesis, an earlier study analyzed the global RNA stability in mouse cells through extended transcriptional inhibition with the aid of Actinomycin D throughout 32h. The time-course analysis revealed that lncRNAs, on average, have higher turnover rates compared to mRNAs, though both classes have a comparable wide range stabilities [302]. The analysis also identified hundreds of short-lived lncRNAs with a half-life of fewer than two hours and other lncRNAs with remarkable stability (half-life > 16h). In accordance, another study applied a novel 5'-bromo-uridine pulse-chase sequencing to study the global RNA stability in a physiological condition [303]. The combined analyses of lncRNAs and mRNAs demonstrated the presence of short-lived and long-lived transcripts. Remarkably, short-lived transcripts were enriched for regulatory functions in response to external signals. The long-lived RNAs, not surprisingly, had housekeeping functions. These studies demonstrate the higher turnover rate of lncRNAs compared to mRNA, on average, but do not entirely correlate this rate with the lower expression levels of lncRNAs. Also, as mentioned earlier, lncRNAs show more tissue-specific expression compared to mRNAs [293]. This specificity is not due to the lower expression levels of lncRNAs; instead, it is associated with higher expression variability. This feature is evidenced further by the striking expression of lncRNAs in brain tissues [304], which may reflect a context-dependent regulation of lncRNAs expression. Recent extended analysis of lincRNAs-associated chromatin marks lent support to the tissue-specific expression notion. As such, the H3K9me3 repressive histone marks show higher enrichment at the promoters of lincRNAs loci than mRNAs

promoters in a tissue-specific manner. Moreover, a genome-wide depletion of most of the transcription binding sites is evident at the promoters of tissue-specific lincRNAs [305]. Therefore, the upstream genetic and epigenetic contexts dictate the expression levels and specificity of lncRNA.

Concerning splicing patterns, 98% of human lncRNA are spliced and tend to have two exons. Such a tendency is evident by the 42% double exonic lncRNAs in comparison to the 9% mRNAs. Although the overall length of protein-coding genes is higher than lncRNAs, both exons and introns of lncRNAs are slightly longer than the exonic regions of protein-coding genes. Remarkably, more than 25% of lncRNAs undergo alternative splicing, possessing at least two isoforms per each gene locus [294]. Nevertheless, recent findings suggest that lincRNAs, in particular, have less efficient splicing capacity compared to mRNAs in mouse and human cells [305]. To a certain extent, lincRNAs have weaker splicing signals and lower binding of the splicing factor U2AF65 than mRNAs. Of note, a subset of noncoding transcripts with specific functions, such as *XIST*, shows higher splicing efficiency. This observation indicates that certain lincRNAs have sequence-related functions, rather than being necessary because of the act of transcription at their loci. This specific point is explained in the following sections with more detailed examples.

1.2.2 Conservation and classification of lncRNAs

Throughout the process of evolution, the protein-coding genes retained more primary sequence conservation compared to the noncoding elements, especially in distally-related species [294]. The majority of noncoding transcripts belonging to vertebrates do not maintain identifiable orthologs [306]. However, the lack of sequence conservation among different species does not exemplify an abstained evolutionary selection or reflect transcriptional noise. Indeed, more than a decade ago, a landmark study revealed that a set of mouse lncRNAs (3122 lncRNAs) exhibits the imprint of purifying selection, considering substitution, insertion/deletion, and splicing signatures [307]. With respect to primary sequences and splice sites, lncRNAs' promoters experience higher selective pressure. This higher conservation suggests the presence of evolutionary-conserved transcriptional regulation machinery, driving the functional aspects of these noncoding elements. Nevertheless, another counterargument correlates the conservation of these promoters to the transcriptional *per se* rather than the noncoding RNAs. In this context, the preserved promoters induce an open chromatin structure that promotes the transcriptional activity of neighboring genes [308]. Hence, the conservation in the regulatory elements is more crucial than

the transcripts' primary sequences [307]. Though the latter postulation may be plausible to a certain extent, it does not explain the higher enrichment of conserved elements within the transcribed regions compared to the intergenic regions [290]. Also, the concordant higher conservation (65%) of the GT-AG dinucleotide splice sites between mouse and human lncRNAs does not comply with the previous model. Notably, such conservation is significantly higher than intronic splice sites (58%; $P = 2.0 \times 10^{-4}$) observed in mouse and human [307]. More supporting evidence emerged from the comprehensive analysis of the GENCODE catalog of human lncRNAs [294]. In this analysis, lncRNAs exons showed a lower degree of conservation compared to protein-coding counterparts, although lncRNAs are more conserved than ancestral repeats. In agreement with earlier studies, the promoters of GENCODE lncRNAs share almost the same degree of conservation with the promoters of protein-coding genes. To evaluate the conservation of individual lncRNA across mammals, the GENCODE consortium applied a multiple genome alignment-independent method. The analysis revealed that nearly 30% of lncRNAs are specific to primates, while only 0.7% (101 lncRNAs) is human-specific. The fast-evolving nature of lncRNAs, based on the primary sequence homology, may obstruct their systematic classification into evolutionary-related families with orthologous functions [294]. In support of the latter notion, the detailed investigation of lincRNAs in zebrafish identified a handful of noncoding RNAs (29 lincRNAs) of detectable sequence homology with mammalian orthologs. Remarkably, on the other hand, direct comparison among vertebrates revealed the prominent significance of positional conservation over sequence conservation [309]. These observations suggest that syntenic conservation comprises, at least to a certain extent, functional relevance. For instance, the genomic locus encoding *NEAT1* (also know as MENβ) and *MALAT1* lncRNAs possess synteny across various vertebrates, where they are in close proximity to FRMD8 and SCYL1 [310]. Regardless of sequence homology, *NEAT1* and *MALAT1* lncRNAs regulate multiple overlapping functions, ranging from organismal development to cancer, in almost all vertebrates [311-314]. Intriguingly, a recent study introduced a promising approach for the classification of evolutionary-unrelated lncRNAs to drive useful functional relevance. Instead of relying on the linear sequence homology, the study implemented *k*-mers comparison of short motifs within the primary sequences of different lncRNAs [315]. In this regard, lncRNAs with similar *k*-mers had comparable features, including subcellular localization and protein binding patterns. Thereby, the direct comparison of linear sequences may neither infer the evolutionary relationship nor the functional homology.

The GENCODE annotation classifies lncRNAs into several biotypes based on their physical positions with respect to the surrounding protein-coding genes [294]. These subtypes are:

lincRNAs: Noncoding transcripts that reside in intergenic loci.

Antisense RNAs: Noncoding transcripts that intersect with any protein-coding exon on the opposite strand.

Sense overlapping RNAs: Noncoding transcripts contain a protein-coding gene within their introns.

Sense intronic RNAs: Noncoding transcripts mapped to the introns of protein-coding genes, but do not overlap with any exon.

Processed RNAs: Any transcript devoid of protein-coding capacity and does not belong to any of the subtypes mentioned above.

A recent update on the lncRNAs diversity and biogenesis suggest the inclusion of new subtypes [316], such as:

PROMPTs: RNAs that are transcribed in the antisense direction within a window of 0.5–2.5 kb upstream of the transcription start site of the neighboring protein-coding genes. PROMPTs are usually 200–600 nucleotide-long with a short half-life and nuclear retention.

eRNAs: Noncoding RNAs transcribed bidirectionally by the act of RNA polymerase II at the enhancers sites. They are less than 2 kb in length, and they lack the typical poly(A) tail. This class of transcripts may contribute to the high order chromatin structure.

circRNAs: As mentioned earlier, circRNAs are back-spliced products of RNA polymerase II, which also lack the poly(A) tail. The spliceosomal machinery usually catalyzes the ligation of the downstream 5’ single strand with the upstream 3’ single strand at the splice junctions of pre-mRNAs. In turn, it results in a circular RNA connected via a 3’-5’ phosphodiester bond.

ciRNAs: This subclass includes the circular intronic ncRNAs, which are products of back splicing as well. However, these products are specifically produced from excised introns, which can not debranch after splicing.

1.2.3 LncRNAs targets and modes of action

The functional heterogeneity governing the lncRNAs-dependent processes restricts the development of a universal mechanistic model of lncRNAs-related outcomes. Considering a historical perspective, the most prevailing concept of lncRNAs-mediated functions relied on the ability of an individual transcript to fine-tune the transcription of neighboring protein-coding genes. Several pioneering studies, investigating the biology of *Xist*, *Kcnq1ot1,* and *HOTAIR* lncRNAs, have nurtured this concept [292, 317-319]. Thereby, these studies elegantly established the pivotal roles of lncRNAs in transcriptional regulation through genomic imprinting and chromatin modulation. Further investigations scrutinized the effect of lncRNAs on modulating the transcriptional programs at a genome-wide level. For instance, *lincRNA-p21* modulates the expression of multiple p53 target genes in response to DNA damage through an association with hnRNPK protein [320]. Similarly, we have also demonstrated that *SCAT7* lncRNA interacts with hnRNPK/YBX1 complex to mediate the expression of oncogenic pro-survival pathways [321]. Of note, the action of lncRNAs is not limited to the transcriptional regulation of target genes; however, lncRNAs contribute to several biological processes, such as rewiring the DDR [322]. Therefore, delineating a single model depicting lncRNAs modes of action may be unfeasible. Instead, a broad classification based on the mechanistic aspects is more reasonable [323].

The first class denotes the act of transcription *per se* of lncRNAs as the main driving force of lncRNAs-dependent functions. In this scenario, as mentioned earlier, the act of transcription modulates the chromatin status of the neighboring genomic loci [308]. A staggering example is the silencing-induced paternal imprinting of the Igf2r gene through *Airn* lncRNA [324, 325]. The long term silencing of *Igf2r* expression does not depend on the sequence composition on *Airn*. However, the act of transcription at the lncRNA transcription start site overlaps with the promoter of Igf2r, which interferes with the recruitment of RNA polymerase II and suppresses *Igf2r* expression. In the absence of inhibitory DNA methylation at the Igf2r promoter, the continuous expression of *Airn* maintains the silencing of *Igf2r* regardless of the developmental stage. Detailed experiments with genomic insertion in *cis* of varying lengths of *Airn* concluded that the lncRNA sequence does not dictate the silencing and subsequent stable imprinting of the genomic locus.

The second class exploits the ability of lncRNAs to recruit various factors and act in *cis* [323]. In contrast to its role in silencing *Igf2r* expression, *Airn*

lncRNA interacts and recruits the H3K9 methyltransferase G9a into the promoter region of Slc22a3 [326]. This action initiates the transcriptional silencing of the target gene in *cis*, leading to further imprinted genomic status. In turn, the elimination of such machinery results in biallelic expression of *Slc22a3*. Concordant with this lncRNA-mediated imprinting in *cis*, the *Kcnq1ot1* lncRNA is another typical example. The 91 kb-long nuclear-retained RNA is transcribed from the Kcnq1 imprinted domain on mouse chromosome 7 [319]. The maternal Kncq1 domain is associated with active transcription of a cluster of genes, comprising several protein-coding genes, over a one megabase region. The maternal domain suppresses the expression of the overlapping *Kcnq1ot* lncRNA by methylating its promoter region. The paternal domain, in contrast, has a methylation-free promoter, which induces the lncRNA transcription. For proper embryonic development, the protein-coding genes lie within the maternal Kcnq1 domain have to undergo stable repression through genomic imprinting. In this context, the lncRNA destines the surrounding domain to localize into the repressive perinucleolar periphery in a cell cycle phase-dependent manner [327]. Parallelly, *Kcnq1ot1* interacts with the DNA methyltransferase 1 (Dnmt1), Polycomb repressive complex 2 (PRC2) members (Ezh2 and Suz12), and G9a histone methyltransferase [319, 328, 329]. This complex network of interactions, rewired by the lncRNA sequence itself, aids in the coordinated repressive DNA and chromatin remodeling. Thus, the paternal expression of *Kcnq1ot1* mediates the bidirectional repression of neighboring maternal genes, resulting in the establishment of lineage-specific genomic imprinting in *cis* [330, 331]. Likewise, *Xist* lncRNA mediates the phenomenal X chromosome inactivation through the association and recruitment of the chromatin remodeler PRC2. This interaction mediates the deposition of the repressive histone mark H3K27me3 over the surrounding genomic loci. However, the lncRNA constitutes various sequence-specific regions associated with distinct functions [332]. The first region of the lncRNA harbors the localization signal that facilitates the transcript-chromatin binding and allows the RNA propagation over distant loci. The other region harbors the A-repeat sequence that produces short *RepA* transcript, promoting the interaction with PRC2 and repression establishment [333]. Though recent findings challenge the specificity of *Xist*-dependent recruitment of PRC2 in mediating the X chromosome inactivation, a comprehensive understanding of *Xist* biology seems more complicated than ever anticipated [334-336].

Albeit the different results, lncRNAs can also modulate the histone vicinity and subsequent transcriptional activities in *cis* via repressor occlusion. The latter case is evident by the action of *PACER* lncRNA, which binds to the repressive subunit p50, occluding it from the active NF-κB complex [337].

The repressor occlusion facilitates the domain-wide histone acetylation and transcription initiation of the inflammation-related gene cyclooxygenase-2 (COX-2).

The third class implicates lncRNAs with the *trans*-acting mode of action. As suggested by the nomenclature, these transcripts regulate cellular processes independently from their transcription sites [323]. These processes may span a broad spectrum of cellular functions, such as transcriptional regulation, nuclear architecture, genomic integrity, and proteins' turnover [321, 338-341]. For the matter of simplicity in the current thesis, albeit it seems repetitive, the *trans*-acting class may comprise two categories. Based on the nature of the interaction, the first category involves lncRNA interacting with protein counterparts. The second category includes lncRNAs with validated RNA-RNA interaction [314].

To start with the RNA/protein-interacting category, the risk factor *NBAT-1* lncRNA serves as a potential example. The lncRNA is transcribed from the 6p22.3 locus and exhibits a differential regulation in different risk groups of neuroblastoma patients. The *NBAT-1*-dependent tumor suppression functions rely on the RNA's ability to recruit the repressive PRC2 members in a *trans* configuration [342]. Thus, the lncRNA represses the transcription of oncogenic factors, including *SOX9* and *NRSF*/*REST*, through epigenetic silencing and deposition of H3K27me3 repressive marks.

Considering the nuclear architecture, *NEAT1* lncRNA represents the most prominent candidate. *NEAT1* localizes to the nuclear compartment and, together with other proteins, forms the nuclear paraspeckles, most likely through phase separation of intrinsically-disordered regions [340, 343]. These paraspeckles are sensitive to RNase treatment, indicating that *NEAT1* forms the architectural base of these nuclear structures. Although the exact functions of paraspeckles are unclear, several studies suggested that the paraspeckles-dependent sequestration regulates the levels of several nuclear RNAs and proteins [344, 345].

In connection with the genome integrity, the abundantly-expressed and highly conserved DNA damage-induced *NORAD* lncRNA serves as a remarkable example. The importance of this transcript stems out from its ability to maintain chromosomal stability and genome integrity by sequestering PUMILIO proteins [338]. The abrogation of *NORAD* expression represses DNA replication and repair factors, resulting in prevalent genomic instability. Further studies elegantly illustrated that *Norad* interacts with PUMILIO2 protein in mouse tissues. The loss of the lncRNA causes

hyperactivation of its target protein that leads to premature aging [346]. Another study lent support to the *NORAD*-PUMILIO interaction framework by implicating the SAM68 protein in facilitating the interaction [347]. However, due to its robust role in ensuring genome integrity, another research group reported contradicting results on *NORAD* interaction in unperturbed and perturbed human cancer cells. Accordingly, *NORAD* binds to RBMX protein to assemble the topoisomerase 1 (TOP1) complex, and hence promoting genomic stability [348]. For further complications on the issue of the *NORAD* interaction, extensive image analysis, and fractionation experiments coupled with rescue experiments indicated that RBMX protein is dispensable for *NORAD* functions [349]. At the moment, a reliable model explaining the *NORAD* mode of action is lacking, at least in terms of interacting protein counterparts.

Despite the prevailing dominance of RNA-protein interaction at the center of the lncRNA research field, the identification of RNA-bind proteins (RBPs) leans on various affinity- or immuno-purification techniques, followed by mass spectrometry analysis [350]. Nevertheless, the experimental setup for characterizing authentic RBPs suffers from irreproducible artifacts, resulting from either technical limitations or unfavorable physiological conditions [314]. The *NORAD* and *Xist* interactomes highlight the current discrepancies in this research area with growing concerns over the reproducibility and reliability. These seemingly contradicting results obstruct the development of a comprehensive model of lncRNAs' interaction, which requires a deep understanding of various interconnected aspects. Such aspects may include the spatio-temporal dynamics and the structural biology of each transcript independently of the associated partners.

Irrespective of RBPs, lncRNAs, and pseudogenes may interact with various species of RNA, including mRNAs and microRNAs (miRNAs). The most dominating model implicates lncRNAs or pseudogenes as decoys that sequester miRNAs away from the respective target. Thus, the lncRNAs act as RNA sponges, or better known as competing for endogenous RNAs (ceRNAs). One of the most well-studied ceRNA is *linc-MD1*, which is indispensable for muscle differentiation in mouse and human myoblasts [351]. The muscle-specific transcript *linc-MD1* sequesters miR-133 and miR-135 miRNAs, permitting positive regulation of MAML1 and MEF2C transcription factors, respectively. Of note, the abrogation of *linc-MD1* expression is a habitual phenotype in Duchenne muscle cells. A similar example is *PTENP1* pseudogene, which is the untranslated version of the *PTEN* tumor suppressor gene [352]. The levels of *PTENP1* correlated with the cellular levels of *PTEN*, antagonizing cellular growth, whereas the

genomic locus of PTENP1 is recurrently lost in cancer. Concordantly, *PTENP1* suppresses PI3K/AKT signaling in cancer [353], which comes in line with the identification of putative PTEN ceRNAs in mouse and human melanoma [354]. Several well-described examples also exist and act in a similar manner, such as *HOXD-AS1* and *HULC* lncRNAs [355, 356]. However, it is needless to say that the precise elucidation of lncRNA-miRNA targets should undergo a thorough investigation beyond the governing base-pairing criteria. As the case in RBPs, the lncRNA-miRNA partners experience escalating issues concerned with specificity and stoichiometry [314, 357]. Hence a new line of research should emerge beyond the irreproducible non-physiological *in vitro* conditions, aiming at more *in vivo* model-oriented studies.

1.2.4 LncRNAs in cell cycle regulation

As delineated in earlier sections of the presented thesis, the cell cycle regulation is highly coordinated and requires a fine-tuning of multiple counterparts. Despite the overwhelming reports on the contribution of hundreds of lncRNAs in cell cycle regulation, rigorous characterization of these transcripts remains insufficient. Given that the cell cycle progresses in a pre-defined spatio-temporal manner, one would expect that each phase favors a unique action of a single lncRNA at a time. However, contradicting reports exist widely on the function or the mode of action of the same lncRNA. For instance, as described in the earlier section, the contradicting mechanisms underlying the *NORAD*-mediated functions serve as an obvious example. Thus, the following section will emphasize the lncRNAs with more defined and reproducible roles in the cell cycle regulation. The section will also highlight some of the conflicts, deducing a simplified overview of each cell cycle phase regulation by the corresponding lncRNA.

1.2.4.1 lncRNAs in G1/S phases

SCAT7: As presented in the current thesis, *SCAT7* lncRNA promotes cell cycle progression and oncogenic signaling through an association with the hnRNPK/YBX complex. *SCAT7* positively rewires the transcription of *CCND1* mRNA (cyclin D1) and modulates the phosphorylation status of RB proteins [321]. *SCAT7*-deficient immortalized human fibroblasts exhibit a remarkable induction of senescence-associated phenotype. Therefore, *SCAT7* elevated expression drives the G1 phase onset and the S phase progression, whereas *SCAT7* ablation reduces the DNA synthesis drastically and affects

the replication fork progression. The latter observations are, in part, due to the crucial role of *SCAT7* in modulating the protein turnover rate of TOP1.

ANRIL: The lncRNA is an antisense transcript of the INK4 member $p15^{INK4b}$ (CDKN2B), transcribed from the INK4/ARF locus in physiological and pathological conditions [358]. Initial observations implicated *ANRIL* in the heterochromatin-mediated, but not DNA methylation, epigenetic silencing of the neighboring CDKN2B gene in different leukemia cell lines [359]. Interestingly, the differentiated mouse embryonic stem cells with exogenous expression of *ANRIL* undergo heterochromatin formation and DNA methylation over the CDK2NB locus. Further studies revealed that *ANRIL* mediates the recruitment of the PRC2 member SUZ12 into the INK4/ARF locus, promoting the establishment of repressive chromatin marks. In turn, the abrogation of *ANRIL* expression results in severe and moderate expression of $p15^{INK4b}$ and $p16^{INK4a}$, respectively [360]. Intriguingly, a seminal study demonstrated that the epigenetic-mediated silencing of the INK4/ARF locus in prostate cancer tissues relies on *ANRIL* interaction with CBX7 [361]. Importantly, the latter protein is a member of the PRC1 chromatin remodeler complex. Of note, both studies confirmed the effect of *ANRIL* silencing on the onset of cellular senescence and the drastic decrease of cell proliferation. Taken together, *ANRIL* may cooperatively bind and recruit both PRC1 and PRC2 to mediate the deposition of repressive heterochromatin marks over the INK4/ARF locus, which may proceed further into stable DNA methylation [359, 362]. Thus, the depletion of *ANRIL* induces cell cycle arrest at the G1 phase.

HULC: The highly up-regulated in liver cancer (*HULC*) transcript is one of the most dysregulated oncogenic factors in hepatocellular carcinoma (HCC) [363]. Higher expression of *HULC* predicts poor survival outcomes in different types of tumors, including osteosarcoma; pancreatic adenocarcinoma; and gastric cancer [364-366]. Further investigations revealed that *HULC* expression was absent in primary colorectal tumors and the corresponding healthy tissues. Also, *HULC* expression is almost undetectable in a wide array of cancer cell lines and bladder cancer tissues. However, *HULC* expression dramatically increases in colorectal carcinomas that metastasize to the liver. Also, parental HCC cell lines free of hepatitis B virus (HBV) lack *HULC* expression, while the derived cells with integrated HBV experience significant upregulation of *HULC* expression [367]. Thus, it is evident that HBV transactivates *HULC* through HBx protein (HBV X protein). Mechanistically, HBx stimulates the CREB transcription factor that triggers the acetylation of the *HULC* promoter, leading to transcriptional activation [368]. Activated *HULC* negatively regulates $p18^{INK4c}$ (*CDKN2C*) in

liver cancer tissues and cell lines. Previous studies indicated the role of $p18^{INK4c}$ as a member of the INK4 family in regulating cell cycle progression and activation of p53 in response to DNA damage [369]. Subsequently, the *HULC* expression positively impacts G1/S phase progression [370]. However, from a mechanistic point of view, it remains unclear how *HULC* expression precisely suppresses $p18^{INK4c}$. Nevertheless, a recent study reported that *HULC* acts ceRNA by sponging miR-372 and miR-373 in cholangiocarcinoma [371]. This study is in line with an earlier report of the role of *HULC* in scavenging miR-372, which in turn reinforces the expression of CREB in hepatocellular carcinoma [356]. Thereby, one would speculate that *HULC* expression promotes a positive feedback loop involving CREB that activates anti-apoptotic factors, and possibly suppresses $p18^{INK4c}$ expression.

pncRNA: The transcript nomenclature stands for promoter-associated noncoding RNA, which is also known as $ncRNA_{CCND1}$. In basal conditions, *pncRNA* is transcribed at low copy number (≈2 copies/cell) from the 5' upstream sequence of cyclin D1 promoter region present. In response to the ionizing radiation-induced DNA damage, *pncRNA* accumulates (≈4 copies/cell) and recruits the RNA-binding protein TLS at the cyclin D1 promoter. The *pncRNA*-TLS complex allosterically inhibits the association of the coactivator CBP/p300 with the cyclin D1 promoter. Such inhibition prevents the deposition of active histone acetylation marks at H3K9 and H3K14 residues, resulting in a reduced *CCND1* expression [372]. Thereby, elevated expression of *pncRNA* arrests the cell cycle at the G1 phase in response to DNA damage.

gadd7: The characterization of *gadd7* relation to cell cycle regulation and DNA damage emerged more than 20 years ago [373]. The RNA nomenclature stands for growth-arrest DNA damage-inducible 7 [374]. Thus, one would expect that *gadd7* expression increases dramatically following the treatment with different DNA damage agents. Upon stimulation, *gadd7* binds to the TDP-43 protein, which associates with the 3' untranslated region of *CDK6* mRNA. Thus, in normal physiological conditions, TDP-43 stabilizes *CDK6* [375], whereas *gadd7* binding with TDP-43 dissociates it from *CDK6* mRNA, promoting *CDK6* degradation [376]. Therefore, *gadd7* overexpression represents an integral part of the DDR machinery that blocks cell cycle progression and induces cell cycle arrest at the G1 phase.

Kcnq1ot1: As mentioned earlier, the *Kcnq1ot1*-dependent expression dictates the imprinting fate of the Kcnq1 genomic locus. Of note, this imprinted locus encodes multiple genes, where $p57^{Kip2}$ (CDKN1C) is one of those imprinted

genes [377]. *Kcnq1ot1* interaction and recruitment of Dnmt1, PRC2, and G9a epigenetic modifiers suppress the expression of $p57^{Kip2}$ [378]. Although most of human and mouse adult tissues have reduced levels of $p57^{Kip2}$, it is strikingly prevalent and highly expressed in adult mouse hematopoietic stem cells [379]. Also, elevated expression of $p57^{Kip2}$ induces G0 quiescence of murine muscle satellite cells [380]. Not surprisingly, *CDKN1C* expression restricts cell proliferation and induces cell cycle arrest at the G1 phase. It also has a remarkable oscillation pattern during the endoreplication of trophoblast giant cells, where $p57^{Kip2}$ protein degrades before the S phase entry and accumulates after successful DNA replication [381]. The diminished expression of $p57^{Kip2}$ associates with several sporadic cancers, including bladder cancer [382, 383]. Hence, the aberrant activity of *Kcnq1ot1* may underlie the cell cycle modulation, most likely G1 phase arrest, and cancer onset indirectly through its imprinted target CDKN1C. However, a recent study demarcated a genuine connection between human *KCNQ1OT1* and cell cycle regulation in glioma tissues and cells [384]. In this context, *KCNQ1OT1* acts as ceRNA that scavengers miR-370 molecules, which targets the 3' UTR of cyclin E2 mRNA (*CCNE2*). Thus, higher expression levels of *KCNQ1OT1* promotes *CCNE2* expression, permitting the G1/S phase progression.

PANDA: As the case in *pncRNA*, the p21-associated ncRNA DNA damage-activated (*PANDA*) lncRNA is transcribed from a promoter region in response to DNA damage [385]. *PANDA* transcription takes place at CDKN1A ($p21^{Cip1}$) promoter in several cell lines. Upon activation, *PANDA* interacts with the nuclear transcription factor Y subunit alpha (NF-YA), which binds to the CCAAT boxes-containing promoters of various target genes. Among these targets, NF-YA controls the transcription of topoisomerase II alpha (*TOP2A*), *CDK1*, *CDC25C, CYCB1,* and other apoptosis-related genes [386, 387]. Interestingly, NF-YA suppresses the transcription of mitotic cyclins and CDKs upon G2 phase arrest in DNA-damaged cells. Consequently, upon DNA damage induction, *PANDA* evicts or prevents NF-YA from binding the promoters of its targets, including pro-apoptotic genes. This was evident by the significant reduction of NF-YA occupancy over the promoter regions of PUMA, NOXA, FAS, and CYCB1 in *PANDA*-depleted cells [385]. Although *PANDA* silencing affected the expression of several cell cycle-related genes, the expression of *CDKN1A* remained unaffected. Seemingly, p53 induces the transcription of *PANDA*; however, such induction does not intersect with *CDKN1A* expression. So, *PANDA* restricts the onset of apoptosis in DNA-damaged cells. Strikingly, further investigation shed light on the functional relevance of *PANDA* expression to cellular senescence [388]. Apart from the interaction with the

NF-YA transcription factor, *PANDA* interacts also with the scaffold-attachment-factor A (SAFA), which sequentially recruits PRC1 and PRC2. The *PANDA*-SAFA-PRC complex deposits H2AK119 ubiquitination and mediates the epigenetic silencing of senescence-associated factors. These factors comprise, among others, *CDKN2A*, *IL6R*, *IL8*, *CCNE1*, and *JUNB*. In a similar line, a recent study reported that the elevated expression of *PANDA* in hepatocellular carcinoma represses the transcription of the senescence-associated factor interleukin 8 (*IL8*) [389]. The expression and release of *IL8* demarcate the onset of cellular senescence, leading to further transcriptional reprogramming and obstruction of cell division. Therefore, in a collective model, *PANDA* preferentially interacts with NF-YA to titrate it away from the promoters of pro-apoptotic genes, whereas the *PANDA*-SAFA-PRC complex epigenetically suppresses cellular senescence. In turn, the resistance to apoptosis and failure of senescence onset in DNA-damaged environment may lead to genomic instability and carcinogenesis [374].

1.2.4.2 LncRNAs in G2/M phases

LY6K-AS: As presented in the current thesis, *LY6K-AS* is transcribed in the antisense direction of the LY6K protein-coding gene, located at chromosome 8. Higher expression of *LY6K-AS* predicts poor survival outcome in lung adenocarcinoma patients. The elevated expression of *LY6K-AS* also contributes to the onset and stepwise progression of lung squamous cell carcinoma [390]. Besides, an earlier study demonstrated that *LY6K-AS* levels decrease significantly upon the onset of tamoxifen-induced senescence in immortalized human fibroblasts [391]. Currently, we have uncovered the role of *LY6K-AS* in mediating faithful chromosomal segregation in lung cancer cells. To do so, *LY6K-AS* associates with the evolutionarily conserved members of the 14-3-3 family of proteins. This association stabilizes 14-3-3 proteins and inhibits their ubiquitin-mediated degradation. Subsequently, the RNA-protein complex stimulates the transcription of kinetochore members and spindle assembly checkpoint proteins. For instance, *LY6K-AS* positively regulates *BUB1*, *BUB1B*, *SPC25*, *MAD2*, *CDK1*, *CYCB1*, *CYCB2*, and *AURKA*. Cells devoid of *LY6K-AS* exhibit a significant increase in mitotic aberrations associated with altered DNA content and karyotype. Notably, *LY6K-AS* depletion does not induce apoptosis; instead, it is associated with mitotic catastrophe and cell cycle arrest.

lincRNA-RoR: The regulator of reprogramming (*lincRNA-RoR*) transcript was first identified in induced pluripotent stem cells (iPSCs) [392]. The elevated expression of *lincRNA-RoR* is an essential constituent in guiding the reprogramming of the parental primary fibroblasts, depending on the activity

of OCT4, SOX2, and NANOG. The transcriptional modulation of *lincRNA-RoR* impacts the onset of pluripotency in fibroblasts but does not affect the proliferation capacity of the investigated cells. Meanwhile, the depletion of *lincRNA-RoR* in iPSCs and embryonic stem cells results in the induction of p53-dependent DNA damage response and apoptosis [392]. Mechanistic studies revealed that *lincRNA-RoR* interferes with *p53* mRNA translation in DNA-damaged MCF-7 cells, while the effect is exceptionally modest in unperturbed cells [393]. Of note, *lincRNA-RoR* interacts through a defined sequence of 28 bases with the cytoplasmic form of hnRNPI protein, which suppresses *p53* post-transcriptionally. Hence, *lincRNA-RoR* silencing arrest cells at the G2/M phase due to p53-mediated DDR [393]. From a cancer perspective, *lincRNA-RoR* increases dramatically in a hypoxic environment, as seen in HCC cells [394]. In gastric and colorectal cancer cell lines, *lincRNA-RoR* occupies the promoter region of the cofactor TESC and acts as a decoy to evict G9a histone methyltransferase [395]. This leads to a drastic decrease in repressive H3K9 histone methylation and subsequent expression of *TESC*. Remarkably, a higher level of *TESC* expression corresponds to the enrichment of cancer stem cells population among lung cancer cells [396]. Intriguingly, it is not clear whether *TESC* expression alters p53 levels in unperturbed or DNA-damaged iPSCs, as well as other cancer model systems.

MANCR: Recent transcriptomic profiling of the non-tumorigenic breast epithelial cell line MCF-10A, estrogen receptor-positive cancer cell line MCF-7, and triple-negative breast cancer cell line MDA-MB-231 reported the dysregulation pattern of the mitotically-associated lncRNA (*MANCR*) [397]. Higher expression of *MANCR*, in addition to copy number gain, is associated with poor prognosis of breast cancer patients. *In vitro* assays indicated that *MANCR* expression is indispensable for survival and cell cycle progression, whereas *MANCR* depletion causes cell cycle arrest at the G2/M phase. Similar to *LY6K-AS*, *MANCR* expression positively drives cell cycle-related processes in multiple cell lines. On the other hand, *MANCR* abrogation reduces the mitotic index and causes defects in cytokinese defects of the replicating cells, leading to altered DNA content and polyploidy. Although *MANCR* empowers proper chromosomal segregation, the mechanistic understanding of the transcript remains elusive.

lncRNA-hPVT1/mPvt1: As depicted by its name, the plasmacytoma variant translocation 1 lncRNA (*lncRNA-PVT1*) represents a common integration site for murine leukemia virus that induces T cell lymphoma [398]. The PVT locus spans >300 kb downstream of c-Myc at chromosome 8q24 in mouse, which encodes several noncoding RNA species. The predicted oncogenic functions of the mouse *lncRNA-mPvt1* led to further discoveries of the

oncogenic properties of its human orthologue *lncRNA-hPVT1*, especially in liver-related cancers [399, 400]. Hence, a systematic transcriptomic profiling of fetal, neonatal, and adult mouse liver demonstrated that *lncRNA-mPvt1* is an oncofetal factor enriched exclusively in fetal liver tissues. It is also associated with stem cell-like properties, and not surprisingly, demonstrates remarkable activation in primary mouse and human tissues of HCC [400]. To mediate its oncogenic functions, *lncRNA-hPVT1* binds and stabilizes the nucleolar protein 2 (NOP2), leading to sustained proliferation and cell cycle progression. Notably, *lncRNA-hPVT1*/NOP2 complex induces the expression of cell cycle-related genes, including mitotic cyclins, CDK1, MCMs, and PLK1. However, unexpectedly, the transcriptional modulation of the lncRNA results in a decrease in the S phase with accumulation in the G1 phase. Intriguingly, it remains unclear how the *lncRNA-hPVT1*/NOP2 complex transactivates multiple cell cycle factors. Also, one would expect a more pronounced effect on mitotic progression and chromosomal segregation as well in *lncRNA-hPVT1*-devoid cells. It is also worth noting that the upstream regulation of the lncRNA is controversial since two proposed models exist. The first model indicated that p53 binds the canonical *cis*-responsive elements at the promoter of PVT1 locus, and transactivates several miRNAs and *lncRNA-hPVT1* in DNA-damaged cells[401]. The second model proposed that human transforming growth factor-beta 1 (TGF-β1) is the primary activator of *lncRNA-hPVT1* [400]. This model further relies on other simultaneous findings that implicate the HBV infection in activating TGF-β signaling [402], whereas HBV readily increases *lncRNA-hPVT1* [400]. Possibly, the upstream regulation of *lncRNA-hPVT1* depends on the cellular status with respect to the DNA damage response.

APAL: A recent investigation reported the overexpression of the Aurora-A/polo-like kinase1-associated lncRNA (*APAL*) in multiple cancers, including breast and lung cancers [403]. Higher expression levels of *APAL* predicts unfavorable prognosis of patients' disease-free survival and relapse-free survival. Complimentary loss- and gain-of-function experiments demonstrated the utmost importance of *APAL* expression in driving the oncogenic proliferation and cell cycle progression of breast cancer triple-negative cells. Importantly, *APAL* silencing induced mitotic catastrophe and unfaithful chromosomal segregation in multiple cell lines accompanied by a significant increase in apoptotic cells. At the mechanistic level, *APAL* interacts with both Aurora A and Polo-like kinase 1 (PLK1), which are the crucial factors in orchestrating the formation and attachment of the microtubules to metaphase chromosome [404]. Thus, *APAL* depletion negatively impacted PLK1 phosphorylation, but not Aurora A, leading to the decreased assembly of PLK1-Aurora A and subsequent chromosomal defects.

In agreement with the observations presented in the current thesis, altering the mitotic progression affects the proliferation capacity of chemoresistant cells. Therefore, the therapeutic interference with the M phase progression confers new strategies for cancer treatment.

1.2.4.3 LncRNAs with multiple modes and phases

MALAT1: The metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*), is also known as nuclear-enriched abundant transcript 2 (*NEAT2*). The lncRNA is transcribed from the 11q13 locus, giving rise to a nearly 8 kb-long transcript, which is exceptionally abundant with high sequence conservation across 33 mammalian species [405]. The elevated expression of the *MALAT1* is associated with highly metastatic lung adenocarcinomas [406, 407] and predicts patients' survival in multiple types of cancer [405, 408]. However, recent investigations revealed a potential tumor suppression-related function of *MALAT1* in glioblastoma by attenuating the ERK/MAPK signaling [409]. In human tissues derived from patients diagnosed with breast or colorectal cancer, *MALAT1* showed a significant reduction compared to healthy counterparts. It also exhibited a significant positive correlation with the expression of the tumor suppressor *PTEN*. Using various breast and colorectal cancer cell lines, *PTEN* silencing diminished *MALAT1* expression, whereas the reciprocal regulation is insignificant. It became evident that *PTEN*, at least in the investigated cell lines, sponges several miRNAs (miR-17, miR-20a, miR-10b), which target the *MALAT1* transcript. Hence, higher expression of *MALAT1* suppresses the migration-inducing proteins, such as integrin β4 (ITGB4) and epithelial cell adhesion molecule (EpCAM) [410]. Importantly, a parallel study generated a knock out mouse model of *Malat1*, using a targeted inactivation strategy [411]. Further elegant genetic breeding experiments in the metastatic breast cancer mouse model revealed the metastasis-suppressive function of *Malat1*. Engrafting human breast cancer cells lacking *MALAT1* exhibited higher metastatic potential than cells with optimal *MALAT1* expression. Interestingly, the genetic add-back of *Malat1* was able to inhibit breast cancer metastasis into mouse lung tissues. These collective observations refer to the tissue specificity and the context-dependent functions of *MALAT1*.

For adding another layer of complexity, there are various proposed mechanisms for *MALAT1*-dependent modulation of the cell cycle progression. The first model involves the active serine/arginine (SR) splicing factors in *MALAT1*-mediated functions. *MALAT1* localizes predominantly into the nuclear speckles, but it is not an essential structural component, and also interacts with SR proteins. Thus, it was proposed that *MALAT1* regulates

alternative splicing of pre-mRNAs within the nuclear speckles [312]. *MALAT1* silencing decreases the phosphorylation levels of SR proteins and affects the alternative splicing machinery. Intriguingly, prolonged depletion of *MALAT1* in HeLa cells results in mitotic aberrations and chromosomal fragmentation in cells progressing into the M phase. Meanwhile, interphase cells were unaffected until the onset of the M phase, indicating the cell cycle phase-specific role of *MALAT1*. However, another model proposed a more diverse role of *MALAT1* in promoting G1 progression and transition into the S phase [412]. For instance, *MALAT1* depletion in human diploid fibroblasts (HDFs) caused a drastic reduction of DNA synthesis associated with an increase in the percentage of cells population in the G1 phase. These observations are in line with the documented role of *MALAT1* in driving the G1 progression through the activation of E2F1 [413]. Concordantly, the depleted cells experienced intrinsic DNA damage and elevated expression of p53, $p21^{Cip1}$, and $p27^{Kip1}$ proteins alongside with cellular senescence. Of note, p53-null or $p16^{INK4a}$ defected cells fail to activate intra S phase checkpoint and progress to the M phase with DNA defects upon *MALAT1* silencing. Also, a drastic decrease in the levels of E2F1 and pRB takes place following the accumulation of p53 in MALAT1-deficient cells. These observations suggest that p53 is an immediate downstream target of *MALAT1*. Detailed analysis indicated that *MALAT1* drives the transition from the quiescence at the G0 phase into the G1 phase, as *MALAT1*-deficient cells do not respond to serum activation. Independently from the G1/S phase regulation, *MALAT1* also is required for proper M phase progression [412]. The latter phenotype points out to the crucial role of the lncRNA in mediating proper alternative splicing via SR proteins. *MALAT1*-depleted cells demonstrate a global alteration of splicing patterns. Among the aberrantly-spliced transcripts, *B-MYB* and *CENPE* are of prime importance due to their activity in mediating the transcription of mitotic genes, and spindle elongation, respectively [414, 415]. Thereby, defected *B-MYB* and *CENPE* interfere with normal mitotic progression and faithful chromosomal segregation, pointing out the indispensable place of *MALAT1* at the core cell cycle circuitry.

LincRNA-p21: As deduced from the terminology, *lincRNA-p21* resides in close upstream proximity of the cell cycle regulator $p21^{Cip1}$ (CDKN1A) in both human and mouse cells. The presence of the active transcription histone marks (H3K4me3) coupled with chromatin immunoprecipitation validations indicated that *lincRNA-p21* is an independent transcription unit expressed in the opposite direction of its neighboring $p21^{Cip1}$ gene [320]. By adopting two independent cell lines derived from engineered mice, harboring either Cre-loxP-activating p53 system or oncogenic K-Ras mutation [416], Huarte et al. identified *lincRNA-p21* as a p53-responsive element [320]. Upon the

exogenous induction of DNA damage, p53 binds a consensus sequence at the promoter region of *lincRNA-p21* and induces the lncRNA transcription. Further transcriptome-wide analyses indicated that p53 and *lincRNA-p21* commonly regulate a broad set of transcripts (930 mRNAs), whereas this shared regulation does not exist in p53-null cells. Although DNA-damaged cells showed an increase in cell viability and DNA synthesis upon p53 silencing, *lincRNA-p21* knockdown affected only the cell viability but not the cell cycle progression. Therefore, the *lincRNA-p21* expression most likely is associated with apoptosis induction. To this end, *lincRNA-p21* binds to the hnRNPK protein through a 780 base-long localization signal at the 5' end of the RNA. The complex then occupies the promoters of p53 target genes and represses their transcriptional activities. Thereby, the *lincRNA-p21*/hnRNPK complex potentiates a pro-apoptotic response upon the induction of DNA damage. Of note, in the presented thesis, we are reporting a contrasting function of hnRNPK, where it enhances the transcription of proliferative and anti-apoptotic genes via the association with *SCAT7*. Based on our studies, the silencing of *hnRNPK* in multiple cell lines, including KRAS-mutated lung cancer cells, induces cell cycle perturbations, and apoptosis. In turn, it is possible that the protein functions in a context-dependent manner, depending on the cell-of-origin. The latter notion is supported by several reports that indicate the multiple modes of action associated with the hnRNPK aberrant expression [417].

Another novel function of *lincRNA-p21* emerges from its critical contribution to somatic cell reprogramming, independently from apoptosis and senescence [418]. In this context, it is worth mentioning the importance of using the pre-iPSC system in delineating the transition from partially programmed to fully programmed pluripotent cells. An earlier study demonstrated the utmost importance of the H3K9 methylation in acting as a barrier against the full reprogramming of pre-iPSCs into iPSCs [419]. Thus, a more in-depth investigation of the pre-iPSCs barrier demarcated the *lincRNA-p21*-mediated establishment of H3K9me3 and/or CpG methylation at the promoters of pluripotency genes [418]. In doing so, *lincRNA-p21* binds to the H3K9 methyltransferase Setdb1 and the DNA methyltransferase Dnmt1, separately. These interactions underlie the epigenetic silencing of the core pluripotency genes, such as *Nanog*, in mouse pre-iPSCs. The silencing of *Hnrnpk* phenocopies the effect of *lincRNA-p21* silencing and enhances the expression of the pluripotency-related genes. Not surprisingly, *Hnrnpk* depletion reduces the occupancy of the Setdb1 and Dnmt1 alongside with the associated repressive H3K9me3 and CpG methylation at the target genes promoters, respectively. Therefore, Hnrnpk mediates the interaction of *lincRNA-p21* with distinct epigenetic remodelers and facilitates the subsequent recruitment

at the pluripotency-associated genes. Of note, in this scenario, *lincRNA-p21* modulation neither alters the *Cdkn1a* expression nor the cell cycle progression.

Apart from its association with *hnRNPK*, *lincRNA-p21* interacts with the RNA binding protein HuR in HeLa cells [298]. This protein modulates the stability or the translation of target mRNAs, affecting a wide array of cellular processes [420]. Surprisingly, the abrogation of *HuR* expression stabilized and increased the half-life time of *lincRNA-p21* in both human and mouse cells. Indeed, HuR permits the binding of let-7/Ago2 to *lincRNA-p21*, facilitating the subsequent decay. Such negative regulation of *lincRNA-p21* enhances the expression levels of *CTNNB1* and *JUNB*, which encode the β-catenin and JunB factors, respectively. Higher expression of these factors promotes cancer progression and migration [421, 422]. Thus, *lincRNA-p21* was proposed to suppress *CTNNB1* and *JUNB* expression. Experimental validations demonstrated that the base-pairing of *lincRNA-p21* with *CTNNB1* and *JUNB* interferes with their translational polysomes. Hence, the HuR/let-7/Ago axis cooperatively downregulates *lincRNA-p21*, conferring more stability and translation of the oncogenic factors *CTNNB1* and *JUNB*.

Collectively, the noncoding portion of the genome contributes significantly to the regulation of vital biological processes. However, the unprecedented increase in the number of functional studies focusing on lncRNAs, especially in cancer, may introduce a lot of bias and false discoveries. Thus, a critical functional dissection coupled with stringent technical and statistical measures may aid in eliminating the present discrepancies. Towards this, we thought of exploiting the cell cycle dependencies in cancer cells as a gateway to a better understanding of lncRNAs dynamics. The following section highlights the main aims and rationale behind the current thesis.

2 AIMS

The main objective of the current thesis is to challenge the predominant protein-centric postulation of cell cycle regulation, especially in cancer. Thus, the collective studies presented in this thesis aim to test the hypothesis that the noncoding portion of the genome, evidenced by lncRNAs, contributes to the complex regulatory network of the cell cycle process. Further, the thesis aims to investigate whether exploiting cell cycle vulnerabilities, through lncRNA transcriptional modulation, provides potential therapeutic regimens for treating various cancer types.

Specific aims:

Paper I: The first study aimed to develop and optimize a nascent RNA capture assay that precisely detects the transcriptional events occurring during the S phase in real-time. The study also aimed to investigate the temporal resolution of replication/transcription conflict during the DNA synthesis phase of the cell cycle.

Paper II: The second study aimed to identify potential cancer-related biomarkers based on the expression profiles of the S phase-enriched lncRNAs. The study further aimed at investigating the role of an uncharacterized lncRNA, termed as *SCAT7*, in promoting oncogenic signaling and cell cycle progression in various cancer models.

Paper III: In connection with the second study, we aimed to unveil the functional significance of *SCAT7* to the DNA replication process and maintenance of genome integrity. Besides, the study aimed at addressing the underlying regulatory mechanism that governs *SCAT7* transcriptional activities in unperturbed cells as well as cells exposed to various stress stimuli.

Paper IV: The study aimed to investigate the prognostic capacity and functions of the *LY6K-AS* lncRNA, which is differentially expressed in lung adenocarcinoma patients. The study also focused on dissecting the transcriptional reprogramming mediated by *LY6K-AS* modulation in association with the oncogenic mitotic progression. Finally, we aimed to study the effect of mitotic inhibition on the acquired chemoresistance, utilizing *in vitro* and *in vivo* models.

3 MATERIALS AND METHODS

3.1 Nascent RNA capture assay

The capture of S phase lncRNAs requires efficient synchronization of the cells at the boundary of the G1/S phase. Thus, we achieved efficient synchronization using the thymidine/hydroxyurea double block method. To start with, 5×10^5 HeLa cells were seeded overnight in a complete medium. For each time point, we designated an individual flask; also, one flask was kept as unsynchronized control. In the next day, the medium was aspirated, the cells were washed with PBS and incubated for 10h in medium supplemented with 2 mM thymidine (Sigma). Then, cells were washed with PBS and incubated overnight with medium supplemented with 1 mM hydroxyurea (Sigma). To allow the progress through the S phase, the medium was aspirated, cells washed with PBS, and a complete medium is added. We collected one flask containing synchronized cells at the start of the block release, and this time point is referred to as "T0". We labeled the nascent RNAs for overlapping 2h time points at different stages of the S phase. At the first time point, the medium was supplemented with EtU (Invitrogen) to a final concentration of 1 mM, and cells were allowed to progress for 2h then harvested. The labeling process was repeated at overlapping periods, defined as follows: T0h–T2h (early S phase), T1.5h–T3.5 h (middle S phase), and T3h–T5h (late S phase). For unlabeled steady-state RNA samples, the cells were collected at the same time points without any external addition of the EtU label. We isolated RNA from all samples with Tri reagent/chloroform (Ambion), followed by DNA digestion with RQ1 DNase I (Promega) for 1h at 37 °C. The RNA content of each sample was separated from the fragmented DNA through another round of Tri reagent/chloroform extraction. The RNA samples were precipitated overnight in absolute ethanol kept at -20 °C. The precipitated RNA samples were collected by centrifugation at maximum speed for 30 min in the cold, then washed twice with 70% ethanol and re-suspended in a minimal volume of nuclease-free water. For each sample, 10 µg RNA was subjected to rRNA depletion using a Ribominus kit (Invitrogen). Next, considering only labeled RNA samples, 3 µg of each rRNA-depleted RNA sample were biotinylated with Click-iT™ Nascent RNA Capture kit (Invitrogen) following the manufacturer's protocol. The biotinylated RNA was captured using streptavidin magnetic beads. We eluted the captured biotinylated RNA by incubating the beads-bound RNA in 200 µl elution buffer (2 mM biotin, 1 M NaCl, 50 mM MOPS, 5 mM EDTA, 2 M 2β-Mercaptoethanol, pH 7.4) for 3 min at 95 °C. The eluted RNA was

immediately recovered from the supernatant and precipitated in 30 µl 3 M sodium acetate (pH 5.2), 1 µl glycoblue (Invitrogen), and 750 µl absolute ethanol. RNA was then re-suspended in nuclease-free water. For unlabeled RNA samples, total RNA was extracted and subjected to rRNA depletion, but not biotinylation and subsequent capture and elution.

3.2 Chromatin oligo-affinity precipitation (ChOP)

For either HeLa or A549 cell lines, 20×10^6 cells were used for each immunoprecipitation. We used two different cross-linking strategies; either direct UV crosslinking or formaldehyde-assisted covalent crosslinking. Considering the UV cross-linking, 3 ml of ice-cold PBS were first added to each culturing dish. Then, all culturing dishes were maintained on ice and exposed to a UV source with a 254 nm wavelength bulb at 2000 mJ/cm^2. Following the UV fixation, cells were scrubbed gently from the culturing dishes on ice and collected in 50 ml tubes. For the formaldehyde fixation, we collected the cells using trypsin, washed them twice with ambient PBS cells, and incubated the washed cells with 10 ml of 1% formaldehyde in PBS for 10 min at room temperature with gentle rotation. The reaction was quenched by adding glycine at a final concentration of 125 mM for 5 min with gentle rotation at room temperature. The fixed cells, either UV or formaldehyde-fixed, were washed twice with ice-cold PBS and spun down for 5 min at 2000 xg in the cold. We used cellular and nuclear lysis buffers, termed as Buffer A and Buffer B, respectively. To start with, 2 ml of buffer A (3 mM $MgCl_2$, 10 mM Tris-HCl; pH 7.4, 10 mM NaCl, 0.5%v/v NP-40, 0.5 mM PMSF and 100 units/ml RNase inhibitor) were incubated with each pellet on ice for 20 min with gentle pipetting every 5 min. We harvested the nuclei by centrifugation at 14,000 × g for 15 min at 4°C. The supernatant was discarded and the pelleted nuclei were resuspended in 1.2 ml of buffer B (50 mM Tris HCl; pH 7.4,10 mM EDTA, 0.5% Triton X-100, 0.1%SDS, 0.5 mM PMSF and 100 units/ml RNase inhibitor). The nuclei were lysed on ice for 40 min with thorough pipetting every 5 min. Following this, we added another 1.2 ml of buffer C (15 mM Tris-HCl; pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5 mM PMSF, and 100 units/ml RNase inhibitor) and incubated the partially-lysed nuclei for 15 min on ice. Lysates were sheared using a Bioruptor sonicator (Diagenode) for 20 cycles at a high pulse. We pooled different biotinylated probes targeting the lncRNA of interest (+ve probes) to pull down the lncRNA and its associated proteins. The probes were designed using LGC Biosearch Technologies' Chromatin Isolation by RNA Purification (ChIRP). The probe designer online tool, used in this method section, is found at

https://www.biosearchtech.com/support/tools/design-software/chirp-probe-designer. We used LacZ biotinylated probes as a negative control in addition to an extra control which is complementary to the +ve probes. Sonicated lysates were incubated overnight on gentle rotation at 4°C with yeast tRNA (100 µg/ml), salmon sperm DNA (100 µg/ml) and each individual set of mixed probes at a final concentration of 10 µM. The lysate/probes mixtures were incubated for 3h at 4°C on rotation with streptavidin-coupled Dynabeads (ThermoFisher Scientific). Beads were then washed twice for 5 min each at 4°C with low salt buffer (20 mM Tris-HCl, pH7.9, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% TritonX-100, 0.5 mM PMSF, and 50 units/ml RNase inhibitor). Then, two more washes with high salt buffer (20 mM Tris-HCl, pH7.9, 500 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100, 0.5 mM PMSF, and 50 units/ml RNase inhibitor) were applied for 5 min each at 4°C. Finally, the beads-bound proteins were washed twice with PBS at room temperature. Different species of RNA-protein complexes were further eluted by heating and vigorous mixing of the beads at 80 °C for 15 min with 150 µl of 0.1% SDS in PBS. The purified eluents were subjected to mass spectrometry analysis.

3.3 Chromatin immunoprecipitation (ChIP)

Following the same formaldehyde fixation steps performed in the ChOP technique, 20×10^6 cells were used for each individual immunoprecipitation. The crosslinked cells were spun down for 10 min at 2000 × g in the cold. Each cells pellet was lysed on ice using 1 ml of SDS lysis buffer (0.1% SDS, 0.5% Triton X-100, 20 mM Tris-HCl, pH 8, and 150 mM NaCl, 1 mM PMSF, and 100 units/ml RNasin) for 30 min with continuous rigorous pipetting every 5 min. The lysed pellets were sonicated at high pulse using a Bioruptor for a total of 40 cycles. The sheared soluble chromatin was cleared maximum speed centrifugation at 4 °C for 10 min, and the supernatant was transferred to a new tube. The size distribution of the sheared DNA verified on 1% agarose gel electrophoresis and Agilent DNA bioanalyzer. The sonication conditions can be modified depending on the used cell line and also the downstream pipeline. In general, optimized immunoprecipitation for qPCR validation may work with higher fragments length; however, ChIP-seq applications require strict DNA fragments distribution in the range of 100-1000 bps. Following the fragments size quality check, 1% volume/volume of the sheared DNA lysate was kept as input and stored at -80°C till the DNA extraction step. Meanwhile, the sonicated DNA was incubated overnight with the respective antibodies for immunoprecipitation at 4 °C on gentle rotation. In addition to the target protein of interest, we used IgG antibody as a

negative control for immunoprecipitation. It is worth mentioning that the IgG control could be either mouse or rabbit to match the protein of interest. For each reaction, 4 μg of antibody, either target or IgG, were used per 1 mg of sonicated DNA. We captured the immunoprecipitates on Protein G/A Dynabeads (ThermoFisher Scientific) for 3 h at 4 °C with gentle rotation. Following the incubation time, the beads-bound complexes were separated from the rest of the lysate by magnetic precipitation. The beads-bound proteins were washed twice with low salt buffer (0.1% SDS, 1% Triton-X 100, 2 mM EDTA, 20 mM Tris-HCl, 150 mM NaCl, 0.5 mM PMSF, and 50 units/ml RNasin) for 5 min in cold. Then, another two washes with high salt buffer (0.1% SDS, 1% Triton-X 100, 2 mM EDTA, 20 mM Tris-HCl, 500 mM NaCl, 0.5 mM PMSF, and 50 units/ml RNasin) were applied for 5 min each in cold. The beads-bound precipitated proteins were eluted by incubating the samples at 55 °C for 30 min with 400 μl of elution buffer (100 mM NaHCO3, 1% SDS, 0.5 mM PMSF and 50 units/ml RNasin) with vigorous shaking. The supernatants were separated from the beads and further processed for DNA isolation by the phenol-chloroform method. Equal volumes of the purified DNA samples were used in qPCR to estimate the relative enrichment of each antibody over the promoter regions of the investigated genes. Multiple sets of overlapping primers were designed to span and amplify different regions upstream and downstream to the transcription start sites. We used the following formula to calculate the relative enrichment of the target protein and IgG separately:

$$\%\ of\ input = \%\ of\ startting\ input\ fraction \times 2^{\wedge[Ct(input) - Ct(IP)]}$$

3.4 RNA immunoprecipitation (RIP)

For each immunoprecipitation reaction, 10×10^6 cells were collected by trypsin and washed with PBS twice at ambient temperature. We used the formaldehyde-assisted crosslinking method to fix the RNA-protein complexes. The cells were incubated with 10 ml of 1% formaldehyde in PBS for 10 min on gentle rotation. The cells were then spun down at 1800 × g for 5 min at room temperature. The cells pellets were washed with 10 ml PBS, spun down, and then re-suspended in 10 ml of glycine (0.125 M) to quench the reaction for 5 min at room temperature with gentle agitation. Following the glycine aspiration, the cells were washed twice with 10 ml ice-cold PBS. The cells were lysed on ice for 20 min using 10 ml (1 ml/10^6 cell) of nuclear isolation buffer (1.25 M glucose, 40 mM Tris HCl; pH7, 20 mM $MgCl_2$, 1× protease inhibitor cocktail, 40 U/ml RNase inhibitor). The lysates were spun down at 2500 × g for 10 min at 4 °C, then re-suspended in 600 μl of RIPA

buffer (150 mM NaCl, 50 mM Tris HCl; pH7, 0.5% sodium deoxycholate, 0.2% SDS, 1% NP-40, 1× protease inhibitor cocktail, 40 U/ml RNase inhibitor). The lysates were sonicated at high power for 10 cycles till the lysates became clear. Following the sonication, the lysates were spun down for 15 min at maximum speed in the cold. The supernatants were transferred to new tubes, and 1% v/v was kept as input. The supernatant in each tube was then divided into two tubes for further immunoprecipitation with the target protein of interest or IgG negative control. To prepare the precipitation reaction, we used Protein G/A Dynabeads (ThermoFisher Scientific) depending on the primary antibody host. In total, 100 µl of the beads were washed twice with 500 µl of RIPA buffer, then re-suspended in 100 µl of the same RIPA buffer. The re-suspended beads were then divided into two tubes where the volume of each tube was adjusted to 600 µl with RIPA buffer and then incubated with 2−5 µg of the primary antibody (target or IgG) for 3h in cold with gentle rotation. After that, the beads were separated on a magnetic rack and the supernatant in each tube was discarded. For each antibody-bound beads, 250 µl of the cleared sonicated lysates were added and the volumes were adjusted to 600 µl with RIPA buffer. The beads/lysate tubes were incubated for 3h in cold with gentle rotation. Then, the beads were separated on a magnetic rack and washed three times with low salt buffer (1× PBS, 0.1% SDS, 0.5% NP-40, 1× protease inhibitor cocktail, 40 U/ml RNase inhibitor) for 5 min each in cold. The bead were washed again twice with high salt buffer (5× PBS, 0.1% SDS, 0.5% NP-40, 1× protease inhibitor cocktail, 40 U/ml RNase inhibitor) for 5 min each in cold. Next, the RNA/proteins crosslinks were reversed by re-suspending and incubating the beads in 35 µl of RNA extraction buffer (100 mM NaCl, 10 mM Tris HCl; pH7, 0.1 mM EDTA, 0.5% SDS, 10 µg/ml proteinase K) for 45 min at 55°C with rigorous mixing, followed by heat inactivation at 95°C for 15 min. The beads were then precipitated and the digested supernatant from each tube was transferred to a new tube. RNA extraction from each immunoprecipitation reaction, as well as the input, was done using Tri reagent/chloroform method followed by DNase treatment. The purified RNA samples were subjected to cDNA preparation where equal volumes, not concentrations of RNA, were considered. The relative RNA enrichment for each immunoprecipitation was calculated using the following formula:

$$\%\ of\ input = \%\ of\ startting\ input\ fraction \times 2^{\wedge[Ct(input) - Ct(IP)]}$$

3.5 Immunoprecipitation of ubiquitinated proteins

To capture the ubiquitinated proteins, proteasomal-mediated degradation has to be prevented. To do so, we treated the cells overnight with 7.5− 10 μM MG132. Then, $3x10^6$ cells were harvested, washed twice with PBS, and lysed with ice-cold lysis buffer (150 mM NaCl, 50 mM Tris, 2.5 mM EDTA, 1% NP-40, 0.1% SDS, 10mM NEM, 1X protease inhibitors, 15μM MG132) for 10 min in cold. The lysed cells were briefly sonicated, and pelleted down by centrifugation for 10 min at 4°C. We stored 1% of the lysate to serve as input. Equal amount of protein lysates were used in subsequent immunoprecipitations. Thus, the lysates were incubated overnight rotating in cold with 5 μg of the respective primary antibodies, representing the target of interest or the IgG negative control. For each immunoprecipitation reaction, we used 50 μl of Dynabeads Protein A/G (ThermoFisher Scientific). The beads were washed three times with lysis buffer, and then incubated with the antibody/lysate mix on rotation at 4°C for 2.5h. After this, the beads were washed twice with 500 μl of lysis buffer for 5 min each at 4°C. Then, we applied stringent washes twice with the lysis buffer supplemented with 800 mM NaCl. To elute the proteins, we boiled the beads in Tthe ris-Glycine SDS sample buffer (ThermoFisher Scientific) for 5 min vigorous agitation. The beads were separated on a magnetic rack, and the supernatant were immediately transferred into new tubes. All samples, in addition to respective inputs, were separated on 10% SDS-PAGE, followed by immunoblotting.

3.6 Immunofluorescence and RNA-FISH

For both immunofluorescence and RNA-FISH staining, cells were cultured on coverslips of high refractive index (≈1.52) in 6-well plates. In the case of immunofluorescence, we fixed the cells using 1% formaldehyde in PBS for 10 min at room temperature. The cells were then permeabilized with 0.25% Triton X-100 in PBS for 30 min. We used 1% BSA in PBS supplemented with 0.1% Triton X-100 (PBS-T) to block the unspecific signals for 1h. The coverslips-grown cells were later incubated with primary antibodies diluted in PBS-T with 1% BSA for 1h at room temperature with gentle shaking. The concentration of the primary antibodies ranged from 1:200− 1:1500, depending on the investigated protein of interest. Following the incubation time, the primary antibodies were aspirated, and the cells were then washed three times with PBS for 5 min each at room temperature. The primary antibodies-bound proteins were conjugated with appropriate Alexa FluorTM 555 or 488 secondary antibodies (Invitrogen) at 1:500 dilution in 1% BSA PBS-T by incubation at room temperature for 1h in dark. We mounted the coverslips with DAPI-containing Vectashield mounting medium (Vector

Laboratories, Burlingame, CA). The mounted slides were left to dry overnight in cold. The slides were further stored at -20°C.

For RNA fluorescent *in situ* hybridization (RNA-FISH) applications, we used custom Stellaris® FISH probes specific to the investigated lncRNA. The RNA-FISH probes were designed to cover the entire non-overlapping length of the lncRNAs. We used the manufacturer's Stellaris® RNA FISH Probe Designer (Biosearch Technologies, Inc., Petaluma, CA) available online at www.biosearchtech.com/stellarisdesigner (Version 4.2). The synthesized probed were conjugated with TAMRA fluorescent label, and we prepared the samples in RNase-free conditions following the manufacturer's standard protocol. Images were acquired using the EVOS™ FL Auto Imaging System (Life Technologies), or ZEISS LSM 800 confocal microscope.

3.7 Cell cycle profiling

We collected the cells to assess their cell cycle profiles at different time points depending on the designated experimental setup. In general, 10^5 cells were harvested, washed twice with ice-cold PBS, pelleted down by centrifugation, and fixed overnight in 70% ethanol at -20°C. Following the fixation step, the fixed cells were spun down for 5 min at ambient temperature, washed with PBS, and then stained either with propidium iodide (PI) or DAPI. For the PI-based staining, the cells were re-suspended in PBS alone and incubated for 30 min at 37°C. Subsequently, the cells were collected by centrifugation and re-suspended in 500 µl of PI solution (Sigma Aldrich) supplemented with 1% RNase A in PBS and kept at 4 °C for 4 h in the dark. For the DAPI staining method, the washed cells were re-suspended vigorously in 500 µl of DAPI solution (Chemometec, Denmark) and incubated for 10 min at 37°C with continuous agitation. The profiles of the PI-stained cells detected using Eclipse single-cell flow cytometry system ec800 and data were analyzed with the manufacturer's software. For DAPI-stained cells, the patterns were assayed using a NucleoCounter NC-3000 platform (Chemometec, Denmark). All samples were assayed at least three times independently, and the statistical analyses were done accordingly.

3.8 EdU incorporation, proliferation, and soft agar assays

We performed the EdU incorporation assay using the Click-iT™ EdU kit with Alexa Fluor™ 488 (ThermoFisher Scientific) according to the

manufacturer's instructions. The cells were cultured overnight on high refractive index coverslips. Depending on the nature of the experiment, the cells were kept in culture until it is appropriate to perform the incorporation assay. We used a short pulse labeling strategy to assess the progression of DNA synthesis. To this end, the cells were incubated with medium containing 10 μM of the EdU analog for 2h. Following the short pulse, the cells were washed with ambient PBS, fixed with 3.7% formaldehyde in PBS for 15 min at room temperature. The fixed cells were then washed twice 3% BAS in PBS and permeabilized for 20 min at room temperature with 0.5% Triton X-100 in PSB. Next, each coverslip was incubated for 30 min in the dark at ambient temperature with Click- iT™ EdU reaction cocktail. Finally, the coverslips were washed once with 3% BSA in PBS and mounted with DAPI-containing Vectashield mounting medium.

We performed proliferation assay either with CellTiter 96® Non-Radioactive Proliferation Assay kit (Promega, USA), or CellTiter-Glo™ luminescent cell viability assay kit (Promega, USA). For the CellTiter 96® Non-Radioactive assay, following each specific experimental setup, the media were aspirated, and cells were washed once with PBS, 425 μl of fresh medium plus 75 μl of MTT dye were added, and cells were incubated at 37 °C in the dark for 4 h. To terminate the reactions, 500 μl of stop solution was added to each well, and the cells were kept overnight in the dark at 4 °C to be lysed and solubilized. We kept empty wells containing the dye and stop solution as background control. The dye intensity was measured using a microplate spectrophotometer at 570 nm. In the case of CellTiter-Glo™ luminescent assay, the media were aspirated, cells were washed with PBS twice, and incubated at ambient temperature for 20 min on shaking with 100 μl of fresh medium and 100 μl of CellTiter-Glo™ reagent. Empty wells containing medium and CellTiter-Glo™ reagent without cells were measured and assigned as background luminescence. The luminescence intensity was measured using a luminescence microplate reader. All experiments were done using at least three independent biological replicates.

The soft agar assay determines the ability of cancer cells to grow and form colonies in an anchorage-independent manner. Thus, we used two different layers of growth agar with varying concentrations. In a standard 24-well growth platform, we prepared the hard layer of agar by mixing 1% molecular biology grade agar with 2 × medium (RMPI 1640 or DMEM) supplemented with 20% FBS in 1:1 ratio. The soft agar layer was left to polymerize for 30 min at sterile conditions. The investigated cells were finely dispersed in the soft agar layer by mixing 2500−5000 cells/well with a 500 μl mix of 1:1 0.6% agar and 2 × medium supplemented with 20% FBS. The soft agar layers

were left for 15 min to polymerize, and then we added 500 µl of standard growth medium supplemented with 10% FBS on the top of the agar layers. Following 7−10 of incubation at 37 °C, the colonies were imaged using an automated Z-stack function of the EVOS™ FL Auto Cell Imaging System (ThermoFisher Scientific). We counted the number of total colonies in each well and estimated the surface area of representative colonies. Appropriate statistical analyses were performed accordingly.

4 RESULTS AND DISCUSSION

4.1 Paper I:

Temporal separation of replication and transcription during S-phase progression

The complexity of the active transcriptional machinery during cell cycle progression aids to function in a spatiotemporal pattern across different phases and genomic loci. The temporal transcriptional events that take place during the S phase have to be well-orchestrated with the DNA replication machinery. Previous studies suggested that simultaneous RNA transcription and DNA replication leads to a collision between the replication machinery and different transcription-elongation complexes with fatal cellular consequences. Although several low throughput-based investigations have shown a temporal distinction between DNA replication and RNA transcription at individual loci, the global temporal pattern remains elusive at a higher resolution state. To this end, we optimized a nascent RNA capture assay coupled with high throughput sequencing to identify the ongoing transcriptional events taking place in different compartments of the S phase. The main findings of the presented study are summarized in the following sections.

4.1.1 Optimizing a nascent RNA capture assay

In order to study the transcriptional dynamics with respect to the DNA replication timing, we divided the S phase in HeLa cells into three overlapping compartments. We denoted these temporal compartments as early, mid, and late stages of the S phase. Using an optimized protocol, we labeled these stages with EtU analog to capture the nascent transcriptional products of each stage in real-time. Simultaneously, we collected the unlabeled transcripts of the respective stage, representing the steady-state transcriptional activities. The steady-state RNAs and the nascent RNAs shared a highly significant correlation at the overall profiles. However, we observed different variations at the levels of the individual genes identified using our optimized nascent RNA capture assay. For instance, the expression profiles of different histone variants, nucleosome assembly genes, and DNA double-stranded break responsive genes exhibited significant enrichment in the early S phase. The actin cytoskeleton-associated genes, however, revealed a predominant transcriptional activity in the mid-S phase. The nucleosome-

associated transcripts demonstrated a gradual accumulation, starting from the early onset and peaking at the mid-S phase. In a similar line, the nascent capture assay could mirror the dynamics of transcriptional stability obtained by earlier Actinomycin D chase experiments. On the contrary, the steady-state RNA capture approach could not provide precise functional enrichment of the temporal transcriptional events occurring in real-time at the DNA synthesis phase.

4.1.2 Replication and transcription timings are inversely correlated

By utilizing the publically available replication timing datasets in HeLa cells, we classified the captured transcripts into early and late-replicating genes. We calculated the average expression patterns for each compartment of the S phase based on steady-state and nascent RNA levels. This integrative analysis confirmed the earlier observations which associate late-replicating genes with lower expression levels compared to early-replicating genes. Nevertheless, the nascent transcribed RNAs, but not the steady-state, demonstrated a significant inverse correlation between replication and transcription timing. The inverse correlation was evident at the individual gene level, as well. Of note, the transcripts with higher S phase-specific expression had a more prominent inverse correlation with the replication timing. We validated further the inverse pattern with quantitative real-time PCR, utilizing both total RNA and nascent RNA material.

Collectively, our study provides an optimized method to capture the nascent transcribed RNAs at high resolution in real-time. In turn, it also provides evidence on the resolution of replication/transcription conflict through a temporal separation of timing.

4.2 Paper II:

PAN-cancer analysis of S-phase enriched lncRNAs identifies oncogenic drivers and biomarkers

The cell cycle regulation has always attracted a "protein-centric" point of view that implicates a complex network of closely related proteins and transcription factors. However, apart from a few well-studied lncRNAs, the roles of lncRNAs in orchestrating the cell cycle transitions and proper progression require adequate interrogation. Given that the DNA replication is the most critical process during cellular division, the regulatory elements governing the S phase progression would be of great importance for cell survival. Thus, in the present study, we utilized our optimized nascent RNA capture assay to identify and investigate the functional implications of the S phase-enriched lncRNAs in HeLa cells. The following sections highlight the main findings of the presented study.

4.2.1 S phase lncRNAs as cancer biomarkers

We initially identified 1,734 lncRNAs, which harbor significant enrichment at different compartments of the S phase over the unsynchronized samples. To investigate the temporal expression patterns of the identified lncRNAs, we applied Short Time-series Expression Miner (STEM) clustering analysis that identified 1,145 lncRNAs with four significant temporal expression patterns. Following that, we analyzed the differential expression patterns of the S phase-enriched lncRNAs across the TCGA datasets that comprise 16 different cancer types with respect to the corresponding healthy tissues. Using stringent filtering criteria (log fold-change ± 2 and FDR < 1E-004 in at least one cancer type), we identified 570 out of the 1,145 S phase lncRNAs with significant differential expression between normal and tumor tissues. Of note, nearly 73% of the differentially expressed lncRNAs demonstrate higher expression levels in the corresponding cancer types. Furthermore, we investigated the prognostic value of the differentially expressed S phase lncRNAs, utilizing a sequential statistical pipeline of Kaplan-Meier (KM) method and regression analyses. The statistical analysis identified 520 S phase lncRNAs that act as independent prognostic biomarkers for survival, at least in once cancer type.

4.2.2 *SCAT7* alters cell cycle progression and cell proliferation

Among the top candidates, we selected eight S phase lncRNAs for functional validation, and we coined them as S phase Cancer-Associated Transcripts (SCATs). We depleted the selected SCATs individually in various cancer cell model systems (HeLa; cervical cancer cells, A549; lung adenocarcinoma cells and Caki-2; renal clear cell carcinoma) with a variety of interfering molecules (siRNAs, shRNAs, and LNA-ASOs). The depletion of the selected SCATs induced significant perturbations in cell cycle progression accompanied by an inhibition of cell proliferation and elevated levels of apoptosis. For further in-depth functional studies, we chose *SCAT7* (also known as *ELF3-AS1* and *RP11-465N4.4*), which exhibits higher expression levels in multiple cancers, and its elevated expression predicts the patients' survival in renal clear cell carcinoma. Thus, we depleted *SCAT7* in different cancer cell lines (MCF-7; breast cancer, HepG2; hepatocellular carcinoma, H2228; lung adenocarcinoma, and 786-O; renal clear cell carcinoma). Of note, the transcriptional modulation of *SCAT7* interfered with most of the cancer hallmarks. For instance, *SCAT7* silencing resulted in significant cell cycle perturbations, induced apoptosis, and reduced cellular growth associated with reduced migration capacity of the investigated cell lines. Interestingly, *SCAT7* knockdown induced a senescence-associated phenotype in the immortalized human fibroblasts cells (BJ-BRAF and TIG3-BRAF). On the other hand, the overexpression of *SCAT7* inhibited the senescence-associated phenotype partially.

4.2.3 SCAT7 regulates the FGF/FGFR signaling pathway

We performed RNA-seq analysis of HeLa, Caki-2, and A549 cells upon *SCAT7* depletion. The subsequent differential expression and molecular pathway analyses demonstrated significant deregulation of the FGF/FGFR signaling pathway in the depleted cells. Also, several pro-survival pathways, such as PI3K/AKT and RAS/MAPK, exhibited significant dysregulation upon *SCAT7* depletion in multiple cell lines. To unveil the molecular mechanism by which *SCAT7* exerts its functions, we performed chromatin oligo-affinity purification (ChOP), followed by a mass-spectrometry analysis to identify *SCAT7*-interacting protein partners. Indeed, we identified 96 proteins, and we selected hnRNPK and YBX1 for subsequent validation and functional studies. Concordant with their known functions, the depletion of either hnRNPK or YBX1 negatively affected the cellular proliferation capacity and interfered with pro-survival cascades, including the FGF/FGFR

signaling pathway. By implementing different molecular techniques such as chromatin immunoprecipitation (ChIP) and RNA immunoprecipitation (RIP), we presented a model that depicts *SCAT7* mode of action. In this model, *SCAT7* interacts with hnRNPK and YBX1 to form a complex which occupies the proximal promoter of different FGFR members, and in turn, facilitates the RNA Polymerase II-based transcription. The elevated expression of *SCAT7* aids in sustaining the proliferative signals mediated by the FGFR RTKs and their associated ligands.

4.2.4 *SCAT7* is a potential therapeutic target for cancer treatment

We engrafted *SCAT7* stable knockdown cells (786-O and A549) subcutaneously into immunocompromised mice and compared their growth parameters to the respective wildtype cells. We observed significant inhibition of the growth parameters of *SCAT7* stable knockdown cells compared to the wildtype cells. Further histological analyses demonstrated a reduction in the proliferation rate accompanied by DNA fragmentation, as indicated by Ki67 staining and TUNEL staining, respectively. We have also implemented a treatment regimen using the LNA-ASOs against *SCAT7* in xenografts engrafted with wildtype A549 cells, resulting in significant inhibition of the growing tumors *in vivo*. Next, we tested a lung adenocarcinoma patient-derived xenograft (PDX) model, where we injected the *SCAT7* ASOs subcutaneously and measured the growth parameters of the implanted tumors. As expected, the *in vivo* depletion of *SCAT7* exhibited a significant reduction in the growth of the treated tumors.

Altogether, the presented study provides a repertoire of potential RNA-based survival biomarkers for prognostic purposes across different types of cancer. Also, the study provides comprehensive clues on the oncogenic role of *SCAT7* in promoting proliferative signaling through the RNA-protein interaction, which represents a potential target for therapeutic intervention in different cancer types.

4.3 Paper III:

SCAT7 lncRNA regulates TOP1 turnover and DNA homology-directed repair in lung cancer

Since *SCAT7* expression demonstrates a significant enrichment in the S phase, we hypothesized that *SCAT7* expression is critical for DNA replication-related processes. This proposed function is independent of the *SCAT7* role in regulating FGF/FGFR signaling. Therefore, we re-investigated the *SCAT7*-associated proteins identified in the previous study. Among the *SCAT7* interactors, we identified several proteins involved in DNA unwinding, sister chromatid cohesion, DNA replication and repair. Subsequent protein network analysis identified five functional groups: DNA repair, infectious disease, transport of mature RNAs to the cytoplasm, FGFR signaling, and epigenetic regulation of gene expression. Hence, we decided to investigate the functional implications of *SCAT7* expression in DNA replication and repair. The summary of the study findings is present in the following sections.

4.3.1 *SCAT7* is crucial for DNA damage response

To investigate the *SCAT7*-associated functions in response to stress stimuli, we exposed A549 cells to several stress conditions, including various genotoxic drugs, specific replication stress-inducing agents, and heat shock. Strikingly, we observed significant induction of *SCAT7* expression in cells treated with cisplatin or camptothecin (CPT). The former drug is a genotoxic non-specific DNA-damaging agent, while the latter drug is a specific topoisomerase I inhibitor. Interestingly, other stress factors did not induce *SCAT7* expression significantly. We performed a time-course estimation of *SCAT* expression in response to cisplatin or CPT treatment, where *SCAT7* exhibited significantly elevated levels at 16h post-treatment. Of note, *SCAT7*-depleted cells demonstrated high levels of intrinsic DNA double-stranded breaks (DSBs), as evident by the accumulation of the phosphorylated γH2A.X histone variant. On the other hand, the overexpression of *SCAT7* reduced the γH2A.X level in cisplatin-treated cells. Hence, we postulate that *SCAT7* is not an immediate DNA damage sensor; however, its expression is crucial to alleviate the DNA damage and promote cellular survival.

4.3.2 *SACT7* mediates DNA homology-directed repair

To dissect the connection between *SCAT7* and DNA repair pathways, we investigated the efficiency of the DNA homologous recombination (HR)

repair and the non-homologous end joining (NHEJ) repair in *SCAT7*-depleted cells. Following the cisplatin treatment and *SCAT7* silencing, we observed significant abrogation of the HR repair, while the NHEJ repair remained unaffected. To confirm the effects of *SCAT7* silencing on HR and NHEJ pathways, we checked the expression levels of different proteins involved in the DNA repair. For instance, we observed a drastic decrease in the phosphorylated levels of the major DNA repair kinases ATM and ATR. Concordantly, *SCAT7* overexpression resulted in higher phosphorylation levels of ATR and its downstream effector CHK1. Also, the ATM co-effectors, including CHK2 and the MRN complex (MRE11, RAD50, and NBS1), exhibited lower phosphorylated status in *SCAT7*-deficient cells compared to *SCAT7*-proficient cells. This set of proteins is known to promote the DNA repair mostly through the HR repair pathway; however, they also overlap with the NHEJ repair pathway. *SCAT7*-deficient cells, on the other hand, did not exhibit alterations in the expression levels of the NHEJ-specific proteins KU70-80, XRCC4, and Ligase IV. We also utilized the DNA combing assay to study the effect of *SCAT7* depletion on the DNA replication fork progression. The analysis revealed an increase in the percentage of staled and newly-fired replication forks, resulting in higher replication stress. Therefore, *SCAT7* expression is critical to promote the DNA homology-directed repair and relief of replication stress through maintaining the phosphorylation levels of ATM and ATR kinases.

4.3.3 *SCAT7* regulates TOP1 turnover

Among *SCAT7*-interacting proteins, topoisomerase I (TOP1) emerged as a potential candidate for mediating *SCAT7*-dependent functions. During the DNA synthesis process, TOP1 forms a transient complex, known as TOP1cc, which is essential for DNA unwinding. The proficient ATM activity promotes the resolution of TOP1cc through ubiquitin-mediated proteasomal degradation. The persistence of the TOP1cc intermediate induces several DNA nicks, resulting in intrinsic DNA DSBs. Thus, we validated *SCAT7* interaction with TOP1 using different immunoprecipitation methods in unperturbed cells. Strikingly, *SCAT7* abrogation caused the accumulation of TOP1 and TOP1cc in CPT-treated cells, indicating the inefficient resolution of TOP1 intermediates. Also, upon sequential CPT and MG132 treatments, *SCAT7*-depleted cells had less effect on TOP1 levels compared to *SCAT7*-proficient cells. So, we performed immunoprecipitation followed by Western blotting, where *SCAT7*-deficient cells exhibited less ubiquitinated TOP1 pool compared to control cells. Alongside with further experiments, we showed that *SCAT7* promotes the resolution of TOP1cc in an ATM-dependent manner through the ubiquitin-mediated degradation machinery.

4.3.4 NF-κB transactivates *SCAT7*

We performed *in silico* analysis of the putative promoter sequence of *SCAT7*, where we found NF-κB consensus binding sites. We validated the NF-κB binding at the *SCAT7* proximal promoter by chromatin immunoprecipitation. Further abrogation of NF-κB activity, either by siRNA or potent inhibitor, resulted in reduced transcriptional activity of *SCAT7*. In a complementary experiment, the overexpression of wildtype form of NF-κB stimulated *SCAT7* expression significantly, whereas mutant forms of NF-κB failed to activate the lncRNA. In DNA-damaged cells, both NF-κB and *SCAT7* demonstrated similar transactivation patterns. Importantly, NF-κB inhibition in CPT-treated cells resulted in a significant reduction of *SCAT7* expression. Therefore, NF-κB underlies the transcriptional activation of *SCAT7* in unperturbed and DNA-damaged cells.

4.3.5 *SCAT7* alters cisplatin-resistant cells

Earlier studies indicated the role of NF-κB in promoting cisplatin resistance. Therefore, we proposed that *SCAT7* may also contribute to the acquired chemoresistance. To test this hypothesis, we developed cisplatin-resistant cells derived from the parental A549 cells through constant exposure to elevated levels of cisplatin. Following this, we estimated the *SCAT7* expression in the fully-resistant cells; however, it did not show any significant increase. Nevertheless, *SCAT7* silencing significantly reduced the proliferation capacity of these resistant cells, whereas additional cisplatin treatment did not add up to the antiproliferative effect of *SCAT7*. For further understanding, we carried out RNA-seq analyses of wildtype cells treated with cisplatin and resistant cells depleted of *SCAT7*. These analyses indicated that *SCAT7* depletion in cisplatin-resistant cells resembles, to a certain extent, the cisplatin treatment in naïve cells. To test the effect of *SCAT7* abrogation on chemoresistance *in vivo*, we engrafted cisplatin-resistant cells into mouse xenografts. We devised different therapeutic regimens to treat the derived tumors, where *SCAT7* silencing alone was able to inhibit the tumors' progression significantly, irrespective of the combined cisplatin.

In conclusion, the presented study provides evidence on the role of NF-κB in the transcriptional activation of *SCAT7*. In turn, *SCAT7* contributes to the maintenance of the genome integrity through regulating the TOP1 turnover and mediating DNA homology-directed repair.

4.4 Paper IV:

_LY6K-AS_ lncRNA regulates mitotic progression and chemoresistance in lung adenocarcinoma cells

In the current study, we set out to investigate the clinical relevance as well as the potential therapeutic targeting of natural antisense lncRNA transcripts (NATs) in lung cancer. Towards this, we utilized the publically available RNA-seq data from the TCGA datasets to identify the top differentially expressed antisense lncRNAs in lung adenocarcinoma. We also integrated the expression patterns of the identified NATs with a high throughput investigation of their independent prognostic capacity. Our systematic analysis identified the *LY6K-AS* lncRNA as a prognostic biomarker for LUAD patients' overall survival. Further functional characterization revealed the crucial role of LY6K-AS in promoting mitotic progression in LUAD cells. The overall findings of the study are present in the subsequent sections.

4.4.1 *LY6K-AS* silencing alters cellular DNA content

LY6K-AS predominantly localizes to the chromatin compartment in LUAD cells. Both transient and stable silencing of the lncRNA reduced the proliferation capacity of various LUAD cell lines and induced significant cell cycle perturbations. Importantly, *LY6K-AS* depletion caused a significant increase in cellular diameter associated with altered DNA contents in the investigated cell lines. Concomitantly, the depleted cells harbored abnormal micronuclei and macronuclei, with many cells exhibiting a multinucleated phenotype. Thus, we carried out metaphase spreading and karyotyping in A549 cells transiently-depleted of *LY6K-AS*, which revealed unusual morphology and karyotype of the mitotic chromosomes. By implementing live-cell imaging and immunofluorescence techniques, we observed a significant increase in chromosomal aberrations upon *LY6K-AS* silencing. Also, the investigated cells demonstrated noticeable misalignment of the mitotic spindle associated with an elevation in mitosis and cytokinesis timing. Therefore, *LY6K-AS* abrogation alters mitotic progression in LUAD cells.

4.4.2 *LY6K-AS* regulates mitosis-promoting factors

The transcriptome-wide analysis of *LY6K-AS*-depleted cells unraveled the regulatory role of the lncRNA in activating various mitosis-related pathways, such as mitotic checkpoints, chromosomes condensation, and resolution of

sister chromatids. At the individual gene level, *LY6K-AS* knockdown significantly modulated the expression of kinetochore members (*BUB1*, *BUB1B*, *SPC25*, *NDC80*, and *MAD2*), mitosis-promoting factors (*CDK1*, *CYCB1*, *CYCB2*, *AURKA*, and *AURKB*), and the cyclin-dependent kinase inhibitor *CDKN1A*. Subsequent *in silico* consensus motif analysis revealed that the downregulated genes harbor the cell cycle homology region (CHR) and E2F motifs. The analysis of *LY6K-AS* expression dynamics indicated a higher transcriptional activity of the lncRNA in the G1 phase of the cell cycle compared to the M phase. Thereby, *LY6K-AS* expression in the G1 phase transactivates several critical factors required for the onset and progression of the M phase in LUAD cells.

4.4.3 *LY6K-AS* stabilizes 14-3-3 proteins

The mass spectrometry analysis identified various members of the 14-3-3 family as *LY6K-AS*-interacting proteins. By utilizing immunoprecipitation techniques, we validated *LY6K-AS* interaction with the 14-3-3 gamma isoform (YWHAG). Interestingly, *YWHAG* expression showed a significant positive correlation with *LY6K-AS* targets. *YWHAG* knockdown also abrogated the transcription of the same *LY6K-AS* targets, and induced cell cycle perturbations associated with altered DNA content in A549 cells. The RNA-seq analysis of *YWHAG*-deprived cells demonstrated significant modulation of cell cycle-related pathways similar to *LY6K-AS*-depleted cells. Importantly, *LY6K-AS* transient and stable knockdown negatively affected the levels of 14-3-3 proteins. However, treating *LY6K-AS*-deficient cells with the proteasome inhibitor MG132 restored the level of 14-3-3 proteins. Using an immunoprecipitation approach, we demonstrated that *LY6K-AS* stabilizes 14-3-3 proteins, preventing their ubiquitin-mediated degradation.

4.4.4 *LY6K-AS* interferes with the chemoresistance

Since the upregulation of mitosis-related processes is a typical phenotype of acquired resistance, we scrutinized the role of *LY6K-AS* in mediating the chemoresistance in LUAD cells. By utilizing different chemoresistant cell lines, we demonstrated the critical role of *LY6K-AS* in promoting mitotic division of these cells. Of note, *LY6K-AS* depletion re-sensitized resistant cells and caused a pronounced effect on the cisplatin resistance. Integrative RNA-seq analyses showed that *LY6K-AS* silencing mimics the effect of cisplatin, and counteracts the acquired resistance to targeted therapy. Further, *In vivo* xenografts demonstrated the potential applicability of *LY6K-AS* targeting in developing new therapeutic intervention approaches.

Taken together, the current study identifies *LY6K-AS* as a prognostic biomarker for overall survival in LUAD patients. The lncRNA interacts with 14-3-3 proteins to regulate the expression of various genes involved in checkpoint signaling and mitosis progression in wildtype and chemoresistance cells.

5 CONCLUSION

Paper I:

- The nascent RNA capture assay provides a comprehensive understanding of the transcriptional dynamics in real-time.
- The DNA synthesis-associated transcription is not homogenous throughout different compartments of the S phase.
- Genes belonging to functionally-distinct classes exhibit different temporal patterns of transcription.
- DNA replication and transcription do not coincide at the same loci simultaneously to avoid the replication fork collapse.

Paper II:

- Long noncoding RNAs provide a potential repertoire of prognostic survival biomarkers for risk stratification in cancer patients.
- The higher expressions levels of *SCAT7* lncRNA independently predicts poor survival outcome in renal clear cell carcinoma.
- The *SCAT7*/hnRNPK/YBX1 complex promotes oncogenic signaling in different cancer model systems to modulate cancer hallmarks, including cellular senescence.
- *SCAT7* targeting represents an innovative therapeutic approach for treating cancer patients.

Paper III:

- *SCAT7* expression promotes the DNA homology-directed repair and genome integrity in response to genotoxic stress.
- *SCAT7* interacts with TOP1 and regulates its turnover in an ubiquitin-dependent manner to permit replication fork progression.
- NF-κB activates *SCAT7* expression in unperturbed and DNA-damages cells.
- *SCAT7* transcriptional modulation alters the proliferation capacity of cisplatin-resistant cells *in vivo* and *in vitro*.

Paper IV:

- *LY6K-AS* lncRNA is an independent prognostic biomarker that promotes the mitotic progression of LUAD cells.
- *LY6K-AS* activates various kinetochore members and the spindle assembly checkpoint to prevent mitotic catastrophe.
- *LY6K-AS* stabilizes the 14-3-3 proteins and inhibit their ubiquitin-mediated degradation to maintain faithful chromosomal segregation.
- *LY6K-AS* transcriptional modulation re-sensitizes cisplatin-resistant cells and interferes with their mitotic progression.

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