G1 PHASE REGULATION IN MOUSE EMBRYONIC STEM CELLS

IN THE CONTEXT OF DNA DAMAGE AND SELF-RENEWAL

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ESSENTIALS

AFFILIATION

- This thesis was elaborated at the Department of Biology of Faculty of Medicine and Dentistry, Palacky University in Olomouc.
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CDK2, cell cycle, centrosome, DNA damage, embryonic stem cells, G1 checkpoint, G1 phase, irradiation, olomoucine II, self-renewal, signal transduction

DECLARATION / VYHLÁSENIE

Hereby I declare that I have written this work on my own under the supervision of Assoc. Prof. Vladimir Divoky, Ph.D., and that all used literature is cited and mentioned in references.

Týmto vyhlasujem, že predloženú prácu som napísala samostatne, pod vedením školiteľa doc. RNDr. Vladimíra Divokého, Ph.D. a s použitím citovanej literatúry.

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ABSTRACT

Mouse embryonic stem cells (mESCs) have been known for their special cell cycle characteristics, including rapid cell cycle progression, a very short G1 phase and lack of G1 arrest after DNA damage, for years. However, the mechanisms driving mESC cycle, underlying G1 checkpoint non-functionality and their relationship to mESC self-renewal have been poorly understood. In this thesis, the results of our studies are presented, which both point to the high CDK2 activity to be crucial in regulation of mESC-specific cell cycle, rapid G1 phase progression and G1 escape after DNA damage, as well as in the machinery, which establishes self-renewal in mESCs. Downregulation of CDK2 activity induces increase in G1 phase cell number, establishes somatic cell-like cell cycle and induces morphology changes and expression of genes associated with differentiation. Also, our model of G1 checkpoint non-functionality, based on ESC-specific localization and cellular compartmentalization of checkpoint and cell cycle regulatory proteins, is presented. We propose that mESCs do not stop in G1 phase after DNA damage due to centrosomal CDK2, which escapes from activated G1 checkpoints pathways and drives G1-S transition even in the conditions of DNA damage. Furthermore, we uncover a new mechanism of CDC25A regulation in response to DNA damage, which is governed by GSK-3β. Finally, we propose a role for centrosomes in cell fate decisions in mESCs.

ABSTRAKT

Myšie embryonálne kmeňové (mEK) bunky vynikajú unikátnym bunkovým cyklom, ktorý sa vyznačuje vysokou rýchlosťou, veľmi krátkou fázou G1 a nefunkčným G1-kontrolným bodom. V tejto práci prezentujem výsledky našich štúdií, ktoré mali za cieľ odhaliť mechanizmy stojace za týmito jedinečnými charakteristikami bunkového cyklu mEK buniek. Obe štúdie poukazujú na centrálnu úlohu CDK2, ktorej vysoká aktivita je kritická pre rýchlu progresiu bunkovým cyklom, krátku fázu G1 a rýchly únik z G1 fázy po poškodení DNA, ako aj pre sebaobnovu mEK buniek. Downregulácia aktivity CDK2 zvyšuje počet buniek vo fáze G1, nastoľuje bunkový cuklus podobný bunkovému cyklu somatických buniek a indukuje zmeny morfológie a expresiu génov asociovaných s diferenciáciou. Prezentovaný je aj náš model nefunkčnosti G1-kontrolného bodu, ktorý sa zakladá na bunkovej kompartmentalizácii a špecifickej lokalizácii proteínov kontrolného bodu a bunkového cyklu v mEK bunkách. Tento model navrhuje, že mEK bunky sa nezastavujú v G1 po poškodení DNA kvôli centrozomálne lokalizovanej CDK2, ktorá uniká regulácii aktivovaným G1-kontrolným bodom a poháňa prechod z fázy G1 do fázy S aj v podmienkach poškodenia DNA. Naviac odhaľujeme i nový mechanizmus regulácie CDC25A v odpovedi na poškodenie DNA prostredníctvom GSK-3β. Vychádzajúc z našich pozorovaní navrhujeme možnú úlohu centrozómov v osudových rozhodnutiach mEK buniek.

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CONTENTS

Part I

THEORETICAL BACKGROUND

1 INTRODUCTION

1.1 EMBRYONIC STEM CELLS

ESCs are pluripotent, self-renewing, immortal cells

Embryonic stem cells (ESCs[1]) are cell lines derived from inner cell mass (ICM) of mammalian blastocysts prior to implantation [1, 2]. They are pluripotent [1, 3], *i.e.* they can give rise to cells of all three germ layers as well as germ cells [4, 5]. ESCs proliferate without apparent limit [6] and can be maintained in culture for extended periods of time, possibly indefinitely, therefore they are often referred to as immortal cells. Pluripotency and indefinite self-renewal distinguish ESCs from tissue stem cells (SCs), which have more limited self-renewal and developmental potentials.

First ESCs were derived from mouse blastocyst [1, 3]. More recently, pluripotent SC lines with properties similar to mouse ESCs (mESCs) have been derived from a variety of developmental stages and mammalian species, as well as from adult cells reprogrammed by ectopic transcription factors. However, their developmental equivalence to the lineages established in the early embryo has been discussed. For instance, it has been suggested that mESCs and human ESCs (hESCs) represent two different stages of embryo development [7, 8]. Based on comparison of the factors required for maintenance of pluripotency, their gene expression profiles and the ability of these cell lines to differentiate into extraembryonic lineages and to the early germ layers of the embryo, it was proposed that there are at least two different pluripotent states represented among the currently available pluripotent SC lines: the ESC-like and the epiblast SC (EpiSC)-like state [9]. They represent cells equivalent to ICM epiblast progenitors (such as mESCs) and to the early postimplantation epiblast (such as hESCs and mouse EpiSCs), respectively [9].

Mouse and human ESCs represent different pluripotent states

1.2 REGULATION OF ESC SELF-RENEWAL AND PLURIPOTENCY

Self-renewal is the process by which a SC divides (asymmetrically or symmetrically) to generate one or two daughter SCs that have a developmental potential similar to the mother cell. This process is under both cell-intrinsic and cell-extrinsic control. Cell-intrinsic regulatory pathways include transcriptional and epigenetic regulation of SC fate: OCT4, SOX2, NANOG and KLF4 elaborate core transcriptional circuitry in ESCs [10–12], working in coordination with Polycomb complexes [13, 14],

Multiple levels of control on self-renewal

1 List of acronyms and abbreviations on page 109

microRNAs [15], histone modification enzymes [16] and chromatin remodeling enzymes of Tip60/p400 and SWI/SNF families [17–21] to stably maintain the expression of pluripotency genes, and to repress lineage determinant genes. Cell-extrinsic regulation is imposed by signals from extracellular environment, percepted by SC receptors. These signals are often produced by specialized cells of SC niche, especially in the case of tissue SCs.

1.2.1 *Transcriptional Regulation of ESC Self-renewal*

Genes that promote self-renewal

Pluripotency and self-renewal of ESCs are maintained by a network of transcription factors including OCT4, SOX2, NANOG, ESRRB, TBX3, TCL1, NAC1, DAX1, SALL4, ZFP281, RIF1 and ZIC3, which participate in auto- and cross-regulatory interactions to increase their own expression and that of other self-renewal genes [10, 12, 22–30].

NANOG imposes ESC phenotype on somatic cells

NANOG, a homeodomain protein, is believed to be central to the maintenance of ESC pluripotency. It is capable of maintaining ESC self-renewal independently of leukemia inhibitory factor (LIF)-STAT3 signaling [31,32]. In cell fusion experiments, NANOG promotes imposition of ESC phenotype on somatic cells [33]. ESCs lacking *Nanog* spontaneously differentiate into primitive endoderm [31, 32], and, conversely, upon differentiation, ESCs lose expression of *Nanog* [32]. To establish ESC identity, NANOG acts in concert with other factors such as OCT4 [34] and SOX2 [35]. *Nanog*, *Pou5f1* (encoding OCT4) and *Sox2* are the earliest-expressed set of genes known to maintain pluripotency [23]. Upon differentiation, NANOG is inactivated by caspase-3-mediated proteolysis [36].

The homeodomain transcription factor OCT4 (OCT3/4, POU5F1) and the high mobility group (HMG) domain DNA-binding protein SOX2 are essential for normal pluripotent cell development and maintenance [34, 35]. OCT4 regulates cell fates in a quantitative manner: it must be maintained at critical concentration to sustain ESC self-renewal [37]. Overexpression of OCT4 triggers differentiation into endoderm lineage and its supression causes ESC to become trophoectoderm [37].

OCT4, NANOG and SOX2 share a substantial proportion of target genes; however, these targets are partially different in mouse and human ESCs. *MYST3* and *Heart and neural crest derivatives expressed 1 (HAND1)* were identified as targets of NANOG and OCT4 in hESCs whereas *Estrogen-related receptor β (Esrbb)* was observed as OCT4/NANOG target only in mESCs [25].

Genes that prevent differentiation

Undifferentiated state of SCs is maintained by repressing the expression of genes that restrict developmental potential or specify differentiation. In ESCs, Ronin inhibits expression of *Gata4* and *Gata6* [38], which promote endodermal differentiation [39–41]. Especially the maintenance of neural SC (NSC) identity appears to depend on the function of many

transcription factors to avoid premature differentiation at various times during development. Differentiation of NSCs is prevented by action of ATF5 [42], SoxB1 family [35, 43], Tailless homolog (TLX) [44] and the nuclear coreceptor (N-coR) [45].

1.2.2 *Epigenetic Regulation of ESC Self-renewal*

The core network of pluripotency-maintaining transcription factors is connected to chromatin modifying complexes, such as NuRD and PRC1, implicated in transcriptional repression [28] as well as to chromatin remodeling complexes, such as SWI/SNF, which are proposed to act as general coordinators of changes in ESC fate, and perhaps an integrator of several distinct modes of epigenetic regulation [21]. It appears that major decisions on pluripotency are regulated on the chromatin level: evidence suggests that changes in chromatin structure, rather than loss of self-renewal gene transcription *per se*, trigger differentiation [21]. It is believed that chromatin remodeling complexes facilitate mESC differentiation by coupling gene repression with global and local changes in chromatin structure [21].

Identification of the pluripotency factor Ronin [38] has supported the hypothesis of epigenetic regulation of ESC self-renewal. Ronin is a zinc-finger DNA-binding protein, which acts through a large protein complex containing HCF-1 (host cell factor-1), SIN3A, HDAC3 and other proteins involved in transcriptional repression or histone modifications and represses expression of differentiation promoting genes *(Gata4, Gata6)* in undifferentiated ESCs and of pluripotency promoting genes *(Pou5f1)* in differentiated ESCs [38]. Upon exit from self-renewal, Ronin is cleaved by caspase-3 [38].

Compared with chromatin of differentiated cell types, chromatin of pluripotent cells is highly dynamic, with loosely associated structural chromatin proteins [46] and in transcriptionally permissive euchromatin state with abundance of acetylated histone modifications [47]. Tissue-specific genes, expected to be silent in undifferentiated cells, are in a semi-permissive transcriptional state in ESCs [48, 49]: they are marked by both positive (histone 3 lysine 4 trimethylation, H3K4me3) and negative (histone 3 lysine 27 trimethylation, H3K27me3) marks for transcription [16, 50]. These dual marks or bivalent domains are present at a large set of developmentally important genes that are silent in ESCs but activated upon differentiation [16, 50]. As methylation of H3K27 is catalyzed by a protein complex that belongs to the Polycomb group (PcG) proteins, a family that was previously known to be important for maintaining gene repression at later stages of development, the existence of bivalent domains indicates a role for PcG proteins in prevention of inappropriate upregulation of tissue-specific genes in ESCs [51].

Key developmental genes contain bivalent domains

ATP-dependent chromatin remodeling enzymes of Tip60/p400 and SWI/SNF families are crucial for maintenance and function of ESCs [17]. Components of mammalian SWI/SNF complex (BRG, BAF155, BAF250A) are indispensable for the proliferation, pluripotency and self-renewal of ESCs [18–20]. Some of these proteins form distinctive SWI/SNF-like BAF complexes (esBAF) in ESCs, which regulate the core transcriptional circuitry in ESC, including PcG proteins and LIF and BMP signaling pathways [19]. On the other hand, upon mESC differentiation, members of the SWI/SNF chromatin remodeling sub-complex, PBAF, are required for the repression of *Nanog* and other self-renewal genes [21]. These observations suggest dual roles for SWI/SNF complex members in maintaining ESC pluripotency: in promoting self-renewal gene expression [19,20] and, *ex adverso*, in providing functions critical for lineage formation [21].

Regulation of self-renewal by miRNAs

MicroRNAs (miRNAs) are also likely to play key roles in ESC gene regulation [52–54]. Recent studies revealed two key groups of miRNAs that are direct targets of OCT4/SOX2/NANOG/TCF3: one group of miRNAs that is preferentially expressed in pluripotent cells (such as miR-302 cluster, miR-290/371 cluster, miR-363 cluster) and a second, Polycomb-occupied group that is silenced in ESCs and is poised to contribute to cell-fate decisions during mammalian development (such as miR-124, miR-155, miR-375, miR-615, miR-708) [15]. miRNAs that are activated in ESCs by OCT4/SOX2/NANOG/TCF3 serve to modulate the direct effects of these transcription factors, tuning levels of key genes and modifying the gene expression program to help poise ESCs for efficient differentiation [15]. Some OCT4/SOX2/NANOG-regulated miRNAs (miR-134, miR-296 and miR-470) have their target sequences in amino acid coding sequence of OCT4, SOX2 and NANOG and are partially conserved in mouse, human and rhesus [55].

Self-renewal of tissue SCs is regulated also by DNA methylation: *DNA methyltransferase 1 (Dnmt1)*-deficient neural progenitors precociously differentiate to astrocytes [56] and HSCs deficient in *Dnmt3a*$^{-/-}$ *Dnmt3b*$^{-/-}$ lack long-term repopulating ability [57].

1.2.3 *Cell-extrinsic Regulation of ESC Self-renewal*

Multiple self-renewal signaling networks subsist in ESCs, with activity dependent upon the cellular context [58]. mESCs can be maintained in an undifferentiated state by culture in defined medium containing LIF, Bone morphogenetic proteins (BMPs) [59] and N2/B27 supplements. Unlike mESCs, hESCs regulate their self-renewal via fibroblast growth factor (FGF)-2 and Activin/Nodal signaling pathways [60,61]. These pathways regulate expression of core intrinsic factors, such as NANOG, that are essential for the maintenance of pluripotency in both species [32].

LIF promotes mESC self-renewal

LIF is a key factor that blocks differentiation of mESCs in culture [62]. It binds to a heterodimeric receptor complex consisting of two related cytokine receptors, LIF receptor (LIFR) and gp130 [63] (Fig. 1.1). Resultant activation of receptor-associated JAK kinases causes recruitment, phosphorylation and dimerization of STAT3 (signal transducer and activator of transcription 3). STAT3 dimers are translocated to the nucleus, where they control the expression of self-renewal regulating genes [64].

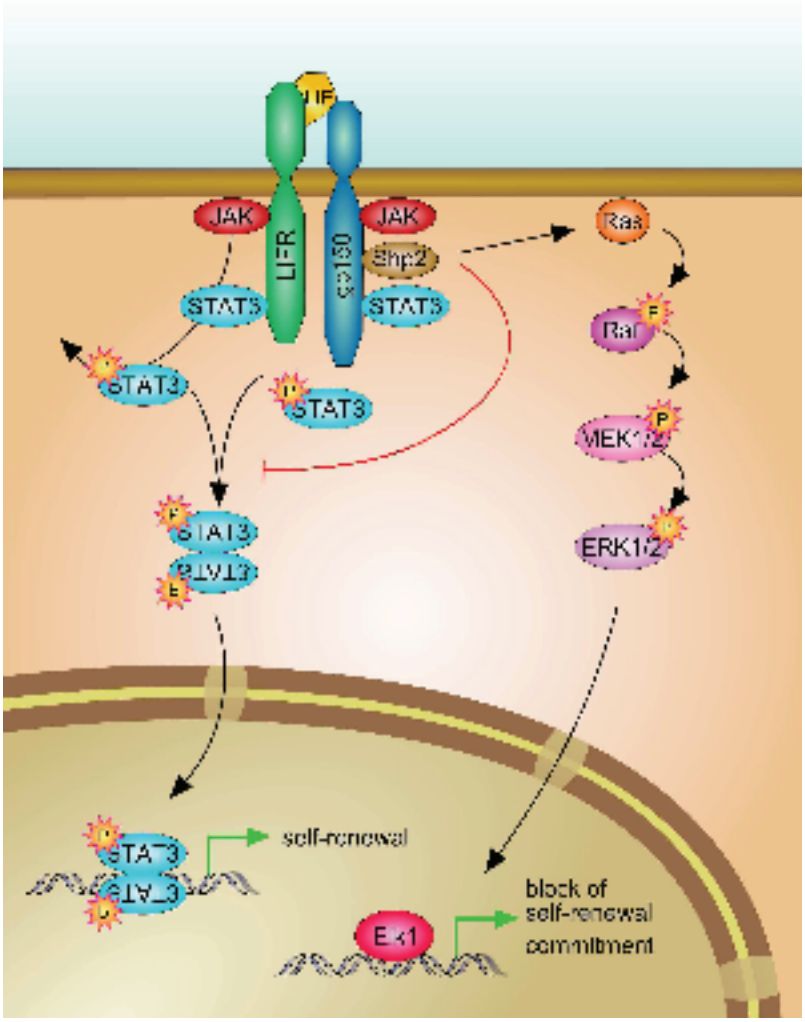

Figure 1.1: Regulation of mESC fate decision by LIF and MAPK pathways.

LIF-dependence of mESC self-renewal is mediated by E-cadherin: *Ecad*$^{-/-}$ mESCs maintain an undifferentiated phenotype when cultured in serum-free medium supplemented with Activin A and Nodal, with FGF-2 required for cellular proliferation [58].

BMP, Activin and Nodal belong to the transforming growth factor (TGF)-β ligand superfamily. They signal by bringing together type I (Alk, activin receptor-like kinase receptors) and type II receptors on the cell

surface (BMPR1A/Alk3 and ActRIIB; ActR-IB/Alk4 and ActR-IIB; Alk4/7 and ActR-IIA/ActR-IIB for BMP, Activin A and Nodal, respectively). Upon ligand-receptor complex formation, type I receptor phosphorylates distinct members of the SMAD family (SMAD2/3 for Activin/Nodal and SMAD1/5/8 for BMPs), although SMAD4 is required as a cofactor in both cascades [65]. SMAD signaling promotes the expression of inhibitor of differentiation (Id), helix-loop-helix domain proteins that dimerize with, and inhibit the function of, helix-loop-helix transcription factors that regulate fate determination [59]. The major effect of BMP4 on the self-renewal of mESCs is accomplished by means of the inhibition of both extracellular receptor kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) pathways [66]; inhibitors of ERK and p38 MAPKs (such as PD98059 and SB203580, respectively) can substitute for BMP signaling in maintenance of mESC pluripotency [66]. In hESCs, Activin signaling directly targets *NANOG* promoter.

Maintenance of mESCs in the artificial milieu of cell culture takes advantage of LIF and BMP (or serum) to block mESCs commitment. However, when differentiation-inducing signaling from MAPK is eliminated, mESCs can self-renew without LIF and BMP [67]. This property suggests that mESCs exhibit an innate program for self-renewal, which may account for their latent tumorigenicity [67].

1.2.3.1 *Wnt Signaling*

Wnt signaling has been implicated in the control over various types of SCs [68–70] and may act as a niche factor to maintain SCs in a self-renewing state [71]. Isolated Wnt proteins are active on a variety of SCs, including SCs of the crypt [68, 72, 73], NSCs [74, 75], hematopoietic SCs (HSCs) [69, 70, 76], epithelial SCs [77–81], mammary SCs [82–86], mesenchymal SCs [87–90] and ESCs [91–100].

Wnts are secret glycoproteins that act as ligands for the seven-pass transmembrane Frizzled (Fz) receptors [101] and an LDL receptor-related protein Lrp5/6 [102, 103]. Fz signaling activates β-catenin-dependent (canonical) and -independent (noncanonical) pathways [104].

In the canonical pathway, which can be induced by Wnt1, Wnt2, Wnt3a, Wnt7a or Wnt8a [105], the Fz downstream signaling leads to inactivation of GSK-3β, resulting in the nuclear accumulation of β-catenin, which in collaboration with T-cell-specific factors (TCF) and lymphoid enhancer factors (LEF) activates the transcription of Wnt target genes [104, 106]. Noncanonical Wnt signaling is mediated by Wnt ligands Wnt4, Wnt5a, Wnt6 and Wnt11 [105] and acts through kinases such as c-Jun NH_2-terminal kinase (JNK) and the calcium-dependent kinases CaMKII (Ca^{2+}/calmodulin-dependent protein kinase II) and PKC (protein kinase C) [107–110].

Canonical Wnt Signaling

The stability of the central player of the canonical Wnt pathway, β-catenin, is regulated by a destruction complex. When Wnt receptors are not engaged, two scaffolding proteins in the destruction complex - the tumor suppressors adenomatous polyposis coli (APC) [111–114] and axin [115–117] - bind β-catenin (Fig. 1.2 A). Then a set of conserved

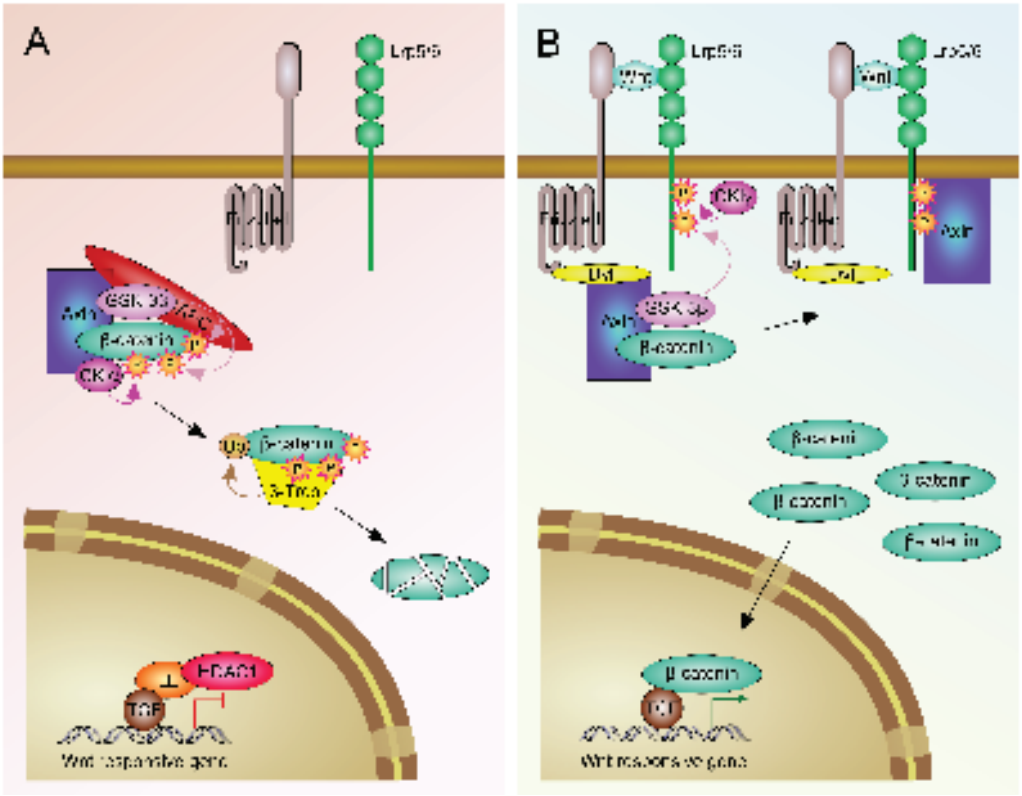

Figure 1.2: Model for Wnt/β-catenin signaling. **A.** In the absence of a Wnt signal, β-catenin is phosphorylated and targeted for proteasome-mediated degradation by a destruction complex that contains axin and GSK-3β among other proteins. **B.** When the Wnt ligand binds to the Frizzled (Fz)-Lrp5/6 receptor complex, Dvl binds to Fz and recruits the destruction complex through interaction with axin. Subsequently, GSK-3β and CKIγ phosphorylate critical sites on Lrp5/6, creating docking sites for axin. Binding of axin to Lrp5/6 inhibits the destruction complex and stabilizes β-catenin.

Ser and Thr residues in the amino terminus of β-catenin is sequentially phosphorylated by glycogen synthase kinase-3β (GSK-3β) [118, 119] and casein kinase Iα (CKIα) [120–122], two kinases residing in the destruction complex. The resulting phosphorylated footprint recruits a β-TrCP-containing E3 ubiquitin ligase, which targets β-catenin for pro-

teasomal degradation [123–125]. When free from nuclear β-catenin, its partner DNA-binding proteins of the TCF/LEF family are bound by Groucho/Transducin-like-enhancer of split (TLE) repressor complexes [126, 127], which recruit histone deacetylase-1 (HDAC1) to inhibit expression of Wnt target genes [128]. Moreover, in the absence of Wnt signaling, the interaction of β-catenin with TCF is negatively regulated by the TCF-binding proteins including NLK (NEMO-like kinase) and CREB binding protein (CBP) [129, 130].

Receptor occupancy inhibits the kinase activity of the destruction complex. Axin binds to the cytoplasmic tail of Lrp5/6 [131] or is bound by activated Dishevelled (Dvl) (Fig. 1.2 B). As a consequence, β-catenin accumulates and translocates into the nucleus where it engages the DNA-binding proteins of the TCF/LEF family and activates expression of target genes [132, 133]. The interaction with β-catenin transiently converts TCF/LEF factors into transcriptional activators. Additional nuclear components, Pygopus (Pygo1, Pygo2) and BCL9 (Legless in *Drosophila*), are involved in Wnt signaling [134–138]. Pygopus is essential for transcriptional activation of TCF/LEF target genes [134, 139], whereas BCL9 seems to bridge Pygopus to TCF-bound β-catenin [134, 140, 141]. In sum, the canonical pathway translates a Wnt signal into the transient transcription of TCF/LEF target gene programme [142].

Targets of the β-catenin-TCF/LEF pathway include: *CCND1* (encoding cyclin D1) [143, 144], *MYC* (encoding c-Myc) [145, 146], *PPARD* (peroxisome proliferator-activated receptor δ; [147]), *GAST* (gastrin) [148], *AXIN2* [149–151], *LGR5/GPR49* (leucine-rich repeat-containing G protein-coupled receptor 5/ G-protein-coupled receptor 49; [152]), *LEF1* [153, 154].

Wnt promotes self-renewal of ESCs

The Wnt/β-catenin signaling pathway has multiple roles in ESC biology, development, and disease [155–157]. It is endogenously active in undifferentiated mESCs and is downregulated upon their differentiation [93]. Activation of the canonical Wnt pathway promotes the undifferentiated phenotype of mESCs [91] and is sufficient to maintain self-renewal of both hESCs and mESCs under conditions that induce differentiation [93, 94, 96, 98, 99, 158]: Through interaction with OCT4, β-catenin upregulates expression of *Nanog* [99]. On the other hand, Wnt pathway has an important role in directing differentiation of ESCs [92, 95, 97]. Wnt pathway influences the balance between pluripotency and differentiation by direct signaling to the core regulatory circuitry of ESCs through T-cell factor-3 (TCF3), which co-occupies promoters throughout the ESC genome in association with the pluripotency regulators OCT4 and NANOG [100]. TCF3 is highly expressed in mESCs, and is critical for early embryonic development [68, 97, 159].

Noncanonical Wnt Signaling

The mechanisms underlying β-catenin-independent (noncanonical) Wnt signaling are not well defined, and may be largely determined by cellular context. They involve the "Wnt-calcium" pathway and the "planar cell polarity" (PCP) pathway. The "Wnt-calcium" pathway utilizes G-protein second-messenger systems to mobilize intracellular calcium stores and activate atypical PKC and other calcium responsive pathways [107,160–162]. The "planar cell polarity" (PCP) pathway couples short-term cytoskeletal reorganization, convergent extension, and control of planar polarity through activation of Rho-type GTPases and Jun N-terminal (JNK) serine/threonine kinase [163]. Many of the noncanonical effects of Wnt signaling depend on signaling pathways that directly or indirectly regulate cytoskeletal dynamics at or near the cell membrane [164].

Noncanonical Wnt signaling has been implicated in regulation of proliferation and differentiation in neural progenitor cells [75] and in regulation of bone differentiation from mesenchymal SCs [88, 89, 165]. In hESCs, noncanonical Wnt signaling controls exit from the pluripotent state and entry toward mesoderm specification and embryonic hematopoiesis [166].

1.2.3.2 *Notch Signaling*

The Notch signaling pathway is an evolutionarily conserved intercellular signaling mechanism essential for regulation of the survival, proliferation and differentiation of a range of cell types throughout the embryonic development and life span of all metazoans [167]. However, it is unnecessary for the very early stage of embryogenesis, including the fertilized egg stage [168].

Notch encodes a single-pass transmembrane protein [169] that functions as a receptor for the ligand present on the cell surfaces of neighboring cells [170]. In mammals, four Notch receptors (Notch1–Notch4) and five Notch ligands (Delta-like1 [also called Delta1], Delta-like3, Delta-like4, Jagged1, and Jagged2) have been identified. Notch ligands are also single-pass transmembrane proteins. Notch receptors undergo intramolecular cleavage of the precursor protein (S1 cleavage by a furin-like convertase) to form heterodimers, composed of an extracellular subunit and a transmembrane subunit, on the plasma membrane [171–173]. Binding of a ligand triggers the cleavage of the extracellular region of the Notch transmembrane subunit (S2 cleavage) [173]. This cleavage facilitates the next cleavage, which occurs within the transmembrane domain (S3 cleavage) [173, 174] and creates the cleaved intracellular domain of Notch (NICD). NICD translocates to the nucleus and associates with the constitutive DNA-binding protein CSL (CBF1/RBP-Jκ, Suppressor of hairless, Lag-1) and thus turns the CSL complex from a transcriptional repressor to a transcriptional activator [175], for which the mastermind

Notch signal is relayed by cleavage of its receptor

adaptor protein is an essential component [176, 177]. The NICD-CSL complex promotes expression of E(spl)/HES (Hairy and enhancer of split) family [178] and their homologs, the Hey (HERP) family [179] of basic helix-loop-helix transcription factors.

SCs are enriched for members of the Notch pathway [180] and cell-cell signaling mediated by Notch has been shown to play a key role in maintaining various mammalian SC systems, notably hematopoietic, neural, skin, intestinal, and skeletal SCs. In these systems, Notch has been shown to regulate proliferation, differentiation, and survival in a context-dependent manner [181].

Role for Notch signaling in adult stem cells

Notch-HES signaling is required for the maintenance and expansion of the neural stem/progenitor cell pool [182–184] and its ectopic activation can be used to promote the survival and expansion of neural SCs both *in vitro* and *in vivo* [185]. Notch1 has an indispensable role in generation of adult-type HSCs [186, 187] and Jagged1-Notch1 pathway might be involved in maintenance of HSCs in their osteoblastic niche [188, 189]. Forced activation of Notch signaling in HSCs (by ectopic expression of a constitutively active form of Notch1 [190, 191] or wild-type HES1 [192]) inhibits murine HSC differentiation and potentially expands the HSCs. Furthermore, Notch signaling inhibits SCs in the bulge from differentiating into epidermal cells and promotes hair formation [193] and functions to maintain intestinal epithelial stem/progenitor cells [194–196].

Role for Notch signaling in ESCs

Notch signaling network appears active in undifferentiated mESCs [197] and upon withdrawal of self-renewal factors (such as LIF and BMP4), it directs mESC differentiation exclusively toward a neuroectodermal fate [197]. Notch1–3 and DLL1 are expressed in hESCs [198]. However, Notch signaling is not active [199], although it can be transiently activated by cell passaging conditions that include cation chelation [199]. Notch signaling is not required for the propagation of undifferentiated hESCs but instead is required for the formation of the progeny of all three embryonic germ layers, but not trophoblast cells [200], and for the maintenance of differentiating cell types [199]. In addition, transient Notch signaling pathway activation enhances generation of hematopoietic cells from committed hESCs [199]. Cell-cell signaling through Notch regulates hESC proliferation [201].

1.3 CELL CYCLE REGULATION

1.3.1 *Cell Cycle Regulation in Somatic Cells. Restriction Point*

Cell cycle progression is driven by cyclin-CDKs

Somatic cell cycle is regulated by sequential activation and inactivation of cyclin-CDK (cyclin-dependent kinase) complexes [202]. Binding of the cyclin subunit is essential for activation and determines the substrate specificity of these complexes. Cyclin D-CDK4 complexes phosphorylate

pocket proteins (pRB, p107, p130) which are further phosphorylated by cyclin E-CDK2 complexes. Activity of cyclin E-CDK2 complexes drives G1-S transition and initiation of DNA replication. Cyclin A-CDK2 regulates DNA replication and controls activation of cyclin B-CDK1, which is required for both entry into and progression through mitosis. However, this simple model of cell cycle regulation has been challenged by studies of cyclin or CDK knockouts that have shown overlapping and substitutionary roles for different types of cyclins as well as CDKs (reviewed in Hochegger *et al.* [203]) and that CDK1 is the only essential cell cycle kinase [204].

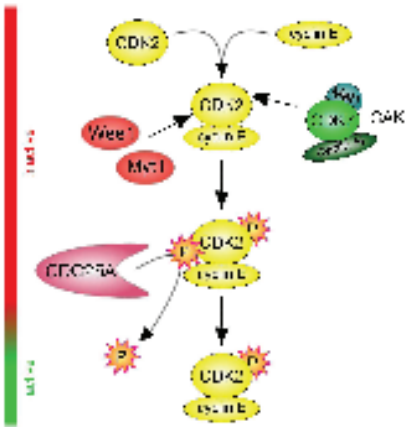

Figure 1.3: Regulation of CDK2 activity by phosphorylation.

Regulation of CDK activity by phosphorylation

Besides association with cyclins, activity of CDKs is regulated by a series of activatory and inhibitory phosphorylations (Fig. 1.3). For proper catalytic activity, CDKs must be phosphorylated on threonine residue (Thr172 in CDK4, Thr160 in CDK2 and Thr161 in CDK1) by CDK7-cyclin H complex (also known as CDK activating kinase, CAK) [205, 206]. Phosphorylation of adjacent threonine and tyrosine residues (Thr14/Tyr15 in CDK2 and CDK1) by Wee1/Myt1 [207, 208] is inhibitory and is relieved by activity of CDC25 phosphatases (CDC25A, CDC25B, CDC25C). Furthermore, CDK4/CDK6 and CDK2/CDK1 complexes are inhibited by proteins of Ink4 ($p15^{Ink4b}$, $p16^{Ink4a}$, $p18^{Ink4c}$, $p19^{Ink4d}$) and Cip/Kip ($p21^{Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$) family, respectively (reviewed in Sherr & Roberts [209]).

Restriction point

During a critical period early in G1 phase, somatic cells are responsive to external signals (*e.g.* growth factors, mitogens) until a point in the cell cycle that is referred to as a restriction point. At this point a decision is made whether the cell will enter the cell cycle and progress to S phase or exit the cell cycle and enter a quiescence state, the Go phase (at

conditions of high cell density and absence of growth factors). The critical regulator of cell cycle entry and the restriction point governing protein is the retinoblastoma protein (pRB): acute downregulation of pRB causes cells to exit quiescence and re-enter the cell cycle [210].

pRB prevents expression of E2F target genes

Hypophosphorylated pRB binds E2F-family transcription factors and recruits chromatin-modifying enzymes that actively repress transcription [211]. Sequential phosphorylation of pRB results in its dissociation from the E2F transcription factors, releasing them to activate target cell cycle genes [212] (Fig. 1.4). In early G1 phase, phosphorylation of pRB is initiated by CDK4/CDK6 in combination with cyclin D1, which accumulates in response to extracellular mitogens [213–216]. In late G1 phase, cyclin E-CDK2 phosphorylates pRB at additional sites [217]. Under stress condition (*e.g.* DNA damage), cell cycle progression is blocked by G1 checkpoint pathways, which inhibit CDK2 activity, thus preventing S phase entry.

The transcriptionally active forms of E2F are a collection of heterodimeric protein complexes [218–221], composed of one E2F protein family subunit (E2F1–E2F6) and one DP protein family subunit (DP1 or DP2). E2F activates a panel of genes involved in progression through the G1 phase, such as *CCNE* (cyclin E) [222], *CCNA2*, *CDC25A*, *CDK2*, *E2F3*, and *RB1*, as well as DNA replication, such as *POLA* (polymerase α), *TOP2A*, *TK1*, *RPA3*, and *RFC2* [223]. Moreover, by transcriptional activation of multiple other targets, E2F integrates cell cycle progression with DNA repair (*UNG*, *RAD54L*, *PRKDC*) and checkpoint pathways (*CHEK1*, *TP53*, *BUB3*) [223].

Genes that promote G1-S transition

1.3.2 *Cell Cycle Regulation in ESCs*

In cell cycle regulation and structure, ESCs demonstrate substantial differences from somatic cells. ESCs proliferate at a fast rate: cell cycle length is about 8-11 h in mESCs [224–226], 15-16 h in hESCs [227] and 12-21 h in rhesus monkey ESCs [228]. The abbreviated cell cycle is owing to shortened gap (G1 and G2) phases; in mESCs, G1 phase takes only approximately 2 h [225] and cell cycle structure consists largely of S phase cells [225]. Furthermore, ESCs can multiply in the absence of serum and are not subject to contact inhibition or anchorage dependence [224]. This has been attributed to predominant expression of the hyperphosphorylated form of the pRB in mESCs [229], indicating that newly formed cells can enter a new phase of DNA replication very shortly after exit from mitosis. pRB family members p107 and p130 are as well expressed [230] and hyperphosphorylated in mESCs and do not associate with E2Fs [226]. mESCs deficient for the three RB family members have no reported cell cycle phenotype [230, 231].

ESCs lack functional restriction point

The repertoire of cell cycle regulatory proteins expressed in mESCs includes cyclins D1, D3, E, A2 and B1 and all CDKs [225, 232–234].

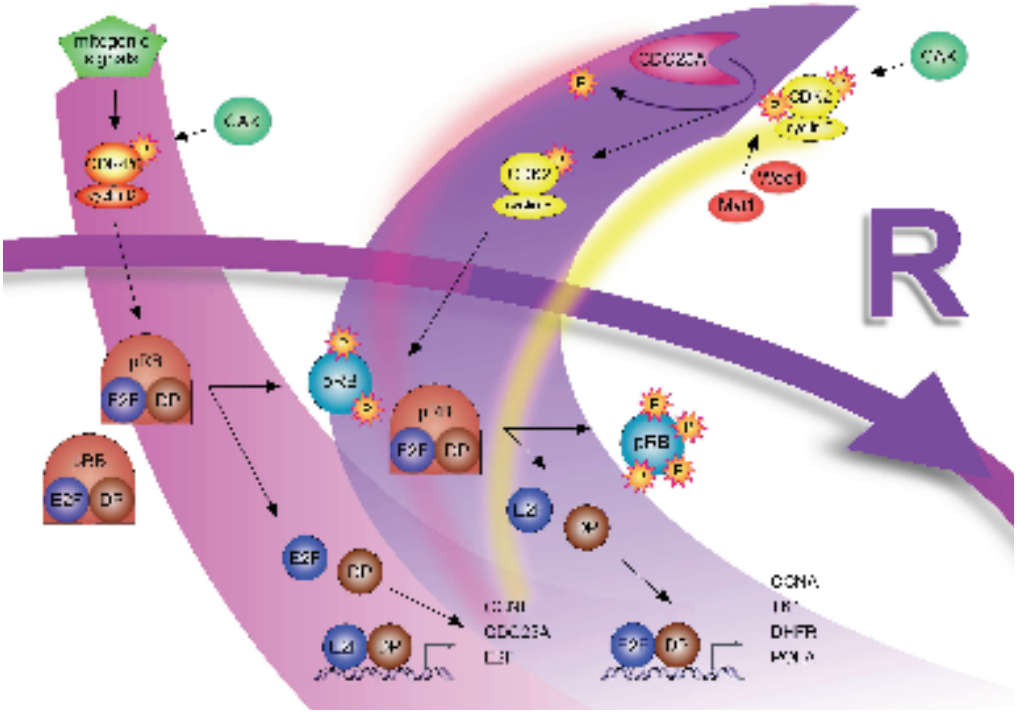

Figure 1.4: Restriction point. In response to mitogenic signals, cell enters early G1 phase (violet). Cyclin D-CDK4/6 complexes phosphorylate pRB, whereby expression of early-G1 E2F target genes, such as for cyclin E and CDC25A, is initiated. In late G1 phase (blue), cyclin E-CDK2 complexes are activated and phosphorylate additional sites on pRB, releasing E2F to activate expression of several S-phase promoting genes, including cyclin A (*CCNA*), dihydrofolate reductase (*DHFR*), polymerase α (*POLA*), and thymidine kinase 1 (*TK1*).

Cyclin levels do not cycle

With the exception of mitotic regulator cyclin B, all cyclins are in mESCs present at comparable levels throughout the cell cycle [225,233]. Moreover, the levels of individual cyclins are significantly elevated compared to somatic cells [234]. In addition to cell-cycle independent CDK activity, mESCs do not express CDK inhibitory molecules of the Ink4 ($p15^{Ink4b}$, $p16^{Ink4a}$, $p18^{Ink4c}$, $p19^{Ink4d}$) and Cip/Kip ($p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$) family [229, 232, 233]. General CDK activity is far higher than that seen in other cell types, including cancer cells, what suggests a decline of CDK activity during differentiation [233]. Most CDK activities in mESCs are active throughout the cell cycle, with the exception of CDK1-cyclin B that becomes selectively activated before mitosis [225], and CDK4-cyclin D1 complexes that exhibit little or no activity in mESCs [233]. It has been suggested that high and precocious CDK2 activity might drive the rapid G1 phase progression in mESCs [225]; however, while inhibition

ESCs do not express CDK inhibitory molecules

of CDK2 activity (by Roo9-3033) slowed down the ESC cycle, it did not change the general cell cycle structure of mESCs and other pluripotent cell types [225].

Studies of hESC cycle regulation showed that hESCs express all G1 type cyclins (cyclins D1, D2, D3 and E) and CDKs (CDK4, CDK6 and CDK2) at variable levels and most of them show cell-cycle dependent expression [227, 235], which is one of the most striking differences between mESCs and hESCs. In contrast to mESCs, where cyclin E levels remain constant throughout the cell cycle, cyclin E protein levels increase around the G1-S transition and cyclin A protein levels are upregulated in late G1-S through G2-M in hESCs [235, 236]. In another study, FACS analysis and immunofluorescence staining showed that cyclin E is constitutively expressed but cyclin A is upregulated in S and G2/M in hESCs [237]; this group was also unable to detect cyclin D1, D2, and D3 in undifferentiated hESCS. The discrepancy between these reports may be due to different sensitivities of detection approaches (Western blot *vs.* immunofluorescence), some contribution from spontaneously differentiated cells, or variability between different hESC lines used in their experiments [238].

Like mESCs, hESCs have a shortened G1 phase, which has been attributed to the elevated mRNA levels of cyclin D2-CDK4 and to low levels of $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ [227, 239, 240]. Also, CDK inhibitors of the Ink4 family ($p15^{Ink4b}$, $p16^{Ink4a}$, $p18^{Ink4c}$, $p19^{Ink4d}$) are expressed at low levels or are not expressed at all [241, 242]. CDK4 and CDK6 have the highest kinase activity in the G1 phase [235]. CDK2 kinase activity culminates in S phase, displaying the highest kinase activity overall in hESCs [235].

pRB, p107 and p130 are expressed in hESCs, with p130 being predominantly expressed on the mRNA level [239]. In response to DNA damage, RB mRNA is modestly increased while p107 and p130 mRNA expression is decreased [239]. pRB exists in both the hyperphosphorylated and hypophosphorylated forms [237].

Regulation of ESC cycle by miRNAs

The length of G1 phase in ESCs is regulated also by miRNAs. miR-290 cluster modulates G1-S transition in mESCs by repressing expression of $p21^{Cip1}$ protein [243]. Similarly, miR-92b regulates $p57^{Kip2}$ protein levels in hESCs [240]. miR-302-367 cluster, expressed in both mESCs and hESCs and regulated by OCT4, SOX2 and NANOG [15, 244, 245], regulates G1-S transition through post-transcriptional negative regulation of cyclin D1 and CDK4 in hESCs [245] and of cyclin D2 in human embryonal carcinoma cells (hECCs) [246]. The critical role of miRNAs in ESC cycle regulation was demonstrated in hESCs, in which knockdown of DICER and DROSHA (the enzymes needed for the processing of mature miRNA) impaired proliferation due to a slow down in cell cycle progression [247]. This defect in hESC proliferation could be partially

restored by introduction of miR-195 and miR-372, negative regulators of Wee1 and $p21^{Cip1}$, respectively [247].

Rather than being at the top of SC hierarchy, miRNAs likely function as crucial intermediate regulators and tuners of cell cycle [248, 249]. Surprisingly, however, the ectopic expression of the complete human miR-302-367 cluster in several human cancer cell lines is sufficient to reprogram them into and to maintain them in a pluripotent ESC-like state [250]; these cells were designated as miRNA-induced pluripotent SCs (mirPSCs).

miRNA-induced pluripotent SCs

1.3.3 *Linking Cell Cycle Regulation to Cell Fate Choices in ESCs*

Upon induction of differentiation, cyclin A2 and cyclin B1, which are highly overexpresssed in mESCs, sharply decrease [234] and cyclin E expression comes under the control of the RB-related family of pocket proteins and, therefore, requires the mitogen-induced activity of cyclin D-CDK4/CDK6 complexes [251]. Also in hESC, protein levels of cyclins D and cyclin B1 increase, while levels of cyclin E and cyclin A decrease with differentiation [235, 237, 245]. The specific patterns of expression of cell cycle regulators in ESCs suggest that some of them have key functions in the proliferative capacity of these cells, the maintenance of self-renewal potential, and the prevention of untimely differentiation [238].

The process of self-renewal requires the coordination of cell cycle progression and cell fate choices (*e.g.* commitment *vs.* self-renewal) [252]. It has been hypothesized that duration of each cell cycle phase in ESCs has a function in maintaining pluripotency and self-renewal [226,235]. An evidence for this hypothesis comes from reprogramming studies, which show that reprogramming of somatic cells to pluripotency (*i.e.* iPSCs) confers similar cell cycle regulation as in mESCs [253].

A link between cell cycle length and the differentiation process has been established in cancer cells, somatic cells and adult SCs, and recently also in hESCs [235, 254, 255]. Neural progenitor cells comprise two subpopulations, differing in the length of their cell cycle: those that are simply undergoing mitotic proliferation have a shorter cycle than those committed to undergo neuronal differentiation [255]. Artificial lengthening of the cell cycle by inhibition of CDKs induced neural progenitors to form neurons [254] and shortening of the cell cycle of neural progenitors prevented their differentiation [255]. Moreover, cyclin D1-CDK4 overexpression studies in neural SCs have shown that G1 lengthening is necessary and sufficient to switch neural progenitors to neurogenesis [256].

A link between cell cycle length and differentiation

In hESCs, two studies indicated that abolishment of CDK2 activity may induce their differentiation: Treatment with a CDK2 inhibitor delayed hESCs in G1 and S phase, resulting in emergence of a subpopulation

Downregulation of CDK2 induces differentiation in hESCs

lacking OCT4 [237], while CDK2 knockdown resulted in G1 arrest and differentiation to extraembryonic lineages [235]. The changes in hESC fate induced by a transient decrease in CDK2 were permanent as expression of genes associated with the pluripotent phenotype never returned to its former level [235]. This observation suggests that reacquisition of a short G1 phase cannot reverse the differentiation marks established during the lengthening of G1 phase [235]. Similar G1 lengthening occurs during spontaneous ESC differentiation and suggests that a longer G1 is perhaps necessary for establishment of epigenetic marks that are necessary for initiation of the differentiation process [257].

Further evidence for the link between cell cycle regulation and ESC fate decisions brought recent study, which presented CDK2-associating protein 1 (CDK2AP1; $p12^{DOC-1}$), an inhibitor of G1-S transition through downregulation of CDK2 [258], as competency factor in mESC differentiation by modulating the phosphorylation level of pRB [259]. In this study, *Cdk2ap1*$^{-/-}$ mESCs were shown to be resistant to LIF withdrawal-induced differentiation and displaying altered pRB phosphorylation [259]. The differentiation competency of the *Cdk2ap1*$^{-/-}$ mESCs was restored upon the ectopic expression of CDK2AP1 or a nonphosphorytable pRB mutant [259]. Essentiality of RB family in mESC differentiation was shown in triple knockout mESCs (*Rb1*$^{-/-}$, *Rb1l*$^{-/-}$, *Rb2l*$^{-/-}$), which were incapable of undergoing proper differentiation [231]. Interestingly, single and double knockout ESCs showed no defect in differentiation, demonstrating the ability of RB family members to compensate for one another in this setting [231].

RB family is essential for mESC differentiation

Together, these observations suggested that G1 phase corresponds to a window of increased sensitivity of SCs to differentiation signals and indicated existence of an intrinsic link between cell cycle regulation and cell fate decisions (self-renewal *vs.* differentiation) in SCs that are likely to be affected by the length of each phase of the cell cycle [235]. This hypothesis is further supported by evidence of key cell cycle regulatory proteins, such as CDK1, cyclin D1, CDK6, CDC7 being targets of pluripotency factors OCT4, SOX2 and NANOG [10]. Furthermore, E2F activity may act as a regulatory co-factor for OCT4 on the promoter of OCT4 target genes and because ORC1L (Origin recognition complex subunit 1-like), a direct E2F target involved in DNA replication, belongs to the core OCT4 regulatory network, E2F and OCT4 activities might be linked [260]. Recently, a direct interaction between pluripotency and cell cycle machinery has been observed: NANOG binds directly to the promoter region of *CDC25A* and intragenic regions of *CDK6*, resulting in their transactivation [242].

A link between cell cycle regulation and cell fate decisions

Very likely many more connections exist between the master regulators of cell cycle and stemness, including via the regulation of microRNAs [261]. c-Myc/E2F driven miR-17-92 cluster, which controls the G1-S

transition, is fundamental for ESC self-renewal and cell proliferation and is downregulated upon ESC differentiation [262]. More recently, LIN28/c-Myc (which regulate miR-17-92) and OCT4/SOX2 regulated miR-302 have been shown to be among a handful of factors shown to be necessary and sufficient to convert differentiated cells to iPSCs [250, 263].

1.3.4 *Cell Cycle Regulation in Adult Stem Sells*

Adult SCs have a functional restriction point

Tissue SCs require a more complex cell cycle regulation because during their life they repeatedly enter periods of quiescence and proliferation. They have active pRB and are dependent on mitogens to activate cyclin D-CDK4/6 so that pRB can be inactivated and cell cycle entered (reviewed by Sherr [264]). RB proteins confer quiescence to HSCs: Deletion of all three RB family genes leads to the proliferation and mobilization of HSCs and to myeloproliferative disease [265]. Dependence of HSCs (and tissue SCs in general) on cyclin D-CDK4/6 activity is demonstrated by lethal phenotypes of *Ccnd1*$^{-/-}$ *Ccnd2*$^{-/-}$ *Ccnd3*$^{-/-}$and *Cdk4*$^{-/-}$*Cdk6*$^{-/-}$ embryos, which die because of defective hematopoiesis [266, 267]. Ink4 family proteins, the inhibitors of cyclin D-CDK4/6 complexes, negatively regulate self-renewal by slowing cell division: Deficiency in *Cdkn2c* ($p18^{Ink4c}$), a CDK4/6 inhibitor, increases HSC frequency and enhances the repopulating capacity of HSCs from young mice in transplantation assays [268]. On the other hand, increase of *Cdkn2a* ($p16^{Ink4a}$) expression with age reduces stem/progenitor cell frequency and function in a variety of aging tissues [269–271].

Under steady-state conditions, SC frequency is negatively regulated by p53. *Tp53* deficiency increases SC frequency and self-renewal in adult NSCs and HSCs [272, 273], perhaps by reducing quiescence [274]. Overactivation of pRB and p53 tumor suppressor pathways can lead to senescence and premature depletion of the SC pool [275]. This is avoided by repression of *Cdkn2a* locus (encoding $p16^{Ink4a}$ and $p19^{Arf}$) mediated by high mobility group protein HMGA2 [276] and BMI-1 [277]. *Bmi1*$^{-/-}$ mice exhibit profound SC self-renewal defects and progressive SC depletion in multiple tissues [277–279] and their phenotype can be partially rescued by deletion of *Cdkn2a* or *Tp53* [277, 280–282]. On the other hand, pRB and p53 pathways reinforce the ability of progenitors to exit the SC state [282, 283].

Importantly, self-renewal of SCs is mechanistically distinct from the proliferation of downstream progenitors (for review, see He *et al.* [284]): some mechanisms preferentially regulate SC self-renewal, whereas other mechanisms preferentially regulate restricted progenitor proliferation [284].

Furthermore, many tissue SCs undergo dynamic changes in their cell cycle status during development and aging to meet the changing

demands of tissue growth and regeneration. Fetal liver HSCs undergo daily symmetric self-renewing divisions to expand the SC pool [285], while adult HSCs are quiescent most of the time [286]: Most adult mouse HSCs asynchronously divide once every 12 days, but a more slowly dividing subset of HSCs divides only once every 55 days or so [287–289]. The rate at which adult HSCs divide further changes during aging [290,291]. Interestingly, primate HSCs seem to be even more quiescent than murine HSCs [292]. Moreover, unlike ESCs, the number of times a single HSC replicates during a lifespan in mammals is limited (80-200 times), suggesting that their self-renewal is intrinsically restricted [293–295]. This limit becomes experimentally evident as exhaustion of their regenerative potential when HSCs are induced to proliferate rapidly in response to environmental stress, such as in serial transplantation or myelosuppressive chemotherapy [296–300].

Quiescence prevents differentiation and exhaustion of SCs

It has been suggested that residing in the quiescent state is the way how HSCs (and possibly also other types of adult SCs) avoid differentiation signals and exhaustion [301] because G1 phase of the cell cycle - particularly the early G1 phase - seems to be a sensitive period during which cell fate decisions are made [202, 254, 302, 303]. Therefore genes that prevent HSCs from entering the cell cycle will tend to preserve their long-term function by reducing their exposure to exhaustion-inducing stimuli in early G1 phase, while genes that facilitate the transition through the G1 phase of the cell cycle, particularly through early G1, might have an impact on the likelihood of particular cell fate decisions by altering the time spent in the sensitive period [301].

Support for these suggestions comes from a number of studies in which proliferation led to the exhaustion of SC function [301]. The result is usually long-term loss of SCs and increased susceptibility to stress-induced exhaustion. In *Cdkn1a*$^{-/-}$ animals, the proportion of primitive hematopoietic cells in the Go phase of the cell cycle was reduced and the number of very primitive cells that gave rise to long-term multipotent colonies in culture was increased [304], their bone marrow was more rapidly exhausted as compared with wild type and also demonstrated the loss of neurosphere-initiating cells with aging [305]. Importantly, the *Cdkn1a*$^{-/-}$ phenotype varies with strain [306] and this could be a result of the strain-dependent cell cycle differences in SC populations [301]. Similar cell cycling and exhaustion phenotypes are displayed by *Gfi1*$^{-/-}$ (growth factor independent 1), *Pten*$^{-/-}$ (phosphatase and tensin homologue) or *Foxo1/3/4*$^{-/-}$ HSCs [307–310]. Deficiency of *Gfi1*$^{-/-}$ HSCs might be, at least in part, caused by severely decreased expression of p21^{Cip1} in *Gfi1*$^{-/-}$ HSCs [307].

Similarly, the mechanisms of HSC deficiency in *Pten*$^{-/-}$ and *Foxo1/3/4*$^{-/-}$ mice are linked: Pten is a negative regulator of phosphatidylinositol 3-kinase (PI3K) signaling and PI3K is known to inhibit the activity of

members of the FoxO family; therefore Pten might regulate SC function by indirectly activating FoxO transcription factors [311]. FoxO family of transcription factors regulate a number of cellular processes including cell cycle arrest and resistance to cell stress (for a review, see Ho *et al.* [312]). Because *Foxo1*, *Foxo3* and *Foxo4* null HSCs, in addition to being decreased in number and increased in cell cycle entry, accumulated elevated levels of reactive oxygen species (ROS) and because this phenotype could be almost completely reversed by treating animals with an antioxidant, it was suggested that ROS are important regulators of HSC quiescence and maintenance [310].

ROS are regulators of HSC maintenance

Other studies have brought evidence for the relationship between adult SC quiescence and the maintenance of SC function. For example, study of $Mef^{-/-}$ (myeloid Elf1-like; or Elf4) mice suggested that MEF is an important negative regulator of HSC quiescence and its deletion results in increased quiescence and the paradoxical HSC expansion that results from reduced HSC exhaustion [313].

In conclusion, to achieve the goal of self-renewal, both ESCs and adult SCs use the same principle of avoiding the early G1 phase of the cell cycle [301]. While ESCs accomplish this by high CDK2 activity, which shortens early G1 phase, adult SCs are maintained in the quiescent state and therefore rarely transit through early G1 [301]. Recently it has been shown that similar mechanisms as in adult SCs are used also in leukemic SCs (LSCs) to prevent their exhaustion [314].

Both ESCs and adult SCs avoid G1 phase to maintain self-renewal

1.4 DNA DAMAGE RESPONSE

1.4.1 *DNA Damage Response in Somatic cells. G1 Checkpoint*

In response to diverse genotoxic stresses, cells activate DNA damage checkpoint pathways to protect genomic integrity and promote survival of the organism [315]. The DNA damage checkpoint network is composed of DNA damage sensors, signal transducers and various effector pathways, and its central components are the phosphoinositide 3-kinase related kinases (PIKKs) ataxia-teleangiectasia mutated (ATM), ATM and Rad3-related (ATR) and DNA-dependent protein kinase (DNA-PK), whose many substrates mediate cell cycle arrest in G1, S or G2 phases, DNA repair and cell death [316–319]. In general, ATM and DNA-PK respond mainly to DNA double strand breaks (DSBs), whereas ATR is activated by single-stranded DNA and stalled DNA replication forks [319]. However, checkpoints induced upon recognition of other types of DNA lesions operate through similar principles and share some of the key elements, including the ATR kinase [315]. In contrast, the downstream checkpoint effectors and their final targets within the cell cycle machinery may differ in G1, S, or G2/M phases [320].

Checkpoint pathways are activated to restore genomic integrity and to prevent formation of mutations

ATM/ATR phosphorylate multiple substrates

Recognizing DNA damage, ATM and/or ATR become activated and phosphorylate a range of substrates, including H2AX (Ser139) [321, 322], p53 (Ser15 in human, Ser18 in mouse) [323–325], MDM2 (Ser395) [326], BRCA1 (Ser1387, Ser1423, Ser1457, Ser1524) [327–329], TopBP1 (topoisomerase-binding protein-1; Ser1131) [330], Nbs1 (Nijmegen breakage syndrome 1; Ser278, Ser343) [331], Kap1 (KRAB domain-associated protein 1; Ser824) [332, 333], and signal messengers Chk1 (Ser317, Ser345) [334] and Chk2 (Ser19, Thr26, Ser33/35, Thr68) [335–337].

Histone H2AX is rapidly phosphorylated in the chromatin microenvironment surrounding a DSB and functions to concentrate repair and signaling factors, such as MRN, BRCA1 and 53BP1, in the vicinity of DNA lesions, forming irradiation induced foci (IRIF) [338]. The assembly of IRIF is potentially mediated by interactions between specific domains of repair/signaling factors and the γ-H2AX tail [339].

BRCA1 is required for ATM/ATR-dependent phosphorylation of p53, c-Jun, Nbs1 and Chk2 following exposure to IR/UV, respectively [340]. BRCA1 phosphorylation activates a process of DNA repair through homologous recombination in cooperation with the BRCA2 [327]. TopBP1 activates ATR-ATRIP complex [341], mediates DNA damage signaling from Nbs1 to ATR and promotes homologous recombination repair [342]. Nbs1 is an integral component of the Mre11/Rad50/Nbs1 (MRN) nuclease complex [343–346], which is important in the repair of DSBs [347]. Kap1 functions as transcriptional corepressor and is involved in regulation of global chromatin compaction [319, 333]. In response to DNA damage, Kap1 is coordinately phosphorylated (at Ser824) and desumoylated, allowing de-repression of the transcription of *CDKN1A*, *GADD45A*, *BAX*, *PUMA*, and *NOXA* [348].

Activated Chk1 and Chk2 impose cell cycle arrest via phosphorylation of their targets, including p53 (Ser20) [349–351] and CDC25 phosphatases [352–354].

MDM2 regulates stability of p53

Phosphorylation of p53 on Ser20 increases its stability because it interferes with binding to the ubiquitin ligase MDM2 [355]. MDM2, one of the transcriptional targets of p53 [356, 357], inhibits p53-mediated transcription, shuttles p53 out of the nucleus [358], and targets p53 for ubiquitin/proteasome-mediated proteolysis [359, 360]; reviewed in Prives [361]).

Tumor suppressor p53, a transcription factor

Activated p53 protein transcriptionally upregulates the expression of downstream target genes, such as *CDKN1A [362]*, *SFN* (encoding 14-3-3σ) [363], *BAX [364]*, *IGF-BP3* [365,366], *FAS* [367], and *TNFRSF10B* (encoding KILLER/DR5) [368], which relay signals that mediate the inhibition of cell growth or the induction of programmed cell death (reviewed by El-Deiry [369]). Following severe DNA damage, an additional phosphorylation at Ser46 increases the affinity of p53 for promoters of proapoptotic genes, such as *TP53AIP1* [370]. The p53-Ser46 phosphorylation is mediated by dual-specificity tyrosine-phosphorylation-

regulated kinase 2 (DYRK2) [371]. p53 has also been implicated in the control of a G2/M checkpoint by transcriptional downregulation of cyclin B1 [372–374] and upregulation of *GADD45* (growth arrest and DNA damage 45) [375].

CDKN1A, which encodes cyclin dependent kinase inhibitor (CKI) $p21^{Cip1}$ [376], is a critical target of p53 [362] in facilitating G1 arrest [377]. $p21^{Cip1}$ binds to CDK2/CDK1-cyclin complexes, resulting in inhibition of their kinase activity [378].

Regulation of CDC25 after DNA damage

Phosphorylation of CDC25A (Ser76, Ser124) causes it to bind β-TrCP (β-transducin repeat-containing protein), a component of the SCF ubiquitin ligase complex, and stimulates SCF-mediated ubiquitination and proteolysis of CDC25A [353, 354]. Phosphorylation of CDC25B (Thr549) and CDC25C (Ser16) either directly inactivates the phosphatase activity or creates a 14-3-3 binding site, leading to their cytoplasmic sequestration, which blocks interaction of CDC25B/CDC25C with their substrate CDK1-cyclin B [379–382]. Also, this mechanism is applied on CDC25A to negatively regulate its mitotic function [383]. In the absence of CDC25 phosphatase activity, the inhibitory phosphorylation of CDK2-cyclin E/A or CDK1-cyclin B kinase complexes persists and the cell cycle is arrested in G1 or G2 phase, respectively [384–391].

G1 checkpoint

G1 checkpoint consists of two paralel pathways

G1 DNA damage checkpoint involves rapid responses targeting cyclin D1 and CDC25A, and a slower response employing p53 (Fig. 1.5). Cyclin D1 is rapidly degraded by ubiquitin/proteasome-dependent mechanisms after DNA damage, resulting in the redistribution of the CDK inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ from CDK4/6-cyclin D1 to CDK2-cyclin A/E [392, 393]. CDK2 is also inactivated by Thr14/Tyr15-phosphorylation after DNA damage-induced degradation of CDC25A (through phosphorylation by Chk1/Chk2) [354]. The slower response of the G1 DNA damage checkpoint is carried out by the activation of p53, which increases the expression of *Cdkn1a* and leads to the inhibition of CDK2-cyclin complexes, preventing expression of S-phase genes [209].

1.4.2 *DNA Damage Response in ESCs*

ESCs lack functional G1 checkpoint

The special features of ESC cycle are reflected in their inability to undergo cell cycle arrest at the G1 checkpoint in response to DNA damage [228, 394–396]. The causes of G1 checkpoint nonfunctionality have been studied mostly in mESCs. They were reported to have functional mechanisms for detection of DNA defects [397], but compromised function of p53 [394] and Chk2 [395].

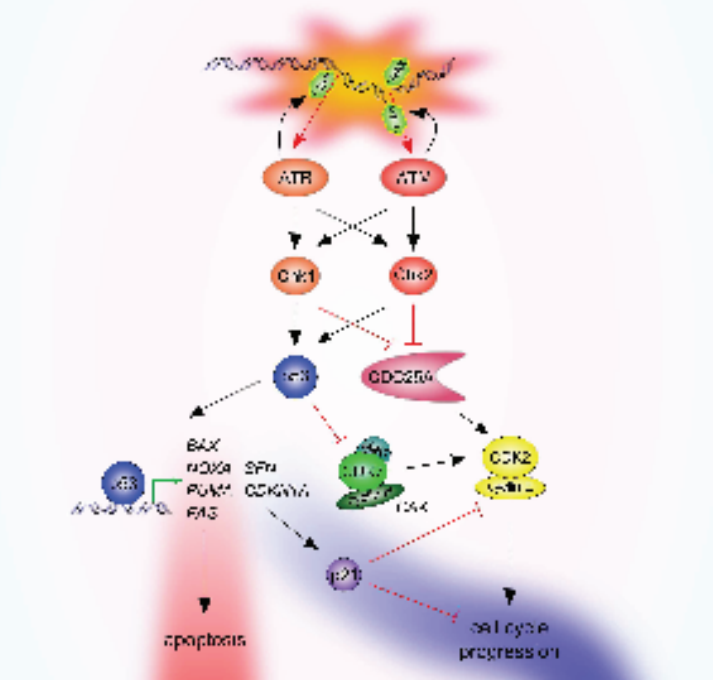

Figure 1.5: Two main G1 checkpoint pathways in somatic cells, the rapid Chk1/2-CDC25A and the delayed p53-p21^{Cip1} pathway.

In mESCs, Chk2 is not intranuclear as in somatic cells, but it is sequestered at centrosomes and thus unavailable for regulation of CDC25A [395]. p53 was found to be abundant and transcriptionally activated after DNA damage, but unable to translocate into the nucleus [394, 395]. Therefore, expression of *Cdkn1a* as well as of proapoptotic genes is not induced and mESCs do not stop in G1 phase and fail to undergo p53-dependent apoptosis in response to DNA damage [394, 398]. However, the concept of p53 inability to translocate into the nucleus has been challenged by the observation that in response to DNA damage, p53 actively suppresses expression of *Nanog* to induce differentiation of DNA-damaged mESCs and thus their elimination from the replicative pool [399]. Moreover, in hESCs p53 is nuclear [396] and expression of *CDKN1A* is robustly induced upon IR [239] while the G1 arrest is not induced [396], suggesting other mechanism(s) prevent functionality of activated checkpoint pathways in ESCs.

The lack of functional G1 checkpoint in ESCs increases the relative importance of internal S and G2 checkpoint pathways for genomic stability. In mESCs, S phase checkpoint is governed by ATR, whose function can be substituted for by p38α [400]. In G2 checkpoint, ATM-Chk2 signaling seems to play essential role: ATM inhibition (with KU55933) abolishes G2 arrest in hESCs [396] and *Chek2*$^{-/-}$ mESCs do not maintain G2 arrest after IR [350].

Part II

ORIGINAL RESEARCH

2

AIMS OF THE THESIS

This study focused on G1 phase regulation in mESCs. It was aimed to

1. unravel the causes of G1 checkpoint non-functionality in mESCs,
2. investigate the role of CDK2 in G1 phase regulation and its potential connections to regulation of mESC fate decisions (*i.e.* self-renewal *vs.* differentiation).

G1 checkpoint

The mechanisms of G1 checkpoint nonfunctionality in ESCs have not been fully resolved. Previous studies suggested that G1 checkpoint might be inactivated by centrosomal sequestration of Chk2 [395] and inability of p53 to translocate to the nucleus [394]. However, the data have been scarce and, especially in the case of p53-p21^{Cip1}pathway, the observations have been rather inconsistent. Therefore, we attempted to elucidate the causes of G1 checkpoint nonfunctionality in mESCs.

Role of CDK2

The results of our study on G1 checkpoint in mESCs made us question involvement of CDK2 in regulation of mESC cycle, namely the classical role of CDK2 in regulation of multiple nuclear processes of G1-S transition (Fig. 2.1) established in somatic cells. Therefore, we investigated the role

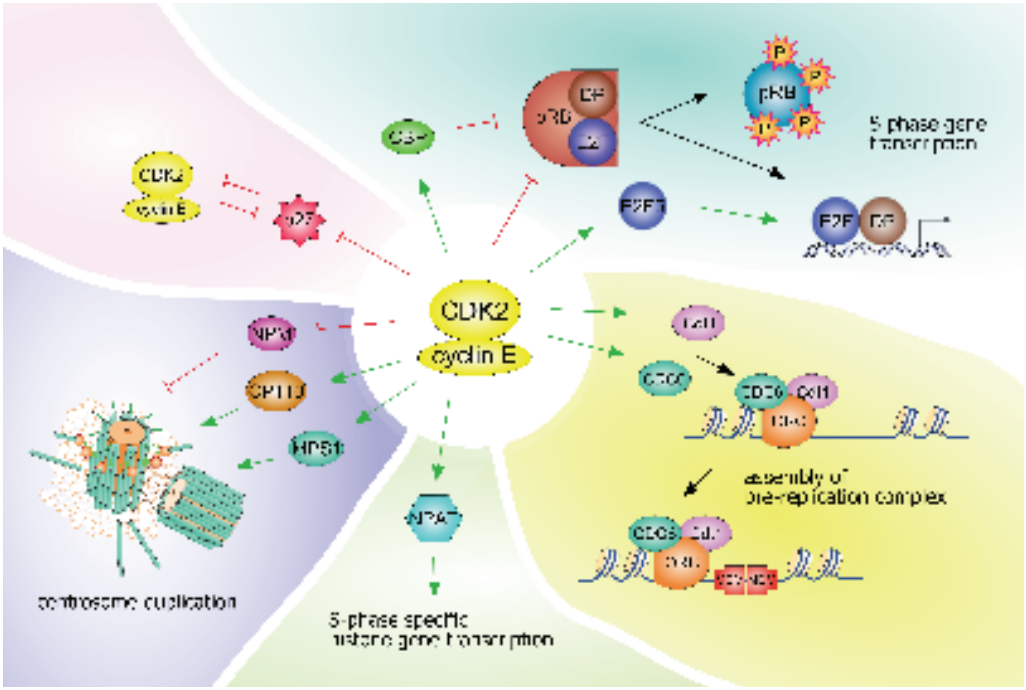

Figure 2.1: Multiple roles for CDK2 in G1-S transition in somatic cells.

of CDK2 in cell cycle regulation in mESCs in greater detail, focusing more deeply on potential role of CDK2 in regulation of self-renewal, which emerged from our observations contemporary with publication of a similar study in hESCs.

3

MATERIALS AND METHODS

Cell culture and synchronization

Culture of mESCs

Two different mESC lines were used in our studies; an inbred HM-1 cell line derived from 129 mouse strain [401] and an F1 (129SvJae×C57BL/6) hybrid line V6.5 (Open Biosystems, Huntsville, AL, USA). The cells were maintained on culture dishes covered with 0.1% gelatin (Sigma-Aldrich, St. Louis, MO, USA) in a humidified atmosphere of 10% CO_2 at 37°C in high-glucose Dulbecco's modified Eagle's medium (D-MEM) with GlutaMAX (Invitrogen/Gibco, Grand Island, NY, USA) supplemented with 15% fetal bovine serum (FBS; Thermo Fisher Scientific/Hyclone, Waltahm, MA, USA), 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco), 0.1 µM β-mercaptoethanol (Serva) and 1000 U/ml LIF (ESGRO, Chemicon, Temecula, CA, USA).

Culture of NIH3T3 and HT29 cells

Mouse embryonic fibroblasts NIH3T3 and human colon adenocarcinoma cell line HT29 (ATCC, Temecula, CA, USA) were maintained in a humidified atmosphere of 5% CO_2 at 37°C in high-glucose D-MEM with GlutaMAX supplemented with 15% (NIH3T3) or 10% (HT29) FBS (Invitrogen/Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin.

Cell synchronization

Cells were synchronized in G2/M phase by treating them with 400 nM nocodazole (Sigma-Aldrich, St. Louis, MO, USA). mESCs were grown in the presence of nocodazole for 12 h, and NIH3T3 cells were grown in its presence for 24 h. After mitotic shake-off, cells were washed three times with phosphate-buffered saline (PBS) with 1% FBS (Invitrogen/Gibco) and plated in standard media.

Cell irradiation

Cells were irradiated with a defined dosage (4 Gy) of gamma irradiation (cobalt irradiator, Teragam, Prague, Czech Republic).

Drugs and inhibitors

Nocodazole was stored as a 400 µM stock solution in DMSO. CEP3891 (Cephalon, Frazer, PA, USA) was stored as a 500 µM solution in DMSO and used at a concentration of 500 nM. The Chk2 inhibitor II [2-(4-(4-chlorophenoxy)phenyl)-1H-benzimidazole-5-carboxamide; Merck Chemicals Limited, Darmstadt, Germany] was stored as 300 µM solution in DMSO and used at 300 nM. LiCl (Sigma-Aldrich) was prepared as a 100 mM solution in PBS and used at 10 mM. The GSK-3 inhibitor X (6-bromoindirubin-3'-acetoxime; Merck Chemicals Limited) was stored as a 2.5 mM stock solution in DMSO and used at 5 µM. Olomoucine II (2-{[2-

((1-R)-1-hydroxymethyl-propylamino)-9-isopropyl-9Hpurin-6-ylamino]-methyl}-phenol) and CAN508 (4-[(3,5-diamino-1H-pyrazol-4-yl)diazenyl]phenol) were synthesized according to published procedures [402, 403]. Both olomoucine II and CAN508 were stored as 100 mM stock solutions in DMSO.

Flow cytometric analysis

Cell cycle distribution was evaluated by BrdU incorporation and propidium iodide staining. Cells were pulsed with 10 µM 5-bromo-2'-deoxyuridine (BrdU) (Sigma-Aldrich) for 30 min, trypsinized (0.5% trypsin-EDTA; Gibco) to obtain a single cell suspension, washed twice in PBS with 1% FBS and resuspended in PBS. The cells were then fixed in ice cold 70% ethanol. After rehydration in PBS with 1% FBS (Gibco), cells were incubated in 2 M HCl with 0.5% (v/v) Triton X-100 for 30 min at room temperature. Following neutralization with 0.1 M $Na_2B_4O_7$, cells were collected by centrifugation and washed with PBS with 1% FBS and 0.5% (v/v) Tween-20. Then they were stained with anti-BrdU FITC-labeled antibody (1:20; Roche Diagnostics, Mannheim, Germany) for 30 min at room temperature in the dark. The cells were then washed with PBS with 1% FBS and 0.5% (v/v) Tween-20 and incubated in 1.1% sodium citrate with 5 ng/µl ribonuclease A (DNA Lego Ribonuclease A; Top-Bio, Prague, Czech Republic) and 60 µg/µl propidium iodide (Sigma-Aldrich) for 30 min at 37°C in the dark. Cells were analyzed by flow cytometry on the Cytomics FC 500 machine using the CXP software (Beckman Coulter, Fullerton, CA, USA). MultiCycle software (Phoenix Flow Systems) was applied to assess cell cycle distribution.

MTT assay

Equal numbers of cells per well (7,000 for mESCs, 5,000 for HT29) were plated on a 96-well plate and incubated for 3 h (mESCs) or 24 h (HT29) under standard conditions. Triplicate samples of these cells were treated with increasing concentrations of inhibitors (range from 10^{-8} to 10^{-4} M) or mock-treated and incubated for 24 h (mESCs) or 72 h (HT29) under standard conditions. After this time, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; Sigma-Aldrich) was added to a final concentration 0.5 mg/ml and the cells were incubated for another 2.5 h under standard conditions. The media was then removed, 100 µl of 10% SDS was added per well and the 96-well plate was incubated overnight at room temperature on a shaker. The absorbance was read at 570 nm. To calculate IC_{50} values (concentrations that produce a 50% of inhibitory effect on cell proliferation), the results from all triplicates were transformed to percentage of controls, and plotted as sigmoid dose-effect

Calculation of IC_{50}

curves using a non linear regression mode and the GraphPad Prism 5 software. Using this software, the IC_{50} values were interpolated.

Western blot analysis

Cells in culture dishes were washed with cold PBS, collected into IP buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% (v/v) glycerol, 0.1% (v/v) Tween 20, 1 μM DTT, 1 μM NaF, 10 μM β glycerophosphate, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 0.1 μM Na_3VO_4, 0.1 μM PMSF), and incubated for 1 h at 4°C. Lysates were cleared by centrifugation at 18,800 g at 4°C for 30 min. Proteins were electrophoretically resolved on SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes (Bio-Rad). Membranes were incubated with primary antibodies at dilutions recommended by the supplier at 4°C overnight, washed in PBS with 0.05% Tween 20, and incubated for 1 h with the appropriate horse radish peroxidase (HRP)-conjugated secondary antibody (DakoCytomation, Glostrup, Denmark). HRP activity was detected with ECL detection kit (Pierce, Rockford, IL, USA) on films (Kodak, Rochester, NY, USA). The following primary antibodies were used: anti-actin, anti-Chk2 (DCS-273), anti-phospho-retinoblastoma (RB) [pSer612] and anti-α-tubulin (Sigma-Aldrich); anti-Chk1 (Stressgen, Ann Arbor, MI, USA); β-catenin (L87A12), Cdc25A (F-6), Cdk2 (M-2), cyclin A (C-19) and p-Cdk2(Thr14/Tyr15)-R (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); anti-p21, c-Nap1, Nek2 (BD Pharmingen, San Diego, CA, USA); phospho-Chk1 (Ser345), phospho-GSK-3β (Ser9) (5B3), p53 (1C12) and phospho-p53 (Ser15) (Cell Signaling Technology, Danvers, MA, USA); and pericentrin, RNA polymerase II antibody [H5], RNA Polymerase II antibody [8WG16] (Abcam). β-actin was used as a loading control.

Antibodies used in Western blots

Immunoprecipitation and kinase assay

Immunoprecipitations were conducted at 4°C. Protein A agarose beads (Sigma-Aldrich) were incubated for 1 h on a rotor in 1 ml IP buffer with 1 μg of CDK2 [Cdk2 (M2)], Chk2 [Chk2 (H-300); both Santa Cruz Biotechnology], CDK1 [anti-cdk1/cdc2, CT, Upstate Biotechnology, Lake Placid, NY, USA] or Chk1 antibody [anti-Chk1 (DCS-316), Sigma-Aldrich]. After washing three times with IP buffer, agarose beads were incubated for 1 h with protein lysates (CDK1/2 kinase assays: 200 μg of proteins for mESCs or 400 μg proteins for NIH3T3; Chk1/2 kinase assays: 100 μg of proteins) in IP buffer on a rotor and finally washed three times with IP buffer. Agarose beads with immunocomplexes were equalized with kinase assay buffer (KAB; 50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 2.5 mM EGTA, 100 μM β-glycerophosphate, 2 μM NaF, 1 μM DTT, 0.1 μM Na_3VO_4). The beads were then resuspended in 30 μl kinase reaction mixture [18 μl KAB, 9 μl 75 μM ATP in KAB, 1 μl [γ-^{33}P]ATP (10 μCi; MP Biochemicals,

Immunoprecipitation

Kinase assay

Irvine, CA, USA) and either 2 µg histone H1 (for CDK1/2 kinase assays; Upstate Biotechnology) or 2 µg Chk1/2 peptide substrate (for Chk1/2 kinase assays; Biaffin, Kassel, Germany)] and incubated for 30 min at 30°C. Reactions were terminated by addition of 12 µl of 4x Loading sample buffer (8% SDS, 40% glycerol, 400 mM DTT, 240 mM Tris, pH 6.8, 0.004% Bromophenol Blue). Samples were boiled and electrophoretically separated on SDS polyacrylamide gels. The radioactivity of dried gels was detected with the bioimager BAS 1800 with a Fuji LAS 1000 CCD Camera System (Fuji Photo Film Co., Ltd., Tokyo, Japan) and quantified with Image Gauge software (Fuji, Valhalla, NY, USA).

Statistical analysis

Statistical evaluation of the data was performed with the independent two-sample t-test using Statistica 8.0 (StatSoft, Inc., Tulsa, OK, USA). $P < 0.05$ was considered statistically significant.

Immunocytochemistry

Cell fixation techniques

Depletion of soluble cytoplasmic protein

Antibodies used for immunostaining

Cells were grown on gelatinized glass coverslips, washed with PBS and fixed for 7 min with a methanol-acetone mixture (1:1, -20°C). Alternatively, to deplete soluble proteins from cytoplasm, the cells were permeabilized for 5 min with 0.5% Triton X-100 in PHEM buffer ([404]; 60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, pH 6.9) at room temperature, washed twice with PHEM buffer, and fixed for 10 min with -20°C methanol. Fixed cells on coverslips were blocked with blocking buffer (1% FBS in PBS), stained with primary antibodies [Cdc25A (M-191), Cdk2 (M2), Chk2 (A-12) (all Santa Cruz Biotechnology); gamma-tubulin (TU-30, Exbio, Prague, Czech Republic); GSK-3β (BD Biosciences, Franklin Lakes, NJ, USA); pericentrin (Abcam, Cambridge, UK); Chk1 antibody (DCS-310.1.1, Sigma-Aldrich)] diluted in blocking buffer for 1 h at room temperature. After washing with PBS, cells were incubated for 30 min with the appropriate fluorophore-conjugated secondary anti-mouse or anti-rabbit antibody [Alexa Fluor® 594, goat anti-rabbit IgG (H+L), Alexa Fluor® 488 donkey anti-mouse IgG (H+L) (Invitrogen, Carlsbad, CA, USA)] in the dark, washed with PBS, dried with ethanol and mounted in Mowiol with DAPI (Sigma-Aldrich). Samples were analyzed using a microscope (Olympus IX 81; Olympus, Tokyo, Japan) fitted with a confocal detection system (Olympus FV 1000; Olympus) and LCS Imaris 5.0.3 software (Bitplane, Zurich, Switzerland). Images were processed using IrfanView, DP Manager (Olympus) and GIMP software.

RNA isolation and quantitative RT-PCR

Total RNA from cells was isolated using TRI Reagent (Applied Biosystems/Ambion, Austin, TX, USA) and treated with DNase (TURBO DNA-free, Ambion). 1 μg of total RNA was used for reverse transcription by First Strand cDNA Transcriptor Synthesis Kit (Roche Applied Science) according to the manufacturer's instructions. The relative quantity of target genes' RNA was determined by real-time/quantitative PCR using a LightCycler 480 (Roche). Gene-specific primer pairs (listed in Supplement on page 113) were designed and evaluated at an annealing temperature of 60°C using freely available web-based software in the Universal ProbeLibrary Assay Design Center. The amplification mix and program were prepared using LightCycler 480 SYBR Green I Master (Roche) according to the manufacturer's instructions. Data were analyzed by using LightCycler 480 Relative Quantification Software (Roche) and expression levels were normalized to *Hmbs* or *Gapdh*.

Rapid isolation of centrosomes

Isolation of centrosomes on density gradient

Cells were treated with 60 ng/ml nocodazole and 1 μg/ml cytochalasin D for 1 h. Then, the cells were successively washed with 1x PBS, 0.1x PBS with 8% sucrose, and 8% sucrose at 4°C. Next, the cells were lysed in ice-cold lysis buffer [1 mM Tris-HCl, pH 8.0, 0.1% β-mercaptoethanol, 0.5% Triton X-100, 1x Complete Protease Inhibitor (Roche)] and the lysates were equalized with HEPES, pH 7.2 and EDTA to a final concentration of 10 mM and 1 mM, respectively. The lysates was separated on a 20% Ficoll cushion (20% w/v Ficoll 400,000, 10 mM HEPES, pH 7.2, 1 mM EDTA, 0.1% Triton X-100) by centrifugation for 35 min at 25,500 g at 4°C. Centrosomal fraction was collected from the interface.

siRNA transfection

mESCs were transfected with 70 nM Stealth Select RNAi for mouse CDK2 (5'- CCCUUUCUUCCAGGAUGUAACUAAA-3'; Invitrogen) or 70 nM Stealth RNAi Negative Control Duplex with Medium GC content (Invitrogen) using X-tremeGENE siRNA Transfection Reagent (Roche) as outlined in the manufacturer's instructions. The cells were analyzed 24 h after transfection.

Quantification of CDK2 protein levels

CDK2 protein levels were quantified from scanned images of Western blots using AlphaEase FC software (Alpha Innotech).

4

RESULTS

4.1 LIST OF PUBLICATIONS AND MEETING CONTRIBUTIONS

This thesis contains data that were presented in publications and on meetings listed below.

Publications

2 publications in journals with IF; 1 publication in preparation

- **Koledova, Z.,** Raskova Kafkova, L., Calabkova, L., Krystof, V., Dolezel, P., Divoky, V.: CDK2 inhibition prolongs G1 phase progression in mouse embryonic stem cells. *Stem Cells and Development*. **19**(2):181-194 February (2010). *(Appendix I)*
- **Koledova, Z.,** Raskova Kafkova, L., Krämer, A., Divoky, V.: DNA Damage-Induced Degradation of CDC25A Does Not Lead to Inhibition of CDK2 Activity in Mouse Embryonic Stem Cells. Stem Cells. January 26 (2010). [Epub ahead of print] *(Appendix II)*
- **Koledova, Z.,** Krämer, A., Raskova Kafkova, L., and Divoky, V.: Cell cycle regulation in embryonic stem cells: centrosomal decisions on self-renewal. [Manuscript in preparation] *(Appendix III)*

Meeting contributions

15 presentations/ abstracts

- Kafkova, L., **Koledova, Z.,** Divoky, V.: Cell Cycle and DNA Damage Response in Murine Embryonic Stem Cells. Research meeting of the project MSM 6198959216, March 18-20 (2006), Ostravice.
- **Koledova, Z.,** Kafkova, L., Divoky, V.: Cell Cycle and DNA-Damage Response in Murine Embryonic Stem Cells. Cancer 2006: From molecular biology to tumor-tailored therapy, August 20-24 (2006), Stará Lesná (Vysoké Tatry), Slovak Republic. (incl. abstract)
- **Koledova, Z.,** Kafkova, L., Divoky, V.: DNA-damage response in mouse embryonic stem cells. Science conference of PhD students, June 11-12 (2006), Faculty of Medicine, Palacky University, Olomouc. (incl. abstract)
- **Koledova, Z.,** Kafkova, L., Divoky, V.: Cell Cycle and DNA Damage Response in Mouse Embryonic Stem Cells. Seminar, December 15-16 (2006), Olomouc.

- **Koledova, Z.,** Kafkova, L., Hennemann, S., Krämer, A., Divoky, V.: Molekulárny podklad nefunkčnosti G1 kontrolného bodu u myších embryonálnych kmeňových buniek. Student science conference, April 18 (2007), Commenius University in Bratislava, Slovak Republic. (incl. abstract)

- **Koledova, Z.,** Divoky, V.: DNA damage response: antitumor barrier versus imortalization of embryonic stem cells. Biotechnics, May 24 (2007), Prague.

- **Koledova, Z.,** Kafkova, L., Hennemann, S., Krämer, A., Divoky, V.: Molekulárny podklad nefunkčnosti G1 kontrolného bodu u myších embryonálnych kmeňových buniek. Science conference of PhD students, September 10-11 (2007), Faculty of Medicine, Palacky University, Olomouc. (incl. abstract) *Best oral presentation award.*

- **Koledova, Z.,** Kafkova, L., Hennemann, S., Krämer, A., Divoky, V.: CDK2 kinase activity is not abrogated after DNA damage in mouse embryonic stem cells. New Frontiers in the Research of PhD Students, November 29 - December 1 (2007), Hradec Králové. (incl. abstract) 3rd *place in Best Oral Presentation Competition.*

- **Koledova, Z.,** Kafkova, L., Hennemann, S., Krämer, A., Divoky, V.: CDK2 Kinase Activity Is Not Abrogated after DNA Damage in Mouse Embryonic Stem Cells. 49th Annual Meeting of American Society of Hematology, December 8-11 (2007), Atlanta (Georgia), USA.

  - abstract in Blood (ASH Annual Meeting Abstracts), November 2007; 110:3371.

- **Koledova, Z.,** Raskova Kafkova, L., Calabkova, L., Krämer, A., Divoky, V.: Štúdium úlohy cyklín-dependentnej kinázy 2 (CDK2) v bunkovom cykle myších embryonálnych buniek. Student science conference, April 23 (2008), Commenius University in Bratislava, Slovak Republic. (incl. abstract)

- **Koledova, Z.,** Raskova Kafkova, L., Calabkova, L., Krämer, A., Divoky, V.: G1 checkpoint pathways are activated but CDK2 activity is not abrogated after DNA damage in mouse embryonic stem cells. XXII. Olomouc hematology days, May 28-31 (2008), Olomouc. (incl. abstract)

- **Koledova, Z.,** Raskova Kafkova, L., Calabkova, L., Krystof, V., Divoky, V.: Cyclin-dependent kinase 2 activity regulates G1 phase duration in mouse embryonic stem cells. Conference of youths, XIII. Work meeting of biochemists and molecular biologists, April 14-15

(2009), Commenius University in Brno. (incl. abstract) 3rd *place in Best Oral Presentation Competition.*

- **Koledova, Z.,** Raskova Kafkova, L., Krämer, A., Divoky, V.: DNA dama-ge-induced CDC25A degradation in mouse embryonic stem cells is regulated by glycogen synthase kinase-3β (GSK-3β). XXIII. Olomouc hematology days, June 24-27 (2009), Olomouc. (incl. abstract)

- **Koledova, Z.,** Raskova Kafkova, L., Calabkova, L., Krystof, V., Divoky, V.: Inhibícia CDK2 kinázy indukuje diferenciáciu myších embryonálnych kmeňových buniek. Science conference of PhD students, September 8-9 (2009), Faculty of Medicine, Palacky University, Olomouc. (incl. abstract) *Best oral presentation award.*

- **Koledova, Z.,** Raskova Kafkova, L., Calabkova, L., Krystof, V., Divoky, V.: CDK2 inhibition prolongs G1 phase progression in mouse embryonic stem cells. Stem Cells 2009, November 19-22 (2009), Antigua, Antigua and Barbuda. (incl. abstract)

4.2 MECHANISMS OF G1 CHECKPOINT NONFUNCTIONALITY IN MESCS

4.2.1 *After DNA damage, mESCs do not arrest in G1 phase*

To study G1 checkpoint response in mESCs, whose typical cell cycle profile consists of about 15-25% cells in G1 phase, 60-70% cells in S phase and 15-25% cells in G2/M phase, it was necessary to increase the proportion of cells in G1 phase. We achieved this goal by synchronization of mESCs with nocodazole treatment in G2/M and subsequent release of mESCs from the block to proceed to G1 phase. Based on cell cycle profile analysis by flow cytometry (Fig. 4.1 A, B) and by Western blots analysis of cyclin A level (Fig. 4.1 C), we set 1.5-h and 3-h time points after nocodazole release as the time points that yield mESCs in early and late G1 phase, respectively. NIH3T3 mouse fibroblasts, which were used as a reference cell line in our DNA-damage checkpoint studies, were treated analogously and the time points 3 h and 7 h after nocodazole release were found to yield cells in early and late G1 phase, respectively (data not shown).

Synchronization of mESCs to G1 phase

Lack of G1 arrest mESCs

The lack of a G1 arrest in studied mESC lines V6.5 and HM-1 was verified by flow cytometric analysis of mESC profiles following ionizing radiation (IR) (Fig. 4.2) and their undifferentiated status was checked by quantitative RT-PCR analysis of expression of differentiation and pluripotency markers (Fig. 4.3).

4.2.2 *CDK2 activity is not abrogated after DNA damage in mESCs*

The major mechanism of G1 arrest after DNA damage in somatic cells is the inhibition of CDK2 kinase activity. Therefore, we investigated CDK2 activity in response to DNA damage in mESCs. Both mESCs and reference NIH3T3 cells were synchronized, IR- or mock-treated in early and late G1 phase and then collected 1.5 and 3 h after IR. CDK2 activity was measured in histone H1 kinase assays. We found it to be unresponsive to DNA damage in mESCs: CDK2 activity did not significantly decrease after DNA damage in mESCs (Fig. 4.4 A, B), as it did in control NIH3T3 cells (Fig. 4.4 C). Correspondingly, pRB phosphorylation level on CDK2-specific site Ser612 did not decrease in mESCs (Fig. 4.5 A, B). Decrease of the pRB-Ser612 phosphorylation level was observed only in NIH3T3 cells (Fig. 4.5 C).

CDK2-(P)Thr14/Tyr15 is not increased after IR in mESCs

Because downregulation of CDK2 activity in response to DNA damage is (in part) regulated by increase of its inhibitory phosphorylation on Thr14/Tyr15 (mediated by degradation of CDC25A phosphatase, which removes Thr14/Tyr15 phosphorylation), we investigated the phosphory-

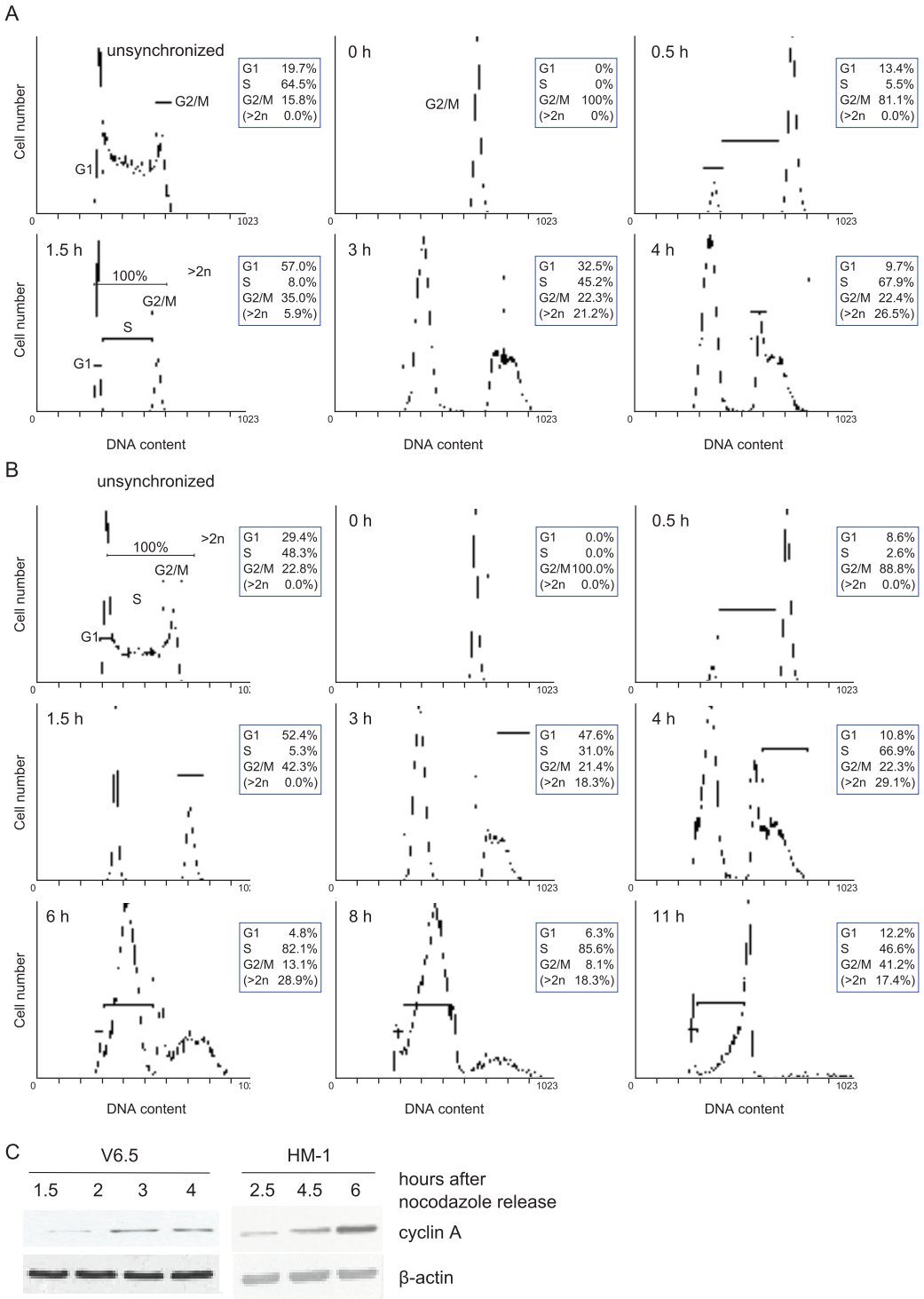

Figure 4.1: Cell cycle phase assessment in mESCs. mESCs (**A.** V6.5, **B.** HM-1) were collected at the indicated time points after release from nocodazole, and their cell cycle progression was monitored by flow cytometry (**A, B**) and Western blots (**C**).

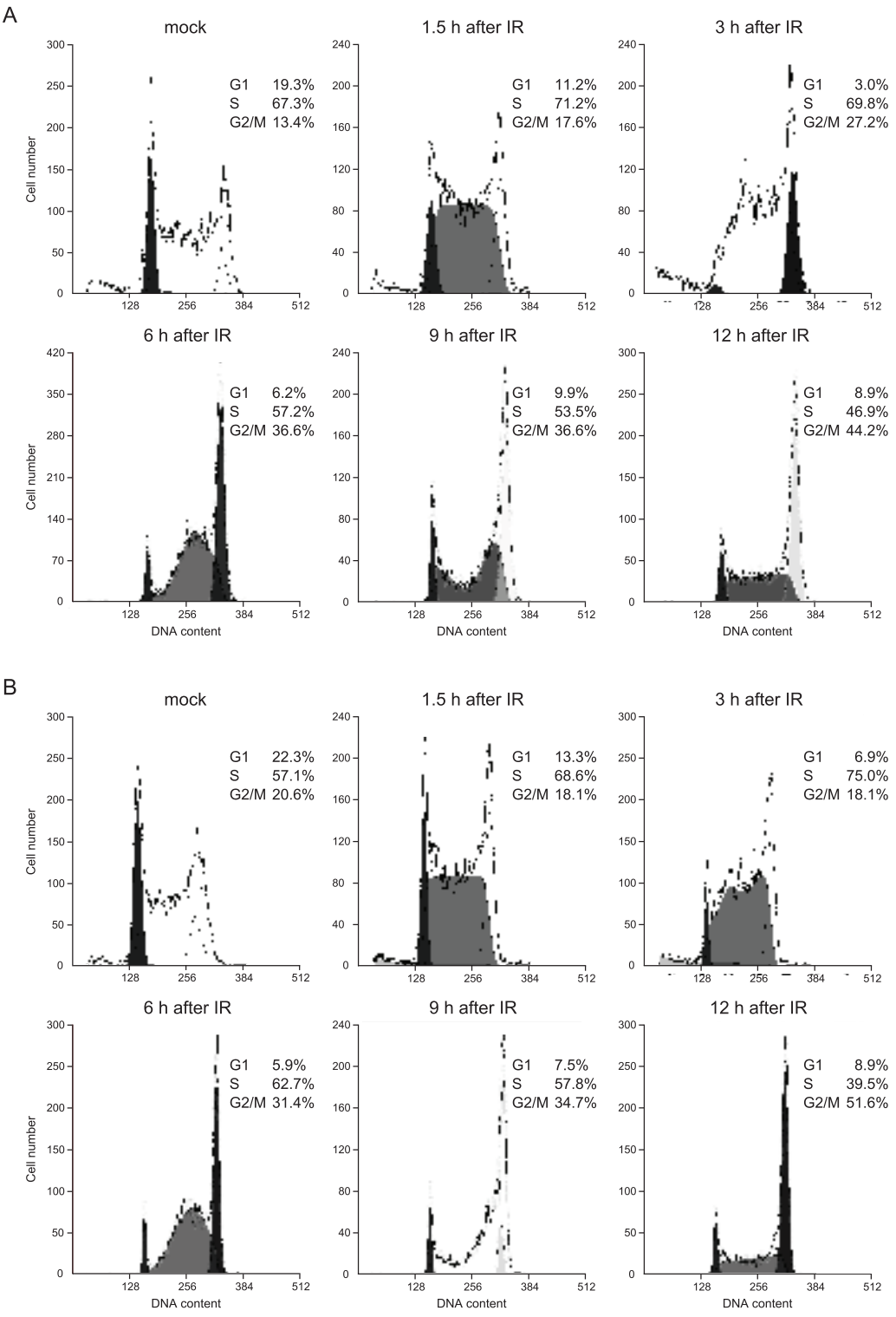

Figure 4.2: mESCs do not arrest in G1 phase after DNA damage. V6.5 (**A**) and HM-1 (**B**) cells were mock- or IR-treated and fixed 1.5, 3, 6, 9, or 12 h after IR, and their cell cycle profile was analyzed by flow cytometry.

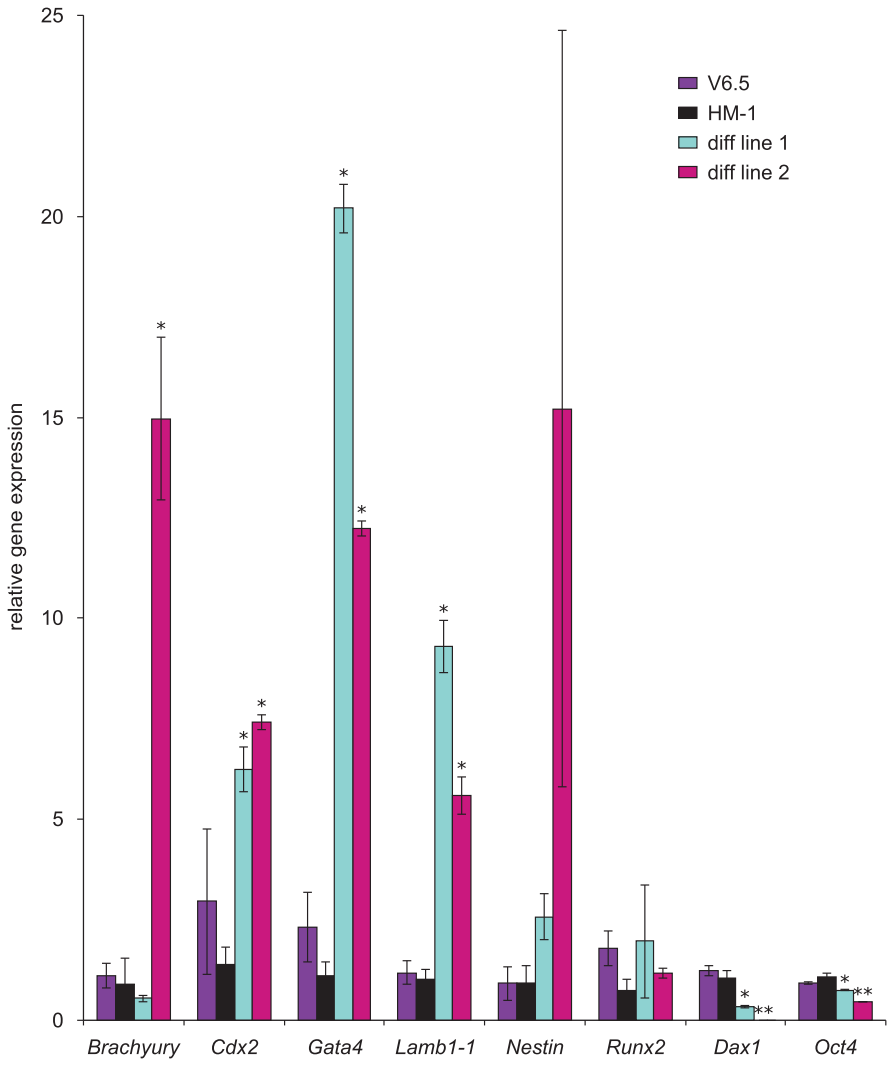

Figure 4.3: Quantitative RT-PCR analysis of differentiation and pluripotency marker expression in V6.5 and HM-1 cells. The expression analysis of differentiation (*Brachyury* to *Runx2*) and pluripotency (*Dax1, Oct4*) markers was performed to check the undifferentiated status of mESCs used in experiments (V6.5 and HM-1). Two mESC lines (derivatives of the V6.5 line: diff line 1 and diff line 2) were differentiated by prolonged cultivation of mESCs in the absence of LIF at low cell density and were used as a positive control for expression analysis of differentiation markers. The data were normalized to *Gapdh* expression and related to expression level of an early passage of undifferentiated HM-1 cells (where 1 = no change from undifferentiated HM-1 cells). The data represent the mean from three independent experiments. Bars, standard deviation (SD); *P $<$ 0.05 and **P $<$ 0.005.

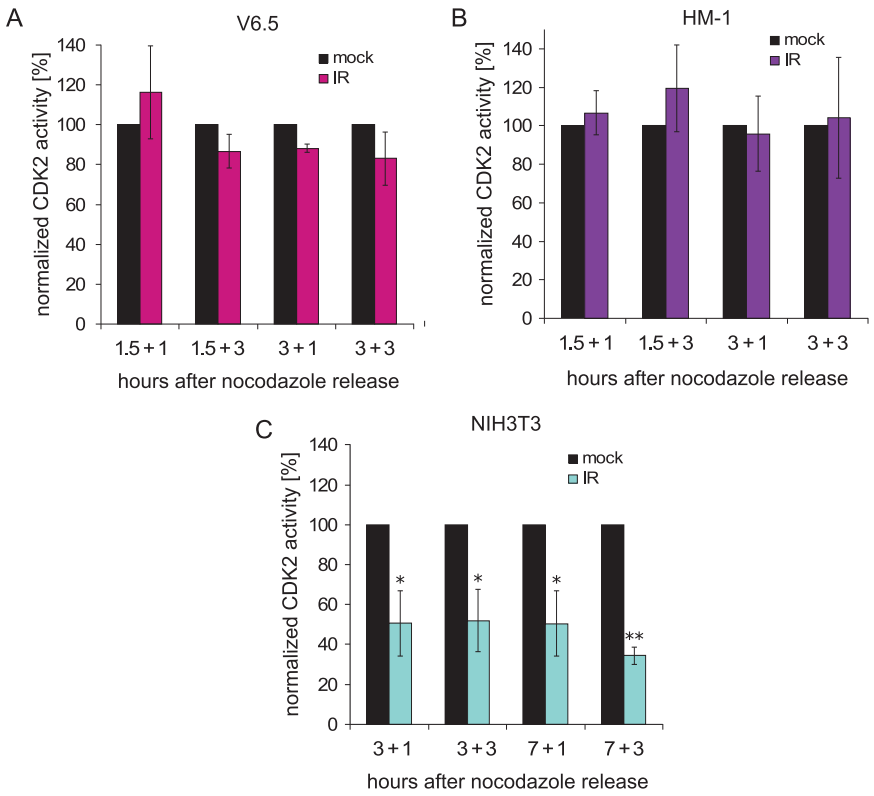

Figure 4.4: Activity of CDK2 is not decreased in response to DNA damage in mESCs. CDK2 kinase activity in synchronized V6.5 (**A**), HM-1 (**B**) and NIH3T3 (**C**) cells after mock- or IR-treatment. The data represent the mean of three independent experiments. Bars, SD; *P $<$ 0.05, **P $<$ 0.005. In V6.5 and HM-1 cells, CDK2 activity changes after IR were not statistically significant: P = 0.18, 0.06, 0.11 and 0.08 for V6.5 and P = 0.21, 0.14, 0.37 and 0.42 for HM-1 for time points 1.5+1, 1.5+3, 3+1 and 3+3, respectively. Designation of time points: time (in hours) after nocodazole release in which cells were irradiated plus time (in hours) after IR in which cells were collected for lysates.

lation level of CDK2-Thr14/Tyr15. It did not increase in mESCs (Fig. 4.6 A, B), while it did in NIH3T3 cells (Fig. 4.6 C) after IR.

4.2.3 *Downregulation of CDK2 activity slows G1 escape after DNA damage in mESCs*

Because CDK2 activity was not abrogated in response to DNA damage in mESCs, we hypothesized that high, DNA-damage unresponsive CDK2 activity might be the driving force for rapid G1 escape of mESCs in the conditions of DNA damage. To test this hypothesis, we inhibited CDK2 activity by olomoucine II, then the cells were IR- or mock-treated and

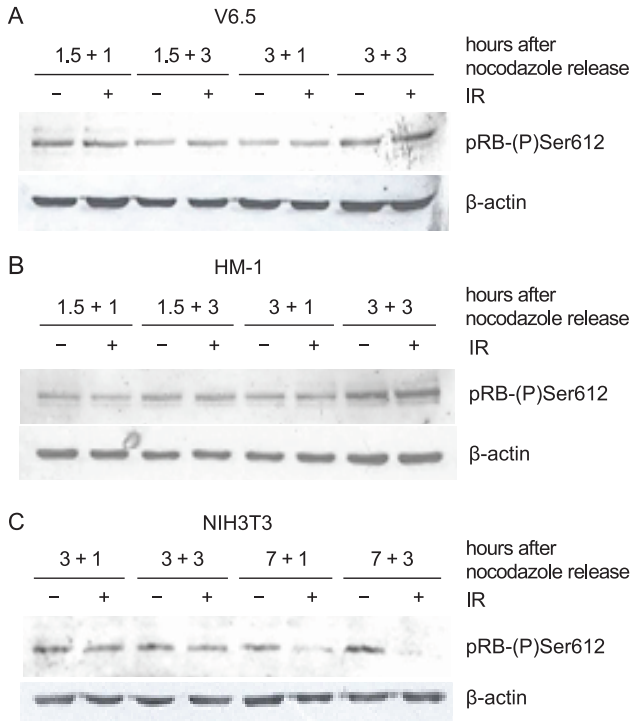

Figure 4.5: pRB phosphorylation on Ser612 does not change after DNA damage in mESCs. Time points as in Fig. 4.4.

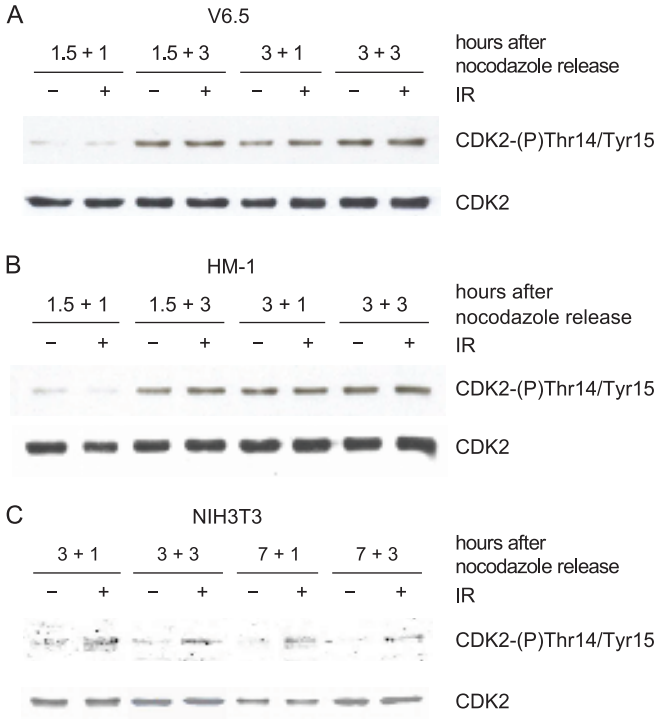

Figure 4.6: Inhibitory phosphorylation of CDK2 on Thr14/Tyr15 does not increase after DNA damage in mESCs. Time points as in Fig. 4.4.

collected 1.5 and 3 h after IR to investigate their cell cycle profiles by flow cytometry analysis. Downregulation of CDK2 activity to 50% by 5 μM olomoucine II (see page 56 and Fig. 4.21) significantly slowed the decrease of number of cells in G1 phase after IR (Fig. 4.7 A, B) and slowed the G1 escape of mESCs. This observation corroborates the central role of high and DNA-damage unimpaired CDK2 activity in the lack of G1 arrest and rapid G1 escape after DNA damage in mESCs.

CDK2 activity underpins G1 escape in mESCs

4.2.4 *G1 checkpoint pathways are activated after DNA damage in mESCs*

To uncover the mechanisms of the lack of G1 arrest in mESCs, we investigated functionality of G1 checkpoint pathways, which are known to operate in somatic cells, in mESCs. We found p53 to be activated after DNA damage by Ser18 phosphorylation in mESCs, as it is in NIH3T3 cells (Fig. 4.8). In addition, expression of *Cdkn1a* ($p21^{Cip1}$), the 53 target gene, increased on both RNA and protein levels in mESCs at comparable levels to those in NIH3T3 cells (Fig. 4.8).

$p21^{Cip1}$ is induced after IR in mESCs

Furthermore, we found Chk2-CDC25A pathway to be functional in mESCs. Chk2 became phosphorylated and CDC25A was degraded in response to DNA damage in mESCs (Fig. 4.9 A, B), resembling the checkpoint activity found in NIH3T3 cells (Fig. 4.9 C). However, unlike Chk2, Chk1 did not become phosphorylated after IR in mESCs: Chk1-Ser345 was found to be constitutively phosphorylated in mESCs and the level of this phosphorylation did not change after DNA damage (Fig. 4.9 D, E). (Data on Chk1 phosphorylation in NIH3T3 are not shown because Chk1 does not become phosphorylated in response to IR in NIH3T3 cells; instead, it is activated in response to DNA damage by UV.) Because a previous study reported Chk2-CDC25A pathway non-functionality in mESCs due to Chk2 centrosomal sequestration [395], we investigated Chk2 as well as Chk1 localization in both V6.5 and HM-1 mESCs. We found both Chk1 and Chk2 to be localized to centrosomes in mESCs (Fig. 4.10).

CDC25A is degraded after IR in mESCs

Chk1/Chk2 are sequestered at centrosomes

4.2.5 *In mESCs, IR-induced CDC25A degradation is regulated by GSK-3β*

Our observation of centrosomal localization of Chk1 and Chk2 made us question their role in degradation of CDC25A after IR in mESCs. To shed more light on this issue, we investigated CDC25A degradation in conditions of both Chk1 and Chk2 activity inhibition (by CEP3891 and Chk2 inhibitor II, respectively; Fig. 4.11 A, B). In NIH3T3 cells Chk1/Chk2 inhibition abrogated CDC25A degradation after IR, as expected (Fig. 4.11 C). However, IR-induced CDC25A degradation was unresponsive to Chk1/Chk2 inhibition in mESCs (Fig. 4.11 C). This observation sug-

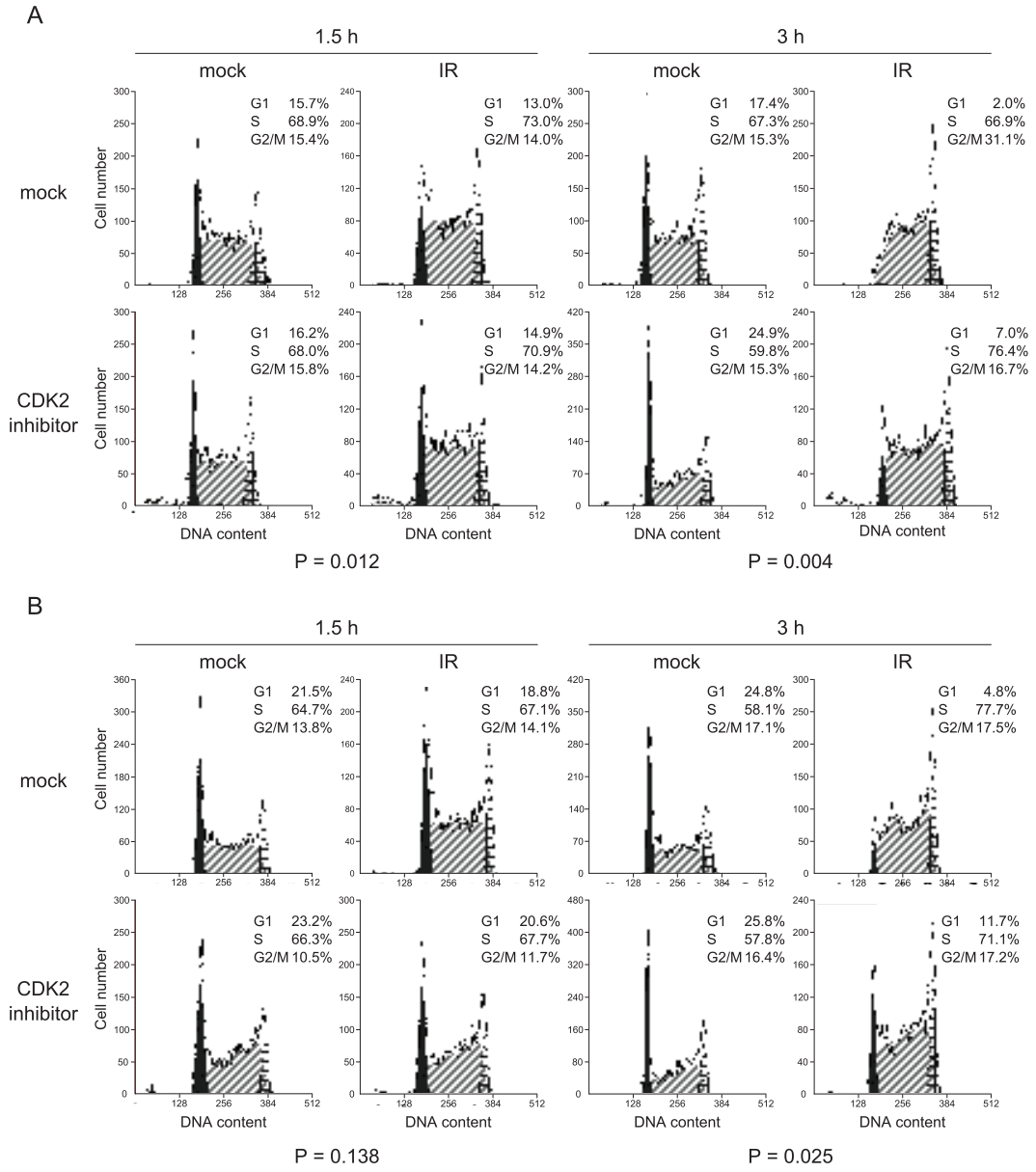

Figure 4.7: Downregulation of CDK2 activity slows G1 escape after DNA damage in mESCs. V6.5 (**A**) and HM-1 (**B**) cells were mock-treated or treated for 1 h with 5 μM olomoucine II, they were then IR- or mock-treated, collected 1.5 h or 3 h after IR and their cell cycle profiles were analyzed by flow cytometry. The data represent three independent experiments. The P value expresses the statistical significance of differences in the number of cells in G1 after IR in CDK2 inhibitor-treated cells compared to mock-treated cells.

gested CDC25A is excluded from regulation by Chk1 and Chk2 after IR in mESCs. We investigated CDC25A localization and found it to be

localized to the nucleus and cytoplasm, but not to centrosomes, in both mESCs and NIH3T3 cells (Fig. 4.12).

In search for IR-induced CDC25A-degradation regulatory molecule we tested GSK-3β, which was shown to target CDC25A for degradation in G1 phase (during unperturbed cell cycle) in somatic cells [409]. We used LiCl and 6-bromoindirubin-3'-acetoxime (BIOac) to inhibit GSK-3β activity and

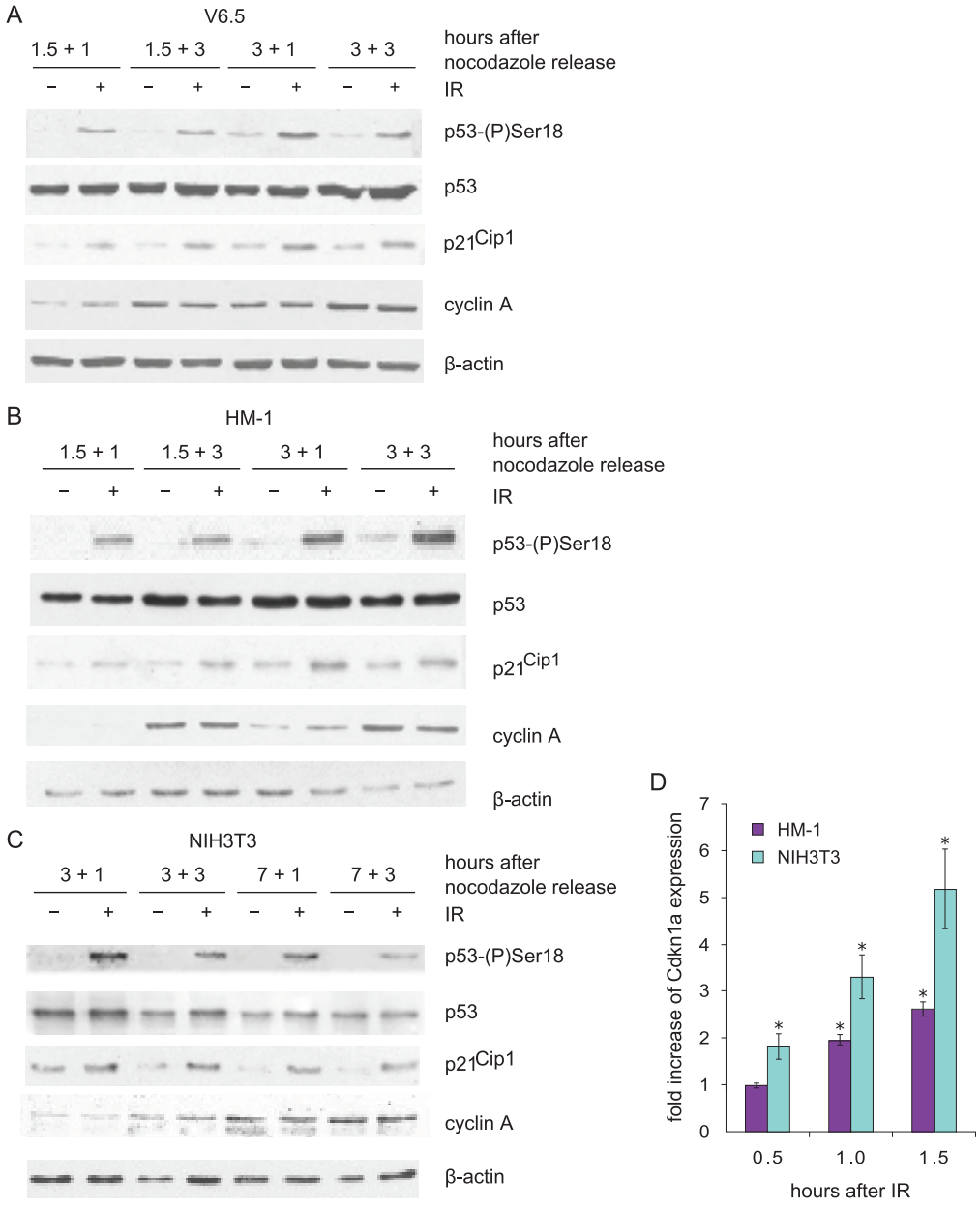

Figure 4.8: Characterization of the p53-p21^{Cip1} pathway in mESCs (**A, B**) and in somatic cells (**C**). **D.** Relative expression of *Cdkn1a* after IR in HM-1 and NIH3T3 cells (normalized to *Hmbs* expression, and plotted as a fold increase of expression after IR relative to mock-treated control). Bars, SD; *P $<$ 0.05.

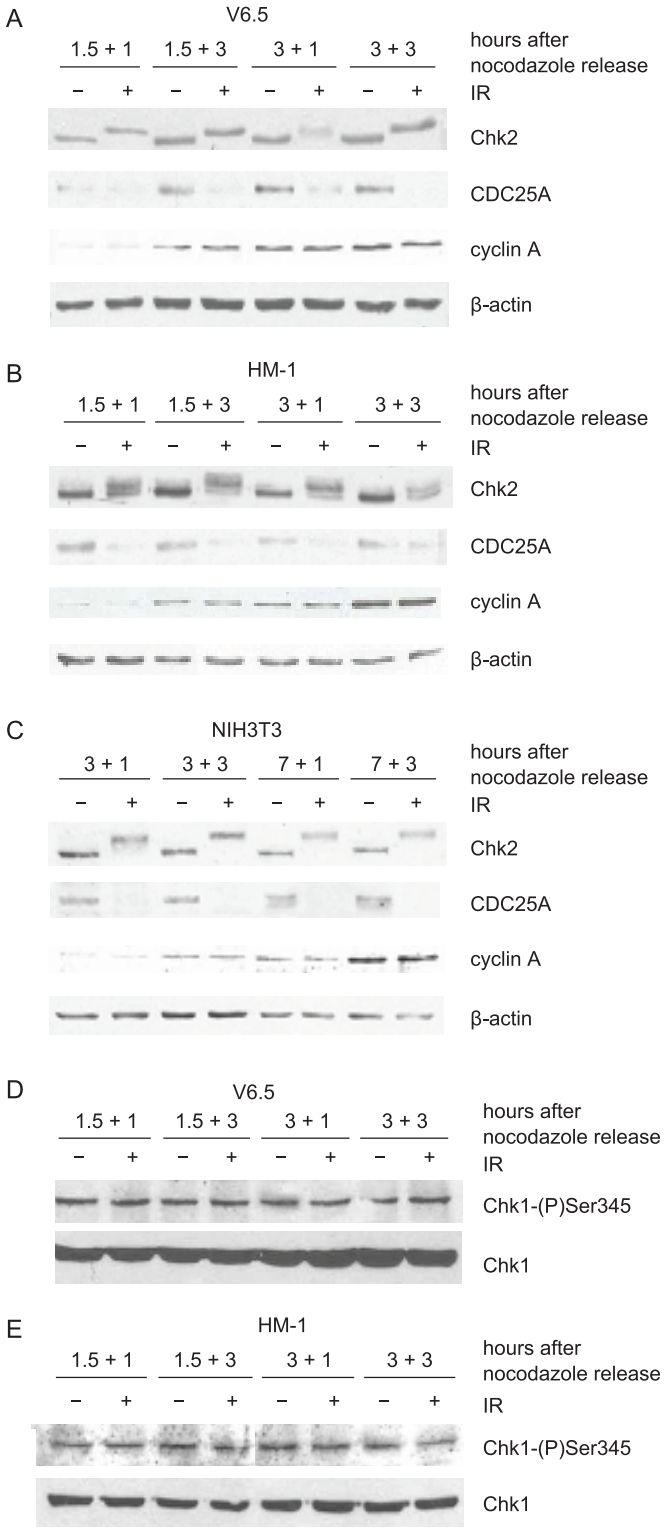

Figure 4.9: Characterization of the Chk1/Chk2-CDC25A pathway. **A-C.** The phosphorylation status of Chk2 was detected by a mobility shift of the Chk2-specific band [405–408]. **D, E.** Chk1 is constitutively phosphorylated in mESCs. Time points as in Fig. 4.4.

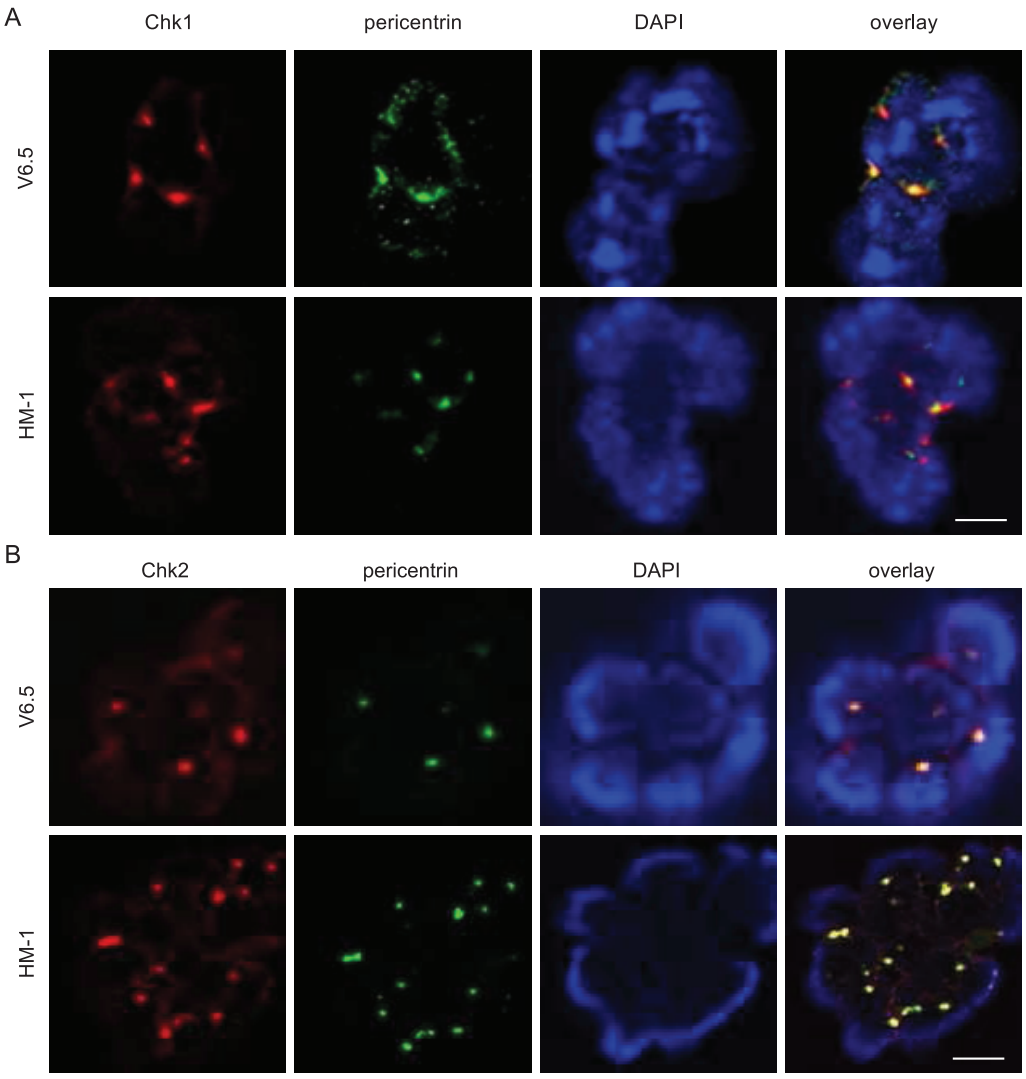

Figure 4.10: Chk1 (**A**) and Chk2 (**B**) entirely localize to centrosomes in mESCs. mESCs cells were depleted of soluble proteins with PHEM treatment and fixed with methanol. Pericentrin was used as a centrosome marker. Nuclei were labeled with DAPI. Scale bar, 5 μm.

A novel role for GSK-3β in mESCs

we investigated CDC25A degradation under these conditions after DNA damage in both mESCs and NIH3T3 cells. The effectiveness of GSK-3β inhibition in our experiments was controlled by monitoring the increase of negative regulatory phosphorylation of GSK-3β at Ser9 and/or increase of β-catenin levels; β-catenin is targeted for degradation by active GSK-3β (Fig. 4.13 A, B). Accumulation of β-catenin corresponded to increased expression of its target genes *Lef1* [153, 154] and *Axin2* [149, 151], while expression of *Axin1*, an *Axin2* homolog insensitive to β-catenin, did not change (Fig. 4.13 C). Inhibition of GSK-3β activity abrogated IR-induced CDC25A degradation in mESCs (Fig. 4.13 A, B). In NIH3T3 fibroblasts,

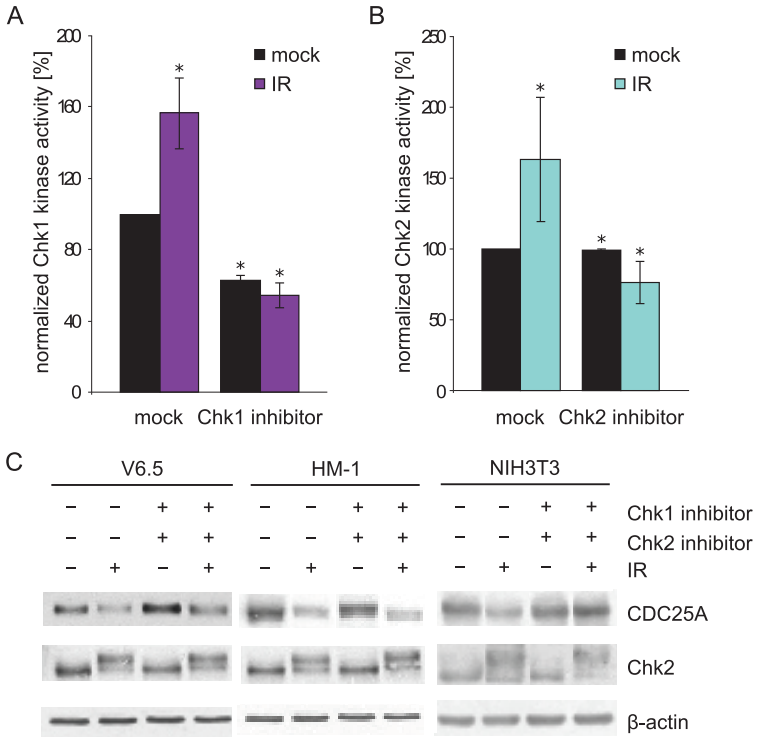

Figure 4.11: CDC25A degradation after DNA damage is not regulated by Chk1/Chk2 in mESCs. **A, B.** Inhibition of Chk1/Chk2 kinase activity with inhibitors CEP3891 and Chk2 inhibitor II was measured in Chk1 (**A**) and Chk2 (**B**) kinase assays, respectively. Bars, SD; *P $<$ 0.05. **C.** Regulation of CDC25A level in response to IR and/or Chk inhibitors. V6.5 and NIH3T3 cells were mock-treated (DMSO) or treated with CEP3891 and Chk2 inhibitor II for 30 min, IR- or mock-treated and collected 1 h after IR.

GSK-3β inhibition had only little impact on CDC25A degradation after IR (Fig. 4.13 A) because in these cells Chk1/Chk2 activity towards CDC25A is intact. Immunolocalization studies of GSK-3β in mESCs revealed that it is localized to the cytoplasm (Fig. 4.14), suggesting involvement of GSK-3β in regulation of cytoplasmic CDC25A level.

4.2.6 *CDK2 is localized predominantly to cytoplasm and centrosomes in mESCs*

Having uncovered the localization discrepancy between Chk1/Chk2 and CDC25A and its consequences on their interaction, we hypothesized that aberrant CDK2 localization in mESCs might be the cause for the observed unresponsiveness of CDK2 activity to CDC25A degradation after IR. Therefore, we investigated the localization of CDK2 in both mESCs and

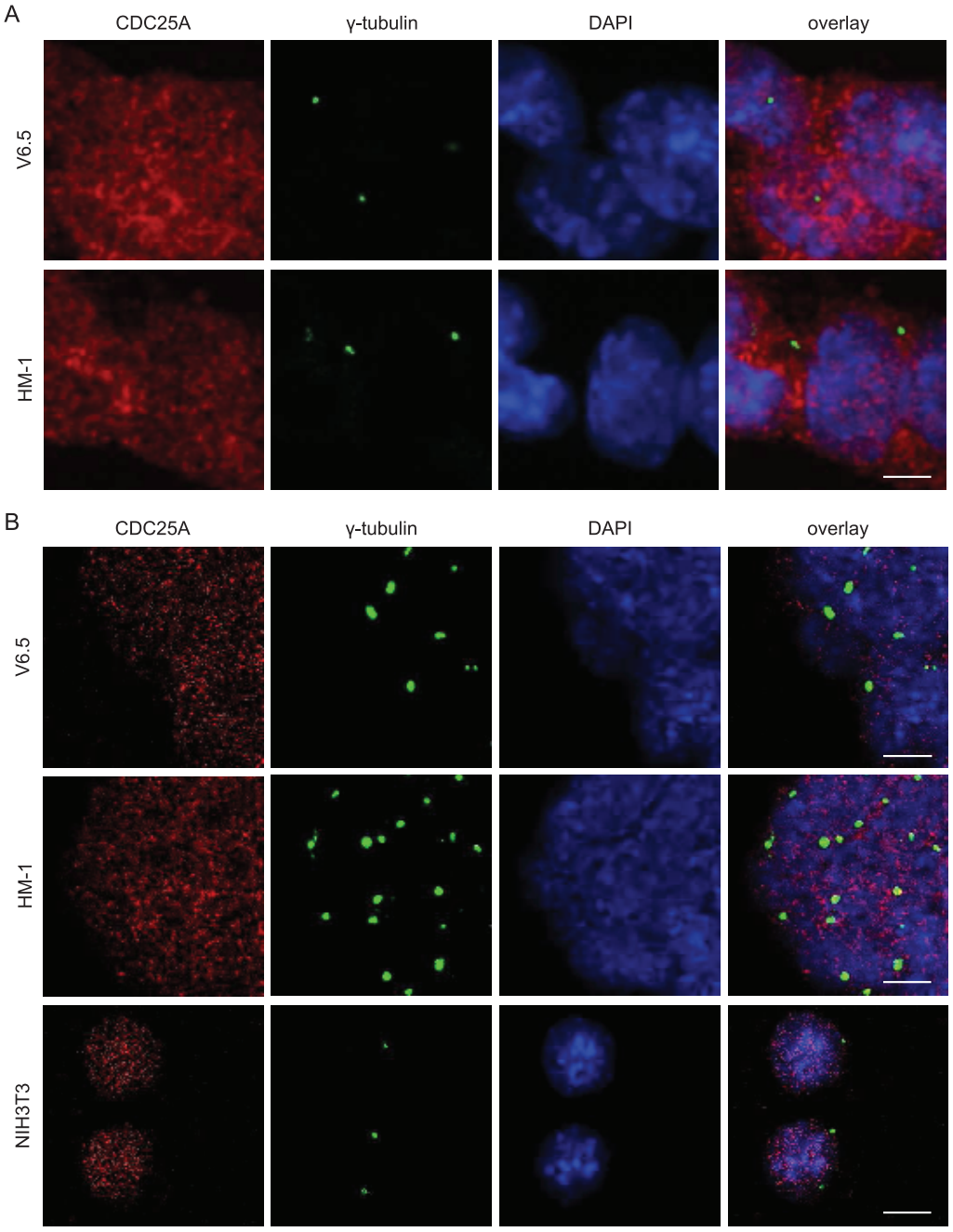

Figure 4.12: CDC25A localizes to the cytoplasm and the nucleus in mESCs. Cells were fixed with methanol-acetone (**A**) or, after extraction of soluble proteins, methanol (**B**). γ-tubulin was used as a centrosome marker. Nuclei were labeled with DAPI. Scale bar, 5 μm.

CDK2 is localized at centrosomes in mESCs

NIH3T3 cells by immunostaining. In NIH3T3 cells, CDK2 was found to be localized to the nucleus, cytoplasm and partially to centrosomes (Fig. 4.15). In mESCs, CDK2 localized predominantly to the cytoplasm and centrosomes (Fig. 4.15 and Fig. 4.16). These observations suggest that

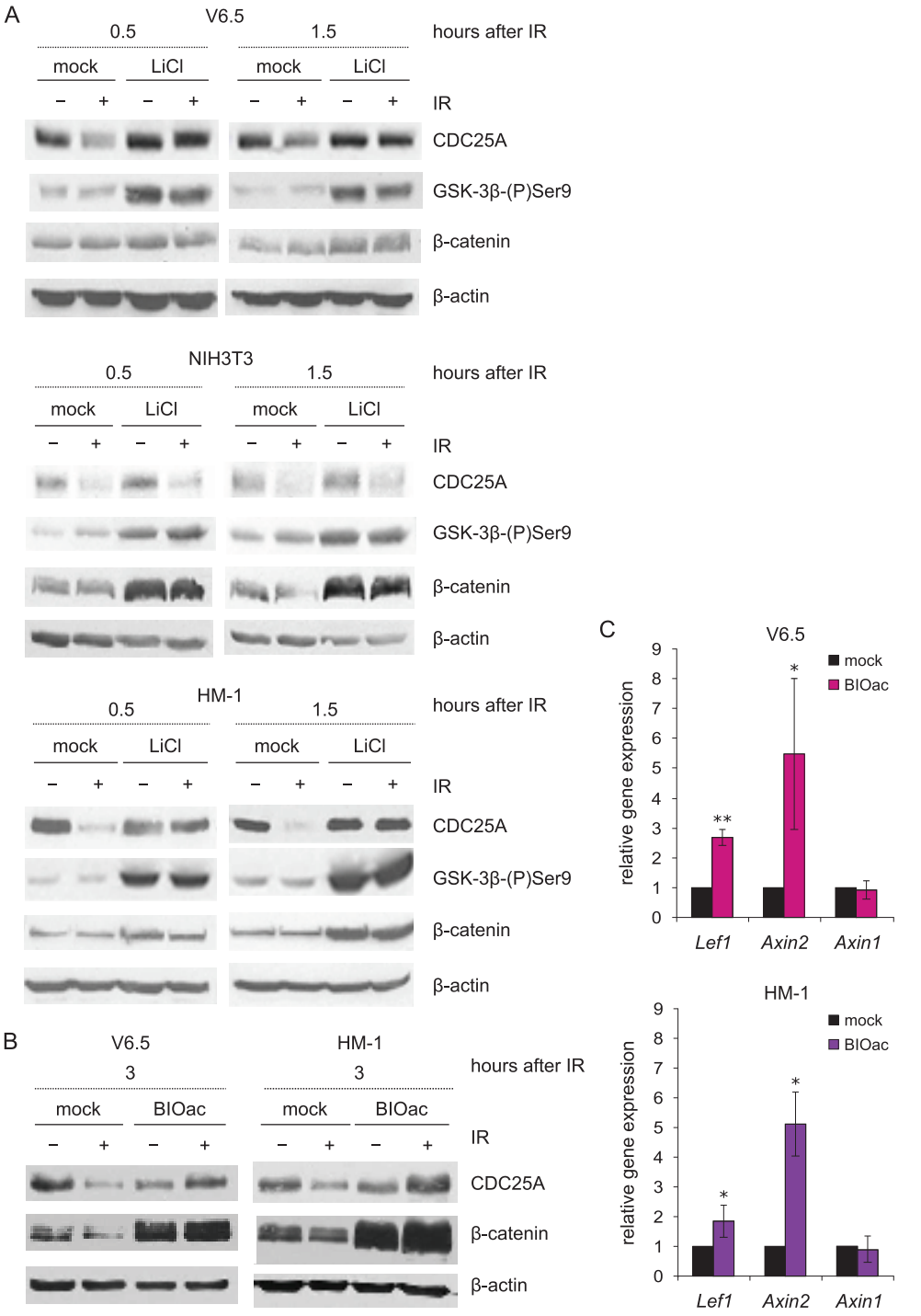

Figure 4.13: CDC25A degradation is regulated by GSK-3β in mESCs. Cells were mock- or GSK-3β inhibitor-treated (**A,** LiCl; **B,** 6-bromoindirubin-3′-acetoxime, BIOac) for 2.5 h. Then, the cells were IR- or mock-treated and lysed either 0.5, 1.5 or 3 h after IR. **C.** Relative expression of β-catenin target genes after BIOac treatment (normalized *Hmbs* expression). The data represent the mean from three independent experiments. Bars, SD; *P $<$ 0.05.

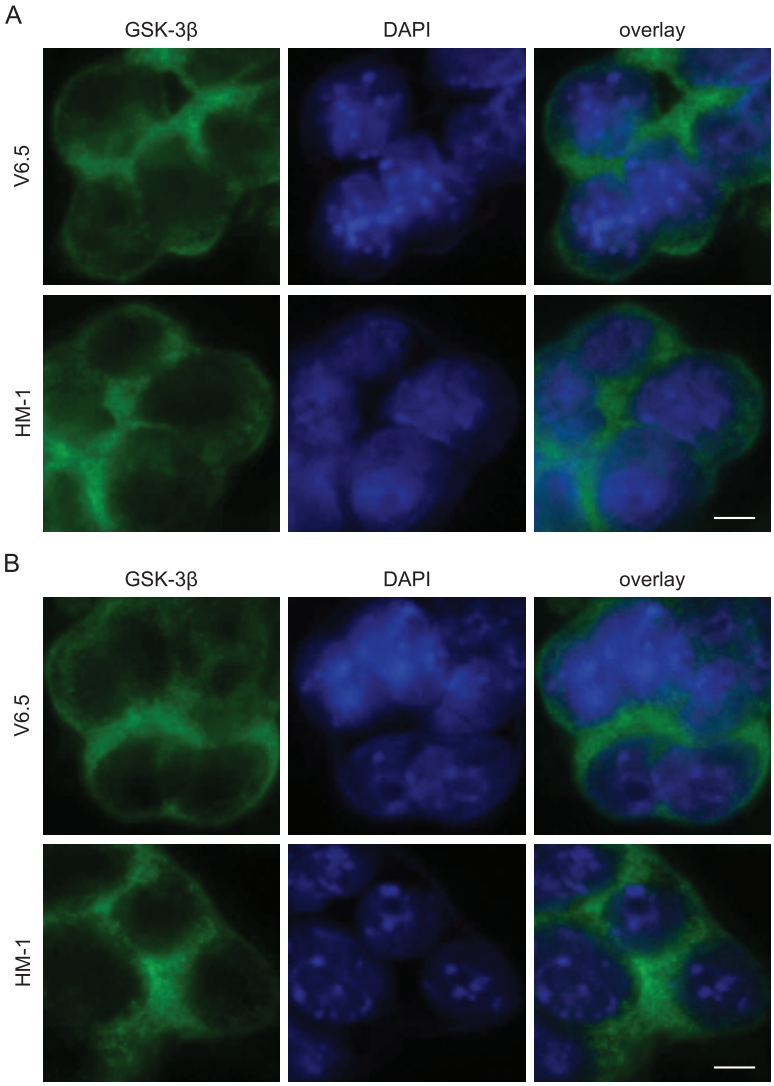

Figure 4.14: GSK-3β is localized to the cytoplasm in mESCs and its localization does not change after DNA damage. mESCs were mock-(**A**) or IR-treated (**B**) and fixed with methanol-acetone. Nuclei were labeled with DAPI. Scale bar, 5 μm.

due to its centrosomal localization and apparent absence from the nuclei, CDK2 might be excluded from the regulation by CDC25A in response to DNA damage in mESCs.

To support our hypothesis of a lack of communication between centrosomal CDK2 and cytoplasmic and/or nuclear CDC25A, we tried to produce centrosomal extracts from mESCs following the strategy for adherent osteosarcoma cells (U2OS) [410]. However, this protocol appeared not to be suitable for separation of centrosomes from mESCs as the procedure did not result in the fractionation of significant amounts of centrosomes as detected by Western blots using antibodies to centrosomal markers including γ-tubulin, Nek2, pericentrin, and c-Nap1 (data not

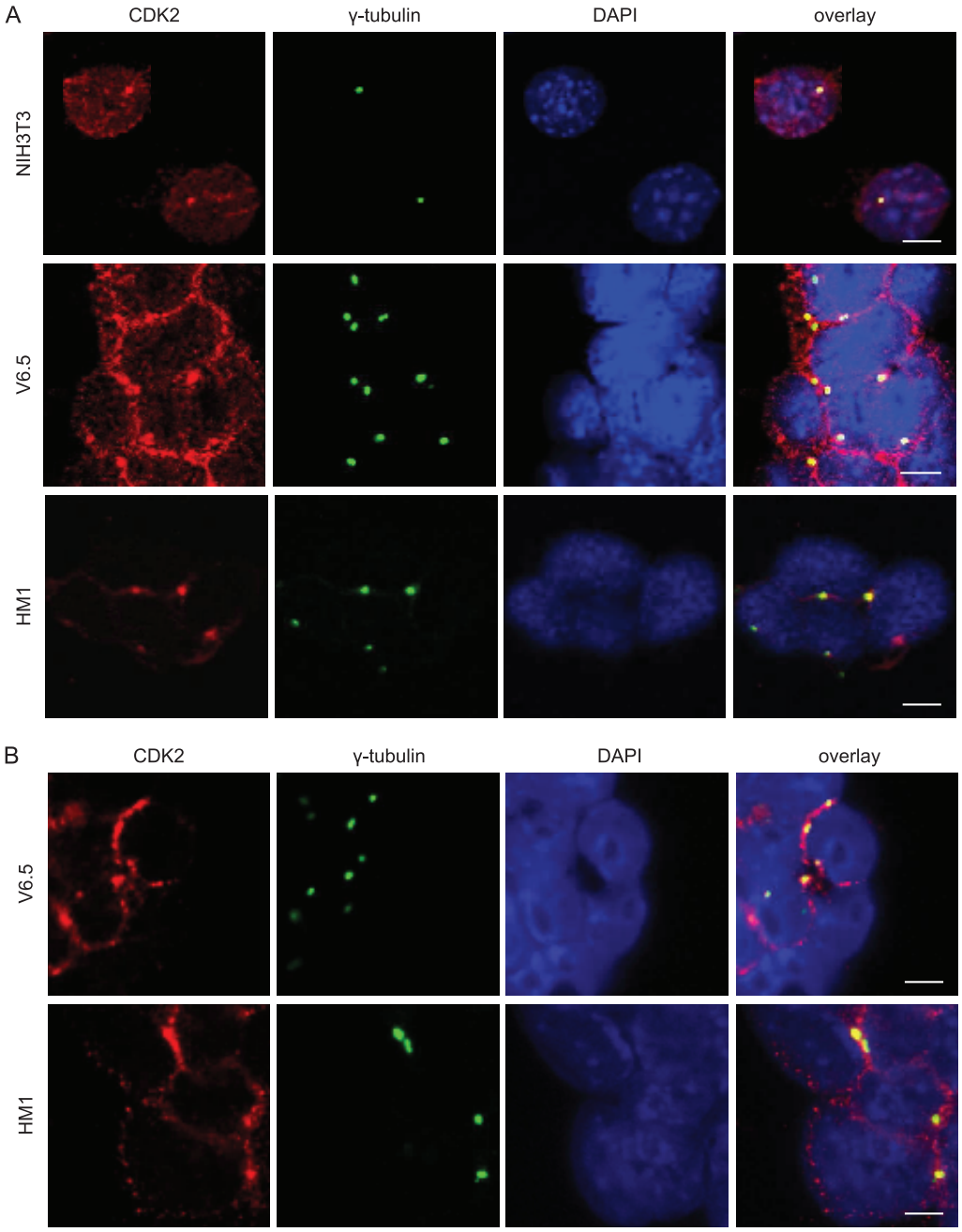

Figure 4.15: CDK2 is localized predominantly to centrosomes and cytoplasm in mESCs. **A, B.** Immunolocalization of CDK2 in mock- (**A**) or IR-treated (**B**) methanol-fixed cells after extraction of the soluble protein pool. Cytoplasmic remnants can be seen in V6.5 and HM-1 cells because the soluble proteins could not be completely removed because of the tight growth of mESCs in colonies. γ-tubulin was used as a centrosome marker, nuclei were labeled with DAPI. Scale bar, 5 μm.

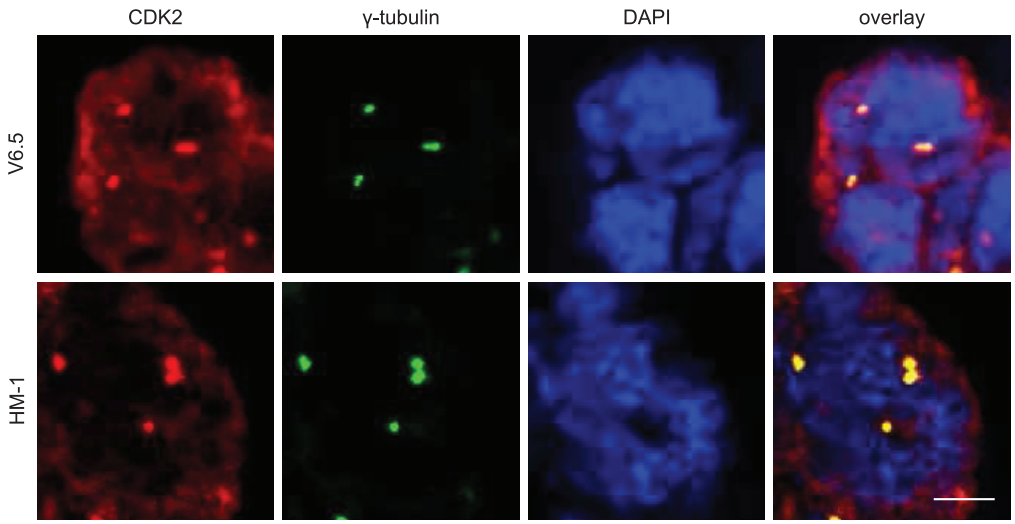

Figure 4.16: CDK2 is localized predominantly to centrosomes and cytoplasm in mESCs. Immunolocalization of CDK2 in mock-treated mESCs, fixed with methanol-acetone and stained for CDK2. γ-tubulin was used as a centrosome marker. Nuclei were labeled with DAPI. Scale bar, 5 μm.

shown). Nevertheless, our study proposes a new model for G1 checkpoint non-functionality in mESCs based on aberrant protein localization with a central role for centrosomes, which might shelter CDK2 from regulation by activated G1 checkpoint pathways.

4.3 ROLE OF CDK2 IN REGULATION OF G1 PHASE IN MESCS

4.3.1 *CDK inhibitor treatment induced significant cell cycle changes in mESCs*

To investigate the role of CDK2 in G1 phase regulation in mESCs, we applied a chemical inhibition approach, based on the use of CDK inhibitors. We used olomoucine II, a potent inhibitor of both CDK2 and CDK9 [411], and CAN508, a selective CDK9 inhibitor [402], to discern the effects of CDK2 inhibition from those of CDK9 inhibition. First, we investigated cytotoxicity of these drugs in studied mESC lines V6.5 and HM-1 as well as in the reference cell line HT29 (Tab. 4.1). Then the cells were treated with olomoucine II or CAN508 (at concentration corresponding to IC_{50}) for times corresponding to, approximately, duration of one cell cycle (11 h for mESCs, 24 h in HT29 cells) and their cell cycle profiles were analyzed by flow cytometry. In HT29 cells, olomoucine II treatment led to a statistically significant increase in G1 phase cell number ($P = 0.003$), while treatment with CAN508 significantly decreased number of cells in G1 phase ($P = 0.009$) and significantly increased number of

cells in S phase (P = 0.02) (Fig. 4.17). Neither olomoucine II, nor CAN508 treatment caused inhibition of DNA replication (Fig. 4.17).

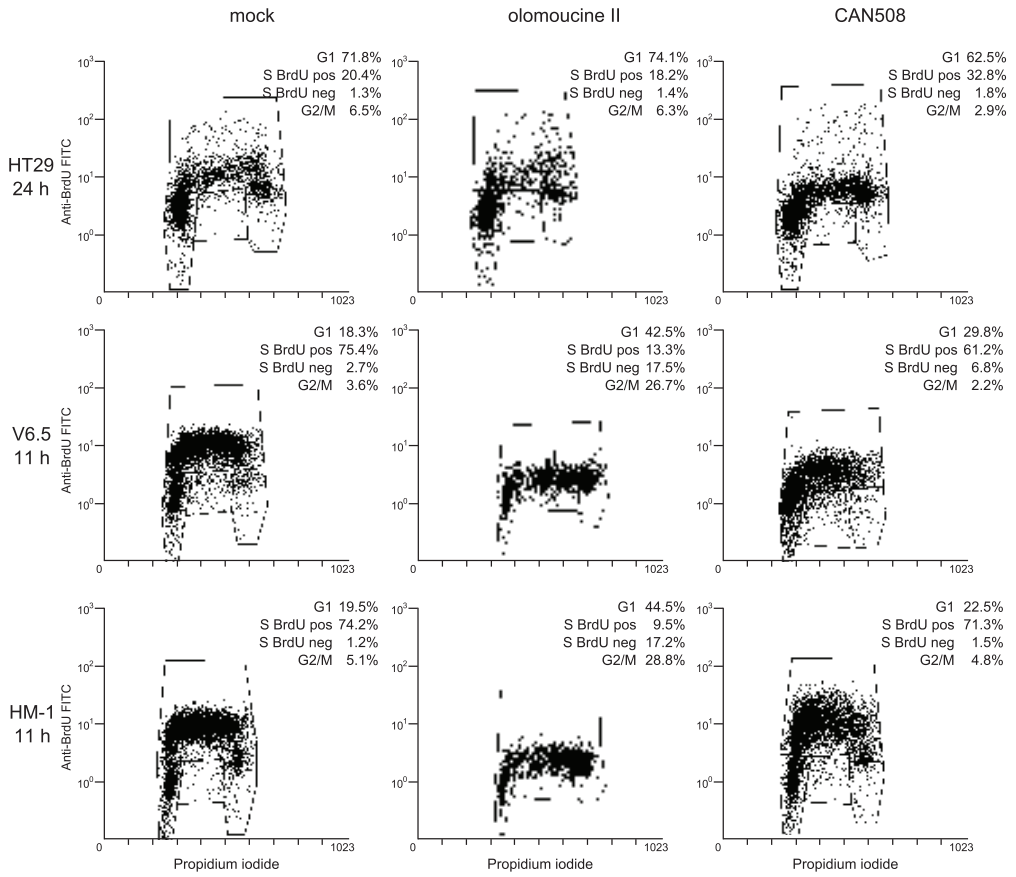

Figure 4.17: Changes of cell cycle profiles after CDK-inhibitor treatment. The cells were treated with inhibitors (at the concentrations corresponding to their IC_{50}s) or mock-treated for the indicated times and their cell cycle profile was analyzed by flow cytometry. The figures are representatives of three independent experiments.

Table 4.1: IC_{50} values for olomoucine II and CAN508. Cytotoxicity of olomoucine II and CAN508 was measured in MTT assays and IC_{50} values were calculated from dose-response curves. The results are means ± SD of at least three experiments.

	olomoucine II [µM]	CAN508 [µM]
V6.5	9.0 ± 0.9	129.0 ± 24.6
HM-1	6.3 ± 0.7	68.1 ± 5.6
HT29	10.7 ± 0.7	54.4 ± 8.2

Olomoucine II induced major changes in ESC cycle profile

mESCs of both cell lines responded to the inhibitor treatment similarly to each other, but differently from HT29 cells. Treatment with olomoucine II caused a significant increase in G1 phase cell number (P = 0.01 and 0.04 for V6.5 and HM-1 cells, respectively) and a significant decrease in S phase cell number (P = 0.03 and 0.05 for V6.5 and HM-1 cells, respectively) in both mESC lines (Fig. 4.17). Moreover, olomoucine II treatment led to a significant inhibition of DNA replication (*i.e.* increase in BrdU negative cell number) (P = 0.02 and 0.01 for V6.5 and HM-1 cells, respectively) in mESCs (Fig. 4.17). CAN508 treatment had similar, but less prominent effects on mESCs: G1 phase cell numbers increased (P = 0.006 for V6.5 and P = 0.37 for HM-1), S phase cell numbers decreased (P = 0.03 for V6.5 and P = 0.06 for HM-1) and the number of BrdU negative cells increased (P = 0.18 and 0.15 for HM-1 and V6.5, respectively) (Fig. 4.17).

4.3.2 *Effects of CAN 508 and olomoucine II on CDK9, CDK2 and CDK1 activities in mESCs*

Next, we investigated the effect of the inhibitors on CDK9, CDK2 and CDK1 activities in mESCs. The effectiveness of CDK9 inhibition by CAN508 or olomoucine II was checked in Western blots for RNA polymerase II (RNA pol II) phosphorylation in the C-terminal domain on Ser2, a CDK9 specific target site. We found RNA pol II-Ser2 phosphorylation (*i.e.* CDK9 activity) to be inhibited by 100 μM CAN508 and 2.5-5 μM olomoucine II (Fig. 4.18 A, B). Effect of olomoucine II on CDK2 and CDK1 activities was measured in histone H1 kinase assays, which showed that 5 μM olomoucine II significantly decreased CDK2 activity (P = 0.002), while CDK1 activity is not significantly decreased up to 10 μM olomoucine II (P = 0.15) (Fig. 4.18 C). These observations suggest that CDK inhibitors olomoucine II and CAN508 have the same targets in mESCs and in somatic cells.

5 μM olomoucine II specifically inhibits CDK2

4.3.3 *Olomoucine II treatment prolongs G1 phase in mESCs*

From kinase assays we concluded that 5 μM is the upper concentration limit of olomoucine II that effectively inhibits CDK2 activity while CDK1 activity remains intact. In view of this observation, we used 1 to 5 μM olomoucine II to investigate the effects of specific downregulation of CDK2 activity on cell cycle in mESCs. To follow the immediate effects of CDK2 inhibition on cell cycle progression, the cells were collected after 6-h treatment for cell cycle analysis by flow cytometry. Olomoucine II treatment significantly increased number of cells in G1 phase (P values for 5 μM olomoucine II; P = 0.01 for V6.5 and P = 0.04 for HM-1 cells), decreased S phase cell numbers (P = 0.03 and 0.04 for V6.5 and HM-1

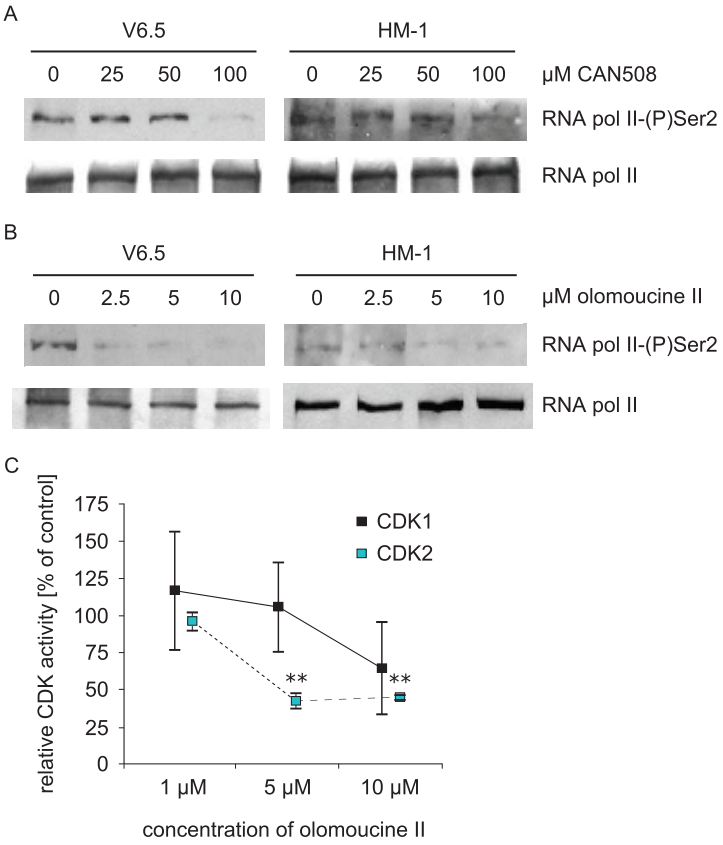

Figure 4.18: Inhibition of CDK9, CDK2 and CDK1 activity by CAN508 or olomoucine II. Phosphorylation of the C-terminal domain of RNA polymerase II is abolished by CAN508 (**A**) and olomoucine II (**B**) treatment. Cells were treated for 11 h with indicated concentration of CDK inhibitors. **C.** Inhibition of CDK1 and CDK2 activity by olomoucine II treatment. After 1-h treatment of V6.5 cells with designated concentrations of olomoucine II, CDK1 or CDK2 activity was measured in histone H1 kinase assays. The results were normalized to control (mock-treated cells). The data are the means of two independent experiments. Bars, 95% confidence interval; $^{**}P < 0.005$.

mESCs, respectively) and inhibited DNA replication (P = 0.03 for V6.5 and 0.01 for HM1 cells) (Fig. 4.19). Prolongation of olomoucine II treatment to 11 h led to cell cycle changes comparable to those after 6-h treatment (Fig. 4.20). mESC treatment with olomoucine II for longer periods of time (up to 96 h) sustained increased G1 phase cell numbers; however, a massive cell death could be observed (data not shown).

The observed increase in proportion of cells in G1 phase after CDK2 inhibition by olomoucine II could be the result of either G1 phase prolongation, or arrest in G1 or G1/S phase. To distinguish between these

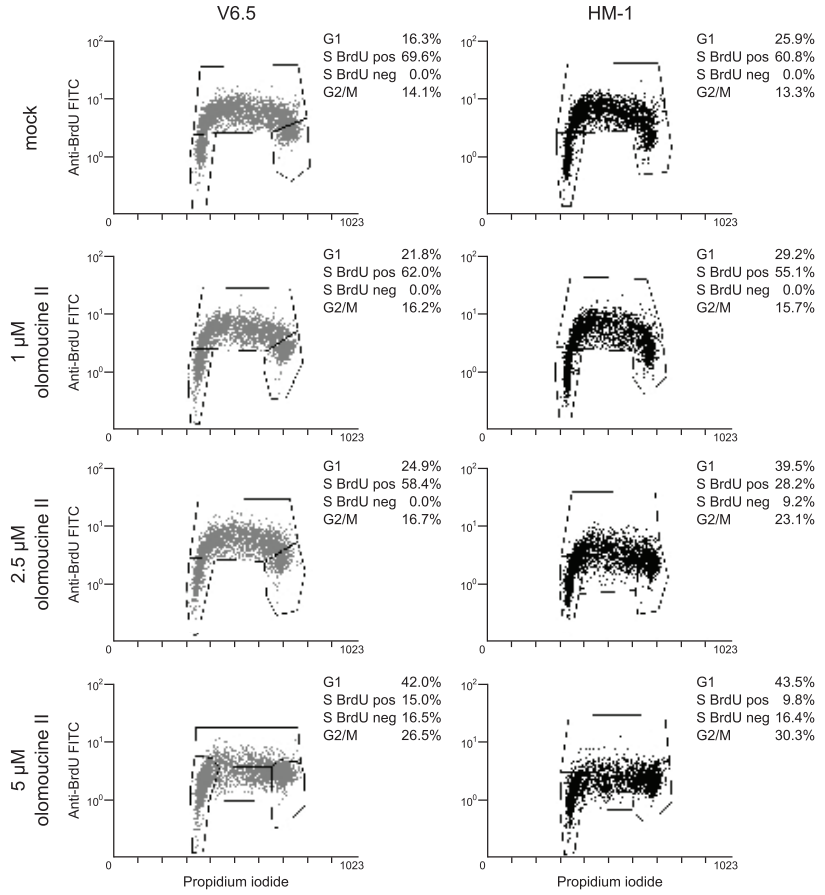

Figure 4.19: Flow cytometry analysis of cell cycle profiles and DNA replication in V6.5 and HM-1 mESCs treated with indicated doses of olomoucine II for 6 h. The figures are representatives of three independent experiments.

CDK2 inhibition slows down G1 phase progression

possibilities and to investigate the effect of CDK2 inhibition on G1/S phase progression in mESC in greater detail, mESCs were synchronized in the G2/M phase by nocodazole treatment and then released from the block to obtain the maximum number of G1 phase cells, which were mock- or 5 µM olomoucine-II treated and their cell cycle progression was followed by flow cytometry. Olomoucine II treatment slowed down G1 phase progression and S phase entry (Fig. 4.21) in mESCs. The G1 phase was prolonged by approximately 2 h (*i.e.* to about 5 h), as shown by the fact that identical proportions of cells in G1 and S phases were found 4 h after nocodazole release in mock-treated cells and 6 h after nocodazole release in olomoucine II-treated cells, respectively.

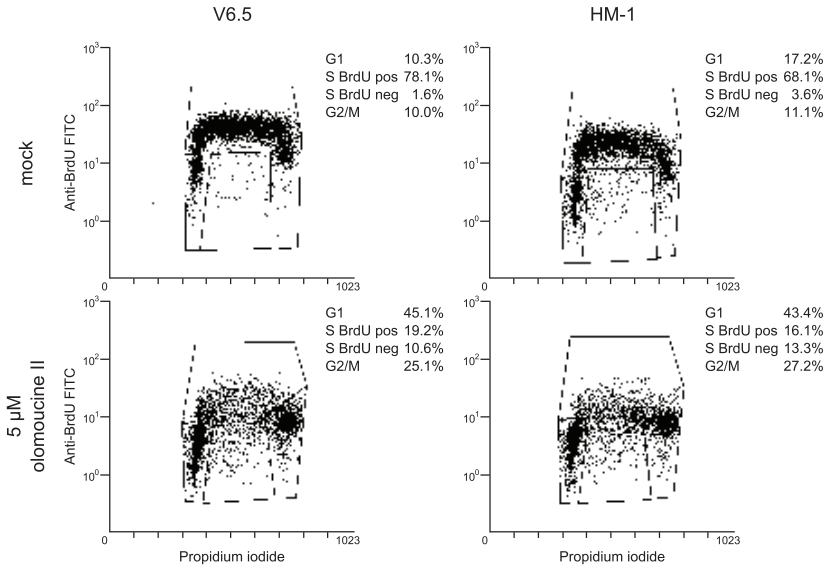

Figure 4.20: Flow cytometry analysis of cell cycle profiles and DNA replication in V6.5 and HM-1 mESCs treated with indicated doses of olomoucine II for 11 h. The figures are representatives of three independent experiments.

4.3.4 *Downregulation of CDK2 activity induces differentiation-associated changes in mESCs*

Downregulation of CDK2 activity in hESCs induces their differentiation [235]. To find out whether this might also be the case in mESCs, we investigated the effects of prolonged CDK2 downregulation by olomoucine II in mESCs. After 24 h of 5 μM olomoucine II treatment, mESCs still exhibited a somatic-cell like profile, *i.e.* a cell cycle profile with a high proportion of cells in G1 phase and a lower proportion of cells in S phase (Fig. 4.22 A). Changes in mESC cycle induced by downregulation of CDK2 activity were accompanied by changes in cell morphology, cell and colony shape and adhesiveness as early as 11 h after 5 μM olomoucine II treatment (Fig. 4.22 B). Examination of pluripotency and differentiation markers by quantitative RT-PCR showed a statistically significant increase in expression of differentiation markers (mesoderm: *Brachyury, Runx2*; ectoderm: *Nestin*; endoderm: *Cdx2, Gata4, Lamb1-1*) and reduced expression of some pluripotency markers (*Dax1*) after 11 h of olomoucine II treatment (Fig. 4.23 A), which was further reinforced after prolonged (24 h) olomoucine II treatment (Fig. 4.23 B). These data indicated induction of mESC differentiation by downregulation of CDK2 activity.

Downregulation of CDK2 activity induces differentiation in mESCs

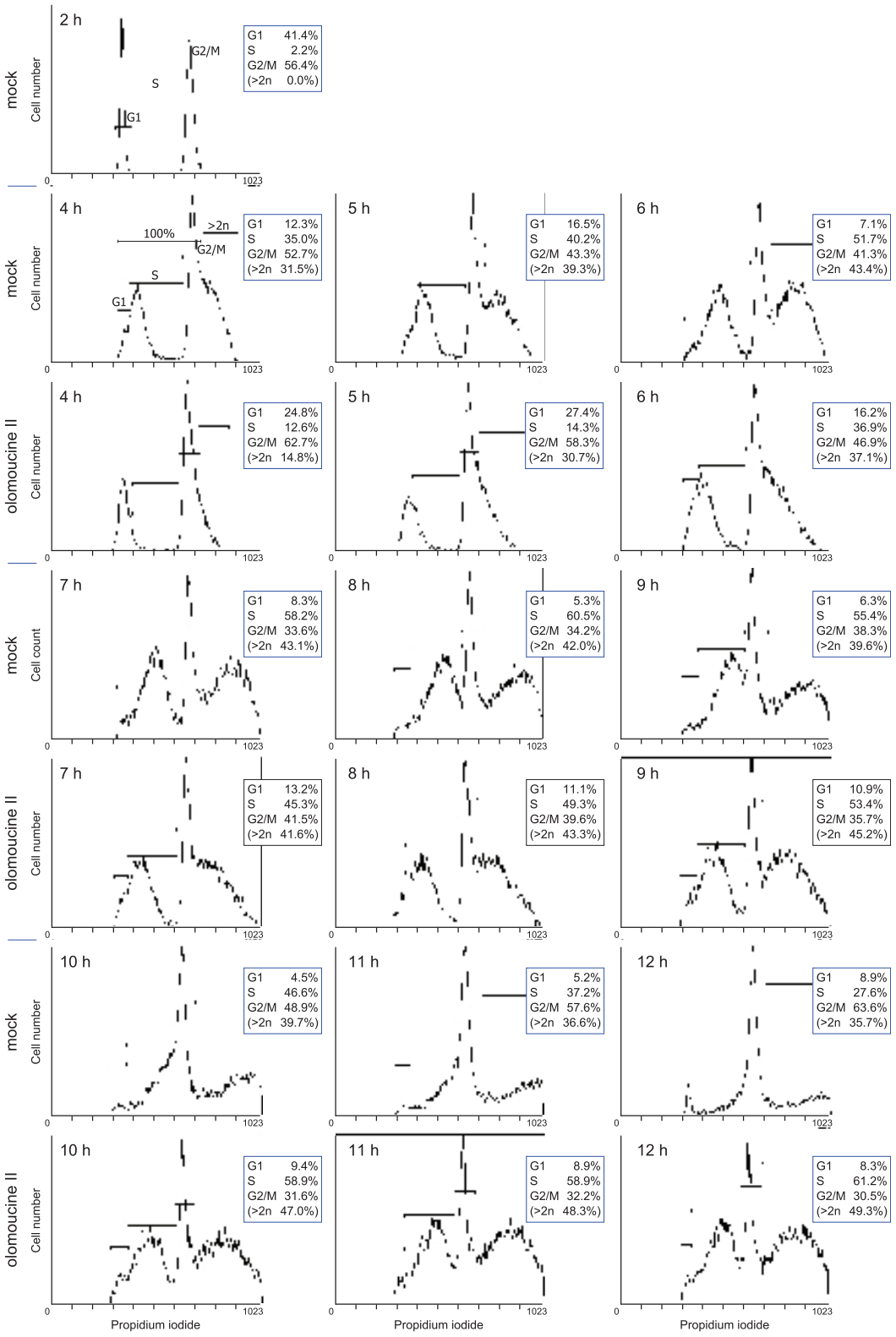

Figure 4.21: Flow cytometry analysis of synchronized mESCs that were treated with 5 µM olomoucine II or mock-treated. Hyperdiploid cells (>2n) were excluded from assessment of cell cycle distribution.

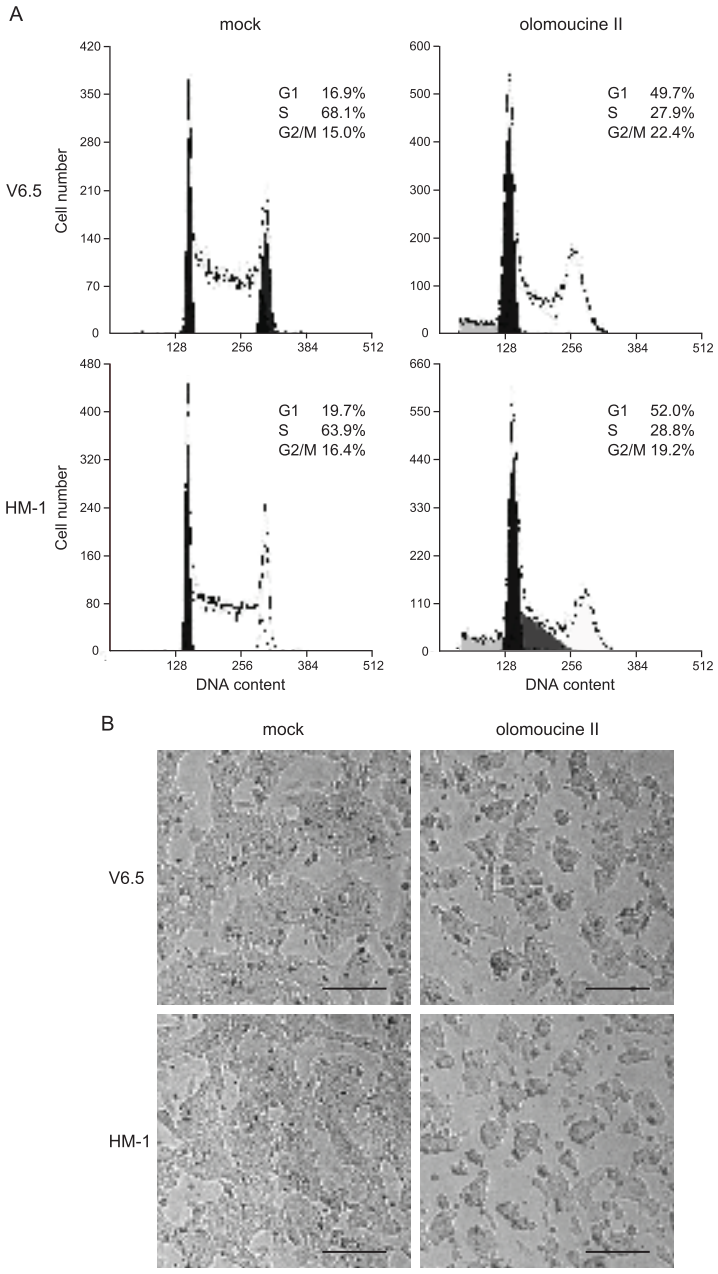

Figure 4.22: Downregulation of CDK2 activity by olomoucine II establishes a somatic cell-like cell cycle profile and induces morphology changes in mESCs. **A.** Cell cycle profile analysis of mESCs treated with 5 μM olomoucine II for 24 h. **B.** The microphotographs of mESCs after 11-h treatment with 5 μM olomoucine II. Scale bar, 200 μm.

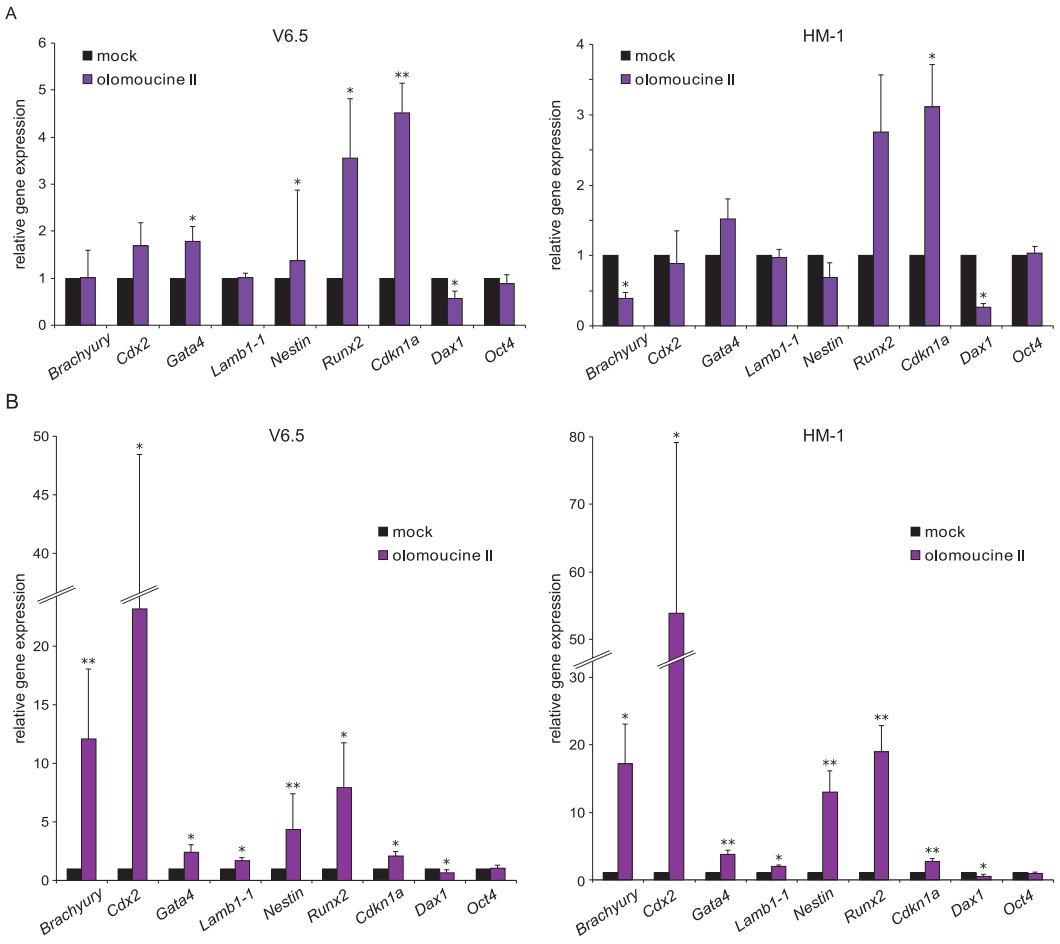

Figure 4.23: Downregulation of CDK2 activity by olomoucine II induces expression of differentiation markers in mESCs. Expression of differentiation and pluripotency markers in mESCs after 11-h **(A)** or **(B)** 24-h treatment with 5 μM olomoucine II, normalized to *Gapdh* expression. The data represent the mean ± SD from three independent experiments, *P < 0.05, **P < 0.005.

4.3.5 *siRNA knockdown of CDK2 has similar effects as CDK2 inhibition*

To verify the observations after olomoucine II-induced CDK2 downregulation, CDK2 expression was downregulated by siRNA approach (43% downregulation on RNA level, 55% downregulation on protein level; Fig. 4.24 A, B). The change in CDK2 expression led to 28% decrease of CDK2 activity (data not shown) and induced increase in G1 phase cell number (P = 0.01) (Fig. 4.24 C). Moreover, siRNA-mediated knockdown of CDK2 induced similar changes in mESC morphology, colony shape and adhesiveness (Fig. 4.24 D) as olomoucine II treatment. These results confirmed that CDK2 is the target molecule for olomoucine II, through

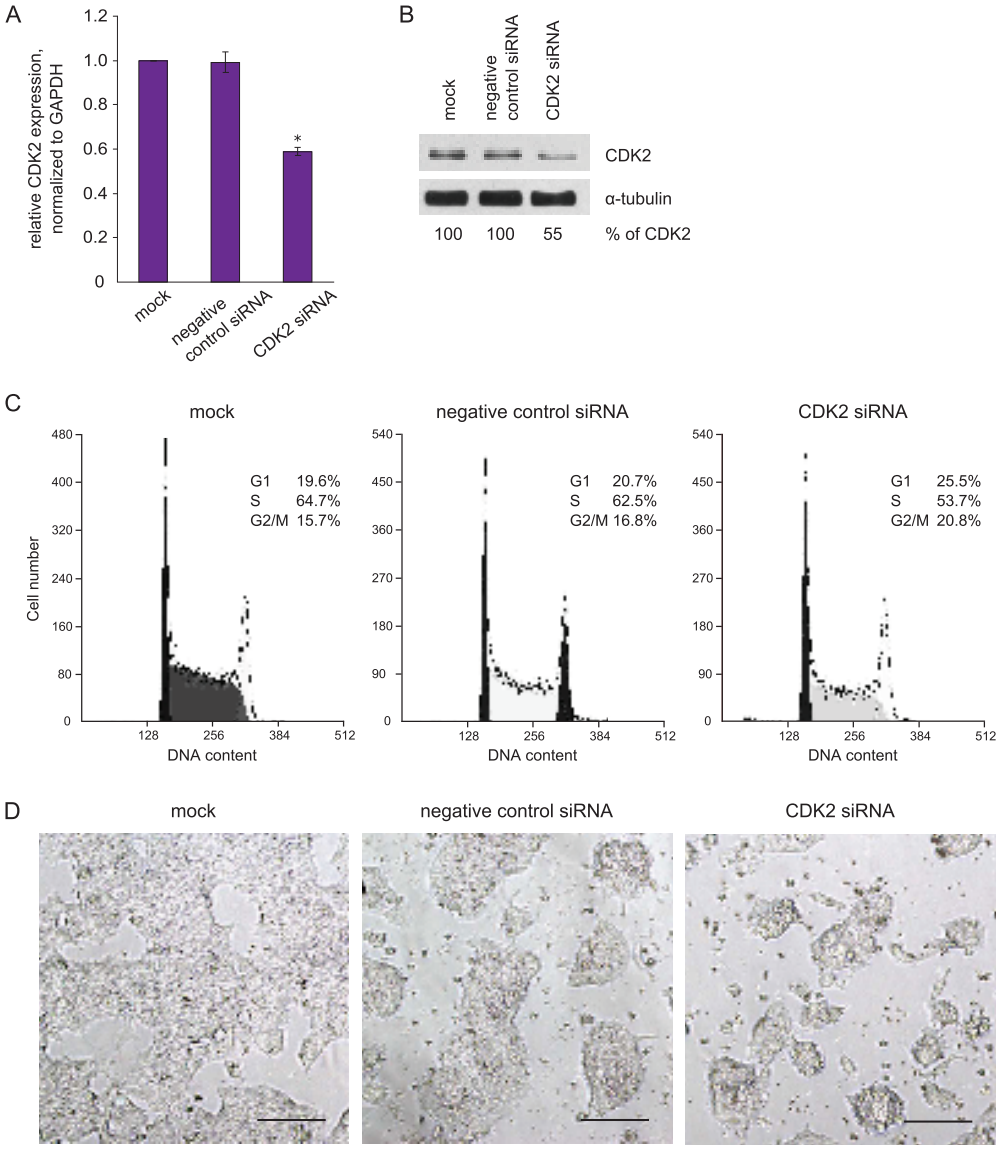

Figure 4.24: CDK2 knockdown leads to similar mESC cycle and morphology changes to those induced by olomoucine II treatment. **A.** Downregulation of CDK2 mRNA level 24 h after CDK2 siRNA transfection. The data represent the means ± SD from two independent experiments. *$P < 0.05$. **B.** Decrease of CDK2 protein level 24 h after CDK2 siRNA transfection. Relative levels of CDK2 were determined from Western blots, normalized to α-tubulin. **C.** Cell cycle profile analysis of CDK2 siRNA transfected mESCs 24 h post-transfection. **D.** The microphotographs of mESCs 24 h after siRNA treatment. Scale bar, 100 μm.

which the observed cell cycle changes and associated modulation of pluripotency are mediated.

5

DISCUSSION

Unusual cell cycle structure and lack of G1 checkpoint response in ESCs have been known for years, but the mechanisms underlying these specific ESC characteristics have remained elusive. In this thesis, results of our studies are presented, which attempted to resolve these phenomena in mESCs.

CDK2 is insensitive to activated G1 checkpoint pathways

In our study we show that the lack of G1 arrest after DNA damage in mESCs is due to insensitivity of CDK2 to activated G1 checkpoint pathways. The persistence of CDK2 activity after IR in mESCs was confirmed by unchanged level of pRB-Ser612 phosphorylation; *i.e.* the phosphorylation of a CDK2 target site. Although it was shown that pRB-Ser612 is also phosphorylated by Chk1/Chk2 after DNA damage [412], this might not be the case in mESCs where Chk1 and Chk2 are sequestered to centrosomes and thus separated from the nuclear pRB. However, Chk1/Chk2 may be responsible for the sustained level of pRB-Ser612 phosphorylation observed in NIH3T3 cells after IR in early G1 phase, which did not correspond to the decreased CDK2 activity observed at the time points 3+1 and 3+3.

A role for DNA-damage refractory CDK2 activity

Sustained CDK2 activity seems to be crucial for G1-S transition in the conditions of DNA damage because downregulation of CDK2 activity by olomoucine II slowed G1 escape after IR in mESCs. As it was recently shown that downregulation of CDK2 activity leads to ESC differentiation [235,413] (also in this thesis), we suggest that DNA damage-refractory CDK2 activity may be important for maintenance of ESC self-renewal and pluripotency in the conditions of DNA damage.

Contrary to previous studies, CDC25A was found to be degraded after DNA damage in mESC

Surprisingly and in contrast to previous reports in mESCs [395], we found CDC25A to be degraded after IR in mESCs. Chk2 was regularly activated in response to IR; in contrast, Chk1 was constitutively phosphorylated on Ser345; this phosphorylation did not change after IR and may play a role in proper localization of Chk1 to centrosomes [414]. The centrosomal sequestration of Chk1 and Chk2 might prevent their interaction with CDC25A in mESCs, which is localized to cytoplasm and nucleus. The lack of Chk1/Chk2-CDC25A signaling was confirmed by dual inhibition of Chk1 and Chk2, which did not prevent CDC25A degradation after IR in mESCs. Moreover, we uncovered a new mechanism of CDC25A regulation after IR in mESCs that is governed by GSK-3β. Phosphorylation of CDC25A by GSK-3β has already been described in somatic cells; however, in these cells GSK-3β participates in CDC25A degradation

during early G1 phase in unperturbed cell cycles [409]. Fittingly, GSK-3β and CDC25A localize to the same cellular compartment in mESCs.

However, although CDC25A was efficiently degraded after IR in a GSK-3β-dependent manner, it was not sufficient to abrogate CDK2 activity in mESCs. Correspondingly, CDK2 phosphorylation at Thr14/Tyr15, the target sites of CDC25A phosphatase, did not change after IR. This might be due to centrosomal localization of a sizable proportion of CDK2, which may be sheltered from any changes in cellular CDC25A levels, as we did not find detectable amounts of CDC25A at the centrosomes by immunofluorescence staining.

Centrosomes as cell cycle control centres

These results lend further support for a role of centrosomes in the regulation of cell cycle progression and checkpoint response in mESCs, as suggested by earlier observations [395]. Also, the concept of centrosomes as cell cycle control centers has long been suggested for somatic cells [415]. The possible (if any) mechanism of G1 phase regulation by centrosomal CDK2 remains elusive. We hypothesize that centrosomal CDK2 (possibly in cooperation with cytoplasmic pool of CDK2) might regulate activity of crucial cell-cycle (*e.g.* pRB-E2F) and cell-fate/self-renewal (*e.g.* SOX2) [416] regulatory proteins that shuttle between cytoplasm and nucleus. Furthermore, in the view of CDK2 role in self-renewal, our observations suggest a role for centrosomes in mESC fate decisions - by sequestration of checkpoint components (Chk1, Chk2) [395, 417] on the one hand and as a shelter for cell cycle-regulatory proteins (CDK2) [417] on the other hand to prevent reactions that might limit self-renewal.

Fate decisions on centrosomes

With regard to the activity of an alternative pathway of CDC25A degradation after DNA damage in mESCs, it is plausible that Chk1/Chk2 might be localized to centrosomes not only to prevent their interaction with CDC25A, but they might play some additional role. They might possibly contribute to sustained total CDK2 activity after DNA damage in mESCs, as Chk1-dependent activation of CDK2 was revealed in somatic cells [418]. Moreover, CDK2 regulates centrosomal duplication [419–423] and DNA damage was shown to induce Chk1-dependent amplification of centrosomes, leading to lethal multipolar mitoses [424]. Possibly, colocalization of CDK2 and Chk1/Chk2 at centrosomes in mESCs might allow for fast and effective centrosome amplification after DNA damage and might act as an additional checkpoint for efficient elimination of cells with damaged DNA from the stem cell pool.

Alternative checkpoint function for Chk1/ Chk2 on centrosomes

To verify our results from immunolocalization studies and the hypothesis of a lack of communication between CDK2 and CDC25A due to their localization to different cellular compartments, we tried to separate centrosomes from mESCs. We followed the only protocol established for centrosome separation from adherent cells. However, the protocol was not suitable for mESCs as the alleged centrosomal fraction was poor for centrosomes and exhibited profound nuclear contamination. This could

be due to different density or molecular weight of mESC centrosomes (what could be caused by differences in localization of various proteins to centrosomes in somatic cells and ESCs) or different "stickiness" of centrosomes to cytoplasmic versus nuclear structures.

Interestingly, although CDC25A is degraded after IR in mESCs, phosphorylation of its target site CDK2-Thr14/Tyr15 does not increase. Wee1/Myt1 phosphorylate CDK2 on Thr14/Tyr15 [208,425,426], thus having a function opposing CDC25A. Because CDC25A is degraded after DNA damage in mESCs, Wee1/Myt1 activity would have to be abrogated for the CDK2-Thr14/Tyr15 phosphorylation level to remain stable when CDC25A is degraded. The abrogation of Wee1/Myt1 activity could be accomplished by several mechanisms, such as direct inactivation of Wee1/Myt1 kinase activity, degradation of Wee1/Myt1 proteins, or delocalization and thus spatial separation from CDK2. In *Xenopus* egg extracts, Wee1 stability is regulated by DNA replication checkpoint. Similarly, an activated DNA replication checkpoint has also been proposed to decrease Wee1 degradation through Hsl7 [427]. Whether Wee1/Myt1 activity is regulated by DNA damage in mESCs and, if so, what is the mechanism of abrogating Wee1/Myt1 activity towards CDK2, remains to be determined.

A role for Wee1/Myt1

The functional status of the p53-p21^{Cip1} G1 checkpoint pathway in mESCs has been disputed. The observations of p53 activation by Ser18 phosphorylation after IR in mESCs are contradictory [395, 397]. It has been reported that the p53-p21^{Cip1} pathway is not functional in mESCs due to the inability of p53 to translocate into the nucleus after DNA damage [394] and due to repression of *Cdkn1a* expression by ESC-specific microRNAs in undifferentiated mESCs [243]. In contrast, another study [399] suggested a p53-dependent suppression of *Nanog* expression as an alternative pathway to maintain genetic stability in mESCs, which would obviously require nuclear localization of p53. Furthermore, unlike in mESCs, p53 is nuclear [396] and p21^{Cip1} is rapidly and robustly induced upon IR in hESCs [239]. Although some of the discrepancies observed by different groups may be explained by the use of different ESC lines, it is difficult to reconcile between these studies.

Nevertheless, our study revealed p53 activation, increased *Cdkn1a* expression and accumulation of p21^{Cip1} after IR in mESCs that is similar to the situation in human ESCs and somatic cells. Because CDK2 activity was unaffected by IR, these findings suggest that CDK2 activity is refractory to both p21^{Cip1} accumulation as well as CDC25A degradation in mESCs. As discussed above, a possible explanation may be the centrosomal delocalization of a significant portion of CDK2 molecules. These molecules could be protected from p21^{Cip1} binding by an unknown mechanism. An alternative explanation may be that the observed p21^{Cip1} protein accumulation in mESCs in our study may be insufficient

p21^{Cip1} is induced after IR in mESCs

to buffer high concentrations of CDK2 because equimolar concentrations of $p21^{Cip1}$ and CDK2-cyclin complexes are required for efficient CDK2 inhibition [428].

The obvious lack of nuclear CDK2 and its centrosomal localization made us question CDK2 involvement in mESC cycle regulation. By specific downregulation of CDK2 activity by 5 μM olomoucine II, we found out that CDK2 is crucial in regulation of rapid G1 phase progression, as its downregulation led to prominent increase in G1 phase cell numbers due to slow-down of mESC progression through G1 phase, *i.e.* prolongation of G1 phase by approximately 2 h. This observation corroborates the role of CDK2 in driving ESC cycles [225]. However, our observation of distinctive cell cycle changes after CDK2 inhibition is novel and suggests that high CDK activity *per se* contributes to a very short G1 phase in mESCs and might be responsible for the unusual cell cycle structure of mESCs.

High CDK2 activity establishes an ESC-specific cell cycle structure

Furthermore, specific downregulation of CDK2 activity established a somatic cell-like cell cycle, led to morphology changes resembling differentiation and induced expression of differentiation markers in mESCs. These observations gave evidence of the hypothesis that specific cell cycle regulation and loss of G1 functions (restriction point and G1 checkpoint) in ESCs might be involved in self-renewal of ESCs [226]. The relationship between cell cycle regulation and pluripotency of ESCs has been under dispute. It was reported that activation of p53 leads to rapid differentiation of ESCs by introducing changes in cell cycle progression, particularly abolishing S phase entry [429]. Recently it was shown that downregulation of CDK2 activity in hESCs induces their differentiation to extraembryonic lineages [235]. Our study in mESCs presents further evidence on the requirement for CDK2 activity for maintenance of the ESC-specific cell cycle structure and for the self-renewal of ESCs.

High CDK2 activity is crucial for self-renewal of ESCs

Also, our study revealed slight differences in effects of CDK2 downregulation between mESCs and hESCs: While hESCs arrest in G1 after downregulation of CDK2 activity, mESCs only slow down their G1 progression. Neither after prolonged 5 μM olomoucine II treatment (48 h or 72 h), nor after treatment with higher doses of olomoucine II (10 μM and higher) did we observe G1 arrest in mESCs (data not shown). Our contrasting observation could be caused by biological differences between mESCs and hESCs. However, more plausible, it might be related to the extent of CDK2 inhibition or to different methods used for downregulation of CDK2 activity. In our study, we primarily analyzed requirements for CDK2 in cell cycle regulation of mESCs using a chemical inhibition approach. To confirm our observations, we also employed CDK2 knockdown by siRNA. Both approaches led, in principle, to the same results, but in the knockdown experiments the G1 cell number was increased less efficiently. This might result from low efficiency of

CDK2 knockdown by siRNA; *i.e.* from the different extent to which CDK2 activity was inhibited. Another possibility is that the knockdown approach introduces a bias through upregulation of other CDKs/cyclins and their potential compensation for S phase promoting functions. Chemical inhibition might not allow for the same compensation [430]. While knockdown of a CDK leaves a pool of its interactory cyclin molecules free and accessible for other CDKs that might bind to them and compensate for the knocked-down CDK, chemical inhibition of a CDK does not leave its partner cyclin pool available.

Knockdown versus chemical inhibition

In general, SCs are more resistant to toxins and various types of drugs due to high expression of specific ABC drug transporters [431]. As this characteristic might be common to "normal" SCs (ESCs and tissue SCs) and "abnormal" (cancer) SCs [431], drug testing using ESCs as a convenient model of cancer SCs might provide important insights into the mechanism(s) by which cancer SCs might respond to cancer therapy. Cytotoxicity of the tested drugs towards mESCs is similar to the cytotoxicity towards cancer cell lines, as the IC_{50} for olomoucine II or CAN508 were similar for mESCs and cancer cells. The only exception was lower sensitivity of V6.5 towards CAN508. This might be due to some unique features of transcription regulation in V6.5 cells [432], rather than due to ABC-mediated drug resistance, as V6.5 sensitivity towards olomoucine II does not significantly differ from that of other cell lines, including the mESC line HM-1.

CDKs are, besides other functions, critical regulators of cell cycle progression and RNA transcription [433] and are frequently deregulated in tumors, as a result of a variety of genetic and epigenetic events [145, 434]. Therefore, CDK inhibitors have appeared as a promising tool for cancer therapy and it has been shown that CDK inhibition can lead to cell cycle arrest and apoptosis [433]. However, because CDK2 activity is dispensable for cancer cell proliferation [435], the suitability of CDK2 as a target for cancer therapy has been called into question. Our study reveals olomoucine II as an effective tool for manipulation of ESC cycle and self-renewal. In this context, it would be interesting to investigate the effects of CDK2 downregulation in cancer SCs because it has been shown that cancer SCs use an ESC-like stemness program to induce and maintain tumors [436, 437]. It is tempting that CDK2 inhibitors might become powerful tools for cancer SC eradication, possibly contributing to their differentiation by modulation of the cell cycle of (cycling) cancer SCs.

CDK2 inhibitors as tools for cancer stem cell eradication

6

SUMMARY/SÚHRN

6.1 SUMMARY

mESCs divide rapidly and have a unique cell cycle structure with a very short G1 phase that lacks restriction point and G1 checkpoint. In this thesis, results of our studies are summarized, which attempted to resolve the mechanisms underlying G1 checkpoint non-functionality in mESCs and investigated the role of the very short G1 phase in mESC self-renewal. Contrary to some previous reports, we found out that in mESC p53-p21^{Cip1} pathway is activated and CDC25A is degraded after DNA damage as in somatic cells. However, mESCs do not stop in G1 after DNA damage because these pathways do not impinge on CDK2 activity. Based on immunolocalization studies, we suggest that CDK2 inactivation by activated G1 checkpoint pathways is prevented by centrosomal localization of CDK2. Furthermore, because our studies have also suggested a role for high CDK2 activity in driving rapid G1 phase progression and in self-renewal in mESCs, we suggest that DNA-damage refractory CDK2 activity might be necessary for maintenance of self-renewal in the conditions of DNA damage. We also described a novel mechanism of CDC25A regulation after DNA damage in mESCs that is governed by GSK-3β instead of Chk1/Chk2. Chk1/Chk2 are sequestered at centrosomes, where they might be involved in an alternative checkpoint pathway. Furthermore, we suggest a role for centrosomes in cell fate decisions in mESCs by preventing interactions that might limit self-renewal. Our studies also point out potential of CDK inhibitors in cancer stem cell eradication by inducing their differentiation.

6.2 SÚHRN

Myšie embryonálne kmeňové (mEK) bunky majú unikátnu štruktúru bunkového cyklu s veľmi krátkou G1 fázou, v ktorej chýba funkčný restrikčný bod a G1 kontrolný bod. V tejto práci sú zhrnuté výsledky našich štúdií, ktoré mali za cieľ odhaliť mechanizmy stojace za nefunkčným G1-kontrolným bodom v mEK bunkách a úlohu krátkej G1 fázy v sebaobnove mEK buniek. Narozdiel od niektorých predchádzajúcich štúdií sme prekvapivo zistili, že dráha p53-$p21^{Cip1}$ je aktivovaná a CDC25A je degradovaná po poškodení DNA u mEK buniek. Avšak mEK bunky sa v G1 fáze po poškodení DNA nezastavili, pretože tieto dráhy nemali dosah na aktivitu CDK2. Na základe imunolokalizačných štúdií predpokladáme, že inaktivácii CDK2 aktivovanými dráhami G1 kontrolného bodu bráni jej centrozomálna lokalizácia. Keďže naše štúdie tiež naznačili úlohu CDK2 v rýchlej progresii G1 fázou a v sebaobnove mEK buniek, aktivita CDK2 odolná voči aktivovaným dráham G1-kontrolného bodu môže byť dôležitá pre zachovanie sebaobnovy mEK buniek v podmienkach poškodenia DNA. Downregulácia CDK2 aktivity totiž vedie k nastoleniu bunkového cyklu podobného somatickému, morfologickým zmenám a expresii markerov spojených s diferenciáciou. Tiež popisujeme nový mechanizmus regulácie CDC25A v odpovedi na poškodenie DNA prostredníctvom GSK-3β namiesto Chk1/Chk2. Chk1/Chk2 sú sekvestrované na centrozómoch, kde môžu byť zapojené do alternatívnej dráhy odpovede na poškodenie DNA. Naviac navrhujeme, že centrozómy môžu zohrávať úlohu v osudových rozhodnutiach mEK buniek tým, že zabraňujú interakciám, ktoré by mohli mať negatívny dopad na sebaobnovu mEK buniek. Naše výsledky tiež vyzdvihujú potenciál CDK inhibítorov v liečbe nádorov a eradikácii nádorových kmeňových buniek indukciou ich diferenciácie.

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Part III

SUPPLEMENTS AND APPENDICES

7

SUPPLEMENTS AND APPENDICES

7.1 ACRONYMS AND ABBREVIATIONS

ABC	ATP-binding cassette
Alk	Activin receptor-like kinase
APC	Adenomatous polyposis coli
ATM	Ataxia-teleangiectasia mutated
ATP	Adenosine triphosphate
ATR	ATM and Rad3-related
BMP	Bone morphogenetic protein
BrdU	5-bromo-2′-deoxyuridine
BUB3	Budding uninhibited by benzimidazoles 3
β-TrCP	β-transducin repeat-containing protein
CAK	CDK activating kinase
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CBP	CREB binding protein
CCNA2	Cyclin A2
CCNE	Cyclin E
CDC25A	Cell division cycle 25A
CDK	Cyclin-dependent kinase
CDK2AP1	Cyclin-dependent kinase 2-associating protein 1
CHEK1	Checkpoint kinase 1
Chk1	Checkpoint kinase 1
CK1	Casein kinase 1
CKI	Cyclin dependent kinase inhibitor

CSL	CBF1/RBP-J, Suppressor of hairless, Lag-1
CTD	C-terminal domain
D-MEM	Dulbecco's modified Eagle's medium
DAPI	4,6-diamidino-2-phenylindole
DSBs	Double strand breaks
DTT	Dithiothreitol
Dvl	Dishevelled
DYRK2	Dual-specificity tyrosine-phosphorylation-regulated kinase 2
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid
EpiSCs	Epiblast stem cells
Erbb	Estrogen-related receptor b
ERK	Extracellular receptor kinase
ESC	Embryonic stem cell
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FoxO	Forkhead box
Fz	Frizzled
GADD45	Growth arrest and DNA damage 45
Gfi1	Growth factor independent 1
GPR49	G-protein-coupled receptor 49
GSK-3β	Glycogen synthase kinase-3β
HAND1	Heart and neural crest derivatives expressed
HCF-1	Host cell factor-1
HDAC1	Histone deacetylase 1
hECCs	human embryonal carcinoma cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid

HES Hairy and enhancer of split

hESC human embryonic stem cell

HRP Horse radish peroxidase

HSC Hematopoietic stem cell

IC_{50} 50% inhibitory concentration

ICM Inner cell mass

iPSCs induced pluripotent stem cells

IR Ionizing radiation

IRIF Irradiation induced foci

Kap1 KRAB domain-associated protein

LEF Lymphoid enhancer factor

LGR5 Leucine-rich repeat-containing G protein-coupled receptor 5

LIF Leukemia inhibitory factor

LIFR LIF receptor

MAPK Mitogen-activated protein kinase

Mef Myeloid Elf1-like

mESC mouse embryonic stem cell

miRNA micro RNA

mirPSCs miRNA-induced pluripotent stem cells

MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NBS1 Nijmegen breakage syndrome 1

N-coR Nuclear coreceptor

NLK NEMO-like kinase

ORC1L Origin recognition complex subunit 1-like

PBS Phosphate-buffered saline

PcG Polycomb group

PI3K Phosphatidylinositol 3-kinase

PIKK	Phosphoinositide 3-kinase related kinase
PKC	Protein kinase C
PMSF	Phenylmethylsulphonyl fluoride
PRKDC	Protein kinase, DNA-activated, catalytic polypeptide
PYgo	Pygopus
pRB	Retinoblastoma protein
Pten	Phosphatase and tensin homologue
RAD54L	RAD54 like
RFC2	Replication factor C2
RPA3	Replication protein A3
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SC	Stem cell
SD	Standard deviation
SDS	Sodium dodecyl sulphate
STAT3	Signal transducer and activator of transcription 3
TCF3	T-cell factor 3
TCFs	T-cell-specific factors
TK	Thymidine kinase
TLE	Transducin-like-Enhancer of Split
Tlx	Tailless
TOP2A	Topoisomerase II alpha
TopBP1	Topoisomerase-binding protein-1
TP53	Tumor protein 53
UNG	Uracil-DNA glycosylase

7.2 SEQUENCES OF PRIMERS USED FOR QUANTITATIVE RT-PCR

Gene	Primers (5′ - 3′)
Axin1	F: ACCCAGTACCACAGAGGACG
	R: CTGCTTCCTCAACCCAGAAG
Axin2	F: TAGGCGGAATGAAGATGGAC
	R: CTGGTCACCCAACAAGGAGT
Brachyury	F: CAGCCCACCTACTGGCTCTA
	R: GAGCCTGGGGTGATGGTA
Cdk2	F: CACAGCCGTGGATATATGG
	R: CATGGTGCTGGGTACACACT
Cdx2	F: CACCATCAGGAGGAAAAGTGA
	R: CTGCGGTTCTGAAACCAAAT
Dax1	F: ACCGTGCTCTTTAACCCAGA
	R: CCGGATGTGCTCAGTAAGG
Gapdh	F: AGCTTGTCATCAACGGGAAG
	R: TTTGATGTTAGTGGGGTCTCG
Gata4	F: GGAAGACACCCCAATCTCG
	R: CATGGCCCCACAATTGAC
Hmbs	F: GAATTCAGTGCCATCGTCCT
	R: CTTCTGGGTGCAAAATCTGG
Lamb1-1	F: TTGCGTGTGTTTGTGATCCT
	R: ATCCAGAGGCACAGTCATCA
Lef1	F: TCACTGTCAGGCGACACTTC
	R: ATGAGGTCTTTTGGGCTCCT
Nes	F: CTGCAGGCCACTGAAAAGTT
	R: TCTGACTCTGTAGACCCTGCTTC
Pou5f1	F: GTTGGAGAAGGTGGAACCAA
	R: CTCCTTCTGCAGGGCTTTC
Cdkn1a	F: TCCACAGCGATATCCAGAGACA
	R: GGACATCACCAGGATTGGAC
Runx2	F: GCCCAGGCGTATTTCAGA
	R: TGCCTGGCTCTTCTTACTGAG

8

LIST OF APPENDICES

Appendix I

Koledova, Z., Raskova Kafkova, L., Krämer, A., and Divoky, V.: DNA Damage-Induced Degradation of Cdc25A Does Not Lead to Inhibition of Cdk2 Activity in Mouse Embryonic Stem Cells. *Stem Cells.* January (2010). [Epub ahead of print]

Appendix II

Koledova, Z., Raskova Kafkova, L., Calabkova, L., Krystof, V., Dolezel, P., and Divoky, V.: Cdk2 inhibition prolongs G1 phase progression in mouse embryonic stem cells. *Stem Cells and Development.* **19**(2), 181-194 February (2010).

Appendix III

Koledova, Z., Krämer, A., Raskova Kafkova, L., and Divoky, V.: Cell cycle regulation in embryonic stem cells: centrosomal decisions on self-renewal. [Manuscript in preparation]

STEM CELLS®

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DNA Damage-Induced Degradation of Cdc25A Does Not Lead to Inhibition of Cdk2 Activity in Mouse Embryonic Stem Cells

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Key words. Cell cycle • embryonic stem cells • irradiation • signal transduction

ABSTRACT

Cyclin-dependent kinase 2 (Cdk2) is the major regulator of the G1/S transition and the target of an activated G1 checkpoint in somatic cells. In the presence of DNA damage, Cdk2 kinase activity is abrogated by a deficiency of Cdc25A phosphatase, which is marked by Chk1/Chk2 for proteasomal degradation. Embryonic stem cells (ESCs) lack a G1 checkpoint response. In this study, we analyzed the G1 checkpoint pathways in mouse ESCs (mESCs) in the presence of DNA double-strand breaks evoked by ionizing radiation (IR). We show that checkpoint pathways, which operate during G1 phase in somatic cells, are activated in mESCs after IR; however, Cdk2 activity is not abolished. We demonstrate that Cdc25A is degraded in mESCs, but this degradation is not regulated by Chk1 and Chk2 kinases because they are sequestered to the centrosome. Instead, Cdc25A degradation is governed by GSK-3β kinase. We hypothesize that Cdc25A degradation does not inhibit Cdk2 activity because a considerable proportion of Cdk2 molecules localize to the cytoplasm and centrosomes in mESCs, where they may be sheltered from regulation by nuclear Cdc25A. We also demonstrate that a high and DNA damage-irresponsive Cdk2 activity is the driving force of the rapid escape of mESCs from G1 phase after DNA damage.

INTRODUCTION

Embryonic stem cells (ESCs) are pluripotent [1, 2], naturally immortal cells derived from the inner cell mass of mammalian blastocysts [3]. These self-renewing cells display a cell cycle structure and regulation that are distinct from those of somatic cells and are also similar, in some features, to those of cancer cells [4]. Among other differences, ESCs proliferate rapidly, have a very short G1 phase, and lack a functional restriction point [5-9]. Moreover, ESCs do not arrest in G1 phase after DNA damage, and their G1 checkpoint has been described as non-functional [9-11].

In somatic cells, the G1/S transition is governed by Cdk2-cyclin E complexes through their action on several targets. The major target of Cdk2-cyclin E is retinoblastoma tumor suppressor protein (pRb) [12]. To be activated, Cdk2 must be phosphorylated at Thr160 [13] by Cdk-activating kinase (CAK) [14, 15]. In contrast, the phosphorylation of two Cdk2 residues (Thr14 and Tyr15) located within the ATP binding loop leads to Cdk2 inactivation

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[13]. The level of the inhibitory phosphorylation is regulated by the activity of Wee1/Myt1 kinases [16-18] and Cdc25A phosphatase [19, 20].

Cdc25A phosphatase is an essential activator of cell cycle progression [21]. It is an unstable protein whose cellular levels are regulated by periodic synthesis and ubiquitin-mediated proteolysis throughout the cell cycle. Cdc25A has an important function in late G1 phase when it accumulates as a result of E2F-1- and c-Myc-mediated transcriptional activation [19, 22, 23]. Cdc25A dephosphorylates Cdk2 at Thr14 and Tyr15, thereby activating Cdk2-cyclin E complexes [20], which further phosphorylate and activate Cdc25A to create an autoamplification loop [24]. Cdc25A also regulates the activity of Cdk2-cyclin A in S phase. During mitosis, Cdc25A contributes to the dephosphorylation and activation of Cdk1 [25].

The G1 checkpoint is controlled via two pathways, the immediate and the delayed response pathways, which converge on Cdk2 to block its activity. The immediate response pathway leads to Chk1/Chk2-mediated phosphorylation and subsequent degradation of Cdc25A [26, 27]. The delayed G1 checkpoint response pathway involves phosphorylation of p53 at several residues, including Ser15 (Ser18 in mouse) and Ser20. Phosphorylation of these residues increases p53 stability and transactivation capacity [28, 29], resulting in the upregulation of the Cdk inhibitory protein $p21^{Cip1}$ [30]. Both G1 checkpoint pathways are non-functional in mouse ESCs (mESCs) because of the inability of mESCs to translocate p53 into the nucleus after DNA damage [10] and also because of the centrosomal sequestration and, thus, spatial separation of Chk2 from its target Cdc25A [11].

In this study, we have further investigated the causes of G1 checkpoint non-functionality in mESCs. For the first time, we describe a lack of Cdc25A-Cdk2 communication in response to DNA damage in mESCs and clearly demonstrate that, although Cdc25A is degraded after DNA damage, this degradation does not inhibit Cdk2 activity. Instead of Chk1/Chk2 kinases, GSK-3β kinase is involved in DNA damage-mediated Cdc25A degradation in mESCs.

MATERIAL AND METHODS

Cell Culture, Irradiation and Synchronization

For our studies, we used two different mESC lines, the inbred cell line HM-1 derived from the 129 mouse strain [31] and the F1 (129SvJae×C57BL/6) hybrid line V6.5 (Open Biosystems, Huntsville, AL, USA). The total *in vitro* culture time of these mESCs at the time of experimental testing was equivalent to passage numbers 22 – 35 of the original mESC line. Both mESC lines were checked repeatedly for chromosome numbers and karyotype abnormalities. mESC culture was carried out following standard procedures [32]. Briefly, the cells were maintained in culture dishes covered with 0.1% gelatin (Sigma-Aldrich, St. Louis, MO, USA) in a humidified atmosphere of 10% CO_2 at 37°C in high-glucose Dulbecco's modified Eagle medium (DMEM) with GlutaMAX (Invitrogen/Gibco, Grand Island, NY, USA) supplemented with 15% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen/Gibco), 0.1 µM β-mercaptoethanol (Serva, Heidelberg, Germany) and 1,000 U/ml leukemia inhibitory factor (ESGRO, Chemicon, Temecula, CA, USA). Mouse embryonic fibroblasts NIH3T3 (ATCC) were maintained in high-glucose DMEM with GlutaMAX supplemented with 15% FBS (Invitrogen/Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were irradiated with a defined dosage (4 Gy) of gamma irradiation (cobalt irradiator, Teragam, Prague, Czech Republic).

Cells were synchronized in G2/M phase by treating them with 400 nM nocodazole (Sigma-Aldrich, St. Louis, MO, USA). mESCs were grown in the presence of nocodazole for 12 h, and NIH3T3 cells were grown in its presence for 24 h. After mitotic shake-off, cells were washed three times with phosphate-buffered

saline (PBS) with 1% FBS (Invitrogen/Gibco) and plated in standard media.

Drugs and Inhibitors

Nocodazole was stored as a 400 μM stock solution in DMSO. CEP3891 (Cephalon, Frazer, PA, USA) was stored as a 500 μM solution in DMSO and used at a concentration of 500 nM. The Chk2 inhibitor II [2-(4-(4-chlorophenoxy)phenyl)-1H-benzimidazole-5-carboxamide; Merck Chemicals Limited, Darmstadt, Germany] was stored as 300 μM solution in DMSO and used at 300 nM. LiCl (Sigma-Aldrich) was prepared as a 100 mM solution in PBS and used at 10 mM. The GSK-3 inhibitor X (6-bromoindirubin-3'-acetoxime; Merck Chemicals Limited) was stored as a 2.5 mM stock solution in DMSO and used at 5 μM. Olomoucine II (2-{[2-((1-*R*)-1-hydroxymethyl-propylamino)-9-isopropyl-9*H*purin-6-ylamino]-methyl}-phenol) was synthesized according to published procedures [33], stored as a 100 mM solution in DMSO and used at 5 μM.

Flow Cytometric Analysis

Cell cycle distribution was evaluated by propidium iodide staining. Cells were trypsinized (0.05% trypsin-EDTA; Invitrogen/Gibco) and resuspended in PBS to obtain a single cell suspension, washed with PBS, and fixed in ice cold 70% ethanol. After rehydration in PBS with 1% FBS (Invitrogen/Gibco), cells were incubated in 1.1% sodium citrate with 5 ng/μl ribonuclease A (DNA Lego Ribonuclease A; Top-Bio, Prague, Czech Republic) and 60 μg/μl propidium iodide (Sigma-Aldrich) for 30 min at 37°C in the dark. Cells were analyzed by flow cytometry on a Cytomics FC 500 machine using CXP software (Beckman Coulter, Fullerton, CA, USA). MultiCycle software (Phoenix Flow Systems) was applied to assess cell cycle distribution.

Western Blot Analysis

Cells in culture dishes were washed with cold PBS, collected into IP buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% (v/v) glycerol, 0.1% (v/v) Tween 20, 1 μM DTT, 1 μM NaF, 10 μM β-glycerophosphate, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 0.1 μM Na_3VO_4, 0.1 μM PMSF), and incubated for 1 h at 4°C. Lysates were cleared by centrifugation at 18,800 *g* at 4°C for 30 min. Proteins were electrophoretically resolved on 10-15% SDS-polyacrylamide gels (for detection of Chk2 mobility shift, 10% SDS-polyacrylamide gels were used) and electroblotted onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Membranes were incubated with primary antibodies at dilutions recommended by the supplier at 4°C overnight, washed in PBS with 0.05% Tween 20, and incubated for 1 h with the appropriate horse radish peroxidase (HRP)-conjugated secondary antibody (DakoCytomation, Glostrup, Denmark). HRP activity was detected with ECL detection kit (Pierce, Rockford, IL, USA) on films (Kodak, Rochester, NY, USA). The following primary antibodies were used: anti-actin, anti-Chk2 (DCS-273) and anti-phospho-retinoblastoma (Rb) [pSer612] (Sigma-Aldrich); anti-Chk1 (Stressgen, Ann Arbor, MI, USA); β-catenin (L87A12), Cdc25A (F-6), Cdk2 (M-2), cyclin A (C-19) and p-Cdk2(Thr14/Tyr15)-R (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); anti-p21 (BD Pharmingen, San Diego, CA, USA); and phospho-Chk1 (Ser345), phospho-GSK-3β (Ser9) (5B3), p53 (1C12) and phospho-p53 (Ser15) (Cell Signaling Technology, Danvers, MA, USA).

Immunoprecipitation and Kinase Assay

Immunoprecipitations were conducted at 4°C. Protein A agarose beads (Sigma-Aldrich) were incubated for 1 h on a rotor in 1 ml IP buffer with 1 μg of Cdk2 antibody [Cdk2 (M-2), Santa Cruz Biotechnology], Chk1 antibody [anti-Chk1 (DCS-316), Sigma-Aldrich] or Chk2 antibody [Chk2 (H-300), Santa Cruz Biotechnology]. After washing three times with IP buffer, agarose beads were incubated for 1 h with protein lysates (Cdk2 kinase assays: 200 μg of proteins for mESCs or 400 μg proteins for NIH3T3; Chk1/Chk2 kinase assays: 100 μg of proteins) in IP buffer on a rotor and finally washed three times with IP buffer. Agarose beads with immunocomplexes were equalized with kinase assay buffer (KAB; 50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 2.5 mM EGTA, 100 μM

β-glycerophosphate, 2 μM NaF, 1 μM DTT, 0.1 μM Na_3VO_4). The beads were then resuspended in 30 μl kinase reaction mixture [18 μl KAB, 9 μl 75 μM ATP in KAB, 1 μl ^{33}P-γ-ATP (10 μCi; MP Biochemicals, Irvine, CA, USA) and either 2 μg histone H1 (for Cdk2 kinase assays; Upstate, Lake Placid, NY, USA) or 2 μg Chk1/2 peptide substrate (for Chk1/2 kinase assays; Biaffin, Kassel, Germany)] and incubated for 30 min at 30°C. Reactions were terminated by addition of 12 μl of 4x loading sample buffer (8% SDS, 40% glycerol, 400 mM DTT, 240 mM Tris, pH 6.8, 0.004% bromophenol blue). Samples were boiled and electrophoretically separated on SDS-polyacrylamide gels. The radioactivity of dried gels was detected with the bioimager BAS 1800 with a Fuji LAS 1000 CCD Camera System (Fuji Photo Film Co., Ltd., Tokyo, Japan) and quantified with Image Gauge software (Fuji, Valhalla, NY, USA).

Statistical analysis

Statistical evaluation of data was performed with the independent two sample t-test using Statistica 8.0 (StatSoft, Inc., Tulsa, OK, USA). $P < 0.05$ was considered statistically significant.

Immunocytochemistry

Cells were grown on gelatinized glass coverslips, washed with PBS and fixed for 7 min with a methanol-acetone mixture (1:1, -20°C). Alternatively, to deplete soluble proteins from cytoplasm, the cells were permeabilized for 5 min with 0.5% Triton X-100 in PHEM buffer ([34]; 60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, pH 6.9) at room temperature, washed twice with PHEM buffer, and fixed for 10 min with -20°C methanol. Fixed cells on coverslips were blocked with blocking buffer (1% FBS in PBS), stained with primary antibodies [Cdc25A (M-191), Cdk2 (M-2), Chk2 (A-12) (all Santa Cruz Biotechnology); gamma-tubulin (TU-30, Exbio, Prague, Czech Republic); GSK-3β (BD Biosciences, Franklin Lakes, NJ, USA); pericentrin (Abcam, Cambridge, UK); Chk1 antibody (DCS-310.1.1, Sigma-Aldrich)] diluted in blocking buffer for 1 h at room temperature. After washing with PBS, cells were incubated for 30 min with the appropriate fluorophore-conjugated secondary anti-mouse or anti-rabbit antibody [Alexa Fluor® 594, goat anti-rabbit IgG (H+L), Alexa Fluor® 488 donkey anti-mouse IgG (H+L) (Invitrogen, Carlsbad, CA, USA)] in the dark, washed with PBS, dried with ethanol and mounted in Mowiol with DAPI (Sigma-Aldrich). Samples were analyzed using a microscope (Olympus IX 81; Olympus, Tokyo, Japan) fitted with a confocal detection system (Olympus FV 1000; Olympus) and LCS Imaris 5.0.3 software (Bitplane, Zurich, Switzerland). Images were processed using IrfanView, DP Manager (Olympus) and GIMP software.

RESULTS

G1 checkpoint deficiency in mESCs is a consequence of persistent Cdk2 kinase activity after DNA damage

In somatic cells, G1 checkpoint activation in response to ionizing radiation (IR) leads to the rapid G1 arrest through inhibition of cyclin E-Cdk2 kinase activity [26]. Because mESCs do not arrest in G1 after DNA damage [9-11], we questioned the functionality of this mechanism in mESCs.

First, we verified the lack of a G1 arrest in V6.5 and HM-1 mESC lines. mESCs were treated with IR (4 Gy), and their cell cycle dynamics was analyzed by flow cytometry 1.5 and 3 h after IR. There were obviously fewer cells in G1 phase and more cells in S and G2/M after IR in both cell lines (Suppl. Fig. 1). The cell cycle profiles of irradiated mESCs clearly show an escape from G1 phase and thereby demonstrate the lack of G1 arrest after IR. The cell cycle analysis also indicated that V6.5 cells escape from G1 phase faster than HM-1 cells, as the V6.5 G1 phase cell numbers decrease (and the G2/M phase cell numbers increase) more rapidly than those of HM-1 cells. The increased speed of G1 escape in V6.5 cells might be due to their shorter generation time when compared to HM-1 cells.

mESCs display a unique cell cycle structure with an extremely short G1 phase [6, 7]. To analyze the DNA damage response in these cells during G1 phase, it was necessary to

increase the number of cells in this cell cycle phase. Therefore, both mESC lines V6.5 and HM-1 were synchronized in M phase by nocodazole treatment. After release from nocodazole arrest, cell cycle progression was monitored by flow cytometric analysis and cyclin A levels (Fig. 1 and Suppl. Fig. 2). Based on the results of these experiments, 1.5- and 3-h time points, corresponding to early and late G1 phase, respectively, were chosen for further analysis. As a control, mouse fibroblasts NIH3T3 were treated analogously. Early and late G1 phase in this cell line occurred 3 and 7 h after nocodazole release, respectively, as judged based on flow cytometric cell cycle progression analysis and cyclin A levels (data not shown).

After release from nocodazole-induced arrest, mESCs as well as control NIH3T3 cells were IR- or mock-treated in early and late G1 phases and harvested 1 and 3 h after the treatment. Subsequently, whole cell lysates were prepared and examined for Cdk2 activity in histone H1 kinase assays. The results were normalized to mock-treated cells for each particular time point. As expected, there was a major decrease in Cdk2 kinase activity after DNA damage in NIH3T3 cells (Fig. 2A). However, Cdk2 kinase activity was not significantly reduced after IR in mESCs (Fig. 2A and Suppl. Fig. 3A). This non-responsiveness of Cdk2 activity to IR in mESCs was corroborated by the analysis of the pRb phosphorylation status at the Cdk2-specific phosphorylation site Ser612 [35]. Whereas the decreased Cdk2 kinase activity after IR corresponded to decreased pRb-Ser612 phosphorylation in control NIH3T3 cells (at time points 7+1 and 7+3), the IR-induced phosphorylation of pRb at Ser612 remained unchanged in mESCs (Fig. 2B and Suppl. Fig. 3B).

Inhibitory phosphorylation of Cdk2 at Thr14/Tyr15 remains unchanged after IR in mESCs

In somatic cells, Cdk2 activity is inhibited in response to DNA damage by increased inhibitory phosphorylation at Thr14/Tyr15 [20, 36]. As shown in Figure 2C and Supplementary Figure 3C, Cdk2 phosphorylation at Thr14/Tyr15 remains unchanged after IR-induced DNA damage in mESCs when comparing the phosphorylation levels of IR-treated/non-treated cells at individual time points. By comparison, control NIH3T3 fibroblasts showed increased Thr14/Tyr15 phosphorylation levels after IR (Fig. 2C), as expected.

The p53-p21^{Cip1} G1 checkpoint pathway is activated after IR in mESCs

Because Cdk2 activity was not abrogated after DNA damage in mESCs, we asked whether the upstream components of G1 checkpoint pathways become activated in these cells after IR. Both mESCs and NIH3T3 cells were synchronized in M phase and, after nocodazole release, were then IR- or mock-treated at time points corresponding to early and late G1 phase as described above. Cell extracts were analyzed by western blot. Previous reports suggested that G1 checkpoint pathways may be compromised in mESCs [10, 11, 37]. However, in our experiments, the p53-p21^{Cip1} pathway was activated in mESCs similar to somatic cells (Fig. 3A) after IR-induced DNA damage. Comparable to somatic cells exposed to IR, p53 was phosphorylated at Ser18. In addition, the level of p21^{Cip1}, the major transcriptional target of p53, increased in mESCs after IR (Fig. 3A and Suppl. Fig. 4A). The marked increase of *p21* gene expression after DNA damage in both mESCs and NIH3T3 cells was confirmed by quantitative RT-PCR analysis (Suppl. Fig. 4B).

To rule out the possibility that differentiation may cause the observed IR-induced p21 expression in mESCs, we checked the differentiation status of both V6.5 and HM-1 mESCs using differentiation and pluripotency marker analysis. The quantitative RT-PCR analysis showed that expression of differentiation markers (*Brachyury, Cdx2, Gata4, Lamb1-1, Nestin, Runx2*) was low and that the expression of pluripotency markers (*Oct4* and *Dax1*) was sustained (Suppl. Fig. 4C), thus confirming the undifferentiated status of the V6.5 and HM-1 cells used in our study.

Chk1 kinase localizes to centrosomes in mESCs, and its phosphorylation at Ser345 is not increased after IR-induced DNA damage

In somatic cells, Chk1 kinase is implicated in the response to DNA damage throughout the cell cycle [26, 38, 39]. Chk1 localizes to the nucleus, and its expression is regulated by E2F [40]. Chk1 is active during unperturbed cell cycles [41, 42] and is significantly phosphorylated at Ser317 [43] and Ser345 [38] after DNA damage. We found that Chk1 is constitutively phosphorylated at Ser345 and that the phosphorylation level of Chk1 at Ser345 does not change after IR-induced DNA damage in mESCs (Fig. 3B and Suppl. Fig. 4D). In NIH3T3 cells, we did not observe any phosphorylation of Chk1 at Ser345 exposed to IR (data not shown), which is in agreement with a predominant role of Chk1 in response to UV irradiation in somatic cells [44]. In agreement with both a recent report on the centrosomal localization of Chk2 kinase in mESC line J11 [11] and our observation that Chk2 localizes to centrosomes in V6.5 and HM-1 mESCs (Suppl. Fig. 4E), we investigated Chk1 localization in these cells by immunofluorescence microscopy. Chk1 was entirely sequestered to the centrosomes in both mESC lines used (Fig. 3C and Suppl. Fig. 4F); its localization did not change after DNA damage (data not shown).

Cdc25A phosphatase is degraded in response to DNA damage, and its degradation is not regulated by Chk1/Chk2 kinases in mESCs

In agreement with previous reports on somatic cells [27, 45], but contrary to a previous report on mESCs [11] in which the Chk2-Cdc25A axis was reported to be non-functional due to centrosomal sequestration of Chk2, our analysis revealed activation of Chk2 in response to IR-induced DNA damage in mESCs; this response was comparable to Chk2 activation in NIH3T3 cells (Fig. 4A and Suppl. Fig. 5A). Correspondingly, we detected Cdc25A degradation after DNA damage in both control NIH3T3 cells and mESCs (Fig. 4A and Suppl. Fig. 5A).

Our observation of centrosomal localization of Chk1 (Fig. 3C and Suppl. Fig. 4F) and Chk2 (Suppl. Fig. 4E) made us question their role in degradation of Cdc25A after IR in mESCs. To shed more light on this issue, 30 min prior to IR, mESCs were treated with 500 nM Chk1 inhibitor CEP3891 [46] and 300 nM Chk2 inhibitor II [47]. The effectiveness of Chk1 and Chk2 inhibition was checked with Chk1 and Chk2 kinase assays (Suppl. Fig. 5B, C). Western blot analysis revealed that Cdc25A degradation after IR is non-responsive to Chk1 and Chk2 inhibition in mESCs (Fig. 4B and Suppl. Fig. 5D). Furthermore, Cdc25A localized to the cytoplasm (Suppl. Fig. 5E) and the nucleus in mESCs (Fig. 4 C and Suppl. Fig. 5F) as well as in NIH3T3 cells (Fig. 4C) and was not detectable at centrosomes in any cells. Taken together, we suggest that Chk1 and Chk2 are not responsible for Cdc25A degradation after IR in mESCs due to their centrosomal sequestration.

Cdk2 is predominantly localized to cytoplasm and centrosomes in mESCs

Having uncovered the localization discrepancy between Chk1/Chk2 and Cdc25A, we investigated the localization of Cdk2, the target of the G1 checkpoint, in both mESCs as well as NIH3T3 cells. In NIH3T3 fibroblasts, Cdk2 localized to the nucleus (Fig. 5) and the cytoplasm (data not shown). Some Cdk2 was also detectable at centrosomes by colocalization with the centrosomal marker γ-tubulin (Fig. 5A). In mESCs, Cdk2 localized predominantly to the cytoplasm (Suppl. Fig. 6) and centrosomes (Fig. 5A), and its localization did not change after DNA damage (Fig. 5B). In the context of Cdk2-Cdc25A interactions, the centrosomal abundance of Cdk2 is of special interest because this centrosomal population of Cdk2 may be excluded from regulation by Cdc25A after IR.

GSK-3β activity is required for IR-induced Cdc25A degradation in mESCs

Recently, glycogen synthase kinase-3β (GSK-3β) was reported to regulate Cdc25A proteolysis in G1 phase in somatic cells [48]. Therefore, we hypothesized that GSK-3β activity may regulate Cdc25A degradation after IR in mESCs. We blocked this kinase

using its inhibitors lithium chloride [49] (10 mM LiCl) or 6-bromoindirubin-3'-acetoxime [50] and investigated Cdc25A degradation under these conditions after DNA damage in both mESCs and NIH3T3 cells. The effectiveness of GSK-3β inhibition in our experiments was controlled by monitoring the increase of negative regulatory phosphorylation of GSK-3β at Ser9 and/or increase of β-catenin levels, which is targeted for degradation by active GSK-3β (Fig. 6A, B, and Suppl. Fig. 7A, B). Accumulation of β-catenin corresponded to increased expression of its target genes *Lef-1* [51, 52] and *Axin2* [53, 54] (Fig. 6C, Suppl. Fig. 7C). In mESCs, inhibition of GSK-3β completely abrogated IR-induced degradation of Cdc25A (Fig. 6A, B, and Suppl. Fig. 7A, B). On the other hand, GSK-3β inhibition had only little impact on Cdc25A degradation after IR in NIH3T3 fibroblasts (Fig. 6A), in which Chk1/Chk2 activity towards Cdc25A is intact.

Further, we investigated the localization of GSK-3β in mESCs and found it in the cytoplasm in both mock-treated (Fig. 6D and Suppl. Fig. 7D) and IR-treated cells (Suppl. Fig. 7E), *i.e.* in the same cell compartment where Cdc25A is localized. These observations collectively suggest a regulatory role for GSK-3β in IR-induced Cdc25A degradation in mESCs.

Downregulation of Cdk2 activity slows escape of mESCs from G1 after DNA damage

Based on our observation that Cdk2 activity is not abrogated after DNA damage in mESC, we hypothesized that high and persistent Cdk2 activity may be the driving force of G1 escape after DNA damage and, thus, the cause of the lack of G1 arrest after DNA damage in mESCs. To test this hypothesis, we used olomoucine II, a potent Cdk2 inhibitor [55], to downregulate Cdk2 activity and monitor the effects of Cdk2 inactivation on mESC cell cycle dynamics after DNA damage. Downregulating Cdk2 activity to 50% by 5 μM olomoucine II (Fig. 7A) significantly slowed the decrease of cells in G1 after IR (Fig. 7B, Suppl. Fig. 8) and slowed down the G1 escape of mESCs. This observation corroborates the central role of unimpaired Cdk2 activity in rapid, DNA-damage non-responsive escape of mESCs from G1 phase.

DISCUSSION

G1 checkpoint pathways are well described in somatic cells. However, to date, there is only little data available with regard to the lack of a G1 checkpoint response in mESCs.

In somatic cells, Cdk2 kinase is the key G1/S transition-promoting enzyme. Its activity towards its substrates, such as pRb, is both rate-limiting and essential for S phase entry. In response to DNA damage, Cdk2 kinase is targeted by the G1 checkpoint to abrogate its activity and, thus, to prevent S phase entry. mESCs are known for their inability to arrest in G1 phase after DNA damage. Instead, mESCs with damaged DNA are preferentially eliminated through apoptosis [10]. Here, we show that the absence of a G1 arrest in mESCs after IR-induced DNA damage is caused by the insensitivity of Cdk2 kinase activity to DNA damage.

Sustained Cdk2 kinase activity in mESCs seems to be crucial for transition from G1 to S phase after DNA damage, as downregulating Cdk2 activity slowed the G1 escape after IR in mESCs. DNA damage-refractory Cdk2 activity may be important for maintenance of pluripotency in ESCs because, as recently showed by Neganova *et al.* [56] and by us [57], downregulating Cdk2 activity in ESCs leads to their differentiation.

Persistent Cdk2 kinase activity after DNA damage in mESCs was confirmed also by unchanged level of phosphorylation at pRb-Ser612, the Cdk2 target site. Recently, it was shown that pRb-Ser612 is also phosphorylated by Chk1/Chk2 after DNA damage [58]. This activity may be responsible for the sustained level of pRb-Ser612 phosphorylation observed in NIH3T3 cells after IR in early G1 phase, which did not correspond to the decreased Cdk2 activity observed at the time points 3+1 and 3+3. Nevertheless, Chk1/Chk2 are probably not involved in the phosphorylation of pRb in mESCs because these proteins

localize to different cell compartments (our observation, data not shown) and Cdk2 activity remains sufficiently high in these cells to fulfill this task.

Cdk2 activity in somatic cells is abrogated by Chk1/Chk2-mediated degradation of Cdc25A phosphatase [26, 27], which is required for Cdk2 activation. We found that both Chk1 and Chk2 kinases are localized to centrosomes in mESCs and, therefore, are unavailable to participate in Cdc25A degradation. Correspondingly, Chk1-Ser345 phosphorylation, which is essential for Chk1-Cdc25A signal transmission [42], did not increase after DNA damage. Instead, Chk1 was constitutively phosphorylated at Ser345, which may be necessary for proper localization of Chk1 to centrosomes [59]. In contrast to Chk1 kinase, Chk2 was regularly activated after IR despite its centrosomal sequestration in mESCs. We confirmed the disconnected signaling between Chk1/Chk2 and Cdc25A by dual inhibition of Chk1 and Chk2, which did not abrogate Cdc25A degradation in mESCs. Instead, we found that GSK-3β regulates the level of Cdc25A phosphatase in response to IR in mESCs. Fittingly and in contrast to Chk1/Chk2 and Cdc25A, GSK-3β and Cdc25A localize to the same cellular compartment in mESCs. GSK-3β activity influences the maintenance of mESCs properties, such as self-renewal, propagation, and differentiation [60]. In addition, phosphorylation of Cdc25A by GSK-3β has already been described in somatic cells, where GSK-3β participates in Cdc25A degradation during early G1 phase [48]. However, which signaling molecules transmit the DNA damage signal to GSK-3β in mESCs remains unknown.

Although Cdc25A was efficiently degraded after DNA damage in a GSK-3β-dependent manner, Cdc25A degradation was not sufficient to abrogate Cdk2 activity in mESCs. Correspondingly, Cdk2 phosphorylation at Thr14/Tyr15, the target sites of Cdc25A phosphatase, remained unchanged after DNA damage. A possible explanation for this finding may be that a sizable proportion of Cdk2 also localizes to centrosomes in mESCs, which may be sheltered from any changes in cellular Cdc25A levels, as we did not find detectable amounts of Cdc25A at the centrosomes by immunofluorescence staining. These results lend further support of a role for centrosomes in the regulation of cell cycle progression and checkpoint response in mESCs, as suggested by earlier observations [11]. Also, the concept of centrosomes as cell cycle control centers has long been suggested for somatic cells [61].

Wee1/Myt1 phosphorylate Cdk2-Thr14/Tyr15 [16-18], thus having a function opposing Cdc25A. Because Cdc25A is degraded after DNA damage in mESCs, Wee1/Myt1 activity would have to be abrogated for the Thr14/Tyr15 phosphorylation level to remain stable when Cdc25A is degraded. Whether Wee1/Myt1 activity is regulated by DNA damage in mESCs and, if so, what is the mechanism of abrogating Wee1/Myt1 activity towards Cdk2 remains to be determined.

In mESCs, the functional status of the alternative G1 checkpoint pathway, p53-$p21^{Cip1}$ is disputed. Results on p53 activation by Ser18 phosphorylation after IR in mESCs are contradictory [11, 37]. Aladjem *et al.* [10] have suggested that the lack of increased p21 expression after DNA damage in mESCs is due to the inability of p53 to translocate into the nucleus after DNA damage. It was also reported that the p53-$p21^{Cip1}$ pathway becomes functional in mESCs upon their differentiation [10] and that p21 expression is repressed in undifferentiated mESCs by ESC-specific micro RNAs [62]. In contrast, another study [63] suggested a p53-dependent suppression of Nanog expression as an alternative pathway to maintain genetic stability in mESCs, which would obviously require nuclear localization of p53. In contrast to mESCs, a rapid and robust induction of $p21^{Cip1}$ upon IR in human ESCs has been reported [64]. Although some of the discrepancies observed by different groups may be explained by the use of different ESC lines, it is difficult to reconcile between these studies. Nevertheless, our study revealed p53 activation, increased *p21* expression and accumulation of $p21^{Cip1}$ after IR in mESCs that is similar to the situation in human ESCs and somatic cells. Because Cdk2 activity was unaffected by IR, these findings suggest that

Cdk2 activity is refractory to both $p21^{Cip1}$ accumulation as well as Cdc25A degradation in mESCs. As discussed above, a possible explanation may be the centrosomal delocalization of a significant portion of Cdk2 molecules. These molecules could be, by an unknown mechanism, protected from p21 binding. An alternative explanation may be the requirement for equimolar concentrations of $p21^{Cip1}$ and cyclin/Cdk2 complexes for efficient Cdk2 inhibition [65]; the observed $p21^{Cip1}$ protein accumulation in mESCs in our study may be insufficient to buffer high concentrations of Cdk2.

CONCLUSION

This study shows that mESCs regularly activate both the immediate and delayed G1 checkpoint pathways known in somatic cells in response to DNA damage. However, these pathways do not impact the activity of Cdk2, which is predominantly localized at centrosomes in mESCs, where it may be sheltered from regulation by nuclear Cdc25A. Furthermore, we show that Cdc25A phosphatase is degraded after DNA damage in mESCs, but this degradation is not mediated by Chk1 and Chk2, which are sequestered to centrosomes. Instead, this degradation seems to be regulated by GSK-3β kinase.

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Figure 1. Monitoring the cell cycle progression of synchronized mESCs. Cells were collected at the indicated time points after release from nocodazole, and their cell-cycle progression was monitored by flow cytometry (A) and western blot analysis (B). A. Cell-cycle profiles of synchronized V6.5 cells. Hyperdiploid cells (>2n) were excluded from assessment of cell cycle distribution. B. Immunoblots for cyclin A in synchronized V6.5 cells. Staining for β-actin was used as a loading control.

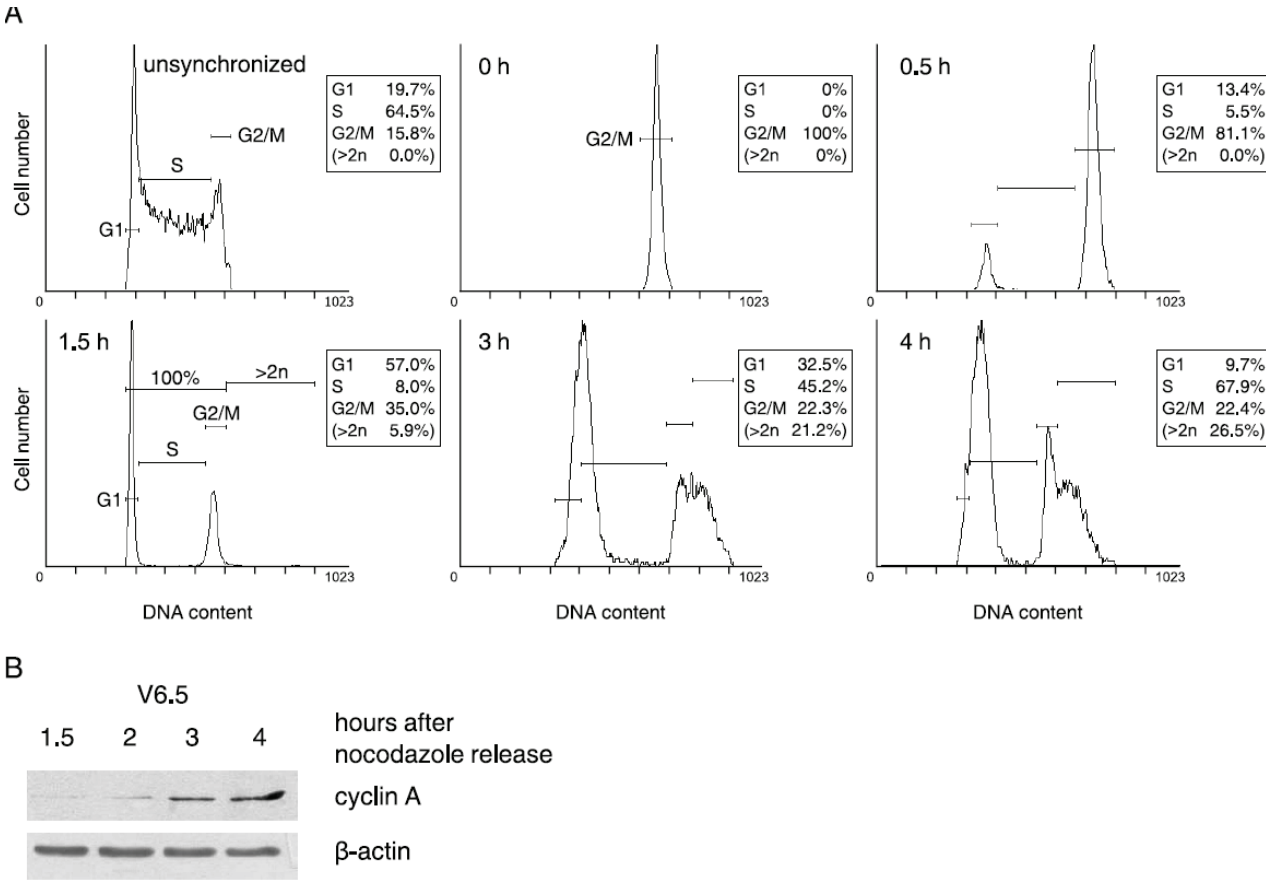


Figure 2. Activity of Cdk2 kinase is not decreased in response to DNA damage in mESCs. A. Cdk2 kinase activity in synchronized V6.5 and NIH3T3 cells after mock- or IR-treatment (black and grey columns, respectively). The data represent the mean of three independent experiments; bars designate standard deviations. *P < 0.05, **P < 0.005. In V6.5 cells, Cdk2 activity changes after IR were not statistically significant: P = 0.18, 0.06, 0.11 and 0.08 for time points 1.5+1, 1.5+3, 3+1 and 3+3, respectively. B. pRb Ser612 phosphorylation [pRb-(P)Ser612] does not change after DNA damage in mESCs. C. Inhibitory phosphorylation of Cdk2 on Thr14/Tyr15 [Cdk2-(P)Thr14/Tyr15] does not increase after DNA damage in mESCs. Designation of time points: time (in hours) after nocodazole release in which cells were irradiated plus time (in hours) after IR in which cells were collected for lysates.

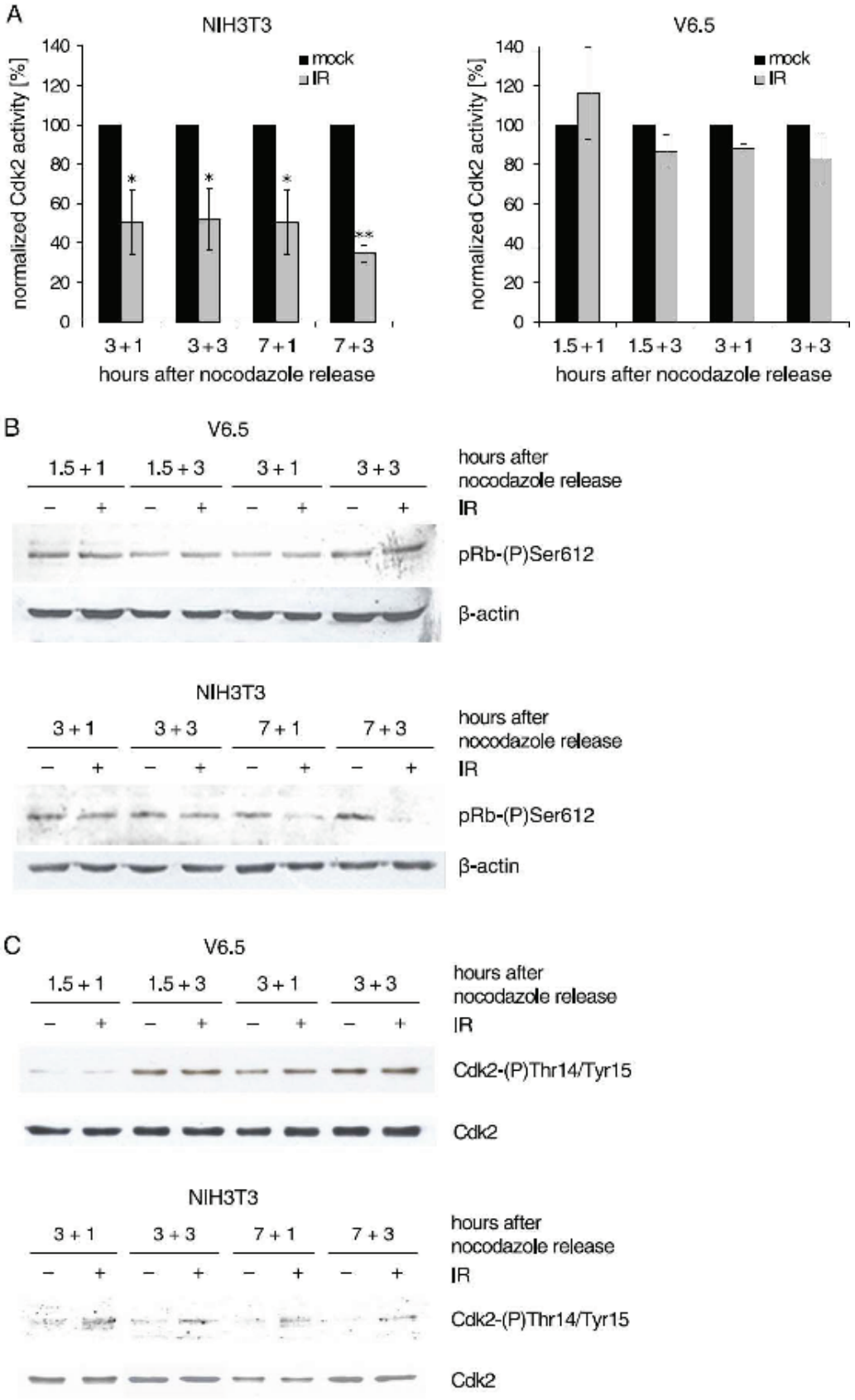
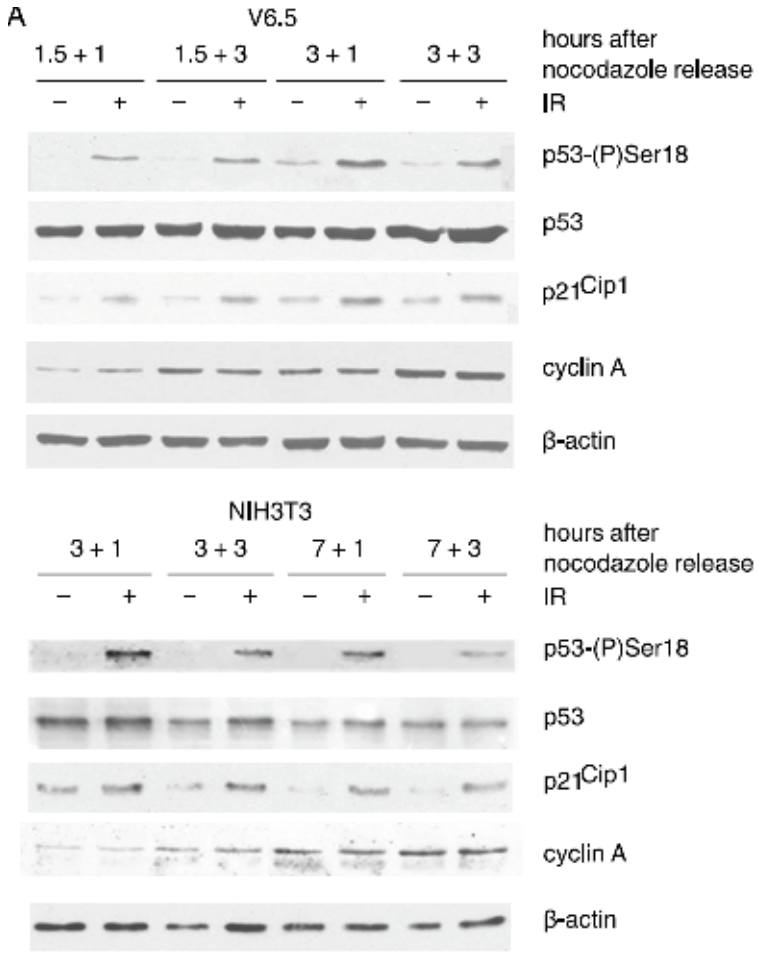
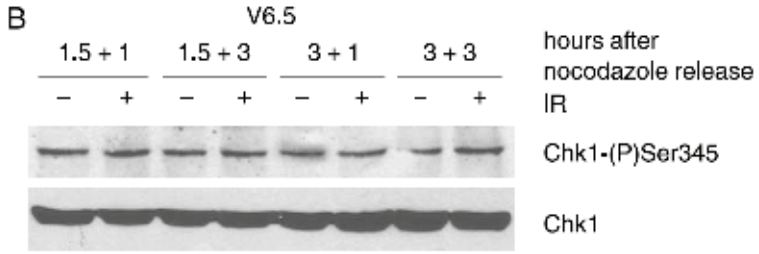

A
NIH3T3
V6.5
mock
IR
normalized Cdk2 activity [%]
0
20
40
60
80
100
120
140
3+1
3+3
7+1
7+3
1.5+1
1.5+3
3+1
3+3
hours after nocodazole release
B
V6.5
hours after nocodazole release
IR
pRb-(P)Ser612
β-actin
NIH3T3
C
V6.5
Cdk2-(P)Thr14/Tyr15
Cdk2
NIH3T3

Figure 3. Characterization of the p53–$p21^{Cip1}$ pathway and Chk1 phosphorylation and localization in mESCs. A. The p53-$p21^{Cip1}$ pathway is activated after DNA damage in both V6.5 mESCs and NIH3T3 cells. Staining for cyclin A and β-actin were performed to check cell cycle phase and protein loading, respectively. Time points are designated as in Fig. 2. B. Levels of Chk1 phosphorylation at Ser345 [Chk1-(P)Ser345] do not change after IR in V6.5 mESCs. C. Chk1 localizes entirely to centrosomes in V6.5 mESCs. After extraction of soluble proteins, V6.5 cells were fixed with methanol. Markers: γ-tubulin - centrosomes; DAPI - DNA. Scale bar, 5 μm.


A
V6.5
1.5 + 1
1.5 + 3
3 + 1
3 + 3
hours after nocodazole release
– + – + – + – +
IR
p53-(P)Ser18
p53
p21Cip1
cyclin A
β-actin
NIH3T3
3 + 1
3 + 3
7 + 1
7 + 3
hours after nocodazole release
– + – + – + – +
IR
p53-(P)Ser18
p53
p21Cip1
cyclin A
β-actin


B
V6.5
1.5 + 1
1.5 + 3
3 + 1
3 + 3
hours after nocodazole release
– + – + – + – +
IR
Chk1-(P)Ser345
Chk1

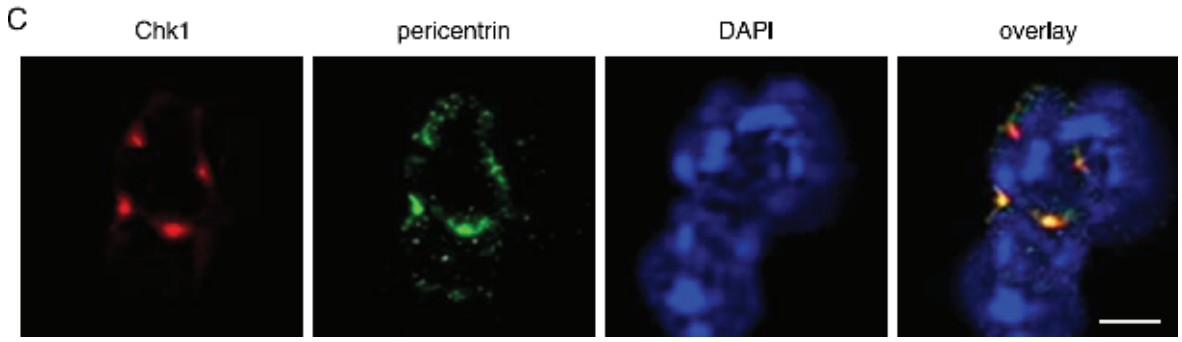

C
Chk1
pericentrin
DAPI
overlay

Figure 4. Characterization of the Chk2–Cdc25A pathway in mESCs. A. Chk2 is activated, and Cdc25A is degraded in mESCs. The phosphorylation status of Chk2 was detected by a mobility shift of the Chk2-specific band [66-69]. Time points are designated as in Fig. 2. B. Cdc25A degradation after DNA damage is not regulated by Chk1/Chk2 in mESCs. V6.5 and NIH3T3 cells were mock-treated (DMSO) or treated with CEP3891 and Chk2 inhibitor II for 30 min, IR- or mock-treated and collected 1 h after IR. C. Cdc25A localizes to nuclei in mESC line V6.5 and NIH3T3 fibroblasts. After extraction of soluble proteins, the cells were fixed with methanol. Markers: γ-tubulin - centrosomes; DAPI - DNA. Scale bar, 5 μm.

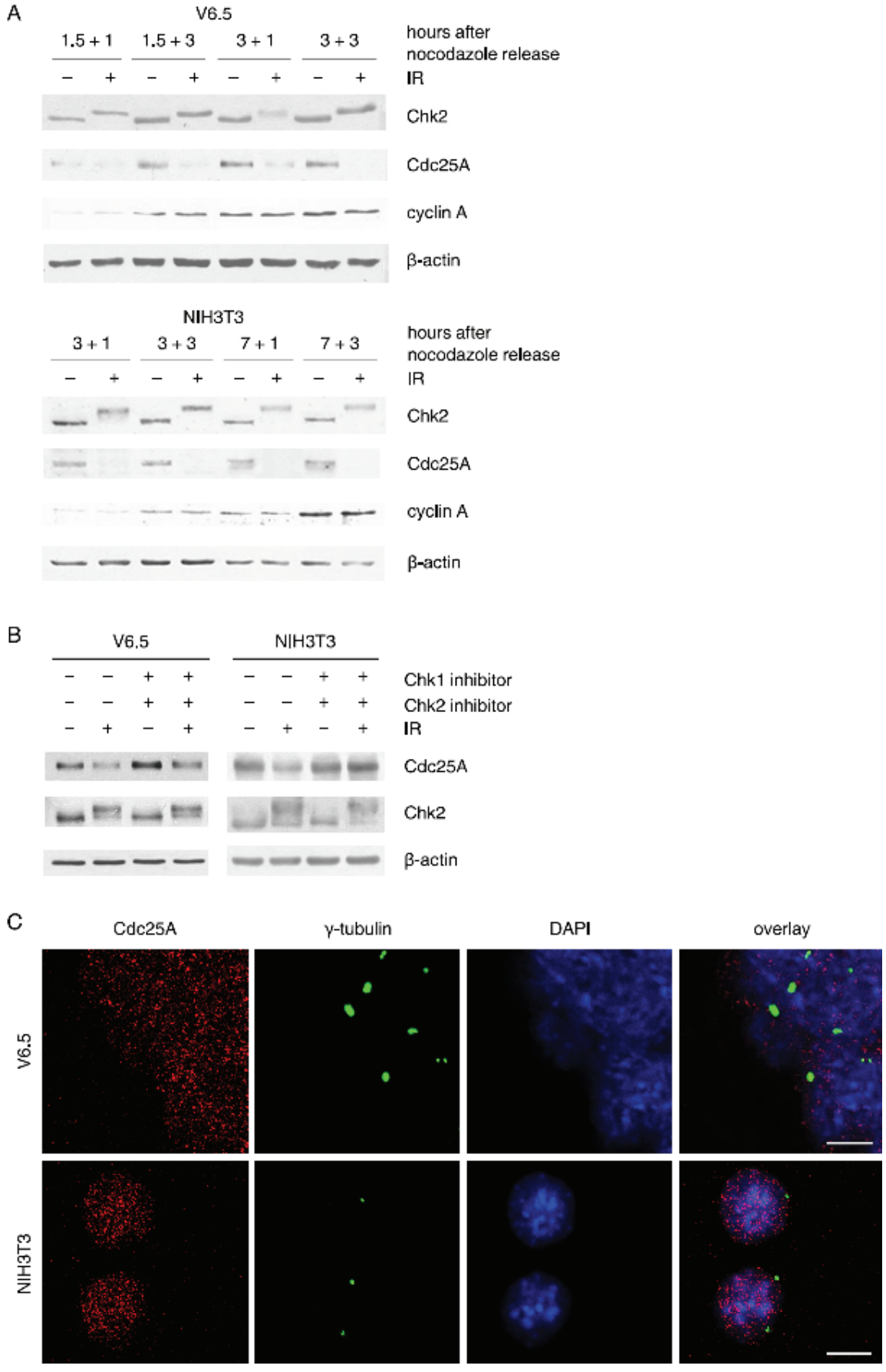

A
V6.5
1.5 + 1
1.5 + 3
3 + 1
3 + 3
hours after nocodazole release
– + – + – + – +
IR
Chk2
Cdc25A
cyclin A
β-actin
NIH3T3
3 + 1
3 + 3
7 + 1
7 + 3
hours after nocodazole release
– + – + – + – +
IR
Chk2
Cdc25A
cyclin A
β-actin
B
V6.5
NIH3T3
– – + + – – + +
Chk1 inhibitor
– – + + – – + +
Chk2 inhibitor
– + – + – + – +
IR
Cdc25A
Chk2
β-actin
C
Cdc25A
γ-tubulin
DAPI
overlay
V6.5
NIH3T3

Figure 5. Cdk2 localizes predominantly to centrosomes in mESCs. Immunolocalization of Cdk2 in methanol-fixed NIH3T3, V6.5 and HM-1 cells after extraction of the soluble protein pool. Prior to fixation, mESCs were either mock- (A) or IR-treated (B). Cytoplasmic remnants can be seen in V6.5 and HM-1 cells because the soluble proteins could not be completely removed because of the tight growth of mESCs in colonies. Markers: γ-tubulin - centrosomes; DAPI - DNA. Scale bar, 5 μm.

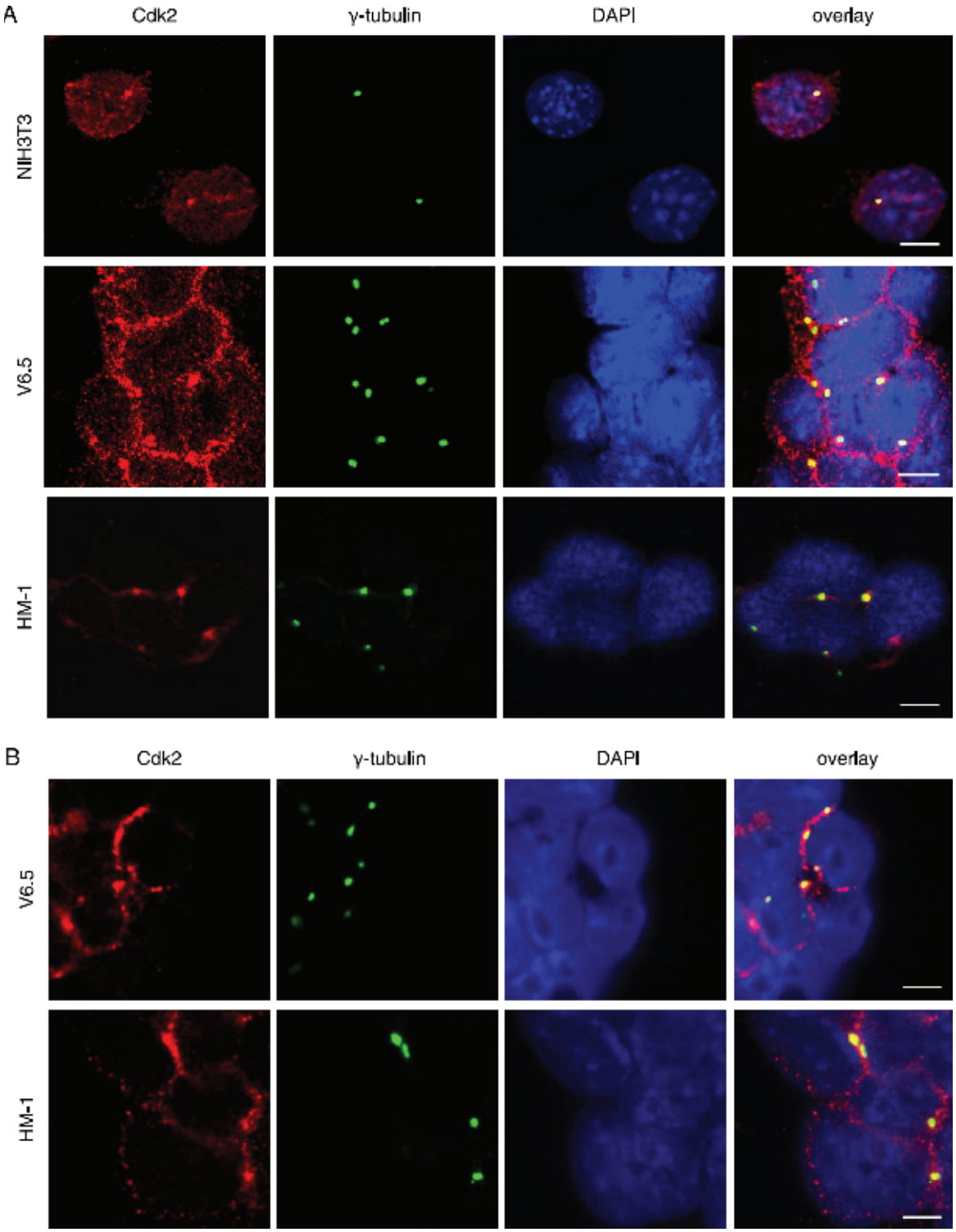


Figure 6. Cdc25A degradation is regulated by GSK-3β in mESCs. A. V6.5 and NIH3T3 cells were mock- or LiCl-treated for 2.5 h. Afterwards, they were IR- or mock-treated and lysed 0.5 or 1.5 h after IR. Abrogation of GSK-3β activity was confirmed by increased levels of GSK-3β-Ser9 inhibitory phosphorylation as well as by the accumulation of β-catenin. B. V6.5 cells were treated with 6-bromoindirubin-3'-acetoxime (BIOac) or mock-treated for 2.5 h, IR- or mock-treated and collected 3 h after IR. Abrogation of GSK-3β activity was confirmed by the accumulation of β-catenin. C. Relative expression of β-catenin target genes after BIOac treatment. *Axin1*, an *Axin2* homolog insensitive to β-catenin [70], was used as a control. Gene expression was analyzed by quantitative RT-PCR and normalized to the expression of the housekeeping gene *Hmbs.* The data represent the mean from three independent experiments, and bars designate standard deviation. Statistical significance was assessed using Student's t-test: *P < 0.05 and **P < 0.005. D. GSK-3β is localized to the cytoplasm in V6.5 cells. V6.5 cells were fixed with methanol-acetone. Nuclei were stained with DAPI. Scale bar, 5 μm.

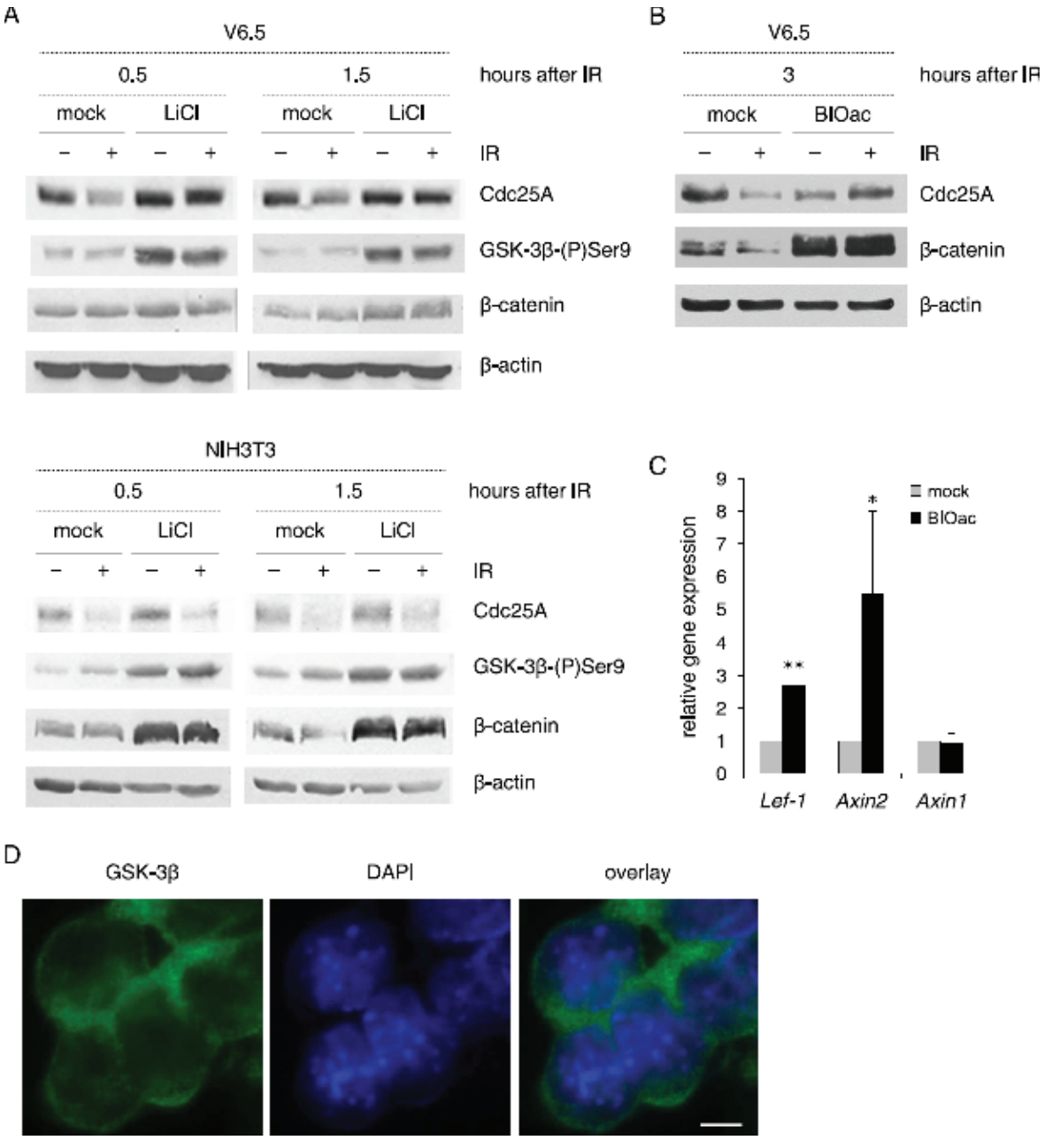
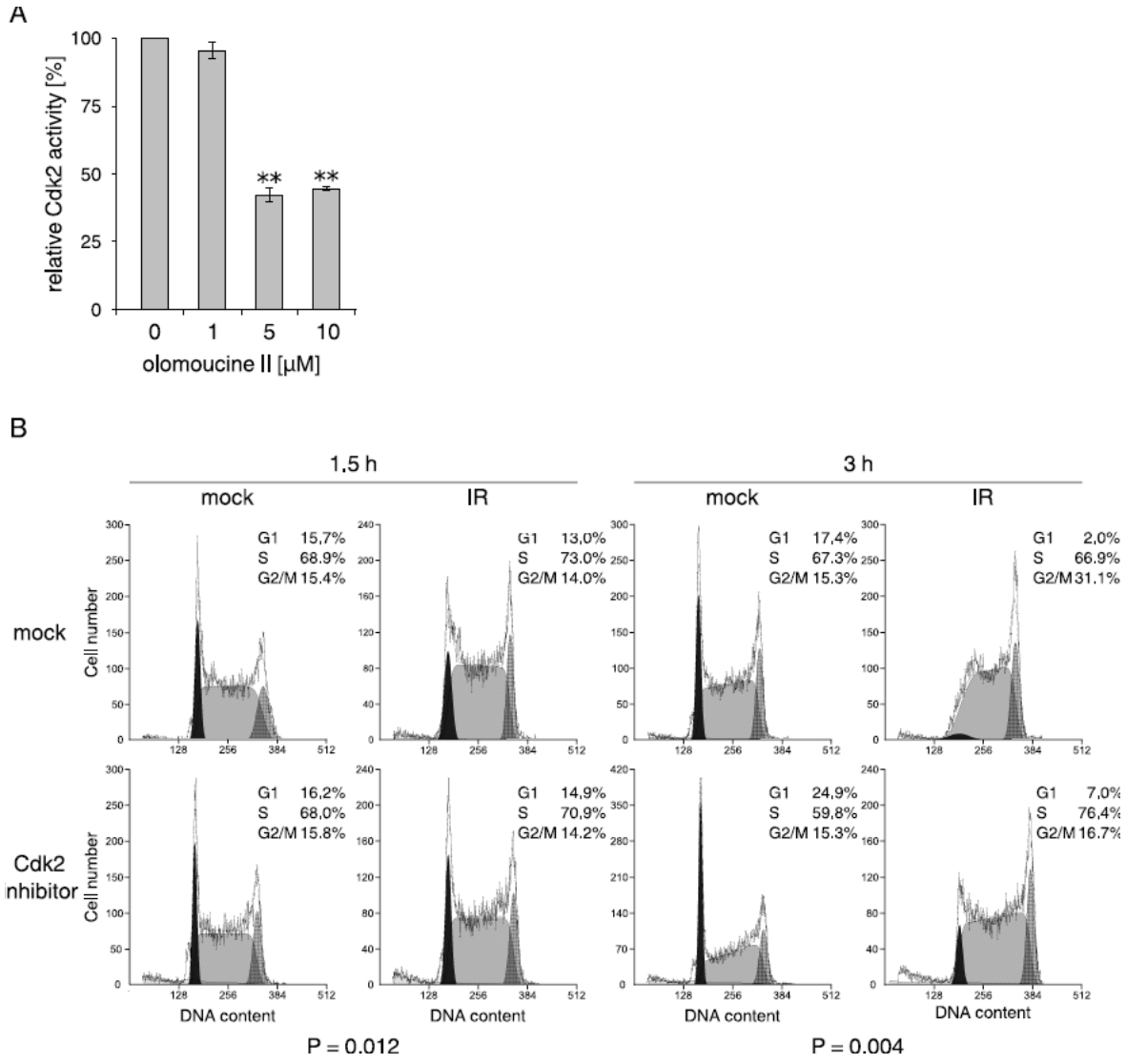


Figure 7. Downregulation of Cdk2 activity slows G1 escape after DNA damage in mESCs. A. Downregulation of Cdk2 activity by olomoucine II. mESCs were treated for 1 h with the indicated concentrations of olomoucine II, a Cdk2 inhibitor, and Cdk2 activity was measured in histone H1 kinase assays. The data represent mean of three independent experiments; bars designate standard deviations. **P < 0.005. B. V6.5 cells were mock-treated or treated for 1 h with 5 μM olomoucine II, they were then IR- or mock-treated, collected 1.5 h or 3 h after IR and their cell cycle profiles were analyzed by flow cytometry. The data represent three independent experiments. The P value expresses the statistical significance of differences in the number of cells in G1 after IR in Cdk2 inhibitor-treated cells compared to mock-treated cells.



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Cdk2 Inhibition Prolongs G1 Phase Progression in Mouse Embryonic Stem Cells

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Embryonic stem cells (ESCs) proliferate rapidly and have a unique cell-cycle structure with a very short G1 phase. Previous reports suggested that the rapid G1 phase progression of ESCs might be underpinned by high and precocious Cdk2 activity and that Cdk2 activity might be crucial for both cell-cycle regulation and cell-fate decisions in human ESCs. However, the actual role of Cdk2 in cell-cycle progression of mouse ESCs (mESCs) has not been elucidated. In this study, we investigated the effects of down-regulation of Cdk2 activity by olomoucine II in 2 mESC lines. Olomoucine II treatment significantly increased the G1 phase cell numbers, decreased the S phase cell numbers, and inhibited DNA replication in mESCs. In nocodazole-synchronized mESCs, we show that specific down-regulation of Cdk2 activity prolongs G1 phase progression. In addition, down-regulation of Cdk2 activity in mESCs established a somatic cell-like cell cycle and induced expression of differentiation markers. Our results suggest that high Cdk2 activity is essential for rapid G1 phase progression and establishment of ESC-specific cell-cycle structure in mESCs and support the hypothesis of a link between cell-cycle regulation and pluripotency maintenance in ESCs. This study reveals olomoucine II to be an effective tool for manipulation of the cell cycle and pluripotency in ESCs and very likely also for the manipulation of other stem cell types, including cancer stem cells.

Introduction

Embryonic stem cells (ESCs) proliferate rapidly and their cell-cycle regulation and structure have unique characteristics that distinguish them from somatic cells. ESCs lack a functional restriction point and do not rely on persistent serum stimulation for continuous proliferation [1–4]. The cell-cycle progression of ESCs is rapid: the generation time of mouse ESCs (mESCs) is ~10 h [2,3]. The cell cycle of ESCs has an unusual structure, as it consists largely of the S phase and has a truncated G1 phase [1–6]. This is reminiscent of the cells of early developing animal embryos, for example, *Xenopus* [7], *Danio* [8], or *Drosophila* [9], where gap phases are completely lacking and cell cycle consists of alternating S and M phases.

At the molecular level, the rapid cell-cycle progression of ESCs in vitro has been described as the result of an unusually high and precocious Cdk2 activity in mouse ESCs (mESCs) that appears to be cell-cycle-independent [3]. In contrast, Cdk2 activity in somatic cells is tightly regulated as Cdk2 is involved in both G1/S transition and initiation of DNA replication [10,11], processes that require proper timing and cellular conditions to ensure flawless genome replication and production of viable progeny [12]. Proliferation in vivo in early embryogenesis appears to be independent of Cdk2 [13,14], suggesting that the G1/S phase-associated Cdks can substitute for each other [15–17]. The study by Stead et al. [3] suggested that the absolute level of Cdk2 activity in mESCs determines cell division rates. A recent study in human ESCs (hESCs) suggested that Cdk2 activity might be crucial for both cell-cycle regulation and cell-fate decisions because siRNA knockdown of Cdk2 resulted in G1 phase arrest and differentiation of hESCs to extraembryonic lineages [18]. But the actual role of Cdk2 in cell-cycle progression of mESCs is not fully understood, and, moreover, Cdk2 is predominantly localized on centrosomes in mESCs (our observation, data not shown), which makes its role in mESC cycle regulation even more obscure.

Selective chemical Cdk inhibition is a useful tool for deciphering the roles and requirements in cells of individual Cdks [19]. In our study, we used this approach to investigate the role of Cdk2 in G1 phase progression of mESCs. We took

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advantage of 2 new generation Cdk inhibitors: olomoucine II and CAN508. We used olomoucine II to inhibit Cdk2 activity in mESCs. As olomoucine II is a potent inhibitor of both Cdk2 and Cdk9 [20], we used also CAN508, a specific Cdk9 inhibitor [21], to distinguish the effects of Cdk2 inhibition from those of Cdk9 inhibition. Cdk2 inhibition by olomoucine II treatment efficiently increases the proportion of G1 cells in mESC culture. In nocodazole-synchronized cells, we show that this is due to decelerated progression of mESCs through the G1 phase. Prolonged down-regulation of Cdk2 activity established a somatic cell-like cell cycle and induced morphology and gene expression changes indicative of mESC differentiation. Based on kinase assays, on comparison of the results of CAN508 and olomoucine II treatment in mESCs, and on the effects of Cdk2 knockdown by siRNA, we suggest that the observed cell-cycle structure changes and induction of differentiation in mESCs is the result of down-regulation of Cdk2 activity after olomoucine II treatment. Our results suggest that the high Cdk2 activity plays an important role in the regulation of rapid G1 phase progression as well as in the establishment of the ESC-specific cell-cycle structure and maintenance of pluripotency in mESCs.

Materials and Methods

Cell culture and synchronization

For our studies, 2 different mESC lines were used; an inbred HM1 cell line derived from 129 mouse strain [22] and an F1 (129SvJae×C57BL/6) hybrid line V6.5 (Open Biosystems, Huntsville, AL). The total in vitro culture time of these mESCs at the time of experimental testing was equivalent to passage number 22–35 of the original mESC line. Both mESC lines were checked repeatedly for chromosome number and possible karyotypic abnormalities. mESC culture was carried out following standard procedures [23]. In brief, the cells were maintained on culture dishes covered with 0.1% gelatin (Sigma-Aldrich, St. Louis, MO) in a humidified atmosphere of 10% CO_2 at 37°C in high-glucose Dulbecco's modified Eagle's medium (D-MEM) with GlutaMAX (Invitrogen/Gibco, Grand Island, NY) supplemented with 15% fetal bovine serum (FBS; Thermo Fisher Scientific/HyClone, Waltham, MA), 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin (Gibco), 0.1 µM β-mercaptoethanol (Serva), and 1000 U/mL leukemia inhibitory factor (ESGRO; Chemicon, Temecula, CA).

Human colon adenocarcinoma cell line HT29 (ATCC) was maintained in a humidified atmosphere of 10% CO_2 at 37°C in high-glucose D-MEM with GlutaMAX supplemented with 10% FBS (Gibco), 100 U/mL penicillin, and 100 µg/mL streptomycin.

mESCs were synchronized in G2/M phase by 400 nM nocodazole (Sigma-Aldrich) treatment for 12 h. After mitotic shake-off, cells were washed 3 times with phosphate-buffered saline (PBS) with 1% FBS (Gibco) and plated in standard mESC media.

Inhibitors

Olomoucine II (2-{[2-((1-*R*)-1-hydroxymethyl-propylamino)-9-isopropyl-9*H*purin-6-ylamino]-methyl}-phenol) and CAN508 (4-[(3,5-diamino-1*H*-pyrazol-4-yl)diazenyl]phenol) were synthesized according to published procedures [21,24]. Both olomoucine II and CAN508 were stored as 100 mM stock solutions in DMSO.

MTT assay

Equal numbers of cells per well (7,000 for mESCs, 5,000 for HT29) were plated on a 96-well plate and incubated for 3 h (mESCs) or 24 h (HT29) under standard conditions. Triplicate samples of these cells were treated with increasing concentrations of inhibitors (range from 10^{-8} to 10^{-4} M) or mock-treated and incubated for 24 h (mESCs) or 72 h (HT29) under standard conditions. After this time, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; Sigma-Aldrich) was added to a final concentration 0.5 mg/mL and the cells were incubated for another 2.5 h under standard conditions. The media was then removed, 100 µL of 10% sodium dodecyl sulfate (SDS) was added per well, and the 96-well plate was incubated overnight at room temperature on a shaker. The absorbance was read at 570 nm. To calculate IC_{50} values (concentrations that produce a 50% of inhibitory effect on cell proliferation), the results from all triplicates were transformed to percentage of controls, and plotted as sigmoid dose–effect curves using a nonlinear regression mode and the GraphPad Prism 5 software. Using this software, the IC_{50} values were interpolated.

Flow cytometric analysis

Cell-cycle distribution was evaluated by 5-bromo-2′-deoxyuridine (BrdU) incorporation and propidium iodide staining. Cells were pulsed with 10 µM BrdU (Sigma-Aldrich) for 30 min, trypsinized (0.5% trypsin–ethylenediaminetetraacetic acid [EDTA]; Gibco) to obtain a single cell suspension, washed twice in PBS with 1% FBS, and resuspended in PBS. The cells were then fixed in ice cold 70% ethanol. After rehydration in PBS with 1% FBS (Gibco), cells were incubated in 2 M HCl with 0.5% (v/v) Triton X-100 for 30 min at room temperature. Following neutralization with 0.1 M $Na_2B_4O_7$, cells were collected by centrifugation and washed with PBS with 1% FBS and 0.5% (v/v) Tween-20. Then they were stained with anti-BrdU fluorescein isothiocyanate (FITC)-labeled antibody (1:20; Roche Diagnostics, Mannheim, Germany) for 30 min at room temperature in darkness. The cells were then washed with PBS with 1% FBS and 0.5% (v/v) Tween-20 and incubated in 1.1% sodium citrate dehydrate with 5 ng/µL ribonuclease A (DNA Lego Ribonuclease A; Top-Bio, Prague, Czech Republic) and 60 µg/µL propidium iodide (Sigma-Aldrich) for 30 min at 37°C in the dark. Cells were analyzed by flow cytometry on the Cytomics FC 500 machine using the CXP software (Beckman Coulter, Fullerton, CA) and following standard procedures. Analysis by MultiCycle (Phoenix Flow Systems) was applied to assess cell-cycle distribution.

Western blot analysis

Cells on culture dishes were washed in cold PBS, collected into IP buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM ethyleneglycoltetraacetic acid (EGTA), 10% (v/v) glycerol, 0.1% (v/v) Tween-20, 1 µM dithiothreitol (DTT), 1 µM NaF, 10 µM β-glycerophosphate, 10 µg/mL leupeptin, 2 µg/mL aprotinin, 0.1 µM Na_3VO_4, 0.1 µM PMSF) and incubated for 1 h at 4°C. Lysates were cleared

by centrifugation at 18,800*g* at 4°C for 30 min. Proteins were electrophoretically resolved on SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes (Bio-Rad). Membranes were incubated at 4°C overnight with primary antibodies [anti-α-tubulin (Sigma-Aldrich); Cdk2 (M2) (Santa Cruz Biotechnology, Santa Cruz, CA); RNA polymerase II antibody [H5], RNA Polymerase II antibody [8WG16] (Abcam)] at dilution recommended by the supplier, washed in PBS with 0.05% Tween, and incubated for 1 h with anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (DakoCytomation, Glostrup, Denmark). HRP activity was detected with an ECL detection kit (Pierce) on films (Bio-Rad, Hercules, CA).

Immunoprecipitation and kinase assay

Immunoprecipitations were conducted at 4°C. Protein A Agarose Beads (Sigma-Aldrich) were incubated for 1 h on a rotor in 1 mL IP buffer with 5 µL Cdk2 antibody (Cdk2 (M2); Santa Cruz Biotechnology) or 4 µL Cdk1 antibody (Anti-cdk1/cdc2, CT; Upstate Biotechnology, Lake Placid, NY). After washing 3 times with IP buffer, the agarose beads were incubated for 1 h with protein lysates (200 µg of proteins) in IP buffer on a rotor and finally washed 3 times with IP buffer. Agarose beads with immunocomplexes were equalized with kinase assay buffer (KAB; 50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 2.5 mM EGTA, 100 µM β-glycerophosphate, 2 µM NaF, 1 µM DTT, 0.1 µM Na_3VO_4). They were then resuspended in 30 µL kinase reaction mixture (18 µL KAB, 9 µL 75 µM adenosine triphosphate (ATP) in KAB, 2 µg histone H1, 1 µL [γ-^{33}P]ATP (10 µCi; MP Biochemicals, Irvine, CA) and incubated for 30 min at 30°C. Reactions were terminated by addition of 12 µL of 4× Loading sample buffer (8% SDS, 40% glycerol, 400 mM DTT, 240 mM Tris, pH 6.8, 0.004% bromophenol blue). Samples were boiled and electrophoretically separated on 8%–12% SDS-polyacrylamide gels. Radioactivity of the dried gels was detected with the bioimager BAS 1800 (Fuji Photo Film Co., Ltd.).

Statistical analysis

Statistical evaluation of the data was performed with the independent 2-sample *t*-test using Statistica 8.0. $P < 0.05$ was considered statistically significant.

RNA isolation and quantitative real-time RT-PCR

Total RNA from cells was isolated using TRI Reagent (Ambion) and treated with DNAse (TURBO DNA-free, Ambion). One microgram of total RNA was used for reverse transcription by First Strand cDNA Transcriptor Synthesis Kit (Roche Applied Science) according to the manufacturer's instructions. The relative quantity of target genes' RNA was determined by real-time quantitative polymerase chain reaction (PCR) using a LightCycler 480 (Roche). Gene-specific primer pairs (listed in Supplementary Table 1; Supplementary materials are available online at http://www.liebertpub.com/) were designed and evaluated at an annealing temperature of 60°C using freely available Web-based software in the Universal ProbeLibrary Assay Design Center. The amplification mix and program were prepared using LightCycler 480 SYBR Green I Master (Roche) according to the manufacturer's instructions. Data were analyzed by using LightCycler 480 Relative Quantification Software (Roche) and expression levels were normalized to the housekeeping gene *GAPDH*.

siRNA transfection

mESCs were transfected with 70 nM Stealth Select RNAi for mouse CDK2 (Invitrogen, Carlsbad, CA; sequence of the duplex: 5′-CCCUUUCUUCCAGGAUGUAACUAAA-3′) or 70 nM Stealth RNAi Negative Control Duplex with Medium GC content (Invitrogen) using X-tremeGENE siRNA Transfection Reagent (Roche) as outlined in the manufacturer's instructions. The cells were analyzed 24 h after transfection.

Quantification of Cdk2 protein levels

Cdk2 protein levels were quantified from scanned images of western blot analyses using AlphaEase FC software, Version 5.0.1 (Alpha Innotech, San Leandro, CA).

Results

Cdk inhibitors are cytotoxic for mESCs

The cytotoxicity of the Cdk inhibitors olomoucine II and CAN508 was tested in MTT assays for 2 mESC lines, V6.5 and HM1, and for the adenocarcinoma cell line HT29. HT29 cells were used as a reference cell line because their response to olomoucine II and CAN508 treatment has been described already [20,21]. mESCs were treated for 24 h and HT29 for 72 h with scaled doses of each inhibitor separately. The duration of inhibitor treatment corresponded approximately to 3 cell cycles in the respective cell lines. The results of MTT tests are summarized in Table 1. The cytotoxic potential of the pan-specific inhibitor olomoucine II was stronger than that of the cytotoxic potential of Cdk9-specific inhibitor CAN508. IC_{50} of olomoucine II was 9.0 µM for V6.5, 6.3 µM for HM1, and 10.7 µM for HT29. The HT29 IC_{50} value is consistent with the observation of Krystof et al. [20]. IC_{50} for CAN508 was 129.0 µM for V6.5, 68.1 µM for HM1, and 54.4 µM for HT29 (this value is consistent with Krystof et al. [21]).

Treatment of mESCs with Cdk inhibitors causes major changes in cell-cycle progression

To investigate the effects of olomoucine II and CAN508 on the cell cycle, mESCs and HT29 cells were treated with olomoucine II or CAN508 at IC_{50} concentrations for the time approximately corresponding to the duration of 1 cell cycle (11 h for mESCs, 24 h for HT29). To investigate more

Table 1. IC_{50} Values for Olomoucine II and CAN508

	Olomoucine II (µm)	*CAN508 (µm)*
V6.5	9.0 ± 0.9	129.0 ± 24.6
HM1	6.3 ± 0.7	68.1 ± 5.6
HT29	10.7 ± 0.7	54.4 ± 8.2

The number of viable cells after 24 h (V6.5 and HM1 cells) or 72 h (HT29 cells) treatment with olomoucine II or CAN508 was quantified in MTT assays and IC_{50} values were calculated from dose–response curves. The results are means (±SD) of at least 3 experiments.

immediate effects of Cdk inhibition, cells of all 3 lines were incubated with the inhibitors for 3 h only. Following incubation, cells were double-stained with BrdU and propidium iodide and their cell-cycle profiles were analyzed by flow cytometry. The results for 1 experiment, representative of 3 independent experiments, are depicted in Figure 1. In the control cell line HT29, the 24-h treatment with olomoucine II caused a slight, but statistically significant increase (from 72% in mock-treated cells to 75% in olomoucine II-treated cells; $P = 0.003$) in the proportion of G1 cells and a minor decrease ($P = 0.06$) in the proportion of S phase cells (Fig. 1). However, the 24-h treatment with CAN508, a specific inhibitor of transcriptional kinase Cdk9, led to a more prominent decrease in G1 phase cell number (from 72% in mock-treated cells to 63% in CAN508-treated cells; $P = 0.009$) and a prominent increase (from 22% in mock-treated cells to 35% in CAN508-treated cells; $P = 0.02$) in S phase cell number (Fig. 1). The same trend to the cell-cycle changes was observed as early as 3 h of inhibitor treatment (Fig. 1). Neither olomoucine II nor CAN508 treatment of HT29 cells led to inhibition of DNA replication (Fig. 1).

mESCs responded to the inhibitor treatment differently from the HT29 cells, and similarly to each other. An 11-h treatment with olomoucine II caused a significant increase in G1 phase cell number in mESCs: from 18% to 43% in mock-treated V6.5 cells ($P = 0.01$; Fig. 1) and from 20% to 45% in mock-treated HM1 cells ($P = 0.04$; Fig. 1). Correspondingly, the proportion of mESCs in S phase dropped significantly after 11-h olomoucine II treatment (from 78% to 31% in V6.5 cells and from 75% to 27% in HM1 cells; $P = 0.03$ and 0.05, respectively). The proportion of G2 phase cells exhibited changes as well, but these changes were not statistically significant ($P = 0.09$ for V6.5 cells and 0.06 for HM1 cells). Moreover, 11-h treatment with olomoucine II caused a 65-fold and a 137-fold increase of BrdU-negative cells in V6.5 and HM1 mESCs, respectively, pointing to a significant inhibition of DNA replication ($P = 0.02$ and 0.01, respectively).

Treatment with the Cdk9 inhibitor CAN508 had similar, but less prominent consequences to those of olomoucine II treatment: G1 phase cell numbers increased ($P = 0.006$ for V6.5 and $P = 0.37$ for HM1), S phase cell numbers decreased ($P = 0.03$ for V6.5 and $P = 0.06$ for HM1), and the proportion of G2 phase cells decreased ($P = 0.28$ and 0.36 for V6.5 and HM1, respectively). The number of BrdU-negative S phase cells increased (Fig. 1), but these slight changes were not statistically significant ($P = 0.18$ and 0.15 for HM1 and V6.5 cells, respectively). Again, the propensity for these cell-cycle changes (observed after 11-h treatment with inhibitor) can be observed as early as 3 h after inhibitor treatment in mESCs.

Effects of CAN508 and olomoucine II treatment on Cdk9, Cdk2, and Cdk1 activities in mESCs

Olomoucine II and CAN508 were reported to be effective inhibitors of Cdk9 both in in vitro kinase inhibition assays and in cancer cell lines: IC_{50} for Cdk9–cyclin T is 0.06 μM for olomoucine II [20] and 0.35 μM for CAN508 [21]. To check the effectiveness of Cdk9 inhibition by CAN508 or olomoucine II, we tested, using western blots, the phosphorylation status of RNA polymerase II on Ser2 in the C-terminal domain (RNA-pol II CTD), the specific Cdk9 target site. We found that CAN508 effectively abolishes Cdk9 activity at the concentrations used in our experiments, as it down-regulates the phosphorylation of RNA-pol II CTD at a concentration of 100 μM in both studied ESC lines (Fig. 2). Cdk9 activity was also inhibited by olomoucine II in mESCs, where a decrease of CTD phosphorylation was detected at 2.5 μM olomoucine II in V6.5 cells and at 5 μM olomoucine II in HM1 cells (Fig. 2).

Besides Cdk9, olomoucine II exhibits specificity for Cdk2 (IC_{50} for Cdk2–cyclin E is 0.1 μM [20]) and a lower specificity for Cdk1 (IC_{50} for Cdk1–cyclin B is 7.6 μM [20]). To determine the activity of olomoucine II against Cdk2 and Cdk1 in mESCs, we undertook a series of histone H1 kinase assays for Cdk2 and Cdk1 activities in mock- or olomoucine II-treated mESCs. The results are plotted in Figure 3. Following 5 μM olomoucine II treatment, the activity of Cdk2 significantly decreased (to 42% of the Cdk2 activity in mock-treated cells; $P = 0.002$). In contrast, the activity of Cdk1 was not significantly decreased by up to 10 μM olomoucine II (64% of the Cdk1 activity in mock-treated cells; $P = 0.15$).

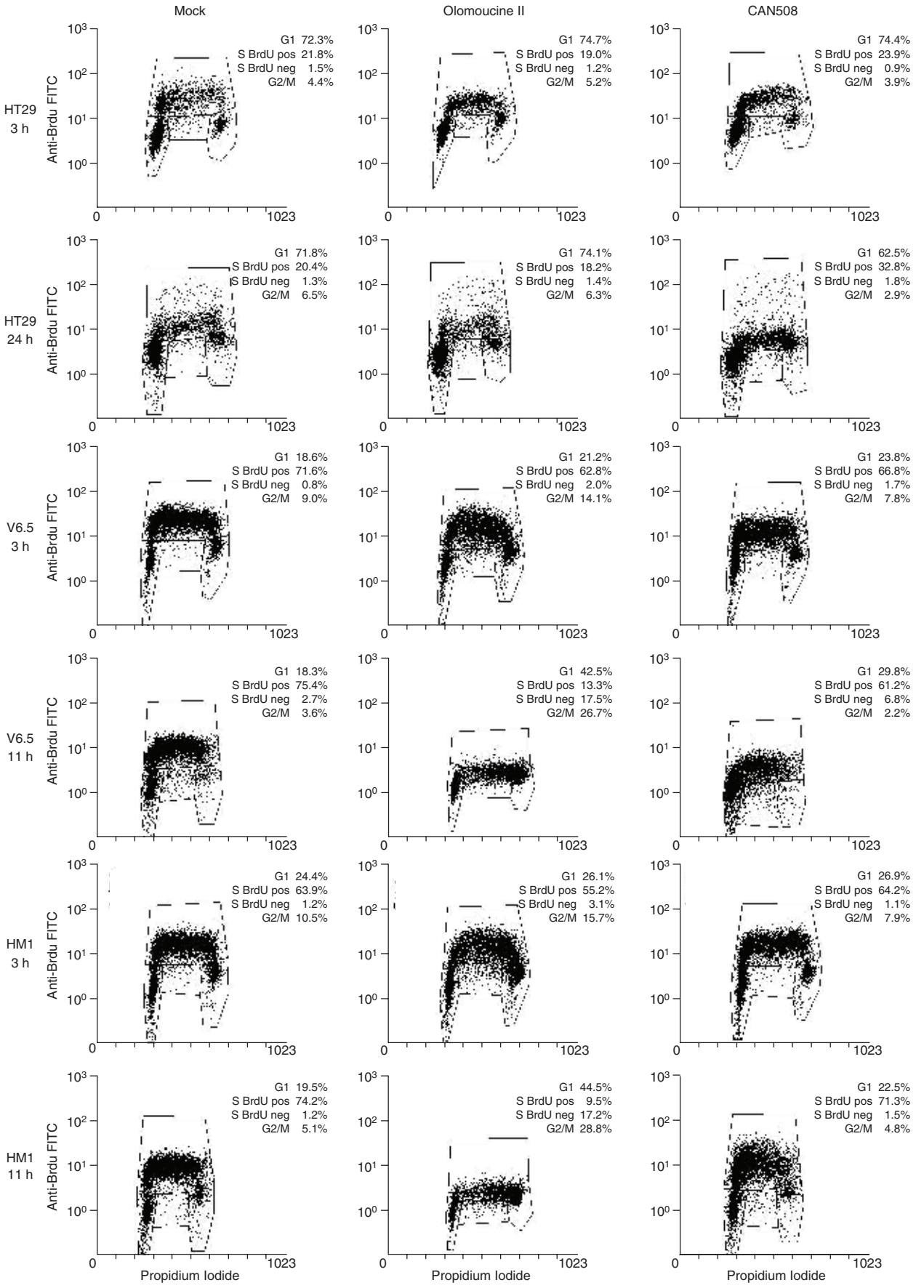
CAN508 was reported to have minor inhibitory effect on Cdk2 (IC_{50} for Cdk2–cyclin E is 3.5 μM [21]) in somatic cells. The specificity of Cdk9 inhibition by CAN508 in mESCs was checked in kinase assays for Cdk1 and Cdk2 activities. CAN508 treatment (up to IC_{50} concentration for the respective mESC lines) had no effect on Cdk1/Cdk2 activity (data not shown).

These observations suggest the Cdk inhibitors olomoucine II and CAN508 have the same targets in mESCs and in somatic cells.

Olomoucine II treatment blocks DNA replication in mESCs

From kinase assays, we concluded that 5 μM is the upper concentration limit of olomoucine II that effectively inhibits Cdk2 activity while not affecting Cdk1 activity (Fig. 3). This results suggested use of 5 μM olomoucine II for more specific down-regulation of Cdk2 activity in mESCs. In view of this observation, both V6.5 and HM1 cells were treated with 1, 2.5, or 5 μM olomoucine II, or mock-treated to investigate the effects of down-regulation of Cdk2 activity on cell-cycle progression in mESCs. The shorter time-point (6 h) of olomoucine II treatment was chosen in order to follow the immediate effects of Cdk2 activity attenuation on mESC cycle progression. After the 6-h treatment, the cells were double-stained with BrdU and propidium iodide and analyzed by flow cytometry. The olomoucine II treatment increased the frequency of mESCs in G1 phase from 16% in mock-treated V6.5 cells up to 42% in 5 μM olomoucine II-treated V6.5 cells ($P = 0.01$) and from 26% up to 44% in HM1 cells ($P = 0.04$) (Fig. 4A).

FIG. 1. Changes of cell-cycle profiles after Cdk inhibitor treatment. HT29, V6.5, and HM1 cells were treated with olomoucine II or CAN508 (at the concentrations corresponding to their IC_{50} values) or mock-treated for the indicated times. Cells were subsequently pulsed with 5-bromo-2′-deoxyuridine (BrdU) for 30 min, fixed, stained with propidium iodide and their cell-cycle profile was analyzed by flow cytometry. The figures are representatives of 3 independent experiments.


Mock
Olomoucine II
CAN508
HT29 3 h
HT29 24 h
V6.5 3 h
V6.5 11 h
HM1 3 h
HM1 11 h
Anti-Brdu FITC
Propidium Iodide
G1 72.3%
S BrdU pos 21.8%
S BrdU neg 1.5%
G2/M 4.4%
G1 74.7%
S BrdU pos 19.0%
S BrdU neg 1.2%
G2/M 5.2%
G1 74.4%
S BrdU pos 23.9%
S BrdU neg 0.9%
G2/M 3.9%
G1 71.8%
S BrdU pos 20.4%
S BrdU neg 1.3%
G2/M 6.5%
G1 74.1%
S BrdU pos 18.2%
S BrdU neg 1.4%
G2/M 6.3%
G1 62.5%
S BrdU pos 32.8%
S BrdU neg 1.8%
G2/M 2.9%
G1 18.6%
S BrdU pos 71.6%
S BrdU neg 0.8%
G2/M 9.0%
G1 21.2%
S BrdU pos 62.8%
S BrdU neg 2.0%
G2/M 14.1%
G1 23.8%
S BrdU pos 66.8%
S BrdU neg 1.7%
G2/M 7.8%
G1 18.3%
S BrdU pos 75.4%
S BrdU neg 2.7%
G2/M 3.6%
G1 42.5%
S BrdU pos 13.3%
S BrdU neg 17.5%
G2/M 26.7%
G1 29.8%
S BrdU pos 61.2%
S BrdU neg 6.8%
G2/M 2.2%
G1 24.4%
S BrdU pos 63.9%
S BrdU neg 1.2%
G2/M 10.5%
G1 26.1%
S BrdU pos 55.2%
S BrdU neg 3.1%
G2/M 15.7%
G1 26.9%
S BrdU pos 64.2%
S BrdU neg 1.1%
G2/M 7.9%
G1 19.5%
S BrdU pos 74.2%
S BrdU neg 1.2%
G2/M 5.1%
G1 44.5%
S BrdU pos 9.5%
S BrdU neg 17.2%
G2/M 28.8%
G1 22.5%
S BrdU pos 71.3%
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G2/M 4.8%

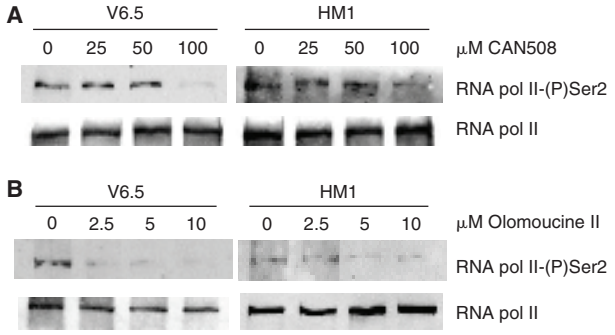

FIG. 2. Phosphorylation of the C-terminal domain of RNA polymerase II is abolished by CAN508 and olomoucine II treatment. Cells were treated for 11 h with the indicated concentration of CAN508 (**A**) or olomoucine II (**B**) or mock-treated, collected and the phosphorylation status of RNA polymerase II on Ser2 was investigated by immunoblotting.

Olomoucine II treatment also led to a decrease in S phase cell numbers and an inhibition of DNA replication. The S phase cell numbers decreased significantly in both V6.5 and HM1 cells treated with 5 μM olomoucine II ($P = 0.03$ and 0.04, respectively; Fig. 4A). Of the S phase cells, about 50%–60% did not incorporate BrdU, that is they were S phase arrested in both mESC lines after 5 μM olomoucine II treatment ($P = 0.03$ for V6.5 and 0.01 for HM1 cells; Fig. 4A). Lower doses of olomoucine II had no effect on DNA replication in V6.5 cells. DNA replication of HM1 cells appeared to be more sensitive to olomoucine II, as inhibition of DNA replication occurred (in 32% of S phase cells; $P = 0.04$) even under 2.5 μM olomoucine II treatment (Fig. 4A).

Prolongation of olomoucine II treatment to 11 h led to cell-cycle changes comparable to those after 6-h treatment (Fig. 4B): G1 phase cell numbers increased ($P = 0.001$ and 0.01 for V6.5 and HM1 cells, respectively), S phase cell numbers decreased ($P = 0.002$ for both V6.5 and HM1 cells), and G2/M phase cell numbers increased ($P = 0.003$ for V6.5 and $P = 0.001$ for HM1 cells) (Fig. 4B). Also, 5 μM olomoucine II treatment inhibited DNA replication in 40%-50% cells ($P = 0.02$ for V6.5 cells and $P = 0.01$ for HM1 cells), as judged from the diminished numbers of BrdU-positive S phase cells ($P = 0.008$ and 0.003 for V6.5 and HM1 cells, respectively) (Fig. 4B). Treatment of mESCs with olomoucine II for longer periods of time (up to 96 h) sustained increased G1 phase cell numbers; however, a massive cell death could be observed (data not shown).

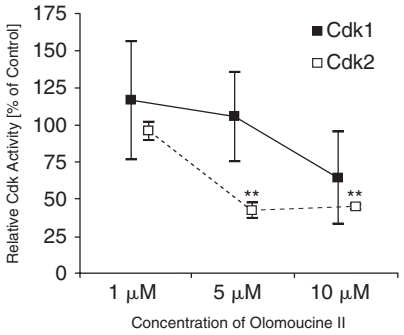

FIG. 3. Inhibition of Cdk1 and Cdk2 activity by olomoucine II treatment. After 1-h treatment of V6.5 cells with designated concentrations of olomoucine II, Cdk1 or Cdk2 activity was measured in histone H1 kinase assays. The results were normalized to control (mock-treated cells). The data are the means of 2 independent experiments. Statistical significance was assessed using Student's *t*-test, $^{**}P < 0.005$. Bars = 95% confidence interval.

Olomoucine II treatment leads to G1 phase prolongation in mESCs

Our data showed that inhibition of Cdk2 activity by olomoucine II in mESC culture increases the proportion of cells in G1 (Fig. 4). This could be the result of either prolongation of the G1 phase, or arrest in the G1- or G1/S phase. To distinguish between these possibilities and to investigate the effect of Cdk2 inhibition on G1/S phase progression in mESC in greater detail, mESCs were synchronized in the G2/M phase by nocodazole treatment and then released from the block to obtain the maximum number of G1 phase cells, which were then followed in their cell-cycle progression under olomoucine II or mock treatment. The maximum number of G1 phase cells (41%) was observed at 2 h after nocodazole release, so at this time-point, olomoucine II was added to a final concentration of 5 μM. Olomoucine II-treated and mock-treated cells were collected at different time-points and their cell-cycle profile was analyzed by flow cytometry (Fig. 5). Olomoucine II treatment slowed down G1 phase progression and S phase entry (Fig. 5) in mESCs. The G1 phase was prolonged by ~2 h (ie, to about 5 h), as shown by the fact that identical proportions of cells in G1 and S phases were found 4 h after nocodazole release in mock-treated cells and 6 h after nocodazole release in olomoucine II-treated cells, respectively. Collectively, these data suggest that a decrease in Cdk2 activity prolongs G1 phase duration from about 3 h in mock-treated cells to ~5 h in 5 μM olomoucine II-treated cells.

Down-regulation of Cdk2 activity establishes a somatic cell-like cell cycle and induces expression of differentiation markers in mESCs

It has been shown that down-regulation of Cdk2 activity in hESCs induces their differentiation [18]. To find out whether this might also be the case in mESCs, we investigated the effects of prolonged Cdk2 down-regulation by olomoucine II in mESCs. After 24 h of 5 μM olomoucine II treatment, mESCs still exhibited a somatic cell-like profile, that is, a cell-cycle profile with a high proportion of cells in G1 phase and a lower proportion of cells in S phase (Fig. 6A). Changes in the mESC cycle induced by down-regulation of Cdk2 activity were accompanied by changes in cell morphology, cell and colony shape, and adhesiveness as early as 11 h after 5 μM olomoucine II treatment (Supplementary Fig. 1A). Examination of pluripotency and differentiation markers by quantitative real-time RT-PCR showed a statistically significant increase in expression of differentiation markers and reduced expression of some pluripotency markers after 11 h of olomoucine II treatment (Supplementary Fig. 1B). This was further reinforced by prolonged olomoucine

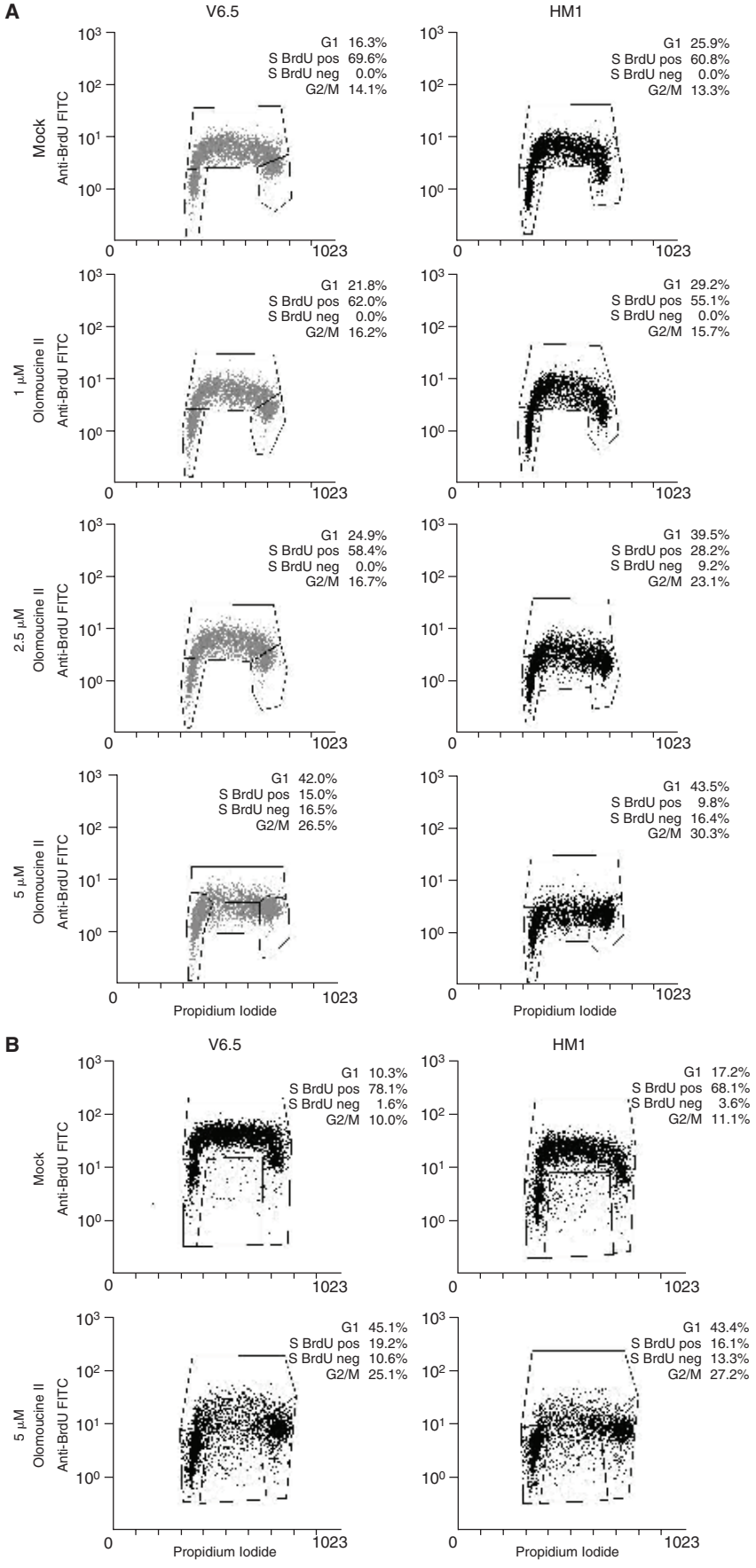

FIG. 4. High Cdk2 activity is required for embryonic stem cell (ESC)-specific cell-cycle profile. Flow cytometry analysis of cell-cycle profiles and DNA replication in V6.5 and HM1 mouse (ESCs) treated with indicated doses of olomoucine II for 6 h (**A**) or 11 h (**B**) and pulsed with 5-bromo-2′-deoxyuridine (BrdU) for 30 min. The figures are representatives of 3 independent experiments.

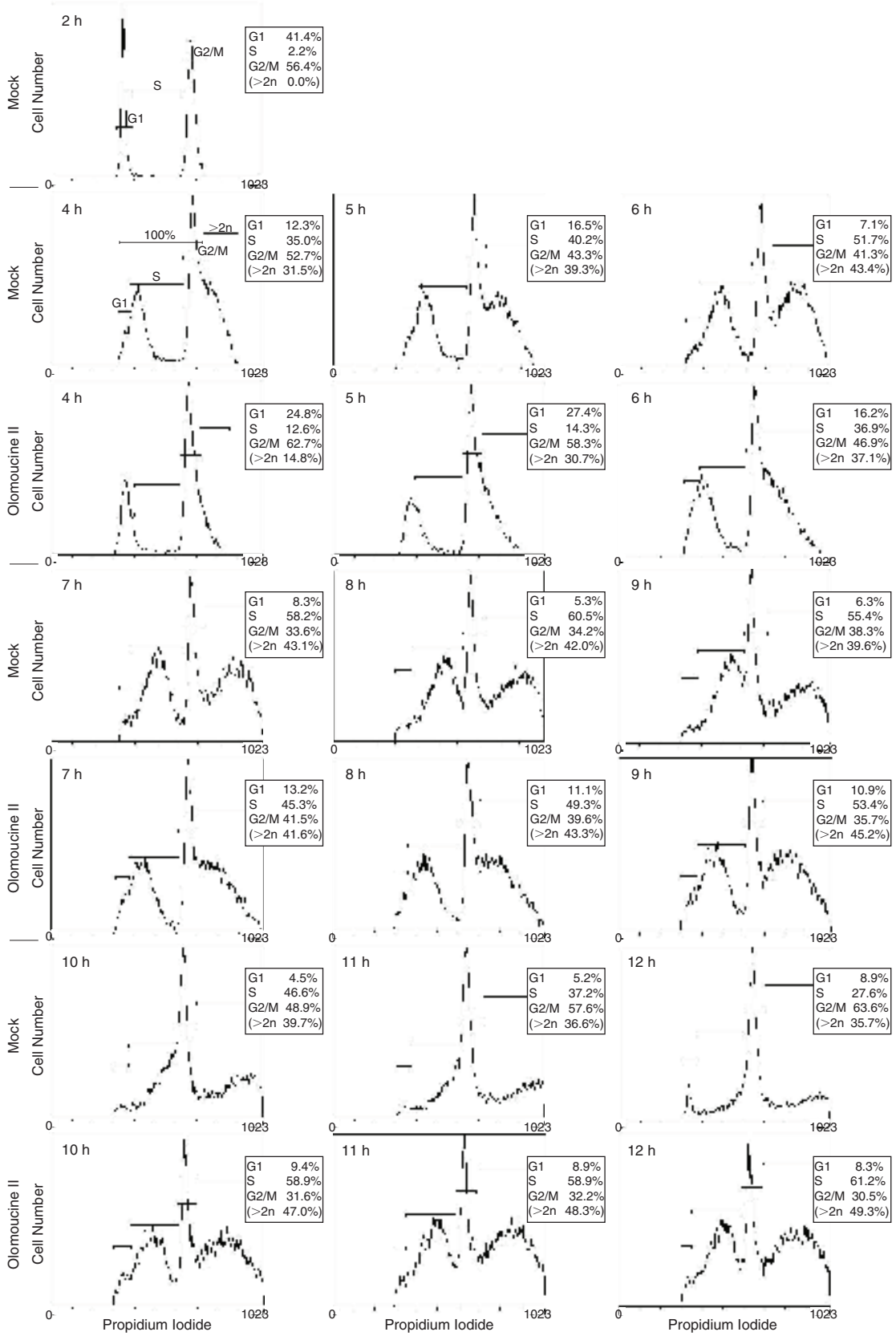

FIG. 5. Down-regulation of Cdk2 activity in mouse embryonic stem cells (mESCs) by olomoucine II leads to prolongation of the G1 phase and delayed S phase entry. V6.5 mESCs were synchronized by nocodazole treatment and 2 h after nocodazole release, the cells were treated with 5 μM olomoucine II or mock-treated. Cells were collected at indicated time-points after nocodazole release and their cell-cycle profiles were analyzed by flow cytometry. Hyperdiploid cells (>2*n*) were excluded from assessment of cell-cycle distribution.

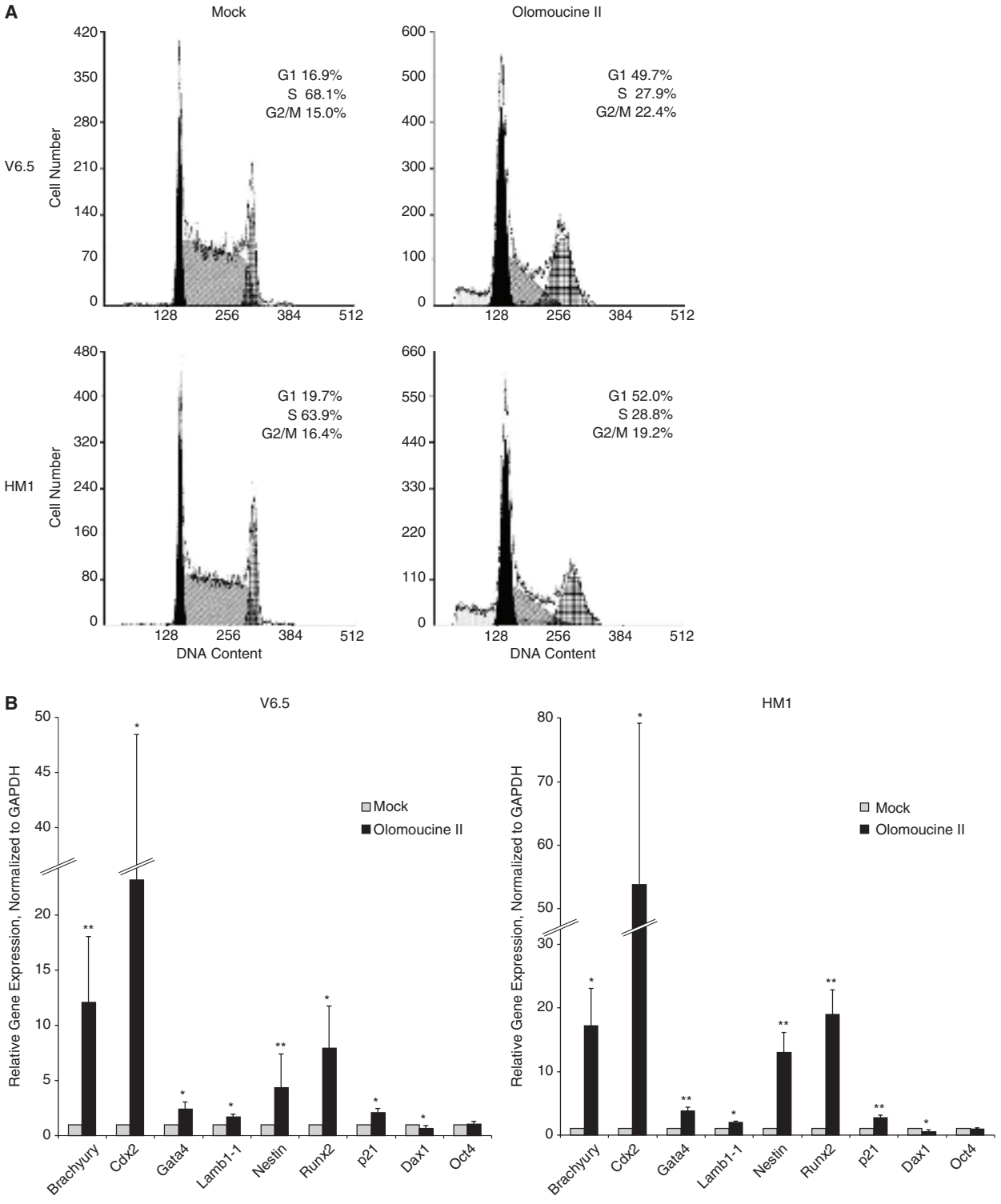

FIG. 6. Down-regulation of Cdk2 activity by olomoucine II establishes a somatic cell-like cell-cycle profile and induces differentiation of mouse embryonic stem cells (mESCs). (**A**) Cell-cycle profile analysis of mESCs treated with 5 μM olomoucine II for 24 h. (**B**) Expression of differentiation (*Brachyury* to *Runx2*) and pluripotency (*Dax1*, *Oct4*) markers and cell-cycle regulatory protein *p21* in mESCs after 24-h treatment with 5 μM olomoucine II. The data represent the mean ± SD from 3 independent experiments. Statistical significance was assessed using Student's *t*-test, $^{*}P < 0.05$, $^{**}P < 0.005$.

II treatment for 24 h (Fig. 6B). Specific down-regulation of Cdk2 activity in mESCs reduced expression of the pluripotency marker *Dax1* and induced expression of trophoblast (*Cdx2*), mesoderm (*Brachyury*), extraembryonic entoderm (*Gata4*), primitive endoderm (*Lamb1-1*), primitive neuroectoderm (*Nestin*), and skeletal (*Runx2*) markers (Fig. 6B). These data are indicative of induction of mESC differentiation by down-regulation of Cdk2 activity.

siRNA knockdown of Cdk2 increases G1 phase cell number, decreases S phase cell number, and induces morphological changes in mESCs

To verify that the observed effects of olomoucine II on mESC cycle regulation were truly caused by down-regulation of Cdk2 activity, we adopted Cdk2 siRNA knockdown approach. Twenty-four hours after Cdk2 siRNA transfection, Cdk2 expression was down-regulated by 43%, as assessed by quantitative real-time RT-PCR (Fig. 7A), and Cdk2 protein level decreased to 55% (Fig. 7B). These changes in Cdk2 expression led to 28% decrease of Cdk2 activity (data not shown) and induced increase of G1 phase cell number from 20.7% in negative control siRNA-treated cells to 26.3% in Cdk2 siRNA-treated cells ($P = 0.01$) (Fig. 7C), which was similar to the effect of olomoucine II on mESC cycle structure. Moreover, siRNA-mediated knockdown of Cdk2 induced similar changes in mESC morphology, colony shape, and adhesiveness (Fig. 7D) as olomoucine II (Supplementary Fig. 1A). These results confirm that Cdk2

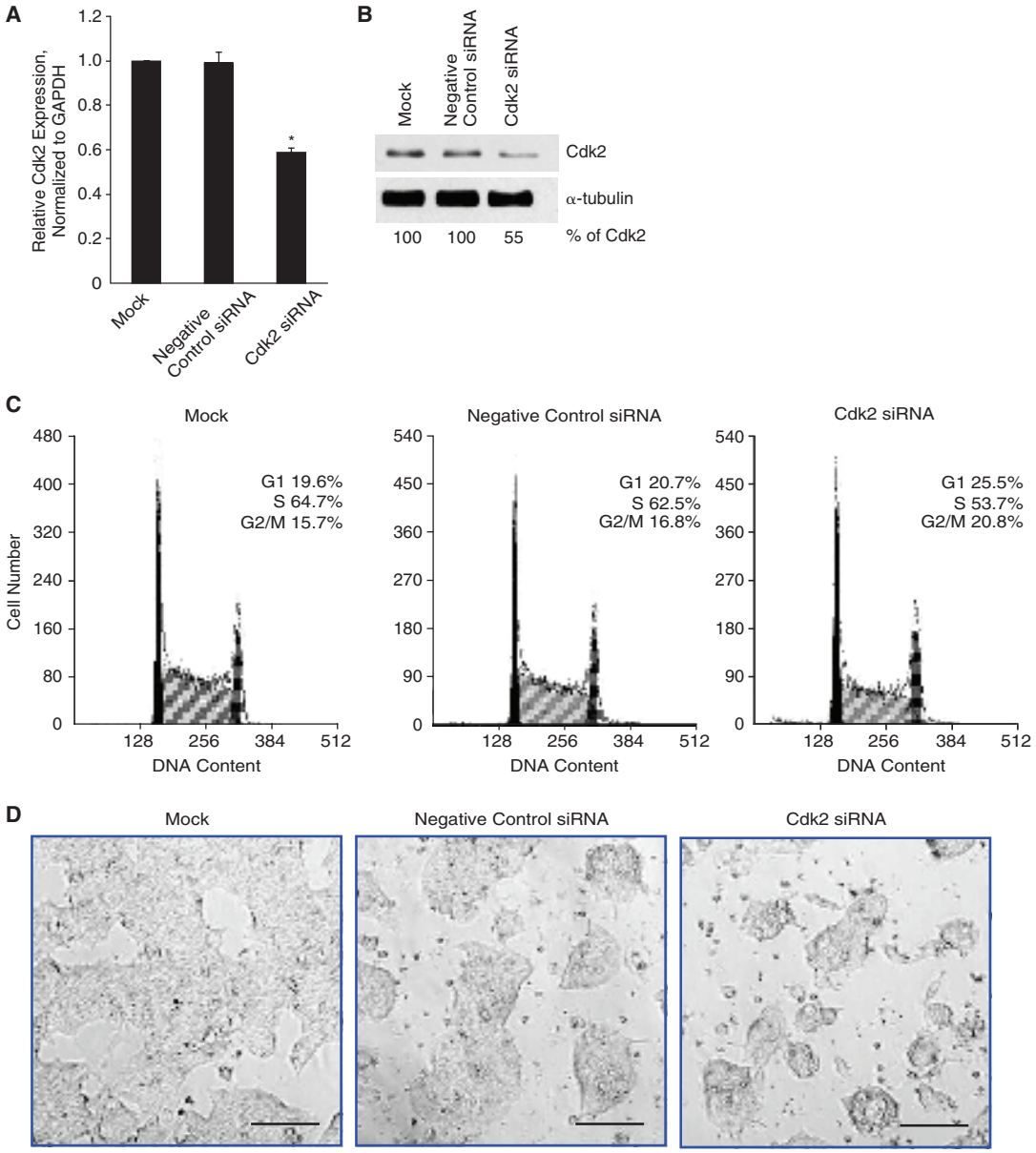

FIG. 7. Cdk2 knockdown leads to similar mouse embryonic stem cell (mESC) cycle and morphology changes to those induced by olomoucine II treatment. (**A**) Down-regulation of Cdk2 mRNA level 24 h after Cdk2 siRNA transfection. The data represent the means ± SD from 2 independent experiments. Statistical significance was assessed using Student's *t*-test, $^*P < 0.05$. (**B**) Decrease of Cdk2 protein level 24 h after Cdk2 siRNA transfection. Relative levels of Cdk2 were determined from western blots using the AlphaEase FC program, normalized to α-tubulin and are indicated beyond the blots. (**C**) Cell-cycle profile analysis of Cdk2 siRNA transfected mESCs 24 h post-transfection. (**D**) The microphotographs of mESCs 24 h after siRNA treatment. Scale bar = 100 μm.

is the target molecule for olomoucine II, through which the observed cell-cycle changes and associated modulation of pluripotency are mediated.

Discussion

In mESCs, high, cell-cycle-independent Cdk2 activity was reported to underpin rapid cell-cycle progression [3] but the role of Cdk2 in the establishment of mESC-specific cell cycle has remained obscure. Moreover, in the context of Cdk substitutionary roles [15–17] and Cdk2 nonessentiality for G1/S transition [13,14], the actual role of Cdk2 in mESC cycle regulation has not been fully elucidated. Finally, the role of Cdk2 in mESC cycle regulation was emphasized by the observation of a predominantly centrosomal localization of Cdk2 in mESCs (our observation, data not shown).

In our model, we used olomoucine II to down-regulate Cdk2 activity in mESCs. As olomoucine II is a Cdk inhibitor with high specificity for both Cdk2 and Cdk9 [20], we used also a Cdk9 inhibitor, CAN508 [21], to distinguish the effects of Cdk9 inhibition from those of Cdk2 inhibition. CAN508 is highly selective for Cdk9 and its effects on Cdk2 are minor. In in vitro kinase assays, CAN508 inhibits Cdk9 activity at least 50 times more effectively than it does Cdk2 activity [21].

mESC treatment with olomoucine II at a concentration equal to its IC_{50} caused major changes in cell-cycle progression: the G1 cell numbers increased and S phase cell numbers decreased (Fig. 1). CAN508 treatment caused similar, but smaller changes to those after olomoucine II treatment corresponding to its lower specificity for Cdk2. Olomoucine II treatment also led to a marked inhibition of DNA replication in mESCs. Overall, these cell-cycle changes in mESCs following olomoucine II or CAN508 treatment were clearly different from the effects of these inhibitors in somatic cells (Fig. 1).

Investigation of Cdk activities in inhibitor-treated mESCs confirmed Cdk9 inhibition in CAN508-treated cells and both Cdk2 and Cdk9 inhibition in olomoucine II-treated cells (Figs. 2 and 3). However, at higher concentrations of olomoucine II, Cdk1 inhibition occurs (Fig. 3) and might be partially responsible for the observed cell-cycle changes when 10 μM (or more) olomoucine is used. Therefore, we investigated cell-cycle progression of mESCs in 5 μM olomoucine II, that is in conditions of specific Cdk2 inhibition. Even under such specificity, G1 phase cell numbers in mESCs increased significantly with a concomitant reduction in S phase cell number and inhibition of DNA replication. In synchronized mESCs, we showed that the increase in the proportion of G1 phase cells after Cdk2 inhibition was caused by slowdown of their progression through G1 phase, that is prolongation of G1 phase by ~2 h (from 3 to 5 h). This observation corroborates the role of Cdk2 in driving ESC cycles [3]. Of particular interest, our observation of distinctive cell-cycle changes after Cdk2 inhibition is novel and adds more depth to the observation of Stead et al. [3]. In their study, inhibition of Cdk2 activity (by treatment with a Cdk2 inhibitor Ro09-3033) did not change the general cell-cycle characteristics of mESCs, although they did observe slowdown of cell-cycle progression after Ro09-3033 treatment as we did after olomoucine II treatment. We suggest that high Cdk activity per se contributes to a very short G1 phase in mESCs and might be responsible for the unusual cell-cycle structure of mESCs.

As the atypical cell-cycle structure with short gap phases is common to all pluripotent cells of embryonic origin [3], it has been proposed that specific cell-cycle regulation and loss of G1 functions (restriction point and G1 checkpoint) in ESCs might be involved in self-renewal of ESCs [25]. The relationship between cell-cycle regulation and pluripotency of ESCs has been under dispute. It was reported that activation of p53 leads to rapid differentiation of ESCs by introducing changes in cell-cycle progression, particularly abolishing S phase entry [26]. Differentiation is a smart way of coping with DNA damage in ESCs [27], which are deficient in a G1 checkpoint response and p53-dependent apoptosis [28,29], by eliminating DNA-damaged ESCs from the replicative ESC pool. Recently, it was shown that down-regulation of Cdk2 activity in hESCs induces their differentiation to extraembryonic lineages [18]. Our study in mESCs presents further evidence on the requirement for Cdk2 activity for maintenance of the ESC-specific cell-cycle structure and for the pluripotency of ESCs. Down-regulation of Cdk2 activity in mESCs by olomoucine II or Cdk2 siRNA knockdown established a somatic cell-like cell cycle (Figs. 6A and 7C, respectively) and induced expression of differentiation markers of lineages of all 3 embryonic layers (Supplementary Fig. 1B and Fig. 6B). This observation is consistent with studies reporting cell-cycle remodeling during differentiation and an increase in G1 cell proportion with the loss of pluripotency [1,3,5,25].

Also, our study revealed slight differences in effects of Cdk2 down-regulation between mESCs and hESCs: while hESCs arrest in G1 after down-regulation of Cdk2 activity [18], mESCs only slowdown their G1 progression (Fig. 5). Neither after prolonged 5 μM olomoucine II treatment (48 or 72 h), nor after treatment with higher doses of olomoucine II (10 μM and higher) did we observe G1 arrest in mESCs (data not shown). Our contrasting observation could be caused by biological differences between mESCs and hESCs, but rather it might be related to the extent of Cdk2 inhibition or to different methods used for down-regulation of Cdk2 activity. In our study, we primarily analyzed requirements for Cdk2 in cell-cycle regulation of mESCs using a chemical inhibition approach. To confirm our observations, we also employed Cdk2 knockdown by siRNA. Both approaches led, in principle, to the same results, but in the knockdown experiments the G1 cell number was increased less efficiently. This might result from low efficiency of Cdk2 knockdown by siRNA; that is, from the different extent to which Cdk2 activity was inhibited. Another possibility is that the knockdown approach introduces a bias through up-regulation of other Cdks/cyclins and their potential compensation for S phase-promoting functions. Chemical inhibition might not allow for the same compensation [19]. While knockdown of a Cdk leaves a pool of its interactory cyclin molecules free and accessible for other Cdks that might bind to them and compensate for the knocked-down Cdk, chemical inhibition of a Cdk does not leave its partner cyclin pool available.

Nevertheless, whatever the modularities of induced cell-cycle changes in different ESC types are, down-regulation of Cdk2 activity commonly results in expression of differentiation markers. In this context, it would be interesting to investigate the effects of Cdk2 down-regulation in cancer stem cells. As cancer stem cells use an ESC-like "stemness" program to induce and maintain tumors [30,31], modulation of the cell cycle of (cycling) cancer stem cells with Cdk inhibitors might become a useful tool for their eradication.

Our study partially addressed the issue of testing Cdk inhibitors on an ESC model. Stem cells, in general, are more resistant to toxins and various types of drugs due to high expression of specific ATP-binding cassette (ABC) drug transporters [32]. As this characteristic might be common to "normal" (embryonic and tissue) stem cells and "abnormal" (cancer) stem cells [32], drug testing using ESCs as convenient model of cancer stem cells might provide important insights into the mechanism(s) by which cancer stem cells might response to cancer therapy.

We report on the cytotoxicity of olomoucine II and CAN508 in mESCs. Cytotoxicity of these drugs toward mESCs is similar to the cytotoxicity toward cancer cell lines, as the IC_{50} for olomoucine II or CAN508 was similar for mESCs and cancer cells. However, the mESC line V6.5 exhibited lower sensitivity toward CAN508, with an IC_{50} 2 to 3 times higher than those for the mESC line HM1 (this study) and for cancer cell lines (reported previously [21]). We suggest this might be due to some unique features of transcription regulation in V6.5 cells [33], rather than due to ABC-mediated drug resistance, as V6.5 sensitivity toward another small molecular Cdk inhibitor, olomoucine II, does not significantly differ from that of other cell lines, including the mESC line HM1. We conclude mESCs do not exert resistance toward Cdk inhibitors olomoucine II and CAN508.

Besides other functions, cyclin-dependent kinases are critical regulators of cell-cycle progression and RNA transcription [34]. As a result of a variety of genetic and epigenetic events, Cdks are frequently deregulated in tumors [35,36]. Chemical Cdk inhibitors have appeared as a promising tool for cancer therapy because Cdk inhibition can lead to cell-cycle arrest and apoptosis [34]. But as Cdk2 activity is dispensable for cancer cell proliferation [37], the suitability of Cdk2 as a target for cancer therapy has been called into question. As modulation of Cdk2 and Cdk1 activity sensitizes survival checkpoint responses after exposure to DNA-damaging agents [38], Cdk2 inhibitors show promising antitumor activity in combination with DNA-damaging drugs [39]. Our study reveals olomoucine II as an effective tool for manipulation of ESC cycle and ESC pluripotency and in this context, it is tempting that Cdk2 inhibitors might become powerful tools for cancer stem cell eradication, possibly contributing to their differentiation.

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Cell cycle regulation in embryonic stem cells: centrosomal decisions on self-renewal

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Abstract

Embryonic stem cells (ESCs) have the intriguing capacity to divide apparently indefinitely while retaining their pluripotency. This is accomplished by specialized mechanisms of cell cycle control which they employ to self-renew. In the last few years, several studies have given evidence for the existence of a direct link between cell cycle regulation and cell fate decisions in stem cells. In this review, we discuss the peculiarities of ESC cycle-control mechanisms, implicate their roles in cell fate decisions and feature centrosomes as important players in the self-renewal versus differentiation roulette.

Pluripotent cells, including mouse and human embryonic stem cells (ESCs) and mouse epiblast stem cells, share unique cell cycle characteristics. They divide rapidly and have a very short G1 phase with most of the cells being in S phase of the cell cycle [1, 2, 3]. This is reminiscent of early embryonic development of lower vertebrates, in which cell/nuclear division is extremely rapid and achieved by skipping gap phases with progression through repetitive rounds of DNA replication followed by chromosome separation (*i.e.* S phase and M phase) [4, 5, 6]. During differentiation, G1 and G2 phases are fully formed and cell division cycles are prolonged, suggesting that a specific cell cycle structure might play a role in the establishment of pluripotency. Multiple studies have provided further evidence for a direct link between cell cycle regulation and cell fate decisions in stem cells. These items will be discussed below.

Cell cycle regulation in somatic cells

The cell cycle, a highly organized process of cell growth, DNA replication, chromosome separation and cell division, is regulated by cyclin-dependent kinases (CDKs). These kinases, among other functions, phosphorylate the retinoblastoma protein (pRB), thereby promoting cell division-cycle entry with progression from G1 to S phase. When unphosphorylated, pRB binds E2F transcription factors, repressing E2F target gene expression. Phosphorylation of pRB by CDKs leads to release of E2Fs and promotes expression of genes, which drive cell cycle progression to S phase (such as cyclin E or CDC25A) and take part in DNA replication (such as thymidine

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kinase or polymerase alpha).

The phosphorylation of pRB is sequential. First, in response to growth factor and/or mitogenic signaling, which induces cyclin D expression, pRB is phosphorylated by cyclin D-CDK4/CDK6 complexes, leading to progression through G1 phase. Induction of E2F-directed cyclin E and CDC25A expression results in formation and activation of cyclin E-CDK2 complexes, which cause further pRB phosphorylation and whose activity drives G1-S transition. Cyclin A-CDK2 then regulates S- and G2-phase events and, finally, cyclin A/B-CDK1 promotes entry into mitosis and controls early mitotic events.

For activation, CDKs have to be bound by cyclins, whose levels vary in a cyclical fashion during the cell cycle. Furthermore, CDK4, CDK2 and CDK1 require phosphorylation on Thr172 in CDK4, Thr160 in CDK2, and Thr161 in CDK1 by CDK-activating kinase (CAK, a complex of CDK7 and cyclin H) [7, 8]. The activity of CDK2 and CDK1 is additionally held in check by an inhibitory phosphorylation on Thr14/Tyr15 through Wee/Myt1 [9, 10], which is relieved by CDC25 phosphatases. Furthermore, CDK4/CDK6 and CDK2/CDK1 complexes are inhibited by proteins of the Ink4 ($p15^{Ink4b}$, $p16^{Ink4a}$, $p18^{Ink4c}$, $p19^{Ink4d}$) and Cip/Kip ($p21^{Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$) families, respectively (reviewed in Sherr & Roberts [11]).

Cell cycle regulation in embryonic stem cells With regard to cell cycle regulation and structure, ESCs demonstrate substantial differences from somatic cells. ESCs proliferate at a fast rate. Progression through a single cell cycle takes about 8-11 h in mESCs [1, 2, 12], 15-16 h in hESCs [13] and 12-21 h in rhesus monkey ESCs as compared to roughly 24 h for many somatic cell types in tissue culture [14]. The short cell cycle duration results, for the most part, from a very short G1 phase that lacks a restriction point and a G1 checkpoint. In contrast to somatic cells, ESCs do not require exogenous growth factors to enter the cell division cycle, are not subject to contact inhibition and do not enter quiescence on growth factor withdrawal [15, 16]. The ESC independence from growth factors (*i.e.* restriction point nonfunctionality) stems from constitutively hyperphosphorylated pRB. Therefore, E2F target genes are constitutively expressed and ESCs progress rapidly to S phase, spending only a very short time (about 2 hours for mESCs) in G1 phase. All interphase cyclins (D1, D3, E, A2) [2, 15, 17, 18] are present in mESCs at comparable levels throughout the cell cycle [2, 17] with cyclin levels being significantly elevated compared to somatic cells [18]. mESCs do not express CDK inhibitory molecules of the Ink4 and Cip/Kip families [19, 15, 17]. Therefore, most CDKs are active throughout the cell cycle in mESCs, with the exception of CDK1-cyclin B that becomes selectively activated before mitosis [2], and CDK4-cyclin D1 complexes which exhibit only little or no activity in mESCs [17]. The driving force of the rapid cell cycle progression in mESCs is high and precocious activity of CDK2 [2]. Chemical inhibition of CDK2 activity prolongs cell cycle duration [2] and slows down the progression of mESCs through G1 phase [20]. Overexpression of the CDK2 inhibitor $p21^{Cip1}$, for example by downregulation of its negative regulator, the mir-290 cluster, has similar effects [21].

In hESCs, a shortened G1 phase has been attributed to elevated mRNA levels of cyclin D2-CDK4 and low levels of $p21^{CipP1}$, $p27^{Kip1}$ and $p57^{Kip2}$ [13, 22, 23], which are controlled by miR-372 ($p21^{Cip1}$) [24] and miR-92b ($p57^{Kip2}$) [23]. Also, CDK inhibitors of the Ink4 family are expressed at low levels or are not expressed at all [25, 26]. Therefore, similar to mESCs, CDKs have very high activity, with CDK2 displaying the highest kinase activity overall in hESCs [27]. Also similar to mESCs, inhibition of CDK2 activity delays G1-S transition [28] and knockdown of CDK2 leads to G1 arrest in hESCs [27], implicating a crucial role for CDK2 for G1 phase regulation in both mouse and human ESCs [20, 27].

Linking cell cycle regulation to cell fate decisions in ESCs Major changes in expression of cell cycle regulatory proteins and cell cycle structure associated with differentiation gave a first hint on the connection between cell cycle regulation and the potency of stem cells with specific patterns of expression

of cell cycle regulators in stem cells might playing a role in maintenance of the stem cell self-renewal potential. Further evidence came from studies in adult stem cells, neural progenitor cells in particular, which suggested that the length of G1 phase directly impacts on cell fate decisions (self-renewal versus differentiation). These studies revealed that proliferating and self-renewing neural progenitors have shorter cell cycles than those committed to neuronal differentiation [29]. Inhibition of CDK activity prolonged their cell cycle and induced formation of neurons [30] whereas shortening of the cell cycle of neural progenitors prevented their differentiation [29]. Finally, cyclin D1-CDK4 overexpression studies in neural stem cells have shown that G1 lengthening is necessary and sufficient to switch neural progenitors from self-renewal to neurogenesis [31].

Recent studies have implicated the existence of similar regulatory connections between cell cycle structure and self-renewal in both hESCs and mESCs and have shown that inhibition of cell cycle progression is sufficient to induce differentiation of ESCs. Treatment of hESCs with nutlin, an activator of p53 (and thus a positive regulator of the CDK2 inhibitor $p21^{Cip1}$) resulted in accumulation of hESCs in G0/G1 that was associated with an increase of differentiation markers and a decrease in pluripotency markers [32]. Downregulation of CDK2 activity in hESCs by either chemical inhibition or knockdown led to G1/S phase delay [28] or G1 arrest [27], respectively, and induced hESC differentiation, as indicated by downregulation of OCT4 [27, 28] and expression of markers of extraembryonic lineages [27]. Likewise, CDK2 inhibition prolonged G1 phase and induced differentiation-associated changes in gene expression and morphology in mESCs [20]. Furthermore, CDK2-associated protein 1 (CDK2AP1; $p12^{DOC-1}$), an inhibitor of G1-S transition through downregulation of CDK2 [33], was shown to act as a competency factor in mESC differentiation by modulating the phosphorylation level of pRB [34]. In this study, *Cdk2ap1*$^{-/-}$ mESCs were shown to be resistant to LIF withdrawal-induced differentiation resulting in altered pRB phosphorylation [34]. The differentiation competency of *Cdk2ap1*$^{-/-}$ mESCs was restored upon ectopic expression of CDK2AP1 or a nonphosphorylatable pRB mutant [34]. Essentiality of RB family proteins in mESC differentiation was demonstrated in triple knockout mESCs (*Rb1*$^{-/-}$, *Rbl1*$^{-/-}$, *Rbl2*$^{-/-}$), which were incapable of undergoing proper differentiation [35]. As single and double knockout ESCs showed no defect in differentiation, RB family members appear to have the ability to compensate for one another in this setting [35]. These observations indicate the existence of an intrinsic link between cell cycle regulation and cell fate decisions (self-renewal versus differentiation) in stem cells that are likely to be affected by the length of each phase of the cell cycle and suggest that G1 phase corresponds to a window of increased sensitivity of stem cells to differentiation signals [27].

This hypothesis is further supported by the observation that core pluripotency factors OCT4, SOX2 and NANOG regulate the expression of key cell cycle regulatory proteins, such as CDK1, cyclin D1, CDK6, CDC25A, and CDC7 [36, 37]. Evidence of another link between cell cycle regulation and self-renewal machineries, namely between E2F and OCT4 activities, came from a study by Chavez and coworkers [38]. This work suggests that E2F may act as a regulatory co-factor for OCT4 at the promoters of OCT4 target genes and that ORC1L (Origin recognition complex subunit 1-like), a direct E2F target involved in DNA replication and mitotic cell cycle progression, belongs to the core OCT4 regulatory network.

Very likely, many more connections exist between master cell cycle regulators and stemness, including the regulation via microRNAs [39]. For instance, the c-Myc/E2F-driven miR-17-92 cluster that controls the G1-S transition, is fundamental for hESC self-renewal and cell proliferation and is down-regulated upon hESC differentiation [40]. Another example is the miR-290 cluster that targets multiple cell cycle-regulatory genes, such as *Cdkn1a* (encoding $p21^{Cip1}$), *Rb1, Rbl1, Rbl2,* and *Lats2* [41]. It is expressed in mESCs, repressed during mESC differentiation, undetectable in adult mouse organs [42, 43, 44], and has recently been shown to be a general characteristic of pluripotent cells [45]. Importantly, LIN28/c-Myc (which regulates miR-17-

92) and OCT4/SOX2-regulated miR-302 have been identified to be among a handful of factors necessary and sufficient to convert differentiated cells into induced pluripotent stem cells [46, 47].

DNA damage response In response to DNA damage, checkpoint pathways are activated to restore genomic integrity and to prevent formation of mutations. These mechanisms halt cell cycle progression when DNA damage has occurred to prevent replication of damaged DNA or passage of damaged chromosomes to progeny in order to avoid mutations and/or genomic instability. Furthermore, DNA damage checkpoint pathways induce the DNA damage repair machinery and, if the damage cannot be repaired, cells are instructed to undergo apoptosis. Accumulation of mutations might lead to aberrant cell function, cellular transformation and tumorigenesis. Major consequences may arise from the disruption of stem cell function, affecting numbers and/or function of stem cells as well as their differentiated progeny, tissue function and homeostasis, and possibly formation of cancer stem cells, leading to premature aging or cancer. Pluripotent stem cells of early embryos (as well as their derivatives, ESCs) must possess especially effective mechanisms of DNA damage response because any malfunction of these cells might cause major malformations of the embryo and jeopardize its health and viability.

DNA damage response in somatic cells In somatic cells, DNA damage is sensed by the phosphoinositide 3-kinase related kinases (PIKK) ataxia-teleangiectasia mutated (ATM), ATM and Rad3-related (ATR) and DNA-dependent protein kinase (DNA-PK). Their many substrates mediate cell cycle arrest in G1, S or G2 phases, DNA repair and cell death [48, 49, 50] in response to various types of DNA damage, with DNA double strand breaks (DSBs) being probably the most dangerous ones. They can be induced by exogenous factors such as ionizing radiation (IR) or genotoxic chemicals including chemotherapeutic drugs, or they can result from endogenous processes such as chromosome breakage, telomere erosion, replication of single strand breaks or action of reactive oxygen species. Activated ATM/ATR phosphorylate multiple substrates, including the signal transducers Chk1/Chk2 and the transcription factor p53. Chk1/Chk2 impose cell cycle arrest by marking CDC25 phosphatases, positive regulators of CDKs, for proteasomal degradation and indirectly by enhancing p53 stability through its phosphorylation on Ser20. This phosphorylation interferes with p53 binding to the ubiquitin ligase Mdm2 that shuttles p53 out of the nucleus and targets it for proteasomal degradation. Phosphorylation of other p53 residues enhances its transactivatory and DNA binding activity, resulting in expression of its downstream targets, including the CDK inhibitor CDKN1A (encoding $p21^{Cip1}$) or proapoptotic genes (*e.g.* PUMA, BAX). Both the immediate Chk1/Chk2-CDC25A pathway and the delayed p53-$p21^{Cip1}$ pathway contribute to core G1 checkpoint mechanisms and converge on CDK2 to inhibit its activity and induce G1 arrest.

DNA damage response in embryonic stem cells Despite the essential role of the G1 checkpoint in maintaining genomic stability, ESCs do not arrest in G1 after DNA damage [51, 52]. Instead, ESCs rely on S and G2/M checkpoints or undergo apoptosis. Why might this be so? Why do ESCs prefer entry into S phase with damaged DNA or cell death over stopping and repairing DNA in G1 phase? There must be some good reason for this strategy.

From the classical point of view, the absence of a G1 checkpoint in ESCs is beneficial for the ESC population as a whole by facilitating removal of ESCs with damaged DNA, thus avoiding mutations that might arise from inaccurate DNA repair and maintaining pristine genetic information of ESCs [53]. Consistent with this hypothesis, ESCs are hypersensitive to DNA damage [54, 55]. Yet mutations through inaccurate DNA repair can arise in later cell cycle phases as well. So why might the G1 checkpoint be inactivated but all other ones, including S and G2/M checkpoints remain intact? And what are the mechanisms that selectively inactivate the G1 checkpoint

but sustain the functionality of other DNA damage checkpoints in ESCs?

Numerous studies have attempted to reveal the mechanisms of G1 checkpoint nonfunctionality in ESCs. First observations suggested that in mESCs, p53 is localized to the cytoplasm and cannot be translocated into the nucleus [51]. Therefore, neither Cdkn1a, nor proapoptotic genes are induced and mESCs do not arrest in G1 or undergo p53-dependent apoptosis in response to DNA damage [51]. However, another study suggested that p53 is functional and revealed p53-mediated downregulation of Nanog expression as a novel mechanism for maintenance of genetic stability in mESCs by inducing their differentiation into other cell types [56]. Nonfunctionality of p53 has been further challenged by a recent study that showed p53 to be activated and Cdkn1a expression induced after IR in mESCs [57]. Furthermore, in hESCs p53 is nuclear [58] and CDKN1A is robustly induced in response to DNA damage [22]. However, despite increased levels of the CDK2 inhibitor p21Cip1 ESCs escape G1 arrest after DNA damage. What is the mechanism of this phenomenon?

With regard to the parallel G1 checkpoint pathway employing the Chk1/Chk2-CDC25A cascade, in two mESC studies Chk2 was reported to be sequestered at centrosomes and thus separated from its substrate CDC25A and therefore unable to mediate its degradation with subsequent G1 arrest [53, 57]. However, these studies diverged in observed phosphorylation patterns of Chk proteins: Chk2 was constitutively phosphorylated in one of the studies, while in the latter its phosphorylation was induced by DNA damage. Koledova and colleagues also reported constitutive phosphorylation of Chk1 on Ser345, which might be consistent with centrosomal sequestration of Chk1 observed in their study as Chk1-Ser345 phosphorylation has been suggested to be important for cytoplasmic and subsequent centrosomal localization of Chk1 on centrosomes [59].

More importantly, CDC25A levels were sustained after IR in the study by Hong and Stambrook [53], but Koledova and colleagues found robust CDC25A degradation, which was mediated by GSK-3β [57]. It is difficult to reconcile between these two studies. Differences in mESC lines studied might account for some of the observed differences; however, it is important to note that the observations of Koledova and collaborators were made in two divergent mESC lines – an inbred and a hybrid line, substantially limiting bias from cell line variability.

Interestingly, neither downregulation of CDC25A levels nor upregulation of p21Cip1 led to a G1 arrest in mESCs because CDK2 activity was not abrogated [57]. An important role for DNA damage-refractory CDK2 activity in driving rapid G1/S phase progression after DNA damage in mESCs was shown by chemical inhibition of CDK2 that slowed down mESCs progression from G1 to S after IR [57].

What might make CDK2 irresponsive to changing levels of its regulators, CDC25A and $p21^{Cip1}$? Immunolocalization studies have suggested that it might be CDK2's ESC-specific localization: CDK2 was found to be localized predominantly to centrosomes, while CDC25A localized to cytoplasm and nuclei and was not detected at centrosomes [57]. Thus, centrosomal CDK2 might be sheltered from varying levels of CDC25A and, probably, also from $p21^{Cip1}$ [57]. Alternatively, levels of $p21^{Cip1}$ might be insufficient to buffer high levels of CDK2 in mESCs as it was shown that equimolar concentrations of $p21^{Cip1}$ and cyclin-CDK2 complexes are required for efficient CDK2 inhibition [60]. The observation of a large CDK2 pool at mESC centrosomes has raised many questions. Among them the possible mechanism of G1 phase regulation by centrosomal CDK2 is most intriguing, although conclusive evidence for its existence remains elusive. We hypothesize that centrosomal CDK2 (possibly in cooperation with the cytoplasmic pool of CDK2) might regulate the activity of relevant cell-cycle (*e.g.* pRB-E2F) and cell-fate/self-renewal (*e.g.* SOX2) [61] regulatory proteins that shuttle between cytoplasm and nucleus. The role of centrosomal pools of Chk1 and Chk2 for G1/S phase regulation, if any, is not understood as well. Are they sequestered to centrosomes to avoid their interaction with CDC25A, as suggested by Hong and Stambrook [53], or do they actually play an active role in maintaining centrosomal

CDK2 activity? Having in mind that even with Chk1/Chk2 at centrosomes CDC25A is targeted for degradation by GSK-3β [57] and that centrosomal Chk1 regulates centrosomal CDK1 activity during unperturbed cell cycles as well as in response to DNA damage in somatic cells [62, 63], it is more probable that the latter might be the case.

In somatic cells, DNA damage induces Chk1-dependent amplification of centrosomes that leads to lethal multipolar mitoses [64]. This mechanism might act as an additional checkpoint in the case when earlier checkpoints fail or are not functional, *e.g.* in ESCs, which naturally are devoid of a G1 checkpoint. Based on the CDK2 requirement for centrosomal amplification [65, 66, 67, 68] and the recent finding that DNA damage causes Chk1-dependent activation of CDK2 [69], we suggest that colocalization of CDK2 and Chk1 on centrosomes might allow for fast and effective centrosome amplification after DNA damage in mESCs with subsequent efficient elimination of damaged cells from the stem cell pool via mitotic catastrophe. Furthermore, Chk1-dependent activating phosphorylation of CDK2 after DNA damage might possibly contribute to sustained total CDK2 activity after DNA damage in mESCs.

In view of CDK2's role in ESC self-renewal [20, 27], maintaining CDK2 activity after DNA damage might be important to sustain pluripotency of ESCs and to avoid their differentiation [57]. Evidence for a tight cooperation of DNA damage and cell cycle regulatory mechanisms in cell fate decisions also comes from studies in muscle stem cells, in which DSBs naturally occur during differentiation [70, 71, 72]. These DSBs are targeted to specific genomic loci, including critical differentiation regulatory genes such as *Cdkn1a*, to induce their expression [73]. It is tempting to speculate that this process may be involved in the development of other tissues as well.

Centrosomes as cell cycle regulatory centers Implicating centrosomes as critical shelters of CDK2 from activated G1 checkpoint pathways in mESCs [57], we suggest a novel role for centrosomes in coordination of fate decisions in stem cells and support the hypothesis of centrosomes as coordination centers of cell cycle regulatory events.

Centrosomes are microtubule organizing centers of animals cells. During G1 phase, there is only one centrosome (consisting of two centrioles and pericentriolar material) that is replicated during S phase. The two centrosomes form a bipolar mitotic spindle during mitosis, thereby allowing for accurate chromosome segregation and cytokinesis. Aberrations in centrosome number and/or function have been associated with genomic instability and tumorigenesis. However, whether these centrosomal aberrations are a cause [74, 75, 76] or a consequence of malignant transformation still remains elusive [77, 78, 79].

Several studies have implicated a role for centrosomes in the regulation of G1-S and G2-M transitions and DNA damage checkpoint signaling with multiple proteins implicated in cell cycle control and DNA damage response are found to localize to centrosomes (for a review, see Doxsey *et al.* [80]). Consecutively, a new concept for centrosomes as cell cycle regulatory centers has arisen, according to which centrosomes might function as "scaffolds" to promote interactions between various regulatory components during the cell cycle [80, 81, 82].

Studies of karyoplasts (acentrosomal cells) revealed that an activity associated with core centrosomal structures is required for cell cycle progression from G1 to S phase, as ablation of centrosomes in late G2 phase led to an arrest at the G1/S boundary of the next cell cycle [83, 84]. This activity might be conferred to cyclin E because its localization to centrosomes is required to promote S phase entry in a CDK2-independent manner [85]. Furthermore, alterations in centrosome composition by knockdown of discrete centrosomal proteins can induce p38-p53-p21$^{\mathrm{Cip1}}$-dependent G1 arrest [86].

The first implication of a role for the centrosome in mitotic entry came from work in oocytes which showed that injection of centrosomes into G2-arrested starfish oocytes can induce progression into mitosis [87], activate maturation promoting factor (MPF; cyclin B-CDK1) and accelerate mitotic entry in Xenopus eggs [88]. Subsequent work in somatic cells

demonstrated the presence of CDK1 [89, 90] and cyclin B [91] at centrosomes. More recently it was shown that an activated cyclin B-CDK1 complex first appears on centrosomes during prophase [92] and this activation is regulated by Chk1, which localizes to centrosomes during interphase and shields centrosomal CDK1 from unscheduled activation by cytoplasmic CDC25B [62]. In response to DNA damage, Chk1 accumulates at centrosomes and induces a G2/M cell cycle arrest [63]. Also, a subset of Chk2 molecules localize to centrosomes and mitotic apparatus, suggesting a role for Chk2 in regulation of mitosis [82, 93]. Together, these observations indicate that centrosomes are sites where cell cycle decisions and checkpoint reactions take place.

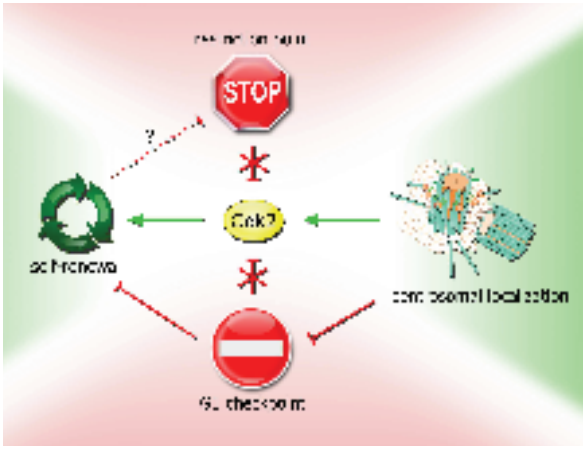

Figure 1.: Regulation of CDK2 activity G1 checkpoint, restriction point and localization to centrosomes in the context of self-renewal. In somatic cells, CDK2 activity is negatively regulated by restriction point and G1 checkpoint. However, because of CDK2's role in self-renewal, these mechanisms are inactivated in mESCs. G1 checkpoint activity is partially abrogated by sequestration of Chk1/Chk2 proteins to centrosomes, separating them from their substrates, and partially by CDK2 localization to centrosomes, where it is protected from others activated G1 checkpoint pathways (p53 – p21; GSK-3β – CDC25A). Also, mechanisms of self renewal may be involved in abrogation of restriction point (dashed line).

Centrosomal decision on self-renewal

The little information available for mESCs suggests a role for centrosomes as sites for sequestration of checkpoint components (Chk1, Chk2) [53, 57] on the one hand and as a shelter for cell cycle-regulatory proteins (CDK2) [57] to prevent reactions that might limit self-renewal (Figure 1). It would be interesting to investigate whether hESCs use similar mechanisms of escape from G1 arrest after DNA damage and whether CDK2 localizes to centrosomes in these cells as well. As mESCs and hESCs differ in cell cycle regulation and because they have been suggested to represent different pluripotent states, equivalent to inner cell mass epiblast progenitors and to the early postimplantation epiblast, respectively [94], the outcome might be surprising.

Another, only recently appreciated role of centrosomes, is regulation of asymmetrical division of stem cells: Although centrosomes are not essential in many somatic cell types, there is compelling evidence that centrosomes are required for the efficient asymmetrical division of stem cells. Well studied examples are the early *C. elegans* and *Xenopus* embryos, *Drosophila* neuroblasts [76] and *Drosophila* male germline stem cells (GSCs) [95], in which perturbations of centrosome function or number compromises asymmetrical division [96, 97]. In some cases, such as in Drosophila male GSCs, the asymmetric outcome of the cellular division is specified by asymmetric placement of daughter cells into microenvironments that provide extrinsic signals to direct cells to different fates [98, 99]. In others, such as in Drosophila neuroblasts, stem cells undergo asymmetric division based on differential segregation of intrinsic fate determinants [100, 101]. Relationships between spindle orientation and symmetricity of stem cell division have also been observed in stem cells from mammalian skin [102], muscle [103], and neuronal stem cells [104], suggesting a broad involvement of centrosomes (through spindle orientation) in the accomplishment of asymmetric stem cell divisions.

Interestingly, it has been shown that Drosophila male GSCs preferentially inherit and retain the mother centrosome and that the asymmetric behavior of mother and daughter centrosomes is the reason for the asymmetric outcome of male GSC divisions [105]. This observation suggests that an "immortal centrosome," retained in the stem cell, could help to determine stem cell fate and raises the possibility that preferential centrosome inheritance is a conserved stem cell mechanism [106].

On the other hand, asymmetrically inher-

ited centrosomes in stem cells could be just innocent bystanders that are differentially segregated because of their role in spindle orientation or they could be carriers of developmental information. One study has already revealed asymmetric inheritance of a fate-determining mRNA that associates with only one centrosome during early embryonic development in a mollusk [107]. The complex role of centrosomes in self-renewal decisions remains to be elucidated.

Embryonic stem cells as a model for embryonic pathways: a handy tool or an artifact? An intriguing issue is to what extent the observations in vitro in ESCs correspond to the situation in vivo in cells of early embryos. Transfer of stem cells from the embryo to the culture dish subjects cells to selective pressure from their new environment and ESCs adapt to culture conditions through self-transformation events involving epigenetic and karyotypic changes [108, 109].

One of the stress factors impinged on embryo-derived stem cells is oxygen: ESCs are routinely cultured under normoxic conditions although it is well known that in the mammalian reproductive tract, the native environment for preimplantation embryos, are hypoxic [110]. Stem cells, including ESCs, might benefit from residing in hypoxic niches where oxidative DNA damage may be less frequent [111]. This hypothesis is supported by the observation of alterations in mitochondrial DNA in late passage hESC lines cultured under high oxygen tension [112]. Also, hESCs were shown to prefer hypoxia for maintenance of a highly proliferative, pluripotent state [113, 114, 115]. However, only short-term hypoxia increased mESC proliferation (through upregulation of cell-cycle regulatory proteins such as Cdk2) [116]. Long-term hypoxia, on the other hand, inhibited self-renewal and led to differentiation of mESCs [117]. The full extent to which ESCs differ from their developmental counterparts in embryos remains elusive. Nevertheless, in the absence of better systems ESCs remain the most suitable tool for modeling of processes in cells of early embryos as well as cancer stem cells.

Concluding remarks In embryonic stem cells, high CDK2 activity drives rapid progression through G1 phase to maintain their self-renewing state by minimizing the potential exposure to differentiation signals. Because downregulation of CDK2 activity is sufficient to induce stem cell differentiation, mechanisms negatively regulating CDK2 activity in somatic cells, such as restriction point and G1 checkpoint, are deactivated in ESCs. Another level of protection of CDK2 activity in ESCs might be its specific localization to centrosomes, where it is sheltered from activated G1 checkpoint pathways. Chk1 and Chk2 proteins are sequestered to centrosomes, to separate them from their substrates and possibly to increase CDK2 protection. Therefore, in the cell fate game, centrosomes appear to play a mediatory role, providing an interface for crucial cell cycle and cell fate decisions to take place.

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