ABSTRACT

LEE, SUNG HYUN. Novel Roles of Cyclin-Dependent Kinases 4/6 in Mouse Keratinocytes. (Under the direction of Dr. Marcelo Rodriguez-Puebla).

The Cyclin-Dependent Kinase 4 (CDK4) and 6 (CDK6) belong to a family of serine-threonine kinases that are regulating cell cycle through the association with D-type cyclins at specific time-points of the cell cycle. It has been widely assumed that CDK4 and CDK6 play redundant roles since they share regulatory subunits and D-type cyclins, and also phosphorylate the same target, the Retinoblastoma protein (pRb). At the same time, however, we have recently shown that CDK4 and CDK6 have non-redundant roles, drawing entirely opposite results in chemical-induced skin tumorigenesis.

We have previously established that overexpression of CDK6 in mouse epidermis (K5CDK6 mice) leads to the reduction of the multiplicity and incidence of skin tumors. Our previous results have shown that CDK6 preferentially binds to cyclin D3. Thus, we hypothesized that the CDK6/cyclin D3 complex negatively impacts skin tumorigenesis. To test this idea, we developed K5CDK6/Cyclin $D3^{-/-}$ mutant mice, and subjected them to a carcinogenesis protocol. Contrary to our hypothesis, lack of cyclin D3 expression does not affect the tumor repression activity of CDK6 since both, K5CDK6 and K5CDK6/$D3^{-/-}$ mice, exhibit a severe reduction of the number of skin tumors. However, ablation of cyclin D3 levels with CDK6 overexpression leads to malignant progression and increment of tumor size compared to siblings. Biochemical analysis of epidermal tumors shows that cyclin D3 ablation results in increased cyclin D1 level, leading to elevated CDK4/cyclin D1 and CDK6/cyclin D1 complex formation in K5CDK6/cyclin $D3^{-/-}$ epidermis. These data suggest

that though ablation of cyclin D3 does not alter the tumor suppressive role of CDK6 at early stages of tumorigenesis, but it alters the susceptibility of CDK6-expressiong tumors to malignant conversion. Therefore, we conclude that CDK6 activity prevents tumor formation during the initial stages of tumorigenesis in a cyclin D3-independent manner and likely in a pRb-independent manner as well.

Unlike CDK6, forced expression of CDK4 in epidermis (K5CDK4 mice) accelerates malignant progression to squamous cell carcinomas (SCC). However, the canonical function of CDK4 inactivating pRb cannot fully explain this result. Therefore, we predict that CDK4 plays a role in malignant progression via pRb-independent pathways by an unknown mechanism. Based in other and our studies, we hypothesize that forced expression of CDK4 results in phosphorylation of centrosome related proteins, leading to centrosome amplification – a driving force of malignant progression. Our results show that CDK4 overexpression leads to an over-amplification of centrosome during carcinogenesis compared to the regular centrosome amplification observed in tumors from wild-type mice. In addition, we suggest 21 putative centrosome-relative substrates for CDK4. Meanwhile, we also hypothesize that CDK4 associates to chromatin and directly regulates the expression of target genes related to centrosome amplification. Supporting our hypothesis, we observed that CDK4 governs the transcription of genes associated with Chromosome Instability (CIN). Interestingly, elevated expression of Aurora B kinase induces supernumerary centrosome in a mouse carcinoma cell line overexpressing CDK4. Collectively, these results suggest that overexpression of CDK4 contributes centrosome amplification by directly regulating CIN related genes at transcriptional levels. In particular, Aurora Kinase B overexpression might

play a unique role in CDK4 overexpressing cells contributing to centrosome amplification and likely in malignant progression.

Novel Roles of Cyclin-Dependent Kinases 4/6 in Mouse Keratinocytes.

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A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Physiology

Raleigh, North Carolina

2015

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ACKNOWLEDGMENTS

I would like to express my most sincere gratitude to Dr. Marcelo Rodriguez-Puebla for their valuable advice and kind guidance on my research. I really appreciate Drs. Philip L. Sannes, Robert C. Smart, and Jun Ninomiya-Tsuji for their valuable comments and advices throughout my degree.

I also thank to my lovely labmates, Dr. Chris Sistrunk, Dr. Xian Wang, Dr. Sun Hye Kim, Adam Ward and Rima Majumdar for having given me support and encouragement.

Finally, I would like to thank my husband and Jinwoo for great supports and love at all times. I am grateful to my family in Korea for your endless support.

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Introduction

1. Canonical roles of G1 phase-Cyclin dependent kinase in Cell cycle Regulation

1.1. Cell cycle Regulation and Cyclin-dependent kinase

The cell cycle is an organized sequence of events in a cell that lead to replication of cell contents containing DNA and organelles and its division into two daughter cells. The regulation of cell cycle progression is obviously crucial to the cell proliferation and differentiation. The cell cycle consists of four distinct phases: G1 phase (first Gap), S phase (synthesis), G2 phase (second Gap) and M phase (mitosis) (Figure 1). In addition, a stage of quiescence called as G0 phase is distinguished for cells that have stopped dividing temporarily or reversibly. When mitogen signals such as growth factor stimulation exist, cells in G0 phase enter to G1 phase. During G1 phase, the biosynthetic activities of the cell rise up to increase in mass and organelle number in preparation for cell division and synthesize various enzymes required in S phase. S phase is the period for synthesis of DNA that typically spends 10-12 hours in mammalian cells and thus, the chromosome content is doubled in this phase. During the G2 phase, the cell synthesizes additional proteins, mainly involving in production of microtubules and continues to increase in size. If protein synthesis in G2 phase is inhibited, the cell fails to undergo mitosis. The segregation of duplicated chromosome in the nucleus into two identical sets (karyokinesis) and cell division (cytokinesis) occur during the M phase. Typically, the mitosis is divided into 4 phases, which include prophase (chromatin condensation and migration toward the cell center), metaphase (disappearance of nuclear membrane and generation of two spindle poles), anaphase (chromosome separation) and telophase (nuclear membranes reform and completion of cytokinesis. Once a cell has completed the cell cycle, it goes back into G1

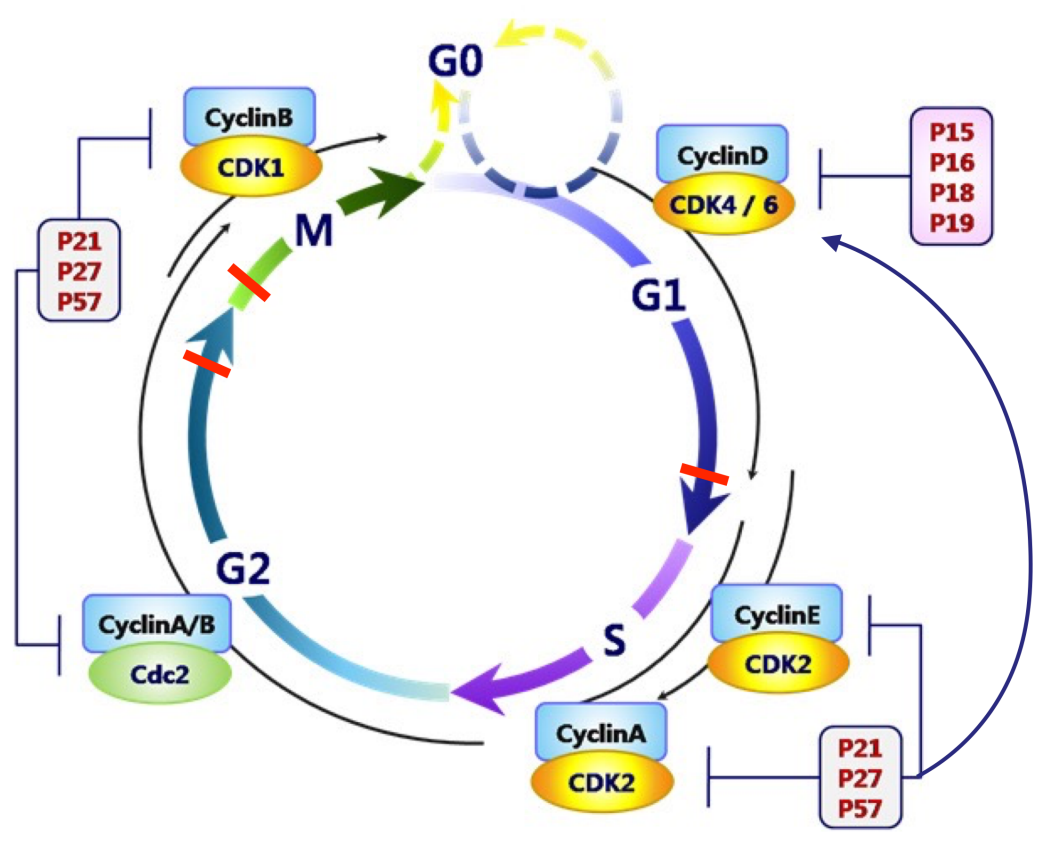

Figure 1. The Cell Cycle and CDKs. The cell cycle is divided into four phases, first gap (G1), synthesis (S phase), second gap (G2) and mitosis (M phase). Cyclin-dependent kinase activity fluctuates throughout the cell cycle and regulates the major process of the cell cycle. Extracellular signals are integrated in G1 phase by D-type cyclins and Cyclin-Dependent Kinases (CDKs), which are negatively regulated by CKIs (Ink4 and Cip/Kip families). The red bars indicate the checkpoint in G1, G2 and mitosis.

phase and repeats the cell cycle again, or places in a G0 phase until the external conditions favor cell replication.

The cell cycle is governed by complexes of Cyclin-dependent kinases (CDKs), a family of serine/threonine kinases, and their count-partners, Cyclins (Evans et al. 1983). Since expression and degradation of Cyclins are occurred at specific points of the cell cycle, activities of CDK fluctuates throughout the cell cycle (Figure 2). To date, at least 29 Cyclins and 11 CDKs have been identified from the human genome, and most are directly and/or indirectly involved in cell cycle regulation (Malumbres & Barbacid 2005). Among them, five CDKs and four different types of Cyclins play their main roles in cell cycle progression: CDK4/6-D type cyclins and CDK2-E type cyclins in G1 phase, CDK2-E/A type cyclins and CDK1-A type cyclin in S phase, CDK1-B type cyclins in G2/M phase (Murray 2004). For instance, D-type cyclins (Cyclin D1, D2, and D3) cooperate with CDK4/6 to initiate G1/S phase transition, whereas, Cyclin E associates with CDK2 to complete G1/S phase transition. CDK2-Cyclin A and CDK1-Cyclin A complex share several substrates involved in DNA replication and check-point. In addition, CDK1-Cyclin B complex phosphorylates broad range of substrates to both the G2/M phase transition and mitosis progression (Malumbres & Barbacid 2005).

1.2. Regulation of CDK4/6 kinase activity

1.2.1. Activation mechanisms

The primary mechanism for activation of CDK4/6 kinase is complex formation with their regulatory subunits D-type cyclins (D1, D2 and D3), therefore, their activities are highly

dependent on the availability of D-type cyclins which is the rate limiting factor (Quelle et al. 1993). It is widely accepted that D-type cyclins serve as a key sensor and integrator of extracellular mitogenic signals through binding to CDK4/6 and diverse transcription factors/co-regulators such as histone acetyltransferase protein p300/CBP (Fu, Wang, et al. 2005b). Many oncogenic signaling pathways are able to induce expression of cyclin D1, which including Ras/MAP signaling, P13K/Akt signaling, Src, ErbB2, β-catenin Tcf/Lef pathway and simian virus 40 small T antigen. Among signaling pathways, induction of cyclin D1 is mostly carried out via Ras-Raf1-MAPK/ERK pathway (Musgrove et al. 2011).

Newly synthesized D-type cyclins quickly form complexes with CDK4/6 and translocate into the nucleus. Unlike cyclins, CDK4/6 expression is relatively consistent throughout the cell cycle and mainly locates in cytoplasm. In vitro, the assembly of purified cyclin D1 and CDK4 occurre with a very low affinity (Kato et al. 1994), because some chaperone proteins play an important role in complex formation. For instance, Hsc70 stabilizes newly synthesized cyclin D1, and consequently increases its availability for assembly with CDK4 (Diehl et al. 2003). Hsp90/CDC37 complex targets CDK4 not bound to D-type cyclins and give stability to CDK4 (Stepanova et al. 1996). Translocalization of D-type cyclins and CDK4/6 complex is regulated by GSK-3β dependent phosphorylation at T286 in cyclin D1. Phosphorylation at T286 in cyclin D1 by GSK-3β promotes the nuclear export during S phase of cell cycle through association of nuclear exportin CRM1 (Alt et al. 2000).

The assembly of D-type cyclins and CDK4/6 is not sufficient to full activation of this complex. In addition to association with cyclins, full kinase activity requires a post-translational modification – an activating phosphorylation on CDK4-T172 by CDK

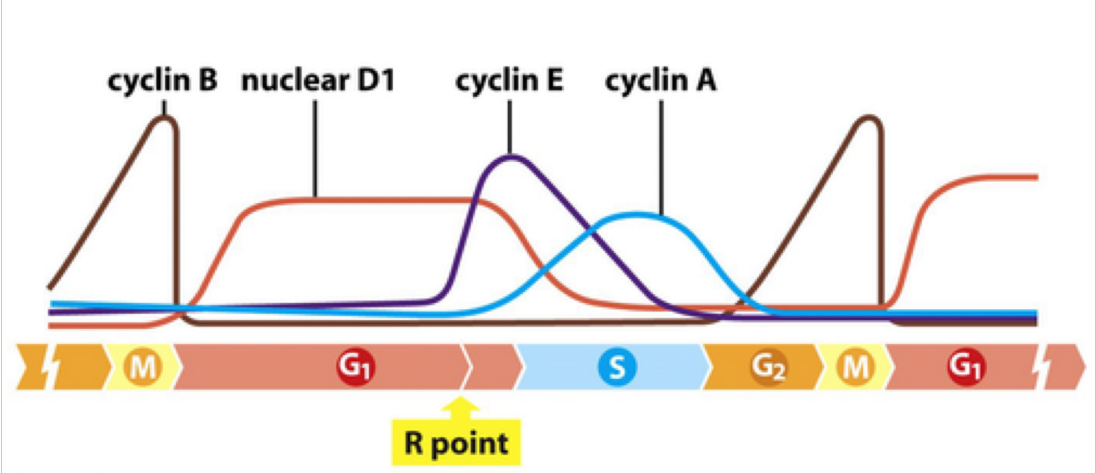

Figure 2. The oscillations of the cyclins through the cell cycle. The concentration of cyclins fluctuates throughout the cell cycle. Extracellular stimuli induce the D-type cyclins expression in early G1 phase. Cyclin E accumulates in late G1 phase, tightly linked to the restriction point (R point). Cyclin A expression occurre during S phase, and degrade during metaphase. Expression of cyclin B starts in G2 phase and reach its highest concentration in anaphase. Catalytic activity of cyclin-CDK complexes is dependent on the concentration of cyclins and post-translational regulation. The figure is from "The biology of Cancer" @ Garland Science, the second edition (Weinberg 2013).

activating kinase (CAK) composed of CDK7/cyclin H/Mat1 (Morgan 1997). The catalytic cleft of CDK for kinase activity is localized between N-lobe and C-lobe, and blocked by two helices, T-loop and PSTAIR helix. Upon cyclin binding, the two helices move inside resulting in exposing an active site on T-loop. Then CAK phosphorylates on the exposed active site allowing proper loading of ATP at the ATP-binding site. Notably, the phosphorylation of CDK4/6-cyclin Ds by CAK effectively increase the catalytic activity 80-300 fold (Coqueret 2002).

1.2.2. Inactivation mechanisms

Direct binding inhibits the activity of cyclin/CDK complexes to Cyclin-dependent kinase inhibitors (CKIs), which consist of two families, the INK4 ($p15^{Ink4b}$, $p16^{Ink4a}$, $p18^{Ink4c}$ and $p19^{Ink4d}$) and Cip/Kip family ($p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip1}$). The Cip/Kip family is considered as broad-spectrum inhibitors due to association with both cyclin D-CDK4/6 and cyclin E/A-CDK2 kinases, whereas, the INK4 family targets CDK4 and CDK6 specifically without affecting the function of the other cyclin/CDK complexes (Ekholm & Reed 2000).

Association of INK4 with the non-catalytic side of CDK4/6 prevents its interaction with D-type cyclins via allosteric changes in the CDK4/6 structure by rotating the C- and N-lobe. In addition, these conformational changes also block the association of Cip/Kip family with CDK4/6. Thereby, substantial INK4 inhibitors cause not only inhibition of CDK4/6, but also redistribution of the CiP/Kip inhibitors released from CDK4/6 which bind and inhibit CDK2/cyclin E, A complexes (Sherr & Roberts 1999).

The role of Cip/Kip family in CDK4/6 is controversial: positive and negative regulation of CDK4/6 activity depending on their phosphorylation status. Unphophorylated p21 and p27 proteins, has been observed under specific conditions such as contact inhibition or serum starvation. These unphosphorylated forms of the Cip/Kip members directly inhibit CDK4/6 activity via blocking a catalytic cleft. On the other hand, phosphorylated p21 (Y76) or p27 (Y88, Y89) is able to interact with active CDK4/6-cyclin D complexes to stabilize their assembly, but they are not required for complex assembly itself (Bagui et al. 2003). Also Cip/Kip proteins mediate nuclear import of CDK4/6-cyclin D complex from cytoplasm (Cheng et al. 1999).

Like activating phosphorylation, CDK inhibitory phosphorylation is also vital for regulation of CDK activity. Two specific kinases, Wee1 and Myt1, phosphorylate CDK4 at Thr14 and it inhibits CDKs activity (Russell & Nurse 1987; Mueller et al. 1995). Inhibitory phosphorylated Cyclin-CDK complexes remain as inactive forms until they are dephosphoylated by the Cdc25 phosphatase family such as Cdc25A, Cdc25B and Cdc25C. Importantly, CDKs also phosphorylate Cdc25 resulting in activation as a positive feedback loop (Morgan 1997).

1.3. G1 phase regulation through the pRb/E2F pathway

Cell division is commonly initiated by extracellular signals such as growth factors and cytokines resulting in synthesis of D-type cyclins working on G1 phase. D-type cyclins form a complex with CDK4 and/or CDK6 followed by transporting to the nucleus. Active complex of CDK4/6-cyclin Ds then initially phosphorylate members of the retinoblastoma

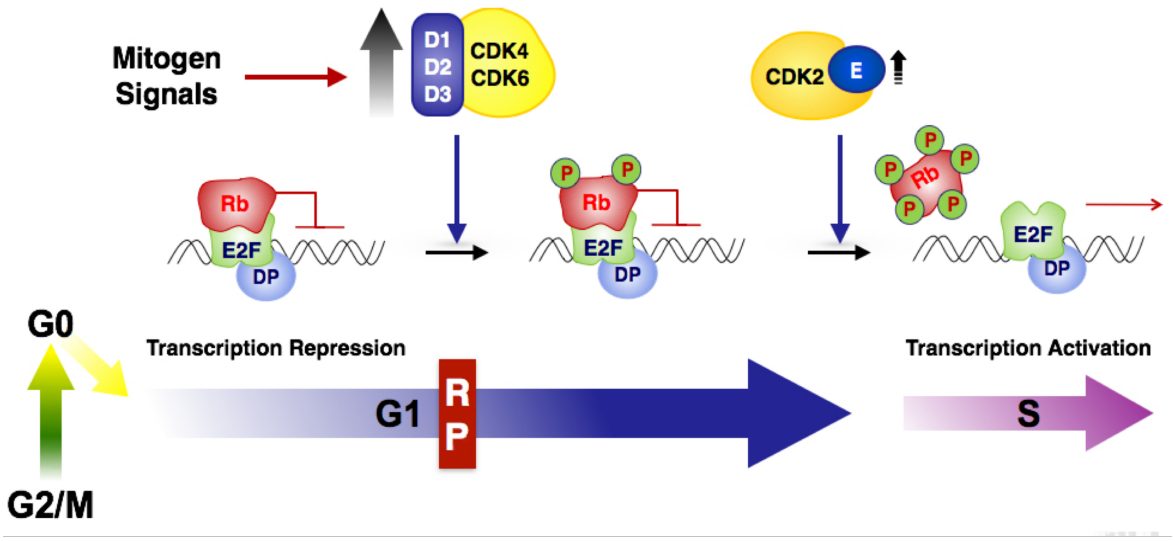

Figure 3. G1/S phase transition and inhibition of the pRb pathway. Extracellular stimuli lead to expression of D-type cyclins, and form complexes with CDK4/6. Cyclin D-CDK4/6 partially phosphorylate pRb followed by expression of cyclin E and A. Active cyclin E-CDK2 complex completes pRb phosphorylation, allowing fully activation of E2F transcriptional activity. Once passing the restriction point (RP) in G1 phase, the cell progress into the cell-cycle independently of mitogenic stimuli.

(Rb) protein family including pRb, p107 and p130. Rb protein family guard the restriction point (R-point), a temporal point in which mitogenic signals are no longer needed for progression into the cell cycle (Sherr 1994). All three members of Rb family inhibit transcription by binding the transcription factors such as E2F family, histone deacetylases and chromatin remodeling complexes (Cobrinik 2005). In detail, pRb associates with E2F-DP dimer and arrest its transcriptional activity in quiescent cells. Upon mitogenic stimulation, quiescent cells exit from G0 into G1 phase and CDK4/6-cyclin D complex is activated. Active CDK4/6-cyclin D complex partially phosphorylate pRb resulting in destabilization of interaction with E2F-DP dimer, and allows partial activation of E2F transcriptional activity. The initial activation of E2F permits the transcription of cycle E followed by formation of CDK2-cyclin E complexes that further phosphorylate pRb. Hyper-phosphorylated pRb completely releases from E2F-DP dimer and E2F starts transcription of critical genes for cell cycle progression (Malumbres & Barbacid 2001) (Figure 3). pRb keeps hyper-phosphorylated state throughout S, G2 and M phases, and it is restored to growth-suppressive hypo-phosphorylated state by Protein Phosphatase 1 (PP1) during M to G1 phase transition (Nelson et al. 1997).

The role of E2F transcription factor in controlling the cell cycle progression has become clear from a large number of functional studies identifying multiple E2F-regulated genes (Dyson 1998). E2F family governs the transcription of various compulsory genes required by DNA replication, DNA repair and DNA-damage checkpoints, and chromatin regulators (Malumbres & Barbacid 2001). Therefore, pRb-CDK4/6-E2F axis is a core of the cell cycle progression, especially during the G1/S phase transition.

2. Non-canonical roles of G1 phase-Cyclin dependent kinase

It is an obvious fact that CDK4/6 are positive regulators of cell division through activation of E2F pathway, but, this canonical model is not sufficient to explain functions of CDK4/6 in tumorigenesis. Recently, several studies have revealed CDK4/6 also has pRb-independent roles affecting tumor development, via direct phosphorylation of targets.

2.1. Activating FOXM1 signaling

Recently, Sincinski's group tenders numerous phosphorylation targets of CDK4 and/or CDK6 by using a systematic screen, based on combination of computational and experimental approaches in vitro (Anders et al. 2011). In this study, the entire human proteome in the protein database SWISS-PROT was scanned with the web-based Scansite (Obenauer et al. 2003) to select putative nuclear substrates, and finally, 71 proteins were picked as in vitro substrates. Interestingly, cyclin D3-CDK6 had broader substrate selectivity than cyclin D1-CDK4: 13 common in vitro substrates for both CDK4 and CDK6 including RB1, 53 for cyclin D3-CDK6, and only 5 for cyclin D1-CDK4.

Forkhead box M1 (FOXM1), defined as the common putative substrate of CDK4 and CDK6, is a typical proliferation-associated transcription factor, promoting G1/S phase transition (Xinhe Wang et al. 2002; I-Ching Wang et al. 2008), suppressing cellular senescence (I-Ching Wang et al. 2005; Park et al. 2009), and intimately contributing tumorigenesis (Wierstra 2013). CDK4/6 can directly phosphorylate at multi-sites on FOXM1, primarily at its C- and N-terminal sequences, but the effects are different (Anders et al. 2011). First, multisite phosphorylation at C-terminus, spanning T600 to S704, releases transactivation

domain (TAD) from auto-repression and leads to activation of FOXM1 transcriptional function. Second, multi-site phosphorylation by CDK4/6 curbs on proteasomal degradation and stabilizes FOXM1. Through experiments with phosphomimic mutation of FOXM1, it is revealed that multiple-phosphorylation spanning whole sequence prevents FOXM1 from proteosomal degradation. Notably, activation and stabilization of FOXM1 by CDK4/6 are pRb-independent because these phenomena also present under ablated pRb in shRNA treated U2OS cells or dysfunctional pRb in Hela cells (Anders et al. 2011).

Activated FOXM1 results in expression of target genes that involved in senescence suppression and G1/S phase transition. To investigate roles of FOXM1 in 19 CDK4/6-regulated genes obtained by microarray analysis from CDK4/6 specific inhibitor-treated U2OS cells, a constitutively active CDK4 mutant was transfected into FOXM1 knockout MEFs followed by comparing 19 genes expression levels to their levels in control groups. As a result, activated FOXM1 by CDK4 influenced 9 out of 19 genes important to DNA replication (*CCNE2*, *MYB*, *MCM2*, *MCM10*, and *CDT1*), DNA repair (*XRCC2*) and mRNA splicing (*SFRS4*).

2.2. Altering TGF-β signaling

Transforming growth factor-β (TGF-β) signaling leads to apoptosis, epithelial-mesenchymal transition and growth arrest at G1 phase in several cell types by Smad protein cascade (Massagué et al. 2000; Derynck et al. 2001). For example, TGF-β signaling induces expression of CKIs such as $p15^{INK4B}$ and $p21^{CIP1}$ (Feng et al. 2000; Pardali et al. 2000; Moustakas et al. 2002), and suppresses the transcription of c-Myc, leading to transcription of

CDK4 and CDC25A directly (Hermeking et al. 2000; Galaktionov et al. 1996). However, the role of TGF-β signaling in tumor development has been controversial: suppression of tumor development in early stage, while, mediation of tumor progression by inducing epithelial-mesenchymal transition in late cancer (Derynck et al. 2001; Grusch et al. 2010).

It has recently been shown that CDK4 and CDK2 can phosphorylate at Smad2/3 linker region and eliminate the anti-proliferative effects of TGF-β signaling (Matsuura et al. 2004; Matsuzaki et al. 2009; Liu & Matsuura 2005) . Matsuura et al showed that CDK2 and CDK4 phosphorylates the linker domain of Smad 3 (Thr278, Ser203, Ser207 and Ser212) and Thr8, resulting in suppression of Smad3 transcriptional activity and anti-proliferative function (Matsuura et al. 2004). In addition, Matsuzaki et al created domain specific antibodies against Smad2/3, detecting phosphorylation sites in linker domain or in C-terminus, and investigated roles of each domain specific phosphorylation (Matsuzaki et al. 2009). According to results, if the linker domain of Smad2/3 is phosphorylated by CDK4 in nuclei, it leads to transcription of c-Myc. Alternatively, c-Jun NH2-terminal kinase (JNK) also phosphorylated the linker domain in the cytoplasm and consequently upregulated up-regulating matrix metalloproteinase-9 (MMP-9), involving tumor invasion mechanism. Thus, phosphorylation in the linker domain of Smad2/3 by CDK4, together with JNK, may twist tumor-suppressive TGF-β signaling to promote malignant progression in late stage of tumors.

2.3. Transcriptional function of CDK6

Although CDK6 promotes cell cycle progression via inhibition of pRb and its overexpression

is commonly found in human cancers, its oncogenic function is still controversial. For example, reduction of CDK6 expression has been founded in some pancreatic tumors, Testicular Germ Cell Tumors and breast tumors (Tomita 2004; Lucas et al. 2004; Schmidt et al. 2001). Supporting these results our laboratory showed that CDK6 overexpression negatively affect tumor development in a mouse skin tumor model (Xian Wang et al. 2011). Although the molecular mechanism by which CDK6 inhibit tumorigenesis is not fully understood, the earlier reports showed that overexpressed CDK6 induces accumulation of growth-suppressing proteins, p53, and p130 (Nagasawa et al. 2001; Lucas et al. 2004).

Two years ago, Kollmann et al revealed unexpected transcriptional role of CDK6, involving in cell cycle inhibition and angiogenesis (Kollmann et al. 2013). Forced expression of CDK6 in BCR-ABL-transformed B acute lymphoid-leukemia cells caused elevation of $p16^{INK4a}$ transcription. Through ChIP assay, they found that a transcriptional complex containing CDK6, D-type cyclin and STAT3 associated on the promoter of $p16^{INK4a}$ and directly regulated expression of $p16^{INK4a}$. Surprisingly, this transcriptional function of CDK6 is kinase activity independent. The kinase inactivated CDK6 mutant also upregulated transcription of $p16^{INK4a}$. As aforementioned, $p16^{INK4a}$ is a CDK4/6 specific inhibitor, therefore, overexpressed CDK6 inhibits cell proliferation and delays tumor formation upon xenograft mice models through this negative feedback loop.

Furthermore, they also examined oncogenic role of CDK6 in T-lymphoid tumor formation. Cdk6-deficient NPM-ALK-transgenic mice shows significantly prolonged latency and resistant to metastasis induced by active Akt/Notch signaling (Hu et al. 2009). These conflicting results, acting as a tumor suppressor in B-lymphoid tumor via induction of

$p16^{INK4a}$ and a driving force of malignant progression in T-lymphoid tumor, can be explained by another CDK6 kinase-independent, transcriptional role (Kollmann et al. 2013). Kollmann et al found NPM-ALK-$CDK6^{+/+}$ lymphoma cells had methylated CpG island in $p16^{INK4a}$ promoter, resulting in loss of expression. On the other hand, CDK6 directly regulated transcription of vascular endothelial growth factor, VEGF-A by association with *Vegf-A* promoter region. Unlike regulation-mechanism of $p16^{INK4a}$, CDK6 formed a transcriptional complex with AP-1 transcription factor c-Jun on *Vegf-A* promoter, but at this time, D-type cyclin was not required to induce transcription. Corresponding with the angiogenic role of VEGF-A, ALCL T-lymphoid tumor expressing high levels of CDK6 presented elevated expression of CD31, an angiogenic marker, and increased density of blood vessels. Therefore, these results suggest that CDK6 transcriptional role is probably a key factor to switch fate of tumorigenesis.

2.4. Centrosome amplification through phosphorylation of NPM1

Chromosomal instability (CIN), a hallmark of tumors, describes as abnormality in chromosome structures (insertion, deletion, recombination, and translocation) and number changes (aneuploidy). Mostly, CIN occurs at early stage of tumors and accelerates tumors malignant progression associated with poor prognosis (Heng et al. 2013). The prominent cause of CIN is supernumerary centrosomes, inducing chromosomal segregation error (Fukasawa 2005). Occasionally, centrosome abnormality occurs by misregulation in the pRb/E2F pathway since E2F targets several genes, related to centrosome-duplication licensing such as cyclin E, cyclin A, Nek2 and RanBPM (Ren et al. 2002; Ishida et al. 2001).

If expression of E2F activators, including cyclin E, cyclin A, cyclin D, CDK4/6 and CDK2, are altered, centrosome amplification can be triggered with/without p53 ablation (Kawamura et al. 2004; Hanashiro et al. 2008; Nelsen et al. 2005; Lee et al. 2014; Adon et al. 2010; Harrison Pitner & Saavedra 2013).

Independently of pRb pathway, hyper-active CDK4 in p53-null cells can mediate centrosome amplification through phosphorylation of Nucleophosmin (NMP1), a licensing centrosome duplication protein (Hinchcliffe & Sluder 2001; Adon et al. 2010; Cuomo et al. 2008). According to Adon et al, *Cdk2*$^{-/-}$ MEF showed similar centrosomes profiles to wild type MEF, while ablation of CDK4 disturbed centrosome duplication: population of MEF with once centrosome was increased, compared to wild-type MEF. However, if CDK2 was overexpressed in *Cdk4*$^{-/-}$ MEF, cells recovered the ability to duplicate centrosome. These results suggest that CDK4 involves in regulation of normal centrosome duplication and CDK2 also compensates for lack of CDK4. In addition, p53 deficiency results in increase of CDK4 or CDK2 activity through reduction of $p21^{Cip1}$, a downstream target of p53. Hyperactive CDK2 and CDK4 in *p53*$^{-/-}$ MEF constitutively phosphorylate NPM at Thr199, resulting in uncontrolled centrosome duplication. Correspondingly, ablation of CDK2 or CDK4 prevented centrosome amplification imposed by p53 knockdown via abrogation of phosphorylation at Thr199 in NPM1 (Adon et al. 2010).

It is already known that regulation of phosphorylation at $NPM1^{T199}$, a target site of cyclin E-CDK2 complex, is critical to licensing centrosome duplication (Okuda et al. 2000; Tokuyama et al. 2001). During early G1 phase, unphosphorylated NPM1 associates with centrosome to prevent premature centriole duplication. Upon phosphorylation at $NPM1^{T199}$, NPM1

detaches from the centrosome, permitting centrosome duplication at G1/S phase transition (Tokuyama et al. 2001). It is worth mentioning that $NPM1^{T199}$ is phosphorylated not only for CDK2 and CDK4, but also CDK6 activated by viral cyclin (v-cyclin) (Sarek et al. 2010). V-cyclin, transcribed from a gene of Kaposi's sarcoma-associated herpes virus (KSHV), has high affinity to CDK6 kinase and activates CDK6 catalytic activity; Cyclin v-CDK6 complex can efficiently phosphorylate pRb and initiate cell cycle progression (Mittnacht & Boshoff 2000; Laman et al. 2001; Coqueret 2003). Although it has not been studied whether cyclin-v-CDK6 complex triggers centrosome amplification, these researches indicate that NPM1, a centrosome duplication licensing protein, is a common substrate of G1 phase CDKs.

3. Deregulation of CDK4/6 in tumors and mouse skin tumorigenesis model

3.1. Alteration of CDK4/6 activity in human tumors

The cell cycle is strictly supervised to avoid uncontrolled cell proliferation. If the cell cycle components are deregulated, it might lead to hyperproliferation and tumor formation. As a master switch of the cell cycle progression, a tumor suppressor protein, pRb has been found dysfunctional in a majority of human cancers. Dysfunction of pRb can be caused by either direct mutational inactivation of itself or alteration of the upstream regulators of pRb activity such as elevated CDKs activity led from overexpression of cyclins/CDKs or impairment of CKI inhibition (Burkhart & Sage 2008).

Hyperactivation of both CDK4 and CDK6 is commonly found in the majority of human neoplasia. For instance, CDK4 is amplified or overexpressed by enforced oncogenic

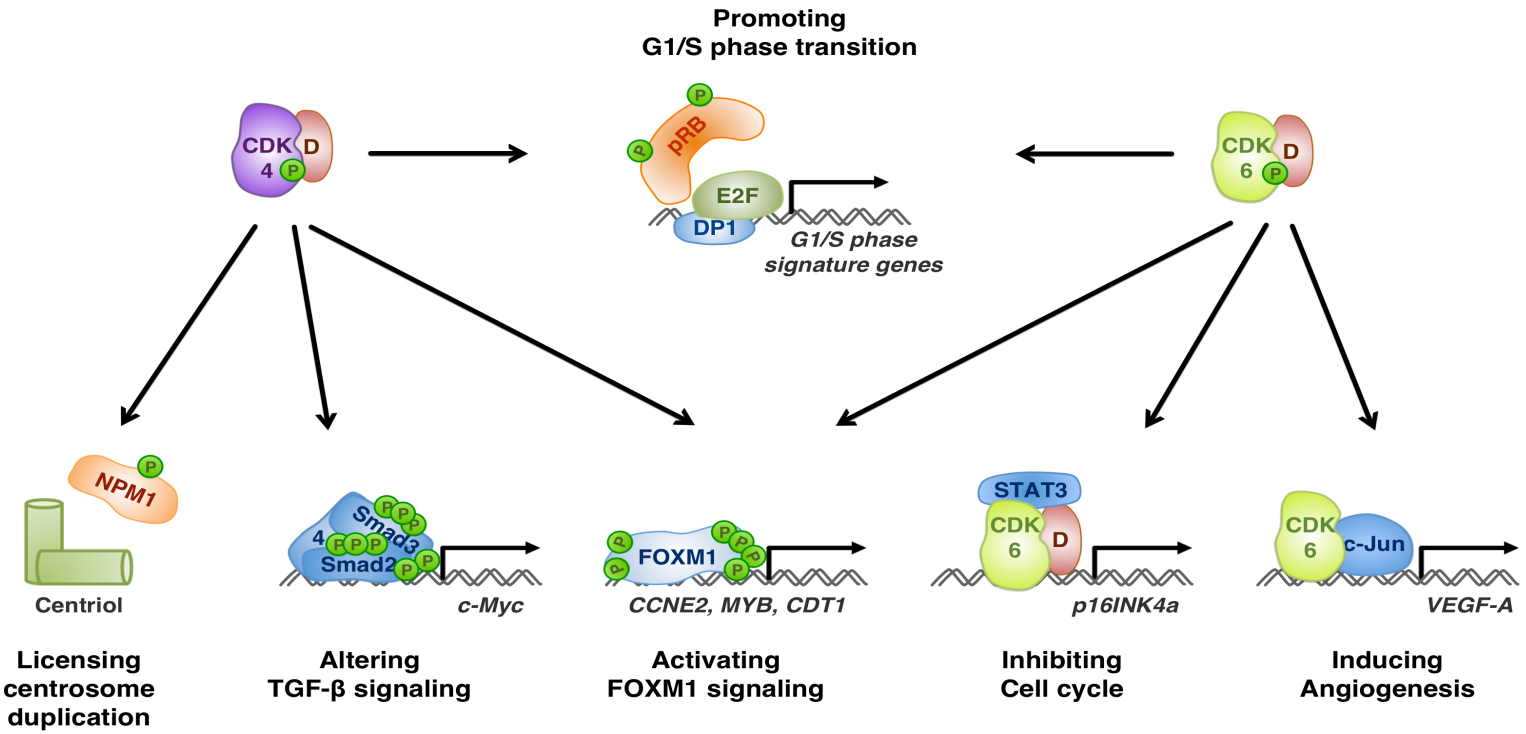

Figure 4. pRb dependent and independent functions of G1-CDKs. Canonically, CDK4/6 phosphorylates pRb and enters G1/S phase transition through expression of S-phase related genes. As pRb-independent mechanisms, CDK4/6 targets multi-sites of FOXM1, resulting in stabilization and activation of FOXM1. In addition, CDK4 phosphorylates the linker domain of Smad2/3, disturbing cell cycle inhibitory effects of TGF-β signaling. CDK4 also catalyzes NPM1 at Thr199, inducing department of NPM1 from centriole and licensing centrosome duplication. In case of CDK6, it has a kinase-independent function as a transcriptional factor. First, CDK6-cyclin Ds-STAT3 complex induces transcription of p16INK4a, negatively influencing on cell cycle progression. Second, in the absence of p16INK4a, CDK6 associates with c-Jun and expresses VEGF-A, triggering angiogenesis of tumors.

signals in gliomas, sporadic breast carcinomas, sarcomas, neuroblastomas and squamous cell carcinomas (Khatib et al. 1993; Ichimura et al. 1996; Holland et al. 1998; An et al. 1999; Easton et al. 1998; Cheung et al. 2001). In case of CDK6, it is also overexpressed in human gliomas, leukemia, squamous cell carcinomas and neuroblastomas (Ichimura et al. 1996; Timmermann et al. 1997; Piboonniyom et al. 2002; Easton et al. 1998). Particularly, activity of CDK4 is elevated in familial melanoma and cytotoxic T-lymphocytes in melanoma due to germline mutation at the p16INK4a binding site in Cdk4 (CDK4R24C) (Wolfel et al. 1995; Zuo et al. 1996).

Misregulation of D-type cyclins also leads to hyperactivation of CDK4/6 in tumors. A large number of studies have implicated abnormal levels of D-type cyclins in the various types of human tumors. Cyclin D1 is well-established proto-oncoprotein: CCND1 gene amplification, translocation and overexpression of cyclin D1 by upstream oncogenic signaling e.g. RAS–MEK–ERK and PI3K pathways are easily found in various tumors, including breast cancer, non-small-cell lung cancer, melanoma, pancreatic cancer, colorectal cancer and head and neck squamous cell carcinoma (Musgrove et al. 2011). Amplification of CCND2 (cyclin D2) or CCND3 (cyclin D3) has also been reported in gliomas and mantle cell lymphomas, but it is relatively lower rate compared to CCND1 (Wlodarska et al. 2008; Cancer Genome Atlas Research Network 2008). Although overexpression of cyclin D2 have also been observed in testicular tumors, gastric cancer and B-cell malignancies (Schmidt et al. 2001; Sicinski et al. 1996; Takano et al. 1999; Delmer et al. 1995), aberrant promoter methylation of CCND2, resulting in lack of cyclin D2 expression, has been frequently found in breast cancer, and prostate cancer (Fischer et al. 2002; Padar et al. 2003). In addition, overexpression of cyclin

D3 has been reported in several human cancers, such as pancreatic adenocarcinoma, laryngeal squamous cell carcinoma, breast cancer, and thymus cancer (Ito et al. 2001; Pruneri et al. 2005; Wong et al. 2001; Filipits et al. 2002).

It is worth mentioning that cyclin D1 also has CDK-independent roles in tumor development. Cyclin D1 interacts with more than 100 proteins in human cancer cell lines, involving in cell cycle control, transcriptional regulation, DNA repair, RNA metabolism, protein folding, cell structure and organization (Jirawatnotai et al. 2011). For example, cyclin D1 independently associate with p21 and p27 without CDK4/6 binding, triggering migration and DNA damage response (Li et al. 2008; Li et al. 2010). In addition, cyclin D1 binds to several transcription regulators, taking charge of cell proliferation and differentiation: nuclear hormone receptor family such as ERα, androgen receptor (AR), peroxisome proliferator-activated receptor- γ (PPARγ) and their co-activators, and transcription factors such as STAT3, b-Myb, DMP1, neurogenic differentiation factor 1 (NEUROD1), MyoD and C/EBPβ (Chenguang Wang et al. 2003; Reutens et al. 2001; McMahon et al. 1999). Moreover, cyclin D1 affects chromatin modification through association with histone deacetylases (HDAC) and p300/CBP (Fu, Rao, et al. 2005a; Fu, Wang, et al. 2005b).

3.2. Mouse skin tumorigenesis model

3.2.1. Physiology of skin and skin cancers

The skin is a complex structure that serves as the first physical barrier from numerous hazardous factors and external stress. The skin is comprised of three compartments,

epidermis, dermis and subcutaneous connective tissue (subcutis or appendage), respectively (Anon 1989).

Epidermis mainly consists of four different layers of epithelial cells: stratum basale (the basal layer), Stratum spinosum (the prickle cell layer), stratum granulosum (the granular cell layer) and stratum corneum (the horny cell layer). The basal layer is mainly made up of keratinocytes in various states including resting with high proliferative capacity (stem cells), proliferating (transit amplifying cells) and early differentiating, and unrelated cells such as melanocytes, Langerhans cells, Merkel cells and T-lymphocytes. Epidermal homeostasis is maintained by proliferation of stem cells periodically, replacing constantly lost cells by turnover or injuries. Divided cells in the basal layer detach from basal lamina, move outward in a columnar fashion with a differentiation process to form the prickle cell layer, the granular cell layer and finally, the horny cell layer, consisting of dead cells.

Skin cancer, generally developing in the epidermis, is the most common human neoplasias finding in about one million Americans each year. Skin cancer is categorized by melanoma and non-melanoma cancer. Melanoma arose from melanocytes, is less common than non-melanoma cancer, but it tends to be more aggressive. The most frequent skin cancer is basal cell carcinoma (BCC). It slowly grows and rarely spreads to distant sites, but it can locally invade nearby tissues. It is believed to develop from the hair follicle due to its histological and biochemical characters (Owens & Watt 2003). The second type of non-melanoma cancer is squamous cell carcinoma (SCC). SCC is more aggressive than BCC, but metastasis is still low rate (Bivens et al. 2006). The origin of SCC is still controversial. Papillomas, benign precursors of SCC, frequently exhibit signs of the interfollicular epidermis

differentiation including the keratin pearls in the tumor, thereby it suggests SCC may develop from the interfollicular epidermis (Owens & Watt 2003). However, studies with mouse skin cancer models have revealed that stem cells in a hair follicle also initiate SCC as well as in the interfollicular epidermis (Morris 2000; Argyris 1985; Morris et al. 1986). Interestingly, active mutation in Ha-ras is very frequent in the SCC, and mutation in K-Ras is also reported in both mouse and human skin SCCs (van der Schroeff et al. 1990; Sutter et al. 1993).

3.2.2. Two-stage carcinogenesis model

The mouse skin model is a useful system to study the multistage nature of carcinogenesis. Through this model, three major sequential stages has been defined: initiation, promotion, and progression. The most frequently used protocol for a chemical-induced carcinogenesis is the two-stage carcinogenesis, consisting of initiation and promotion steps. In the initiation step, a single low dose of a chemical mutagen such as 7,12-dimethybenz [α] anthrcene (DMBA) is topically applied to the mice dorsal skin. Cytochrome p450 metabolizes DMBA into DMBA-3,4-diol, 1,2,-epoxide that leads to the specific mutation in codon 61 of the Ha-ras gene, an extremely strong selective advantage to carcinogenesis (Fujiki et al. 1989). The initiated cells can response to the promotion step even one year after initiation, thus, the initiation event is considered irreversible. In the promotion stage, mice are treated with continuous application of non-mutagenic chemical, stimulating cell proliferation, such as 12-O-tetradecanoylphorbol 13-acetate (TPA), also known as phorbol 12-myristate13-acetate (PMA). TPA transiently activates protein kinase C, resulting in colonial expansion of initiated cells. Commonly, papilloma appears 6-8 weeks after the initial application of TPA

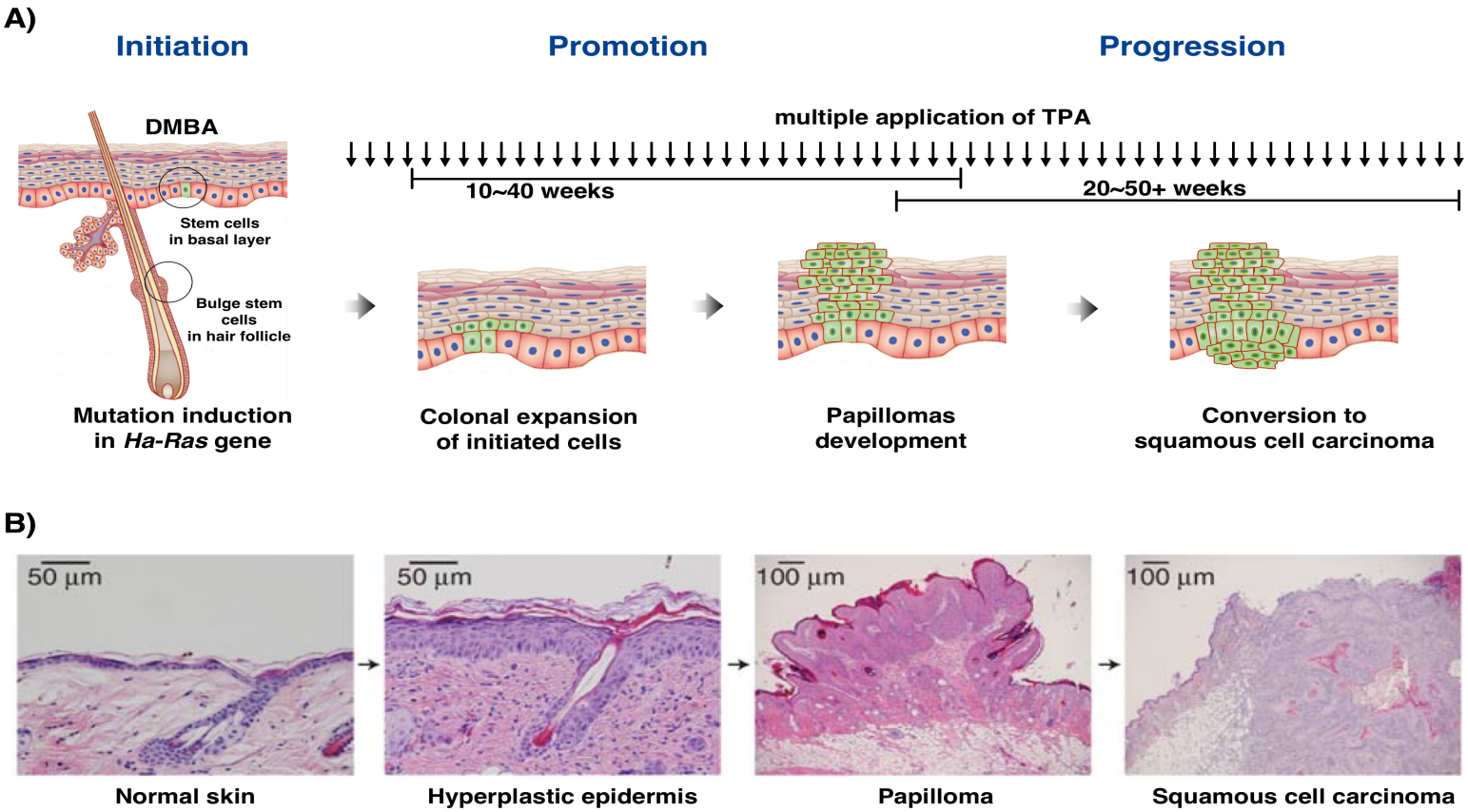

Figure 5. Chemical induced carcinogenesis in mouse skin (A) For initiation, DMBA applies on the mouse dorsal skin, and it induces active Ha-Ras mutation in keratinocyte stem cells. After 3 weeks from initiation, a promoting reagent, TPA, is continuously treated by termination of an experiment. Typically, papilloma appears after 6-8 weeks of promotion and begins to conversion of SCC after 20 weeks, but it dependent on genetic backgrounds. (B) Representative mouse skin sections stained with H&E: normal skin, hyperplastic epidermis, a papilloma and a SCC. The figures are modified from previously published figures (Wong & Chang 2008; Abel et al. 2009).

and a small percentage of papillomas, approximately 5-10%, progress to malignant carcinomas, SCC after 25-30 weeks promotion. However, appearance of tumors and malignant progression are dependent on the genetic background of the mouse strain. For example, the FVB strain is very sensitive to chemically induced malignant conversion (SCC) while C57BL/6 is relatively resistant to tumor formation (Woodworth et al. 2004).

3.2.3. Experimental mouse skin model of D-type cyclin/CDKs in G1 phase

Our laboratory has investigated the specific role of G1 regulators in skin carcinogenesis using mouse models. Through two-stage carcinogenesis, we have shown that deregulation of CDK4 plays a major role in skin tumor development and malignant progression. Ablation of CDK4 suppresses skin tumor development, but it does not alter normal keratinocyte proliferation (Rodriguez-Puebla et al. 2002). In contrast, forced overexpression of CDK4 under the control of the K5 promoter (K5-CDK4) results in a dramatic increment in the rate of malignant progression to SCC. Biochemical analysis of tumors from K5CDK4 reveals that overexpression of CDK4 results in increased association with p21/p27 that are released from CDK2 leading to indirect activation of CDK2 kinase (Miliani de Marval et al. 2003). However, overexpression of CDK2 (K5-CDK2) did not result in increased malignant progression as observed in CDK4 (Macias et al. 2008; Macias et al. 2007). Interestingly, CDK6 overexpression mice model, K5-CDK6, shows an inhibitory effect on skin tumor development due to the induction of elevated apoptosis in the hair follicle (Xian Wang et al. 2011). The conclusion of this experiments indicates that CDK4 plays a unique role in skin carcinogenesis, especially in malignant progression.

We also have studied about roles of D-type cyclins in skin tumorigenesis. Deficiency of cyclin D1 exerts a strong influence on ras-associated tumor development negatively, resulting in up to an 80% decrease in tumorigenesis (Robles et al. 1998). However, cyclin D1 overexpression does not affect skin tumor development despite increasing CDK4/6 activity and cell proliferation in vivo (Rodriguez-Puebla et al. 1999). In a case of cyclin D2 and D3, we reveal that cyclin D2 and cyclin D3 play opposite roles in skin carcinogenesis. Overexpression of cyclin D2 (K5-D2) results in an increased number of papillomas and malignant progression, while, expression of D3 (K5-D3) shows suppression of skin tumor, presumably through down-regulation of cyclin D2 expression (Rojas et al. 2007). Importantly, overexpression of cyclin D3, and the consequent reduction of cyclin D2 level, synergize with lack of cyclin D1 (K5D3/D1$^{-/-}$ mice) preventing mouse skin tumor development (Xian Wang et al. 2012).

4. Research Focus

As aforementioned, CDK4/6 plays a central role in G1/S phase transition through inhibition of pRb, leading to activation of E2F transcriptional function. Functionally inactivation of the pRb pathway by either direct mutation of pRb or elevated activity of G1-CDKs leads to unscheduled cell proliferation, chromosome instability and genomic instability (Sherr 1994; Malumbres & Barbacid 2001). Therefore, elevated activity of CDK4 or CDK6 has been found in various human and experimental tumors.

Our laboratory has investigated the specific role of G1 phase CDKs in the chemical-induced carcinogenesis using genetically modified mouse models. Surprisingly, we have observed that overexpression of CDK6 (K5CDK6) or overexpressed CDK4 (K5CDK4) brings about completely opposite results. In fact, inhibition of tumorigenesis was determined in K5CDK6 mice (Xian Wang et al. 2011) vs. induction of malignant progression in K5CDK4 mice (Miliani de Marval et al. 2003)

K5CDK6 mice had shown that overexpression of CDK6 in mouse skin results in decreased skin tumor development (Xian Wang et al. 2011). As expected, elevated CDK6 activity triggered epidermal proliferation, but also induced apoptosis in the hair follicle, an event that affect tumor development negatively. Also, we noticed elevated levels of CDK6/cyclin D3 complexes in epidermis compare to other D-type cyclins. This inhibitory effect on tumorigenesis also had been observed in cyclin D3 overexpressed mouse models, K5D3, and K5D3/cyclin $D1^{-/-}$. We previously reported that forced expression of cyclin D3 in an epidermis (K5D3) decreased expression of cyclin D2 as a compensatory mechanism and suppressed skin tumorigenesis (Rojas et al. 2007). Moreover, the K5D3/cyclin $D1^{-/-}$ compound mice, presenting undetectable levels of cyclin D1 and cyclin D2 in an epidermis showed the strongest repression of tumor development (Xian Wang et al. 2012). Interestingly, elevated cyclin D3 in the epidermis is prone to associate CDK6, as well as elevation of CDK6/cyclin D3 complex formation in the epidermis of K5CDK6 mice (Xian Wang et al. 2011). Therefore, ***we hypothesized that the association of CDK6/cyclin D3 and its enzymatic activity would mediate suppression of tumor development.***

On the other hand, overexpression of CDK4 in epidermis (K5CDK4) resulted in epidermal hyperplasia and increased susceptibility to malignant progression to SCC (Miliani de Marval et al. 2003). However, the canonical role of CDK4, activating E2F transcriptional function by phosphorylation of pRb, cannot fully explain the malignant progression because K5D1 and K5D1/K5CDK4 double transgenic mice did not present earlier or higher rate of SCC development compared to K5CDK4 mice (Miliani de Marval et al. 2003). Furthermore, the indirect activation of CDK2 through sequestration of $p27^{Kip1}$ and $p21^{Cip1}$ by CDK4, is not responsible for the malignant progression since elevated CDK2 kinase activity in K5CDK2 mice did not enhance skin tumorigenesis (Macias et al. 2008). Moreover, deficiency of pRb from epidermis did not increase malignant progression (Ruiz et al. 2005). Thus, ***we hypothesize that a pRb-independent role of CDK4 might lead to malignant conversion.*** One of the causes of tumor progression is centrosome amplification, contributing chromosomal instability. Interesting, CDK4 has also been involved in a regulation of centrosome duplication. Therefore, ***we hypothesize that forced expression of CDK4 contributes to centrosome amplification, leading to malignant progression of epidermal tumors.*** In this study, we concentrate our efforts to determine novel roles of CDK6 and CDK4 in skin carcinogenesis.

Altogether, to test our hypothesis, we pursued two main research aims:

1) First, we investigated whether the catalytic activity of CDK6/cyclin D3 complex negatively affects tumorigenesis (Chapter 2). To address this question, we generated

K5CDK6/Cyclin D3$^{-/-}$ mice and subjected them to the two-stage carcinogenesis protocol.

2) Second, we examine whether forced expression of CDK4 induces centrosome amplification in skin epidermal tumors and what are the molecular mechanisms involved leading in supernumerary centrosome. To determine these questions, we utilized proteomic analysis to identify centrosome related substrates of CDK4 (Chapter 3), and performed ChIP-assay to discover novel roles of CDK4 regulating CIN-related genes (Chapter 4).

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CHAPTER 2

Deficiency of cyclin D3 increases the malignant progression of CDK6-dependent skin papillomas.

Abstract

The Cyclin-Dependent Kinases (CDKs) are a family of essential serine-threonine kinases that are regulated by an association with cyclins at specific time-points of the cell cycle. Specifically, D-type cyclins form complexes and activate CDK6 and CDK4. We have previously established that transgenic expression of CDK6 in mouse epidermis (K5CDK6 mice) altered an early stage of tumorigenesis leading to the substantial reduction of the multiplicity and incidence of skin tumors. Our previous results have shown that CDK6 preferentially binds to cyclin D3, and that overexpression of cyclin D3 also decreases tumor development in mouse epidermis. Thus, we hypothesized that the association of CDK6/cyclin D3 and its enzymatic activity negatively impact tumor development. To verify this, we developed K5CDK6/Cyclin $D3^{-/-}$ mutant mice and subjected them to the two-stage carcinogenesis protocol. Contrary to our hypothesis, lack of cyclin D3 expression does not affect the tumor repression activity of CDK6 since both K5CDK6 and K5CDK6/cyclin $D3^{-/-}$ mice, exhibit a severe reduction in the multiplicity and incidence of skin tumors compared to wild-type siblings. However, histopathological analysis of skin tumors revealed that 29% of the total observed skin tumors in K5CDK6/cyclin $D3^{-/-}$ mice were classified as squamous-cell carcinomas I (SCC) with a variable degree of squamous differentiation; whereas wild-type or K5CDK6 siblings developed 15% of SCC, and cyclin $D3^{-/-}$ siblings exhibit only benign tumors. Importantly, biochemical analysis of epidermal tumors shows that cyclin D3 ablation results in a rise in cyclin D1 protein levels, leading to elevated CDK4/cyclin D1 and CDK6/cyclin D1 complex formation in K5CDK6/cyclin $D3^{-/-}$ epidermis. These data suggest that though cyclin D3 expression does not alter the tumor suppressive role of CDK6 at early

stages of tumorigenesis, but it does affect the expression of cyclin D1, resulting in the increased rate of malignant progression. We conclude that CDK6 activity can prevent tumor formation during the initial stages of tumorigenesis in a cyclin D3-independent manner and likely in a pRb-independent manner as well. However, variations in the expression of cyclin D3 affected cyclin D2 and/or cyclin D1 levels, directly altering the susceptibility of CDK6 expressing tumors to malignant conversion. Therefore, therapies directed to reduce D-type cyclins expression should be considered within the context of the expression level of CDK6 and CDK4 to define the potential effect in the malignant progression of skin tumors.

1. Introduction

Cell proliferation and differentiation are sharply regulated by a numerous cell cycle regulators to avoid hyperplastic and neoplastic growth, and especially proper controls of restriction-point passage by retinoblastoma (pRb) are critical for cell cycle progression (Sherr 1994; Besson et al. 2008). However in cancer cells, frequent alterations of pRb/CDKs/cyclins/p16 pathway occur through functional inactivation of pRb which is the result of elevated CDKs activities caused by overexpression of G1-phase CDKs, D or E-type cyclins or loss of CDK inhibitors (Malumbres & Barbacid 2009). Therefore, misregulation of CDKs mediate cell cycle defects acquiring key features of cancer cells such as uncontrolled proliferation.

D-type Cyclin-dependent kinases 4/6 (CDK4/6) are responsible for G1/S phase transition through initiating phosphorylation of pRb (Sherr 1994). CDK4 and CDK6 share 71% of

amino acid identity, and they are family members of Serine/Threonine kinase (Meyerson & Harlow 1994). They are expressed ubiquitously through the cell cycle, but their activities are dependent on complex formation with D-type cyclins (Choi & Anders 2014). Both kinases are considered as major oncogenic drivers due to being frequently overexpressed or hyperactivated in various human cancers (Ortega et al. 2002; Malumbres & Barbacid 2001).

Although CDK4 and CDK6 share similar functional characteristics, in the last decade, relevant differences of between them have been reported. For example, CDK6 has difference in cell-type specific expression (Lucas et al. 1995), subcellular localization (Ericson et al. 2003), preference for phosphorylation sites in the pRb (Takaki 2005), and regulation mechanism by CDK-Activating kinase (CAK) (Bockstaele et al. 2009).

In addition, CDK6 plays a particular role in differentiation of various types of cells; down-regulation of CDK6 must preceded to differentiate osteoclast, osteoblast, myeloid, leukemic cells, and erythroid cell (Grossel & Hinds 2006; Matushansky et al. 2003; Fujimoto et al. 2007; Ogasawara et al. 2004; Choe et al. 2010). Human clinical data also have revealed that both CDK4 and CDK6 are overexpressed in gliomas (Costello et al. 1997; Perry et al. 1999; Holland et al. 1998; Reifenberger et al. 1996), whereas only CDK6 but not CDK4, is targeted to certain types of tumors such as lymphoid tumors (Chilosi et al. 1998; Lien et al. 2000; Corcoran et al. 1999), neuroblastomas (Easton et al. 1998), and squamous cell carcinomas (SCCs) (Timmermann et al. 1997; Piboonniyom et al. 2002).

Contrary to the oncogenic functions of CDK6, several reports show that overexpression of CDK6 negatively affects cell growth or tumor development (Nagasawa et al. 2001; Kollmann et al. 2013; Wang et al. 2011). Forced expression of CDK6 in NIH3T3 cells

induced accumulation of p53 and p130 and resulted in growth suppression and hypersensitivity to UV-irradiation (Nagasawa et al. 2001). Furthermore, overexpressed CDK6 in a model of p185BCR-ABL+ B-acute lymphoid leukemia, inhibited proliferation through its kinase-independent transcriptional function inducing expression of $p16^{INK4a}$ (Kollmann et al. 2013). Our previous study also has shown a strong inhibitory effect of overexpressed CDK6 in a mouse model of skin carcinogenesis (Wang et al. 2011).

The two-stage carcinogenesis is well-established model to understand the multistage nature of tumor progression through initiation and promotion. In this model a mutation in Ha-ras oncogene occurred in epidermal cells by a single topical application of a carcinogen such as 7,12- dimethylbenz(a)anthracene (DMBA). Upon multiple topical applications of mouse epidermis with 12-0-tetradecanoylphorbol-13-acetate (TPA) the initiated cells are clonally expanded and promote the growth of exophytic benign tumors called papillomas. Finally, some of the papillomas progress to squamous cell carcinomas (SCC) during the stage of malignant progression. Using this protocol, our group have reported the effects of up- and down-regulation CDKs and D-type cyclins in epithelial tissues of transgenic mice models (Rodriguez-Puebla et al. 2002; Rodriguez-Puebla et al. 1999; Rodriguez-Puebla et al. 1998; Macias et al. 2008; Macias et al. 2007; Wang et al. 2012; Rojas et al. 2007; Miliani de Marval et al. 2003; Rojas et al. 2009; Robles et al. 1998; Wang et al. 2011; Rodriguez-Puebla et al. 2000). One of our mice model, K5CDK6 mice, had shown that overexpression of CDK6 in mouse skin inhibited skin tumor development due to the induction of elevated apoptosis in a hair follicle (Wang et al. 2011). Moreover, we noticed that elevated CDK6 is prone to associate cyclin D3 comparing to other D-type cyclins.

D-type cyclins, D1, D2, and D3, are critical regulators of CDK4/6 because the activity of CDK4/6 is dependent on complex formation with D-type cyclins (Matsushime et al. 1992; Meyerson & Harlow 1994). The three members of D-type cyclins have highly conserved amino acid sequence (Sherr 1994). They are major downstream targets of mitogen stimuli such as the Ras signaling (Peeper et al. 1997). Therefore, initial reports covered their growth-promoting functions as activators of CDK4/6. Consistent with their role in cell cycle progression, overexpression of D-type cyclins or deregulation of their functions by genetic mutations (Musgrove et al. 2011) have been reported in several types of human tumors, for instance, breast carcinomas, uterine cervix, head and neck squamous cell carcinomas, testicular tumors, and B cell malignancies (Dickson et al. 1995; Peters et al. 1995; Fantl et al. 1995; Cheung et al. 2001; Fujii et al. 2001; Sicinski et al. 1996; Delmer, Ajchenbaum-Cymbalista, Tang, Ramond, Faussat, Marie & Zittoun 1995a; Delmer, Ajchenbaum-Cymbalista, Tang, Ramond, Faussat, Marie & Zittoun 1995b; Filipits et al. 2002). However in the last decade, it has been revealed that they are expressed in tissue-specific manners (Sherr 1995; Ciemerych et al. 2002), and also they have CDK-independent activities (Bernards 1999; Pestell 2013).

Among D-type cyclins, cyclin D3 shows the broadest expression patterns among human cultured cell types including normal diploid cells and tumor cell lines (Bartkova et al. 1998). Unlike cyclin D1 and D2, cyclin D3 has not been considered as oncogene due to its controversial functional aspects. Several papers have revealed that cyclin D3 functions as a balancer between proliferation and differentiation of pro-B cells, myeloid progenitor cells and skeletal muscle stem cells (De Luca et al. 2013; Filipits et al. 2002; Gurung & Parnaik

2012; Bartkova et al. 1998; Kato & Sherr 1993; Peterson et al. 2005). It has been reported that cyclin D3 knockout mice exhibit deficient in maturation of granulocytes in the bone marrow and show reduced levels of neutrophil granulocytes in their peripheral blood (Sicinska et al. 2006), which supports the previous observations in human clinical data. Despite recent research achievements, unique roles of cyclin D3 have not been fully understood.

Our group previously reported the negative effects of overexpressed cyclin D3 on skin tumor susceptibility (Rojas et al. 2007). Using the two-stage carcinogenesis model, we have shown that forced expression of D3 in an epidermis (K5D3 transgenic mice) suppressed skin tumor, presumably through down-regulation of cyclin D2 expression (Rojas et al. 2007). Similarly, the K5D3/cyclin $D1^{-/-}$ compound mice, having ablation of cyclin D1 and reduction of cyclin D2 in the epidermis, showed a stronger inhibition of tumor development (Wang et al. 2012). We also observed tumor repression effects in overexpressed CDK6 mice (K5CDK6) (Wang et al. 2011). Interestingly, we noticed the elevation of CDK6/cyclin D3 complex formation in their epidermis and skin tumors of K5CDK6 mice. According to these observations, *we hypothesized that the association of CDK6/cyclin D3 and its enzymatic activity negatively impacts mouse skin tumor development.*

To address this question, we generated K5CDK6/$D3^{-/-}$ compound mice, which overexpress CDK6 with lack of cyclin D3 expression and applied a standard two-stage carcinogenesis protocol.

2. Materials and Methods

2.1 Mouse Models

The cyclin D3 knockout mice model were developed by Sicinski's group (Sicinska et al. 2003) and the generation of K5-CDK6 transgenic mice which overexpression of human CDK6 in the mouse epidermis were reported in our previous paper (Wang et al. 2011). K5-CDK6 transgenic mice were mated to cyclin $D3^{+/-}$ transgenic mice to obtain K5-CDK6/cyclin $D3^{+/-}$, and sequentially K5-CDk6/cyclin $D3^{+/-}$ mice were backcrossed with cyclin $D3^{+/-}$ mice to acquire K5CDK6/cyclin $D3^{-/-}$ double transgenic mice and their siblings; wild type, K5CDK6, and cyclin $D3^{-/-}$.

Mouse genotype was confirmed by transgene-Specific PCR. For the detection of K5-CDK6 transgene, forward primer CTGACCAGCAGTTACAATG and a reverse primer were GAGTCCAATCACGTCCAAG for beta-globing intron sequence were used. For D3 knockout mice screening, we utilized the same primers designed by Sincinski's group (Sicinska et al. 2003). Genomic PCR was performed using KAPA2G Fast PCR kit (KAPA Biosystems, MA) by denaturation at 95°C for 3 min, followed by 35 cycles of amplification: denaturation at 95°C for 30 sec, annealing at 60°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 5 min.

2.2. Two-stage chemical carcinogenesis

After setting up experimental mice groups, 10-13 mice of each group were used for two-stage carcinogenesis experiments. For experiments, newborn mice were initiated at day 3 after birth with a single application of 50μg of 7,12-dimethybenz [α] anthrcene (DMBA) in

50 µl of acetone on the dorsal skin. Three weeks later, they were promoted with 4.19µg of 12-o-tetradecanoylphorbol 13-acetate (TPA) in 200µl of acetone twice a week for 25 weeks. Skin tumors larger than 1 mm in diameter were counted every week until the termination of experiments. Traced tumors were converted to Multiplicity and Incidence and compared among different groups. Collected tumors were fix in 10% of a formalin buffer and sent to the histopathology lab to generate paraffin-embedded blocks.

Malignant progression to SCC was determined by histopathological analysis of paraffin-embedded H&E stained cross sections.

2.3. Pathological analysis and Immunohistochemical staining

Formalin fixed paraffin embedded epidermis samples were stained with hematoxylin and eosin stain (H&E stain) by the histopathology lab at NCSU.

For the measurement of epithelial cell proliferation, 60µg/g body weight of 5-bromodeoxyuridine (BrdU) was injected 30 min before the mice were euthanized. BrdU incorporation was detected by immunohistochemical staining of paraffin-embedded skin sections with 1:250 of mouse anti-BrdU (Ab-2) monoclonal antibody (Vector Laboratories Inc., CA), 6µg /ml of biotin-conjugated anti-mouse antibody (Vector Laboratories Inc., CA), and an avidin-biotin peroxides kit (Vectastain Elite ABC system, Vector Laboratories, Inc., CA). Stained sample was developed by diaminobenzidine (DAB) (DAB Substrate kit, Vector Laboratories Inc., CA) and counter-stained with Hematoxylin.

Apoptotic cells were identified by terminal deoxynucleotidyl transferase-mediated dUTP mimic-end labeling assay (Tunel Assay) with the FragEL DNA Fragmentation Detection Kit

(Calbiochem; EMB Millipore, Germany) according to the manufacturer's instructions. Briefly, the terminal deoxynucleotidyl transferase (TdT enzyme) binds to the exposed 3'-OH ends of a DNA fragment generated by an apoptosis progression and catalyzes the addition of biotin-labeled and unlabeled deoxynucleotides. Biotinylated nucleotide was detected using a streptavidin-horseradish peroxidase conjugate. Counterstaining with methyl green allows for quantification of normal and apoptotic cells.

The numbers of proliferating or apoptotic cells in the tumors were determined in section of 250μm^2 with a reticule grid. To analyze normal epidermal skin without any treatment, labeled cells in the interfollicular epidermis were counted and normalized by total cell numbers. In all cases, At least 10 fields were counted per section on a total 10-12 paraffin-embedded sections, representing 5 mice per genotype.

2.4. Preparation of Lysate and Western Blots

Shaved dorsal epidermal skins or tumors were snap-frozen in liquid nitrogen when mice experiments were terminated. To extract protein lysate from epidermis, skin was spread with the dermis down side over a clean glass on ice and scraped with RIPA buffer [25mM Tris-HCl (pH 8), 150mM NaCl, 1% NP-40, 5% sodium deoxycholate, 0.1% SDS] containing 1 x protease inhibitor cocktail (Sigma-Aldrich Co. LLC, MO). Scrapped skin was transferred to a 1.5ml tube and matched volume of RIPA buffer around 500μl per sample. Tumor was crushed with a pestle and mortar in liquid nitrogen, and transferred to 1.5ml tube. Powder of tumor was resuspended with 500μl of RIPA buffer. Epidermal scrapes or ground tumors were homogenized with a Poltroon PT10 homogenizer for 3 sets of 10-second, sonicated

with a Brason sonifer 450 for 3 sets of 20-second pulses at setting 2, and centrifuged at maximum speed for 5 min. Supernatant was transferred to a new tube and the protein concentration was measured with the *DC*™ Protein Assay system (Bio-Rad laboratories, CA). Protein lysate was boiled in laemmli sample buffer for western blot analysis.

50 μg of Protein lysates were analyzed through 10% SDS-PAGE and resolved samples were transferred onto nitrocellulose membranes. The membrane was blocked for 1 hour with 5% nonfat powdered milk in 0.1% Tween20-TBS buffer prior to overnight incubation with the primary antibody in 1% milk. The following primary antibodies were used: Rabbit polyclonal antibodies against CDK4 (C-22), CDK6 (C-21), cyclin D1 (C-20), cyclin D2 (M-20), p16 (M-156), p21 (M-19), p27 (C-19) and b-actin (I-19) (Santa Cruz Biotech, CA), and mouse monoclonal antibody against cyclin D3 (DCS-22) (NeoMarker, CA). Membranes were washed and incubated with secondary antibodies: goat anti-rabbit-HRP secondary antibodies (sc-2004), donkey anti-goat-HRP secondary antibodies (sc-2020) (Santa Cruz Biotech, CA) or goat anti-mouse-HRP secondary antibody (#31430) (Pierce Biotechnology Inc., ThermoFisher Scientific Inc., IL). Enhanced chemiluminescence (ECL detection kit, GE health Care, NJ) was used for immunoblotting detection.

2.5. Co-Immunoprecipitation

Fresh protein lysates were prepared with NP-40 co-IP buffer [50mM Tris-HCl (pH 7.5), 150mM NaCl, 0.5% NP-40, 50mM NaF] containing 1 x protease inhibitor cocktail instead of RIPA buffer. Scrapped skins or crushed tumors were homogenized, sonicated and centrifuged. To reduce non-specific binding, supernatants were filtrated through 0.45μm of

syringe filter (Corning Inc., NY). 250μg of protein samples were pre-cleared by 50ul of Sepharose CL4B bead (Sigma-Aldrich Co. LLC, MO) for 30 min with rotation, spun down at 3000rpm and supernatants were transferred to new tubes for co-immunoprecipitation. 2μg of polyclonal antibody against CDK6 (C-21), CDK4 (C-22), or normal rabbit IgG (sc-2027) were added to samples and incubated for overnight at 4C with constant rotation. After then, 30μl of pre-washed protein A agarose (Pierce Biotechnology Inc., ThermoFisher Scientific Inc., IL) per each sample were added and rotated for 4 hours, and then, spun down at 3000rpm. After washing three times with extraction buffer, co-IP samples were analyzed by Western blot as described above. 50μg of Protein lysate was loaded as a control input.

3. Results

3.1. Generation of K5CDK6/D3$^{-/-}$ mice and histological evaluation of mouse normal epidermis

We have recently reported that the constitutive overexpression of CDK6 (K5CDK6) or cyclin D3 (K5D3) on the basal cell layer of the epidermis had similar inhibitory effects on skin tumor development. Notably, CDK6 preferentially bind to cyclin D3 in keratinocytes of both CDK6 and Cyclin D3 transgenic mice. Therefore, to determine whether a formation of CDK6/cyclin D3 complex is required to maintain epidermal homeostasis and suppression of tumor development, we generated K5CDK6/cyclin D3$^{-/-}$ compound mice.

It is already reported that K5CDK6 or cyclin D3$^{-/-}$ mice did not display any obvious developmental abnormalities or defects in skin development compared to wild-type siblings

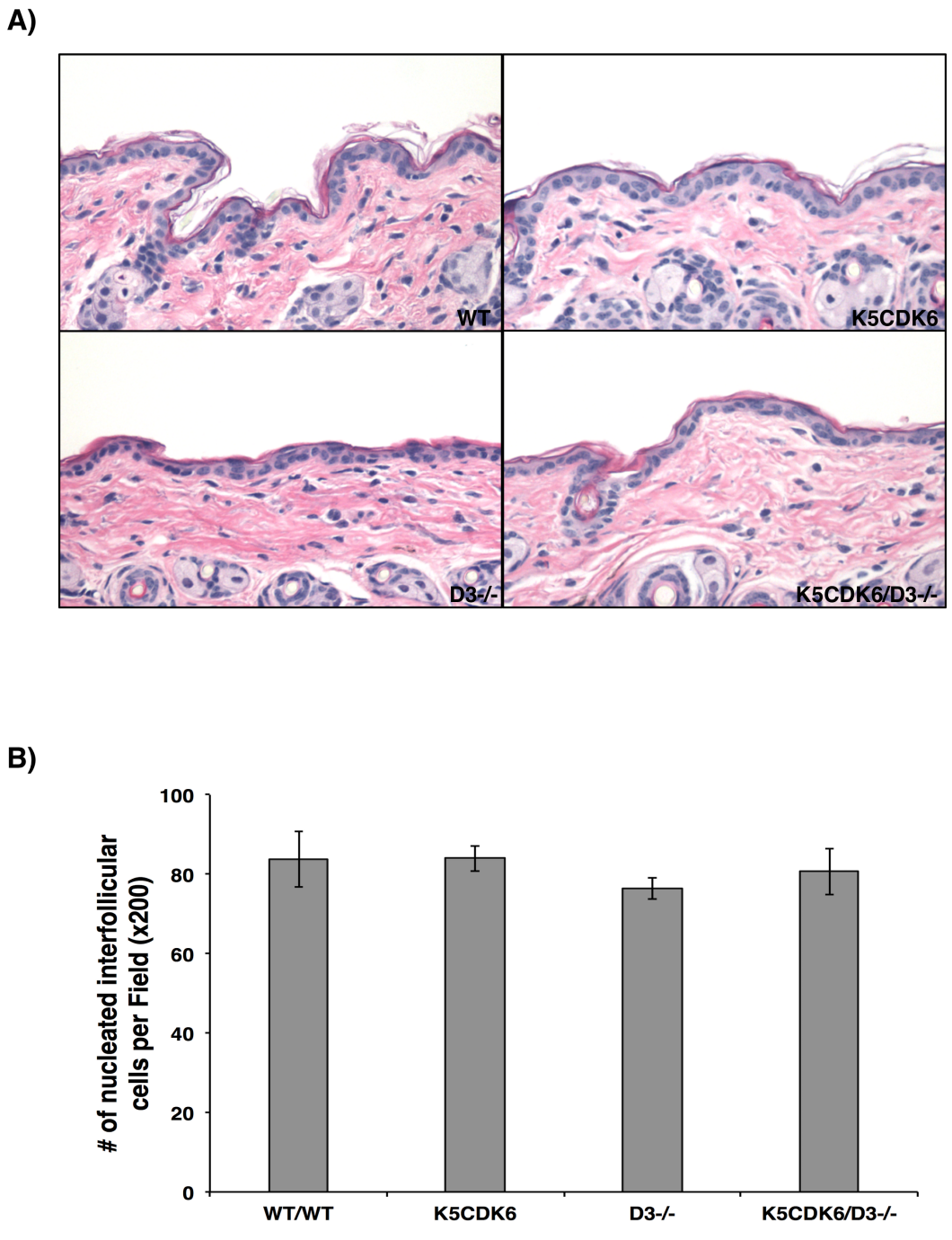

Figure 6. Histological analysis of mouse epidermis. (A) Representative paraffin-sections of skin from wild-type (WT), K5CDK6, cyclin D3$^{-/-}$(D3$^{-/-}$), K5CDK6/cyclin D3$^{-/-}$(K5CDK6/D3$^{-/-}$) siblings stained with H&E. (B) Number of nucleated cells in interfollicular epidermis. n=3 per each group, Bars; ± Standard Deviation.

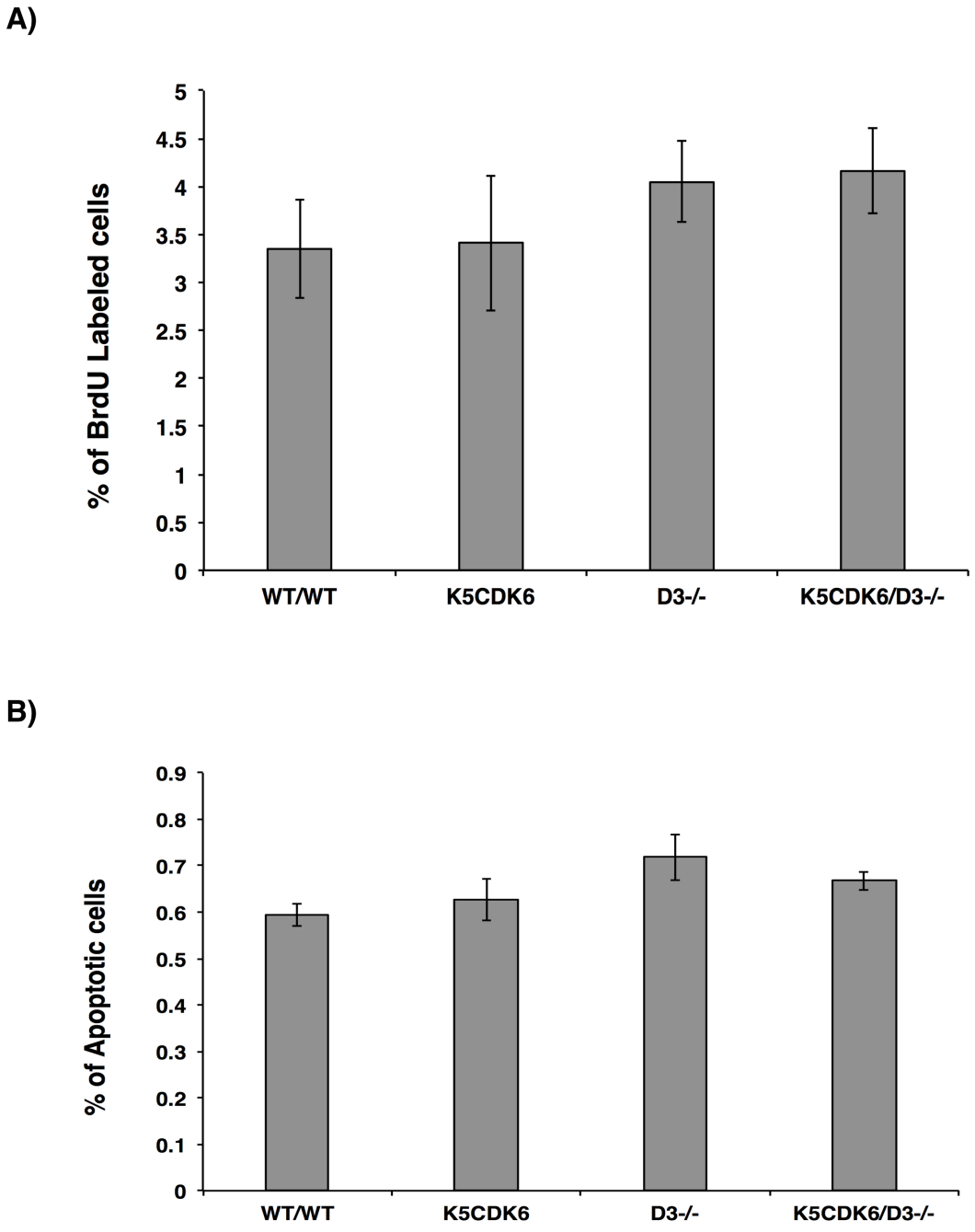

Figure 7. Keratinocyte proliferation and apoptosis in interfollicular epidermis of transgenic mice (A) The BrdU label index of the interfollicular epidermis of the four genotypes analyzed (B) Quantification of Apoptotic cells in interfollicular epidermis from WT, K5CDK6, cyclin $D3^{-/-}$ ($D3^{-/-}$), K5CDK6/cyclin $D3^{-/-}$ (K5CDK6/$D3^{-/-}$). n=3 per each group, Bars; ± Standard Deviation.

(Sicinska et al. 2003; Wang et al. 2011). Consistent with these data, our compound mice, K5CDK6/cyclin D3$^{-/-}$ were born at the expected frequency (data not shown). In addition, H&E staining of the dorsal epidermis did not show modifications in the morphology of follicular and interfollicular epidermis among wild type, K5CDK6, cyclin D3$^{-/-}$ and K5CDK6/cyclin D3$^{-/-}$ siblings. Supporting these data, there was no significant difference in the number of nucleated cells in the interfollicular epidermis at the first telogen (postnatal day 23) (Figure 6A, B). To identify proliferation status of keratinocytes, BrdU incorporation was carried out, and the ratio of positively stained cells was analyzed in the interfollicular epidermis. As a result, we detected slightly increment of BrdU positive cells in interfollicular epidermis of cyclin D3$^{-/-}$ and K5CDK6/cyclin D3$^{-/-}$ (1.2-fold), but it was not statistically significant compared with BrdU index of wild-type siblings ($p=0.332$, t-test). Inconsistent with previous observations (Wang et al. 2011), proliferation rate in the basal cell layer of K5CDK6 did not show the difference from wild-type siblings (Figure 7A). Moreover, no significant differences were observed in the number of apoptotic cells in an epidermis of the four genotypes analyzed (Figure 7B). Apoptotic keratinocytes in the interfollicular epidermis were counted in hematoxylin and eosin stained sections and scored positive if the following criteria were present: dark pyknotic nuclei and absence of cellular contacts. According to these data, ablation of cyclin D3 did not affect the number of nucleated cells and keratinocyte proliferation / apoptosis in the interfollicular epidermis of both, cyclin D3$^{-/-}$ and K5CDK6/cyclin D3$^{-/-}$ mice. Therefore, we conclude that a lack of cyclin D3 does not severely affect mice epidermal function.

3.2. Biochemical analysis of mouse normal epidermis

Because cyclin D3 ablation does not bring out defect in epidermal structure, we expect that an absence of cyclin D3 would be compensated by other D-type cyclins. Although D-type cyclins are expressed from different genes (CCND1, CCND2, and CCND3) and induced in cell lineage-specific manner (Sherr 1994), they are able to compensate for a loss of each one (Chen & Pollard 2003; Lam et al. 2000; Zhang et al. 2011; Mataraza et al. 2006; Wang et al. 2012). In the case of cyclin D3, the expression level is increased to compensates loss of cyclin D1 during ErbB2-induced breast tumor development, or loss of cyclin D2 in mouse B-cell lymphocytes (Zhang et al. 2011; Lam et al. 2000). Furthermore, ablation of cyclin D3 was counterbalanced by accumulated cyclin D2 during normal proliferation of B-1a cells (Mataraza et al. 2006).

To figure out whether disruption of cyclin D3 alters the expression of other cell cycle regulators in G1 phase, we performed biochemical analysis of epidermal keratinocytes (Figure 8A). Western blot analysis of mouse epidermal lysate showed that cyclin D3 ablation did not affect the expression of CDK6 in K5CDK6/cyclin $D3^{-/-}$ compound mice, which was still much higher that wild-type and cyclin $D3^{-/-}$ siblings. Forced expression of CDK6 also did not disturb expression of CDK4. Interestingly, lack of cyclin D3 expression resulted in the obvious increment of cyclin D1, but not cyclin D2. This result suggests compensation between cyclin D3 and cyclin D1. We also analyzed whether the protein level of the two families of cyclin-dependent kinase inhibitors were modified. The level of $p16^{INK4a}$ was slightly increased in K5CDK6 transgenic mice compared to wild type siblings. Other inhibitors of CDK/cyclin complex such as $p21^{Cip1}$ and $p27^{Kip1}$ were not affected by

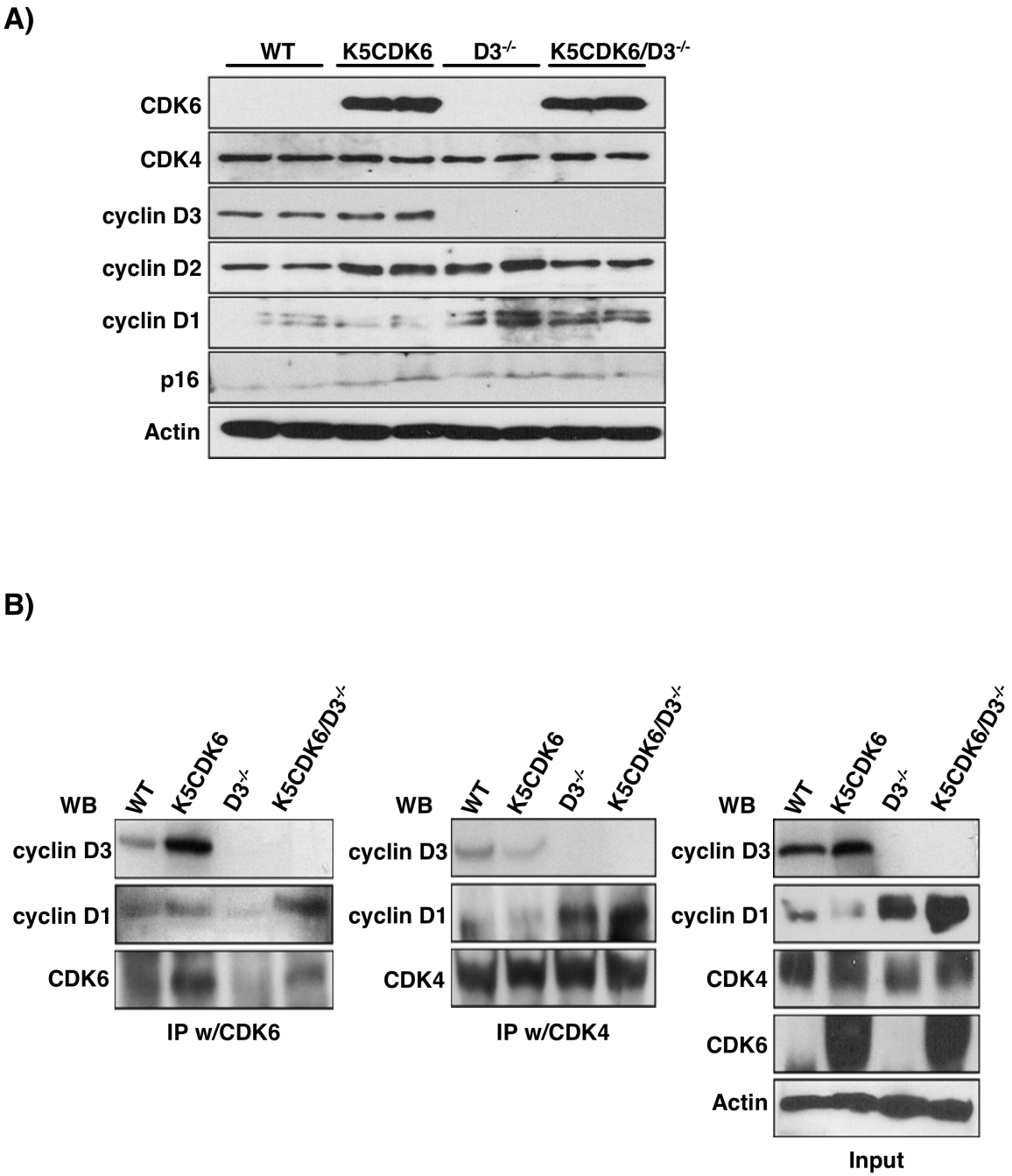

Figure 8. Biochemical analysis of Cell cycle regulators in mouse epidermis. (A) Immunoblotting analysis of wild-type (WT), K5CDK6, cyclin D3$^{-/-}$ (D3$^{-/-}$), and K5CDK6/cyclin D3$^{-/-}$ (K5CDK6/D3$^{-/-}$) epidermal lysates for CDK4, CDK6, cyclin D1, D2, D3, p16 and β-actin as loading control. (B) Epidermis lysates from each group were immunoprecipitated with CDK6 or CDK4 antibody and blotted with antibodies against cyclin D1 and cyclin D3. Input denoted protein levels in each epidermal lysate.

overexpression of CDK6 or cyclin D3 deficiency (Data not shown).

To define whether overexpression of CDK6 and/or deletion of cyclin D3 altered the cell cycle regulation at the level of CDK-cyclin complex formations, we carried out co-immunoprecipitation assay with antibodies against CDK6 or CDK4 (Figure 8B). As expected, cyclin D3$^{-/-}$ or K5CDK6/cyclin D3$^{-/-}$ mice did not show CDK6/cyclin D3 complex in epidermis due to an absence of cyclin D3. Consistent with our previous results, we observed increased level of K5CDK6/cyclin D3 complex in epidermis of K5CDK6 mice (Wang et al. 2011). Interestingly, K5CDK6/cyclin D3$^{-/-}$ epidermis showed an elevated level of CDK6/cyclin D1 and CDK4/cyclin D1 complex formation compared to other siblings. It is worth mentioning that CDK4/cyclin D1 complex was also elevated in cyclin D3$^{-/-}$ mice epidermis. Therefore, we conclude that the absence of cyclin D3 induced overexpression of cyclin D1 resulting in increased CDK6/cyclin D1 and/or CDK4/cyclin D1 complex formation. These findings suggest that compensatory effect of cyclin D1 would offset effect of cyclin D3 ablation through CDK4/cyclin D1 complex formation in mouse epidermis.

3.3. Kinetics of Tumor formation in K5CDK6/D3$^{-/-}$ compound mice

In order to investigate the role of CDK6/D3 complex in skin tumorigenesis, we performed a two stage-carcinogenesis protocol. Four groups of mice (wild type, K5CDK6, cyclin D3$^{-/-}$ and K5CDK6/cyclin D3$^{-/-}$) were utilized. This protocol is a well-suited model to study the multistage nature of tumorigenesis. Skin tumors are induced by a single application of a carcinogen such as DMBA, following by multiple applications of tumor promoter agent such as TPA. This stimulus promotes the generation of benign tumors, so called papillomas, by

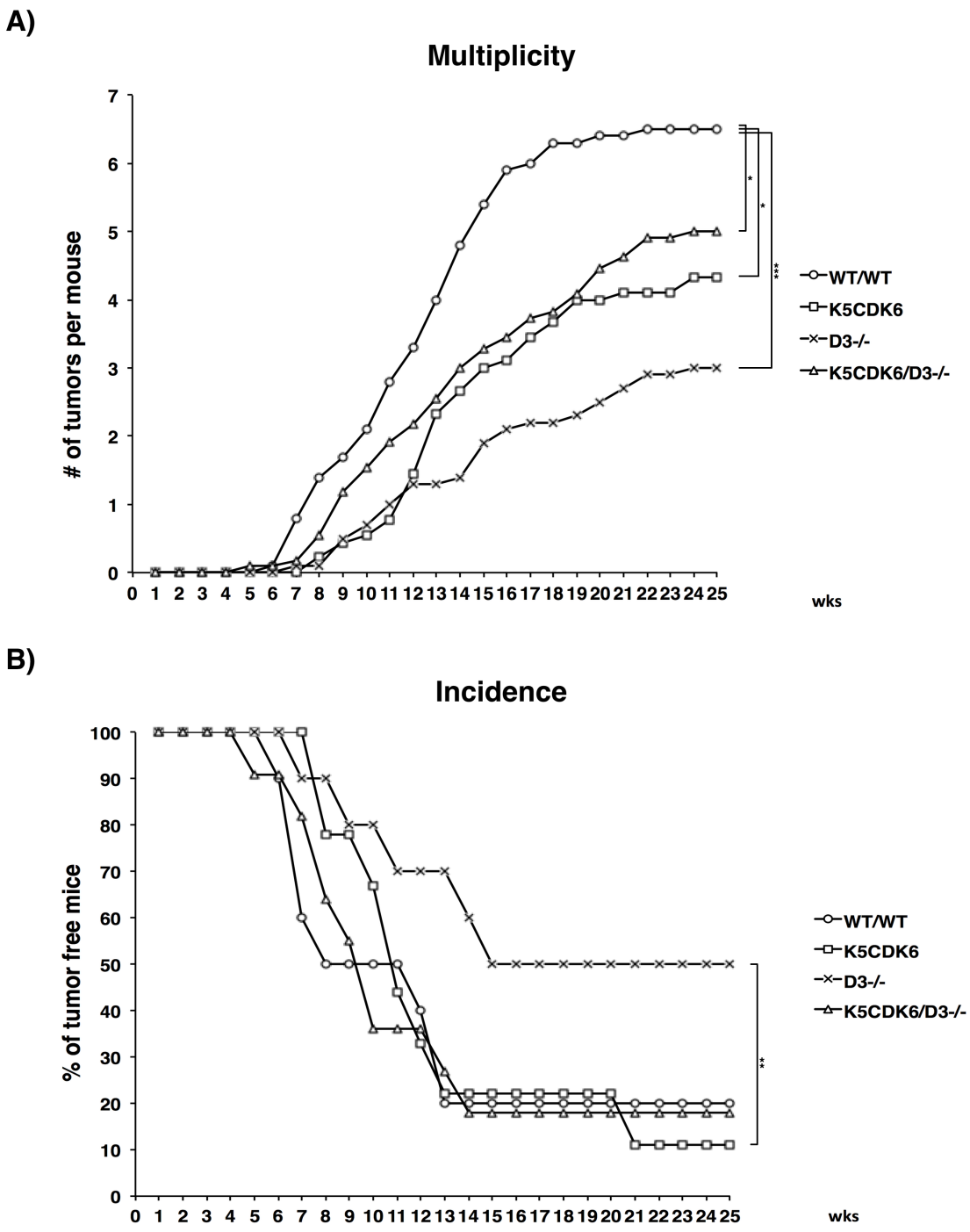

Figure 9. Kinetics of Tumor formation in WT, K5CDK6, cyclin D3$^{-/-}$ (D3$^{-/-}$) and K5CDK6/cyclin D3$^{-/-}$ (K5CDK6/D3$^{-/-}$) compound mice. Mice were initiated with DMBA and promoted with multiple application of TPA on dorsal skin. n=10-13 per group (A) Average number of tumors per mouse as functional weeks of study (B) Percentage of mice without tumors as functional weeks of study. $P < 0.05$; *, $P < 0.005$; **, $P < 0.0005$; ***, t-test

clonal expansion of the initiated cells. The incidence (percentage of mouse with tumors) and multiplicity (number of tumors per mouse) of papillomas were scored in each group for 25 weeks (Figure 9A, B).

As previously reported, K5CDK6 mice displayed a significantly lower number of tumors per mouse compared with wild-type mice (33% reduction at 25 weeks, p=0.01, t-test). Importantly, cyclin $D3^{-/-}$ mice showed the strongest inhibition of tumor development among the four groups (54% reduction, p= 0.0002, t-test). Thus, we conclude that ablation of D3 negatively affects an early event in tumorigenesis such as initiation and/or clonal expansion of initiated cells.

Importantly, the incidence and multiplicity of papillomas in K5CDK6/cyclin $D3^{-/-}$ mice was similar to K5CDK6 mice (25% reduction, p=0.042, t-test). Although, tumor inhibition was also observed in K5CDK6/cyclin $D3^{-/-}$ mice compared with WT siblings, no synergistic effect between ablation of cyclin D3 and overexpression of CDK6 was established (Figure 9A).

In addition, the incidence of papilloma formation was different in cyclin $D3^{-/-}$ compared to other groups. Wild-type, K5CDK6, and K5CDK6/Cyclin $D3^{-/-}$ mice developed visible tumors between 5-8 weeks (latency) and reached a 50% of penetrance by ~8-10.5 weeks. Although the latency of cyclin $D3^{-/-}$ mice were similar to other siblings (at 7 weeks), the 50% of penetrance in cyclin $D3^{-/-}$ mice is severely delayed by 15 weeks (p=0.001, t-test). Consequently, the incidence of papilloma formation reached a plateau of 90-80% in wild-type, K5CDK6, and K5CDK6/Cyclin $D3^{-/-}$ mice. In contrast, Cyclin $D3^{-/-}$ mice reached only 50% of papilloma incidence at the end of the experiment. These results show that ablation of

cyclin D3 severely affects tumor development (Figure 9B). These results indicates that ablation of cyclin D3 cause severe tumor repression, however it did not affect the tumor inhibition activity of CDK6 because both K5CDK6 and K5CDK6/cyclin $D3^{-/-}$ compound mice exhibit a substantial reduction in the multiplicity compared to wild type siblings. Therefore, contrary to our initial hypothesis, we conclude that the lack of cyclin D3 and forced expression of CDK6 trigger independent tumor suppressor mechanisms.

3.4. Biochemical and immunohistological analysis

To determine whether simultaneous increase of CDK6 and lack of cyclin D3 affects keratinocyte proliferation and/or apoptosis in tumors, we quantified the BrdU incorporation and analyze the presence of apoptotic cells by Tunel Assay. Ablation of cyclin D3 did not affect keratinocyte proliferation or apoptosis in normal epidermis, whereas, both proliferation and apoptosis were significantly different in tumors among the four groups analyzed (Figure 10, 11). BrdU labeling showed that the keratinocyte proliferation increased 1.3-fold in both K5CDK6 and K5CDK6/cyclin $D3^{-/-}$ tumors compared with wild-type tumors ($p=0.026$ for K5CDK6, $p=0.0015$ for K5CDK6/cyclin $D3^{-/-}$, t-test). In contrast, the number of proliferating cells was significantly reduced in cyclin $D3^{-/-}$ papillomas compared with the wild-type tumors (0.70-fold, $p=0.011$, t-test). Based on these results, we conclude that ablation of cyclin D3 decreased keratinocyte proliferation in skin tumors, but it did not dismiss the increment of proliferation by forced expression of CDK6 (Figure 10B).

Interesting, all tumors from transgenic mice show elevated apoptosis compared to WT tumors (Figure 11A,B). Consistent with our previous report (Wang et al. 2011), we observed

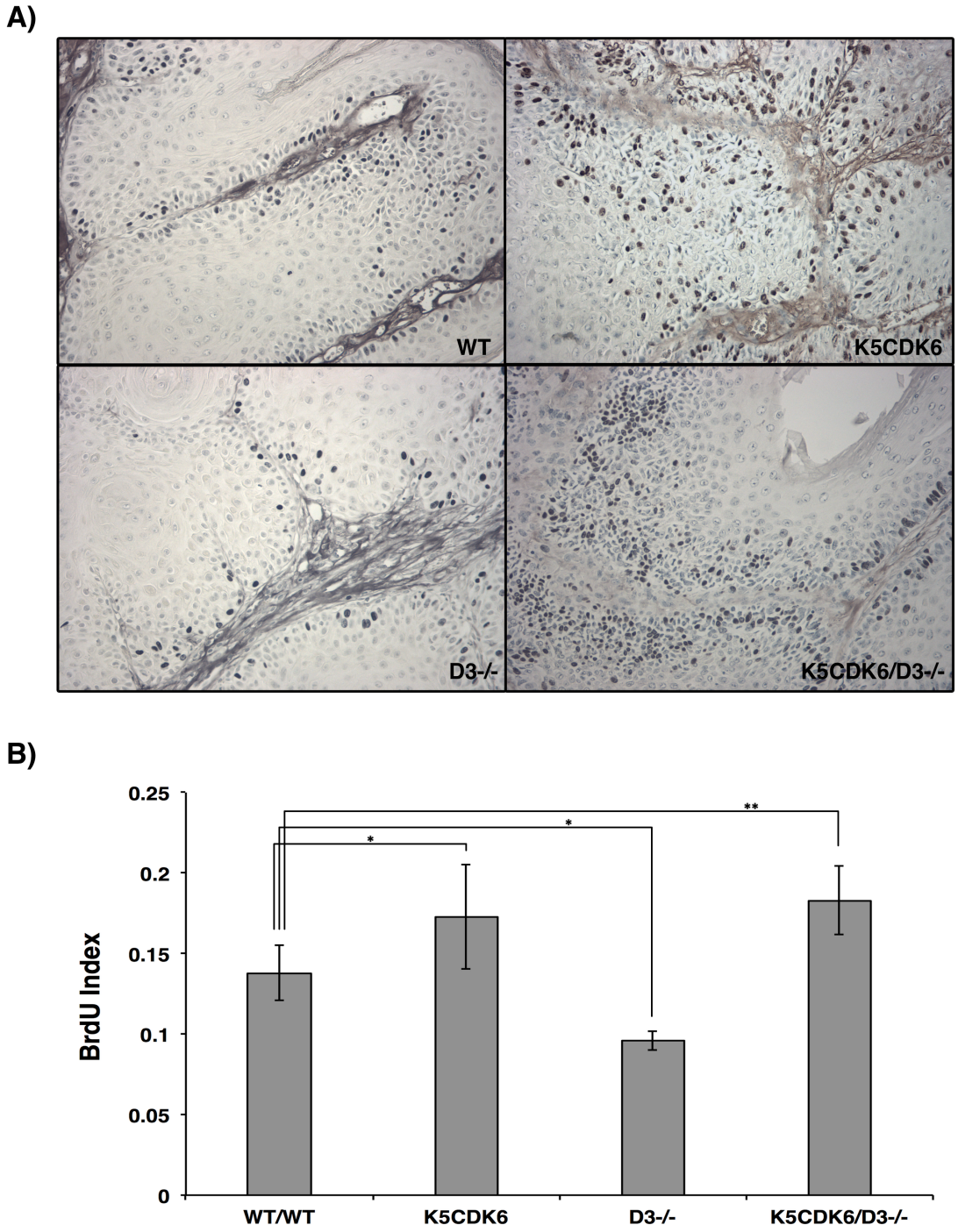

Figure 10. Keratinocyte proliferation in skin tumors. (A) BrdU immunostaining of tumors from wild type (WT), K5CDK6, Cyclin D3$^{-/-}$ (D3$^{-/-}$), K5CDK6/Cyclin D3$^{-/-}$ (K5CDK6/D3$^{-/-}$) mice. (B) The BrdU label index of the tumors of four genotypes analyzed. 10~15 tumors per each group were analyzed, Bars; ± Standard Deviation. $P < 0.05$; *, $P < 0.005$; **, t-test

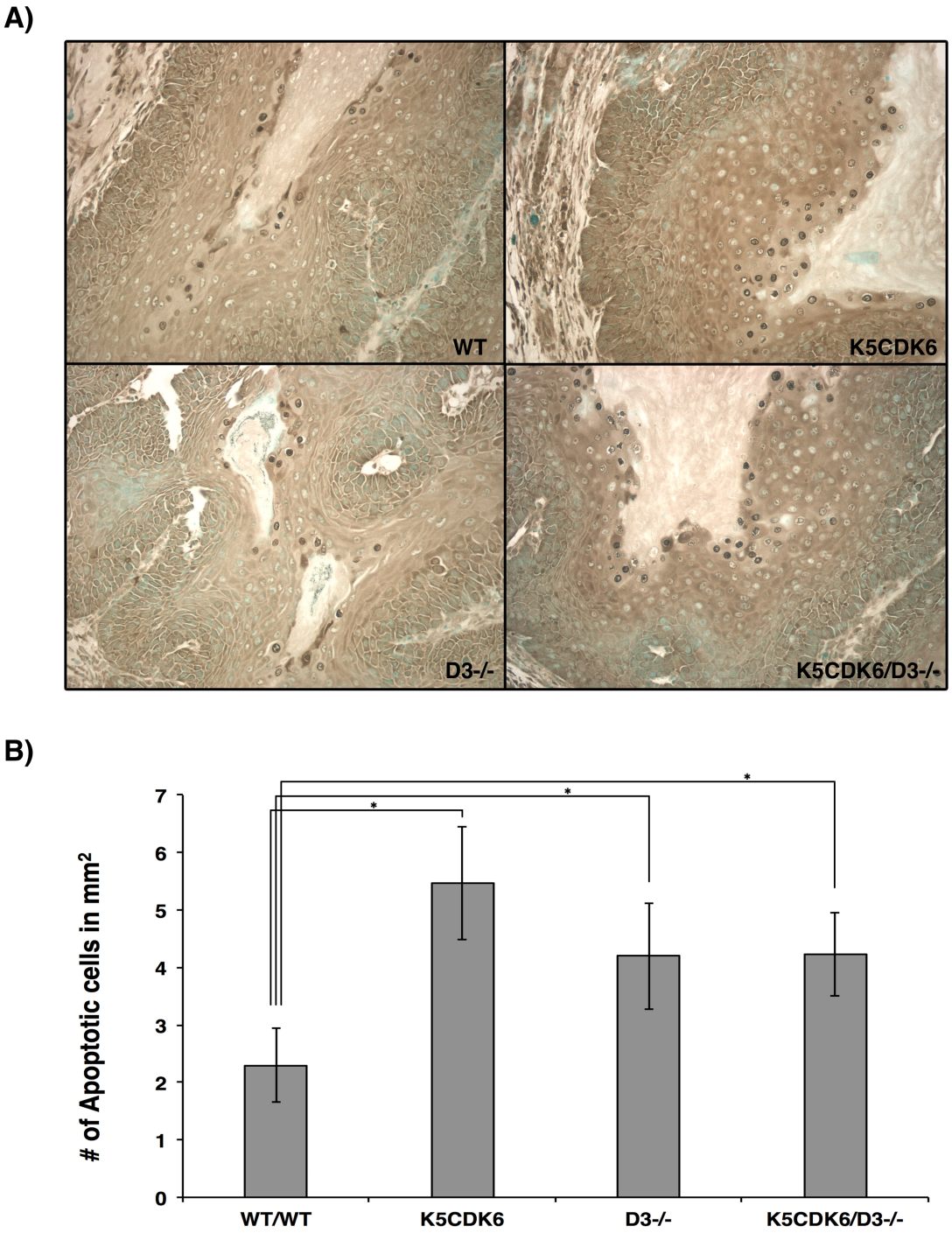

Figure 11. Increase apoptosis in keratinocytes of transgenic mice. (A) Tunel Assay of tumors from wild-type (WT), K5CDK6, cyclin $D3^{-/-}$ ($D3^{-/-}$), K5CDK6/cyclin $D3^{-/-}$ (K5CDK6/ $D3^{-/-}$) mice. (B) Quantification of apoptotic cells in skin papillomas of wild-type (WT/WT), K5CDK6, Cyclin $D3^{-/-}$ ($D3^{-/-}$) and K5CDK6/$D3^{-/-}$ mice. 8 tumors per each group were analyzed, Bars; ± Standard Deviation. $P < 0.00001$; *

2.4-fold increased apoptosis in K5CDK6 ($p<0.00001$, t-test) and 1.8-fold increased apoptosis in both cyclin $D3^{-/-}$ and K5CDK6/cyclin $D3^{-/-}$ ($p<0.00001$, t-test) compared to wild-type tumors. Therefore, we conclude that the lack of D3 lead to increasing apoptosis, but it did not result in the synergetic effect of overexpression of CDK6 (Figure 11B).

In order to determine whether overexpression of CDK6 and/or deletion of cyclin D3 alter the expression of other cell-cycle regulators in skin tumors, we perform biochemical analysis of papillomas from the four groups of mice (Figure 12). CDK6 protein levels remain significantly higher in K5CDK6 and K5CDK6/cyclin $D3^{-/-}$ tumors compared with wild-type papillomas regardless the presence of cyclin D3. In some case, expression of CDK4 seemed to decrease in K5CDK6 tumors, however, these differences were tumor specific. Also, CDK inhibitors such as $p27^{Kip1}$, $p21^{Cip1}$ and $p16^{Ink4b}$ displayed no homogeneous expression levels through tumors. As determined in normal epidermis, compensatory expression of cyclin D1 was also observed in tumors from cyclin $D3^{-/-}$ and K5CDK6/cyclin $D3^{-/-}$ mice. Therefore, we speculate that elevation of CDK4/cyclin D1 and CDK6/cyclin D1 complex levels in tumors of K5CDK6/cyclin $D3^{-/-}$ would positively affect higher keratinocyte proliferation than in wild-type tumors.

3.5. Malignant progression of tumors from K5CDK6/$D3^{-/-}$

Both ablation of cyclin D3 and forced expression of CDK6 significantly altered keratinocyte proliferation and apoptosis in tumors, thus, we speculate that changes in the kinetic of proliferation/apoptosis will influence the rate of malignant progression.

To determine the influence of the CDK6 and cyclin D3 in the rate of malignant progression,

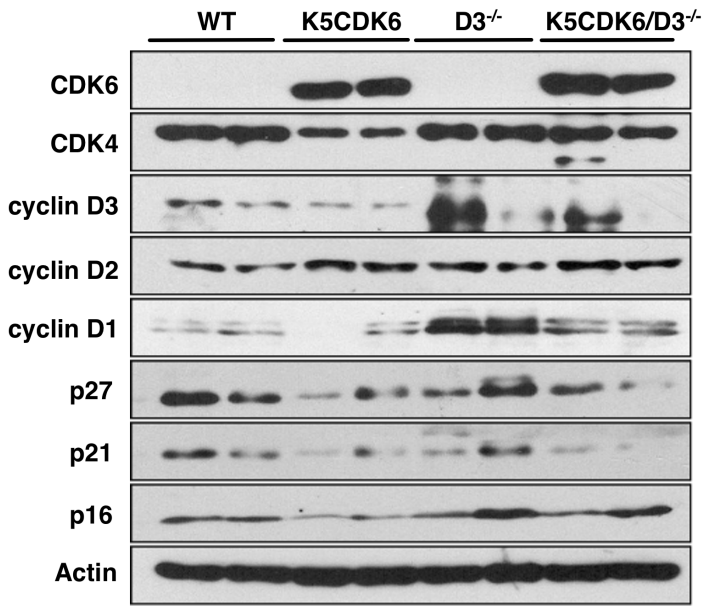

Figure 12. Biochemical analysis of cell cycle regulators in tumors from wilt-type (WT), K5Cdk6, Cyclin D3$^{-/-}$ (D3$^{-/-}$)_ and K5Cdk6/cyclin D3$^{-/-}$ (K5CDK6/ D3$^{-/-}$) mice. Protein lysates from skin papillomas obtained from wildtype (WT), K5CDK6, D3$^{-/-}$ and K5CDK6/D3$^{-/-}$ mice were separated by SDS-PAGE, transferred onto a nitrocellulose membrane. Primary antibodies against CDK4, CDK6, cyclin D1, D2, D3, p27, p21 and p16 were used for immunoblot analysis. β-Actin used as a loading control

we perform histopathological analysis of skin tumors collected at the end of the experiment (25 weeks of promotion) (Table 1). All tumors of cyclin $D3^{-/-}$ mice were classified as well-differentiated papillomas with no atypia in the basal layers. On the other hand, 15% of the analyzed tumors from wild-type and K5CDK6 mice, and 29% of K5CDK6/cyclin $D3^{-/-}$ tumors were classified as SCC or carcinoma in situ (CIS) with areas of infiltration (Table I).

In order to determine whether the differences in apoptosis and proliferation among the different genotype influence the total grow (size) of the tumors, we measured the size of tumors from each genotype groups (Figure 13). Most of skin tumor did not reach more than 50 mm^3 (~78% of wild-type, 82.5% of K5CDK6, 93.3% of cyclin $D3^{-/-}$, and 74% of K5CDK6/cyclin $D3^{-/-}$ tumors). However, 16% of the K5CDk6/cyclin $D3^{-/-}$ tumors are larger than 100mm^3, whereas, only 9% of wild-type tumors and 10% of K5CDK6 tumors reached that size. Importantly, cyclin $D3^{-/-}$ mice had the lowest number of tumors measuring over 100 mm^3 (6.6%) and the highest number of tumors measuring less than 1 mm^3.

Based on these results, we speculate that once the initiated cells overcome the inhibitory barrier established by overexpression of CDK6 or ablation of cyclin D3 at an early stage of tumorigenesis, the tumor size depends on the rate of proliferation and apoptosis of keratinocytes. Supporting this idea, simultaneous reduction in the rate of proliferation and elevated apoptosis in tumors of cyclin $D3^{-/-}$ mice would lead to slow growth of tumors compared to the other groups.

Therefore, we conclude that the negative balance established by elevated apoptosis and reduce proliferation in cyclin $D3^{-/-}$ tumors might not only influence the tumor size, but also inhibit malignant progression. However, ablation of cyclin D3 within the context of CDK6

Table 1. Histopathological Analysis of Skin Tumors

Mice	*No. of analyzed tumors / group*	*No. (%) of tumors classified as:*	
		Papilloma [a]	SCC [b]
Wild Type	21	18 (85.7%)	3 (14.3%)
K5CDK6	20	17 (85.0%)	3 (15.0%)
Cyclin $D3^{-/-}$	10	10 (100%)	0 (0%)
K5CDK6/Cyclin $D3^{-/-}$	24	17 (70.8%)	7 (29.2%)

[a] No atypia in basal layers, basal cell hyperplasia, and no invasion of epidermal cells into the dermis.
[b] Differentiated or highly undifferentiated SCC with expansion of basal and spinous layer, loss of polarity, and cords of epidermal cells contiguous to the basal layer invading the dermis.

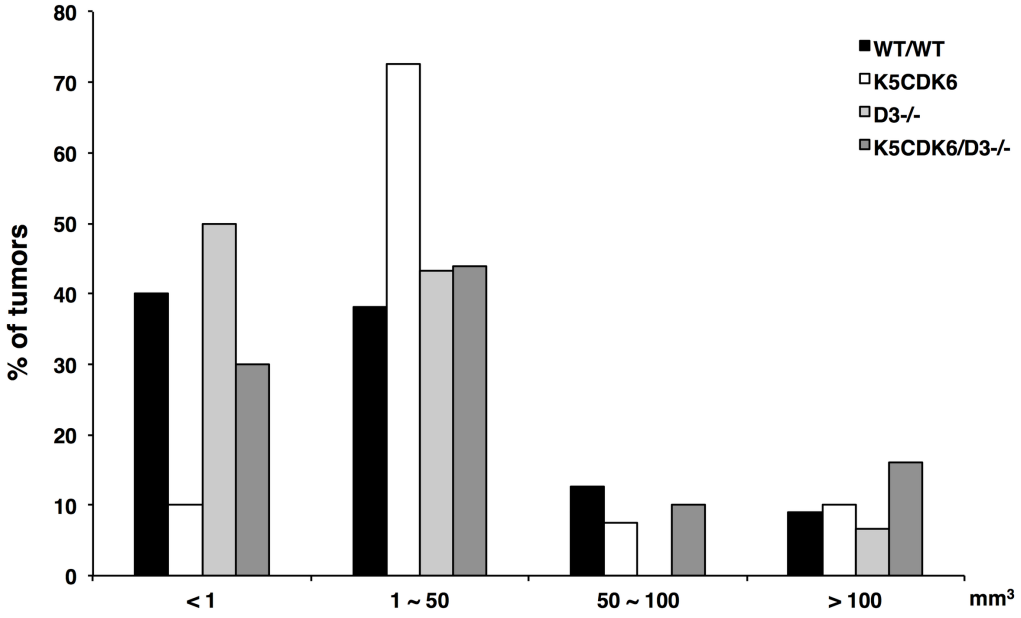

Figure 13. Reduction of tumor size by ablation of cyclin D3 in absence of CDK6 overexpression. Skin papillomas were classified according to their volume and depicted as percentage of the total number of tumors analyzed in each genotype.

overexpression did not inhibit malignant progression likely due to the increase proliferation observed in CDK6 overexpressing keratinocytes. Also, we suggest that increased level of cyclin D1 with overexpressed CDK6 might play a positive role in the progression of K5CDK6/cyclin D3$^{-/-}$ tumors through formation of CDK6/cyclin D1 complexes and probably CDK4/cyclin D1 complexes.

4. Discussion

It is widely accepted that D-type cyclins and CDK4/6 play pivotal roles as proto-oncogenes in various human and mice tumors. However, we have reported that forced expression of CDK6 (K5CDK6) or cyclin D3 (K5D3) in mouse skin epidermis significantly inhibits skin tumorigenesis under chemically induced tumor protocol (Wang et al. 2011; Rojas et al. 2007). Interestingly, we observed the elevated CDK6/cyclin D3 complex formation in both transgenic mouse epidermis and skin tumors. Thus, we hypothesized that the association of CDK6/cyclin D3 negatively affects tumorigenesis. Our hypothesis implies that both CDK6 and cyclin D3 are necessary for skin tumor inhibition.

Contrary to our hypothesis, lack of cyclin D3 expression does not affect the tumor repression activity of CDK6 since both K5CDK6 and K5CDK6/cyclin D3$^{-/-}$ mice exhibit a severe reduction in the multiplicity and incidence of skin tumors. Therefore, our results support a model in which CDK6 and cyclin D3 play independent roles as inhibitors of tumorigenesis, likely affecting an early stage of skin tumorigenesis, such as tumor initiation and/or clonal

expansion of the initiated cells. It is worth mentioning that, similar to cyclin $D1^{-/-}$ and cyclin $D2^{-/-}$ mice, ablation of cyclin D3 resulted in a severe reduction in the number of tumors. Histopathological analysis of skin tumors showed that lack of cyclin D3 leads to inhibition of tumor progression, as all the cyclin $D3^{-/-}$ tumors were classified as well-differentiated skin papillomas. At the same time, however, it also revealed that overexpression of CDK6 in absence of cyclin D3 (K5CDK6/cyclin $D3^{-/-}$ mice) causes malignant progression. Our results show that lack of cyclin D3 affects two stages of skin carcinogenesis, in which the reduce number of tumors suggest an inhibitory effect in an early stage of tumorigenesis, but the cyclin $D3^{-/-}$ tumors which overpasses the early inhibition develop as benign papillomas does not progress to squamous cell carcinomas. Therefore, these results suggest that, in the absence of cyclin D3 with forced expression of CDK6, CDK6 plays a unique role in malignant progression. Additionally, keratinocytes from K5CDK6/cyclin $D3^{-/-}$ mice showed increased level of CDK4/cyclin D1 and CDK6/cyclin D1 complexes. Based on this, it can be speculated that cyclin D1 overexpression in tumors of K5CDK6/cyclin $D3^{-/-}$ might positively affect keratinocyte proliferation, leading to greater tumor size in comparison to other siblings.

The role of cyclin D3 in skin keratinocyte is still unclear, because any structural or functional abnormality by ablation of cyclin D3 has not been observed in this study. The total number of nucleated cells or keratinocyte proliferation/apoptosis upon lack of cyclin $D3^{-/-}$ remains comparable to that of wild-type mice. However, our biochemical analysis revealed that the absence of cyclin D3 results in upregulation of cyclin D1 level in mouse epidermis. Importantly, the increased level of cyclin D1 leads to elevated formation of CDK4/cyclin D1

complex, compare with wild-type and K5CDK6 epidermis. These results suggest that cyclin D1 expression compensate the lack of cyclin D3 in mice skin, allowing to maintain epidermal homeostasis.

Compensation for altered expression of D-type cyclins has been reported, but its regulation mechanism is not understood (Chen & Pollard 2003; Lam et al. 2000; Zhang et al. 2011; Mataraza et al. 2006; Wang et al. 2012). Double knockout mice retaining only a single D-type cyclin developed normally until late gestation, but died beyond that point due to loss of tissue-specific expression and insufficient upregulation of the remaining cyclin in most of their tissues (Ciemerych et al. 2002). Expression of cyclin D3 was induced in mouse B-lymphocytes of cyclin D2-null, which activated via the antigen receptor and CDK40 (Lam et al. 2000). In addition, Cyclin D2 completely compensated for the loss of cyclin D1 in the proliferative responses of mouse uterine epithelial cells to estrogens (Chen & Pollard 2003), and disruption of cyclin D3 was compensated by cyclin D2 to mask the normal functions in mitogenesis of B-1a cells (Mataraza et al. 2006). Consistent with these reports, our previous studies also showed that forced expression of cyclin D3 in mouse skin resulted in decreased level of cyclin D2 (Rojas et al. 2007).

The mechanism by which CDK6 suppress tumor promotion is not entirely understood, but several unexpected kinase-independent roles of CDK6 were recently revealed by Kollmann's group (Kollmann et al. 2013). Overexpressed CDK6 in BCR-ABL-transformed B acute lymphoid-leukemia cells forms a transcriptional complex with D-type cyclin and STAT3 and, this complex induces expression of $p16^{INK4a}$, an inhibitor of CDK4/6 kinase. Through this

negative feedback loop, overexpressed CDK6 inhibits cell proliferation and delays tumor formation upon xenograft mice models. They show that cyclin D2 and CDK6 are present at $p16^{INK4a}$ promoter. However, if $p16^{INK4a}$ is silent by methylation of its promoter, CDK6 can act as an oncogenic protein through promoting proliferation by pRb inactivation and stimulating angiogenesis by induction of VEGF-A in T-cell lymphoma. Consistent with this study, our biochemical analysis showed slightly increased level of $p16^{INK4a}$ in normal epidermis of K5CDK6 and K5CDK6/$D3^{-/-}$. Actually, the existence of $p16^{INK4a}$ itself is a critical factor to decide tumor suppression by forced expression of CDK6. Thus, it is speculated that overexpressed CDK6 suppresses tumor initiation by forming a negative feedback loop with $p16^{INK4a}$.

There are other studies supporting a transcriptional and kinase-independent function of CDK6. CDK6 associates with a promoter region of prostate-specific antigen gene with androgen receptor (AR), and enhances its transcription in LNCaP prostate cancer cells (Lim et al. 2005). In addition, recently a large number of CDK4/6 substrates are uncovered by Sicinski's group (Anders et al. 2011). Throughout this *in silico*, they identify that many of the 71 *in vitro* substrates share a transcriptional regulation function. Therefore, determining whether CDK6 shares transcriptional function with novel substrates will allow us to establish tumor suppressive mechanism by overexpressed CDK6.

Furthermore, we found that ablation of cyclin D3 also leads to the significant reduction of papilloma initiation and carcinoma progression. These are conflicting results with tumor inhibitory effects of forced expressed cyclin D3 under the same protocol (Wang et al. 2012;

Rojas et al. 2007). According to these previous studies, overexpression of cyclin D3 with or without cyclin D1 (K5D3 or K5D3/cyclin $D1^{-/-}$) in mouse keratinocytes induces down-regulation of cyclin D2 and results in the decrement of skin tumorigenesis. Therefore, it was expected that $D3^{-/-}$ mice would show similar or elevated tumor development compared to wild type, due to following upregulated cyclin D2 by the absence of cyclin D3. Instead, we found that cyclin D1, but not cyclin D2 was overexpressed. However, cyclin $D3^{-/-}$ mice showed the lowest multiplicity and incidence of analyzed groups. These results suggest that overexpressed cyclin D1 can compensate for the absence of cyclin D3 in normal mouse epidermis that show normal epidermal homeostasis, but it is not enough to overcome the inhibitory barriers induced by ablation of cyclin D3 in tumorigenesis.

Although the increased level of cyclin D1 is insufficient to elevated tumor development, it definitely plays a positive role in a progression of tumors. Consistent with a previous study, forced expressed CDK6 in K5CDK6 mice does not affect malignant progression to SCC compared to wild-type siblings. However, we observed 2-fold increase in the rate of SCC progression in K5CDK6/$D3^{-/-}$ mice, compared to those in wild type and K5CDK6 siblings. Biochemical analysis of normal epidermis and tumors reveals elevated level of CDK4/cyclin D1 and CDK6/cyclin D1 complex in K5CDK6/$D3^{-/-}$, whereas, CDK6/cyclin D3 complex is dominant over other cyclin complexes in K5CDK6. Enhancement of malignant conversion precisely corresponds with results in K5CDK4 mice under the same protocol (Miliani de Marval et al. 2003). Dramatic changes in the incidence of SCC were observed in K5CDK4 mice compared to wild-type sibling, and increased expression of CDK4 resulted in elevated CDK4/cyclin D1 complex formation in late tumors. As well, many studies about effects of

cyclin D1 in skin tumorigenesis suggested that cyclin D1 gives advantage on malignant conversion to SCC (Robles & Conti 1995; Yamamoto et al. 2002; Bianchi et al. 1993; Wang et al. 2012; Fujii et al. 2001).

In summary, we have demonstrated that CDK6 and cyclin D3 have independent tumor suppression mechanisms, acting on early stage of tumor development. Nevertheless, once the initiated cell overcomes the inhibitory barrier induced by ablation of cyclin D3, CDK6 favors the tumor growth and malignant progression through compensatory expression of cyclin D1.

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CHAPTER 3

Accelerate centrosome amplification by forced expression of CDK4 during chemical induced skin tumorigenesis.

Abstract

Elevated activity of Cyclin-dependent kinase 4 (CDK4) is common in the majority of human neoplasia. We have previously demonstrated that forced expression of CDK4 in mouse epidermis induces malignant conversion to squamous cell carcinomas (SCC) in chemically induced skin tumors. We predicted that CDK4 plays an additional role in malignant progression via pRb-independent pathways. However, the mechanisms by which changes in CDK4 expression levels control skin tumorigenesis have not been established. Centrosome amplification is a well-known driving force for malignant progression of solid tumors through triggering aneuploidy. Thus, we hypothesized that the forced expression of CDK4 leads to phosphorylation of centrosome related proteins, and contributes centrosome amplification. We concluded that the increase of CDK4 expression intensify centrosome amplification in skin tumors during tumor promotion and malignant progression. Moreover, in order to investigate the putative phosphorylation targets of CDK4, we analyzed centrosome related proteins via mass-spectrometry, and finally suggested 21 putative centrosome-relative substrates for CDK4. The identification of CDK4 as a regulator of centrosome cycle may help to develop a strategy for preventing malignant progression of skin tumors.

1. Introduction

A large body of literatures during the past decades has shown the importance of Cyclin-dependent kinase (CDKs) regulation in tumor development. Frequent alterations of the

retinoblastoma (pRb) pathway occur through functional inactivation of pRb that is the result of elevated CDK activities caused by overexpression of CDKs, cyclins or loss of CDK-inhibitors. Therefore, misregulation of CDKs mediate cell cycle defects acquiring key features of cancer cells such as unscheduled proliferation, genomic instability (GIN) and chromosome instability (CIN) (Malumbres & Barbacid 2009).

Cyclin-dependent kinase 4 (CDK4) is essential for G1 phase regulation in cell cycle and its kinase activity is acquired by association with D-type cyclins. CDK4 plays a canonical role phosphorylating the tumor suppressor pRb during the G1/S phase transition (Zarkowska 1999). Notably, it has been found elevated CDK4 activity by mutation in the binding sites of cyclin-dependent kinase inhibitors (CDKI), gene amplification, or loss of CDK-inhibitors such as $p16^{INK4a}$. These alterations are common in particular human neoplasias such as, familial melanoma, gliomas, sporadic breast carcinomas, sarcomas, neuroblastomas and squamous cell carcinomas (Khatib et al. 1993; Wolfel et al. 1995; Ichimura et al. 1996; Holland et al. 1998; An et al. 1999; Easton et al. 1998; Cheung et al. 2001).

Our laboratory has investigated the specific role of CDK4 in skin carcinogenesis using genetically modified mouse skin models. We have previously demonstrated that forced expression of CDK4 in mouse epidermis (K5CDK4 mice) resulted in a dramatic increment in the rate of malignant progression to squamous cell carcinomas (SCC) in the chemical-induced carcinogenesis. This result can be interpreted through hyperphosphorylation and inactivation of pRb by overexpressed CDK4. However, the canonical role of CDK4 is not sufficient to explain it due to several reasons. First of all, overexpression of other CDKs such as CDK2 or CDK6 in mouse epidermis (K5CDK2 or K5CDK6) results in pRb

phosphorylation, but the tumors does not show malignant progression (Macias et al. 2008; Wang et al. 2011). In addition, overexpressed cyclin D1, an activator of CDK4, in mouse epidermis (K5D1) did not induce earlier higher rate of SCC development than K5CDK4 mice (Miliani de Marval et al. 2003). Moreover, deletion of pRb from epidermis does not increase tumor susceptibility nor malignant progression (Ruiz et al. 2005). Thus, we predict that CDK4 has pRb-independent roles leading to SCC malignant progression, however, the molecular mechanisms by which changes in CDK4 expression levels contributing to malignancy have not been established.

Chromosome instability (CIN) including insertion, deletion, recombination, translocation and aneuploidy (chromosome loss and gain) is the driving force for tumor progression, because it induces rapid accumulation of many specific mutations required for malignant transformation (Loeb 1991). Thus, abnormal centrosome amplification (CA) is considered as a cause of aneuploidy by triggering chromosome segregation errors (Fukasawa 2005; D'Assoro et al. 2002).

The centrosome is a non-membranous organelle composed of a pair of centrioles (mother and daughter) and pericentriolar materials (PMC) surrounding the centrioles. As the primary function of the centrosome is to organize mitotic spindles during mitosis, it is referred as a microtubule organizing center (MTOC). Normal centrosome cycle is tightly regulated through a cell cycle. Centrosome cycle is synchronized with cell cycle progression (Rieder et al. 2001). Centriole duplication begins in late G1/early S-phase, separates and elongates in the G2-phase, and migrates to the spindle pole and generates spindle followed by segregation during mitosis and cytokinesis. pRb/CDKs/$p16^{INK4a}$ pathway plays a significant role in the

centrosome duplication, because E2F regulated the transcription of several chromosomal related genes such as cyclin E, cyclin A, Nek2 and RanBPM (Ren et al. 2002; Ishida et al. 2001). Therefore, deregulation of CDKs on G1/S phase or loss of cell cycle checkpoint function can trigger centrosome amplification.

Additionally, CDK2 in G1 phase promote centrosome duplication via modulation of several potential targets. For example, Monopolar spindle 1 (Mps-1), an essential centrosomal kinase for mitotic spindle assembly, can be stabilized by direct action of CDK2/cyclin A, and consequently accumulated Mps-1 participates in centrosome duplication (Fisk & Winey 2001; Fisk et al. 2003; Kasbek et al. 2007). Other potential targets of CDKs are Centriolar coiled-coil protein 110kDa (CP110) and Plk4, involving in regulation of centriole duplication and centrosome separation. CP110 is phosphorylated by various CDKs such as CDK2/cyclin E, CDK2/cyclin A, and CDK1/cyclin B, but its activity-regulation mechanism is not entirely understood (Chen et al. 2002). In a case of Plk4, it is not the direct phosphorylation target of CDK2, but Plk4 cannot initiate centriole duplication without CDK2 activity (Habedanck et al. 2005). Importantly, CDK2 and CDK4 directly target Nucleophosmin (NPM) which licensing centrosome duplication (Okuda et al. 2000; Okuda 2002). Absence of CDK2 or CDK4 prevents centrosome amplification and chromosomal instability in p53-null MEFs via abrogating phosphorylation of NPM at Thr199 (Adon et al. 2010).

Like CDK2, CDK4 has also involved in centrosome amplification. In a recent report, the Saavedra's group suggests a model to explain how excessive activity of CDK4 triggers centrosome amplification. As aforementioned, they show that hyperactive CDK4, by down-regulated $p21^{Cip1}$, hyperphosphorylates NPM at Thr199 and induces CA (Adon et al. 2010).

Furthermore, they demonstrate that Nek2 kinase (a regulator of centrosome separation) act downstream of CDK4/cyclin D1; Thus, overexpression of CDK4/cyclin D1 in Her2+ breast cancer cell, and/or c-Myc breast cancer cells resulted in abundant Nek2. Overexpressed Nek2 brings out binucleation through failed cytokinesis and leads to CA (Zeng et al. 2010; Harrison Pitner & Saavedra 2013). Despite these advances, the molecular mechanisms linking the pRb-independent function/s of CDK4 is poorly understood due to little knowledge of potential substrates of CDK4 involved in centrosome regulation.

Based on our own results utilizing chemical-induced skin carcinogenesis in K5CDK4 mice, *we hypothesize that the forced expression of CDK4 lead to phosphorylation of centrosome related proteins and contributes centrosome amplification.*

2. Materials and Methods

2.1. Immunofluorescence analysis of centrosome

Paraffin-embedded sections of skin tumors were obtained from previous experiments of two-stage carcinogenesis protocol (Macias et al. 2007). Normal skin and hyperplastic skin were prepared at post-natal 21 days, and hyperplastic skin was induced by four times-TPA treatments for two weeks. For immunofluorescence, tissue cross sections were deparaffinized, rehydrated, and permeabilized using citrate based antigen unmasking solution (Vector Laboratories Inc., CA). After antigen retrieval process, sections were incubated in cold methanol for 10 min and blocked with PBS containing 10% normal goat serum, 3% BSA, and 0.5% gelatin for 1 hr. Then, centrosome was stained with 1:500 of rabbit-anti-γ-

tubulin polyclonal antibody (T5192, Sigma-Aldrich Co. LLC, MO) followed by incubation with 1:1000 of Alexafluor 488 goat-anti-rabbit secondary antibody (Molecular Probes, OR). Sections were counterstain with To-pro3 (blue) (Molecular Probes, OR), visualized under the Olympus IX81 confocal microscope and analyzed by ImageJ software.

2.2. Centrosome Isolation

To purify centrosome from NIH3T3 cells, a sucrose density-gradient centrifugation protocol was used as previously described (Meigs & Kaplan 2008). Briefly, 75-80% confluent cells on 10 petri dish plates were prepared, treated with 70ng/ml of nocodazole (Sigma-Aldrich Co. LLC, MO) in culture medium for 16 hrs and added 20μg/ml of cytochalasin B (Sigma-Aldrich Co. LLC, MO) for 90min before washing with cold PBS, 8% of sucrose (w/v) in 0.1xPBS, 8% of sucrose (w/v) in DW and 1mM of Tris (pH8.0) containing β-mercaptoethanol. Cells were extracted with lysis buffer [1mM Tris-HCl pH8.0, 0.5% NP-40 buffer, 0.1% β-mercaptoethanol, 50μM PMSF, 1mM Na_3VO_4 and 1x protease inhibitor cocktail] and harvested in one conical tube and gently mixed by rotation at 4°C for 30 min. Then 1/50 volume of 50x PE buffer [500mM PIPES pH7.2, 0.5M EDTA] was added, and the mixed solution centrifuged at 1000 rpm at 4°C for 5 min. The supernatant was filtrated through a 40μm pore size of nylon mesh strainer (Thermo Fisher Scientific, IL). 14ml of the filtrated supernatant was loaded on top of 1 ml of sucrose cushion [60% of sucrose (w/v), 1x PE buffer, 0.1% β-mercaptoethanol] in a thin-wall ultracentrifuge tube (Beckman Coulter, Inc., CA) and centrifuged at 12000rpm at 4°C for 40 min in the SW28.1 swing-bucket ultracentrifuge rotor (Beckman Coulter, Inc., CA). The sample was discarded all but 2ml

was applied on a 60% sucrose cushion and mixed with the sucrose cushion in the bottom to make 20% sucrose. A discontinuous sucrose gradient in the ultracentrifuge tube was made by gently overlaying the lighter sucrose solution onto the dense sucrose solution; 5ml of 70%, 3ml of 50% and then 3ml of 40% sucrose in 1x PE buffer containing 0.1% β-mercaptoethanol. 20% sucrose sample solution was overlaid on the top of the gradient sucrose solution and centrifuged at 25000rpm 4°C for 80 min. The bottom of the tube was punctured by a 22-gauge of needle and fractions were collected 250μl each in the microcentrifuge tubes. Protein concentration of each fraction was measured by BCA protein assay (Thermo Fisher Scientific Inc., IL) for further studies.

2.3. Western blot analysis

To identify which fractions had enriched centrosome, western blot analysis was performed. SDS sample buffer added into 10μl of each fraction and analyzed through 10% SDS-PAGE. The membrane was blocked with 5% non-fat milk in 0.1% Tween20-TBS buffer, incubated with 1:1000 of the rabbit-anti-γ-tubulin polyclonal antibody (T5192, Sigma-Aldrich Co. LLC, MO) and 1:2000 of Goat-anti-rabbit-HRP secondary antibody (sc-2004, Santa Cruz Biotech, CA).

2.4. Kinase Assay

To find centrosome related proteins as the putative target of CDK4, we performed kinase assay with the centrosome enrichment-fraction. 500μg of fresh protein lysate from NIH3T3 cells was prepared with Tween-20 buffer [50mM Hepes (pH7.5), 150mM NaCl, 0.1mM

EDTA, 25mM EGTA, 10% glycerol, 0.1% Tween-20, 1mM NaF, 0.1mM Na_3VO_4, 1mM DTT and 0.1mM PMSF] and immunoprecipitated with 2μg of CDK4 antibody conjugated with agarose (sc-260 AC, Santa Cruz Biotech, CA) for 2hrs at 4°C with constant rotation. Incubated beads were washed twice with lysis buffer and once with kinase assay buffer [50 mM Hepes (pH7.0), 10mM $MgCl_2$, 5mM $MnCl_2$]. Then the bead pellet was resuspended with 30μl of kinase buffer containing 5μCi of [γ-32P] ATP (6000 Ci/mmol), 1mmol/L DTT, 5μmol/L ATP, and the protein sample such as 1μg of pRb substrate (Upstate Biotechnology Inc., Charlottesville, VA), 5 or 10μg of centrosome enrichment-fraction, or 1ug of whole lysate from NIH3T3 cell. The mixtures were incubated at 30°C for 30min with gentle tapping. SDS sample buffer was added, and each sample was boiled for 3min to stop the reaction. Kinase assay samples were electrophoresed through polyacrylamide gels and exposed to X-ray film at -80°C. We selected several bands with strong signal intensity on the centrosome fraction to analyze by Mass-spectrometry. The size of selected bands was calculated compared to known molecular weight of the protein ladder.

2.5. Mass-spectrometry and data analysis

In parallel with the kinase assay, 80μg of centrosome enrichment-fraction in SDS sample buffer was electrophoresed, stained with coomassie brilliant blue R-250 solution, and destained until the protein ladder and other bands were observed in the polyacrylamide gel. We remove the bands of the sample with the same size as the bands phosphorylated by CDK4 in the kinase assay. The excised bands were analyzed at the NC State Mass

Spectrometry facility (NCSU MSF). In parallel, the band of the same size from a control (unloaded) lane was removed and analyzed as a negative control.

The LC/MS data was analyzed by the proteomics database search-algorithm of MASCOT (Matrix Science) (Perkins et al. 1999), as well as ProteoIQ software (NuSep, BioInquire, Athens, GA). The proteins were categorized by their function, cellular location and relationship with centrosome as based on their protein functional information (http://www.uniprot.org, http://www.ncbi.nlm.nih.gov/pubmed). After then, putative phosphorylation sites by CDKs of classified proteins were analyzed by using CDK consensus sites (S/T*-P-X-R/K) on http://www.phosphosite.org and http://scansite3.mit.edu.

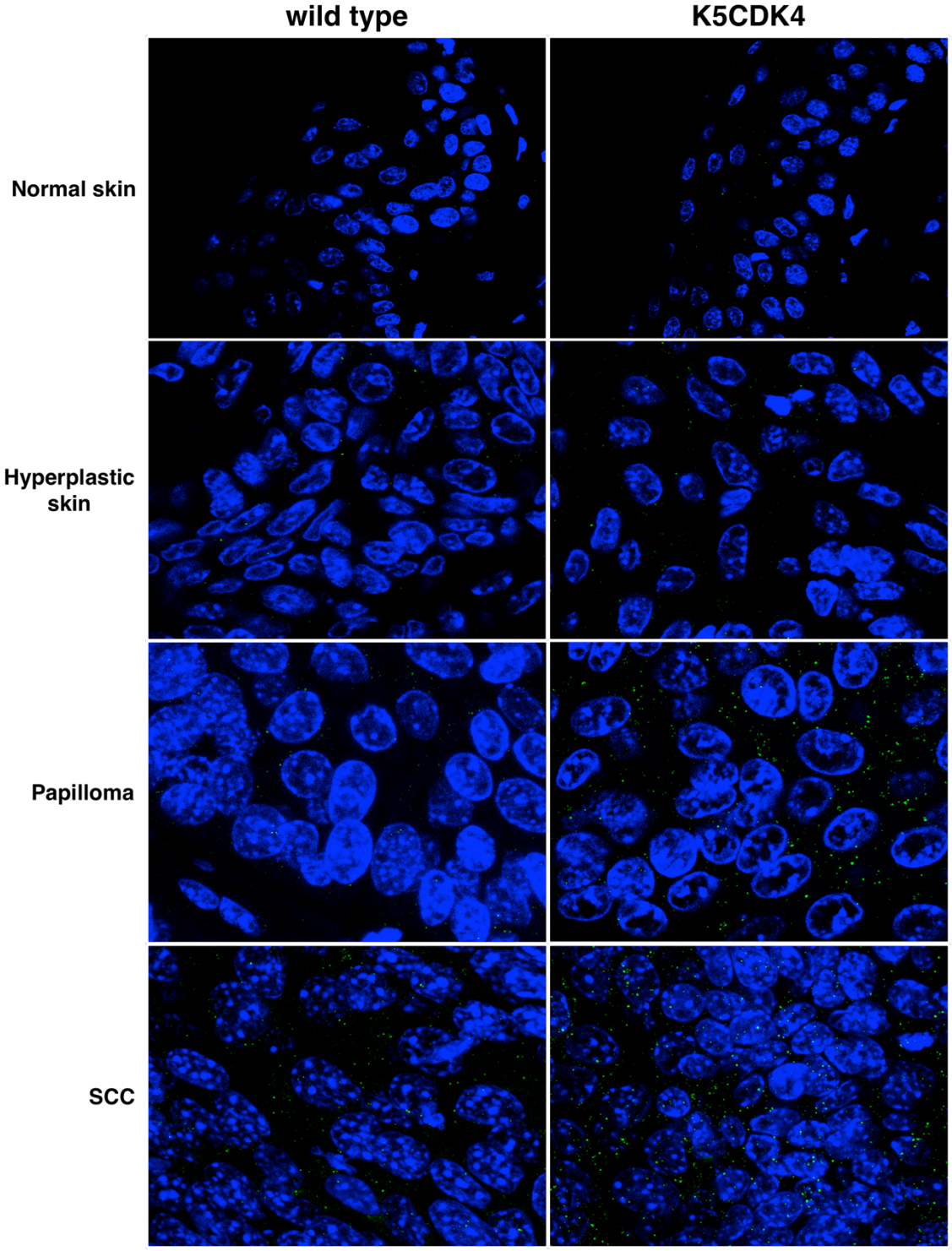
3. Results

3.1. Accelerate centrosome amplification in skin tumors overexpressing CDK4

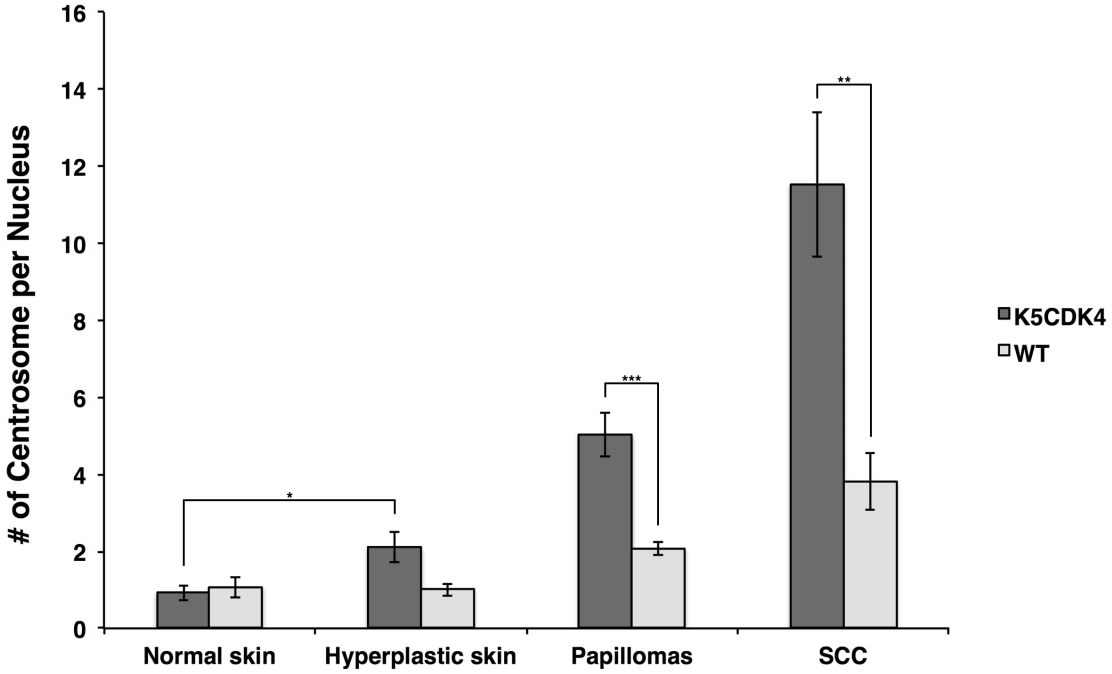
In order to determine the effect of overexpressed CDK4 on centrosome amplification, we analyzed the normal dorsal skin, hyperplastic skin (TPA treated), chemically induced papillomas and SCC from K5CDK4 mice and wild-type siblings. Paraffin-embedded sections were sectioned and utilized for immunofluorescence analysis with γ-tubulin, a marker of centrosome (Figure 14A). The total number of centrosomes normalized by the number of the nucleus counted in the same section. Analysis of both, K5CDK4, and wilt-type, samples showed a trend to increase the number of centrosomes (CA) during tumor progression (from papillomas to SCC) (Figure 14B). Centrosome amplification has been reported in most tumors (Chan 2011). Correspondently, our results show that the number of centrosome per

Figure 14. Immunofluorescence staining of centrosome in mouse skins and tumors. (A) Confocal image of γ-tubulin staining labeling centrosome (green dots) and nucleus stained with To-pro3 (Blue). Normal skin was prepared at post-natal 21 days. TPA-induced hyperplastic skin was used as hyperproliferative control. Papillomas and SCC were chemically induced via 2-stage carcinogenesis protocol. (B) Quantification of centrosome amplification normalized by the number of nucleus in the same field. Bars; ± Standard Deviation. $P < 0.05$; *, $P < 0.005$; **, $P < 0.0005$; ***, t-test

A)


wild type
K5CDK4
Normal skin
Hyperplastic skin
Papilloma
SCC

B)


# of Centrosome per Nucleus
16
14
12
10
8
6
4
2
0
*
***
**
Normal skin
Hyperplastic skin
Papillomas
SCC
K5CDK4
WT

nucleus was increased in tumors from both wild-type or K5CDK4 mice compared to centrosome number in normal or hyperplastic dorsal skins. Normal skins from wild-type or K5CDK4 showed comparable numbers of centrosome (about 1 centrosome/nucleus).

Interesting, the hyperplastic epidermis of K5CDK4 mice displayed increased number of centrosome compared to their normal skin (2.1 centrosome/nucleus, $p=0.049$, t-test) (Figure 14B). Therefore, it is tempting to speculate that overexpression of CDK4 affects centrosome amplification in normal tissues, but the effect was exacerbated during the tumorigenesis process.

Although, an increasing number of centrosomes was observed in papillomas and SCC from wild-type mice, centrosome amplification was greatly accelerated in papillomas and SCC of K5CDK4. The number of centrosome per nucleus in papillomas from K5CDK4 mice (5.0 centrosome/nucleus) was 2.4-fold amplified compared to wild type (2.1 centrosome/nucleus, $p<0.0001$, t-test). In addition, we observed 3.1-fold increased the number of centrosome per nucleus in SCC from K5CDK4 mice (11.5 centrosome/nucleus) compared in SCC from wild-type mice (3.8 centrosome/nucleus, $p=0.0015$, t-test). Therefore, we conclude that forced expression of CDK4 in mouse epidermal tumors intensify centrosome amplification observed during tumor promotion and malignant progression.

3.2. Analysis of CDK4 substrate candidates related to centrosome function

To test our hypothesis, we look for CDK4-targets on centrosome related proteins. In the first step, centrosomes were isolated and utilized for CDK4-kinase assay. We purified centrosomes by sucrose gradient centrifugation and performed western blotting analysis with

γ-tubulin antibody to confirm the fraction/s enriched in centrosome. Centrosomes concentrated in the fraction number 10, a portion containing 48%-60% sucrose after the final centrifugation (Figure 15A). γ-tubulin signals in Fractions 20-24 indicated cytosolic γ-tubulin (Meigs & Kaplan 2008; Reber 2011).

Furthermore, CDK4 was immunoprecipitated from NIH3T3 cells and utilized in a kinase assay with the isolate centrosome fraction as substrate. pRb peptide was utilized as the positive control of CDK4 kinase activity (Figure 15B). Through this experiment, we had confidence that there were some substrates in the centrosome enriched-fraction, because some bands were distinct compared with equivalent band in the control and same sizes to bands in CDK4 immunoprecipitated sample from NIH3T3 lysate that distinctly visible compared with control IgG sample (Data is not shown). Then, we decided to analyze four areas in the sample line including 100~120kDa, 55~60kDa, 45~50kDa, and 33~38kDa (Figure 15C).

To identify CDK4 substrates, we categorized 550 of proteins from Mass spectrometry analysis through their subcellular location. Furthermore, we screened the Phosphosite Plus® and Scansite3 databases to determine whether these proteins have at least one phosphorylation sites with CDK consensus site. At the same time, we ascertained the relativeness of the screened proteins with centrosome through published papers. Our final screen revealed 21 putative centrosome relative substrates including CLASP1, Cytoplasmic dynein 1 (DYNC1I2 and DYNC1H1), Dynactin, Centrosomin, Myristoylated alanine-rich C-kinase substrate (MARCKS), and Myb-binding protein 1A (Mybbp1a) (Table 2).

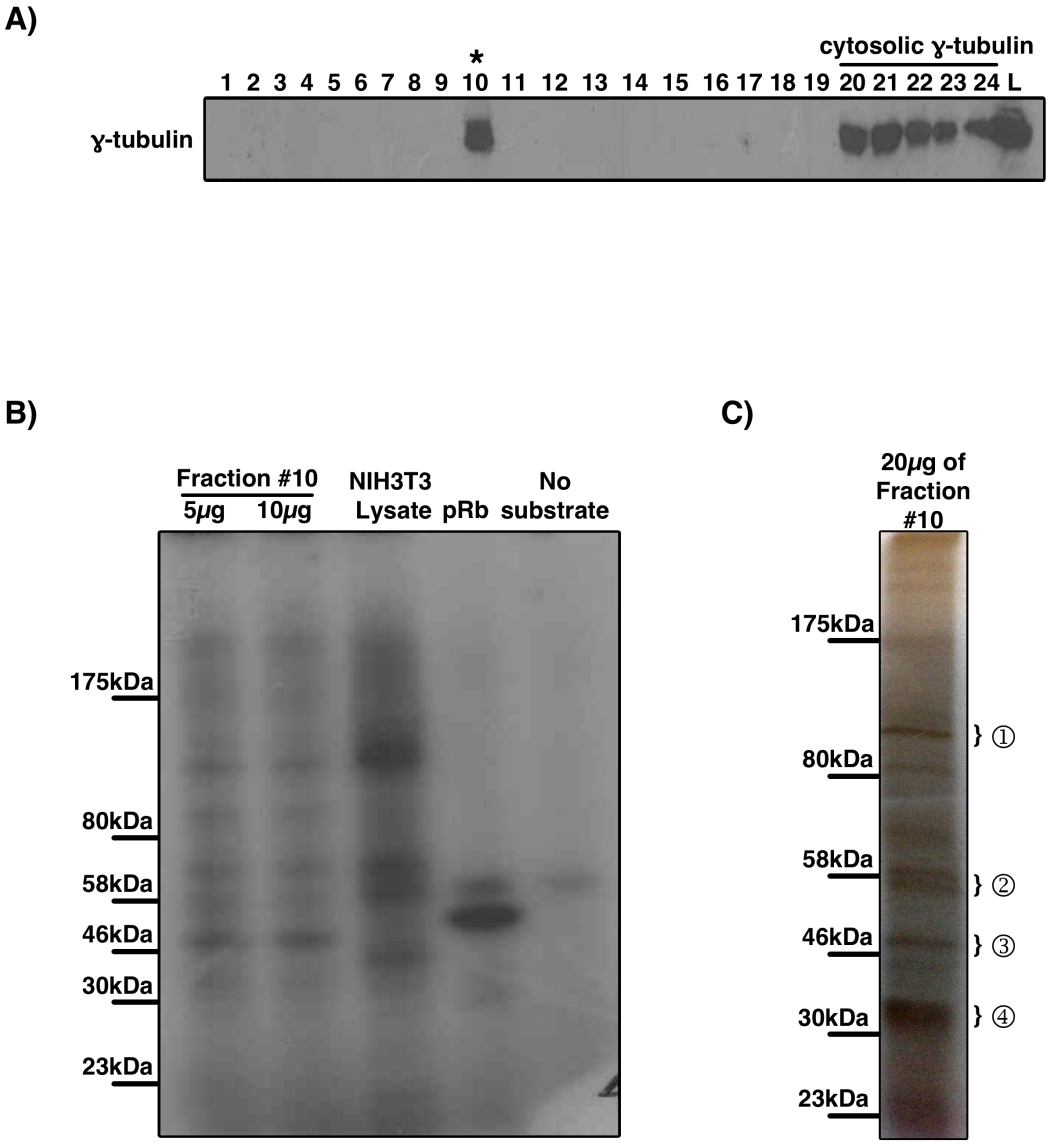


Figure 15. Centrosome purification and CDK4-kinase assay. (A) Centrosome fraction was isolated from nocodazole-arrested mitotic cells by using sucrose-density centrifugation. Centrosome-positive fraction was determined by western blotting analysis with γ-tubulin antibody. Whole lysate of NIH3T3 cells was used as positive control (L). (B) In vitro CDK4-phosphorylation assay using protein preparation of fraction 10. Protein lysate of NIH3T3, and a pRb peptide were utilized as CDK4 substrates. No substrate indicates the background of kinase assay. (C) Silver staining of fraction 10. Four areas were selected for further proteomic analysis.

Table 2. The list of CDK4 substrate candidates relating with centrosome function

Sequence Id	*Protein Name*	*Protein Weight (kDa)*
sp|Q7TPV4|MBB1A_MOUSE	Myb-binding protein 1A OS=Mus musculus GN=Mybbp1a	151.9
sp|P26645|MARCS_MOUSE	Myristoylated alanine-rich C-kinase substrate OS=Mus musculus GN=Marcks	29.6
sp|Q61532|MK06_MOUSE	Mitogen-activated protein kinase 6 OS=Mus musculus GN=Mapk6	82.1
sp|Q8K298|ANLN_MOUSE	Actin-binding protein anillin OS=Mus musculus GN=Anln	122.6
sp|Q80TV8|CLAP1_MOUSE	CLIP-associating protein 1 OS=Mus musculus GN=Clasp1	169.1
sp|Q6PDL0|DC1L2_MOUSE	Cytoplasmic dynein 1 light intermediate chain 2 OS=Mus musculus GN=Dync1li2	54.1
sp|Q9JHU4|DYHC1_MOUSE	Cytoplasmic dynein 1 heavy chain 1 OS=Mus musculus GN=Dync1h1	531.6
sp|O08539|BIN1_MOUSE	Myc box-dependent-interacting protein 1 OS=Mus musculus GN=Bin1	64.4
sp|P09405|NUCL_MOUSE	Nucleolin OS=Mus musculus GN=Ncl	76.6
sp|Q9QXS1|PLEC_MOUSE	Plectin OS=Mus musculus GN=Plec	533.8
sp|P08553|NFM_MOUSE	Neurofilament medium polypeptide OS=Mus musculus GN=Nefm	95.8
sp|Q61595|KTN1_MOUSE	Kinectin OS=Mus musculus GN=Ktn1	152.4
sp|P11276|FINC_MOUSE	Fibronectin OS=Mus musculus GN=Fn1	272.3
sp|O70318|E41L2_MOUSE	Band 4.1-like protein 2 OS=Mus musculus GN=Epb41l2	109.8
sp|O08788|DCTN1_MOUSE	Dynactin subunit 1 OS=Mus musculus GN=Dctn1	141.5
sp|Q3ULF4|SPG7_MOUSE	Paraplegin OS=Mus musculus GN=Spg7	85.9
sp|Q8BTM8|FLNA_MOUSE	Filamin-A OS=Mus musculus GN=Flna	281.0
sp|Q8K2Z4|CND1_MOUSE	Condensin complex subunit 1 OS=Mus musculus GN=Ncapd2	155.5
sp|P70335|ROCK1_MOUSE	Rho-associated protein kinase 1 OS=Mus musculus GN=Rock1	158.0
sp|P23116|EIF3A_MOUSE	Eukaryotic translation initiation factor 3 subunit A OS=Mus musculus GN=Eif3a	161.8
sp|Q920A5|RISC_MOUSE	Retinoid-inducible serine carboxypeptidase OS=Mus musculus GN=Scpep1	50.9

4. Discussion

Investigations performed in the last two decades have shown that CDK4/6 behaves as oncogenic drivers in various human and experimental neoplasia. Thus, most of the studies of CDK4 focused on its proliferative role, via suppression of pRb, as the primary determinant of the oncogenic properties. However, recent researches have also shown that CDK4/6 has pRb-independent mechanisms such as blockage of TGF-β growth inhibition signaling (Matsuura et al. 2004; Matsuzaki et al. 2009) or activation of FOXM1signaling involved in cell cycle progression and suppression of senescence (Anders et al. 2011). Our laboratory also suggested that CDK4 plays a key role in malignant progression during skin cancer tumorigenesis, but it does not bear on pRb pathway (Macias et al. 2008; Macias et al. 2007; Miliani de Marval et al. 2003; Wang et al. 2011).

In this chapter, we focused on centrosome amplification as a driver for malignant progression. Several studies have suggested a correlation between CDK4 and centrosome amplification (Harrison Pitner & Saavedra 2013; Chen et al. 2002; Zeng et al. 2010; Adon et al. 2010), but despite these achievements, its working mechanisms have not been established. Thus, the aim of our study was to test whether CDK4 overexpression triggers centrosome amplification and to define the centrosome related substrates contributing centrosome abnormality. Our study demonstrated that the forced expression of CDK4 leads to intensify centrosome amplification in mouse skin carcinogenesis. Furthermore, we suggested 21 putative centrosome relative substrates for CDK4 via mass spectrometry analysis of putative substrates of CDK4.

Among candidates on our list, 9 proteins are involved in cytoskeletal filament organization, assembly or cross-linkage: Plectin, MARCKS, Anillin, EPB41L2, CLASP1, Neurofilament Medium Polypeptide, Filamin-A, ROCK1 and Eukaryotic translation initiation factor 3 subunit A (alternative name: Centrosomin (Cnn)). It also contains motor proteins such as components of cytoplasmic Dynein 1 (DNCLI2, DNCLI1, and Cytoplasmic dynein 1 heavy chain 1) and Dynactin 1.

- One of the interesting candidates is cytoplasmic Dynein-Dynactin complex because they mediate transportation of variable proteins to centrosome or spindle pore, and plays a significant role in mitosis. For example, nuclear mitotic apparatus protein (NuMA) that is essential for the organization and stabilization of spindle poles, associates with Arp-1, a subunit of the Dynein/Dynactin complex and moves to the spindle poles. In the case of Dynein dysfunction, accumulation of NuMa in the spindle pole is strongly inhibited, resulting in disassembled of the spindle pole (Merdes et al. 2000; Gaglio et al. 1996). In addition, there are evidences that cytoplasmic Dynein recruits integral components of pericentriolar material (PCM), Pericentrin and PCM-1 to centrosome. Pericentrin is transferred by direct binding to Dynein light intermediate chain, and it serves as a scaffold protein for connecting various centrosome proteins including γ-Tubulin and PCM-1 (Tynan et al. 2000; Doxsey et al. 1994; Li et al. 2001; Zimmerman et al. 2004). Therefore, ablation of Pericentrin in cells induces mitotic spindle disorganization, chromosome misalignment and aneuploidy (Rauch et al. 2008). In case of PCM-1, it indirectly associates with Dynein motor through Huntington-associated protein (HAP1) or

Pericentrin, but the detailed mechanism is still unknown (Engelender et al. 1997; Flory et al. 2000). Moreover, cytoplasmic Dynein localizes on kinetochores that play important roles in mitotic chromosome alignment, and deeply engages in microtubule attachment and the spindle assembly checkpoint with several candidate binding partners including Dynactin, Zw10, Lissencephaly-1 (LIS-1), nuclear distribution protein E/C (NudE/NudC) and Spindly (Bader & Vaughan 2010). The post-translational modification on Dynein intermediate chain by CDK1 is necessary to regulate mitotic spindle assembly (Huang et al. 1999; Sivaram et al. 2009). Unfortunately, effect of Dynein/Dynactin phosphorylation by CDK4 in spindle poles is still unknown, but we anticipated that overexpressed CDK4 would lead to improper phosphorylation of Dynein/Dynactin triggering misregulation in centrosome.

- We are also interested in CLASP1, a centrosome associated protein that involving in kinetochore-microtubule attachment (Maiato et al. 2003; Pereira et al. 2006; Mimori-Kiyosue et al. 2006). In addition, CLASP1/2 plays independent roles of kinetochore-microtubule attachments on maintaining a spindle pole integrity. If CLASPs functions are disturbed, it leads to monopolarity, short spindles, multipolarity and aneuploidy (Logarinho et al. 2012). CLASP1 has multi-phosphorylation sites by CDKs, but phosphorylation effects or mechanisms are still unknown. However, a recent study has revealed that CLASP2, an alternative splicing form of CLASP1, requires multisite phosphorylation by CDK1 to succeed in phosphorylation of CSK3β motifs, and these phosphorylations on CLASP2 completely disrupts CLASP2 plus-end tracking during mitosis (Kumar et al. 2012). According to this study, we

expected hyper- or inappropriate phosphorylation by overexpressed CDK4 would interrupt CLASP1 function related to centrosome or spindle pole assembly.

Although our results suggested several candidates phosphorylated by CDK4, our analysis has a limitation. We screened the protein list with CDK consensus sequence (S/T*-P-X-R/K) (Anders et al. 2011). This motif applies typically to all members of the CDK family, not specific for CDK4. Unlike other CDKs target a broad range of substrates, CDK4/6 are fastidious about substrate selectivity (Choi & Anders 2014). Even, we do not know whether different complexes between CDK4 and the three D-type cyclins phosphorylate the same targets or distinct substrates. Thus, further studies will be necessary to validate actual substrates for CDK4 in centrosome abnormality during tumorigenesis.

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CHAPTER 4

CDK4 regulates the mitosis-related gene Aurora B kinase at transcriptional levels in mouse epidermal keratinocyte cell line.

Abstract

Misregulation of cyclin-dependent kinases (CDKs) mediates cell cycle defects, acquiring key features of cancer cells such as unscheduled proliferation, genomic instability (GIN) and chromosome instability (CIN). Especially, in the chapter 3, we showed that overexpressed CDK4 intensified centrosome amplification, leading to CIN in chemically induced skin tumors. In order to address the molecular mechanism in which CDK4 involves, we hypothesized that CDK4 plays a role regulating CIN relative genes through association with chromatin. According to our results, CDK4 associated with chromatin regardless of pRb presence and regulated expression of target genes such as *Cenpp* and *Aurkb*. Thus, we concluded that CDK4 regulates the CIN-related genes at transcriptional levels in mouse epidermal keratinocyte cell line. In particular, the result that only CDK4 overexpressing 308 cells presented centrosome amplification, led us to suggest that the elevated level of Aurora Kinase B in CDK4 overexpressing 308 cells might play a positive role in contributing to centrosome amplification. Therefore, defining the role of CDK4 in regulation of Aurora B kinase expression will provide the better strategy for treatment of skin cancers.

1. Introduction

Chromosomal instability (CIN) resulted from errors in chromosome segregation is a crucial characteristic of tumor and a driving force of malignant tumor progression (Lengauer et al. 1998). Chromosome mis-segregation during mitosis is caused by impairment of spindle assembly checkpoint (SAC), errors in kinetochore-microtubule attachments, centrosome

amplification (CA), and alteration of centromere geometry (Orr & Compton 2013). These mitotic defects leading to CIN deeply relates to activities of Cyclin-dependent kinases (CDKs) that govern cell cycle. For example, clinical analysis of 12 cancer data sets with CIN, revealed that CDK1, cyclin E, A and B were frequently overexpressed and correlated with CIN (Carter et al. 2006). In addition, decreased activity of CDK1 by small molecule inhibitors or upregulation of CDK inhibitors (CKI) such as $p21^{Cip1}$ or $p27^{Kip1}$, interfered with SAC and promoted CIN (Rajagopalan & Lengauer 2004).

In case of the G1-phase CDKs/cyclins complexes (CDK4, 6/cyclin Ds, CDK2/cyclin E complex), alteration in their activities induces centrosome abnormality since they affect licensing and centriole duplication in the centrosome cycle (Harrison Pitner & Saavedra 2013; Chen et al. 2002; Fukasawa 2005; Hanashiro et al. 2008; Harrison et al. 2011; Adon et al. 2010). It is well known that disturbed p53-$p21^{Waf1}$-CDK2/cyclin E pathway during mitosis result in centrosome amplification (CA) (Duensing et al. 2006; Tarapore et al. 2002; Hanashiro et al. 2008; Cheng et al. 2008). When $p21^{Cip1}$ or cyclin E introduced into $p53^{-/-}$ cells with CA, the cell restored normal centrosome duplication cycle (Tarapore et al. 2001; Hanashiro et al. 2008). Absence of CDK2 in $p53^{-/-}$ MEFs also prevented CA and CIN through abrogating phosphorylation of Nucleophosmin (NPM1) (Adon et al. 2010). Additionally, the CDK-canonical function, through inhibition of pRb and activation of E2F, results in centrosome regulation since E2F regulate several centrosome related genes such as Nek2 and Ran BPM known for inducing CA by their overexpression (Ren et al. 2002; Ishida et al. 2001). Moreover, activated CDK2 directly phosphorylates centrosome proteins affecting centrosome regulation (Adon et al. 2010; Tarapore et al. 2002; Okuda et al. 2000;

Chen et al. 2002; Habedanck et al. 2005). For example, CDK2 targeted NPM1, Monopolar spindle 1 (Mps-1), Centriolar coiled-coil protein 110kDa (CP110) and Plk4.

As well as CDK2, it has also been revealed that elevated CDK4 activity also triggers CA. Many studies suggested that upregulated cyclin D1, a catalytic active partner of CDK4, has correlation with chromosome abnormalities in several different mammary tumors and in vitro cell models (Collecchi et al. 2000; Lung et al. 2002; Rennstam et al. 2001). In addition, transient expression of cyclin D1 in normal hepatocytes triggered CA, abnormal mitotic spindle and aneuploidy (Nelsen et al. 2005). The Saavedra's group demonstrated overexpression of cyclin D1 by c-Myc, K-Ras^{G12D} or Her2/Neu triggered CA in breast cancers through increment of Nek2 kinase (Harrison Pitner & Saavedra 2013; Zeng et al. 2010). Nek2 kinase is a downstream target of E2F. Hence, increased CDK4/cyclin D1 activity resulted in abundant expression of Nek2 kinase that regulates centrosome separation contributing to CA. If the expression of cyclin D1, CDK4 or Nek2 was abolished by siRNA, Ras-induced or Her2/Neu-induced CA was abrogated. Controversially, ectopic expression of CDK4 in normal oral keratinocytes is not sufficient to lead centrosome abnormality (Piboonniyom et al. 2003), while, elevated activity of CDK4 can induce CA in mammary epithelial cells through active Ras oncogenic signaling (Zeng et al. 2010). In addition, the Saavedra's group also showed that CDK4 mediate CA in p53-Null mouse embryonic fibroblasts through phosphorylation of NPM, a suppressor of centriole duplication (Okuda et al. 2000; Adon et al. 2010). However, NPM1 is a primary target of CDK2, not CDK4, since phosphorylation of NPM1 is more efficiently carried out by CDK2 than CDK4 (Adon et al.

2010). Despite several studies have been performed in this field, the molecular mechanism in which CDK4 involves, is still poorly understood.

Recently, it was identified a new pRb independent function of cyclin D1 in chromosomal instability. Through genome-wide ChIP sequencing in embryonic fibroblasts from a cyclin D1 knock-in mouse, Pestell's group uncovered that cyclin D1 occupied the regulatory region of genes governing chromosomal stability including *Cenpp*, *Aurkb*, *Ckap2*, *Top2A*, *Zw10* and *Mlf1ip* (Casimiro et al. 2012). Moreover, transcriptional and protein levels of target genes were increased by expression of cyclin D1. In this model, elevated expression of *Aurkb* consequently led to increased phosphorylation of H3S10, a target of Aurora kinas B. They also observed that forced expression of Cyclin D1 in $Ccnd1^{-/-}$ MEFs (Cyclin D1-null) induces chromosomal aberration, centrosome amplification, polyploidy, and aneuploidy. Interesting, transgenic mice overexpressing cyclin D1 in the mammary gland, developed more tumors with elevated CIN profiles. These data indicates that cyclin D1 induces CIN and tumorigenesis through direct regulation of CIN related genes transcription.

Many studies have demonstrated that cyclin D1 plays a significant role in the recruitment of various transcription factors or chromatin remodeling proteins such as SUV39, HP1α, HDAC, and p300/CBP in cortex of local chromatin (M. Fu et al. 2004; Hulit et al. 2004; C. Wang et al. 2003; M. Fu et al. 2005; Bienvenu et al. 2005; Reutens et al. 2001). Relevantly, Casimiro et al also demonstrated that cyclin D1 enriched DNA sequence contained several transcription factor-binding sites including Ctcf, Zfx, Sp1, Mizf, Esr1, ERa, E2f1, Creb1, and Hif1α/Arnt (Casimiro et al. 2012).

Although association of cyclin D1 with chromatin or transcriptional coregulation is independent of CDK-binding domain (C. Wang et al. 2003; M. Fu et al. 2005), the involvement of CDK4/6 in transactivation of CIN related gene has not been determined.

In the chapter 3, we have shown that similar to cyclin D1, forced expression of CDK4 also lead to centrosome amplification (Figure 14). Therefore, *we hypothesize that CDK4 play a role regulating CIN relative genes through association with chromatin*. In order to test our hypothesis, we performed CDK4-ChIP analysis to determine whether CDK4 associates with the regulatory sequences of CIN related genes. We observed that CDK4 associated to with regulatory sequences of *Cenpp*, *Aurkb*, and *Zw10* in pRb independent manner and upregulated expression of CIN relative genes. Increased Aurora B kinase by CDK4 triggered centrosome amplification in mouse skin papilloma 308 cell lines. Overall, our data suggest that overexpression of CDK4 contributes centrosome amplification by directly regulating transcriptional levels of specific genes such as Aurora B kinase.

2. Materials and Methods

2.1. Isolation of Adult keratinocyte and cell lines

Adult epithelial keratinocytes were collected from dorsal skins of 8 weeks wild-type mice as following previously described protocol (de Marval et al. 2014). Briefly, fat and underlying subcutis of hair removed-dorsal skin was carefully scraped off with a razor blade. The skin was cut into several pieces and soaked into cold trypsin solution [0.25 % Trypsin, without EDTA, without phenol red (Sigma-Aldrich Co. LLC, MO)] with the dermis down overnight

at 4°C. After incubation, the dermis side of skin strips was removed by gently scraping and then flipped to an epidermal side to collect keratinocytes. The epidermis was scraped off with adding with adding low Ca2+ media [10% Chelex Fetal bovine serum (FBS (Gemini Bio-products, CA) chelated with analytic grade Chelex 100 resin (Bio-Rad Laboratories, CA), 1x Penicillin-Streptomycin (Mediatech Inc, VA) in EMEM (Gibco®, Thermo Fisher Scientific Inc., IL)] until the shine dermis was exposed. Scraped cells collected into a small beaker filled with 30ml of media, pipetted up and down several times, and stirred slowly for 30min. Media containing keratinocytes was filtered through a 100μM nylon cell strainer (Thermo Fisher Scientific, IL) and 100μM nylon cell strainer sequentially. It was spun down at 1000rpm for 7min, and the supernatant was carefully removed by aspiration. Cells resuspended with low Ca2+ media and plated on 100mm-cell culture dishes laying PLL-coated coverslips (1x107 cells per dish) for immunofluorescence staining.

NIH3T3 (the murine embryonic fibroblast cell line), HeLa (human cervical cancer cell) or Saos-2 (human osteosarcoma cell line) was cultured in Dulbecco's modified Eagle's medium (DMEM, Mediatech Inc, VA) containing 10% of newborn calf serum (NCS, Mediatech Inc, VA) and 1x penicillin-Streptomycin. Keratinocytes cell lines, 308 derived from benign skin papillomas and CH72 derived from malignant SCC grew in Eagle's Minimum Essential Medium (MEM) containing 1% NCS or 4% NCS.

2.2. Retroviral infection and generation of stable cell lines

To generated CDK4 overexpressed cell lines, cDNA encoding murine Cdk4 subcloned into the pLPCX retroviral vector (Clontech Laboratories, Inc., CA) using primers containing NotI

restriction sites. The pCS-CDK4-HA (subcloned murine Cdk4 with a Human influenza hemagglutinin (HA) tag into the pCS retroviral vector) was present from Dr. Chrispino (Muntean et al. 2007). pLPCX-Cdk4 vector was amplified in DH5α competent E.coli cells, and pCS-Cdk4-HA vector was purified from Stbl3 competent E.coli cells (Invitrogen Corp., Thermo Fisher Scientific Inc., IL). Both retroviral vector transiently transfected into the Platinum Retroviral Packaging Cell line (Plat-E cell) with psPAX2 packing vector (Addgene, MA) using FuGENE® 6 Transfection Reagent (Promega Corp., WI). On the day following transfection, the culture medium changed to fresh medium. After 48hrs or 72hrs from transfection, the virus-containing medium was filtered through a through 0.45μm of syringe filter (Corning Inc., NY). Harvested retroviruses infected into targeted cells. 50% diluted the virus-containing media added onto targeted cells (40% confluent) with a final concentration of 4μg/ml of hexadimethrine bromide (polybrene, Sigma-Aldrich Co. LLC, MO) and incubated overnight. Infecting media was removed by aspiration, and replaced with fresh normal media. After 4hrs, infection repeated once more using the same procedure to cells and stayed them overnight. Following a replacement with fresh normal media, cells were grown in normally. The pCS-Cdk4-HA retrovirus stably transfected into NIH3T3 cells (NIH3T3-CDK4) and the pLPCX-Cdk4 retrovirus incorporated in 308 cell line (308-CDK4) selected by 2μg/ml of puromycin (Sigma-Aldrich Co. LLC, MO).

2.3. CSK buffer extraction and Immunofluorescent staining

To visualize chromatin-bound proteins, unbound nuclear proteins, cytosolic and cytoskeletal proteins were removed prior to fixation with cytoskeletal extraction buffer (CSK buffer). 70-

80% confluent cells were grown on poly-L-lysine (Sigma-Aldrich Co. LLC, MO) coated coverslips used for immunofluorescence staining. For CSK extraction, cells were washed with DPBS (Mediatech Inc, VA) twice and incubated with CSK buffer [100mM NaCl, 300mM sucrose, 3mM $MgCl_2$, 10mM PIPES (pH.8), and 0.5% Triton X-100] containing 1x protease inhibitor cocktail (Sigma-Aldrich Co. LLC, MO) for 2 min on ice. After extraction, cells were fixed in 10% formalin for 20min at RT. For in situ DNase extraction, cells were permeabilized with CSK buffer as above, washed with DPBS twice, and treated 10U of Optizyme Recombinant DNase I (Thermo Fisher Scientific Inc., IL) for 30min at 37°C followed by fixation with 10% formalin.

Immunofluorescence staining performed with these processing cells. Cells were blocked with 10% goat serum diluted in 0.01% Triton X-100/PBS solution for 30min at RT, incubated with primary antibodies against CDK4 (C-22) and HDAC1 (10E2) (Santa Cruz Biotech, CA) at 4°C overnight. Cells were washed with PBS six times and placed in fluorescence conjugated secondary antibodies for 2hrs; Goat anti-rabbit-FITC conjugated (Pierce Biotechnology Inc., Thermo Fisher Scientific Inc., IL) for CDK4 staining, and Goat anti-mouse-alexafluor 488 (Molecular Probes, OR) for HDAC1 staining. DNA counterstained with DAPI in mount solution (Vector Laboratories Inc., CA). Stained sections examined under a Nikon Eclips E400 fluorescence microscope (Nikon Corporation, Japan), and images were collected by Qcapture software (QImaging, Canada).

2.4. Fractionation of Chromatin bound protein

To isolate chromatin-bound proteins and analyze by western blot, we utilized CSK extraction. 5 plates of each cell lines were prepared at 80-90% confluence on 10cm culture dishes. Cells were harvested by scraping, gathered in a microcentrifuge tube, spun down at 2000rpm for 3min. Cells incubated in 100µl of cold CSK buffer containing 1x protease inhibitor cocktail (Sigma-Aldrich Co. LLC, MO) on ice for 20min with occasionally tapping and centrifuged at 3600rpm for 3min. The supernatant transferred to a new tube (solution fraction), and the pellet (chromatin fraction) was washed with 200µl of CSK buffer without Triton X-100 twice. The washed pellet was resuspended in 60µl of CSK buffer and divided by two; 30µl chromatin fraction sample for western blot and 30µl chromatin fraction for DNase I treatment. 6U of DNase I added in the chromatin fraction, incubated at 37°C for 30min, and spun down at 12000rpm for 10min. The supernatant (proteins released from chromatin) transferred into a new tube. These processes were illustrated in Figure 17A. The pellet (debris) was washed CSK buffer twice. For western blot analysis, 5µl of 6xSDS loading buffer added in 25µl of each sample. The chromatin fraction and the debris sonicated with a Brason sonifer 450 for 2 sets of 10-second pulses at setting 2 before loading on SDS-PAGE.

2.5. Western blot analysis

Cell lines were lysed in RIPA buffer [150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris-HCl (pH8.0)] containing 1x protease inhibitor cocktail. The protein concentration measured with the DC™ Protein Assay system (Bio-Rad laboratories, CA), and the same amount of proteins loaded on 10% SDS-PAGE gel (25µg for detection of

CDK4 and Aurora B, and 150μg for Cenpp). The membrane was blocked for 30min with 5% nonfat milk in 0.1% Tween20-TBS buffer followed by overnight incubation with the primary antibodies; CDK4 (C-22), β-actin (I-19) (Santa Cruz Biotech, CA), Cenpp (PA5-31186), and Aurora B (MA5-17226) (Pierce Biotechnology Inc., Thermo Fisher Scientific Inc., IL). Membranes were washed and incubated with goat anti-rabbit-HRP secondary antibodies (sc-2004), donkey anti-goat-HRP secondary antibodies (sc-2020) or goat anti-mouse-HRP secondary antibody (#31430).

2.6. Co-immunoprecipitation

Proteins released from chromatin were prepared using 10 plates of CH72 cell lines following the same procedure described above. After DNase I treatment and centrifugation, the supernatant was diluted in 1ml of CSK buffer and pre-cleared by 50ul of Sepharose CL4B bead (Sigma-Aldrich Co. LLC, MO) for 30 min with rotation. After spinning down at 3000rpm, the supernatant was divided into two tubes containing 20μl of agarose conjugated CDK4 (C-22 AC, Santa Cruz Biotech, CA) antibody or agarose conjugated normal rabbit IgG (sc-2345, Santa Cruz Biotech, CA) and incubated at 4°C for 2 hrs with constant rotation. Samples were spun down at 3000rpm, washed three times with CSK buffer and resuspended in 2x SDS loading buffer to perform western blot and silver staining.

2.7. Silver staining

Co-immunoprecipitated proteins were identified by silver staining with Pierce® Silver Stain Kit according to the manufacturer's instructions. SDS-PAGE gel fixed in 30% ethanol: 10%

acetic acid solution, washed with ultrapure water twice and sensitized with incubation in sensitizer working solution. After washing with water, the gel was stained with silver nitrate (AgNO3) for 30min, washed with water shortly, and developed until bands appeared. Reaction stopped with 5% acetic acid solution.

2.8. ChIP Assay

We utilized the SimpleChIP® Enzymatic chromatin IP kit (Cell Signaling Technology, Inc., MA). In brief, approximately $4x10^7$ cells of NIH3T3 or NIH3T3-CDK4 were prepared for ChIP assay. Cell culture media replaced with 10ml of fresh media containing 1% formaldehyde to crosslink proteins to DNA and incubation for 10min at RT on the rocker. This reaction was stopped by addition of glycine, a 0.125M final concentration in media. Cells washed with PBS, scraped, collected into one conical tube, and centrifuged at 1500rpm. Chromatin was released by adding lysis buffer containing DTT, protease inhibitors, and PMSF and fragmented by partial digestion with micrococcal nuclease to obtain chromatin fragments of 1 to 5 nucleosomes in size. Nuclei were pelleted by centrifugation at 13000rpm and resuspended in 1x ChIP buffer containing SDS, protease inhibitor, and PMSF. Pellet was sonicated (3 sets of 20-second pulses at setting 2, a Brason sonifer 450) to break nuclear membrane and clarified by centrifugation at 10000rpm for 10min, and then the supernatant was kept in -80°C for further use. To analyze chromatin digestion and concentration, RNAs and proteins in 50µl chromatin sample were digested by mixed with 2µl RNase A at 37°C for 30min and then, 2µl Proteinase K at 65°C for 2hrs. DNAs were purified using spin columns enclosed in the kit. After purification, 10µl sample was used to electrophoresis on 1%

agarose gel to check fragmentation and DNA should be digested in approximately 50-900bp. In addition, DNA concentration was measured with a NanoDrop 1000 (Thermo Fisher Scientific Inc., IL).

10µg chromatin DNA diluted in 500µl of 1x ChIP buffer containing protease inhibitor cocktail used for each immunoprecipitation. 2µg of each rabbit antibodies against CDK4 (C-22), normal IgG (New England Biolabs Ltd, UK) or Histone H3 (D2B12) (New England Biolabs Ltd, UK) added in DNA samples and incubated overnight at 4°C with rotation. Subsequently, 30µl of ChIP-grade protein G agarose beads (Cell Signaling Technology, Inc., MA) added in the ChIP sample and incubated for 2hrs at 4°C followed by centrifugation at 6000rpm for 1min. The supernatant transferred to a new tube and processed immunoprecipitation repeatedly (total 3 times, Figure 19A). The agarose pellet was washed with 1x ChIP buffer three times and 1x ChIP buffer with high salt, adding extra 350mM NaCl. Chromatin was eluted from antibody/protein G bead through 30min incubations at 65°C with gently vortexing, separated by centrifugation at 6000rpm for 1min and transferred to a new tube. RNA and proteins in Eluted DNA were removed through RNase A and proteinase K treatment, and DNA was purified through the spin column as mentioned above.

Standard PCR was performed using 50ng DNAs of each sample and 2% input, specific primers for genes of *Cenpp*, *Aurkb*, *Capk2*, *Zw10*, *Top2a*, and *Mlf1ip* reported elsewhere (Casimiro et al. 2012) (Table 3.) with KAPA2G Fast PCR kit (KAPA Biosystems, MA) by initial denaturation at 95°C for 5min, followed by 34 cycles of amplification: denaturation at 95°C for 30sec, annealing at 50°C (*Cenpp*, *Aurkb*), 56.8°C (*Mlf1ip*) and 62°C (*Zw10*, *Top2a*) for 30sec, extension at 72°C for 30sec, and a final extension at 72°C for 5min.

Table 3. List of primers for ChIP

Genes	Sequence
Ckap2	Forward ACAAAGCTCTCCCAACTGGA Reverse TCCAAAGATCATTCGGGAAC
Mlf1ip	Forward AAAGGCAGGGACTCCAAACT Reverse AGGCCGGGGAGTTCTAAAT
Zw10	Forward GGGAGGGCCATAAAGGATTA Reverse GCGTCTAGAAGGCACCAAAG
Cenpp	Forward CTTGAACCGGAAATCAGGAA Reverse GGCAAGTGCATTTTCCTTTC
Aurkb	Forward GACGGGGAGAAGTGCTTTTT Reverse CCCTGCAAGGATTTCTTCTG
Top2A	Forward ATCACCGACTCGCTCTCATT Reverse GCACATGGACCTTCCTCATT

2.9. siRNA treatment

siRNAs were transfected into cells with Lipofectamine® RNAiMAX Reagent (Invitrogen, Life Technologies, CA) according to the manufacturer's instructions. 308 or 308-CDK4 Cells cultured in 60mm cell culture dishes at 70-80% of confluence. We utilized commercial available CDK4 specific siRNA (sc-29262) and control siRNA (sc-37007) (Santa Cruz Biotech, CA). In brief, 60pmole of siRNA was diluted in 300µl Opti-MEM® (Invitrogen, Life Technologies, CA), mixed with 18µl lipofectamine in 300µl Opti-MEM®, and incubated for 5min at RT. The mixture was added to the cells in 4ml of culture media without antibiotics. To reduce apoptosis, media was replaced with fresh media after 24hrs, and cells were harvested at 96 hrs after transfection began.

RNA was isolated using TRIzol® Reagent (Ambion, Life Technologies, CA). Cell culture media removed and 1ml TRIzol® Reagent directly added to the cells followed by transferred

to a new tube. After the incubation for 5min, 0.2ml chloroform was added, vortexed vigorously, and stayed for 3min at RT. The mixture was separated by centrifugation at 12000rpm for 15min at 4°C into a lower red phenol phase, an interphase, and an upper aqueous phase. About 500µl of the aqueous phase containing RNA placed into a new tube, 0.5ml of isopropanol was added to the aqueous phase, and incubated for 10min at RT subsequently. After that, the sample was spun down at 12000rpm for 10min and the pellet was washed with 1ml of 75% ethanol twice. Dried RNA pellet resuspended in 40µl of 0.1% Diethylpyrocarbonate (DEPC)-DW and measured concentration with NanoDrop 1000.

For western blot analysis, cells culture dishes were washed with PBS twice, and PBS was aspirated as much as we can. 30µl of RIPA buffer containing 1x protease inhibitor cocktails directly added to cells scraped and transferred to a new tube. Protein lysate was sonicated with 2 sets of 6-second pulses and diluted with SDS sample loading buffer. Procedures of SDS-PAGE and western blot described above.

2.10. Quantitative PCR

Synthesis of cDNA with total RNA was performed using an iScript cDNA synthesis kit (Bio-Rad laboratories, CA). 2µg total RNA mixed with the reverse transcriptase H in reaction buffer and incubated following procedures: 5min at 25°C, 30min at 42°C, and 5min at 85.

The iQTM SYBR® Green Supermix (Bio-Rad laboratories, CA) was used for quantitative real-time PCR. Same primers listed in Casimiro et al. 2012 used for this analysis and mouse GAPDH selected as the reference gene (Table 4.). PCR amplification performed with a 20µl reaction mixture containing 2µl of cDNA, 300nM of each primer, and final 1x iQTM

SYBR® Green supermix (Bio-Rad laboratories, CA). The PCR condition was as follows: initial denaturation at 95°C for 3min, followed by 40 cycles of denaturation at 95°C for 15sec, annealing and extension at 60°C for 30sec. Transcriptional level of target genes was normalized by transcriptional level of GAPDH and it compared with control siRNA treated 308 cells or normal cell line not overexpressing CDK4 (NIH3T3 or 308) according to the algorithms $2^{-(\Delta\Delta Ct)}$, respectively.

Table 4. List of primers for qPCR

Genes	Sequence
Ckap2	Forward ATTAAGCGATGGCAGAGTCC Reverse TTTCTTTGTTCTCGGAAGGC
Zw10	Forward GAAGTGCCAGGATGTGATTG Reverse AGCTTGTGATCAGCATCAGG
Cenpp	Forward CATGGAGATCCGCAGTACC Reverse CATCCCTTCCTCATCGATTT
Aurkb	Forward CCCAGAGAGTCCTACGGAAG Reverse TGTTCTCAGCCAACTTCTGG
Top2A	Forward ATCACCGACTCGCTCTCATT Reverse GCACATGGACCTTCCTCATT

2.11. Centrosome immunofluorescent staining

Cultured cells were grown on the poly-L-lysin coated coverslips to 80% confluence, and they were washed twice with PBS followed by fixation using 100% methanol at -20°C overnight. The samples moved into 24-wells, washed with PBS for 5min, and incubated in blocking solution [10% Goat serum in 0.01% Triton X-100/PBS] at RT for 1hr. 1:500 of rabbit-anti-γ-tubulin polyclonal antibody (T5192, Sigma-Aldrich Co. LLC, MO) was incubated overnight

to stain centrosomes, and washed 6 times with 0.01% Triton X-100/PBS on the rocker. 1:1000 of Alexafluor 488 goat-anti-rabbit secondary antibody (Molecular Probes, OR) used followed by washing 6 times. The nucleus was counterstained with DAPI incorporated in the mount solution (Vector Laboratories Inc., CA). For the analysis of centrosome alteration, the percentage of cells with 1, 2, or more than 2 (≥3) centrosomes were calculated with at least 200 cells from three independent experiments.

2.12. *In silico* Analysis of gene regulatory region

To anticipate the putative phosphorylation targets of CDK4 that affect transcriptional levels of genes, the promoter regions of *Cenpp*, *Aurkb*, *Capk2*, *Zw10*, and *Top2a* were analyzed on a basis of information from published papers, (Casimiro et al. 2012) and (Anders et al. 2011). First, the sequence of CDK4 bound promoter regions was obtained from http://www.informatics.jax.org by using given primer sequences for ChIP assay. We analyzed the potential transcription factor binding sites using TRANSFAC®, a database of eukaryotic transcription factors (http://www.biobase-international.com/product/transcription-factor-binding-sites) with the default parameters.

3. Results

3.1. CDK4 associates to Chromatin in pRb-independent manner

To investigate whether CDK4 associates with chromatin, immunofluorescence staining was performed with mouse primary keratinocytes and 308 mouse papilloma cell line in which

soluble proteins were previously extracted (Figure 16). As a positive control of chromatin association, we performed immunostaining of histone deacetylase 1 (HDAC1) which normally bind to chromatin. Originally CDK4 localizes to the cytoplasm and is imports into the nucleus with cyclin Ds during G1 phase. After removing soluble proteins of the cytoplasm and nucleus with cytoskeleton (CSK) buffer extraction, we observed that abundant CDK4 still remained associated with cellular structures (cellular ghosts) (Figure 16). In order to determine whether the observed CDK4 binds to chromatin or other cellular structure, we eliminate the cellular DNA with DNAase I. We expect that proteins linked to chromatin will be extracted from the cellular ghosts upon the DNAse I treatment. Successful digestion of chromatin was indicated by loss of DAPI signals and release of chromatin-bound protein-HDAC1. The intensity of the fluorescent of CDK4 drastically reduced when DNase I treatment (Figure 16C, F). This remarkable reduction demonstrates that the CDK4 localized in the nucleus interacts with chromatin in normal epidermal keratinocytes and cancer cells.

Association of CDK4 with chromatin was also confirmed by immunoblotting. Cytosolic and nuclear fractions were isolated using CSK buffer and analyzed by western blots. Western blot analysis revealed that CDK4 protein remained in the insoluble chromatin fraction in CH72 (mouse squamous cancer cell), Hela (human cervical cancer cell), and SaOS-2 (human osteosarcoma cell) cell lines (Figure 17A). Importantly, CDK4 was release from the chromatin fraction upon DNA digestion (Figure 17B). Interestingly, CDK4 interact with chromatin in a pRb-independent manner. According to the current model of cell-cycle regulation, we expected that most of the CDK4 will be associated with the chromatin fraction through pRb, since the primary function of CDK4/cyclin Ds complexes is inhibition of pRb

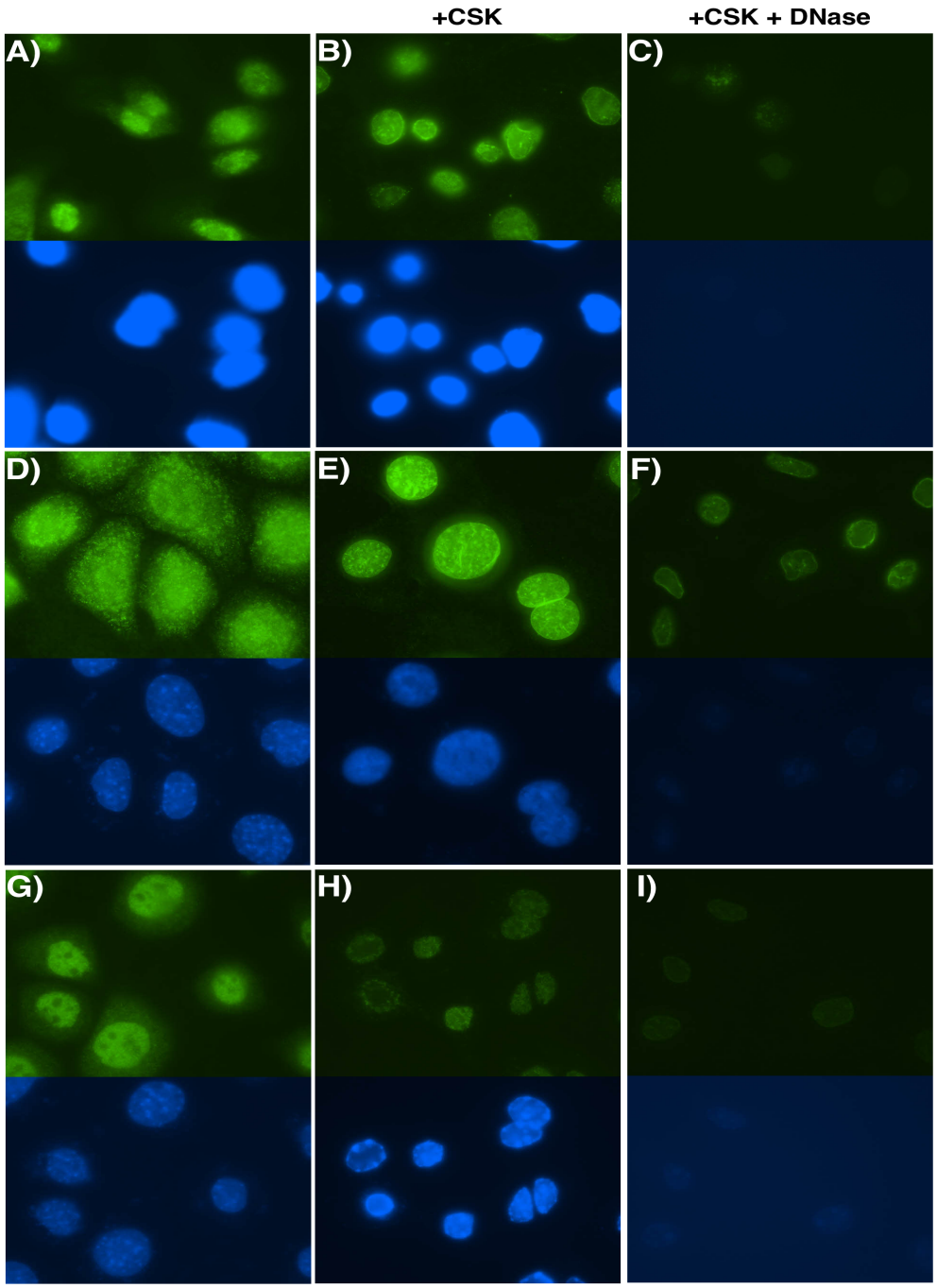

Figure 16. Association of CDK4 to chromatin. Immunofluorescence (IF) analysis of CDK4 (green) in epithelial keratinocytes (A, B, C) and 308 cells (D, E, F). HDAC1 in 308 cells was use as a positive control of chromatin bound proteins (green) (G, H, I). +CSK denote treatment with cytoskeletal extraction buffer (CSK buffer) (B, E, H), while, +CSK +DNase denote incubation of DNase I after CSK buffer treatment (C, F, I). DAPI was used as nuclear counterstain (blue).

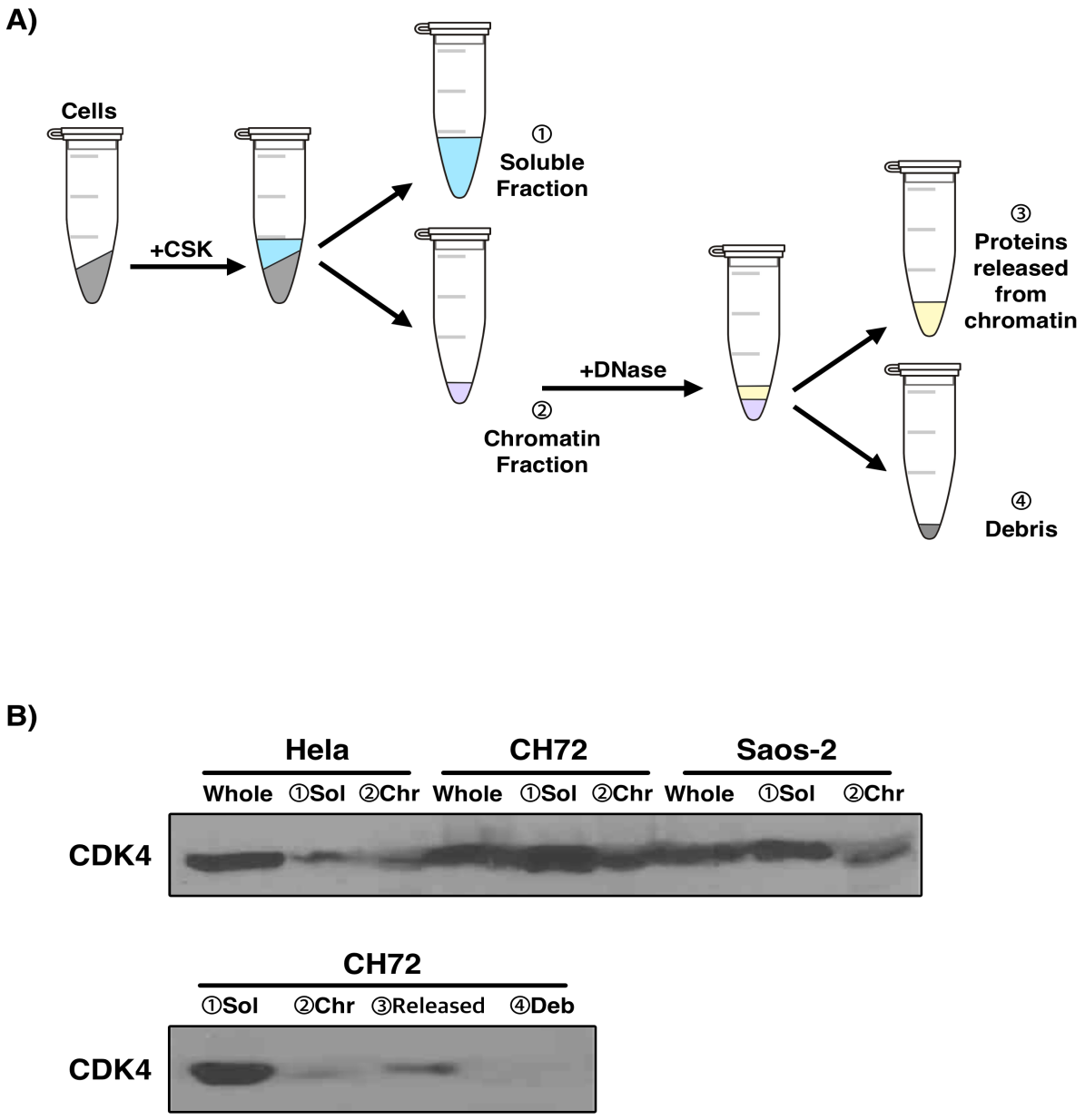

Figure 17. Interaction of CDK4 with the chromatin fraction of mouse and human cell lines. (A) Scheme of CSK extraction. Each fraction denotes ① soluble proteins of cytoplasm or nucleus, ② chromatin bound proteins, ③ chromatin bound proteins released from chromatin and ④ cell debris containing cytoskeletons. (B) Soluble, chromatin, and released protein fractions of Hela, Saos-2 and CH72 cells were analyzed by western blotting with anti-CDK4 antibody. Whole lysate of each cell line was used as positive control (Whole).

through phosphorylation. However, Hela cells (with an inactive form of pRb) and Saos-2 (with a truncated form of pRb) showed that CDK4 bind to the chromatin fraction (Figure 17B) (Shew et al. 1990). Thus, existence of chromatin-bound CDK4 in Hela and Saos-2 cells implies that its association is not mediated through the canonical role of CDK4/cyclin Ds complexes. Based on these data, we conclude that a considerable proportion of CDK4 associates with chromatins in pRb-independent manner.

3.2. CDK4 localize in the regulatory sites of genes associated with chromosomal stability.

Dr.Pestell's group has shown that cyclin D1 bind and regulate CIN related genes at a transcriptional level; however, whether the CDK subunits also involved in this regulation was not reported (Casimiro et al. 2012). Newly synthesized cyclin D1 strongly forms complexes with CDK4/6 and translocate into the nucleus. In addition, free cyclin D1 is relatively unstable and quickly degraded in comparison to cyclin D1 complexed with CDK4 (Germain et al. 2000).

To determine the putative participation of CDK4 regulating CIN genes, we performed ChIP assay with antibodies against CDK4, rabbit IgG (a negative control for detection of nonspecific bound), and histone H3 (a positive control for detection of success of ChIP). We studied the putative association of CDK4 to the regulatory sites of *Cenpp*, *Aurkb*, *Ckap2*, *Zw10*, *Top2A*, and *Mlf1ip* through PCR analysis with set of primers previously described by Casimiro et al. 2012 (Casimiro et al. 2012). Among the tested genes, we observed that CDK4 associated with the regulatory sites of *Cenpp*, *Aurkb*, and *Zw10*, but not with *Capk2, Top2a,* and *Mlf1ip* (Figure 18). This result indicates that CDK4, similar to cyclin D1, also

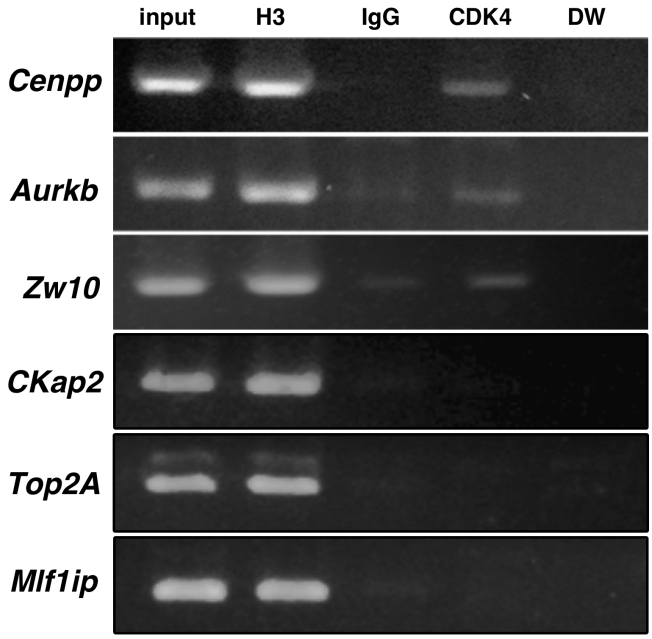

Figure 18. Association of CDK4 to the regulatory sites of CIN related genes. ChIP analysis of NIH3T3 cells were performed with anti-CDK4 antibody (CDK4). Chromatin input (input) and ChIP with anti-H3 antibody (H3) were used as positive controls. ChIP with normal IgG was used as negative control. DW indicated the blank for PCR reaction.

bind to these regulatory regions and suggests that the catalytic activity of CDK4 might affect the expression of target genes.

3.3. Overexpression of CDK4 increases *Cenpp* and *Aurkb* transcription levels and upregulates the proteins levels.

In order to determine whether overexpression of CDK4 lead to increasing binding to the regulatory regions of target genes, we carried out ChIP assay in three consecutive immunoprecipitation (Figure 19A) and compared the intensity of the amplified bands between NIH3T3 and NIH3T3-CDK4 cells. Band intensities quantified with imageJ software, all PCR band intensities from NIH3T3-CDK4 chromatin were higher than intensities from NIH3T3 chromatin: *Cenpp* gene showed, 2.6-, 4.7-, and 9-fold increase in the 1^{st}, 2^{nd}, 3^{rd} ChIP analysis. *Aurkb* gene showed 2.4-, 7.2-fold increase in the 1st and 2nd ChIP analysis, and no PCR signal were detected in third (Figure 19B). This result clearly indicates that overexpressed CDK4 lead to increasing binds to the regulatory regions.

We also asked whether overexpression of CDK4 change the transcriptional levels of these genes. Therefore, we carried out quantitative RT-PCR with SYBR green. The transcriptional level of genes regulated by CDK4 was normalized to the transcriptional level of *Gapdh*, and compared with NIH3T3 cells. Moreover, to determine whether CDK4 regulated these genes in additional cell lines, we also utilized a mouse cell line, 308, derived from mouse skin tumors. Our results demonstrated that CDK4 regulate *Cenpp* and *Aurkb* at transcriptional levels. Forced expression of CDK4 occurred in 1.5- and 2.2-fold increase of *Cenpp* and *Aurkb* in NIH3T3-CDK4 cells ($p=0.02$ for *Cenpp*, $p=0.003$ for *Aurkb*). Similarly,

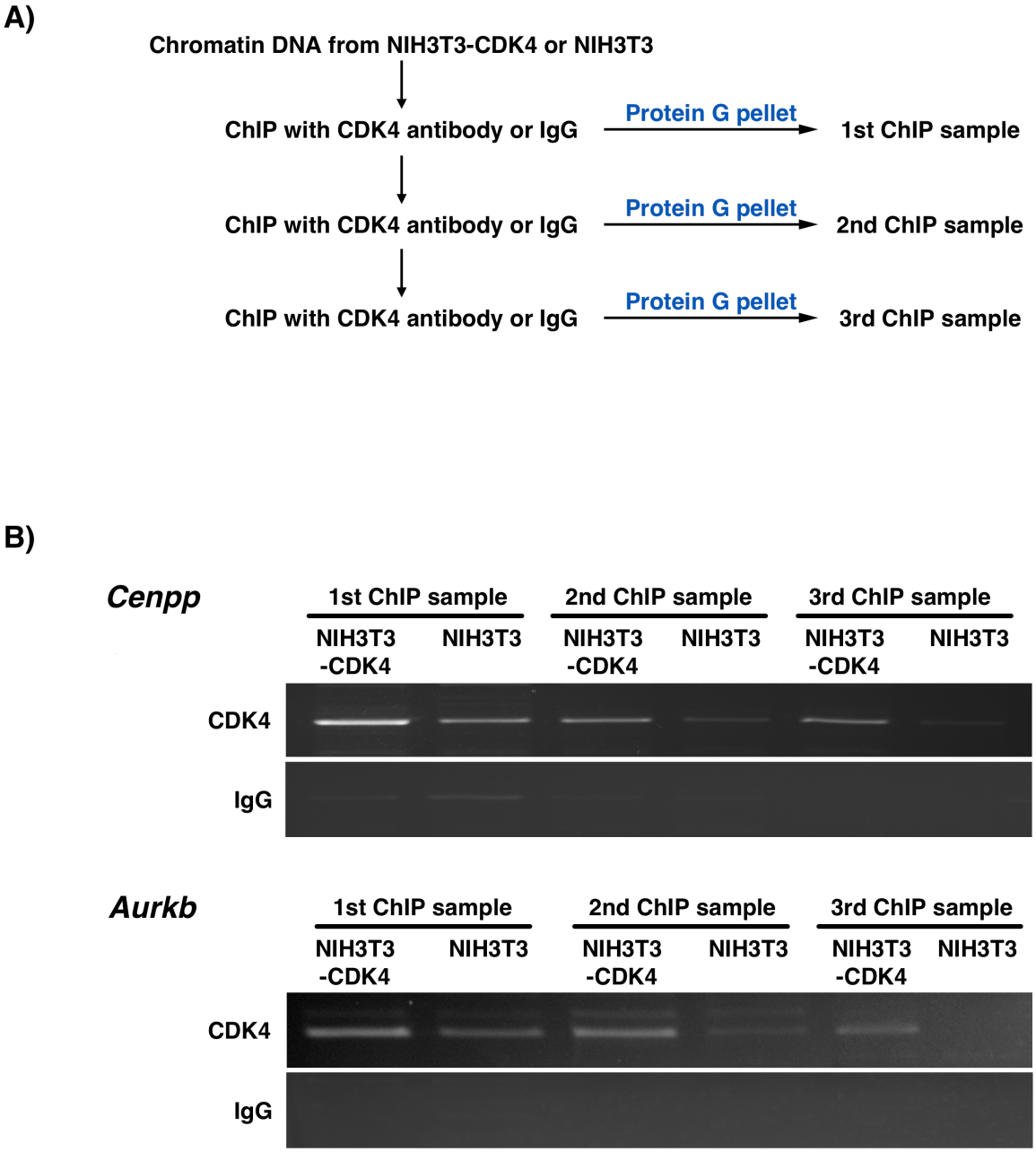

Figure 19. Increased interaction of CDK4 to *Cenpp* and *Aurkb* regulatory regions by forced expression of CDK4. (A) Experimental scheme. (B) ChIP analysis was performed in 3 consecutive immunoprecipitations with anti-CDK4 antibody and chromatin from NIH3T3 or NIH3T3-CDK4. Normal IgG was used as control of ChIP analysis.

we observed 2.2-and 3.5 increase levels of *Cenpp* and *Aurkb* in 308-CDK4 epithelial cells (p=0.004 for *Cenpp*, p=0.006 for *Aurkb*). In the case of *Zw10* gene, its level was not altered in NIH3T3 cells or rather decreased about 0.5 fold in 308 cells by overexpression of CDK4 (p=0.01 in 308-CDK4). The transcriptional level of *Ckap2*, a CDK4 non-associated gene, was 1.3 fold increased in NIH3T3-CDK4 cells (Not significant), but was not changed in 308-CDK4 cells. However, *Top2A* transcriptional level did not show notable changes. (Figure 20A).

In addition, we examined whether transcriptional regulation of these genes also results in elevated protein levels. We analyzed only CENPP and Aurora B kinase since transcriptional changes of these genes were conspicuous and consistent with NIH3T3 and 308 cells. Forced expressed CDK4 in 308 cells resulted in increased protein levels of both CENPP and Aurora B kinase (Figure 20B). CENPP expression level was also increased in NIH3T3-CDK4 cells, whereas, the level of Aurora kinase B showed a little elevation compared to NIH3T3 cells (Figure 20B). Therefore, we conclude that overexpressed CDK4 increases expression of Cenpp and Aurkb via binding to their regulatory regions. Furthermore, elevation of Aurora kinase B in 308-CDK4, but not in NIH3T3-CDK4 cells suggests that this cancer cell line carry additional alterations required to increase Aurora B Kinase. It is worth mentioning that 308 cells, carrying an activating mutation in codon 61 of the Ha-ras gene were generated from chemically induced mouse skin tumors (Balmain et al. 1984).

3.4. Ablation of CDK4 reduces *Cenpp* and *Aurkb* expression levels.

Overexpression of CDK4 increases the transcriptional level and protein levels of CENPP and

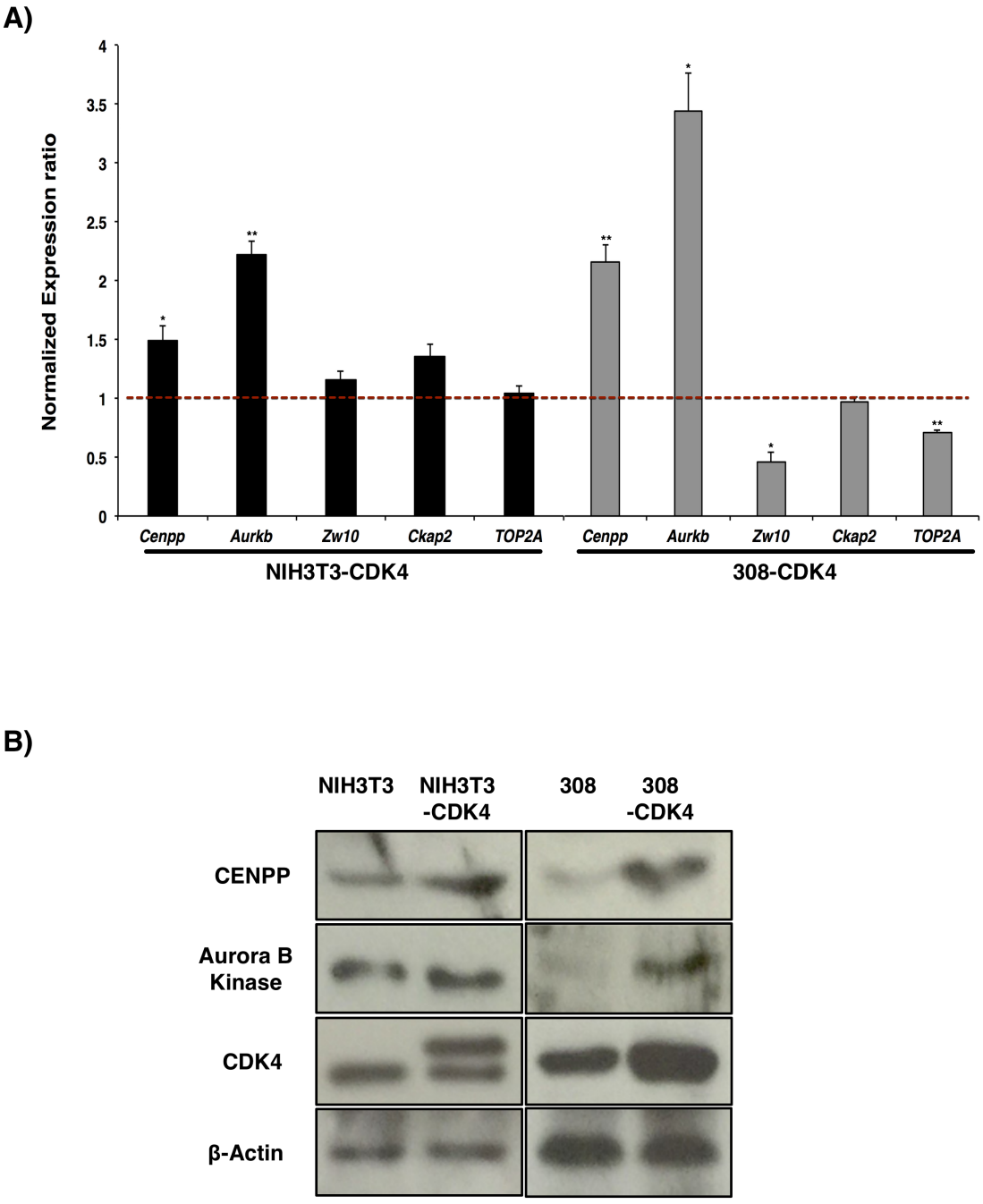

Figure 20. Increased protein levels of CENPP and Aurora kinase B by overexpression of CDK4. (A) Quantitative PCR analysis. The transcriptional levels of genes in CDK4 overexpressing cells are normalized with Gapdh expression. Three independent experiments, Bars; ± Standard Deviation. $P < 0.05$; *, $P < 0.005$; **, t-test (B) Western blot analysis of CENPP and Aurora B kinase. β-Actin is used as loading control.

Aurora kinase B. Thus we expected that knockdown of CDK4 will reduce the level of these genes. Therefore, we utilized CDK4 specific siRNA to down-regulate CDK4 level in 308-CDK4 and 308 cells. This siRNA was not effectively reducing CDK4 level in NIH3T3-CDK4 cell lines, probably caused by a technical problem (Data is not shown).

We performed real-time PCR to quantitate the transcriptional levels of *Aurkb* and *Cenpp* 96 hr after siRNA transfection. Transcriptional levels normalized to the transcriptional level of Gapdh, and the compared with the level of control siRNA transfected 308 cells. From this experiment, we observed that mRNA levels of *Aurkb* and *Cenpp* decreased in both 308-CDK4 and 308 cell lines treated with CDK4 siRNA (Figure 21A). Upon siRNA transfection, the mRNA level of *Cenpp* was reduced 24% in 308 cells (308 CDK4 siRNA) compared with control siRNA (308 cont siRNA) ($p=0.002$, t-test). Importantly, the CDK4 siRNA also reduce (51%) the CDK4 level in 308 cells overexpressing CDK4 (308-CDK4 cdk4 siRNA) compared to control cells (308-CDK4 cont siRNA) ($p=0.043$, t-test). *Aurkb* transcriptional levels were decreased 46% in 308 CDK4 siRNA cells ($p=0.01$, t-test) and 78% in 308-CDK4 cdk4 siRNA cells ($p=0.0005$, t-test) compared to control groups. Therefore, these results support our previous data in which CDK4 regulates gene expression by association with regulatory sequences. Through the western blot analysis, we confirmed that CDK4 protein level was knockdown and consequently the protein level of Aurora kinase B decrease 80% in 308-CDK4 cells (Figure 21B).

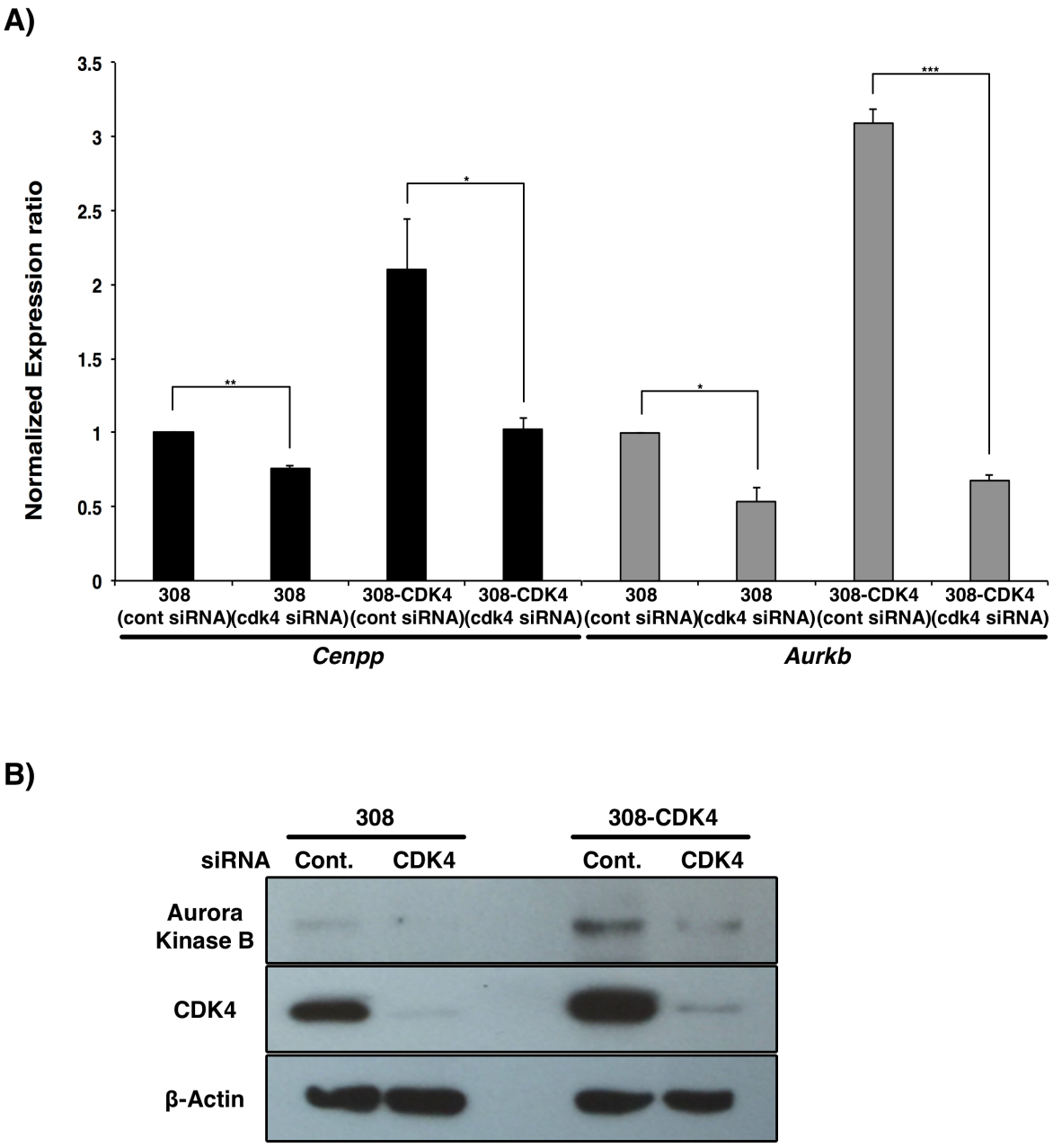

Figure 21. Reduction of CENPP and Aurora kinase B via knockdown of CDK4 by siRNA treatment. (A) Quantitative real-time PCR. Transcriptional levels were normalized with GAPDH level and compared with 308 control siRNA cells. Three independent experiments, Bars; ± Standard Deviation. $P < 0.05$; *, $P < 0.005$; **, $P < 0.0005$; ***, t-test (B) Western blot analysis of CENPP and Aurora B kinase. β-Actin is used as loading control. (B) Western blot analysis of Aurora Kinase B. Reduction of CDK4 and Aurora kinase B detected in both 308 and 308-CDK4 cells. β-Actin used as a loading control.

3.5. Forced expression of CDK4 induces Centrosome amplification in 308 cell lines, but not in NIH3T3 cells.

Both CENPP and Aurora B kinase involve in mitotic progression and chromosomal segregation. Therefore, our next question was whether altered expression of these proteins results in CIN through centrosome amplification.

To determine centrosome abnormality, we performed immunofluorescence staining with γ-tubulin antibody followed by quantification of the frequencies of cells carrying 1, 2, or more than 3 centrosomes in NIH3T3, NIH3T3-CDK4, 308 and 308-CDK4 cells. Interestingly, overexpression of CDK4 did not lead to centrosome amplification in NIH3T3 cell lines; the percentage of NIH3T3-CDK4 cells with more than 3 centrosomes (1.05%) was similar to the percentage of NIH3T3 cells (2.01%). However, the proportion of 308-CDK4 cells with centrosome amplification (9.26%) was 4-fold higher that the percentage of 308 cells (1.96%) ($p=0.0013$, t-test) (Figure 22B).

Our data demonstrated that CDK4 regulated CENPP in both, NIH3T3, and 308 cell lines; whereas Aurora B kinase is upregulated only in 308-CDK4 cells. Therefore, these results suggest that Aurora Kinase B overexpression might play a unique role in CDK4 overexpressing cells contributing to centrosome amplification and likely in malignant progression.

3.6. Prediction of transcription factor binding sites on CDK4-regulated genes

We demonstrated that CDK4 associated to CIN related genes and directly regulated transcription levels of *Cenpp* and *Aurkb*. Our next question is whether CDK4 interacts with

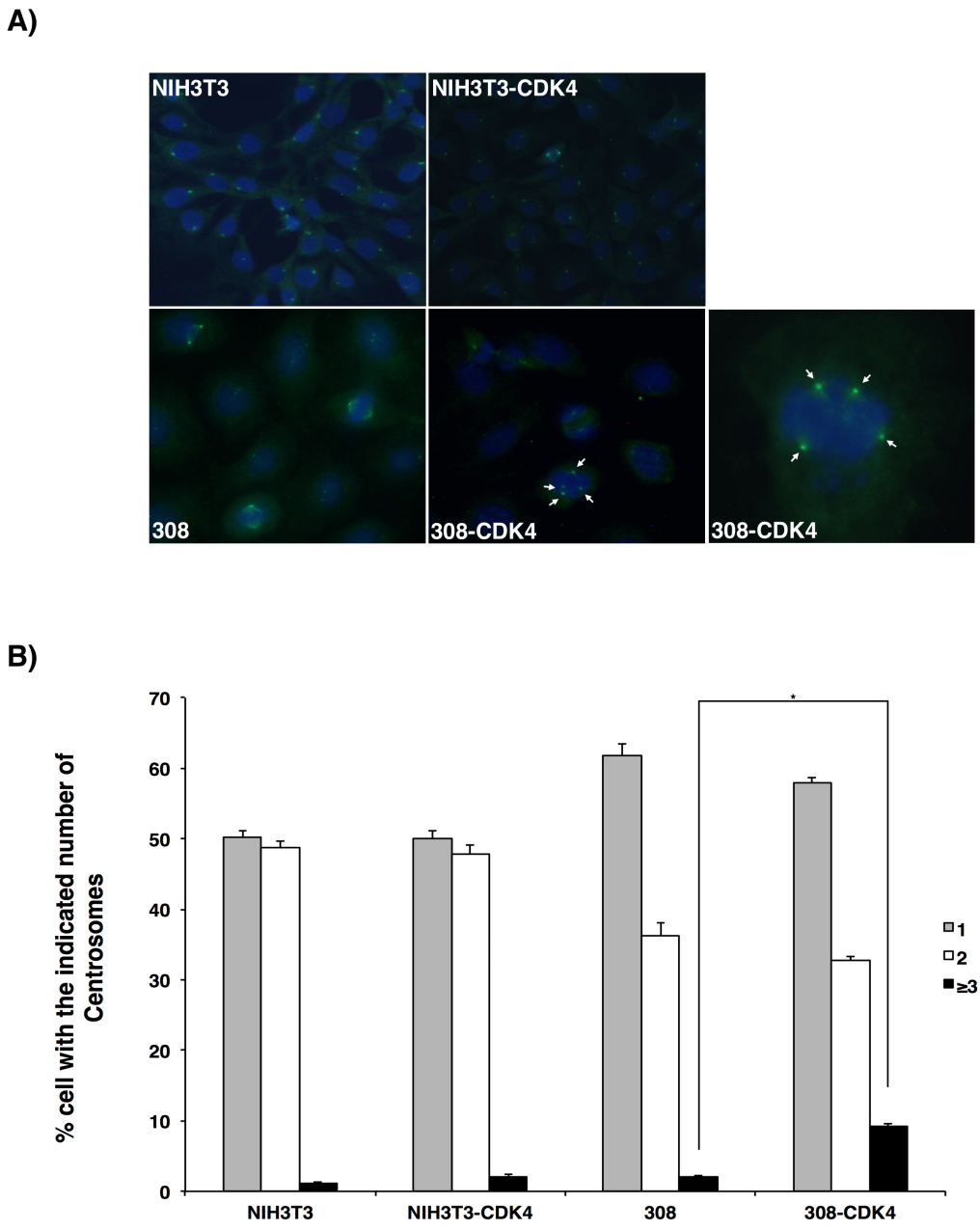

Figure 22. Induction of centrosome abnormality by forced expression of CDK4. (A) γ-tubulin staining denotes centrosome (green dots). Nucleus was visualized with DAPI (Blue). Cells were plated on poly L-lysine-coated cover glass and grow up to 80% confluence. White arrow points supernumerary centrosomes in 308-CDK4 cells. (B) The number of cells with specific centrosome numbers were counted and presented as percentage of the total number of cells counted per field. The number of centrosomes ≥3 represents abnormal centrosome numbers. Bars; ± Standard Deviation. P =0.0013; *, t-test

other transcription factors on chromatin. To determine, chromatin fraction isolated from NIH3T3 cells through treatment of CSK buffer as described in chapter 3 followed by co-immunoprecipitation with CDK4 antibody (Figure 23A). After co-immunoprecipitation, these samples were analyzed by silver staining to figure out whether there are putative binding partners of CDK4 or not. In comparison to bands appeared in the negative control lane, there were several distinct bands in the CDK4 co-IP sample lane (Figure 23B). Especially, three bands of 32kDa, 40kDa and 52kDa were clearly distinguishable. Therefore, we conclude that CDK4 binds to several proteins locating on chromatin. However, this approach does not rule out the possibility that some of this proteins belong to know CDK4-binding proteins such as D-type cyclins (~33 kDa).

In order to investigate whether putative CDK4 substrates on the chromatin are regulatory factors participating in the regulation of *Cenpp* and *Aurkb*, we examined what transcription factors bound to the promoter regions of those genes (-10kbp ~ +1000bp from transcription start site). We performed In silico analysis with the TRANSFAC® database for DNA Transcription Factor Binding Site Prediction (Wingender 1988 Through consensus binding sequence analysis (positional weight matrices), we determined that the approximately 330 putative transcription factor binding sites were present in each promoter region including Zinc finger protein 333 (ZFP333), Krueppel-like factor 6 (Klf6, Cpbp), Nuclear factor of activated T-cells (NF-AT1), Homeobox protein CDX-2 (Cdx-2) and AT-rich interactive domain-containing protein 3A (Arid3a, Dri1). Transcription factor binding motif that commonly appeared on both promoters of *Cenpp* and *Aurkb* were sorted by the frequency on the promoter in descending order (Table 5). In addition, there were several experimentally-

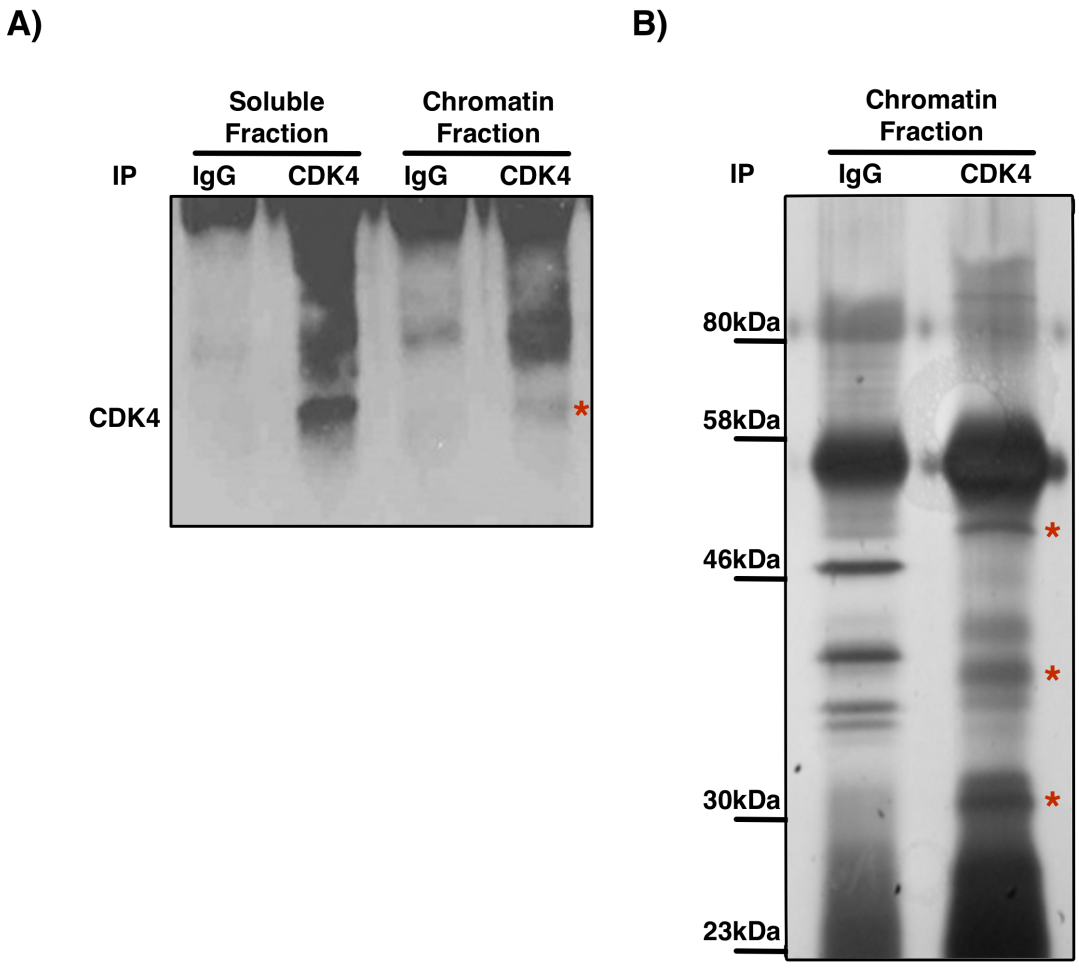

Figure 23. Analysis of transcription factor binding sites on regulatory regions of *Cenpp* and *Aurkb*. (A) Immunoprecipitation using cytoplasmic/nuclear soluble proteins and chromatin bound proteins with anti-CDK4 antibody and normal IgG used as a negative control. CDK4 was detected by western blot (*). (B) ChIP samples with normal IgG and anti-CDK4 antibody stained with silver. (*) Distinct bands compared to IgG IP sample

Table 5. The list of common transcription factors predicted on *Cenpp* and *Aurkb* gene

			Cenpp	*Aurkb*
	Factor	**Classification**	**Sites/Sequence[a)]**	**Sites/Sequence[b)]**
	ZNF333	ZFC2H2	40	25.67
	SRY	HMG	30	27.33
	CPBP	ZFC2H2	11	28.67
	NF-AT1	REL	19	14.67
*	CDX-2	HOX	16	14.33
	DRI1	ARID	9	11.33
	CRX	HOX	9	11.33
	ING4	ZFPHD	6	11.33
	Xvent-1	HOX	12	4.67
	FAC1	ZFPHD	7	7
	HMGIY	ATHOOK	7	5.33
*	Ctcf	CTCF	9	1
*	c-Myc	MYC	7	1.33
*	GKLF	ZFC2H2	4	6.33
*	C/EBPalpha	BZIP	7	3.33
	Nanog	HOX	6	4
	ATF-2	BZIP	6	4
	TATA	TATA	7	2.67
*	E2F	E2F	6	1
*	Esrrb	HOX	8	1
	Freac-3	FORKHEAD	5	4
	SP100 secondary motif	SAND	3	5.33
	BBX secondary motif	HMG	6	2
	POU6F1	HOX	6	1.67
	RUSH-1alpha	ZFRING	5	2.67
	LEF-1	HMG	5	2.67
	BEN	BHLH	2	5.33
	RFX	RFX	4	2.67
	HNF-3beta	FORKHEAD	3	3.67
	Churchill	CHCH	2	4.67
	BRCA1:USF2	ZFRING	5	1.33
	c-Myb	MYB	3	3.33
*	N-Myc	MYC	3	1
*	Brg1	HOX	3	1
	Nkx2.5	HOX	4	2
	Ikaros	ZFC2H2	3	3
	LRH-1	ZFC4-NR	3	3
	Ebox	BHLH	2	4
	NF-1A	SMAD	3	2.33

Table 5. Continued

	Factor	Classification	*Cenpp* Sites/Sequence[a)]	*Aurkb* Sites/Sequence[b)]
	myogenin	BHLH	2	3.33
	GEN_INI	GENINI	2	3.33
*	Zfx	ZFC2H2	3	1
	YY1	ZFC2H2	4	1
	TEF-1	TEA	2	3
	Pit-1	HOX	2	3
	Tbx5	TBX	2	3
	RREB-1	ZFC2H2	3	1.67
	TTF-1	HOX	2	2.67
	CDP CR1	HOX	3	1.5
	ER-alpha	ZFC4-NR	2	2.33
	CREB1	BZIP	3	1
	GLI	ZFC2H2	3	1
	AP-2alphaA	BHSH	2	2
	SF-1	ZFC4-NR	1	3
	ZFP105 secondary motif	ZFC2H2	1	3
	NF-1	SMAD	2	1.67
	MZF1	ZFC2H2	1	2.67
	HNF-1alpha	HOX	2	1.33
	dlx-3	HOX	2	1.33
	Pbx	HOX	1	2.33
	POU2F1	HOX	2	1
	Muscle initiator	GENINI	2	1
	STAT1	STAT	1	2
	HSF1	HSF	1	2
	AIRE	ZFPHD	1	2
	CP2	GRAINY	1	2
	MAF	BZIP	1	1.67
	REST	ZFC2H2	1	1.33
	AP-1	BZIP	1	1
	MAFA	BZIP	1	1
	Smad4	SMAD	1	1
	Blimp-1	ZFC2H2	1	1
	Gfi1	ZFC2H2	1	1

* Experimentally determined transcription factor by ChIP-seq

Sites/Sequence –The frequency of TF motif hit in one relevant gene sequence. [a)] Inspecting promoter sequence is PM000512208. [b)] 3 different promoters of *Aurkb* gene are used, PM000522308, PM000522307, and PM000522309. (All provided by TRANSFAC®)

proven binding sites determined by ChIP-Seq (Wingender 2008), for example, CCCTC-binding factor (Ctcf), E2F transcription factor 1 (E2F-1), Zinc finger protein X (Zfx), Cdx-2, c-Myc, and Krueppel-like factor 4 (Klf4, GKLF) within the Cenpp and Aurkb gene (Table 5). Importantly, we found that 6 of transcription factors in the analysis result were regarded as putative substrates of CDK4 (Anders et al. 2011): the c-Myc, GLI1, Myeloid zinc finger 1 (MZF-1), c-Myb, Heat shock factor protein 1 (HSF-1), B-cell lymphoma 6 (Bcl-6) and GATA2. These putative substrates of CDK4 will be determined which is actually interacting with CDK4 by co-immunoprecipitation.

4. Discussion

We have continually addressed how forced expression of CDK4 promotes malignant progression during a chemical-induced carcinogenesis. One of the putative mechanisms is the induction of centrosome abnormality by CDK4, a well-known driving force for malignant progression. Our previous study in the chapter 3 demonstrated that the forced expression of CDK4 leads to intensify centrosome amplification in mouse skin tumors compared to tumors from wild-type mice. In this chapter, we focused on the role of CDK4 in the regulation of CIN related genes, as described by Casimiro et al. (Casimiro et al. 2012). First, we showed that CDK4 associated with chromatin regardless of pRb presence. Importantly, we have described a novel role of CDK4, which binds the same regulatory region of CIN related genes as described for cyclin D1. Expression levels of CDK4 bound genes, *Cenpp*, *Aurkb*, were up- or down-regulated according to cellular levels of CDK4, whereas, CDK4 unbound

genes, *Ckap2*, and *Top2A*, did not change significantly. This result suggests that CDK4 can associate gene regulatory regions with cyclin D1, and catalytic activity of CDK4 might affect expression of target genes. Moreover, overexpression of CDK4 triggered centrosome amplification in 308 cells. Therefore, we suggested that forced expression of CDK4 directly regulates transcription of CIN related genes and contributes centrosome amplification through elevation of Aurora B kinase in 308 cells.

Aurora B kinase is a member of Aurora kinase family that highly conserved Ser/Thr kinase and phosphorylates several effector molecules about chromatin condensation, cytokinesis, and microtubule- kinetochore attachment. In early G2 phase, Aurora B kinase phosphorylates histone H3 on Ser10 to condense chromosomes (Hendzel et al. 1997). During mitosis, Aurora B kinase forms a chromosome passenger complex (CPC) with INCENP, Survivin and Borealin, and it coordinates kinetochore-microtubule attachment (Ruchaud et al. 2007). Aurora B kinase in CPC complex also recruits checkpoint proteins such as MAD2 and BUBR1 to ensure spindle checkpoint until all chromosomes achieve bipolar orientation (Lens et al. 2003). Although amplification or mutation of *Aurkb* gene has not been reported in human tumors, Aurora B kinase is overexpressed in various types of cancers including prostate cancer, testicular cancer, hepatocellular cancer, breast cancer and colorectal carcinoma (Baldini et al. 2010; Lin et al. 2010; Chieffi et al. 2006; Chieffi et al. 2004; Katayama et al. 1999; Bischoff et al. 1998). Elevated level of Aurora B kinase correlates with cancer proliferation, aggressive phenotypes and poor prognosis in oral cancer, endometrial carcinoma, thyroid carcinoma, and hepatocellular cancer (Qi et al. 2007; Kurai

et al. 2005; Sorrentino et al. 2005; Lin et al. 2010). Correlation between overexpressed Aurora B kinase and tumor progression could explain by induction of CIN. Multinucleation and polyploidy caused by errors in cytokinesis and chromosome segregation are representative phenomena occurred by overexpressed Aurora B kinase (Ota et al. 2002). Moreover, increased Aurora B kinase leads to centrosome amplification and these abnormal phenotypes were exacerbated by ablation of p53 (Araki et al. 2004; Meraldi et al. 2002; Hontz et al. 2007; J. Fu et al. 2007). Despite abnormal expression of Aurora B kinase contributes tumorigenesis, the molecular mechanism is still poorly understood.

In this chapter, we have observed that the increased level of CDK4 upregulated the expression of *Aurkb* gene. However, raised transcriptional level did not lead to elevation of protein level in NIH3T3 cell lines, whereas, 308-CDK4 cell lines presented increment of Aurora B protein level and contributed centrosome amplification. It is worth mentioning that overexpression of CDK4 was not sufficient for induction of centrosome amplification in normal oral keratinocytes (Piboonniyom et al. 2003). In addition, it was shown in the previous chapter that normal or hyperplastic epidermis from K5CDK4 mice did not present centrosome amplification, but tumors did. Therefore, the increased level of CDK4 and the consequent overexpression of Aurora B kinase did not behave as an initiating event. These results suggest that other alterations are required in 308 cancer cells to increase Aurora B kinase level. 308 cells derived from chemically induced early benign skin tumors, and they carry an activating mutation in codon 61 of Ha-ras gene. Thus, we hypothesized that overexpressed CDK4 and activated Ras collaborate to increase *Aurkb* transcription and/or

stabilization of protein. The potential mechanism of synergetic effects between Ha-ras and CDK4 might involve the FOXM1 transcription factor. FOXM1, a member of the mammalian Forkhead box transcription factor family, is critical for G1/S transition and essential for mitotic progression (Wierstra 2013). Activated proto-oncoproteins such as H-Ras, c-Myc, c-Myb, EGFR or HER2/Neu promote FOXM1 expression, whereas, tumor suppressors such as pRb, PTEN, Cdh1, GATA3, or p53 decrease FOXM1 expression (Wierstra 2013). In addition to activation of FOXM1 by Ras oncoprotein, CDK4/cyclin D1 also activates FOXM1 transcriptional function and stabilizes FOXM1 protein level by phosphorylation on multiple sites (Anders et al. 2011). Importantly, FOXM1 is essential for expression of Aurora B kinase through direct association with human Aurkb promoter region at -730bp to -742 and -652bp to -740bp (I.-C. Wang et al. 2005). Knockdown of FOXM1 by siRNA treatment reduced Aurora B kinase, whereas, elevated FOXM1 increased Aurora B (I.-C. Wang et al. 2005; Bonet et al. 2012). Thus, we anticipate that activated H-Ras and overexpressed CDK4 increase expression and transactivity of FOXM1, followed by elevation of Aurkb transcription in 308-CDK4 cells compared to NIH3T3-CDK4 cells.

Apart from FOXM1, we analyzed transcription factor binding sites in regulatory regions of *Cenpp* and *Aurkb* around a CDK4 binding site to investigate potential CDK4 substrates (Table 5). We demonstrated that CDK4 bound to CIN related genes and regulated their transcriptional levels. Basically, catalytic activity of CDK4 starts with complex formation with D-type cyclins. Furthermore, because cyclin D1 regulates *Cenpp* and *Aurkb* genes at transcriptional levels, we hypothesized that CDK4/cyclin D1 might also have catalytic

activity and phosphorylate adjacent substrates on chromatin. Anders et al. (Anders et al. 2011) reported a list of putative substrates for CDK4 sharing transactivity. Among the candidates, c-Myc, c-Myb, MZF-1, HSF-1, Gli, GATA2 and Bcl-6 might be possible to associate with *Aurkb* gene and *Cenpp* gene. However, the role of these transcription factors in regulating Cenpp or Aurkb remains to be determined.

In summary, we have demonstrated that CDK4 plays a unique and novel role regulating some particular genes at transcriptional levels. We showed that CDK4 associates to CIN genes such as *Cenpp* and *Aurbk,* and upregulates their expression. Increased Aurora B kinase in 308-CDK4 cells leads to centrosome amplification, a hallmark of malignant transformation. Therefore, our results highlight a novel pathway by which CDK4 might induce malignant progression in mouse skin tumors. Overall, our data suggest that overexpression of CDK4 contributes centrosome amplification by directly regulating transcriptional levels of genes, especially Aurora B kinase in 308 cells.

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CHAPTER 5

General Discussion

1. General discussion

The primary objective of this thesis was to study alternative roles of CDK4 and CDK6 to the well-known function in the pRb pathway and define the effect of these putative novel roles in skin tumorigenesis.

Therefore, the goal of our studies was to address three central questions:

1) Does the catalytic activity of CDK6/cyclin D3 complex negatively affect a tumorigenesis?
2) Does forced expression of CDK4 induce centrosome amplification in skin epidermal tumors?
3) What are the molecular mechanisms involved leading in supernumerary centrosome?

In chapter 2, **we hypothesized that the association of CDK6/cyclin D3 and its enzymatic activity would mediate suppression of tumor development** since we had found elevation of CDK6/cyclin D3 complex formation in K5CDK6 mice epidermis. To address this hypothesis, we generated K5CDK6/cyclin $D3^{-/-}$ compound mice to eliminate CDK6/cyclin D3 complex in epidermis and carried out two-stage carcinogenesis. Contrary to our prediction, lack of CDK6/cyclin D3 complex in K5CDK6/$D3^{-/-}$ mice did not result in the increase of tumor development compared to K5CDK6 mice. In fact, both K5CDK6 and K5CDK6/cyclin $D3^{-/-}$ transgenic mice exhibited a similar reduction of papillomas' number in comparison to wild-type sibling. Therefore, **we conclude that forced expressed CDK6 suppresses skin tumor development by affecting an early stage of tumorigenesis by cyclin D3 independent mechanisms.** In addition, our analysis showed that ablation of

cyclin D3 affects two stages of tumorigenesis. First, the result that cyclin D3$^{-/-}$ mice also presented severe repression of tumor development suggests an inhibitory effect in an early stage of tumorigenesis such as tumor initiation and/or clonal expansion of the initiated cells. Second, no squamous cell carcinomas in cyclin D3$^{-/-}$ mice indicate that lack of cyclin D3 negatively affects tumor progression. On the other hand, K5CDK6/cyclin D3$^{-/-}$ mice exhibited an increased rate of malignant progression. Thus, we conclude that overexpression of CDK6 may play different roles in different carcinogenesis stages. During the early stage, CDK6 plays an inhibitory role in cyclin D3 independent manner. However, once the initiated cells successfully overpass the inhibitory barrier in early stages, overexpressed CDK6 in absence of cyclin D3 accelerates malignant tumor progression.

Interestingly, ablation of cyclin D3 is well tolerated by keratinocytes without any noticeable abnormality. Our biochemical analysis reveals that cyclin D3 ablation results in the elevated level of cyclin D1, and it suggests that cyclin D1 expression might compensate the deficiency of cyclin D3 in mouse epidermis. However, elevated cyclin D1 level is not sufficient to overcome the inhibitory effects observed upon ablation of cyclin D3 or CDK6-overexpression in early stage of tumorigenesis (cyclin D3$^{-/-}$ and K5CDK6/cyclin D3$^{-/-}$ mice). As aforementioned, overexpression of cyclin D1 in Cyclin D3$^{-/-}$ keratinocytes might play an essential role in malignant progression. Earlier studies of our laboratory have shown that CDK4 and CDK6 preferentially bind to cyclin D1 and D2, but not to cyclin D3, during papilloma promotion and malignant progression (Rodriguez-Puebla et al. 1998; Rojas et al. 2007). Therefore, we speculate that absence of cyclin D3 is responsible for the early

inhibition of tumor development, but the tumor that overpass this blockage accelerates malignant progression due to the increased level of cyclin D1.

Although we showed that inhibitory role of CDK6 is independent of cyclin D3, the molecular mechanism in which CDK6 is involving still remains to be determined. Our results suggest that CDK6 inhibits an early stage of tumorigenesis leading to reduced number of initiated cells that carrying Ha-Ras mutation in codon 61 and/or fail in the clonal expansion of initiated cells. Thus, future directions should include the analysis of whether forced expression of CDK6 alters susceptibility to Ha-Ras mutation or differentiation/senescence of initiated cells. In addition, several recent studies suggest that CDK6 has transcriptional regulation functions (Lim et al. 2005; Anders et al. 2011; Kollmann et al. 2013). Therefore, addressing which genes are regulated by CDK6 via ChIP-sequencing technology will allow us to establish tumor suppressive mechanism by overexpressed CDK6. We expect that upon progress in these directions, we will understand the novel role of CDK6 in skin tumorigenesis to approach CDK6 better as a therapeutic target.

Unlike inhibitory effects of CDK6, overexpressed CDK4 in epidermis (K5CDK4 mice) increases susceptibility to malignant progression to squamous cell carcinoma upon chemical-induced carcinogenesis via unknown pRb independent mechanisms (Miliani de Marval et al. 2003). CDK4 involves in license of centrosome duplication at G1 phase. Therefore, **we hypothesize that forced expression of CDK4 contributes to centrosome amplification, leading to malignant progression of epidermal tumors.** To determine the second question, we analyzed the centrosome number in the normal dorsal skin, hyperplastic skin, chemically

induced papillomas and SCC from K5CDK4 mice and wild-type siblings in the chapter 3. Coincident with our prediction, the number of centrosome per nucleus in papillomas and SCC from K5CDK4 mice is greater than in tumors from wild-type mice. Thus, **we conclude that overexpression of CDK4 accelerates centrosome amplification during chemically induced skin tumorigenesis.** This result suggests that pRb-independent function of CDK4 would alter the network of centrosome related proteins, but its working mechanisms have not been established. Thus, we have tried to figure out what molecular mechanisms are involved leading in supernumerary centrosome (the third question). It is important to mention that overexpression of CDK4 results in supernumerary centrosomes in hyperplastic skin compared with normal skin. Therefore, we speculate that the increasing number of centrosome trigger by forced expression of CDK4 is independent of the initiation of the tumorigenesis process. However, increased number of centrosomes may lead to elevating chromosome instability and potentiate the malignant progression of papillomas of K5CDK4 mice.

In chapter 3**, we hypothesize that the forced expression of CDK4 leads to phosphorylation of centrosome related proteins and contributes centrosome amplification.** To address this question, we isolated centrosome-enriched fraction from mouse fibroblast NIH3T3 cells, followed by performing mass spectrometry to identify centrosome related substrates of CDK4. As a result, we suggest 21 putative centrosome relative substrates including cytoplasmic Dynein 1 and CLAPS1. However, our analysis has a technical limitation such as screening with CDK consensus sequence, not particular for CDK4. Unlike CDK1, CDK2, and CDK6, CDK4 has showed very narrow selectivity to

substrates (Anders et al. 2011; Choi & Anders 2014). Thus, further biochemical studies are needed to validate actual substrates for CDK4 through co-immunoprecipitation analysis and kinase assay with CDK4. Also, it should be determined whether hyperphosphorylation of substrates by overexpressed CDK4 triggers centrosome amplification.

Recently, transcriptional roles of cyclin D1 in chromosomal instability (CIN) related genes are newly defined (Casimiro et al. 2012). This study gave us a clue that CDK4 would involve in the regulation of CIN-related genes since cyclin D1 strongly associate with CDK4 in nucleus, in general (Kato et al. 1994). Thus, **we hypothesize that CDK4 plays a role regulating CIN relative genes through association with chromatin.** In the chapter 4, we performed ChIP-assay against CDK4 to determine transcriptional regulation functions of CIN-related genes. Supporting our hypothesis, we observed that CDK4 associates with chromatin regardless of pRb presence and directly regulates expression of genes such as *Cenpp* and *Aurkb*. Specifically, Aurora Kinase B overexpression in CDK4 overexpressing 308 cells might play a positive role in contributing to centrosome amplification and likely in malignant progression. Hence, **we conclude that CDK4 regulates the CIN-related gene *Aurkb* at transcriptional levels in mouse epidermal keratinocyte cell line.** According to our result, CDK4 occupies the same site of gene regulatory region where cyclin D1 also associates. Since CDK4/cyclin D1 complex has catalytic activity, we speculate that CDK4/cyclin D1 can phosphorylate adjacent substrates in the gene promoter region and affect expression of a gene. *In silico* analysis of the regulatory regions of *Cenpp* and *Aurkb* genes allowed us to compare the list of the predictive transcription factors binding to those

regulatory regions with a list of putative substrates for CDK4 reported by Anders et al. (Anders et al. 2011). The list of putative substrates of CDK4 includes the transcription factors c-Myc, c-Myb, MZF-1, HSF-1, Gli, GATA2 and Bcl-6. Therefore, we propose that the role of these transcription factors regulating *Cenpp* or *Aurkb*, and the potential effect of CDK4 phosphorylation needs to examine in the further studies. It is worthy of notice that CDK4 induces Aurora kinase B leading to centrosome amplification only in 308 cells representing the early benign papilloma, but not in NIH3T3 cells. These results might support our data (chapter 3) that only tumors from K5CDK4 mice present enhanced centrosome amplification, but not in normal skin or hyperplastic skin. Collectively, these results suggest that other oncogenic alterations, such as Ha-Ras mutation, synergize to increase *Aurkb* expression and/or stabilization of protein. Hence, we expect that further investigations of collaborative mechanisms for regulation of Aurora B kinase will give us more detailed information about a unique role of CDK4 in tumor malignant progression.

In addition, future studies to examine the contribution of Aurora B kinase in overexpressed CDK4 to skin tumor malignant progression should be warranted. To address this, skin-specific Aurora B kinase-deficient mice need to be generated by genetic ablation of Aurora B kinase since Aurora B kinase-null mouse is embryonic lethality (Fernández-Miranda et al. 2011). From the two-stage carcinogenesis with skin-specific Aurora B-deficient/K5CDK4 compound mouse, we anticipate that we can determine the adverse effect of Aurora B kinase on the SCC conversion. Therefore, further studies about effects of Aurora B kinase ablation in epidermis of K5CDK4 mouse will provide more significant *in vivo* evidences about the role of CDK4 in skin tumor malignant progression. Overall, we think that the continuation

of these studies aimed at identifying novel roles of CDK4 and CDK6 in skin carcinogenesis will provide insights into how to approach CDK4/6 as therapeutic targets.

2. References

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