2014-01-24

A Novel Role of Cdk5 in the Cell Cycle

Law, Vincent

UNIVERSITY OF CALGARY

A Novel Role of Cdk5 in the Cell Cycle

by

Vincent Law

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF MEDICAL SCIENCE

CALGARY, ALBERTA

JANUARY, 2014

Abstract

Cyclin-dependent kinase 5 (Cdk5) is a small 33 kDa serine/threonine kinase belonging to the cdk family. It was originally identified based on the structural similarity to Cdk1 (Cdc2) and Cdk2, key regulators of eukaryotic cell cycle progression. Unlike other members of the cdk family however, Cdk5 is not activated by cyclins, but rather by non-cyclin proteins p35 and p39. The majority of reports regarding Cdk5 relate to neuronal development, particularly in the brain, and so whether Cdk5 functions in cell division has remained a mystery. Further studies by various groups revealed additional roles of Cdk5 outside the neuronal context, including functions in cancer progression. Recently, Cdk5 was shown to play a role in modulating the cell cycle. Here in this study, we further explore the potential function of Cdk5 in cell cycle progression, particularly in non-neuronal cells. Using both siRNA and genetic approaches, we found that the absence of Cdk5 enhances cell proliferation. The loss of Cdk5 causes an upregulation of Cyclin D1 expression, while levels of the cyclin-depdent kinase inhibitor proteins p21 and p27 are reduced. Furthermore, based on flow cytometry analysis, cells lacking Cdk5 results in cells moving from G1 to S faster than normal cells, indicating a potential function in the G1/S transition. Ionizing radiation (IR) studies also reveal that the absence of Cdk5 reduces p53 phosphorylation, and maintains the presence of γH2AX foci, indicating a disruption in DNA repair process, which may cause genomic instability. Collectively, these results support the idea that Cdk5 may play an important role in regulating the cell cycle of non-neuronal cells, particularly at the G1/S transition.

Acknowledgements

I would like to thank my supervisor Dr. Ki-Young Lee for his mentorship and guidance from the beginning to my completion of the MSc program. It was also an honor to have the support from my supervisory committee members: Dr. Karl Riabowol, Dr. Jennifer Cobb, and Dr. Donald Welsh.

In addition, it was a great pleasure to work with the members (past and present) in Dr. Ki-Young Lee's lab, and who had helped me with troubleshooting difficult experiments: Dr. Tom Kim, Hassan Tariq, Zach Levacque, Kyle Sorenson, Dr. Ashley Mummery and Dr. Susie Rosales.

Table of contents

LIST OF FIGURES

List of Abbreviations

Akt: Protein kinase B

APC/C: Anaphase promoting complex

ATM: Ataxia telangiectasia mutated

ATP: Adenosine 5'-triphosphate

Bax: Bcl-2-like protein 4

Bcl-2: B-cell lymphoma 2

CAK: Cdk activating kinase

Cdc: Cell division cycle

Cdk: Cyclin-dependent kinase

CKI: Cyclin-dependent kinase inhibitor

DCX: Doublecortin

Egr1: Early growth response gene 1

Eme1: Crossover junction endonuclease 1

EphA: Ephrin A

ERK: Extracellular signal-regulated kinase

FAK: Focal adhesion kinase

HU: Hydroxyurea

INK4: Inhibitor of kinase 4

MEK1: Mitogen-activated protein kinase kinase 1

Myt1: Myelin transcription factor 1

Nudel: NudE-like, a homologue of *Aspergillus* nuclear distribution molecule

ODF1: Outer dense fiber of sperm tail 1

PBS: Phosphate buffered saline

PCNA: Proliferating cell nuclear antigen

PI3K: Phosphatidylinositol 3-kinase

pRb: Retinoblastoma protein

PUMA: p53 upregulated modulator of apoptosis

Ser: Serine

STAT3: Signal transducer and activator of transcription 3

Thr: Threonine

Tyr: Tyrosine

RhoA: Ras homolog gene family, member A

XPC: Xeroderma pigmentosum, complementation group C

Chapter 1: Introduction, Hypothesis and Objectives

1. INTRODUCTION

Cell division is a cyclic process in which one cell replicates itself into two daughter cells. This process is fundamental throughout the life span of multi-cellular organisms, and failure in normal cell division can lead to devastating outcomes such as birth defects and development of disease. One of these diseases is cancer, where rapid cell division is a major characteristic. Both genetic and environmental factors are involved with shaping the overall progression of this disease. The status of cancer cells varies between different tissue origins and its stromal surroundings, and is very complex and dynamic. Universally however, they all exhibit the loss of cell cycle control. One way to study cancer cell behavior and to implement treatment strategies against it is by understanding the molecular basis behind cell cycle progression, and proteins that are important for maintaining a normal cell cycle status.

1.1 Cdks and their roles in cell cycle regulation

The mammalian cell cycle consists of four major phases in sequential order: growth 1 (G1), synthesis (S), growth 2 (G2), and mitosis (M). Each is tightly regulated by specific proteins, ensuring that the next phase does not begin until the one prior is complete. A family of proteins called the Cyclin-dependent kinases (cdk) is one of the key players to cell cycle regulation.

Cdks are evolutionary conserved serine/threonine kinases that are expressed in all eukaryotes. The first member in this family was found in *Saccharomyces cerevisae* and was named the Cdc2, a major regulator for yeast cell division. The human equivalent of Cdc2, called Cdk1, was subsequently cloned[1,2]. To date, there are at least eleven cdk family members identified (Cdk1 – Cdk11)[3] where Cdk1 – 4, 6 and 7 are regarded as important for cell cycle regulation, while Cdk8 and 9 are shown to play a role in gene transcription regulation[4-7]. Similar to a circular relay track, each cdk protein is responsible for a part of the cell cycle, and cdk members are activated in sequence to ensure the complete cycle progresses in an ordered fashion. Specifically, Cdk4 and 6 regulates entry into the cell cycle during G1, and prepare for G1/S and S where Cdk2 activity is most prominent. As cells enter G2 and M phases, Cdk2 activity is diminished and in exchange Cdk1 activity is elevated to further regulate and complete the cell cycle.

Generally cdks are expressed across all tissue types but remain inactive. The key determinants of cdk activities are the binding of cdk to another family of cell cycle regulatory proteins called cyclins. Each cdk member has its unique cyclin binding partner(s). For example, Cdks 4 and 6 binds with Cyclin D1, 2 and 3, Cdk2 with Cyclins E and A, and Cdk1 with Cyclins A and B. In addition to cyclin binding partners, cdk also requires the phosphorylation by cdk activating kinase (CAK) in order to become fully active. Phosphorylation occurs at the threonine residue within the T-loop, adjacent to cdk's catalytic active site[8]. Active cdk-cyclin complexes target a range of protein substrates for phosphorylation. It is characteristically on serine (S) and threonine (T) amino acid residues of the protein target, that are usually directed by a proline (P) and

typically followed by a lysine (K) or an arginine (R) following to a random amino acid (X), hence the sequence motif [S/T]PX[K/R][9].

While cdk activating phosphorylations are not regulated, inhibitory phosphorylation sites on cdks do serve an important role monitoring the activities of cdk-cyclin complexes. Threonine and tyrosine residues 14 and 15 respectively are located near ATP-binding sites of the cdk. Wee1 is one of the protein enzymes that phosphorylates Tyr15 site. The result of this phosphorylation is causing protein conformational changes that interfere ATP phosphate orientation. In vertebrates, Myt1 is another enzyme that phosphorylates both Thr14 and Tyr15 sites. Release from active cdk's "inhibitory state" is achieved via Thr14 and Tyr15 dephosphorylations by Cdc25 phosphatase. Together, Wee1 and Cdc25 act like molecular switches turning cdk activity on-and-off.

As mentioned before, a specific cdk becomes the dominantly active cdk member at a given phase during cell cycle progression and together the cdks coordinate with each other to complete the process. One of the mechanisms by which this coordination is accomplished is by regulating the level of cyclins. Depending on the phase of the cell cycle, different cyclins, which partner to specific cdks, are either expressed or degraded. While cyclins and CAKs regulate cdk activities and help with the transition through cell cycles, there are also protein regulators that control the level of cdk activities by acting as cdk suppressors. An example would be the cdk inhibitor (CKI) class of proteins, which inhibit cdk-cyclin complex, and is a mechanism that cells adapt to modulate the timing of specific cdk protein activation.

Two major families of CKIs, one of which is the cip/kip family including members p21 and p27. Both p21 and p27 can control multiple cdk-cyclin complexes by associating to both cdk and cyclin, and primarily function to regulate cdk-cyclin activities during G1/S (Cdk4, 6-Cyclin D and Cdk2-Cyclin E) and S-phases (Cdk2-Cyclin E, A)[10,11]. Interestingly, in addition to cdk-cyclin inhibitory function, cip/kip has also been shown to play a role activating Cdk4, 6-Cyclin D by enhancing the protein complex formation, and to initiate the start of cell cycle progression at G1. Another major CKI family, INK4 on the other hand preferably targets against Cdk4 and 6 monomers, disrupting their associations with Cyclin D, and as a result cell cycle progression is arrested (Fig 1).

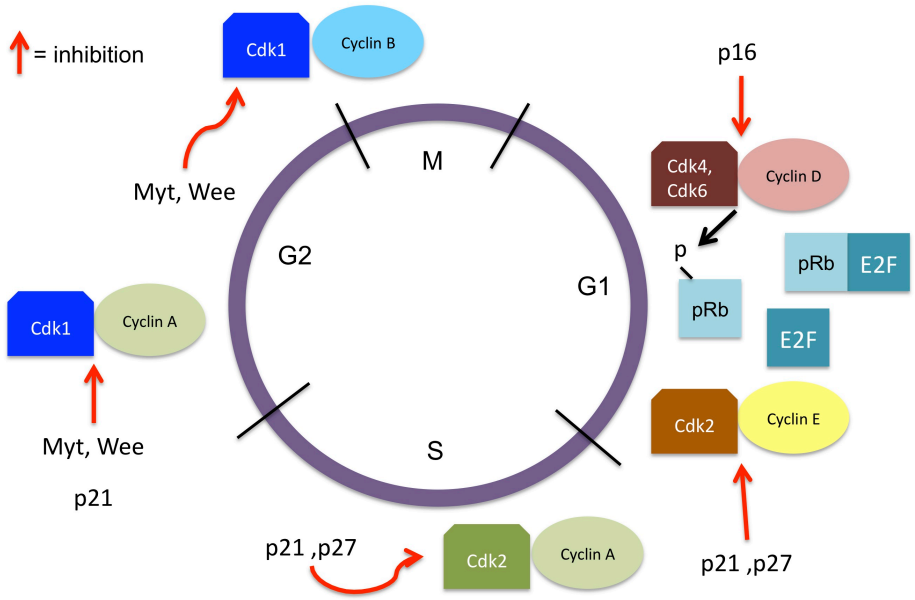


Fig. 1

Fig 1: A simplified diagram of the cell cycle and its key players.

Although, as discussed, cdk activity relies on cyclin binding and mainly regulates cell cycle progression of proliferating cells. There is however, cdk member that is distinctive to other cdks because it has its own set of binding partners and functions beyond dividing cells in post-mitotic condition. This unique cdk family member is called Cdk5.

1.2 Cdk5

1.2.1 Cdk5 discovery and functions

Cdk5, also known as neuronal Cdc2-Like Kinase (NCLK), is a small 33kDa proline-directed serine and threonine protein kinase belonging to the cdk family based on structural similarities with Cdk1 and Cdk2. Discovered nearly two decades ago, Cdk5 was found to exhibit unique properties that were unlike any other cdk members[12,13]. One of the distinctive features that differentiate Cdk5 from other cdks is that Wee1 and Myt1 kinase inhibitory phosphorylations of Thr14 and Tyr15 residues respectively have no effect on the activities of Cdk5. Instead phosphorylated Tyr15 residue in Cdk5 increases its enzymatic activity, and it has been shown that this phosphorylation site is targeted by c-Abl, an adaptor protein of Cables and Fyn[14,15]. Interestingly, although phosphorylations of Cdk5 produces different functional outcome than other cdks, phosphorylation sites such as Thr14 and Tyr15 residues remained as conserved sequences among the cdk family.

While most cdks are activated by cyclins, Cdk5 activation is distinctively through the binding to p35[16] and p39[17], which share approximately 57% homology. In addition to being activators for Cdk5, p35 and p39 also regulate its subcellular distribution. The amino-terminal myristoylation motif within p35 and p39 allows both activators to associate with the plasma membrane as well as cytoskeleton, thus sequestering Cdk5 at different subcellular locations. Active Cdk5/p35 and Cdk5/p39 complexes can phosphorylate a wide range of protein substrates, and induce various cellular functions[18,19] that are uncommon to other cdks. For example, Thr286 residue of MEK1, an upstream kinase of ERK in the mitogen-activated protein kinase (MAPK) signaling pathway can be phosphorylated by Cdk5[20,21]. The MAPK pathway is responsible for multiple cellular functions such as cell differentiation, proliferation, survival and apoptosis. ERK activity upregulates the gene expression of p35 through activating its responsible transcription factor Egr1[22]. Cdk5/p35 phosphorylation of MEK1 at Thr286 acts like a molecular switch, which "switches off" the activity of MEK1, thereby controlling the duration of ERK activity, a negative feedback mechanism of the MAPK pathway.

Active Cdk5 possesses both pro-survival and death-inducing functions. The absence of Cdk5 activity during embryogenesis causes a detrimental outcome, as evident in mice lacking the *cdk5* gene that exhibit pre- or perinatal mortality[23,24]. The major cause of death of *cdk5*$^{-/-}$ embryos in utero was due to severe neurological defect, especially the brain[25], suggesting the importance of Cdk5 regulation during early central nervous system development. Cell survival function of Cdk5 extends beyond embryogenesis. For

instance, Cdk5 protects neuronal cells from apoptosis when exposed to stress stimuli. Several pathways were proposed, including the PI3K/Akt pathway, ERK modulation of the pro-survival Bcl-2 and STAT3 phosphorylation and transcriptional activation[26]. On the other hand, hyperactivation of Cdk5 can also pose harmful effects to cells. Cdk5 hyperactivation occurs when there is abnormal production of p25, a cleavage product of p35 by a calcium-dependent proteolytic enzyme Calpain[27,28]. Unlike p35 where it is mostly located in the cell periphery, p25 moves freely in the cytosol to nuclear and perinuclear regions[18], giving it more accessibility to Cdk5 monomers. Over production of Cdk5/p25 complex caused by ER stress in the nucleus of post-mitotic neurons could lead to a detrimental outcome such as triggering the apoptotic pathway[29-31]. Another negative effect in which hyperactivation of Cdk5 has on cells is the alteration of cytoskeleton remodeling, which may destroy its structural integrity[32].

One of the many functions of Cdk5 is its ability to influence the dynamicity of microtubules and microfilaments[33]. There are several microtubule related proteins that are Cdk5 substrates. Tau, a microtubule stabilizing protein that is abundant in the brain, is phosphorylated by Cdk5. Hyperphosphorylation of Tau by Cdk5 causes a deconstruction of microtubule organization, leading to the formation of amyloid aggregates and neurofibrillary tangles, and as a result leading to neuronal cell death[34,35]. In fact, brains of patients with Alzheimer's disease often display abnormally elevated levels of Cdk5 activity and expression of p25[36]. Evidently, the balance of Cdk5 activity and the timing when it is activated directly relates to the overall survival outcome of neuronal cells. Hence, the importance of Cdk5 regulation and function in neurons is extensively studied.

1.2.2 Neuronal roles of Cdk5

Cdk5's involvement in neuronal differentiation is another unique feature by which Cdk5 is considered an atypical member in the cdk family. In developing neurons, Cdk5/p35 has been shown to be important for modulating the direction of growing axons and organizing its cytoskeleton framework[37]. During axonal regeneration, Cdk5 is recruited to the growth cones of projecting axons and phosphorylates a number of microtubule-associated proteins (MAPs), such as MAP1B, to stabilize the elongation process. To further emphasize the impact of Cdk5 activity on microtubule dynamics, Cdk5 also plays a critical role in neuronal migration[33,37,38]. Studies revealed mouse embryos depleted of Cdk5 showed defective cortical lamination (layering) in the neocortex, cerebellum and hippocampus[38]. This was believed to be due to the lack of direct Cdk5 phosphorylation of Nudel, FAK, and DCX, all of which were important for microtubule arrangement and believed to be responsible for migrating "newborn" neurons from the outermost layer to the granule cell layer.

In mature neurons, Cdk5 is involved in synaptic plasticity (memory and learning)[39] and the regulation of neurotransmission[40]. It seems that the modulation of Cdk5 activity is directly associated with learning abilities as implicated in transgenic *in vivo* model where hippocampus-dependent spatial learning was found to be significantly impaired in $p35^{-/-}$ mice[41]. Memory gain is a result of long term synaptic transmission between two neurons, termed long term potentiation (LTP), which highly depends upon the dynamics of dendritic spines. The exact molecular mechanism by which Cdk5 is involved in LTP[39,42]

is unclear but several findings suggest it may have been related to dendritic spine morphogenesis, possibly through modulation of the RhoA signaling pathway, which is responsible for altering dendritic spine morphology. When EphA tyrosine kinase receptor is activated by the ligand Ephrin, it recruits Cdk5/p35 to its receptor site and further enhances Cdk5/p35 activity via Cdk5 Tyr15 phosphorylation. Cdk5/p35 subsequently regulates RhoA activity through phosphorylating Ephexin1, a Rho-guanine nucleotide exchange factor, mediated by the EphA/Ephrin activation[43,44].

In addition to helping shape neurons, Cdk5 also plays a pivotal role in regulating neurotransmitter release at the presynaptic terminal of neurons. Reports have suggested that the presence of Cdk5 hinders presynaptic potentiation function, and that inhibition of Cdk5 facilitates neurotransmitter release. Plausible mechanisms were proposed to explain this. Cdk5 suppresses Ca^{2+} influx by interfering the interaction of pro-neurotransmitter release factors with P/Q type Ca^{2+} channels[40]. The exchange of intracellular Ca^{2+} through Ca^{2+} gated channels directly links to endocytosis process. Evidence also revealed that Cdk5 could phosphorylate the α_{1A} subunit of P/Q type Ca^{2+} channel, as a result blocking its interaction with SNAP-25 and Synaptotagmin I, which are important for inducing neurotransmitters release[40]. Furthermore, when Cdk5 activity is depleted by siRNA or pharmacological treatment, the inhibition of synapse terminals was relieved. Endogenously, this can be accomplished by balancing the activities of Cdk5 and its counteracting proline-directed Ser/Thr phosphatase Calcineurin (CN) at the nerve terminal, where increasing CN activity will result in accumulating larger pools of vesicles readily for release and recycle during neurotransmission[45].

1.2.3 Non-neuronal roles of Cdk5

Cdk5's multi-cellular functions extend beyond neurons. Substantial evidence shows Cdk5 activity is not restricted in the nervous system, but also in different types of tissues. Depending on the physiological properties of each cell type, Cdk5 may exert different effects on its cellular activities. It appears that, as in neuronal cells, Cdk5 can alter microtubule and actin filament remodeling of non-neuronal cells, either through direct or indirect contribution[46]. One of the earliest implications of non-neuronal function of Cdk5 is myogenesis demonstrated in C2 myoblast cells[47]. Such involvement includes multiple signaling pathways and their crosstalk, which tightly regulate Cdk5 activity. For instance, the PI3K-p70S6K pathway is activated during myoblast differentiation, and upregulates the expression of p35 by increasing Egr1 transcription factor. This signaling mechanism is simultaneously linked to Akt activation, while Cdk5/p35 stabilizes Nestin, an intermediate filament protein that modulates the pace of myoblast differentiation[48]. Nestin in turn also balances the level of Cdk5 activity. High level of Nestin was found to reduce Cdk5 activity, which could halt the myogenesis process. In addition, protein kinase C zeta (PKCζ) activates Calpain cleavage of p35 into p25, a more potent activator form for Cdk5, during early myoblast differentiation to increase Cdk5 activity.

The testis is another organ beyond the brain that expresses high levels of p35, implicating the potential significance of Cdk5 activity in this tissue. Evidently, spermatogenesis in the testis is a process in which Cdk5 was found to play a role. It was observed in sertoli cells and meiotic metaphase germ cells that Cdk5 could modulate cell functions and

development through interacting with microtubules and microfilaments. Although the exact molecular mechanism is unclear, it is appreciated that Cdk5 activation is not exclusive to post-mitotic cells. In regards to Cdk5 activity in the testis, our laboratory have also showed that Cdk5 activity may control the assembly and disassembly of sperm tails through a timely outer dense fibre 1 (ODF1) substrate phosphorylation[49]. The dynamicity of the sperm tail is dependent upon the proximal to distal assembly of ODF1 along the length of the tail axonome. An elevation of Cdk5 activity hence coincides with sperm tail elongation in spermatids. In $p35^{-/-}$ mice, ODF1 Ser193 phosphorylation is noticeably reduced. This phosphorylation residue was suggested to enhance ODF1 association with E3 ubiquitin ligase family of interacting proteins. It was therefore speculated that Cdk5 activity controls the assembly and disassembly of sperm tails through ODF1 phosphorylation.

1.2.4 Role of Cdk5 in cancer

The pathological consequences related to Cdk5 mutation have been investigated extensively since the discovery of this protein kinase. Various studies that focus on cancer biology have reported the connection between Cdk5 and disease progression, which includes cancer cell proliferation control, cell death pathways, and chemosensitivity[50]. In cervical carcinoma, Cdk5 has been shown to interact with and phosphorylates ectopically expressed p53 tumor suppressor protein under non-genotoxic condition. When p53 was overexpressed in HPV positive cancer cells lacking the p53 gene, neither cell cycle arrest nor apoptosis occurred. However, when Cdk5

phosphorylated Ser20 and Ser46 residues on p53, it activated p53 binding to promoter regions of p21 and Bax, upregulating the expression of these proteins to initiate cell cycle arrest and pro-death signals[51].

In conditions where neuronal cells were exposed to genotoxic stress such as irradiation and DNA damaging agents, Cdk5 had been shown to phosphorylate Ser794 residue on ATM, and induce ATM-dependent DNA damage response[52]. The Cdk5-ATM partnership was important for attenuating DNA damage-induced cell cycle re-entry, and to upregulate cell cycle suppressors, DNA repair machinery, and cell death regulators. Although in other study using colorectal cancer cells, Cdk5 was instead found associated with STAT3 and phosphorylate its Ser727 residue[53] to trigger transcriptional activation of DNA repair genes, such as Eme1[53], an endonuclease involved in DNA repair.

In breast cancer cells, Cdk5 plays a role regulating their proliferation. As reported by Upadhyay et al[54], a dramatic reduction in cell number of both MCF-7 and MDA MB231 breast cancer cell lines were observed after treatment with Cdk inhibitor Roscovitine and Cdk5-specific inhibitor Cdk2/5. In addition, when MCF-7 cells were treated with Carboplatin, hyperactivation of Cdk5 occurred in response to robust ERK activation, as a result triggering pro-death signaling pathway. Similarly, this observation was found in prostate cancer cells study, where over activation of Cdk5 in response to drug treatment caused detrimental effects to their survival. Further investigation reveals Cdk5 also has the ability to regulate the stability of androgen receptors (AR) in prostate cancer cells.

The interaction between Cdk5 and AR enhances prostate cancer growth and significantly increases its proliferation[55].

Cdk5's impact on the possible pharmacological outcome to cancer cells after drug treatment can be seen in studies with poly (ADP-ribose) polymerase (PARP) inhibitor, which blocks PARP's early sensing of single strand DNA (ssDNA) breaks. Several lines of cancers are dependent upon PARP for survival, hence making PARP a favorable target for cancer treatment. Specifically, PARP inhibitor is coupled with other cancer therapy or drugs to induce synthetic lethality in cancer cells. Turner and colleagues'[56] investigation identified Cdk5 as a determinant of PARP inhibitor sensitivity, where the depletion of Cdk5 increases the killings of cancer cells due to PARP inhibition. Similar result was obtained by Bolin et al.[57] However further in their study, they found that Cdk5-depleted cells were able to religate ssDNA breaks despite of level differences of PARP sensor recruitment at the ssDNA break sites. This suggests Cdk5 might not directly affect PARP's function to facilitate PARP inhibitor killing, but perhaps work via an alternative pathway, which has to do with processing DNA damage response.

Further emphasis of Cdk5's role in drug sensitivity can be seen in a study with multiple myeloma (MM) cells. The presence of Cdk5 protects MM cells from the killing effect of the proteasome inhibitor Bortezomib, possibly by modulating the expression of one of the proteosome subunit PSMB5. The omission of Cdk5 in MM cells reverses the response of MM cells to Bortezomib, increases its sensitivity to the drug treatment[58]. Collectively, all of these findings conclude that Cdk5 can impose both positive and negative outcomes on

the progression of cancer, and modulates a wide range of cellular functions that promote both cell survival and death.

1.2.5 Current interest of Cdk5 and its potential role in cell cycle

Cdk5 can be described as a "double edge sword", where its spatial and temporal localizations may vary the final outcome of cell survival and death[50]. Many Cdk5-associated studies primarily focused on its role in neural development, and considered it only important in post-mitotic condition. Often the role of Cdk5 in proliferating cells and whether it plays a role in cell cycle regulation remain unclear and is disregarded. However, there are now some clues and substantial evidence that suggest differently. Several recent reports have addressed the potential role of Cdk5 in cell cycle regulation, mainly in neuronal cells. For example, Zhang et al[29] have shown that neurons could dedifferentiate, triggering cell cycle reentry from its quiescent state if exposed to stress stimuli, and it seems that Cdk5 degradation is one of the requirements leading to this process. Further investigation by the same group revealed that Cdk5 degradation was possibly due to a translocation of Cdk5 from the nucleus to the cytoplasm[29,59,60]. In the cytoplasm, Cdk5 is ubiquitinated by E3 ligase APC-Cdh1, a tag for proteolytic degradation. Degradation of Cdk5 leads to differentiated neurons re-entering cell cycle progression, an unfavorable outcome that ultimately triggers apoptosis[61]. The sequestration of Cdk5 in the nucleus prevented cell cycle re-entry, occurred through the interaction with cell cycle suppressor p27[29,59]. Although the exact mechanism is not well understood, current evidence suggests p27 is a substrate for Cdk5 phosphorylation.

Specifically, Cdk5 phosphorylates Thr187 and Ser10 of p27, securing the protein in the nucleus, thus help to maintain neurons in a state of cell cycle arrest. This type of interaction of Cdk5 with a cell cycle regulating partner is one of the first line of evidence suggesting a possible cell cycle inhibitory function of Cdk5.

Evidence from another study demonstrated that Cdk5 might interact with E2F transcription factor. Cdk5-E2F binding interferes with E2F from forming a heterodimer complex with DP1, which is required for stabilizing E2F and DNA binding[59]. This indicates that Cdk5 may inhibit neurons from entering cell cycle by sequestering E2F, hence keeping them at G0 quiescent phase.

Recently, our laboratory has mapped both Cdk5 and its activator p35 in centrosomes of HeLa cells[62]. Because centrosomes are cellular organelles located in the microtubule-organizing centre (MTOC) and play key functions during different phases of the cell cycle, therefore the presence of both Cdk5 and its activator proteins in proliferating cells may indicate a purpose for cell cycle regulation that remains to be elucidated. Collectively along with reports that indicate Cdk5 as a potential candidate for cell cycle checkpoint regulation[56], DNA damage repair[52], and genomic instability[63], there is now substantial evidence that implicate Cdk5 in additional and novel functions, specifically in the cell cycle of non-neuronal cells. Still, how Cdk5 affects cell proliferation, and its exact function in monitoring, perhaps as an inhibitor of cell cycle progression, is still largely unknown and further investigations are needed.

In the present study, I investigated the possible role of Cdk5 in cell cycle progression outside the neuronal system. The consequence of losing Cdk5 in proliferating cells was explored in both *in vitro* and *in vivo* methods. In addition, the specific phase of the cell cycle that may be controlled by Cdk5 was analyzed. Our result revealed that cell proliferation was greatly enhanced in the absence of Cdk5 in Retinal Pigmented Epithelial cell line as well as primary Mouse Embryonic Fibroblast (MEF) cells. Increased mitotic cell population due to the depletion of Cdk5 was evident in $Cdk5^{-/-}$ mouse embryos, where they also displayed larger body size and mass. Interestingly, the abnormal proliferation rate found in $Cdk5^{-/-}$ cells could be restored to normal by introducing Cdk5 protein into these cells. Further investigation via cell synchronization techniques and flow cytometry analysis revealed that $Cdk5^{-/-}$ cells progressed through cell cycle faster than wt cells, and our data indicated a potential mechanism involving G1 and G1/S regulation. These findings challenge the notion that Cdk5 does play an important role in non-neuronal cell cycle and contradict the current theory that it primarily functions in neuronal system. Our study sheds novel information about Cdk5 that leads to better understanding the basis behind cell cycle, and gives insight for future studies to derive therapeutic strategies that requires manipulating progression of cancer cells.

2. HYPOTHESIS

My hypothesis is that Cdk5, like other cdk members, plays a role in cell proliferation, specifically serving as an inhibitor of cell cycle progression.

3. OBJECTIVES

The objective of my study is to investigate the potential role of Cdk5 in regulating the cell cycle.

My specific aims are:

1. To investigate how Cdk5 affects cell proliferation.
2. To investigate the specific phase(s) of the cell cycle inhibited by Cdk5.
3. To elucidate the molecular mechanisms by which Cdk5 may inhibit the cell cycle.

Chapter 2: Materials and Methods

2.1 Cell culturing

RPE cells were cultured in Dulbecco's Modified Eagle Medium/ Nutrient Mixture F-12 (DMEM/ F12), containing 10% Fetal Bovine Serum (FBS) and 100 U/ml Penicillin Streptomycin (PS). RPE cells were maintained in 5% CO_2, 21% O_2, 37^{o}C cell culture incubator.

For primary MEF cells, cells were maintained at optimal culturing condition as described by the standard protocol in the literature[64], where O_2 was reduced to 3% to minimize oxidative stress to cells, protecting it from early senescence. Cells were cultured in DMEM medium containing 10% FBS and 100 U/ml PS.

2.2 Western blot analysis

For all western blot experiments, 40 – 80μg of proteins were separated by SDS-PAGE, and subsequently transferred on to nitrocellulose membrane (100V, 60 min). Transferred membrane was first preceded with blocking step with 5% skim milk for 30 min, then incubated with 1:1000 diluted primary antibodies (anti-Cdk5, anti-GST, anti-Cyclin D1, anti-Cyclin A, anti-Cyclin B1, anti-Cdk4, anti-p21, anti-Actin (Santa Cruz Biotechnology); anti-p27 (Invitrogen); anti-phospho-Chk1 S345, anti-Chk1, anti-phospho-Rb S807/811, anti-phospho-p53 S15 (Cell Signaling Technology); anti-γH2AX, anti-phospho-Histone H3 (Millipore)) overnight on shaker at 4 ^{o}C. Membrane was washed four times with TBS-T followed by incubation with 1:2500 dilution of secondary

antibody conjugated with HRP for 50 min. Membrane was washed again four times with TBS-T before adding ECL substrate reagent (GE Healthcare). Protein bands were visible on developed X-ray films (GE Healthcare) that were exposed to the reported chemiluminescent signals.

2.3 siRNA transfection

For siRNA transfection, RPE cells were seeded to 60% confluence in 35 mm dishes before transfection. 20 nM of mock- or cdk5-siRNAs were transfected into RPE cells by siLentFect Lipid (Bio-Rad Laboratories). Cdk5-siRNAs were conjugated at the 5' with HEX Dye Phosphoramidite, and was used to determine under florescent microscope (Olympus IX71) the presence of dye (535 nm absorption; 556 nm emission) in cell cytoplasm to confirm positive transfection. Cdk5 depletion by siRNA was assessed by Western blot analysis where samples were collected by adding lysis buffer on days 1, 3 and 6 post-transfection.

2.4 Mouse embryos isolation and genotyping

Cdk5$^{-/-}$ embryos and its wt littermates were produced from intercrossing *cdk5*$^{+/-}$ mice, which were obtained from the Jackson Laboratory. Breeding mice were maintained at the University of Calgary Animal Facility. At E13.5, pregnant mice were taken out and sacrificed by surgical dislocation. The embryos were washed with PBS, weighed, and

photographed. Small pieces of limbs from each embryo were cut to isolate genomic DNA and protein samples for genotyping purpose.

2.5 Whole mouse embryo cryosection

The embryos were then placed in OmniSette tissue cassettes embedded in Optimum Cutting Temperature (O.C.T.) compound (Fisher Scientific). The tissue cassettes were submerged in pre-cooled by dry ice 2-methyl butane for 3 min., and frozen samples were kept in -80 ^{o}C. For tissue sections, frozen whole embryos were cut at sagittal plane using a cryostat under -20 ^{o}C temperature condition. Sagittal sections were 10mm in thickness and were fixed on glass slides (VWR Scientific).

2.6 Primary MEF cells harvestation

E13.5 mouse embryos and its wt littermates were obtained from *cdk5*$^{+/-}$ pregnant mouse as indicated above. Embryos were cut into small pieces and digested with Trypsin-EDTA (Invitrogen) at 37^{o}C agitator for 30 min. Incubation time was extended if required. Digested tissues were added with fresh DMEM media, centrifuged at x1000 RPM for 5 min. MEF cell pellets were resuspended and washed two more times with fresh DMEM media, and platted into 100 mm tissue culture dishes containing DMEM growth media (10% FBS, 100U/ml PS). The following day, leftover undigested tissues were removed sterilely with forceps.

2.7 Proliferation assay

For RPE cells proliferation assay, same number (1.0×10^5) of cdk5-depleted (after 24 hrs post-transfection) and control RPE cells were seeded into 35 mm tissue culture dishes. Growing cells were collected on days 1, 3 and 6 via trypsinization (Trypsin EDTA). Subsequently, trypan blue exclusion assay was performed to recount the total number of cells. All cell counting procedure was performed using a Hemocytometer under the light microscope (Olympus CK40).

To compare proliferation rates of *cdk5*$^{-/-}$ and wt MEF cells, early passages were used. 7×10^4 *cdk5*$^{-/-}$ and wt MEF cells were seeded into 60 mm dishes. On days 1, 3 and 5 cells were trypsinized for recounting. The total number of cells was determined by trypan blue exclusion assay, counted by using the Hemocytometer under a light microscope (Olympus CK40).

2.8 Delivery of GST-Cdk5 into Cdk5$^{-/-}$ cells

GST-Cdk5 fusion proteins were generated in our laboratory as previously described[65]. Early passages of *cdk5*$^{-/-}$ MEF cells were introduced with either GST-Cdk5 or GST using the BioPORTER (Genlantis) reagent. Protein delivery procedure was carried out according to the product instruction manual. The presence of GST-Cdk5 and GST in *cdk5*$^{-/-}$ MEF cells was confirmed by Western blot analysis using Cdk5 and GST specific antibodies.

2.9 Cell synchronization

Primary MEF cells synchronization at G1 and S phases of the cell cycle were achieved by the following: For G1 synchronization, MEF cells were grown to 100% confluence to induce cell contact growth inhibition. Adhered cells were trypsinized and replate to new tissue culture dishes at 50% confluence. For S-phase synchronization, growth contact inhibited MEF cells were seeded into DMEM growth media (100% FBS, 100U/ml PS) containing 10 μM HU for 24 hrs. To release cells from S-phase, HU treatment was removed and washed with PBS three times, then replaced with fresh DMEM growth medium.

2.10 Cell cycle flow cytometry analysis

MEF cells that were released from synchronization (as described above) were trypsinized at specific time points. For G1 synchronization, released MEF cells were collected at 8, 19, 27, 33, 41 and 51 hrs. For HU-induced S synchronization, MEF cells were collected at 1 and 5 hrs, while Nocodazole-induced G2/M synchronized MEF cells were collected every hr up to 4 hrs after growth medium release. Cells were fixed with ice cold 70% ethanol and its DNA were subsequently stained with 50 mg/ml Propidium Iodide (PI) solution which contained 200 mg/ml RNAse1 and 0.1% Triton X-100 for 30 min at room temperature. Samples were submitted to the flow cytometry facility at the University of Calgary for flow cytometry analysis.

2.11 Ionizing radiation and protein collection

Confluent *cdk5*$^{-/-}$ and wt MEF cells were trypsinized and reseeded to approximately 60% confluence in 60 mm tissue culture dishes. Cells were then exposed to 4 Gy of ionizing radiation to induce double strand DNA break damage and placed back into tissue culture incubator (37^{o}C, 5% CO2, 3% O2) to allow recovery. Protein samples from ionized radiated MEF cells were collected with lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophophate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 mM PMSF] at 1, 2, 5 and 24 hrs. Cell lysates were centrifuged at x14,000 RPM for 15 min at 4^{o}C. Supernatent was removed and pellet was resuspended with 100 μl of 1% SDS. The pellet samples were then placed water bath sonicator (Branson 1510) for 15 min. After sonication, samples were completely dissolved by boiling at 100 ^{o}C for 10 min.

2.12 Kinase assay and autoradiogram

HsH1 kinase activity of the reconstituted enzymes was measured by incubating the reaction mixture, which contained 20 mM MOPS, pH 7.4, 30 mM $MgCl_2$, 100 μM ATP [γ-^{32}P] (1000 cpm/pmol), and 100 μM HsH1 peptide P^{9}KTPKKAKKL^{18}, at 30°C for 20 min. [γ-^{32}P]phosphate incorporation into the substrate peptide was quantitated by liquid scintillation using a Beckman LKB 1215 scintillation counter. RB phosphorylating activity was determined using the expressed p70$^{N\text{-}RB}$ peptides (0.1 μg/μl) as substrates

under the same conditions. Samples were analyzed by SDS-PAGE followed by autoradiography.

2.13 Immunocytochemistry

MEF cells on cover slips were fixed with 3.7% paraformaldehyde for 15 min and then rinsed three times with PBS. Prior to adding primary antibody, fixed cells were first permeabilized by adding 0.2% Triton X-100 diluted in PBS, and were washed again three times with PBS. 100 ml of primary antibody at 1:150 diluted in 2% BSA was added to fixed and permeabilized cells and incubated for 1 hr at room temperature. Fluorescently-conjugated secondary antibody (anti-Rabbit IgG Alexa Fluor 488) (Molecular Probes) was diluted at 1:200 in 2% BSA, and subsequently added to cover slips for 20 min at room temperature, after primary antibody was removed with rinsed with three times with PBS. Subsequently cover slips were covered with 2 ml of 0.1 mg/ml of 4', 6-diamidino-2-phenylindole (DAPI) for 10 min under room temperature, washed three more times with PBS, then mounted on glass slides using 10 ml of Mountant PermaFluor (Thermo Scientific). As background control, cover slips without primary antibody incubation was used. All procedures were performed in the dark. Immunoflorescence was detected by using Olympus IX71 microscope and images were taken using the ImagePro Express program.

2.14 Immunohistochemistry

Fixed tissue sections on glass slides were first incubated in peroxidase block (Dako) to block endogenous peroxidase activity. Avidin and biotin were subsequently blocked by using the Avidin/Biotin blocking kit (Zymed). To eliminate non-specific binding of primary antibody, serum free protein block was performed (Dako) on tissues. 1:100 dilution of Ki67 antibody (Abcam ab92353) was added to the samples and incubated for 2 hrs. Diluted secondary antibody directly against primary Ki67 (Jackson Immuno) was added for 40 min. Each step was rinsed with TBST for 2 min in between. To visualize Ki67 positive cells, 100 ml of Vectastain® ABC Reagent was first added for 30 min. Subsequently equal volume of DAB (prepared from kit reagents by Zymed) was added for 5 min. Tissue sections were analyzed and photographs were taken using a light microscope.

2.15 Statistical Analysis

Data was analyzed by Student's T test (two tailed). $p < 0.05$ was taken as statistically significant.

Chapter 3: Results

3.1 Cdk5 modulates cell proliferation.

Previously in our laboratory, we noted that Cdk5 localizes around the area of the sperm-connecting piece, pointing to the possibility that it associates with the basal body, a structure that recapitulates the structure and function of the centrosome. Indeed, we have recently shown evidence that Cdk5 and p35 exist in centrosomes all through the cell cycle. Cdk5 and p35 also remained associated with centrosomes isolated from nocodazole-treated cells, specifically localizing in the pericentriolar material of the centrosome, indicating that their association with centrosomes is independent of microtubules, and that they are integral centrosome components. Together, these findings suggest a role for Cdk5 in the cell cycle. Indeed, it has been shown that Cdk5 serves to suppress the neuronal cell cycle, particularly in G1/S. There is increasing evidence that shows Cdk5 plays a role beyond the brain tissue. Cdk5 activity can be detected in non-neuronal proliferating cells, but the role of Cdk5 regulating their cell cycle progression remains to be investigated. First, I asked whether the loss of Cdk5 would affect cell proliferation. To address this question, I depleted Cdk5 in RPE cells using Cdk5 specific siRNA. RPE cells were previously shown in our laboratory to exhibit Cdk5 expression and activity (Fig. 2). The Cdk5 siRNA was conjugated at the 5' with HEX Dye Phosphoramidite, which was used to visualize siRNA's localization. After 24 hrs of transfection, nearly all of the RPE cells incorporated HEX Dye, observed under a florescent microscope (Fig. 3A). To validate that Cdk5 siRNA was able to deplete Cdk5 expression in RPE cells, samples (40 μg of protein cell lysates) were collected on day 1, 3 and 6 post-transfection for western blot analysis. There was almost a complete

knockdown of Cdk5 in RPE cells 3 days after Cdk5 siRNA treatment, and this status was maintained on day 6, while no effect was observed with RPE cells treated with mock-siRNA (Fig. 3B). After confirming Cdk5 depletion, 1.0 x 10^5 of both Cdk5-depleted and control RPE cells were seeded into 35 mm dishes for comparing their proliferation rates over 6 days period. It was found that in the absence of Cdk5 expression, the number of RPE cells increased significantly (Fig. 3C), indicating an increase in cell proliferation rate.

To eliminate the possibility of siRNA off-target effect in our proliferation assay, I sought to recapitulate the result using a genetic approach. Since $Cdk5^{-/-}$ mice exhibit pre- and perinatal mortality, we required utilizing mouse embryos generated by crossing $cdk5^{+/-}$ mice for subsequent studies. Paired E13.5 WT and $Cdk5^{-/-}$ mouse embryos were isolated, and their genotypes were confirmed by western blot analysis (Fig 4A). We harvested primary MEF cells from these mouse embryos, which were cultured in hypoxic (3% O_2) condition as suggested in the literature[64]. An equal number of WT and $Cdk5^{-/-}$ MEF cells (1.0 x10^3) were seeded in 35 mm dishes, and were recounted on days 1, 3 and 6. Supporting our RPE cell proliferation assay data, $Cdk5^{-/-}$ MEF cells displayed a significantly faster proliferation rate compared to WT cells (Fig 4B). Based on the both results from the RPE cell line and primary MEF cells, we determined that the loss of Cdk5 enhances cell proliferation.

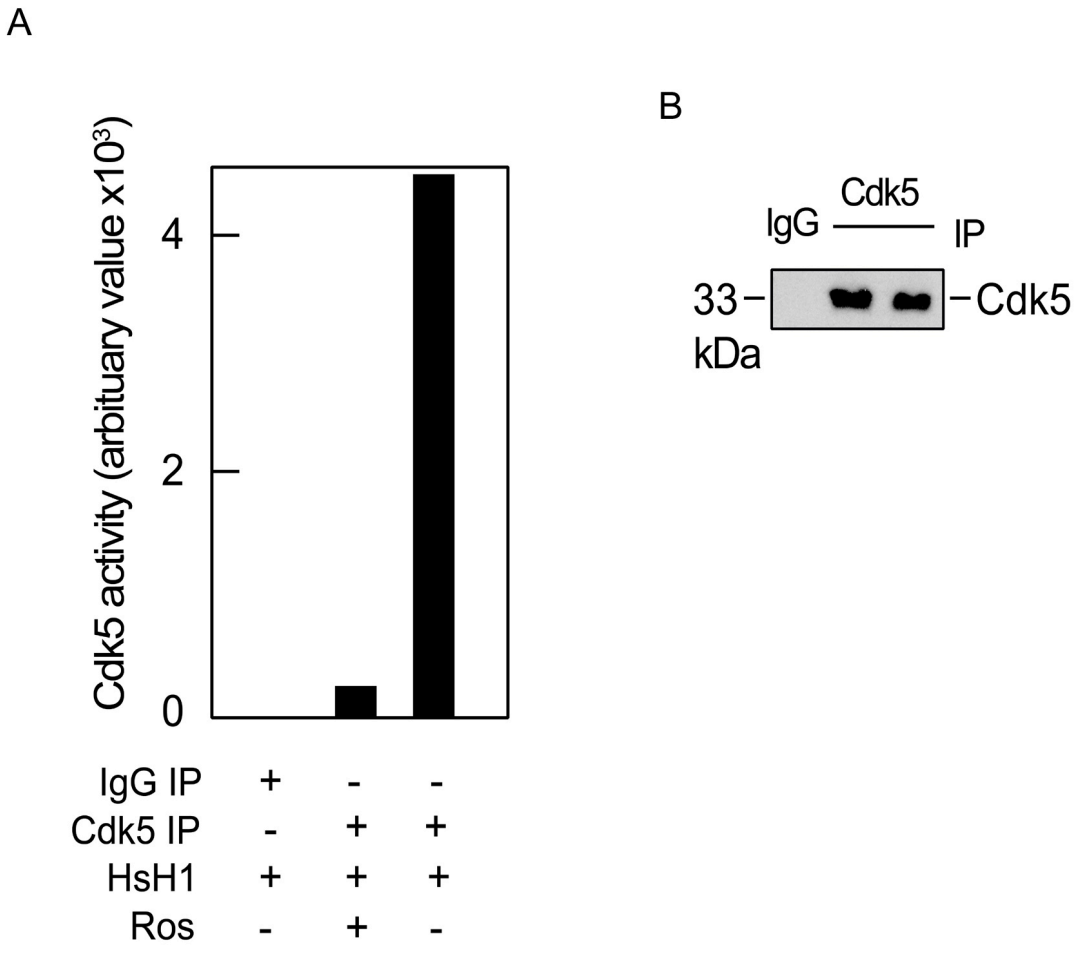

A
B
Cdk5 activity (arbituary value x10³)
4
2
0
IgG IP + - -
Cdk5 IP - + +
HsH1 + + +
Ros - + -
IgG
Cdk5
IP
33
kDa
Cdk5

Fig. 2

Fig. 2: RPE cells exhibit Cdk5 activity.

(A) RPE cell lysates were incubated with anti-Cdk5-agarose beads and anti-IgG-agarose beads for IP. Subsequently, Cdk5 kinase assay was performed. IP'd samples were added to substrate HsH1 with ATP [γ-^{32}P]. For another negative control, Cdk5 inhibitor Roscovitine (Ros) was used. The amount of phospho-HsH1 was measured using scintillation counter. (B) Confirmation of Cdk5 in Cdk5-IP samples using western blot analysis.

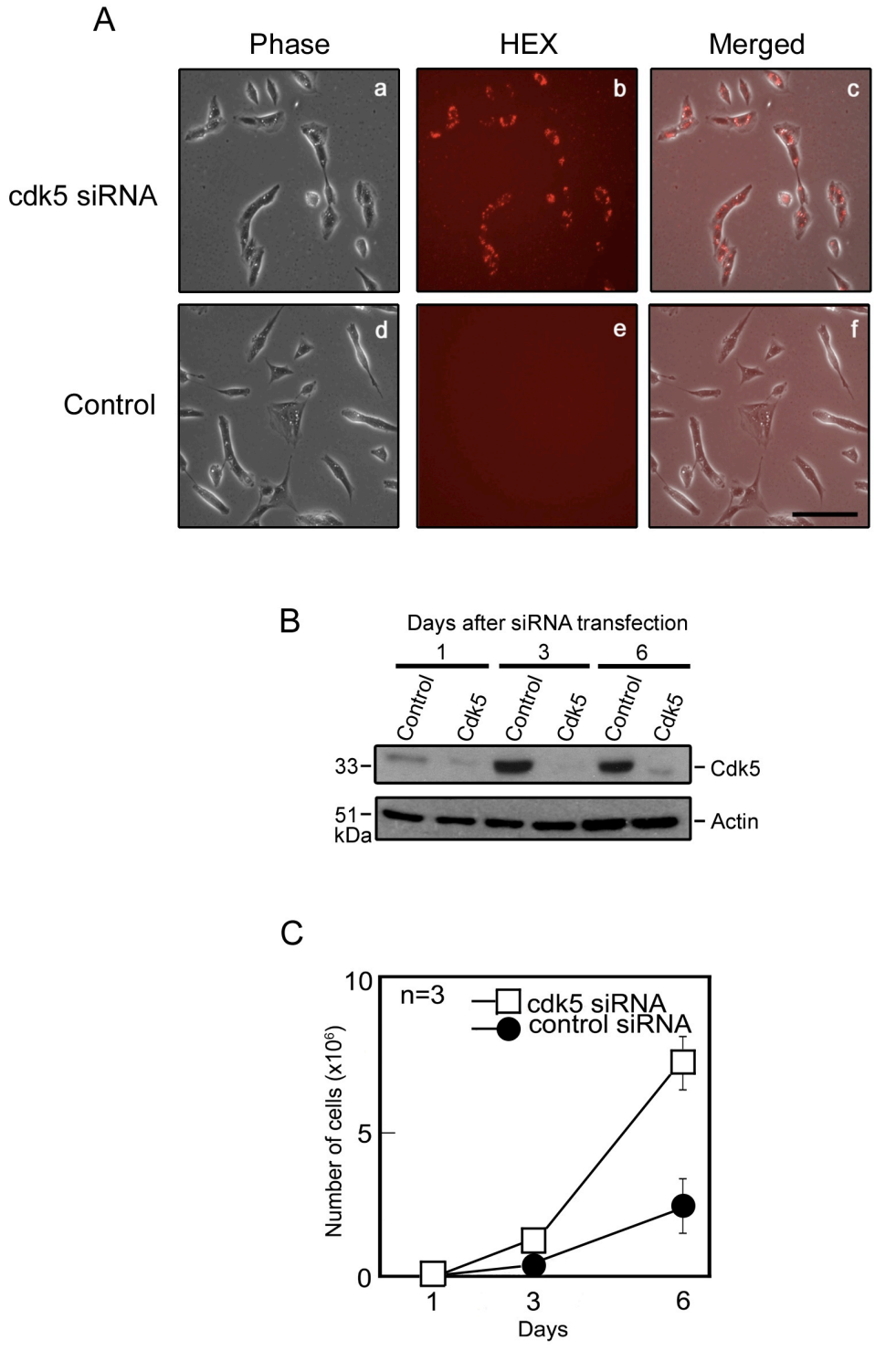
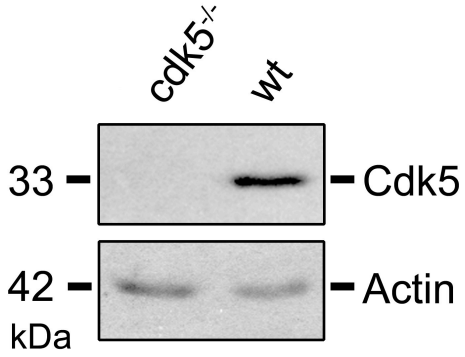

A
Phase
HEX
Merged
cdk5 siRNA
Control
a
b
c
d
e
f
B
Days after siRNA transfection
1
3
6
Control
Cdk5
Control
Cdk5
Control
Cdk5
33
Cdk5
51
kDa
Actin
C
n=3
cdk5 siRNA
control siRNA
Number of cells (x10⁶)
10
5
0
1
3
6
Days

Fig. 3

Fig. 3: Depletion of Cdk5 increases RPE cell proliferation.

(A) Images of RPE cells transfected with Cdk5-siRNAs (a, b and c) and control-siRNAs (d, e and f). Cdk5 siRNA is 5' linked with HEX Dye Phosphoramidite. Panel b shows the detection of HEX in cell cytoplasm, which indicates successful incorporation of Cdk5 siRNAs into RPE cells. Bar = 50 μm. (B) Equal number of cells were seeded for siRNA transfection procedure. siRNA-transfected RPE cells were harvested on day 1, 3 and 6 and cells were lysed with lysis buffer. Whole cell lysate samples were tested for the depletion of Cdk5 expression using western blog analysis. Actin was used for protein loading control for each corresponding day. (C) The rates of cell proliferation of Cdk5-depleted and control RPE cells were compared (n = 3).

A


cdk5-/-
wt
33
Cdk5
42
Actin
kDa

B

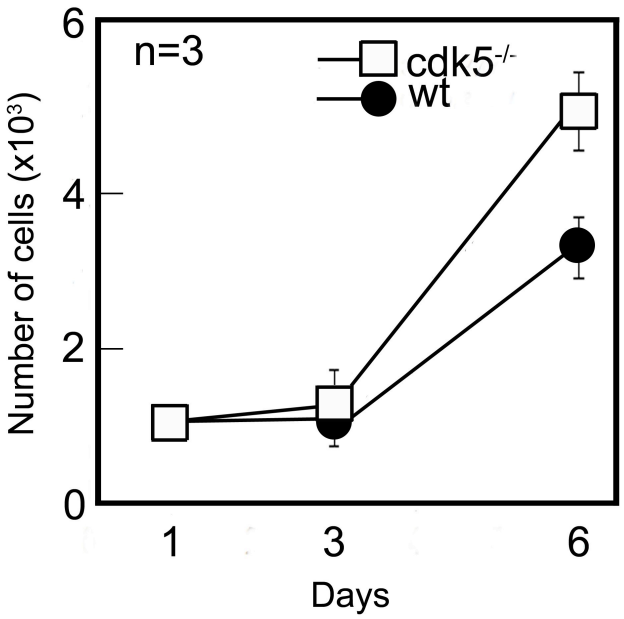

n=3
cdk5-/-
wt
Number of cells (x10³)
0
2
4
6
1
3
6
Days

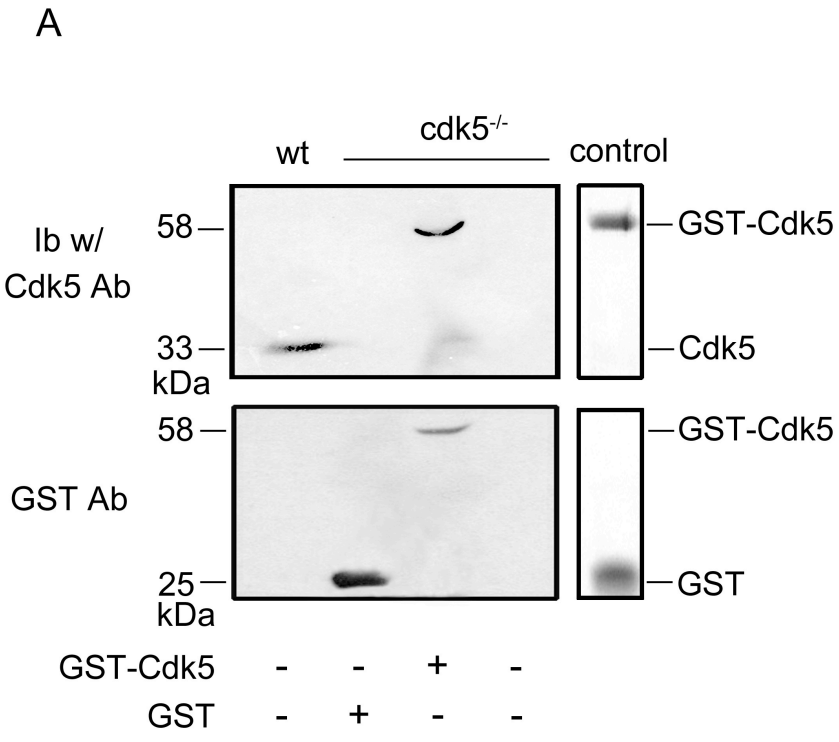
Fig. 4

Fig. 4: Loss of Cdk5 enhances MEF cell proliferation.

E13.5 mouse embryos generated by intercrossing *cdk5*$^{+/-}$ breeding pairs were isolated. MEF cells were harvested by trypsinization method and cells were maintained according to the 3T3 protocol. Early passages of MEF cells were used for the study. (A) The absence of Cdk5 expression in Cdk5$^{-/-}$ MEF cells was confirmed by Western blot analysis. Actin was used as loading control. (E) Proliferation rates comparison between Cdk5$^{-/-}$ and WT MEF cells spanning a 6-day period (n = 3).

3.2 Introduction of Cdk5 converts Cdk5$^{-/-}$ cells to normal phenotype.

My next question was whether the faster than normal cell proliferation rate seen in the Cdk5-absent condition could be rescued by introducing exogenous Cdk5 proteins. By utilizing the BioPORTER reagent, GST-tagged Cdk5 recombinant protein (GST-Cdk5), which was previously generated in our laboratory, was delivered into Cdk5$^{-/-}$ MEF cells. The BioPORTER reagent lipid can capture the protein of interest and transport it into living cells without altering its structure and function. In addition to GST-Cdk5 fusion proteins, for controls GST-alone peptides transfected into Cdk5$^{-/-}$ MEF cells and untreated Cdk5$^{-/-}$ and WT MEF cells were also used. By western blot analysis detecting against Cdk5 and GST, the presence of GST-Cdk5 in Cdk5$^{-/-}$ MEF cells was confirmed (Fig. 5A). To test whether the introduction of exogenous Cdk5 would decrease the growth of Cdk5$^{-/-}$ MEF cells, the same number (7.0×10^4) of GST-Cdk5 or GST-alone Cdk5$^{-/-}$ MEF cells along with untreated WT and Cdk5$^{-/-}$ MEF cells were seeded into 35 mm dishes. Because primary MEF cells tend to have a longer doubling time as reported in different studies[66,67], the total number of cells were recounted after 72 hrs using trypan blue exclusion assay. The data showed that compared to untreated Cdk5$^{-/-}$ MEF cells, Cdk5$^{-/-}$ MEF cells treated with exogenous GST-Cdk5 resulted in significantly reduced cell number. GST-alone did not have an effect on the fast growing Cdk5$^{-/-}$ MEF cells (Fig. 5B). This suggested that an enhanced cell proliferation phenotype found in our Cdk5$^{-/-}$ MEF cells could be converted to normal by introducing exogenous Cdk5 into them.


A
wt
cdk5-/-
control
Ib w/
Cdk5 Ab
58
33
kDa
GST-Cdk5
Cdk5
GST Ab
58
25
kDa
GST-Cdk5
GST
GST-Cdk5 - - + -
GST - + - -

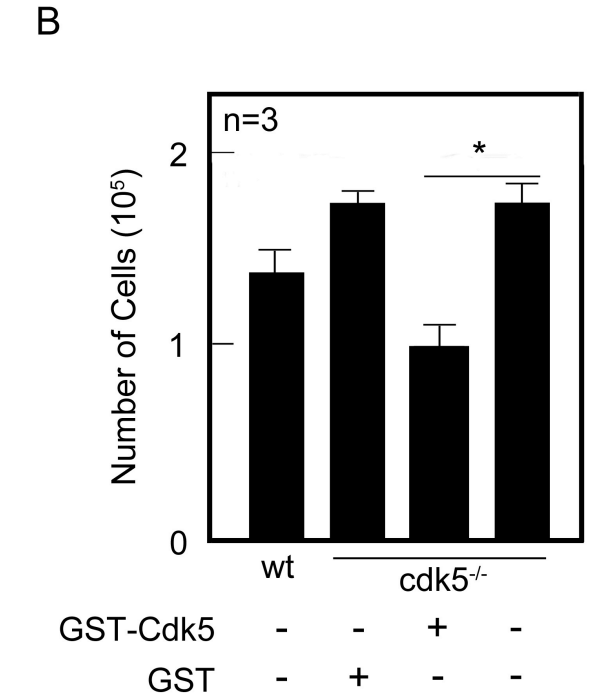

B
n=3
*
Number of Cells (10^5)
2
1
0
wt
cdk5-/-
GST-Cdk5 - - + -
GST - + - -

Fig. 5

Fig. 5: Introduction of GST-Cdk5 into Cdk5$^{-/-}$ MEF cells slows their growth

Either GST-Cdk5 recombinant protein and GST-alone was delivered into Cdk5$^{-/-}$ MEF cells using BioPORTER reagent. (A) Western blot analysis of cell lysates (30 μg) showing successful incorporation of GST-Cdk5 and GST-alone into Cdk5$^{-/-}$ MEF cells. Cdk5 and GST specific antibodies were used. (B) Equal number of cells for each of the condition: WT (lane 1), Cdk5$^{-/-}$ + GST (lane 2), Cdk5$^{-/-}$ + GST-Cdk5 (lane 3) and Cdk5$^{-/-}$ (lane 4) were seeded. 3 days after BioPORTER treatment total number of cells were counted using trypan blue exclusion assay (n = 3). Statistical analysis was performed by Student's T test (two tailed). * is considered significant where p-value is < 0.05.

3.3 Cdk5$^{-/-}$ mouse embryos display larger body size and greater weight compared to control embryos.

The presence of Cdk5 activity is crucial in embryonic development as cell proliferation and differentiation are regulated in a coordinated manner[68]. Any adverse changes in Cdk member functions during this period can alter total cell number and size of embryos[69]. Because my data showed that depletion of Cdk5 causes faster proliferation rate and as a result more total number of cells, therefore I sought to examine whether Cdk5$^{-/-}$ embryos' size and weight would also be different compared to their littermates. Moreover, previous report by Qiu et al[70] on p27 cell cycle suppressor study using mice genetic model had correlated larger organ size with high cell proliferation rate. Hence based on these findings I predicted that Cdk5$^{-/-}$ embryos would exhibit larger size compared to normal. For the experiment, I isolated embryos from Cdk5$^{+/-}$ pregnant mice during its early E13.5 pregnancy. E13.5 embryos were used due to the fact that the *cdk5*$^{-/-}$ condition could display prenatal mortality during the late embryonic stage. Paired WT and Cdk5$^{-/-}$ mouse embryos from five separate *cdk5*$^{+/-}$ breeding pairs were collected. The embryos were cleaned, weighed and photographed. WT and Cdk5$^{-/-}$ statuses were confirmed by PCR method (data not shown) and Cdk5 specific western blot analysis (Fig. 6B). It was observed that there was a noticeable size difference between Cdk5$^{-/-}$ versus WT embryos (Fig. 6A), where ones without the c*dk5* gene displayed bigger body size compared to its littermates. I found that the average weight of Cdk5$^{-/-}$ embryos was also greater than their WT (Fig. 6C). To further investigate whether the increased body size and weight in Cdk5$^{-/-}$ embryos was most likely due to increasing proliferating cells and not due to cell size,

the total amount of genomic DNA content was compared. The total amount of genomic DNA in the $Cdk5^{-/-}$ was higher than WT (data not shown). This indicates that increased body size and weight found in $Cdk5^{-/-}$ embryos is likely due to a defect in cell proliferation.

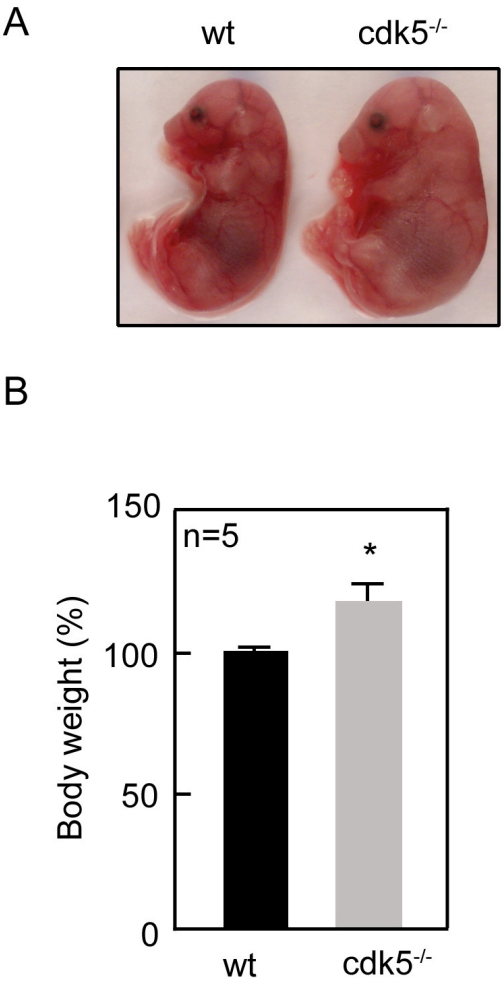

A
wt
cdk5-/-
B
150
n=5
*
100
50
0
Body weight (%)
wt
cdk5-/-
C
wt
cdk5-/-
33
Cdk5
42
Actin
kDa

Fig. 6

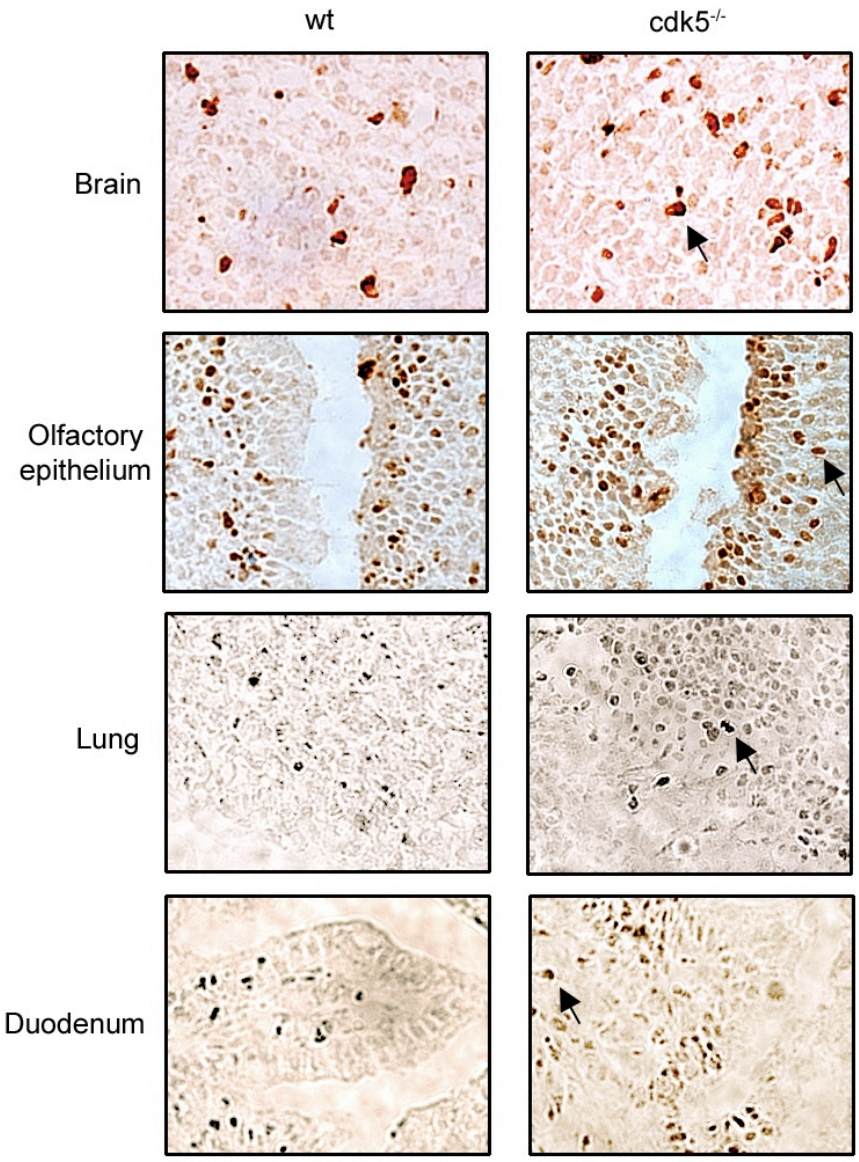
Fig. 6: Cdk5$^{-/-}$ mouse embryos exhibit larger body sizes and are heavier.

(A) Representative photo showing size difference comparing paired WT and Cdk5$^{-/-}$ mouse embryos. (B) Combined data of weight comparison between E13.5 WT and Cdk5$^{-/-}$ mouse embryos, collected from n = 5 cdk5+/- breeding pairs. Statistical analysis was performed by Student's T test (two tailed). * is considered significant where p-value is < 0.05. (C) Genotypes of embryos were validated by Cdk5 specific western blot analysis. 20 μg of tissue homogenlysates were loaded. Actin was used as loading control.

3.4 Loss of Cdk5 results in larger population of proliferating cells *in vivo*.

To better visualize cell proliferation statuses *in vivo* in response to Cdk5 mutation, it was decided to compare the population size of proliferating cells between c*dk5*$^{-/-}$ and WT. Given the difficulty and unsuccessful isolation of specific organs in E13.5 mouse embryos, whole-mount embryo sectioning was performed instead to examine different organs separately. Tissue samples of whole mouse embryos were cut to 10 μm in thickness, fixed with 4% PFA, and subsequently stained with Ki67 antibodies, a marker to detect cells that were undergoing cell cycle progression. As determined by observing the proportion of Ki67 positive cells in similar regions found in *cdk5*$^{-/-}$ and WT mouse embryos, it was discovered that the *cdk5*$^{-/-}$ overall displayed a higher population of proliferating cells (Fig 7A). The most obvious organ was the brain, given an average of 18.3% in cdk5$^{-/-}$ and 7.3% in WT, which supported previous study shown in cultured neurons the function of Cdk5 suppressing neuronal cell cycle[29,71]. This perhaps has to do with Cdk5's well known function in early brain development and critical role for maintaining the integrity of the central nervous system[72]. There were several other tissue types that exhibited noticeable increase in cell proliferation when Cdk5 was mutated. These apparent differences were found in the olfactory epithelium (26.0% *cdk5*$^{-/-}$ vs. 15.3% WT), the lung (29.7% *cdk5*$^{-/-}$ vs. 23.4% WT) and the duodenum (13.2% *cdk5*$^{-/-}$ vs. 6.9% WT) (Fig. 7B). Other organs such as the kidney and liver were not conclusive (data not shown). Nevertheless, it was interesting to discover that cell proliferation in tissues other than the brain could also be affected by the absence of Cdk5. Combined with our

initial *in vitro* data showing the loss of Cdk5 enhances cell proliferation, it is speculated that Cdk5 can function as a cell cycle inhibitor in non-neuronal cell cycle.



	% Ki67 positive cells	
	wt	cdk5$^{-/-}$
Brain	7 ± 0.3	18 ± 0.3
Olfactory epithelium	15 ± 0.3	25 ± 1.0
Lung	23 ± 0.4	29 ± 0.7
Duodenum	6 ± 0.9	13 ± 0.2

Fig. 7

Fig. 7: Cdk5$^{-/-}$ mouse embryos display more proliferating cells.

Representative images of paired WT and Cdk5$^{-/-}$ mouse embryos tissue sections, 10 μm in thickness are shown. Whole sectioned embryos were stained with Ki67 cell proliferation marker. Various regions of Cdk5$^{-/-}$ mouse embryos were compared with the corresponding regions in the WT mouse embryos. Images of sections were photographed and five were randomly selected for quantification. Percentages of Ki67 cells were calculated from the number of Ki67 positive cells divided by total number of cells in the field. Standard error for counting was also calculated.

3.5 Absence of Cdk5 promotes G1/S activation

As discovered in post-mitotic neurons, Cdk5 has been shown to prevent the occurrence of dedifferentiation or S-phase re-entry by maintaining their G1 cell cycle arrest status[73,74]. This information allowed us to identify the possibility of Cdk5 regulating G1/S transition in non-neuronal mitotic cells, which is still unknown. Some of the key events that orchestrate cells to migrate from G1 to S-phase include the activation of the STAT3 and MAPK pathways, as well as shifting the balance of Cyclins and CKIs. Since my primary $Cdk5^{-/-}$ MEF cells proliferate at a much faster rate than its paired WT, I sought to compare the differences in expressions of proteins that were responsible for G1/S transition. It was found that the level phospho-ERK1/2 of the MAPK pathway was enhanced in the absence of Cdk5. Previously Cdk5 has been shown to negatively regulate ERK1/2 activation[20]. Cyclin D1, responsible for promoting G1/S transition by interacting with Cdk4 and 6, was also found upregulated. Conversely, the known target phosphorylation site of Cdk5, S727 residue on STAT3[75] was reduced. Similar to p53, STAT3 can mediate the expression of CKIs, p21[76] and p27[77], which are cell cycle suppressors mostly active during G1 phase. My data revealed that both of these proteins were downregulated in the absence of Cdk5 (Fig 8A & B). This evidence demonstrates a potential function of Cdk5 for G1/S transition of non-neuronal cell cycle.

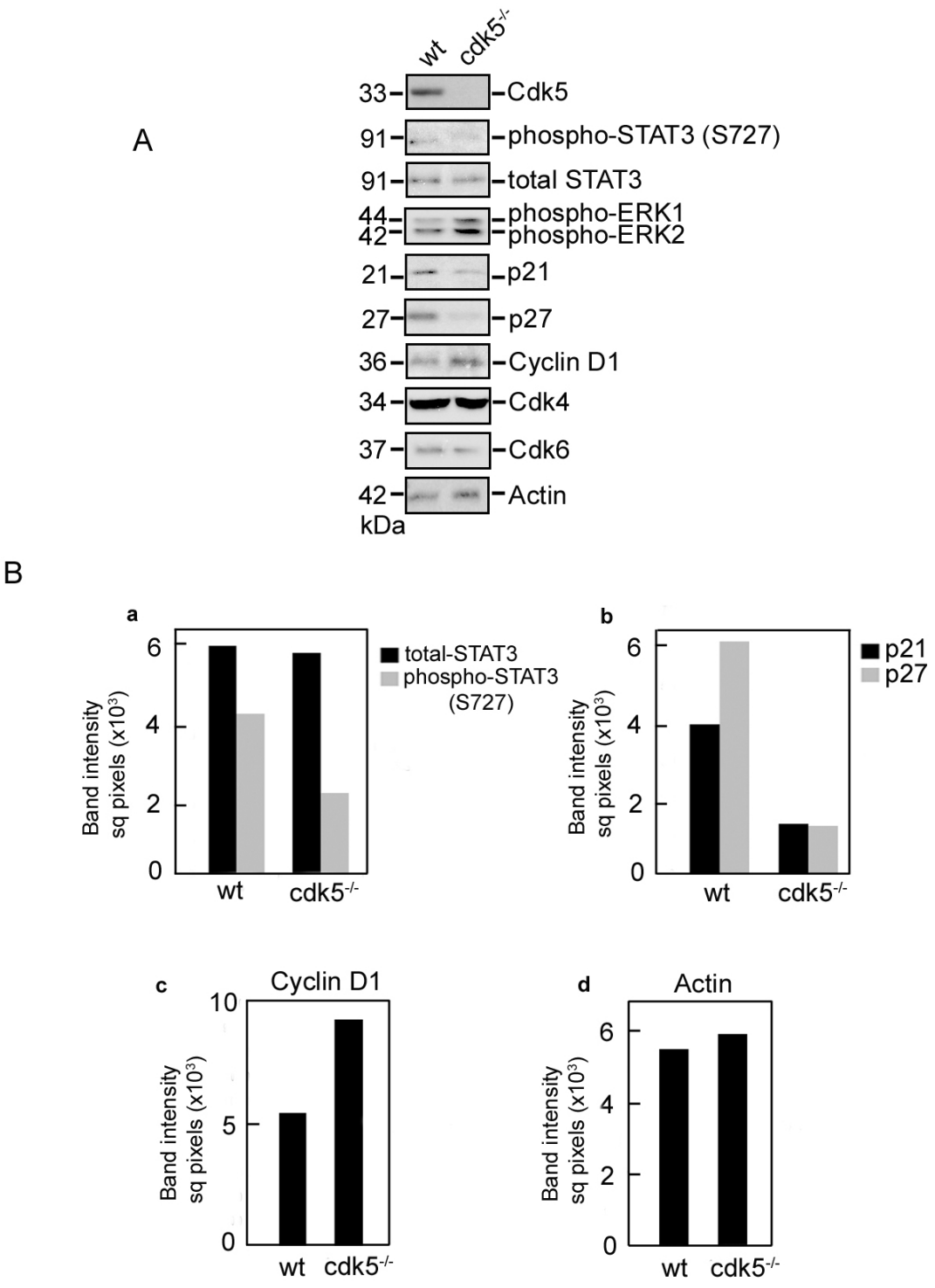

A
wt
cdk5-/-
33
Cdk5
91
phospho-STAT3 (S727)
91
total STAT3
44
42
phospho-ERK1
phospho-ERK2
21
p21
27
p27
36
Cyclin D1
34
Cdk4
37
Cdk6
42
Actin
kDa
B
a
Band intensity sq pixels (x10³)
total-STAT3
phospho-STAT3 (S727)
wt
cdk5-/-
b
p21
p27
c
Cyclin D1
d
Actin

Fig. 8

Fig. 8: Cdk5 downregulates pro-cell cycle progression proteins and upregulates cell suppression proteins.

Western blot analysis of 40 μg total cell lysates (A) Comparison of phopho-S727 STAT3, total-STAT3, phospho-ERK1/2, p21, p27, and Cyclin D1 protein expression profiles in WT and $Cdk5^{-/-}$ MEF samples. Cdk5, 4 and 6 levels were also analyzed. Actin was used as loading control. (B) Quantification of some of the proteins in (A) using the ImageJ program, which measures protein band intensity.

3.6 Cdk5$^{-/-}$ cells exhibit higher S-phase and aneuploidy.

To further illustrate the outcome of losing Cdk5 function in the cell cycle, and how it may affect G1 and S populations of cells, flow cytometry analysis of unsynchronized primary Cdk5$^{-/-}$ and WT MEF cells was performed to characterize their cell cycle profiles. Fixed Cdk5$^{-/-}$ and WT MEF cells were stained with PI, and subsequently sorted based on its ploidy and DNA amount, which translated into specific phases of the cell cycle. Considering only diploid populations, Cdk5$^{-/-}$ MEF cells displayed a significantly higher S-phase population compared to WT MEF cells. The G2/M population was also slightly higher, though not shown statistically significant, in the absence of Cdk5 (Fig. 9C). This result was consistent with n = 3 of primary MEF cells isolated from three sets of breeding pairs. Interestingly, the lack of Cdk5 also generated a higher frequency of aneuploidy cells (Fig. 9A & B), suggesting while both Cdk5$^{-/-}$ and WT cells were grown under the same condition, Cdk5$^{-/-}$ cells were probably more prone to genomic instability.

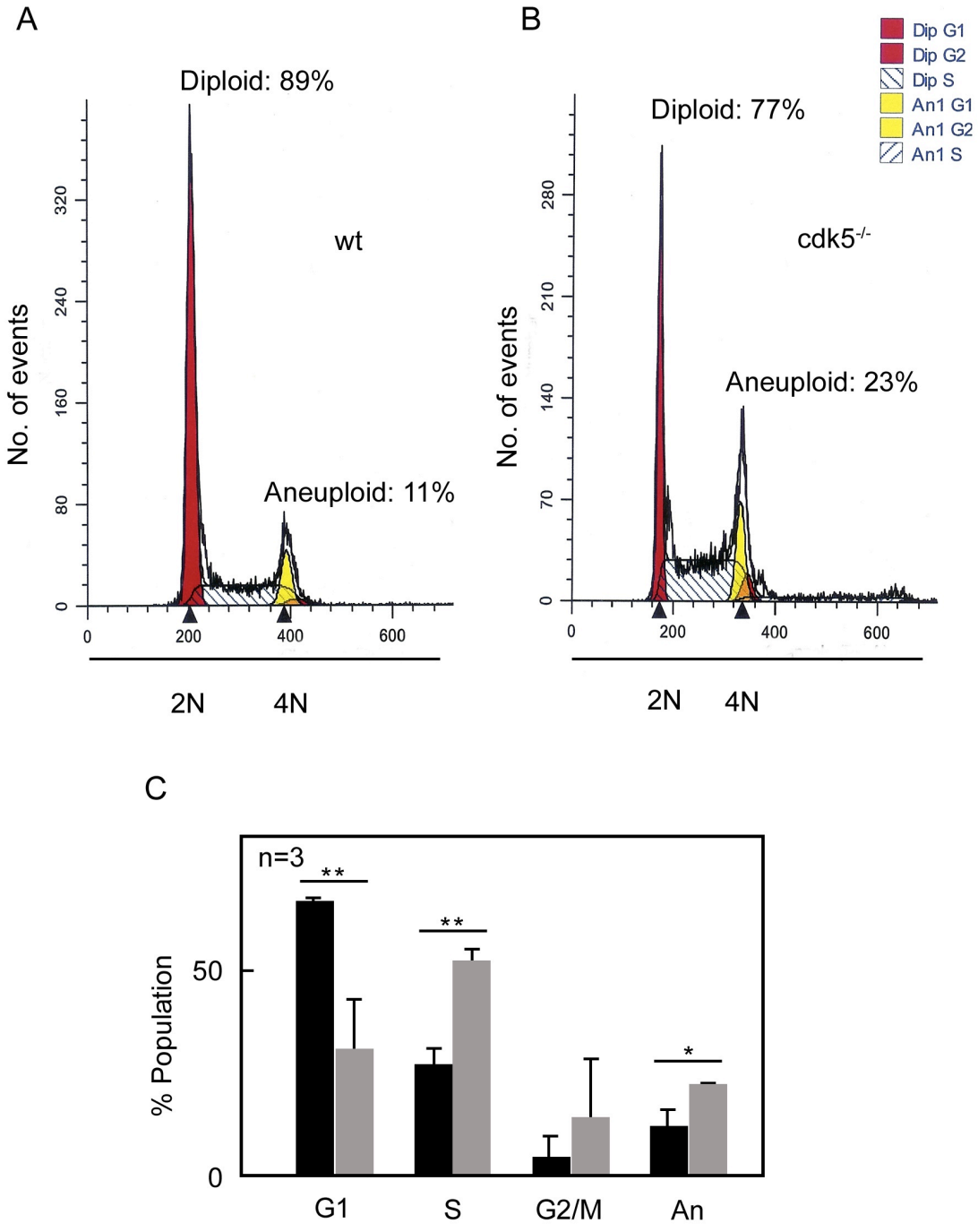

A
Diploid: 89%
wt
Aneuploid: 11%
No. of events
0
80
160
240
320
0
200
400
600
2N
4N
B
Dip G1
Dip G2
Dip S
An1 G1
An1 G2
An1 S
Diploid: 77%
cdk5-/-
Aneuploid: 23%
No. of events
0
70
140
210
280
0
200
400
600
2N
4N
C
n=3
**
**
*
% Population
0
50
G1
S
G2/M
An

Fig. 9

Fig. 9: Cdk5$^{-/-}$ MEF cells exhibit significantly higher S-phase and aneuploidy population.

Flow cytometry analysis of unsynchronized (A) WT and (B) Cdk5$^{-/-}$ MEF cells. (C) Cell cycle phases (G1, S and G2/M) and aneuploidy distribution of unsynchronized WT and Cdk5$^{-/-}$ MEF cells. For G1, S and G2/M, only diploid population was considered. Data was derived from n = 3 batches of MEF cells from three separate breeding pairs. Statistic was performed using Student's T-test, ** = p-value < 0.005; * = p-value < 0.05.

3.7 The lack of Cdk5 results in early S-phase entry and faster progression through the cell cycle.

To further validate the effects of losing Cdk5 on G1/S transition, cell cycle patterns of $Cdk5^{-/-}$ and WT MEF cells starting from G1 were compared. To synchronize $Cdk5^{-/-}$ and WT MEF cells to the starting point at G1 phase, they were grown to full confluence, which induced growth-contact inhibition. Trypsinization and replate in growth media would re-initialize cell cycle progression. Cells were harvested at various time points after G1 release, starting from 8 hrs to 51 hrs. Since my earlier data showed that the proliferation rate of $Cdk5^{-/-}$ MEF cells was significantly higher compared to WT and also, given the high percentage of S-phase population resulted from the loss of Cdk5, it was expected $Cdk5^{-/-}$ MEF cells would migrate through G1 to S-phase faster than WT MEF cells. Indeed, through PI staining and flow cytometry analysis of my collected samples, $Cdk5^{-/-}$ MEF cells displayed early and increased S-phase entry (Fig. 10A). By 19 hrs, there were almost twice as many $Cdk5^{-/-}$ MEF cells (41%) that entered S-phase compared to WT cells (24%). For $Cdk5^{-/-}$ MEF cells, population of cells situated at S-phase peaked after 24 hrs (55%) (Fig. 10A & B), and a second round of S-phase occurred after 40 hrs (54%). On the other hand, WT MEF cells displayed a slower increase of S-phase population, from 13% at 8 hrs to 32% at 51 hrs (Fig. 10A), indicating *cdk5*$^{-/-}$ progress through G1 and transits into S-phase faster than normal.

Cyclin D1, an activator for Cdk4 and 6, is required for G1/S transition. The downstream target is pRb, where phosphorylation of the pRb protein is critical to release the E2F

transcription factor preparing cells entering S-phase. Hence, to further solidify the observation that Cdk5 plays a role in G1/S transition, $Cdk5^{-/-}$ and WT samples 8 hrs after G1 release were taken to measure the levels of Cyclin D1-Cdk complex activity, which would correlate with the amount of pRb phosphorylation. Cyclin D1 in $Cdk5^{-/-}$ and WT samples were initially immuno-precipitated, and subsequently incubated with $p70^{N\text{-}RB}$, a 70 kDa N-terminal portion of pRb that contained Cyclin D1-Cdk complex phosphorylation sites. Based on my kinase assay data, it was shown phosphorylation of $p70^{N\text{-}RB}$ was visibly higher in the $Cdk5^{-/-}$ cells, 8 hrs after G1 release (Fig. 11).

Next, protein expression of Cyclin A, and B1 in proliferating $Cdk5^{-/-}$ and WT MEF cells was analyzed (Fig. 12). Cyclin A expression occurs in S-phase and peaks during late S-phase entering G2-phase of the cell cycle[78]. As observed by western blot analysis, detectable Cyclin A protein occurred at 19 hrs after G1 release in $Cdk5^{-/-}$ MEF cells, as compared to 27 hrs in WT MEF cells. The level of Cyclin A expressed was also higher (Fig 12B). Cyclin A level was shown markedly lower after 27 hrs and oscillated at 44 hrs, while WT MEF cells exhibited a slower peak of Cyclin A at 27 hrs, and later protein expression returned at 51 hrs. Similarly, western blot analysis of the G2/M transition regulator Cyclin B1 also revealed early and enhanced expression pattern in the absence of Cdk5 (Fig. 12C).

Additionally, $Cdk5^{-/-}$ and WT MEF cells were synchronized into G1 to S boundary by using HU. Interestingly, upon releasing these cells from arrest, it was discovered that $Cdk5^{-/-}$ MEF cells escaped from G1/S boundary into S-phase at ease compared to WT

MEF cells (Fig. 13). Further testing is needed to determine whether Cdk5 also has regulating function the moment entering S phase or within S phase. Collectively however, my data strongly implicates that Cdk5 plays a role in G1/S transition during cell cycle progression, where the absence of it causes cells to enter S-phase much earlier, and hence faster cell cycle progression.

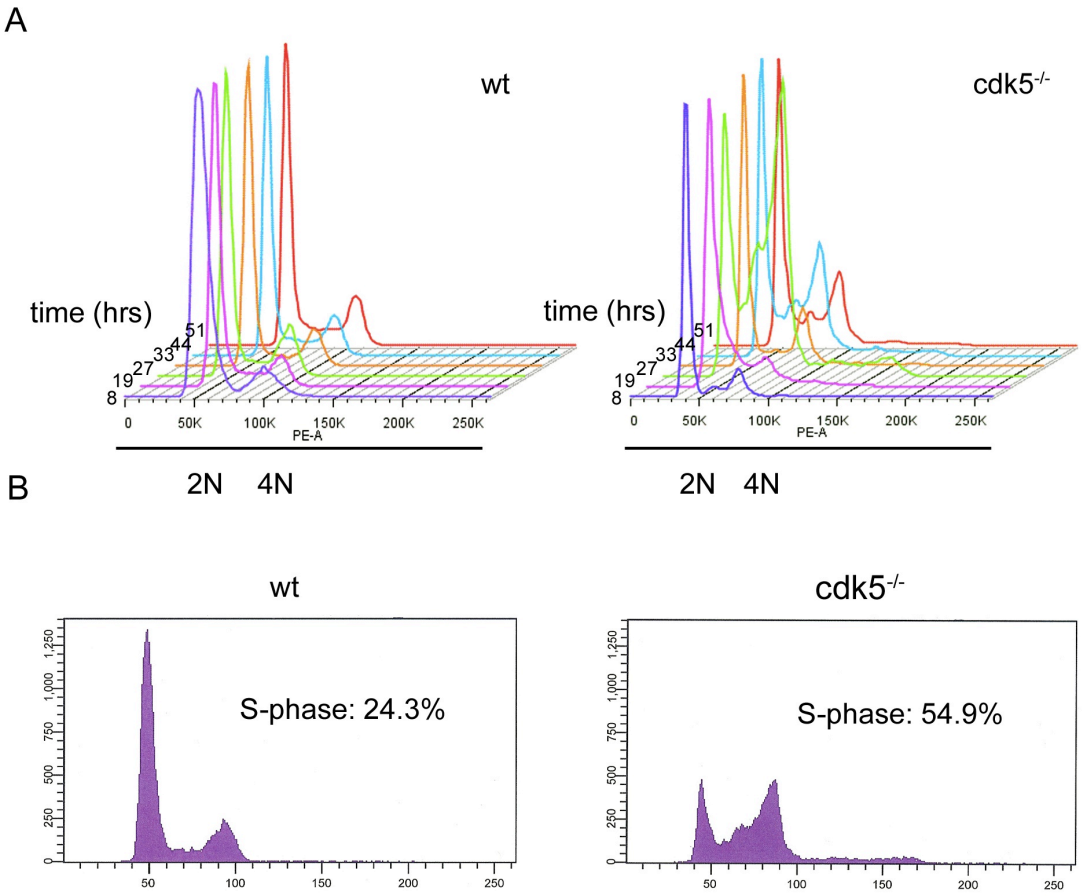

A
wt
cdk5-/-
time (hrs)
8
19
27
33
44
51
0
50K
100K
150K
200K
250K
PE-A
2N
4N
B
wt
cdk5-/-
S-phase: 24.3%
S-phase: 54.9%
0
250
500
750
1000
1250
50
100
150
200
250

Fig. 10

Fig. 10: Loss of Cdk5 enhances early entry into S-phase and oscillation of the cell cycle.

(A) Flow cytometry analysis of cell cycle progressing paired WT and $Cdk5^{-/-}$ MEF cells. Cells were G1 synchronized using growth contact inhibition method. G1 arrest release occurred when cells were trypsized and reseeded. Cell cycle progressing cells were collected at different times between 8 to 51 hrs. (B) S-phase distribution comparison of synchronized WT and $Cdk5^{-/-}$ MEF cells 27 hrs after G1 released. Only diploid population was considered.

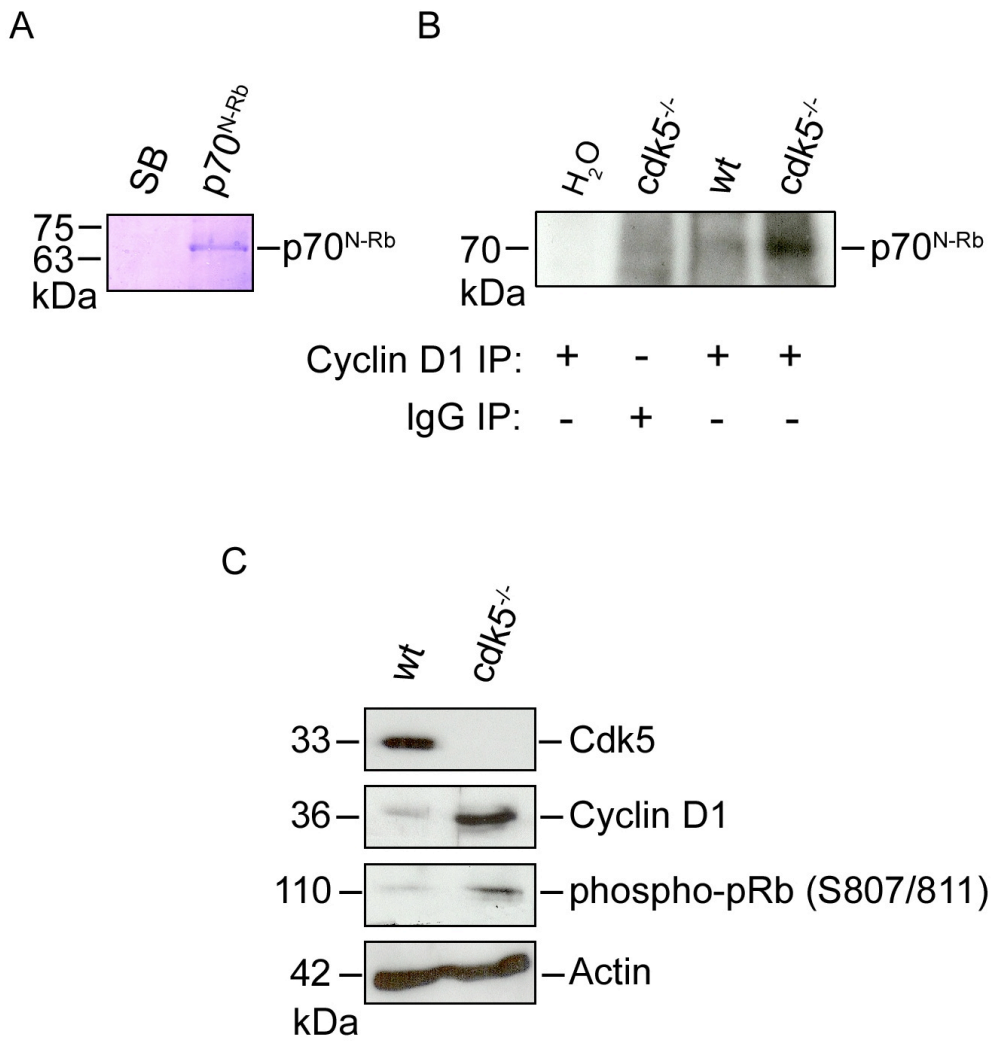

A
SB
p70N-Rb
75
63
kDa
p70N-Rb
B
H2O
cdk5-/-
wt
cdk5-/-
70
kDa
p70N-Rb
Cyclin D1 IP: + - + +
IgG IP: - + - -
C
wt
cdk5-/-
33
Cdk5
36
Cyclin D1
110
phospho-pRb (S807/811)
42
Actin
kDa

Fig. 11

Fig. 11: Absence of Cdk5 results in enhanced G1/S transition indicated by upregulation of pRb phosphorylation.

(A) A coomassie blue stained SDS-PAGE gel showing the presence of $p70^{N\text{-}RB}$ substrate, the N-terminal portion of pRb. (B) Autoradiogram showing an upregulation of $p70^{N\text{-}RB}$ phosphorylation due to Cyclin D1-Cdk complex in the absence of Cdk5. $Cdk5^{-/-}$ and WT MEF cells were synchronized to G1 by cell contact inhibition. Cells were trypsinized, lysed 8 hrs after replate. Cell lysates (1.5 mg) were incubated with either Cyclin D1-agarose beads or IgG-agarose beads for IP. IP'd samples were added to $p70^{N\text{-}RB}$ substrates containing ATP [γ-^{32}P]. SDS-PAGE and nitrocellulose membrane transfer were performed. The appearing phosphorylation was detected by X-ray film exposure. (C) Western blot analysis of $Cdk5^{-/-}$ and WT MEF samples visualizing the levels of Cyclin D1 and phospho-pRb (S807/811). Actin was used as a loading control.

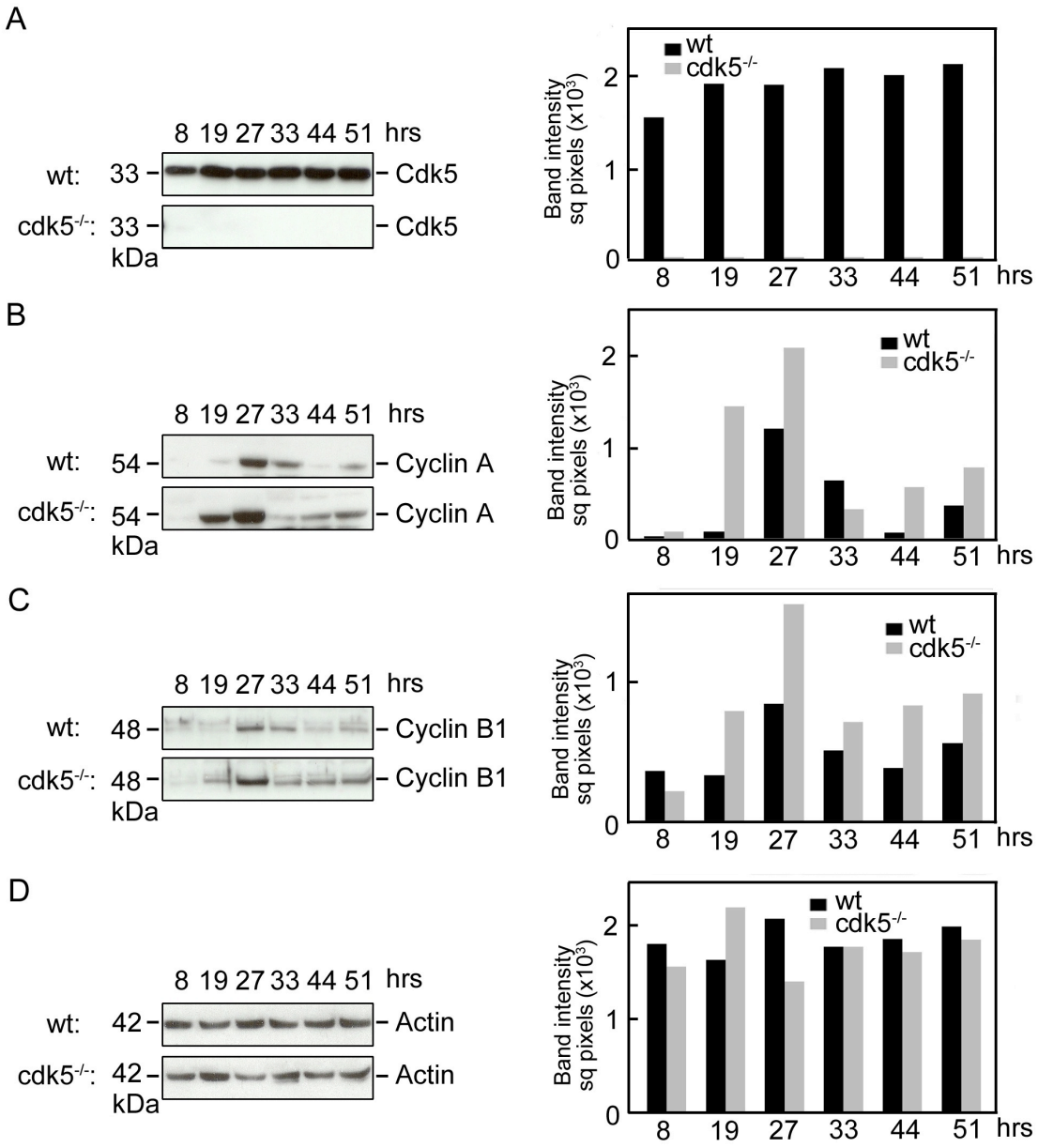

A
8 19 27 33 44 51 hrs
wt: 33 kDa
Cdk5
cdk5-/-: 33 kDa
Cdk5
wt
cdk5-/-
Band intensity sq pixels (x10³)
0
1
2
8 19 27 33 44 51 hrs
B
8 19 27 33 44 51 hrs
wt: 54 kDa
Cyclin A
cdk5-/-: 54 kDa
Cyclin A
wt
cdk5-/-
Band intensity sq pixels (x10³)
0
1
2
8 19 27 33 44 51 hrs
C
8 19 27 33 44 51 hrs
wt: 48 kDa
Cyclin B1
cdk5-/-: 48 kDa
Cyclin B1
wt
cdk5-/-
Band intensity sq pixels (x10³)
0
1
8 19 27 33 44 51 hrs
D
8 19 27 33 44 51 hrs
wt: 42 kDa
Actin
cdk5-/-: 42 kDa
Actin
wt
cdk5-/-
Band intensity sq pixels (x10³)
0
1
2
8 19 27 33 44 51 hrs

Fig. 12

Fig. 12: Loss of Cdk5 results in early entry into S-phase.

G1 Synchronized (A) WT and Cdk5$^{-/-}$ MEF cells were harvested with lysis buffer at specific time points 8, 19, 27, 33, 44, and 51 hrs after release from cell cycle arrest. 40 μg of each protein sample was used for western blotting analysis. Change in levels of (B) Cyclin A, and (C) B1 were analyzed. (D) Actin was used as a loading control.

P4 = G1
P6 = G1/S
P5 = S

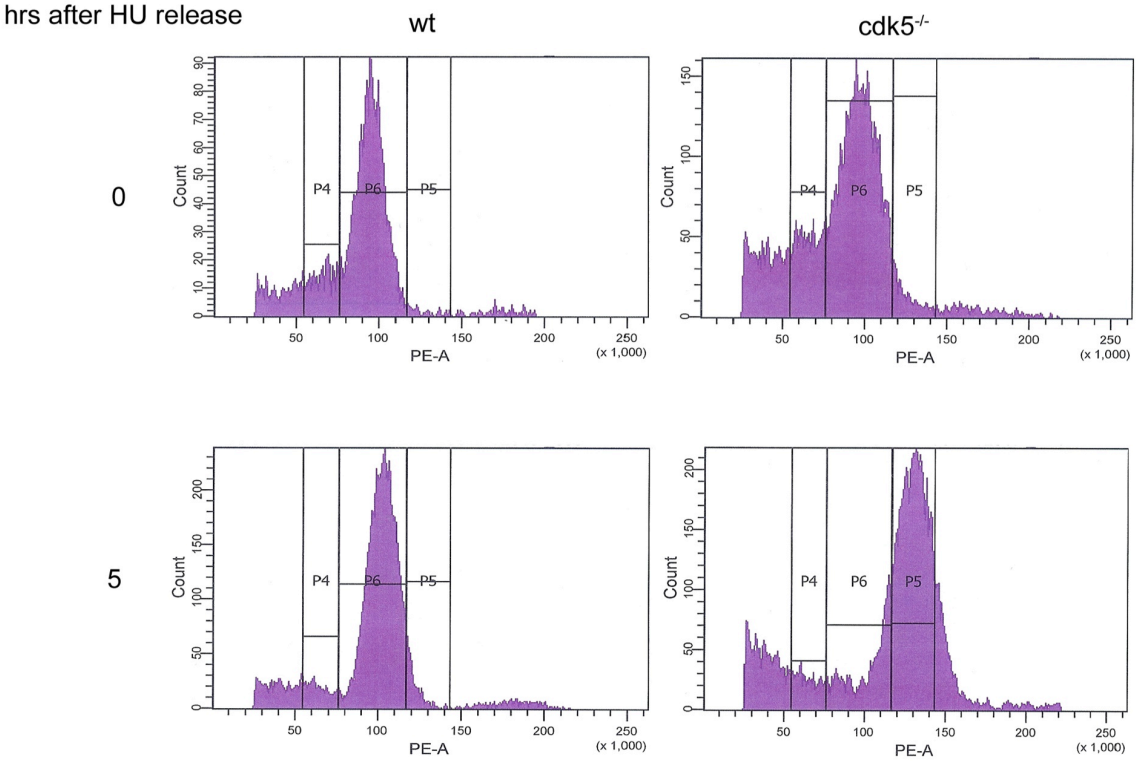


Fig. 13

Fig. 13: Loss of Cdk5 results in early migration of G1 to S-phase.

$Cdk5^{-/-}$ and WT MEF cells were synchronized to S-phase by HU. Samples were collected 5 hrs after HU release, and flow cytometry analysis was performed.

3.8 Loss of Cdk5 causes cells to become more susceptible to DNA damage.

To explore the molecular mechanism by which Cdk5 regulates the cell cycle, specifically during G1 to S-phase, I decided to investigate how the loss of Cdk5 affects G1/S through DNA damage. Based on my FACS analysis of $Cdk5^{-/-}$ and WT MEF cells, loss of Cdk5 function seemed to cause a higher frequency of generating aneuploidy cells, hinting that a faulty DNA repair pathway may be involved. Although it had previously been suggested that Cdk5 regulation of ATM was required for dsDNA break repair mechanism, but given its critical function protecting neuronal cells from dedifferentiation, I predicted that Cdk5's critical role in G1/S control was to maintain cell cycle inhibition under genotoxic stress conditions. Both primary $Cdk5^{-/-}$ and WT MEF cells that were synchronized to G1 by trypsinization and replate after growth contact inhibition were exposed with 4 Gy of IR, which induced dsDNA breaks. DNA damage in $Cdk5^{-/-}$ and WT cells was analyzed by detecting the level of γH2AX foci, an indicator for dsDNA breaks. Compared to WT, $Cdk5^{-/-}$ MEF cells revealed higher basal level of γH2AX as detected by western blot analysis and immunocytochemistry (Fig. 14A & C). This might correspond to the higher population of aneuploidy cells detected in my FACS analysis. Both $Cdk5^{-/-}$ and WT cells showed drastic elevation of γH2AX, 1 hr post IR-treatment (Fig. 14A). As expected, γH2AX levels dropped, closer to pre-IR treatment, after 24 hrs. This was an indication of DNA damage recovery. In contrast, $Cdk5^{-/-}$ MEF cells strikingly did not show reduction, but instead elevated level of γH2AX signals after 24 hrs (Fig 14B). Conversely, phospho-Ser15 p53 level was significantly lower in the absence of Cdk5 (Fig. 14A). While WT MEF cells showed expected transient upregulation of p53 phosphorylation at Ser15 site

after IR, phospho-Ser15 p53 in Cdk5$^{-/-}$ cells was minimal. This supports previous knowledge that p53 is target substrate of Cdk5. However, this is also the first evidence demonstrating using genetic approach that deletion of Cdk5 increases the likelihood of maintaining damaged DNA, indicated by sustained γH2AX foci. It is possible that lacking Cdk5, in part, reduces the phosphorylation of p53, which is required to maintain p53 stability[79]. Since p53 is a transcriptional activator for cell cycle suppressor protein p21, he "lesser-stable" p53 may contribute to the previously observed reduced expression of p21 found in Cdk5$^{-/-}$ cells.

We found that phospho-Ser15 p53 was dramatically reduced in the case of complete Cdk5 depletion. Overall, this led us to believe that the lack of Cdk5 impairs the DNA damage response and this, at least in part, is responsible for leading to an increasing cell proliferation rate and aneuploidy through genomic instability.

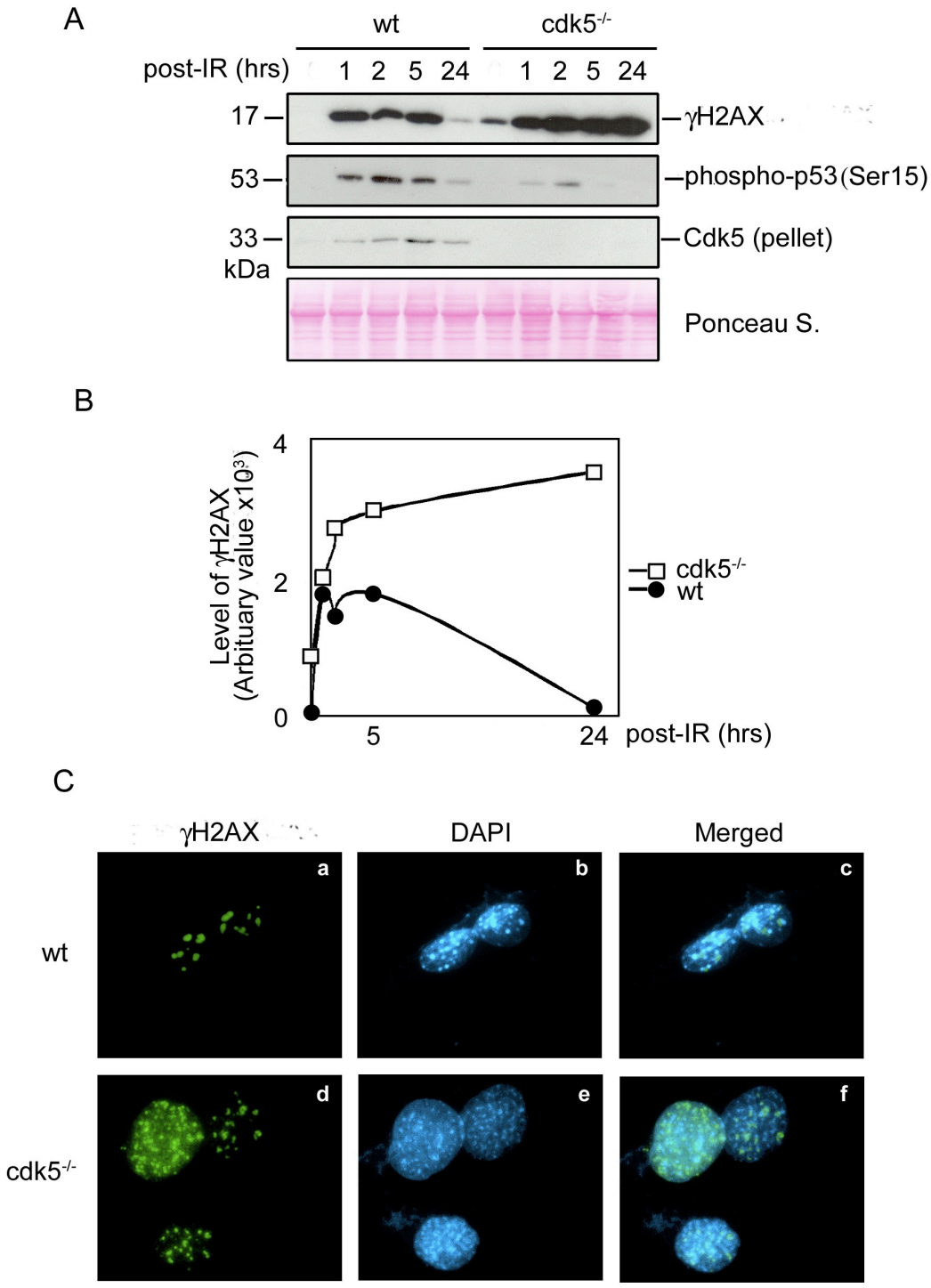

A
wt
cdk5-/-
post-IR (hrs)
1 2 5 24 1 2 5 24
17
53
33
kDa
γH2AX
phospho-p53(Ser15)
Cdk5 (pellet)
Ponceau S.
B
Level of γH2AX (Arbituary value x10^3)
4
2
0
5
24
post-IR (hrs)
cdk5-/-
wt
C
γH2AX
DAPI
Merged
wt
cdk5-/-
a
b
c
d
e
f

Fig. 14

Fig. 14: Loss of Cdk5 increases DNA damage and impairs DNA breaks repair process.

$Cdk5^{-/-}$ and WT MEF cells were synchronized to G1 by cell contact inhibition. Cell cycle was initiated by trypsinization and replate. Subsequently, $Cdk5^{-/-}$ and WT MEF cells were exposed to 4 Gy of IR. Cells were collected 1, 2, 5 and 24 hrs post-IR treatment by lysis buffer. (A) Detection of γH2AX, phospho-p53 and Cdk5 by western blot analysis. (B) The level of γH2AX in (A) was quantified by ImageJ program, based on protein band intensity. (C) Untreated $Cdk5^{-/-}$ and WT MEF cells were stained with γH2AX antibody (a and d), and DAPI (b and d). Representative images were taken under florescent microscope.

Chapter 4: Discussion and Future Direction

4.1 Discussion

Although Cdk5 was initially identified as a homologue of Cdk1 and Cdk2, most of the initial studies related to this protein kinase have been leaning towards its functions in differentiated neurons and brain development. The structural similarities of Cdk5 with other cdk family members allows it to bind to cell cycle regulators such as Cyclin D[80] and E[81], and PCNA[82], however none of these complexes exhibit activity, hence its involvement in cell cycle progression is a mystery. For the first time, however, our investigation found that lack of Cdk5 evidently affects cell proliferation outside of the neuronal context and causes cells to divide much faster than normal cells. Interestingly, similar observation was vaguely mentioned in previous report using *in vitro* Cdk5-siRNA system, but their data was not shown. In addition, our data also showed accelerated cell divisions as a result of Cdk5-depletion and can be slowed down when Cdk5 is introduced into cells. This suggests a potential role of Cdk5, perhaps as an inhibitor, to surveillance cell cycle progression.

Several studies performed in neurons have convincing evidence that suggest "new" functions of Cdk5 in cell cycle regulation. In particular, Kawauchi et al[83] revealed p27 protein stabilization and sublocalization depends on direct Cdk5/p35 binding. Two amino acid residues in p27, Ser10 and Thr187, were shown to be in *vitro* phosphorylation sites of Cdk5. The latter may be responsible for signaling p27 translocation from the nucleus to cytoplasm for degradation. Ser10 phosphorylation, on the other hand, is believed to stabilize p27 in the nucleus. My data revealed, in primary MEF cells, that p27 protein

expression was downregulated upon deletion of the c*dk5* gene. Although I was unable to successfully detect phospho-Ser10 p27 in my samples using western blot analysis, reduced p27 level supports Kawauchi et al's indication that the presence of Cdk5 is important for the stability of this protein. In addition, as p27 functions to suppress the cell cycle during G1 phase and entering S phase, the lower p27 level found in $Cdk5^{-/-}$ MEF cells compared to WT cells correlate with my observations that cell proliferation increases in the absence of Cdk5.

Similar to p27, another cip/kip family member p21, was also found downregulated in $Cdk5^{-/-}$ MEF cells. Like p27, p21 interferes with cell cycle progression through suppressing the migration of cells from G1 to S phase by inhibiting the activities of S phase cdk-cyclin complexes. Corresponding to this analysis, it was found that under unsynchronized conditions, $Cdk5^{-/-}$ MEF cells displayed a higher population of S phase cells. Although p27 and p21 may have compensatory roles, it has been proven that when both are depleted cell proliferation becomes highly aggressive. It is unclear whether the absence of Cdk5 directly contributes to p27 and p21's transcriptional activation or is due to a combination of other mechanisms. While STAT3 Ser727 can be phosphorylated by various protein kinases including Cdk5, promotes p21 and potentially p27 expressions. The downregulation of phospho-S727 STAT3 in the absence of Cdk5 activity may in part contribute to lowering the capacity for cell cycle inhibition. Cdk5 has also been shown to inhibit G1 genes that promote cell cycle progression, given our $Cdk5^{-/-}$ samples revealed explicitly high Cyclin D1 expression. Subsequently, in upregulated pRb phosphorylation, it is clear that the involvement of Cdk5 in the cell cycle is not dependent upon one single

cellular pathway. Nonetheless, the data obtained from my current study suggests that the lack of Cdk5 causes a deregulation in the cell cycle, possibly within G1 and/or S phase.

Further evidence from my flow cytometry analysis revealed that cell cycle phase transition from G1 to S happened much earlier in the $Cdk5^{-/-}$ than in the WT. This data was validated by $Cdk5^{-/-}$ samples displaying early and enhanced expression of Cyclin A following G1 release, and continuing faster cell cycle progression as indicated by the early presence of Cyclin B1 expression. This is comparable to studies in neuronal cells during development, Cdk5 may be important for helping the cell cycle to remain arrested. Reported by Cicero et al[84], abnormally high expressions of Cyclin A and D were found in the neocortex of $Cdk5^{-/-}$ E16.5 mouse embryos. Further shown *in vitro*, neurons without Cdk5 continued to express cell cycle-related proteins and enhanced uptake of BrDU proliferation marker[71]. Corresponding to our observation, $cdk5^{-/-}$ embryos exhibited increasing population of proliferating cells in the brain region, indicated by Ki67 proliferative marker. In both *in vitro* and *in vivo* events, neurons failed to stop cell divisions and begin differentiation. The importance of Cdk5 maintaining neuronal cell cycle control extends to adulthood. One of the major cellular events of neurodegeneration leading to Alzheimer's Disease was the unfavorable cell cycle reentry (dedifferentiation) of differentiated neurons[85]. A dramatic shift of Cdk5 localization from the nucleus to the cytoplasm was observed in the Aβ-treated cultured primary neurons[73], hence suggesting a pivotal role of Cdk5 localizing in the nucleus to associate with other cell cycle regulating proteins.

In our study we expected to see in the brain region of *cdk5*$^{-/-}$ embryos that would contain more Ki67 positive cells than its normal littermates. However, we were surprised to find similar characteristics in other tissues not related to the nervous system. It demonstrates that the novel cell cycle inhibitory function of Cdk5 also works in non-neuronal environment and regulates the proliferation of extraneuronal cells.

To understand the exact mechanism(s) of how Cdk5 participates in cell cycle inhibition particularly during G1 and G/S transition in non-neuronal cells may seem challenging due to Cdk5's ability to associate with many different protein substrates and influence multiple signaling pathways. One of the ways to address this was by looking at my data showing a Cdk5$^{-/-}$ condition that resulted higher frequency and more population of aneuploidy. This suggests that the absence of Cdk5 may facilitate genomic instability. To preserve genomic stability, cells have adopted a complex response system that enables cell cycle arrest and allows damaged DNA to repair properly. Hence, the mechanism(s) by which Cdk5 is involved in the cell cycle is possibly linked to cell cycle inhibition in response to detrimental genotoxic stress signals that are imposed to cells. Cells that fail to arrest may result in bypassing the surveillance needed for them to restore to normal function.

As Cdk5 has been demonstrated as an active player for DNA damage repair pathways, such as non-homologous end-joining (NHEJ) of dsDNA breaks, it was necessary to investigate the characteristics of Cdk5$^{-/-}$ MEF cells under this condition. NHEJ is the pathway in eukaryotic cells for repair of most IR-induced dsDNA breaks and is the chief

dsDNA break repair pathway in G0, G1[86]. As normal primary MEF cells were able to recover from 4 Gy of IR, indicated by the decreasing levels of γH2AX foci (a marker for dsDNA breaks), it became apparent that knocking out Cdk5 would diminish this ability. This observation supports previous findings where breast carcinoma cells CAL51 showed increasing numbers of γH2AX foci upon Cdk5 depletion by siRNA[56]. There are several plausible mechanisms suggested by which Cdk5 may take part in response to DNA damage. For example, DNA damage response protein p53 stabilization has been shown directly connected to Cdk5's phosphorylation on its Ser15, Ser33 and Ser46 residues[87]. Stably active p53 triggers transcriptional activation of genes that are responsible for cell cycle arrest, DNA repair and apoptosis. Examples of these response elements include p21[88], XPC[89], and PUMA[90] respectively. Upon IR-treatment, G1 released $Cdk5^{-/-}$ MEF cells expectedly showed reducing levels of Ser15 phospho-p53 suggesting the lack of Cdk5 disrupts the p53 mediated DNA damage response process. Therefore, the decreased p21 expression observed in our $Cdk5^{-/-}$ samples and the resulting increase in cell proliferation may be explained in part by the lack of Cdk5 phosphorylation of p53. Alternatively, p53 is regulated through ATM activation, which can also be modulated by Cdk5. In response to a DNA damage signal, Cdk5 is activated and phosphorylates ATM Ser794, a prerequisite required for ATM to activate autophosphorylation at Ser1981[52]. Fully activated ATM subsequently phosphorylates downstream targets such as p53 and γH2AX. For neuronal cells, the importance of Cdk5-ATM association was suggested to protect any damaged neurons from triggering cell cycle re-entry and the apoptotic signaling pathway and allowed the time needed for repair. The absence of Cdk5 may therefore diminish Cdk5-ATM association hence that ability. Moreover, Cdk5 regulation

of ATM activation was recently shown not only exclusive in the neuronal context, but also in cancer cells. It was discovered that abrogation of Cdk5 activation after DNA damage could induce spontaneous dsDNA breaks.

Conversely, DNA repair mechanisms regulated by Cdk5 have been found in another study suggesting it was via STAT3 activation. HT29 cells that were exposed to genotoxic agents, in this case the Topoisomerase I inhibitor, caused Cdk5 activation resulting in the phoshporylation of Ser727 of STAT3. They showed that phospho-Ser727 STAT3 subsequently bound to the promoter region of Eme1, an endonuclease involved in DNA repair[91]. Contradictory to other findings, Cdk5's activation of STAT3 would inactivate p53 and transcriptional activation of p21. However, it was also noted that Cdk5 regulation of STAT3 activation might have extended function beyond G1 phase and G1/S transition, but also regulate DNA repair mechanism during G2 phase. It was apparent in our study that both STAT3 and p53 phosphorylation were affected in the absence of Cdk5. Collectively, these data further emphasize the complexity of cellular function and network in regards to Cdk5's involvement. Our study demonstrated using both *in vitro* and *in vivo* systems that Cdk5 plays an important role in the cell cycle. Specifically with a potential cell cycle checkpoint function during G1 to S transition as well as protecting genomic stability. It also changed our initial beliefs that Cdk5 plays no role in non-neuronal and normal dividing cells.

4.2 Future Direction

Our current data showed normal cell cycle regulation requires the presence of Cdk5, and the lack of Cdk5 causes an irregular proliferation rate, as well as inducing an increased frequency of genomic instability. Furthermore, the data from this study have also indicated Cdk5's cell cycle function towards G1, G1/S. Cdk5 regulation of G1 and/or G1/S transition is possibly via p53 and STAT3 pathways, regulating the expressions of cell cycle suppressor proteins such as p21 and p27 (Fig. 15). Proper cell cycle arrest induced by genotoxic stress may be abrogated in the absence of Cdk5. Without responding to cell cycle arrest signals, cells are not given the time required to repair its damaged DNA. Multiple rounds of this process may lead to a devastating outcome. Cells with malfunctioning cell cycle control can potentially lead to the development of cancer.

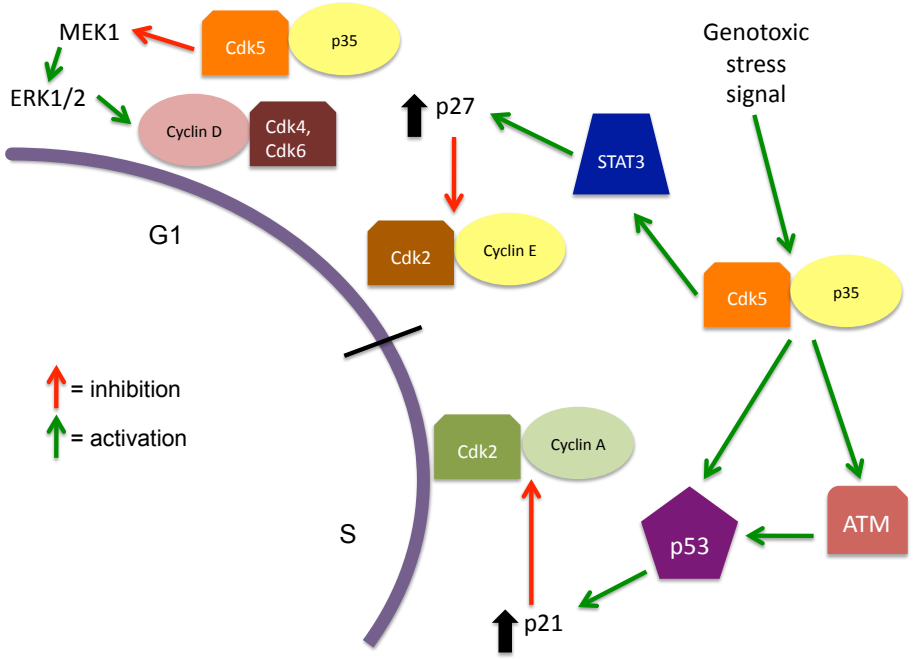


Fig. 15

Fig. 15: A schematic diagram of how Cdk5 may contribute to regulating G1/S of the cell cycle.

It was also interesting to observe that Cyclin D1 was enhanced significantly in our Cdk5$^{-/-}$ samples. A study by Maestre C et al[92] has shown that Cdk5 modulates the activity of E3 ubiquitin ligase APC/C through the phosphorylation of Ser40, Thr121, and Ser163 of Cdh1 (a direct activator of APC/C). Cdk5 modulated APC/C-Cdh1 was sufficient to regulate the stability of Cyclin B1. Since the APC/C-Cdh1 complex occurs late in the M phase replacing APC/C-Cdc20 entering G1, it would be worthwhile to investigate whether Cdk5 directly involves this process linking to Cyclin D1 protein stabilization at G1.

Based on our initial flow cytometry analysis utilizing HU to synchronize MEF cells into S phase, we found supported evidence that Cdk5$^{-/-}$ MEF cells progressed into S-phase significantly faster than WT MEF cells. We cannot rule out the possibility that Cdk5 also plays a role in regulating the S-phase and/or intra-S checkpoint. The subsequent experiments require focusing on intra-S checkpoint functions by treating S-phase synchronized cells with IR inducing DNA damage, and analyzing the activated levels of related proteins such as ATM, Chk1, Chk2 and p53. Interestingly, Turner et al have also previously suggested a novel function of Cdk5 regulating the intra-S (and possibly G2/M) checkpoint in regards to DNA damage response[56,93]. Alternatively, intra-S checkpoint activation can be achieved by treating cells with methyl methanesulfonate (MMS). MMS is an alkyating agent that causes dsDNA breaks. Similarly, FACS analysis should also be performed to confirm whether depletion of Cdk5 would bypass the intra-S checkpoint function. Furthermore, as we suspect that dsDNA breaks are increased in the absence of

Cdk5, DNA damage quantification method such as the comet assay should be performed to validate our prediction.

In conclusion, I present my findings that Cdk5 is a cell cycle regulation. My results have opened new areas of studying Cdk5 as a cell cycle inhibitor. Further investigation in the future on the role of Cdk5 in the cell cycle may provide new insight about this unique cdk family member, this information can be used to better understand the basis behind cell cycle progression, and possibly derive new therapeutic strategies in the future to impose on uncontrollable cancer cells growth.

References

1 Lee, M. G. & Nurse, P. Complementation used to clone a human homologue of the fission yeast cell cycle control gene cdc2. *Nature* **327**, 31-35, doi:10.1038/327031a0 (1987).

2 Lee, M. G. & Nurse, P. Cell cycle genes of the fission yeast. *Sci Prog* **71**, 1-14 (1987).

3 Sridhar, J., Akula, N. & Pattabiraman, N. Selectivity and potency of cyclin-dependent kinase inhibitors. *AAPS J* **8**, E204-221, doi:10.1208/aapsj080125 (2006).

4 Harper, J. W. & Adams, P. D. Cyclin-dependent kinases. *Chem Rev* **101**, 2511-2526, doi:cr0001030 [pii] (2001).

5 Fisher, R. P. Secrets of a double agent: CDK7 in cell-cycle control and transcription. *J Cell Sci* **118**, 5171-5180, doi:118/22/5171 [pii] 10.1242/jcs.02718 (2005).

6 Galbraith, M. D., Donner, A. J. & Espinosa, J. M. CDK8: a positive regulator of transcription. *Transcription* **1**, 4-12, doi:10.4161/trns.1.1.12373 (2010).

7 Romano, G. & Giordano, A. Role of the cyclin-dependent kinase 9-related pathway in mammalian gene expression and human diseases. *Cell Cycle* **7**, 3664-3668, doi:7122 [pii] (2008).

8 Liu, J. & Kipreos, E. T. Evolution of cyclin-dependent kinases (CDKs) and CDK-activating kinases (CAKs): differential conservation of CAKs in yeast and metazoa. *Mol Biol Evol* **17**, 1061-1074 (2000).

9 Zhang, L., Fried, F. B., Guo, H. & Friedman, A. D. Cyclin-dependent kinase phosphorylation of RUNX1/AML1 on 3 sites increases transactivation potency and stimulates cell proliferation. *Blood* **111**, 1193-1200, doi:blood-2007-08-109702 [pii]
10.1182/blood-2007-08-109702 (2008).

10 Nakayama, K. Cip/Kip cyclin-dependent kinase inhibitors: brakes of the cell cycle engine during development. *Bioessays* **20**, 1020-1029, doi:10.1002/(SICI)1521-1878(199812)20:12<1020::AID-BIES8>3.0.CO;2-D [pii]
10.1002/(SICI)1521-1878(199812)20:12<1020::AID-BIES8>3.0.CO;2-D (1998).

11 Denicourt, C. & Dowdy, S. F. Cip/Kip proteins: more than just CDKs inhibitors. *Genes Dev* **18**, 851-855, doi:10.1101/gad.1205304
18/8/851 [pii] (2004).

12 Beaudette, K. N., Lew, J. & Wang, J. H. Substrate specificity characterization of a cdc2-like protein kinase purified from bovine brain. *J Biol Chem* **268**, 20825-20830 (1993).

13 Kobayashi, S. *et al.* A cdc2-related kinase PSSALRE/cdk5 is homologous with the 30 kDa subunit of tau protein kinase II, a proline-directed protein kinase associated with microtubule. *FEBS Lett* **335**, 171-175, doi:0014-5793(93)80723-8 [pii] (1993).

14 Poon, R. Y., Lew, J. & Hunter, T. Identification of functional domains in the neuronal Cdk5 activator protein. *J Biol Chem* **272**, 5703-5708 (1997).

15 Zukerberg, L. R. *et al.* Cables links Cdk5 and c-Abl and facilitates Cdk5 tyrosine phosphorylation, kinase upregulation, and neurite outgrowth. *Neuron* **26**, 633-646, doi:S0896-6273(00)81200-3 [pii] (2000).

16 Tsai, L. H., Delalle, I., Caviness, V. S., Jr., Chae, T. & Harlow, E. p35 is a neural-specific regulatory subunit of cyclin-dependent kinase 5. *Nature* **371**, 419-423, doi:10.1038/371419a0 (1994).

17 Cai, X. H. *et al.* Changes in the expression of novel Cdk5 activator messenger RNA (p39nck5ai mRNA) during rat brain development. *Neurosci Res* **28**, 355-360, doi:S0168-0102(97)00063-1 [pii] (1997).

18 Hisanaga, S. & Saito, T. The regulation of cyclin-dependent kinase 5 activity through the metabolism of p35 or p39 Cdk5 activator. *Neurosignals* **12**, 221-229, doi:74624

74624 [pii] (2003).

19 Dhariwala, F. A. & Rajadhyaksha, M. S. An unusual member of the Cdk family: Cdk5. *Cell Mol Neurobiol* **28**, 351-369, doi:10.1007/s10571-007-9242-1 (2008).

20 Sharma, P. *et al.* Phosphorylation of MEK1 by cdk5/p35 down-regulates the mitogen-activated protein kinase pathway. *J Biol Chem* **277**, 528-534, doi:10.1074/jbc.M109324200

M109324200 [pii] (2002).

21 Zheng, Y. L. *et al.* Cdk5 Modulation of mitogen-activated protein kinase signaling regulates neuronal survival. *Mol Biol Cell* **18**, 404-413, doi:E06-09-0851 [pii]

10.1091/mbc.E06-09-0851 (2007).

22 Harada, T., Morooka, T., Ogawa, S. & Nishida, E. ERK induces p35, a neuron-specific activator of Cdk5, through induction of Egr1. *Nat Cell Biol* **3**, 453-459, doi:10.1038/35074516

35074516 [pii] (2001).

23 Hellmich, M. R., Pant, H. C., Wada, E. & Battey, J. F. Neuronal cdc2-like kinase: a cdc2-related protein kinase with predominantly neuronal expression. *Proc Natl Acad Sci U S A* **89**, 10867-10871 (1992).

24 Ohshima, T. *et al.* Targeted disruption of the cyclin-dependent kinase 5 gene results in abnormal corticogenesis, neuronal pathology and perinatal death. *Proc Natl Acad Sci U S A* **93**, 11173-11178 (1996).

25 Ohshima, T. *et al.* Cdk5 is required for multipolar-to-bipolar transition during radial neuronal migration and proper dendrite development of pyramidal neurons in the cerebral cortex. *Development* **134**, 2273-2282, doi:dev.02854 [pii]

10.1242/dev.02854 (2007).

26 Wang, C. X. *et al.* Cyclin-dependent kinase-5 prevents neuronal apoptosis through ERK-mediated upregulation of Bcl-2. *Cell Death Differ* **13**, 1203-1212, doi:4401804 [pii]

10.1038/sj.cdd.4401804 (2006).

27 Camins, A., Verdaguer, E., Folch, J., Canudas, A. M. & Pallas, M. The role of CDK5/P25 formation/inhibition in neurodegeneration. *Drug News Perspect* **19**, 453-460, doi:1043961 [pii]

10.1358/dnp.2006.19.8.1043961 (2006).

28 Su, S. C. & Tsai, L. H. Cyclin-dependent kinases in brain development and disease. *Annu Rev Cell Dev Biol* **27**, 465-491, doi:10.1146/annurev-cellbio-092910-154023 (2011).

29 Zhang, J. *et al.* Nuclear localization of Cdk5 is a key determinant in the postmitotic state of neurons. *Proc Natl Acad Sci U S A* **105**, 8772-8777, doi:10.1073/pnas.0711355105

0711355105 [pii] (2008).

30 Modi, P. K., Komaravelli, N., Singh, N. & Sharma, P. Interplay between MEK-ERK signaling, cyclin D1, and cyclin-dependent kinase 5 regulates cell cycle reentry and apoptosis of neurons. *Mol Biol Cell* **23**, 3722-3730, doi:10.1091/mbc.E12-02-0125

mbc.E12-02-0125 [pii] (2012).

31 Saito, T. *et al.* p25/cyclin-dependent kinase 5 promotes the progression of cell death in nucleus of endoplasmic reticulum-stressed neurons. *J Neurochem* **102**, 133-140, doi:JNC4540 [pii]

10.1111/j.1471-4159.2007.04540.x (2007).

32 Bu, B., Li, J., Davies, P. & Vincent, I. Deregulation of cdk5, hyperphosphorylation, and cytoskeletal pathology in the Niemann-Pick type C murine model. *J Neurosci* **22**, 6515-6525, doi:20026692

22/15/6515 [pii] (2002).

33 Xie, Z., Samuels, B. A. & Tsai, L. H. Cyclin-dependent kinase 5 permits efficient cytoskeletal remodeling--a hypothesis on neuronal migration. *Cereb Cortex* **16 Suppl 1**, i64-68, doi:16/suppl_1/i64 [pii]
10.1093/cercor/bhj170 (2006).

34 Noble, W. *et al.* Cdk5 is a key factor in tau aggregation and tangle formation in vivo. *Neuron* **38**, 555-565, doi:S0896627303002599 [pii] (2003).

35 Takahashi, S., Saito, T., Hisanaga, S., Pant, H. C. & Kulkarni, A. B. Tau phosphorylation by cyclin-dependent kinase 5/p39 during brain development reduces its affinity for microtubules. *J Biol Chem* **278**, 10506-10515, doi:10.1074/jbc.M211964200
M211964200 [pii] (2003).

36 Shukla, V., Skuntz, S. & Pant, H. C. Deregulated Cdk5 activity is involved in inducing Alzheimer's disease. *Arch Med Res* **43**, 655-662, doi:10.1016/j.arcmed.2012.10.015
S0188-4409(12)00297-4 [pii] (2012).

37 Lambert de Rouvroit, C. & Goffinet, A. M. Neuronal migration. *Mech Dev* **105**, 47-56, doi:S0925477301003963 [pii] (2001).

38 Chae, T. *et al.* Mice lacking p35, a neuronal specific activator of Cdk5, display cortical lamination defects, seizures, and adult lethality. *Neuron* **18**, 29-42, doi:S0896-6273(01)80044-1 [pii] (1997).

39 Angelo, M., Plattner, F. & Giese, K. P. Cyclin-dependent kinase 5 in synaptic plasticity, learning and memory. *J Neurochem* **99**, 353-370, doi:JNC4040 [pii]
10.1111/j.1471-4159.2006.04040.x (2006).

40 Tomizawa, K. *et al.* Cdk5/p35 regulates neurotransmitter release through phosphorylation and downregulation of P/Q-type voltage-dependent calcium channel activity. *J Neurosci* **22**, 2590-2597, doi:20026252
22/7/2590 [pii] (2002).

41 Ohshima, T. *et al.* Impairment of hippocampal long-term depression and defective spatial learning and memory in p35 mice. *J Neurochem* **94**, 917-925, doi:JNC3233 [pii]
10.1111/j.1471-4159.2005.03233.x (2005).

42 Li, B. S. *et al.* Regulation of NMDA receptors by cyclin-dependent kinase-5. *Proc Natl Acad Sci U S A* **98**, 12742-12747, doi:10.1073/pnas.211428098
211428098 [pii] (2001).

43 Cheng, Q. *et al.* Cdk5/p35 and Rho-kinase mediate ephrin-A5-induced signaling in retinal ganglion cells. *Mol Cell Neurosci* **24**, 632-645, doi:S1044743103002203 [pii] (2003).

44 Fu, W. Y. *et al.* Cdk5 regulates EphA4-mediated dendritic spine retraction through an ephexin1-dependent mechanism. *Nat Neurosci* **10**, 67-76, doi:nn1811 [pii]
10.1038/nn1811 (2007).

45 Kim, S. H. & Ryan, T. A. CDK5 serves as a major control point in neurotransmitter release. *Neuron* **67**, 797-809, doi:10.1016/j.neuron.2010.08.003
S0896-6273(10)00613-6 [pii] (2010).

46 Rosales, J. L. & Lee, K. Y. Extraneuronal roles of cyclin-dependent kinase 5. *Bioessays* **28**, 1023-1034, doi:10.1002/bies.20473 (2006).

47 Lazaro, J. B. *et al.* Cyclin dependent kinase 5, cdk5, is a positive regulator of myogenesis in mouse C2 cells. *J Cell Sci* **110 (Pt 10)**, 1251-1260 (1997).

48 Pallari, H. M. *et al.* Nestin as a regulator of Cdk5 in differentiating myoblasts. *Mol Biol Cell* **22**, 1539-1549, doi:10.1091/mbc.E10-07-0568
mbc.E10-07-0568 [pii] (2011).

49 Rosales, J. L. *et al.* ODF1 phosphorylation by Cdk5/p35 enhances ODF1-OIP1 interaction. *Cell Physiol Biochem* **20**, 311-318, doi:000107517 [pii]
10.1159/000107517 (2007).

50 Liebl, J., Furst, R., Vollmar, A. M. & Zahler, S. Twice switched at birth: cell cycle-independent roles of the "neuron-specific" cyclin-dependent kinase 5 (Cdk5) in non-neuronal cells. *Cell Signal* **23**, 1698-1707, doi:10.1016/j.cellsig.2011.06.020
S0898-6568(11)00192-6 [pii] (2011).

51 Ajay, A. K. *et al.* Cdk5 phosphorylates non-genotoxically overexpressed p53 following inhibition of PP2A to induce cell cycle arrest/apoptosis and inhibits tumor progression. *Mol Cancer* **9**, 204, doi:10.1186/1476-4598-9-204
1476-4598-9-204 [pii] (2010).

52 Tian, B., Yang, Q. & Mao, Z. Phosphorylation of ATM by Cdk5 mediates DNA damage signalling and regulates neuronal death. *Nat Cell Biol* **11**, 211-218, doi:10.1038/ncb1829
ncb1829 [pii] (2009).

53 Lin, H., Chen, M. C., Chiu, C. Y., Song, Y. M. & Lin, S. Y. Cdk5 regulates STAT3 activation and cell proliferation in medullary thyroid carcinoma cells. *J Biol Chem* **282**, 2776-2784, doi:M607234200 [pii]

10.1074/jbc.M607234200 (2007).

54 Upadhyay, A. K., Ajay, A. K., Singh, S. & Bhat, M. K. Cell cycle regulatory protein 5 (Cdk5) is a novel downstream target of ERK in carboplatin induced death of breast cancer cells. *Curr Cancer Drug Targets* **8**, 741-752 (2008).

55 Hsu, F. N. *et al.* Regulation of androgen receptor and prostate cancer growth by cyclin-dependent kinase 5. *J Biol Chem* **286**, 33141-33149, doi:10.1074/jbc.M111.252080

M111.252080 [pii] (2011).

56 Turner, N. C. *et al.* A synthetic lethal siRNA screen identifying genes mediating sensitivity to a PARP inhibitor. *EMBO J* **27**, 1368-1377, doi:10.1038/emboj.2008.61

emboj200861 [pii] (2008).

57 Bolin, C. *et al.* The impact of cyclin-dependent kinase 5 depletion on poly(ADP-ribose) polymerase activity and responses to radiation. *Cell Mol Life Sci* **69**, 951-962, doi:10.1007/s00018-011-0811-6 (2012).

58 Zhu, Y. X. *et al.* RNAi screen of the druggable genome identifies modulators of proteasome inhibitor sensitivity in myeloma including CDK5. *Blood* **117**, 3847-3857, doi:10.1182/blood-2010-08-304022

blood-2010-08-304022 [pii] (2011).

59 Zhang, J. *et al.* Cdk5 suppresses the neuronal cell cycle by disrupting the E2F1-DP1 complex. *J Neurosci* **30**, 5219-5228, doi:10.1523/JNEUROSCI.5628-09.2010

30/15/5219 [pii] (2010).

60 Chen, B. B., Glasser, J. R., Coon, T. A. & Mallampalli, R. K. FBXL2 is a ubiquitin E3 ligase subunit that triggers mitotic arrest. *Cell Cycle* **10**, 3487-3494, doi:10.4161/cc.10.20.17742

17742 [pii] (2011).

61 Zhang, J., Li, H., Zhou, T., Zhou, J. & Herrup, K. Cdk5 levels oscillate during the neuronal cell cycle: Cdh1 ubiquitination triggers proteosome-dependent degradation during S-phase. *J Biol Chem* **287**, 25985-25994, doi:10.1074/jbc.M112.343152

M112.343152 [pii] (2012).

62 Rosales, J. L., Rattner, J. B. & Lee, K. Y. The primary microcephaly 3 (MCPH3) interacting protein, p35 and its catalytic subunit, Cdk5, are centrosomal proteins. *Cell Cycle* **9**, 618-620, doi:10597 [pii] (2010).

63 Lee, K. Y., Mummery, A., Park, J., Tariq, H. & Rosales, J. L. Localization of CDK5 in the midbody and increased aneuploidy in CDK5-/- cells. *Cell Cycle* **9**, 3629-3630, doi:10.4161/cc.9.17.12722

12722 [pii] (2010).

64 Parrinello, S. *et al.* Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts. *Nat Cell Biol* **5**, 741-747, doi:10.1038/ncb1024

ncb1024 [pii] (2003).

65 Lee, K. Y., Helbing, C. C., Choi, K. S., Johnston, R. N. & Wang, J. H. Neuronal Cdc2-like kinase (Nclk) binds and phosphorylates the retinoblastoma protein. *J Biol Chem* **272**, 5622-5626 (1997).

66 Kirley, S. D., Rueda, B. R., Chung, D. C. & Zukerberg, L. R. Increased growth rate, delayed senescense and decreased serum dependence characterize cables-deficient cells. *Cancer Biol Ther* **4**, 654-658, doi:1732 [pii] (2005).

67 Castiel, A. *et al.* The Stil protein regulates centrosome integrity and mitosis through suppression of Chfr. *J Cell Sci* **124**, 532-539, doi:10.1242/jcs.079731 jcs.079731 [pii] (2011).

68 Zhu, L. & Skoultchi, A. I. Coordinating cell proliferation and differentiation. *Curr Opin Genet Dev* **11**, 91-97, doi:S0959-437X(00)00162-3 [pii] (2001).

69 Santamaria, D. *et al.* Cdk1 is sufficient to drive the mammalian cell cycle. *Nature* **448**, 811-815, doi:nature06046 [pii] 10.1038/nature06046 (2007).

70 Qiu, J. *et al.* p27Kip1 constrains proliferation of neural progenitor cells in adult brain under homeostatic and ischemic conditions. *Stem Cells* **27**, 920-927, doi:10.1002/stem.1 (2009).

71 Zhang, J., Li, H. & Herrup, K. Cdk5 nuclear localization is p27-dependent in nerve cells: implications for cell cycle suppression and caspase-3 activation. *J Biol Chem* **285**, 14052-14061, doi:10.1074/jbc.M109.068262 M109.068262 [pii] (2010).

72 Cruz, J. C. & Tsai, L. H. A Jekyll and Hyde kinase: roles for Cdk5 in brain development and disease. *Curr Opin Neurobiol* **14**, 390-394, doi:10.1016/j.conb.2004.05.002

S0959438804000698 [pii] (2004).

73 Lopes, J. P., Oliveira, C. R. & Agostinho, P. Cdk5 acts as a mediator of neuronal cell cycle re-entry triggered by amyloid-beta and prion peptides. *Cell Cycle* **8**, 97-104, doi:7506 [pii] (2009).

74 Futatsugi, A. *et al.* Cyclin-dependent kinase 5 regulates E2F transcription factor through phosphorylation of Rb protein in neurons. *Cell Cycle* **11**, 1603-1610, doi:10.4161/cc.20009

20009 [pii] (2012).

75 Fu, A. K. *et al.* Cyclin-dependent kinase 5 phosphorylates signal transducer and activator of transcription 3 and regulates its transcriptional activity. *Proc Natl Acad Sci U S A* **101**, 6728-6733, doi:10.1073/pnas.0307606100

0307606100 [pii] (2004).

76 Hawthorne, V. S. *et al.* ErbB2-mediated Src and signal transducer and activator of transcription 3 activation leads to transcriptional up-regulation of p21Cip1 and chemoresistance in breast cancer cells. *Mol Cancer Res* **7**, 592-600, doi:10.1158/1541-7786.MCR-08-0316

7/4/592 [pii] (2009).

77 de Koning, J. P. *et al.* STAT3-mediated differentiation and survival and of myeloid cells in response to granulocyte colony-stimulating factor: role for

the cyclin-dependent kinase inhibitor p27(Kip1). *Oncogene* **19**, 3290-3298, doi:10.1038/sj.onc.1203627 (2000).

78 Yam, C. H., Fung, T. K. & Poon, R. Y. Cyclin A in cell cycle control and cancer. *Cell Mol Life Sci* **59**, 1317-1326 (2002).

79 Ashcroft, M., Kubbutat, M. H. & Vousden, K. H. Regulation of p53 function and stability by phosphorylation. *Mol Cell Biol* **19**, 1751-1758 (1999).

80 Xiong, Y., Zhang, H. & Beach, D. D type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. *Cell* **71**, 505-514, doi:0092-8674(92)90518-H [pii] (1992).

81 Miyajima, M., Nornes, H. O. & Neuman, T. Cyclin E is expressed in neurons and forms complexes with cdk5. *Neuroreport* **6**, 1130-1132 (1995).

82 Zhang, H., Xiong, Y. & Beach, D. Proliferating cell nuclear antigen and p21 are components of multiple cell cycle kinase complexes. *Mol Biol Cell* **4**, 897-906 (1993).

83 Kawauchi, T., Chihama, K., Nabeshima, Y. & Hoshino, M. Cdk5 phosphorylates and stabilizes p27kip1 contributing to actin organization and cortical neuronal migration. *Nat Cell Biol* **8**, 17-26, doi:ncb1338 [pii]
10.1038/ncb1338 (2006).

84 Cicero, S. & Herrup, K. Cyclin-dependent kinase 5 is essential for neuronal cell cycle arrest and differentiation. *J Neurosci* **25**, 9658-9668, doi:25/42/9658 [pii]
10.1523/JNEUROSCI.1773-05.2005 (2005).

85 Lopes, J. P., Oliveira, C. R. & Agostinho, P. Cell cycle re-entry in Alzheimer's disease: a major neuropathological characteristic? *Curr Alzheimer Res* **6**, 205-212 (2009).

86 Shrivastav, M., De Haro, L. P. & Nickoloff, J. A. Regulation of DNA double-strand break repair pathway choice. *Cell Res* **18**, 134-147, doi:cr2007111 [pii]

10.1038/cr.2007.111 (2008).

87 Lee, J. H., Kim, H. S., Lee, S. J. & Kim, K. T. Stabilization and activation of p53 induced by Cdk5 contributes to neuronal cell death. *J Cell Sci* **120**, 2259-2271, doi:120/13/2259 [pii]

10.1242/jcs.03468 (2007).

88 Elbendary, A. A. *et al.* Relationship between p21 expression and mutation of the p53 tumor suppressor gene in normal and malignant ovarian epithelial cells. *Clin Cancer Res* **2**, 1571-1575 (1996).

89 Adimoolam, S. & Ford, J. M. p53 and DNA damage-inducible expression of the xeroderma pigmentosum group C gene. *Proc Natl Acad Sci U S A* **99**, 12985-12990, doi:10.1073/pnas.202485699

202485699 [pii] (2002).

90 Nakano, K. & Vousden, K. H. PUMA, a novel proapoptotic gene, is induced by p53. *Mol Cell* **7**, 683-694, doi:S1097-2765(01)00214-3 [pii] (2001).

91 Courapied, S. *et al.* The cdk5 kinase regulates the STAT3 transcription factor to prevent DNA damage upon topoisomerase I inhibition. *J Biol Chem* **285**, 26765-26778, doi:10.1074/jbc.M109.092304

M109.092304 [pii] (2010).

92 Maestre, C., Delgado-Esteban, M., Gomez-Sanchez, J. C., Bolanos, J. P. & Almeida, A. Cdk5 phosphorylates Cdh1 and modulates cyclin B1 stability in excitotoxicity. *EMBO J* **27**, 2736-2745, doi:10.1038/emboj.2008.195

emboj2008195 [pii] (2008).

93 Levacque, Z., Rosales, J. L. & Lee, K. Y. Level of cdk5 expression predicts the survival of relapsed multiple myeloma patients. *Cell Cycle* **11**, 4093-4095, doi:10.4161/cc.21886

21886 [pii] (2012).