Elucidating the roles of Aurora B kinase in neurons

Presented by

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Abstract

To reveal molecular determinates that underlie the intrinsic molecular pathways within neurons that support regeneration after injury, a DNA microarray study was performed on axotomized neuronal clusters that were maintained in culture and free from glial and astrocytes contamination. The microarray data indicated that post-injury regenerative sprouting requires two distinct pathways; a cell survival response to protect against pernicious secondary processes and a regenerative response driven by modulation of the neuronal cytoskeleton. From the transcriptomic data, cell cycle associated aurora B kinase (Aurkb), which was significantly up-regulated but never investigated in the context of neurons, was identified for further work. Immunohistochemical studies revealed that Aurkb is expressed extensively and cell-specifically in neurons of certain brain structures such as the cortex, hippocampus and amygdala. Its elevated expression in the embryonic brain cortex as compared to that of an adult implies that it may be involved in the process of brain maturation. Interestingly, the changing localization of Aurkb within developing cultured neurons and particularly its localisation outside of the nucleus at various stages of neuronal maturation further suggests that it may have direct roles neurite outgrowth. Indeed, impairing Aurkb activity in cultured neurons via different experimental approaches resulted in several key neuronal deficits. Generally, neurons with inactive Aurkb were found to have either shorter or no elaborated axons. They also possessed abnormally swollen cell bodies. Enlargement of the cell body, independent of nucleus size, was related to a substantial increase in microtubule mass within the area between the nucleus and axon hillock region. Furthermore, their expanded cell bodies are bordered by several aberrant, thin, frayed and highly disorganised neuritic processes. Next, yeast 2 hybrid identified INCENP as a binding partner of Aurkb in neurons. Subsequent phosphoproteomics studies coupled with functional
analysis of protein associations have further revealed that inhibition of neuronal Aurkb affected a cluster of proteins and kinases that are major players of neuronal cytoskeleton regulation and organisation. In conclusion, this is the first comprehensive study of Aurkb in the brain and neurons. Specifically, the phosphoproteomic, pharmacological and molecular knockin and knockout studies provided considerable evidence that Aurkb has key roles in neurite cytoskeleton modulation. Taken together, the work in this thesis has clearly identified a novel and alternate cell cycle independent function of Aurkb in post-mitotic neurons.
Declarations

Declaration of Originality
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Jian Ming Jeremy NG

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Preface

This thesis consists only of my original work towards the Ph.D. degree, and I received significant assistance for the following:

Chapter 3
Dr Jacqueline Leung and A/P. Roger Chung (University of Tasmania) have rendered tremendous assistance in the culturing of rat primary cortical neurons, axotomy of neuronal clusters and harvesting of RNA.

Chapter 4
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Next, I would like to thank my co-supervisor, A/P Roger S Chung, whom I’m greatly indebted to. He has provided great perspective, assistance and guidance, especially during the writing process. His support and words of encouragement were my pillars of strength and he is an inspirational mentor. I’m also extremely grateful for his help in some of the experiments, the invaluable career advice he has provided along the way and the time he spent on sharing his personal experience and journey to being an outstanding researcher.

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Publications and presentations

Publications derived from this thesis


Other publications


Conferences

The Neurobiology of Dementia Research School 2011 (Hobart, Tasmania, Australia): Transcriptional insights on the regenerative mechanics of axotomized neurons in vitro, Jeremy Ng, J.M., Cheung, N.S., Chung, R.S.

The 3rd Australia-China Biomedical Research Conference; Inflammation and chronic diseases: arthritis and beyond (Hobart, Tasmania, Australia): Organising committee and in attendance.
# List of abbreviations

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<tr>
<td>+TIP</td>
<td>Plus-end tracking protein</td>
</tr>
<tr>
<td>A2</td>
<td>2 month old adult mouse</td>
</tr>
<tr>
<td>A8</td>
<td>8 month old adult mouse</td>
</tr>
<tr>
<td>AD</td>
<td>Activation domain</td>
</tr>
<tr>
<td>Adcy</td>
<td>Adenylate cyclase</td>
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<tr>
<td>Add</td>
<td>Adducin</td>
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<tr>
<td>Aurka</td>
<td>Aurora A kinase</td>
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<td>Aurkb</td>
<td>Aurora B kinase</td>
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<tr>
<td>Aurkc</td>
<td>Aurora C kinase</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BLA</td>
<td>Basolateral amygdala anterior</td>
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<td>BLV</td>
<td>Basolateral amygdala ventral</td>
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<tr>
<td>BMA</td>
<td>Basomedial amygdala anterior</td>
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<tr>
<td>BMP</td>
<td>Basomedial amygdala posterior</td>
</tr>
<tr>
<td>Bmp</td>
<td>Bone morphogenetic proteins</td>
</tr>
<tr>
<td>C</td>
<td>Cortical plate</td>
</tr>
<tr>
<td>CA1</td>
<td>Field CA1 of the hippocampus</td>
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<td>CA2</td>
<td>Field CA2 of the hippocampus</td>
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<td>CA3</td>
<td>Field CA3 of the hippocampus</td>
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<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<tr>
<td>Cdks</td>
<td>Cyclin-dependent kinases</td>
</tr>
<tr>
<td>CeC</td>
<td>Central amygdaloid nucleus</td>
</tr>
<tr>
<td>CGN</td>
<td>Cerebellar granule neuron</td>
</tr>
<tr>
<td>Clasp</td>
<td>Cytoplasmic linker associated protein</td>
</tr>
<tr>
<td>CLIPs</td>
<td>Cytoplasmic linker proteins</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CPC</td>
<td>Chromosomal passenger complex</td>
</tr>
<tr>
<td>Crmps</td>
<td>Collapsin response mediator proteins</td>
</tr>
<tr>
<td>Cspgs</td>
<td>Chondroitin sulfate proteoglycans</td>
</tr>
</tbody>
</table>
CSSG   Oxidised glutathione
DAPI   4’,6-Diamidino-2-phenylindole dihydrochloride
DAVID  Database for Annotation, Visualization and Integrated Discovery
DBD   DNA binding domain
Dbn   Debrin
Dclk1  Doublecortin-like kinase 1
DG    Dentate gyrus
E15   Day 15 embryonic mouse
EcR   Ecdysone receptor
Ect   Ectorhinal cortex
Efn   Ephrins
EGFP  Enhanced green fluorescence protein
Eifs  Eukaryotic initiation factors
Eph   Ephrin receptors
ERLIC Repulsion-hydrophilic interaction chromatography
EVC   Empty vector control
FDR   False discovery rate
Gap43 Growth associated protein 43
GFAP  Glial fibrillary acidic protein
Gpr   G-protein coupled receptor
GrDG  Granular layer of dentate gyrus
GSH   Glutathione
Gsk3β Glycogen synthase kinase 3 beta
Hdac  Histone deacetylases
Hif   Hippocampal fissure
HPLC High performance liquid chromatography
Hsp   Heat shock protein
IAP   Inhibitor of apoptosis protein
ic    Internal capsule
ILK   Integrin-like kinase
INCENP Inner centromere protein
IZ    Intermediary zone
LMol  Lacunosum moleculare layer of the hippocampus
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTP</td>
<td>Long term potentiation</td>
</tr>
<tr>
<td>M2</td>
<td>Supplementary motor cortex</td>
</tr>
<tr>
<td>Map2</td>
<td>Microtubule associated protein 2</td>
</tr>
<tr>
<td>MCS</td>
<td>Multiple cloning site</td>
</tr>
<tr>
<td>MePV</td>
<td>Posteroventral medial amygdala</td>
</tr>
<tr>
<td>MoDG</td>
<td>Molecular dentate gyrus</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>MT</td>
<td>Microtubule</td>
</tr>
<tr>
<td>Mtap1b</td>
<td>Microtubule associated proteins 1b</td>
</tr>
<tr>
<td>MTOC</td>
<td>Microtubule organizing centre</td>
</tr>
<tr>
<td>Ncam</td>
<td>Neural cell adhesion molecule</td>
</tr>
<tr>
<td>NeuN</td>
<td>Neuronal nuclei</td>
</tr>
<tr>
<td>NgR6s</td>
<td>Nogo-66 family receptors</td>
</tr>
<tr>
<td>Ni-NTA</td>
<td>Nickel-nitrilotriacetic acid</td>
</tr>
<tr>
<td>NP-40</td>
<td>Nonidet P-40</td>
</tr>
<tr>
<td>Nrp</td>
<td>Neuropilins</td>
</tr>
<tr>
<td>ns</td>
<td>Nigrostriatal fibers</td>
</tr>
<tr>
<td>NSC</td>
<td>Non silencing control</td>
</tr>
<tr>
<td>NTC</td>
<td>No template control</td>
</tr>
<tr>
<td>Ntrkb</td>
<td>Bdnf receptor</td>
</tr>
<tr>
<td>Opt</td>
<td>Optic tract</td>
</tr>
<tr>
<td>Or</td>
<td>Oreins layer hippocampus</td>
</tr>
<tr>
<td>ORC</td>
<td>Origin recognition complex</td>
</tr>
<tr>
<td>P3</td>
<td>Postnatal day 3 mouse pup</td>
</tr>
<tr>
<td>Pak1</td>
<td>p21 protein activated kinase 1</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PCD</td>
<td>Programmed cell death</td>
</tr>
<tr>
<td>Pde</td>
<td>Phosphodiesterase</td>
</tr>
<tr>
<td>Pdpk1</td>
<td>3-phosphoinositide dependent protein kinase 1</td>
</tr>
<tr>
<td>PES</td>
<td>Polyethersulfone</td>
</tr>
<tr>
<td>Pir</td>
<td>Piriform cortex</td>
</tr>
<tr>
<td>Plk2</td>
<td>Polo-like kinase 2</td>
</tr>
<tr>
<td>Plxd</td>
<td>Plexins</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>Po</td>
<td>Posterior group of thalamus</td>
</tr>
<tr>
<td>PoDG</td>
<td>Polymorph layer of the dentate gyrus</td>
</tr>
<tr>
<td>Prdx</td>
<td>Peroxiredoxins</td>
</tr>
<tr>
<td>PTight</td>
<td>Tet-responsive promoter</td>
</tr>
<tr>
<td>Py</td>
<td>Pyramidal cell layer of the hippocampus</td>
</tr>
<tr>
<td>Rad</td>
<td>Radiatum layer of the hippocampus</td>
</tr>
<tr>
<td>RE</td>
<td>Restriction enzyme</td>
</tr>
<tr>
<td>RGCs</td>
<td>Retinal ganglion cells</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RSA</td>
<td>Retrosplenial agranular cortex</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>rTA-Advance</td>
<td>Modified Tet protein</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>S1</td>
<td>Primary somatosensory cortex</td>
</tr>
<tr>
<td>S2</td>
<td>Second somatosensory cortex</td>
</tr>
<tr>
<td>SAC</td>
<td>Spindle assembly checkpoint</td>
</tr>
<tr>
<td>SC</td>
<td>Subcortical plate</td>
</tr>
<tr>
<td>Sema</td>
<td>Semaphorins</td>
</tr>
<tr>
<td>Sf9</td>
<td><em>Spodoptera frugiperda</em> 9</td>
</tr>
<tr>
<td>Slit</td>
<td>Slits</td>
</tr>
<tr>
<td>SMI312</td>
<td>Pan-axonal neurofilament marker</td>
</tr>
<tr>
<td>Sod</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>Spg</td>
<td>Spastin</td>
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<tr>
<td>STh</td>
<td>Subthalamic nucleus</td>
</tr>
<tr>
<td>Stmn</td>
<td>Stamin</td>
</tr>
<tr>
<td>STRING</td>
<td>Search Tool for the Retrieval of Interacting Genes</td>
</tr>
<tr>
<td>TBI</td>
<td>Traumatic brain injury</td>
</tr>
<tr>
<td>tGFP</td>
<td>Turbo green fluorescence protein</td>
</tr>
<tr>
<td>TLK-1</td>
<td>Tousled-like kinase</td>
</tr>
<tr>
<td>Tops</td>
<td>DNA topoisomerase</td>
</tr>
<tr>
<td>TuJ1</td>
<td>Neuron-specific class III β-tubulin</td>
</tr>
<tr>
<td>U6</td>
<td>Human U6</td>
</tr>
<tr>
<td>VL</td>
<td>Ventrolateral thalamic nucleus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>--------------------------------------------------</td>
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<tr>
<td>VPL</td>
<td>Ventral posterolateral thalamic nucleus</td>
</tr>
<tr>
<td>VPM</td>
<td>Ventral posteromedial thalamic nucleus</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>Y2H</td>
<td>Yeast two-hybrid</td>
</tr>
</tbody>
</table>
CHAPTER 1

Literature Review
1.1 Traumatic brain injury (TBI)

1.1.1 What is TBI?

Traumatic brain injury (TBI) is a result of non-congenital damage to the brain. It is often caused by external forces inflicted by motor vehicle accidents, falls, assaults, mishaps, contact sports and explosive blasts. TBI is a major cause of death and disability worldwide. According to statistics provided by the Australian Institute of Health and Welfare, approximately 1 in every 50 Australians currently suffers from, or had suffered from some form of TBI. A brain injury can last a lifetime and most often, people with TBI also suffer from various neuropsychological disabling conditions which include for example, short term memory loss, depression, disorientation and excessive compulsive disorder (Lux 2007). Because of this, TBI victims often have complex needs and require a range of support services to cope with daily physical activities and sudden emotional changes. In terms of injury cost, TBI often puts a financial strain on families and it is gradually becoming a substantial economic burden for many nations.

1.1.2 Current lack of effective therapeutic treatments for TBI

There is currently a lack of effective therapeutics to treat TBI. Most available therapeutic approaches for TBI victims are actioned in the acute clinical setting, aimed at stabilizing an individual and preventing post-injury exacerbation of damage. These include medical procedures that ensure proper oxygen blood flow to the brain after injury, prescription of drugs to minimize secondary damage such as glutamate-induced cell swelling/neuronal death and establishment of rehabilitative care centres where teams of professionals provide
specialized care for trauma victims (Tomberg, Toomela et al. 2007). Although after-injury treatments and care are important, it is also necessary to develop strategies to limit and salvage primary damage that occurs within days of a head trauma. To be able to do that, the initial molecular and cellular changes that take place in the brain immediately after injury that progress to neuronal death and regeneration need to be understood. This thesis will focus specifically upon the regenerative response of the brain to injury.

1.2 Factors that underlie neurite regeneration following axonal injury

1.2.1 The environment of the adult mammalian CNS is inhibitory to regeneration

In most cases of TBI, axonal connections are badly disrupted and/or severed upon trauma, and this is believed to be a major cause of cognitive dysfunction (Buki and Povlishock 2006). It is now clear that the injured adult mammalian brain has limited regenerative capabilities. Initial studies revealed that this was due to a non-permissive central nervous system (CNS) environment created by surrounding cells like glial, astrocytes and oligodendrocytes after injury (figure 1.1). Some of these molecular inhibitors include chondroitin sulphate proteoglycans (CSPGs) produced by reactive astrocytes, and myelin associated proteins (such as nogo, myelin-associated glycoproteins) produced by intact oligodendrocytes and leftover myelin debris of injured axonal tracts (Mukhopadhyay, Doherty et al. 1994; GrandPre, Nakamura et al. 2000; Morgenstern, Asher et al. 2002). In addition, major repulsive guidance cues that determine axonal pathfinding during development such as ephrins and semaphorins were identified in CNS myelin and they have also been regarded as inhibitors of neuronal sprouting after CNS injury (Moreau-Fauvarque, Kumanogoh et al. 2003; Benson, Romero et al. 2005). The inhibitory actions of this broad range of molecules are via a distinct series of
receptor mediated signalling mechanisms. Although the receptor mechanism for CSPGs inhibition remains unclear, the inhibitory effects cause by myelin associated proteins are transduced primarily by Nogo-66 family receptors (NgRs) and co-receptors (p75, TROY, LINGO1) which then activate downstream signals such as RhoA to disrupt growth cone dynamics (Lehmann, Fournier et al. 1999; Fournier, GrandPre et al. 2001; Domeniconi, Cao et al. 2002; Yamashita, Higuchi et al. 2002; Fournier, Takizawa et al. 2003; Yamashita and Tohyama 2003; Mi, Lee et al. 2004; Park, Yiu et al. 2005). Indeed, limiting the expression of these molecules, their receptors and downstream targets such as RhoA has been reported to partially enhance neurite growth and regeneration after injury (Fournier, Takizawa et al. 2003; Kim, Liu et al. 2004; Li, Liu et al. 2004). It is important to note that most of these studies have been performed in spinal cord injury models, but it is currently believed that similar mechanisms are present in the injured brain.

Figure 1.1: Inhibitory central nervous system (CNS) environment prevents neurite regrowth after brain trauma.
1.2.2 Intrinsic regenerative ability of mature adult mammalian neurons

It was also believed that the failure of severed axons to adequately regenerate to restore loss of function could also relate to an intrinsic inability of injured axons to sprout regenerative processes. However, further research revealed that axotomized neurons do have the innate ability to undergo regenerative sprouting but this is often impeded by the inhibitory CNS environment. This is highlighted by the seminal studies of Aguayo and colleagues, who demonstrated that injured adult spinal cord nerves could sprout into PNS tissue grafts (David and Aguayo 1985). Although bearing the limitation of being one-dimensional, in vitro experimental models have proven to be very insightful in this area of research. A particular advantage of these approaches is the ability to specifically evaluate intrinsic regeneration of injured axons in the absence of glial cells or inhibitory substrates and molecules. Elegant studies have demonstrated that severance of individual axons of CNS neurons that have been maintained to relative maturity (> 2 weeks in vitro) results in a rapid regenerative response (Taylor, Blurton-Jones et al. 2005; Taylor, Berchtold et al. 2009).

Likewise, axotomy of thick fasciculated axonal bundles of relatively mature (21 days in vitro) clusters of cultured cortical neurons is routinely performed in our lab to investigate the intrinsic regenerative capabilities of injured neurons (figure 1.2). Previous studies based on this model revealed that within minutes after axotomy, reactive changes including cytoskeleton alterations that resulted in ring- and bulb-like accumulations of neurofilaments were seen. These reactive changes resolved over time and were replaced by regenerative sprouting events that occurred approximately 8 hrs after injury and by 24 hrs post injury, numerous regenerating processes had traversed the lesion site. After 3 weeks in culture,
clusters of neurons interconnected by bundles of regenerated axons were observed and this was amenable to the state prior to transection (Dickson, Adlard et al. 2000; Chuckowree and Vickers 2003). Noting these regenerative changes is crucial as they demonstrate that neurons possess the intrinsic ability to regenerate quite significantly in the absence of an inhibitory extracellular environment inflicted by other cell types.

Figure 1.2: Established in vitro model for studying axotomy induced neurite sprouting.
1.3 DNA microarray studies identify Aurkb as a novel molecule associated with intrinsic regenerative responses to injury

As discussed, it is apparent that in order to promote efficient neurite regeneration, it is essential to first limit the presence of inhibitory molecules in the CNS environment and at the same time manipulate molecules that are involved in intrinsic regenerative processes within neurons. Several studies have applied DNA microarray approaches to identify key gene expression changes associated with axonal injury and regenerative sprouting in experimental models including spinal cord injury and brain injury. However, a notable technical limitation to these approaches is that it is not possible to distinguish between neuron-specific and glial-derived gene expression changes.

To investigate the molecular determinates that specifically underlie neuronal regeneration at a transcriptional level, a DNA microarray analysis was performed on the established neuronal culture model highlighted earlier (Figure 1.2), in which axonal bundles of relatively mature clusters of cortical neurons were severed in vitro, to study injury-induced neurite regeneration. Details on the procedure and results are discussed in Chapter 2 and 3. Briefly, it was found that post-injury regenerative sprouting is an intricate process that requires two distinct pathways, namely restructuring of neurite cytoskeleton and a cell survival response whereby genes are regulated to protect neurons against pernicious secondary processes.

Intriguingly, the microarray data showed that several cell cycle associated genes were differentially regulated in the absence of apoptotic genes upon axotomy. These cell cycle regulators, in the context of neuronal injury, could potentially have functions outside their normal role in cell division. The results also complemented the findings of others for
example; both Klf4 and HDAC6 identified in this study are known regulators of cell cycle and they were recently found to play important roles in neurite outgrowth (Moore, Blackmore et al. 2009; Rivieccio, Brochier et al. 2009). In particular, cell cycle associated aurora B (Aurkb), a member of the aurora kinase family, appeared to be an interesting target for follow up study since it was up-regulated by an average of 4 folds.

Aurora kinases are known for their roles in cell proliferation. Notably, existing literatures clearly indicated that aurora kinases are involved in the regulation of cytoskeletal structures necessary for mitosis. It is probably because of this involvement that Aurka, a sister kinase of Aurkb, was recently implicated with neurite extension and neuronal polarity, processes that involve complex cytoskeleton organization (Khazaei and Puschel 2009; Mori, Yamada et al. 2009). On the other hand, even though Aurkb was never investigated in the field of neuroscience research, it may also hold a yet discovered neuronal function just like Aurka since it has the ability to modulate changes in the mitotic cytoskeleton. In this case, a possible neuronal function of Aurkb is supported by the microarray data. Thus, the rest of this review will focus upon outlining the current understanding in the field of how Aurka and Aurkb are regulated, their exact roles in mitosis and how they are or might be involved in neuronal processes based on findings and inference drawn from existing literatures.

1.4 Aurora kinases and cell cycle

1.4.1 Introduction and structure of aurora kinases

Based upon review of the mammalian kinome, aurora kinases are a unique group of serine/threonine kinases. Although they are distinctly different from other kinases, they
possess catalytic domains that share some conformational similarities to the AGC family of kinases. There are 3 members of aurora kinase family, aurora A (Aurka), aurora B (Aurkb) and aurora C (Aurkc) and they have been mapped on chromosomes 20q13.2, 17p13.1, and 19q13.3 respectively in humans (Bernard, Sanseau et al. 1998; Bischoff, Anderson et al. 1998; Kimura, Matsuda et al. 1998). All 3 kinases possess a similar domain structure which includes a N-terminal domain that contains 39-132 residues, a kinase domain and a short C-terminal domain of about 15-20 residues (figure 1.3). The catalytic domains are highly conserved among the kinases and across higher species. However, their N-terminal domains vary in size and sequence and this could possibly account for the individual kinase selective protein-protein interactions and localization of the various aurora kinases within the cell.

Basically, members of the aurora kinase family serve central roles during cell proliferation. While Aurka is involved in regulating centrosomes and assembling microtubule spindle, Aurkb is recognised for maintaining bi-orientation and driving cytokinesis during mitosis. On the other hand, the functions of Aurkc are currently poorly understood, and will only be described briefly in this review. Briefly, studies have shown that Aurkc is specifically expressed in mammalian testis and commonly associated with meiosis (Tang, Lin et al. 2006). In addition, there is some evidence suggesting that Aurkc might complement Aurkb function to support mitotic progression (Sasai, Katayama et al. 2004; Slattery, Mancini et al. 2009).
Figure 1.3: Organisation of human aurora A, B and C kinases -

The catalytic domains of the three aurora kinases are very similar and highly conserved. Positions of A-box, D-box and activation loop in respective kinases are as depicted. A and D-boxes are associated with proteasome mediated degradation of the kinases. Overall sequence similarities among Aurka & b, Aurkb & c and Aurka & c are 57%, 75% and 60% respectively.

1.4.2 Regulation of aurora A kinase (Aurka)

1.4.2.1 Main regulator of Aurka: TPX2

Aurka interacts with various factors within the cell and among them; TPX2, a microtubule associated protein, is a keynote player in its regulation. The mechanism on how Aurka is
activated by TPX2 has been extensively characterized. First, autophosphorylation of Aurka at threonine 288 (Thr288) located within its activation loop partially activates it (Walter, Seghezzi et al. 2000). Crystal studies revealed that subsequent binding of TPX2 to Aurka facilitates optimal substrate binding and prevents its dephosphorylation at Thr288 by protein phophatase 1 (PP1) (Bayliss, Sardon et al. 2003). In this manner, TPX2 stabilizes and fully activates Aurka. Interaction of TPX2 and Aurka was mapped to the N terminal and C-terminal kinase domain of respective proteins. Intriguingly, a peptide containing the N-terminal 1-43 residues of TPX2 was sufficient to fully activate and protect Aurka from dephosphorylation. In addition, binding of TPX2 to Aurka via its N terminal domain is required for its interaction during spindle formation. Evidence have shown that truncated form of TPX2 lacking the first 33 amino acids of the N terminus failed to bind and target Aurka to MTs of spindle which then led to mitotic defects (Bird and Hyman 2008).

1.4.2.2 Other factors that positively regulate Aurka

Apart from TPX2, Aurka is also positively regulated by a series of other co-factors and kinases. Briefly, HEF1, Ajuba and Bora regulate Aurka by stimulating its autophosphorylation at Thr288 (Hirota, Kunitoku et al. 2003; Hutterer, Berdnik et al. 2006; Pugacheva and Golemis 2006). Protein phosphatase inhibitor 2 (I-2) which is known for maintaining Aurka in its active form by binding and inhibiting PP1 activity could also directly stimulate Aurka activity via its C terminal domain (Satinover, Leach et al. 2004). In addition, while integrin-like kinase (ILK) promotes Aurka-TACC3/ch-TOG interactions to organise spindle poles (Fielding, Dobreva et al. 2008), PAK1 further activates Aurka itself to drive centrosome maturation (Zhao, Lim et al. 2005).
1.4.2.3 Molecules that negatively regulate Aurka

In contrast, Aurka is negatively regulated by protein phosphatases namely PP1 and PP2A. PP1 binds Aurka and deactivates it by dephosphorylating Thr288 during mitosis (Katayama, Zhou et al. 2001) and PP2A determines Aurka degradation by dephosphorylating serine 51 that is located in its A box (Horn, Thelu et al. 2007). PP2A could also dephosphorylate and stabilize PTTG1, a securin protein that binds separase to prevent cohesin cleavage, to inhibit Aurka activity both in vitro and in vivo (Tong, Ben-Shlomo et al. 2008). Besides phosphatases, Aurka is tightly regulated by p53. Here, p53 mediates expression of GADD45a which binds and antagonizes Aurka to deter centrosome amplification (Shao, Wang et al. 2006). Interestingly however, the binding of TPX2 to Aurka could shield and prevent it from p53-induced inhibition (Eyers and Maller 2004).

1.4.2.4 Degradation of Aurka

Apart from phosphorylation and dephosphorylation events, Aurka is degraded by proteasomes via both ubiquitin dependent and independent pathways. At the end of mitosis, Aurka is ubiquitinated by Cdh1 activated APC/C and this marks it for proteolysis. Notably, this process requires the recognition of both C terminal D box and N terminal A box of Aurka (Castro, Vigneron et al. 2002). In particular, dephosphorylation of serine 53 at the A box of Aurka is crucial since its mutation to aspartic acid to mimic phosphorylation totally blocks Cdh1-APC/C mediated proteasomal destruction (Littlepage and Ruderman 2002). Unlike APC/C induced proteolysis via ubiquitination, binding of AURKAIP1 to Aurka destabilizes it and promotes its degradation by proteasomes through an ubiquitin-independent pathway (Lim
and Gopalan 2007). Overall, amidst the complexity of Aurka regulation, it has to be regulated in a timely fashion to ensure smooth progression of mitosis.

Figure 1.4: Regulatory partners of aurora A & B kinases -
These findings were obtained from various research work done on dividing cells. Protein kinases are indicated in red and phosphorylation events by red arrows. Protein phosphatases are indicated in blue.
1.4.3 Regulation of aurora B kinase (Aurkb)

1.4.3.1 Main regulator of Aurkb: INCENP

Like Aurka, Aurkb interacts with several proteins to facilitate mitosis and of which, inner centromere protein (INCENP) is of utmost importance. Aurkb associates with INCENP to form the catalytic core of the chromosomal passenger complex (CPC) which plays vital roles in cell division. Here, INCENP catalytically increases Aurkb autophosphorylation and activation. This involves a two-step process. First, Aurkb binds to the “IN” box of INCENP located at the C terminal end. This binding promotes autophosphorylation of threonine 232 (Thr232) located at the activation loop and partially enhances Aurkb activity. Following which, phosphorylation of INCENP’s TSS motif (a.a 848-850) by Aurkb via a feedback mechanism fully elicits the complex activity. This mechanism is delicately presented by (Sessa, Mapelli et al. 2005) as depicted in figure 1.5. In the absence of INCENP C terminus phosphorylation, F837 of INCENP pushes on L138 of Aurkb and causes downward rotation of Aurkb αC helix. This movement then pushes E141 of Aurkb down, stretches the activation loop and prevents its bonding with K122. Opening of the catalytic cleft, a consequence of this downward movement, is further maintained by C terminal end of Aurkb and this keeps Aurkb in a partially active state. Conversely, phosphorylation of INCENP’s TSS motif by AurkB through a feedback mechanism releases the pressure of F837. With that, αC helix of AurkB rotates back to its normal position and this closes the catalytic cleft, allowing the establishment of an essential ion interaction between E141 and K122. At the same time, C terminal brake is released and Aurkb becomes fully activated. Studies have further shown that by regulating Aurkb activity, INCENP also modulates CPC localization and function during mitosis (Xu, Ogawa et al. 2009).
1.4.3.2 Survivin and borealin, two other members of the chromosomal passenger complex (CPC)

Besides INCENP, two other important proteins that interact and round up the CPC include survivin and Borealin. Briefly, survivin is a member of the inhibitor of apoptosis protein (IAP) family and its primary roles are to bind the other CPC components and enhance targeting of the entire complex to appropriate positions such as centromere and midbody within the cell during mitosis (Vader, Kauw et al. 2006). Whether or not survivin has an influence on Aurkb activity is debatable. While some groups demonstrated that survivin
binding to Aurkb enhanced its activity both \textit{in vitro} and \textit{in vivo} (Bolton, Lan et al. 2002; Chen, Jin et al. 2003), another group reported that survivin does not have an impact on Aurkb activation (Honda, Korner et al. 2003). As for borealin, its interaction with the other members of the CPC is vital for timely mitotic progression and accurate kinetochore-spindle attachments (Gassmann, Carvalho et al. 2004). Recently, it was discovered that phosphophorylation of borealin at its threonine residues by Mps1 enhanced Aurkb activity. Cells depleted of Msp1 exhibited chromosomes alignment abnormalities and diminished Aurkb activity but these defects were restored upon the expression of phosphomimetic forms of borealin (Jelluma, Brenkman et al. 2008).

\textbf{1.4.3.3 Other factors that positively regulate Aurkb}

In addition to members of the CPC, two other kinases that directly enhance Aurkb activity include Tousled-like kinase (TLK-1) and serine/threonine kinase Chk1. TLK-1 contributes to Aurkb-mediated chromosome segregation by being a substrate and activator of Aurkb but it does that in a way that is independent of its kinase function (Han, Riefler et al. 2005; Riefler, Dent et al. 2008). Chk1 also prevents spontaneous chromosome missegregation by phosphorylating Aurkb at serine 331. By doing so, it fully induces Aurkb activity and promotes optimal phosphorylation of INCENP’s TSS motif as well as survivin interaction with CPC (Petsalaki, Akoumianaki et al. 2011). Activation of Aurkb is also dependent on cooperation between co-factors such as TD60 and microtubules. Here, TD60 aids in the recruitment of CPC and Haspin kinase to centromeres which then regulates Aurkb at various levels (Rosasco-Nitcher, Lan et al. 2008).
1.4.3.4 Phosphatases negatively regulate Aurkb

Like Aurka, inactivation of Aurkb is mediated by both PP1 and PP2A via dephosphorylation of the kinase T loop (Sugiyama, Sugiura et al. 2002). However, the difference is that Aurkb physical interaction with EB1 could protect it from being dephosphorylated by PP2A but not PP1. This method of protection is unique and strictly limited to Aurkb since EB1 does not protect Aurka from its inactivation by PP2A (Sun, Gao et al. 2008). These findings were intriguing and distinctly reflected on the complexity and specificity of aurora kinases regulation.

1.4.3.5 Degradation of Aurkb

Level of Aurkb has to be tightly maintained to allow error-free progression of cell cycle. Aurkb is ubiquitinated by Cdh1 or Cdc20 activated APC/C at specific stages of mitosis and this tags it for degradation by proteasomes. Mutational studies informed that this degradation process is mediated by both A and D boxes located at the N and C terminus of Aurkb respectively (Nguyen, Chinnappan et al. 2005; Stewart and Fang 2005).

1.4.4 Roles of Aurka in cell division

1.4.4.1 Mitotic entry

Aurka is a driver of cell cycle progression. Specifically, cells depleted of Aurka exhibited late commitment to mitosis (Marumoto, Hirota et al. 2002). Its control over G2-M phase transition is via interaction and activation of various protein partners. For example, Aurka
associates with LIM protein Ajuba to elevate its own activation. With that, it then phosphorylates and brings cyclin B1-Cdk1 complex to the centrosome to initiate mitotic entry (Hirota, Kunitoku et al. 2003). In addition, synergistic interaction between Bora and Aurka is required to activate Plk1 which then activates Cdk1 to promote mitotic entry (Seki, Coppinger et al. 2008). This was further confirmed by a separate study in which Aurka was found to phosphorylate Plk1 at threonine 210 specifically to commit cell’s entry to mitosis after a checkpoint arrest (Macurek, Lindqvist et al. 2008)

1.4.4.2 Centrosome maturation and mitotic spindle formation

Centrosome is the key microtubule organizing centre (MTOC) in animal cells. Prior entry to mitosis, it is essential for centrosomes to mature and increase their microtubule nucleation capability to facilitate subsequent mitotic spindle formation (Piehl, Tulu et al. 2004). Microtubule nucleation is dependent on the existence of several pericentriolar matrix (PCM) proteins which have to be recruited to the centrosomes’ immediate surroundings (Nigg and Stearns 2011). Aurka contributes to this process by bringing several major PCM players such as γ-tubulin, Lats2, Ndel1 and TACC to the centrosome through direct phosphorylation. These players are required for accurate assembly of the MT spindle. For example, TACC, a microtubule associated protein; helps to modulate and stabilize astral microtubules (Giet, McLean et al. 2002) while Lats2, a serine/threonine kinase, recruits γ-tubulin which is a master nucleator that mediates and maintains the link between spindle microtubules and centrosomes (Abe, Ohsugi et al. 2006). Without Aurka, centrosomes never fully mature and this gives rise to aberrant mitotic spindle. Though this does not stop cell cycle progression, it often results in serious mitotic defects.
1.4.4.3 Spindle bipolarity and stabilization

After centrosomes maturation, Aurka facilitates their separation to spindle poles. Accurate localization of centrosomes to opposite poles is important for the establishment of a biopolar spindle and this movement is greatly dependent on cortical forces and antiparallel MT sliding. Aurka is thought to control both cortical forces and MT sliding by regulating astral MT growth and phosphorylating motor molecules such as kinesin 5 respectively (Barr and Gergely 2007). Apart from centrosomes driven spindle formation, chromatin kinetochore could also induce spindle assembly. Interestingly, it was recently reported that Aurka has a positive influence on this pathway as well and in this case, its inactivation in the absence of poleward MT flux, inhibited bipolar spindle assembly in an additive manner (Toso, Winter et al. 2009).

Positioning of Aurka to microtubules and centrosomes at the spindle poles is regulated mainly by Plk1 and Tpx2 (Kufer, Sillje et al. 2002; De Luca, Lavia et al. 2006). There, Aurka phosphorylates various previously mentioned PCM molecules such as TACC and Ndel1 to initiate bipolar spindle assembly. In particular, Aurka also phosphorylates MCAK, a tubulin depolymerase, located at the spindle poles at different sites to first promote microtubule aster formation and later to relocate it to MT spindle to aid in the achievement of bipolarity (Zhang, Ems-McClung et al. 2008). First, to promote aster organisation, Aurka inactivates MCAK by phosphorylating it at serine 196 at early time points to favour MT nucleation. Next, to facilitate bipolar spindle formation, Aurka decreases MCAK binding to aster microtubules and brings it to the spindle poles by phosphorylating it at serine 719. Addition of phosphomimetic MCAK (S719E) which not only significantly shortened the time taken for bipolar spindle establishment but also resulted in a fourfold increase in spindle
formation further confirmed the importance of Aurka activity in regulating MCAK to establish spindle bipolarity. Furthermore, it was suspected that upon spindle formation, Aurka stabilized it until anaphase onset by keeping MCAK in check.

![Image of aurora A and B kinases localization](Carmena et al. 2007)

**Figure 1.6: Localization of aurora A (green) & B (red) kinases in HeLa cells.**

### 1.4.5 Roles of Aurkb in cell division

#### 1.4.5.1 Chromosome structuring and condensation

Phosphorylation of histone H3 at serine 10 by Aurkb is conserved across diverse species (Hsu, Sun et al. 2000). This, together with the fact that Aurkb could regulate and recruit
condensins onto chromosomes, led to the hypothesis that Aurkb is directly responsible for chromatin condensation during prophase (Giet and Glover 2001). Basically, condensins are protein complexes that facilitate chromatin condensation and assembly during mitosis through DNA supercoiling and knotting (Hudson, Marshall et al. 2009). However, this idea in which AurkB controls chromosome condensation during prophase was questioned as subsequent studies revealed that its inactivation did not greatly affect overall chromosome condensation (Hauf, Cole et al. 2003; Takemoto, Murayama et al. 2007). As such, it is more accurate to state that AurkB regulates chromatin structure rather than condensation during early mitosis.

However, through a chromosome condensation study in yeast cells that used mutant forms of AurkB, it was reported that Aurkb is required for the maintenance of chromosome condensation during mid-mitosis, especially after anaphase onset (Vas, Andrews et al. 2007). These observations were in concordance with a few other early studies whereby inhibition of AurkB with specific chemical inhibitors like ZM447439 and hesperidin did not affect chromosome condensation in prophase but led to an early and accelerated chromosome de-condensation during cell’s exit from mitosis (Hauf, Cole et al. 2003; Gadea and Ruderman 2005). It was later found that inactivation of Cdc48/p97 deterred ubiquitin-mediated proteolysis of AurkB and this delayed de-condensation at the end of mitosis. In support, this delay could be restored by the depletion of AurkB (Ramadan, Bruderer et al. 2007). Collectively, these findings demonstrated that AurkB regulates chromatin structures during prophase, prevents cells’ premature exit from mitosis by maintaining and antagonizing chromosome condensation during and after anaphase and its inactivation promotes chromosome de-condensation which is essential for nucleus reformation at the end of mitosis.
1.4.5.2 Correction of aberrant microtubule (MT)-kinetochore attachments

Accurate and precise attachment of spindle MTs to kinetochores of sister chromatids during metaphase is a prerequisite for proper separation of chromosomes during mitosis. This process is known as biorientation. With correct biorientation, tension ensues across centromeres as a result of the pulling forces exerted by spindle MTs from opposite directions. This tension is sensed and it stabilizes proper kinetochore-MT attachments. Upon incorrect attachments that result in the loss of tension, AurkB comes into play where it destabilizes these attachments, giving sister chromatids a new opportunity to reform and biorient for accurate segregation (Liu and Lampson 2009). This was clearly demonstrated in a study whereby inhibition of AurkB by Hesperidin, stabilized incorrect attachments and promoted mono-oriented chromosomes with syntelic attachments (Hauf, Cole et al. 2003). Conversely, removal of this inhibitor reversed improper chromosome alignment through selective disassembly of incorrect attachments (Lampson, Renduchitala et al. 2004).

Subsequent studies performed on a molecular level revealed that AurkB does that by phosphorylating a series of substrates that interact or regulate interactions between kinetochores and MTs. For example, interaction of Dam1 and Nde80 promotes the coupling of kinetochores to MTs. However, in the presence of activated AurkB, Dam1 becomes phosphorylated and detached itself from Nde80 and that resets aberrant attachments (Tien, Umbreit et al. 2010). Separately, the mechanisms by which how AurkB regulates MCAK, a member of kinesin-13 family with a MT depolymerisation activity, to correct chromosome-MT attachments are more intricate. Phosphorylation of MCAK at Ser196 by AurkB inhibits its depolymerisation activity and this promotes stabilization of correct MT attachments for properly aligned chromosomes (Lan, Zhang et al. 2004). Conversely, AurkB activity is also
important in directing MCAK to centromeres where they help to depolymerize aberrant MT attachments of misaligned chromatid pairs (Andrews, Ovechkina et al. 2004). In a more recent publication, AurkB was shown to phosphorylate MCAK at different sites to regulate its activity and localization in a spatially and temporal manner depending on kinetochore-MTs attachment status (Zhang, Lan et al. 2007).

More intriguing is the question of how incorrect attachments are selectively disassembled by AurkB during metaphase. This was recently deciphered by a team, from which AurkB was found to be a tension sensor as previously believed (Liu, Vader et al. 2009). Briefly, phosphorylation of a substrate at the kinetochore by Aurkb depends on its proximity from the kinase itself which is located at the inner centromere. In the absence of tension, substrates at kinetochore which are near to the inner centromere get phosphorylate by AurkB. Phosphorylation of these substrates reduced their affinities for MT and destabilized incorrect attachments (Cheeseman, Chappie et al. 2006; DeLuca, Gall et al. 2006; Ciferri, Pasqualato et al. 2008). However, when MTs are properly attached to kinetochores, centromere tension which result from the opposite pulling forces of the spindle MTs separates these kinetochore substrates from AurkB in the inner centromere. This prevents phosphorylation by Aurkb and at the same time provides the substrates a chance to be dephosphorylated by phosphatases. In this case, dephosphorylation increases substrates’ affinities for MT and stabilizes spindle attachments (Gestaut, Graczyk et al. 2008). It was further shown placement of AurkB closer to the kinetochore destabilized and impaired bi-orientation. Overall, the way AurkB interacts and phosphorylates diverse partners to regulate spindle MT and its attachments to kinetochore for accurate and successful mitosis is rather elegant and yet complex.
1.4.5.3 Reinforcement of the spindle assembly checkpoint (SAC)

SAC is one of the few checkpoints that cells have in place to prevent missegregation and aneuploidy. In normal cells, SAC remains active until all sister chromatids are arranged in a bipolar manner along the mitotic spindle, ready for anaphase. Proteins involved in the regulation of SAC include Bub, Mad, CENP-E, Mps1 kinase, and several dynein interacting proteins. These proteins interact with kinetochores and elicit signals that delay anaphase until biorientation is achieved. AurkB indirectly controls and reinforces SAC by regulating some of these proteins. For example, Mad2 and BubR1 which are localized to unattached kinetochores, bind and inhibit cdc20-APC/C complex to prevent anaphase, whose activation destroys securin and cyclin B and then drives anaphase (Fang 2002). AurkB is indirectly involved in this process as it holds a role in the recruitment of BubR1 to the kinetochore (Ditchfield, Johnson et al. 2003). CENP-E is a mitotic checkpoint protein responsible for microtubule capture at kinetochores (Abrieu, Kahana et al. 2000). It was recently demonstrated that AurkB regulates CENP-E to mediate chromosome congression and promote timely anaphase onset (Kim, Holland et al. 2010; Maia, Feijao et al. 2010). Although the exact roles of AurkB in SAC signalling remain unclear, it is becoming more evident that Aurkb does have an indirect influence in its maintenance.

1.4.5.4 Regulation of cytokinesis

During anaphase when chromatids separate, AurkB moves from centromeres to the spindle midzone. This migration process first involves the activation of Cdc14 by seperase. With that, Cdc14 dephosphorylates INCENP, a protein partner that associates with AurkB, and directs the entire AurkB-INCENP complex to the spindle midzone (Pereira and Schiebel 2003).
Central spindle assembly starts with the bundling of antiparallel overlapping non-kinetochore MTs. Here, AurkB is responsible for the recruitment and phosphorylation of several proteins to facilitate this process. For example, centraspindlin is a known complex that aids in the bundling of MTs at central spindle (Mishima, Kaitna et al. 2002; Bowerman 2010). It is made up of a kinesin-6 protein (ZEN-4/MKLP1) and a Rho-family GAP (CYK4/MgcRacGAP) and its localization to spindle midzone is dependent on AurkB (Kaitna, Mendoza et al. 2000). Through in vitro and in vivo studies, it was found that phosphorylation of ZEN-4/MKLP1 by AurkB stabilized interactions between cell cortex and midbody that are essential for successful cytokinesis (Guse, Mishima et al. 2005).

Following central spindle becoming compact and maturing into the midbody, this sends signals to initiate the formation of an actinmyosin ring around the cell cortex that later constricts to form a cleavage furrow that is essential for subsequent pinching of the cell into two (Glotzer 2005). During this process, AurkB regulates and ensures proper function of the contractile ring, making sure that cleavage furrow occurs at the right time by phosphorylating substrates as vimentin and myosin light chain at specific sites. (Murata-Hori, Fumoto et al. 2000; Goto, Yasui et al. 2003; Yokoyama, Goto et al. 2005).

Aurkb is also necessary for the recruitment of multiple components to the midbody apparatus to complete cytokinesis. Recently, it was found that AurkB interacts and phosphorylates Nlp at Ser448 and Ser585 and this recruits it to the midbody apparatus. This process is pivotal as depletion of Nlp impaired cytokinesis and resulted in cells with multiple nuclei (Yan, Jin et al. 2010). Separately, phosphorylation of RASSF1A at Ser203 by AurkB mediates its interaction with Syntaxin 16 which then brings the complex to the midbody. Depletion of
AurkB impaired localization of Syntaxin 16 and resulted in cytokinesis defects. This mislocalization could be prevented through the overexpression of phosphomimetic RASSF1A mutant (Song, Kim et al. 2009). As seen, it is intriguing to know that AurkB plays diverse roles in cytokinesis alone, a process that involves dynamic modulation of cytoskeletal structures.

Figure 1.7: Roles of aurora A & B kinases during different phases of mitosis.
1.5 Aurora kinases and neuronal processes

Having known that aurora kinases regulate cytoskeletal elements to form structures such as microtubule spindle and cleavage furrow during mitosis, it is hence not surprising if they are also involved in neuronal processes since neurons greatly depend on cytoskeletal mechanics to develop, mature and form networks with surrounding cells. The rest of this review is to discuss the possible neuronal functions of aurora kinases based on existing findings and with much inference from extensive research performed in cell division for Aurkb.

1.5.1 Neuronal functions of Aurka

1.5.1.1 Neuronal plasticity

The pioneering work on Aurka in neurons involved localized translation of dendritic mRNA to functional proteins at neuronal synapses. Here, stimulation of NMDA receptors activates AurkA; which in turn phosphorylates CPEB, a RNA-binding protein, to steer polyadenylation-induced translation of αCaMKII (Huang, Jung et al. 2002). αCaMKII is a kinase implicated with long term potentiation (LTP) events. It does that by regulating signalling cascades to control synaptic strengths during neurotransmission. This experiment was interesting as it established the first link between the different roles of Aurka in cell proliferation and neuronal processes.
1.5.1.2 Neurite extension

Apart from its roles in neuronal plasticity, the first evidence pointing to the fact that Aurka directs neuronal cytoskeletal processes came from a report by Mori D et al. in 2009. Using dorsal root ganglion neurons, the authors demonstrated that phosphorylation of Aurka at threonine 287 by aPKC augments its interaction with TPX2. The complex then phosphorylates Ndel1 at serine 251 and recruits it to the centrosome to spur neurite extension (Mori, Yamada et al. 2009). Suppression of aPKC by inhibitor, Aurka by RNAi knockdown and disruption of Ndel1 by Cre-mediated recombination in a conditional knockout mouse all resulted in severe impairment of neurite extension. Furthermore, using an EB3-GFP, a microtubule plus-end marker, the authors found that disruption to aPKC-Aurka-Ndel1 pathway specifically affected microtubule emanation from the microtubule organizing centre (MTOC) but not the overall growth speed. Together, these findings showcased Aurka as a vital player in microtubule modulation necessary for neurite extension.

1.5.1.3 Neuronal polarity

Separately, another group reported that Aurka is required for neuronal polarity. Its suppression in hippocampal neurons by RNAi disrupted the formation of axon and resulted in the growth of multiple neurites that were of undefined identity. Here, Aurka ability to interact and phosphorylate Par3 at serine 962 is paramount for the establishment of neuronal polarity (Khazaei and Puschel 2009). The fact that this loss in neuronal polarity could be rescued by phospho-mimic (S962D) but not non-phosphorylatable (S962A) form of Par3, further confirmed the hypothesis and adds Aurka to the growing list of proteins that underlie neuronal polarity.
1.5.1.4 Cilia disassembly

Another unexpected non-mitotic role of Aurka is its involvement in controlling cilia disassembly. Briefly, Aurka is activated by the prometastatic scaffolding protein HEF1/Cas-L/NEDD9 in hTERT-immortalized retinal pigment epithelial cell. Here, its activation first resulted in phosphorylation follow by activation of HDAC6, a tubulin deacetylase, which is responsible for cilia disassembly (Pugacheva, Jablonski et al. 2007). More relevant, a fast expanding literature suggests that primary cilium is crucial in neuronal signalling associated with processes such as neurogenesis, cell polarity and axonal guidance (Lee and Gleeson 2010; Louvi and Grove 2011). As such, the point to highlight is that because Aurka could regulate cilia disassembly in epithelial cells, it could also possibly do the same in neurons to dictate neuronal processes that are dependent on ciliary functions.

1.5.2 Is there a role for Aurkb in neurons?

1.5.2.1 Cell cycle regulatory partners of Aurkb that hold neuronal functions

Aurora kinases interact and regulate diverse molecules during cell division. Apart from the recent findings on Aurka as discussed earlier, nothing was reported on Aurkb in the context of neuronal research as at today. Interestingly however, recent reports have revealed that several components that were regulated by Aurkb during cell division do possess novel functions in various neuronal processes and as such, they provide indirect insights on a possible role of Aurkb in neurons. Some examples are discussed as follow.
Retinoblastoma protein (Rb) is a tumour suppressor. It prevents excessive cell growth by inhibiting cell cycle progression until mechanisms are in place for cells to divide. Being a phosphoprotein, it is tightly regulated by phosphorylation and misregulation often results in the formation of polypoid cells. Notably, cells treated with AZD1152, a potent inhibitor of Aurkb, contained under-phosphorylated Rb, escaped G1 checkpoint and underwent endoreduplication of DNA after mitosis which subsequently gave rise to polypoid nuclei. Here, it was found that Aurkb phosphorylated Rb at serine 780 to repress E2F induced endoreduplication after aberrant mitotic exit (Nair, Ho et al. 2009). By regulating Rb, Aurkb protects the integrity of mitotic processes and keeps cell cycle in check. More relevant is that Rb, apart from its role in cell cycle progression, also mediates neuronal migration by repressing E2F induced neogenin expression. In this case, both absence of Rb and overexpression of neogenin greatly impaired neuronal migration. These findings delineate a novel pathway for Rb where it orchestrates transcription of a gene that deviates from the classical gene targets of E2F to regulate processes totally different from cell cycle progression (Andrusiak, McClellan et al. 2011). Furthermore, the point that Rb could regulate neuronal migration supports the possibility that it might also determine the expression of other genes involved in a similar but steady state event such as neurogenesis. Exactly how Rb is being regulated to drive target genes expression in neurons remains unknown but as a phosphoprotein, it could possibly be regulated by Aurkb via a mechanism akin to that during cell cycle progression as mentioned.

Cohesin is a four subunit complex that attaches to and glues pairs of replicated chromosomes, known as sister chromatids during mitosis. Its timely release from the sister chromatids is vital for anaphase onset and accurate segregation of the chromatid pair. Release of cohesin from sister chromatids occurs in 2 stages, first from chromosome arms during prophase to
metaphase and then from centromere prior to anaphase. In both stages, its release is controlled and determined by Aurkb. Here, inhibition of Aurkb reduced dissociation of cohesin from chromosome arms and prevented opening of chromosome arms during prometaphase (Gimenez-Abian, Sumara et al. 2004). Aurkb also regulates cohesin removal from the centromeres to loosen up attached sister chromatids prior to anaphase by delocalizing Shugoshin, a protein that prevents removal of centromeric cohesin (Dai, Sullivan et al. 2006). Furthermore, Aurkb mediates the recruitment of separase to mitotic chromosomes to cleave cohesins that were located at the centromeres to facilitate metaphase transition (Yuan, Li et al. 2009). As such, failure to appropriately regulate cohesins during mitosis often causes segregation defects that ultimately result in cell abnormalities. Interestingly, apart from its roles in cell proliferation, 2 separate studies using Drosophila neurons found that cohesin also serves vital roles in neuronal morphogenesis and its mutation severely disrupted axon pruning. Axon pruning, a procedure to remove excessive neuronal branches and connections, is vital for the correction and formation of proper neural circuits during early development. First, using modified fruit flies whose Rad21 subunit of cohesin could be cleaved by TEV protease, Pauli et al. showed that the absence of functional cohesin caused expected missegregation of chromosomes in dividing cells. Something novel and unexpected was that its cleavage also resulted in lethal axon pruning defects in neurons (Pauli, Althoff et al. 2008). Likewise, Schuldiner et al. performed a piggyback-based mosaic screen and found that mutations of 2 subunits of cohesin, namely SMC1 and SA, blocked axon pruning in mushroom body of Drosophila gamma neurons (Schuldiner, Berdnik et al. 2008). This phenomenon was seen and further confirmed in SMC1-/- neurons. Alongside, a significant reduction in ecdysone receptor (EcR), a key regulator of axon pruning, was noted in SM-/- neurons. With support from a previous study (Misulovin, Schwartz et al. 2008), the current data suggested that the entire cohesin complex regulates postmitotic components,
such as EcR, at a transcriptional level. In addition, the authors went on to demonstrate that
SMC1 is also involved in dendrite targeting of olfactory projection neurons. This clearly
indicated that its function is not strictly limited to axon pruning and further illustrated the
importance of cohesin in neuronal processes. Although these studies suggested that cohesin
dictates the structure and function of postmitotic neurons in addition to its central role in
mitosis, the direct mechanisms behind its regulation remain to be elucidated. A study that
showed co-ordinated expression of separase, securin and cohesin is pivotal in brain
development supports a plausible regulatory role for Aurkb in neurons since it was shown to
regulate both separase and cohesin during cell division as discussed earlier (Pemberton,
Franklyn et al. 2007).

1.5.2.2 Possible neuronal function of Aurkb: implications drawn from research on
dividing cells

With inference from studies on Aurkb performed in dividing cells, it is apparent that Aurkb
possesses the ability to directly and/or indirectly orchestrate and modulate cytoskeletal
structures to support precise cell division.

One of which is spindle establishment, a structure that is dependent on intricate microtubule
organization. Here, Aurkb can modulate microtubule spindle disassembly depending on the
cell bioorientation status. In the absence of bioorientation, it will phosphorylate a series of
proteins, for example Dam1, Ndc80 and MCAK, at specific sites to destabilize erroneous
spindle microtubule-kinetochore attachments. This then provides the cell with a fresh chance
to establish the correct MT spindle (Zhang, Lan et al. 2007; Tien, Umbreit et al. 2010).
Details on how phosphorylation of such molecules by Aurkb results in MT destabilization
were discussed earlier in section 1.4.5.2. Even though Aurkb activation is less commonly associated with microtubule stabilization, there is still evidence showing that it could participate in the generation and maintenance of stable microtubule spindle. One specific example is its inhibition of stathmin (Op18), a microtubule destabilizing protein, via hyperphosphorylation (Gadea and Ruderman 2006). Notably, it is often inappropriate to associate Aurkb activation solely with either microtubule stabilization or destabilization events. Very recently, it was found that mDia3 ability to bind EB1 is pivotal in metaphase chromosome alignment and its phosphorylation by Aurkb exhibited reduced ability to bind and stabilize microtubules. However, mDia3 mutants that could not be phosphorylated by Aurkb showed defects in chromosome alignment and this further showed that Aurkb phosphorylation in the first place is vital for mDia3 activation and its initial ability to capture EB1 (Cheng, Zhang et al. 2011). As seen, MT spindle stabilization or destabilization is not a simple process and it is very much dependent on the balance as well as the temporal and spatial activity of Aurkb within the cell. In addition, apart from indirectly modulating microtubules networks through its association with diverse protein partners, Aurkb can also directly interact with microtubules and well known microtubule associated proteins such as EB1 to control spindle formation and maintenance during mitosis (Rosasco-Nitcher, Lan et al. 2008; Sun, Gao et al. 2008).

Aurkb is also involved in the formation of cleavage furrow during cytokinesis, a structure that first relies on the bundling of midbody non-kinetochore microtubules and subsequent contraction of a bundle of aligned actin filaments and myosins (Straight and Field 2000; Pollard and Wu 2010). During this process, Aurkb recruits several proteins, one of which being centraspindlin, to the central spindle to aid in microtubule bundling (Kaitna, Mendoza et al. 2000). Following which, it ensures proper function of the actomyosin contractile ring by
phosphorylating cytoskeletal elements such as vimentin and myosin light chains at specific sites in a timely fashion (Murata-Hori, Fumoto et al. 2000; Goto, Yasui et al. 2003; Yokoyama, Goto et al. 2005). These were also discussed in details earlier in section 1.6.5.4. Hence, failure to form appropriate cytoskeletal structures due to Aurkb dysregulation often results in mitosis and cytokinesis defects.

In neurons, reorganization of cytoskeleton to achieve specific structures, a process akin to cell division, is instrumental for neurite growth and development. Since Aurkb has the ability to orchestrate various cytoskeletal structures in dividing cells as mentioned, it is possible that it could also be involved in the modulation of neuronal cytoskeleton. In this case, using an established primary cell culture model to examine transcriptomic changes that were responsible for neurite regeneration upon axotomy as reported in chapter 3, it was found that several cell cycle genes were differentially regulated in the absence of apoptotic genes, consistent with cell death being not prominent (Jeremy Ng, Chen et al. 2011). Very interestingly, Aurkb happened to be one of the significantly regulated genes. Its gene expression was up-regulated by an average of 4 folds throughout and this trend was further validated by RT-PCR. This piece of information, together with the recent discovery that its sister kinase (Aurka) is involved in several novel neuronal functions as highlighted earlier, further supported the possibility that Aurkb might also possess a neuronal function that is unrelated to its role to cell division. As such, part of this thesis was to address these intriguing questions: Is Aurkb present in the brain and neurons? If so, does it play a role in neuronal processes, for example neuronal development or neurite regeneration?
1.6 Research aims

In summary, both intrinsic and extrinsic (extracellular) factors contribute directly to the failure of neurite regeneration in the adult mammalian CNS. The focus of this thesis is to identify molecular pathways associated with the intrinsic capacity of neurons to regeneratively sprout after axotomy. This was undertaken using an established *in vitro* axonal injury model, coupled with high throughput screening DNA microarray technology, to identify molecules whose expression are significantly altered followed axonal injury. These experiments identified the cell cycle regulator aurora B kinase (Aurkb) as being highly upregulated in regeneratively sprouting cultured neurons. Because the role of Aurkb in neurons had not been reported in the literature, this study undertook a series of experimental studies to define the role of Aurkb in neurite outgrowth.

Overall, the work in this thesis was done to address the following research aims:

1) To gain mechanistic insights into the key molecular determinates that specifically underlie neuronal regeneration at a transcriptomic level.

2) To determine the expression and localization of Aurkb in mouse brain and cortical neurons.

3) To elucidate the biological and functional relevance of Aurkb in cortical neurons.

4) To screen for possible binding partners and direct/indirect substrates of Aurkb in neurons.
CHAPTER 2

Materials and Methods
2.1 Introduction

The following methods described in this chapter are the generalised methods used for the study of various research objectives in this thesis.

2.2 Animal ethics

All animal experimentation was performed under the guidelines stipulated by the University of Tasmania Animal Ethics Committee, which is in accordance with the Australian code of practice for the care and use of animals for scientific purposes.

2.3 Cell culture

2.3.1 Rat PC12 culture

Rat PC12 cells (# CRL-1721, ATCC) obtained from a collaborator (Dr Qiao Xin Li from University of Melbourne) were grown in complete culture medium (DMEM supplemented with 10% fetal calf serum and 1% penicillin and streptomycin) at an initial cell density of $1.36 \times 10^5$ cells per cm$^2$ of culture area. The cultures were maintained in a humidified 5% CO$_2$ incubator at 37°C. A complete medium change was performed every 2 – 3 days.
2.3.2 Mouse primary cortical neuronal culture

Primary neocortical neurons obtained from embryos of pregnant C57BL/6 mouse were cultured as described (Cheung, Pascoe et al. 1998). Cortices from the brains of gestational day 15 – 16 fetuses were microdissected and subjected to trypsin digestion and mechanical trituration. Dissociated cells were harvested by centrifugation and resuspended in complete medium (Neurobasal medium with 2.5% B-27 supplement, 0.25% GlutaMAX-I supplement and 1% penicillin and streptomycin). Cells were then seeded at a density of $1.36 \times 10^5$ or $2.75 \times 10^4$ cells/cm$^2$ of culture area on plastic surface or glass coverslips respectively, coated with 100 mg/ml of poly-D-lysine. The cultures were maintained in a humidified 5% CO$_2$ incubator at 37°C. Complete medium change was performed on Day 3 and every 2 days after for cultures kept longer than 3 days. Immunocytochemical staining of the cultures for MAP2 and GFAP indicated > 95% of the cells were neurons with minimal contamination by glia (Cheung, Pascoe et al. 1998).

2.3.3 Rat primary cortical neuronal cluster culture

Rat cortical neuron cultures were prepared according to previously published protocols (Chung, Vickers et al. 2002; Chuckowree and Vickers 2003; Chung, Staal et al. 2005). Briefly, cortical tissue was isolated from E17 hooded Wistar rat embryos and incubated in 0.1% trypsin (in 10 mM HEPES buffer) for 15 mins. Following trituration and filtration through a 20 um gauze filter, neurons were plated into 12-well tissue culture plates pre-coated overnight with 1 mg/ml of L-lysine in borate buffer, pH 7.4, at a cell density of $4.5 \times 10^5$ cells/well of a 24-well culture plate. Neuronal cultures were maintained at 37°C in humidified air containing 5% CO$_2$ for 21 days prior to experimental axotomy. Neurons were
initially plated into a culture medium consisting of Neurobasal\textsuperscript{TM} medium (Gibco), supplemented with 10% foetal bovine serum, 0.1% B-27 supplement (Gibco), 0.1 mM L-glutamine (Gibco), and 200 U/ml gentamicin (Gibco). After 24 hrs, the media was replaced with medium lacking foetal bovine serum, and replaced every three or four days.

2.4 Experimental procedures undertaken for Chapter 3

2.4.1 Axotomy of rat primary cortical neuronal clusters

Rat neurons were maintained in culture for 21 days, at which time they had formed large spherical clusters of neurons and an interconnected network of thick fasciculated bundles of axons. Using a fine blade microscalpel, axonal bundles were completely severed at approximately half way along the axonal bundles. All axonal bundles in each culture well were severed. For control (no axotomy) and at the appropriate time points after axotomy (8, 15, 24 hrs), RNA from neuronal cells were collected, representing neurons both proximal and distal to the axotomy. Six biological replicates were obtained for control and three for each of the four time-points after injury.

2.4.2 Total RNA extraction and isolation from cultured rat neuronal clusters

RNA from rat neuronal clusters was extracted and collected in eppendorf tubes using RNeasy Mini Kit (Qiagen Cat. No. 74104) as per the manufacturer’s instructions. The whole procedure was done using RNase-free filtered pipette tips. The following procedures were suited for 1 million cultured cells per sample.
2.4.3 Determination of RNA concentration

The pedestal of the Nanodrop ND-1000 Version 3.2.1 was thoroughly cleaned with distilled water using laboratory wipe prior to use. Blanking was done with distilled water. Total RNA sample was mixed well by gentle pipetting. 1.5 µl of the RNA sample was then added onto the pedestal of the equipment. Absorbance reading was taken at 260 nm and 280 nm. One unit of OD260 nm reading was equivalent to 40 µg/ml of RNA. Concentration of RNA was calculated by multiplying 40 to the absorbance reading at 260 nm and then by 50 (dilution factor). Ratio of OD260 nm: OD280 nm would give the purity of the RNA sample.

2.4.4 Checking of RNA quality

In a sterile PCR tube, 1 µl of total RNA sample was mixed with 1 µl RNA loading buffer (Sigma-Aldrich Cat. No. 1486). The mixture is heated at 70°C for 4 mins on a heat block and then spun down to collect any condensation. The total volume of the mixture was topped up to 10 µl with the RNA Dilution buffer and mix by gentle pipetting. Sample mixtures were run and analysed immediately on the E-gene HDA-GT12 System. Quality of RNA is determined by two bands, both 18S and 26S ribosomal RNA, observed at a ratio of 1:2 respectively.

2.4.5 cDNA synthesis/ reverse transcription (for RT-PCR)

Reverse transcription was carried out using Taqman reverse transcription reagents (Applied Biosystems) and according to manufacturer instructions. In a PCR tube, a reaction mix was first prepared. The volumes of each component in the reaction mix as indicated in the table below are for 1 RNA sample.
Chapter 2: Materials and methods

<table>
<thead>
<tr>
<th>Component</th>
<th>Per Sample (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X RT-buffer</td>
<td>1</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>2.2</td>
</tr>
<tr>
<td>deoxyNTPs Mixture</td>
<td>2</td>
</tr>
<tr>
<td>Random Hexamers</td>
<td>0.5</td>
</tr>
<tr>
<td>Rnase Inhibitor</td>
<td>0.2</td>
</tr>
<tr>
<td>Reverse Transcriptase (50 U/µl)</td>
<td>0.625</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>6.525</strong></td>
</tr>
</tbody>
</table>

Following, in a labelled microcentrifuge tube (MicroAmp Optical Reaction Tube), volume corresponding to 200 µg of each RNA sample was added to the reaction mix meant for one sample (6.525 µl). This was then topped up with RNase-free water (DEPC-treated) to give a final reaction volume of 10 µl. The tube was capped and centrifuged. All reaction tubes were then loaded into a thermal cycler. The parameters used for reverse transcription were as follow:

<table>
<thead>
<tr>
<th>Step</th>
<th>Hexamer Incubation</th>
<th>Reverse Transcription</th>
<th>Reverse Transcription Inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Temperature</strong></td>
<td>HOLD</td>
<td>HOLD</td>
<td>HOLD</td>
</tr>
<tr>
<td>25°C</td>
<td>37°C</td>
<td>95°C</td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>10 mins</td>
<td>60 mins</td>
<td>5 mins</td>
</tr>
<tr>
<td>Volume</td>
<td>10 µl</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A primer incubation step at 25°C for 10 mins is necessary to maximize primer-RNA template binding when random hexamers were used for first strand cDNA synthesis. Incubating the reaction at 37°C for 60 mins facilitates reverse transcription. The reaction was then stopped by keeping it at 95°C for 5 mins. After thermal cycling, all cDNA samples were stored at -15 to -25°C until the next step.
2.4.6 Real-time Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed using different Taqman gene probes purchased from Applied Biosystems and the cDNA obtained earlier from different samples. The procedure was repeated 3 times for each probe on each sample alongside three no template controls (NTC). All genes tested were normalised against an internal loading controls, 18S rRNA. A PCR reaction master mix was prepared based on the No. of reactions \times 20 \mu l (excluding the cDNA) as shown in the table below:

<table>
<thead>
<tr>
<th>Reaction component</th>
<th>Per reaction (ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taqman Universal Master Mix (2X)</td>
<td>12.5</td>
</tr>
<tr>
<td>20X Assay Mix of Gene of Interest</td>
<td>1.25</td>
</tr>
<tr>
<td>20X 18S RNA Assay Mix</td>
<td>1.25</td>
</tr>
<tr>
<td>cDNA (total 100 ng)</td>
<td>5</td>
</tr>
<tr>
<td>DEPC-treated Water</td>
<td>5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>25</strong></td>
</tr>
</tbody>
</table>

20 \mu l of the PCR reaction master mix was pipetted to the bottom of each well of the optical 96-well fast reaction plate. 5 \mu l of cDNA (100 ng) or DEPC-treated water (NTC) was added to the designated reaction well. The reaction plate was sealed with an Optical Adhesive Cover. The plate was then centripeted at 4000 rpm for 5 mins to remove air bubbles and to ensure the solution was kept to the bottom of each well. The plate was then ran and read by the 7000 Fast Real-Time PCR System with the following parameters:

<table>
<thead>
<tr>
<th>PCR Setup</th>
<th>Carryover decontamination via UNG</th>
<th>AmpliTaq Gold Pre-activation</th>
<th>Melting Point</th>
<th>Anneal/Extend Step (Combined)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Temperature</strong></td>
<td>50°C</td>
<td>95°C</td>
<td>95°C</td>
<td>60°C</td>
</tr>
<tr>
<td><strong>Time</strong></td>
<td>2 mins</td>
<td>10 mins</td>
<td>15 secs</td>
<td>1 min</td>
</tr>
<tr>
<td><strong>No. of cycles</strong></td>
<td>1</td>
<td>1</td>
<td>40</td>
<td></td>
</tr>
</tbody>
</table>
2.4.7 DNA microarray experiment using Illumina® Rat Ref12 Ver1 hybridization beadchip arrays

500 ng of total RNA from each sample was made up to a start volume of 11 ul with RNase-free water. Using T7 Oligo(dT) primer, RNA was reverse transcribed to form first strand cDNA containing a T7 promoter sequence. Subsequently, this was used for the second strand cDNA synthesis with the addition of DNA polymerase. Rnase H was also added simultaneously to degrade and remove any RNA. Purification of cDNA was done to remove any remaining RNA, primers, enzymes, and salts that would hinder in vitro transcription. Finally in vitro transcription was carried out to generate multiple copies of biotinylated cRNA from the double-stranded cDNA templates. All procedures were performed using Illumina® TotalPrep RNA Amplification Kit and according to manufacturer instructions. After which, the yield of cRNA was quantitated using Nanodrop ND-1000 Version 3.2.1.

750 ng cRNA from each sample was made up to a volume of 5 µl with RNase-free water. This was mixed with 10 µl of hybridization buffer and preheated at 65°C for 5 mins. Each assay mixture was then carefully loaded into the large sample port located at the side of each beadchip array. Following, the loaded beadchip array was placed in a humified hybridization chamber which was then was incubated in a 58°C oven for 17 hrs. After the incubation period, the IntelliHyb seal on the beadchip array was removed. The arrays were then washed with various buffers, blocked, labelled with streptavidin-Cy3 and dried. With that, each beadchip array was scanned on the Illumina scanner using Bead Studio software at Scan Factor = 0.65.
The Illumina beadchip arrays (Rat Ref12 Ver.1) used in my study employs the single-channel (one-sample) format. Each array contains multiple replicates of gene-specific probes of the specified organism (in this case, it was rat). The array enables the detection of transcriptional regulatory change for a total of 22,519 well-annotated RefSeq transcripts pooled from various databases as depicted below.

<table>
<thead>
<tr>
<th>Probe Content Source:</th>
</tr>
</thead>
<tbody>
<tr>
<td>BeadChip</td>
</tr>
<tr>
<td>NCBI-RefSeq Database</td>
</tr>
<tr>
<td>(Release 16 NM)</td>
</tr>
<tr>
<td>NCBI-RefSeq Database</td>
</tr>
<tr>
<td>(Release 16 XM)</td>
</tr>
<tr>
<td>NCBI-RefSeq Database</td>
</tr>
<tr>
<td>(Release 16 XR)</td>
</tr>
<tr>
<td>UniGene</td>
</tr>
<tr>
<td><strong>Total</strong></td>
</tr>
</tbody>
</table>

2.4.8 Microarray data collection and analysis

Initial analysis of the scanned images was performed using BeadScan (Illumina®). For absolute analysis, each chip was scaled to a target intensity of 1000-2000, and probed. The absolute data (signal intensity, detection call and detection P-value) were exported into GeneSpringGX 7.3 (Agilent Technologies, CA, USA) software for analysis by parametric test based on cross-gene error model (PCGEM). One-way ANOVA approach was used to identify differentially expressed genes.
Array data were globally normalized using GeneSpring® V7.3 software. Firstly, all measurements on each chip were divided by the 50th percentile value (per chip normalization). Secondly, each gene was normalized to the baseline value of the control samples (per gene normalization) using median. Following, genes were filtered for a minimum fold change of ±1.5 fold in at least one of 4 time-points after axotomy against controls to facilitate observation of gene regulatory trend. One-way ANOVA ($P$-value <0.05) and Benjamin-Hochberg false discovery rate (FDR) Correction were used to seek differentially expressed genes. Genes which were differentially expressed are annotated according to Gene Ontology-Biological Process provided by the online bioinformatics resources Database for Annotation, Visualization and Integrated Discovery (DAVID) 6.7 (http://david.abcc.ncifcrf.gov/) (Dennis, Sherman et al. 2003; Huang da, Sherman et al. 2009). The microarray data reported here are described in accordance with MIAME guidelines, and has been deposited in the NCBIs Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Super-Series accession number GSE 23653.

### 2.4.9 Statistical analysis

All experiments were repeated at least three times. Data were analysed using post-hoc Tukey test with one-way analysis of variance (ANOVA) to assess significant differences in multiple comparisons. Values of $p$<0.05 were considered as statistically significant and presented as mean ± SE.
2.5 Experimental procedures undertaken for Chapter 4

2.5.1 Primary antibodies used

Monoclonal antibodies used included NeuN (MAB377; Millipore), GFAP (MAB360; Millipore), TuJ1 (G7121; Promega), Map2 (MAB3418; Millipore) and SMI312 (SMI-312R; Covance) while polyclonal antibody used included total Aurkb (ab2254; Abcam) and phospho aurora kinases (#2914; cellsignal). Immunizing peptide (ab13569) used for blocking of total Aurkb antibody was purchased from Abcam. Rabbit (X0903) and mouse (X0931) serums used for negative controls were both from Dako. Antibodies dilutions used for each procedure were as follow a) western blotting: total Aurkb (1:1500); phospho aurora kinases (1:1500), b) immunohistochemistry: Aurkb (1:100); GFAP (1:1000) and NeuN (1:1000) and c) immunocytochemistry: Aurkb (1:150); NeuN (1:600); MAP2 (1:1000); SMI312 (1:1000) and TuJ1 (1:4500). Rabbit and mouse serums were used at 1: 10,000 and 1: 1000 respectively.

2.5.2 Preparation of tissue and whole neuronal cell homogenates

For preparation of brain tissue homogenates, portions of the cerebral cortex were taken from E15 embryonic mouse and A2 adult mouse and each lysed with 500 ul of 5X SDS sample buffer. As for whole cell homogenates, a 24-well plate of cultured primary cortical neurons, maintained for a certain number of days and plated at a cell density of 2.45 x 10^5 cells per well, was lysed with 400 ul of 5X SDS sample buffer. Both types of homogenates were subsequently sonicated and heat-denatured at 95 °C for 5 mins prior to SDS – PAGE.
2.5.3 Isolation, fixation and preparation of brain tissues.

For isolation of brains from A2 (2 month old) and A8 (8 month old) adult C57BL/6 mouse (each n=3), animals were anaesthetized and subjected to cardiac perfusion with 100 ml of 0.1 M PBS followed by 10% neutral buffer formalin (NBF). After which, brains were removed and kept in 10% NBF overnight. Separately, P3 (postnatal day 3) pups (n=3) were put on ice, decapitated and the brains removed were kept in 10% NBF overnight. As for isolation of brains from E15 (embryonic day 15) embryonic pups (n=3), a pregnant mother was sacrificed by CO₂ asphyxiation and its embryos were removed. Brains were then carefully isolated from the embryonic pups and immersed in 10% NBF overnight. The fixed brains were then transferred and kept in 70% ethanol until they were placed on a tissue processor for dehydration, clearing and infiltration. Processed brains were embedded in paraffin wax, vibratome sectioned at ~5 – 7 um and mounted on positively charged APES glass slides.

2.5.4 Generation and purification of recombinant protein from Sf9 insect cells

Rodent His-tag Aurkb wild type (His-Aurkb-WT) recombinant protein was generated in Spodoptera frugiperda 9 (Sf9) insect cells as described in (Chan, Lio et al. 2010). Sf9 insect cells were first co-transfected with Bac-PAK6 baculoviral DNA and pBacPAK9-His-Aurkb-WT (customized order from GeneArt) by calcium phosphate transfection procedures according to manufacturer’s instruction (Clonetech). Recombinant His-Aurkb-WT baculovirus were then isolated; plaque purified three times, propagated and titred. One litre of Sf9 cells were allowed to grow in a 5 litres spinner flask (Bellco Glass) at 27°C to a density of 0.7–1.0 x 10⁶ cells per ml in Grace’s medium (Invitrogen) with 10% (v/v) of foetal bovine serum. The Sf9 cells were then infected with the recombinant His-Aurkb-WT baculovirus at a
multiplicity of infection (MOI) ≥ 10. 50 hrs after infection, cell were harvested and lysed in 30ml of lysis buffer (20 mM Tris pH 7.0, 10% (v/v) glycerol, 50 mM β-glycerophosphate, 1% (v/v) Nonidet P-40 (NP-40), 0.2 mg/ml benzanidine, 0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF), 2 mM β-mercaptoethanol and 0.1 mg/ml trypsin inhibitor). Protein was purified by nickel-affinity (Ni-NTA) chromatography with an increasing elution gradient of imidazole (80, 100, 120, 150, 180, 220, 250 and 300 mM). Elution fractions from 7 – 12 were pooled, divided into 100 ul aliquots and stored at -80 °C.

2.5.5 Coomassie brilliant blue staining and western blotting

10% acrylamide gels were used unless otherwise stated. Following electrophoresis, the gels were stained with coomassie brilliant blue and destained with destaining solution (20% (v/v) methanol, 10% (v/v) acetic acid top up with distilled H2O) to allow visualization of separated proteins and quantitation. For western blot analysis, after SDS – PAGE, proteins were electrophoretically transferred to PVDF membranes. The membranes were first blocked with 5% (v/v) skim milk in 1X TBST for 1hr at room temperature (RT) and incubated overnight with primary antibody that was diluted accordingly in 1% (v/v) BSA in 1X TBST at 4 °C with shaking. After 3 times of 5 mins washing in 1X TBST, the membranes were incubated for 1 hr at RT with secondary antibody (HRP-conjugated polyclonal goat anti-rabbit IgG antibody, Millipore) diluted 1:1000 in blocking buffer. Each membrane was developed with 2 ml of detection buffer (0.04% (v/v) luminol, 0.007% (v/v) p-coumeric acid, both dissolved in DMSO and top up with 100 mM Tris buffer, pH 9.35) and 1 ul of H2O2 using Image Reader Las-3000 (Fujifilm).
2.5.6 Immunohistochemistry

Dewaxing of paraffin wax embedded tissue sections was carried out using xylene followed by varied concentration (100%, 95%, 70% (v/v)) of ethanol. Heat retrieval was done in 0.01 M citrate buffer, pH 6 at 100 °C for 20 mins in a pressure cooker to expose intracellular antigens. Double-labelled immunohistochemistry was performed as followed. Sections were blocked with serum-free protein block (Dako) for 30 mins and incubated with polyclonal primary antibody (Aurkb) that was diluted with antibody diluent (Dako) for 2 hrs at RT. Sections were then incubated with biotinylated secondary antibody, followed by streptavidin conjugated alkaline phosphatase and developed with liquid permanent red substrate-chromogen (Dako). Excess staining solution was washed off and the sections were quenched in 3% H₂O₂ for 15 mins to block endogenous peroxidase activity. Diluted monoclonal primary antibody (NeuN or GFAP) was applied and the sections were incubated for another 2 hrs at RT. After which, sections were incubated with HRP labelled polymer conjugated anti-mouse secondary antibody (Dako) and developed with either brown diaminobenzidine (DAB) chromogen (Invitrogen) for GFAP or Vina Green chromogen (Biocare Medical) for NeuN. Vina Green instead of DAB was used for double staining of Aurkb and NeuN to allow distinct colour visualization of both target proteins. Single-labelled immunohistochemistry was performed as according to the first half of the procedures described in double immunolabelling. All nuclei were stained with Mayer’s Hematoxylin solution for 3 mins at RT. Excess staining solution was washed off and sections were dried on a 60 °C hotplate prior to mounting in glass cover slips with Pertex medium. Negative controls were done alongside every experiment using either rabbit or mouse serum instead of primary antibodies. A separate control was performed to illustrate Aurkb antibody specificity in fixed tissue sections by using antibody that was pre-blocked with its immunizing peptide.
2.5.7 Immunocytochemistry

Primary cortical neurons grown on glass coverslips were fixed in 4% paraformaldehyde in 1X PBS, pH7.4 at RT for 10 mins. Fixed cells were first blocked in 5% BSA in 1X PBS for 40 mins at RT and then incubated with respective primary antibodies diluted to the appropriate concentration in 5% BSA in 1X PBS for 2 hrs at RT. Target proteins were then detected using anti-rabbit antibody conjugated to Alexa 594 Fluor® and/or anti-mouse antibody conjugated to Alexa Fluor® 488 (both from Invitrogen) while DAPI was used to stain nuclei of cells. Coverslips were then briefly dipped in distilled H₂O to remove any salt particles due to earlier PBS wash. Subsequently, coverslips were mounted on glass slides with DAKO fluorescence mounting medium. Stained cells were then visualized under fluorescence microscope at excitation wavelengths of 350nm, 495 nm and 590 nm for DAPI, Alexa Fluor® 488 and Alexa Fluor® 594 respectively. Experiments were performed alongside negative controls using either rabbit or mouse serum instead of primary antibodies. A separate control was performed to illustrate Aurkb antibody specificity in fixed cells by using antibody that was pre-blocked with its immunizing peptide.

2.6 Experimental procedures undertaken for Chapter 5

2.6.1 Primary antibodies used

Beside the antibodies specified in section 2.4.1, a few additional antibodies were used for experiments pertaining to chapter 5. Monoclonal antibody used included α-tubulin (T6074,
Sigma Aldrich) while polyclonal antibodies used included GFP (A-6455, Invitrogen) and FLAG (F7425, Sigma Aldrich). Antibodies dilutions used for each procedure were as follows a) western blotting: GFP (1:1000); α-tubulin (1:1500) and b) immunohistochemistry: FLAG (1:1000).

2.6.2 Drug treatment of neurons

ZM447439 (Cat #2458, Tocris Bioscience), a widely used and specific chemical inhibitor of Aurkb, was dissolved in cell culture grade DMSO to give stock of 25 mM. This was aliquoted into separate microfuge tubes and stored at -80°C. To use, the stock was first diluted 10X with DMSO and further diluted with sterile 1X PBS to obtain the desired concentration for cell treatment. 0.8 uM of ZM447439 was added to freshly plated (2 hrs after plating) cultured mouse primary cortical neurons on Day 0 to inhibit neuronal Aurkb. Control neurons were treated with diluted DMSO. Both control and treated neurons were monitored over the next 72 hrs. They were then fixed with 4% paraformaldehyde in 1X PBS, pH 7.4 on Day 3 and kept in 4°C until immunocytochemistry.

2.6.3 Cloning of pLVX-Aurkb-WT/K109R-EGFP and pLVX-EGFP (control)

Rat Aurkb-WT (wild type) and KR (lysine 109 mutated to arginine) sequences found in separate pEGFP-N1 vectors given by collaborator, Dr Maki Murata-Hori, were PCR out using a set of designed primers (details mentioned in the next paragraph). The PCR products were then ligated between MluI and EcoRI restriction enzyme (RE) cut sites found within the multiple cloning site (MCS) of pLVX-tight-puro lentiviral vector. As for cloning of pLVX-EGFP, PCR was not required since EGFP sequence could be cut directly from an empty
pEGFP-N1 vector and shuttered into pLVX-tight-puro using BamHI and NotI RE (see chapter 5 for further details)

2.6.4 Polymerase chain reaction (PCR) for cloning pLVX-Aurkb-WT/K109R-EGFP

Primers designed for PCR (Forward: GGTAC CACGC GTATG GCTCA GAAAG AGAAC GTCTAC, Reverse: GAGCT CGAAT TCCTA TTACT TGTAC AGCTC GTCCA TGCC) were made by GeneWorks, SA, Australia. PCR was performed according to the reaction mix and parameters specified below.

<table>
<thead>
<tr>
<th>Reaction component</th>
<th>Per reaction (ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X PCR buffer</td>
<td>2</td>
</tr>
<tr>
<td>Forward primer (10uM)</td>
<td>0.5</td>
</tr>
<tr>
<td>Reverse primer (10uM)</td>
<td>0.5</td>
</tr>
<tr>
<td>Plasmid DNA template</td>
<td>1</td>
</tr>
<tr>
<td>50mM MgCl₂</td>
<td>0.5</td>
</tr>
<tr>
<td>10mM deoxyNTPs mixture</td>
<td>0.5</td>
</tr>
<tr>
<td>Taq DNA polymerase (Invitrogen)</td>
<td>0.5</td>
</tr>
<tr>
<td>DNAse free water</td>
<td>14.5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>20</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PCR Setup</th>
<th>DNA denaturation</th>
<th>Annealing</th>
<th>Polymerization</th>
<th>Anneal/Extend Step (Combined)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>94°C</td>
<td>55°C</td>
<td>72°C</td>
<td>4°C</td>
</tr>
<tr>
<td>Time</td>
<td>30 secs</td>
<td>1 min</td>
<td>2 mins</td>
<td>∞</td>
</tr>
<tr>
<td>No. of cycles</td>
<td>40</td>
<td></td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>
2.6.5 DNA ligation

Plasmids and PCR products were digested separately with the appropriate restriction enzymes at 37°C for 2 hrs. Digested plasmid was further dephosphorylated by treatment with alkaline phosphatase. DNA ligation reaction mix was set up as shown in the table below. The reaction was carried out at 16°C overnight. Reaction was stopped by incubating the mixture at 65°C for 5 mins.

<table>
<thead>
<tr>
<th>Reaction component</th>
<th>Per reaction (ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X ligase reaction buffer</td>
<td>2</td>
</tr>
<tr>
<td>RE digested PCR products</td>
<td>1</td>
</tr>
<tr>
<td>RE digested plasmid</td>
<td>1</td>
</tr>
<tr>
<td>T4 DNA ligase (NEB)</td>
<td>1</td>
</tr>
<tr>
<td>DNAse free water</td>
<td>15</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>20</strong></td>
</tr>
</tbody>
</table>

2.6.6 Restriction enzyme (RE) digestion and agarose gel electrophoresis

Digestion of plasmid DNA was carried out using appropriate REs (NEB) and RE buffer. The mix per reaction was indicated in the table below. Digestion was carried out at 37°C for 2 hrs. Reaction was stopped by incubating the mixture at 65°C for 5 mins. Digested contents and DNA marker were then run alongside each other on a 1% agarose gel which was made up with few drops of Ethidium Bromide at 100 volts for 30 – 90 mins depending on the size of the DNA. After which, the gel was viewed under a UV light box at 300 nm illumination to confirm the right plasmids.
### Chapter 2: Materials and methods

<table>
<thead>
<tr>
<th>Reaction component</th>
<th>Per reaction (ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X RE buffer (NEB)</td>
<td>2</td>
</tr>
<tr>
<td>RE 1 (NEB)</td>
<td>1.25</td>
</tr>
<tr>
<td>RE 2 (NEB)</td>
<td>1.25</td>
</tr>
<tr>
<td>100X BSA (if required)</td>
<td>0.2</td>
</tr>
<tr>
<td>Plasmid DNA</td>
<td>1.5</td>
</tr>
<tr>
<td>DNAse free water</td>
<td>14 or 13.8</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>20</strong></td>
</tr>
</tbody>
</table>

#### 2.6.7 PCR and DNA sequencing to verify pLVX-Aurkb-WT/K109R-EGFP and pLVX-EGFP (control)

Verification of clones via PCR was performed as according to the procedure mentioned previously but using a different set of primers specially designed for pLVX-tight-puro clones. DNA sequencing for all 3 clones was outsourced to Australian Genome Research Facility (AGRF), Melbourne (see chapter 5 for further details).

#### 2.6.8 Synthesis of pLVX-Flag-Aurkb WT/ K109R/ T235A

Synthesis of pLVX-Flag-Aurkb WT (wild type) and two other kinase dead mutants K109R (lysine 109 mutated to arginine) and T235A (threonine 235 mutated to alanine) was outsourced to GeneArt, Invitrogen, Life Technologies. Flag tag (MDYKDDDK) was added to the N terminal of Aurkb for all 3 clones and each of these gene sequences was inserted between BamHI and EcoRI RE sites found in the MCS of pLVX-tight-puro. DNA sequencing was finally performed to verify the clones (see chapter 5 for further details).
2.6.9 Lentiviral clones used for Aurkb knock-down studies

Lentiviral vectors containing Aurkb shRNAmir were purchased from Open Biosystems. These shRNAmir were offered in two different types of lentiviral vector, namely pGIPz and pLKO.1. Basically, the differences between the two vectors are that pGIPz expresses a turbo GFP (tGFP) which allows easy identification of cells that were successfully transduced by the virus and its shRNA transcript is driven by a CMV promoter while pLKO.1 does not contain a GFP marker and its shRNA transcript is driven by a Human U6 (U6) promoter. The clones purchased for Aurkb knockdown studies were pGIPz (no 433575 or 433576) and pLKO.1 (no. 28840 and 28756) (see chapter 5 for further details).

2.6.10 Bacterial transformation

Transformation was performed using 100 ng of plasmid and 50 ul of competent DH5α E. coli cells. Firstly, competent cells removed from -80°C were thawed on ice for 2 mins. Plasmid was added to the cells and allowed to stand for 30 mins on ice. The cells were then subjected to heat shock at 42°C for 90 secs to facilitate uptake of plasmid DNA. Transformed cells were placed on ice for 2 mins to recover. Following, 500 ul of LB broth was added and the cells were incubated at 37°C for 1 hr with shaking. Cells were spun down at 5000 rpm for 2 mins. Majority of the medium was removed leaving around 50 ul. Cells were re-suspended by gentle pipetting and plated on LB agar containing the appropriate antibiotic selection marker. Subsequently, the agar plate was incubated overnight and a single bacterium colony was selected and picked for up-scale in 5 ml of LB broth with antibiotics; followed by a mini-preparation of plasmid purification. In cases where more plasmids (e.g lentivirus packaging plasmids) were required, a medium scale plasmid purification was done by taking 1ml of the
bacteria culture and growing them up in 200 ml of LB broth. Some of these bacteria culture which were grew up from a single colony were frozen at -80°C for future use after the addition of some sterile glycerol (~20%).

2.6.11 Plasmid purification

Depending on the bacterial culture volume, purification of plasmids was performed using either a mini (#A1223) or midi (#A2492) plasmid purification kit offered by Promega, according to manufacturers’ instructions.

2.6.12 Determination of plasmid concentration

Plasmid concentration was determined using Nanodrop ND-1000 Version 3.2.1. The pedestal of the equipment was cleaned and blanking was done, both with distilled water. 1.5 µl of DNA plasmid was then added onto the pedestal and absorbance reading was taken at 260 nm and 280 nm. One unit of OD260 nm reading was equivalent to 40 µg/ml of double-stranded DNA. Concentration of plasmid was calculated by multiplying 40 to the absorbance reading at 260 nm and then by 50 (dilution factor). Ratio of OD260 nm: OD280 nm would give the purity of the DNA plasmid and the ideal reading will be from 1.8-2.0.

2.6.13 Production of lentivirus in HEK293T cells

Lentiviruses were produced according to instructions specified in Clonetech Lenti-X™ Tet-On® Advanced inducible gene expression system (Cat. No. 632162) with slight modifications. Briefly, instead of using the packaging plasmids and transfection reagent
provided in the kit, pMD2.G and pSPAX2 purchased from Addgene were used for virus packaging while Lipofectamine 2000 (Invitrogen) was chosen as the transfection reagent as they are more cost efficient in the long run. 2.5 ug of pMD2.G and 6.5 ug of pSPAX2 were used alongside every 3 ug of plasmid DNA (pLVX-response vector carrying GOI, pLVX-Tet On-regulator or pLKO.1/ pGIPz shRNA plasmids constructs). These plasmids in mentioned proportion were mixed with 36 ul of Lipofectamine 2000 in 3 ml of Opti-MEM® (Invitrogen) and incubated for 20 mins at room temperature to allow the DNA-Lipofectamine 2000 complexes to form. Transfection was done by adding the mixture into HEK293T cells (ATCC No. CRL-11268™) which were grown up to ~80% confluence in a T75 tissue culture flask. The transfected cells were maintained at 37°C in humidified air containing 5% CO₂ overnight. Approximately 8-10 hrs after transfection, the medium was aspirated and replaced with complete culture medium. The first virus harvest was done 24 hrs after medium change; followed by a second harvest at 48 hrs. The virus supernatant was then spun down at 1000 rpm for 1 min to remove large pieces of cell debris. The supernatant was subsequently filtered through a 0.22 um polyethersulfone (PES) membrane filter to ensure complete removal of any remaining minute cell debris. The virus could then be aliquot into microfuge tubes and kept at -20°C for storage or left at 4°C till the next virus concentration step. Aliquot of virus prior to storage is essential as virus titers can drop as much as 2 – 4 folds with each freeze-thaw cycle.

2.6.14 Virus concentration

SW 32 Ti swinging-bucket rotor from Beckman Coulter was used for concentrating virus. First, approximately 34 ml of unconcentrated virus supernatant was added to an Ultra Clear Thinwall centrifuge tube (item no: 344058, Beckman). Using a 13 gauge needle, 4 ml of
Optiprep medium (D1556, Sigma Aldrich) was slowly injected into the bottom of the centrifuge tube containing the virus supernatant. Following which, the tubes were spun at 17,000 rpm for 2 hrs. The viruses which are concentrated just above the Optiprep medium normally appear as a ring-like layer of cloudy off-white precipitate. Majority of the supernatant was aspirated, leaving around 2 ml of banded virus mixture that lay just above the Optiprep medium. The concentrated viruses were then carefully pipetted into a sterile 2 ml microcentrifuge tube (as little as possible of Optiprep was removed during the process). Collected virus mixture was then spun at 6000 rpm at 4°C for approximately 6 hrs to pellet down the viruses. The supernatant was aspirated and the virus pellet was resuspended in 100 – 200 ul of sterile 1X PBS. Resuspended virus was then split into separate microcentrifuge tubes in aliquots of 5 – 10 ul and stored at -80°C until use. It is important to note that virus titers can drop as much as 2 – 4 folds with each freeze-thaw cycle.

**2.6.15 Overexpression of target protein in primary cortical neurons using lentivirus**

5 ul of concentrated pLVX-response and regulator viruses was each diluted 10 times with 1X PBS. 1ul of each diluted virus was then added to cultured neurons. For overexpression studies of Aurkb WT/KR-EGFP, EGFP control and Flag-aurkb-WT/KR/TA in growing neurons, viruses were added 2 hrs after the cells were plated. Approximately 12 hrs after transduction, existing medium was replaced with fresh neurobasal medium containing 800 ng/ml of doxycycline to induce protein expression. This was done con-currently for appropriate controls in which no doxycycline was added. Transduced neurons were then either harvested on Day 5 for western blotting analysis or fixed on Day 3 for cytoimmunochemistry. Specific details on lentivirus-mediated overexpression of different forms of Aurkb were described in Chapter 5.
2.6.16 Overexpression of target protein in PC12 cells using lentivirus

Transduction of PC12 cells with Aurkb WT/KR-EGFP and EGFP control viruses was carried out as according to the procedure specified for primary cortical neurons above. Unlike neurons in which virus was added 2 hrs after plating on Day 0, lentivirus was added to cultured PC12 cells when they are about 60% confluent. Transduced cells were then harvested 3 days after for western blotting analysis.

2.6.17 Knock-down of target gene in primary cortical neurons using lentivirus

5 ul of concentrated lentiviruses carrying either pGIPz or pLKO.1 shRNAmir clones was diluted 10 times with 1X PBS. 1ul of each diluted virus was then added to cultured neurons. For knock-down studies of Aurkb in growing neurons, viruses were added 2 hrs after the cells were plated. Approximately 12 hrs after transduction, existing medium was replaced with fresh neurobasal medium. This is a non-inducible system and no doxycycline is required to drive shRNA transcript expression. As such, gene expression will initiate shortly after transduction. Appropriate controls were done con-currently using virus that carries either an empty vector or non-sense shRNA sequence. Transduced neurons were then either harvested on Day 5 for western blotting analysis or fixed on Day 3 for cytoimmunochemistry. Specific details on lentivirus-mediated knock-down of endogenous Aurkb were described in Chapter 5.
2.6.18 Others

Imunochemistry, western blotting and coomassie brilliant blue staining experiments related to this chapter were all performed as according to the procedures specified in earlier sections.

2.7 Experimental procedures undertaken for Chapter 6

2.7.1 Yeast-2-hybrid

The yeast two-hybrid screen using Aurkb as bait was carried out by Dualsystems Biotech AG, Zurich, Switzerland. The bait construct for yeast two-hybrid screening was made by subcloning a cDNA encoding full length mouse Aurkb into the vector pLexA-DIR (Dualsystems Biotech AG, Zurich, Switzerland). The bait construct was transformed into the strain NMY32 (MATa his3Δ200 trp1-901 leu2-3,112 (lexAop)8-ADE2 LYS2::(lexAop)4-HIS3 URA3::(lexAop)8-lacZ GAL4) using standard procedures (Gietz and Woods 2001). Correct expression of the bait was verified by western blotting of cell extracts using a mouse monoclonal antibody directed against the LexA domain (Dualsystems Biotech, Switzerland). The absence of self-activation was verified by co-transformation of the bait together with a control prey and selection on minimal medium lacking the amino acids tryptophan, leucine and histidine with 2.5 mM of 3-aminotriazole (selective medium). For the yeast two-hybrid screen, the bait was co-transformed together with either mouse brain (Dualsystem Biotech) or mouse primary cortical neuron (created by Dualsystem Biotech using the RNA sample we sent over) cDNA library into NMY32. For screen using mouse brain cDNA library, 5.4 \times 10^6 transformants were screened, yielding 14 transformants that grew on selective medium. Positive transformants were tested for β-galactosidase activity using a PXG β-galactosidase
assay (Dualsystems Biotech). 12 of the 14 initial positives showed β-galactosidase activity and were considered to be true positives. As for screen using mouse primary cortical neuron cDNA library, 8.1 x 10^5 transformants were screened, yielding 14 transformants that grew on selective medium. Positive transformants were tested for β-galactosidase activity using a PXG β-galactosidase assay (Dualsystems Biotech). 10 of the 14 initial positives showed β-galactosidase activity and were considered to be true positives. Library plasmids were isolated from positive clones. The identity of positive interactors was determined by sequencing.

2.7.2 Immunohistochemistry

Single-immunohistochemical labelling was performed on E15 and P3 vibratome sectioned brain slices according to procedure specified in section 2.5.6 using polyclonal INCENP antibody (ab36453, Abcam) at a dilution factor of 1:200.

2.7.3 Collection of neuronal samples for phosphoproteomics

Cultured primary cortical neurons were either vehicle-treated (DMSO) or treated with 0.8 μM of ZM447439 on Day 5. Both treated and control cells were harvested with lysis buffer optimized for phosphoproteomics at 8 hrs after drug treatment. The same experiment was done over 3 separate cultures to obtain a total of 2 mg protein for each condition. Harvested samples of the same conditions were pooled for analysis. At the same time, this normalized biological culture variations.
2.7.4 Phosphoproteomics

Cells were harvested in a lysis buffer made up of 50 mM HEPES (pH 7.5), 8 M urea, 75 mM NaCl, a mixture of both phosphatase and protease inhibitor cocktail (PhosSTOP and COMPLETE from Roche Applied Science). The samples were reduced with 10 mM DTT at 37°C for 1 hr and then alkylated with 55 mM iodoacetamide in the dark for 30 mins at room temperature before trypsin digestion. Prior to trypsinization, samples were first diluted 8 times with 50 mM ammonium bicarbonate to 1 M urea and adjusted to pH8. Small portions of samples obtained from each condition were taken for protein quantitation using bicinchoninic acid (BCA) assay kit purchased from Thermo Scientific. Lyophilized sequencing grade modified trypsin (Promega) was re-suspended in buffer supplied by and according to manufacturer’s instructions. Subsequently, 1 mg of protein sample for each condition was trypsinized in a 1:200 (trypsin/protein) mass ratio. A 2-step trysinization was performed whereby firstly, protein samples were trypsinized at 37°C for 12 hrs and after that; fresh trypsin was added to reactions and further incubated at 37°C for another 4 hrs. Acetic acid was then added to each sample to stop trypsinization and they were adjusted to pH3. Each sample was then speed vac till 50 ul prior to desalting using Sep Pak ® Vac RC C18 cartridge columns (Waters). Steps for desalting were performed as follow. Each desalting column was washed with 10 ml of Buffer A (70% acetonitrile with 0.1% formic acid) and conditioned with 10 ml of Buffer B (3% acetonitrile with 0.1% formic acid). Protein samples were then loaded onto the desalting columns, washed with 10 ml of Buffer B and finally eluted with 2 ml of Buffer A. Following which, eluted samples were speed vac until dry. A yellow film-like peptide layer should be observed at the bottom of each microfuge tube and that was resuspended in 200 ul of 70% of Buffer A. Samples were then centrifuged at 2000 g for 10 mins at 4°C and supernatants but not the sediments were transferred to clean glass vials.
meant for HPLC. Here, electrostatic repulsion-hydrophilic interaction chromatography (ERLIC) was used for phosphopeptides enrichment. This was done by passing the samples through a PolyWAX LP column made up of weak anion-exchange silica-based material (4.6 x 200 mm, 5 um particle size, 300 A pore size, PolyLC) on a Shimadzu Prominence UFLC unit. Each sample was eluted into approximately 80 fractions, at a flow rate of 1 ml/min using the following parameters: a) 100% Buffer A for 10 mins, b) gradient from 0-100% Buffer B over a period of 40 mins, c) 100% B for the last 10 mins. The column was then re-used by washing and re-equilibrating with Buffer A for 60 mins between runs of different samples. The UV detection was monitored at a wavelength of 280 nm. Each sample fraction was then speed vac to a final volume of 50 ul and different fractions for the same sample were pooled according to protein peaks to obtain approximately 20 fractions (instead of the original 80). These final peptide fractions were then speed vac till dry, each re-suspended in 100 ul of 0.1% formic acid, vortex and then centrifuged at 2,000 g for 10 mins. Finally, the supernatant but not the sediments from each fraction was carefully transferred into a clean HPLC glass vial and stored at -80˚C until they were ready to be loaded for mass spectrometry. LC-MS/MS followed by comparative phosphoproteomes analysis were done by our collaborator, A/P Siu Kwan Sze from Nanyang Technological University in Singapore. Further information regarding procedures of LC-MS/MS as well as basic comparative analysis could be found in previous reports (Gan, Guo et al. 2008; Datta, Park et al. 2010; Singh, Gan et al. 2011). The list of differentially regulated proteins which passed One-way Anova analysis and with a *p*-value of < 0.05 was finally passed through STRING version 9.0, online software, to establish functional protein association networks.
Chapter 3 has been published as:

CHAPTER 4

Expression and localization of
Aurkb in mouse brain and cortical
neurons
4.1 Introduction

It is intriguing that several cell cycle associated genes were differentially regulated in the absence of apoptotic genes upon axotomy as seen from the microarray data reported in the previous chapter. For example Klf4, which was significantly down-regulated, is a key cell cycle transcription factor that was recently found to also play a novel and vital role in neurite outgrowth (Moore, Blackmore et al. 2009). In accordance with the data, a recent review also highlighted the importance of several key cell cycle regulators in directing various neuronal processes that are independent of their cell cycle functions (Frank and Tsai 2009).

Interestingly, a literature search performed on Aurkb, whose gene expression was up-regulated by an average of 4 folds as seen from the microarray study described in Chapter 3, revealed that it was never reported in the context of neurons. As such, to verify Aurkb gene expression, RT-PCR was performed using the same samples and the results concurred with the microarray data. With that, it seems that Aurkb is an interesting target to look at in neurons since no prior reports were made. For a start, it is imperative to first examine whether Aurkb is expressed in the brain and particularly in neurons. As such, this chapter of my thesis was to address these questions via various techniques which included immunohistochemistry, immunocytochemistry and western blotting. Briefly, I characterized the expression and localization of Aurkb in mouse brains of various ages. In addition, I also looked at Aurkb localization, its expression as well as its activity in cultured primary cortical neurons across several days. Overall, the findings were intriguing and further suggested that Aurkb might serve a vital role in neuronal development.
4.2 Experimental design

First, western blotting, immunohistochemistry and immunocytochemistry were performed to confirm whether human-sequence derived polyclonal Aurkb antibody could detect rodent Aurkb. Specificity of the antibody was further determined with the same techniques by pre-blocking it with immunizing peptide. Following which, the presence and expression of Aurkb in brain across age were verified by immunohistochemistry using brains slices obtained from day 15 embryonic pup (E15), post-natal day 3 pup (P3), 2 month-old adult (A2) and 8 month-old adult (A8) mouse. In addition, various cell markers were used to determine the types of cell in which Aurkb was found in the brain. A detailed localization study on Aurkb was then performed using developing primary neurons that were grown in culture. Lastly, expression and activity of Aurkb in developing neurons were determined through western blotting analysis.

4.3 Results

4.3.1 Specificity of Aurkb antibody (ab2254)

Polyclonal Aurkb antibody (Abcam, ab2254) was raised against a synthetic peptide derived from the first hundred amino acids of human Aurkb sequence. To confirm the specificity of this antibody for use on rodent tissues, recombinant His-tag Aurkb wild type (His-Aurkb-WT) derived from rodent cDNA was produced in Sf9 insect cells using a baculovirus expression system and purified via a Ni-NTA column. Recombinant His-Aurkb-WT eluted with an increasing concentration gradient of imidazole was ~90% free from contaminating proteins, fractions E7 – E12 as circled in red in figure 4.1A.
Specificity of ab2254 for rodent Aurkb was first confirmed by western blotting using various dilutions of recombinant His-Aurkb-WT, pooled from fractions E7 – E12. As seen in figure 4.1B, Ab2254 managed to pick up rodent Aurkb in the absence of peptide blocking. No Aurkb was detected when the antibody was pre-blocked with its immunizing peptide. This clearly illustrated its specificity.

To further confirm the antibody specificity, immunohistochemistry and immunocytochemistry were performed on rodent brain tissue sections and fixed cultured primary cortical neurons respectively, with and without ab2254 immunizing peptide blocking (figures 4.1C and 4.1D). The lack of Aurkb immunoreactivity in both experiments upon peptide blocking further informed that the antibody is specific and it is suitable for use in detecting rodent Aurkb.
Figure 4.1
Figure 4.1: Specificity of Aurkb antibody (ab2254) -

(A) Ni-NTA column purified rodent recombinant His-Aurkb-WT was ~90% free from contaminating proteins as shown through coomassie brilliant blue stained SDS-PAGE gel (B) Immunoblot of Aurkb using various concentration of recombinant His-Aurkb-WT, with and without ab2254 immunizing peptide blocking. (C) Aurkb immunoreactivity in CA3 region of the hippocampus (2-month old mouse) with and without immunizing peptide blocking. (D) Fluorescent immunostaining of Aurkb in Day 3 cultured primary cortical neurons with and without immunizing peptide blocking. Scale bar: 10μm.

4.3.2 Localization of Aurkb in the adult mouse brain

Immunohistochemistry was performed with Aurkb antibody to examine the distribution of Aurkb in coronal sections from 2 month-old adult (A2) mouse brain. There were obvious
differences in the intensity of Aurkb immunoreactivity within different regions of the brain (figure 4.2) and the most salient features are described in the following paragraphs.

Overall, strong Aurkb staining was seen in various regions of the cerebral cortex, hippocampus and amygdala. In contrast, its staining intensity in basal ganglia and thalamus varied from light to moderate. Generally, Aurkb is localized mainly in the nuclear bodies of cells. On closer examination, some of these cells also have light to moderate staining of Aurkb observed in the nucleus border although it is difficult to precisely determine whether this staining was indeed expressed just outside the nucleus. Some cells on the other hand have a very distinct perinuclear staining of Aurkb.

Cerebral cortex: Basically, no Aurkb labelling was observed in layer I of the cerebral cortex while layer II to IV showed a moderate to strong intensity of Aurkb immunoreactivity (figure 4.2a – 4.2f). In RSA and M2, Aurkb staining was relatively stronger in layer II-III as compared to the rest of the layers and its immunoreactivity was mainly limited to the nuclei of cells (figure 4.2a, b). As for S1 and S2, moderate to strong Aurkb immunoreactivity was seen evenly across the different layers. Unlike cells in layer IV-VI where Aurkb was localized in the nucleus, some cells found along layer II-III possessed a distinct perinuclear staining pattern (figure 4.2c, d). The Ect showed a moderate intensity of Aurkb staining across all layers. Interestingly, Aurkb labelling for most of the cells in layer II-III were distinctly perinuclear while cells with a nuclear stain were restricted only to layer IV-VI (figure 4.2e). Strong Aurkb immunoreactivity was found in pir and dorsal edopiriform cortex. Here, it was represented by a mixed population of cells that were either stained in the nuclei or perinuclear regions (figure 4.2f).
Hippocampus: Intense Aurkb immunoreactivity was observed at pyramidal cell layers found in CA1, CA2 and CA3 regions of the Ammon’s horn and within the granular cell layers of the dentate gyrus (figure 4.2h). AurkB in these regions appeared as a combination of speckle and punctate nuclear body stain. Little or no Aurkb labelling was found in Or, Rad, LMol, MoDG and PoDG.

Amygdala: Aurkb immunoreactivity was seen extensively in many nuclei of cells found in BLA, BLV, BMA, MePV and MePD of the amygdala. Comparatively, scattered Aurkb immunostaining was observed in the perinuclear area of some cells that were located around BMP of the amygdala (figure 4.2i).

Basal ganglia: In the basal ganglia, there was very sparse AurkB staining in opt and white matter lining the ic. However, moderate AurkB immunoreactivity was noticed in STh and ns. Apart from being stained mainly in the nuclei, the staining of cells in both of these regions also appeared stratified (figure 4.2g).

Thalamus: As for the thalamus, little or very weak AurkB labelling was noted in various regions including VPL, VPM and VL (figure 4.2j).

4.3.3 Aurkb is localized in neurons but not astrocytes of adult mouse brain

To determine the type of cells in which AurkB was present, immunohistochemistry was performed separately on mouse brain tissue sections using NeuN and GFAP anti-bodies which are diagnostic markers commonly used to identify neurons and astrocytes respectively. As observed, Aurkb immunostaining pattern at different brain regions closely resembled that
of NeuN antibody staining and this showed that AurkB was localized primarily in neuronal cells (figure 4.3A). Conversely, Aurkb distribution in comparison to GFAP antibody staining was vastly different and this distinctly demonstrated that AurkB was not found in astrocytes (figure 4.3A). These observations based on single-antibody staining were subsequently confirmed by the co-localization of AurkB in NeuN positive cells but not GFAP positive cells as shown through double-labelled immunohistochemistry (figure 4.3B).
Figure 4.2: Localization of Aurkb in A2 mouse brain -

(A) Schematic representation on half of a coronally sectioned mouse brain, highlighting the different brain regions captured in (a-i). (a) cortex – RSA, (b) cortex – M2, (c) cortex – S1, (d) cortex – S2, (e) cortex – Ect, (f) cortex – pir, (g) basal ganglia, (h) hippocampus, (i) amygdala, (j) thalamus. Enlargements of the boxed regions were presented at the side and/or the bottom of the main pictures. Roman letters in (b – e) referred to the different cortical layers. See abbreviation list. Scale bars: ⊦ = 100um, — = 25um.
Figure 4.3: Presence of Aurkb in neurons but not astrocytes in brain tissue section -

(A) Single immunohistochemical labelling of Aurkb, NeuN and GFAP in CA3, DG and S2 regions of A2 mouse brain sections. (B) Double immunohistochemical labelling of Aurkb with either GFAP or NeuN in CA3, pir and S1 regions of A2 mouse brain sections. Because brown and red colours are hard to distinguish, Vina Green instead of DAB was used alongside permanent liquid red as chromogens for the labelling of NeuN and Aurkb respectively to allow distinct colour visualization of both target proteins. Black arrow heads: absence of Aurkb in GFAP positive cells. Black arrows: presence of Aurkb in NeuN positive cells. See abbreviation list. Scale bar: 25um.
4.3.4 Varied expression of Aurkb in cortex at different brain developmental stages

A series of Aurkb immunohistochemical studies were undertaken on mouse brains obtained from gestational Day 15 embryonic pup (E15), post-natal Day 3 pup (P3) and 8 month-old adult (A8), in addition to what was already reported in A2 adult mouse brain. In particular, striking differences were seen across the cerebral cortices. Generally, Aurkb immunoreactivity was detected in all of the cortex tissue sections obtained from mice at different developmental stages but its staining intensity varied from moderate to strong.

Intense Aurkb labelling was observed in the cortical plate and ventricular zones but in comparison low levels were observed in the subcortical plate and intermediary zone of E15 parietal cortex (figure 4.4A). In contrast, there was a gradient-like stain of Aurkb in P3 cortex whereby stronger labelling was seen in layer II and III as compared to layer IV – VI, with the exception of layer I where no staining was observed. For both E15 and P3 cortex, the presence of Aurkb was limited mainly to cell nuclei with very low levels observed in the surrounding parts of the cell. Aurkb immunoreactivity was distinctively different in A8 cortex whereby its staining was moderate and more evenly spread across all cortical layers. In addition, some cells with prominent perinuclear Aurkb labelling were found along layer I – III of the adult cortex but were absent from both pre and early post-natal cortex. Overall, Aurkb immunoreactivity in A8 cortex was very similar to those observations reported earlier in A2 cortex.

Notably, there seemed to be an association between decreased expression of Aurkb and brain development. This was based upon the varying intensities of Aurkb immunoreactivity seen across cerebral cortex of different ages, in which its staining appeared to be the strongest in
E15 and the weakest in adult. This was confirmed by western blot analysis from which significantly more Aurkb was detected in E15 cortical tissue homogenates as compared to that of adult, according to the total amount of proteins loaded for each sample as quantified through coommasie brilliant blue staining (figure 4.4B).

Figure 4.4
Figure 4.4: Varied Aurkb expression in different age cortex -

(A) Aurkb immunoreactivity in different cortex tissue sections acquired from E15, P3, A2 and A8 mouse. (B) Western blot analysis of total Aurkb in Day 5 neuronal cell lysate and tissue homogenates of adult and E15 cortex. Recombinant His-Aurkb-WT was used as positive control and total protein loaded for each sample was quantified by commassie brilliant blue staining. Abbreviations: C, cortical plate; IZ, intermediary zone; SC, subcortical plate; SV, subventricular zone; VZ, ventricular zone. Roman letters referred to the different cortical layers. Scale bar: 30um.
4.3.5 Varied expression of Aurkb in hippocampus of different aged brain

It was demonstrated earlier that in the adult brain, apart from the cortex, Aurkb was also localized primarily in the hippocampus. Through immunohistochemistry performed on different aged brain tissue slices, a few key changes in its expression within and around the hippocampus region were noted across the different brain developmental stages.

In the developing hippocampus of E15, some cells were labelled more brightly than others. The stains in these cells appeared punctated and they are localized primarily in the cell body, presumably in the nucleus as seen in figure 4.5. In P3 brain where the hippocampus begun to take shape, intense Aurkb immunoreactivity was observed in the pyramidal cells occupying the Ammon’s horn. In comparison, a significantly weaker but moderate labelling was detected in the dentate gyrus marked by (‡) in figure 4.5. Interestingly, this observation was “switched” in the matured hippocampus of A2 and A8 mouse, from which slightly stronger Aurkb immunoreactivity was observed in the dentate gyrus as compared to CA 1, 2 and 3 regions of the Ammon’s horn.

Another key difference was the presence of Aurkb immunoreactivity in the white matter tracts of the early developing brain. This was evident from the staining found along the intermediary zone and the region just above the Ammon’s horn of E15 and P3 mouse brain respectively, both marked by (*) in figure 4.5. In contrast, Aurkb immunoreactivity in this brain region disappeared with age as it was not observed in A2 and A8 mouse.
Figure 4.5: Varied Aurkb expression in different age hippocampus -

Aurkb staining at the dentate gyrus increases with age (marked by ‡). Aurkb immunoreactivity is present in white matter tracts at E15 and P3 (marked by *), but disappears from the latter in the adult. Scale bar: 60um.
4.3.6 Localization of Aurkb in cultured primary cortical neurons

To properly characterize AurkB localization and further validate its presence in neurons, immunocytochemistry was carried out using primary cortical neurons cultured on glass coverslips in vitro. Its localization in developing neurons varied with neuronal maturation (figure 4.6A), concordant with the results seen earlier in the developing mouse brain. In neurons that were just plated and without any processes, Aurkb was localized mainly in the nucleus. However, in early stage neurons with budding but non-definitive cytoskeletal processes, majority of the Aurkb accumulated as an intense spot just right outside the nucleus borderer, around or overlapping the region where microtubules tend to accumulate as shown by Tuj1 staining. This phenomenon was particularly evident in Day 1 cultured neurons, a phase when majority of the cells had their somata surrounded by segmented lamellipodia and microtubules that were not yet properly and/or fully assembled into an orderly array. In neuron with defined and properly extended processes (Day 2 and onwards), bulk of Aurkb was found not only in the nucleus but also in the cell soma, around the neuronal hillock region. Small amount of Aurkb could also be seen sparsely distributed along some of the neuritic processes. Immuno-labellings of Aurkb in developing neuronal cells were repeated on separate occasions with various neuronal markers namely Tuj1, NeuN, SMI312 and Map2 which stained for neuronal microtubules, nucleus, axon and dendrites respectively. The results were consistent throughout and representative images were as presented in Figure 4.6A.

These observations from fluorescence microscopy were further confirmed by confocal imaging as seen in Fig 4.6B. From the Z stack images, it was obvious that Aurkb co-existed
on the same planes with NeuN, a marker for neuronal nuclei. This distinctly demonstrated that Aurkb was localized in the nucleus. Likewise, from the Z stacks images of Aurkb and Tuj1 double labelling, it was clear that some Aurkb found in the neuronal soma and along processes interacted with the neuronal cytoskeleton as they co-localized with Tuj1 stains.

Figure 4.6
Figure 4.6
Figure 4.6: Localization of Aurkb in cultured cortical neurons -

(A) Normal fluorescent microscopy images illustrating the localization of Aurkb in Day 0, 1, 3, 5, 7 cultured primary cortical neurons. Aurkb (red) was counterstained with the following neuronal markers (green); Tuj1, SMI312, NeuN and Map2. Nuclei were stained with DAPI (blue). Aurkb accumulated into a small dense region outside the nuclei of Day 1 cultured neurons (white arrow). In neurons with elaborated processes, portion of the Aurkb localized to the neuronal hillock region (white arrow heads) and neurite processes (white dashed arrows). (B) Confocal microscopy images confirmed the presence of Aurkb in nucleus,
neuronal hillock region (white arrow heads) and neurite processes (white dashed arrows). *, †, ‡, § represented z stacks view of selected image from different axis. Scale bar: 10um in (A) and 5um in (B).

4.3.7 Expression and activity of Aurkb in cultured growing cortical neurons

To re-confirm and examine the expression of Aurkb in developing neurons, western blotting was performed on whole cell lysates of cultured mouse primary cortical neurons that were harvested on Day 1, 3, 5, 7 and 9. As seen in figure 4.7A, Aurkb was expressed in neurons as early as Day 1 and this level of protein expression was maintained throughout the days. The results mirrored the observations seen in immunocytochemistry. Interestingly however, upon probing with a phospho aurora kinases antibody which marks Aurkb activity through its phosphorylation at Threonine 235 (T235), a gradual increase in phosphorylation level was noted overtime. This trend was made palpable when the protein bands representing total and phosphorylated Aurkb for each day were quantitated using Image J and the individual ratio of phosphorylated Aurkb over total Aurkb was plotted across day (figure 4.7B). The ratios plotted for each day were the averages obtained from 3 separate experiments.

It is important to note that the phospho aurora kinases antibody used in this case could also pick up phosphorylated forms of Aurka and Aurkc. In spite of that, it was clear that the bands observed in this case belonged to Aurkb since its size did not match that of Aurka and Aurkc as according to the antibody data sheet. This was further supported by its size comparison to recombinant Aurkb (WT), which was loaded as a positive control (figure 4.7A). Together; these results revealed that Aurkb expression and activation were vital for neuronal development and growth, especially during the period when neuritic processes were formed.
Figure 4.7: Expression and activity of Aurkb in developing cortical neurons -

(A) Western blotting results demonstrated that Aurkb was expressed as early as Day 1 and this level of expression was maintained over days. An increase in phosphorylation level as seen overtime highlighted that its activity is vital for neuronal development and growth. (B) Total and phosphorylated Aurkb for each day were quantitated using Image J and the individual ratio of phosphorylated Aurkb over total Aurkb was plotted across day. These ratios were the averages obtained from 3 separate experiments.
4.4 Discussion

In the previous chapter, transcriptomic studies found that Aurkb gene expression was elevated after axotomy, indicating that it might play a possible role in the regenerative sprouting response of injured neurons. However, because Aurkb has never been investigated in the context of neurons, it is imperative to first determine its expression profile in the brain as well as to characterise its expression and localization in cultured primary cortical neurons. As such, experiments were undertaken in this chapter to address these questions.

First, a series of in vitro and in vivo experiments were performed to ensure that human-sequence derived Aurkb antibody (Ab2254) is specific and suited for used in rodent tissues and cells. In vitro, Aurkb-specific immunoreactivity was examined via western blotting and immunocytochemistry using recombinant His-tagged Aurkb protein derived from rodent cDNA and cultured primary cortical neurons respectively. In vivo, the specificity of Aurkb antibody was confirmed through immunohistochemistry performed on brain sections of 2-month old adult mouse. Importantly, Aurkb immunoreactivity was absent in all experiments in which antibody binding was blocked with an immunizing peptide, indicated that the tested antibody is specific and suited for use in detecting rodent Aurkb. The effort to ensure the specificity of the Aurkb antibody is necessary because the subsequent experiments undertaken were dependent upon the accurate detection of total Aurkb.

Next, immunohistochemistry was performed on coronally sectioned brain slices of 2 month-old adult mouse, and it revealed that Aurkb was distributed across various brain regions. The most intense and prominent expression of Aurkb was in the cerebral cortex, piriform cortex, hippocampus and amygdala. Generally, the staining around these regions appeared punctate
and Aurkb was localized mainly in the nuclear bodies. In some of these cells, moderate amount of Aurkb could also be observed along the nuclei border. Interestingly, some cells located in the upper layers of certain cortical regions possessed a different and distinct perinuclear staining pattern. These Aurkb immuno-positive cells were later identified to be neurons based on single and double-label immunohistochemistry performed using two different types of antibody markers, namely GFAP and NeuN which distinguish between astrocytes and neurons respectively. Together, the results from these experiments demonstrate that Aurkb, a cell cycle associated kinase, is indeed highly expressed in certain brain structures and selectively by neurons.

The expression of Aurkb in different aged brains was then investigated via immunohistochemistry, using brains slices obtained from day 15 embryonic pup (E15), postnatal day 3 pup (P3) and 8 month-old adult (A8) mouse. These were undertaken in addition to the experiments performed on 2 month-old adult (A2) reported previously. As observed in A2 brain, intense Aurkb immunoreactivity was found in the cortex and hippocampus of these different age brain sections. However, the key difference that was noted was that expression of Aurkb decreased with brain maturation, such that Aurkb immunoreactivity was strongest in E15 brain and the weakest in adult brain. This observation was confirmed by western blot analysis of cortical tissue homogenates obtained from E15 and adult mouse brain. This finding was interesting because it suggests that Aurkb may carry out specific roles in the maturation process of neurons during brain development.

Because Aurkb is highly expressed during the early stages of brain development, this indicated that it might be involved in similar but steady state events such as neurite establishment and growth. To investigate this further, immunocytochemistry was performed
with various neuronal markers to study Aurkb localization within developing cultured primary cortical neurons. In freshly plated neurons that were without any processes, Aurkb was observed solely in the nuclei. However when the neurons began to elaborate processes, a stage characterised by segmented lamellipodia and newly formed microtubules, the majority of the Aurkb labelling was not in the neuronal nuclei but had accumulated into a small dense region that was in close proximity to the nuclei. In subsequent stages when neurites were well extended, Aurkb was found not only in the nuclei but also around the cytoplasmic axon hillock regions. In addition, small puncta of Aurkb labelling were observed along some of the neuritic processes. These observations were further validated by confocal imaging. The changing localization of Aurkb within developing neurons and particularly its localisation outside of the nucleus at various stages of neuronal maturation suggests that it may have direct roles in neurite outgrowth.

Together, the studies from brain sections and cultured neurons suggest a possible role for Aurkb in neuronal maturation, and in particular neurite outgrowth because the accumulation of Aurkb at an intense spot located right outside the nucleus at an early stage of neuronal development indicates that it could be interacting with specific structure such as the centrosome, which is known to serve as an early nucleation point for microtubules destined for growing neuritic processes (Baas and Yu 1996). This was probably true because in cells with undefined and immature protrusions, the region where Aurkb consolidated actually overlapped with the areas where microtubules tend to accumulate as observed through Tuj1 labelling. In this regard, Aurkb could serve as an important mediator linking microtubule-derived pulling forces generated in the leading processes and the microtubule-based transportation network surrounding the nucleus to orchestrate neuritic growth.
In summary, this is the first comprehensive study of Aurkb in the brain and neurons. The fact that it is found in various structures of an adult brain and highly expressed in the immature brain indicates that Aurkb might serve novel cell cycle independent functions in brain development and plasticity. More specifically, Aurkb could be involved in the maturation process of neurons since it is dynamically localized, expressed and activated in developing cultured primary cortical neurons. The next chapter of this thesis investigates this hypothesis by using different experimental approaches to interfere with Aurkb function in growing neurons.
CHAPTER 5

Functional role of Aurkb in neurite outgrowth of cultured cortical neurons
5.1 Introduction

It is clear from the studies described in chapter 4 that Aurkb is expressed in the brain and more specifically in neurons. Based on several other findings in chapter 3, it is highly probable that Aurkb plays a role in orchestrating growth of neuritic processes since it's localisation within neurons changes dependent upon maturation, and it is highly expressed during the early stages of neuronal development. This could also be inferred from the fact that it possesses the ability to regulate cytoskeletal structures during mitosis as discussed earlier. With that, the research objective in this chapter is to investigate whether Aurkb has a functional role in cultured primary cortical neurons during the development and extension of neuritic processes. Both pharmacological and loss of function experiments were used as tools for investigation.

5.2 Experimental design

The overall scope of this chapter was to use several different experimental approaches (pharmacological, expression of kinase-dead AurkB and knockdown of AurkB expression) to interfere with Aurkb function in neurons as a means of identifying the role of AurkB in neurite outgrowth. For the pharmacological study, cultured neurons were treated immediately after plating with ZM447439, a specific chemical inhibitor of Aurkb, and morphological changes over several days were characterised by immunocytochemistry and quantitated. To further identify the functional roles of Aurkb, loss of function studies were performed. This involved expressing kinase dead variants of AurkB and knocking-down endogenous AurkB in growing cultured cortical neurons via a lentivirus-mediated approach. For knock-down experiments, a set of lentiviral vectors carrying Aurkb shRNA mir sequences
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purchased from OpenBiosystems was used. For the expression of kinase dead AurkB, an inducible lentivirus system from Clontech was adopted, in which expression of target protein was driven by doxycycline.

5.3 Results

5.3.1 Inhibition of Aurkb in cultured neurons using a specific chemical inhibitor

ZM447439 is a potent and specific chemical inhibitor of aurora kinases and it does not interfere with other kinases when used up to 5uM concentrations. Although it inhibits both Aurka and Aurkb activity, it is 20-fold more selective for Aurkb. Hence, at the 0.8uM concentration used in this study, it is expected that ZM447439 is primarily acting upon AurkB. This drug is widely used to study AurkB inhibition in many experimental models (Gadea and Ruderman 2006; Famulski and Chan 2007; Kasuboski, Bader et al. 2011). Importantly, it has been demonstrated that HeLa cells treated with 2uM of ZM447439 specifically replicate the cellular phenotypes seen in Aurkb RNA interference experiments, indicating the specificity of ZM447439 when used at 0.8uM concentration for this experiment. (Ditchfield, Johnson et al. 2003). In this chapter, cultured primary cortical neurons were treated with ZM447439 on Day 0, approximately 2 hrs after plating. By Day3, there were distinct differences in neuronal morphology between control cells that were treated with DMSO (vehicle) and ZM447439 treated cells. To properly characterise these morphological differences, the cells were fixed and cytoimmunochemistry was performed using various cytoskeletal antibody markers, namely SMI312, MAP2 and Tuj1.
Control and drug-treated neurons were first stained with SMI312, an axonal marker. As seen in figure 5.1A, most of the control cells possessed elaborated and defined axons while treatment with ZM447439 significantly reduced the number of cells with elaborated axons. While some ZM447439 treated cells did sprout axons, these processes appeared shorter as compared to those of control cells. To quantitate these observations, a total of 6 images from 3 different coverslips were captured and analysed by automated tracing using HCA-Vision software. Maximum neurite length for each cell was measured in pixels and those with a length of <50 pixels (which is approximately the width of a neuronal cell body) were classified as cells without axonal processes. The average of maximum neurite length was 207 and 101 pixels for control and treated group respectively. A two-sample t-test was performed and calculations indicated that the difference was statistically significant with a p-value < 0.0001. Likewise, quantitative analysis revealed that a larger percentage of ZM447439 treated cells (45%) failed to establish distinct axonal processes (maximum neurite length < 50 pixels) as compared to control cells (22%). These quantitative results clearly confirmed the general morphological observations that ZM447439 treatment not only reduced axonal length but also decreased the number of cells with elaborated axons. The neurons were also stained with MAP2, a dendritic marker. From figure 5.1B, most cells in both control and treated groups contained established dendrites and there did not appear to be any notable morphological differences. Quantitative measurements from 6 photos from 3 coverslips were analysed by automated tracing using HCA-Vision software. Because it is normal for most neurons to contain more than one dendrite, total neurite length instead of maximum neurite length was measured and compiled. A minimum threshold of 50 pixels (one cell body width) was used to distinguish cells without distinct dendritic processes. As described in Figure 5.1B, ZM447439 had no statistically significant effect upon dendrite outgrowth (p > 0.05).
In addition to distinct axonal and dendritic changes caused by Aurkb inhibition, changes to the entire microtubule network within control or ZM447439 treated neurons were visualized using an antibody that stained for neuron-specific βIII-tubulin (Tuj1) as shown in figure 5.1C. There were a few key morphological observations made. First, most vehicle-treated neurons possessed a single, distinct and elongated process (in accordance with SMI-312 labelling described earlier, this is presumably the axon) while a substantial proportion of drug-treated cells lacked this primary process, or it was substantially shorter. This in direct concordance with the observations reported with SMI312 staining. Second, the majority of the cells in both groups had a few short and distinct processes surrounding their cell bodies. These structures were most likely dendrites, as reflected by the MAP2 staining described in Figure 5.1B. Third, numerous ZM447439 treated cells had several thin, frayed and scattered neuritic processes that were very much disorganised surrounding their cell bodies. The images in figure 5.1C were over-exposed to reveal these mentioned observations that were obviously absent from control cells. Notably, these features were only observed with the microtubule marker and not SMI312 and MAP2 staining, suggesting that ZM44739 is specifically altering microtubule organisation in neurons. Lastly, drug-treated cells displayed abnormally shaped and substantially expanded cell bodies as compared to control. Enlargement of the cell body, independent of nucleus size (as observed with DAPI stain), was due to a substantial increase in microtubule mass within the area between the nucleus and axon hillock region. Notably, this region is where AurkB localisation was most pronounced in cultured cortical neurons as described in chapter 4. Overall, immunocytochemistry performed with different antibodies that stained for various cytoskeletal structures revealed that inhibition of Aurkb via a chemical inhibitor caused significant changes to the neuritic cytoskeleton of developing neurons and the findings reflected on the results reported in Chapter 4, in which Aurkb was highly expressed in the developing brain and dynamically localized within a growing neuron.
Figure 5.1

**A**

Day 3

**SMI312, DAPI**

Average of maximum neurite length (pixels)

- Ctrl (n=115): 206.9
- 0.8uM ZM447439 (n=69): 100.8

% of cells with maximum neurite length <50 pixels

- Ctrl (n=115): 22%
- 0.8uM ZM447439 (n=69): 45%

**B**

Day 3

**MAP2, DAPI**

Average of total neurite length (pixels)

- Ctrl (n=120): 237.1
- 0.8uM ZM447439 (n=61): 210.9

% of cells with total neurite length <50 pixels

- Ctrl (n=120): 15%
- 0.8uM ZM447439 (n=61): 22%
Figure 5.1: Inhibition of neuronal Aurkb with ZM447439 -

(A) Immunolabelling of control and treated cells with SMI312, an axonal marker. Treated cells had a lower average of maximum neurite length and a higher percentage of cells with maximum neurite length < 50 pixels. The difference was significant with a p-value < 0.0001, represented by (*). (B) Quantitative analysis of MAP2 staining revealed that there was no significant difference for total dendrites length (p-value > 0.05) or percentage of cells with total dendrites length <50 pixels between both groups. (C) Immunolabelling with Tuj1 coloured the entire microtubule networks in both control and treated cells. Additional differences were observed in treated cells, they possessed an enlarged cell body (white arrow head) bordered by a series of thin, frayed and disorganised neuritic processes (white arrow). Scale bar = 10um.
5.3.2 Lentiviral expression of wild-type and kinase-dead AurkB in cultured neurons

To verify the observations reported with the use of ZM447439 using a different methodological technique, a lentiviral approach was used to introduce expression of kinase dead variants of Aurkb into cultured neurons.

Briefly, a lentiviral strategy utilising the Tet-On advance inducible gene expression system offered by Clonetech was adopted. This system consists of 2 separate lentiviral vectors namely pLVX-TetOn-Advanced and pLVX-tight-puro. pLVX-TetOn-Advanced is a regulatory component that stably expresses a modified Tet protein known as rtTA-Advanced while pLVX-tight-puro is a response lentiviral plasmid in which target gene is inserted. Basically, although rtTA-Advanced binds to Tet-responsive promoter (PTight) found in pLVX-tight-puro, gene transcription is repressed. However, this repression is lifted upon the addition of doxycycline, which binds to and completes the rtTA-Advanced complex. This then drives transgene expression as shown in figure 5.2A.

Subsequently, both plasmids, each together with packaging plasmids, were transfected separately into HEK293T cells to generate respective lentiviruses (one that expresses rtTA-Advanced and one that expresses target protein under the control of rtTA-Advanced and doxycycline). Neurons were then simultaneously transduced with both viruses and doxycycline was added whenever to induce transgene expression as depicted in figure 5.2B. Details on preparation and concentration of viruses as well as how and when neurons were transduced were mentioned in Chapter 2 (Materials and Methods).
Figure 5.2
Figure 5.2: Concept on how Lenti-X™ Tet-On® Advanced inducible gene expression system works -

(A) Using regulator and response lentiviral vectors, protein expression is driven in the presence of doxycycline. (B) Generation of separate lentiviruses in HEK293T cells and subsequent virus transduction of cultured cortical neurons.

5.3.3 Generation of pLVX-Aurkb-WT/K109R-EGFP and pLVX-EGFP (control) constructs

The Aurkb-WT (wildtype) and Aurkb-KR (lysine 109 mutated to arginine) sequences were obtained from vectors generously provided by Dr Maki Murata-Hori. In the vectors provided, Aurkb WT and KR were inserted between EcoRI-ApaI and EcoRI-KpnI restriction enzyme (RE) cut sites found within the multiple cloning sites (MCS) of the host pEGFP-N1 vector respectively. pEGFP-N1 vector encodes a red-shifted variant of wild-type GFP which has been optimized for brighter fluorescence and higher expression in mammalian cells. Fusion of target protein to the N terminus of EGFP retains the fluorescent properties, allowing the study of the localization of the fusion protein in vivo.

To overexpress protein of interest in neurons using the mentioned inducible lenti-X gene expression system, gene sequences of Aurkb-WT/KR, each fused to EGFP were cloned out from pEGFP-N1 via PCR using a set of designed primers (Forward: GGTACCACGCTATGGCTCAGAAAGAGAACGTCTAC, Reverse: GAGCTCGAATTCTATTTACTTGTACGTGCTCAAGAAGTCTAAGG). Each of the PCR products was then inserted within MluI and EcoRI RE cut sites of pLVX-tight-puro, a vector containing all viral processing elements.
necessary for the production of infectious but replication-incompetent lentivirus. Because of the sites Aurkb WT and KR were inserted within pEGFP-N1, direct cut and paste of Aurkb tagged to EGFP sequences from pEGFP-N1 into pLVX-tight-puro using just RE was not possible as there were no common and in sequence RE cut sites found in both plasmids.

Alongside, a control vector, pLVX-tight-puro containing just EGFP, was created. In this case, unlike the cloning of Aurkb-WT/KR-EGFP sequences into pLVX-tight-puro, EGFP alone could be cut directly from an empty pEGFP-N1 vector and shutt into pLVX-tight-puro using BamHI and NotI RE.

These clones were subsequently verified via PCR and RE digestion followed by DNA gel electrophoresis as well as DNA sequencing. PCR and DNA sequencing were carried out using a set of pLVX-tight-puro sequencing primers (Forward: GAGGT AGGCG TGTAC GGTGG, Reverse: CTGCT AAAGC GCATG CTCCA). An additional primer (GTAGG TCAGG GTGGT CACGA) was required for the full sequencing of pLVX-Aurkb-WT/KR-EGFP. The cloning and verification processes described for pLVX-Aurkb-WT/KR-EGFP and pLVX-EGFP control constructs were illustrated in figure 5.3 and 5.4 respectively.

![Figure 5.3](image-url)
D) DNA sequencing data

- Aurkb WT – EGFP (rat) in pLVX-tight-puro:

![DNA sequencing data diagram]

![PCR using pLVX-tight-puro sequencing primers diagram]

Figure 5.3
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Figure 5.3

-Aurkb K109R – EGFP (rat) in pLVX-tight-puro:

1 MluI  M A Q K E N V Y P W Y G S K T S Q
1 acgcgtatgtgccagaagagacactctaccctcggctgcctccacagcttc
2 SGLNTLPQRVLREPAVTAP
61 ttctggcctacaacctggccacagagtcctacagagagagagctgctgcaacctcc
4 QALMRNSNSQSTAVPGQKLT
61 cagaccctctactgaaacgtgttcacacagcttccacactttcaccatgac
161 E NKGATALQGQSRQPFTID
181 gagaaacaggtgccccactctgctggaagagccagccagccagcccctaccattgac
181 NFEIGRPLGKGFKGNYVLP
241 acatccgttgggtggtcttggtgtctgccagttgctggtctggtctggtctgtg
101 EKKSRFIVALILFKSQUIE
301 gagaaacaggtgccccactctgctggaagagccagccagccagcccctaccattgac
361 EGVHELQRLEREIEIQAHKL
361 gagaaacaggtgccccactctgctggaagagccagccagccagcccctaccattgac
141 NILQLYNYFYDQQRIYLIE
421 aatattttctagctgtaacactctttctagtacctagcagagacagctgtgaagtactgagaa

- EcoRI
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Figure 5.3

Legend:
- Blue letters: Aurkb protein (uppercase) and gene (lowercase) sequences.
- Orange letters: Linker peptide (uppercase) and gene (lowercase) sequences.
- Green letters: EGFP protein (uppercase) and gene (lowercase) sequences.
- Red letters: MluI restriction enzyme site
- Purple letters: EcoRI restriction enzyme site
- Single underlined (black): sequence verified by pLVX forward sequencing primer (GAGGT AGGCG TGTAC GGTGG)
- Dashed underlined (black): sequence verified by additional reverse sequencing primer (GTAGG TCAGG GTGGT CACGA)
- Double underlined (black): sequence verified by pLVX reverse sequencing primer (CTGCT AAAGC GCATG CTCCA)
- Highlighted in green: Lysine 109 (K109)
- Highlighted in blue: Lysine 109 mutated to arginine (R109)

**Figure 5.3: Cloning of pLVX-Aurkb WT/K109R-EGFP -**

(A) Because no common and in sequence RE sites were found in both pEGFP-N1 and pLVX-tight-puro vectors, as circled in red (WT) and green (KR), each gene sequence had to be PCRed out using a set of designed primers and then ligated into pLVX-tight-puro vector. pLVX-Aurkb WT/KR –EGFP clones were then verified by (B) RE digestion (C) PCR and (D) DNA sequencing.
C) DNA sequencing data

- EGFP control in pLVX-tight-puro:

```plaintext
1 BamHI M V S K G E E L F T G V V P I L V E
1 ggatccatggtgagcaaaggccagagctgtttacccgaggggtgtggtcccattcctgtgtcagg
2 L D G D V N G H K F S V S G E E G D A
61 ctgacggccagtaaacggccacaaattcagcgctgttccggcagggcgagggcgatgcc
41 T Y G K L L T L K F I C T T G K L P F V P W
121 accaccggaagtctgacccttgatctcaaccacgcaagctgtccctgcctgtggtcccctgq
61 F T L V T T L T Y G V Q C F S R Y P D H
181 ccacccctctgtggaccacccttgacacctcgctgctgctgttcaaggctacctgccacccgacacc
81 M R Q H D F F K S A M F E G Y V Q E R T
241 atgaagcagcaagctctctcaggtccggcatacccgaaggtcagtccagaggagccacc
101 I F F K D D G N Y K T R A E V K F E G D
301 atctctctctaaaccgaccaacctaaagaccgccgacggtgaactgctgqaggccacac
121 T L V N R I E L K G I D F K R D E G N I L
361 acctcggtgacccgcatactgagctgacgagggcacaacctgaccggaagccacaacctctgq
141 G H K L E Y N N S H V Y I M A D K Q
421 ggaccaagctgtcagtaacacgacagcccaaacgtctatctatagcggacagcgccac
161 K N G I K V N F K I R H I E D G S V Q
481 aagacgcaagctctctcaggtccggcatacccgaaggtcagtccagaggagccacc
181 L A D H Y Q Q N T P I G D G P V L L P D
541 ctgctggcagacactaccgagacacccatactgcggcacggccctgctgctgqccac
201 N R Y L S T Q S A L S K D E K R D H
601 acctcgctctgqacacgataccctgctgagctgacgagggcacaacctgaccggaagccacaacctctgq
221 M V L E F T A A G I T L G M D E L Y
661 atggtcctgctggagttcgtgaccgccggggatcactctggtctgggtgtgatgtcac
241 K * * NotI
721 aagtaaagcgccgcc
```

Legend:

- Green letters: EGFP protein (uppercase) and gene (lowercase) sequences.
- Red letters: BamHI restriction enzyme site
- Purple letters: NotI restriction enzyme site
- Single underlined (black): sequence verified by pLVX forward sequencing primer (GAGGT AGGCCG TGTAC GGTGG)
- Double underlined (black): sequence verified by pLVX reverse sequencing primer (CTGCT AAAGC GCATG CTCCA)

Figure 5.4: Cloning of pLVX-EGFP (control) -

(A) Because common and in sequence RE sites were found in both pEGFP-N1 and pLVX-tight-puro vectors, as circled in blue, EGFP gene sequence could be cut out using RE and then ligated into pLVX-tight-puro vector. pLVX-EGFP (control) was then verified by (B) RE digestion and (C) DNA sequencing.
5.3.4 Lentivirus-mediated expression of Aurkb-WT-EGFP, Aurkb-K109R-EGFP and EGFP control in cultured mouse primary cortical neurons

Lentiviruses carrying the clones (namely pLVX-Aurkb-WT/KR-EGFP and pLVX-EGFP control) were generated as according to the procedures specified earlier. Respective viruses were added to cultured primary cortical neurons 2 hrs after plating. Complete medium change was performed 12 hrs after transduction and 750ng/ml of doxycycline was added to drive expression of target proteins. In a further experimental control, no doxycycline was added to cells that had been transduced with both viruses. The cells were then observed under fluorescent microscope for EGFP on Day 5. The expression of the target protein was doxycycline-treatment specific, indicating no inappropriate expression of the target protein. Generally, the transduction efficiency ranged from 60 - 70%. As seen in figure 5.5A, neurons expressing only EGFP appeared normal, with well-defined cell bodies and elaborated primary neuritic processes. However, neurons expressing either Aurkb-WT/KR-EGFP appeared distorted with enlarged and irregularly shaped cell bodies. Notably, no visible morphological differences were observed between cells expressing either forms of EGFP tagged Aurkb.

Validation of target protein expression was assessed by immunoblotting of total cell lysates from lentiviral-infected neurons, using an Aurkb antibody as depicted in figure 5.5B. From which, a 65kDa protein band which was made up of EGFP (~26kDa) and Aurkb (41k Da) could be seen and circled in blue. The functionalities of the expressed AurkB proteins in neurons were then determined. According to past reports, phosphorylation of Aurkb at Threonine 235 (T235) is necessary for its activation (Yasui, Urano et al. 2004). As such, western blotting was performed using a commercially available phospho-aurora kinases antibody which detects Aurkb only when it is phosphorylated at T235. As expected, unlike
endogenous Aurkb which was phosphorylated, overexpressed Aurkb-KR-EGFP (kinase
deaf) remained unphosphorylated. Surprisingly however, overexpressed WT forms of Aurkb
were also not phosphorylated as circled in red, shown in figure 5.5B. The lack of
phosphorylation at T235 clearly showed that Aurkb-WT-EGFP was not functional. With that,
it might actually mimic the kinase dead variant of Aurkb and this could possibly explain why
no obvious morphological differences were observed between neurons that overexpressed
either WT or kinase dead (KR) forms of Aurkb. There were 2 probable reasons as to why
Aurkb-WT-EGFP was not functional. First, this might be due to species differences in which
rat Aurkb was expressed in mouse neurons. However, this is unlikely since the structures of
rat and mouse Aurkb share a 93% sequence homology. Second and more likely, the EGFP
tag might hinder the phosphorylation of T235 which is required for Aurkb activity. As such,
construction of lentiviral constructs with non-GFP tagged Aurkb was performed to test these
speculations.

![Figure 5.5](image)
Figure 5.5: Expression of AurkB WT/KR-EGFP (rat) in cultured mouse primary cortical neurons -

(A) Successful virus transduction of neurons. However, little or no differences could be observed between neurons overexpressing AurkB WT or KR tagged to EGFP. (B) Overexpression was confirmed by western blotting. Both total (circled in blue) and phosphorylated AurkB were probed. Intriguingly, AurkBWT-EGFP was not functional since it was not phosphorylated (circled in red). Scale bar = 50um.

5.3.5 Lentivirus-mediated expression of AurkB-WT-EGFP, AurkB-K109R-EGFP and EGFP control in rat PC12 cells

To determine whether the loss of activity observed in rat AurkB-WT-EGFP was due to its expression in mouse neurons, lentivirus carrying rat pLVX-AurkB-WT-EGFP, pLVX-AurkB-
KR-EGFP and pLVX-EGFP control were used to transduce PC12, cells derived from pheochromocytoma of rat adrenal medulla. Through immunoblotting with total Aurkb antibody, a 65 kDa band representing the overexpressed protein was observed for both Aurkb-WT and KR transduced cells. This was shown in figure 5.6 and circled in blue. Intriguingly, the lack of phosphorylation at T235 in Aurkb-WT-EGFP even when it was expressed in a rat cell line, as circled in red in figure 5.6, clearly illustrated that its loss in activity was not due to species differences. Instead, most likely it was caused by the EGFP tag. Although EGFP has proven to be an excellent protein-tag that allows relatively straightforward visualization of target protein expression, its size (~26kDa) may be causing a steric hindrance that limits the phosphorylation and ultimately the activity of Aurkb.
Figure 5.6: Overexpression of Aurkb WT/KR-EGFP (rat) in rat PC12 cells -
Overexpression was confirmed by western blotting. Both total (circled in blue) and phosphorylated Aurkb were probed. AurkbWT-EGFP remained unphosphorylated; indicating its loss in activity was not due to species differences (circled in red).

5.3.6 Synthesis of pLVX-Flag-Aurkb WT/ K109R/ T235A

At this point, despite the problems faced with pLVXxAurkbxEGFP clones, it was noted that the inducible lentivirus-mediated transduction system worked well for inducible expression of target proteins in cultured primary cortical neurons. Hence, the pLVX system was retained, but the EGFP tag was replaced with a smaller tag. Flag tag (MDYKDDDK) was considered an appropriate replacement since it is a small peptide that comprises just 8 amino acids (~1kDa). This time, a commercial service from GeneArt (Invitrogen) was engaged to synthesize the required cDNA clones. In addition to WT and KR mutant, an additional kinase dead variant of Aurkb was made by mutating T235 to alanine (A). For all clones, codon optimization was performed to enhance protein expression in mammalian cells. Flag tag was added to the N terminal of Aurkb for all 3 clones and each of these gene sequences was inserted between BamHI and EcoRI RE sites found in the MCS of pLVX-tight-puro. Finally DNA sequencing was performed to verify the sequence of each clone and these data were presented in figure 5.7.
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A) **pLVX-Flag-Aurkb-WT**

1 BamHI M D Y K D D D D K M A Q K E N V Y P
1 ggatccatggactacaagggagcagacgagaagagatggccagaaagaaaaagctgtaacc
21 WPYGSKTSQSLNTPQRVL
61 tgccccctacgcaagaacaacgccagagcgcctgtagaacccctgccccgcaggtgctg
41 RKEPATPAAQLAMNRSSNQS
121 agaaaaagaaacctgctgtacccctgccagccgtatgaaccgtcactcactcatctg
61 TAVFGQKTNKGTALQQGS
181 actgccgtgcagccgaggtaccgtaccgaacaccggtacgtccacgagcgc
41 QSRQPFTIDNFEIGRPLGKG
241 cagttcagacagccccttcaccatcgaaacctcactgactagccagacccccctggcaagggc
101 KFGNYYLAREKKKSRIVAL
301 agatctgaggcatgtctacctgctggagagagatccagtctgacctctgctggctg
121 IILFKSIKEKGEVQLRREI
361 atctctgttcaagagccagatcggagaaagagccgtggagacgagacgtgtg
41 EIQAHLKHPNILQLYNFYD
421 gagatccagccagccctgtagaaaccacactcctgcaagctacaacttctcatac
161 QQRILYLILEYAPRGEYKEL
481 cagatcctgcagagctctgtagatgagccccagagagctgtagctcaaaaaagctg
181 QKSTFDQERTATIMEELSD
541 cagagttcggcagcttctgcagacagagaaacgagccaccatctggaagaactgagcagac
201 AMLMYCKKKVIHDRIKPENL
601 gctccctgtatctgccaaagaagatgtacctccagggacatccagacggagaacctg
221 LGLQGELKIADFGSVHAP
661 tgtgctgaggctcgagggagagctgaagctctctctgtgtagacctgcattgccccccc
241 SLRRLCMGTLDYLPPEMIE
721 agatctgagagagagttgtgctggagccccctgccaggtatcctggagagctgagag
261 GRMHENMVDBLCIGVLCYEL
781 ggcctgatcagacagagatgtgtggacctggtgtcatcgcccttgctgtgstacgagtc
281 MVSFESPSHSHSETYRRIV
841 atgtctgagcagccccatccattcagagccccacacagcagacatcctgccgagctgtg
301 KVDLKFPSSMPMPALGAKDSLK
901 aaggtgacctgaaagttccccccagacagcatctccctggtgccgaaagccaacctgtagacgcaag
321 LLKHNPSRLPLEQVSAHPW
961 ctgctgagcaaaccccccagccccagactgtgcccccttggaacaggtgtctgccccatccccgg
341 VRANSSRRVLPSSALL* EcoRI
1021 gtctggccaaatatccgccagagtagctgctgccccccagccccctgtgataggaattc

B) **pLVX-Flag-Aurkb-K109R**

1 BamHI M D Y K D D D D K M A Q K E N V Y P
1 ggatccatggactacaagggagcagacgagaagagatggccagaaagaaaaagctgtaacc
21 WPYGSKTSQSLNTPQRVL
61 tgccccctacgcaagaacaacgccagagcgcctgtagaacccctgccccgcaggtgctg
41 RKEPATPAAQLAMNRSSNQS

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C) pLVX-Flag-Aurkb-T235A

```
BamHI

```

```
EcoRI

```

Figure 5.7
Legend

- Blue letters (uppercase): protein sequence
- Black letters (lowercase): gene sequence
- Red letters: BamHI restriction enzyme site
- Green letters: EcoRI restriction enzyme site
- Highlighted in yellow: Flag peptide (uppercase) and gene (lowercase) sequences
- Highlighted in green: Lysine 109 (K109)
- Highlighted in magenta: Threonine 235 (T235)
- Highlighted in blue: Lysine 109 mutated to arginine (R109)
- Highlighted in red: Threonine 235 mutated to alanine (A235)

Figure 5.7: Verification of pLVX clones via DNA sequencing -

The clones are (A) pLVX-Flag-Aurkb WT, (B) pLVX-Flag-Aurkb K109R and (C) pLVX-Flag-Aurkb T235A.
5.3.7 Lentivirus-mediated expression of Flag-Aurkb-WT, Flag-Aurkb-K109R and Flag-Aurkb-T235A in cultured mouse primary cortical neurons

Lentiviruses were generated for each of the clones, namely pLVX-Flag-Aurkb WT, pLVX-Flag-Aurkb K109R and pLVX-Flag-Aurkb T235A. Respective viruses were used to transduce cultured primary cortical neurons, 2 hrs after they were plated (Day 0). A complete medium change was performed 12 hrs after transduction and 750ng/ml of doxycycline was added to induce protein expression. As a further series of controls, a subset of cells that were transduced with viruses received no doxycycline treatment. The neurons were maintained in culture for a further 2 days and on Day 3, they were harvested for immunoblotting and some were fixed for immunocytochemistry.

As seen in figure 5.8A, the expression of target protein was specific to only cells treated with doxycycline (circled in blue), while cells that were not treated with doxycycline expressed basal levels of endogenous Aurkb. To determine the activities of the expressed target proteins, immunoblotting was carried out with a phopho-aurora kinases antibody and α-tubulin was used as a loading control. This time, phosphorylation at T235 was observed for WT (circled in red) but not in the kinase dead variants of Aurkb as shown in figure 5.8A. From these results, it was clear that Flag tag did not compromise the activity of Aurkb-WT, and that EGFP was the cause for the loss of activity described in section 5.3.4.

To assess differences in neuronal morphology between cells expressing either WT AurkB or kinase dead mutants, immunocytochemistry was used to visualise the cytoskeletal morphology of neurons expressing the different forms of Aurkb as depicted in figure 5.8B.
From immuno-labelling of Tuj1, Flag and DAPI stain, it was clear that control neurons which were transduced with viruses but were not treated with doxycycline appeared normal. They possessed defined cell bodies as well as elaborated and properly extended processes. Neurons that overexpressed Flag-Aurkb-WT looked kind of similar to controls with well-defined cell bodies and extended dendrites and axons which were slightly thicker. Notably, distinct morphological differences were observed in neurons that expressed kinase dead variants of Aurkb. Most prominent was a significant effect upon the arrangement of MT cytoskeleton that was in close proximity to the neuronal nucleus. Neurons that overexpressed either Flag-Aurkb-K109R or Flag-Aurkb-T235A displayed broader cell bodies which were filled with a frayed, scattered and disorganised MT cytoskeleton network bordering the nucleus as seen from the immunoreactivity of neuron specific marker, β-III tubulin (Tuj1). Because of this defect, quite a large population of these neurons failed to establish elaborated and properly extended dendrites. These morphological shortcomings were most obvious in neurons that expressed T235A kinase dead variant of Aurkb and this concurred with the western blot results whereby a mutation at T235 to alanine had a substantially greater inhibitory effect upon Aurkb phosphorylation in comparison to K109R mutant. Generally, the axons of neurons that expressed Flag-Aurkb-TA mutant were shorter than those of control, WT and K109R.

Overall, these observations were in direct accordance with the morphological changes seen in ZM447439 treated neurons and provide further evidence that Aurkb plays a vital role in neuronal morphology during neuronal maturation.
Chapter 5: Aurkb in neurite outgrowth

Figure 5.8

A

Without Doxycycline (-)  

With Doxycycline (+)

<table>
<thead>
<tr>
<th>Untreated Control</th>
<th>PLVX-Flag-Aurkb(WT) (ra)</th>
<th>PLVX-Flag-Aurkb(K108R) (ra)</th>
<th>PLVX-Flag-Aurkb(T235A) (ra)</th>
<th>PLVX-Flag-Aurkb(WT) (ra)</th>
<th>PLVX-Flag-Aurkb(K108R) (ra)</th>
<th>PLVX-Flag-Aurkb(T235A) (ra)</th>
<th>Recombinant His-Aurkb WT (ra) unch</th>
<th>His-Aurkb WT (ra) unch</th>
</tr>
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<tr>
<td>Aurkb (41kDa)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospho Aurkb</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>(42kDa)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>α-tubulin</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td></td>
</tr>
</tbody>
</table>

Cultured primary cortical neurons (mouse)

B

Aurkb (WT), - doxycycline  

Aurkb (WT), + doxycycline

Tuj1, Flag, DAPI
Figure 5.8: Lentivirus-mediated overexpression of Flag-Aurkb WT/ K109R/ T235A in cultured primary cortical neurons -

(A) Immunoblotting results indicated that different forms of Flag-Aurkb were successfully overexpressed in cortical neurons upon the addition of doxycycline. In comparison to WT, functionalities of kinase dead AurkB variants were verified through the lack of
phosphorylation. (B) Immunocytochemistry demonstrated that overexpression of kinase dead Aurkb affected neuronal morphologies during development. These cells possessed enlarged cell bodies that were filled and surrounded by frayed and disorganised microtubules. Processes such as dendrites in particular were not as elaborated as those in controls. Scale bar = 10um

5.3.8 Lentivirus-mediated knock-down of endogenous Aurkb in cultured mouse primary cortical neurons (using pGIPZ shRNAmir)

To further confirm the observations of the previous experiments, lentivirus-mediated knock-down of endogenous AurkB expression was undertaken. A set of pre-validated Aurkb shRNAAmirs in pGIPz lentiviral vector was purchased from Open Biosystems. pGIPz vector was specially chosen because it expresses a turbo GFP (tGFP) which allows easy identification of cells that were successfully transduced by the virus. Notably, this is a non-inducible system and shRNA transcripts are driven by a CMV promoter (details depicted in Figure 5.9A).

Lentiviruses carrying Aurkb shRNAAmir (Cat # 433575 or 433576) and non-silencing shRNAAmir control (NSC) were generated as according to procedures specified earlier. These were then used to transduce cultured primary cortical neurons, 2hrs after plating. Neurons were maintained in culture for 4 days after transduction and then lysed for western blot analysis. Surprisingly, no knock-down of Aurkb (both total and phosphorylated forms) was observed as highlighted in Figure 5.9B. This was unexpected, most likely due to the very poor (~10%) transduction efficiency as shown in Figure 5.9C.
To confirm that the viruses generated were indeed infectious, virus transduction was carried out on both HEK293T (dividing cells) and primary cerebellar granule neurons (CGN) (non-dividing cells). The efficiencies in both cases were quite good, averaging around 95% and 70% for HEK293T and CGN respectively (Figure 5.9C). It was suspected that the substantial difference in gene knockdown efficiency in cortical neurons could be due to the type of promoter present in pGIPz vector. In this regard, a recent paper in which the authors reported that CMV promoter directed robust transgene expression in CGN but worked poorly in primary cortical neurons may explain these outcomes (Li, Husic et al. 2010). To overcome this apparent problem, a different promoter was selected for use.

Figure 5.9

- Non-inducible
- Expresses turbo GFP
- Target shRNAmir expression driven by CMV promoter
- CMV is a relatively weak promoter for driving gene expression in cortical neurons (also reported by Li M et al. 2010)
Figure 5.9: Lentivirus-mediated knock down of Aurkb in primary neurons using pGIPz clones -

(A) Information on pGIPz lentiviral vector. (B) Western blotting data showed that pGIPz Aurkb shRNAmir failed to effectively knockdown total and phosphorylated Aurkb in primary neurons. (C) Varied transduction efficiencies were observed in different cell types despite addition of same amount of pGIPz lentivirus. It was suspected that CMV is not a strong promoter in driving transgene expression in cortical neurons. Scale bar = 10µm.
5.3.9 Lentivirus-mediated knock-down of endogenous Aurkb in cultured mouse primary cortical neurons (using pLKO.1 shRNAmir)

To overcome the poor expression of the CMV promoter in primary cortical neurons, the pLKO.1 shRNAmir which is driven by Human U6 (U6) promoter was selected. Notably however, this vector does not express turbo GFP (tGFP), meaning that it would not be possible to assess transduction efficiency by fluorescence approaches (figure 5.10A).

Notably, when neurons were transduced with pLKO.1 Aurkb shRNAmirs (Cat # 28840 and 28756), a substantial knock-down in total and phosphorylated Aurkb was seen through western blot analysis (circled in blue and red respectively in figure 5.10B). These observations were made in comparison to the amount of total and phosphorylated Aurkb from untreated control and cells that were transduced with virus carrying empty vector (pLKO.1 EVC). Furthermore, α-tubulin was probed to ensure approximately equal amount of proteins were loaded for each conditioned samples. The results indicated that pLKO.1 shRNAmir which is driven by U6 promoter is better suited for knock-down of target gene in primary cortical neurons as compared to the pGIPz clones.

Because pLKO.1 clones do not express EGFP, immunocytochemistry was required using total Aurkb antibody to identify neurons that were successfully knocked-down. However, it was quite difficult to definitively identify cells with lower levels of Aurkb upon treatment of Aurkb shRNAmir because the difference in Aurkb staining among cells on the same coverslips was subtle. This might be due to the varied levels of Aurkb knock down in cells. In spite of this fact, from figure 5.10C, it was obvious that in some neurons the fluorescent staining of Aurkb in cells treated with lentivirus carrying Aurkb shRNAmir was very much
less intense as compared to those in controls, given that both were exposed for the same amount of time and stimulated at a similar and constant excitation wavelength. This clearly indicated that Aurkb was effectively knock-down in cortical neurons with the use of pLKO.1 Aurkb shRNAmir lentivirus.

To observe the morphologies of these Aurkb knock-down cells, Tuj1 staining was performed. As shown in figure 5.10C, they possessed irregular-shaped cell bodies and were surrounded by highly disorganised processes in which the microtubules appeared frayed and loosely bundled, mirroring those neurons with overexpressed kinase dead variants of Aurkb as seen earlier. In contrast, control neurons looked normal with defined cell bodies and elaborated neurites.

To further substantiate the observations from immunocytochemistry, phase contrast images of untreated control and neurons transduced with pLKO.1 empty vector control (EVC), 28756 and 28840 shRNAmir on Day 1 and fixed on Day 5 were captured and shown in figure 5.12D. Generally, the morphologies of neurons transduced with Aurkb shRNAmir were substantially different from both untreated control and EVC transduced neurons. Untreated control or EVC transduced neurons possessed defined cell bodies and much elaborated, organized and neater primary and secondary projections. In contrast, neurons transduced with Aurkb shRNAmir had slightly enlarged, irregular shaped cells bodies with very much frayed and disorganised projections bordering the nucleus. This might be the cause for less elaborated dendrites. Notably, for each condition, these observations were made from individual cell or a group of 2-3 cells that were distinctly away from the others (highlighted in dash white box in figure 5.10D). By doing so, this ascertained that the observed mesh of scattered projections surrounding the nucleus were not from any cells that were nearby.
Overall, the effect of Aurkb knockdown in neurons complemented those observations seen in chemical inhibitors and overexpression studies. This clearly illustrated that the neuronal morphologies seen in the absence of active Aurkb were real for all the experiments that were undertaken to determine its functional and biological relevance in neurons in this chapter.

Figure 5.10
Figure 5.10
Figure 5.10
**Figure 5.10: Lentivirus-mediated knock down of Aurkb in primary neurons using pLKO.1 clones -**

(A) Information on pLKO.1 lentiviral vector. (B) Western blotting data showed that pLKO.1 Aurkb shRNAmir managed to effectively knockdown total and phosphorylated Aurkb in primary neurons. (C) Aurkb knock-down was further confirmed by immunocytochemistry. Neurons with diminished Aurkb expression were characterized by irregularly-shaped cell bodies that were bordered by frayed and highly disorganised MT processes. (D) Phase contrast images showed that neurons transduced with virus carrying Aurkb shRNAmir*s in general, looked different from controls. There were more neurons with loosely shaped cell bodies surrounded by scattered and messed up processes. Scale bar = 10μm.

### 5.4 Discussion

The main objective of this chapter is to investigate the functions of Aurkb in neurite outgrowth and neuronal maturation using a range of different experimental approaches to interfere with Aurkb activity. In this regard, inhibition of Aurkb with ZM447439, a specific chemical inhibitor, in cultured primary cortical neurons revealed several key observations. First, drug treatment not only reduced maximum axonal length but also decreased the number of cells with elaborated axons. Next, treated cells possessed abnormally enlarged cell bodies. Enlargement of the cell body, independent of nucleus size, was linked to a substantial increase in microtubule mass within the area between the nucleus and axon hillock region. In addition, cell bodies of these treated cells were bordered by several aberrant, thin, frayed and highly disorganised neuritic processes. Importantly, these cytoskeletal changes were also reflected in both functional knock-in of kinase dead Aurkb and knock-down of endogenous Aurkb in
cultured neurons via a lentivirus-mediated approach. Together, these overlapping observations from several different experimental approaches used to interfere with Aurkb functions in neurons have provided compelling evidence that Aurkb plays a novel and vital role in neurite outgrowth during neuronal maturation.

The key finding from this chapter is that inhibition of neuronal Aurkb activity (either pharmacologically or at the molecular level) caused significant cytoskeletal changes that distorted establishment and growth of neuritic processes. This is the first report describing any function for Aurkb in neurons, and hence it is not possible to compare these discoveries with existing literature in this field. However, it is possible to extrapolate how Aurkb might alter cytoskeletal dynamics in neurons by comparison with the mechanisms through which Aurkb contributes to cytoskeletal organisation during mitosis.

**Aurkb acts as a tension sensor and cytoskeletal organiser during mitosis:** As already reviewed earlier in Chapter 1, during bio-orientation of mitosis when kinetochores are properly attached to spindle microtubule, tension that ensues across centromeres stabilizes MT spindle and promotes segregation of sister chromatids. However, when sister chromatids are incorrectly attached to the spindle, loss of centromeric tension is sensed by Aurkb. This activates Aurkb and causes it to phosphorylate a series of microtubules-associated proteins such as Dam1 and Ndc80 to correct wrongful kinetochore-MT attachments or MCAK to directly destabilize impaired MT spindle (Zhang, Lan et al. 2007; Alushin, Ramey et al. 2010; Tien, Umbreit et al. 2010). Collapse of these structures then provides the cells a fresh chance to re-form the correct microtubules spindles.
Importance of tension in cytoskeletal organisation and neurite outgrowth: Neuronal development and regeneration involve intricate cytoskeletal growth dynamics. These processes resemble a labyrinth, where several neuritic processes within a neuron grow and compete with each other to become the axon, even when total neurite length is as short as 50um. This competition will only cease when a neurite manages to extend beyond a critical length, indicating that the neuron has polarized. More important is that during the competition process prior to establishment of neuronal polarity, not all neurites that protrude from the cell body are maintained but often, most of these neurites collapse and retract. Retraction in this case prevents aberrant neurite projections and promotes accurate establishment of future inter-cells connections. In this regards, dynamic growth behaviours of neurons are greatly dependent on a series of factors, including external growth environment and molecular mechanisms and forces that are evoked intrinsically. Among which, cytoskeletal tension is a major determinant.

Numerous studies which used calibrated glass needles to measure and apply tension at neurite tips have demonstrated that tension is a regulator of neurite initiation, elongation and retraction, processes commonly associated with neuronal maturation (Zheng, Lamoureux et al. 1991; Lamoureux, Ruthel et al. 2002; Lamoureux, Heidemann et al. 2011). These studies have shown that growth rate of a neurite is directly proportional to the tensile strength applied on the neurite and further supported the growth model in which tensional forces generated as a result of cytoskeletal and membrane dynamics taking place within the axial microtubule bundle and the advancing growth cone, are acting like tractors, pulling on the neurites and driving elongation. Likewise, it was suggested that when active cytoskeletal tensional forces generated by neurite shafts are below a certain threshold, failure to maintain projections occurs and causes neurite retraction (Heidemann and Buxbaum 1994).
Aurkb serves as a tension sensor and cytoskeletal organiser in neurons: Both processes, namely establishment of microtubule structures during mitosis and dynamic growth behaviours of neuritic projections require cytoskeleton generated tensional forces for the maintenance or growth of cytoskeletal structures. Because Aurkb is a known tension sensor of mitosis, it could also possibly serve as a temporally and spatially controlled sensor of cytoskeletal tension in neurons to determine whether neurites that protruded during the mentioned competitive phase of neuronal maturation should elongate or retract. If this is true and neuronal Aurkb works in a fashion that is very much similar to its role in mitosis, this implies that insufficient or absence of cytoskeletal tension evoked by a particular group of neurites could activate a localized pool of Aurkb. In this scenario, activated Aurkb will directly phosphorylate various neuronal substrates or indirectly enhance relevant signalling cascades to promote destabilization and elimination of selected neuritic projections, shaping neuronal cytoskeletal networks in the process. Based on this argument, inhibition of neuronal Aurkb on the opposite end will cause failure in the collapse and retraction of these neuritic projections during neuronal maturation. This could hence account for the morphological observations reported in this chapter, in which cells bodies of developing neurons with inactive Aurkb were filled and bordered by several aberrant, thin, frayed and highly disorganised neuritic processes as compared to control cells.

Furthermore, neuritic projections are often preceded by cytoskeletal structures such as perinuclear microtubule cage-like networks that originate from microtubule organization center (MTOC) of neurons (Tsai and Gleeson 2005; Higginbotham and Gleeson 2007). Following the same principle suggested earlier, when cytoskeletal tracks originating from for example, MTOC of neurons, are improperly formed and orchestrated due to Aurkb inhibition,
Figure 5.11: Aurkb serves as a tension sensor to organise cytoskeletal networks in neurons.
transport of cytoskeletal proteins destined for primary processes such as axon and dendrites may become hindered and cause the proteins to get “dam up” around the cytoplasmic region between nucleus and axon hillock. This might then explain the increase in microtubule mass within the expanded cell body of neurons with inactive Aurkb as seen through Tuj1 staining performed for all pharmacological and molecular knockin and knockout studies. In addition, uncontrolled formation of neuritic processes due to inactive Aurkb and possibly perturbation in the transport of cytoskeletal proteins could negatively affect axon establishment and growth, relating the axonal defects observed upon Aurkb inhibition in neurons.

Together, the hypothesis of Aurkb being a tension sensor to regulate and organise neuronal cytoskeleton was summarised in figure 5.11. Even though the mechanisms suggested based on existing knowledge remain to be validated, they have provided a reasonable and convincing explanation on how inhibition of neuronal Aurkb might be driving cytoskeletal changes reported in this thesis.

Overall, inhibition of neuronal Aurkb resulted in abnormal neuritic projections and this clearly suggested that it plays a pivotal role in the orchestration of cytoskeletal networks in developing neurons. Next, it will be interesting to account for these morphological changes on a molecular level since identification of any neuronal molecules associated with Aurkb inhibition could then form the basis for further research. In this regards, yeast 2 hybrid and phosphoproteomics were undertaken in the next chapter to screen for binding partners and downstream signalling substrates of Aurkb in neurons.
CHAPTER 6

Identification of binding partners and signaling substrates of Aurkb in neurons.
Chapter 6: Neuronal partners and substrates of Aurkb

6.1 Introduction

The overarching aim of this chapter was to undertake preliminary studies to identify the potential signaling pathways associated with AurkB mediated changes in neurite growth. This was undertaken in two parts; i) a yeast two-hybrid (Y2H) study to identify direct binding partners and activators of AurkB, and ii) a phosphoproteomics screen to identify the downstream targets of AurkB activation.

Y2H is a powerful tool for identifying physical interactions between a target protein and other proteins. In brief, the target protein (Aurkb) is used as bait in which it is fused to the DNA binding domain (DBD) of a transcription factor. DBD of the complex will bind to operator sequences upstream of reporter genes but transcription of reporter genes is suppressed as sequences require for transcription are still missing. On the other side, unknown protein Y (prey) is fused to the activation domain (AD) of the same transcription factor previously fused to the bait. This 2nd complex does not promote transcription since without DBD; it could not be recruited to the promoter sites. However, when both target protein (bait) and unknown protein (prey) are co-expressed in yeast, interaction between the two reconstitute a “hybrid” transcriptional factor, where AD of prey could be then recruited to the promoter sequences to initiate transcription of reporter genes. Transcription of reporter genes in most cases account for enzymes that allow yeast to grow on selective medium lacking amino acids like histidine and/or adenine or give it the ability to cleave X-gal, an organic compound that upon cleavage will turn from colourless to blue. Yeasts that transcribe reporter genes are considered true positives and they will be isolated and amplified in E.coli, from which the plasmids will be purified for DNA sequencing. These sequences will then be
translated in all 3 reading frames and searched against online databases such as BlastX coupled with Swissprot to reveal identities of the interactors. Apart from possible false negatives and false positives, cell toxicity due to Aurkb overexpression is less likely to be an issue for Y2H since it is endogenously expressed in yeasts (Biggins, Severin et al. 1999)

The second aim of this chapter is to determine substrates that are directly affected by and/or involved in Aurkb signalling in neurons. Because Aurkb is a kinase, this means that it has the ability to phosphorylate specific protein substrates. Generally, protein phosphorylation plays vital roles in a wide range of cellular processes. Not only could it alter protein expression and activity levels, it could also affect its subcellular localization. More important, proteins phosphorylated by kinases often form complexes with other protein partners which then further activate or inhibit certain molecules to ignite a series of signalling cascades that result in cellular changes. As such, to obtain a broad but meaningful overview on how inhibition of Aurkb affects biological signalling in neurons on a molecular level, phosphoproteomics was carried out.

Unlike normal proteomics which generate only general information on the expression profiles of proteins, phosphoproteomics provide a more targeted piece of information. Apart from identifying proteins that are significantly regulated due to drug treatment, it further informs the user about proteins’ phosphorylation status and the sites involved. This renders a more accurate knowledge on protein activity and highlights important signalling pathways that are much more relevant based on the collated protein phosphorylation profiles. At the same time, it helps in the revelation of potential curative protein targets associated with a pathological condition as exemplified by the use of selected kinase inhibitor such as ZM447439, which targets Aurkb specifically. Overall, while phosphoproteomics will greatly expand knowledge
about the numbers and types of phosphoproteins, its greatest promise are the speedy analysis of entire phosphorylation based signalling networks.

6.2 Experimental design

Yeast 2 hybrid (Y2H) screening was performed by DualSystem Biotech, Zurich, Switzerland and the procedures were specified in Chapter 2 section 2.7.1. Two different Y2H screens were done, one using total mouse brain cDNA library and the other using mouse primary cortical neuron cDNA library. Here, mouse brain cDNA library was already available from the company itself but for the latter, it was specially created by DualSystem Biotech on our behalf, using RNA that was harvested from cultured primary cortical neurons on our side. The main purpose of doing two different screens was that the interactors identified from mouse brain cDNA library screen might not be true interactors of Aurkb in neurons since the cDNA library was derived from a range of different cell types which include astrocytes, microglial and oligodendrocytes. As such, an additional screen using a cDNA library derived from pure cultures of primary cortical neurons was performed as a precautionary measure. Common partners identified in both screens will most likely be a true and specific interactor of Aurkb in neurons.

For phosphoproteomics studies, Day 5 neuronal lysates were collected from control and treated cells, 8 hrs after treatment with either DMSO (vehicle) or ZM447439, a known chemical inhibitor of Aurkb, according to procedures specified in Chapter 2. Because quite a significant amount of protein per condition (2mg) had to be collected for the experiment, this was done over a few cultures. For this experiment, it was more logical to inhibit neuronal Aurkb using a specific chemical inhibitor since it was relatively impossible in terms of cost
and time to produce enough lentivirus to knock-down Aurkb in neurons to obtain that amount of protein required for phosphoproteomics. Enrichment of phosphopeptides was done via electrostatic repulsion-hydrophilic interaction chromatography (ERLIC), in which the samples were passed through a PolyWAX LP column. Phosphopeptides enriched from each sample were sequenced by LC-MS/MS and their identities were confirmed upon comparison with protein databases. Comparative phosphoproteomes coupled with bioinformatics analysis was then performed to reveal proteins that were differentially expressed between control and ZM447439 treated samples. Identified proteins were then passed through STRING analysis to reveal functional protein association networks. This allowed easy prediction of possible neuronal pathways that were affected by Aurkb inhibition.

6.3 Results

6.3.1 Interactors of Aurkb identified from Y2H screens

Bait strain (Aurkb) was mated with prey strains (derived from cDNA library) and a total of $5.4 \times 10^6$ and $8.1 \times 10^5$ mated transformants were screened from experiments performed with cDNA library obtained from mouse brain and mouse primary cortical neurons respectively. In each of these screens, 14 primary interactors were found. For the screen using mouse brain cDNA library, 12 out of the 14 interactors were sequenced successfully and of which, 11 were identified as inner centromere protein (INCENP) and 1 as ATP synthase-coupling factor 6. As for the screen with cDNA library derived from just primary cortical neurons, 10 out of the 14 interactors were sequenced successfully and of which, 7 were identified as INCENP while the remaining 3 were found to be ferritin light chain, glycosyltransferase 8 domain-containing protein 1 and nucleoporin GLE1. Among all the interactors identified, only
INCENP was classified as a class A interactor which meant that it had been rescued more than three times and would most likely be a true interactor of Aurkb in neuron. Rest of the proteins identified were classified as class C interactors which meant that each of them was found only once in the screens and for that, they may represent either true interactors of Aurkb or common false positives. These data were summarised in figure 6.1.

6.3.2 Presence of INCENP in E15 and P3 mouse brain

From the Y2H data, it seemed that INCENP is a real interactor of Aurkb in neuron and to know whether it is expressed in the brain, immunohistochemistry was performed on E15 and P3 brain slices using an INCENP antibody. Relatively stronger INCENP immunoreactivity was seen in specific regions such as the cerebral cortex and hippocampus of E15 and P3 mouse brains as shown in figure 6.2. This clearly indicated that INCENP is expressed in neurons since these brain regions in which it was found are predominantly made up of neurons and more indicatively, its immunoreactivity resembled the staining patterns of Aurkb and NeuN, a known neuronal marker, as seen previously in Chapter 4. Immunohistochemistry performed in this case served as a preliminary study of INCENP in the brain. Nonetheless, more experiments are required to confirm its presence and interactions with Aurkb in neurons but these are beyond the scope of this thesis.
### Chapter 6: Neuronal partners and substrates of Aurkb

#### Figure 6.1: Result overview for Y2H screens using cDNA library derived from either mouse brain or mouse primary cortical neurons.

<table>
<thead>
<tr>
<th></th>
<th>Using mouse brain cDNA library</th>
<th>Using mouse primary cortical neurons cDNA library</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Report strain</strong></td>
<td>NMYS1 bearing bait plasmid pMyc-mAuroraB-LexA</td>
<td>NMYS1 bearing bait plasmid pMyc-mAuroraB-LexA</td>
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<tr>
<td><strong>Screening medium</strong></td>
<td>SD-his-leu-trp + 2.5 mM 3-AT</td>
<td>SD-his-leu-trp + 2.5 mM 3-AT</td>
</tr>
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<td><strong>Complexity of the cDNA library</strong></td>
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<td>$7.4 \times 10^6$</td>
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<tr>
<td><strong>Average insert size</strong></td>
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<td>1.9 kb</td>
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<td>Sequential transformation</td>
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</tr>
<tr>
<td><strong>Transformants screened</strong></td>
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<td>$8.1 \times 10^5$</td>
</tr>
<tr>
<td><strong>Number of primary interactors</strong></td>
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<tr>
<td><strong>Plasmid isolation from</strong></td>
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</tr>
<tr>
<td><strong>Successful plasmid rescue from 5' junction sequencing primer</strong></td>
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<td>10</td>
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<tr>
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<td>INCENP (identified 7 times, class A)</td>
</tr>
<tr>
<td></td>
<td>ATP synthase-coupling factor 6 (identified 1 time, class C)</td>
<td>Ferritin light chain (identified 1 time, class C)</td>
</tr>
<tr>
<td></td>
<td>Glycosyltransferase 8 domain-containing protein 1 (identified 1 time, class C)</td>
<td>Nucleoporin GLE1 (identified 1 time, class C)</td>
</tr>
</tbody>
</table>

**Definition of interactors’ classification:**

**Class A:** Interactors which have been rescued more than three times. They represent highly likely interactors of the bait.

**Class B:** Interactors which have been identified two times. They represent highly likely interactors of the bait.

**Class C:** Interactors that were found only once in the screen (“singletons”). Although some of those may indeed represent true interactors of the protein of interest, others represent common false positives.

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Figure 6.1: Result overview for Y2H screens using cDNA library derived from either mouse brain or mouse primary cortical neurons.
Figure 6.2: Presence of INCENP in E15 and P3 mouse brain -
Single immunohistochemical-labelling was performed and INCENP was found predominantly in the cortex and hippocampus. Scale bar = 50um.

6.3.3 Differentially regulated proteins identified from phosphoproteomics

Comparison between phosphoproteomes of control and ZM447439 treated cells was made. There were a total of 270 differentially regulated proteins from either control or treated group.
Chapter 6: Neuronal partners and substrates of Aurkb

with a minimum mascot score of 40 that passed One-way ANOVA analysis and with a statistical significance of p < 0.05. Because the list is too large for functional association analysis, all hypothetical and unidentified proteins were removed and only proteins with a combined mascot score of >150 from both control and treated groups were used. The list was shortened by combined mascot score instead of percentage change between samples because a higher mascot score, which indicated more peptide fragments from a certain protein were identified, provided a higher level of confidence that the particular protein was indeed present in both/either samples. With that, this came down to 77 proteins as shown in table 6.1, where mascot scores of individual proteins from control and treated groups as well as their % change (treated / control) were indicated. A number of these proteins turned out to be key regulators of neuronal cytoskeleton and they included Crmps, Gap43, Clasp, Mtap, Mapt, Dbn, Ncam, Add and Ppp1r9a. In addition, kinases that are known to evoke signalling cascades to modulate neuronal cytoskeletal structures were also found and they included, GSK3β, Gsk3α, Pak1, and Pdpk1. In general, it appeared that inhibition of neuronal Aurkb was causing significant cytoskeleton changes and the data supported the results found in earlier chapters.

6.3.4 Prediction of functional protein association networks

Together with Aurkb and INCENP (identified from Y2H), the proteins presented in Table 6.1 were passed through an online database resource known as Search Tool for the Retrieval of Interacting Genes (STRING) version 9.0, for association analysis. STRING is a database of known and predicted protein interactions and it is widely reviewed and used for studying protein-protein networks (Choudhary, Kumar et al. 2009; Fridlich, Delalande et al. 2009; Gaballa, Newton et al. 2010; Szklarczyk, Franceschini et al. 2011). The interactions made by STRING include direct (physical) and indirect (functional) associations and they are
derived from four sources, namely genomic context, high-throughput experiments, co-expression studies and previous knowledge from existing literature. STRING version 9.0 quantitatively integrates interaction data from these sources for a large number of organisms, and transfers information between these organisms where applicable. The database currently covers more than 5 million proteins from approximately 1200 organisms.

Among the 77 analysed proteins, 36 of them were found to establish some forms of association and this is shown in figure 6.3. Importantly, the interactome revealed 4 distinct groups of proteins, each commonly associated with a functional pathway. First, a cluster of molecules and kinases that hold key roles in orchestrating remodelling of the neuronal cytoskeleton was found and they included Gsk, Pdpk, Mtap, Mapt, Crmp, Gap, Add, Dbn, Ncam and Dxc. Next, it involved a group of proteins (Clasp, Rangap, Prkar, Djnc and Macf) that has direct and/or indirect associations with Aurkb and INCENP. The third group of proteins identified are factors that regulate protein synthesis and they were Eif, Abcf, Eef and Ssrp. Finally, the network also highlighted a series of proteins that are involved in RNA and DNA processing, namely; Dhx, Cstf, Srrm, Hnrnp, Psip, Top and Ptbp. These groups of proteins were circled in red as depicted in figure 6.3.
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Chapter 6: Neuronal partners and substrates of Aurkb

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Table 6.1
Table 6.1: List of 77 differentially regulated proteins identified from comparative phosphoproteomes analysis of control and ZM447439 treated neuronal samples, 8 hrs after drug treatment -

These proteins have a combined mascot score of > 150. They also passed one-way ANOVA and are significant with $p < 0.05$. Individual protein mascot scores as well as its percentage change (treated / control) across samples are indicated.
Protein associations are made based on four sources, namely genomic context, high-throughput experiments, co-expression studies and previous knowledge from existing literatures.

Note: Associations are made by blue lines. Stronger associations are represented by thicker lines.
Figure 6.3: Functional protein association networks predicted using STRING version 9.0 -

Protein associations are made based on four sources, namely genomic context, high-throughput experiments, co-expression studies and previous knowledge from existing literatures. Four distinct groups of proteins, each involved in a known functional pathway, are circled in red. The pathways include modulation of neuronal cytoskeleton, Aurkb regulation, protein synthesis and RNA and DNA processing.

6.4 Discussion

6.4.1 Implications of INCENP as a binding partner of Aurkb in neurons

The discovery of INCENP as a binding partner of Aurkb from Y2H screens was not surprising since it had already been identified by others who work in the field of cell cycle research to be a regulator of Aurkb. To briefly recall what was already discussed in Chapter 1, binding of INCENP to Aurkb in human promotes autophosphorylation of threonine 232 (T232) found within the kinase activation loop and through a feedback mechanism, Aurkb further phosphorylates INCENP TSS motif located at the C terminal end which then results in a series of structural changes to fully elicit Aurkb activity. These findings from dividing cell clearly illustrated the importance of the interaction of INCENP with Aurkb, through which it has a significant impact on Aurkb activity. More importantly, this is also probably true in post-mitotic cells since INCENP was identified more than 5 times in each of the Y2H screens performed using cDNA library derived from either whole mouse brain or pure cultures of primary cortical neurons. Besides, INCENP is also expressed in the brain, particularly in
neurons, as shown through immunohistochemistry. Furthermore, this is supported by the fact that overexpression of a kinase dead variant of rat Aurkb in which threonine 235 located in its activation loop, an amino acid that is dependent on INCENP-mediated autophosphorylation, when mutated to alanine (T235A) caused evident and significant neuritic changes in developing neurons as reported in Chapter 5. Together, these findings provided indirect but reasonable supportive evidence suggesting that INCENP could possibly interact and regulate Aurkb function in neurons to evoke a series of activities that orchestrate neuronal cytoskeleton networks.

Identification of INCENP through Y2H also provided interesting insights on the dynamic localization of Aurkb within a cell. In a proliferating cell, Aurkb is localized to specific mitotic structures but prior to nuclear envelope breakdown, apart from residing mainly in the nucleus, some of it can be found in the nucleocytoplasmic compartment. It is puzzling how Aurkb is transported outside the nucleus since it does not have localization signals that transport receptors could recognise. It was however reported that its transport is mediated by interacting partners such as INCENP which contains multiple nuclear localization signals (Rodriguez, Lens et al. 2006). This has important implications because it highlights the point that if INCENP does bind Aurkb in neurons, it could possibly mediate and explain the cytoplasmic localisation of Aurkb during the various stages of neuronal maturation as reported and discussed in Chapter 4.

In addition, Y2H screens did not pick up survivin and borealin which are 2 other components of the chromosomal passenger complex (CPC) as reviewed in Chapter 1. This makes sense because it was reported that both survivin and borealin interact with the N terminus of INCENP instead of binding directly to Aurkb (Ruchaud, Carmena et al. 2007). As seen,
INCENP is an important linker that holds various components of the CPC together. With that, the results from Y2H raised a few interesting questions: Are survivin and borealin expressed in neurons? If so, do they bind INCENP and Aurkb to form the CPC complex which then carries out alternate neuronal functions? If not, does that mean the activity of Aurkb-INCENP complex in neurons is independent of both survivin and borealin interactions and unlike cell cycle, it could function on its own where it interacts and phosphorylates a unique class of neuronal substrates to dictate neuritic changes?

6.4.2 Proteins that modulate neuronal cytoskeleton

Phosphoproteomics coupled with functional analysis of protein associations have provided interesting insights on pathways that are directly associated with Aurkb inhibition in neurons. Among which, the most significant discovery was a cluster of proteins and kinases that is commonly associated with the regulation of microtubule networks and actin filaments. The identification of these proteins is important because it is in complete accordance with the findings in previous chapters demonstrating that Aurkb has direct actions upon cytoskeletal organisation in developing neurons. When considered altogether, the phosphoproteomic, pharmacological and molecular knockin and knockout studies provide considerable evidence that Aurkb has key roles in neurite cytoskeleton modulation. Here, the functions of the particular proteins highlighted in this group are briefly discussed in regards to phosphorylation events associated with modulation of neuronal cytoskeleton based on existing literatures.
6.4.2.1 Growth associated protein 43 (Gap43)

Gap43 is a major neuronal protein that plays multiple roles in various processes which include neurite formation, regeneration, development and plasticity. Its importance is evident from the fact that mice lacking Gap43 die within days after birth due to axon pathfinding defects (Strittmatter, Fankhauser et al. 1995). Gap43 is a substrate of many kinases such as PKC and phosphorylated Gap43 are associated with growth cones that are highly motile and growing but not those that are actively retracting or had already collapsed (Dent and Meiri 1998). Particularly, phosphorylation of Gap43 at Ser41 was implicated with branching and growth of neuritic processes as well as events of plasticity such as long term potentiation. Apart from Ser41, Gap43 possesses many other phosphorylation sites. However, the answers to how and why these sites are phosphorylated remain to be determined even until today (Spencer, Schuh et al. 1992). For that, it will be interesting to find out how these sites are phosphorylated and what sort of neuronal events are ignited or inhibited by these phosphorylations upon Aurkb inhibition.

6.4.2.2 Collapsin response mediator proteins (Crmp1, Crmp2 and Crmp3)

Three forms of Crmp (type 1, 2 and 3) were identified from phosphoproteomics. Crmps are a family of cytosolic phosphoproteins expressed highly in the brain. By responding to various extracellular cues, they play vital roles in modulating cytoskeletal structures and contribute to neuronal development and maturation. In particular, they are important mediators of Sem3A, a known repulsive guidance cue that can cause actin depolymerisation and crumbling of growth cones. Changes in Crmp activation states, often due to phosphorylation by a variety of
kinases in response to Sem3A and other signalling factors, evoke drastic neuritic changes. For example, non-phosphorylated Crmp2 has the ability to promote axonal growth and branching, possibly by facilitating microtubule assembly via direct binding to tubulin heterodimer (Fukata, Itoh et al. 2002). However, sequential phosphorylation of Crmp2 by Cdk5 and Gsk3β upon Sem3A stimulation reduced its binding affinity for tubulin and promoted growth cone collapse (Uchida, Ohshima et al. 2005). Likewise, phosphorylation of Crmp2 by Rho kinase led to the same phenomenon (Arimura, Menager et al. 2005). Specifically, phosphorylation of Crmp2 at Thr514 by Gsk3β impaired neuronal polarization and deterred neurite outgrowth. In contrast, treatment with neurotrophic factors such as neurotrophin-3, promoted axonal growth by decreasing phosphorylated Crmp2 and elevating active forms of non-phosphorylated Crmp2 (Yoshimura, Kawano et al. 2005).

Like Crmp2, Crmp1 plays vital roles in various neuronal processes. Phosphorylation of Crmp1 by Cdk5 is vital for mediating Sema3A induced spine development in the cerebral cortex (Yamashita, Morita et al. 2007). Apart from that, Crmp1 also participates in neurotrophins induced neurite outgrowth in sensory neurons (Quach, Duchemin et al. 2004). Interestingly, a novel splice variant of Crmp1 with an extended N terminus could prevent neurite retraction by binding and inhibiting RhoA binding kinase (Leung, Ng et al. 2002). Furthermore, Crmp1 is involved in radial neuronal migration through Reelin signalling in the developing cerebral cortex and its loss has a devastating effect on LTP maintenance and spatial learning and memory (Yamashita, Uchida et al. 2006; Su, Chien et al. 2007).

Crmp3 on the other hand was less investigated. However, recent work performed on Crmp3 knockout mouse brain demonstrated that it is involved in neuronal plasticity and dendrite arborisation. Its absence resulted in several abnormalities of dendrites and spine
morphogenesis within the CA1 region of hippocampus (Quach, Massicotte et al. 2008). A more recent paper showed that overexpression of Crmp3 prevented PrP peptide induced dendrite dystrophies in cultured hippocampus neurons, possibly by maintaining proper Ca2+ trafficking and signalling (Quach, Wang et al. 2011). This showed that Crmp3 might be important for reducing hippocampal dendritic dystrophy associated with neurodegeneration. Together, these findings highlighted that Crmps are key modulators of neuritic processes and their actions are greatly regulated by phosphorylation.

### 6.4.2.3 Microtubule associated proteins (Mtap1b)

Map1b is a major neuron-specific microtubule associated protein. It is linked to various neuronal processes that involve dynamic control of microtubule stability. First, it has a role in brain development since it is highly expressed in newborn rodent brain but decreases in expression with age. Map1b is also implicated with neuronal differentiation and migration. Phosphorylation of Map1b at Ser1269 and Thr1265 by Gsk3β is spatially restricted to growing axons and in a gradient that is highest at the distal end (Trivedi, Marsh et al. 2005). This showed that its phosphorylation is a vital determinant of neuronal differentiation events such as axon establishment. Next, its relevance in neuronal migration was evident from the abnormalities seen in various brain regions of Map1b-deficient mice. These observations happened to mimic migration defects seen in the brains of Reelin knockout mice. This was interesting because Reelin could induce phosphorylation of Map1b by activating Gsk3β and Cdk5 both in vivo and in vitro (Gonzalez-Billault, Del Rio et al. 2005). Unlike Crmp2, phosphorylation of Map1b by Gsk3β activates it and destabilizes microtubules to create a dynamic state necessary for migration.
6.4.2.4 Debrin (Dbn)

Drebrin is an F-actin binding protein that regulates actin dynamics. It disrupts actin-myosin interactions by inhibiting the ATPase activity of myosin and competes with other proteins such as tropomyosin and α-actinin for binding to F-actin. Drebrin A is a major isoform found in neurons whereby it plays a pivotal role in directing dendritic spine morphogenesis and plasticity (Takahashi, Sekino et al. 2003; Ivanov, Esclapez et al. 2009). Overexpression of drebrin A caused the formation of enlarged megapodia in immature neurons and lengthened spines in mature neurons (Hayashi, Ishikawa et al. 1996; Mizui, Takahashi et al. 2005). On the other hand, its suppression not only reduced spine density and resulted in the appearance of thin, under-developed spines but also inhibited the overall number of synapses in hippocampal neurons (Takahashi, Mizui et al. 2006). Binding of drebrins located on F actin in proximal region of filopodia to EB3 that were located at the tips of microtubules invading filopodia aided in the establishment of F actin – microtubules interactions necessary for neuritogenesis (Geraldo, Khanzada et al. 2008). Interestingly however, being a protein with multiple phosphorylation sites, its regulation by kinases was never thoroughly investigated in neurons and as such, this is a worthy area that warrants further investigations.

6.4.2.5 Neural cell adhesion molecule (Ncam)

Ncam is a cell-cell adhesion protein expressed on the surface of neurons. However, its roles in neurite outgrowth do not rely as much on its adhesion function but instead are more dependent on its ability to activate a complex network of intracellular signalling cascades (Doherty, Williams et al. 2000). For example, Ncam is vital in promoting
autophosphorylation of Fgfr, a receptor tyrosine kinase, which then evokes localized calcium signalling to stimulate axonal guidance and growth (Saffell, Williams et al. 1997). Calcium signalling in this case should not be undermined as it then promotes phosphorylation of Gap43 which has an additive effect on neurite outgrowth responses (Doherty, Williams et al. 2000). In addition, immunoprecipitation demonstrated that Ncam could associate with Fyn, a non-receptor tyrosine kinase, which then further activates another non-receptor tyrosine kinase (Fak), to promote neurite outgrowth (Beggs, Soriano et al. 1994; Beggs, Baragona et al. 1997). Even though Ncam does not have any motifs for binding SH2 or 3 domains of Fyn, it may complex with another protein such as Rptpα, a receptor tyrosine phosphatase to activate Fyn (Bodrikov, Leshchyns'ka et al. 2005). More interesting was that phosphorylation of Gap43 by PKC is important for Ncam induced neurite outgrowth and this involves functional association between Ncam and Gap43 coupled with spectrin. Although the phosphorylation of Ncam itself remains obscure, it appears that its roles in diverse intracellular signalling necessary for neurite growth might explain why it was significantly regulated in this phosphoproteomics study.

### 6.4.2.6 Adducin (Add)

Basically, adducins are a group of membrane cytoskeleton associated proteins that are encoded by 3 closely related genes (α, β, γ). They are known substrates of PKC and depending on phosphorylation; they play vital roles in actin-spectrin network assembly (Matsuoka, Li et al. 2000). In dividing platelets and epithelial cells for example, dephosphorylated adducins stabilize spectrin skeleton and prevent filopodia formation by capping actin filaments. However, upon phosphorylation by PKC, they are released from the filaments and loss of actin capping activity then favours actin-filopodia extension. Likewise,
similar functions of adducins were recently observed in neurons whereby phosphorylation of synaptic adducins determined its levels and localization which in turn influenced synaptic remodelling via actin-based synapse elaboration and/or spectrin-based synapse stabilization (Pielage, Bulat et al. 2011). Notably, β-adducin (Add2) is expressed at high levels in brains. Apart from \textit{PKC}, a very recent study identified β-adducin as a novel substrate of Gsk3β \textit{in vitro} and \textit{in vivo}. Through overexpressing wild type β-adducin in neurons, the team further demonstrated that its phosphorylation by Gsk3β promotes neurite outgrowth (Farghaian, Turnley et al. 2011).

\textbf{6.4.2.7 Glycogen synthase kinase 3 beta (Gsk3β)}

Gsk3β plays diverse roles in neuritic process formation. Its activity is largely determined by phosphorylation. In regards to neuronal polarity, inactivation of Gsk3β via phosphorylation causes neurons to form multiple axons. For that, a group specifically suggested that this was caused by localized phosphorylation of Gsk3β at Ser9 by Akt/PKB (Jiang, Guo et al. 2005). However another group, which used neurons from a knock-in mouse in which Ser9 of Gsk3β was mutated to alanine, argued that this was not mediated by Akt/PKB pathway but rather it was due to an accumulation of APC proteins in the neurite tips (Gartner, Huang et al. 2006). In addition to neuronal polarity, Gsk3β is also associated with neurite outgrowth. While it was reported that inhibition of Gsk3β strongly facilitates neurites growth in myelin treated neuronal cells (Seira, Gavin et al. 2010), there were also studies demonstrating that myelin could directly phosphorylate and inactivate Gsk3β to inhibit neurite outgrowth (Alabed, Pool et al. 2010). As seen, the mechanism of how Gskβ regulates neurite formation is complex yet intriguing. However, putting aside these contrasting findings, what more important is that
these research evidence point to the fact that Gsk3β is a vital regulatory kinase for neurite growth and modulation.

6.4.2.8 p21 protein activated kinase 1 (Pak1)

Pak1 is a key kinase that regulates cytoskeletal structures. It is highly expressed in postmitotic neurons (Zhong, Banerjee et al. 2003). Localized activation of Pak1 in neuron is vital in determining polarity and morphology. Overexpression of catalytically active Pak1 resulted in multiple neurites with axonal like identity but caused a significant reduction in outgrowth of the longest neurite. Knockdown of Pak1 evoked abnormal neuronal soma protrusions that were made up of curved and distorted acetylated microtubules bordered by aberrant and extensive lamellipodia. Further evidence suggested that Pak1 induced cytoskeletal changes were due to activation of Rac1 and inhibition of cofilin, both of which are well-known actin associated molecules (Jacobs, Causeret et al. 2007). It was also reported that Pak1 could control stathmin via phosphorylation, a microtubule destabilizing protein, to prevent catastrophic breakdown of microtubules (Wittmann, Bokoch et al. 2004). As seen, its ability to regulate both cytoskeletal elements associated molecules might explain the changes seen in neuronal F-actin and microtubule skeletal network. Temporal and spatial regulation of Pak1 also plays a big role in cortical development. Overexpression of constitutively active Pak1 caused neurons to possess confused and disorientated projections and to arrest in the intermediate zone (IZ). In contrast, loss of Pak1 causes majority of the neurons to accumulate in both IZ and deep cortical layers but fails to appropriately cease migration with some neurons projecting into cell-sparse marginal zone (Causeret, Terao et al. 2009). Apart from its roles in neuronal polarity and cortical development, Pak1 also has the ability to regulate dendritic spines. This could be seen from the fact that it is able to limit
cofilin and prevent Aβ oligomer induced degradation of drebrin, an F-actin binding protein associated with spine morphogenesis and plasticity that was discussed earlier (Zhao, Ma et al. 2006).

### 6.4.2.9 Doublecortin-like kinase 1 (Dclk1) and 3-phosphoinositide dependent protein kinase 1 (Pdpk1)

Dclk1 and Pdpk1 were also identified. Briefly, Dclk1 works through calcium signalling pathway to regulate doublecortin, a microtubule associated protein that is involved in neuronal development and migration (Friocourt, Liu et al. 2007). As for Pdpk1, it is a master kinase that regulates several other kinases including Gsk, Akt and PKC, all of which play crucial roles in neurite outgrowth, elongation and branching (Larsson 2006; Read and Gorman 2009). Notably, Pak1 which was mentioned earlier has a kinase independent function where it acts as a scaffold for Akt recruitment to the membrane and facilitates its activation by Pdpk1 (Higuchi, Onishi et al. 2008).

### 6.4.3 Proteins associated with Aurkb

In addition, a series of proteins that forms direct and/or indirect associations with Aurkb and INCENP was identified. This involved Clasp, dynein, Rangap and Macf1. During mitosis, recruitment of Clasps to the outer kinetochore is necessary for accurate kinetochore – spindle microtubule attachments and this process is mediated by events fired by Aurkb phosphorylation (Maiato, Fairley et al. 2003; Schmidt, Kiyomitsu et al. 2010). Because Clasps are also commonly associated with dynein and Macf1, this indirectly links both proteins to Aurkb. This linkage is interesting because dynein, being a motor molecule that mediates cargo...
transport along microtubule tracks of cells, is not only involved in kinetochore assembly (Kasuboski, Bader et al. 2011) but also in neuronal processes such as axoplasmic transport (Goldstein and Yang 2000). As for Macf1, it is a linker that bridges both microtubules and actin filaments and for that, it is involved in many cellular processes including cell migration and neuronal growth (Sanchez-Soriano, Travis et al. 2009; Zaoui, Benseddik et al. 2010). In particular, recent studies have further demonstrated that knockout of Macf1 resulted in multiple developmental defects in mouse brain (Goryunov, He et al. 2010). Interestingly, both proteins are regulated by phosphorylation. While anterograde transport of membrane bound organelles by dyneins, at least those in the brain, are regulated via phosphorylation (Dillman and Pfister 1994), Macf1 involvement in wound healing is mediated specifically by GSK3β (Wu, Shen et al. 2011).

6.4.3.1 Cytoplasmic linker associated proteins (Clasp2)

Clasp2 is particularly interesting among these identified proteins. Apart from mitosis, it is also involved in cell migration and neuronal growth cone dynamics. Because Clasp2 is closely associated with Aurkb as seen from the network map, it appeared to be the most relevant protein that links Aurkb to other well-known regulators of neuronal cytoskeleton mentioned in previous section.

Basically, Clasp2 is a microtubule plus-end tracking protein (+TIP) that interacts primarily with cytoplasmic linker proteins (CLIPs) to promote growth and stabilization of microtubules at certain cellular sites in response to signalling cues. (Akhmanova, Hoogenraad et al. 2001). During polarised cell migration, non-phosphorylated Clasp2 accumulates and binds EB1 at the plus ends of microtubules. This asymmetrical distribution of Clasp2 along microtubules
then facilitates its binding to Iqgap1, an effector of Rac1/Cdc42. By binding both EB1 and Iqgap1, Clasp2 serves as a connector to hold microtubule and actin filaments together at the leading edge to drive cell migration. Here, it was found that phosphorylation of Clasp2 at specific sites by Gsk3β could dissociate its binding from Iqgap1, EB1 and microtubules. Dissociation of proteins and subsequent loss of asymmetrically distributed Clasp2 caused migration to stall (Watanabe, Noritake et al. 2009). These results complemented another finding in which expression of constitutively active Gsk3β in epithelial cells destabilized lamella MTs by disrupting lateral microtubule interaction within the polarized cell cortex. This destabilizing effect of Gsk3β could be salvaged by overexpression of non-phosphorylatable Clasp2 (Kumar, Lyle et al. 2009). These studies showed that active Gsk3β suppresses the affinity of Clasp2 for microtubules while its inhibition promotes Clasp2-microtubule binding. This could possibly account for why Gsk3β was also differentially regulated in this phosphoproteomics study as mentioned earlier.

Intriguingly, Clasp2 is abundantly expressed in the brain. Akin to how it binds microtubules in dividing cells to drive cell migration, it preferentially labels the ends of neuronal microtubules that probe and penetrate growth cone leading edge, implying a function in axon growth and navigation. Overexpression of Clasp2 in neurons could hyper stabilize microtubules to impede microtubule growth and hinder growth cone progression (Lee, Engel et al. 2004). Based on these findings, it seemed that Clasp2 has to be tightly regulated to allow optimum advancement of neuritic processes. However, the answer on exactly how it is regulated to evoke neuronal changes remains to be elucidated. Possibly, this might be dependent on its binding to microtubules which is controlled by kinases induced phosphorylation events, for example by Gsk3β as inferred from the findings in migrating cells. Overall, it is reasonable to relate and suspect that phosphorylation of neuronal Clasp2
by kinases is vital for mediating its affinity for microtubules which then has a direct impact on its localization and results in subsequent alterations of neuritic processes.

6.4.4 Proteins involved in protein synthesis

Proteins involved in protein translation were also identified and they consisted of Eif, Eef, Ssrp and Abcf. Most of these translation factors exist in many subtypes and isoforms and even though the specific types found in this phosphoproteomics study were not well reported in the context of neuron, existing literatures on other isoforms do however inform about how these factors were regulated by phosphorylation to facilitate or deter neuronal protein synthesis. Taking for example, eukaryotic initiation factors (Eifs) are scaffolding proteins involved in the initial phase of protein translation. It was shown that BDNF, a neurotrophic factor, promoted protein synthesis in cortical neurons by inducing phosphorylation of Eif4 and it did that by activating multiple signalling cascades that involved PI3K, mTOR and MAPK (Takei, Kawamura et al. 2001). Conversely, activation of Gsk3β in the presence of 6-OHDA that resulted in neuronal cell death was implicated with phosphorylation of Eif2 (Chen, Bower et al. 2004). This probably applies to the rest of the identified proteins as well. Despite the need for further validation, the main idea that can be drawn from this analysis is that protein synthesis, which plays a major part in growth and maintainence of neuritic processes, might be misregulated in neurons when Aurkb is inhibited (Rodriguez, Czaplinski et al. 2008).

6.4.5 Proteins involved in RNA and DNA processing

The next group of proteins which included Ssrm, Hnrnp, Cstf, Dhx, Top and Psip are commonly associated with RNA and DNA processing. These proteins are involved in diverse
cellular events, including neuronal processes and are regulated by phosphorylation. In regards to RNA processing, Hnrnps are a set of proteins that bind and help package pre-mRNA into functional complexes for splicing events, gene transcription and post transcriptional modifications. Specifically, Hnrnpk which was identified in this study, is a vital regulator of various neuronal cytoskeletal RNAs such as NF-L, NF-H, Gap43 and p21 (Irwin, Baekelandt et al. 1997; Yano, Okano et al. 2005; Thyagarajan and Szaro 2008) and its loss not only impedes axonal growth in xenopus embryos but also inhibits neurite establishment in culture (Liu, Gervasi et al. 2008; Liu and Szaro 2011). Because Hnrnpk consists of up to 73 potential phosphorylation sites, it is dynamically regulated by multiple kinases and phosphatases (Bomsztyk, Denisenko et al. 2004).

Speaking of DNA processing, DNA topoisomerase (Tops) are enzymes that alleviate cellular stress cause by topological problems that arise during DNA replication, transcription and repair. They do that by resolving DNA knots and tangles through transient breakage and subsequent rejoining of DNA strands. For that, they serve vital roles in ensuring smooth and timely transcription of selected genes during various cellular processes. There are two main classes of Tops (α and β) and akin to Hnrnp, they are regulated by phosphorylation (Pommier, Kerrigan et al. 1990; Cardenas and Gasser 1993; Yu, Khan et al. 2004; Li, Wang et al. 2008). Of which, Top2b, which was also differentially regulated in this case, determines neuronal cell fate by regulating expression of certain genes involve in a variety of neuronal processes such as differentiation, development, migration and axon guidance (Sano, Miyaji-Yamaguchi et al. 2008; Heng and Le 2010).

In summary, because processing of RNA and DNA is an event closely associated with protein translation, this reflected on why factors involved in protein synthesis were also identified as
mentioned earlier. Importantly, this part of the data suggested that misregulation of neuronal proteins, under the influence of Aurkb inhibition, occurred at a transcriptional level.

### 6.4.6 Concluding remarks on phosphoproteomics

There are a few limitations of phosphoproteomics even though it provides a high-throughput identification of proteins that are related to Aurkb signalling in neurons. First, protein detected by this method might not be a direct substrate of Aurkb and some relevant proteins will be missed since no extraction condition is all encompassing. It is possible that proteins with low stoichiometry of phosphorylation, in very low abundance and/or phosphorylated as a target for rapid association or degradation will be lost. Furthermore, targets discovered from this process have to be further validated by experimental procedures such as western blotting, RT-PCR, immunochemistry, kinase assay and/or immunoprecipitation.

Nevertheless, although phosphoproteomics did not clearly dissect the sequential molecular events that took place upon Aurkb inhibition in neurons, it did provide an insightful glimpse on pathways that were affected by its inhibition. More important, protein association analysis identified specific clusters of potential targets that are well-known and establish regulators of neuritic processes for future studies. Overall, the data from phosphoproteomics supported the findings in previous chapters by illuminating the fact that inhibition of Aurkb in neurons was indeed causing changes to molecules that are major determinants of neuronal cytoskeleton regulation and organisation.
CHAPTER 7

Conclusions and future directions
7.1 Conclusions and future directions

7.1.1 Summary of findings

The work performed in this thesis serves as an excellent example of how DNA microarray technology can be used as a screening tool to reveal biological pathways associated with a pathological situation and to shortlist novel and interesting gene targets for future work. Hence, the transcriptomic studies described in Chapter 3 have not only provided several interesting mechanistic insights into the intrinsic regenerative sprouting ability of axotomized neurons in vitro, but also identified Aurkb, a cell cycle associated kinase that has never been reported in the context of neurons, for further work. This was based upon a literature review that revealed that Aurkb has a central role in regulating cytoskeletal remodelling that underlies mitosis and spindle formation and separation. It was predicted that Aurkb may have a similar role in regulating cytoskeletal remodelling associated with neurite outgrowth and regeneration. In this context, the remaining goals of this thesis were subsequently to reveal insight into the roles of Aurkb in neurons, the outcomes of which are summarised in the following paragraph.

Detailed immunohistochemical studies in Chapter 4 revealed that Aurkb was expressed extensively in selected brain structures such as the cortex, hippocampus and amygdala and specifically by neurons. Aurkb expression was highest in E15 brain cortex, and substantially lower in the adult brain adult indicating that Aurkb might carry out specific roles in the maturation process of neurons during brain development. In support of this, changing localization of Aurkb accompanied by increased levels of the phosphorylated (active) form of Aurkb within developing cultured neurons in culture further suggested that Aurkb could be
involved in the growth and developmental process of neurons. To test this hypothesis, in Chapter 5 the activity of Aurkb in cultured neurons was negatively manipulated by several different experimental approaches, which encompassed the use of a specific chemical inhibitor as well as molecular knock-in of kinase dead Aurkb and knock-down of endogenous Aurkb in neurons via a lentivirus-mediated approach. It was found that interference with Aurkb activity resulted in neurons having either shorter or no elaborated axons, and substantially swollen cell bodies with increased microtubule mass that were at the same time bordered by several aberrant, thin and highly disorganised neuritic projections. Together, these loss-of-function studies have provided considerable evidence that Aurkb has key roles in neurite outgrowth of cultured cortical neurons. Moreover, the discovery of INCENP as a binding partner of neuronal Aurkb from Y2H (Chapter 6) implies that it may regulate Aurkb to orchestrate neuronal cytoskeleton networks. Lastly, phosphoproteomics (Chapter 6) coupled with functional analysis of protein associations have identified a cluster of proteins and kinases that is commonly associated with the regulation of microtubule networks and actin filaments. The identification of these proteins was in accordance to findings in previous chapters, suggesting that inhibition of Aurkb in neurons caused changes to molecules that are major determinants of neuronal cytoskeleton regulation and organisation. In summary, all the experiments undertaken in this thesis have clearly identified an important role for Aurkb in regulating cytoskeletal remodelling during the process of neurite formation and extension.

7.1.2 Possible future work

The findings presented in this thesis provide a range of directions for future research. First, because this thesis presents evidence that Aurkb regulates the maturation process of neurons in culture and it is highly expressed in immature mouse brain, it is possible that Aurkb may be
involved in corticogenesis, a process that involves orchestrated migration of neurons through the cortical layers during neuronal development. Future studies to test this hypothesis in vivo in embryonic mice could be undertaken using in utero electroporation (Saito 2006). For this methodology, Aurkb shRNA or overexpression plasmids would be microinjected into the lateral ventricle of E11-13 embryos. Transfection of DNA plasmids into surrounding brain cells is mediated by electroporation involving the delivery of square-wave electric pulses through forcep-type electrodes. Following electroporation, the utero embryos will be carefully placed back into the abdominal cavity of the pregnant mother. Brains of mouse embryos at later stages of embryonic development (E18 or 19) or postnatal pups can then be harvested for immunohistochemical analysis to reveal whether loss or over expression of Aurkb affects corticogenesis.

Next, because this thesis provides strong evidence that Aurkb is required for neurite outgrowth of developing neurons, it is also possible that it has a role in neurite regeneration after axonal injury since both of these processes involve cytoskeletal remodelling and neurite extension. In fact, a recent study which compared gene changes during neurite outgrowth and regeneration in embryonic superior cervical ganglia (SCG) and dorsal root ganglia (DRG) neurons, has reported that there are many commonalities in gene expression profiles between these two processes (Szpara, Vranizan et al. 2007). More important, a plausible function of Aurkb in neurite regeneration is supported by its elevated gene expression after axotomy as reported in Chapter 3. As such, one interesting experiment to follow up would be to examine the role of Aurkb in post-injury neurite regrowth after brain injury in vivo (Chung, Vickers et al. 2003; Chung, Penkowa et al. 2008). Briefly for this procedure, a focal cortical brain injury would be performed on rodents using a 25 gauge Hamilton needle. Immediately after injury, compounds can be injected directly into the lesion site to investigate the importance of AurkB
in neuronal sprouting associated with recovery. These possible compounds include specific chemical inhibitors of Aurkb, and lentiviruses that either overexpress different variants of Aurkb or knock-down endogenous Aurkb in the brain. Between 3-7 days after injury, brains of injured mice would be fixed and then be sectioned for immunohistochemical analysis to reveal the precise roles of Aurkb in the regenerative sprouting response of injured brain.

Also, it will be important to follow-up and validate whether INCENP is a binding partner of neuronal Aurkb via co-immunoprecipitation and in vitro binding assays. Likewise, potential targets identified from phosphoproteomics require verification via immunoblotting and in vitro kinase assays. These biological targets can then be further manipulated via molecular means to reveal their exact roles in neuronal outgrowth and regeneration and how they are linked to Aurkb signalling in neurons.

7.1.3 Emerging importance of cell cycle regulators in neuronal processes

It is important to note that the primary function of Aurkb is generally considered to be involved in regulating cell cycle. Notably, several recent reports have identified that several other key cell cycle regulators possess alternative functions in various neuronal processes including neuronal migration, synaptic plasticity and neuronal growth and maturation (Frank and Tsai 2009).

One example of this are the molecules Cdh1 and Cdc20, which work antagonistically by targeting APC/C to specific substrates at different phases of cell cycle, have been implicated with axonal growth and dendrite morphogenesis respectively (Konishi, Stegmuller et al. 2004; Kim, Puram et al. 2009). Also, emerging evidence has revealed that Cip/Kip family of
cyclin-dependent kinases inhibitors consisting of p21, p27 and p57, which were once viewed solely as nuclear proteins that bind and inactivate cyclin-CDK complexes to prevent inappropriate cell cycle progression, are expressed within neuron-rich brain regions and are essential components of neuronal migration. While the role of p21 in neurons remains unclear, both p27 and p57 loss of functions impair neuronal migration (Nguyen, Besson et al. 2006; Itoh, Masuyama et al. 2007). In particular, cortical neuronal migration was affected upon p27 stabilization by neuronal specific Cdk5 which resulted in cofillin-mediated disassembly of F-actins in neuronal processes (Kawauchi, Chihama et al. 2006). In addition, certain components of the hexameric origin recognition complex (ORC), a complex that is involved in DNA replication and heterochromatin formation, were found in somatodendritic compartments of neurons, defining unexpected roles in neuronal plasticity. Here, knockdown of these ORC subunits drastically decreased dendritic branching and greatly retarded dendritic spine development (Huang, Zang et al. 2005).

More importantly, a growing body of evidence further suggested that cell cycle kinases in particular, are key mediators of various neuronal processes. For example, polo-like kinase 2 (Plk2), a cell cycle associated protein, was found not only to have a role in shaping dendritic spines formation in mature neurons but also to be involved with BDNF-induced dendritic arborisation in immature neurons (Pak and Sheng 2003; Guo, Tan et al. 2011). Similarly, cyclin-dependent kinases (Cdks), which ensure systematic progression of cell cycle, are implicated with microtubule-based neuroplasticity in differentiated pyramidal neurons (Schmetsdorf, Arnold et al. 2009). Furthermore, the levels of the protein kinase Wee1, which regulates cell entry into mitosis, must be tightly regulated in neurons to facilitate the establishment of neuronal polarity (Muller, Lutter et al. 2010). As seen, these studies clearly illustrated the increasing recognition of the vital roles of cell cycle protein kinases in neuronal
development. Likewise, recent reports together with research findings from this thesis strongly suggested that Aurkb, which regulate diverse events during mitosis, also serve unique and novel neuronal functions that are independent of its cell cycle roles.

7.1.4 Concluding remarks

Overall, parallel functions of various cell cycle regulators in neuronal processes illuminate an underlying commonality between these two very distinct biological pathways. One explanation for this redundancy in function might be that it is wasteful for cells to invest energy in making proteins that serve only one biological process. In this context, it is reasonable to assume that many of these cell cycle components are designed or have assimilated and evolved over time to take up certain functions in postmitotic neurons.

Here, Aurkb which is involved in multiple aspect of mitosis was investigated for the first time in the brain and neurons. Specifically, the phosphoproteomic, pharmacological and molecular knockin and knockout studies provided considerable evidence that Aurkb has key roles in neurite cytoskeleton modulation. Taken together, the work in this thesis has clearly identified a novel and alternate cell cycle independent function of Aurkb in post-mitotic neurons. Based upon these findings as well as the work performed by others, research on cell cycle regulators in neurons is becoming increasingly vital and relevant. In conclusion, identifying and revisiting the functions of cell cycle proteins such as Aurkb will assist in the revelation of novel concepts and provide a lucid association between their distinctly different cell cycle and neuronal functions.
Supplementary data
### Supplementary Table 1: Unfiltered MS/MS Data

List of 270 differentially regulated proteins from either control or treated group with a minimum Mascot score of 40 that passed one-way ANOVA analysis and with a statistical significance of p < 0.05.

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References
References


References


Castro, A., S. Vigneron, et al. (2002). "The D-Box-activating domain (DAD) is a new proteolysis signal that stimulates the silent D-Box sequence of Aurora-A." EMBO Rep 3(12): 1209-1214.


References


References


