# GLYCOGEN SYNTHASE KINASE-3 IS REQUIRED FOR AXON GROWTH AND DEVELOPMENT

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# ABSTRACT

XINSHUO WANG: GSK-3 is Required for Axon Growth and Development
(Under the direction of Dr. William Snider)

Glycogen Synthase Kinase-3 (GSK-3) is a critical downstream regulator in multiple signaling pathways including neurotrophin/PI3K and Wnt/β-catenin pathways. GSK-3 signal has been strongly implicated in the regulation of neuronal polarity and axon growth *in vitro*. However, how GSK-3 is regulated and whether it is required for mammalian nervous system development is unknown. Here, I assessed the role of Par6 signaling in the regulation of GSK-3 activity and NGF-induced axon growth. My results show that Par6 is required for GSK-3 phosphorylation and inhibition. Elimination of Par6 inhibits NGF-induced axon growth. We have also generated nervous system specific GSK-3 knockout mice. By studying GSK-3 null cortical neurons, my results show that GSK-3 is required for axon growth *in vivo* and the deletion of GSK-3 mainly affects the microtubule dynamics which prevents the axon growth. We conclude that GSK-3 is a key regulator of axon growth and development both *in vivo* and *in vitro*.

# ACKNOWLEDGEMENT

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# LIST OF ABBREVIATIONS

BAC: Bacterial artificial chromosome

BSA: Bovine serum albumin

CNS: Central nervous system

CRIB: Cdc42/Rac-interactive binding

CRMP2: Collapsin response mediator protein-2

DIX: *DI*shevelled and A*X*in domain

DMSO: Dimethyl sulfoxide

EDTA: Ethylene-diamine-tetra-acetic acid

EGFP: Enhanced green fluorescent protein

FGF: Fibroblast growth factor

GMP: Guanosine monophosphate

GPCR: G-protein coupled receptor

GTP: Guanosine-5'-triphosphate

MDCK: Madin-Darby canine kidney cell line

NFAT: Nuclear factor of activated T cells

NGF: Nerve growth factor

PB1: Phox and Bem1 domains

PBS: Phosphate buffered saline

PDZ: *P*SD95, *D*lg, *Z*O-1 domains

PI3K: Phosphatidylinositol 3-kinase

PKC: Protein kinase C

PNS: Periphery nervous system

PVDF: Polyvinylidene fluoride

RT-PCR: Reverse-transcript polymerase chain reaction

RNA: Ribonucleic acid

siRNA: small interfering RNA

SEM: Standard error of the mean

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

# INTRODUCTION

Glycogen synthase kinase-3 (GSK-3) is a widely expressed and highly conserved serine/threonine kinase. It was originally identified as key regulatory kinase that phosphorylates and inhibits glycogen synthase downstream of insulin signaling (Embi et al., 1980). However, it has since been shown to play far broader roles in many cellular processes (Cohen and Frame, 2001; Doble and Woodgett, 2003). There are two mammalian GSK-3 isoforms encoded by distinct genes: GSK-3$\alpha$ and GSK-3$\beta$. The two proteins share 97% sequence similarity within their kinase catalytic domains, but differ significantly from one another outside this region (Woodgett, 1990).

GSK-3 is unusual in that it is constitutively active in resting cells, and is primarily regulated through inhibition of its activity. GSK-3 phosphorylates a large number of substrates including cytoskeletal proteins, transcription factors, and metabolic regulators. It thus has prominent roles in the regulation of cellular morphology, gene expression, cell division, cell fate decisions, and apoptosis (Frame and Cohen, 2001; Jope and Johnson, 2004). Because of its importance, the activity of GSK-3 must be carefully regulated both temporally and spatially by mechanisms individually tailored for each substrate. The first chapter of my thesis describes a previously unknown regulatory mechanism of GSK-3 by the conserved

Par3/6-aPKCζ polarity complex in nervous system.

In multicellular organisms, GSK-3 functions in several distinct signaling pathways (Doble and Woodgett, 2003; Woodgett, 2001). In response to growth factors such as FGF and NGF, phosphorylation of GSK-3 (serine-21 on GSK-3α and serine-9 on GSK-3β) by protein kinase B (PKB, aka Akt) causes GSK-3 inactivation. GSK-3 also plays a key inhibitory role in the canonical Wnt pathway. In unstimulated cells, GSK-3 phosphorylates β-catenin, thereby targeting it for ubiquitylation and proteasomal degradation. Exposure of cells to Wnts leads to inactivation of GSK-3 through an as yet unclear mechanism involving the protein Dishevelled (Dvl). As a result, β-catenin is dephosphorylated and escapes the destruction machinery. Unphosphorylated β-catenin accumulates in the cytoplasm and translocates to the nucleus, where it becomes a transcriptional activator. The Hedgehog (Hh) pathway is somewhat similar to the Wnt pathway. In the absence of an Hh signal, GSK-3, together with other kinases, phosphorylates Cubitus interruptus (Ci) in flies or Gli in mammals to target it for proteolytic processing. The truncated Ci or Gli functions as a transcriptional repressor. Activation of Hh signaling results in translocation of unprocessed Ci or Gli to the nucleus where it activates Hh target genes. There are also studies showing that GSK-3 inhibition modulates signaling in Notch pathways *in vitro* (Espinosa et al., 2003; Foltz et al., 2002; Jin et al., 2008) and BMP pathways in *Xenopus* embryos (Fuentealba et al., 2007; Sapkota et al., 2007). More recently, the Akt/GSK-3 cascade has been found to be a new modality of G protein-coupled receptor (GPCR) signaling which provides a better biological understanding of psychiatric disorders (Beaulieu et al., 2007). All these pathways in which GSK-3 acts

as a key regulator, when dysregulated, have been implicated in the development of multiple human diseases. Given this association, GSK-3 has emerged as an attractive therapeutic target for the treatment of neurodegenerative diseases, non-insulin-dependent diabetes mellitus, inflammation and cancer (Cohen and Goedert, 2004; Meijer et al., 2004).

Lithium was the first discovered GSK-3 inhibitor (Stambolic et al., 1996). Despite the fact that millimolar concentrations (20-30mM) of lithium are required to inhibit GSK-3 in cells, it has been broadly used to treat patients with mood disorders and in trials for treatment of Alzheimer's disease and Amyotrophic Lateral Sclerosis (Fornai et al., 2008; Zarate et al., 2006; Zhong and Lee, 2007). Besides lithium, more than 50 inhibitors of GSK-3 have been identified and this list is growing (Ougolkov and Billadeau, 2008). GSK-3 inhibitors also have diverse effects on embryonic stem cells (ESCs). Sato et al. has shown that GSK-3 inhibition through 6-bromoindirubin-3'-oxime (BIO) could maintain pluripotency of mouse and human ESCs (Sato et al., 2004). Further, Doble et al. reported enhanced retention of ESC markers in GSK3-$\alpha/\beta$ double knockout ESCs under differentiation conditions which further supports the idea (Doble et al., 2007). However, it is important to emphasize that the specificity of these compounds may not be restricted to GSK-3. For example, lithium has many other effects, including inhibiting adenylate cyclase and inositol monophosphatase (Quiroz et al., 2004; Williams et al., 2004). SB 216763, SB 415286 and the Chiron inhibitors (Cline et al., 2002; Nikoulina et al., 2002) have been classed as more selective inhibitors of GSK-3. But this does not prove complete specificity (Martinez et al., 2002). A major difficulty in interpreting effects of GSK-3 inhibitors is

that there have been no genetic studies eliminating GSK-3 in the developing nervous system. Thus the effects of GSK-3 deficiency in the mammalian nervous system are not established. The second chapter of my thesis describes results of conditional mutagenesis experiments will address this issue.

# Chapter 1

**Regulation of GSK-3β activity and embryonic DRG axon growth by the Cdc42-Par3/6-aPKCζ polarity complex**

## INTRODUCTION

Recently, GSK-3β has emerged as a key regulator of axon morphogenesis downstream of many extracellular cues, such as neurotrophins, Wnts and Semaphorin 3A (Chadborn et al., 2006; Del Rio et al., 2004; Eickholt et al., 2002; Ito et al., 2006; Lucas et al., 1998; Zhou et al., 2004). Jiang et al. (Jiang et al., 2005) and Yoshimura et al. (Yoshimura et al., 2005) have shown that specification of rapidly elongating axonal processes versus more slowly growing dendritic processes in hippocampal neurons is regulated by GSK-3β activity. Spatially localized inactivation of GSK-3β by PI3K in a single immature neurite was necessary for future axon specification and maintenance, whereas global inactivation of GSK-3β led to the formation of multiple axons. We have also shown that in dorsal root ganglion (DRG) neurons, localized inactivation of GSK-3β at the growth cone is required for rapid axon elongation induced by NGF (Zhou et al., 2004) and complete inhibition of GSK-3 activity or loss of GSK-3 proteins prevent axon extension (Kim et al., 2006).

In spite of its importance, we still have a limited understanding of how regulation of GSK-3β activity occurs in neurons. Previous work in our lab has identified a pathway that links PI3K signaling to axonal microtubule assembly (Zhou et al., 2004). In this work, Zhou et al. showed that in an extending axon, PI3K is only activated at the distal tip and growth cone. The spatially localized PI3K signaling is then conveyed downstream through a similarly localized inactivation of GSK-3β. These two spatially coupled kinases control axon assembly by regulating a microtubule binding protein, adenomatous polyposis coli (APC). Although it is widely accepted that Akt is the key mediator of the serine-9 phosphorylation and inactivation of GSK-3β downstream of PI3K signaling (Cross et al., 1995), our unpublished data raise the possibility that alternative pathways may exist which also play important roles in regulation of GSK-3 function. For example, intergrin-linked kinase (ILK) has been shown to mediate NGF-induced axon growth and axon-dendrite polarity by leading to GSK-3β inactivation (Guo et al., 2007; Mills et al., 2003; Zhou et al., 2004). Cyclic GMP signaling can also regulate axon development through phosphorylation of GSK-3 (Zhao et al., 2009).

Another way for PI3K to access GSK-3β may be through the conserved Par3/6-aPKCζ polarity complex. The partitioning-defective (Par) genes were initially identified in *C. elegans* for their roles in directing asymmetric cell division during early development (Cowan and Hyman, 2004; Kemphues et al., 1988). Par3, Par6 and atypical PKCζ form a large multiprotein complex which is activated by GTP bound Cdc42 (Henrique and Schweisguth, 2003; Joberty et al., 2000). Recent progress has been made in addressing the role of the polarity pathways in axon/dendrite

specification and spine morphogenesis where tight regulation of cytoskeleton dynamics is essential. In hippocampal neurons, the Par3/6-aPKCζ complex is recruited to the growth cone of one single neurite that will become future axon in a PI3K dependent manner (Shi et al., 2003). When overexpressed, Par3 and Par6 fail to localize correctly, leading to defects in polarization (Nishimura et al., 2004; Nishimura et al., 2005; Shi et al., 2003). Inhibition of aPKCζ activity prevents axon formation (Shi et al., 2003), whereas active (phosphorylated) aPKCζ can be seen at the tips of growing axons (Schwamborn and Puschel, 2004). Moreover, Par3 and Par6-aPKCζ have been shown to regulate dendritic spine morphogenesis in hippocampal neurons. Par3 is required for spine maturation (Zhang and Macara, 2006) whereas Par6-aPKCζ is essential for spine biogenesis and maintenance (Zhang and Macara, 2008).

Functionally, the Par3/6-aPKCζ complex is able to regulate astrocyte migration by controlling centrosome positioning (Etienne-Manneville and Hall, 2003a). Interestingly, GSK-3β has been placed downstream of the complex and the inactivation of GSK-3β is dependent on Cdc42 and aPKCζ activity. Phosphorylation of GSK-3β occurs specifically at the leading edge of migrating astrocytes and induces the interaction of APC with microtubules, which promotes cell polarization. These data begin to define a pathway for PI3K regulation of GSK-3β in astrocytes, however, no publications have addressed whether this pathway is also present in neurons.

In the polarity complex, Par6 associates with aPKCζ through its N-terminal PB1 domain, with GTP-bound Cdc42 through a partial CRIB motif and with Par3 through its PDZ domain. It is the critical adaptor which mediates the activation of the

whole polarity complex (Etienne-Manneville and Hall, 2003b; Henrique and Schweisguth, 2003). Solecki and colleagues initially addressed the role of Par6 in neurite outgrowth in cerebellar granular neurons using Par6 siRNA (Solecki et al., 2004). They found disrupted axon extension in Par6-null neurons. Although this work suggested that blockade of axon growth in Par6 deficient neurons may be mediated by prevention of centrosome orientation, no downstream target of Par6 was studied. These data provide the initial basis for studying Par6 signaling in axon growth.

**The aim of this study was to assess the role of Par6 signaling in the regulation of GSK-3β activity and NGF-induced axon growth. This study used three systems: E14 DRG neurons plated on laminin in the presence of NGF, rat pheochromocytoma PC12 cells plated on poly-D-lysine (PDL) in the presence of NGF and mouse catacholaminergic CAD cells.**

# RESULTS

## Par6α is the only Par6 isoform that is expressed in DRG neurons, PC12 and CAD cells

Four Par6 isoforms (α, β, δ and γ) have been identified in nonneuronal MDCK epithelial cells, but only α, β, γ have full length published mammalian sequences (Gao and Macara, 2004). Western blots have shown only a single band corresponding to Par6 in DRG neurons, PC12 cells and CAD cells. In order to determine which Par6 isoform(s) is/are present in the systems we were studying, we performed RT-PCR experiment. We designed isoform-specific primers to each of the three isoforms and ran RT-PCR on RNAs isolated from various tissues. Interestingly, we found only Par6α is expressed in DRG neurons, PC12 cells and CAD cells, whereas all three isoforms were expressed in varying degree in other tissues (Figure 1).

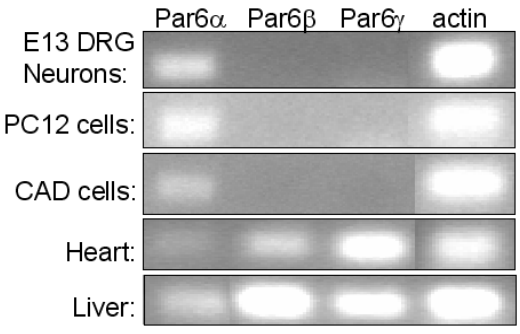


Figure 1 Distribution of Par6 isoforms in various tissues. Isoform-specific primers were used for RT-PCR on RNAs isolated from various tissues. Actin was used as a loading control.

This result allowed us to design a Par6α specific siRNA to directly assess the effect of Par6 deficiency on GSK-3β activity and axon extension.

**Par6α knockdown reduce NGF-induced GSK-3β inactivation**

Since Par6α is the only isoform expressed in DRG neurons, PC12 and CAD cells, we designed a Par6α specific siRNA construct to knockdown Par6α protein without affecting other Par6 isoforms. siRNA was introduced using the pSuper vector (Brummelkamp et al., 2002) which is engineered to harbor an EGFP expression cassette. Cells expressing this construct therefore expressed the EGFP reporter. The efficacy of the siRNAs was tested in PC12 and CAD cells. Par6 protein was dramatically reduced by four days post-transfection (Figure 2). Differentiated PC12 cells were used in these experiments because we were unable to obtain high enough rate of transfection in DRG neurons to perform western blots.

Upon NGF stimulation, GSK-3β is inactivated via phosphorylation on serine-9 position. Re-addition of NGF to NGF-starved DRG or PC12 cultures induces dramatic upregulation of GSK-3β phosphorylation (Zhou et al., 2004). We used this model to test whether GSK-3β inactivation is mediated via Par6 signaling. PC12 cells were electroporated with Par6α siRNA and allowed to differentiate for 4 days in the presence of NGF. Differentiated Par6α-null PC12 cells were serum- and NGF-starved overnight and stimulated with NGF for a short period (15 minutes). Cell lysates were then collected and tested with western blots. In control cells, NGF stimulation induced huge elevated levels of GSK-3β serine-9 phosphorylation. However, Par6α deficient cells showed no significant changes on GSK-3β phosphorylation compared to baseline in response to NGF treatment (Figure 2).

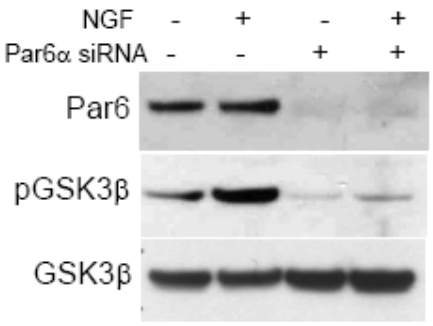


Figure 2 GSK-3β activity is regulated by Par6. Top lane: Western blot of PC12 cells electroporated with control or Par6α siRNA and cultured for 4 days with NGF shown efficient knockdown of Par6α. Middle lane: Par6α knockdown significantly reduces NGF-induced phosphorylation of GSK-3β. Bottom lane: Total GSK-3β was used as a loading control.

These data demonstrate that Par6 is required for NGF-induced serine-9 phosphorylation of GSK-3β.

**Par6 is required for NGF-induced axon growth**

In order to address the effects of Par6 on axon growth in primary embryonic DRG neurons, E14 DRG neurons were electroporated with Par6α siRNA and cultured for 4 days in the presence of NGF. Neurons were then re-plated and cultured again with NGF. Par6α protein is strongly reduced over the 4 day period. Thus, re-plating allows the axon growth response to NGF in the setting of Par6α deficiency to be assessed. 24-hours following re-plating, the lengths of the longest axon were measured. We found that neurons deficient in Par6α grew axons only half as long as control transfected neurons (Figure 3, provided by Dr. Mark Walzer). Since the Par6β is resistant to the Par6α siRNA, we were able to co-transfect Par6α siRNA with full-length Par6β plasmid to rule out the off-target effects of the Par6α siRNA. As

shown in Figure 3, neurons expressing both Par6α siRNA and full length Par6β plasmid have a similar amount of axon growth. Furthermore, western blots done with PC12 cell lysates show the full recovery of Par6 protein expression after co-transfection of Par6β plasmid (Figure 4, provided by Dr. Mark Walzer). These results confirm that Par6α function is required for NGF-induced axon growth.

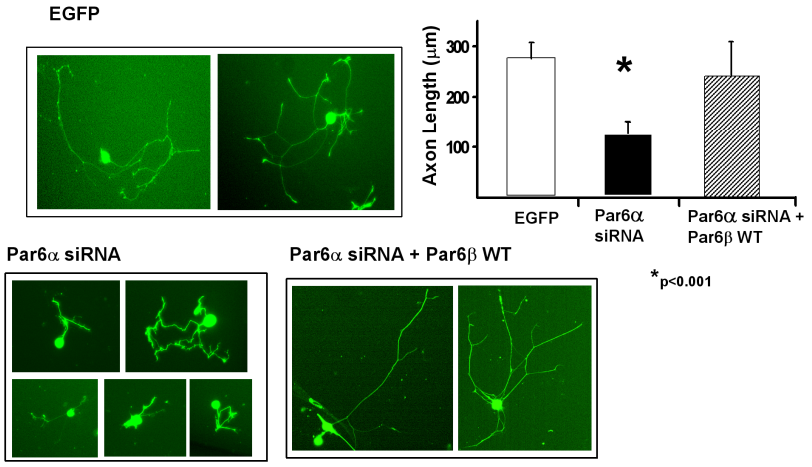


Figure 3 Par6α is required for efficient NGF-induced axon growth in E14 DRG neurons. EGFP, Par6α siRNA-EFGP or Par6α siRNA-EGFP+Par6β-EGFP expressing DRG neurons were plated on PDL/laminin coated coverslips in the presence of NGF. Graphical quantification of the length of the longest axon (υm) 24h following re-plating shows significant reduction of axon growth after Par6α knockdown. Re-expression of Par6β into Par6α-null neurons rescues the axon growth. Data shown are means+SEMs. Figure is provided by Dr. Mark Walzer.

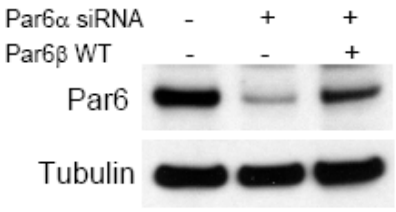


Figure 4: Introduction of Par6β to Par6α-decificent PC12 cells results in near normal amount of Par6 protein expression as in control cells. Figure is provided by Dr. Mark Walzer.

Similar effects were also seen in Par6α deficient PC12 cells and CAD cells. PC12 cells respond to NGF by differentiation and elaboration of neurites (Greene and Tischler, 1976). Four days after transfection, control EGFP-expressing PC12 cells showed significant neurite growth while cells expressing the Par6α siRNA demonstrated little growth (Figure 5, top). CAD cells differentiate upon serum withdraw (Qi et al., 1997). Knocking down of Par6α in CAD cells causes significantly reduction in axon growth (Figure 5, bottom).

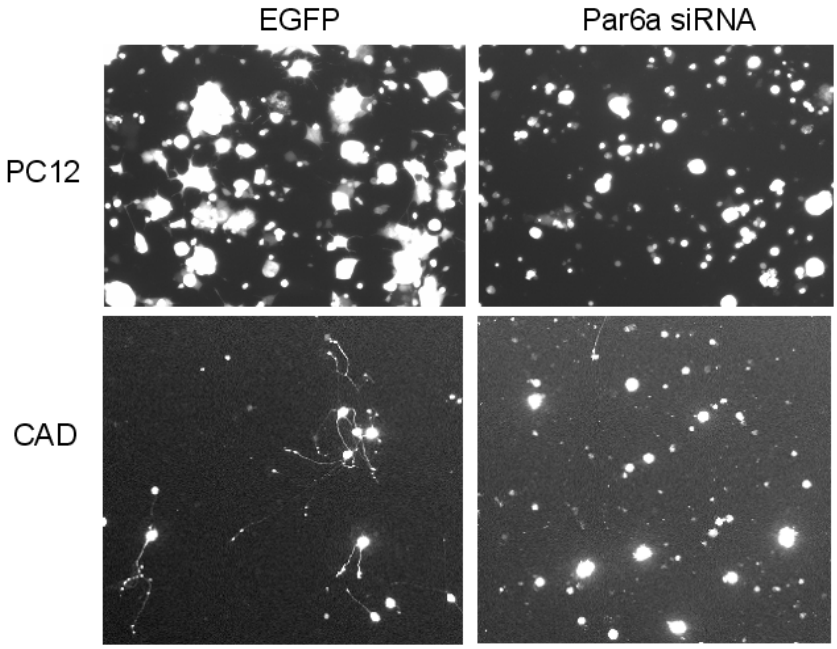


Figure 5: Top: Par6 is required for neurite outgrowth in PC12 cells. Par6α siRNA-EGFP expressing PC12 cells do not elaborate neurites in response to NGF. Bottom: CAD cells differentiated by serum deprivation show reduction of neurite outgrowth compared with EGFP expressing cells. Quantification is not shown.

These data suggest that Par6α is required for efficient elaboration of neurites by cell lines in response to differentiation-inducing treatments.

**Par6 knockdown does not interfere with Tau phosphorylation.**

Tau is a major neuronal microtubule associated protein (MAP). One of its established functions is promotion of assembly and maintenance of microtubules structure (Drubin and Kirschner, 1986; Weingarten et al., 1975). The most important post-translational modification of tau is phosphorylation. The degree of phosphorylation regulates its microtubule-binding and tubulin-polymerizing activities (Lindwall and Cole, 1984). Hyperphosphorylated tau is unable to bind to microtubules thus leading to microtubule instability and altered axonal transport (Ballatore et al., 2007). GSK-3 is a key kinase involved in tau phosphorylation. Hence we asked whether tau phosphorylation is affected by Par6$\alpha$ knockdown.

GSK-3$\beta$ phosphorylates tau at the serine 199/202 sites. The GSK-3 inhibitor 6-bromoindirubin-3'-oxime (BIO) had a dose-dependent effect on tau phosphorylation at these sites (Figure 6, left panel). We expected to see hyperphosphorylated tau in Par6 null cells since GSK-3$\beta$ activity is elevated in these cells. However, after Par6 knockdown, no significant change of Tau phosphorylation on serine 199/202 sites was observed (Figure 6, right panel).

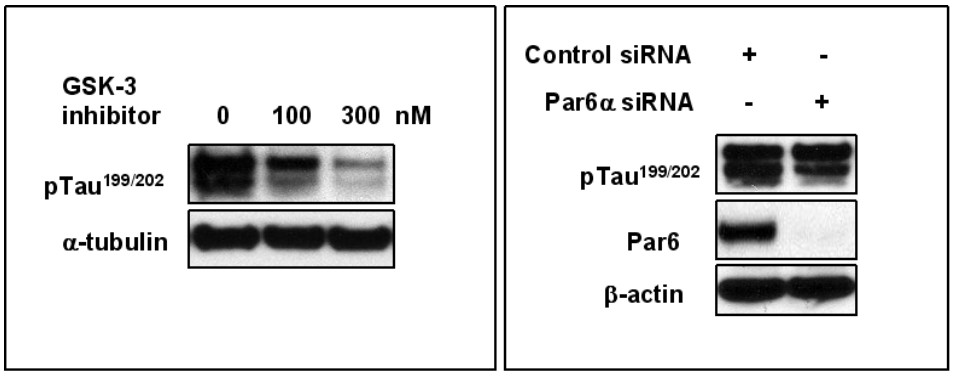


Figure 6 Regulation of GSK-3$\beta$ activity by Par6 signaling does not affect tau phosphorylation. Left panel shows that phosphorylation of Tau at serine 199/202 is regulated by GSK-3 activity.

Addition of GSK-3 inhibitor (BIO) shows dose-dependent inhibition on tau phosphorylation. Right panel shows that knocking down of Par6 does not impair Tau phosorylation at GSK-3 sites. $\alpha$-tubulin and $\beta$-actin was used as loading controls.

These results mean that at this moment, we cannot tie Par6$\alpha$ regulation of GSK-3 activity to a specific cytoskeletal mechanism.

**Generation of a Dvl-2 conditional knockout mouse**

To explore other mechanisms that might be responsible for GSK-3 regulation in the setting of axon growth, we generated Dvl-2 floxed allele mice. Dishevelled (Dvl) proteins, of which three have been identified in humans and mice (Klingensmith et al., 1996; Pizzuti et al., 1996; Semenov and Snyder, 1997; Sussman et al., 1994; Tsang et al., 1996) are highly conserved component of both the canonical Wnt and planar cell polarity (PCP) signaling cascades (Wallingford and Habas, 2005). In canonical Wnt pathways, dishevelled proteins function as essential scaffold proteins that transduce the signal activated by Wnt and inactivate GSK-3. Mouse knockouts for each of the Dvl genes have been generated. Distinct phenotypes of those mice reveal separate functions for each Dvl proteins. Dvl-1 knockout mice are viable and fertile, but display social interaction abnormalities and defects in sensorimotor gating (Lijam et al., 1997). By contrast, cardiac outflow tract abnormalities and rib/vertebral malformations are observed in Dvl-2 knockout mice (Hamblet et al., 2002). Dvl-3 knockout mice also have cardiac outflow tract abnormalities and misorientated stereocilia in the organ of Corti as well (Etheridge et al., 2008). However, functional redundancy among Dvl genes is also suggested from their overlapping expression patterns and high degree of conservation. In support of this idea, Dvl double mutants

display phenotypes that were not observed and much more severer than the single Dvl knockouts, including a complete opening of the neural tube (Etheridge et al., 2008; Wang et al., 2006).

Functional redundancy among Dvl isoforms and early embryonic lethality in double knockout mice prompted us to generate a tissue specific Dvl double knockout mouse. Since Dvl-1 knockout mice are viable, fertile, and structurally normal; Dvl-2 and Dvl-3 knockout mice have very similar phenotypes, we have decided to generate a Dvl-2 floxed allele line. With the help from the BAC engineering core facility in UNC neuroscience center, we have successfully generated the Dvl-2 conditional allele in which the region between exon 2 and exon13 on Dvl-2 gene has been floxed (Figure 7). Deletion of those exons will result in a reading frame shift with no functional protein production. Currently, these mice are being bred to Dvl-1 null mice and different Cre lines to produce neuronal specific Dvl1/2 double knockout mice.

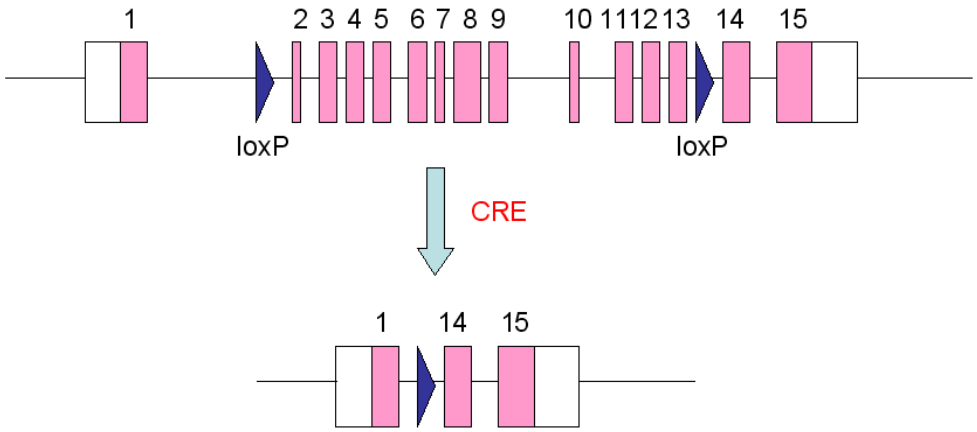


Figure 7 Dishevelled-2 conditional knockout strategy. Diagrammatic representation of the construct used for generating a null allele (top), and the inactivated gene after homologous recombination (bottom).

# DISCUSSION

## NGF-promoted axon growth requires Par6 signaling

NGF has long been shown to be required for axon growth of DRG neurons both *in vitro* and *in vivo* (Tucker et al., 2001). It is well accepted that activation of specific transcription factors and the subsequent gene expression downstream of neurotrophin signaling pathways is necessary for efficient and sustained axon growth (Graef et al., 2003; Lonze et al., 2002). More recently, however, locally mediated transcription independent events have been identified which regulate axon growth at the level of cytoskeleton regulation.

Growth cones at the tips of growing axons are the major sites where neurons receive and integrate extracellular signals to direct axonal cytoskeletal assembly (Baas and Luo, 2001). Axon elongation is initiated with growth cone advancement via actin polymerization at the leading edge. Subsequent microtubule polymerization and bundling are also the major events that mediate the formation of axons (Gordon-Weeks, 2004). For example, APC binds to the plus ends of microtubules and stabilize their growing ends (Zumbrunn et al., 2001). Zhou and colleagues found that NGF-induced axon growth requires phosphorylation of APC by GSK-3β, which abolished its stabilization properties (Zhou et al., 2004). Although the cytoskeletal mechanisms underlying axon growth and guidance have been extensively studied, how extracellular signals control the cytoskeletal dynamics, especially the microtubules remains a significant question. My work has characterized some of the upstream components that are required to regulate GSK-3β activity in the setting of NGF-induced axon growth.

Cdc42 is a member of the Rho GTPase subfamily and participates in many signaling pathways, but is particularly important in cytoskeletal remodeling (Etienne-Manneville and Hall, 2002). The Cdc42 interaction with a Par3/6-aPKCζ complex is conserved throughout animal evolution and is particularly required to specify and maintain the polarity of the nascent axon (Shi et al., 2003). Interestingly, Etienne-Manneville and Hall found that in migrating astrocytes, GSK-3β is present in a complex with Par6 and aPKCζ. Wounding results in a Cdc42-dependent inhibition of GSK-3β through phosphorylation on serine-9, which is in correlation with the dissociation of GSK-3β from Par6. APC also localizes to the leading edge in response to wounding, and this depends on activation of Cdc42 and aPKCζ or inactivation of GSK-3β (Etienne-Manneville and Hall, 2003a). This establishes a signaling pathway from Cdc42-Par6-aPKCζ-mediated inhibition of GSK-3β to the potential regulation of microtubule dynamics by APC. We found that similar signaling pathway exists in DRG neurons. Par6 likely plays the role of a scaffolding protein where upon activation via Cdc42, it allows for increased association of aPKCζ with GSK-3β in a multiprotein complex (our unpublished data). We also found Par6 is required for serine-9 phosphorylation of GSK-3β and NGF-induced axon growth. These data for the first time identify NGF as an extracellular signaling mediator that can access the polarity complex and show that the polarity complex is able to mediate microtubule dynamics through inactivation of GSK-3β. These are functions for the polarity complex that go beyond their established role in regulating the actin cytoskeleton.

Our finding is in slight contrast to that of Solecki and colleagues who showed

that in Par6 deficient cerebellar granular neurons, axon extension was completely absent. They proposed that this was due to a lack of centrosome reoritation (Solecki et al., 2004). In our Par6 deficient DRG neurons, an axon still forms but axonal extension was significantly slowed. This discrepancy may be attributed to different neuron types with different signaling cascades responding to extracellular signals. In DRG neurons, NGF signaling is required for efficient axon extension whereas other extracellular signals may regulate initial elaboration of the axon in cerebellar neurons. In addition, Selocki et al. also showed that overexpression of Par6 disrupted axon extension in cerebellar neurons. This latter finding suggests that tight control of Par6 levels in neurons is critical for axon growth.

**Regulation of GSK-3 phosphorylation and activity**

Zhou and colleagues' work from our lab reported the GSK-3β-APC complex, normally considered a component of the Wnt pathway, can be accessed by neurotrophin signaling. This result is in conflict with the current view that GSK-3β in the Wnt complex appears to be insulated from signaling triggered by receptor tyrosine kinases (Cohen and Frame, 2001; Patel et al., 2004). Wnt treatment of HEK293 cells, which was effective in stabilizing β-catenin, caused a reduction in GSK-3β activity. However, this reduction in activity was not as a result of serine-9 phosphorylation. Insulin treatment, although causing a reduction in GSK-3β activity and increasing serine-9 phosphorylation, did not affect β-catenin levels in the same cells (Ding et al., 2000). Experiments using *Drosophila* cells also came to the same conclusions (Matsubayashi et al., 2004; Papadopoulou et al., 2004). However, how

this compartmentalization is achieved and how broadly it applies is not clear. Although our results clearly shown that the cell polarity signaling complex could interact with the Wnt pathway and regulate GSK-3β activity via serine-9 phosphorylation; this did not affect downstream Tau phosphorylation at serine 199/202 which is GSK-3β site and does not require priming. This result indicates that Par6 regulation of GSK-3β might lead to some more different effects on microtubules, such as regulation of plus-end binding protein, APC. Another possibility is that GSK-3 activity is also regulated by mechanisms other than serine-9/21 phosporylation.

Recent mouse genetics show that when wild type GSK-3α and GSK-3β were replaced by mutants that cannot be phosphorylated at their N-terminus *in vivo*, the resulting mutant mice developed normally with no overt phenotype in the nervous system, although a defect in insulin signaling was present (McManus et al., 2005). There is also increasing evidence highlighting the distinct roles of GSK-3α in multiple aspects of cellular functions (Matsuda et al., 2008; Phiel et al., 2003), which indicates that phosphorylation of GSK-3α at the comparable serine-21 site may also play an important role in the regulation of GSK-3 functions.

Besides the Cdc42-Par3/6-aPKCζ polarity signaling we have discussed above, dishevelled (Dvl) protein might also be an important regulator upstream of GSK-3 to study. Several lines of evidence make it promising. First, Dvl has multiple binding partners that are polarity proteins. One of them is the Lethal giant larvae (Lgl) protein which is required for the asymmetric targeting of cell fate determinants in neuronal precursors (Betschinger et al., 2003; Dollar et al., 2005; Klezovitch et al., 2004). aPKCζ-dependent phosphorylation of Lgl has been shown to be essential for

Lgl function in cellular polarity (Hutterer et al., 2004; Plant et al., 2003). More recently, Dvl-1 was found to be colocalized with aPKCζ at the tips of hippocampal neurites and both of them are later enriched at the tip of the nascent axon. Dvl-1 association with aPKCζ increased its kinase activity *in vitro* (Zhang et al., 2007). MARK2/Par1 is known to regulate microtubule assembly in response to polarity cues and is one of the downstream mediators of aPKCζ to regulate neuronal polarity (Chen et al., 2006). Dvl-1 not only interacts with Par1 (Sun et al., 2001), but may inhibit MARK2 activity through aPKCζ activation as well (Zhang et al., 2007). This evidence suggests that Dvl might function in a large polarity complex. Second, Dvl also regulates GSK-3 function via Axin, a negative regulator of the canonical Wnt pathway (Wallingford and Habas, 2005). Thus, Dvls may regulate axon growth via two mechanisms, an interaction with the polarity complex and via direct regulation of GSK-3 and its downstream target APC.

## SUMMARY

The current work provides evidence for an important role of the Par3/6-aPKCζ polarity complex in regulating GSK-3β activity during NGF-induced axon growth in DRG neurons and PC12 cells. We found a significant reduction of NGF-induced axon extension coinciding with increased GSK-3β activity following knockdown of Par6. These results identified the Par3/6-aPKCζ polarity complex as a previously unrecognized mediator of NGF-induced axon growth potentially through acting as an upstream regulator of GSK-3.

# Chapter 2

**GSK-3 is a critical regulator of axon growth *in vivo***

## INTRODUCTION

GSK-3 is a downstream convergent point for many axon growth regulatory pathways (Kim et al., 2006). Experimentally altering levels of GSK-3 activity has been shown to profoundly regulate neuronal morphology *in vitro*. Globally increasing GSK-3 activity dramatically reduces neurotrophin-induced DRG and hippocampal axon growth and interferes with hippocampal dendrite/axonal specification (Jiang et al., 2005; Yoshimura et al., 2005; Zhou et al., 2004). Globally inhibiting GSK-3 activity with pharmacological inhibitors also profoundly influences axonal and dendritic morphology of both PNS and CNS neurons. However, the studies with GSK-3 inhibitors have inconsistent outcomes. Several studies have indicated that inhibition of GSK-3 activity by Wnts or lithium decreased axon elongation and induced axon branching in DRG and cerebellar neurons (Goold and Gordon-Weeks, 2005; Krylova et al., 2002; Lucas et al., 1998; Owen and Gordon-Weeks, 2003). Consistent with these results, Shi et al. (Shi et al., 2003) observed that global inhibition of GSK-3 activity led to a defect in axon development in hippocampal neurons. However, Wnts

also reportedly stimulate the extension of postcrossing commissural axons (Lyuksyutova et al., 2003). Further, two recent studies showed that formation of multiple long axons was induced in hippocampal cultures in which GSK-3 activity was inhibited (Jiang et al., 2005; Yoshimura et al., 2005), consistent with the idea that global elimination of GSK-3 activity enhances axon growth. Thus, from the published work to date, it is not entirely clear whether global inhibition of GSK-3 enhances or inhibits axon growth. This confusion may arise from two aspects. Firstly, different pharmacological reagents used in these studies may have different efficacy to inhibit GSK-3 activity. Those differential effects may further affect GSK-3's activity toward its substrates. This issue has been studied thoroughly and resolved by our lab (Kim et al., 2006). Secondly, we still do not quite understand what the mechanisms that regulate GSK-3 activity are and how they interact with each other.

Despite many pharmacological studies showing important roles for GSK-3 through development, GSK-3 single knock-out mice do not show very severe phenotypes. GSK-3$\alpha$ deficient mice are viable and only display enhanced glucose and insulin sensitivity accompanied by reduced fat mass (MacAulay et al., 2007). GSK-3$\beta$ deficient mice did not exhibit an embryonic lethality until gestation; either via massive liver degeneration caused by an unexpected NF-$\kappa$b-mediated hepatocyte apoptosis (Hoeflich et al., 2000) or via congenital heart defects caused by blunted cardiomyocyte differentiation (Kerkela et al., 2008). More importantly, dramatic defects in morphogenesis of nervous system and other organs have not been reported. Consistently, our lab has shown that the neurotrophin-induced axon growth, development of hippocampal neuronal polarity, and gross nervous system

development all occur surprisingly normally in GSK-3β null mice (Kim et al., 2006). The lack of effect of eliminating either isoform on morphological development has suggested the compensatory role from the other isoform.

To further investigate the functions of GSK-3 on early brain development and axon growth in mammals, we generated GSK-3α null (MacAulay et al., 2007) and GSK-3β floxed allele mice (Patel et al., 2008) and crossed these with a Nestin-cre line (Tronche et al., 1999). The Nestin-cre line drives expression of Cre recombinase in CNS progenitor as early as E10 (Yokota et al., 2009). Neither GSK-3$\alpha^{-/-}$ nor GSK-3$\beta^{F/F}$Nestin-cre mice exhibit major brain developmental malformations (our unpublished data). However, after crossing with the Nestin-cre line, conditional double null progeny die shortly after birth and have dramatic defects in brain development.

**The aim of this study is to understand how GSK-3 functions *in vivo* in the regulation of early brain development and axon growth**

# RESULTS

## GSK-3 deletion leads to massive hyperproliferation of neural progenitors

Deletion of GSK-3$\alpha$ and GSK-3$\beta$ in neural progenitors leads to massive hyperproliferation of neural progenitors. These results have been generated by Dr. Woo-Yang Kim and submitted for publication (Woo-Yang Kim, Xinshuo Wang, Yaohong Wu, Bradley Doble, Satish Patel, James Woodgett and William Snider. GSK-3 is a master regulator of neural progenitor homeostasis in mammals.). Although our intent was to study effects of GSK-3 deletion on axon growth *in vivo*, the massive disruption of brain development prompted me to take an *in vitro* approach for the initial studies.

## GSK-3 deletion leads to inhibition of axon formation

In culture, cortical neuritogenesis follows a well defined set of stages (de Lima et al., 1997) similar to hippocampal neurons (Dotti et al., 1988). Stage 1 cortical neurons produce extensive lamellipodia and filopodia around their periphery. Stage 2 neurons possess multiple neurites, but not an established axon. Stage 3 neurons extend a single axon from one of their multiple neurites.

To explore a role for GSK-3 in neuritogenesis and/or axonogenesis, we cultured cortical progenitor cells from GSK-3 double mutant (GSK-3$\alpha^{-/-}$ GSK-3$\beta^{F/F}$ Nestine-Cre) and control littermate (GSK-3$\alpha^{+/-}$ GSK-3$\beta^{F/W}$ Nestine-Cre) brains at E14. Cells were grown on PDL/laminin coated coverslips in Neurobasal A media supplemented with B27 and glutamate for 5 days. Representative fields of cells fixed

on day 3 and day 5 are shown in Figure 8. Strikingly, after 48h in culture, the majority of cortical neurons (up to 90%) from double mutant brains remained in stage 1 or 2, with less than 10% reaching stage 3. However, those 10% neurons, which do proceed to stage 3, are positive for neuronal markers such as Tuj-1 and dendritic marker MAP2 (data not shown). This was in sharp contrast to cells from control brains, where less than 10% of neurons were in stage 2, and more than 90% developed to stage 3 (Figure 8). These effects were seen at every stage from E13 to E19. In addition, the growth cones of the longest processes were greatly enlarged in the double mutant neurons (Figure 9). The enlarged growth cones in the mutant neurons have clear filopodia protrusions, but are filled with long curved microtubules, whereas control growth cones were only partially penetrated by bundled microtubules (figure not shown). This is consistent with our previous pharmacology inhibitor results showing that when GSK-3 activity is completely eliminated by BIO at 100-300nM concentration, axon growth is inhibited and growth cones are greatly enlarged (Kim et al., 2006).

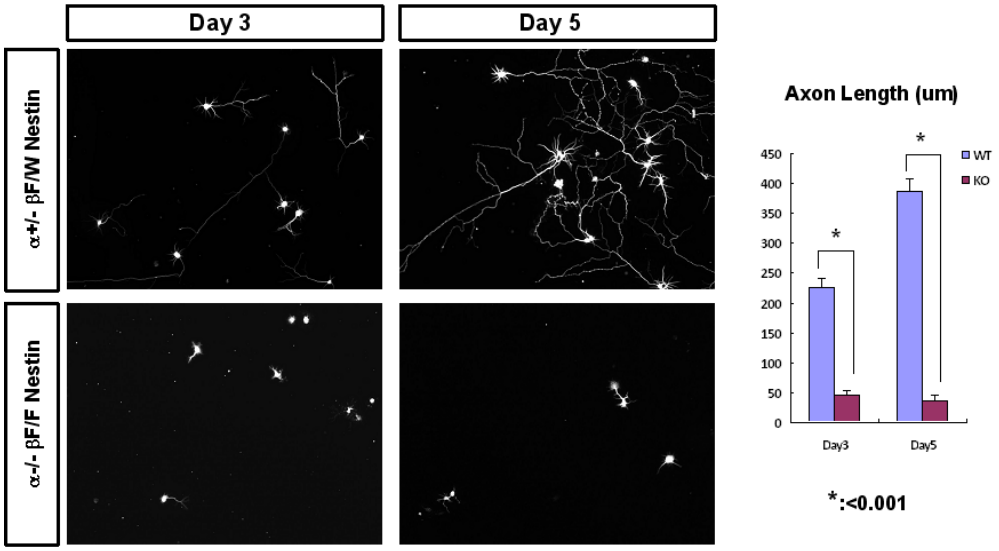

Figure 8 Deletion of GSK-3 inhibits the cortical axon growth. Cortical progenitors from GSK-3 double mutant or control brain were dissociated and cultured on PDL/laminin coated coverslips for up to 5 days. Cells were fixed and stained with α-tubulin to visualize the morphology. Quantification of the length of the longest axon extended from each cell was shown on the right. Data shown are means+SEMs.

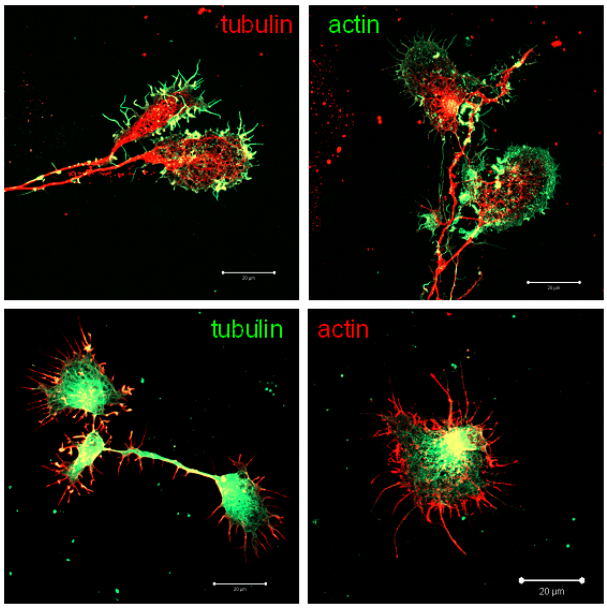


Figure 9 GSK-3 null neurons have aberrant growth cone morphology. Growth cones from GSK-3 deficient cortical neurons were stained with α-tubulin (red in upper panel and green in lower panel) and F-actin (green in upper panel and red in lower panel).

Similar axon growth and growth cone formation defects were also observed in DRG neurons taken from the double mutant embryos and cultured *in vitro* on PDL/laminin coated coverslips (figure not shown). These results suggest that GSK-3 is required for axon growth and growth cone formation in cortical and DRG neurons.

**The defects in axon growth are cell autonomous**

To examine whether the observed axon growth defects were cell autonomous and specifically caused by GSK-3 ablation, we transfected wild-type GSK-3β plasmid into the GSK-3 null neurons by electroporation and cultured them for 4 days for

protein expression. As shown in Figure 10, mutant neurons transfected with GSK-3β were rescued in their ability to form axons while mutant neurons transfected with EGFP alone remain axonless.

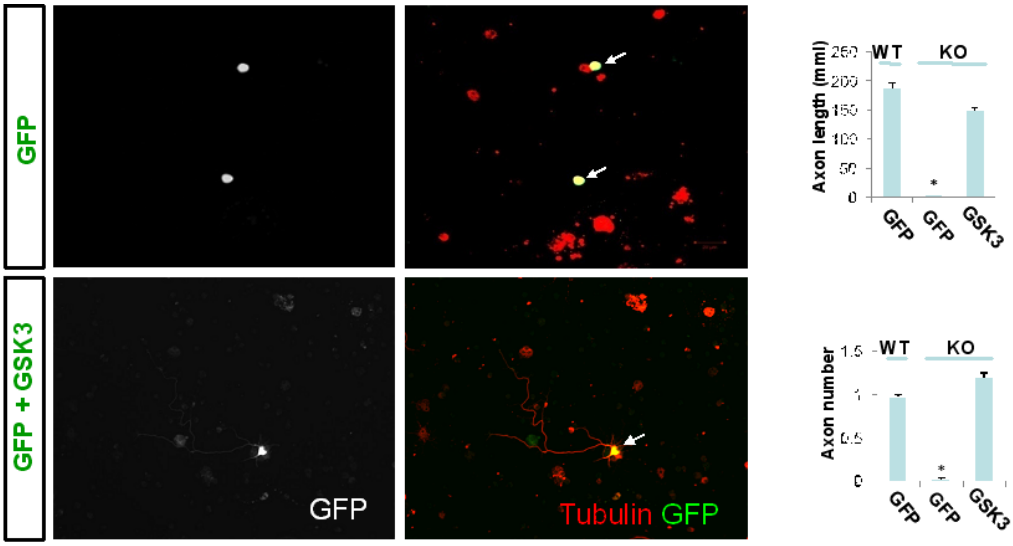


Figure 10 Axon growth defects in GSK-3 null neurons can be rescued by re-expression of GSK-3. Cortical progenitors taken from the double mutant brain were transfected with full length GSK-3β plasmid plus GFP or GFP alone as control. GSK-3β expression rescued the axon growth defects. Quantification of the length of the longest axon and numbers of axons from each GFP-positive cell was shown on the right. Data shown are means+SEMs.

We also cultured cortical progenitor cells from GSK-$3\alpha^{-/-}$ GSK-$3\beta^{F/F}$ and GSK-$3\alpha^{+/-}$ GSK-$3\beta^{F/W}$ brains and overexpressed plasmid encoding the Cre recombinase to see whether Cre-mediated GSK-3 deletion *in vitro* had a similar effect on axon growth as seen *in vivo*. Results shown in Figure 10 confirmed the cell-autonomous effect of GSK-3 on axon growth. Only overexpression of Cre in GSK-$3\alpha^{-/-}$ GSK-$3\beta^{F/F}$ cells, which removes all GSK-3 alleles, inhibited the axon growth. In control neurons, where GSK-3α and GSK-3β were still expressed, axons grow normally (Figure 11, provided by Dr. Woo-Yang Kim).

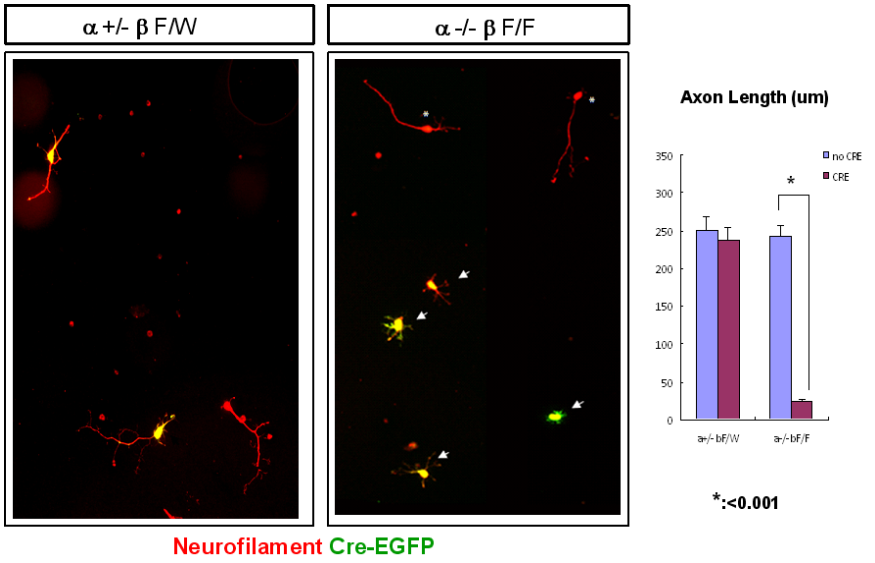


Figure 11 Overexpression of Cre *in vitro* mimics axon growth defects *in vivo*. Cortical progenitors taken from the GSK-3$\alpha^{+/-}$ GSK-3$\beta^{F/W}$ or GSK-3$\alpha^{-/-}$ GSK-3$\beta^{F/F}$ brains were transfected with Cre-EGFP plasmid or GFP as control. Cre overexpression in GSK-3$\alpha^{-/-}$ GSK-3$\beta^{F/F}$ cells causes inhibition of axon growth as seen *in vivo*. Quantification of the length of the longest axon extended from each cell was shown on the right. Data shown are means+SEMs. Figure is provided by Dr. Woo-Yang Kim.

These results suggest that GSK-3 functions cell autonomously, within cortical neurons, to mediate axon growth.

## Overexpression of β-catenin does not phenocopy the axon growth defects in GSK-3 null neurons

GSK-3 is a key negative regulatory component in the canonical Wnt signaling pathway. In all cell types studied to date, GSK-3 acts to suppress cellular β-catenin levels by phosphorylating the protein and targeting it for proteosomal degradation. In GSK-3 double mutant brain, β-catenin is increased substantially (data not shown). To determine whether increased β-catenin levels in GSK-3 null neurons are responsible

for the axon growth defects, we overexpressed a stabilized β-catenin construct into wild-type neurons to mimic a GSK-3 null environment. This mutant form of β-catenin was made in such a way that it cannot be phosphorylated by GSK-3 thus it will not be degraded. Interestingly, we found overexpression of the stabilized β-catenin does not affect axon growth (Figure 12). Instead, it enhances the dendritic development (see arrows in Figure 12) with an increase in dendrite length and no change in dendrite numbers (Quantification not shown). This result is consistent with previous finding which identified β-catenin as a critical mediator of dendritic morphogenesis (Yu and Malenka, 2003).

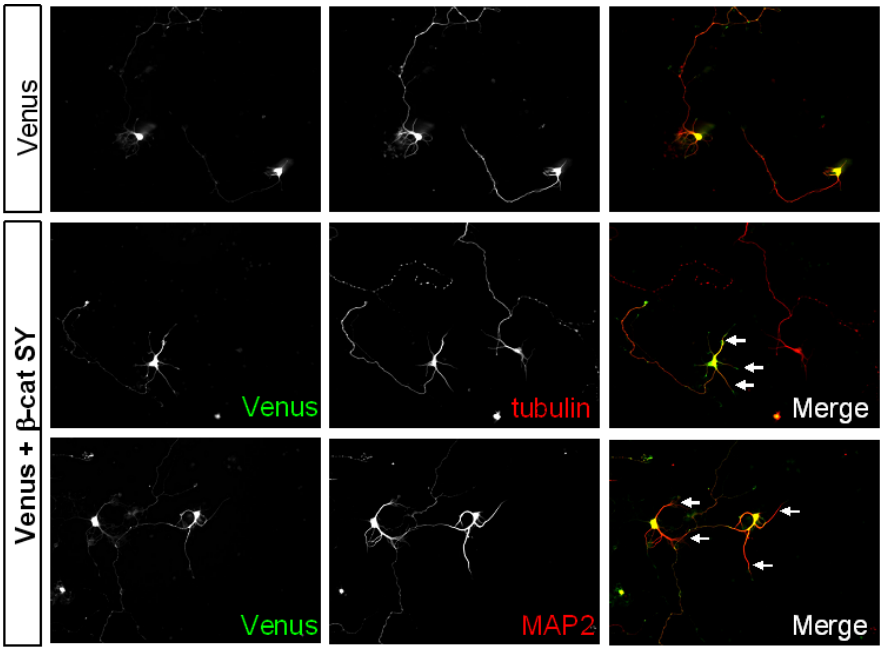


Figure 12 β-catenin overexpression in wild-type neurons does not mimic the axon growth defects in GSK-3 null neurons. Cortical progenitors taken from the wild type brain were transfected with stabilized β-catenin plus GFP or GFP alone as control and cultured for 4 days. Cells were fixed and stained with α-tubulin or MAP2 in red.

These results suggest that defects of axon growth in GSK-3 null neurons

were not due to increased cellular levels of β-catenin.

**GSK-3 null neurons have excessive bundled microtubule structures in axons**

Microtubules play an important role in axon specification. Axonal microtubules show increased stability and such stabilization probably allows microtubules to protrude with their dynamic ends more distally, thereby promoting axon formation (Witte et al., 2008). Many GSK-3 substrates are microtubule-associated proteins (MAPs) including APC, CRMP2, MAP1b, mictotubule-associated protein 2 (MAP2), tau, and neurofilament. GSK-3 phosphorylation of those MAPs will modulate their affinities to microtubule, which further regulate microtubule growth and stability. To determine whether the axon growth defects were caused by altered microtubule dynamics, GSK-3 null neurons were stained with α-tubulin, one of the components of tubulin dimers. In control neurons, axonal microtubules have an intact structure. However, in mutant neurons, axons were filled with excessively bundled microtubules (Figure 13). Consistent with the hypothesis that GSK-3 deficiency leads to unstable microtubules, the level of acetylated tubulin (aged, stable form of tubulin) (Westermann and Weber, 2003), is also reduced in GSK-3 deficient neurons (data not shown).

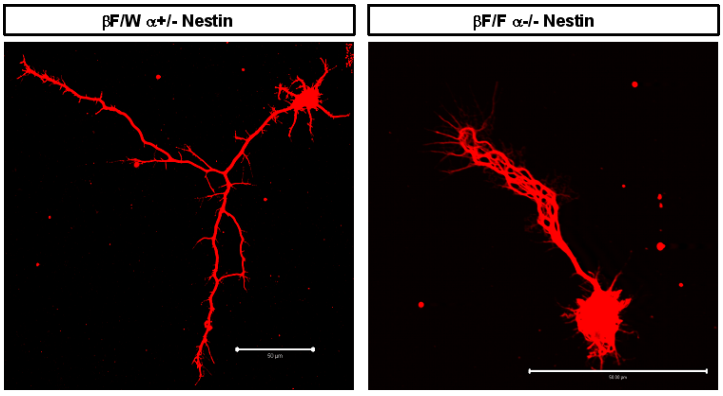

Figure 13 GSK-3 null neurons have excessively bundled microtubules in axons. Cortical neurons taken from wild type and double mutant brain were cultured *in vitro* for 4 days and stained with α-tubule. Scale bars are shown.

These results suggest that microtubule instability might be the major reason for axon growth inhibition in GSK-3 null neurons.

# DISCUSSION

### Loss of GSK-3 inhibits axon growth

Although the role of GSK-3 in axon morphogenesis has been well established (Jiang et al., 2005; Yoshimura et al., 2005; Zhou et al., 2004), most of the supporting evidence comes from either treating cultured neurons with different pharmacological inhibitors or overexpressing various GSK-3 mutant constructs. There was no genetic evidence available in mammals until our group firstly characterizes the nervous system development in GSK-3β null mice (Kim et al., 2006). However, the brain, spinal cord, and DRGs of the null mice did not appear grossly different from the controls. The development of hippocampal neuron polarity was also normal.

The potential compensation by GSK-3α is obvious since GSK-3α is abundantly expressed in the entire nervous system (Kim et al., 2006). We therefore used a conditional knockout strategy in a GSK-3α null background to target GSK-3β specifically in the developing nervous system by employing a mouse strain expression Cre recombinase under the control of the neural specifying elements of the Nestin promoter. The Nestin-cre transgene induces widespread recombination in the CNS neural progenitors from around embryonic day 10 (Yokota et al., 2009;

Zhong et al., 2007) and there is a complete depletion of GSK-3β in the nervous system in mice homozygous for a floxed GSK-3β allele (Dr. Woo-Yang Kim and Dr. William Snider, unpublished data).

Severe failure of the brain development was found in the double mutant mice (Dr. Woo-Yang Kim and Dr. William Snider, unpublished data). Characterization of these double mutants reveals that the brain and spinal cord exhibit abnormalities in progenitor proliferation and axon growth and guidance (Dr. Woo-Yang Kim and Dr. William Snider, unpublished data). To further analyze how deletion of GSK-3 affects axon growth, we dissociated cortical progenitors from control and double mutant brain and cultured them *in vitro* on PDL/laminin coated coverslips. GSK-3 null neurons attached normally and the initial neurite sprouting within 24h was not significantly different from control neurons. After 24h, however, differences began to show. One of the neurites in control neurons extended much quickly than others and became an axon. In GSK-3 null neurons, none of the MAP2 positive neurites were able to extend further and form a Tau-1-positive axon.

A possible reason for the lack of axon elaboration is loss of polarity cues. According to previous findings, localized inhibition of GSK-3 at one of the neurites is required for axon-dendrite specification. With no GSK-3 expression in these double mutant neurons, axon growth is strongly suppressed since no polarity cues were available. Re-expression of GSK-3β back into these double mutant neurons almost fully rescues the axon growth inhibition. Cre-mediated *in vitro* GSK-3 removal also faithfully mimiced the axon growth defects. These results present the first analysis of the physiological role of GSK-3 in the establishment of neuronal polarity and confirm

the critical requirement for GSK-3 in axon growth. These results also conform well with our previous studies of GSK-3 inhibitors and GSK-3 knockdown using siRNA *in vitro* (Kim et al., 2006).

**Beta-catenin or microtubule?**

β-catenin has dual functions in cells. It is an essential component of the canonical Wnt signaling system that controls decisive steps in development (Clevers, 2006). Together with other members of the cadherin/catenin family, β-catenin also forms a major transmembrane signaling complex through which extracellular signals can influence the actin cytoskeleton (Gumbiner, 1996; Yap et al., 1997). In the nervous system, cadherin/catenin complex is present in neuronal processes during early development (Benson and Tanaka, 1998) and appears to play multiple roles during neural circuit formation and maturation (Abe et al., 2004; Togashi et al., 2002; Yu and Malenka, 2003).

In the GSK-3 double mutant brain, β-catenin is increased substantially which is in consistent with the critical importance of GSK-3 in phosphorylating β-catenin in the destruction complex that mediates canonical Wnt signaling. It is thus reasonable to speculate that this aberrantly high level of β-catenin might be responsible for axon growth defects in GSK-3 null neurons. However, when a stabilized form of β-catenin is overexpressed in wild type neuron, the axon growth is largely unaffected (see Figure 12). There is only a moderate increase in dendritic length with no change in dendrite numbers. Yu et al. (Yu and Malenka, 2003) also shown that increasing the intracellular levels of β-catenin enhances dendritic arborization in rat hippocampal

neurons. They thought the effect was due to increased formation of the cadherin/catenin complex and likely tied to the effects on the actin cytoskeleton.

GSK-3 also regulates microtubule growth and stability (Zhou and Snider, 2005). It phosphorylates many microtubule associated proteins such as Tau, MAP1b, CRMP2 and APC. Mutations or the absence of these proteins modifies the axon growth and formation (Dawson et al., 2001; Gonzalez-Billault et al., 2001; Takei et al., 1997; Takei et al., 2000; Teng et al., 2001; Yokota et al., 2009). GSK-3 phosphorylation of microtubule associated protein such as MAP1b and tau appears to reduce their binding to microbutules, therefore maintaining a population of dynamically unstable microtubules (Trivedi et al., 2005). Thus, GSK-3 phosphorylation can render the microtubules more dynamic, favoring axon growth. In turn, absence of GSK-3 stabilizes microtubules thus blocks the axon growth. This is exactly what we saw in the GSK-3 null neurons. Our result suggests that disruption of microtubule dynamics in the absence of GSK-3 might be the major cause of axon growth inhibition in GSK-3 null neurons.

However, at this point, we still don't know the exact mechanism for the lack of axon growth in GSK-3 null neurons. We favor dysregulation of the cytoskeletal dynamics but we don't know the full differentiation status of the GSK-3 deficient neurons. There could be many other molecular abnormalities that contribute. Moreover, some of my unpublished results shown that PNS axon growth may bypass the requirement of GSK-3. Thus, the most important next step is to establish the relevance of these findings to axon growth *in vivo*. To that end, we have set up a number of crosses with different neuronal-specific Cre lines to assess axon growth

and targeting, such as sensory neuron specific Advillin-Cre (Hasegawa et al., 2007), pyramidal neuron specific Nex-Cre (Goebbels et al., 2006), retinal ganglion neuron specific Math5-Cre (Yang et al., 2003).

## SUMMARY

This study examined the role of GSK-3 in neural development by conditional inactivation of GSK-3 in neural progenitor cells of the developing cortex. The current work provides strong evidence for the critical role of GSK-3 in neuronal axon growth and development. We found that deletion of GSK-3 inhibits axon growth in cortical and DRG neurons and the effect of GSK-3 is cell-autonomous. Loss of GSK-3 results in disrupted microtubule structure which might be the major reason for axon growth defects. These findings provide insights into the regulation of axon growth and the role of GSK-3 during nervous system development.

# MATERIALS AND METHODS

## Antibodies

The following primary antibodies were used: anti-pGSK-3β [$Ser^{9}$] (Cell Signaling), anti-GSK-3β (BD Bioscience), anti-Par6 (N-18, Santa Cruz Biotechnology), anti-pTau [$S^{199/202}$] (Invitrogen), anti-β-actin (Cell signaling), anti-α-tubulin (Sigma), anti-NF200 (Sigma) and anti-MAP2 (Millipore).

The following secondary antibodies were used: HRP-conjugated donkey anti-mouse IgG (Dako), anti-rabbit IgG (Cell signaling) and bovine anti-goat IgG (Santa Cruz Biotechology). Alexa fluor-555 or Alexa fluor-488 conjugated phalloidin, anti-mouse and anti-rabbit antibodies (Molecular Probes).

## Plasmids

Par6β wild-type plasmid is provided by Dr. Jeffrey Pessin (State University of NY at Stony Brook). Full length GSK-3β plasmid is provided by Xiao-Fan Wang (Duke University). Cre-EGFP plasmid is provided by Dr. Franck Polleux (UNC-Chapel Hill). Constitutively active β-catenin plasmids are generously provided by Dr. Bert Vogelstein (Johns Hopkins University)

## Animals

Mice were cared for according to animal protocols approved by the University of North Carolina and University of Toronto. Nervous system-specific conditional GSK-3 double knockout mice (GSK-$3\alpha^{-/-}$ GSK-$3\beta^{F/F}$ Nestin-Cre) were generated by mating GSK-3α null mice (McManus et al., 2005), floxed-GSK-3β mice (Patel et al.,

2008), and Nestin-Cre mice (Tronche et al., 1999). Littermate GSK-3a$^{+/-}$ GSK-3b$^{F/W}$ Nestin-Cre mice served as controls.

**Inhibitors**

GSK3 inhibitor X (BIO), (2′*Z*, 3′*E*)-6-Bromoindirubin-3′-acetoxime was obtained from Calbiochem.

**Supplements**

Mouse NGF was obtained from Harlan Bioproducts.

**Oligos**

RT-PCR primer sequences are: Par6γ 5' CTGCAGCGCTGTGGAAGTC, Par6γ 3' TCTTCAAACTTCCCAGGCTTGT, Par6β 5' GACAACTACCACAAGGCGGTTT, Par6β 3' CCAAAGGCACTGTAGTCAGCTTCT, Par6α 5' AAGAAAGGGCTCCTGCTACGA, Par6α 3'GACACCTGGCGGAAATCTTG

The Par6α siRNA target sequence GCTGCTGGCGGTCAGTGATGAGATCCTTG, resides from 679-707bp downstream from the initiating methionine within the open reading frame of the mouse Par6α mRNA. The Par6 siRNA oligonucleotide sequence is as follows: GCUGCUGGCGGUCAGUGAUUU and was obtained from Dharmacon RNA Technologies.

**Total RNA isolation and RT-PCR**

Total RNA from cultured cells, embryonic DRG neurons or tissues was isolated using RNeasy Mini Kit (Qiagen) or Trizol (Invitrogen), as described by the manufacturer. Reverse-transcript PCR was performed by using Omnicript RT kit (Qiagen) and random primers (Invitrogen), as described by the manufacturer.

**Cell Culture and Transfection**

Dissecting and culturing of mouse DRG neurons was performed as described previously (Markus et al., 2002). Briefly, pregnant CF-1 mice were euthanized and embryos removed at E13-14. DRGs were dissected and digested with collagenase (Roche) (1 mg/ml) for 15 min followed by trypsin-EDTA (0.25%) (Sigma) for 5 min at 37℃. DRGs were then dissociated with a 1 ml pipette ti p in plating medium (MEM containing L-glutamine with 5% fetal bovine serum and 1X penicillin/streptomycin, Invitrogen) supplemented with antimitotics (20 mM 5-fluoro-2-deoxyuridine, 20 mM uridine, Sigma), electroporated with an Amaxa Nucleofector apparatus (Amaxa) then plated onto 24-well plastic culture dishes coated with poly-D-lysine (100 mg/ml) (Sigma) and laminin (10 mg/ml) (BD Bioscience) and cultured in the presence of NGF (50 ng/ml). DRGs were lysed with standard RIPA lysis buffer supplemented with protease and phosphatase inhibitors (Sigma) and stored at –80ºC for immunoblot analysis or fixed in 4% paraformaldehyde, coverslipped and analyzed for axon growth and morphology.

For cortical neuron isolation, the two telencephalic hemispheres were isolated under sterile conditions in $Ca^{2+}$/$Mg^{2+}$-free PBS. Tissues were incubated for 5min at 37℃ in 0.25% Trypsin/EDTA (Sigma). The tissue was then dissociated mechanically with 1ml Pasteur pipette, and plated in Neurobasal A medium supplemented with 1XB27, 2mM L-glutamate and 1X penicillin/streptomycin (all from Invitrogen). Cortical primary cells were then electroporated with an Amaxa Nucleofector apparatus (Amaxa). Electroporated cells were cultured on coverslips coated with poly-D-lysine/laminin same as for DRG neurons.

PC12 cells were maintained in DMEM/F12 (1:1) with 10% horse serum/5% fetal bovine serum on poly-D-lysine (100 mg/ml) coated culture dishes. For studies involving acute NGF treatment, cells were differentiated for 4 days in NGF (50ng/ml), serum and NGF starved overnight in DMEM/F12 (1:1) plus 0.3% BSA and acutely treated with NGF (50-100ng/ml). Cells were washed with PBS and lysed with standard RIPA lysis buffer supplemented with protease and phosphatase inhibitors (Sigma) and stored at –80ºC for immunoblot analysis.

CAD cells were maintained in DMEM/F12 (1:1) with 8% fetal bovine serum on culture dishes. To induce differentiation, serum-containing medium was changed to serum-free medium and cells were further cultured for 3-4 days.

**Immunohistochemistry**

Following fixation in 4% paraformaldehyde for 15 min, embryonic DRG neurons or cortical neurons were blocked for 1 hour at room temperature and incubated in primary antibodies (see above) overnight at 4 degree. All antibodies were diluted in blocking buffer which is 1XPBS containing 2% BSA and 0.3% Triton X-100. The following day, neurons were washed in PBS, incubated in Alexa Fluor-conjugated secondary antibodies for 1 hr at room temperature, washed again with PBS and coverslipped with anti-fade mounting media for visualization.

**Image Capture and Axon Growth Analysis**

Images were taken with a Zeiss LSM confocal microscope or a Nikon Eclipse epifluorescence microscope. For image analysis, Zeiss LSM 510 image browser and Metamorph software (Molecular Devices Corporation) were used. For axon length quantification, the longest axon from each transfected or control neuron was

measured by manually tracing with Metamorph software. At least 50 neurons with axons were selected in each experiment. All data are reported as mean+SEM followed by an unpaired Student's t test to determine the significance of the data between groups.

**Immunoblots**

Samples were separated on precast 4-12% SDS-PAGE gels (Invitrogen) and transferred to PVDF membranes (Amersham). The membranes were then blocked with 5% Blotting Grade Blocker Non-Fat Dry Milk (Bio-Rad) in PBS with 0.1% Tween-20 (Sigma), and incubated with primary antibody in blocking solution at 4℃ overnight. The following day, membranes were wash with PBST and incubated with HRP-conjugated secondary antibody for 1 hour at room temperature in blocking solution and detected with ECL plus (Amersham) followed by exposure to Hyperfilm (Kodak).

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