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Phosphorylated Tyr142 β-Catenin signaling in axon morphogenesis and centrosomal functions

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PHOSPHORYLATED TYR142 β-CATENIN SIGNALING IN AXON MORPHOGENESIS AND CENTROSOMAL FUNCTIONS

Ph.D Dissertation

For the fulfillment of Doctoral degree

by

DEEPSHIKHA BHARDWAJ

Under the supervision of

Dr. Judit Herreros Danés

Lleida, 2014

Judit Herreros Danés, Ph.D in Biological Sciences and Associate Professor, Department of Basic Medical Sciences of University of Lleida, as supervisor of this thesis,

Hereby certify that,

Deepshikha Bhardwaj (Passport number: H5059226)**,** with Bachelor of Science (B.Sc) in Life Sciences (University of Delhi, India) and Master of Science (M.Sc) in Biotechnology (Choudhary Charan Singh University-Meerut, India), has completed the experimental work entitled **"Phosphorylated Tyr142 β-catenin signaling in axon morphogenesis and centrosomal functions"** under my direct supervision**.**

This work, including the thesis has been completed to my level of satisfaction to meet the requirements for the presentation before the corresponding defence tribunal and, so be it, to obtain **Doctor of Philosophy degree (Ph.D)** from the University of Lleida.

Signed:

Dra. Judit Herreros Danés

Lleida, 29th of October 2014

Dedicated to my mother

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INDEX

ABBREVIATIONS

µM	Micromolar
7-TM	Seven-Transmembrane
Ac CoA	Acetyl Coenzyme A
ACM	Astrocyte Culture Media
AD	Alzheimer's Disease
APC	Adenomatous Polyposis Coli
aPKC	atypical protein kinase C
Arg	Arginine
ATP	Adenosine Triphosphate
Aur A	Aurora kinase A
BDNF	Brain Derived Neurotrophic Factor
bFGF	Basic Fibroblast Growth Factor
CA	Cornu Ammonis
CaMKII	Ca^{2+} /Calmodulin-dependent protein kinase II
cAMP	Cyclic Adenosine Monophosphate
CDC25	Cell Division Cycle 25
CDK	Cyclin-Dependent kinase
cDNA	Complementary Deoxyribonucleic Acid
C-domain	Central-domain
CENPJ	Centromere Associated Protein J
CGE	Caudal Ganglionic Eminences
CINs	Cortical Interneurons
CK-1	Casein Kinase-1
C-Nap1	Centrosomal-Nek2 Associated Protein 1
CNS	Central Nervous System

CP	Cortical Plate
CPNs	Cortical Projection Neurons
CRD	Cysteine-Rich Domains
CREB	cAMP Response Element-Binding protein
Ct	Cycle threshold
DCX	Doublecortin
DG	Dentate Gyrus
DIV	Days In Vitro
DKK-1	Dickkopf-1
DMEM	Dulbecco´s Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide
DRG	Dorsal Root Ganglia
Ds Red	Discosoma Red Fluorescent Protein
Dvl	Dishevelled
EC50	Half maximal effective concentration
ECM	Extracellular Matrix
EDTA	Ethylene Diamine Tetraacetate
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial Mesenchymal Transition
ESCs	Embryonic Stem Cells
F-actin	Filamentous-actin
FBS	Foetal Bovine Serum

FH535	T cell-factor inhibitor
Fz	Frizzled
g/l	Grams/Litre
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GBM	Glioblastoma multiforme
GCL	Granule Cell Layer
GE	Ganglionic Eminence
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
GICs	Glioma initiating cells
Glu	Glutamic Acid
GnRH-1	Gonadotrophin Releasing Hormone-1
GS	Glutamine Synthase
GSK-3β	Glycogen Synthase Kinase-3β
h	Hours
H	Hoechst
HBSS	Hank's Balanced Salt Solution
HD	Huntington's disease
HGF	Hepatocyte Growth Factor
HGFA	Hepatocyte Growth Factor Activator
HL	Hairpin Loop
HP	Hippocampal Formation
HS	Horse Serum
IC50	Half maximal inhibitory concentration
IF	Immunofluorescence

IgG	Immunoglobulin G
IP3	Inositol Triphosphate
IPT	Immunoglobulin-like fold shared by Plexins and Transcriptional factors
ITAMs	Immunoreceptor Tyrosine- based Activation Motifs
IZ	Intermediate Zone
JNK	c-Jun N Terminal kinase
kDa	Kilodalton
KL domain	Kringle domain
LFT	Lipofectamine
LRP5/6	Low density lipoprotein-Related Protein-5/6
LV	Lateral Ventricle
Lys	Lysine
M Phase	Mitotic Phase
MAPK	Mitogen Activated Protein Kinase
MAPs	Microtubule-Associated Proteins
MEM	Minimum Essential Media
MET	Mesenchymal Epithelial Transition
MGE	Medial Ganglionic Eminences
min	Minutes
ml	Millilitre
mM	Milimolar
mRNA	Messenger Ribonucleic Acid
MT	Microtubule
MTOC	Microtubule Organizing Center
MW	Molecular Weight

MZ	Marginal Zone
NA	Numerical Aperture
NGF	Nerve Growth Factor
NI	Non-Infected
nM	Nanomolar
NSCs	Neuroepithelial Stem Cells
NT	Neurotrophins
NT-3	Neurotrophin-3
NT-4	Neurotrophin-4
OB	Olfactory Bulb
$p75^{NTR}$	p75 Neurotrophin Receptor
PBS	Phosphate Buffered Saline
PCM	Pericentrioler Material
PCP	Planar Cell Polarity
PCR	Polymerase Chain Reaction
PDGFR	Platelet-Derived Growth Factor Receptor
PDL	Poly-D Lysine
P-domain	Peripheral-domain
PEI	Polyethilenimine
Perv	Sodium Pervanadate
PFA	Paraformaldehyde
Phe	Phenylalanine
PI3K	Phosphatidylinositol-3-kinase
Pic	Piceatannol
PIP3	Phosphatidylinositol-3,4,5-Triphosphate

PKA	Protein Kinase A
PLC	Phospholipase C
Plk1	Polo-like kinase 1
PLO	Poly-L-Ornithine
PMet	Phosphorylated Met
PNS	Peripheral Nervous system
PP	Transient Plate
PP1α	Protein Phosphatase 1α
PSI	Plexins Semaphorins Integrins
PTyr142 β-cat	Phosphorylated Tyrosine142 β-catenin
RG	Radial Glia
RGCs	Radial Glial Cells
RMS	Rostral migratory Stream
RMT	Room Temperature
RNA	Ribonucleic Acid
RPM	Revolutions Per Minute
RT	Reverse Transcriptase
RTK	Receptor Tyrosine Kinase
SB225002	Antagonist of CXCR2 (receptor of CXCL2)
SB328437	Antagonist of CCR3 (only receptor of CCL20 and one of the receptor of CCL5)
Scr	Sramble
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
sec	Seconds
Ser	Serine

sFRP	Secreted Frizzled Related Protein
SGCs	Satellite Glial Cells
SGZ	Subgranular Zone
SH2	Src homology 2
Shh	Sonic Hedgehog
shRNA	Short hairpin RNA
SOS	Son Of Sevenless
SP	Subplate
SPH	Serine Protease Homology
sq-PCR	Semi-Quantitative Polymerase Chain Reaction
SU11274	Met Inhibitor
SVZ	Subventricular Zone
Syk	Spleen Tyrosine Kinase
T0	Artificial scratch creation time point
T24	24h post artificial scratch
TABTN	Total Axonal Branch Tip Number
TBS-T	Tris Buffered Saline-Tween 20
TCF/LEF	T Cell Factor/Lymphoid Enhancing Factor
T-domain	Transition-domain
TF	Transcription Factors
TGF β	Transforming Growth Factor β
Thr	Threonine
Trk	Tropomyosin receptor kinase
Tyr	Tyrosine
Tyr142Phe	β-catenin non-phosphorylable at Tyrosine142

VZ	Ventricular Zone
WB	Western Blot
WIF	Wnt Inhibitory Factor
WT	Wild Type
WT β-cat	Wild Type β-catenin
ZAP-70	Zeta-Activated protein of 70kDa
α-N catenin	Neural α-catenin
α-tubulin	α-tub
β-cat	β-catenin
β-TrCP	β-transducin repeat-containing-protein
γ-tub	γ-tubulin
γ-TURC's	γ-tubulin ring complexes

ABSTRACT

Abstract

β-catenin is a multifunctional protein, key component of adherent junctions and effector of the Wnt canonical pathway, was recently implicated in centrosomal functions. In the canonical Wnt pathway, when Wnt is present in the system, β-catenin escapes degradation, accumulates in the cytosol and translocates to the nucleus where, together with T-cell Factor (TCF) transcription factors, it regulates transcription of Wnt targets. Switching from adhesive to signaling functions (independent of Wnt) is achieved in part through phosphorylation of β-catenin at Tyr142 that promotes detachment of β-catenin from the adhesion complex and promotes migration by transcriptional regulation of target genes. Met receptor tyrosine kinase (the receptor for Hepatocyte Growth Factor (HGF)), is one of the kinases regulating β-catenin phosphoryation at Tyr142 during cell migration and axon outgrowth stimulated by HGF. On the other hand, β-catenin phosphorylation at Ser/Thr regulates β-catenin degradation and has been demonstrated to affect centrosomal cohesion/separation and spindle formation.

Here we focus on PhosphoTyrosine142 β-catenin (PTyr142 β-cat) signaling. First, we demonstrate that chemokines of CC and CXC families promote axon outgrowth. Furthermore, chemokine signaling acts downstream to HGF/Met/β-catenin/TCF signaling to regulate axon morphogenesis in developing hippocampal neurons. We also show that CXCL2 promotes axon branching and is involved in sensory axon outgrowth from dorsal root ganglia. In the second part of the work, we find for the first time that phosphorylated Tyr142 β-catenin localizes to centrosomes in primary astrocytes and glioma cells, and that centrosomal levels drop in mitosis. We also demonstrate the novel centrosomal localization of Met phosphorylated at Tyr1234/35. Aiming at identifying which is the kinase(s) regulating centrosomal PTyr142 β-cat, we show that a Met inhibitor does not affect it. However, an inhibitor of Spleen Tyrosine Kinase (Syk) decreases centrosomal PTyr142 β-cat, suggesting that Syk regulates the phosphorylation of Tyr142 β-catenin at centrosome. In addition, β-catenin is involved in the correct positioning of centrosomes during astrocyte migration and phosphorylation of β-catenin at Tyr142 is needed for HGF-stimulated cell migration.

Collectively, this work demonstrates the multiple roles of PTyr142 β-cat signaling, influencing axon morphogenesis (via regulation of chemokines expression) as well as centrosomal functions, cell polarity and migration.

Resumen

β-catenina es una proteína multifuncional, componente clave de las uniones adherentes y efector de la vía canónica Wnt, recientemente implicada en funciones centrosomales. En la señalización por Wnt, cuando Wnt está presente, β-catenina se acumula en el citosol y transloca al núcleo donde, junto con factores TCF, regula la transcripción de genes diana. La interelación entre funciones adhesivas y señalizadoras (independientes de Wnt) de β-catenina se logra, en parte, a través de la fosforilación de β-catenina en Tyr142, que promueve la desunión de β-catenina del complejo de adhesión y la migración a través de la regulación transcripcional. El receptor tirosina quinasa Met (receptor del Factor de Crecimiento Hepático (HGF)) induce la fosforilación de β-catenina en Tyr142 durante la migración y el crecimiento axonal estimulados por HGF. Por otra parte, la fosforilación de β-catenina en Ser/Thr regula la degradación de β-catenina y afecta a la cohesión/separación de los centrosomas y la formación del huso mitótico.

Aquí nos centramos en la señalización por β-catenina fosforilada en Tyr142. En primer lugar, demostramos que quimiocinas de las familias CC y CXC promueven el crecimiento axonal y que las quimiocinas actúan en la señalización inducida por HGF/Met/β-catenina/TCF durante la morfogénesis del axón. También mostramos que CXCL2 promueve la ramificación del axón en neuronas hipocampales y el crecimiento de axones sensoriales de los ganglios de la raíz dorsal. En segundo lugar, demostramos que β-catenina fosforilada en Tyr142 localiza en centrosomas en astrocitos primarios y células de glioma, y que estos niveles centrosomales disminuyen durante la mitosis. También mostramos la localización centrosomal de Met activo. Con objeto de identificar cual es la quinasa que regula la fosforilación de Tyr142 β-catenina en el centrosoma, mostramos que un inhibidor de Syk disminuye los niveles centrosomales de esta forma de β-catenina, lo que sugiere que Syk fosforila β-catenina en Tyr142 en el centrosoma. Además, β-catenina está implicada en el posicionamiento del centrosoma durante la migración de astrocitos y la fosforilación de β-catenina en Tyr142 es necesaria en la migración celular estimulada por HGF.

En conjunto, este trabajo ilustra las múltiples funciones señalizadoras de β-catenina fosforilada en Tyr142 en la morfogénesis del axón (a través de la expresión de quimiocinas), así como en funciones centrosomales y en polaridad celular y migración.

Resum

β-catenina és una proteïna multifuncional, component clau de les unions adherents i efector de la via canònica Wnt, recentment implicada en funcions centrosomals. En la senyalització per Wnt, quan Wnt està present β-catenina s'acumula en el citosol i transloca al nucli on, juntament amb factors TCF, regula la transcripció de gens diana. La interrelació entre funcions adhesives i funcions senyalitzadores (independents de Wnt) de β-catenina s'aconsegueix en part a través de la fosforilació de β-catenina en Tyr142, que promou la desunió de β-catenina del complex d'adhesió i la migració mitjançant la regulació transcripcional. El receptor tirosina quinasa Met (receptor del Factor de Creixement Hepàtic (HGF)) regula la fosforilació de β-catenina en Tyr142 durant la migració cel · lular i el creixement axonal estimulat per HGF. D'altra banda, la fosforilació de β-catenina en Ser/Thr regula la degradació de β-catenina i afecta la cohesió/separació centrosomal i la formació del fus mitòtic.

Aquí ens centrem en la senyalització per β-catenina fosforilada en Tyr142. En primer lloc, demostrem que quimiocines de les famílies CC i CXC promouen el creixement axonal i que la senyalització per quimiocines és necessària en la senyalització induïda per HGF/Met/β-catenina/TCF durant la morfogènesi axonal en neurones de l'hipocamp. També mostrem que CXCL2 promou la ramificació de l'axó i que aquesta quimiocina està involucrada en el creixement d'axons sensorials dels ganglis de l'arrel dorsal. A la segona part, demostrem que β-catenina fosforilada en Tyr142 es localitza en els centrosomes en astròcits primaris i cèl · lules de glioma, i que els seus nivells centrosomals disminueixen durant la mitosi. A més, demostrem la localització centrosomal de Met actiu. Amb l'objectiu d'identificar quina és la quinasa que regula els nivells centrosomals de fosfo-Tyr142 β-catenina, mostrem que un inhibidor de Syk disminueix els nivells centrosomals de fosfo-Tyr142 β-catenina, el que suggereix que Syk fosforila β-catenina en Tyr142 al centrosoma. A més, β-catenina està implicada en el posicionament del centrosoma durant la migració d'astròcits i la fosforilació de β-catenina en Tyr142 és necessària en la migració cel.lular estimulada per HGF.

En conjunt, aquest treball demostra les múltiples funcions senyalitzadores de β-catenina fosforilada en Tyr142, en la morfogènesi de l'axó (a través de la regulació de l'expressió de quimiocines), així com en funcions centrosomals i en polaritat cellular i migració.

INTRODUCTION

A. Neuronal development and axon outgrowth

1. Neurons and Glia

The adult vertebrate central nervous system (CNS) comprises of four major cell types: neurons, oligodendrocytes, astrocytes and ependymal lining of the ventricle. These cells are derived originally from the early neuroepithelium that forms the neural plate along the midline of developing embryo. As the development advances, single layer of pseudostratified epithelium folds to form the neural tube, where differentiation of neuroepithelial stem cells (NSCs) into neurons and glia occurs in a temporal specific manner (Rao, 1999), which happens in three phases (Figure 1).

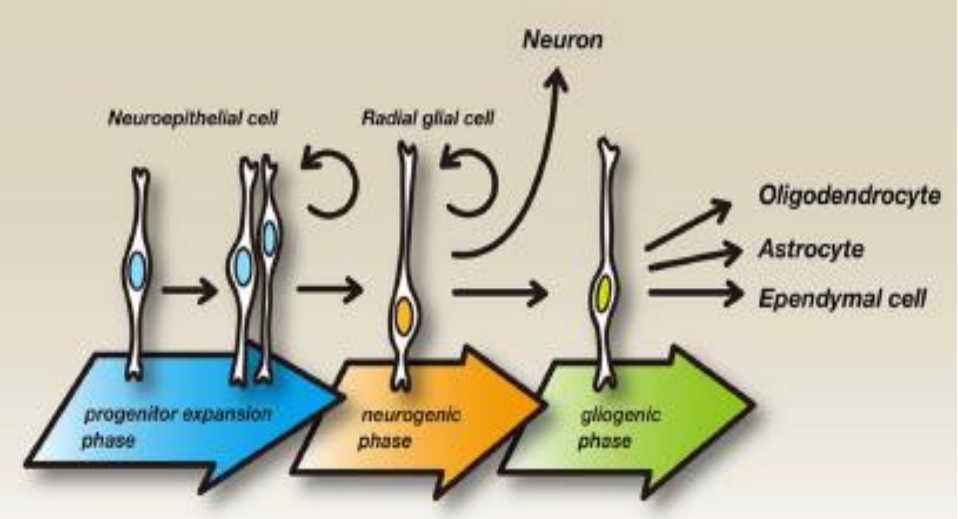


***Figure1**. **Illustration showing neural stem cells (NSCs) lineage (Shimojo, Ohtsuka, & Kageyama, 2011).** NSCs divides symmetrically and expand their population in progenitor expansion phase, followed by the elongation of the cells to form radial glial cells (RGCs). RGCs later gives rise to immature neurons in neurogenic phase and glial cells (oligodendrocytes, astrocytes and ependymal cells) in gliogenic phase.*

The first phase starts with progenitor expansion, where each NSC of neural tube divides symmetrically into two NSCs (Miller, & Gauthier, 2007) followed by the elongation of NSCs and formation of RGCs, which have cell bodies in the ventricular zone and radial fibres in the pial surface. The RGCs further undergo asymmetric division, giving rise to RGC and immature neuron or basal progenitor (neurogenic phase or neurogenesis) (Miyata et al., 2001; Noctor et al., 2001). The immature neurons later migrate outside of the ventricular zone alongside the radial fibres into the cortical plate and attain maturity,

whereas basal progenitors migrate into subventricular zone (SVZ) proceeded by proliferation and formation of more neurons. Later RGCs add different types of neurons to the deep as well as superficial layers. Following the production of neurons, gliogenic phase takes place where RGCs give rise to oligodendrocytes, ependymal cells and astrocytes.

Until 1990´s, neurogenesis was believed to occur only during embryonic and prenatal stages in an adult mammalian brain (Ming, & Song, 2005) with a widely accepted explanation that brain is too complex to allow generation and incorporation of newly born neurons. Many striking studies then demolished the prevailing hypothesis and brought into light the existence of "adult neurogenesis" in human brain throughout life, but only in selected regions (Neurogenic regions) (Altman, & Das, 1965; Eriksson et al., 1998; Curtis et al., 2003, 2007). New neurons are continuously being generated in the adult brain in selected regions- the subgranular zone (SGZ) in the dentate gyrus (DG) of the hippocampus, where NSCs generate cells that differentiate into newborn granule cells and the SVZ of the lateral ventricles, where new neurons are generated, which then migrate to rostral migratory stream (RMS) towards the olfactory bulb (OB) and differentiate into different types of olfactory neurons (Gage, 2000) (Figure 2). Neurogenesis in other adult regions is believed to be very limited under normal conditions but can be induced after injury (Gould, 2007).

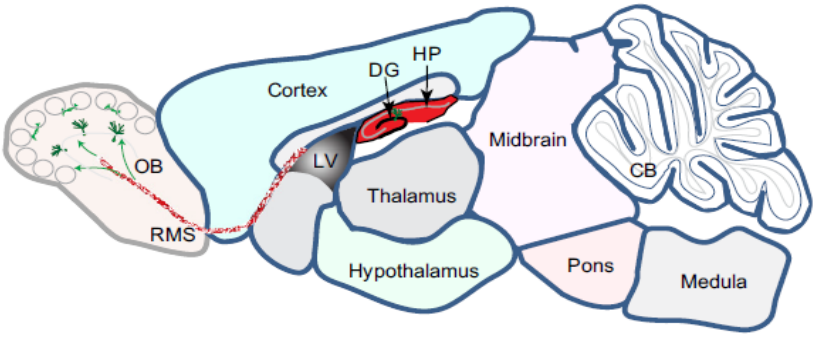


Figure 2. Neurogenic regions of an adult rodent brain (Ming, & Song, 2011)*. A sagittal section view highlighting the two restricted regions that exhibit active adult neurogenesis: dentate gyrus (DG) in the hippocampal formation (HP), and the lateral ventricle (LV) to the rostral migratory stream (RMS) to the olfactory bulb (OB).*

This piece of information raises future scope of utilizing the property of the cells from these regions to yield new ones for the replacement to those parts of brain damaged as a

result of neurodegenerative disease, brain injury or some external factors like stress. Nonetheless, till date the major regions of brain where this replacement was tested did not show promising results with significant replacement of large projection neurons (Eriksson et al., 1998; Curtis et al., 2003, 2007).

1.1 Types of neurons

A nerve cell with all its processes is called "neuron", the structural and functional unit of nervous system. A neuron has a cell body called as soma and two types of processes: dendrites, which are the short cytoplasmic processes that receive the nerve impulse transmitted by a neighbouring cell towards the cell body and; axon, which is the long cytoplasmic process of the cell body, transmitting impulse (or action potential) from cell body to other neuron (Figure 3).

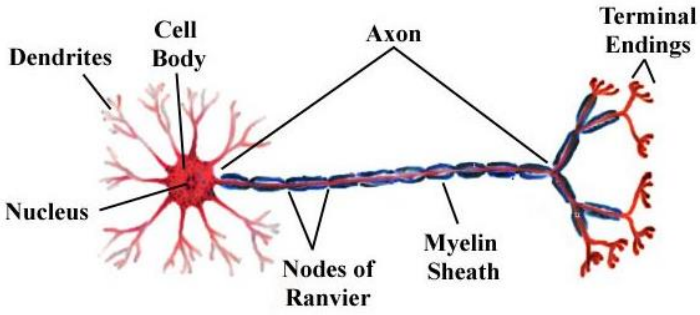


Figure 3. Structure of a typical CNS neuron.

The axon arises from the cell body in a conical elevation called axon hillock. Axons are covered by a myelin sheath formed by Schwann cells (in the Peripheral Nervous System, PNS) or by oligodendrocytes (in the CNS), and the region in between where myelin sheath is absent is the junction of adjacent myelinated segment called Node of Ranvier. Neurons communicate to each other by synapses through the presynaptic release of neurotransmitters, which trigger the action potential at the postsynaptic cell. Action potentials are propagated as electrical signals by exploiting the electrically excitable membrane of the neuron.

Based on the variations in processes (Figure 4), neurons are divided as: multipolar neurons, which consists of one axon and two or more dendrites, like motoneurons, interneurons and pyramidal neurons; bipolar neurons, composed of one axon and one dendrite, example: neurons of retina in eye, inner ear and olfactory membrane; unipolar

neurons, which have single process, and pseudounipolar neurons, which share characteristics with both unipolar as well as bipolar cells. They have a single process that extends from soma (like unipolar cells) and branches later into two distinct structures (like bipolar cells), for example dorsal root ganglia neurons.

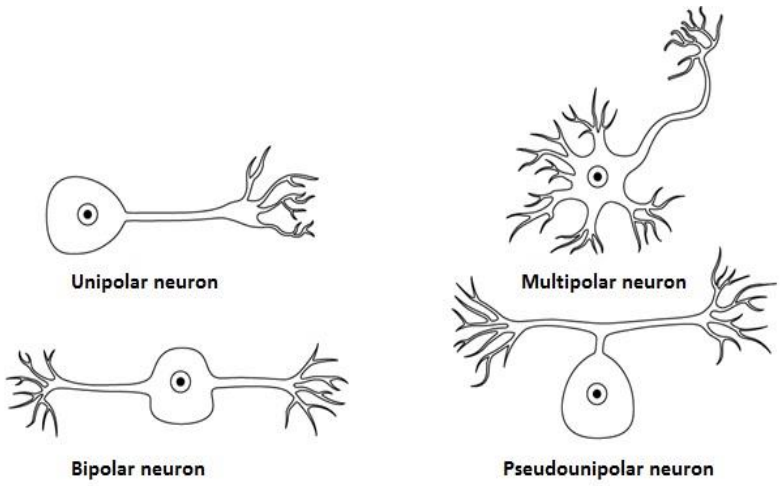


Figure 4. Types of neurons based upon the number and placement of axons.

Hippocampal neurons

The Hippocampus is a neural structure in the medial temporal lobe of the brain that has a distinctive, curved shape resembling the sea horse. It is a part of limbic system, present at the surface of cerebral hemisphere in an indentation, where it attaches to the midbrain. Regarding the neural circuitry underlying the hippocampus, the first zone is the DG, where a tightly packed layer of small *granule cells* wrap around the end of the hippocampus proper, forming a pointed wedge in some cross-sections. Next comes a series of *Cornu Ammonis (CA)* areas: first CA4 (which underlies the dentate gyrus), then CA3, then a very small zone called CA2 and then CA1. The CA areas are filled with densely packed pyramidal cells similar to those found in the neocortex. After CA1 comes an area called the subiculum. After this comes a pair of ill-defined areas called the presubiculum and parasubiculum, then a transition to the cortex proper (mostly the entorhinal area of the cortex) (Figure 5). One edge of the "U" shape of hippocampus, field CA4, which is embedded into a backward facing strongly flexed V-shaped DG and comprises molecular, granular, subgranular cell layers and poly-morph layer called hilus. The major pathways of signal flow through the hippocampus combine to form a loop called as trisynaptic circuit, formed of perforant path-DG-CA3-CA1.

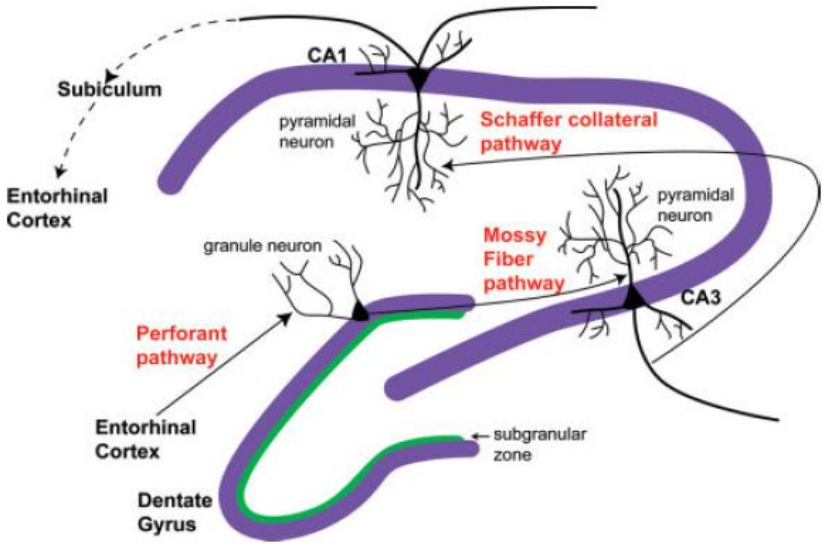


Figure 5. Neuronal circuitry in the hippocampus (Mccaffery, Zhang, & Crandall, 2005)*. Representative pyramidal neurons are drawn in the hippocampal CA1 and CA3 regions (shaded purple) and the granule neurons of the dentate gyrus (shaded purple), which can be renewed from progenitor cells situated in the subgranular zone (shaded green). The pathway crucial to memory formation leads from the association areas of the cortex passing via the entorhinal cortex to the hippocampus and returning to the entorhinal cortex via the subiculum. The three steps within this pathway compose the tri-synaptic circuit (labeled in red: the perforant, mossy fiber, and Schaeffer collateral pathways).*

Different regions of hippocampus are characterized by different types of neurons. DG is predominantly characterized by granular cells, whose cell body is located in the granular layer and dendrites branch to the molecular layer. CA layers display pyramidal neurons as the principle cell type, where the neuronal soma is located in the granular layer. From their apical pole a thick dendrite arises that can branch profusely in the molecular layer and from its basal pole emerges a set of small dendrites, which ramify in the polymorphic layer and the axon. The granule neurons and pyramidal neurons are excitatory and glutamatergic, whereas interneurons harboured by the hippocampus are GABAergic.

1.2 Glia

The term “glial cells” denotes a broad category of cells made of many subtypes. They out-number neurons and make up a large part of the nervous tissue. Glial cells, discovered first as the neuron supporting cells, now have well defined roles like homeostasis of the

extracellular environment by providing appropriate conditions for neurons and synapses (Theodosis, Poulain, & Oliet, 2008); Myelination (Colman, Pedraza, & Yoshida, 2001); transmitting signals over long distances in form of Ca^{2+} waves (Perea, & Araque, 2005); regulation of synaptic transmission (reviewed in Araque, 2008) and promoting regeneration factors to the injured nervous system (Fu, & Gordon, 1997; Houle, & Tessler, 2003).

The main glial subtypes in CNS are astrocytes, oligodendrocytes and microglia; and Schwann cells, enteric glial cells and satellite cells in PNS

1.2.1 Glial cells in the CNS

a. Oligodendrocytes. Oligodendrocytes are the myelinating cells of the CNS. There are different subtypes of oligodendrocytes in spinal cord and brain derived from specialized domains of ventral ventricular zone in spinal cord and from medial ganglionic eminence and anterior entopeduncular area in ventral brain. They are derived from precursors by a complex cell lineage process that renders mature cells producing the insulating myelin sheath (Takebayashi et al., 2002; Zhou, & Anderson, 2002). Oligodendrocyte injury and degeneration is involved in the pathology of Multiple Sclerosis (Lucchinetti, & Lassmann, 2001).

b. Astrocytes. Classically considered as supporting cells in the brain, astrocytes have been recently acknowledged for there role as dynamic regulators of many brain processes, including synaptogenesis and synaptic efficacy (Ullian et al., 2001; Christopherson et al., 2005); supporting adult neurogenesis (Pixley, 1992; Song, Stevens, & Gage, 2002); and for acting as neural stem cells in the adult brain (Garcia et al., 2004; Sanai et al., 2004). Thus astrocytes's emerging role as the key regulator in brain function and plasticity highlights the critical need to better characterize the heterogeneity and developmental specification of different subpopulations of astrocytes both within adult neurogenic regions and throughout brain (Imura et al., 2006; Sakaguchi et al., 2006). Astrocytes demonstrate heterogeneity at morphological, ultrastructural and molecular levels including growth factor receptor expression, proliferation capacity and electrophysiological properties within the SVZ and SGZ (Doetsch et al., 1997, 2002; Seri et al., 2001, 2004; Filippov et al., 2003; Kronenberg et al., 2003; Garcia et al., 2004): Radial astrocytes (nestin positive that extend a process into Granule Cell Layer (GCL)), Horizontal astrocytes (extending basal processes under the GCL and nestin negative) in the SGZ

(Kronenberg et al., 2003; Seri et al., 2004). In the SVZ, B1 and B2 types of astrocytes are present which differ in their location, cellular ultrastructure and proliferation profiles (Doetsch et al., 1997). In addition, based on its presence in white matter or grey matter (Ramon, & Cajal, 1909), they are divided into: 1) Protoplasmic astrocytes, which populate grey matter and have more irregular processes and less glial filaments (reviewed in Freeman, 2010); 2) Fibrous astrocytes, which populate the white matter and are characterized by more regular shapes and cylindrical processes exhibiting a "starlike" appearance with dense glial filaments (Figure 6).

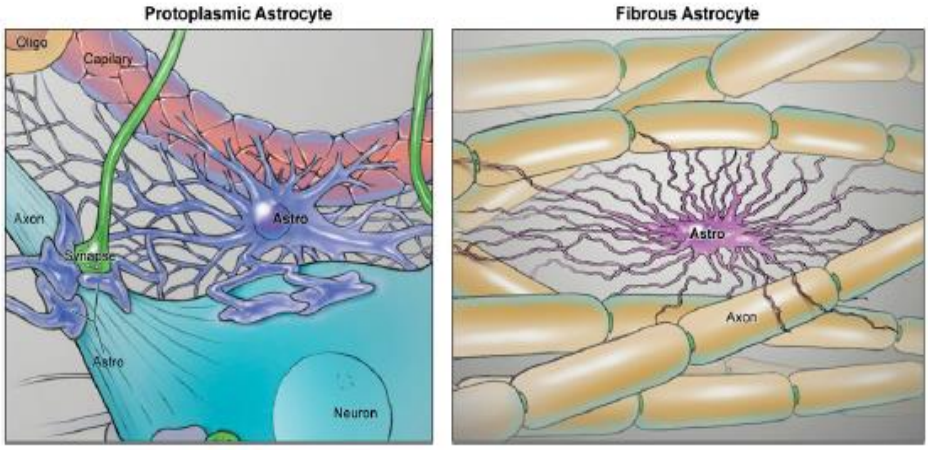


Figure 6. Subtypes of astrocytes in grey and white matter *(Molofsky et al., 2012). The protoplasmic astrocyte (left), shown in close association with neuron in grey matter and fibrous astrocytes (right), in the white matter.*

With their end terminals surrounding blood vessels, astrocytes form gap junctions and are closely associated with vasculature and its basal lamina in the adult SVZ and SGZ. Astrocytes express a number of secreted factors, including cytokines (Barkho et al., 2006) and Wnt factors (Lie et al., 2005) that regulate proliferation and fate specification of adult neural precursors, as well as neuronal migration and synapse formation, maturation and plasticity (reviewed in Freeman, 2010). Thus, astrocytes express a range of neurotransmitter receptors, transporters and ion channels which let them sense neuronal activity and direct morphological changes (Verkhratsky, & Steinhäuser, 2000; Malarkey, & Parpura, 2009).

During mammalian nervous system development, neural precursors generate neurons followed by the differentiation of astrocytes and glia (Freeman, 2010). In spite of the generation and expansion of astrocytes being completed by early postnatal stages, elaboration and refining their processes continues even after birth during active period of

synaptogenesis (Ullian et al., 2001). Astrocytic morphogenesis initiates with the cellular processes appearing on the 1st week of postnatal development in nature. By 3-4 weeks, astrocytic processes increase bifurcation and distal appendages acquire a much thinner look (Bushong, Martone, & Ellisman, 2004). At postnatal day 7, astrocytes exhibit significant overlap of processes with the neighbouring astrocyte but by postnatal day 14-21 they are pruned back along with the discrete border (Bushong, Martone, & Ellisman, 2004) (Figure 7). This tendency called as "Tiling" is the way for complete coverage of the brain.

Recent progress in the last two decades demonstrated that astrocytes are responsible for a wide variety of essential functions for a healthy CNS, including primary roles in synaptic transmission and information processing by neural circuit development (Clarke, & Barres, 2013). Consequently, the loss of normal astrocytes is involved in CNS pathologies including trauma, viral or bacterial infections and neurodegeneration (Mucke, & Eddleston, 1993), where astrocytes undergo a reaction called "astrogliosis" with increased cell division and protein expression, and a characteristic morphological change (the hypertrophy of their cellular processes) associated with an increased motility (Ridet et al., 1997).

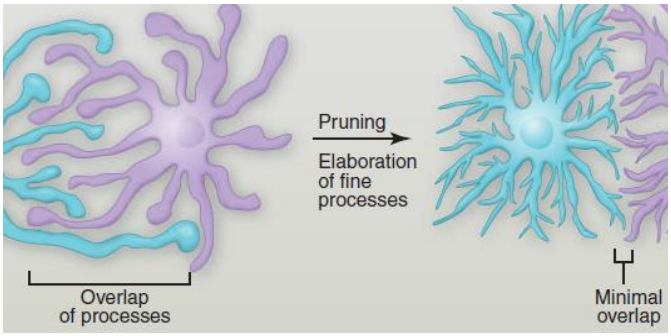


Figure 7. Coordination of astrocyte morphological growth and pruning (adapted from Freeman, 2010). *Astrocytes initially extend large, filopodial processes that overlap significantly with neighboring astrocytes; however, by postnatal day 21, astrocytes refine their morphology to occupy unique spatial domains and elaborate fine processes that closely associate with synapses (Bushong et al., 2004)*

Astrogliosis and brain injury. The mechanism by which within hours of any type of brain injury, survived astrocytes of the affected region exhibit hypertrophy and proliferation is termed as reactive astrogliosis (Ridet et al., 1997). This process is usually

completed by the migration of microglia and macrophages to the affected area. Reactive astrocytes then increase the expression of its structural proteins: Glial Fibrillary Acidic Protein (GFAP) and vimentin (Eng, Ghirnikar, & Lee, 2000) and many others, for example copper-zinc superoxide dismutase, glutathione peroxidase and metallothionein are increased in reactive astrocytes upon ischemia (Liu et al., 1993; Takizawa et al., 1994; Neal et al., 1996) indicating reduction of reactive oxygen species (ROS). Also astrocytes express inducible form of Heme oxygenase in response to brain insults (Geddes et al., 1996; Takeda et al., 1996), which is the first step of heme metabolism, which might be important in preventing heme iron precipitation after conditions such as trauma that liberate hemoglobin into the brain parenchyma.

Astrocytes release a variety of trophic factors under normal conditions which have a positive influence on neuronal survival (Ridet et al., 1997). Reactive astrocytes increase the expression of several trophic factors, especially Nerve Growth Factor (NGF), basic Fibroblast Growth Factor (bFGF), Brain Derived Neurotrophic Factor (BDNF) and Neuregulins, which also have well defined roles in neurite outgrowth (Schwartz, & Nishiyama, 1994; Strauss et al., 1994; Mocchetti, & Wrathall, 1995; Tokita et al., 2001). Also chemokines of CXC and CC families especially CXCL1, CXCL2, CXCL12, CCL2, CCL3 are produced by astrocytes upon brain and spinal cord injury (reviewed in Jaerve, & Müller, 2012), thus involving astrocytes in brain and spinal cord trauma.

1.2.2 Glial cells in the PNS

a. Schwann cells. These are the major glial cell type in PNS. Named after the german biologist Theodor Schwann, there are a variety of Schwann cells (myelinating and non-myelinating) which keep peripheral nerves alive. The myelinating Schwann cells form insulating sheath around the axon similarly as done by oligodendrocytes in CNS, whereas the non-myelinating ones are similar to astrocytes and likely to play metabolic and mechanical support functions.

b. Olfactory ensheathing cells. They resemble non-myelinating Schwann cells and associate both with CNS and PNS primary olfactory axons.

***c. Enteric glia cells*.** They are found in the autonomic ganglia of the gut (enteric system), share similarity to astrocytes in both structure and biochemistry and are involved in the formation of synaptic interactions in the enteric system.

d. Satellite glial cells (SGCs). These cells cover the surface of nerve cell bodies in sensory, sympathetic and parasympathetic ganglia (Hanani, 2005, 2010). Literature of SGCs with respect to sensory ganglia is discussed here. Sensory ganglia contain the cell bodies of neurons that transmit sensory information from the periphery to CNS (spinal cord) (Aldskogius, Elfvin, & Forsman, 1986; Matthews, & Cuello, 1982). Most of the sensory signals are transmitted to CNS by Dorsal Root Ganglia (DRG) located near the entrance of spinal cord. An interesting feature of sensory ganglia is that somata of sensory neurons are wrapped by a layer of SGCs (Figure 8) (Hanani, 2005).

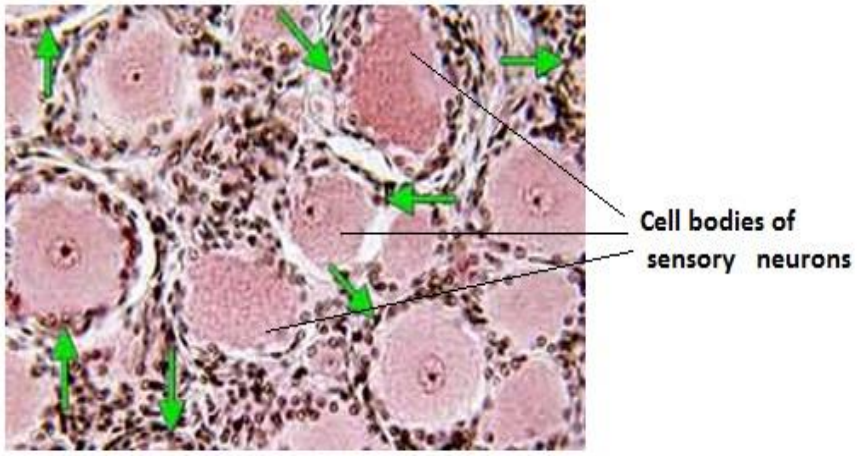


Figure 8. Satellite glial cells surrounding sensory neurons (http://vanat.cvm.umn.edu/). *Figure is showing spinal ganglion (H & E stain), where neuronal cell bodies are surrounded by satellite glial cells (shown by arrows in green).*

In general each sensory neuron has its own SGCs sheath, which usually consists of several SGCs, and thus the neuron and its surrounding SGCs form a distinct morphological and probably functional unit. These units are separated by regions containing connective tissue. However, a small percentage of neurons (5.6% in rat DRGs) form small groups of 2-3 cells enclosed in a common connective tissue, separated from each other by SGC sheets (Pannese et al., 1991) which are characterized best and specifically by a molecular marker glutamine synthase (GS).

SGCs have been implicated in Neurotrophins (NTs)-regulated growth and survival of sensory neurons (Pannese, & Procacci, 2002), in NTs-mediated sympathetic nerve fibre sprouting upon injury to DRG neurons (Zhou et al., 1999) and in neural development and maintenance (De Koninck, Carbonetto, & Cooper, 1993; Sjogreen, Wiklund, & Ekstrom, 2000).

2. Neuronal migration

The migration of newly born neurons is a precisely regulated process, essential for the development of proper brain circuitry. In the cerebral cortex, the two major types of neurons- glutamatergic excitatory projection (pyramidal) neurons and GABAergic inhibitory interneurons, arise from distinct sets of progenitor cells within the telencephalon and adopt two main strategies to disperse throughout CNS: **Radial migration and Tangential migration** (Figure 9) (Hatten, 1999; Marín, & Rubenstein, 2003).

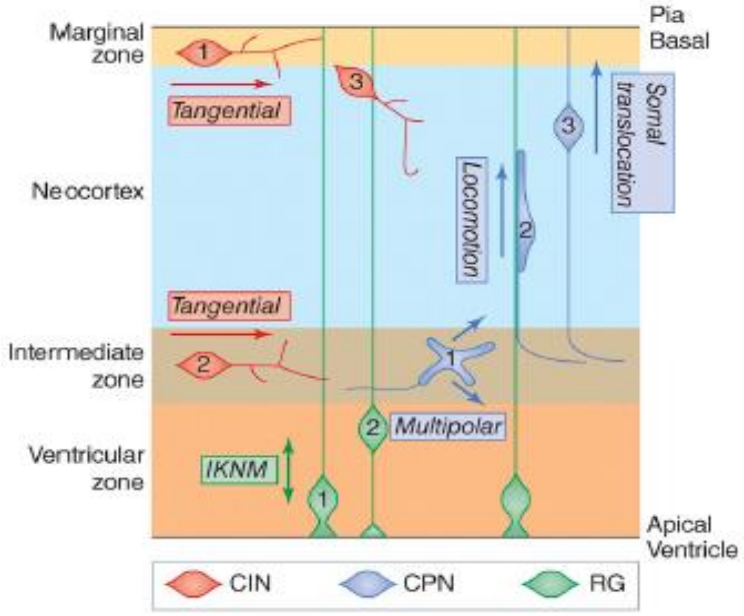


Figure 9. Major neuron migrations in neocortex (Cooper, 2013). *Transverse section through the developing rodent brain, showing cortical interneurons (CINs, red), migrating tangentially along the marginal zone* ***(1)*** *and the intermediate zone* ***(2)*** *from their origins in the basal forebrain, which later migrate into the cortical plate* ***(3)****. The same figure is also demonstrating the cortical projection neurons (CPNs, blue), migrating radially by three phases: mutipolar* ***(1)****, locomotion* ***(2)*** *and somal translocation* ***(3).*** *Supporting cells for radial migration: radial glia (RG, green) is shown undergoing interkinetic nuclear movement (IKNM) with mitosis apical (1) and S phase basal (2).*

Neurons following radial migration move perpendicular to the ventricular surface and alongside the radial glial fibres, which are used as substrate. On the other hand, tangentially migrating neurons follow trajectories parallel to ventricular surface and orthogonal to radial glia, without needing any support. Correct positioning of newly born neurons in the six layered neocortex is crucial for the appropriate functional connectivity

between right types and number of neurons. The best structure illustrating both types of migration is the cerebral cortex; therefore, cortical neurons are discussed in the following part.

Cortical projection neurons (CPNs) arise from undifferentiated NSCs in the ventricular zone (VZ) and SVZ of the telencephalon and reach their target location by radial migration (Ayala, Shu, & Tsai, 2007; Bystron, Blakemore, & Rakic, 2008). The earliest-arriving neurons form the transient plate (PP) followed by the formation of cortical plate (CP) by neurons. The CP neurons, split the PP into superficial marginal zone (MZ) and the subplate (SP), which is located near the newly formed cortical layer (Ayala, Shu, & Tsai, 2007). Successively migrating neurons add up to more superficial cortical layers. Hence, neurons belonging to the deepest cortical layers are generated first and arrive to their destination first followed by the neurons which will reside in the upper layers. Radial migration occurs by soma translocation and locomotion, with earliest formed neurons employing first mode and most of the cortical neurons using the latter to migrate respectively. Apart from this, several studies have also identified multipolar neurons in intermediate zone (IZ) and SVZ, which possess multiple thin processes that extends and retracts in a random fashion (Tabata, Kanatani, & Nakajima, 2009). These neurons do not require the support of RGCs, and have been suggested critical for the progressive emergence of different neuronal layers identities and proper cortical lamination (Miyoshi, & Fishell, 2012; Ohshima et al., 2007).

Inhibitory interneurons of the cerebral cortex arise from the medial and caudal ganglionic eminences (MGE and CGE) and the preoptic area within ventral telencephalon and migrate tangentially into the developing neocortex (Figure 9) (Batista-Brito, & Fishell, 2009; Kriegstein, & Noctor, 2004). Migration of CINs involves oriented exit from ganglionic eminence (GE) towards the cortex as well as migration within cortex towards specific positions. Once they reach the cortex, different subpopulations opt for different modes of migration. The first mode, helps dispersing interneurons across the neocortex to achieve proper laminar organization (Tanaka et al., 2006), where significant number of interneurons acquire multidirectional tangential migration in multiple zones of cortex (Yokota et al., 2007) so as to migrate long distance and in different directions. The second mode involves a subpopulation of interneurons exhibiting ventricle oriented migration (Nadarajah et al., 2002), where interneurons within the IZ migrate first towards the ventricle before migrating radially to their position within the CP, to obtain layer

information for correct cortical positioning. Finally, there is the third mode, where tangentially migrating streams of interneurons switch to radial migration as they move towards specific locations within the CP (Faux et al., 2012; Nadarjah et al., 2002).

Guidance cues regulating neuronal migration represent a diverse class of secreted or substrate-bound molecules that act as either chemotactic, chemoattractant, or chemorepellent guides, some of which are: Class 3 Semaphorins (Sema3A-3G) that behave both as chemorepellent (Kolodkin, & Tessier-Lavigne, 2011) as well as chemoattractant (Chen et al., 2008); Slit proteins that repel interneurons from ganglionic eminence so as to initiate their migration towards the neocortex (Hu, 1999; Wu et al., 1999); Neuregulin 1 growth factor, which by binding to ErbB receptor tyrosine kinases controls the exit of MGE-derived interneurons towards the dorsal cortex (Flames et al., 2004) and Netrin 1, which serves as both repellent as well as attractant, depending on the receptor binding (Marin, & Rubenstein, 2003). Apart from these, some of the secreted molecules used in this work have also been implicated in neuronal migration including Hepatocyte Growth Factor (HGF) and chemokines, which are described in next sections.

Proper complex control of neuronal migration underlies the correct formation and physiology of the brain. Thus, mutations in α and β tubulin genes and the Microtubule (MT) binding protein doublecortin (DCX) among others, which disrupt neuronal cortical migration, have been implicated in Lissencephaly (lack of brain infoldings), Pachygyria (thick brain convolutions) and Polymicrogyria (excessive number of small brain convolutions) neurological syndromes in humans (Keays et al., 2007; Jaglin et al., 2009). Mutations in X-linked gene DCX that result in a large population of neurons failing to migrate, cause subcortical band heterotropia (Manent et al., 2009). Loss of excitatory/inhibitory neuron balance in specific brain circuitries has also been implicated in psychiatric disorders like schizophrenia, anxiety or depression (Di Cristo, 2007).

3. The Centrosome: Introduction and its role in neural development

The formation of the mammalian brain circuitry requires careful coordination of proliferation, migration and differentiation programs between distinct neuronal populations. The spectacular morphological modification of a neuroblast into a mature neuron and the establishment of well developed axonal and dendritic arbours points at a

strictly regulated cytoskeletal reorganization (Higginbotham, & Gleeson, 2007; Hoogenraad, & Bradke, 2009). Therefore it is not surprising that many neurodevelopmental disorders are the result of mutations in cytoskeletal proteins, including tubulins (Thornton, & Woods, 2009; Tischfield et al., 2010).

Indeed, a central component of the neuronal cytoskeletal structure is the microtubule (MT) array, the centrosome is the MT-organizing center (MTOC) of the cell. The centrosome is a membrane-less organelle typically 1µm that distinguishes prokaryotes from eukaryotes (Marshall, 2009). Eukaryotic cells have one centrosome, consisting of a pair of barrel-shaped centrioles (composed of nine-tripled MTs) surrounded by pericentriolar material (PCM) (Bettencourt-Dias, & Glover, 2007; Bornens, 2012). MTs are the hollow tubes composed of 13 protofilaments of α and β tubulin dimers organized in a head to tail manner. Tubulin in MTs polymerizes in a way, such that the α-subunits of one tubulin dimer contacts the β-subunit of the next. Hence in each protofilament there will be, one end with α-subunits exposed while the other end will have β-subunit exposed, which are designated as the minus (-) and plus (+) ends respectively (Walker et al., 1988). PCM is organized around the centriole and contains MT nucleation factors, such as γ-tubulin, pericentrin, ninein and NEDD1, and MT nucleation complexes called γ-tubulin ring complexes (γ-TuRCs) (Figure 10) (Fu, & Glover, 2012; Lawo et al., 2012).

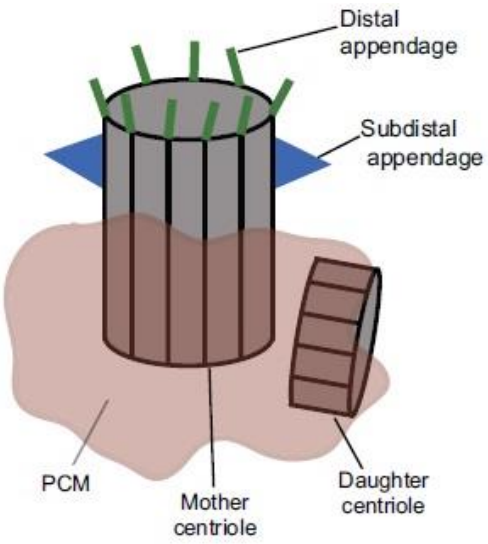


Figure 10 . Anatomy of the vertebrate centrosome (Tang, & Marshall, 2012). *Centrosome is composed of two parallely placed centrioles surrounded by PCM, of which mother centriole can be distinguished by the two sets of appendages: the subdistal and distal (Dawe, Farr, & Gull, 2007). Upon exit from the cell cycle, the mother centriole acts as a nucleation site for the growth of primary cilia.*

The centrosome nucleates a radial array of MTs, whose minus ends (−) are anchored at the centrosome and plus ends (+) extend into the cell periphery (Conde, & Caceres, 2009). In vertebrate neurons, axons have a uniform arrangement of microtubules with + ends distal to the cell body (+ ends out), whereas dendrites have equal numbers of + and − end-out

microtubules. The centrosome not only provides structural foundation for the MT array but also the major MT nucleation site, involved in many different cell processes like cell division, cell migration and differentiation (Azimzadeh, & Bornens, 2007; Bettencourt-Dias, & Glover, 2007; Doxsey, McCollum, & Theurkauf, 2005; Nigg, & Raff, 2009). Recruitment of MT nucleation proteins is regulated in part by the cell cycle–dependent protein Plk1 (Polo-like kinase 1; Casenghi et al., 2003; Haren, Stearns, & Lüders, 2009; Eot-Houllier et al., 2010). Inhibition, depletion or mislocalization of Plk1 during mitosis perturbs bipolar spindle formation and leads to mitotic failure, in part through centrosome-mediated defects.

The centrosome cycle, ensures the equal distribution of centrosomes to daughter cells during cell division. It initiates during the early phase of cell cycle, so that by the time mitosis occurs there are two centrosomes. The centrosome cycle consists of four phases which are synchronized to cell cycle (Figure 11), that includes: centrosome duplication during G1 and S phase, centrosome maturation in G2 phase, centrosome separation in mitotic phase and centrosome disorientation/splitting in the late mitotic phase, that refers to the loss of orthogonality between the mother and daughter centrioles.

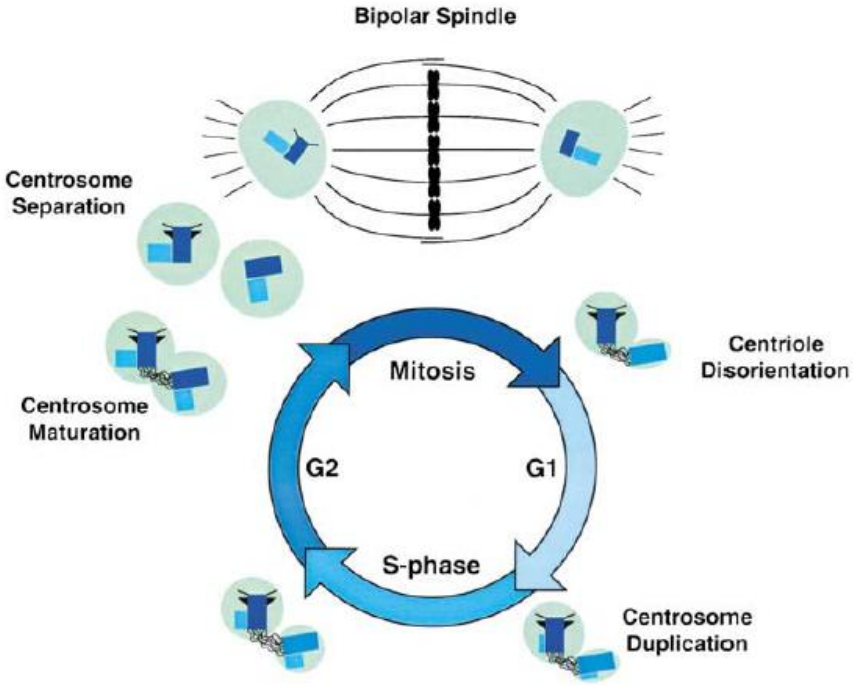


Figure 11. Schematic view of centrosome cycle (Meraldi, & Nigg, 2002). *It initiates upon centriole disengagement in G1 phase which licenses centrioles for duplication in S phase along with the nucleation of daughter centrioles. Later the procentrioles mature in*

G2 phase followed by building up of poles of bipolar mitotic spindles resulting in their separation in M phase (Mitotic phase). Mature centrioles are shown in dark blue, immature centrioles in blue, pro-centrioles in light blue and PCM in green.

The first phase of centrosome cycle is centrosome duplication which is highly regulated by Cyclin-Dependent Kinase 2 (Cdk2) and its binding partners cyclin E (Matsumoto, & Maller, 2004) and cyclin A (Meraldi et al., 1999) in embryonic cells and somatic cells, respectively. Nucleophosmin (NPM/B23) and Monopolar spindle-1 (Mps1) have been demonstrated as the candidate substrates downstream to Cdk2 for centrosome duplication (Loncarek, & Khodjakov, 2009). Apart from Cdk2, two other protein kinases have also been implicated in centrosome duplication, namely ZYG-1 (O'Connell et al., 2001) and Calcium-calmodulin kinase II (CaMKII) (Matsumoto, & Maller, 2002). The next phase is centrosome maturation, which is defined by the increase of γ-TuRCs upto three to five fold (Khodjakov, & Rieder, 1999) that allows the mature centrosome to have a greater ability to nucleate MTs. Polo-like kinase (Sillibourne et al., 2010), Aurora kinases (Hannak et al., 2001) and Nek2 (Prigent, Glover, & Giet, 2005) also plays very important role in the recruitment of γ-tubulin and other proteins to form PCM around the centrioles. Next comes the separation of duplicated centrosomes into distinct MTOCs at G2/M transition in two distinct steps during centrosome separation phase, where demolition of connection between the parental centrioles occurs in the first step, followed by complete centrosome separation via microtubule motor proteins in the second step (Meraldi, & Nigg, 2002). Centrosomal-Nek2-associated protein1 (C-Nap1) and Rootletin has been described to work as the physical linker between the two centrosomes, which upon phosphorylation dissociate from the centrosomes, resulting into centrosomal separation (Yang, Adamian, & Li, 2006). The last phase of the centrosome cycle occurring during the late mitosis/early G1 exhibits the striking loss of orthogonal orientation between the centrioles. Once disorientation occurs, mature centriole begins to move towards the cleaving furrow, marking the termination of cell cycle and re-establishment of linker between the parent centrioles (Mayor et al., 2000) which was disassembled in the previous cycle

Mutations in centrosome-localized proteins are associated with pathologies such as Huntington disease and Lissencephaly (Sathasivam et al., 2001; Badano, Teslovich, & Katsanis, 2005; Kuijpers, & Hoogenraad, 2011). Here, I will discuss briefly the role of centrosome positioning during different stages of neural development.

3.1 Centrosome positioning during neurogenesis

During corticogenesis, apical progenitor cells either undergo proliferative symmetrical divisions or neurogenic asymmetric divisions. The balance between these two types of divisions determines the size of the progenitor cell pool and ultimately the number of neurons generated in the brain. One hypothesis proposes that centrosomes are required for proper neural progenitor cell fate specification through the regulation of mitotic spindle orientation such that vertical cleavage correlates with asymmetrical divisions and horizontal cleavage favors symmetrical divisions. NSCs are attached to the apical and pial surfaces, polarized along the apico-basal axis. During S-phase DNA replication, the nucleus of the NSCs moves basally and returns to the apical surface (Figure 12; i-iv) before entering into mitosis, but centrosomes retain their position at apical surface during G1, S and G2 phases. As neurogenesis initiates, NSCs divide by symmetrical proliferative division (Figure 12; v,vi) where they complete cytokinesis and bisects the apical membrane such that both daughter cells receive a centrosome and apical membrane proteins, but remain attached to each other while adopting neuroepithelial fate. This is followed by asymmetrical division, where only one daughter cell receives apical membrane and adherens junction and remains a NSC, while the other one detaches from apical surface and migrates sub-ventrically, developing into either a basal progenitor or a neuron (Figure 12; viii,ix).

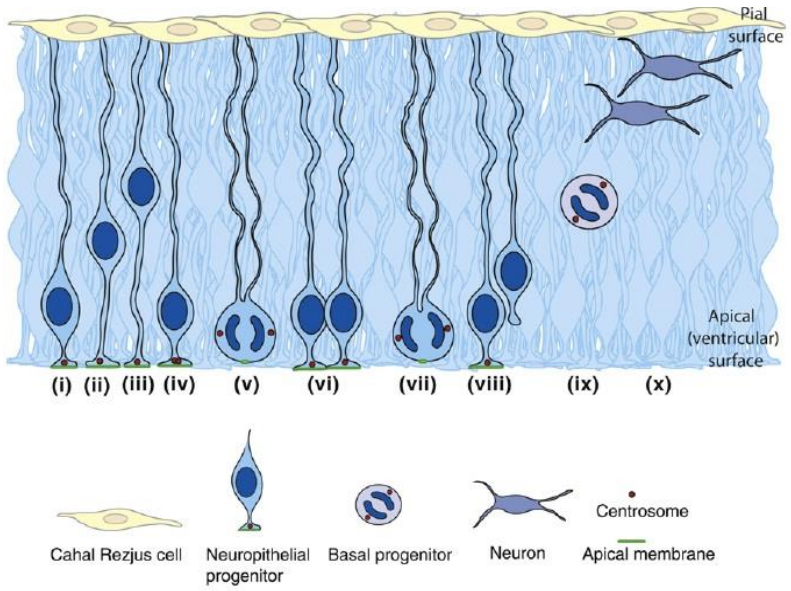


Figure 12. Variation in centrosome positioning during neurogenesis (Thornton, & Woods, 2009). *Processes of neuroepithelial cells (blue) contact the apical (ventricular)*

and pial (basal) surfaces. The nuclei (nuclei) migrate basally during G1 (i,ii) undergoes S phase (iii); and migrate apically during G2 (iv), but centrosomes (red dots) stay at apical membrane (green). When mitosis occurs at apical surface, centrosomes form the spindle poles. Symmetrical division leads to production of two identical neuroepithelial cells (v,vi), whereas asymmetrical division (vii) leads to the production of one neuroepithelial and other one detaches from the membrane (viii) and becomes either a basal progenitor (ix) or neuron. Basal progenitors (ix) lack processes and polarity and predominantly divide, resulting in the production of two neurons (x)

Both apico-basal polarity and regulation of cell cleavage plane depend on the MT network and centrosome function. Several centrosomal proteins regulating mitotic spindle orientation in neuronal progenitors have been identified (Fietz, & Huttner, 2011). A complete control of centrosome over its position during the spindle pole orientation is very important for the proper distribution of fate factors in order to maintain a balance between propagation of progenitor pool and neurogenesis. A slight imbalance can lead to disorders like Microcephaly (Cox et al., 2006; Chenn, & Walsh, 2002). The human gene Centromere-associated protein J (CENPJ) is mutated in some patients with autosomal recessive primary microcephaly, characterized by hypoproliferation of neuronal precursors.

3.2 Centrosome positioning during neuronal migration

In many non polarized cells, such as interphase fibroblasts, the centrosome is located near the cell center and is physically linked to the nucleus, with MTs radiating out to the cell cortex (Figure 13A). The centrosome is often found in close proximity to the neurite that becomes the axon, suggesting a role in determining the site of axon outgrowth. The first observation regarding the centrosomal localization ahead of the nucleus in migrating neurons was made in 1970's (Gregory et al., 1988; Rakic, 1972). Migrating neurons undergo major morphological changes regulated by changes in the cytoskeleton structure and centrosome positioning (Higginbotham, & Gleeson, 2007). In migrating neurons, the centrosome is sometimes positioned ahead of the nucleus, suggesting that it drives the forward movement of the nucleus along microtubules. However, live imaging of radial migration of granule cells in cultured developing mouse cerebellum demonstrated that nucleus migration is not always correlated with the movement of the centrosome (see for details Tang, & Marshall, 2012). The implication of the centrosome in migration has been

reported in tangentially migrating cortical interneurons (Vitalis, & Rissier, 2011; Wonders, & Anderson, 2006), where failure to maintain the proper positioning of centrosome in facial branchiomotor neurons leads to ecotopic migration (Figure 13A) (Grant, & Moens, 2010). Experiments in radially migrating cortical pyramidal neurons (Kriegstein, & Noctor, 2004) and other motile cells like endothelial cells and astrocytes (Yvon et al., 2002) support the same conclusion, but not those performed in leukocytes and keratocytes (Huang, Silverstein, & Malawista, 1991; Verkhovsky, Svitkina, & Borisy, 1999), where centrosome is not required for cell movement or chemotaxis but considered important in specific case of wound healing (Wakida et al., 2010). Wound healing experiments have demonstrated that centrosome becomes oriented between the nucleus and the leading edge (Gotlieb et al., 1981) along with Golgi complex and the endocytic recycling compartment in several cell types (Koonce et al., 1984; Yvon et al., 2002) (Figure 13). However, even within a single cell type, the relative position of the centrosome and the nucleus can vary as a function of myosin II activity (Szabó et al., 2011) or the type of substrate (Schütze, Maniotis, & Schliwa, 1991).

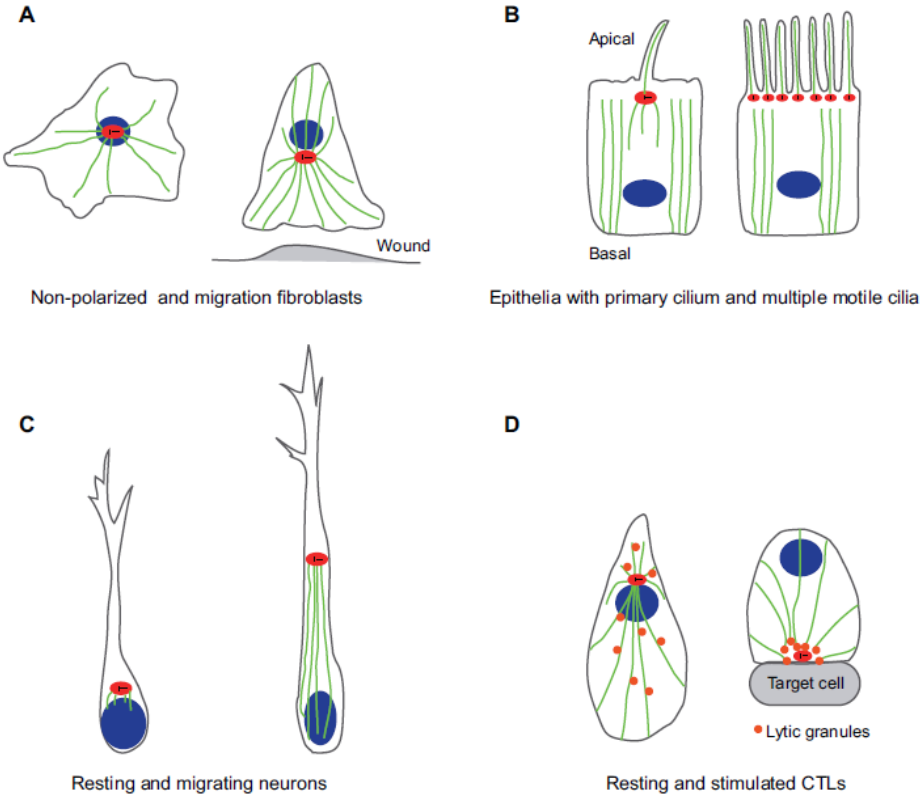


Figure 13. Centrosome positioning dependence upon cell type and cell state. (Tang, & Marshall, 2012). *Figure is demonstrating:* ***(A)*** *Fibroblasts in non-polarized state (left)*

where centrosome is physically linked to the nucleus and located near the centre of the cell, with MTs radiating from the centrosome to cell cortex. Wound healing (right), makes centrosome reorient between the nucleus and the leading edge. ***(B)*** *Epithelial cells in the polarized state (left) locates centrosomes on the apical surface of the cell, accompanied by a loss of radial MT organization and the formation of a predominantly apical-basal array of MTs. The mother centriole of the centrosome becomes a basal body, which gives rise to a primary cilium. In multiciliated epithelial cells (right), hundreds of centrioles are assembled at once in a single cell, leading to the formation of multiple cilia.* ***(C)*** *Resting neurons (left) show centrosome proximity to the neurite destined to become an axón, whereas in migrating neurons (right) centrosome positions ahead of the nucleus with MTs connecting centrosome and the nucleus.* ***(D)*** *In the unstimulated Cytotoxic T lymphocytes (CTLs)(left), centrosome is located near the nucleus and lytic granules are distributed all long the MTs. However upon stimulation (right), their centrosome directs the delivery of lytic granules by moving along the MTs to the plasma membrane followed by the point of secretion for release of lytic granules to the immunological synapse. Red ovals indicates centrosome, centrioles are shown as black lines, Blue ovals are showing nuclei and Green lines are indicating MTs.*

Cell migration mechanism (Neural and non-neural)

Cell polarization during migration involves the asymmetrical distribution of signaling molecules and the cytoskeleton, in addition to directed membrane trafficking. A polarized migrating cell has a single leading edge and filopodia at the front; MTOC and Golgi apparatus orienting towards the direction of migration and; temporal capture and stabilization of MT plus ends near the leading edge (Figure 14).

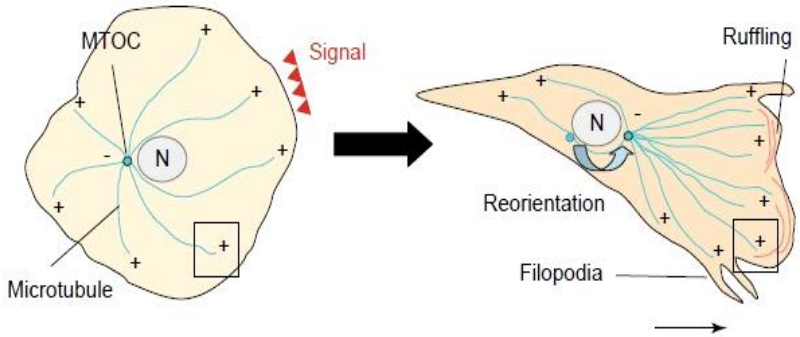


Figure 14. Microtubule polarization in migrating cells (Watanabe, Noritake, & Kaibuchi, 2005). *Figure demonstrates the rearrangement of MTs upon transforming from*

a resting state (left) to a polarized state (right). In resting cells, the minus ends of MTs are anchored at the MTOC, which reorients towards the direction of migration, once encountered with the signal. During cell migration, selective stabilization of Plus ends of MTs occurs, enabling MTOC to reorient towards the leading edge, resulting into polarized MT which facilitates cell migration. During migration, MT plus ends get curved (MT reffuling). N: nucleus

Cell migration requires the coordination of processes, especially the reorganization of cytoskeleton and MTs (Hall, 2012; Goode, Drubin, & Barnes, 2000). MTs minus ends localize predominantly at MTOC (Figure 14), with their plus ends alternating between two phases of growth and shrinkage (a state of dynamic instability) to explore intracellular spaces (Kirschner,& Mitchison, 1986). Via this search process, plus ends are captured and stabilized at target destinations like kinetochores on the mitotic spindle and cell cortex. This enables the MTOC to reorient towards the leading edge, resulting in a polarized MT array which facilitates cell migration.

4. Neuronal differentiation: establishment of the neuronal morphology

After neurons are born and differentiate, they break their previous symmetry, change shape dramatically and two compartments are structurally and functionally established: axon and dendrites, which differ from each other in the composition of proteins and organelles. Microtubule-associated proteins (MAPs) are thought to be involved critically in the development of polarity. Tau is a MAP protein typically confined to the axon, whereas MAP-2 is localized to the soma and dendrites (Binder, Frankfurter, & Rebhun, 1986). Axons are typically long and thin, with a uniform width and branching at right angles from the cell body. On the other hand, dendrites are relatively shorter, appears thicker as they emerge from the cell body, thinner as the distance is increased from the cell body and bifurcates into Y-shaped branching. Axons contain synaptic vesicles from which neurotransmitters are released in response to electric signals, whereas dendrites exhibit spines, which contain receptors for neurotransmitters.

In vitro dissociated neuronal cultures greatly contributed to our understanding of neuronal polarity (Goslin, & Banker, 1989; Craig, & Banker, 1994). Cultured cortical and hippocampal neurons show morphology transition through several stages (Figure15) from

freshly plated stage-1 cells to stage-5 (achieved from about 2 weeks in culture), when they show a well developed axon and dendrites, dendritic spines and functional synapses (Dotti, Sullivan, & Banker, 1988; Craig, & Banker, 1994; Arimura, & Kaibuchi, 2007). This work has focused on stage-3 (DIV2) hippocampal neurons, in which the longest cell process present typically corresponds to the axon.

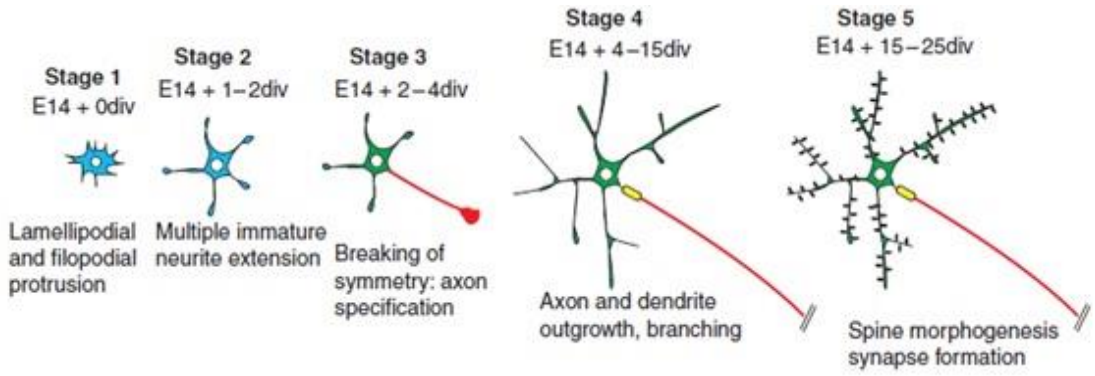


Figure 15. Events leading to the polarization of hippocampal neurons in vitro (Polleux, & Snider, 2010) *. Changes in the morphology of neurons upon plating and during the development (days in vitro, DIV) of the culture. Immature neurons display lamellopodial and filipodial protrusions (stage 1, 0DIV), which lead to immature neurites budding out (stage 2). This is followed by the breaking of the symmetry (stage 3), when one neurite grows faster and becomes the axon, while other neurites will develop later (from 4DIV) and become dendrites. Axon and dendrites can be distinguished by specific immunostainings against tau and MAP-2, respectively. Stage 4 is characterized by rapid axonal and dendritic outgrowth, leading to the complex morphology of terminally differentiated neurons that display dendritic spines and functional synapses (stage-5).*

Axon development includes three important phases: 1) Axon specification during neuronal polarization, 2) Axon outgrowth and guidance, and 3) Axon branching and presynaptic differentiation (Barnes, & Polleux, 2009; Donahoo, & Richards, 2009).

Neuronal polarization is the process of breaking symmetry in the newly born cell so as to form axon and dendrites (Dotti, & Banker, 1987). Among the several important signaling pathways involved in axon specification is the phosphatidylinositol-3-kinase (PI3K) pathway (Arimura, & Kaibuchi, 2007; Barnes, & Polleux, 2009), where PI3K and its lipid product- phosphatidylinositol-3,4,5-triphosphate (PIP3), accumulate at the tip of the prospective axon, as well as the mPar3/mPar6/aPKC polarity complex (Shi, Jan, & Jan, 2003). Roth et al., recently reported tension induced signaling to be significant in axon

specification by the use of micropatterns of curved paths, where it was demonstrated that the neurite which grows on a straight path and subjected to largest tension was most likely to be fated as the axon (Roth et al., 2012). In addition, Cytochalasin D (an actin depolymerising drug) applied to stage 2 neurons causes multiple axon-like neurites, suggesting that instability of actin filaments is necessary for axon formation (Bradke, & Dotti, 1999).

An instructive role for the centrosome in axon specification has been proposed in hippocampal neurons, cerebellar granule cells (Witte, & Bradke, 2008) and cortical neurons (de Anda et al., 2010). The idea behind this was that asymmetrical stabilization of MTs occurs only in one neurite, which is specified as the future axon (Witte, Neukirchen, & Bradke, 2008). Extra numerary centrosomes led to the formation of two axon-like neurites (de Anda et al., 2010). During neuronal development, as centrosome position coincides with the localization of the Golgi complex, it was also hypothesized that centrosome position in front of the future axon directs polarized MT-dependent secretory transport (Sutterlin, & Colanzi, 2010). However, studies in zebrafish and mutant flies disagreed with this hypothesis both *in vitro* and *in vivo*, suggesting a predominant role for extrinsic cues (Distel et al., 2010; Zolessi et al., 2006; Basto et al., 2006). If neurite outgrowth is generated several hours after the last mitosis, the positioning of the centrosome may not be important for initial neuronal polarization (Conde, & Caceres, 2009).

Followed by axon specification, axon growth continues which is the product of two opposite forces: slow axonal transport and polymerization of MTs providing a pushing force from the axonal shaft; and the retrograde flow of actin providing a pulling force at the front of the growth cone (Letourneau, Shattuck, & Ressler, 1987; Suter and Miller, 2011). At the leading edge of the growth cone, MTs tend to persist in growth but do not grow into lamellipodia. However if actin is blocked, neurite extension still continues through MTs extension into lamellipodia that bend against the plasma membrane (Andersen, & Bi, 2000). As per Anderson and Bi, feedback loops (positive and negative) specify axon and its sustained growth. The positive feedback includes: physical components (cytoskeletal assembly and addition of new membrane), accumulation of vesicles for the future axon and preferential exocytosis (Ca^{2+} , receptor tyrosine kinases and phosphatases) at axonal growth cone (Anderson, & Bi, 2000). Negative loop is characterized by the nature of inhibitory signals, which could be high Cyclic Adenosine

Monophosphate (cAMP) concentrations (Zheng, Zheng, & Poo, 1997; Mattson, Taylor-Hunter, & Kater, 1988) with the possible involvement of cAMP/PKA pathway. Propagation of inhibitory signals results into decrease in actin dynamics, MT polymerization and membrane insertion in the processes destined to be dendrites,

Axons reach their targets with the help of a dynamic structure at the tip of the axon called "Growth cone" (Ramon, & Cajal, 1890). A growth cone consists of the leading edge with dynamic finger-like filopodia, considered as the guiding sensors at the front line of the growth cone (Mattila & Lappalainen, 2008), separated by the sheets of membrane in between called lamellipodia (Figure 16).

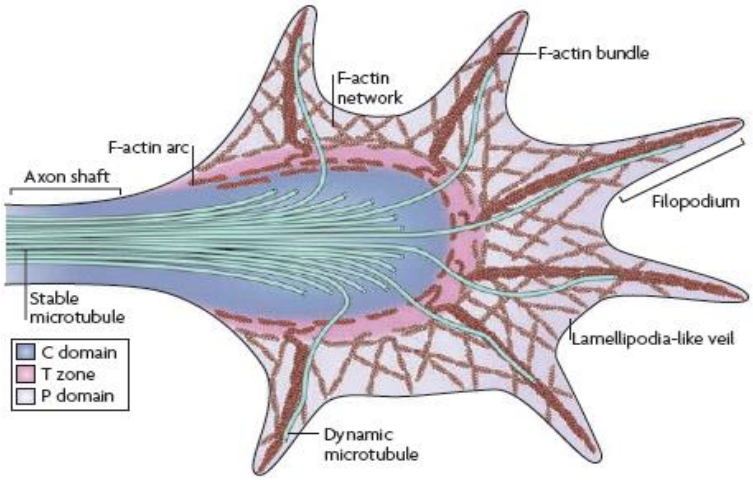


Figure 16. Structure of a Growth cone (Lowery, & Van Vactor, 2009) *consists of filopodia, separated in between by lamellipodia-like veils. The cytoskeleton elements on the "P" domain are F-actin bundles, with "C" domain harboring stable bundled MTs that enter the growth cone and "T" zone, which lies at the interface between P and C domains.*

Based on the cytoskeletal distribution, the growth cone can be divided into three domains (Dent & Gertler, 2003): The peripheral (P) domain containing long, bundled actin filaments {Filamentous-actin (F-actin) bundles} which form filopodia as well as mesh-like branched F-actin network giving rise to lamellipodia-like veils; the central (C) domain, which encloses stable, bundled MTs that enter the growth cone from the axon shaft; and the transition (T) zone, present at the interface between P and C domains, where actin arcs lie perpendicular to F-actin bundles and form hemicircumference rings (Schaefer, Kabir, & Forscher, 2002).

The growth cone, with the help of its cytoskeleton moves into the direction of attractive cues in three stages, protrusion, engorgement and consolidation (Goldberg, & Burmeister, 1986; Dent, & Gertler, 2003) (Figure17), which initiate when the distal end of the growth cone comes in contact with an adhesive substrate (Figure 17a). Adhesive substrate includes adhesive molecules on neighbouring cell's surface (Maness, & Schachner, 2007) or molecules embedded into the extracellular matrix (Evans, 2007), providing a defined surface to which the growth cone can adhere and activate intracellular signaling pathways. This results in a rapid extension of filopodia and lamellar extensions along the leading edge of the growth cone in the "protrusion phase". It anchors the actin with respect to the substrate, such that F-actin polymerization continues in front and the lamellipodia-like veils and filopodia of the P domain move forward to extend the leading edge (Mogilner, 2006) (Figure 17b). After actin clears off from the corridor between the substrate and the C domain, engorgement occurs where F-actin arcs reorientate from the C domain towards the site of new growth (Suter & Forscher, 2000, 2001; Lee & Suter, 2008), followed by migration of C domain MT's into this region (Figure 17c). Finally, consolidation of the newly migrated C domain at the growth cone neck forms a new part of axon shaft (Figure 17d).

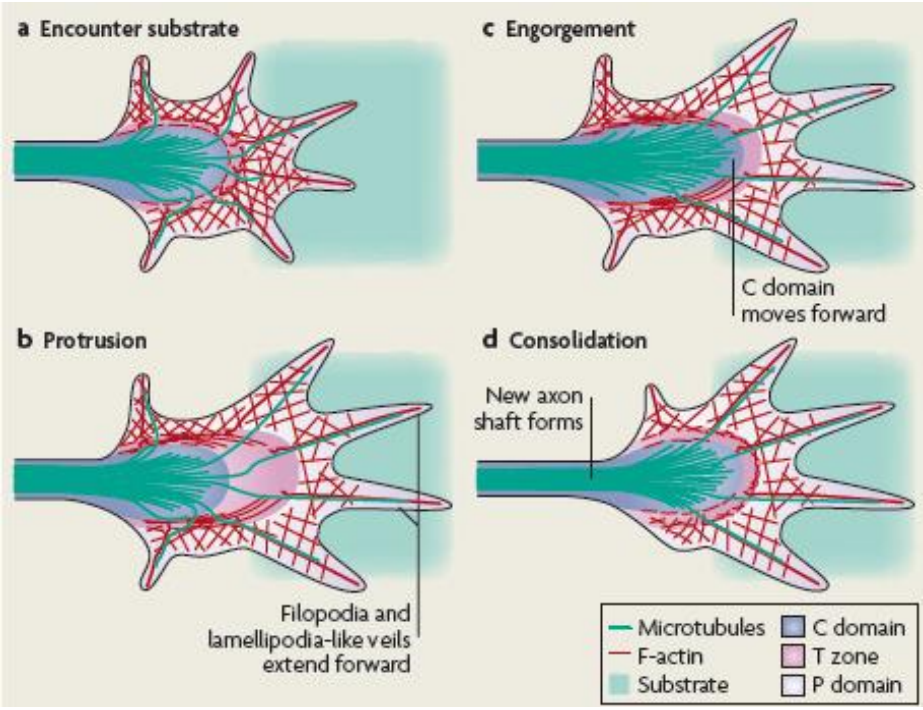


Figure17. Description of axon outgrowth stages (***Lowery & Van Vactor, 2009***). ***(a)*** *Distal end of the growth cone encounters adhesive substrate resulting in the activation of*

intracellular signaling that links actin cytoskeleton to the substrate. ***(b)*** *F-actin polymerization continues in front making P domain move forward to extend the leading edge.* ***(c)*** *Followed by clearing of actin from the space between adhesion and C domain, F actin is removed leading C domain MTs into this region.* ***(d)*** *Finally happens the consolidation of recently advanced C domain as the proximal part of the growth cone to form a new segment of the axon shaft.*

The last step of the axon development is terminal branching, which allows a single axon to connect to a multiple set of targets. Axon branching witnesses similar F-actin and MT reorganization as described in axon elongation except that the growth cone "splits" during the engorgement phase (Dent, & Kalil, 2001) (Figure18). Briefly, F-actin reorganization gives rise to protrusion, followed by MTs invasion of the transient structure for consolidation, before the mature branch starts elongating through MT bundling.

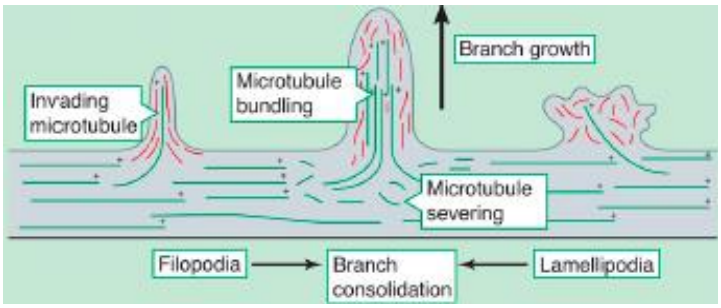


Figure 18. Cytoskeletal changes during branch formation in the axon (Lewis, Courchet, & Polleux, 2013)*. Similarly to axon growth mechanism, filopdia and lamellipodia get invaded by MTs resulting in elongation upon MT bundling.*

Signaling molecules involved in neurite outgrowth

A range of secreted molecules, including growth factors and morphogens promote axon and neurite outgrowth, among which Neurotrophins (NTs), Hepatocyte growth factor (HGF) and Wnts (Maina, & Klein, 1999; Korhonen et al., 2000; Chao, 2003; Yu, & Malenka, 2003; Ciani, & Salinas, 2005; Nakano et al., 2007; Park & Shen, 2012). Wnt family of secreted proteins have been reported to function as axon pathfinding cues and target-derived factors that regulate axon terminal arborization, and act retrogradely for the induction of axon remodelling (characterized by axon spreading and growth cone enlargement) and synapse formation (Hall, Lucas, & Salinas, 2000; Ciani, & Salinas, 2005; Zou, 2006; Purro et al., 2008). In addition, adhesion molecules like β-catenin, a component of the cell–cell adhesion complex and effector of canonical Wnt signaling, also plays key roles in axon outgrowth, dendritogenesis and synapse formation (Murase,

Mosser, & Schuman, 2002; Bamji et al., 2003, 2006; Yu, & Malenka, 2003; Lu et al., 2004; David et al., 2008). These molecules and evidence implicating them in the regulation of neurite outgrowth will be described here in more detail.

5. HGF and Met

Hepatocyte Growth Factor (HGF) also known as Scatter Factor, was originally identified as a molecule that could trigger motility, proliferation and morphogenesis in a variety of epithelial cells (Birchmeier, & Gherardi, 1998). Its well described roles in cell migration and tumor invasion came from its involvement in neural induction during gastrulation (Birchmeier et al., 1997; Jeffers, Rong, & Vande Woude, 1996).

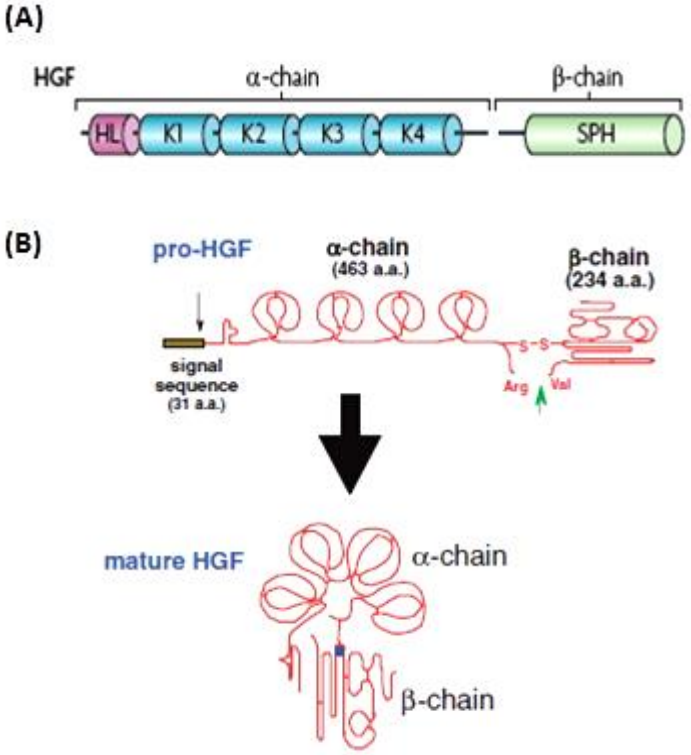


Figure 19.(A) Structural domain of HGF (Trusolino, Bertotti, & Comoglio, 2010) *showing amino-terminal hairpin loop domain (HL), four kringle domains (K1-K4) and a serine protease homology (SPH) domain, and* ***(B) conversion from inactive precursor to active and mature HGF (Nakamura, & Mizuno, 2010).*** *Secreted pro-HGF is cleaved by HGF activators to convert it into the mature and active form, whit the α and β chains.*

It is a multidomain protein similar to plasminogen, a circulating proenzyme that promotes the lysis of fibrin blood clots in its active form as plasmin. HGF is synthesized as a single-chain inactive precursor, converted by serine proteases into an active form with two subunits – α and β with molecular weight of 69 kDa and 34 kDa linked by a disulfide bond

(Naka et al., 1992; Naldini et al., 1992) (Figure 19B). HGF consists of six domains: an amino-terminal hairpin loop domain (HL), four kringle domains (K1-K4) and a serine protease homology (SPH) domain, which lacks enzymatic activity (Figure 19A).

The HGF receptor Met, a tyrosine kinase receptor first identified in 1980's as an oncogene (Cooper, 1984; Park et al., 1986; c-Met), is a disulfide-linked heterodimer, comprised of an extracellular α-subunit and a transmembrane β-subunit that contains the enzymatic activity (Figure 20).

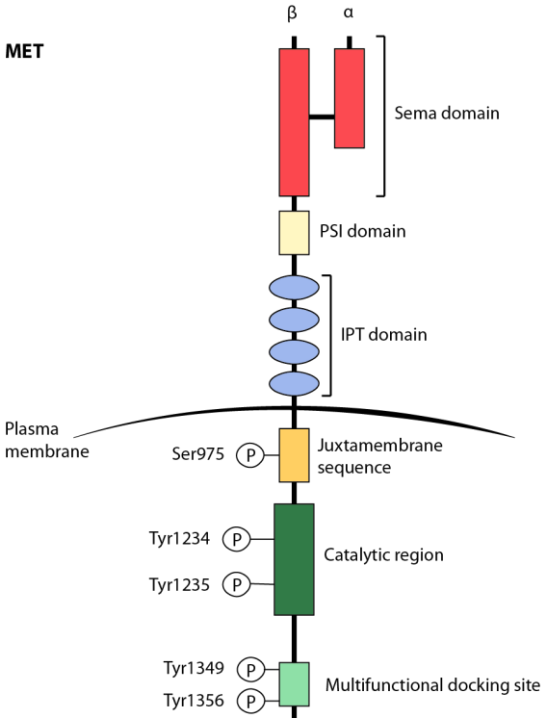


Figure 20. Structure of HGF receptor (Met) (Faria, Smith, & Rutka, 2011)*. It is composed of extracellular α-subunit and transmembrane β subunit. The extracellular unit is formed of 3 domains (sema, PSI and IPT), whereas the intracellular part is composed of juxtamembrane sequence, catalytic region (with docking sites at Tyr 1234 and Tyr 1235), and multifunctional docking site involved in the recruitment of adaptors*.

The extracellular region of Met is composed of three domains: the sema domain which spans the first 500 residues at the N terminus covering the complete α-subunit and a part of β-subunit; the PSI domain (also present in plexins, semaphorins and integrins) covering approximately 50 residues and containing four conserved disulfide bonds; the remaining 400 residues, connecting the PSI domain to the transmembrane helix are organized into four IPT (Immunoglobulin-like fold shared by plexins and transcriptional factors)

domains. The intracellular segment is composed of three portions: a juxtamembrane sequence that downregulates kinase activity followed by phosphorylation at Ser975; a catalytic region positively modulating kinase activity following trans-phosphorylation at Tyr1234 and Tyr1235; and a carboxy terminal multifunctional docking site that contains two docking tyrosines (Tyr1349 and Tyr1356) which are involved in the recruitment of several transducers and adaptors.

Met receptor gets activated upon binding of the active HGF. Precursor HGF is converted into active HGF by a protease called HGF activator (HGFA) (Miyazawa et al., 1993). HGF activation is regulated by serine protease inhibitors called SPINT1 and SPINT2 (Kawaguchi et al., 1997; Shimomura et al., 1997) (Figure 21).

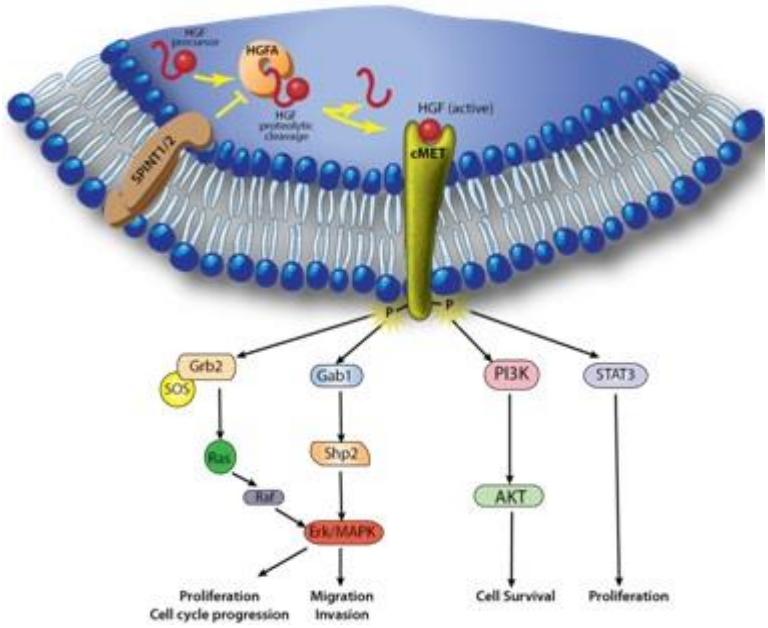


Figure 21. Pathways downstream to the HGF-Met activation (Faria, Smith, & Rutka, 2011) and the promoted cellular effects.

Kinase activity of Met gets activated upon HGF binding, receptor dimerization and trans-phosphorylation of two tyrosine residues in the catalytic region (Tyr1234 and Tyr1235) followed by the phosphorylation of two additional tyrosines in the carboxy-terminal tail (Tyr1349 and Tyr1356). The latter phosphorylated tyrosine residues create docking sites for a number of adaptor proteins including the growth factor receptor-bound protein 2 (Grb2), Grb2-associated adaptor protein (Gab1), son of sevenless (SOS), SRC homology protein tyrosine phosphatase 3 (Shp3), PI3K and signal transducer and activator of transcription 3 (STAT3). which leads to the activation of MAPK, PI3K/AKT and STAT

pathways mediating Met dependent cell proliferation, survival, migration and invasion (Faria, Smith, & Rutka, 2011).

Met mutation is causative for familial carcinomas, such as renal carcinoma or head-and-neck carcinoma in humans (Schmidt et al., 1997). In tumors, HGF is often produced by stromal cells, while Met is overexpressed by cancer cells, which was suggested as a paracrine loop that determines a malignant behavior (Nakamura et al., 1997; Matsumoto et al., 1996; Vermeulen et al., 2010).

5.1 HGF/Met signaling in neurite morphogenesis

A number of studies involving different neuronal cellular types described the implication of HGF signaling in neurite elongation and guidance. HGF was reported to increase neurite outgrowth and enhance dendritic maturation in the CNS: in hippocampal neurons (Lim & Walikonis, 2008; Korhonen et al., 2000), cortical neurons (Gutierrez et al., 2004; Hamanoue et al., 1996) and thalamic neurons (Powell et al., 2003). In addition to its role as a neutrophic factor, HGF serves as a chemoattractant to a subset of motoneuron axons expressing Met and guides them towards their targets in the mesenchyme (expressing HGF) (Ebens et al., 1996). In the PNS, HGF/Met have been described for their role in sensory neuron development by promoting neurite outgrowth induced by NGF in DRG explants (Maina et al., 1997). Thus, mutant mice embryos lacking Met, showed enhanced apoptosis in DRG as well as a reduction in sensory fibres innervating the skin of the limbs and thorax (Maina et al., 1997).

Classical downstream pathways to HGF/Met implicate the activation of Akt in promoting dendritic maturation in hippocampal neurons (Lim, & Walikonis, 2008). HGF signaling activates Akt, which makes GSK-3β inactive by phosphorylation and reduces MAP-2 phosphorylation, resulting in dendritic elongation, which was further confirmed by utilizing Met and Akt inhibitors (Lim, & Walikonis, 2008).

Decreased Met expression has been linked to autism susceptibility, thus highlighting the importance of Met signaling in neuronal development (Campbell et al., 2006).

5.2 HGF and Met signaling in cell migration

HGF and Met are expressed not only in neurons but also non-neuronal cells within nervous system like microglia (Di Renzo et al., 1993), Schwann cell (Krasnoselsky et al., 1994), oligodendrocytes (Yan & Rivkees, 2002; Ohya et al., 2007) and astrocytes (Shimazaki et al., 2003). HGF/Met motogenic activity is a highly conserved mechanism regulating migration of cortical neurons (Powell, Mars, & Levitt, 2001; Powell et al., 2003), Gonadotrophin releasing hormone-1 (GnRH-1) secreting neurons (Giacobini et al., 2007) and Spinal motor neurons (Ebens et al., 1996, Wong et al., 1997). Mice lacking tissue type plasminogen activator or urokinase plasminogen activator receptor (key components of HGF activation) exhibited deficient scattered activity in forebrain due to abnormal interneuron migration from the ganglionic eminescence (Powell, Mars, & Levitt, 2001) and reduced migration of GnRH-1 neurons in the forebrain (Giacobini et al., 2007).

Outside the nervous system, HGF induces migration of retinal endothelial cells (Cai et al., 2000), and mesenchymal stem cells (Forte et al., 2006). HGF binding to Met, activates signaling cascades downstream amongst which ERK1/2 and PI3K/Akt signaling particularly have been reported in HGF-mediated migration, invasion and metastasis (Menakongka, 2010; Ye et al., 2008) , by regulating PI3-kinase (Royal, & Park, 1995).

6. Neurotrophins (NTs) and NT receptors

NTs are secreted proteins with central functions in the CNS ranging from differentiation and neuronal survival to synaptogenesis and activity-dependent forms of synaptic plasticity (Lewin & Barde, 1996; Huang & Reichardt, 2001). In the mammalian brain, the four identified Neurotrophins are: Nerve Growth Factor (NGF), Brain Derived Neurotrophic Factor (BDNF), Neurotrophin-3 (NT-3) and Neurotrophin-4 (NT-4), which act by binding to two distinct classes of receptor: p75 neurotrophin receptor ($p75^{NTR}$) and the Tropomyosin receptor kinase (Trk) family of tyrosine kinase receptors (TrkA, TrkB, TrkC) (Dechant, & Barde, 2002; Huang, & Reichardt, 2003; Kaplan, & Miller, 2000; Chao, 2003).

Similar to other proteins, NTs also arise from precursors termed as pro-neurotrophins (30-35 kDa), which are proteolytically cleaved to produce mature proteins (12-13 kDa) (Seidah et al., 1996). Unusually, the pre-neurotrophins are not inactive as they have been

shown to bind with high affinity to $p75^{NTR}$, which was underestimated as a low affinity NT receptor. Mature NTs bind to specific Trk receptors with high affinity and to p75NTR with low affinity (Lu, Pang, & Woo, 2005).

The Trk receptors are transmembrane glycoproteins of ~140 kDa. They are the tyrosine kinase receptors containing extracellular immunoglobulin G (IgG) domains for ligand binding and a catalytic tyrosine kinase sequence in the intracellular domain. The extracellular portion of $p75^{NTR}$ contains four cysteine-rich repeats and the intracellular part contains death domain (Chao, 2003) (Figure 22). Whereas the tyrosine kinase domains are highly related (80% amino acid identity), the extracellular domains are more divergent (30%). Binding of NTs to Trk receptors occurs mainly through IgG domains, with the domain closer to transmembrane region playing a prominent role (Perez et al., 1995; Urfer et al., 1995; Arevalo et al., 2001). The preferred receptor for NGF is TrkA, TrkB for BDNF and TrkC for NT-3 (Barbacid 1994), but these specificities are not absolute as NT-3 is also a ligand for TrkA and TrkB.

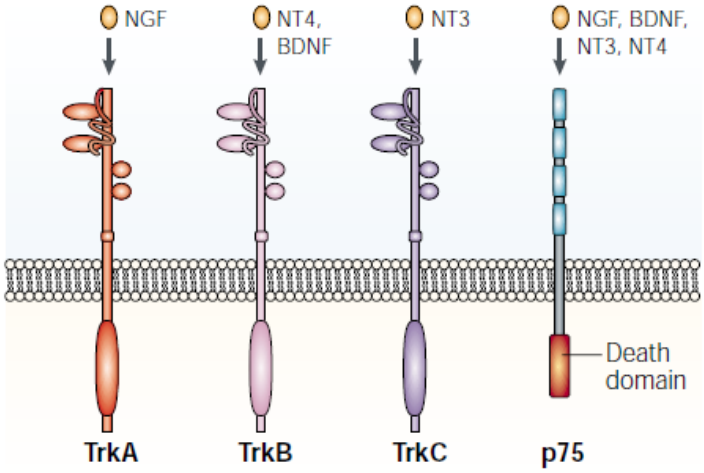


Figure 22. NTs and their receptors: Trk and $p75^{NTR}$ (Chao, 2003) *. Each NT receptor has its specific affinity towards different NTs, NGF binds to TrkA, BDNF and NT-4 to TrkB, and NT-3 to TrkC with high affinity. ProNTs and mature NTs can also bind to p75NTR with low affinity.*

Binding of NTs to the Trk receptors leads to receptor dimerization and phosphorylation in trans of the receptors, which leads to the recruitment of different adaptors and activation of several pathways downstream among which the Shc-Ras-MAPK, Rap-MAPK, PI3K-Akt, and PLCγ- PKC pathways are the best studied (Figure 23) (Chao, 2003).

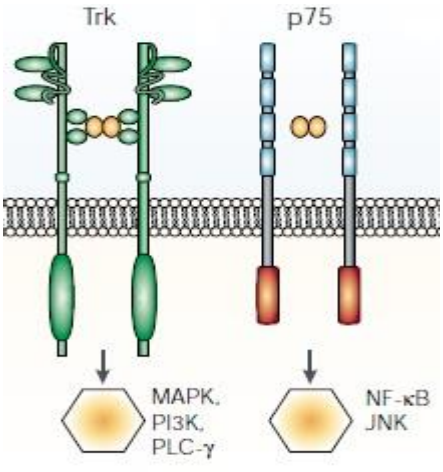


Figure 23. Signaling pathways activated upon NT receptor dimerization (Chao, 2003). *NTs binding to their specific receptors leads to receptor dimerization, activating several transduction pathways downstream like MAPK, PI3K, Phospholipase C* (*PLC-γ) (downstream to Trk activation) and NF-κB and JNK (downstream to p75).*

These signaling pathways are common for other receptor tyrosine kinases also, but the unique combination of Trk receptor docking site, recruitment of different adaptors and enzymes results in specific responses for NTs. Two specific phosphorylated tyrosine residues, located in the juxtamembrane domain and in the C terminus (Tyr490 and Tyr785 in TrkA), serve as docking sites for adapter molecules (Obermeier et al., 1994; Stephens et al., 1994).

On the other hand, $p75^{NTR}$ is the founding member of the TNF (Tumor Necrosis Factor) family of receptors. Although it does not have a catalytic intracellular tyrosine kinase domain, it is capable of mediating the NT signals. This happens both by modulating TrkA signaling and independently of Trks (Kaplan, & Miller, 2000). The ligand binding to p75NTR increases the binding to TrkA, enhances TrkA autophosphorylation and the selectivity for NT ligands. The Trk-independent pathway of $p75^{NTR}$ increases intracellular ceramide levels and further activates NFkB transcription factor (Carter, Lewin, & Delbru, 1997) and c-Jun N terminal kinase (JNK) (Casaccia-Bonnefil et al., 1996). Conversely, TrkA activation can inhibit $p75^{NTR}$-mediated signaling.

NTs are known for their wide range of roles in the nervous system, including survival, cell fate, axon growth and guidance, dendrite structure and pruning and synaptic plasticity (Chao, 2003; Park, & Poo, 2013). Here their role in neurite morphogenesis and neuronal survival is further discussed.

6.1 Role in neurite morphogenesis

NTs have strong neuritogenic properties, both in neuron development and after injury (Boyce, & Mendell, 2014). They have been shown to regulate neuritic outgrowth, dendritic and axonal morphology both *in vitro* (Bosco, & Linden, 1999; Gavazzi et al., 1999) and *in vivo* (Coumans et al., 2001; Yip, & So, 2001; Altschuler et al., 1999). Following deafening and deafferentiation, NT-3 and BDNF promote axon outgrowth within the cochlea (Altschuler et al., 1999). Both NT-3 and BDNF also enhance supraspinal axonal outgrowth following spinal cord transection in the adult rat (Coumans et al., 2001). The role of NT signaling in axon growth appears to be mediated by an increased polymerizationof of F-actin in growth cones and axon shafts, as indicated by experiments using NT-3, NGF and BDNF in growth cones of sensory neurons (Paves, & Saarma, 1997). In addition, axons in contact with NT-coated beads showed localized induction of axonal filopodia rich in F-actin (Gallo, & Letourneau, 1998). Regulation of cytoskeletal-associated proteins like ADF/cofilin (Meberg et al., 1998), Rho family members like Rac1 and Cdc42 (Gallo, & Letourneau, 2000) downstream of NT signaling have also been reported. It has been postulated that NTs regulate axon cytoskeleton by binding with their Trk receptors and activation of downstream signaling cascades. Classical effectors downstream of Trk signaling implicated in neurite outgrowth are PKA, PI3-K and PLC-γ. NGF- mediated neurite outgrowth in PC12 cells have been shown to be promoted by the activation of PLC-γ downstream to TrkA (Inagaki, Thoenen, & Lindholm, 1995). Also, NT-3 influenced the localization of β-actin to growth cones in a manner dependent on the activation of PKA (Zhang, Singer, & Bassell, 1999). Furthermore, the increase in axonal sprouting in DRG cultures by NGF-coated beads required PI3K and upon treatment with Wortmannin (specific blocker of PI3K) the response of NGF was inhibited (Gallo, & Letourneau, 1998). Additional work supports the role of PI3K in NGF mediated signaling in neurite outgrowth in PC12 cells (Jackson et al., 1996) and retinal ganglion cells (Lavie, Dybowski, & Agranoff, 1997). Moreover, NGF has been shown to affect axon outgrowth by the localized inactivation of Glycogen Synthase Kinase 3β (GSK-3β) downstream to PI3K signaling pathway and MT (+) end protein Adenomatous Polyposis Coli (APC) regulation (Zhou et al., 2004). Previous work of our group also demonstrated a crosstalk between NTs/Trk signaling and β-catenin signaling in axon outgrowth and branching in hippocampal neurons, resulting in the

increased phosphorylation of Tyr654 β-catenin upon TrkB and C activation (David et al., 2008).

6.2 Role in neuronal survival

In the PNS

In vivo experiments of loss or gain of function of NGF demonstrated that NGF is a survival factor for sympathetic neurons and a subpopulation of DRG neurons (those sensing thermal stimuli that express TrkA receptor) (Ceni et al., 2014). Single allele loss of NGF gene reduces the survival of TrkA-expressing DRG neurons, whereas mice overexpressing NGF showed increased survival of TrkA expressing neurons, both in wild type (Albers, Wright, & Davis, 1994) and NGF null mice (Harrison et al., 2004)

DRG neurons that sense stimuli from the mechanical displacement of muscle and joints express TrkC upon neurogenesis. These neurons and their end organs are lost in NT-3 and TrkC mutants (Ernfors et al., 1994; Klein et al., 1994). Their dependence on NT-3 occurs before final target innervation, as these neurons die immediately after neurogenesis in NT-3 null mutants (Farinas et al., 1996).

In the CNS

In contrast to PNS, CNS responds to NTs modestly (Huang, & Reichardt, 2001; Rauskolb et al., 2010). Except NGF, all the NTs can promote survival of purified motor neurons *in vivo* (Sendtner, Holtmann, & Hughes, 1996). These neurons could however survive if they were lacking one of the factors BDNF, NT-3, NT-4/5 *in vivo* (Conover et al., 1995; Ernfors, Lee, & Jaenisch, 1994; Ernfors et al., 1994; Farinas et al., 1994; Jones et al., 1994). In fact, only 20% deficit occurred in facial and spinal motor neurons from the triple mutant mice, lacking NT-3, BDNF and NT-4/5 (Agerman et al., 2000). Surprisingly, mice lacking TrkA and C showed only slight reduction in motor neurons, whereas null mice for TrkB exhibited a dramatic decline in motor neurons in facial nucleus and lumbar spinal cord. Other neurons from forebrain and cholinergic neurons showed alterations in neuronal differentiation (Smeyne et al., 1994) in NGF null mice, but perinatal survival was not affected.

7. Wnt signaling

The development and specification of different organs and tissues taking place during embryonic development is coordinated by signaling molecules known as Morphogens which includes Sonic hedgehog (Shh), Transforming Growth Factor β (TGF β), Fibroblast Growth Factor (FGF) and Wnt factors. The Wnt signaling pathway is a conserved pathway in metazoan animals. The name Wnt is a resultant from a fusion of the name of the *Drosophila* segment polarity gene *wingless* and the name of the vertebrate homolog, *integrated or int-1* (Wodarz, & Nusse, 1998). Despite the discovery of Wnt signaling about thirty years ago (Nusse, & Varmus, 1982), the first successful purification of a Wnt (Wnt-3a) happened two centuries later, which revealed that Wnts are lipid modified (Willert et al., 2003) secreted morphogens that regulate cell fate decision, cell polarity and embryonic patterning (Parr, & McMahon, 1994; Wodarz, & Nusse, 1998; Nusse, & Varmus, 2012). A great deal of evidence has demonstrated their requirement in early patterning by acting as posteriorizing signals for neural crest induction (reviewed in Mulligan & Cheyette, 2012). In recent years, the critical involvement of Wnt signaling in neural circuit development, including regulation of neural precursor proliferation, neurogenesis, neuronal migration, axon guidance, synapse formation, dendritic development and synaptic plasticity, has come into light (for reviews see Ciani, & Salinas, 2005; Inestrosa, & Arenas, 2010¸ Toledo, Colombres, & Inestrosa, 2008). The relevance of Wnt signaling in brain development and physiology was highlighted by the discovery that deregulation of Wnt signaling associates to neurodegenerative diseases, such as Alzheimer disease, and to other brain pathologies like schizophrenia and autism (Dickins & Salinas, 2013) (discussed later).

The complexity and specificity in Wnt signaling is in part achieved by the abundance of Wnt ligands (nineteen identified; Logan, & Nusse, 2004), which are cysteine-rich proteins of approximately 350–400 amino acids that contain an N-terminal signal peptide for secretion (He et al., 2004). Interaction with their Frizzled (Fz) receptors and Low density lipoprotein-Related Protein-5/6 (LRP5/6) co-receptors is required to mediate Wnt canonical signaling (see below). In addition to the large number of Wnt factors, there are ten mammalian Fz proteins, which are seven-transmembrane (7TM) receptors and have large extracellular N-terminal cysteine-rich domains (CRD; Bhanot et al., 1996) that provide a primary platform for Wnt binding (Dann et al., 2001; Janda et al., 2012). Fzs cooperate with a single-pass transmembrane molecule of the LRP family, known as Arrow

in *Drosophila* (Wehrli et al., 2000) and LRP5 and 6 in vertebrates (Pinson et al., 2000; Tamai et al., 2000). Regulation of Wnt signaling at the extracellular membrane is also modulated by a number of antagonists (Cadigan, & Liu, 2006). Upon Wnt binding to the receptor complex, the signal is transmitted to the cytoplasmic phosphoprotein Dishevelled (Dvl) and at this level, Wnt signal branches into two main signaling cascades: canonical/β-catenin signaling and non-canonical/β-catenin independent pathway (Planar Cell Polarity and Wnt/Calcium signaling pathways).

7.1 Canonical Wnt/β-catenin pathway

β-catenin is a central effector of the canonical Wnt signaling pathway, where its intracellular levels and phosphorylation status determines the downstream cascade (Angers, & Moon, 2009) . Wnts and the receptors involved in canonical Wnt pathway (Wnt-1, Wnt-3a, Wnt-7a) are different from the ones involved in non-canonical Wnt pathways (Wnt-4, Wnt-5, Wnt-11) (Angers, & Moon, 2009). Similarly not all the Fz proteins are canonical, for example mammalian Fz7 acts both in canonical and non-canonical Wnt pathway (reviewed in Cadigan & Liu, 2006) , and in flies Fz1 and Fz2 acts redundantly in Wnt/β-catenin signaling (Chen, & Struhl, 1999). At present, there are two models for Wnt signaling, the classical one accepted for many years (current Wnt model) (Figure 24A) and a new model (proposed by Li et al., 2012) (Figure 24B), both of which will be explained here.

In the current Wnt canonical model (Figure 24A), when **Wnt is absent**, β-catenin is degraded by the proteasome upon formation of the "destruction complex" (comprising Dvl, Axin, APC), β-catenin, casein kinase-1 (CK-1 and Glycogen-Synthase-Kinase-3β (GSK-3β). Within this complex, β-catenin is phosphorylated by the Ser/Thr kinases CK-1 and GSK-3β, which is then recognized by E3 ubiquitin ligase β-transducin repeat-containing protein (β-TrCP). β-catenin phosphorylation targets it for proteasomal degradation, thus helping to maintain the cytosolic levels of β-catenin low (Salic et al., 2000; Price, 2006; Wu, & Pan, 2010). **When Wnt is present**, It binds to Fz and its coreceptor LRP5/6. Subsequently, CK-1 binds to the receptor complex, leading to the activation of scaffold protein Dvl that recruits APC and Axin to the receptor complex (MacDonald, Tamai, & He, 2009; Rosso, & Inestrosa, 2013). Thus, the destruction complex is disassembled and GSK-3β is inhibited. Therefore, β-catenin is stabilized,

accumulates in the cytosol and translocates to the nucleus (Gordon, & Nusse, 2006). In the nucleus, β-catenin associates with Transcription factor T Cell Factor/Lymphoid Enhancing Factor (TCF/LEF) and regulates the expression of Wnt targets, including Axin, c-Myc and cyclinD1 (Maretzky et al., 2005; Gavert et al., 2007; Clevers, & Nusse, 2012; Gordon, & Nusse, 2006) among many other genes (see the "Wnt web page").

A new modified model for canonical Wnt signaling was put forward in 2012 (Figure 24B) (Li et al., 2012). Here, in the **absence of Wnt**, the destruction complex residing in the cytoplasm binds and phosphorylates β-catenin resulting in the ubiquitination of β-catenin by β-TrCP. The proteasome then recycles the complex by degrading β-catenin. When **Wnt is present**, it induces the association of intact complex with phosphorylated LRP. After binding to LRP, the destruction complex (including GSK-3β) still captures and phosphorylates β-catenin, but β-catenin ubiquination is inhibited. Accumulation of newly synthesized β-catenin in the cytosol, results in its nuclear translocation resulting in the regulation of Wnt target genes.

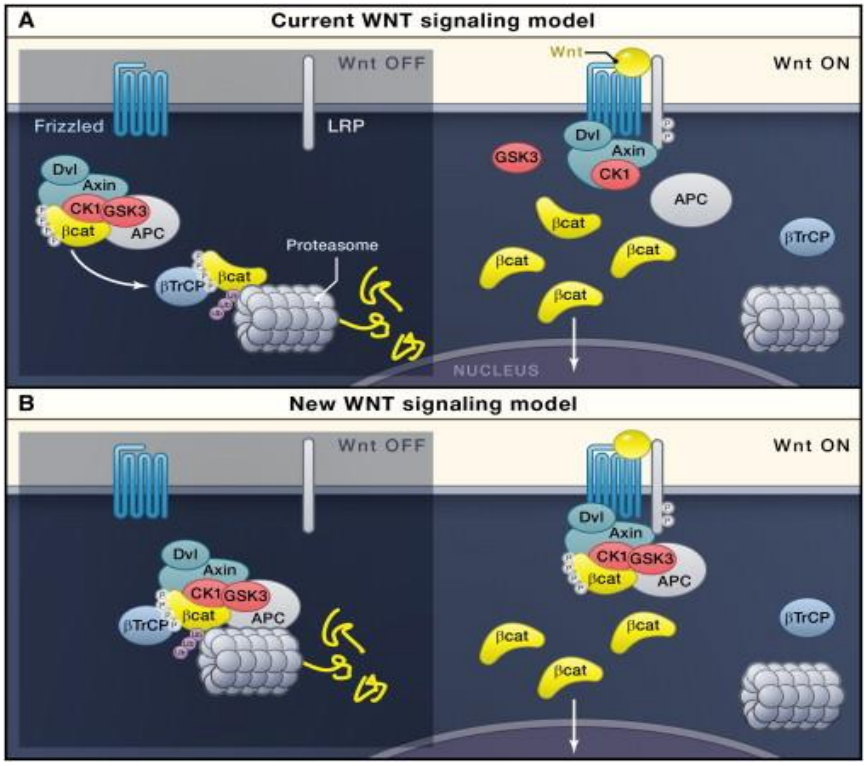


Figure 24. Canonical Wnt pathway (current and new). ***(A)*** *Current model: when the Wnt is absent, destruction complex stays inact in cytoplasm and results in the Ser/Thr phosphorylation of β-catenin, which gets ubiquitinated by β-TrCP and degraded by the proteasome system. The presence of Wnt induces the association of axin with*

phosphorylated LRP-1, leading to the disassembly of the destruction complex and β-catenin stabilization. ***(B)*** *In the new model (Li et al., 2012) absence of Wnt lets destruction complex free to phosphorylate β-catenin, which is degraded through the proteasome, followed by recycling of destruction complex. In the presence of Wnt, it induces association of the destruction complex to LRP and the receptor complex, where β-catenin is still phosphorylated. A pool of newly synthesized β-catenin accumulates in the cytoplasm followed by its translocation to nucleus and transcriptional regulation of target genes.*

Finally, another model proposes that upon Wnt signaling activation, GSK-3β and the "Wnt signalosome" (comprising LRP5/6, Axin and Dvl) are internalized into acidic vesicles/ multivesicular bodies, in which the enzymatic activity of GSK-3β is inhibited (Taelman et al., 2010; Vinyoles et al., 2014), therefore allowing β-catenin accumulation.

7.2 Non- Canonical/β-catenin independent Wnt pathways

There are two β-catenin independent pathways: the Planar Cell Polarity pathway (Wnt/PCP or Wnt/JNK pathway) and the Calcium pathway (Wnt/Ca^{2+} pathway) (Angers, & Moon, 2009), both initiating upon Wnt binding to its receptor Fz and alternate single transmembrane receptor tyrosine kinases (RTKs) Ror1/2 and Ryk (Van Amerongen, Mikels, & Nusse, 2008)

a. Planar Cell Polarity pathway

In this pathway (Figure 25, left branch), a Wnt ligand binds to its receptors and activates the scaffolding protein Dv1, followed by activation of Rho/Rac small GTPase and c-Jun-N-terminal kinase (JNK), leading to changes in both actin and MT reorganization (Gordon, & Nusse, 2006; Rosso et al., 2005; Yamanaka et al., 2002). This pathway is responsible for asymmetric distribution of cytoskeleton and cell polarization (Huelsken, & Held, 2009).

b. Wnt/Ca^{2+} pathway

In this signaling cascade (Figure 25, right), Wnt binding to its receptors Fz and RTK leads to the production of IP3 and DAG from membrane-bound phospholipid phosphatidyl inositol 4,5-bisphosphate via the action of membrane-bound enzyme phospholipase-C (PLC). Intracellular levels of Ca^{2+} increased, resulting in the activation of Ca^{2+} sensitive kinases like Ca^{2+} /Calmodulin-dependent protein kinase II (Camk II) and Protein Kinase C

(PKC), which further activates the nuclear translocation of transcription factors: Nuclear Factor of Activated T cells (NFAT) and cAMP Response Element-Binding protein (CREB) (Oliva,Vargas, & Inestrosa, 2013; Kohn, & Moon, 2005; Montcouquiol, Crenshaw, & Kelley, 2006). In CNS, this pathway has so far been associated with axon outgrowth and guidance, dendrite development, and synapse function (Ciani et al, 2011; Hutchins, Li, & Kalil, 2011, 2012; Li, Hutchins, & Kalil, 2009; Varela-Nallar et al, 2010).

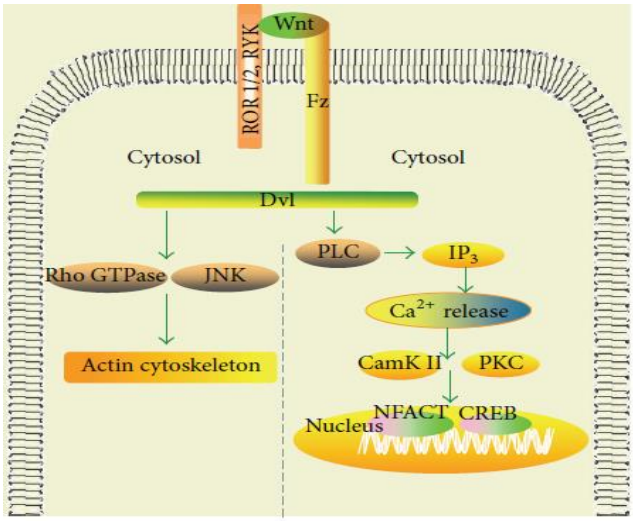


Figure 25. Planar cell polarity (PCP) and Wnt/Ca^{2+} Pathway (Wan et al., 2014). *In the PCP pathway, Wnt binding to Fz activates the scaffolding by activation of Rho GTPases (Rho and Rac) and JNK, which leads to changes in actin and MT reorganization. In the Wnt/Ca^{2+} Pathway, Wnt binding to DVL, releases intracellular Ca^{2+} and activates Ca^{2+} /Calmodulin-dependent protein kinase II (Camk II) and protein kinase C (PKC).*

7.3 Wnt signaling in neurodegenerative diseases.

Dysfunctional Wnt signaling has been related to a number of neurological disorders including autism, schizophrenia, bipolar disorders, Alzheimer's disease (AD) and Parkinson's disease (Inestrosa, & Arenas, 2010; Okerlund, & Cheyette, 2011). The common feature shared by these neurological disorders is aberrant synapse function, disassembly of which often precedes or even triggers neuronal death (a hallmark of many neurodegenerative disorders) (Saxena, & Caroni, 2007; Rosen, & Stevens, 2010; Kessels, Nabavi, & Malinow, 2013). AD is a degenerative disorder characterized by progressive deterioration of cognitive functions caused by synaptic dysfunction and damage of specific brain regions (Mattson, 2004; Toledo, Colombres, & Inestrosa, 2008). More than a decade

ago, a voice in favour of a strong relationship between an impaired Wnt signaling and neuronal damage in AD was raised (De Ferrari and Inestrosa, 2000; Inestrosa et al., 2000; Garrido et al., 2002; De Ferrari et al., 2003; Inestrosa, & Arenas, 2010). A number of studies have now demonstrated alterations in Wnt signaling components in AD (Zhang et al., 1998; Inestrosa et al., 2002; Caricasole et al., 2004; Ghanevati, & Miller, 2005; De Ferrari et al., 2007; Magdesian et al., 2008) including β-catenin (Zhang et al., 1998), overexpression of the Wnt antagonist Dkk-1 (Caricasole et al., 2004; Rosi et al., 2010; Purro, Dickins, & Salinas, 2012), LRP5/6 (Caricasole et al., 2004; Killick et al., 2012) in addition to the pivotal role of GSK3β that is known to phosphorylate Tau (Alvarez et al., 2004). Also Apo-lipoprotein E (apoE) allele 4, which has been linked to the increased risk for AD, has been shown to inhibit canonical Wnt signaling upon stimulation with Wnt-7a (Caruso et al., 2006).

7.4 Wnt signaling in neurite morphogenesis

Canonical Wnt-3a and Wnt-7a affect axon morphogenesis by targeting MTs. Loss of Wnt-7a or its effector Dvl causes defects in terminal remodeling of axons *in vivo*, leading to defective recruitment of proteins to the synapse and alterations in synaptic function (Ahmad-Annuar et al., 2006; Hall et al., 2000) (Figure 26A). Wnt-3 increases axon branching and growth cone size in NT-3 responsive sensory neurons, but not in NGF responsive sensory neurones (Krylova et al., 2002) (Figure 26B).

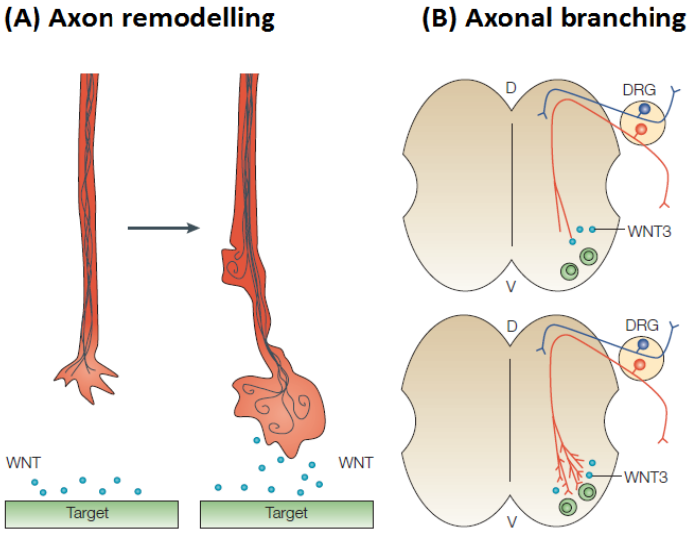


Figure 26. Wnts regulate the behavior of axons (Ciani, & Salinas, 2005). *(a) Wnts function as the retrograde signals, which are released by the target for the modulation of*

axon behavior. Here Wnts inhibit axon extension but enhanced growth cone size and branching, with regulation of MTs dynamics and reorganization. (b)Wnt-3, released by spinal lateral motor neurons (green) located at the limb levels, regulates the arborization and presynaptic differentiation of NT-3 sensory neurons (red) but not NGF-responsive ones (blue).

Wnt-3a increases neurite outgrowth in DRG explants (Lu et al., 2004) and spinal neurons (David, Cantí, & Herreros, 2010). Wnt-3a decreases the speed of growth cone while increasing the growth cone size, a behaviour of growth cones typically observed in the proximity of target cells during synapse formation (Purro et al., 2008). In this process, Dvl increases MT stability by locally inhibiting GSK-3β and MAP1B (Ciani et al., 2004). Indeed, many protein regulators of cytoskeleton dynamics like MAP1B, Tau, MAP2 (Berling et al., 1994; Lucas et al., 1998) and APC (Zumbrunn et al., 2001; Zhou et al., 2004) are targets of GSK3β. Phosphorylation of these proteins by GSK3β affects MT stability (Gonazalez-Billault et al., 2004; Zhou et al., 2004; Baas, & Qiang, 2005), which might contribute to axon specification and growth. In addition, Wnt signaling regulates MTs by removing APC from the MT + plus at growth cones, leading to MT looping and increased growth cone size (Purro et al., 2008). On the other hand, Wnt-5a (non canonical) promotes axon outgrowth and branching in developing sympathetic neurons by regulating the expression of NGF (Bodmer et al., 2009). Wnt-5a promotes axon morphogenesis in hippocampal neurons also, but with Dvl and atypical protein kinase C (aPKC) (in complex with PAR3 and PAR6) as the downstream effectors (Zhang et al., 2007), where Wnt-5a effect on axonal differentiation was attenuated upon the inhibition of Dvl or aPKC. Wnt-5a has been shown to promote axon outgrowth as well as repulsion axon guidance cue in cortical neurons by acting through distinct Wnt receptors (Ryk and Fz) and different calcium signaling pathways (IP3 and Transient receptor potential channels (TRP)) (Li, Hutchins, & Kalil, 2009). Similar to this, a novel role of Wnt/Ca^{2+} implicates Wnt-5a through regulation of calpain and calcium activity in the forward movement of growth cones (Yang et al., 2011).

Since evidences accumulated in support of the role of Wnt signaling in axon morphogenesis, several studies also started coming up pointing to the role of Wnts in the regulation of the dendritic tree morphology (reviewed in Rosso, & Inestrosa, 2013). The first study in this field, postulated that β-catenin was critical for the dendritic morphology (Yu, & Malenka, 2003). Neuronal activity in cultured hippocampal neurons enhances

dendritic complexity by increasing Wnt-2 expression, mediated by Ca^{2+}signaling (Wayman et al., 2006). Wnt-7a has been demonstrated to promote excitatory synapse formation by inducing the formation and growth of dendritic spines, which was deficient in the CA1 and CA3 regions of the hippocampus of Wnt-7a or Dvl mutant mice (Ciani et al., 2011). Although the mechanism by which Wnt-7a acts remains to be elucidated, it was proven that spine growth is induced through Dvl1 and local activation of CaMKII at dendritic spines (Ciani et al., 2011). Also, in hippocampal neurons Wnt-7b/Dvl signaling has been shown to be involved in modulation of dendritic outgrowth and branching through changes in the activity of Rho GTPases and JNK (Rosso et al., 2005) (Figure 27).

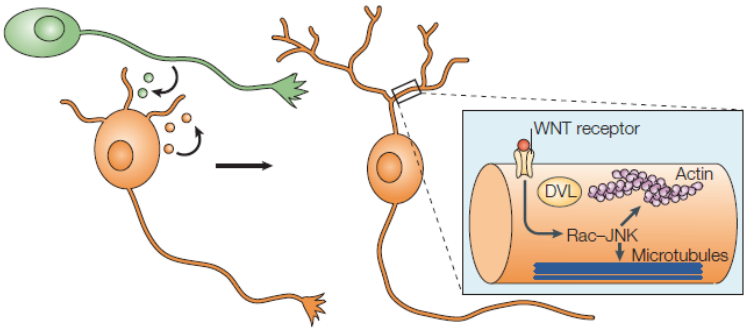


Figure 27. Wnt signaling regulates dendritic morphogenesis (Ciani, & Salinas, 2005). *On the left, figure is showing the regulation of dendritic outgrowth and branching through Wnts, either in an paracrine manner (released from an incoming axons (green)) or autocrine manner (released by the responding neuron (orange)) the involvement of PCP pathway through Dvl, Rac and JNK in stimulation of growth and branching of dendrites. Upon Wnt binding to its receptor, PCP pathway involving Dvl, Rac and JNK, regulates dendritic morphogenesis by cytoskeleton regulation (right).*

Therefore, this Wnt/Dvl regulation of dendritic morphogenesis was "non-canonical" as the activation of GSK3β or inhibition of β-catenin was not involved (Rosso et al., 2005).

8. β-catenin signaling

β-catenin is a multifunctional protein that plays an essential role in cell-cell adhesion, Wnt signaling and the centrosome cycle (Figure 28). Although its role in cell-cell adhesion and Wnt signaling (Nelson, & Nusse, 2004) has been extensively demonstrated, its function at the centrosome is poorly understood.

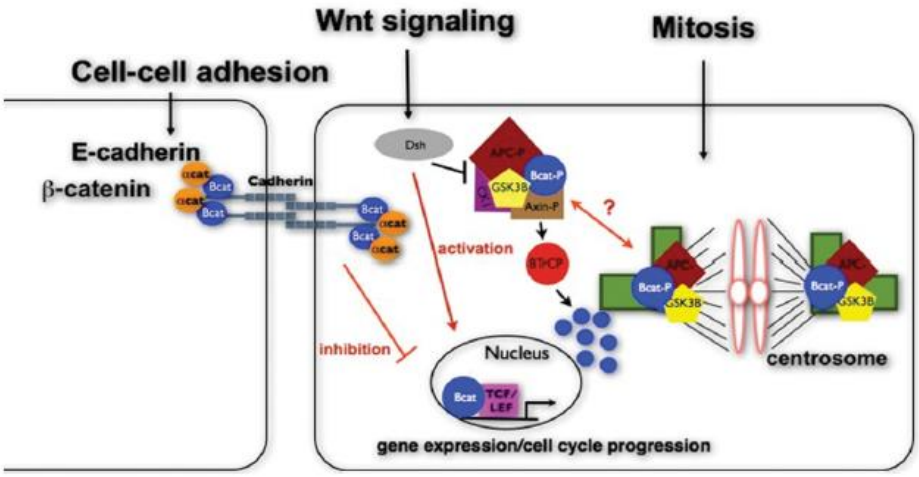


Figure 28. β-catenin involvement in cell adhesion, Wnt signaling and centrosome (Mbom, Nelson, & Barth, 2013)*. The figure is illustrating the presence of β-catenin at cell-cell contacts (associated to Cadherins and α-catenin), cytoplasm (involved in the destruction complex that controls its degradation), nucleus (acting as a cotranscritional activator of TCF/LEF) and centrosome (associated to other Wnt pathway components), suggesting a crosstalk between these distinct pools of β-catenin.*

In the late 1980's, β-catenin was independently discovered based on its different functions structural and signaling by the group of Rolf Kemler. It was isolated together with two other molecules: α-catenin and γ-catenin, as proteins associated with E-cadherin (Valenta, Hausmann, & Basler, 2012). The signaling ability of β-catenin was revealed through its *Drosophila* orthologue *Armadillo,* discovered in the seminal screens for mutations affecting segmentation of the *Drosophila* embryo (Wieschaus, Nüsslein-Volhard, & Jürgens, 1984). Further analysis of Armadillo revealed the conservation of its structural function in adherens junction (McCrea, Turck, & Gumbiner, 1990; Peifer, & Wieschaus, 1990; Orsulic, & Peifer, 1996). Epistatic analysis later divulged Armadillo segmentation function regulation by Wingless (Riggleman, Schedl, & Wieschaus, 1990), which was a major breakthrough for describing the Wnt/β-catenin signaling pathway. In 1990's the signaling function of β-catenin in the nucleus mediated by TCF/LEF transcription factors was demonstrated (Behrens et al., 1996; Huber et al., 1996).

How can a single molecule mediate so many different pathways? The answer lies behind its structural conformation (Figure 29). β-catenin has a central armadillo (arm) repeat domain spanning 141 to 664 residues composed of 12 imperfect armadillo repeats. Each arm repeat of the central region comprises ~42 residues, which form three helices arranged

in triangular shape. The 12 contiguous repeats form a superhelix that features a positive charged groove, serving binding sites for many of the β-catenin binding partners. The N-terminal region refuges the binding sites for α-catenin as well as GSK3β (at Ser33 and Ser37) (Meggy et al., 2005a,b; Wu et al., 2003) and CK-1, which are recognized by β-TrCP ubiquitin ligase (Hart et al., 1999; Kitagawa et al., 1999). The N and C terminal domains are trypsin sensitive, hence are structurally flexible leaving the rigid scaffold for central domain (Huber, Nelson, & Weis, 1997), which serve as an interaction platform for many binding partners of β-catenin, in the cytosol and nucleus (Huber, Nelson, & Weis, 1997).

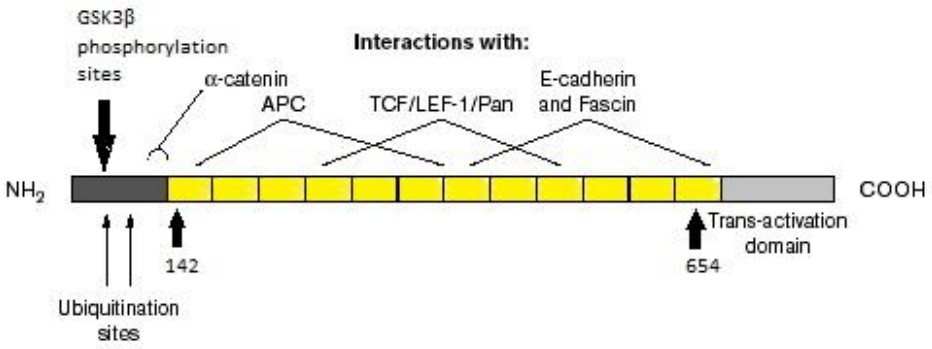


Figure 29. The interaction domains of β-catenin*. GSK3β phosphorylates β-catenin at Ser33/37, recognized by β-TrCP as ubiquitination sites; α-catenin binds at the N terminal and the first two armadillo repeats; central domain (in yellow colour) harbors the binding sites for APC, TCF/LEF-1 and E-cadherin. The position of two main tyrosine phosphorylation sites (Tyr142 and Tyr654) on β-catenin are indicated by the arrows.*

8.1 β-catenin regulation during cell-cell adhesion

The majority of β-catenin is located in the cytoplasm as a component of cadherin based cell-cell junction in the absence of Wnt. These cadherin based cell junctions are important to form polarized epithelial tissues, which is necessary to maintain the organism integrity (Meng, & Takeichi, 2009). Cadherins are single pass transmembrane glycoproteins engaged in Ca^{2+} dependent homotypic interactions through their extracellular regions. They are named based upon their tissue location like E-Cadherin (Epithelial Cadherin). The newly synthesized E-cadherin associates with β-catenin (in a region comprising Tyr 654 on the C-terminal domain of β-catenin), thus protecting β-catenin from the destruction complex (involved in β-catenin dependent Wnt signaling) (Figure 30) (Huber, & Weis, 2001).

In the cell-cell adhesion complex, β-catenin also binds to α-catenin, in a region at the N-terminus of β-catenin including Tyr142 (Pokutta, & Weis, 2000; Xing et al., 2008). Binding of α-catenin stabilizes β-catenin in the hinged form such that E-cadherin can bind simultaneously (Pokutta, & Weis, 2000; Huber, & Weis, 2001). In fact the β-catenin binding site on α-catenin and α-catenin's homodimerization interface overlaps with each other, hence α-catenin can bind to β-catenin only as a monomer (Drees et al., 2005; Yamada et al., 2005). As a homodimer (when α-catenin cannot bind to β-catenin), α-catenin interacts with actin and promotes bundling of actin filaments (Benjamin et al., 2010). Therefore, binding of α-catenin to the cadherin complex negatively regulates actin polymerization mediated by α-catenin (Yamada et al., 2005).

β-catenin function can be regulated through tyrosine phosphorylation. Of particular importance in this work are Tyr142 in the first armadillo repeat and Tyr654 in the last armadillo repeat. The region of β-catenin including Tyr142 is crucial for α-catenin binding (Piedra et al., 2003; Pokutta, & Weis, 2000; Brembeck et al., 2004). β-catenin phosphorylated at Tyr142 (PTyr142 β-cat) by Fyn, Fer or the RTK Met significantly reduces α-catenin binding and detaches PTyr142 β-cat from the adhesion complex, resulting in reduced cell adhesion and increased cell migration (Piedra et al., 2003; Brembeck et al., 2004; Bustos et al., 2006). PTyr142 β-cat translocates to nucleus, where it binds to cofactors like BCL9-2/Legless and TCF4 resulting in increased transcription in epithelial cells and in neurons (Brembeck et al., 2004; David et al., 2008) (Figure 30).

On the other hand, Tyr654 phosphorylation also reduces adhesion by interfering with the binding to cadherin and has been reported to enhance β-catenin mediated transcription (Piedra et al., 2001). In neurons, β-catenin phosphorylation at Tyr654, induces the detachment of β-catenin from N-cadherin. PTyr654 β-cat was found associated to the actin and MT cytoskeleton at growth cones, leading to the promotion of axon growth and branching (David et al., 2008; Figure 30).

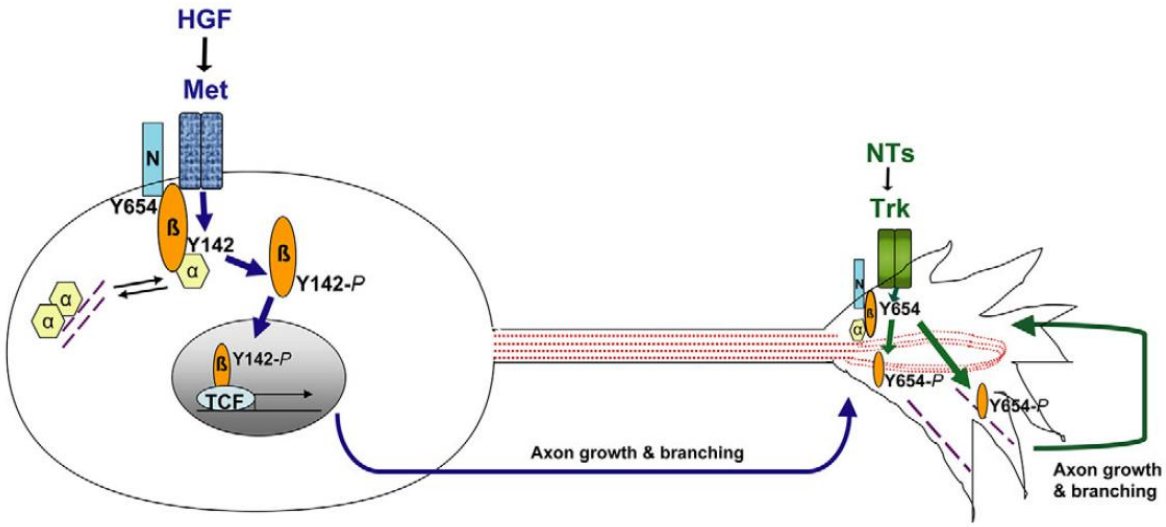


Figure 30. Model for β-catenin tyrosine phosphorylation downstream of growth factor signaling in axon outgrowth and branching (David et al., 2008). *β-catenin (β) phosphorylation at Tyr142 upon HGF-Met signaling induces the detachment of β-catenin from the adhesion complex and targets it to nucleus, where it regulates TCF4-mediated transcription to promote axon morphogenesis. On the contrary, activatation of NT-Trk signaling results in the phosphorylation of β-catenin at Tyr654, detaching it from N-cadherin (N) that then associates to the cytoskeleton to promote axon outgrowth and branching. Tyr indicates Tyrosine; P is Phospho*

Regarding the involvement of β-catenin in neural progenitor migration, coupling of migration and neurogenesis is severely disrupted by the absence of β-catenin (Fancy et al., 2009; Feigenson et al., 2009; Ye et al., 2009; Yang et al., 2012; Dai et al., 2014). β-catenin conditional inactivation in the mouse cortex and hippocampus leads to abnormalities in the organization of the neuroepithelium, which include disrupted interkinetic nuclear migration, loss of adherens junctions, impaired radial migration of neurons toward superficial layers and decreased cell proliferation (Machon et al., 2003). β-catenin plays a critical role deciding whether cortical progenitors should continue proliferating or differentiate (Zechner et al., 2003; Woodhead et al., 2006). In β-catenin overexpression, progenitor cells exit the cell cycle less frequently and continue to proliferate (Zechner et al., 2003). On the contrary, β-catenin depletion causes cortical progenitors to prematurely exit cell cycle and to differentiate into neurons that migrate to the cortical plate (Woodhead et al., 2006).

In glial cells, a role of β-catenin in wound-healing in astrocytes was related to the acquisition of an astrocytoma phenotype (Yang et al., 2012). In was shown that upon creating a wound in an astrocyte monolayer, destabilization of the cadherin-catenin complexes occurs, involving phosphorylation of Ser675 β-catenin that would to translocate to the nucleus and induce a malignant phenotype (Yang et al., 2012).

8.2 β-catenin signaling in neurite morphogenesis

Previous work from our group linked β-catenin dissociated from the adhesion complex to the promotion of axon outgrowth ("axon migration") (David et al., 2008), where it was demonstrated that stimulation of Trks and Met by NTs and HGF induces the phosphorylatation of β-catenin at Tyr654 and Tyr142, respectively, in hippocampal neurons leading to promotion of axon outgrowth. Axon morphogenesis promoted by PTyr654 β-cat was TCF4-independent and likely involves cytoskeletal regulation (David et al., 2008). In contrast, HGF/Met signaling induced β-catenin phosphorylation at Tyr142, leading to the nuclear translocation of β-catenin and promoting axon outgrowth and branching by a TCF4-dependent transcriptional mechanism (David et al., 2008).

In hippocampal neurons, β-catenin (as a component of the adhesion complex) has been acclaimed as a critical mediator of dendritic morphology, where its overexpression was shown to increase dendritic arborization through the interaction with N-cadherin and α-Ncatenin (neural α-catenin) (Yu, & Malenka, 2003). Sequestering of endogenous β-catenin or the addition of DKK-1 (Dickkopf-1, extracellular antagonist of Wnt) was shown to block the dendritogenic effect of depolarization by high K^+, suggesting that Wnt expression regulation by neuronal activity in turn modulated dendritic arborization via β-catenin (Yu, & Malenka, 2003). Conditional knock out of β-catenin from new-born neurons in post-natal hippocampal DG lead to defects in dendritic morphology such that neurons could not extend proper dendrites and resulting in neuronal death (Gao et al., 2007). Depolarization critically increases the pool of β-catenin at dendritic spine synapses by regulating the phosphorylation of Tyr654 β-cat, which when dephosphorylated associates tighly to cadherins to influence synaptic size and strength (Murase, Mosser, & Schuman, 2002).

9. Chemokines and chemokine receptors

Chemokines (short term for chemoattractant cytokines) belong to a superfamily of small (8-14 kDa) secreted proteins, best known for their ability to regulate leukocyte migration during inflammatory responses. They have been highly conserved during evolution, indicated by sequence homology of chemokines expressed in mammals, birds and fish (reviewed in Cartier et al., 2005). Till date more than 50 chemokines have been identified (Rossi, & Zlotnik, 2000; Murphy et al., 2000; Murphy, 2002). Chemokines can be divided into subfamilies according to the position of a pair of cysteines located near the N terminus of each protein (Rossi & Zlotnik, 2000; Proudfoot, 2002; Proudfoot et al., 2002) as CXC, CC, C, and CX3C chemokines (Figure 31). Less commonly, these subfamilies are also referred to as the α, β γ and δ chemokines, respectively. CXC, CC and CX3C chemokines have four conserved cysteines, whereas C chemokines have only two, which correspond to the second and fourth cysteines present in the other groups. The first two cysteines in the CC chemokines are adjacent, whereas in the CXC and CX3C chemokines they are separated by one (CXC) or three (CX3C) amino acids. Considering their roles in the immune system, chemokines have also been classified as inflammatory and homeostatic (reviewed in Sallusto, Mackay, & Lanzavecchia, 2000). Inflammatory chemokines like CCL5 and CXCL2, which are expressed in inflamed tissue in response to pro-inflammatory cytokines or to pathogens recruit effector cells (monocytes, granulocytes and effector T cells), whereas homeostatic chemokines like CXCL12 are constitutively expressed in different environments within tissues and are involved in trafficking of cells that belong to adaptive immune system.

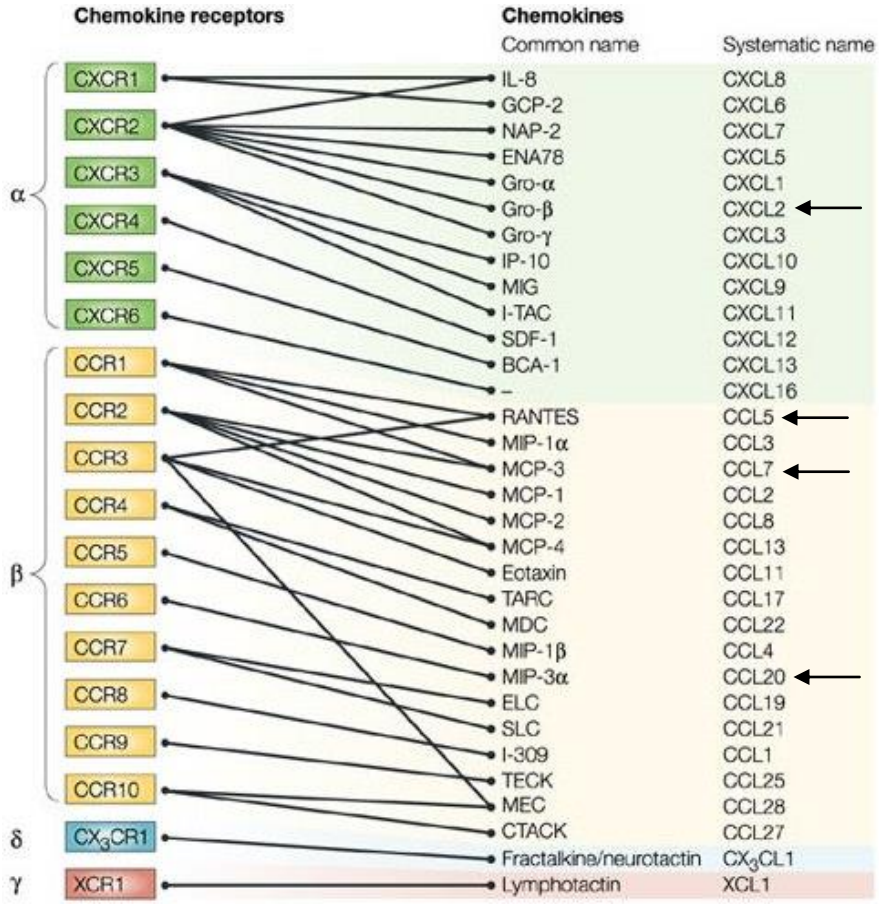


Figure 31. The different subfamilies of chemokine receptors and chemokine ligands (adapted from Tran, & Miller, 2003). *Table listing the chemokines divided into different subfamilies (α, β, γ and δ). It includes their "systematic name" as well as "common name" along with their receptor. Chemokines in the focus of this thesis work have been marked with the arrows. C- Cysteine residue and X - Amino acid.*

Chemokines carry out their functions by interaction with G protein-coupled receptors (GPCR's), which are members of a superfamily of seven-transmembrane domain receptors (TM 1-7) that mediate intracellular signals through heterotrimeric GTP-binding proteins (Figure 32). In GPCR's, the seven transmembrane domains are preceeded by an extracellular (ECL 1-3) and intracellular loops (three of each) and followed by an intracellular C-terminal domain.

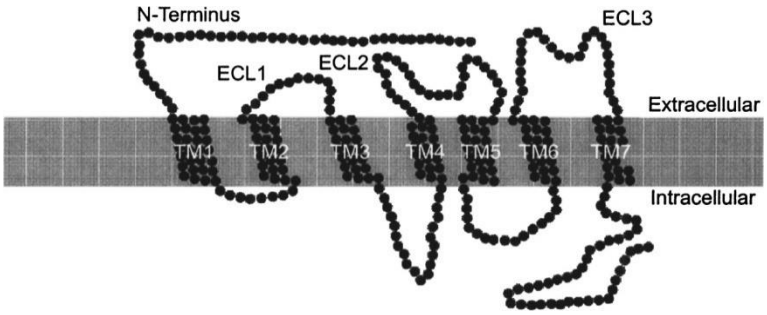


Figure 32. Structure of chemokine receptor (Fernandez, & Lolis, 2002) *showing N-terminal, seven transmembrane domains (TM 1-7), extracellular domains (ECL 1-3) and intracellular loops (folds).*

The **chemokine receptors** have been classified as CC, CXC, XC or CX3C according to the chemokine class by which they are activated, followed by the letter "R" denoting receptor (as opposed to "L" in the case of the chemokine ligand). Nineteen chemokine receptors have been known so far, including six CXC receptors, ten CC receptors and two single receptor each for CX3CL1 and CXCL1 chemokines. It should be noted, as illustrated in Figure 31, that many chemokines can bind to several receptors and that chemokine receptors usually act as such for more than one chemokine.

Chemokine binding to the corresponding receptor results in the activation of G proteins, which leads to the dissociation of the G protein heterotrimer into its α and βγ subunits (Figure 33). Activation of phosphatidyl-inositol specific Phospholipase C (PLC β2 and β3) leads to the generation of DAG and IP3, which upon binding to its specific receptor in the endoplasmic reticulum, triggers Ca^{2+} release from intracellular stores. DAG, in conjunction with Ca^{2+}, activates various PKC enzymes (Figure 33). Temporary Ca^{2+} elevation is the best characterized effect of chemokine stimulation and it has been widely used to study functional expression of chemokine receptors for a variety of chemokines (Bajetto et al., 1999; Boutet et al., 2001; Gillard et al., 2002).

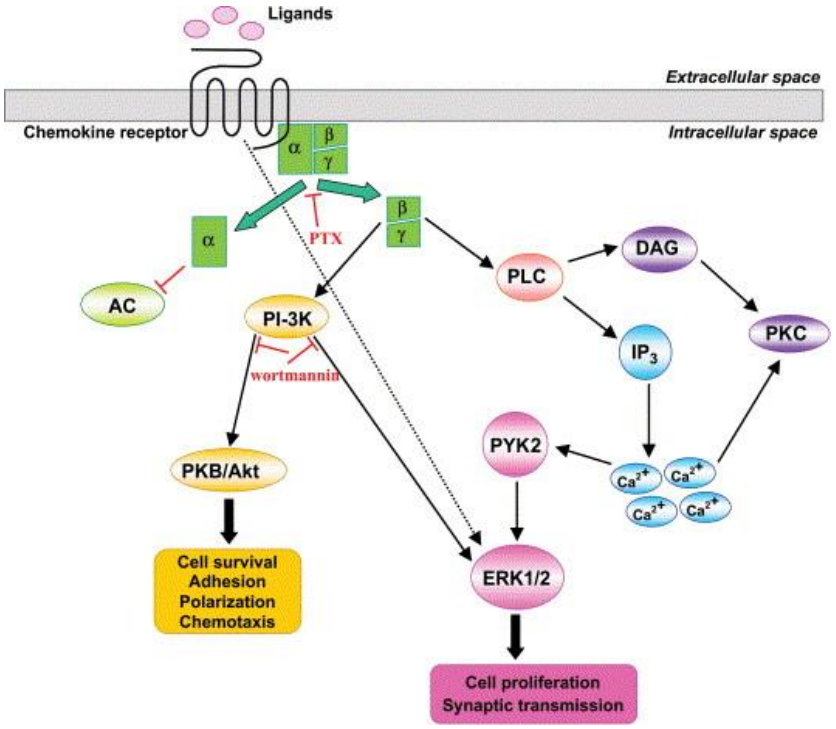


Figure 33. Signaling pathways downstream to chemokine receptor activation along with the effects promoted (Cartier et al., 2005). *AC: adenylyl cyclase; DAG: diacylglycerol; ERK1/2: extracellular signal-regulated kinase; IP3: inositol triphosphate; PI-3K: phosphoinositide 3-kinase; PKB-Akt: protein kinase B; PKC: protein kinase C; PLC: phospholipase C; PTX: pertussis toxin; PYK2: proline-rich tyrosine kinase 2.*

Chemokine-induced activation of P13K has also been described (Figure 33) (Sotsios et al., 1999; Thelen, Uguccioni, & Bosiger, 1995) and knock out experiments in mice (Hirsch et al., 2000; Sasaki et al., 2000) have revealed it to be centrally involved in the cellular responses to chemokines, including cell polarization and chemotaxis.

Several chemokines have been reported to activate Mitogen-Activated Protein Kinase (MAPK) cascade involving Extracellular signal-Regulated Kinase (ERK)-1/2 (Ganju et al., 1998; Knall, 1996; Bajetto et al., 2001;Wang & Richmond, 2001) and the calcium dependent-protein kinase Pyk2 (Del Corno et al., 2001; Dutt, Wang, & Groopman, 1998; Hatch et al., 1998) (Figure 33).

Chemokine function in immune cell trafficking both in pathological and physiological conditions is well documented (Baggiolini, 1998; Moser, & Loetscher, 2001; Rot, & Andrian, 2004). Importantly, chemokine expression in the CNS links to their crucial roles in brain development, synaptic transmission and injury. Indeed, chemokine signaling has been specifically involved in neurogenesis, neuronal migration and axon elongation and

branching (Charo, & Ransohoff, 2006; Ubogu et al., 2006; Bertollini et al., 2006; Lysko et al., 2011; Pujol et al., 2005, Yang et al., 2013; Edman, Mira, & Arenas, 2008; Edman et al., 2008). In this work, chemokines have been studied in the context of axon outgrowth, so the focus here will be restricted to it and briefly in connection to brain injury.

9.1 Chemokines in brain injury

Chemokine expression have been mainly observed in different glial cell types during CNS injury, amongst which the chemokines studied in this work- CXCL2 (Pineau et al., 2009; Ma et al., 2002; Szmydynger-Chodobska et al., 2009), CCL5 (Rice at al., 2007; Benton et al., 2008; Stefini et al., 2008), CCL7 (Ma et al., 2002; Helmy et al., 2009; Wang et al., 1999), CCL20 (Mc Tigue et al., 1998; Terao et al., 2009).

CXCR2 ligands (**CXCL2**, one amongst eight other members of CXC family) recruit neutrophils, expressing the CXCR2 receptor to the site of CNS injury, which is in agreement with the report demonstrating less recruitment of neutrophils to the injury site, less tissue damage and neuronal apoptosis in CXCR2 deficient mice (Semple et al., 2010) thus indicating CXCR2 antagonism as a beneficial therapy in posttraumatic inflammation.

On the other hand, there are mixed views regarding a beneficial role of **CCL5** and its receptors (CCR1, CCR3 and CCR5) (Braunersreuther et al., 2007) in the damaged CNS. Not only CCL5 but also CCR5 is upregulated in astrocytes, microglia, endothelial cells and neurons following CNS injury (Spleiss et al., 1998; Tripathy et al., 2010a, b). Its detrimental effects involves increased cerebral damage by secondary induction of pro-inflammatory cytokines like Interleukin-6 (Shahrara et al., 2006), whereas beneficial ones include neuronal protection and survival (Tripathy et al., 2010a, b) and protection from excitotoxic NMDA-induced apoptosis (Eugenin et al., 2003). Furthermore, the interaction of **CCL20** with CCR6 has been described to attract inflammatory monocytes and activate microglia, contributing to neuroinflammation followed by brain injury (Terao et al., 2009).

In sum, a huge amount of information is now available regarding chemokine expression, regulation and signaling upon injury or stroke. Several therapeutic paradigms with chemokines in experimental CNS trauma models of spinal cord injury and traumatic brain injury have shown improvements in axon outgrowth, which might facilitate the development of therapies in CNS repair (Jaerve & Müller, 2012). Finally, the chemoattractant function of chemokines can be exploited as a thereapeutic intervention in neuroinflammation by suppressing or changing the course of infiltration of inflammatory cells. The success of chemokines in clinical transfer, however, depends on the elucidation

of their cell-subtype-specific action and cellular signaling pathways in CNS injury in order to identify selective drug targets.

9.2 Chemokines in neurite morphogenesis

Until recently not a lot of reports involved chemokine signaling in neurite outgrowth. Among the chemokines reported to be involved in neurite outgrowth there is mainly CXCL12/SDF-1 (Pujol, Kitabgi, & Boudin, 2005; Lysko, Putt, & Golden, 2011; Opatz et al., 2009). Other works implicated CCL2, CCL5 and CCL7 (Edman, Mira, & Arenas, 2008; Chou et al., 2008). CXCL12 and its receptors (CXCR4 and CXCR7) promote neurite outgrowth by overcoming myelin proteins induced inhibition (Opatz et al., 2009), which is among the major hindrance to the neuronal impair in the injured adult mammalian CNS (Filbin, 2003; Schwab et al.,2005). CXCL12 reduces axon elongation, but increases axonal branching in hippocampal neurons (Pujol, Kitabgi, & Boudin, 2005). CXCL12 has also been shown to modulate the branching of the leading process of interneurons, affecting the speed of migration (Lysko, Putt, & Golden, 2011). CXCL12 reduces the branching frequency of the leading process, thus maintaining the stream migration of neuroblasts from the ventral ganglionic eminence. In contrast, blocking CXCL12 leads to increase in the branching frequency, stream exit and cortical plate invasion (Lysko, Putt, & Golden, 2011). In addition, CCL2 and CCL7 have been identified as pro-differentiation factors, promoting neuritogenesis in midbrain dopaminergic neurons (Edman, Mira, & Arenas, 2008). Defects in transcription and release of CCL5 from astrocytes has been reported to contribute to neuronal dysfunction in Huntington's disease (HD) (Chou et al., 2008). In the same work, a correlation between the amount of secreted CCL5 and neurite maturity (quantified as the differences in the length of neurites as well the number of sprouted branches) in primary cortical neurons was also demonstrated (Chou et al., 2008).

9.3 Chemokine involvement in neural and non-neural migration

Besides chemokines well studied roles in the immune system, chemokines and their receptors are expressed by a variety of cell types in the CNS, where they are clearly involved in neuronal migration (Mélik- Parsadaniantz, & Rostène, 2008; Mithal, Banisadr, & Miller, 2012; Zhu, & Murakami, 2012). Projection neurons in the intermediate zone (IZ)/SVZ of the cortex express CXCL12 (Stumm, & Höllt, 2007; Tiveron et al., 2006) that controls the tangential migration of CXCR4-expressing GABAergic interneurons (López-

Bendito et al., 2008; Tiveron et al., 2006). In addition, CXCL12 is persistently expressed in the meninges (Paredes et al., 2006; Stumm et al., 2007), where it also regulates the tangential migration of GABAergic interneurons (López-Bendito et al., 2008) and hem-derived Cajal-Retzius cells throughout the marginal zone of the cortex (Borrell, & Marín, 2006; Paredes et al., 2006). CXCL12/CXCR4 signaling is also responsible for the migration, assembly and positioning of cerebellar granule, Purkinje (Ma et al., 1998), precerebellar (Zhu et al., 2009) and olfactory neurons (Miyasaka, Knaut, & Yoshihara, 2007), as well as facial motoneurons (Sapède et al., 2005). Moreover, CXCL12 signaling involving both CXCR4 and CXCR7 is required for guiding the cortical interneurons (Wang et al., 2011; Abe et al., 2014; Sánchez-Alcañiz et al., 2011), whereas CXCL12 and CXCR4 regulate the initial trajectory of ventral motoneurons (Lieberam et al., 2005) and axon pathfinding of retinal ganglion cells and olfactory neurons (Li et al., 2005; Miyasaka, Knaut, & Yoshihara, 2007). Similarly, the migration and final position of trigeminal and DRG cells, as well as their target innervation, also require CXCL12/CXCR4 (Balabanian et al., 2005; Knaut et al., 2005; Odemis et al., 2005). Thus, CXCL12/CXCR4 signaling regulates the three key processes essential for the establishment of neural networks in different neuronal systems: neuronal migration, cell positioning and axon wiring.

In cancer, a list of chemokines and their receptors are expressed and endogenously produced by tumor cells, including CXCL7 and CXCR1/CXCR2, CCL5 and CXCL12 among others (Karatsu et al., 1993; Desbaillets et al., 1994, 1997; Barnes, Jones, & Perez, 1997; Zhou et al., 2002; Barbero et al., 2003). Amongst all the chemokines, CXCL12/CXCR4 has been studied in glioma cell invasion (Zhou et al., 2002; Bajetto et al., 2006; Zhang, Sarkar, & Yong, 2005; Ehtesham et al., 2006) , thus highlighting the role of chemokines in migration during development and in pathology.

10.1 Role of centrosome in migration: centrosome reorientation

In this work, centrosome positioning has been studied using primary rat astrocytes as the cell model, so the mechanism involved in this cell type is discussed. Centrosome re-orientation has been studied *in vitro* using wound-healing assay where astrocytes are grown to reach confluency, followed by formation of a scratch. Astrocytes migrate towards the wound created in the monolayer by extending pseudopodium-like structure and re-orienting the centrosome and Golgi towards the wound (Etienne-Manneville & Hall, 2001). In astrocytes, centrosome re-orientation banks upon integrin stimulated

activity of the Rho-GTPase Cdc42, which regulates signaling from cell-surface receptors to actin and MT cytoskeleton (Raftopoulou, & Hall, 2004) (Figure 34). At the leading edge, Cdc42 recruits and activates mPar6-PKC complex, which phosphorylates and inactivates GSK-3β. GSK-3β inactivation causes APC bound to MT plus-end to bind to MT minus-end, thus facilitating MT capture at the leading edge by dynein (Etienne-Manneville & Hall, 2001; Palazzo et al., 2001). The MT minus end motor activity of dynein, pulls captured MTs towards the leading edge and orients centrosome within the protrusion into the wound.

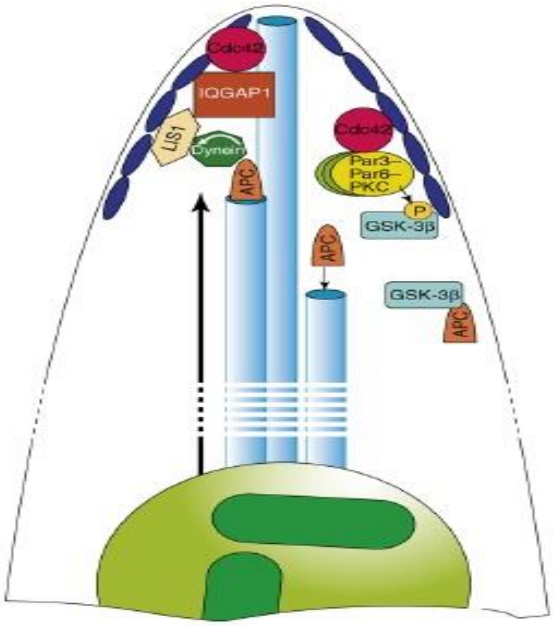


Figure 34. Proposed model for Centrosome positioning mechanism (Higginbotham & Gleeson, 2007). *Through Cdc42 in the leading process, guidance cues stimulate actin (purple ovals) polymerization. The par complex is targetted by Cdc42 to the leading edge, where it phosphorylates and inactivates GSK3β resulting in APC binding to MT ends and stabilization of them. IQGAP1 is the link between actin and plus end MT proteins like APC. Pulling force on MTs, which orients centrosome within the leading process, is done by LIS1anddyenein*

A similar mechanism for centrosomal position applies to the process of cell migration and neurogenesis in the cerebral cortex (Higginbotham, & Gleeson, 2007).

10.2 Wnt/β-catenin signaling at centrosome

β-catenin's recognition as a regulator of centrosome function came from the experiments, where its overexpression resulted in centrosome disorganization and loss of cortical anchoring of MTs (Ligon et al., 2001). Total β-catenin levels follow very well synchronized changes during different stages of the cell cycle in non-transformed epithelial cells, demonstrating an increase in S-phase, followed by maximum at late G2/M and an abrupt reduction in G1 (Olmeda, Castel, & Cano, 2003). Nevertheless, β-catenin levels do not appear to change significantly in cancer cell lines (Hadjihannas et al., 2012). The involvement of β-catenin in recruiting γ-tubulin to centrosomes has been pointed out, since the depletion of β-catenin inhibits MT nucleation (Huang, Senga, & Hamaguchi, 2007) and β-catenin null neuronal progenitors lack γ-tubulin containing centrosome or MT network (Chilov et al., 2011). β-catenin siRNA expression suppresses the astral MT organization, an effect that was mimicked by the GSK-3β inhibitor, lithium chloride (Huang, Senga, & Hamaguchi, 2007). In addition, expression of stabilized mutant β-catenin, which mimics mutations found in cancer, results in extra non-microtubule nucleating structures that contain a subset of centrosome proteins including γ-tubulin and centrin (Bahmanyar et al., 2010). These results indicate that β-catenin is required for centrosome amplification, and mutations in β-catenin might contribute to the formation of alterations of centrosomes observed in cancers (Bahmanyar et al., 2010). Regarding the establishment of bipolar mitotic spindle, loss of β-catenin leads to increased number of cells with monopolar spindles (Kaplan et al., 2004). Finally, a role for β-catenin regulating centrosome separation during mitosis has also been demonstrated, where activation of the Ser/Thr kinase Nek2 (by Polo-like kinase 1, Plk1) results in the phosphorylation of centriolar linker proteins Rootletin and C-Nap1 (centrosomal Nek2-associated protein 1), which is thought to cause the separation of centrosomes (Bahmanyar et al., 2008). β-catenin binds to and is phosphorylated by Nek2, and is in a complex with Rootletin. In interphase, β-catenin colocalizes with Rootletin at the proximal end of centrioles. However, in mitosis, when Nek2 activity increases, β-catenin localizes to centrosomes at spindle poles independent of Rootletin. Increased Nek2 activity disrupts the interaction of Rootletin with centrosomes and results in binding of β-catenin to Rootletin-independent sites on centrosomes, an event that is required for centrosome separation (Bahmanyar et al., 2008) (Figure 35).

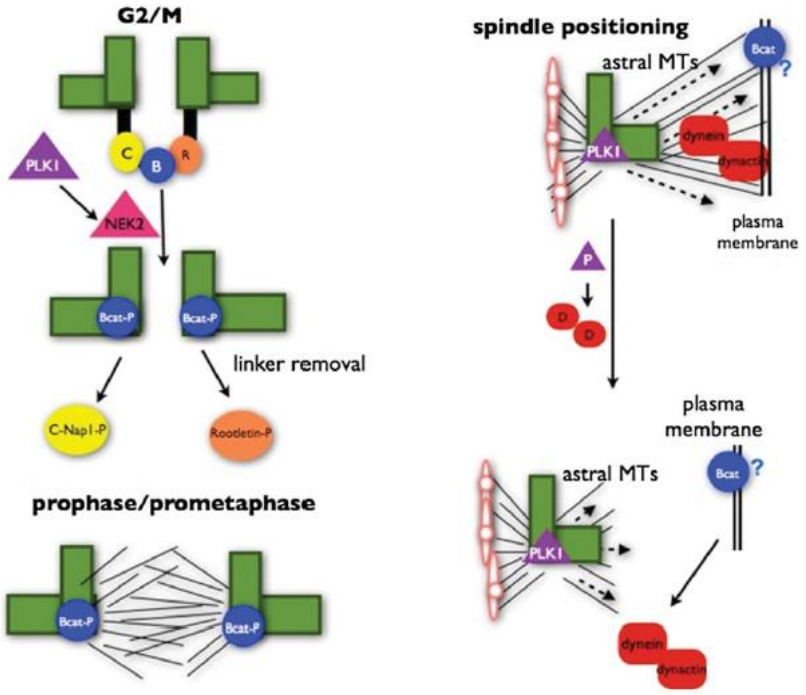


Figure 35. β-catenin and its interactor molecules at centrosome (Mbom, Nelson, & Barth, 2013). *During G2/M transition (left), Nek2 phosphorylates the centrosome linker proteins C-Nap1 and Rootletin, resulting into centrosomes separation and bipolar spindle formation, in parallel with the increased expression of β-catenin at centrosome (especially during the centrosome seperation). PLK1 works as the upstream factor to Nek2 during this phase. During the spindle positioning (right), β-catenin gets involved in cortical anchoring of MTs in complex with dynein, and PLK1 negatively regulates the dynein-dynactin complex at the cell cortex, ensuring the proper spindle positioning. This way centrosomes are separated and mitotic spindle formation takes place.*

Interestingly, expression of stabilized forms of β-catenin (lacking N-terminal regions that regulate its degradation, thus showing longer life-times at centrosomes) leads to increasing distance between centrioles/centrosomes and higher number of cells with separared (split) centrosomes, as similarly found for Nek2A overexpression (Bahmanyar et al., 2008). These results indicate that like Nek2, β-catenin is a negative regulator of centrosome cohesion.

Apart from total β-catenin, phosphorylated β-catenin has also been reported at the centrosome. β-catenin phosphorylated at Ser33/Ser37/Thr41 (phospho-β-catenin), which should be targeted for degradation by the proteasome in the canonical Wnt signalling

pathway, accumulates in the centrosome. This form localizes preferentially to mother centrosome during interphase and is recruited to daughter centrosome during mitosis (Huang, Senga, & Hamaguchi, 2007). Expression of non-phosphorytable β-catenin mutants (Ser33Ala) cause disruption in MT regrowth and MT array, whereas overexpressing phospho-mimetic mutants of β-catenin (Ser33/Ser37/Thr41 mutated to Glu) leads to multiple centrosomes and aberrant denser radial MT arrays (Huang, Senga, & Hamaguchi, 2007).

Interestingly, phospho-β-catenin increases during cell cycle in synchronized cells and remains associated to the centrosome during mitosis (Huang, Senga, & Hamaguchi, 2007; Hadjihannas, Brückner, & Behrens, 2010). The levels of phosphoSer/Thr β-catenin parallel those of axin/conductin2, a negative regulator of Wnt signalling (Hadjihannas, Brückner, & Behrens, 2010) and are involved in centrosome cohesion. Conductin/axin2 levels peak at G2/M followed by a rapid decline during return to G1. In line with this, Wnt/β-catenin target genes are low at G2/M and high at G1/S, and β-catenin phosphorylation oscillates during the cell cycle in a conductin dependent manner (Hadjihannas, Brückner, & Behrens, 2010). In the proposed model, Wnt signaling and dephospho-Ser/Thr β-catenin thus promote centrosome separation or splitting (Hadjihannas, Brückner, & Behrens, 2010).

In neuronal progenitor cells, reduced levels of phospho-Ser/Thr β-catenin affected the orientation of the mitotic spindle, increased asymmetric cell divisions and caused premature differentiation, highlighthing a function for centrosomal phospho-Ser/Thr β-catenin in maintaining MT and the polarity in neuronal progenitors (Chilov et al., 2011).

Wnt signaling components modulate different aspects of mitosis like MT dynamics, spindle formation and centrosome division by localizing to centrosome. GSK-3β influences MT dynamics as its long term inhibition abolishes MT growth similar to axin ablation. On the contrary, its short term inhibition accelerates reorganization of MTs (Huang, Senga, & Hamaguchi, 2007). This results in stabilization of the MTs and mitotic spindle (Wakefield, Stephens, & Tavare, 2003; Fumoto, Hoogenraad, & Kikuchi, 2006) by reducing the phosphorylation of MT associated proteins (Hanger et al., 1992; Fumoto, Hoogenraad, & Kikuchi, 2006).

Interestingly, in addition to β-catenin, APC, Axin and other Wnt pathway components like GSK-3β localize to the centrosome (Olmeda, Castel, & Cano, 2003; Mbom, Nelson, & Barth, 2013) and regulate mitotic progression. Axin1 is involved in MT nucleation at the centrosome (Fumoto et al., 2009). In addition, GSK-3β, Axin2 and APC regulate mitotic spindle positioning and chromosome segregation (Tighe et al., 2007; Fodde et al., 2001; Hadjihannas et al., 2006), which in case of mutations in APC causes chromosome instability by impairing the linkage between MTs and kinetochores (Fodde at al., 2001). In a similar way, Dvl in collaboration with Fzd and LRP6 coreceptors are important to establish spindle orientation (Kikuchi et al., 2010).

B. Glioblastoma multiforme

Any tumor arising from the the glial cells or the supportive tissue in brain is called "Glioma". Gliomas are the most common type of primary brain tumors, which are classified histologically and immunohistochemically as astrocytomas, oligodendrogliomas and oligoastrocytomas (sharing features of both astrocytes and oligodendrocytes). The incidence rate of primary brain tumors worldwide is approximately seven per 100,000 individuals per year (Huang et al., 2007).

The World Health Organization (WHO) classified the CNS tumors based on the malignancy as on a scale of I to IV (StLouis et al., 1999; Kleihues, Burger, & Scheithauer, 1993). Grade I tumors are benign in nature which can be cured if surgically removed. Grade II astrocytoma tumors are characterized as low-malignant, but their diffuse infiltration render them incurable by surgery. Grade III Anaplastic astrocytoma exhibits increased anaplasia and proliferation over Grade II, and the Grade IV Glioblastoma multiforme (GBM), which is the most devastating and malignant astrocytic glioma is characterized by a high degree of cellularity, vascular proliferation, tumor cell chemoresistance and necrosis (Ohgaki, & Kleihues, 2005a, b). GBM is nowadays considered an incurable malignancy even after aggressive chemotherapy and neurosurgical resection (Denysenko et al., 2010). On the basis of clinical presentation, GBM is subdivided into primary and secondary GBM (Figure 36). Primary GBMs accounts the majority of cases in older patients with no evidence of prior symptoms or antecedent lower grade pathology whereas, secondary GBMs are the rare ones accounting for occurrence in patients below the age of 45 years and around 70% of cases are derived from the

progressive transformation of lower grade astrocytoma like grade II transforming into grade III/IV.

The existence of brain tumor stem cells was proposed a decade ago, followed by the the advances in the stem cell field and the discovery of adult neurogenesis (Vescovi, Galli, & Reynolds, 2006; Ignatova et al., 2002; Gu, Janoschka, & Ge, 2013). GBM have been characterized by Glioma initiating cells (GICs, a type of cancer cells), exhibiting common features of neural stem cells including: the expression of CD133 (prominin), neurosphere forming ability and the reproduction of tumors (Singh et al., 2003, 2004). GBM, like any other cancer is characterized by genetic instability and complex alterations in chromosome structure and copy number. The somatic copy number alterations found in malignant gliomas include broad or regional alterations spanning segments or whole arms of entire chromosomes as well as focal events involving one or a few genes. A great amount of information compiled recruiting advanced techniques like Genomic Identification of Significant Targets in Cancer (GISTIC) (Beroukhim et al. 2007; Mermel et al. 2011) and Genomic Topography Scan (GTS) (Wiedemeyer et al. 2008), came out with somatically mutated genes that include TP53 (42%), PTEN (33%), Neurofibromatosis-1 (NF1) (21%), EGFR (18%), RB1 (11%), PIK3R1 (10%), and PIK3CA (7%) (Figure 36). Some of these mutations are more abundant in particular subclasses of GBMs (proneural, neural, classical and mesenchymal) (Verhaak et al., 2010).

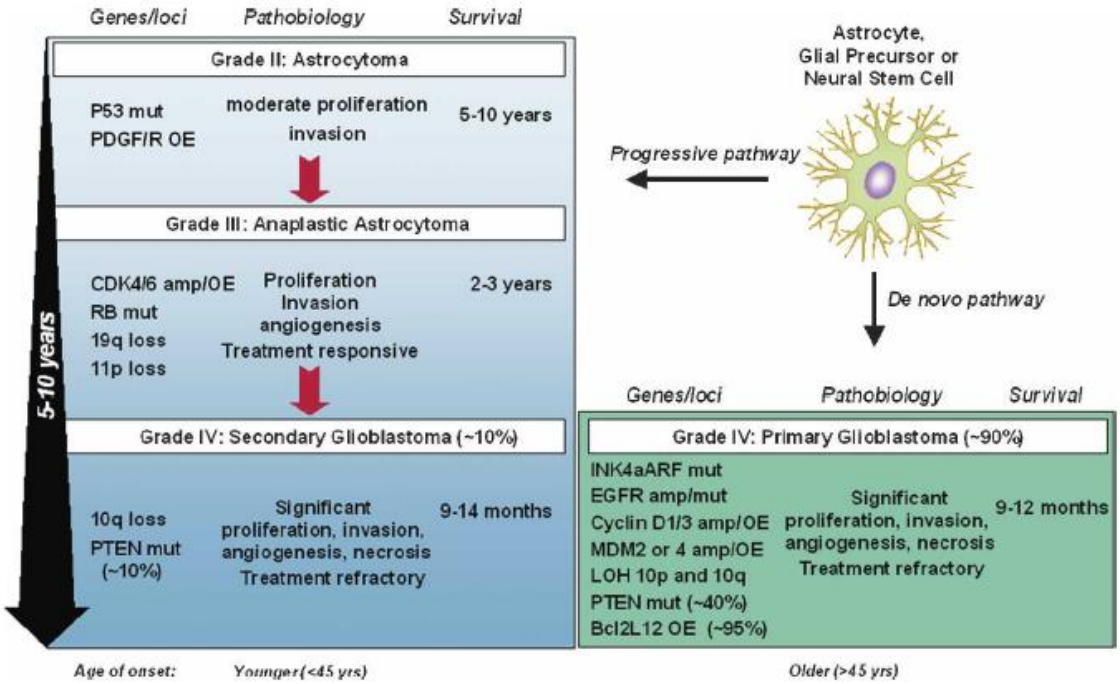


Figure 36. Chromosomal and genetic aberrations underlying GBM (Furnari et al., 2007). *Figure shows the relationship between survival, pathobiology and the underlying molecular pathways in Grade II and III astrocytomas and leading to the formation of primary (de novo) and secondary (progressive) GBMs.* (OE) Overexpressed; (amp) amplified; (mut) mutated.

The comprehensive studies on genomics of GBM, revealed a set of core signaling pathways activated in GBM which are: the P53 pathway, the Retinoblastoma (RB) pathway and RTK/Ras/PI3K pathway (TCGA, 2008; Parsons et al., 2008). These pathways exhibit the majority of mutations, which enhance cell proliferation and survival, allowing the tumor to escape from cell cycle checkpoints, senescence and apoptosis. Specifically, p53 signaling was impaired in 87% of the samples through CDKN2A deletion (49%), MDM2 (14%) and MDM4 (7%) amplification, and mutation and deletion of TP53 (35%). Likewise, Rb signaling was impaired in 78% of the samples through CDKN2 family deletion; amplification of CDK4 (18%), CDK6 (1%), and CCND2 (2%); and mutation or deletion of RB1 (11%). Finally, evidence of RTK/RAS/PI3K activation was found in 88% of tumors, including contributions from unexpected mutations or deletions in NF1 (18%) and PIK3R1.

Mutations in tumor suppressor gene neurofibromatosis type-1 (NF1) have been reported to increase the incidence of malignant glioma (Zhu et al., 2005). In addition to the core signaling pathways, heterozygous deletion of NFKB inhibitor A (NFKBIA) gene was also observed to be present in a quarter of GBM samples (Bredel et al., 2011).

The information deduced from the efforts put forward to get into more details regarding the GBM over two decades characterized six intracellular events occuring in combination to cause and sustain GBM (Nakada et al., 2011). The very first event is the cell cycle control, where glioma cells develop various means for evading the cell cycle control so as to get the growth benefit. Alteration in atleast one of the component of (CDK)-4/RB pathway has been reported in anaplastic astrocyoma and the majority of gliomas. Second event involves the overexpression of growth factor and their receptors, amongst which Epidermal Growth Factor Receptor (EGFR) and Platelet-Derived Growth Factor Receptor (PDGFR) are best characterized. Other events include angiogenesis (formation of new blood vessels from the exisiting ones), involving growth factors like Vascular Endothelial Growth Factor (VEGF); invasion and migration, abnormality of apoptosis and genomic instability.

1. Epithelial-mesenchymal transition (EMT) in tumor invasion

EMT, described first by Elizabeth Hay in chick primitive streak (Hay, 1995) is a biological process which allows a polarized epithelial cell to undergo the biochemical changes to assume the mesenchymal phenotype, which includes enhanced migratory capacity, invasiveness, elevated resistance to apoptosis and increased production of ECM components (Kalluri, & Neilson, 2003). This transition from epithelial to mesencymal phenotype is important during embyogenesis and organ development, tissue repair and pathological stress and in initiating the invasive and metastatic behavior of epithelial cancers (Kalluri & Weinberg, 2009). The reverse process-mesenchymal epithelial transition (MET) involving the conversion of mesenchymal cells to epithelial derivatives also occurs, which is associated with kidney formation (Lipschutz, 1998; Rothenpieler, & Dressler, 1993).

EMT is clearly associated with tumor progression and metastasis. Uncontrolled epithelial cell proliferation and angiogenesis marks the initiation and early growth of primary epithelial cancers (Hanahan, & Weinberg, 2000). Many studies proposed EMT activation as the critical requisite to acquire the malignant phenotype by epithelial cancer cells (Thiery, 2002). The most convincing report regarding the EMTs in cancer came from the observation that EMT-derived migratory cancer cells establishes the secondary colonies at distant sites, resembling histopathologically primary tumors from which they arose (Kalluri, & Weinberg, 2009). Cancer cells undergo MET after extravasation into the parenchyma of distant organ because of the absence of heterotypic signals which were responsible for the induction of EMT in primary tumor (Thiery, 2002; Jechlinger, Grunert, & Beug, 2002; Bissell et al., 2002). Summarizing these reports, leads one to consider the induction of EMT to be central for the progression of carcinomas to metastatic stage and embroil MET for the further colonization process (Figure 37).

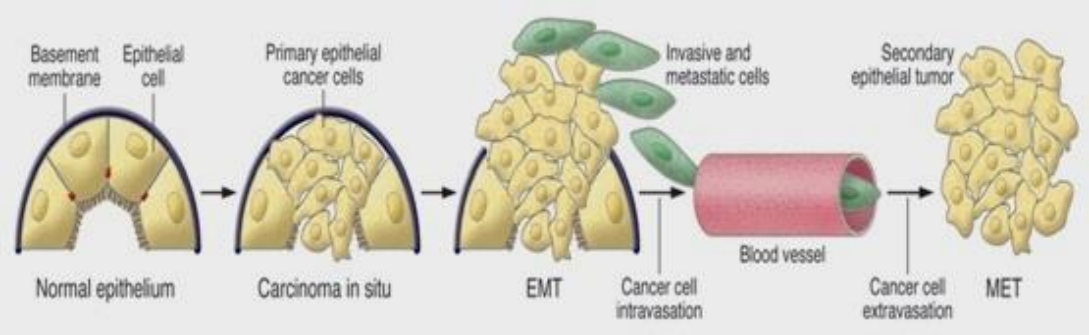


Figure 37. EMT contribution to cancer progression (Kalluri, & Weinberg, 2009). *The development from normal epithelium to invasive carcinoma proceeds through several stages, which initiates when epithelial cells loose their polarity and detache from basement membrane and involves alteration in ECM-interactions and signaling networks. This is followed by an angiogenic switch, facilitating malignancy and enabling cancer cells to enter the circulation and exit the blood stream at a remote site, where they may form micro and macro-metastases. This may also involve METs (reversion to the epithelial phenotype)*

Studies have reported HGF, EGF, PDGF and TGF-β (Transforming Growth Factor) as EMT-inducing signals and identified EMT transcription factors, notably Snail, Slug, Zinc finger E-box Binding homeobox1 (ZEB1), Twist, Goosecoid and FOXC2 (Thiery, 2002; Jechlinger, Grunert, & Beug, 2002; Shi, & Massague, 2003; Niessen, 2008; Medici, Hay, & Olsen, 2008; Kokudo et al., 2008). The respective signaling pathways involved are ERK, MAPK, Akt, Smads, RhoB, β-catenin, LEF, Ras and c-Fos as well as cell surface proteins, like β4 integrins, α5β integrin and αVβ integrin (Tse, & Kalluri, 2007).

Apart from the mentioned factors, disruption of cell-cell adherens junctions and the cell-ECM adhesions mediated by the integrins also activate EMT (Yang, & Weinberg, 2008; Gupta et al., 2005; Mani et al., 2008; Hartwell et al., 2006). The concept in favour of the link between E-cadherin loss by cancer cells and induction of EMT has been well established (Tepass et al., 2000; Edelman et al., 1983). Reorganization of cell-cell adhesion complexes and suppression of proliferation occurs when cytoplasmic portion of E-cadherin (containing β-catenin binding site) is expressed, forcing cells to loose the mesencymal phenotype (Eger et al., 2000; Reichmann et al., 1992). Hence, association of β-catenin to Cadherin in cytoplasm is important for the preservation of epithelial features of cancer cells and, in contrast, its movement to the nucleus (associated with the loss of E-cadherin) implies acquisition of an invasive phenotype (Kim, Lu, & Hay, 2002; Thiery, 2002).

2. Wnt signaling in GBM

The aberrant activation of canonical Wnt signaling pathway in variety of tumors (Polakis, 2000; Reya, & Clevers, 2005) did not come as surprise after the discovery of its crucial involvement in the induction of self renewal properties in embryonic stem cells (ESCs) and adult stem cell regulation (Reya, & Clevers, 2005; Wend et al., 2010; Inestrosa, &

Arenas, 2010). Wnt-target genes thus include ESC genes – Nanog, Oct-4, Sox-2 and c-Myc- that have been mentioned for their association with both poorly differentiated as well as aggressive GBM (Ben-Porath et al., 2008). PLAGL2 (a novel protooncogene) promotes GICs proliferation and gliomagenesis by enhancing the expression of Wnt-6, Fz-9 and Fz-2 (Zheng et al., 2010).

Wnt pathway components (Dvl-3, FRAT-1, Pygo-2, TCF3 and Lef-1) (Sareddy et al., 2009; Wang et al., 2010; Guo et al., 2010) and target genes (cyclin D1 and c-myc) (Sareddy et al., 2009; Liu et al., 2010) show increased expression in high grade astrocytoma and GBM (Figure 38) and have been involved in glioma proliferation. Keeping aside canonical Wnts, the role of non-canonical Wnt-5a has also been demonstrated in U251 cell line in regards to stimulation of cell proliferation (Yu et al., 2007). Silencing β-catenin, Wnt-2 and Pygo (Pygopus)-2 affected proliferation of U251 cell line in a negative manner (Wang et al., 2010; Pu et al., 2009). Apart from the association of Wnt members with glioma, expression of Wnt antagonists, including tumor suppressor Wnt Inhibitory Factor (WIF) have also been shown to be reduced in astrocytomas, linked to aberrant promoter hypermethylation (Yang et al., 2010). In addition, hypermethylation of sFRP and DKK (two more Wnt antagonists) has been linked to primary and secondary GBM, respectively (Gotze et al., 2010).

Consistent with the role of non-canonical Wnt-5a in invasion and metastasis in other cancers (Pukrop et al., 2006; Weeraratna et al., 2002; Moon et al., 2004), Wnt-5a also enhances glioma cell migration by regulating Matrix Metalloproteinase (MMP)-2 expression (Kamino et al., 2011), and its silencing reduces glioma cell invasion in U251 glioma cell line (Pu et al., 2009; Kamino et al., 2011)

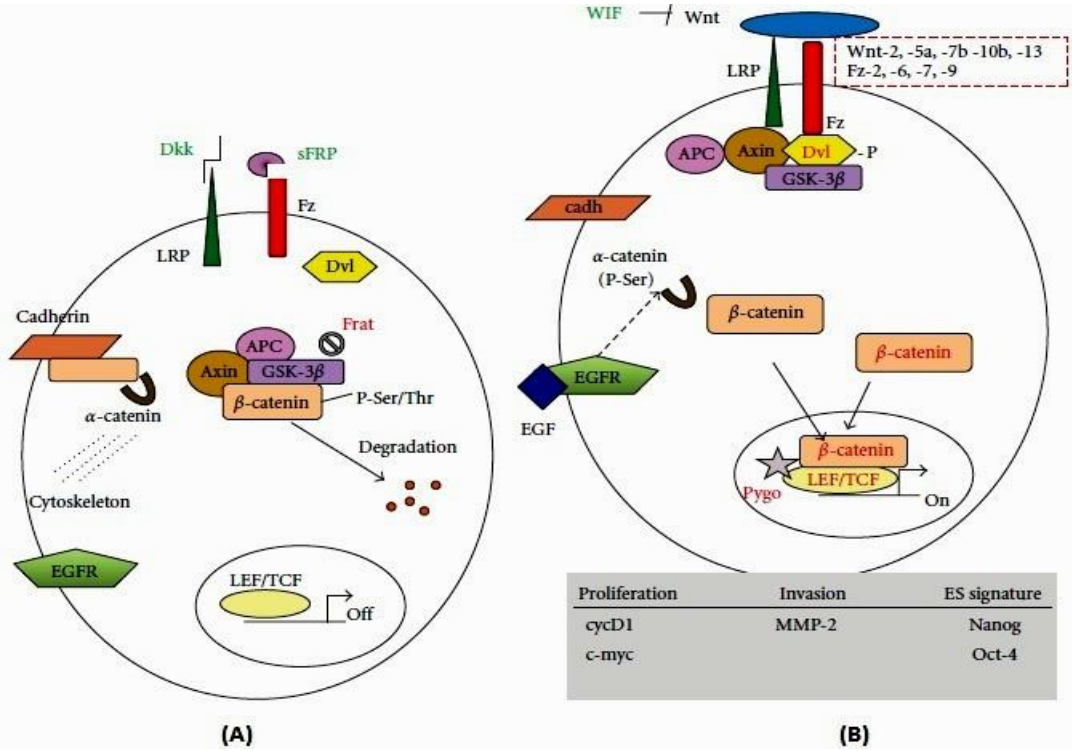


Figure 38. Wnt/β-catenin and β-catenin signaling in glioma cells (Nager et al., 2012). *Absence* ***(A)*** *and presence* ***(B)*** *of Wnt influences β-catenin localization in the nucleus, followed by the transcription of Wnt target genes (as described in figure 24). The box shows the Wnt target genes implicated in proliferation and invasion of glioma cells or conferring ES cell signature to GICs (related to aggressive growth and recurrence). Text in red indicates Wnt pathway components that are overexpressed and green indicates Wnt antagonists repressed in high-grade astrocytomas and GBM. Wnt factors and Fz that have been reported to be upregulated in high-grade astrocytomas and GBM are shown in the figure.*

3. β-catenin signaling in GBM

β-catenin has been proposed as a prognostic marker of malignancy in GBM at both mRNA and protein levels, which increase in high- grade astrocytomas as well as GBM (Sareddy et al., 2009; Liu et al., 2010, 2011).

In GICs, protooncogene PLAGL2's amplification correlates with increase in β-catenin levels in GBM (Zheng et al., 2010). Also PEG3 (paternally expressed gene 3 with tumor suppressing activity) (Jiang et al., 2010) and transcription factor Forkhead box M1 (Zhang et al., 2011) both utilize β-catenin in promoting proliferation and maintainance of GICs.

β-catenin has been reported as an important component of adherent junctions where it binds with E-cadherin and modulates cell-cell adhesion and migration (Clevers, 2006). The role of β-catenin in facilitating EMT of tumor cells is well described (Sánchez-Tilló et al., 2011; Schmalhofer, Brabletz, & Brabletz, 2009) (Figure 39).

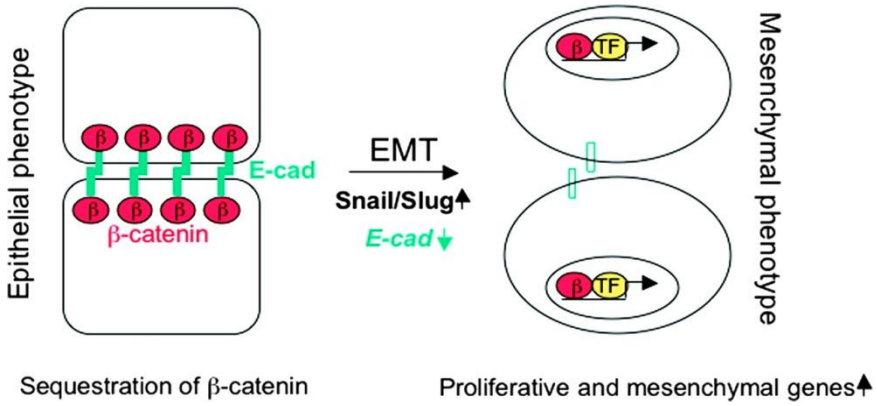


Figure 39. β-catenin in EMT (Dietrich, & Kaina, 2010). *Figure is showing cell-cell adhesion in the epithelial phenotype, mediated by the interactions of β-catenin and E-cadherin (left). Upon the overexpression of master regulators of EMT like Snail and Slug, leads to the association of β-catenin to the transcription factors (TF) of the TCF/LEF family in the nucleus, resulting in the induction of transcription of proliferative and mesenchymal genes and a mesenchymal phenotype.*

However, signals for the nuclear translocation of β-catenin in tumor development are not only conveyed by Wnt factors (Lu, & Hunter, 2004), but also by growth factor/RTK signaling. Phosphorylation of β-catenin at specific tyrosine residues is expected to increase migration (Lilien, & Balsamo, 2005; David et al., 2008). One such example is EGF/EGFR signaling, where via ERK 1/2 and casein kinase -2 (CK-2), α-catenin is phosphorylated at Ser 641 thus promoting β-catenin transactivation and glioma cell invasion (Ji et al., 2009).

4. HGF and Met signaling in GBM

HGF and Met are expressed in a variety of tumors, with their higher levels correlating with poor prognosis (Birchmeier et al., 2003). The implication of HGF/Met signaling in glioma is based upon the findings that their expression positively correlated with the glioma grade with the coexpression of HGF/Met more prevalent in high grade gliomas (Koochekpour et al., 1997; Moriyama et al., 1998; Rosen et al., 1996). HGF gene transfer to glioma cells enhanced their tumorigenecity, tumor growth and angiogenesis (Laterra et al., 1997a, b) and its inhibition lead to reduction of *in vivo* tumor formation and growth

(Abounader et al., 1999, 2002). These evidences were further confirmed by both overexpression (Abounader et al., 2004; Laterra et al., 1997a, b) as well as loss of function experiments (Abounader et al., 1999, 2002) for HGF and Met.

Cellular level visualizes the effects of HGF/Met pathway on glioma by induction of cell cycle progression; tumor cell migration, invasion and angiogenesis; and tumor apoptosis inhibition (Abounader, & Laterra, 2005) (Figure 40).

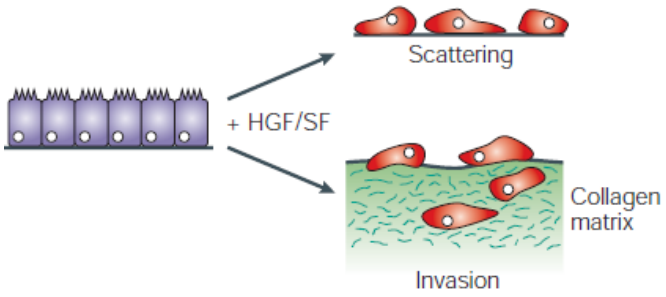


Figure 40. Met-induced cell scattering and invasion (Birchmeier et al., 2003). *Figure depicts HGF-induced scattering and invasion of epithelial cells. In the absence of HGF, epithelial cells adhere to each other, are polarized and form tight cell sheets (left). Upon HGF treatment, epithelial cells decrease cell-cell adhesion, assume a mesenchymal morphology, express mesenchymal markers and detach from the neighbouring cells (right, top). When HGF-treated cells are grown on top of a collagen matrix, they migrate and invade the extracellular matrix (right, bottom).*

HGF induced proliferation has been shown in many brain tumor cell lines including those derived from glioma, medulloblastomas and neuroblastoma (Abounader et al., 2004; Hecht et al., 2004; Koochekpour et al., 1997). Beyond its name, HGF is now recognized as an essential organotrophic factor in almost all tissues (Nakamura, & Nizuno, 2010). It induces mitogenic, motogenic and morphogenic activities in various types of cells via its functional receptor, Met (Bottaro et al., 1991; Higuchi et al., 1992). As a component with mitogenic ability, HGF/Met activation allows the cells to escape G1/G0 arrest and alters multiple cell cycle regulators like p27, phosphor-Rb, E2F-1 and c-myc (Walter et al., 2002). Furthermore, HGF have been identified as the most potent stimulator of glioma cell migration (Brockmann et al., 2003) with the supporting evidence demonstrating high content of HGF in extracts in brain tumor cyst fluid and brain tumor tissue and neutralizing antibodies against HGF reducing glioma cell migration upto 50% (Lamszus et al., 1998).

Another mode by which HGF/Met signaling contributes to malignancy is inhibition of tumor cell death and apoptosis. Activation of HGF/Met signaling along with PI3K and Akt

dependent pathways have been shown to protect GBM cells and tumor xenografts from DNA-damaging agents (Bowers et al., 2000), which was further confirmed by inhibiting HGF and Met in vivo (Abounader et al., 2002).

5. Centrosomal alterations in GBM

The connection between centrosomal abnormalities and cancer has been proposed since long (Boveri, 1914) and had been observed in different types of cancers accompanied by extensive chromosome aberrations (D'Assoro et al., 2002; Pihan et al., 2003). Although genetic and epigenetic changes resulting in mitotic dysregulation has been recognised in many types of cancer cells (Wang et al., 2004b), only countable number of studies described it in glioma (Dietzmann et al., 2001; Roymans et al., 2001; Reichardt et al., 2003; Klein et al., 2004; Katsetos et al., 2006; Saito et al., 2008). Centrosomes are regulated by many mitotic kinases like Aurora kinase A (AurA), Polo-like kinase 1 (Plk1) and Cyclin dependent kinase 1 (Cdk1) (Nigg, & Stearns, 2011), the inhibitors of which have been implicated as potential cancer therapeutics (Harrison, Holen, & Liu, 2009) . Plk1 is overexpressed in glioma (Dietzmann et al., 2001) and its inhibition for the selective killing of glioma stem cells enriched populations have been strategic (Lee et al., 2012); AurA overexpression has been shown in GBM (Loh et al., 2010; Lehman et al., 2012) and its expression levels have been correlated with prognosis (Barton et al., 2010). Apart from this, centrosome positioning in considered to determine cell polarity, which is widely disturbed in tumor cells (Humbert et al., 2003; Iden, & Collard, 2008). N-cadherin regulates centrosome reorientation in astrocytes, as the silencing of N-cadherin does not allow centrosomes to reorient in the direction of the wound in glioma cells (Camand et al., 2012).

6. Spleen tyrosine kinase (Syk)

Syk along with Zeta-activated protein of 70kDa (ZAP-70) constitutes an autonomous family of non-receptor tyrosine kinases (Zhu et al., 2007). It was first identified as a 40 kDa proteolytic fragment derived from a p72 tyrosine kinase present in spleen, thymus and lungs (Zioncheck et al., 1998). Originally cloned from porcine spleen (Taniguchi et al., 1991), it has been studied intensively in hematopoietic cells (B and T lymphocytes, natural killer cells, mast cells, macrophages and platelets) (Sada et al., 2001). Recently, Syk expression has also been detected in other cell types including hepatocytes, fibroblasts, endothelial cells and neuronal cells, depicting it as as ubiquitinous signaling molecule

(Yanagi et al., 2001). Apart from its presence in cytoplasm and plasma membrane (Ma et al., 2001; de Virgilio et al., 2004; Stupack et al., 1999), Syk is also present in nucleus, where it affects transcription (Wang et al., 2005). Syk at adherens junctions influences intercellular adhesion (Larive et al., 2009; Zhang et al., 2009) and in centrosomes, it affects cell division (Zyss et al., 2005; Sulimenko et al., 2006; Uckun et al., 2010; Xue et al., 2012).

Structurally, Syk is composed of two adjacent Src homology 2 (SH2) domains and a kinase domain interrupted by two interdomains A and B (Coopman, & Mueller, 2006) (Figure 41). SH2 domains are the structural motifs that bind phospho-tyrosine to promote protein-protein interactions (Liu et al., 2006). Each of the SH2 domains of Syk binds proteins containing the sequence pYXXL/I, where pY is phosphotyrosine, L and I are leucine and isoleucine respectively, and X denotes any amino acid. These SH2 domains are juxtaposed in such an orientation that allows them to interact simultaneously as a linker sequence, which contains two of these pYXXL/I cassettes separated by 6-10 amino acids (Fütterer et al., 1998). These high-affinity Syk-binding sites are known as immunoreceptor tyrosine-based activation motifs (ITAMs) because they are present in many receptors that are important in immune cells (Abram, & Lowell, 2007).

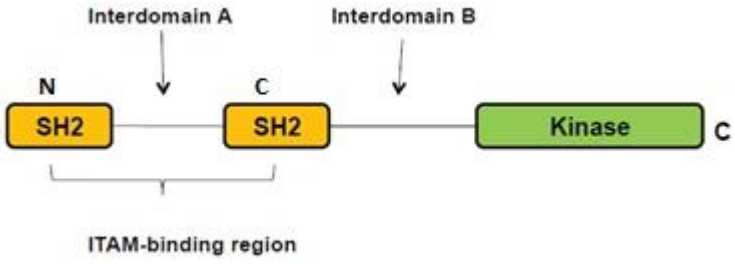


Figure 41. Structure of spleen tyrosine kinase (Syk) protein (Pamuk, & Tsokos, 2010). *Syk consists of two tandem SH2 domains and a tyrosine kinase domain. There are two interdomains A and B, where A lies between the two SH2 domains and B is between between C-terminal SH2 domain and the tyrosine kinase domain. Ligands for Syk activation are diphosphorylated ITAM of immunoreceptors, which binds to SH2 domains (N and C).*

Syk is activated on the binding of its tandem SH2 domains to biphosphorylated immunoreceptor tyrosine-based activation motifs present in the intracellular part of immunoreceptors, leading to its autophosphorylation and phosphorylation of its effectors (Coopman, & Mueller, 2006). Signals from the Syk-immunoreceptor complex are

transmitted further through the phosphorylation of adaptor proteins such as B cell linker (BLNK)/ SLP-65 (SH2 domain leukocyte-specific phosphoprotein 65 kDa), SLP-76, and LAT (linker for activation of T cells) (Mócsai, Ruland, & Tybulewicz, 2010; Yablonski, & Weiss, 2001). When phosphorylated, these proteins serve as scaffolds to which effectors dock with SH2 or other related phosphotyrosine-binding motifs. Effectors include members of the Tec-family of tyrosine kinases, lipid kinases, phospholipases and guanine nucleotide exchange factors that further propagate the signal allowing for the activation of multiple pathways including PI3K/Akt, Ras/ ERK, PLC/ NFAT, Vav-1/Rac, and the IKK/ NF-kB pathway (Geahlen, 2009; Mócsai, Ruland, & Tybulewicz, 2010).

Syk has been implicated in the pathogenesis of hematological malignancies including B-lineage leukemias and lymphomas; autoimmune, allergic and neoplastic disorders (D'Cruz, & Uckun, 2012; Pamuk & Tsokos, 2010). In addition, Syk has been associated with tumor biology. Its ecotopic expression in breast cancer (Coopman et al., 2000; Toyoma et al., 2003; Moroni et al., 2004), melanoma (Hoeller et al., 2005) and pancreatic adenocarcinoma (Layton et al., 2009) cells inhibits motility and metastasis (Sung et al., 2009) and therefore a tumor suppressor role was assigned (Coopman et al., 2000). Carcinomas exhibiting reduced Syk levels have also been documented, including classic Hodgkin lymphoma (Ushmorov et al., 2006), gastric (Wang et al., 2004a), ovarian (Dhillon et al., 2004), urinary bladder (Kunze, Krassenkova, & Fayyazi, 2008), hepatocellular (Yuan et al., 2006), oral squamous (Ogane et al., 2009) and cervical cancer (Zhao et al., 2010). Although the level of Syk expression alone is not prognostic for breast cancer survival, a signature set of Syk "interacting" genes effectively predicts patient outcome (Blancato et al., 2014).

Syk is also a mitotic kinase that localizes to the centrosome and interacts with the key centrosomal component γ-tubulin and affects mitotic progression, by regulating γ-tubulin mediated MT (Zyss et al., 2005; He et al., 2002), yet another way of its involvement in cancer. Its overexpression has been reported to lead to abnormal multipolar mitotic spindles and multinucleated cells (Coopman et al., 2000; Moroni et al., 2004), which may be due to Syk hyperactivity at centrosomes (Zyss et al., 2005). Suppression of tumor growth by the reintroduction of Syk in athymic mice due to aberrant mitosis and cytokinesis has been described in a legendary work by Coopman and collegues, where overexpression of wild-type Syk in a Syk negative breast cancer cell line inhibits tumor growth and metastasis. Tumor growth is reverted back upon overexpression of kinase-deficient Syk in Syk-positive breast cancer cell line (Coopman et al., 2000). Results from

the same group demonstrated that Syk is a centrosomal kinase, the centrosomal levels of which drop for the normal progression of mitosis and abnormal cell division state is adquired upon the sustained expression of Syk (Zyss et al., 2005).

The downstream signaling events modulated by activated Syk are still under investigation, but activation of PI3K/Akt pathway is a likely contributor. Acute myeloid leukemia and B cell lymphomas involves the activation of mechanistic target of rapamycin (mTOR), which lies downstream of Akt, correlated with presence of activated Syk (Carnevale et al., 2013; Leseux et al., 2006). Both retinoblastoma and B-cell chronic lymphocytic leukemia shows elevation in Syk expressing cells due to PKCδ and PI3K/Akt pathway (Baudot et al., 2009; Zhang et al., 2012; Gobessi et al., 2009). In diffuse large B cell lymphoma, Syk and Akt promotes B Cell Receptor (BCR) signaling by repressing the expression of pro-apoptotic protein Harakiri and upregulating of cholesterol biosynthesis (Chen et al., 2013).

OBJECTIVES

β-catenin is a key component of the cell-cell adhesion complex, a critical Wnt effector in the Wnt canonical pathway (Nelson & Nusse, 2004) and a component of the centrosome (Kaplan et al., 2004; Mbom, Nelson, & Barth, 2013). In the Wnt canonical pathway, β-catenin translocates to the nucleus and activates transcription of Wnt targets. Modulation of β-catenin at the adhesion complex is achieved in part by Tyrosine phosphorylation of β-catenin, which results in decreased adhesion and increased cell migration. Our group demonstrated that PhosphoTyrosine142 β-catenin (PTyr142 β-cat) is induced by HGF/Met signaling during axon outgrowth in hippocampal neurons (David et al., 2008). In this context, PTyr142 β-cat translocates to the nucleus and may regulate expression of genes involved in cell migration. Here, on one hand, I studied the involvement of HGF/Met/PTyr142 β-cat axis in axon outgrowth by identifying which are the target genes regulating axon morphogenesis.

On the other hand, β-catenin has been found at centrosomes and its phosphorylation in Ser/Thr acts by maintaining centrosome cohesion (Bahmanyar et al., 2008). On the contrary, β-catenin dephosphorylated on Ser/Thr and Wnt signaling activation promotes centrosome separation (Hadjihannas, Brückner, & Behrens, 2010), which is required during mitosis. I investigated the localization of PTyr142 β-cat at the centrosome and aimed at identifying the kinase(s) responsible(s) for maintaining this phosphorylated form at the centrosome.

The summarized objectives therefore are:

1. To investigate the target genes regulated downstream of HGF/Met and β-catenin signaling during axon morphogenesis in hippocampal neurons.

1.1. Role of the identified target genes (i.e., chemokines) in axon morphogenesis in hippocampal neurons.

- Role of recombinant chemokines in axon morphogenesis.
- Effects of blocking chemokine signaling (chemokine receptor antagonists and anti-chemokine antibodies).

1.2. To study chemokine expression by HGF and β-catenin/TCF signaling.

1.3. To study a possible involvement of chemokines in dorsal root ganglia neurite outgrowth.

2. To investigate the localization of PTyr142 β-cat at the centrosome.

2.1. To study the localization of PTyr142 β-cat in unsynchronized cells and in mitosis, using different fixation protocols.

2.2. To explore the role of β-catenin in the mechanism of cell migration and centrosome re-orientation during migration.

2.3. To search for the tyrosine kinases that could be associated to the centrosome and may be responsible for phosphorylating PTyr142 β-cat at the centrosome (Met and Syk).

MATERIALS

Table 1. Reagents

Product category	Product and manufacturer's details
Tissue culture reagents	**All reagents were purchased from Invitrogen:** ▪ Minimum Essential Media. ▪ Dulbecco´s Modified Eagle Medium containing 4.5 g/l and 1 g/l glucose. ▪ Neurobasal media. ▪ Hank's balanced salt solution. ▪ Opti-MEM. ▪ Glutamine. ▪ Sodium pyruvate. ▪ Horse serum. ▪ Foetal bovine serum. ▪ Penicillin/Streptomycin. ▪ N2 and B27 supplements.
Growth factors, recombinant proteins and inhibitors	Working concentrations ▪ Hepatocyte growth factor (*Peprotech)* -50 ng/ml ▪ Wnt-3a (*Millipore*) -100 ng/ml ▪ SU11274, c-Met inhibitor (*Sigma*) - 2 µM ▪ FH535, TCF inhibitor (*Sigma*) - 10 µM ▪ Piceatannol, Syk inhibitor (*Sigma*) - 200 µM ▪ SB225002 (CXCR2 antagonist)(*Tocris*) - 1.25 nM ▪ SB328437 (CCR3 antagonist)(*Tocris*) - 20 nM ▪ Chemokines: cc5, ccl7 - 10, 300 and 1000 ng/ml ccl20 (*R&D systems*) cxcl2 (*Peprotech*).

Table 1. continued

Product category	Product and manufacturer's details	
	▪ Neurotrophin-3 (R&D Systems)	- 50 ng/ml
	▪ Nerve Growth Factor (R&D Systems)	- 50 ng/ml
		Working concentrations
Miscellaneous	▪ Protein-G-sepharose beads (*Sigma*)	- 50%
	▪ Nocodazole (MT depolymerazing agent) (*Millipore*)	- 0.2 µM
	▪ Cytochalasin-D inhibitor of actin polymerization(*Sigma*)	- 1 µg/ml
	▪ Poly-D-lysine (*Sigma*)	- 500 µg/ml
	▪ poly-L-ornithine (*Sigma*)	- 1.5 µg/ml
	▪ Lipofectamine 2000 (*Invitrogen*)	
	▪ Hoescht 33258, cell permeable (*Sigma*)	

Table 2. Antibodies

Antibody	Manufacturer	Purpose of use	Dilution	Moleculr weight (kDa)
Primary Antibodies				
▪ APC	Santacruz Biotechnology	WB	1: 100	260
▪ α-tubulin	Sigma	WB/IF	1: 2000	50
▪ α-cxcl2	R & D systems	IF	1: 2.5	
▪ α-ccl20	R & D systems	IF	1: 2.5	
▪ βIII-tubulin	Covance	IF	1: 3000	50-55
• β-actin	Sigma	WB	1: 10,000	42

▪ γ-tubulin	Sigma	WB/IF	1:10,000/1:25	50
▪ β-catenin	BD transduction	WB/IF	0	92
▪ PTyr142 β-cat	Abnova	WB/IF	1: 500 1:1000/1:200	92
▪ Phopho-Tyr	Upstate	IF		
▪ Syk	SantacruzBiotechnology	WB/IF	1: 200 1: 500/1: 100	72
Secondary Antibodies				
▪ Anti-Mouse HRP	Sigma	WB	1: 10,000	
▪ Anti-Rabbit HRP	Sigma	WB	1: 10,000	
▪ Alexa488 fluor goat anti-mouse IgG	Invitrogen	IF	1: 600	
▪ Alexa595 fluor goat anti-rabbit IgG	Invitrogen	IF	1: 600	

Table 3. Oligonucleotides used to amplify transcripts of chemokine genes

Gene	Sequence	Length (bp)	Annealing temp (°C)
CXCL2	Forward: AGGGTACAGGGGTTGTTGTG Reverse: TTTGGACGATCCTCTGAACC	203	55
CCL5	Forward: ATATGGCTCGGACACCACTC Reverse: CCCACTTCTTCTCTGGGTTG	167	55
CCL7	Forward: GGGACCAATTCATCCACTTG Reverse: CCTCCTCAACCCACTTCTGA	172	55
CCL20	Forward: GCTTACCTCTGCAGCCAGTC Reverse: CGGATCTTTTCGACTTCAGG	169	55

Table 4. Real time (q PCR) probes used for chemokine genes

Gene	Reference number	Manufacturer´s details
CXCL2	Rn00586403	Applied Biosystems
CCL5	Rn00579590	Applied Biosystems

METHODS

1. Cell culture

1.1 Primary cell culture

Cells from primary cultures were obtained from rat embryonic as well as postnatal stages according to the protocols. All animal procedures were in agreement with guidelines of ethical committee for research in Spain, following European standards.

1.1.1 Hippocampal neurons

To isolate rat hippocampal neurons, pregnant rat females were sacrificed at 18-19 days of gestation, embryos were removed and hippocampi isolated and kept in cold Hank's balanced salt solution (HBSS). Subsequently they were trypsinized, triturated manually with narrowed pipettes and resuspended in DMEM (4.5 g/l glucose) supplemented with 10% HS, 1mM sodium pyruvate, 2mM glutamine and 20 units/ml of penicillin/streptomycin (P/S). After three hours of cells plating, media was replaced by DMEM (4.5 g/l glucose) supplemented with N2 and B27. Neurons were plated on poly-D-lysine (PDL) coated (500µg/ml) glass coverslips for immunocytochemistry (40 cells/mm^2) or on plastic (1000-1500 cells/mm^2) for RNA isolation, or lentiviral transduction.

Experimental design

Axon length quantification for different treatments: Cells were treated overnight from 1DIV to 2DIV with recombinant chemokines (CXCL2, CCL20, CCL5, CCL7 at different concentrations of 10, 300 and 1000 ng/ml), blocking antibodies against chemokines (α-CCL20 and α-CXCL2 at 40µg/ml) and chemokine receptor antagonists [SB225002 (antagonist of CXCR2) and SB328437 (antagonist of CCR3) at 1.25nM and 20 nM respectively], HGF 50ng/ml, SU11274 2µM or FH535 10µM.

Pervanadate was prepared as following:

First, 30% Hydrogen peroxide (H_2O_2) was diluted in sterile water in a ratio of 1: 83.3 (for example 12µl of H_2O_2 in 988 µl sterile water). Equal amounts of H_2O_2 mix and 100 mM stock of sodium orthovanadate were then mixed so as to dilute sodium orthovanadate to 50mM. Finally, pervanadate was added to the cell media to a final molarity of 1mM for 10-15 min at 37°C.

Semi-quantitative PCR (sq-PCR): Neurons were treated overnight from 1DIV to 2DIV with HGF 50 ng/ml.

Quantitative real time PCR (qPCR): For evaluating CXCL2 and CCL5 expression levels, cells were either treated from 1DIV to 2DIV (HGF 50ng/ml, SU11274 2µM, FH535 10µM) or from 3DIV to 4DIV (HGF 50 ng/ml) in the case of cells transduced with lentiviruses expressing shRNAs against β-catenin.

1.1.2 Striatal astrocytes

The method used for the isolation and purification of rat striatal astocytes was described by Etienne-Manneville (2006). Before starting the dissection, 100-mm tissue culture petridishes were coated with 1.5µg/ml poly-L-ornithine (PLO) for 1h at 37°C, washed once with water followed by pouring Astrocyte Culturing media (ACM) (DMEM 1g/l glucose, 2mM glutamine, 20 units/ml P/S and 10% heat-inactivated FBS) into the petridish. P0-P1 (Postnatal day 0-1) rat pups were taken, skulls opened and brains placed in a Petridish on ice containing HBSS solution. After removal of the meninges, striata were dissected and transferred to cold HBSS. Following one wash with HBSS, striata were transferred to warm ACM. They were cut into pieces and mechanically dissociated into a cell suspension with the help of narrowed glass pipettes (up and down at least 15 times). The suspension was allowed to sediment for 5 min, the top 1 ml suspension transferred to another tube and the rest of the suspension further dissociated. After decantation, suspension from this tube was pooled with the previous one and cell aggregates were discarded. This was later centrifuged at 800 rpm for 8 min, supernatant discarded and pellet resuspended in warm ACM. Cells were plated based on an estimation of 1 pup per petridish and were maintained at 37° in a moist 5% CO2, 95% air atmosphere. After one week, petridishes were flushed ten times with PBS to release unwanted debris, oligodendrocytes and microglial cells and returned with the fresh ACM to the incubator. They were ready to be used for experiments within 10-12 days.

Experiment design

Striatal astrocytes were used for immunocytochemistry, in vitro scratch assay and centrosome reorientation assay. Whether it was glass coverslips or plastic, surface was always coated with PLO (1.5 µg/ml) before plating. For immunocytochemistry, cells were plated at a density of 50 cells/mm^2 and treated with Pervanadate (15 min, 37°C) or with Piceatannol (Pic; 200µM). For scratch and centrosome reorientation assays, cells were plated at 300 cells/mm^2.

1.1.3 Dorsal root ganglia (DRG) explant culture

Before initiating the culture, M24 well plates were made ready by coating them with collagen (1:10 dilution in water; see below) for 1h at 37°C followed by aspiration and complete drying in the laminar air flow hood. To begin with the culture, P0 pups were sacrificed and spinal column was opened to access spinal cord. DRGs at either side of the spinal cord were picked. They are round with small pieces of nerve root on either side, which were trimmed. After isolation DRGs were kept in HBSS. One ganglia/M24 well was placed followed by addition of Neurobasal culture media supplemented with N2 and B27. After letting ganglia attach for 2-3h, Nerve Growth Factor (NGF) and Neurotrophin-3 (NT-3) were added to the media together with the treatments (CXCL2 1000 ng/ml; CXCL2 1000 ng/ml along with SB225002 1.25nM) for 48h.

Collagen purification from rat tails: 5 tails of 2-3 months old rats were taken. They were washed in 70% ethanol for 30 min and later for 15 min in sterile water. With the help of spencer tweezers inside a culture hood, tails were broken to expose collagen fibers. Fibres were cut and placed in 100 ml of acetic acid (diluted 1: 100 in sterile water). They were left under agitation for 2 days at room temperature (RMT). Finally the volume was brought up to 250 ml with acetic acid and the mix centrifuged at 13,000 rpm for 15 min in sterile tubes. Supernatant was aliquoted and freezed at -20°C.

1.2 Cell lines culture

All the cell lines used are available from ATCC and were maintained as recommended.

1.2.1 Human Embryonic Kindney 293T (Hek293T)

This cell line is rapidly growing and easy to transfect. It was grown in media containing DMEM (4.5 g/l glucose) with 10% FBS, 10% NEAA, 1mM sodium pyruvate and 20 units/ml P/S. Cells were maintained at 37°C and 5% CO2 and were split in 1:10 dilution on reaching 80-90% confluency. This cell line was used for lentiviral production, *in vitro* scratch assay and centrosomal immunostainings.

1.2.2 Glioblastoma cell lines (U251MG and U87MG)

These cell lines propagate well in media containing MEM, 10% FBS, 10% NEAA, 1mM sodium pyruvate, 2mM glutamine, 20 units/ml P/S. They were split at a dilution of 1:3 upon reaching confluency. These cells were exploited for immunocytochemistry and centrosome isolation.

For future use, cell line aliquots were frozen in storage vials in 850 µl of FBS followed by dropwise 150 µl DMSO in liquid nitrogen tanks.

2. Transfection

2.1 Transfection with Polyethilenimine

Polyethilenimine (PEI) cell transfection just like usual transfection did not include any serum or antibiotics. This method was used to prepare lentiviral particles in Hek293T cells. DNA was prepared in sterile NaCl 150mM as well as PEI solution, which was diluted to a final concentration of 10µM. PEI was added to DNA vial, after mixing for a minute and left to incubate for 10 min at RMT so as to form PEI-DNA complex. The resulting transfection mix was added dropwise to the Hek293T culture plate containing DMEM (4.5 g/l glucose). After 3h media was aspirated and full-containing media added.

2.2 Transfection with Lipofectamine-2000

The day before the transfection, cells were plated such that they are 90% confluent while initiating transfection. Lipofectamine-2000 (Invitrogen; LFT) was used with a LFT : DNA ratio different for different cell types. For Hek293T, ratio was 2 µl LFT : 1µg DNA for M24 well. However, for glioma cell lines, ratio was 1,5 µl LFT : 1µg of DNA for M24 well. To examine the transfection efficiency, Green fluorescent protein (GFP) DNA was co-transfected along with the specific DNA. In that case, amount of specific DNA was reduced to 0.8 µg/M24 well along with 0.3 µg of GFP DNA so as to make a total of 1.1 µg DNA/M24 well.

To start with the procedure, LFT was added to 50 µl of warm opti-MEM and incubated for 5 min at RMT. DNA (specific as well as GFP) were added to another vial of 50 µl opti-MEM. Both the vials were mixed and incubated at RMT for 15-20 min to form DNA-LFT

complexes. Before adding dropwise the transfection mix to the cells, M24 well media was aspirated and replaced by 200 ul DMEM 4.5 g/l glucose (Hek293T) or opti-MEM (Glioma cell lines). After 3-4 h of transfection, media was aspirated and replaced by the complete media. Within 10h, cells observed under the fluorescence microscope started to show GFP expression. Transfection efficiency observed for Hek293T was around 90% whereas for glioma cell lines it was ~60% .

3. Lentivirus production and transduction

3.1 Lentiviral constructs

shRNA primers specific for rat β-catenin were designed and chosen using algorithms available on Promega and Invitrogen websites. The primers selected correspond to 5_-GTTTGTGCAGTTGCTTTAT-3_ (shRNA#1) and 5_-GGGTTCCGATGA -TATAAAT-3_ (shRNA#2). Primers were designed for cloning into pSUPER plasmid using the *Bgl*II and *Hin*dIII sites. They were subcloned into the pLVTHM plasmid (from D. Trono, Geneva) together with the H1 promoter for RNA polymerase III.

3.2 Lentiviral production

Lentiviruses were produced as described (Naldini L et al., 1992). Vectors of 2nd generation were used (from D.trono). First set of vectors were pEIGW/pLVTHM, used to overexpress or inhibit gene expression, respectively. In order to produce lentiviral siRNA construct, pLVTHM was designed in such a way that H1 Pol III promoter can be easily replaced by H1-siRNA cassette from Psuper.retro.puro using EcoRI-Cla I.

Second vector psPAX2 codes for viral packaging proteins. It contains a very efficient promoter (CAG), which allows the expression of viral packaging compounds of which TAT protein, DNA polymerase and Reverse Transcriptase are the most relevant. Third vector, pM2G, codes for the virus envelope.

For lentiviral production shRNA vectors specific for rat β-catenin were transfected into Hek293T cells together with the plasmids psPAX2 and pMD2G, as previously described (David et al., 2008). Briefly, Hek293T cells were plated at high density on 0.1% gelatin coated 100mm tissue culture plates, so that the next day they were 90% confluent. At confluency they were transfected with:

Vector pWPI/pLVTHM	20µg
PSPAX2	13µg
pM2G	7µg

Tranfection was performed by PEI transfection protocol. Hek293T cells were allowed to produce lentiviruses for 48h followed by the centrifugation of medium at 4000g for 5 min, discarding dead cells present in the supernatant and further clarification through 45µm filter. Lentiviruses were concentrated by centrifugation at 50,000g for 3h. To further obtaining higher infection efficiency, lentiviruses were finally suspended in a solution of 1% BSA in PBS. The biological titer of the viral preparation was expressed as a number of transducing units per ml (TU/ml) and was determined by transducing Hek293T in different amounts. After 48h incubation, the percentage of GFP positive cells were counted and viruses at $5x10^8$-$1x10^9$ TU/ml were used in the experiments.

3.3 Transduction

For lentiviral transduction different cell types were transduced at different times and were left with the viruses for different incubation times**.** ***Hippocampal neurons*** were infected after 3h of plating, incubated overnight and media changed on DIV1. Cells were lysed and run for Western blotting to check the percentage of β-catenin silencing. ***Striatal astrocytes*** were transduced for centrosome reorientation experiment on achieving 60% confluency for 11-12h, proceeded by media change.

4. Western blotting

For cell lysis, cells were first washed with PBS, lysed in 2% SDS and 62.5 mM Tris HCl pH 6.8, buffer and collected with a scrapper. Protein was quantified by Lowry assay. Cell lysates were resolved on Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) (usually 8% or 10% resolving gel and 5% stacking gel). Proteins were electrotransferred from the gel to a polyvinyledene difluoride (PVDF) Immobilon-P transfer membrane (Millipore, Billerica, MA) using a semi-dry Trans-Blot apparatus (Hoefer, Amersham Pharmacia Biotech). Membranes were blocked with Tris-buffered saline with Tween 20 (TBS-T) (20mM Tris-HCl pH 7.4, 150mM NaCl and 0.05% Tween 20) containing 5% non fat dry milk for 1h at RMT, washed with TBS-T and incubated overnight with the primary antibody (diluted in TBS-T as per manufacturer´s instructions). Peroxidase-conjugated secondary antibodies (1:10000; Sigma) diluted in 5% milk- TBS-T were incubated with the membrane for 45 min. Membranes were developed with either

Enhanced chemiluminescence (ECL) (Thermoscientific, USA) or Supersignal (Millipore, Billerica, MA) on Super RX Fuji medical X-Ray film with the help of Optimax X-Ray film processor (Germany). Protein loadings were quantified with respect to β-actin (used as the loading control) using scion image software.

5. Immunofluorescence

Cells were plated on PDL-coated coverslips (500 µg/ml for hippocampal neurons; 25 µg/ml for Glioma and Hek293T cell line) and PLO coated coverslips (1.5 µg/ml for striatal astrocytes) followed by washes with water and addition of media as per cell type. Most of the treatments were overnight (HGF 50 ng/ml; SU11274 2µM; FH535 10µM; recombinant chemokines, antibodies against chemokines) except (piceatannol 200µM for 6h; pervanadate for 15 min). After the treatments cells were fixed either fixed with 4% Paraformaldehyde (PFA) for 15-20 min at RMT, with ice cold methanol (for centrosomal immunostainings; 5 min at -20°C) or with detergent fixation (that allows optimal cytoskeletal immunostaining: 0.3% PFA, 0.2% glutaraldeyde, 0.2% Triton X-100 and 10 mM EGTA pH 7.2; 10 min at 37°C, (as described by Ciani & Salinas, 2007). After fixation, cells were washed thrice with Phosphate buffered saline (PBS) and later blocked and permeabilized with PBS containing 5% FBS, 5% HS, 0.2% Glycine and 0.1% Triton X-100 for 1h at RMT. In methanol-fixed cells Triton X-100 was omitted. This step was followed by incubation with primary antibodies (βIII-tubulin, PTyr142 β-cat, γ-tubulin, APC, α-tubulin, Phospho-Tyrosine (PTyr)) overnight at 4°C diluted in the same blocking solution. Subsequently, coverslips were washed with PBS and provided with secondary antibodies coupled to Alexa-488 or Alexa-564 (diluted 1:600 in blocking buffer) for 45 min at RMT in the dark. Finally, cells were washed with PBS and mounted on Moviol (Moviol 488, glycerol 50%, 0.2M Tris-HCl buffer pH-8.5). Images were obtained using an inverted Olympus IX70 microscope (10x, 0.3 numerical aperture (NA; 20x, 0.4 NA; 32x, 0.4 NA) equipped with epifluorescence optics and a camera (Olympus OM-4 Ti). FluoView^{TM} FV1000 Confocal Microscope (60x) was used to obtain centrosome immunostaining pictures and z-stacks. DPM Manager software was used together with Olympus IX70 microscope, whereas FV10-ASW was used for confocal images.

6. Immunofluorescence intensity quantification

Relative IF intensities of PTyr142 β-cat (immunostained in red) and γ-tubulin (immunostained in green) in each single cell (U87MG and U251MG cell lines) were measured from merge images (overlapping PTyr142 β-cat and γ-tubulin immunostainings) in different conditions using the RGB plugin of ImageJ software (freely available online). The ratio of PTyr142 β-cat immunofluorescence intensity to γ-tubulin immunofluorescence intensity was also calculated.

7. Axon length and branching quantification

Hippocampal neurons cultured on glass coverslips were provided with treatments 1DIV to 2DIV as mentioned before. Neurons were immunostained using mouse anti-βIII tubulin antibody (Covance) and Alexa-488-couple anti-mouse IgG. The axon was identified as the longest neurite at this stage (2DIV) of the hippocampal neuron development. Axon length was measured using Adobe Photoshop software. Axon branching was measured by counting Total Axonal Branch Tip Number (TABTN) (Yu, & Malenka, 2003). Typically, 15–20 neurons were measured/condition in ≥ three independent experiments.

8. Scratch assay

Scratch / wound healing assay is a method to study cell migration *in vitro* adapted from (Liang, Park, & Guan, 2007). This method is based upon the idea that upon creating of an artificial gap or "scratch" on a confluent cell monolayer, the cells on the edge will migrate towards the scratch until new cell to cell contacts are established again. The standard procedure includes the comparison of acquired images of scratch when it is created (T0) and later time intervals depending on the cell type in order to study the differences in migration upon different treatments.

8.1 Scratch assay on non-transfected cells

This was performed using striatal astrocytes by allowing them to grow on coverslips. On reaching confluency, scratch was created using a p200 pipette tip. Debris was removed followed by one wash with the ACM media. Phase-microscopy images were taken at this point of time as time 0 (T0) for all the conditions including control. Next, cells were treated with HGF50 ng/ml with or without Met inhibitor (SU 11274 2μM). After 24 hours

of scratch (T24), images were re-acquired and the percentage reduction in the width of scratch at T24 with respect to T0 was calculated with the assistance of Image J software. Width of the scratch in each image was the average of three measurements taken randomly. For each condition, 20 images were evaluated.

8.2 Scratch assay on transfected cells

To study cell migration on transfected cells using the scratch/wound-healing assay, Hek293T cell line was used as a cell model. Cells were grown on coverslips (coated with 25ug/ml PDL) until they reached a confluency of 80%. Cells were transfected using LFT 2000 reagent and GFP plasmid DNA (0.3 µg) together with WT (0.8 µg) or Tyr142Phe (0.8 µg) β-catenin plasmids . These plasmid´s cDNAs were obtained and cloned into pcDNA3.1 as described (Piedra et al., 2003) and were a kind gift of Dr. Mireia Duñach. After 6-7 hours of transfection, scratch was created, cells washed with Hek293T media and treated. Images acquired at T0 under fluorescent microscopy were compared with final images taken at 24 hours (T24) using Image J software.

9. Centrosome reorientation assay

In a confluent astrocyte monolayer, centrosome is usually located near the nucleus at a random position. When a scratch in the monolayer is produced, centrosome localizes ("re-orients") in front of the nucleus facing the wound (Etienne-Manneville, & Hall, 2001) . Having plated rat striatal astrocytes at a density of 300 cells/mm^2 on glass coverslips and letting them reach around 60% confluency, lentiviruses carrying the shRNA against β-catenin or scrambled shRNA were added for 11-12h (0DIV). After this period, lentivirus were removed and fresh complete media added. Non-infected coverslips were used as controls. At 5DIV after lentiviral transduction, the scratch was created using a 200 ul pipette tip for 10h followed by fixation with 4% PFA and immunostaining using anti-γ-tubulin antibodies. Hoescht was used to stain the nucleus. Images were captured (40x , 0.55 NA) using Olympus 1X51 microscpe. Only the front row of cells at the edge of to scratch was analyzed. To score which cells present their centrosomes oriented towards the wound, the cell is divided into four equal quadrants starting from the center of the nucleus and one quadrant facing the scratch, as described (Etienne-Manneville, 2006). Thus, a cell was considered as "reoriented" if the centrosome was located in the quadrant facing the scratch. Number of cells re-oriented vs non-oriented were calculated.

10. Mitosis arrest

Mitosis arrest was carried out in U251MG and U87MG glioma cell lines. Procedure started from plating cells on coverslips coated PDL at a density (50 cells/mm^2) in complete media. After a few hours of plating, cells were deprived of serum for 20h followed by complete media addition for the next 16-20h. Next, cells were fixed with methanol and immunostained using antibodies against PTyr142 β-cat, γ-tubulin and α-tubulin. Hoescht was used to stain the nucleus. Images were captured using an inverted Olympus IX70 microscope (32x, 0.40 NA) equipped with epifluorescence optics and camera (Olympus OM-4Ti) and confocal microscope FV10-ASW (60x objective).

11. Centrosome isolation

Centrosomal isolation was performed as previously described (Hsu, & White, 1998) with minor modifications. Exponentially growing striatal astrocytes and U251MG cells were treated with Nocodazole (0.2 μM) and Cytochalasin D (1μg/ml) for 1h at 37°C to depolymerize actin and microtubule filaments. Cells were harvested after trypsinisation and lysed in 4 ml lysis buffer composed of 1 mM Hepes (pH-7.2), 0.5% Igepal, 0.5 mM $MgCl_2$ and 0.1% β-Mercaptoethanol containing complete protease inhibitor and phosphatase inhibitors (40mM β-Glycerophosphate, 1mM Sodium Orthovanadate and 25mM Sodium Fluoride). Swollen nuclei and chromatin aggregates were removed by centrifugation at 5500 rpm for 10 min and supernatant was filtered through 70 μm mesh. To the filtered lysate 9mM Hepes and DNAse I (2 units/ml) were added and left to incubate on ice for 30 min. Typically, 4 ml of the lysate was then underlaid with 0,5 ml of 60% sucrose solution (60% w/w sucrose in 10mM Pipes pH-7.2, 0.1% Triton X-100, 0.1% β-Mercaptoethanol) and centrifuged at 12,500 rpm for 30 min (JA14 rotor, Beckman coulter Avanti J-26 XP centrifuge) in order to sediment the centrosomes into sucrose cushion . The protocol was continued by discarding the top 3 ml of supernatant and the remaining 1,5 ml was loaded into a discontinuous gradient consisting of: 500 μl of 70% sucrose, 300 μl of 50% sucrose and 300 μl of 40% sucrose in the above Pipes buffer. Tubes (Beckman polyallomer centrifuge tubes, 2.2 ml) were centrifuged at 35,000 rpm for 30 min (TLS55 rotor, Beckman coulter optima ultracentrifuge). Fractions were collected from the top: 500μl for fraction number 8 (top) and the rest of the fractions, named 7-0, had 200μl/fraction. Remaining stuff not collected as it was too viscous. Fractions were

diluted in 10mM Pipes buffer, pH 7.2 to make each of them upto 1ml. Trichloracetic acid (6.5%, final concentration) was added to all the fractions in order to precipitate the protein and incubated for 20 min at RMT followed by centrifugation and removal of supernatant. Pellets were dissolved in loading buffer (without β-Mercaptoethanol) with the addition of Tris pH 8. After boiling the samples, they were loaded into 10% SDS-PAGE and Western blotted.

12. Luciferase assay

To determine β-catenin transcriptional activation status, luciferase assay was performed following transfection of the TOP-Flash plasmid that carries a synthetic promoter containing three copies of the TCF-4 binding site upstream of a firefly luciferase reporter gene (a kind gift of Dr. Garcia de Herreros, IMIM, Barcelona, Spain). Hek293T cells were plated at a density of 100 cells/mm^2 in 24-well plates and transfected with Lipofectamine 2000 on the day next to plating. Treatments were given on the following day for 24 h (HGF 50 ng/ml; Wnt-3a 100 ng/ml; FH535 8μM and SU11274 2 μM). After 48 h of transfection, cells were lysed in lysis buffer 25mM glycylglycine, pH 7.8, 15mM Mg_2SO_4, 1% Triton X-100, 5mM EGTA and left for rocking on ice for 15 min. Luciferase activity in the lysates was determined in Luciferase Buffer (25 mM glycylglycine, 15 mM $KHPO_4$, pH 7,8, 15 mM Mg_2SO_4, 1% Triton X-100, 5 mM EGTA, 1 mM dithiothreitol, 2mM ATP, 100 mM acetyl-coenzymeA (Ac CoA), and 100mM luciferine) using a microplate luminometer. Luciferase activity was normalized for the total protein concentration measured by Lowry method in each well.

13. Polymerase chain reaction (PCR)

Chemokine expression was analyzed by semi-quantitative (sq) PCR and real-time PCR (qPCR) in hippocampal neurons control or treated with HGF 50 ng/ml; SU11274 2μM; FH535 10μM or pervanadate (overnight from 1DIV to 2DIV).

13.1 RNA isolation and cDNA synthesis

Primarily, Mesenger RNA (mRNA) was extracted using Nucleospin RNA II Kit (Macherey-Nagel) as per manufacturer's instructions. RNA was quantified using nanodrop (ND-1000) spectrophotometer. mRNA was reverse-transcribed (RT) to cDNA using random hexamers and Superscript II reverse transcriptase (Applied Biosystems) in a PCR device (25°C for 10 min, 42°C for 60 min, and 95°C for 5 min). Negative control RT-minus reactions were carried out to confirm absence of DNA contamination in RNA.

13.2 Semi-quantitative PCR (sq-PCR)

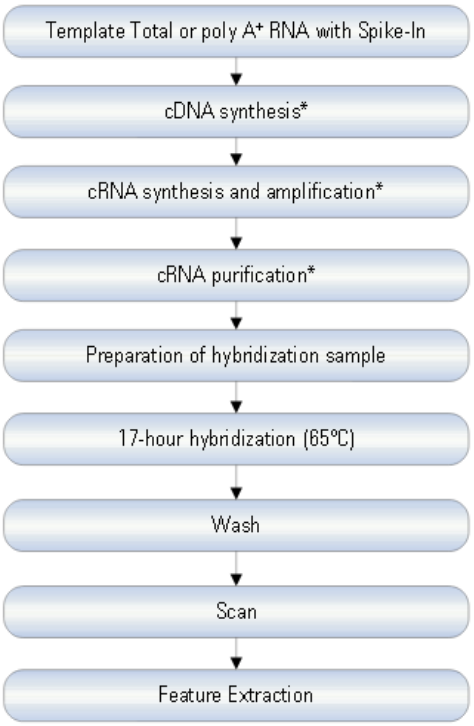
Equal volumes of cDNA were amplified by PCR using a couple of specific primers expanding at least two exons within the gene of interest. Sequences of the primers used are mentioned in Table3. 10µl aliquots were taken from 25, 30, and 35 PCR cycles (CXCL2 and CCL7), 30, 34, and 38 PCR cycles (CCL5) or 24 and 28 PCR cycles (CCL20). PCR products were analysed in 3% agarose gel. Densitometry of the DNA bands was performed using the Scion Image software (Scion Corporation) and comparing measurements from non-saturated PCR products. Loading was checked by amplification of the GAPDH transcript. Transcript analysis was performed from at least three independent samples.

13.3 Real time PCR (qPCR)

qPCR amplification was carried out taking cDNA (processed from 1µg of RNA) as the template along with gene primers for rat chemokine genes (CXCL2 and CCL5) and an endogenous control (GAPDH in an ABI prism 7000 sequence detection system (Applied Biosystems). This detection system determines the absolute quantity of a target nucleic acid sequence in a test sample by analyzing the cycle to cycle change in fluorescent signal as a result of amplification being carried out. Expression of the transcript levels were analysed using a FAM-labeled CXCL2 or CCL5 probes and compared to that of GAPDH, used as a loading control. The results were obtained in the form of a Ct value (cycle threshold) representing the cycle number of the PCR during which the exponential growth growth of PCR product starts. Therefore, the higher the expression of the gene in the sample, lower will be the Ct value. The relative quantity of mRNA for every gene was calculated as: ΔCt = Ct of the target gene – Ct of GAPDH gene; $\Delta(\Delta Ct)$ = ΔCt of sample - ΔCt of control. Relative mRNA levels were calculated and expressed as fold induction over contralateral controls (value = 1.0) using the formula $2^{-\Delta(\Delta Ct)}$. 1µl aliquot of each cDNA was used per well and samples were run in triplicate.

14. Array processing and Array data analysis

RNA from control and HGF 50ng/ml treated samples was obtained from 2DIV rat hippocampal neurons. Array experiments were performed in collaboration with Drs. A. Dopazo and A. Benguria (CNIC, Madrid, Spain).



RNA samples (800 ng) were amplified and labeled with Cy3-CTP using the One-Color Microarray-Based Gene Expression Analysis Protocol (Agilent Technologies, Palo Alto, CA, USA) and hybridized to Whole Rat Genome Microarray 4 × 44K (G4131F, Agilent Technologies). Raw data files from the scanned arrays were extracted using Feature Extraction software version 9 (Agilent Technologies). Data files from Feature Extraction software were imported into GeneSpring® GX software version 9.0. (Agilent Technologies). Quantile normalization was performed (Bolstad et al., 2003) and expression values (log2 transformed) were obtained for each probe. Probes were also flagged (Present, Marginal, Absent) using GeneSpring® default settings. Probes with signal values above the lower percentile (20th) and flagged as Present or Marginal in 100% of replicates in at least one out of the two conditions under study, were selected for further analysis. Paired t-test was performed between conditions to be tested for

differential expression analysis. Raw p-values were corrected for false discovery rate control using Benjamini–Hochsberg's method (Benjamini and Hochberg, 1995). The steps followed while performing array shown in the flow chart.

15. Statistical analysis

Significance was calculated by the Student T test (paired). Asterisk (*) indicates statistical significance compared to the corresponding untreated control and hash (#) compared to stimulated controls.

RESULTS

Chapter 1.

HGF signaling regulates chemokine expression in developing hippocampal neurons

1.1 Chemokines of the CC and CXC families are upregulated in hippocampal neurons upon stimulation with HGF.

To identify the genes regulated downstream to HGF signaling during axon morphogenesis, microarray analysis was performed in collaboration with the Genomic Unit of CNIC (Madrid, Spain). Total RNA was obtained from rat hippocampal neurons untreated or treated with HGF (50 ng/ml for 24h from 1DIV to 2DIV; n=4). RNA was amplified, labeled and hybridized to Whole Rat Genome Microarray 4 × 44K (Agilent Technologies) representing about 41000 rat genes and transcripts.

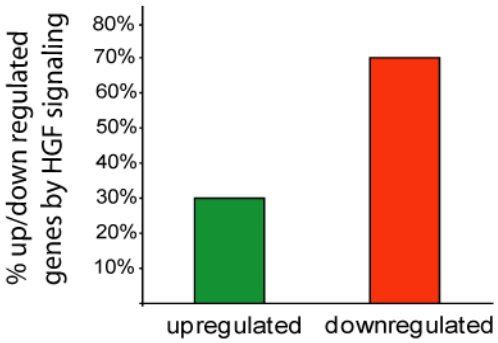
Array data analysis, upon discarding noise signals, provided 27982 probes that were statiscally (t-paired test) analyzed. Normalized expression signal for individual genes in "Control" and "HGF" was in log2 scale. Fold changes were calculated (HGF vs control sample) in lineal scale (1 meaning no change). p-values and corrected p-values (corrected for False Discovery Rate) were calculated. Differentially expressed genes were filtered as showing corrected p values <0.25 (showing at this stage 1792 genes of the total genes in the array). In addition, among these genes only those showing fold changes >1.5 were selected, resulting in a list of 233 genes/probes containing both upregulated and downregulated genes in response to treatment with HGF.

To our surprise, array results pointed to several chemokines of the CC and CXC families as a group of genes upregulated by HGF signaling. In addition, chemokine genes were ranked among the genes showing higher fold increase values vs control samples. As shown in (Figure 1A), array results indicate that upregulated genes in HGF-treated vs control hippocampal neurons include CCL5, CCL7, CCL13, CCL20, CCL19 and CXCL2. Colony-stimulating-factor-1 (CSF-1) and Bone Morphogenetic Protein-6 (BMP-6) were other secreted molecules identified by the array as upregulated by HGF signaling, with fold increases vs. control neurons of 2,07 and 1,78, respectively (p values = 0,002 and 0,012, respectively).

(A)

Gene	Fold Change	p value	p corrected
CCL5	4.086	0.01571	0.24870692
CCL7	3.700	0.00278	0.18946882
CCL13	3.358	0.00660	0.21122499
CCL20	2.730	0.01363	0.24347675
CXCL2	2.695	0.00580	0.21108855
CCL19	1.571	0.00266	0.24479723

(B)



(C)

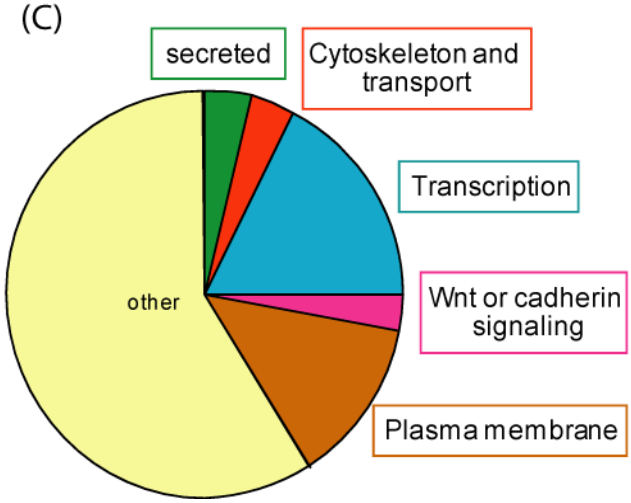
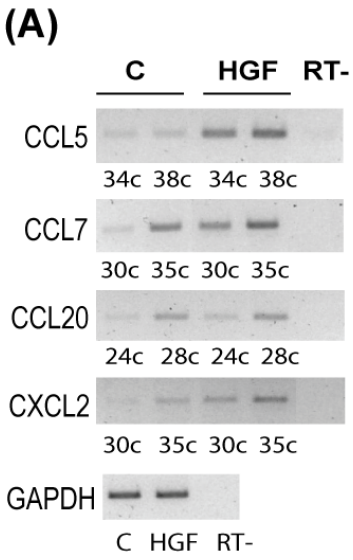


Figure1. Array data analysis revealed the genes upregulated downstream to HGF signaling in hippocampal neurons. (A) *Table summarizing the chemokine genes, which amongst the other genes (not mentioned) showed a higher fold increase upon HGF stimulation (50 ng/ml, 24h, 1DIV to 2 DIV) vs control in hippocampal neurons.* ***(B)*** *Graphical representation of the percentage of genes identified downstream to HGF signaling categorised as upregulated or downregulated upon 24h treatment with HGF (50 ng/ml) vs control in hippocampal neurons. Of the total genes, 30% were upregulated and 70% were downregulated.* ***(C)*** *Pie chart classifying the target genes downstream to HGF signaling deduced from the array based upon their function. Of all, 4% were secreted molecules, 3.2% referred as cytoskeletal and transport related proteins, 13% included plasma membrane proteins and receptors, 2.8% were the Wnt or cadherin/catenin signaling components and 17% were transcriptional regulators and nuclear proteins.*

The majority of the identified genes were downregulated upon HGF signaling (70%) with only a 30% of genes being upregulated (Figure 1B). We have attempted a simple functional classification of the target genes listed in the array (Figure 1C): 4% of the genes

can be classified as secreted molecules (all chemokine genes, BMP6, CSF-1), 3.2% of genes refer to cytoskeletal and transport related proteins (Microtubule Associated Protein-9 and -2, ankyrin2, tubulin β6, kinesin family member 1A), 13% of genes include plasma membrane proteins and receptors (Fas, CD83, syntaxin-11, SV2B, ICAM1), 2.8% of genes are components of Wnt or cadherin/catenin signaling (APC, TCF12, cadherin-4, protocadherin-7, and protein phosphateses like PTPN11, PTPRD, PPP1R9A) and 17% of genes refer to transcription regulators and nuclear proteins (including FOSL1, KLF7, cyclin-dependent-kinase inhibitor 1 and 2B, splicing factor SFRS2IP, histone 1 family member 4, EIF3A, Calcium/Calmodulin kinase 4, REST corepressor 1).



(B)

Gene	Fold Change ± sem
CCL5	2.970 ± 0.67
CCL7	1.692 ± 0.34
CCL20	1.572 ± 0.31
CXCL2	2.484 ± 0.72
CCL19	1.487 ± 0.17

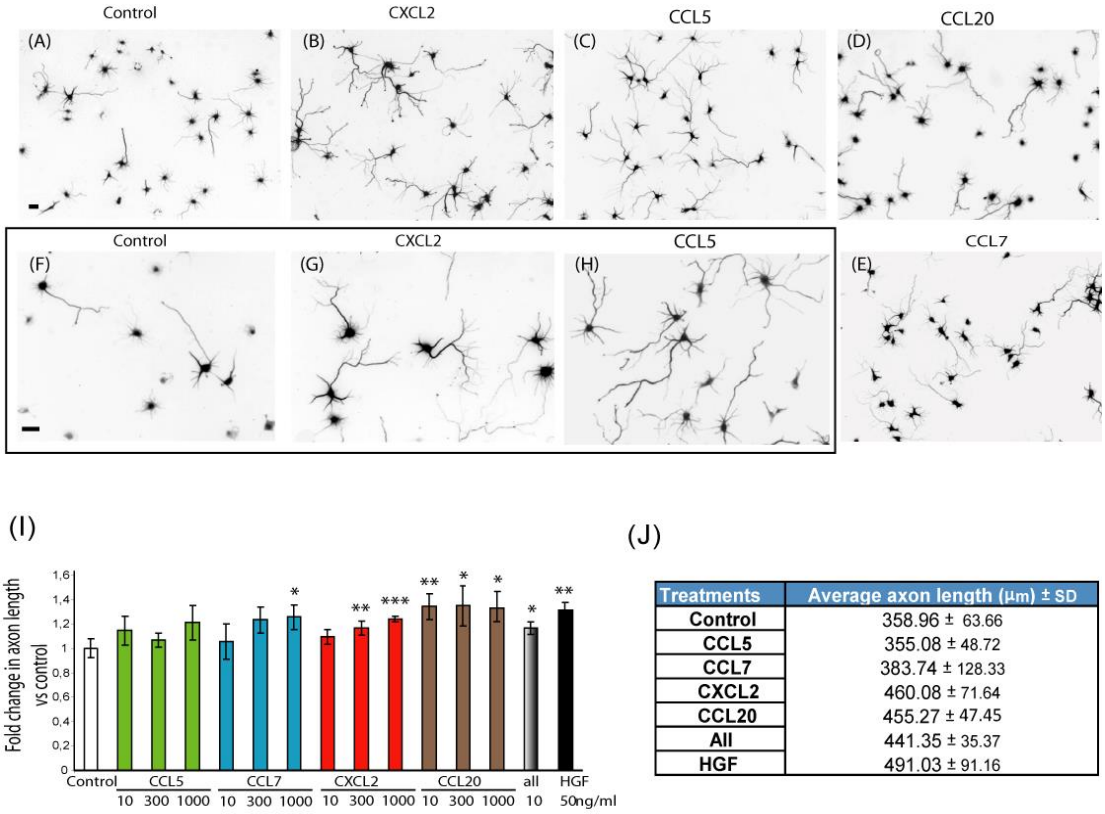
Figure 2. Upregulation of chemokines genes upon HGF stimulation demonstrated by sq-PCR. (A) *sq-PCR was run for chemokine genes (CCL5, CCL7, CCL20 and CXCL2) comparing their expressional levels in control and HGF (50 ng/ml, 24h, 1DIV to 2DIV) treated hippocampal neurons. For different genes, PCR was run for different number of cycles (as mentioned). GAPDH was used as the loading control (Image corresponds to 30 pcr cycles). RT- indicates the samples in which reaction was run without RT enzyme.* ***(B)*** *Summary table for the quantification of sq-PCR showing the fold change achieved for chemokine genes upon treatment with HGF vs control ± sem. c corresponds to the number of pcr cycles*

To confirm the array results, we ran sq-PCR for the chemokine genes upregulated in the array (Figure 2A). A summary of the changes in the expression obtained upon normalization of the intensity of the product band in HGF vs control sample are shown in Figure 2B.

In-silico analysis of the region 2 kb upstream of the ATG in the identified chemokine genes, confirmed the presence of several copies of TCF binding site **A/T CAAAG G/C** or the reverse and complementary sequence **G/C CTTTG A/T** (Van de Wetering et al., 1991; Giese, Amsterdam,& Grosschedl, 1991) (data not shown). The identification of the chemokines as molecules regulated by HGF signaling in hippocampal neurons, prompted us to investigate their possible role in the regulation of axon morphogenesis.

1.2 Chemokines promote axon outgrowth in hippocampal neurons

In order to test a possible role of chemokines in the regulation of axon outgrowth as suggested by the array data, different concentrations of recombinant chemokines were tested (CCL5, CCL7, CCL20, CXCL2 ranging from 10-1000 ng/ml) in cultured hippocampal neurons. Treatments were applied to the culture from 1DIV to 2DIV. Upon fixation, anti βIII-tubulin immunostaining was used to reveal the neuron morphology. At this stage (2DIV) of the hippocampal neuron culture, the longest neurite is unequivocally identified as the axon, while the future dendrites remain as short processes. Axon length measurements of neurons treated with different concentrations of chemokines indicated that CCL7 (1000 ng/ml), CXCL2 (300 and 1000 ng/ml) and CCL20 (10, 300 and 1000 ng/ml) significantly increase axon length versus untreated control neurons (Figure 3I, J). CCL5 showed a tendency to increase axon length, but results were not statistically significant.



(J)

Treatments	Average axon length (µm) ± SD
Control	358.96 ± 63.66
CCL5	355.08 ± 48.72
CCL7	383.74 ± 128.33
CXCL2	460.08 ± 71.64
CCL20	455.27 ± 47.45
All	441.35 ± 35.37
HGF	491.03 ± 91.16

Figure 3. Recombinant chemokines increase axon length. (A-H) *Hippocampal neurons (2DIV) control and treated with CXCL2, CCL5, CCL7, CCL20 (1000 ng/ml) immunostained using anti βIII-tubulin antibody. Immunofluorescence pictures have been inverted for a better visualization of the neuron morphology.* ***(A-E)*** *Images acquired at 10x and* ***(F-H)*** *at 20x. Bars = 30 µm.* ***(I)*** *Graphical representation comparing axon length achieved on treating hippocampal neurons with different recombinant chemokines at different doses to axon length of control neurons. "All" refers to the cocktail of the four chemokines (10ng/ml each). (J) Table summarizing the average axon length of hippocampal neurons quantified in µm, following treatment with the corresponding chemokine (1000 ng/ml), HGF (50 ng/ml) or a cocktail of chemokines at 10 ng/ml versus untreated neurons. * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$. n=10.* shows the statistical significance versus untreated controls.*

The increase in axon length achieved after treatment with chemokines was in the range of the increase produced by HGF stimulation (50 ng/ml, 24h) (David et al., 2008), of around a 30-35% over the length of axons of untreated neurons. We reasoned that perhaps the simultaneous treatment with a mix of chemokines could produce a bigger increase in axon

length. We treated hippocampal neurons with a cocktail of all the same chemokines at 10 ng/ml, which however promoted axon outgrowth in a similar manner (Figure 3).

Among the chemokines tested, it was noted that CXCL2 seemed to promote axon arborization. Therefore, axon branching was measured by counting Total Axonal Branch Tip Number (TABTN) (Yu, & Malenka, 2003). Axon branching measurements confirmed that CXCL2 not only promotes axon outgrowth but also significantly increases axon branching at all the concentrations tested (10-1000 ng/ml) (Figure 4). The other chemokines did not produce significant axon branching at the concentrations used. Together, these results demonstrate that chemokines promote axon outgrowth (CCL5, CXCL2 and CCL20) as well as axon branching (CXCL2) in developing hippocampal neurons.

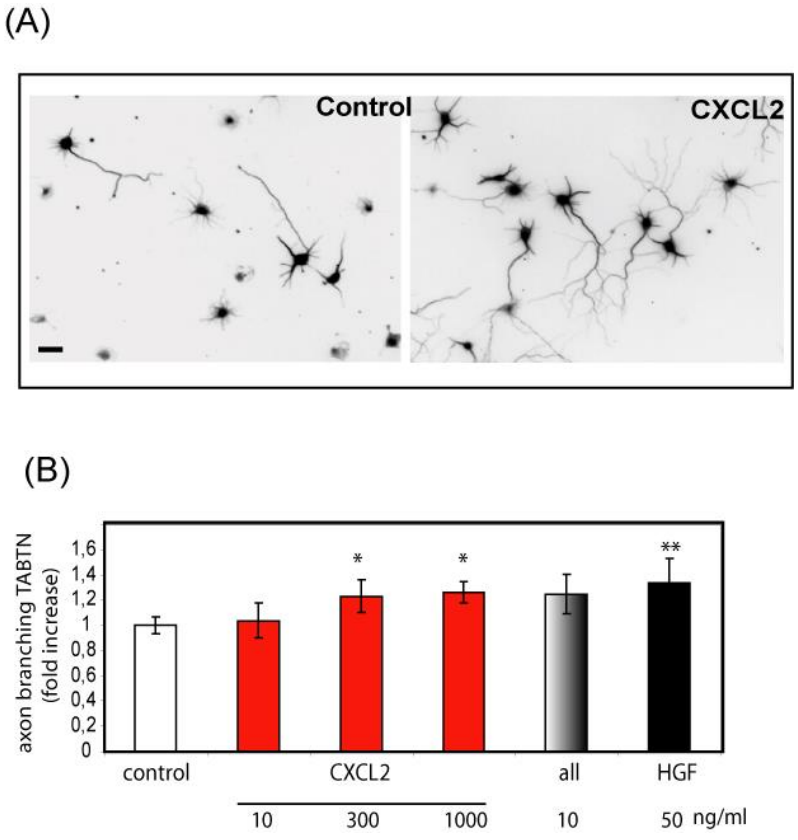


Figure 4. CXCL2 induces axon branching in hippocampal neurons. (A) *Images acquired at 20x showing control and cxcl2 treated hippocampal neuron immunostained with anti βIII-tubulin. Bars = 30 µm.* ***(B)*** *Graphical representation showing fold increase in axon branching in CXCL2-treated neurons (10, 300 and 1000 ng/ml) vs. control. "All" represents the cocktail of all the recombinant chemokines (10 ng/ml each). HGF (50 ng/ml) was used as the positive control. * $p \leq 0.05$.*

Having proven that chemokines can promote axon morphogenesis when added to the culture medium in hippocampal neurons, we decided to block chemokine signaling to confirm a role of chemokine signaling downstream of the HGF signaling cascade. We focused ourselves on CCL20 as this chemokine produced a significant increase in axon length at all the tested concentrations and in CXCL2 because of its unique effect on axon branching. Thus, we used blocking antibodies against the chemokines (anti-rat CXCL2 and anti-rat CCL20) and chemokine receptor antagonists (SB225002 and SB328437) (Figure 5 and 6).

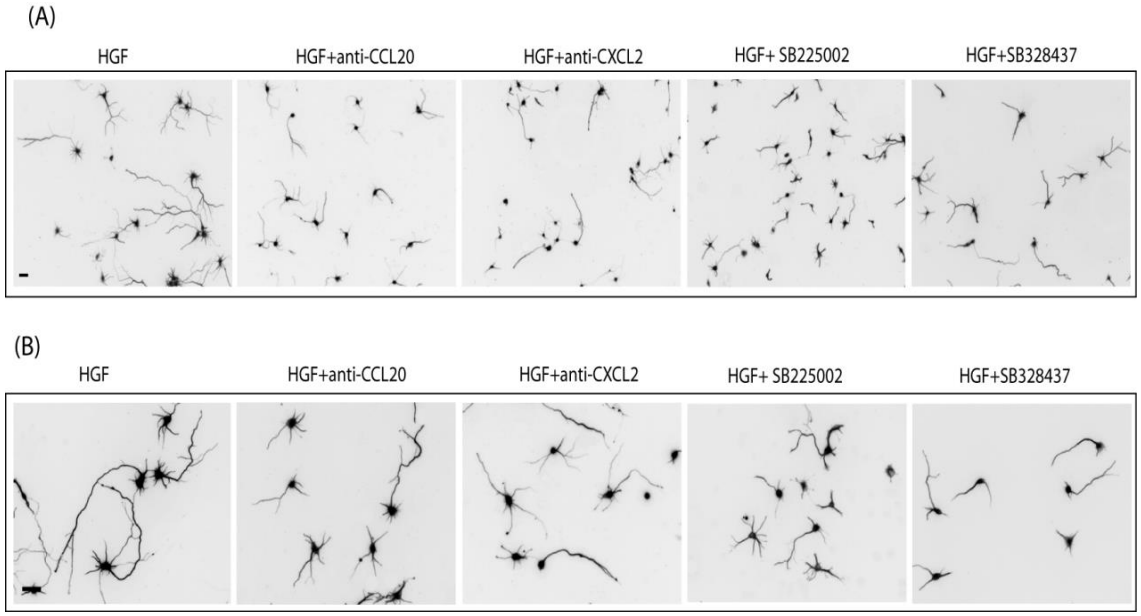


Figure 5. Chemokine signaling is involved in axon morphogenesis downstream to HGF signaling in hippocampal neurons. *Neurons were treatments with antibodies against chemokines (anti-CXCL2 and anti-CCL20, 40µg/ml) and chemokine receptor antagonists (SB225002 1.25 nM and SB328437 20 nM) together with HGF (50 ng/ml) from 1DIV to 2DIV. Cells were fixed and immunostained using βIII-tubulin antibodies. Bars = 30 µm. Images were acquired at 10x* ***(A)*** *for axon length quantification and 20x* ***(B)*** *for better visualization of the neuron morphology.*

In the blocking antibodies experiments, neurons were incubated with HGF together with antibodies against CXCL2 or CCL20 (40 µg/ml). Control neurons were treated with the same concentration of a non-relevant protein (ovalbumin) added to the culture medium together with HGF to confirm the specificity of a possible effect of the antibodies. Axon length and branching were measured. As expected, HGF promoted axon length was not affected by the presence of ovalbumin. Neurons treated with HGF (50 ng/ml) together

with the anti-CCL20 or anti-CXCL2 antibodies (40 µg/ml) from 1DIV to 2DIV displayed axons significantly shorter (by 32% and 33% respectively) than those of neurons stimulated with HGF alone or with HGF and ovalbumin (40 µg/ml) (Figure 5 and 6). In fact, axon length values in the presence of blocking antibodies were below those of control untreated neurons (Figure 6).

On the other hand, we used chemokine receptor antagonists. We chose SB225002 to block CXCL2 signaling through its receptor CXCR2 (White et al., 1998) and SB328437 (White et al., 2000) that acts on CCR3, the only known receptor for CCL20 that also acts as one of the receptors of CCL5. Incubation of hippocampal neurons with HGF (50 ng/ml) and SB225002- 1.25 nM or with SB328437- 20 nM from 1DIV to 2DIV inhibited axon outgrowth by 72% and 34% respectively, as compared to treatment with HGF alone (Figure 6A).

To evaluate the role of a putative chemokine signaling as downstream of HGF signaling in axon arborization, axon branching was measured in HGF stimulation compared to HGF treatment together with blocking antibodies or chemokine receptor antagonists. As shown in Figure 6B, anti-CXCL2 and CCL20 antibodies inhibited the axon branching induced by HGF treatment and reduced to that of control untreated neurons. On the other hand, chemokine receptor antagonists SB225002 1.25 nM or SB328437 20 nM significantly blocked the axon branching induced upon HGF stimulation and reduced to values below those of control neurons (Figure 6B). These results suggest that the chemokines are produced and secreted by the hippocampal neurons upon HGF stimulation in order to aid for axon outgrowth and branching. Together, these findings indicate that CCL20 and CXCL2 signaling is involved in axon morphogenesis downstream of HGF signaling in hippocampal neurons, in agreement with data pointed out by the array study.

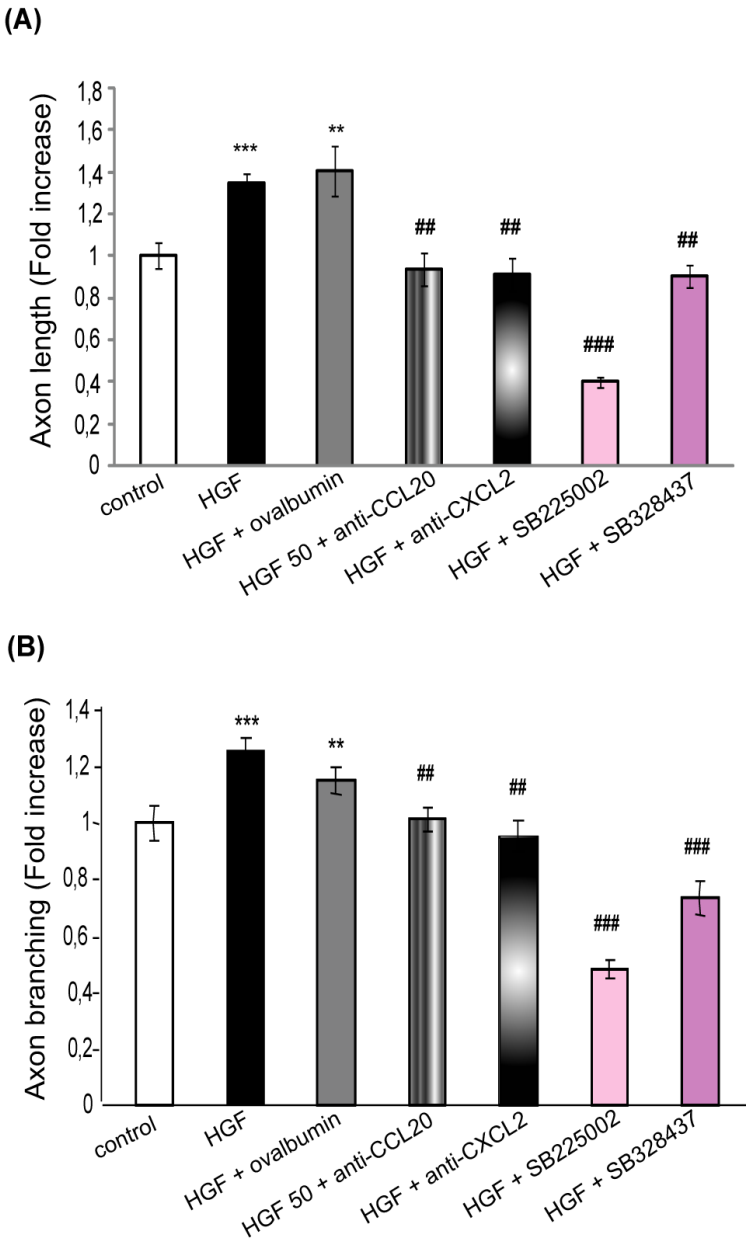


Figure 6. Chemokine signaling is involved in axon morphogenesis promoted by HGF. *Graphical representation exhibiting the fold change in axon length as well as branching, upon treating hippocampal neurons with antibodies against CXCL2 and CCL20 and the receptors antagonist of CXCR2 and CCR3, respectively. HGF was taken as the positive control. ** $p \leq 0.01$ and *** $p \leq 0.001$. $^{\#\#}$ $p \leq 0.01$ and $^{\#\#\#}$ $p \leq 0.001$. * and # shows the statistical significance in comparison to untreated controls and stimulated controls respectively.*

1.3 Met and TCF inhibition reduce the axon outgrowth induced by HGF signaling.

Next, we wanted to investigate the pathway regulating chemokine downstream of HGF signaling. We have previous demonstrated that HGF signaling increases PTyr142 β-cat, which promotes TCF-4-dependent transcriptional regulation of target genes during the axon morphogenesis (David et al., 2008). We used SU11274, which inhibits Met activity (Berthou et al., 2004) by targeting the ATP-binding site of Met to block HGF-dependent Met activation (Ma et al., 2003; Ma et al., 2005) and FH535, which inhibits TCF/β-catenin by blocking the recruitment of β-catenin to the promoter of the target genes (Handeli, & Simon, 2008).

First, we studied β-catenin transcriptional activation using luciferase assay. We transfected Hek293T cells with the TOP-FLASH reporter plasmid that carries a synthetic promoter containing three copies of the TCF-4 binding site upstream to the luciferase reporter gene (Figure 7A). This is a widely used reporter system to monitor β-catenin transcriptional activity in mammalian cells. The bioluminescent reaction catalyzed by luciferase requires luciferin (the substrate), ATP, Mg^{2+} and Ac CoA. Mixing these reagents with the cell lysates of transfected cells from different experimental conditions results in a flash of light, which is detected by a luminometer. Higher the β-catenin transcription, more will be the binding to TCF sites resulting in activation of the reporter gene (luciferase) read by luminometer.

Hek293T cells were transfected with the TOP-FLASH reporter plasmid. HGF/Met signaling is active in this cell line (Royal, & Park, 1995). Although no stimulation of luciferase reporter activity was obtained when treating cells with HGF (50 ng/ml, 24h) vs control, treatment with SU11274 and HGF reduced β-catenin transcriptional activation to values below of those of untreated cells. This finding suggests a basal activation of HGF/Met signaling (probably by an autocrine production of HGF) in Hek293T cells. Furthermore, treatment with FH535 (10 μM 24h) together with HGF reduced luciferase activity significantly, indicating the involvement of TCF in the HGF/Met signaling cascade. Wnt-3a was used as positive control and produced a clear activation of luciferase activity that was inhibited by FH535 (Figure 7B). These results confirmed FH535 as an effective TCF/β-catenin inhibitor and illustrate that HGF/Met signals through TCF/β-catenin in Hek293T cells (among other cell systems) (Monga et al., 2002).

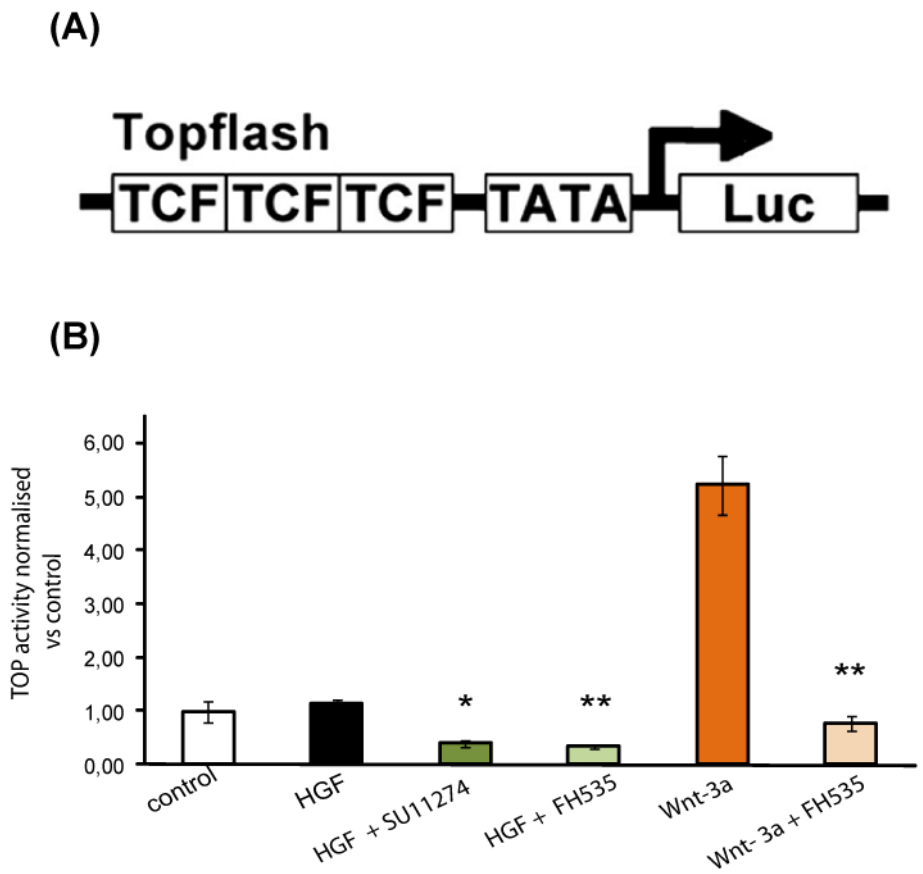


Figure 7. β-catenin transcriptional activity in transfected HEK293T cells upon HGF/Met signaling regulation. ***(A)*** *Schematic representation of TOP-FLASH reporter plasmid carrying synthetic promoter containing three copies of the TCF-4 binding site upstream to luciferase reporter gene.* ***(B)*** *Graphical representation of the relative luciferase activity quantified in Hek293T cells upon transfection with TOP reporter plasmid for 48h and treated for the last 24h with HGF (50 ng/ml), SU11274 (2 µM), Wnt-3a (100 ng/ml) and FH535 (10 µM). * $p \leq 0.05$, ** $p \leq 0.01$. * shows the statistical significance in comparosin to untreated controls.*

In order to get deeper into the signaling pathway activated by HGF in hippocampal neurons in axon outgrowth, neurons were also treated with SU11274 and FH535. Both inhibitors inhibited the axon outgrowth induced by HGF signaling (Figure 8A and B). Wnt-3a was used as the positive control, which produced an increase in axon outgrowth vs untreated neurons that was inhibited by the TCF inhibitor FH535, as expected. Interestingly, FH535 treatment also blocked the axon outgrowth promoted by HGF, rendering the axon length values below the control neurons. This result confirms that HGF

signaling is dependent on TCF-driven transcription in hippocampal neurons (David et al., 2008).

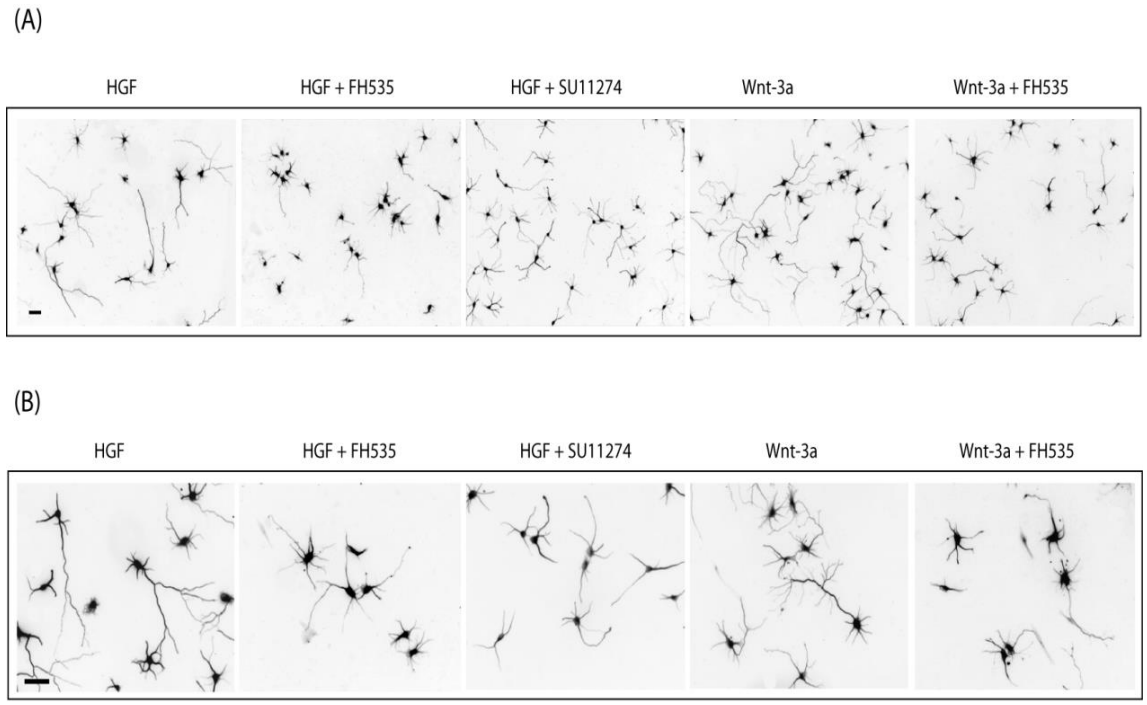


Figure 8. Met and TCF inhibition reduce axon outgrowth induced by HGF in hippocampal neurons. *Images acquired at 10x* ***(A)*** *and 20x* ***(B)*** *of 2DIV hippocampal neurons demonstrating increased axon outgrowth upon HGF (50 ng/ml 1DIV to 2DIV) stimulation, which is lost upon treating them with HGF and TCF inhibitor (FH535 10 µM) or Met inhibitor (SU11274 2 µM). Wnt-3a (100 ng/ml), used as the positive control, promoted axon outgrowth, which was reduced by treatment with FH535 10 µM. Neurons were immunostained using anti βIII-tubulin antibodies and axon length was measured. Images are inverted for the better visualization of the neuron morphology. Bars = 30 µm.*

1.4 CXCL2 and CCL5 expression is regulated by HGF signaling through TCF/β-catenin signaling

We next asked whether chemokine expression is controlled by TCF/ β-catenin downstream of HGF/Met signaling. For this we chose two chemokines, CXCL2 (as it was involved in both axon outgrowth as well as axon branching) and CCL5 (as a member of CC family of chemokines). The expression of both chemokines was analyzed by real time qPCR in control neurons or in neurons treated with HGF alone or together with SU11274 2 μM or with FH535 10 μM (overnight from 1DIV to 2DIV). Expression of CXCL2 was increased by 1.6 fold in HGF-treated vs untreated neurons, and was reduced to below control values in neurons treated HGF plus SU11274. HGF together with FH535 also decreased CXCL2 expression in comparison to that in neurons treated only with HGF, suggesting that it is mediated through TCF/β-catenin pathway (Figure 9A). Pervanadate (a tyrosine phosphatase inhibitor) was used previously to stabilize the PTyr142 form of β-catenin (David et al., 2008) and we reasoned that perhaps could promote a larger increase in the upregulation of CXCL2. However, the increase of CXCL2 expression in neurons treated with HGF and pervanadate was similar to that obtained in neurons treated with HGF alone (Figure 9B). In addition, CXCL2 expression was also analysed in hippocampal neurons transduced with lentiviruses carrying shRNA against β-catenin. The silencing of β-catenin achieved on transducing neurons with lentiviruses from day of plating up to 4DIV was around 40-50% as reported earlier and detected by Western-blot (David et al., 2008) (Figure 9E). Of note, some authors have shown that silencing of β-catenin seems to impact significantly the nuclear pool of β-catenin, whereas cytoplasmic levels remain largely unaltered (Eichhoff et al., 2011). Treating neurons with HGF (50 ng/ml overnight from 3DIV to 4DIV), increased CXCL2 mRNA levels in neurons expressing scrambled shRNA. However, the increase in CXCL2 mRNA levels was lost when neurons transduced with shRNA against β-catenin were treated with HGF (Figure 9D).

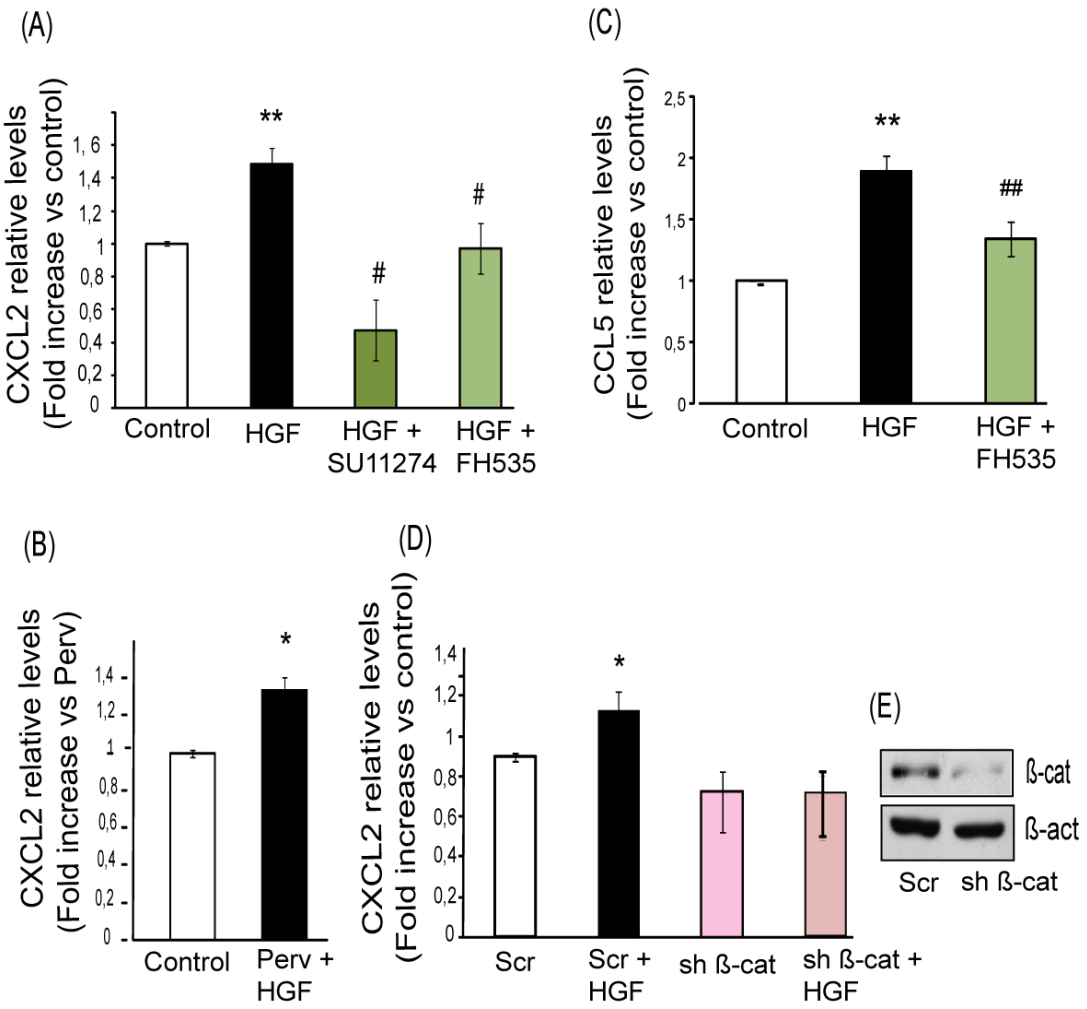


Figure 9. CXCL2 and CCL5 expression is regulated by HGF signaling via TCF/β-catenin pathway. (A) *Graphical representation of relative CXCL2 levels (2DIV) in hippocampal neurons showing increase in CXCL2 expression achieved upon treatment with HGF (50 ng/ml) overnight, which was decreased below the control when HGF was accompanied with FH535 (10μM) or SU11274 (2μM).* ***(B)*** *CXCL2 relative levels in control neuron vs HGF (50 ng/ml overninght) plus pervanadate (Perv) (last 2h) treated neurons.* ***(C)*** *CCL5 relative levels (4DIV) increased upon stimulation with HGF and were significantly reduced upon co-treatment with HGF and FH535.* ***(D)*** *CXCL2 relative levels at 4DIV in hippocampal neurons transduced with lentiviral vectors driving the expression of scrambled or β-catenin shRNAs. Upon treatment with HGF, scrambled (scr) shRNA transduced hippocampal neurons show an increase in CXCL2 levels, which is lost in the case of shRNA β-catenin transduced neurons.* ***(E)*** *Western-blot showing the β-catenin silencing achieved on transducing hippocampal neurons with shRNA against β-catenin from 0DIV-4DIV. β-actin (β-act) was used as the loading control and silencing efficiency*

*was quantified with the help of Scion image software. * and # shows the statistical significance in comparison to untreated controls and stimulated controls respectively. * $p \leq 0.05$,** $p \leq 0.01$, # $p \leq 0.05$, ## $p \leq 0.01$.*

Furthermore, CCL5 expression was also analysed by real time qPCR in neurons treated with HGF with or without FH535. HGF treatement increased by nearly 2 fold the expression of CCL5, which was reduced by ~ half in the presence of FH535 (*Figure 9C).* In summary, these results confirmed the initial findings of the array and indicate that CCL5 and CXCL2 are, at least in part, regulated by TCF/β-catenin downstream of HGF signaling.

1.5 CXCL2 promotes neurite outgrowth in DRG explants.

Upon revealing the role of chemokines in axon morphogenesis in hippocampal neurons, we investigated a putative effect of the chemokines in the regulation of neurite outgrowth in the PNS. We focused these experiments on CXCL2. To address this possibility we prepared DRG explants from newborn rat pups (P1-P2), which were grown on collagen in the presence of NT-3 and NGF (50ng/ml) required for sensory neuron survival (Figure 10A and 10B). Neurite extension from DRG sensory neurons was evaluated in control untreated conditions, CXCL2 (1000 ng/ml), and CXCL2 (1000 ng/ml) + SB225002 (1.25nM). Phase contrast images (4x) were taken after 48h and 96h of treatments (Figure 10).

Even though the neurites did show some outgrowth from the untreated explant, outgrowth of the neurites was prominent in CXCL2-treated DRG explants in comparison to untreated control explanted. This was consistent in both NT-3 as well as NGF conditions. Furthermore, cells coming out of the explants were observed already in control explants, which increased in CXCL2-treated DRG explants and were significantly reduced in number when SB225002 was added together with CXCL2 (again observed in both NT-3 and NGF-treated explants; Figure 10). These observations were accounted for both the time points: 48h as well as 96h of treatment.

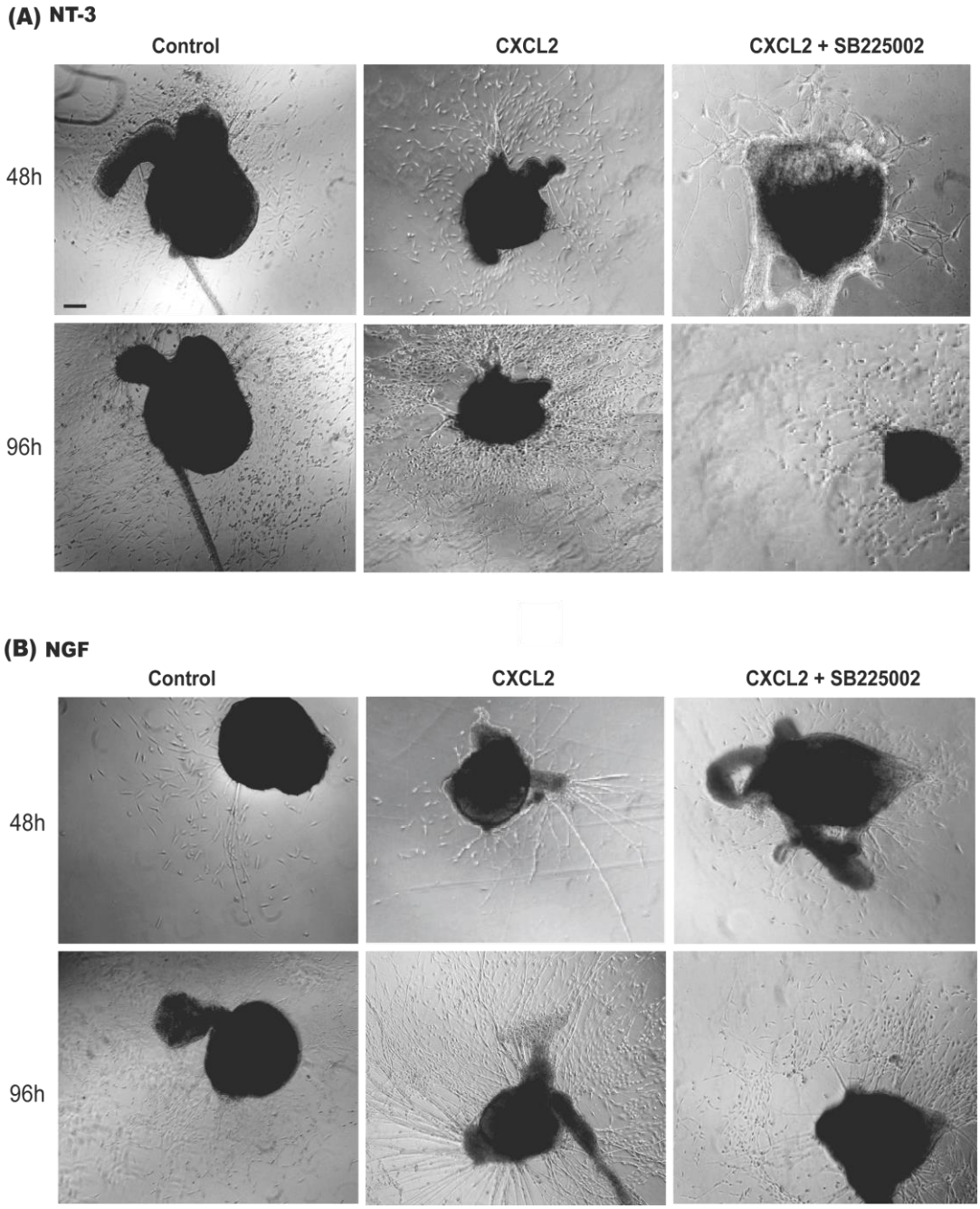


Figure 10. CXCL2 promotes neurite outgrowth of DRG sensory neurons, which is inhibited by a CXCR2 antagonist. *Figure shows the phase contrast images taken at 4x at 48h and 96h following the treatments in the presence of NT-3 (A) or NGF (B) to support neuron survival. An enhancement in the outgrowth of neurites coming out of the DRG explants was seen in both NT3- and NGF-treated explants, upon addition of CXCL2 (1000 ng/ml) vs control that was reduced following the addition of CXCL2 (1000 ng/ml) plus SB225002 (1.25nM). Bars = 8 µm.*

To support the observations made by Phase contrast microscopy, we immunostained the DRG explants with an antibody specific for βIII-tubulin and costained them with Hoescht to visualize the individual cells coming out of the explants (Figure 11).

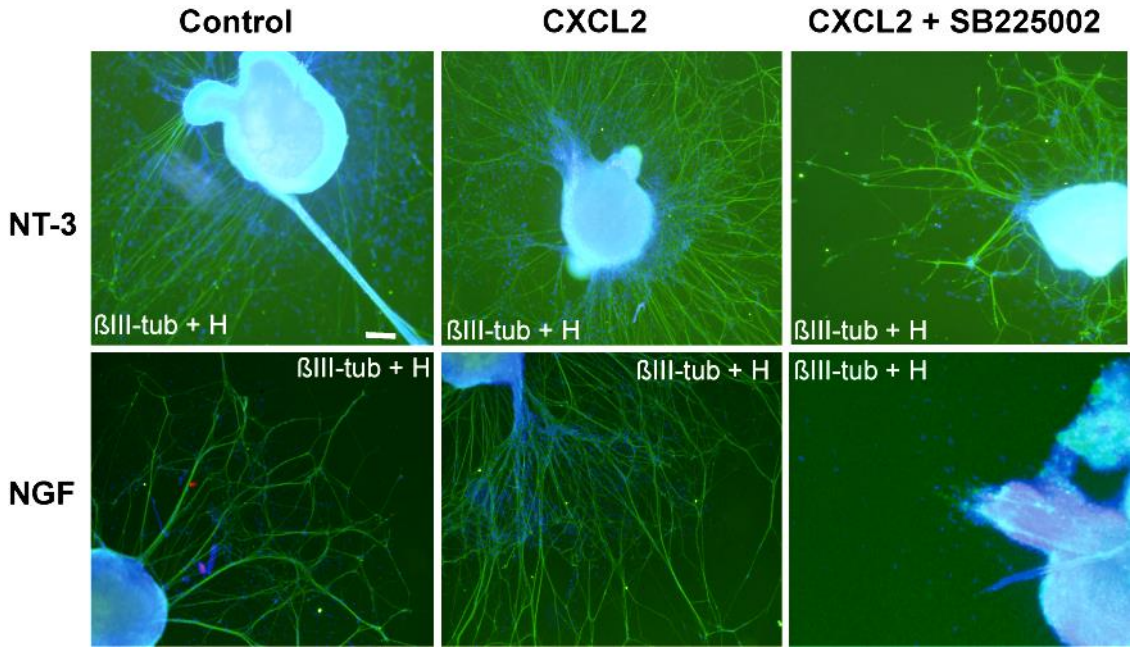


Figure 11. βIII-tubulin immunostaining reveals that CXCL2 increases neurite outgrowth, which is reduced by SB225002, in DRG explants. *Figure is demonstrating the immunofluorescence images taken at 4x after 96h of treatment, in both NT-3 and NGF-treated explants. Although control explants show neurite outgrowth, upon CXCL2 (1000 ng/ml) a denser mesh of neurites is observed coming out from the explants, which is significantly inhibited by the addition of SB225002 (1.25 nM), in both NT-3 (top) as well as NGF- treated (bottom) explants. Neurites are shown immunostained with βIII- tubulin (in green) and nuclear staining by Hoechst in blue. Bars = 8 µm.*

Images captured at 4x using a fluorescence microcope exhibited βIII-tubulin immunostaining to all the neurites coming out of the explants that helped visualizing the neurite outgrowth effect promoted by CXCL2 (Figures 11). This was further strengthened by the results obtained upon co-treatment with CXCL2 and SB225002, showing inhibition of the outgrowth observed upon treatment with CXCL2 alone. Together, these findings suggest that CXCL2 promotes neurite outgrowth of regenerating sensory axons, which is inhibited upon the addition of its receptor antagonist.

We aimed at getting further understanding about the cells coming out from the explants, which we thought could be SGCs. To visualize SGCs we immunostained the explants with anti-GFAP antibody (as described by Garrison et al., 1991; Souza et al., 2013) . Chemokines have been demonstrated to actívate SGCs by enhancing GFAP expression during the sensitivity towards neuropathic pain (Souza et al., 2013). DRG explants immunostained for GFAP and counterstained by Hoechst after 96h of treatment confirmed that the cells coming out of the explants were GFAP, and likely SGCs (Figure 12).

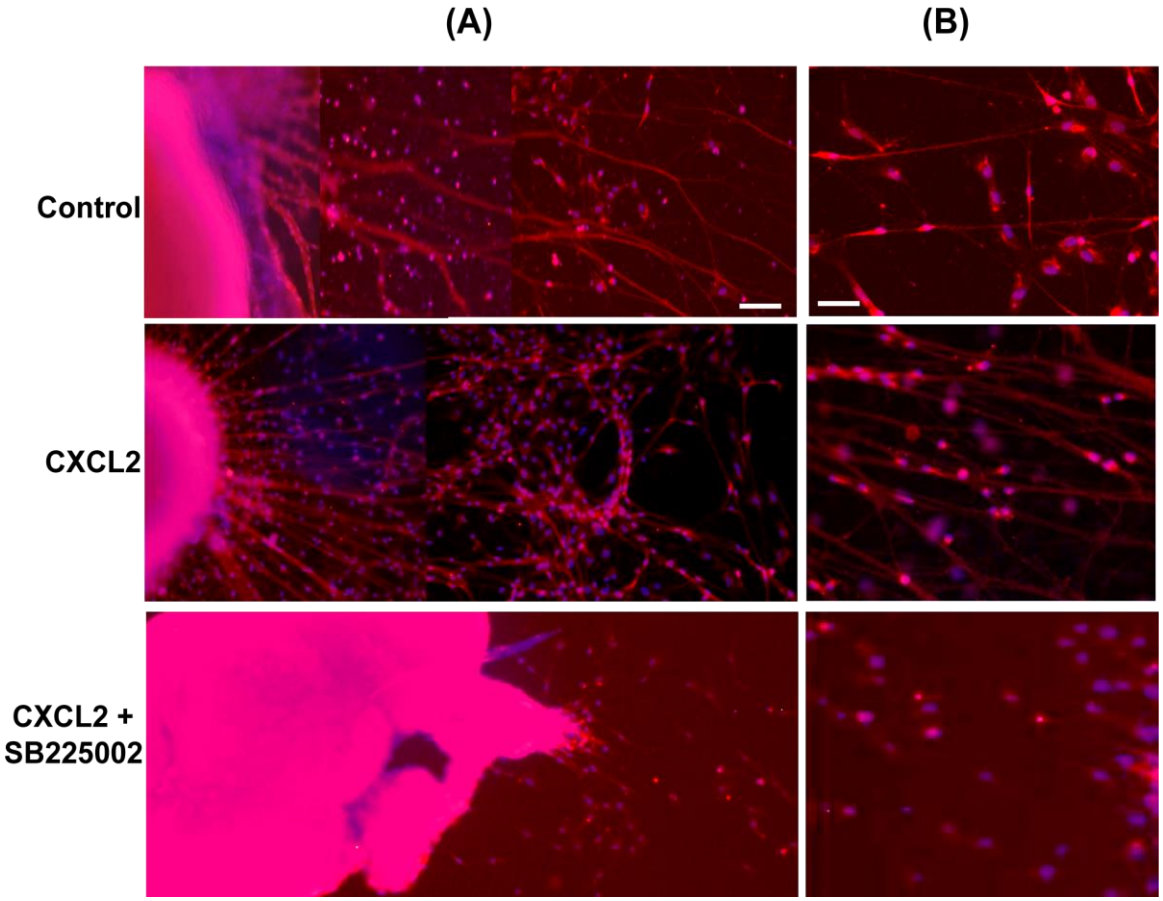


Figure 12. CXCL2 promotes SGCs to proliferate and/or migrate out of the explant, which may support neurite outgrowth of sensory neurons. *The figure is showing immunofluorescence images captured at 10x (**A**) and 20x **(B)** for the DRG explants, immunostained by an antibody against GFAP (red) following 96h of treatments. The number of SGCs expressing GFAP, increases upon the treatment with CXCL2 (1000 ng/ml) in comparison to untreated explants and this effect of CXCL2 is lost upon the co-treatment with SB225002 (1.25nM). Note in the panels in B (control and CXCL2) that SGCs appeared aligned on top of the neurites. GFAP antibody seems to induce some*

background staining at the explant and on neurites. Hoechst staining is in blue. Bars = 11 µm.

Immunofluorescence images captured at 10x and 20x showed the presence of SGCs (confirmed by immunostaining with anti-GFAP antibodies) out from the explants (Figure 12), consistent with the Phase contrast images (Figure 10). Although SGCs were present in both untreated as well as CXCL2-treated explants, sitting on top or around the neurites, their numbers clearly increased by treatment with CXCL2 and decreased by the cotreatment with CXCL2 and SB225002 (Figure 12) (as observed before). These findings suggest that CXCL2 promotes neurite outgrowth in DRG explants at least in part by inducing the proliferation or migration of SGCs. These observations raise the question as to whether the effect of CXCL2 in neurite outgrowth is by directly acting on the neurite and/or by regulating the number of SGCs, which give support to neurite and could release trophic factors promoting more outgrowth (see Discussion).

Chapter 2.

Role of β-catenin and the phosphorylation of its Tyrosine residue 142 at centrosome

2.1 PhosphoTyrosine142 β-catenin (PTyr142 β-cat) localizes to the centrosome in different cell types and cofractionates with γ-tubulin.

We studied the subcellular localization of PTyr142 β-cat by immunocytochemistry using the antibody specific for it in Hek293T (epithelial kidney cell line), primary striatal astrocytes and glioma (U87MG and U251MG) cell lines (Figure 13).

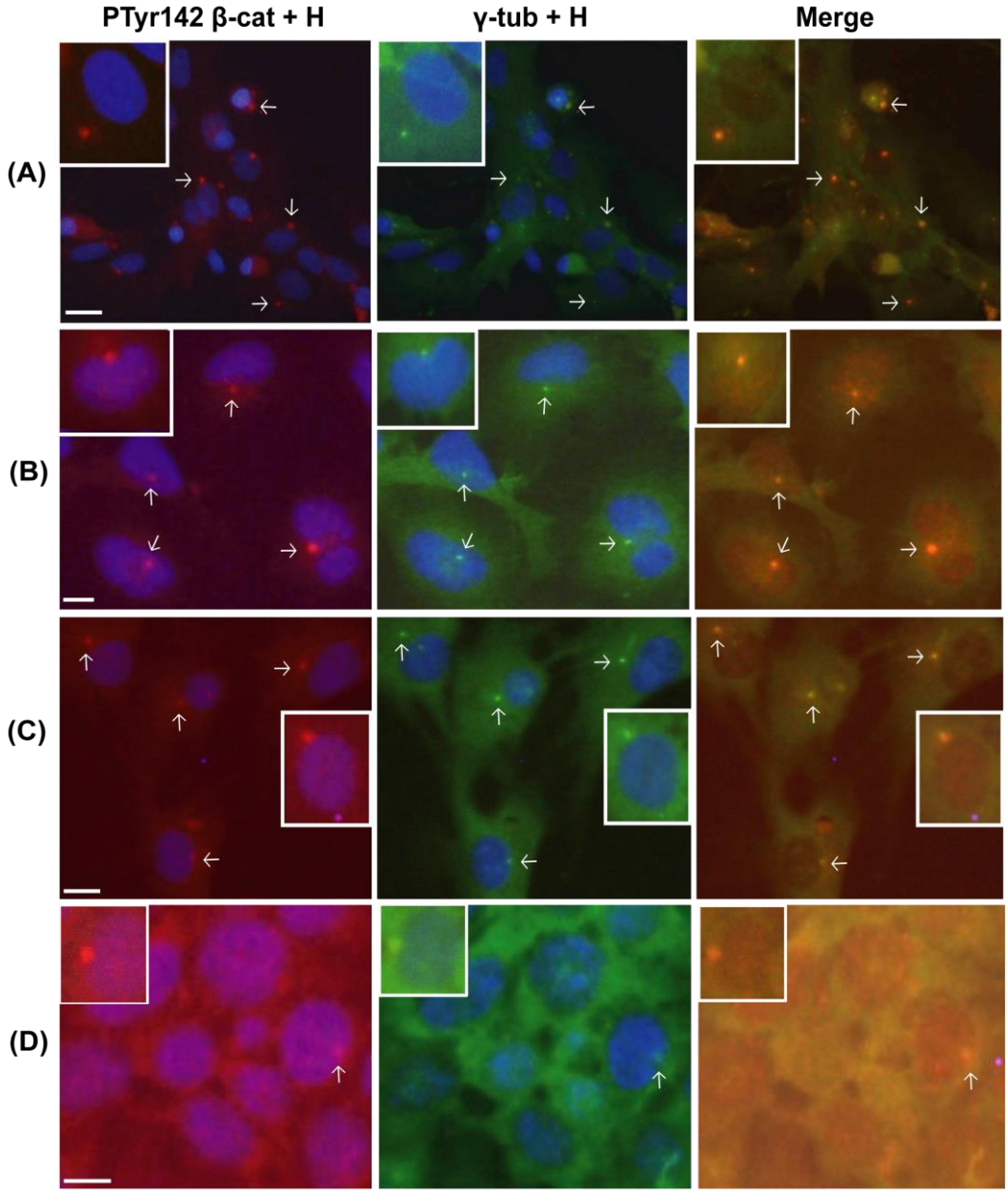


Figure 13. Centrosomal localization of PTyr142 β-cat in different cell types. *Figure shows PTyr142 β-cat localization at centrosome in primary striatal astrocytes* ***(A)****, U251MG* ***(B)****, U87MG* ***(C)*** *and Hek293T cells* ***(D)****, confirmed by colocalization with γ-tub*

immunostaining (arrows). Insets are showing the enlargements of a single cell showing specific immunostainings (as mentioned). Images were captured using a fluorescence microscope (20x, 0.4NA). Abbreviations used: γ-tub (in green) stands for γ-tubulin; H (in blue) stands for Hoechst. Bars for A, B, C, D are 16 µm, 11 µm, 15µm and 21 µm respectively.

PTyr142 β-cat was present at the centrosome in all the studied cell types. This was confirmed by its colocalization with γ-tubulin, a crucial component of the pericentriolar material responsible for the nucleation of MTs, in a dot pattern observed at the proximity of the nucleus (depicted by Hoechst staining).

Next, different fixation protocols were tried to try to identify fixation conditions that would provide us with the best centrosomal immunostainings, because different fixation methods have been developed for it (Ciani, & Salinas, 2007) for which striatal astrocytes were fixed either with 100% methanol, PFA (4%) or by the "detergent fixation" protocol (Figure 14) (see Methods). The latter method has been described to provide best immunostainings for cytoskeletal proteins. PFA fixation showed brighter and nitid PTyr142 β-cat immunostaining at the centrosome (as addressed by colocalization with γ-tubulin immunostaining). Methanol and detergent fixation produced weaker PTyr142 β-cat immunostainings at the centrosome, in agreement with weaker γ-tubulin immunostaining, suggesting the partial extraction of centrosomal components upon methanol or detergent fixation of the cells. Therefore, PFA fixation provided the best results (Figure 14) and was used in all the PTyr142 β-cat immunostainings except where co-immunostainings with Syk was fixed by methanol as recommended (Zyss et al., 2005).

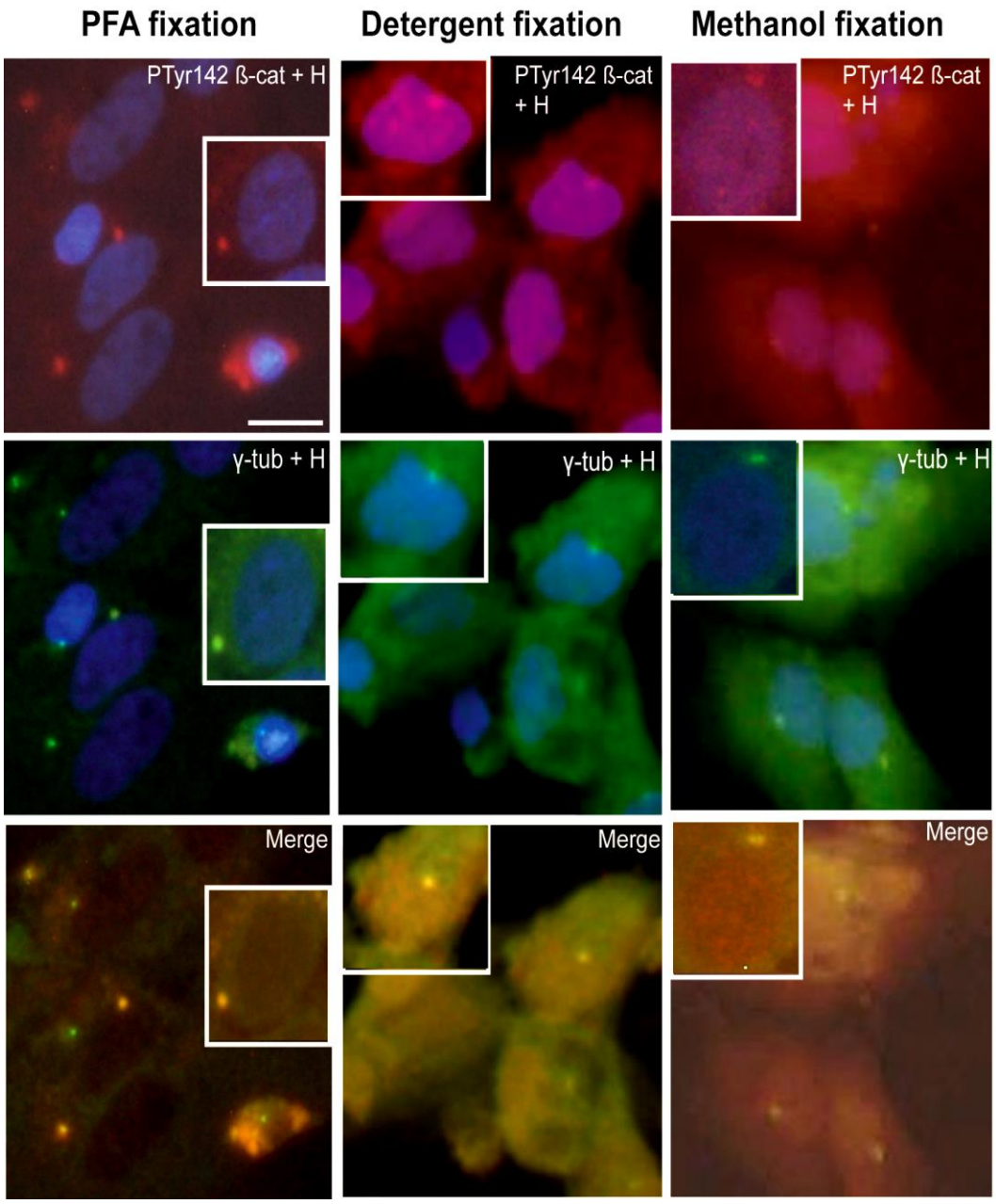


Figure 14. PTyr142 β-cat centrosomal immunostaining upon different fixation protocols. *The panel shows PTyr142 β-cat immunostaining results obtained following the three fixation methods in striatal astrocytes. PFA fixed cells showed more intense and nitid PTyr142 β-cat and γ-tub immunostainings at centrosome. Instead, detergent fixed and especially methanol fixed cells showed weaker, more diffuse PTyr142 β-cat and γ-tub immunostainings and higher background. Insets are showing the enlargements of cells showing immunostaining at centrosomes. Images were captured using a fluorescence microscope (32x, 0.4NA) showing PTyr142 β-cat in red, γ-tub in green and H in blue. Bars = 25 µm.*

Since immunocytochemical results strongly suggested that PTyr142 β-cat accumulates at the centrosome, next we aimed to confirm these findings by biochemical means, using sucrose gradients to separate centrosomal fractions. Centrosomal fractions were isolated from exponentially growing unsynchronized U251MG cells and striatal astrocytes, using discontinuous sucrose gradient centrifugation method (Hsu, & White, 1998) and subjected to SDS-PAGE and Western blot (WB) analysis. For these procedues, cells were harvested after a brief treatment with nocodazole and cytochalasin D to disrupt MTs and actin cytoskeleton. Fractions were collected from the top, with top fraction being named as 8 and the lowest fraction as 0 (Figure 15). In the same figure, the stage at which a sample of the lysates was collected (as control) is also shown.

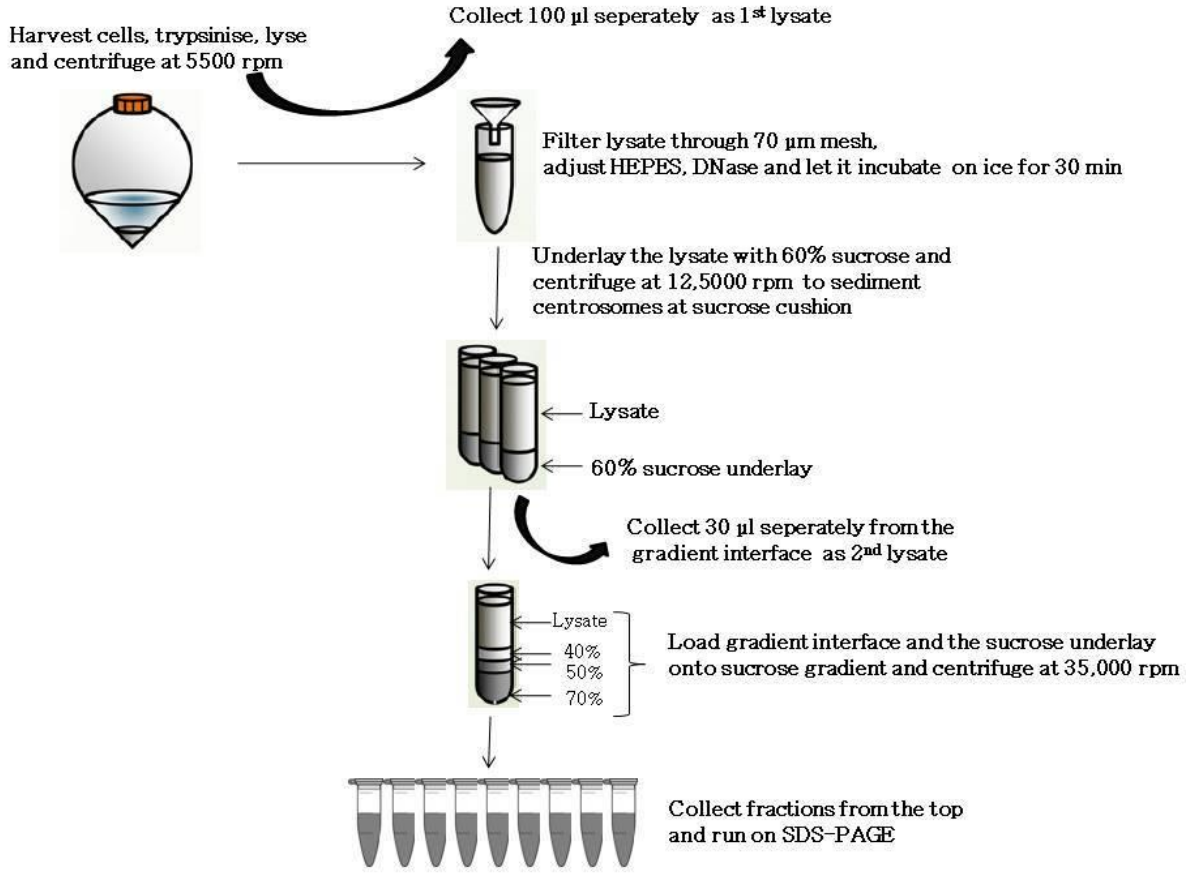


Figure 15. Scheme depicting the centrosome isolation protocol and the different stages of collection of lysates.

Fraction number 2 and 3 were considered centrosomal fractions, corresponding to ~ 50-60% sucrose fractions (Hsu, & White, 1998), as identified by a peak of γ-tubulin. Co-fractionation of PTyr142 β-cat as well as total β-catenin was detected by WB in the γ-

tubulin-positive centrosomal fractions in striatal rat astrocytes as well as U251MG glioma cells. Together with the immunostaining results, these results thus confirmed the finding of PTyr142 β-cat being localized at the centrosome (Figure 16). Other centrosomal components that co-fractioned with γ-tubulin and β-catenin were APC (in striatal astrocytes) (Louie et al., 2004) and Syk (Zyss et al., 2005) (Figure 16).

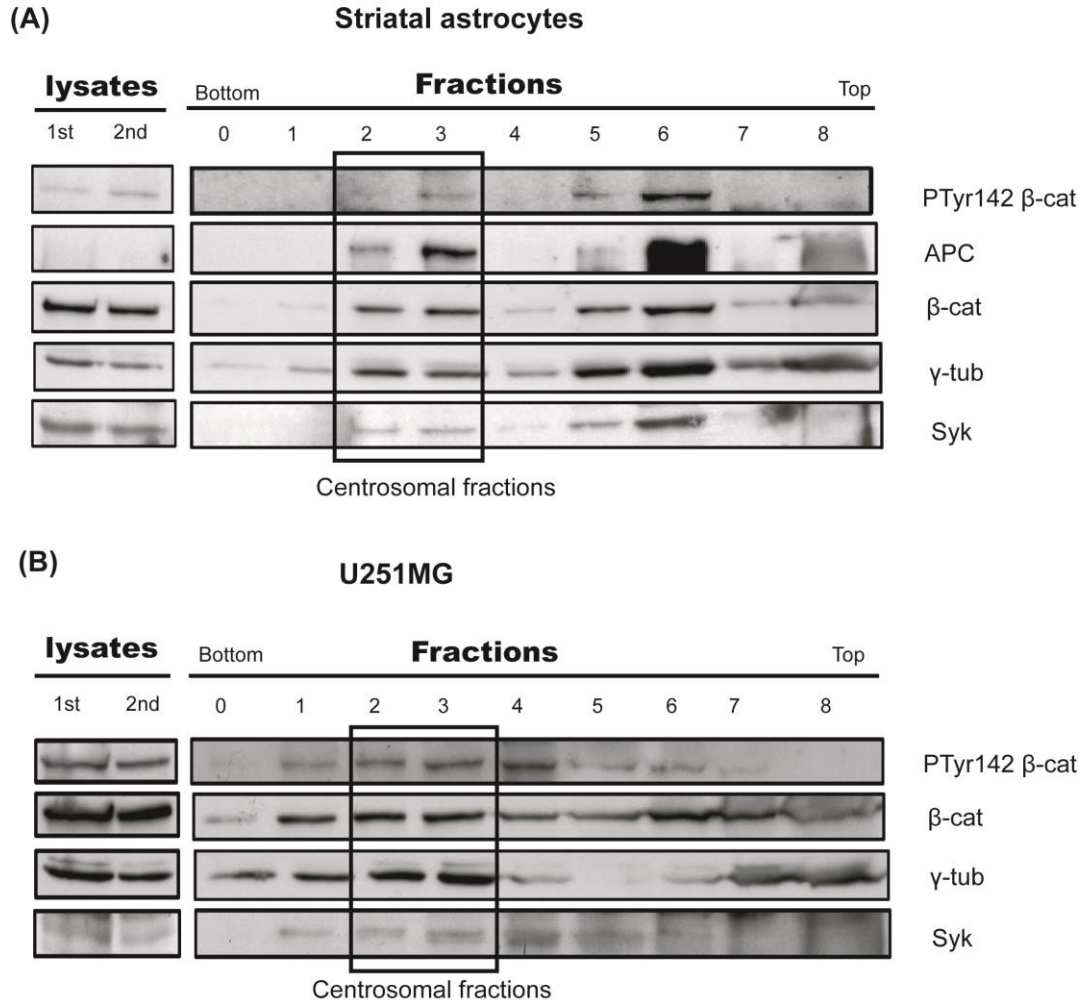


Figure 16. Centrosome fractionation shows cofractionation of PTyr142 β-cat in the centrosome-enriched fractions. *Cell lysates of rat striatal astrocytes* ***(A)*** *and U251MG cells* ***(B)*** *treated with cytochalysin D and nocodazole (see Methods) were processed for sucrose gradient subfractionation to isolate centrosomal-enriched fractions. Upon collection, gradient fractions (0-8) were subjected to SDS-PAGE and WB using specific antibodies (as indicated). WB for γ-tub identifies fractions 2-3 (50-60% sucrose) as centrosomal-enriched fractions. Nevertheless, γ-tub can also be found in other fractions as similarly described by other authors (Fumoto et al., 2009). PTyr142 β-cat and total β-catenin (β-cat) cofractionate in these fractions in both cell types. Centrosomal fractions also show cofractionation of APC in astrocytes and Syk. On the left, samples of the total*

lysates (1^{st} and 2^{n} lysates) are shown. Levels of PTyr142 β-cat and total β-cat in the lysates are higher in U251MG glioma cells that in rat astrocytes.

To try to confirm the specificity of the PTyr142 β-cat immunostaining at the centrosome, we also performed an immunocytochemical study using a pan anti-PhosphoTyrosine (PTyr) antibody in Hek293T, U251MG and U87MG cell lines (Figure 17).

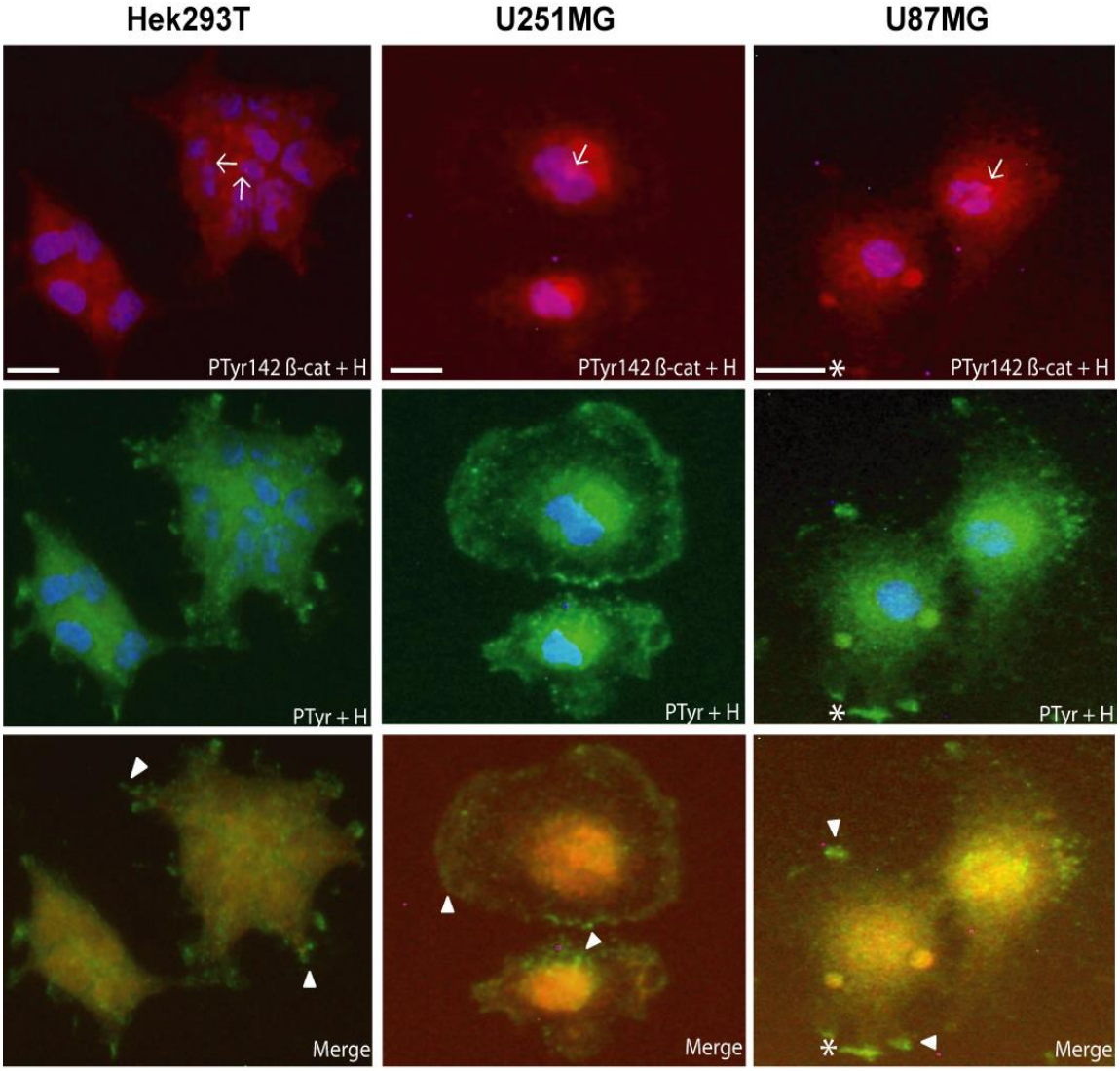


Figure 17. Differential subcellular localization of PTyr142 β-cat and pan-PTyr immunostainings. *The figure shows the immunolocalization of PTyr142 β-cat and PTyr in Hek293T, U251MG and U87MG cells. Whereas PTyr142 β-cat localization can be seen in the cytosol, nucleus and centrosome (arrows) of U251MG and U87MG and Hek293T cells (costained by Hoechst), PTyr was seen mainly at cell to substrate contacts (arrow head). Apparent colocalization between the two immunostainings is observed in the nucleus of glioma cell lines and some PTyr142 β-cat immunostaining at lamella on the plasma*

membrane of U87MG cells (asterisks). Images were taken in a fluorescence microscope (32x, 0.4NA) staining PTyr in green, H in blue and PTyr142 β-cat in red. Bars for Hek293T and U251MG are 17 µm and; for U87MG is 23 µm.

However, we found that PTyr142 β-cat and PTyr immunostainings differentially localized at different cell compartments. Whereas PTyr immunostaining was found more on the plasma membrane and cell-substrate contacts and less in the cytoplasm and nucleus, PTyr142 β-cat immunostaining decorated only some lamella in U87MG cells and immunostained the cytoplasm, nucleus and putative centrosome in all the three cell types. The unability to address the double immunocytochemistry, using anti-PTyr and γ-tubulin antibodies, due to the fact that both are mouse monoclonals, made it difficult to unequivocally identify whether PTyr immunostaining is found at the centrosome. Nevertheless, these findings highlight that PTyr142 β-cat, unlike other PTyr-proteins, mainly signals away from the adhesion and cell-substrate contacts.

Considering the presence of PTyr142 β-cat at the centrosome, we next asked if it was regulated during mitosis. To this aim, we used U251MG cells because they are bigger in size and the centrosome is easier to visualize. Cells plated on coverslips were cell cycle-arrested by serum deprivation for 20h followed by a period of 16-20h of release in complete media (period in which mitotic figures are easily observed). Using immunocytochemistry, variations in PTyr142 β-cat levels and its localization at the centrosome during different mitotic phases (M-phases) were detected by the antibody against PTyr142 β-cat. The different M-Phases were identified using Hoechst dye (to monitor the DNA condensation status) and α-tubulin (α-tub, to reveal the MT reorganization) (Figure 18). Although PTyr142 β-cat appeared intensely centrosomal in cells in interphase (shown by arrows in Figure 18), its levels at centrosome decreased abruptly in metaphase and continued to be absent from centrosomes till the end of telophase. PTyr142 β-cat levels in the neighbouring interphase cells were compared to those observed in the cells found in mitosis and used as internal standards (Figure 18).

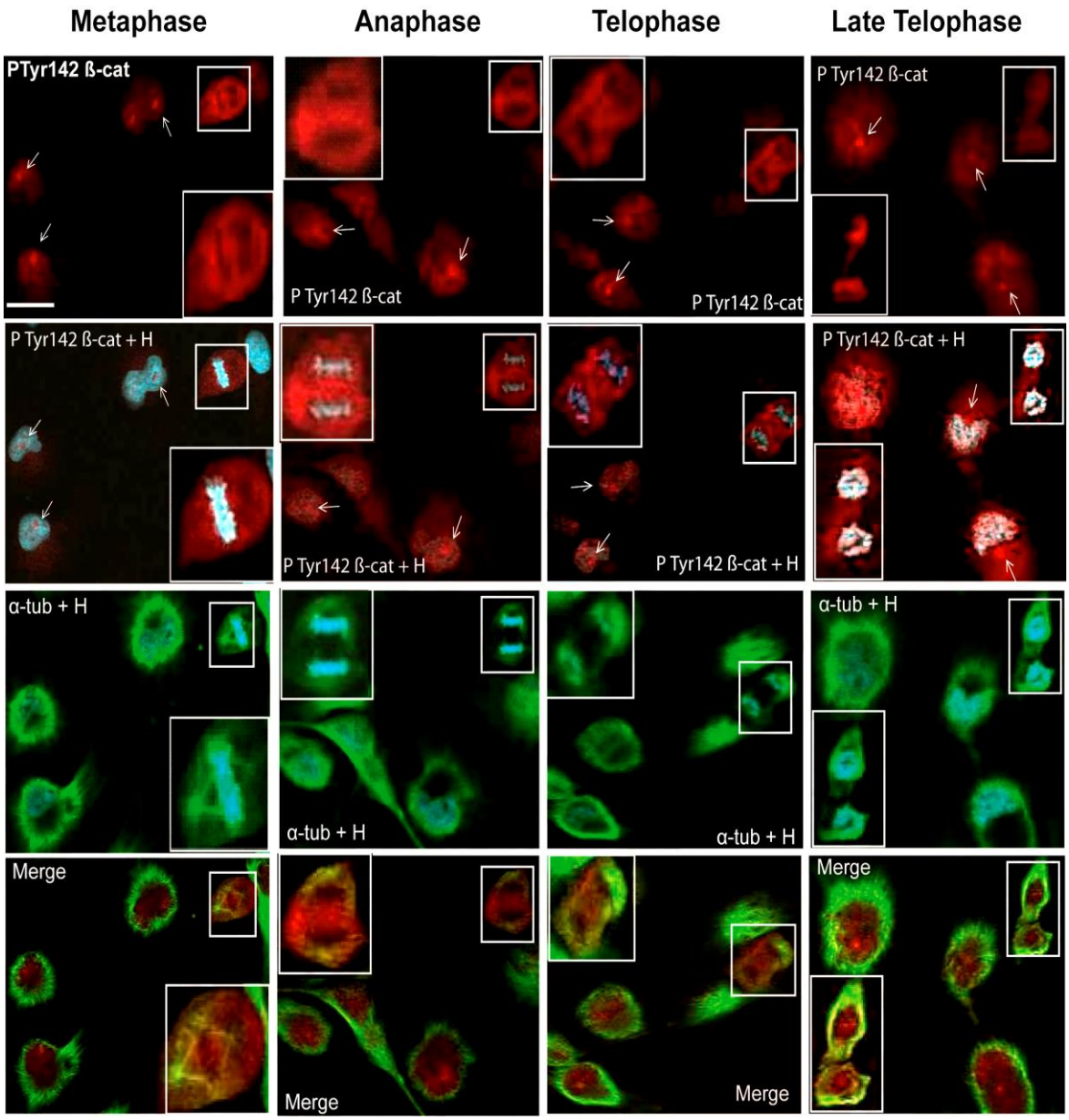


Figure 18. Variation in centrosomal levels of PTyr142 β-cat during mitosis. *Figure demonstrates the absence of PTyr142 β-cat from the centrosomes during all the stages of mitosis in U251MG cells. Images correspond to confocal sections taken at 60X showing PTyr142 β-cat in red, α-tub (α-tubulin) in green, and H in blue. Bars = 16 µm.*

These observations suggest that PTyr142 β-cat at the centrosome possibly imposes a break for mitosis progression.

2.2 β-catenin and its phosphorylation at Tyr142 residue are involved downstream to HGF signaling during cell migration and polarization.

The establishment and maintenance of cell polarity is a characteristic feature of all eukaryotic cells. Upon establishing the presence of PTyr142 β-cat at centrosome, we moved our study to the direction of cell polarity because the centrosome is the major organizer of MTs and of crucial relevance importance in determining cell shape, polarity and motility (reviewed in Tang, & Marshall, 2012). Two approaches have been used to address it. On one hand, we performed wound-healing assay on monolayers of rat primary astrocytes, which has been described and used as a simple model to study cell migration (Etienne-Manneville, & Hall, 2001) and Hek293T cells, kidney epithelial cells which are easy grow and manipulate. On the other hand, we also analyzed orientation of the centrosome (MTOC) during cell migration.

Since published work demonstrates that Met RTK phosphorylates β-catenin at Tyr142 (Brembeck et al., 2004; David et al., 2008), we wanted to address the connection between HGF/Met signaling and β-catenin signaling in cell migration. Met expression has already been reported in astrocytes (Shimazaki et al., 2003). First, we studied cell migration in astrocytes upon formation of a scratch, which stimulates its migration in the direction perpendicular to the wound. We investigated the involvement of Met signaling in astrocyte migration by treating astrocytes with HGF (50 ng/ml, 24h) and/or a Met inhibitor (SU11274; 2 μM, 24h), after creating an artificial "scratch" or "wound". Phase-contrast images of the scratch at the time of creating it (0h) and after 24h were taken. Width of the scratch was measured. For each treatment condition, duplicates were taken and in each wound image, three readings were taken (to avoid the discrepancies because of the variation in the width of scratch). As expected, HGF treatment stimulated cell migration in comparison to control untreated cells, demonstrated by a reduction of ~28% of the width of the wound after 24h (Figure 19). In contrast, co-treatment of HGF and SU11274 blocked cell migration, as demonstrated by a width of the scratch after 24h similar to that of control cells (18% wider, suggesting the retraction of the monolayer; Figure 19). These results clearly indicate that HGF signaling promotes cell migration in rat astrocytes, which is inhibited by the Met inhibitor SU11274.

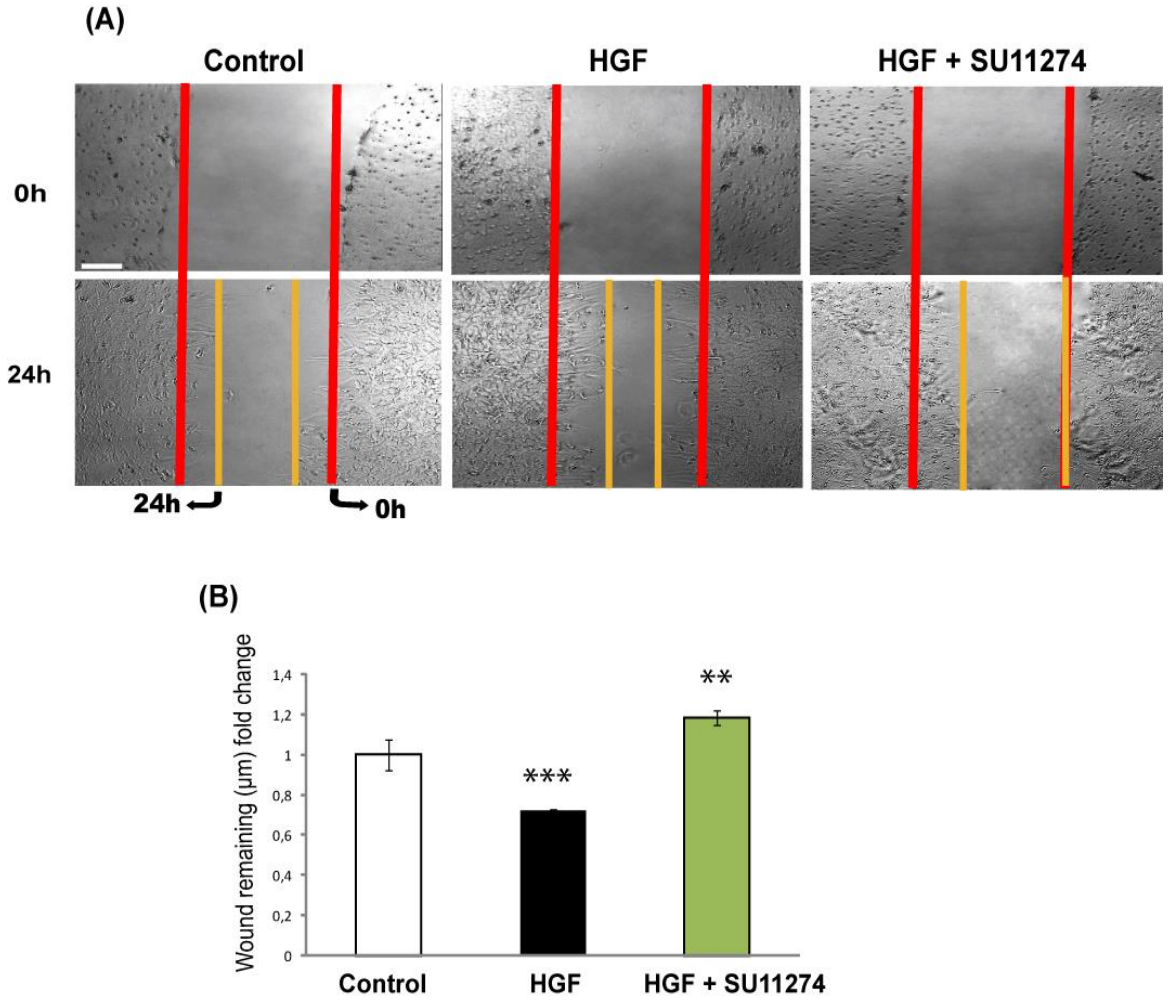


Figure 19. Quantification of cell migration in rat primary striatal astrocytes upon Met stimulation or inhibition utilizing in vitro scratch assay. (A) *Phase contrast images of the striatal astrocytes monolayer, were compared from 0h (Red line, when the scratch was given, top images) to 24h (Yellow line, scratch after 24h of cell migration, lower images) in different conditions (control, HGF 50 ng/ml, HGF 50 ng/ml + SU11274 2µM). Images were captured at 4x.* ***(B)*** *Graphical representation showing the width of the scratch in µm after 24h of treatments in comparison to the scratch at 0h. Width of the scratch was measured with the help of image J software. Results are normalized versus control and shown as fold change. Minimum three expreriments were taken and the width of the space created by scratch in each image was measured in triplicates. ** $p \leq 0.01$ and *** $p \leq 0.001$, where * shows the statistical significance versus untreated control. Bars = 15 µm*

In order to study the role of β-catenin Tyr142 residue downstream to HGF signaling while promoting cell migration, Hek293T cells were used for transfection experiments. For this, Wild Type (WT) or mutant Tyr142Phe β-cat (β-catenin non-phosphorytable at Tyr142)

(kind gifts from Dr. Mireia Duñach) were expressed together with GFP. Considering the time 0 (T0) as the time when scratch was given to the cell monolayer followed by treatments, images were taken in the fluorescent microscope using 4x objective (Figure 20). At this time 40-50% of cells showed GFP expression, which increases upto more than 90% by 24h (T24). Scratch images from T0 and T24 were measured and compared for the wound left after 24h of scratch in different conditions.

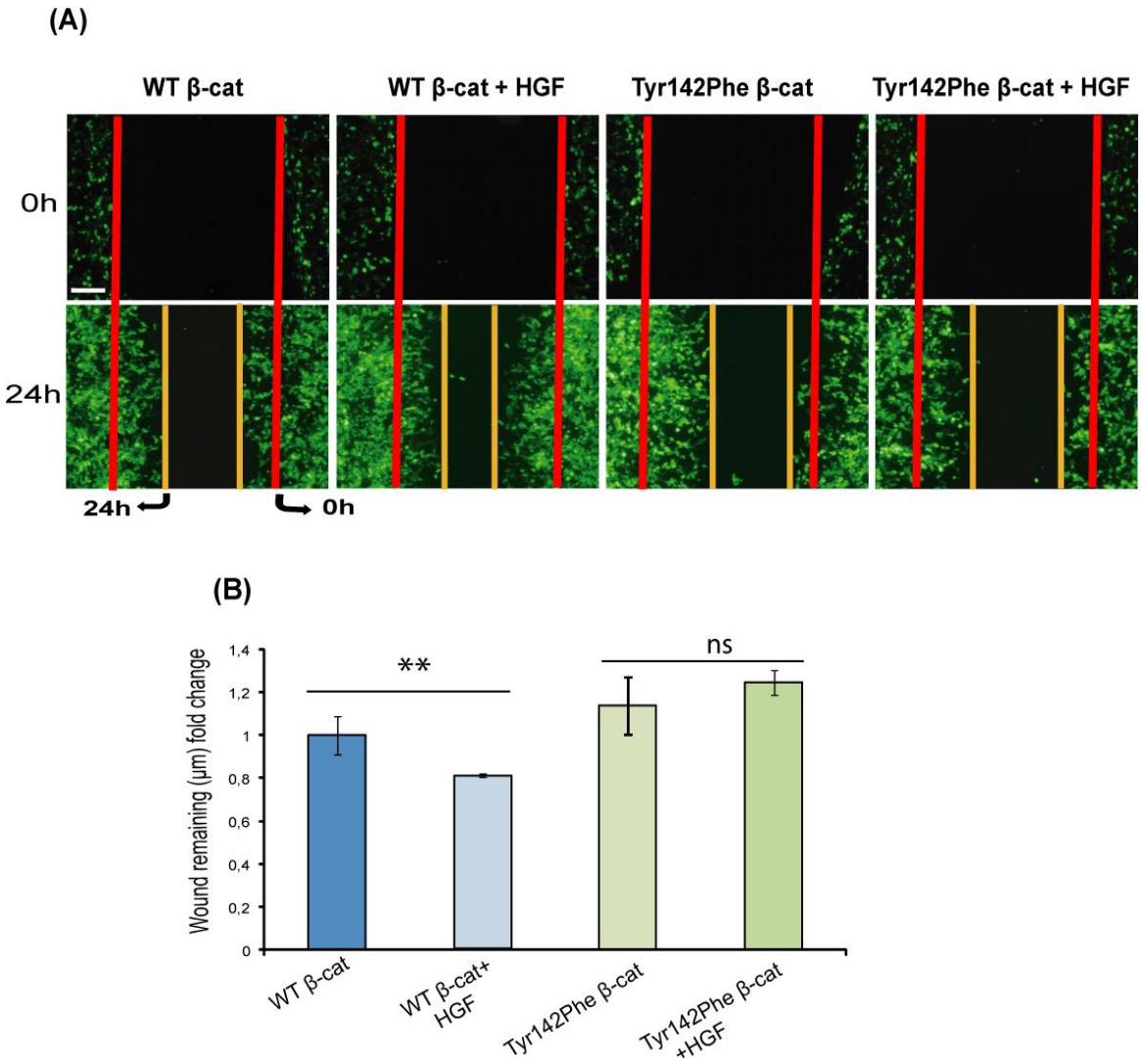


Figure 20. Cell migration induced by HGF signaling is inhibited in Hek293T cells expressing Tyr142Phe β-cat. (A) *Images of GFP fluorescence captured at 4x, from monolayers of cells expressing GFP+WT β-cat (untreated – or treated with HGF 50 ng/ml) or expressing GFP+Tyr142Phe β-cat (untreated – or treated with HGF 50 ng/ml). Following transfection of WT or mutant β-cat, cells were left untreatred or treated with HGF and the width of the scratch was quantified at 0h (red line, when the scratch was created) and 24h (yellow line, scratch after 24h)* ***(B)*** *Plot is showing the graphical*

*representation of the wound width values (in μm) at 24h in the different conditions. Values are normalized versus WT β-cat (fold change). Plot includes results of ≥ three experiments in each condition, performed in duplicates. ** $p \leq 0.01$, where * shows the statistical significance versus untreated control. Differences between Tyr142Phe β-cat-expressing cells treated with HGF vs untreated cells were not significant (n.s.). Bars = 13 μm.*

In cells overexpressing WT β-cat, HGF stimulation promoted cell migration and significantly reduced the width of the wound left after 24h by 20% in comparison to untreated WT β-cat-expressing cells (Figure 20). However, there was no reduction in the width of the wound when Tyr142Phe β-cat-expressing cells were treated with HGF, as compared to Tyr142Phe β-cat control cells (Figure 21). These results indicate that PTyr142 β-cat is required for the promotion of cell migration induced by HGF/Met signaling in Hek293T cells.

Next, we aimed at investigating the role of β-catenin in centrosome reorientation during cell migration. To this end, we used primary rat striatal astrocytes. It has already been shown that in migrating astrocytes centrosome localizes in front of the nucleus in the direction of migration (Etienne-Manneville, & Hall, 2001; Etienne-Manneville, 2006) (Figure 21A). In addition, the correct position of the centrosome during migration controls the direction of migration in astrocytes, a process in which N-cadherin is involved (Camand et al., 2012) Thus, silencing of N-cadherin in glioma cells resulted in a higher percentage of cells presenting incorrectly positioned centrosomes and erratic movement (with cells constantly changing the direction of migration) (Camand et al., 2012). To elucidate the role of β-catenin in this process, lentiviral infection allowing the expression of shRNA directed towards β-catenin was used (as previously described in David et al., 2008). Non-Infected (NI) astrocytes and those expressing Scrambled (Scr) or shRNA against β-catenin (shRNA β-cat) were compared (Figure 21B). γ-tubulin immunostaining was used to reveal the position of centrosome in response to scratch and Hoechst to stain the nucleus. The efficiency of silencing of β-catenin achieved by transducing rat primary astrocytes with shRNA β-cat was around 30% (as similarly reported in neurons; David et al., 2008; David et al. 2010). To evaluate the centrosome reorientation upon wound, cells of first and second rows were only selected as the ones beyond this will not be affected by the wound (as described in Etienne-Manneville, 2006). Duplicates for each condition were

taken and counted for centrosome reorientation (as described in Etienne-Manneville, 2006).

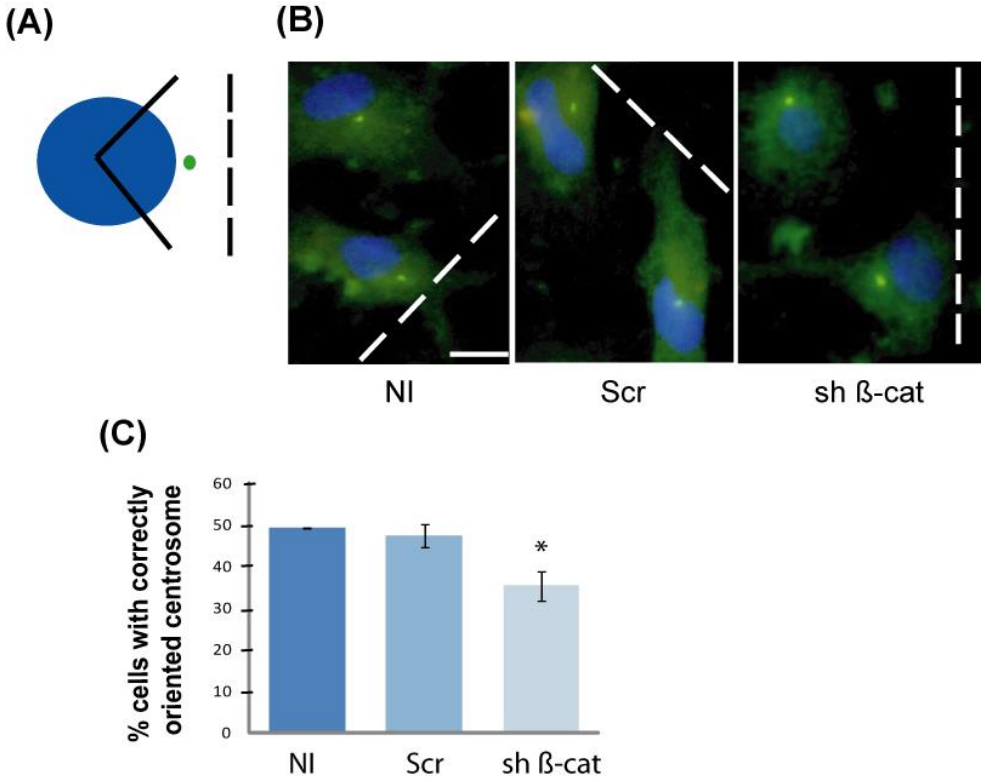


Figure 21. Centrosome reorientation in striatal astrocytes is altered by β-catenin silencing. (A) *The scheme illustrates that the centrosome (in green) is considered as correctly positioned if it lies within the quadrant (black line) of the nucleus (in blue) facing the scratch (black, dotted line).* ***(B)*** *Immunofluorescence images showing the centrosome (immunostained by γ-tub antibody) position versus the scratch (white dotted lines) in NI or Scr and sh β-cat-expressing striatal astrocytes. Images were taken at 40x using a fluorescence microscope. Note that the cells shown in shRNAβ-cat displaycentrosomes incorrectly positioned vs the scratch according to A.* ***(C)*** *Graphical representation showing the % of cells displaying correctly positioned centrosomes in the different experimental conditions. Minimum three experiments were included and 25-30 images were counted per condition. * $p \leq 0.05$, where * shows the statistical significance versus untreated control. Bars = 22 µm.*

Whereas NI showed 49,22 % of cells showing correctly positioned centrosomes towards the direction of the scratch, Scr showed values similar to NI with 47,22 % of cells showing correctly positioned centrosomes. In contrast, in cells expressing shRNA against β-cat, the percentage of cells displaying correctly positioned centrosome was significantly reduced

(by ~15%) in comparison to the values in NI and Scr expressing cells (Figure 21C). This finding demonstrates that β-catenin is involved in regulating the position of the centrosome during cell migration, although it is not the only parameter.

2.3 Met and Syk as possible tyrosine kinases involved in the phosphorylation of Tyr142 β-cat at the centrosome.

Having demonstrated that PTyr142 β-cat is found at centrosomes in interphase cells and the involvement of β-catenin in cell polarization , we aimed at identifying the Tyr kinase responsible for maintaining the phosphorylation of Tyr142 at centrosomes, which may be putatively involved in the control of centrosome cycle and/or cell migration. Our previous work demonstrated that Met directly phosphorylates PTyr142 β-cat (David et al., 2008). Therefore, Met was a strong candidate kinase that could regulate centrosomal PTyr142 β-cat. Moreover, although Met has not been previously localized to the centrosome, recent reports demonstrated that overexpression of Met leads to supernumerary centrosomes (Nam et al., 2010), implying a connection between Met and centrosomal regulation. We investigated whether phosphorylated Met could be found in centrosomes. We checked various anti-PhosphoMet antibodies: a rabbit polyclonal and a rabbit monoclonal, D26, antibodies recognizing phosphorylated tyrosines (PTyr)1234/1235 (in the catalytic region of Met) and a rabbit polyclonal antibody against PTyr1349 Met (in the multifunctional docking site). U87MG cells were fixed with methanol (15 min, -20°C), which maintains good centrosomal immunostainings, and doubly immunostained using the corresponding Met antibody and a mouse antibody against γ-tubulin (to confirm the immunolocalization to the centrosomes). Interestingly, immunostaining results clearly revealed the presence of PTyr1234/1235 Met (detected by the D26 antibody), active Met, at the centrosome (overlapping with γ-tubulin immunostaining) (Figure 22B). A weaker PTyr1234/35 Met immunostaining at centrosome was also observed when using the rabbit polyclonal against PTyr1234/1235 Met (Figure 22A). However, immunostaining obtained using anti-PTyr1349 Met did not label the centrosomes (Figure 22C). Cells incubated with secondary antibodies only were used as a negative control (Figure 22D). These results indicate the presence of active Met at centrosomes.

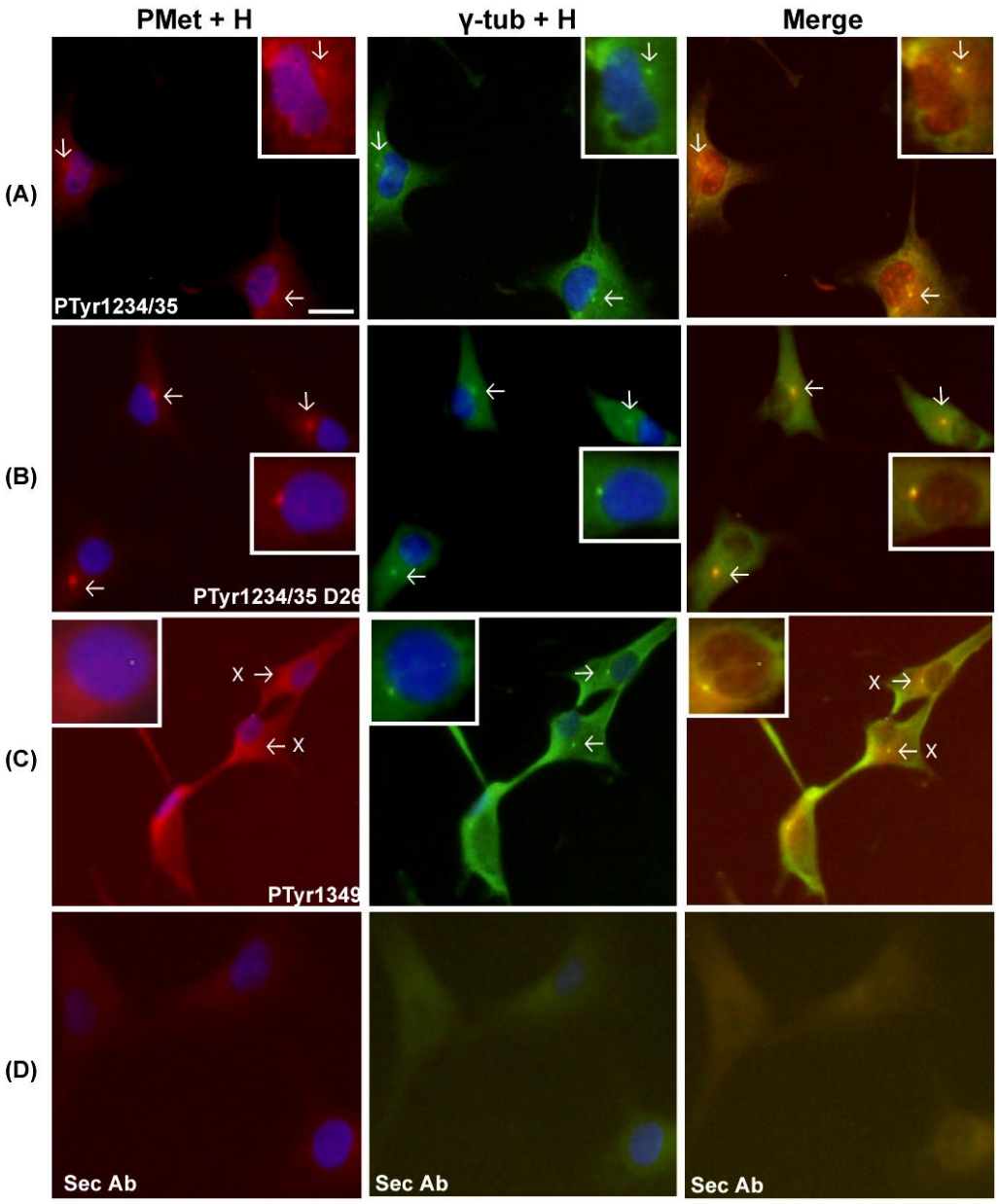


Figure 22. PTyr1234/1235 Met localizes at the centrosome in U87MG cells. *Immunofluorescence images (20x) comparing the immunostainings obtained using different anti-PMet antibodies: rabbit polyclonal against PTyr1234/1235 Met (**B**), rabbit monoclonal against PTyr1234/1235 Met, D26 (**B**), and rabbit polyclonal against PTyr1349 Met (**C**). Results with D26 antibody showed a bright PTyr1234/1235 Met immunostaining at the centrosome, colocalizing with γ-tub. In contrast, a polyclonal antibody against PTyr1234/1235 Met showed only weak centrosomal immunostaining. Immunostaining using anti-PTyr1349 Met antibodies does not localize to the centrosome. Images obtained from cells incubated with secondary antibodies only (no primary antibody; Sec Ab) **(D)** are shown as negative control. Insets show the enlarged views of*

cells displaying the immunostainings at centrosome. Positive centrosome stainings are marked with arrows (→) and its absence is marked as (X). H stands for Hoechst. Phospho-Met immunostainings are visualized in red, γ-tub in green and H in blue. Bars = 15 µm.

Having observed the presence of PTyr1234/1235 Met at centrosome, we wanted to investigate the effect of a Met inhibitor, SU11274 (that inhibits ATP binding to Met) on the centrosomal levels of PTyr1234/1235 Met and of PTyr142 β-cat. U87MG cells were treated with SU11274 (2µM, overnight) and then routine immunostaining was performed. However, we could not detect any change in the levels of PTyr1234/1235 Met at centrosomes in SU11274 treated cells compared to control cells (Figure 23).

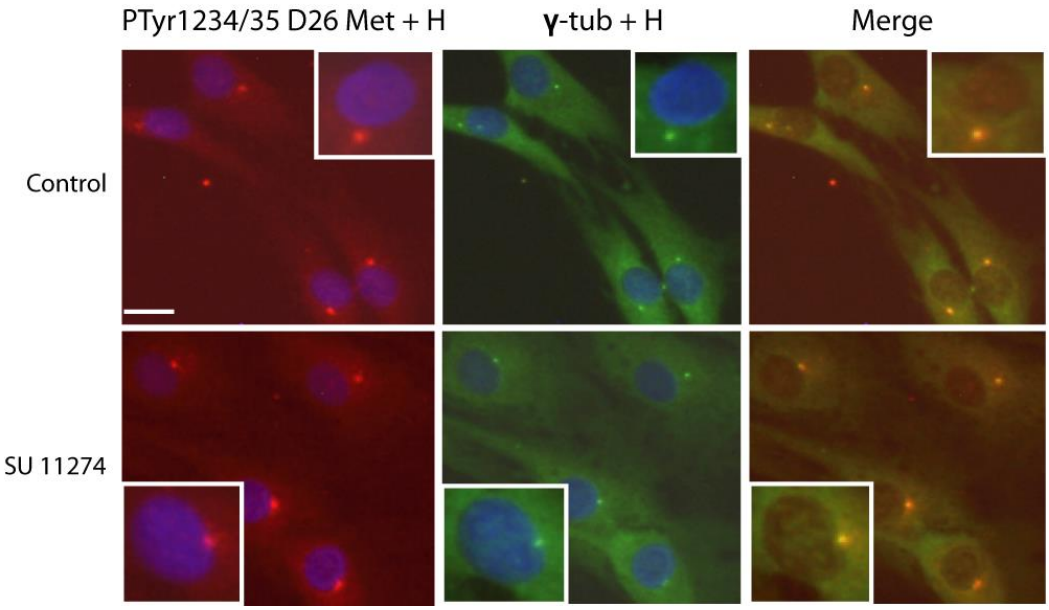


Figure 23. Met inhibitor, SU11274, does not alter centrosomal PTyr1234/1235 Met levels in U87MG cells. *Immunofluorescence images (20x) demonstrate that there is no change on the centrosomal levels of PMet Tyr1234/1235 upon inhibition of Met by SU11274 (2µM , overnight) compared to control cells. Insets are showing the enlarged view of centrosomal immunostainings. PMet 1234/1235 is visualized is red, γ-tub in green and H in blue. Bars = 15 µm.*

Effective inhibition of Met by SU11274, immunodetected by a significant reduction of PTyr1234/1235 Met levels was confirmed in U87MG total cell lysates (results not shown). These results localize for the first time active Met (PTyr1234/1235 Met) at centrosomes. However, centrosomal active Met could not be inhibited by SU11274. These results

correlated with levels of centrosomal PTyr142 β-cat being unaffected upon treatment with SU11274 (Figure 24).

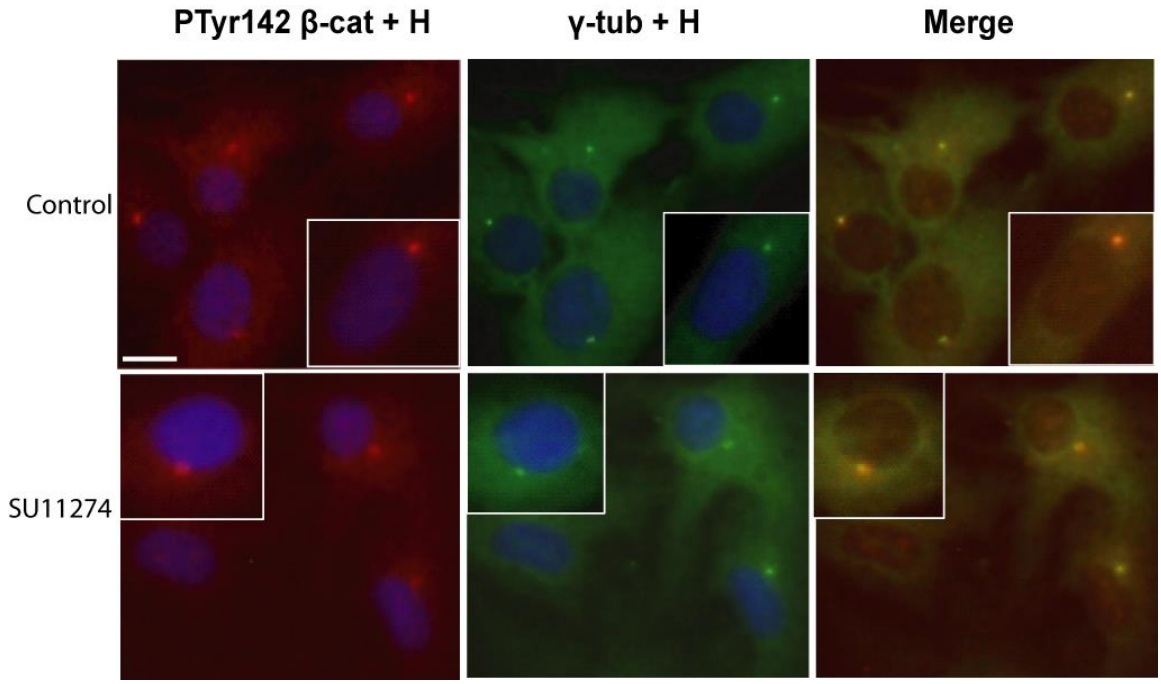


Figure 24. PTyr142 β-cat levels are not altered upon Met inhibition. *Consistent with the Met inhibitor (SU11274 2 μM) not altering PTyr1234/35 Met at centrosome (in the previous figure), PTyr142 β-cat centrosomal levels in U87MG were also unaffected upon treatment with SU11274 (2μM). Images were captured at 20x, showing PTyr142 β-cat immunostained in red and γ-tub in green. Bars = 15 μm.*

Another candidate Tyr kinase was Syk, which was found in centrosomal fractions also (Figure 17). Syk is known to localize at the centrosome affecting mitosis progression, which levels drop in mitosis (Zyss et al., 2005) as similarly observed for PTyr142 β-cat (Figure 19). To investigate the connection between PTyr142 β-cat and Syk at the centrosome, we transfected glioma cell lines (U251MG and U87MG) with WT and mutant (Tyr130Glu and Lys402Arg) Syk plasmids fused to *Discosoma* Red (Ds Red) fluorescent protein (a kind gift from P. Coopman, Montpellier, France). The two mutant forms were: a) a kinase-dead mutant in which the ATP-binding domain was inactivated (Lys402Arg) and absent from centrosomes, but present at membrane and b) a mutant possessing increased intrinsic kinase activity (Tyr130Glu) that basally localizes to the centrosome . The altered catalytic activities of these mutants was verified by evaluating their phosphorylation capacity, hence as expected Lys402Arg mutant lacked any detectable

kinase activity, whereas Tyr130Glu mutant showed an increased capacity to phosphorylate itself and other proteins (Zyss et al., 2005).

Syk-transfected cells were fixed with methanol (15 min, -20°C; which allows a good visualization of the DsRed fluorescence reporting Syk expression), followed by immunostaining against PTyr142 β-cat. Expression of WT and mutant Syk in glioma cell lines showed a pattern consistent with the distribution of Syk in different subcellular compartments in a breast cancer cell line (Zyss et al., 2005). WT Syk was present at both plasma membrane extensions and centrosomes; Lys402Arg (kinase-dead mutant) was present exclusively at membrane but no longer present at centrosomes; and the constitutively active mutant (Tyr130Glu) was predominantly present at the centrosomes (Figure 25, 26).

We reasoned that if Syk was responsible for phosphorylating PTyr142 β-cat, overexpression of basally active or inactive Syk mutants would increase or reduce, respectively, PTyr142 β-cat levels at the centrosome. Images taken under a fluorescence microscope revealed the colocalization of Tyr130Glu Syk and PTyr142 β-cat at centrosome in U87MG cell line, which was absent in the mutant Lys402Arg (Figure 25) (as described). However, there was no apparent change in the centrosomal of PTyr142 β-cat when overactive Tyr130Glu Syk was expressed (Figure 25).

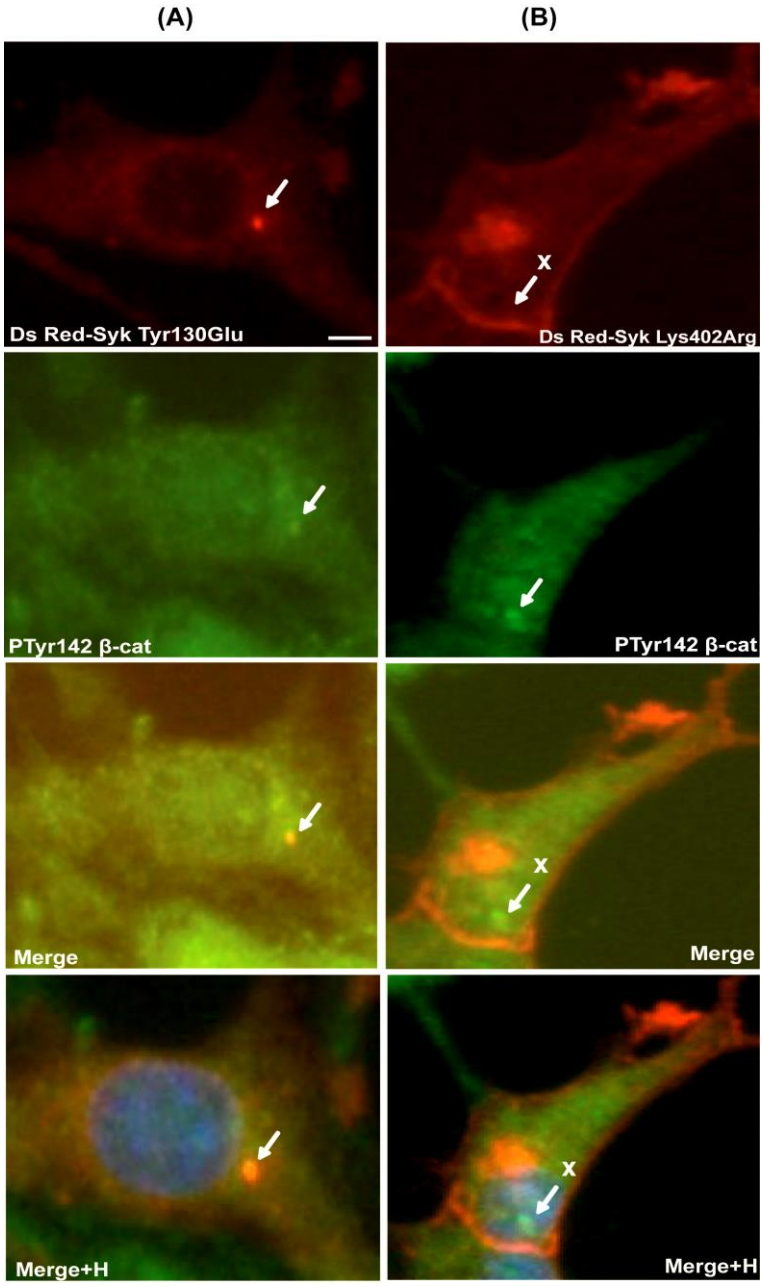


Figure 25. Overexpression of hyperactive or inactive Syk mutants does not seem to affect centrosomal PTyr142 β-cat in U87MG cells. *DsRed fluorescence or immunofluorescence images acquired at 20x show the localization of DsRed-Syk constitutive active (Tyr130Glu,* ***A****) or kinase-dead (Lys402Arg,* ***B****) mutants. Whereas Tyr130Glu is found almost exclusively at the centrosome (where it colocalizes with PTyr142 β-cat), kinase-dead Lys402Arg Syk localizes to the plasma membrane and cytoplasm, and it is absent from the centrosome. PTyr142 β-cat localizes to centrosome (arrows) also in kinase-dead syk mutant-expressing cells. PTyr142 β-cat is visualized in green and H in blue. Bars = 15 µm.*

Similarly, in U251MG cells, WT and Tyr130Glu Syk (Figure 26A and B) localized to the centrosomes together with PTyr142 β-cat. However, when inactive Syk was found at the centrosome, PTyr142 β-cat was still observed at centrosomes in both glioma cell lines (Figure 25B and 26C).

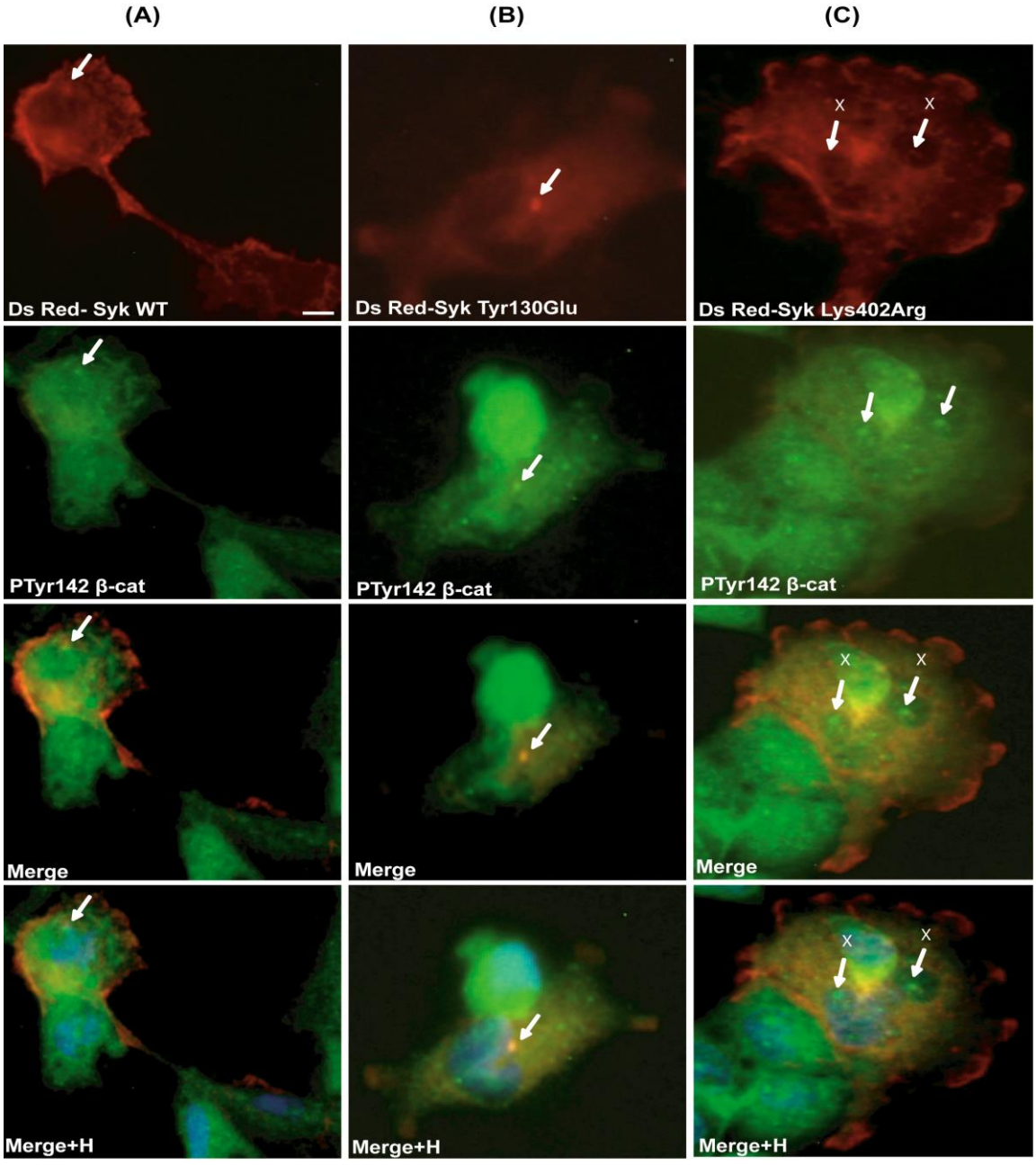


Figure 26. Overexpression of WT, active or inactive Syk does not affect PTyr142 β-cat at the centrosome in U251MG cells. *DsRed fluorescence or imunofluorescence images acquired at 20x demonstrate the presence of WT Syk at plasma membrane as well as centrosome* ***(A)****, preferential expression in the centrosome of constitutive active mutant Tyr130Glu* ***(B)*** *and expression restricted to the plasma membrane of the kinase-dead*

*mutant Lys402Arg **(C)**. However, expression of the different Syk mutants does not affect the localization of PTyr142 β-cat to the centrosome. Arrows point to centrosomal localization and (X) to the absence of Syk expression at the centrosome. PTyr142 β-cat is visualized in green and H in blue. Bars = 11 μm.*

These results indicate that modulating Syk activity by expression of mutant Syk forms at the centrosome does not affect in a significant manner the levels of PTyr142 β-cat, suggesting that Syk may not be the kinase responsible for this phosphorylation. However, endogenous Syk expressed by glioma cells could explain why we were not able to see changes in the levels of centrosomal PTyr142 β-cat upon overexpression of Syk forms.

Thus, we took as an alternative approach the use of Piceatannol (Pic) to specifically inhibit Syk (Larive et al., 2009; Seow, Chue, & Wong, 2002) and then investigate the possible changes in the centrosomal levels of PTyr142 β-cat. For these set of immunostainings, a short methanol fixation (5 min, -20°C) was performed, which provides good PTyr142 β-cat and γ-tubulin results (similar to PFA fixation). In addition to treatments with Pic (200μM, 6h at 37°C), we also treated glioma cells with the Tyr phosphatase inhibitor sodium pervanadate (1mM, 15 min at 37°C prior fixation), to try to potentiate centrosomal PTyr142 β-cat levels. In pervanadate-treated U87MG cells, PTyr142 β-cat was dominantly defined at centrosome (colocalizing with γ-tubulin; Figure 27). Interestingly, upon cell treatment with Pic, centrosomal PTyr142 β-cat levels decreased and colocalization with γ-tub diminished in most of the cells (Figure 27).

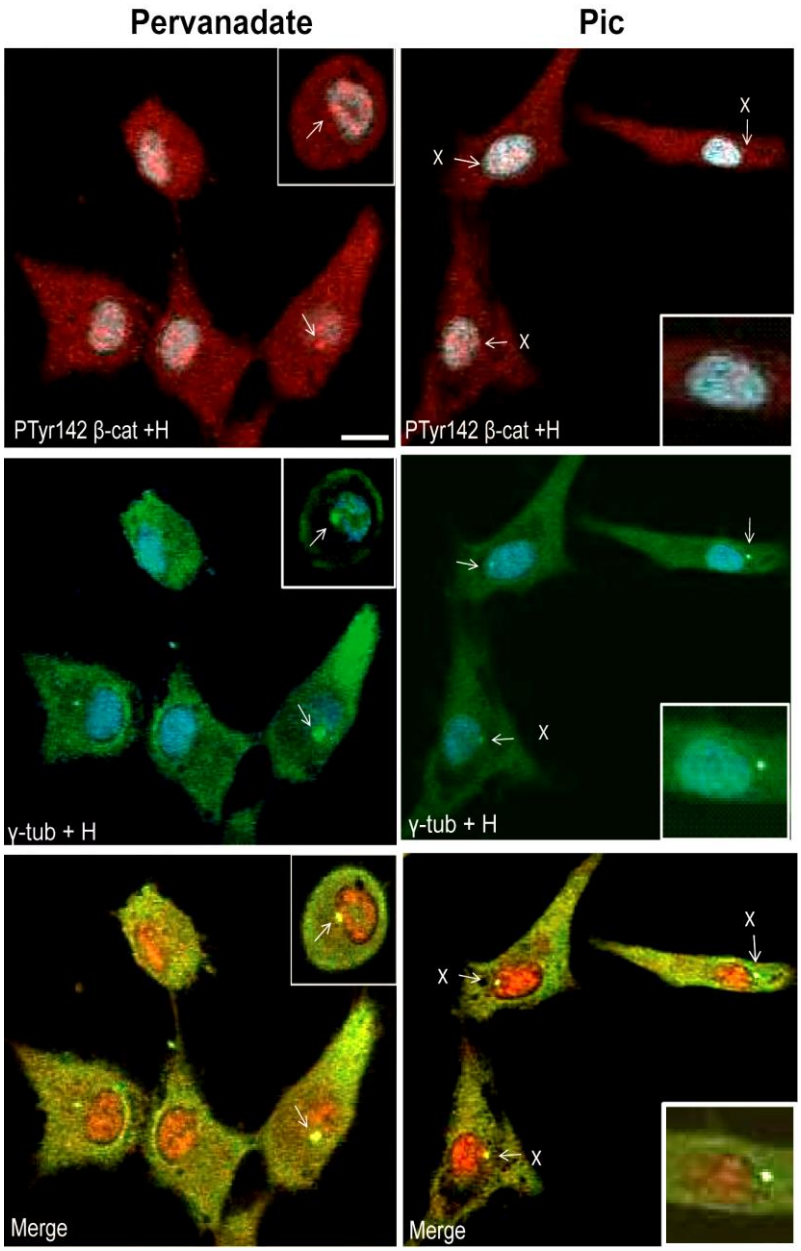


Figure 27. Syk inhibition decreases centrosomal PTyr142 β-cat in U87MG cells. *The figure shows the immunofluorescence images acquired at 60x (confocal microscope), demonstrating the decrease of PTyr142 β-cat from the centrosome (immunostained by γ-tub antibody in green) in Pic-treated (200 µM) cells in comparison to the cells treated with pervanadate (1mM). Arrows indicate either the colocalization or no colocalization (X) of PTyr142 β-cat with γ-tub at centrosome. Insets are showing detailed views of cells displaying centrosomal immunostainings. H is in blue. Bars = 15 µm*

Similar results were obtained in U251MG cells (Figure 28). Whereas control untreated and pervanadate-treated cells did show centrosomal immunostaining for PTyr142 β-cat, centrosomal PTyr142 β-cat was reduced or completely absent in the majority of Pic-

treated cells, suggesting that Syk may be playing a role in regulating centrosomal levels of PTyr142 β-cat.

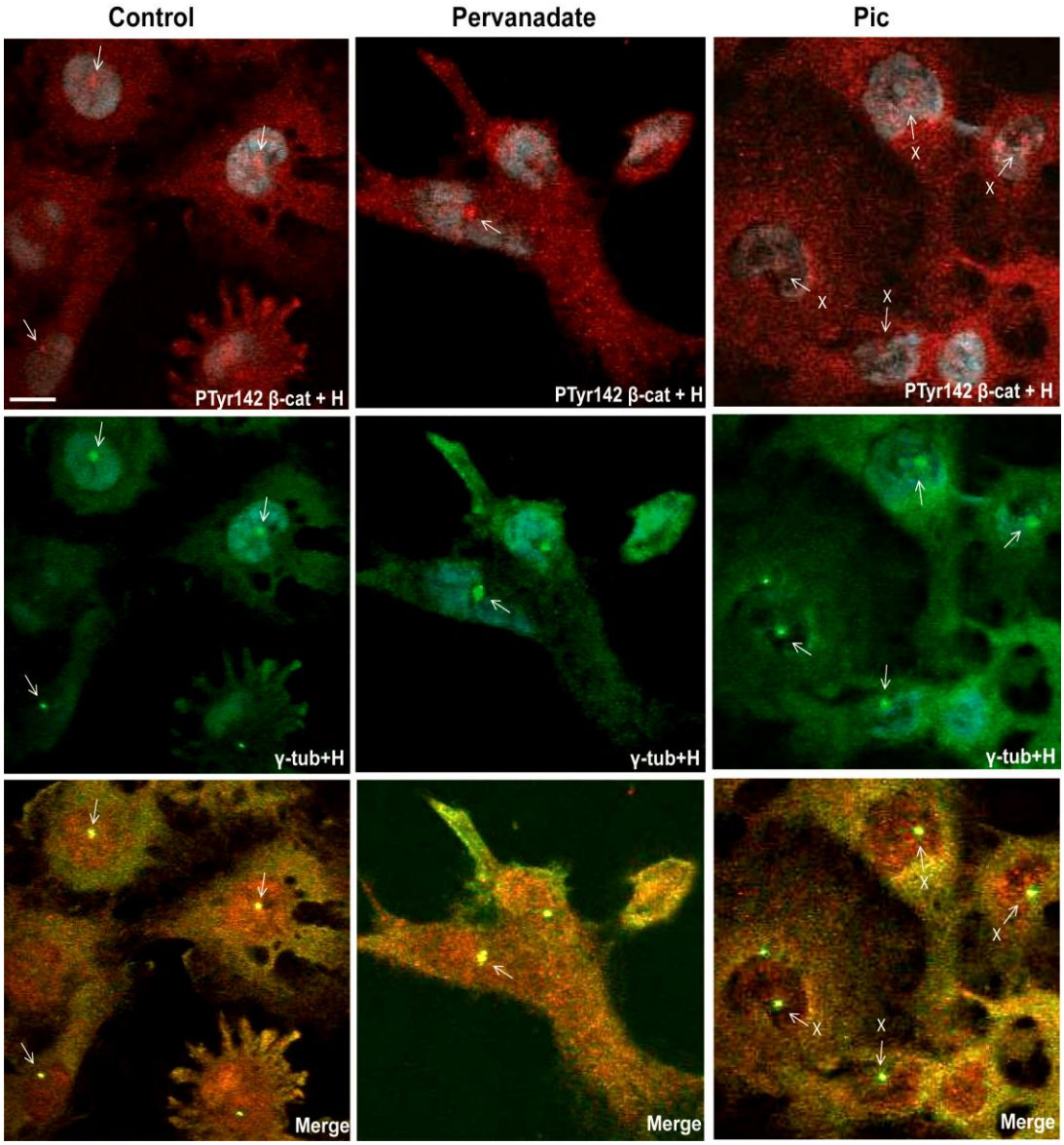


Figure 28. Centrosomal levels of PTyr142 β-cat decrease upon treatment with Pic in U251MG cells*. Figure shows immunofluorescence images acquired at 60X (confocal microscope), showing centrosomal PTyr142 β-cat immunostaining (in red) in control, pervanadate- (1mM) or Pic-treated (200 µM) cells. Centrosomal PTyr142 β-cat immunostaining diminishes upon treatment with Pic compared to control untreated cells. γ-tub is immunostained in green and H in blue. Bars = 16 µm.*

Apart from this, we also noticed some pervanadate-treated cells showing apparently bigger centrosomes compared to control cells (as reported by larger intense dotted PTyr142 β-cat and γ-tubulin immunostainings).

To support our obervations, we quantified immunofluorescence intensities of centrosomal PTyr142 β-cat and γ-tubulin obtained under different treatment conditions in U87MG and U251MG cells (Figure 29).

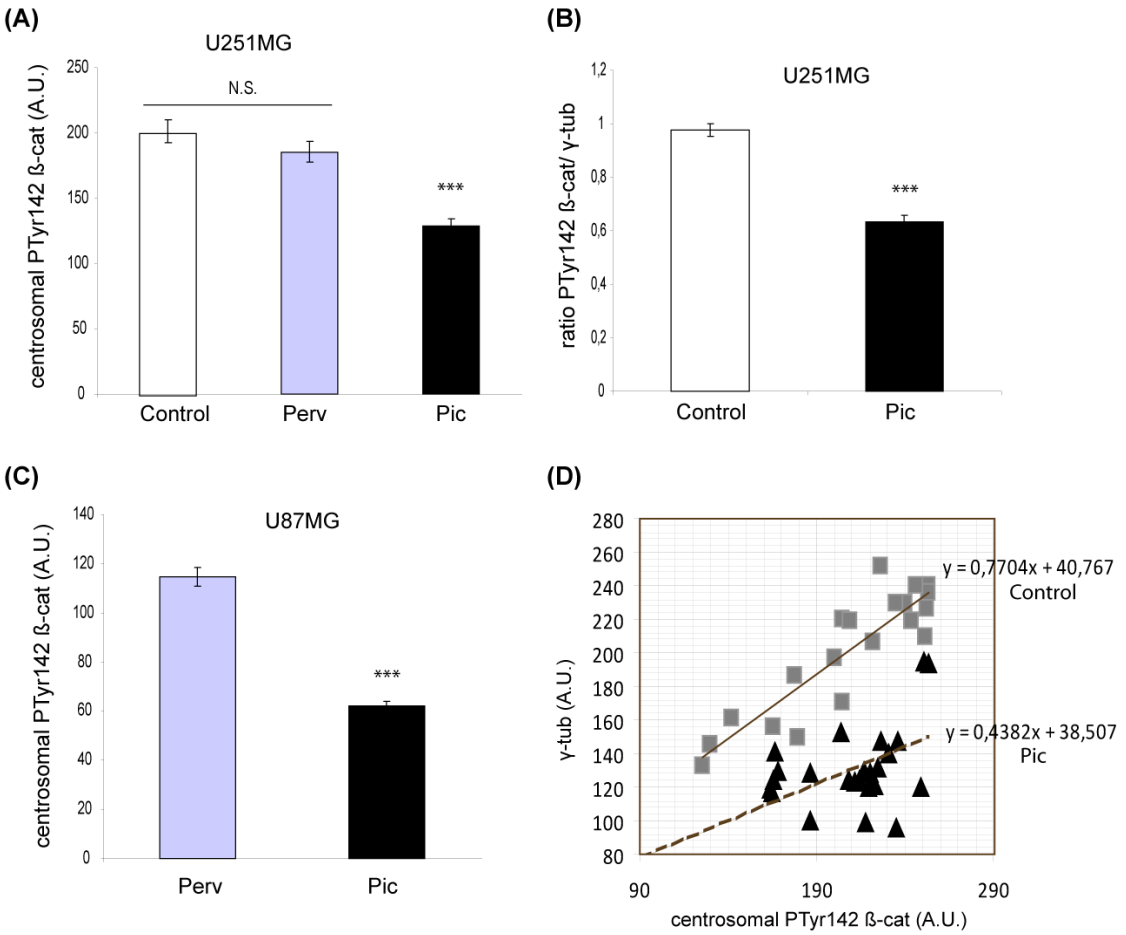


Figure 29. Immunofluorescence intensity quantification confirms the reduction of PTyr142 β-cat levels at centrosome following treatment with Pic in glioma cell lines. *Immunofluorescence intensities of PTyr142 β-cat and γ-tub at centrosome were measured using the RGB plugin of ImageJ (available online) in U251MG (A, B and D) and U87MG (C) cells. Plots in A and C represent the intensity values obtained for centrosomal PTyr142 β-cat, which is significantly reducted in Pic-treated cells (200 µM, 6h) compared to control or Pervanadate-treated (Perv; 1mM, 15 mins) cells. Differences between control and Pervanadate were not significant (N.S.). *** $p \leq 0.001$ indicates statistical significance versus control. Plot in C represents the values for the*

*"colocalization ratio" (PTyr142 β-cat/γ-tub) at centrosome) in each cell, which is also significantly reduced in Pic-treated cells versus control cells (*** $p \leq 0.001$). Plot in D summarizes graphically the correlation between PTyr142 β-cat and γ-tub intensity values in untreated and Pic-treated U251MG cells. The linearizations obtained for control (grey squares) and Pic-treated (black triangles) cells illustrate the dispersion, with lower PTyr142 β-cat values in Pic-treated cells ($p \leq 0.001$). A.U., arbitrary units.*

The immunofluorescence intensity quantifications revealed a siginificant reduction in PTyr142 β-cat levels at centrosome following treatment with Pic with respect to control in U251MG and U87MG cell lines. In contrast, γ-tubulin intensive values were not affected by treatment with Pic. The ratio depicting the immunofluorescence intensity of PTyr142 β-cat to γ-tubulin in each cell was also reduced significantly in U251MG cells treated with Pic versus untreated cells (Figure 29).

The immunostainings revealing the regulation of centrosomal PTyr142 β-cat by Pic, were further supported by confirming the centrosomal localization of endogenous Syk in glioma cells. Double immunocytochemistry was performed using anti-Syk antibodies together with PTyr142 β-cat antibodies in both U87MG and U251MG cells lines. In U87MG control cells, PTyr142 β-cat and Syk colocalize at the centrosomes (Figure 30). In contrast, in Pic-treated cells PTyr142 β-cat was absent from the centrosomes as described above, while total Syk levels at the centrosome remain as in control cells as expected. This finding is illustrated by the merge pictures depicting either "yellow" (colocalization of PTyr142 β-cat and Syk) or "green" (displaying only Syk immunostaining) centrosomes in control and Pic-treated cells, respectively (Figure 30)

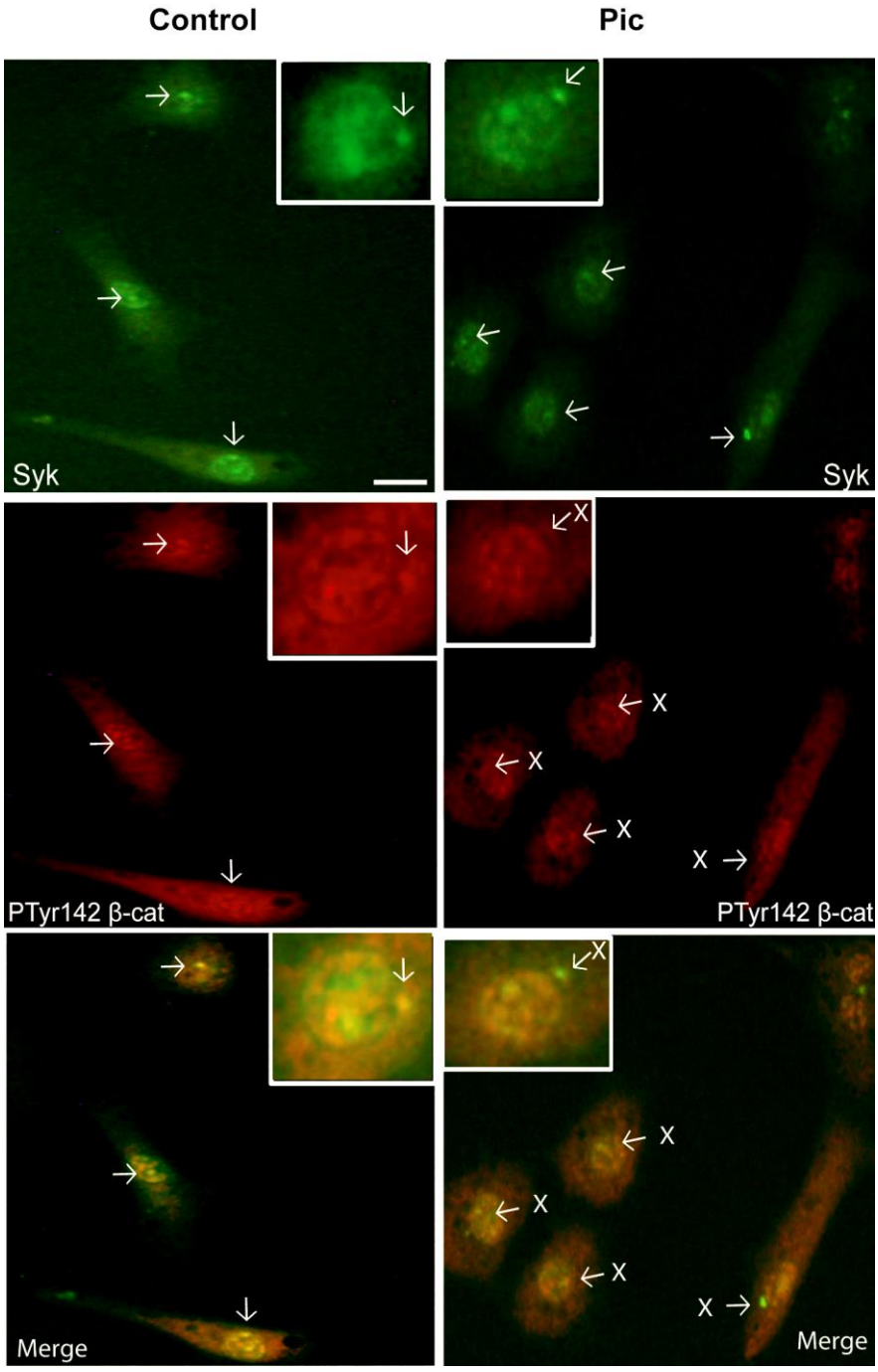


Figure 30. Centrosomal localization of endogenous Syk in U87MG cells. *Immunofluorescence images acquired at 20X demonstrate the colocalization of both PTyr142 β-cat (in red) and Syk (in green) in control cells and Pic-treated cells. In cells treated with Pic, PTyr142 β-cat levels diminish but centrosomal Syk levels remain similar to control. Positive centrosomal stainings are illustrated by arrows, whereas absence or reduction in the centrosomal levels are marked as (X). H is immunostained in blue. Bars = 15 µm.*

In U251MG cells, whereas centrosomal expression of both Syk and PTyr142 β-cat was observed upon treatment with pervanadate, Syk persisted at centrosomes upon treatment with Pic. However, again, centrosomal PTyr142 β-cat was decreased by Pic (Figure 31).

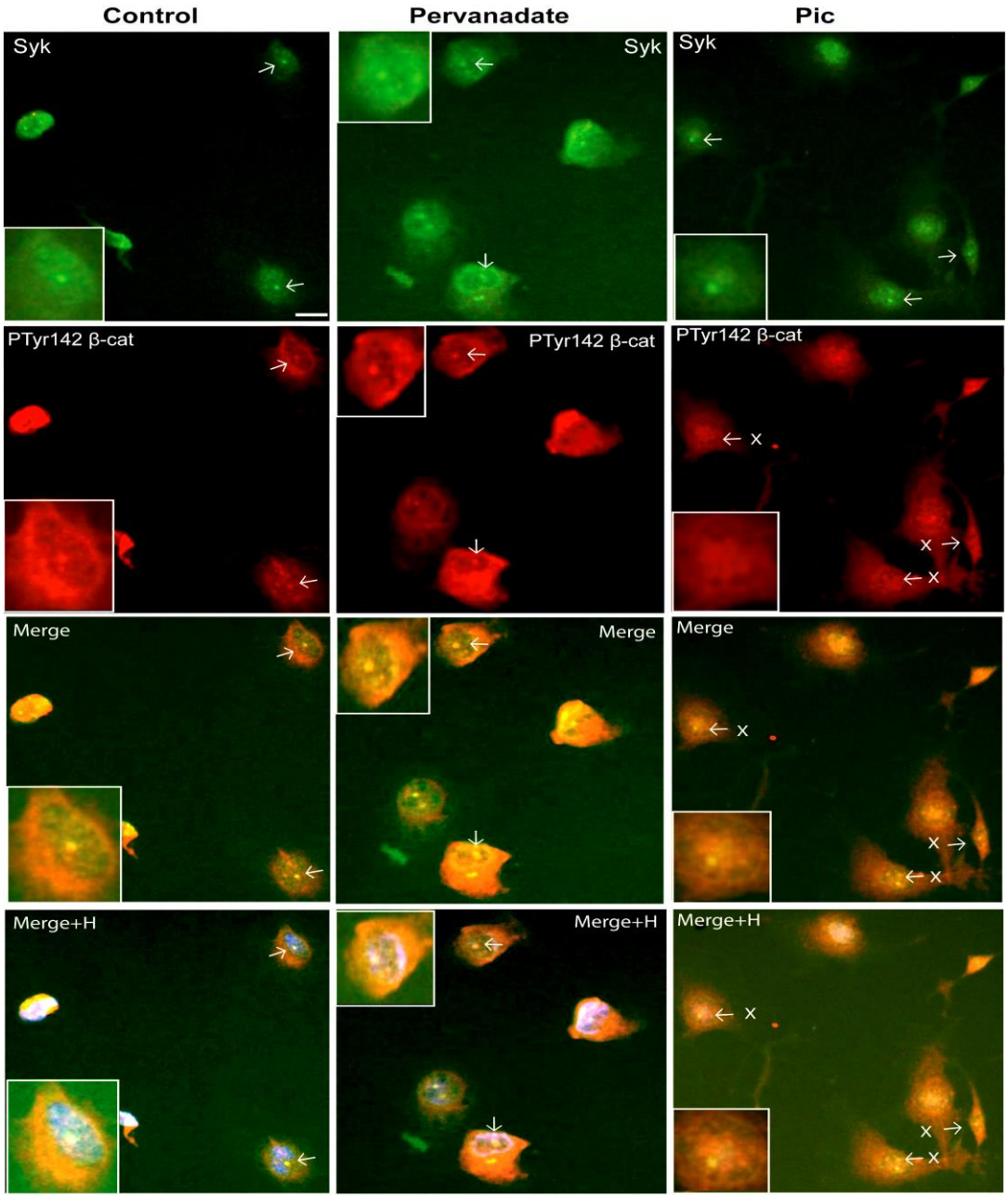


Figure 31. Comparison of centrosomal levels of PTyr142 β-cat and Syk upon pervanadate (1mM) and Pic (200 µM) treatments to U251MG cells. *Immunofluorescence images acquired at 20x demonstrate the colocalization of PTyr142 β-cat and Syk at centrosome in control conditions. Treatment with Pic diminishes PTyr142 β-cat centrosomal levels. Positive PTyr142 β-cat centrosomal staining is illustrated with the*

help of arrows, whereas its absence is marked with (X). H is immunostained in blue. Bars = 11 µm.

DISCUSSION

β-catenin plays essential and well-established roles in cell–cell adhesion and Wnt canonical signaling (Nelson, & Nusse, 2004). Moreover, β-catenin's implication in bipolar spindle formation and centrosomal functions came into light recently (Kaplan et al., 2004; Huang et al., 2007; Bahmanyar et al., 2008). Of note, phosphorylation of β-catenin by different kinases plays key roles in regulating the level, localization, and signaling function of β-catenin in different cellular processes.

β-catenin plays an important role in the structural and functional organization of adherens junctions in cell–cell adhesion. It binds tightly to the cytoplasmic domain of type I cadherins and to the actin-binding protein α-catenin (Weis, & Nelson, 2006). The catenin–cadherin complex is regulated by Ser/Thr phosphorylation of β-catenin (Bek, & Kemler, 2002) and E-cadherin (Aberle et al., 1994). In axon outgrowth, synapse development and pathological states, Tyr phosphorylation of β-catenin disrupts the cadherin–catenin complex, causing reduction of cell–cell adhesion and increasing cytoplasmic β-catenin (Roura et al., 1999; Piedra et al., 2003; David et al., 2008, Brembeck et al., 2004; Murase, Mosser, & Schuman, 2002). In the case of β-catenin phosphorylated at Tyr142, this signaling form is induced by HGF in neurons (David et al., 2008). PTyr142 β-cat translocates to the nucleus and regulates expression of target genes in a TCF-dependent manner. Here we identified several chemokines as novel targets of HGF/Met/β-catenin/TCF signaling. In addition, we showed the involvement of chemokine signaling in the definition of the morphology of axon arbours during the establishment of neuronal connectivity in brain development.

β-catenin also plays a key role in the canonical Wnt signaling pathway (Nelson, & Nusse, 2004), in which regulation of the level of cytoplasmic β-catenin is the central element. In addition to its roles in Wnt signaling and cell–cell contact, β-catenin is a component of the centrosome (Kaplan et al., 2004). During interphase, the centrosome coordinates an astral array of MTs that participates in intracellular trafficking, cell motility, adhesion and polarity (Azimzadeh, & Bornens, 2007). β-catenin localizes to centrosomes in interphase and in mitosis and controls centrosome separation and bipolar spindle formation (Kaplan et al., 2004; Bahmanyar et al., 2008). Depletion of β-catenin leads to an increase in monopolar spindles and impaired centrosome separation (Kaplan et al., 2004; Huang, Senga, & Hamaguchi, 2007). On the contrary, stabilized β-catenin (with alanine substitutions of GSK-3β/CK1 phosphorylation sites Ser33/Ser37/Thr41 and Ser45) also localizes to centrosomes (Bahmanyar et al., 2008, 2010). Stabilization of β-catenin results

in premature centrosome separation (Bahmanyar et al., 2008), an effect also proposed for β-catenin dephosphorylated at Ser/Thr ("Wnt-active") (Hadjihannas, Brückner, & Behrens, 2010). Here we demonstrate that PTyr142 β-cat localizes to centrosome both in cancerous and non-cancerous cells. We also propose that Syk regulates centrosomal PTyr142 β-cat. Centrosomal aberrations are common in cancer, including centrosome amplification (extranumerary centrosomes), leading to monopolar or multipolar spindles and chromosome instability. As Wnt/β-catenin signaling is widely implicated in cancer, the localization of this novel β-catenin signaling form at the centrosome points at new functional implications of β-catenin (in addition to regulation of transcriptional targets, self-renewal, proliferation and migration) that may clearly contribute to its oncogenic roles.

Chemokines in axon morphogenesis

Chemokine signaling during neurogenesis and neuronal migration

Our cDNA array data identified several chemokines as target genes of HGF signaling in developing hippocampal neurons (Bhardwaj et al., 2013). Chemokines, were initially recognized as the proteins engaged in diverse functions in the immune system (Rossi, & Zlotnik, 2000) including regulating leukocyte migration and neuroinflammation (Hesselgesser, & Horuk, 1999; Ransohoff, & Tani, 1998). The insights into their functions were thought to be obtained through their evolutionary ancestry with the immune system. However, it is now clear that the rapid expansion of the chemokine family only accompanied the evolution of immune system, proven by their roles in nervous system (discovered later). In the recent years, numerous studies have demonstrated the expression of chemokines as well as their receptors in the CNS, where they have been shown crucial in physiological and pathological conditions such as neuronal development, synaptic transmission, brain homeostasis, injury and disease-associated neuroinflammation (reviewed in de Haas et al., 2007). Phenotypic inspection of CXCL12 and CXCR4 knock-out mice indicated the original function of chemokines in regulating migration of stem cells (Miller, Banisadr, & Bhattacharyya, 2008). Both α chemokines (CXCL1, CXCL6 and CXCL8 and their receptors CXCR1 and CXCR2) and β-chemokines (CCL2 and CCL7) have been studied for their the task in regulating proliferation, neurogenesis and differentiation of dopaminergic precursors in the midbrain (Edman et al., 2008; Edman,

Mira, & Arenas, 2008). An age dependent role of CCL2, CCL3 and CXCL1 have been shown to influence both migration rate and fate of SVZ-derived neural precursor cells (Gordon et al., 2012). But the role of chemokines is not only restricted to developmental stages, as chemokines like CXCL12, CCL2, CCL3 and CXCL1 acts as chemoattractant and guide adult neuronal precursors during migration (Gordon, McGregor, & Connor, 2009; Tran et al., 2004, 2007). CXCL12/CXCR4 signaling is involved in the guidance of motoneuron axons (Lieberam et al., 2005) and downstream of Sonic Hedgehog signaling in retinal ganglion cell axon path finding (Stacher Hörndli & Chien, 2012). In agreement with our array data, *in situ* hybridization data (freely available online) reveal the expression of chemokines in the mouse hippocampus during embryonic and adult life (Genepaint and Allen Brain Atlas webpages). Further strengthening the role of chemokines during development in hippocampus, CXCL12/CXCR4 was shown to promote the extension of perforant-fibres from the entorhinal cortex to DG (Ohshima et al., 2008), which was further complemented by demonstrating the CXCR4-knockout mice exhibiting the defect in the structure of DG for the poor migration of the granule cells (Bagri et al., 2002; Lu, Grove, & Miller, 2002). Interestingly, CXCL12 has also been involved in the modulation of branching for the leading process of interneurons, which inversely affects the speed of migration of interneurons during their travel from the ganglionic eminescence towards the cortical plate (Lysko, Putt, & Golden, 2011). CXCL12 either produces growth cone repulsion or attraction depending on the levels of cGMP (Xiang et al., 2002). Remarkably, CXCL12 signaling regulates the migration of a variety of neuron and neuronal progenitor populations: gonadotropin-releasing hormone-1 neurons emerging from the nasal placode (Casoni et al., 2012), interneurons moving from the medial ganglionic eminence towards the cortical plate (Lopez- Bendito et al., 2008; Lysko, Putt, & Golden, 2011), cerebellar progenitors (Zou et al.,1998; Vilz et al., 2005) and sensory neuron progenitors towards the DRGs (Belmadani et al., 2005).

Involvement of chemokine signaling in neurite outgrowth

As opposed to the well established role of some chemokines in the regulation of neuronal progenitor or neuronal migration, not many research reports were available demonstrating their involvement in neurite outgrowth. Nevertheless, CXCL12/SDF-1 is one of the best studied in this new role also. CXCL12 signaling via its receptor CXCR4 has been involved in axon morphogenesis, where it reduces axon elongation while increasing branching, such

that it reduces growth cone number in hippocampal neurons (an effect restricted to the axon) (Pujol, Kitabgi, & Boudin, 2005). CC chemokines including CCL2 and CCL7 via their receptors CCR1 and CCR2, have been identified as the factors promoting the differentiation and neuritogenesis of midbrain dopaminergic neurons (Edman, Mira, & Arenas, 2008). Another CC chemokine, CCL5 is secreted by astrocytes and regulates neurite morphogenesis in cortical neurons (Chou et al., 2008). Furthermore, decreased secretion of CCL5 by astrocytes expressing mutant Huntingtin protein contributes to the neuronal dysfunction in Huntington's disease (Chou et al., 2008),

To our knowledge, our work is the first one reporting the induction of axon morphogenesis by several CC chemokines and CXCL2 in hippocampal neurons (Bhardwaj et al., 2013). We identified several chemokines in a cDNA array comparing the expression pattern of control and HGF-treated developing rat hippocampal neurons (at 2DIV). Similar to the role of CXCL12 in promoting axon branching in dissociated hippocampal neurons (Pujol, Kitabgi, & Boudin, 2005), we found that CXCL2 enhances neurite morphogenesis by increasing axon length and remarkably axon branching, suggesting that the CXC family of chemokines induce branch formation. Further supporting the evidence in favour of chemokines regulating axon development, mice lacking chemokine receptor CX3CR1 showed impairments in hippocampal cognitive function and synaptic plasticity (Rogers et al., 2011).

The above summarized observations in the CNS have been paralleled by the important roles identified for the chemokines in the PNS too, where effort has been put to investigate chemokine signaling in different chronic pain syndromes resulting from damage to the nervous system or infections from agents such as HIV-1 (Wallace et al., 2007; White, Jung, & Miller, 2007). Generally, two distinct targets in PNS are chosen to study the actions of chemokines: nociceptive sensory (DRG) neurons and microglia within the dorsal horn of the spinal cord (SC) (reviewed in Miller et al., 2008). The best studied example amongst all the chemokines in this field is CCL2/CCR2 (reviewed in Miller et al., 2008), followed by CX3CL1 and CCL1, the attenuation of which has been proposed as an important target for the drug development against neuropathic pain (Zhu et al., 2014; Solinski et al., 2013 ; Akimoto et al., 2013; Zhu et al., 2013) and; CXCL1/CXCR2 signaling (Zhang et al., 2013). These molecules do not exhibit high levels in the DRG or SC, but are strongly upregulated in in the state of chronic pain (White et al., 2005; Sun et al., 2006). Upregulation of CXCL12/CXCR4 as well as CCL2/CCR2 signaling has been

seen upregulated upon chronic morphine treatment and in a state of chronic pain (Wilson et al., 2008; White et al., 2005; Sun et al., 2006). To our knowledge, from the CXC family of chemokines only CXCL12 have been demonstrated in promoting neurite outgrowth from DRG neurons (PNS) on inhibitory CNS myelin (Opatz et al., 2009), followed by CXCL2 (this work). In this study, CXCL2/CXCR2 signaling has been suggested as promoting axon outgrowth in neurite outgrowth developed by DRG explants, which was reduced upon the addition of an inhibitor of the CXCR2 receptor (SB225002). Neurite outgrowth from DRG explants (plated on collagen) was promoted by CXCL2 treatment compared to control, both in the presence of NT-3 or NGF (allowing survival of propioceptive or nocioceptive sensory neurons, respectively). As DRG explants were prepared from neonatal DRGs whose sensory axons are broken in the process of extraction, this finding suggests that CXCL2 may promote of axon outgrowth of injured "regenerating" axons. In addition, some cells (presumably SGCs) were observed migrating out of the control DRG explants, but their number out of the explant increased in the presence of CXCL2. We were not able to distinguish whether exogenously added CXCL2 promoted the neurite outgrowth or this chemokine was also responsible for the proliferation/migration of SGCs and subsequently these SGCs secreted molecules responsible for the outgrowth. SGCs are the most important cell type of glial cells in sensory ganglions that respond to sensory damage or inflammation by proliferating and expressing GFAP (Souza et al., 2013). CX3CL1 and CXCL12 have been shown to enhance sensitivity towards pain by the activation of SGCs (Souza et al., 2013; Bhangoo et al., 2007). Chemokine receptors like CXCR4 are expressed by the DRG SGCs (Bhangoo et al., 2007). Nonetheless, the expression of CXCR2 in sensory neurons of DRG has also been demonstrated previously, while showing it to have important pro-nociceptive effects on neurons (Wang et al., 2008). Co-immunostainings with GFAP and βIII tubulin in DRG explants showed that SGCs are immunostained for GFAP and already out of the explant in control conditions. However, upon CXCL2 treatment the number of SGCs out of the explant significantly increases (possibly by CXCL2 regulating the proliferation/migration of SGCs) and this correlates with increased neurite outgrowth (as confirmed by βIII tubulin immunostaining). Interestingly, GFAP-immunostained SGCs appeared aligned on top of the neurites, suggesting that SGCs support neurite outgrowth. Alternatively, SGCs could provide also some trophic suppot (by secreting cytokines or other molecules) that could regulate neurite outgrowth. Indeed, inhibition of CXCR2 (the

only receptor for CXCL2) by SB225002 significantly inhibited neurite outgrowth, and only few SGCs exhibiting GFAP immunostaining were seen.

In sum, our work reveals that chemokine signaling plays a role in axon morphogenesis in developing hippocampal neurons and in the outgrowth of regenerating sensory axons.

HGF and β-catenin signaling in chemokine expression

This study has established a relationship between HGF and chemokine signaling in hippocampal neurons during axon morphogenesis (Bhardwaj et al., 2013). In agreement with our cDNA array study, HGF-treated neurons exhibited enhanced chemokine expression, especially CCL5, CCL7, CCL20 and CXCL2. Antibodies directed against CCL20 and CXCL2, and CXCR3 and CXCR2 antagonists SB328437 and SB225002 both displayed reduction in axon outgrowth and branching promoted by HGF, which indicates that chemokine signaling acts downstream to HGF signaling. Furthermore, SB225002 and SB328437 treatment resulted in reduction of axon length values below those of untreated neurons indicating that endogenous chemokine production by hippocampal neurons also affects axon development. Nonetheless, antibodies against CCL20 or the treatment with SB328437 decreased axon branching in the presence of HGF. However, CCL20 at the tested concentrations (10-1000 ng/ml) did not promote significant axon branching, suggesting that promotion or inhibition of axon branching exhibit different EC50/IC50 (Half maximal effective concentration/Half maximal inhibitory concentration) values. Perhaps CCL20 concentrations lower than tested may induce axon branching. It is noteworthy that our array study on HGF-treated neurons identified other secreted proteins, in addition to chemokines, putatively involved in axon morphogenesis including CSF-1 and BMP-6.

β-catenin is a central component of Wnt signaling cascade which promotes transcription of Wnt targets upon binding to Lef/TCF (reviewed in Valenta, Hausmann, & Basler, 2012). Both HGF and β-catenin have been reported to be critically involved in axon morphogenesis and dendritic development in hippocampal neurons (reviewed in Akamura & Izuno, 2010; David et al., 2008). Apart from this, the interaction between Met and β-catenin has been demonstrated (Purcell et al., 2011; David et al., 2008; Monga, 2002) that results in the phosphorylation of β-catenin at Tyr142, leading to PTyr142 β-cat translocation to the nucleus and regulating axon morphogenesis through TCF4-dependent transcriptional activation (David et al., 2008). In cancer cells, β-catenin and α-catenin

phosphorylation downstream of RTKs and/or Src is also emerging as a Wnt independent mechanism that promotes β-catenin transcriptional activation (Ji et al., 2009; Xi et al., 2013). In this work, chemokines were indentified as transcriptional targets of HGF signaling in developing hippocampal neurons. CXCL2 and CCL5 expression analysis confirmed that these chemokines are upregulated by HGF signaling. Furthermore, TCF inhibition or silencing of β-catenin blocked the upregulation of CXCL2 by HGF, suggesting that chemokines are at least in part responsible for the axon morphogenesis promoted by HGF/Met/β-catenin signaling (Bhardwaj et al., 2013). In support of this, a previous paper reported upregulation of cytokines and chemokines including CXCL2 by Wnt-3a and β-catenin signaling in activated microglia (Halleskog et al., 2011). Moreover, Wnt/β-catenin and Met signaling synergistically activate CXCL12 expression in breast cancer cells, further highlighting the connection between β-catenin, Met and chemokine signaling in cancer (Holland et al., 2013). Real time PCR analysis also showed that TCF inhibition reduced the expression of CCL5 promoted by HGF (by ~30% compared to HGF treated neurons) indicating in part regulation of CCL5 through TCF/β-catenin downstream of HGF signaling. HGF signaling could be activating another pathway, e.g. NFκB pathway, which has been shown to regulate CCL5 expression in a Huntington's disease model (Chou et al., 2008).

Hence, our findings add the chemokines to the growing list of secreted molecules that modulate axon outgrowth in CNS and PNS. Chemoattractive cytokines are widely involved in brain injury and post-ischemic repair (Wang et al., 2012), orchestrating the recruitment of inflamatory cells. In addition, chemokines promote stem cell attraction, survival and differentiation. Chemokines might thus indirectly participate in remyelination, neovascularization and neuroprotection that are important for CNS repair after trauma (reviewed in Miller et al., 2008; Jaerve, & Müller, 2012). CXCL12 was demonstrated to regulate neurite outgrowth in the presence of growth inhibitory CNS myelin and to enhance axonal sprouting after spinal cord injury (Jaerve, Schira, & Müller, 2012). Our work opens the possibility that other chemokines of CC and CXC families regulate axon outgrowth in a general manner, outside of the hippocampus and in adult life. As proposed for CXCL12, it will be exciting to investigate whether other chemokines could improve nerve regeneration *in vivo.*

Phosphorylated Tyrosine142 residue of β-catenin at centrosomes

Involvement of β-catenin and its phosphorylation at Tyr142 in cell polarization and migration

Evidence in favour of HGF/Met/β-catenin signaling in axon outgrowth, cell migration and invasion (David et al. 2008; Monga et al., 2002; Brembeck et al., 2004) took us to investigate this signaling axis in migrating astrocytes and glioma cells. We performed wound-healing assays in rat astrocytes treated with HGF or with HGF and the Met inhibitor, SU11274. From these experiments, we concluded that HGF works as a migratory factor, as the width of the wound in astrocytes treated with HGF was smaller that in controls after 24h of treatment. In contrast, in astrocyte monolayers treated with HGF and the Met inhibitor, the width of the wound remained similar or wider than in controls. Hence, we show that HGF/Met signaling operates *in vitro* in astrocyte migration induced by the artificial formation of a wound to the astrocyte monolayer.

Further, to address the role of β-catenin downstream to HGF signaling in cell migration, we performed *in vitro* wound-healing assays on Hek293T cells expressing WT or Tyr142Phe β-cat. The addition of HGF to cells expressing WT β-cat increased cell migration versus untreated cells overexpressing WT β-cat. However, cell migration (measured as the reduction in the width of the wound) was similar in cells expressing Tyr142Phe β-cat untreated or treated with HGF, indicating the requirement for PTyr142 β-cat downstream to HGF/Met signaling during cell migration in Hek293T cells. Similar to our work, phosphorylation of β-catenin at Ser675 (causing the dissociation of β-catenin from adherens complex) has been proposed to be involved in the promotion of astrocyte activation, migration and acquisition of an astrocytoma phenotype upon astrocyte injury (Yang et al., 2012). Since β-catenin signaling appears to operate downstream of HGF signaling in astrocytes, manipulation of this pathway could be helpful in brain injury to modulate migration and repopulation of the site of injury by astrocytes.

We also investigated the role of β-catenin in centrosome positioning during cell migration in primary astrocytes. It is well known that during migration, astrocytes localize the centrosome in front of the nucleus, in the direction of migration (Etienne-Manneville, &

Hall, 2001; Etienne-Manneville, 2006). Here we showed the effect of silencing β-catenin in rat astrocytes, where shRNA against β-catenin reduced the percentage of cells bearing correctly positioned centrosomes in the direction of wound. In contrast, the percentage of cells with correctly positioned centrosomes was similar in non-infected as well as in scrambled shRNA conditions, which highlights the specificity of the shRNA β-catenin. In line with our results, other cell adhesion molecules like N-cadherin have also been recognized important for the correct positioning of centrosome in migrating astrocytes (Camand et al., 2012). Thus, silencing of N-cadherin increases the percentage of cells with incorrectly positioned centrosomes and promotes erratic movement, a condition that resembles the situation of GBM cells (expressing low levels of N-cadherin and typically showing non-directed migration; Camand et al., 2012). Although, β-catenin's presence at centrosome has been previously demonstrated by immunocytochemical as well as biochemical approaches (Kaplan et al., 2004; Olmeda, Castel, & Cano, 2003; see below), its role in centrosome reorientation during migration was not described. Thus, this work shows β-catenin's involvement in cell polarity by guiding the correct positioning of centrosomes to regulate directionality during cell migration, possibly through interaction with the MTs. β-catenin is overexpressed in GBM (Sareddy et al., 2009; Liu et al., 2010, 2011; Ji et al., 2009), where centrosomal PTyr142 β-cat has been observed (this work). Further work is needed to address whether centrosomal PTyr142 β-cat signaling could be related to centrosome reorientation in astrocyte and astrocytoma cell migration.

Total, PSer/Thr and PTyr142 β-cat localize to centrosomes.

β-catenin, a multifaceted protein with critical structural and signaling functions in adhesion and Wnt pathway, extends its arms into cell-cell contacts, nucleus, cytosol and, lately, centrosomes (reviewed in Mbom, Nelson, & Barth, 2013). β-catenin cytoplasmic and nuclear levels were demonstrated to increase in S phase, reaching maximum at late G2/M followed by the abrupt reduction as cells re-enter G1 after mitosis (Olmeda, Castel, & Cano, 2003) in transformed keratinocytes. Another report, however, showed unchanged total β-catenin levels in synchronized colorectal cell lines in G2/M phase (Hadjihannas et al., 2012). One of the first evidences in favour of β-catenin's role in regulating centrosomal functions, came up with the demonstration that overexpression of β-catenin leads to centrosome and MT array disorganization (Ligon et al., 2001), while β-catenin silencing inhibits MT nucleation from centrosomes (Huang, Senga, & Hamaguchi, 2007).

Later, β-catenin was recognized for its role in recruiting centrosomal proteins and contributing to centrosome maturation, bipolar spindle formation and centrosome separation (reviewed in Mbom, Nelson, & Barth, 2013). β-catenin localization to intherphase centrosomes is mediated by centrosomal linker proteins (Rootletin and C-Nap), which become phophorylated by Nek2 at the onset of mitosis thus providing means for centrosome separation. In mitosis, β-catenin localizes at spindle poles independent of these linker proteins (Bahmanyar et al., 2008). We observed total β-catenin co-fractionating with γ-tubulin in centrosomal fractions isolated both from striatal astrocytes as well as U251MG glioma cell line, which was accompanied by the Wnt pathway component APC in striatal astrocytes as reported (Louie et al., 2004; Lui et al., 2012, Olmeda, Castel, & Cano, 2003; Hadjihannas et al., 2012).

Not only total β-catenin, but also phosphorylated β-catenin forms has been localized at centrosome, with different roles to play. Immunofluorescence studies using the antibody recognizing β-catenin phosphorylated at Ser33/Ser37/Thr41 (PSer/Thr) localized this form to the centrosome (Chilov et al., 2011), with preferential localization at mother centrosome in interphase and to daughter centrosome during cell division (Fuentealba et al., 2008). Increased PSer/Thr β-catenin and conductin (also known as Axin2; a negative regulator and target of Wnt signaling) levels accumulate in G2/M phases in synchronized colorectal cells (Hadjihannas et al., 2012). Reduced levels of PSer/Thr β-catenin have been linked to mitotic spindle defects and, centrosomal and MT defects could be rescued upon expressing a phospho-mimetic mutant (Chilov et al., 2011). These results were obtained in neuronal progenitor cells, and mice expressing mutant β-catenin (mutated at Ser33/Ser34/Thr41) displayed defects in MTs and polarity (Chilov et al., 2011). Importantly, PSer/Thr β-catenin is found at bipolar spindle in mitosis (Bahmanyar et al., 2008; Mbom et al., 2014). Interestingly, Nek2 appears as the the main kinase regulating PSer/Thr β-catenin at centrosome and GSK-3β inhibitors do not affect centrosomal PSer/Thr β-catenin (Mbom et al., 2014). These authors propose that Nek2 binding to β-catenin (observed both for active and kinase-dead Nek2) interferes with binding of the E3 ligase to β-catenin and therefore with its ubiquitination and degradation (Mbom et al., 2014). This explains the apparent contradiction that PSer/Thr β-catenin (usually targeted for degradation in the Wnt pathway) is stable at centrosomes. In addition, a role for PSer/Thr β-catenin with regards to centrosome separation has also been proposed, where during mitosis Plk-1 activates Nek2 and Nek2-mediated β-catenin binding to centrosomes is needed for centrosome

separation (Bahmanyar et al., 2008) through the modulation of β-catenin interaction with the centrosomal linker Rootletin. Centrosomal PSer/Thr β-catenin is retained during mitosis and centrosomal disjunction (Mbom et al., 2014). On the other hand, it was shown that expression of a stabilized form of β-catenin (with impaired N-terminal-regulated degradation) leads to increasing distance between centrosomes, suggesting that β-catenin acts as a negative regulator of centrosome cohesion (Bahmanyar et al., 2008). In agreement with this, it was proposed that Wnt signaling or inhibition of GSK-3β promotes centrosome splitting by altering the phosphorylation status of β-catenin to dephospho Ser/Thr β-catenin (Hadjihannas, Brückner, & Behrens, 2010). The mechanisms by which β-catenin promotes centrosome disjunction remain therefore nowadays unclear. β-catenin may regulate centrosomal disjunction through different mechanisms involving interactions with different partners.

In addition to the described presence of centrosomal PSer/Thr β-catenin, our work demonstrates for the first time the centrosomal localization of β-catenin phosphorylated at a tyrosine residue. Here we show, using different types of cell fixation and a phosphospecific antibody, that β-catenin phosphorylated at Tyr142 (PTyr142 β-cat) localizes to centrosome in different cell types, including Hek293T kidney cells, striatal astrocytes and glioma cell lines. PTyr142 β-cat appears to be "stable" at centrosomes as it was immunodetected under basal conditions. Centrosome fractionation further confirmed our immunofluorescence results, showing the presence of PTyr142 β-cat (co-fractionating with γ-tubulin) in centrosomal fractions. PTyr142 β-cat levels were higher in the U251MG glioma cell line than in striatal astrocytes, in agreement with a role for this phosphorylated form of β-catenin in GBM invasion (Nager et al., unpublished results). We also found that the immunostainings obtained using anti-PTyr142 β-cat or pan-PTyr antibodies were different. PTyr142 β-cat immunostaining concentrated in the cytosol and nucleus (as similarly found in neurons; David et al., 2008), whereas pan-PTyr immunostaining localized preferentially to the plasma membrane in lamella in glioma cells.

Our study of PTyr142 β-cat levels at the centrosomes in glioma cells synchronized by serum-deprivation and subsequent release in serum-containing media allowed us to investigate the changes in centrosomal PTyr142 β-cat during mitotis. We observed that PTyr142 β-cat levels significantly drop from centrosomes during mitosis, in contrast to what was described for total and PSer/Thr β-catenin (Kaplan et al., 2004; Huang, Senga, & Hamaguchi, 2007; Mbom et al., 2014). This suggests that centrosomal PTyr142 β-cat

might be affecting cell division in a negative manner, such that decline in its levels (or dephosphorylation) is needed for cell division. Hence, our results show that PTyr142 β-cat is present in interphase centrosomes and is downregulated during cell division. Furthermore, PTyr142 β-cat was absent from mitotic spindles, suggesting that this β-catenin form would be in principle not necessary for spindle functions.

A balance between the activities of centrosomal kinases and phosphatases determines centrosome dynamics during cell cycle. Generally, phosphorylation of centrosome linker proteins (e.g., C-Nap1) leading to disruption of centrosome linking, will be a result of either activation of kinase or the inhibition of a phosphatase. One such phosphatase is Protein Phosphatase 1α (PP1α), known to suppress Nek2 activity as well as to dephosphorylate C-Nap1 thus suppressing centrosome splitting (Cohen, Holmes, & Tsukitani, 1990). In addition, PP1α is inhibited at the onset of mitosis, when Nek2 is activated (Puntoni, & Villa-Moruzzi, 1997). We could not clarify the mechanism by which PTyr142 β-cat was degraded or dephosphorylated during mitosis. Little is known about the implication of tyrosine kinases (in contrast to Ser/Thr mitotic kinases; Wang, Jiang, & Zhang, 2014) in the coupling of the centrosome and the cell cycles that ensures bipolar mitotic spindle fidelity. Intriguingly, a decline in centrosomal Syk tyrosine kinase during mitosis has been described, through its regulation by the ubiquitin-proteasome system (Zyss et al., 2005). On the other hand, Cell division cycle 25 (CDC25) phosphatases dephosphorylate Thr and Tyr residues and activate cyclin-dependent kinase (CDK) cyclin complexes. CDC25A, B and C are activated by mitotic kinases like Plk and Aur A (Boutros, Lobjois, & Ducommun, 2007) and control entry and progression into mitosis. CDC25B is thought to be responsible for the initial activation of CDK1–cyclin B at the centrosome that contributes to MT network reorganization and mitotic spindle assembly. CDC25C localizes to the centrosome in a cell cycle-dependent manner (late S phase, G2 and mitosis) (Bonnet, Coopman, & Morris, 2008). CDC25B localizes asymmetrically to mother centrosome during interphase, becomes evenly distributed by G2 and is required for centriole duplication (Boutros, & Ducommun, 2008). CDC25s could be candidate phosphatases that dephosphorylate PTyr142 β-catenin in mitosis. Alternatively, decreased centrosomal Syk could result in lower centrosomal levels of PTyr142 β-cat in mitosis (see below).

Apart from the mitotic downregulation of PTyr142 β-cat, the putative involvement of this β-catenin form in centrosome separation can not be ruled out, considering the roles of β-

catenin in this step of the centrosome cycle (Bahmanyar et al., 2008; Hadjihannas, Brückner, & Behrens, 2010). Indeed, centrosomes separation is a requirement for cells to enter into mitosis.

Tyrosine kinases phosphorylating β-catenin localize to centrosomes

Having localized PTyr142 β-cat at centrosomes, we aimed at identifying the putative kinase(s) regulating centrosomal PTyr142 β-cat. Until now different tyrosine kinases have been reported to modify different tyrosine residues in β-catenin, which dictate signaling activation in different cellular contexts. For example, Src phosphorylates β-catenin at Tyr86, (Roura et al., 1999) and Fer phosphorylates β-catenin at Tyr142 (Piedra et al., 2003). Here we focused on Met as one such candidate, as Met is overexpressed in GBM cells (Koochekpour et al., 1997) and we and other authors have demonstrated that Met phosphorylates β-catenin at Tyr142 *in vitro* and downstream of HGF signaling (David et al., 2008; Brembeck et al., 2004; Monga et al., 2002). Although Met was not localized to centrosomes before, overexpression of constitutively-active Met lead to extra numerary centrosomes, aberrant MT spindles and chromosome instability (Nam et al., 2010). We demonstrated that PTyr1234/35 (active) Met is found in centrosomes in basal conditions in glioma cells. PTyr1234/35 Met levels at centrosomes were however unaffected upon treatment with a Met inhibitor (SU11274) in U87MG cells or by stimulation with HGF (results not shown), the Met ligand. This correlated with PTyr142 β-cat centrosomal levels remaining apparently unaffected upon addition of HGF (not shown) or Met inhibitor in comparison to untreated cells. The reason for this lack of regulation of active Met at centrosomes is not known, but it could be part of the deregulated Met signaling operating in GBM. Therefore, these results do not allow us to conclude whether PTyr1234/35 Met and PTyr142 β-cat at centrosomes are functionally related.

Another candidate tyrosine kinase that could be related with centrosomal PTyr142 β-cat is Syk. The presence of active Syk at centrosomes has been established (Zyss et al., 2005), where the increased kinase activity (at Tyr130Glu residue of Syk) appears critical for both its centrosomal localization and its transportation from the plasma membrane via dynein/dynactin-dependent MT motors (Fargier et al., 2013). In addition, centrosomal Syk controls cell division and it is lost during mitosis (as similarly observed for PTyr142 β-

cat). Overexpression of stable Syk forms leads to abnormal mitosis, aberrant cell division and multipolar spindles (Zyss et al., 2005). A recent report searching for potential Syk substrates identified centrosomal Nek9 as a Syk substrate in breast cancer cells (Xue et al., 2012). To investigate whether Syk could regulate the centrosomal pool of PTyr142 β-cat, we expressed WT and Syk mutants in glioma cells: a kinase-dead mutant (Lys402Arg) which does not localize to centrosomes, and a mutant possessing increased intrinsic kinase activity (Tyr130Glu) that basally and almost exclusively localizes to the centrosome. Transfecting U251MG and U87MG cells with these mutants, however, did not significantly affect the centrosomal levels of PTyr142 β-cat. Thus, exogenously modulating Syk activity by expression of active or inactive Syk forms did not produce apparent changes in centrosomal PTyr142 β-cat. This made us examine the possible variations in PTyr142 β-cat levels upon inhibition of endogenous Syk by cell treatment of cells with the Syk inhibitor, Pic (Larive et al., 2009; Seow, Chue, & Wong, 2002). Pic suppresses proliferation of a variety of tumor cells (including leukemia, lymphoma, cancer of breast, prostate, colon and melanoma), promotes apoptosis and inhibits NFκB pathway (Piotrowska, Kucinska, & Murias, 2012). Interestingly, immunostainings demonstrated a decrease in centrosomal PTyr142 β-cat levels in cells treated with Pic, in comparison to control cells, in the two glioma cell lines. Quantification of the intensity of centrosomal PTyr142 β-cat levels confirmed these observations. Changes in the centrosomal levels of PTyr142 β-cat following treatment with pervanadate (a tyrosine phosphatase inhibitor and Syk activator; Nagai et al., 1995) were not significant (versus control). We also performed co-immunostaining for Syk and PTyr142 β-cat that illustrated their colocalization at centrosomes, as expected because Syk was also present in centrosomal fractions isolated from U251MG cells. These findings suggest that Pic inhibition of Syk results in reduced PTyr142 β-cat from the centrosome and point to Syk as a kinase regulating centrosomal PTyr142 β-cat. More experiments, like silencing of Syk, would be needed to further support this result. Syk was proposed as a tumor supressor (Coopman et al., 2000) and its high expression in astrocytes and Hek293T cells (results not shown) could thus account for the centrosomal PTyr142 β-cat pools in those cells. Interestingly, Syk has been reported to associate and phosphorylate other cell-cell adhesion components such as E-cadherin and α-catenin (Larive et al., 2009).

The localization of PTyr142 β-cat, active Syk and active Met at centrosomes reinforces the view of centrosomes as more than MT-organizing centers, but as important signaling

platforms that regulate the coupling of the centrosome cycle and the cell cycle. Alteration of the phosphorylation status of centrosomal proteins likely contributes to centrosomal abnormalities and affects the complex coordination of both cycles. EGF signaling has been recently implicated in centrosome separation and accelerating mitosis with fewer errors, a finding with implications in EGFR-positive cancers (Mardin et al., 2013). Understanding centrosomal PTyr142 β-cat signaling in normal and cancer cells in the context of cell cycle and cell migration control could provide us with new hypothesis for cancer therapy.

A general view of the results presented in this thesis work is summarized in Figure 32.

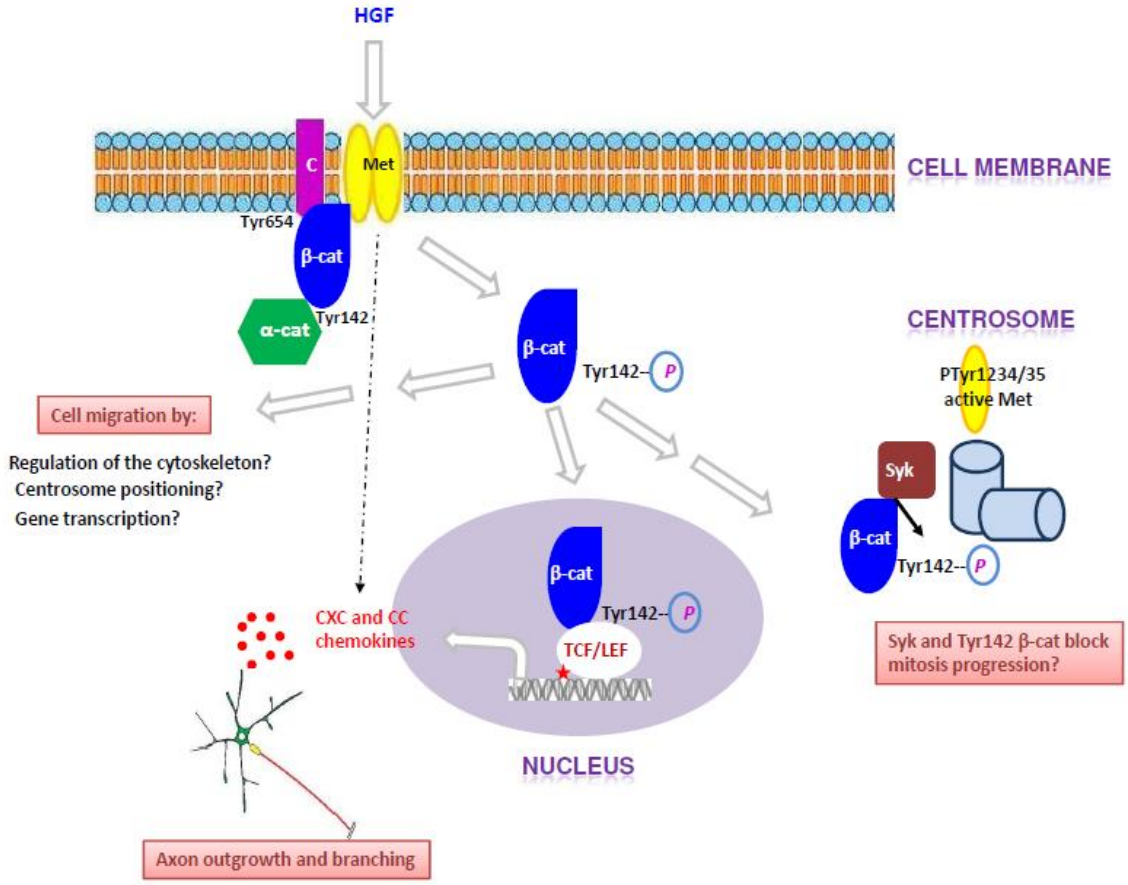


Figure 32. Summary model for the role of β-catenin and PTyr142 β-cat in different cell compartments. *β-catenin interacts with cadherins (C) (in a region including Tyr654 β-cat) and with α-catenin (α-cat) (in a region including Tyr142 β-cat) at cell-cell junctions. Results obtained in hippocampal neurons indicate that upon HGF binding to Met, Met activation leads to phosphorylation of β-catenin at Tyr142 that promotes its detachment from the adhesion complex and translocation to the nucleus, where it acts by activating transcription of TCF target genes. Here we showed that chemokines are targets of*

HGF/Met/β-catenin/TCF signaling that promote axon outgrowth and branching in hippocampal and DRG neurons. Dashed arrow indicates other pathways downstream of HGF/Met regulating chemokine expression. A pool of PTyr142 β-cat localizes to centrosome (in addition to the nucleus) in glioma cells. At the centrosome, Syk tyrosine kinase helps in maintaining the phosphorylation status of PTyr142 β-cat. Since both Syk and PTyr142 β-cat are decreased at centrosomes during mitosis, we postulate that they would block mitotic progression. Active Met (PTyr1234/35 Met) also localizes to centrosome. We also demonstrated the role of β-catenin in the correct orientation of centrosome during cell migration, and the requirement of PTyr142 β-cat in cell migration downstream of HGF signaling. Whether PTyr142 β-cat signaling in cell migration implicates gene transcription, centrosomal function and/or regulation of the cytoskeleton is not known.

We studied PTyr142 β-cat in the context of neuron develepment, where it was previously proposed to regulate transcription of genes important for axon morphogenesis downstream to HGF/Met signaling. In chapter 1, we identified several CC chemokines and CXCL2 as novel secreted molecules regulating axon outgrowth and branching. In chapter 2, we addressed the involvement of β-catenin and PTyr142 β-cat in cell migration. We demonstrated the participation of β-catenin in the positioning of the centrosome during cell migration. Last but not least, we localized PTyr142 β-cat at centrosome, where β-catenin is likely phosphorylated by Syk at Tyr142. Centrosomal Syk and PTyr142 β-cat are downregulated in mitosis, suggesting that they impose a blockade on mitotic progression.

CONCLUSIONS

1. Chemokines of CC and CXC families are upregulated downstream to HGF signaling in developing hippocampal neurons.

2. Recombinant chemokines CCL5, CCL7, CCL20 and CXCL2 promote axon outgrowth in hippocampal neurons.

3. CXCL2 promotes significant axon branching in hippocampal neurons.

4. Endogenous chemokine signaling acts downstream to HGF during axon outgrowth, as blocking antibodies against CCL20 and CXCL2 and SB225002 and SB328437 (chemokine receptor antagonists) reduce the HGF-induced axon morphogenesis.

5. Met and TCF inhibition reduce the axon outgrowth promoted by HGF in hippocampal neurons.

6. CXCL2 and CCL5 expression are regulated by HGF/Met/β-catenin/TCF pathway in hippocampal neurons.

7. CXCL2 promotes neurite outgrowth from the sensory neurons of DRG, directly or through stimulation of SGC proliferation / migration.

8. HGF signaling promotes the migration of rat primary astrocytes in wound-healing assays, which is inhibited by the Met inhibitor SU11274.

9. Phosphorylation of β-catenin at Tyr142 is important in the promotion of cell migration downstream to HGF signaling in Hek293T cells.

10. β-catenin plays a role in the orientation of centrosomes during directional migration in rat primary astrocytes.

11. PTyr142 β-cat localizes to centrosome and co-migrates with centrosomal-enriched fractions in different cell types, including astrocytes and glioblastoma cells.

12. PTyr142 β-cat centrosomal levels decrease during mitosis, suggesting that they regulate cell cycle progression in a negative manner.

13. PTyr1234/35 Met is found at centrosomes in glioblastoma cell lines.

14. The Syk inhibitor Piceatannol decreases PTyr142 β-cat from the centrosomes, suggesting that Syk regulates centrosomal PTyr142 β-cat.

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ANNEXURE

frontiers in
CELLULAR NEUROSCIENCE

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Chemokines induce axon outgrowth downstream of Hepatocyte Growth Factor and TCF/β-catenin signaling

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Axon morphogenesis is a complex process regulated by a variety of secreted molecules, including morphogens and growth factors, resulting in the establishment of the neuronal circuitry. Our previous work demonstrated that growth factors [Neurotrophins (NT) and Hepatocyte Growth Factor (HGF)] signal through β-catenin during axon morphogenesis. HGF signaling promotes axon outgrowth and branching by inducing β-catenin phosphorylation at Y142 and transcriptional regulation of T-Cell Factor (TCF) target genes. Here, we asked which genes are regulated by HGF signaling during axon morphogenesis. An array screening indicated that HGF signaling elevates the expression of chemokines of the CC and CXC families. In line with this, CCL7, CCL20, and CXCL2 significantly increase axon outgrowth in hippocampal neurons. Experiments using blocking antibodies and chemokine receptor antagonists demonstrate that chemokines act downstream of HGF signaling during axon morphogenesis. In addition, qPCR data demonstrates that CXCL2 and CCL5 expression is stimulated by HGF through Met/b-catenin/TCF pathway. These results identify CC family members and CXCL2 chemokines as novel regulators of axon morphogenesis downstream of HGF signaling.

Keywords: beta-catenin, axon, neurite outgrowth, chemokine, hippocampal neurons, hepatocyte growth factor

INTRODUCTION

The establishment of the neuronal morphogenesis is a complex process by which neurons extend and branch out an axon and dendrites, resulting in the proper assembly of the neuronal circuitry. A range of secreted molecules, including growth factors and morphogens, promote axonal, and dendrite outgrowth. Among them, the Neurotrophins (NT), Hepatocyte Growth Factor (HGF), and Wnts regulate neuronal survival, neurite outgrowth, synaptogenesis, and synaptic plasticity (Maina and Klein, 1999; Korhonen et al., 2000; Chao, 2003; Yu and Malenka, 2003; Ciani and Salinas, 2005; Nakano et al., 2007; Park and Shen, 2012). HGF signaling through its tyrosine kinase receptor Met provides neurotrophic signals to hippocampal neurons (Korhonen et al., 2000) and promotes axon outgrowth (David et al., 2008). How neurons interpret this variety of signals to develop unique axon arbor morphologies is just beginning to be understood.

β-catenin, a component of the cell–cell adhesion complex and an effector of canonical Wnt signaling, plays a key role in axon outgrowth, dendritogenesis, and synapse formation (Murase et al., 2002; Bamji et al., 2003, 2006; Yu and Malenka, 2003; Lu et al., 2004). Briefly, canonical Wnt signaling results in the cytosolic stabilization of β-catenin, which in the absence of Wnt is degraded through the ubiquitin-proteasome system (Li et al., 2012). Stabilized β-catenin translocates to the nucleus and together with Lymphoid Enhancer Factor-1 (LEF-1)/T-cell factor (TCF) transcription factors regulates the expression of Wnt target genes (Behrens et al., 1996; Molenaar et al., 1996). On the other hand, β-catenin binding to the adhesion complex components cadherin and α-catenin is altered by β-catenin tyrosine phosphorylation, resulting in the downregulation of cell adhesion and the promotion of migration (Nelson and Nusse, 2004; Heuberger and Birchmeier, 2010). We previously showed a requirement for β-catenin phosphorylation at Y654 and Y142 in the axon outgrowth promoted by the NT and HGF signaling, respectively. HGF signaling induces the phosphorylation of β-catenin at Y142 (PY142), which translocates to the nucleus and promotes axon morphogenesis through TCF4/β-catenin-dependent transcription of target genes (David et al., 2008). These findings highlight the relevance of β-catenin forms producing transcriptional regulation independent of Wnt signaling (Monga et al., 2002; Zeng et al., 2006; Heuberger and Birchmeier, 2010; Xi et al., 2012).

Chemotactic cytokines ("chemokines") are small proteins classified into four subgroups referred to as CXC/α, CC/β, CX_3C/δ, or C/δ families (Zlotnik and Yoshie, 2000; Tran and Miller, 2003) according to the position and spacing of cysteine residues important for their tri-dimensional structure. Chemokines are best known for their role in leukocyte migration in host immune surveillance and inflammatory responses. However, chemokines and their G-protein-coupled receptors are also expressed by neurons and glia in the nervous system. Interestingly, chemokines of the CXC and CC families have been implicated in proliferation, neurogenesis, and neuronal differentiation of neural precursors

(Tran and Miller, 2003; Edman et al., 2008a,b; Wu et al., 2009). Meningeal CXCl12/Sdf-1 signaling through its receptor CXCR4 regulates the migration of cerebellar progenitors (Zou et al., 1998; Reiss et al., 2002) and Cajal-Retzius cells (Borrell and Marin, 2006). CXCL12 also controls interneuron migration during cortical development (Stumm et al., 2003; Lopez-Bendito et al., 2008; Lysko et al., 2011; Sanchez-Alcaniz et al., 2011; Wang et al., 2011). Furthermore, CXCL12 reduces axon outgrowth and branching in hippocampal neurons (Pujol et al., 2005). Importantly, neuronal migration and morphogenesis are coordinated processes that appear inversely regulated by CXCL12 signaling. CXCL12 increases the rate of interneuron migration while reducing neurite branching, whereas blocking CXCL12 signaling enhances neurite branching, thus explaining how interneurons switch from migratory streams to invade the cortical plate and branch out extensively (Lysko et al., 2011). Moreover, astrocyte-secreted CCL5/Rantes induces the outgrowth of cortical neuron neurites (Chou et al., 2008). CCL5 secretion is suppressed in astrocytes from a Huntington mouse model (Chou et al., 2008), indicating that chemokine signaling is involved in neuronal physiology and pathology.

Here we asked which are the genes regulated by HGF/β-catenin signaling during axon morphogenesis. We observe that expression of CC and CXC chemokines is upregulated by HGF signaling in hippocampal neurons. We find that chemokines promote axon outgrowth in hippocampal neurons, the most remarkable one being CXCL2 that also stimulates axon branching. Experiments using chemokine blocking antibodies and pharmacological inhibitors of chemokine receptors demonstrate that chemokines act downstream of HGF signaling. We also show that chemokine expression is reduced upon Met and TCF inhibition. These results identify the chemokines as novel regulators of axon morphogenesis downstream of HGF and β-catenin/TCF signaling.

MATERIALS AND METHODS

MATERIALS

HGF was purchased from Peprotech, Wnt-3a from Millipore, Hoescht-33258, SU11274, and FH535 were from Sigma, and SB225502 and SB324837 from Tocris. Antibodies were purchased from the following companies: βIII-tubulin from Covance, β-actin from Sigma, β-catenin from Becton-Dickinson and anti-rat CCL20 and CXCL2 antibodies from R&D. Rat CCL5, CCL7, CCL20 were from R&D Systems and CXCL2 from Peprotech.

HIPPOCAMPAL CULTURES

Rat primary hippocampal neurons were isolated from 18–19 day embryos and cultured in DMEM medium supplemented with N2 and B27. Neurons were plated on poly-D-lysine coated (500 μg/ml) glass coverslips for immunostaining (40 cells/mm^2) or on plastic (1000–1500 cells/mm^2) for RNA isolation.

IMMUNOFLUORESCENCE AND AXON MEASUREMENTS

Neurons plated on coverslips were treated at the first day in vitro (1DIV) and fixed at 2DIV. Treatments were as follows: 10, 300, or 1000 ng/ml for chemokines; HGF 50 ng/ml; Wnt-3a 100 ng/ml; SU11274 2 μM; FH535 10 μM; SB225502 1.25 nM; SB324837 20 nM; blocking antibodies against rat CCL20 and CXCL2 (40 μg/ml) or ovalbumin at the same concentration. Neurons were fixed with 4% paraformaldehyde (PFA) for 20 min at RT. Cells were then washed with phosphate buffer saline (PBS) and blocked and permeabilized in PBS containing 5% Foetal calf serum, 5% Horse serum, 0.2% glycine, and 0.1% Triton X100, before incubation with βIII-tubulin antibody. Secondary antibodies were Alexa Fluor488 or Fluor594 (Molecular Probes). Coverslips were mounted on Mowiol. Micrographs were obtained using an inverted Olympus IX70 microscope (10×, 0.3 NA, or 20×, 0.4 NA) equipped with epifluorescence optics and a camera (Olympus OM-4 Ti). Images were acquired using DPM Manager Software and processed using MacBiophotonics ImageJ software (www.macbiophotonics.ca). Axon length was measured using Adobe Photoshop software and the axon was identified as the longest neurite at this stage (2 DIV) of the hippocampal cell development. Images were inverted using Photoshop and are shown on a white background for a clearer visualization of their morphology. Branching was measured by counting Total Axonal Branch Tip Number (TABTN) (Yu and Malenka, 2003). Typically, 15–20 neurons were measured/condition in ≥ three independent experiments. Axon length and branching plots represent values compared to the corresponding untreated control, shown as average ± s.e.m. Significance was calculated by the Student's *t*-test. Asterisk (*) indicates statistical significance compared to the corresponding untreated control and hash (#) compared to stimulated controls (see legends for details).

LUCIFERASE ASSAY

To determine β-catenin transcriptional activation status, luciferase assay was performed following transfection of the TOP-Flash plasmid that carries a synthetic promoter containing three copies of the TCF-4 binding site upstream of a firefly luciferase reporter gene. Hek293T cells were plated at a density of 100 cells/mm^2 and transfected with Lipofectamine 2000 (Life Technologies) on the day next after plating. Treatments were given on the following day for 24 h (HGF 50 ng/ml; Wnt-3a 100 ng/ml; FH535 8 μM and SU11274 2 μM). After 48 h of transfection, cells were lysed in lysis buffer 25 mM glycylglycine, pH 7.8, 15 mM Mg_2SO4, 1% Triton X-100, 5 mM EGTA and rocket on ice for 15 min. Luciferase activity in the cell lysates was determined in Luciferase Buffer (25 mM glycylglycine, 15 mM $KHPO_4$, pH 7,8, 15 mM Mg_2SO_4, 1% Triton X-100, 5 mM EGTA, 1 mM dithiothreitol, 2 mM ATP, 100 mM acetyl-coenzymeA, and 100 mM luciferine) using a microplate luminometer. Luciferase activity was normalized for the total protein concentration in each condition.

shRNA EXPRESSION BY LENTIVIRAL INFECTION

shRNA vectors specific for rat β-catenin were transfected into Hek293T cells together with the plasmids psPAX2 and pMD2G, as previously described (David et al., 2008). The Hek293T medium was collected after 48 h of transfection and centrifuged at 50,000×g for 3 h. The viral pellet was re-suspended in sterile PBS plus 2% Bovine Serum Albumin. Hippocampal neurons were transduced 3–4 h after plating. According to GFP expression driven by the lentiviral vector ~90% of neurons were transduced.

Neurons were treated with HGF alone or together with pharmacological inhibitors at 3 DIV and collected at 4 DIV for mRNA purification (RNA isolation kit; Macherey-Nagel). Efficiency of the silencing induced by shRNA was evaluated by Western-blotting of 4DIV hippocampal neuron cell lysates. Densitometric analysis of the bands was performed using Scion software and β-catenin levels were normalized to the intensity of β-actin band.

RNA ISOLATION

For RNA isolation, treatments were performed as for immunofluorescence studies. Pervanadate was applied for the last 2 h of HGF stimulation. RNA was isolated using Nucleospin RNA II kit (Macherey-Nagel), including a DNase digestion step to remove contaminant DNA.

ARRAY PROCESSING AND ARRAY DATA ANALYSIS

RNA samples (800 ng) were amplified and labeled with Cy3-CTP using the One-Color Microarray-Based Gene Expression Analysis Protocol (Agilent Technologies, Palo Alto, CA, USA) and hybridized to Whole Rat Genome Microarray 4 × 44K (G4131F, Agilent Technologies).

Raw data files from the scanned arrays were extracted using Feature Extraction software version 9 (Agilent Technologies). Data files from Feature Extraction software were imported into GeneSpring® GX software version 9.0. (Agilent Technologies). Quantile normalization was performed (Bolstad et al., 2003) and expression values (log2 transformed) were obtained for each probe. Probes were also flagged (*Present*, *Marginal*, *Absent*) using GeneSpring® default settings. Probes with signal values above the lower percentile (20th) and flagged as *Present* or *Marginal* in 100% of replicates in at least one out of the two conditions under study, were selected for further analysis. Paired *t*-test was performed between conditions to be tested for differential expression analysis. Raw *p*-values were corrected for false discovery rate control using Benjamini–Hochsberg's method (Benjamini and Hochberg, 1995).

REVERSE-TRANSCRIPTASE PCR

mRNA was reverse-transcribed (RT) to cDNA (25°C for 10 min, 42°C for 60 min, and 95°C for 5 min) using random hexamers and Superscript II reverse transcriptase (Applied Biosystems). Negative control RT-minus reactions were carried out to confirm absence of DNA contamination in RNA.

SEMI-QUANTITATIVE PCR

To detect the relative expression of different chemokine genes in HGF-treated and untreated samples, semi-quantitative (sq) PCR was run. Equal volumes of cDNA were amplified by PCR using a couple of specific primers expanding at least two exons within the gene of interest. Sequences of the primers used were: CCL5 forward atatggctcggacaccactc, CCL5 reverse cccacttcttctctgggttg, CCL7 forward gggaccaattcatccacttg, CCL7 reverse cctcctcaacccacttctga, CCL20 forward gcttacctctgcagccagtc, CCL20 reverse cggatcttttcgacttcagg, CXCL2 forward agggtacaggggttgttgtg, CXCL2 reverse tttggacgatcctctgaacc. Ten microlitre aliquots taken from 25, 30, and 35 PCR cycles (CXCL2 and CCL7), 30, 34, and 38 PCR cycles (CCL5) or 24 and 28 PCR cycles (CCL20) were analyzed in 3% agarose gel. Densitometry of the DNA bands was performed using the Scion Image software (Scion Corporation) and comparing measurements from non-saturated PCR products. Loading was checked by amplification of the GAPDH transcript. Transcript analysis was performed from at least three independent simples.

REAL TIME PCR (qPCR)

cDNA processed from 1 μg RNA was used as the template. One microlitre aliquot of each cDNA was used per well. Samples were run in triplicate. Expression of the transcript levels were analysed using a FAM-labeled CXCL2 or CCL5 probes and compared to that of GAPDH, used as a loading control, in a ABI Prism 7000 HT sequence detection system (Applied Biosystems). Relative expression was calculated using the ΔΔC1 method.

RESULTS

CHEMOKINES OF THE CC AND CXC FAMILIES ARE UPREGULATED BY HGF TREATMENT

Previous work demonstrated that HGF signals through PY142 β-catenin and TCF4 to regulate the expression of target genes

A

	Array		sqPCR
Gene	Fold change	p value (n=4)	Fold change
Ccl5	4,086	0,01571	2,970 ± 0,67
Ccl7	3,700	0,00278	1,692 ± 0,34
Ccl13	3,358	0,00660	ND
Ccl7	3,298	0,00761	1,692 ± 0,34
Ccl20	2,730	0,01363	1,572 ± 0,31
Cxcl2	2,695	0,00580	2,484 ± 0,72
Ccl19	1,571	0,00266	1,487 ± 0,17

B

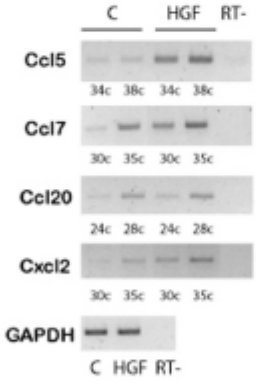

FIGURE 1 | Chemokine genes are upregulated by HGF signaling in 2DIV hippocampal neurons. (A) Summarized array data (left) indicating the chemokine genes that are upregulated in HGF-treated (50 ng/ml, 24 h) compared to untreated hippocampal neurons. (Right) Summary of the quantification of sqPCR experiments. Values indicate fold change of the chemokine expression in HGF-treated vs. untreated samples ± s.e.m. (≥3 experiments). **(B)** Representative sqPCR of samples taken at the indicated PCR cycle to compare the expression of chemokines in untreated and HGF-treated hippocampal neurons. GAPDH was used as a housekeeping gene (Image corresponds to 30 PCR cycles). RT-indicates samples in which reaction was run without RT enzyme.

during hippocampal neuron development (David et al., 2008). We questioned which are the genes regulated upon HGF stimulation in axon morphogenesis. We performed array experiments using control and HGF-treated hippocampal neuron samples. Array results revealed an upregulation of several chemokine genes in HGF-treated neurons compared to untreated neurons (**Figure 1A**), which was confirmed by sqRT-PCR (**Figures 1A,B**). *In silico* analysis of the 2 kb region upstream of the ATG in the identified chemokine genes showed the presence of several copies of putative TCF-binding sites, as predicted for β-catenin/TCF-target genes (data not shown). These findings indicated that chemokines may be involved in the HGF-induced axon morphogenesis.

CHEMOKINE SIGNALING PROMOTES AXON MORPHOGENESIS

To address this possibility, we first tested whether chemokines induce axon outgrowth and branching. Hippocampal neurons were treated with CCL5, CCL7, CCL20, or CXCL2 at different concentrations (10–1000 ng/ml). CCL5 (10 ng/ml), CXCL2 (300 and 1000 ng/ml), and CCL20 (10 and 1000 ng/ml) significantly increased the total length of the axon compared to axon length values of untreated neurons (**Figure 2**). A cocktail of all the chemokines (10 ng/ml) also increased axon outgrowth (**Figure 2I**). The increases in axon length were in the range of that obtained by HGF stimulation (**Figure 2I**). In addition to increasing axon length, CXCL2 also produced axon branching (**Figure 2J**). Axon branching was not significant for the other studied chemokines at the tested concentrations (data not shown).

Having showed that exogenously added chemokines induce axon morphogenesis in hippocampal neurons, we studied whether blocking chemokine signaling would inhibit the effect of HGF on axon morphogenesis. To this end, we used blocking antibodies against the chemokines as well as the chemokine receptor antagonists SB2250002 and SB328437 (White et al., 1998, 2000). Neurons incubated with HGF together with antibodies against rat CXCL2 or CCL20 (40 μg/ml) displayed axon length and branching values similar to those of untreated neurons (**Figure 3**). However, the increase in axon length promoted by HGF was not affected by the presence of ovalbumin at the same concentration than the antibodies (40 μg/ml). Furthermore, treatment with HGF and the antagonist for the receptor of CXCL2 (CXCR2)

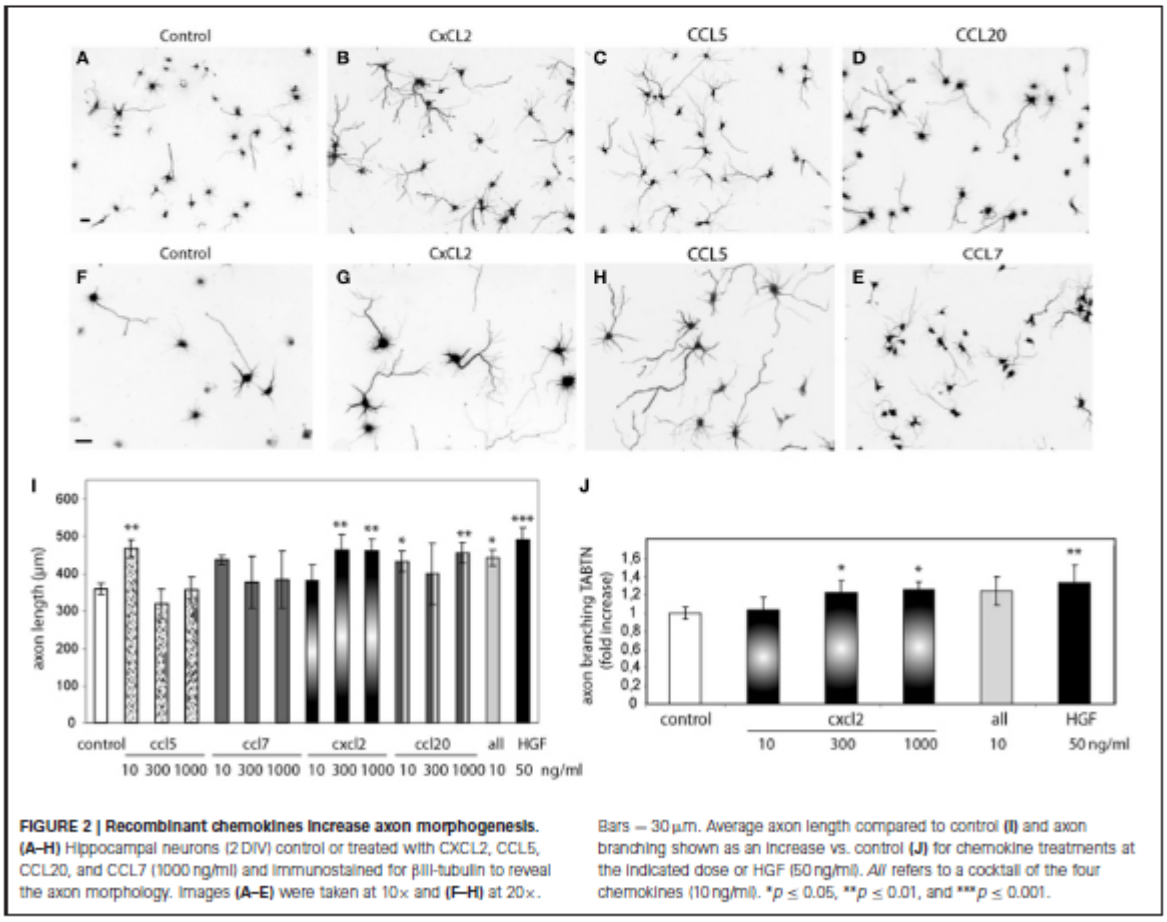

FIGURE 2 | Recombinant chemokines increase axon morphogenesis. **(A–H)** Hippocampal neurons (2 DIV) control or treated with CXCL2, CCL5, CCL20, and CCL7 (1000 ng/ml) and immunostained for βIII-tubulin to reveal the axon morphology. Images **(A–E)** were taken at 10× and **(F–H)** at 20×. Bars = 30 μm. Average axon length compared to control **(I)** and axon branching shown as an increase vs. control **(J)** for chemokine treatments at the indicated dose or HGF (50 ng/ml). *All* refers to a cocktail of the four chemokines (10 ng/ml). $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$.

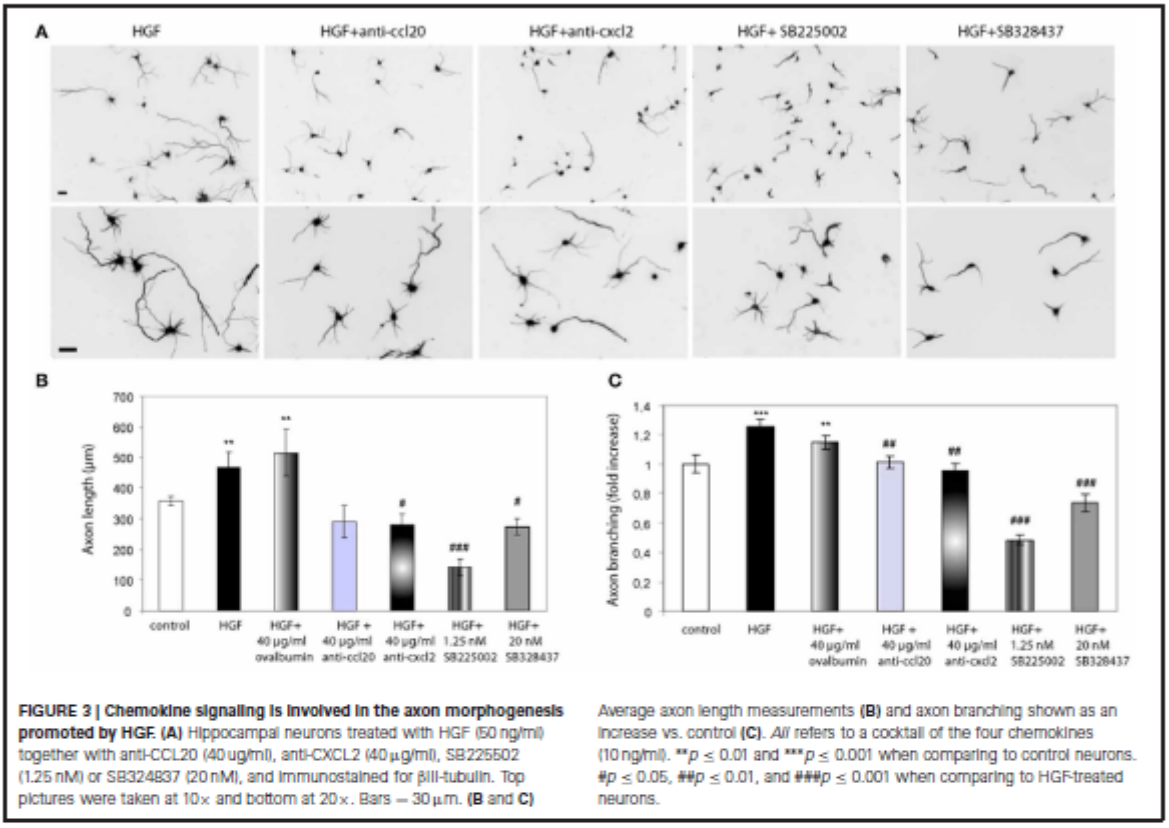

FIGURE 3 | Chemokine signaling is involved in the axon morphogenesis promoted by HGF. (A) Hippocampal neurons treated with HGF (50 ng/ml) together with anti-CCL20 (40 ug/ml), anti-CXCL2 (40 μg/ml), SB225502 (1.25 nM) or SB324837 (20 nM), and immunostained for βIII-tubulin. Top pictures were taken at 10× and bottom at 20×. Bars = 30 μm. **(B** and **C)** Average axon length measurements **(B)** and axon branching shown as an increase vs. control **(C)**. *All* refers to a cocktail of the four chemokines (10 ng/ml). $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$ when comparing to control neurons. $\#p \leq 0.05$, $\#\#p \leq 0.01$, and $\#\#\#p \leq 0.001$ when comparing to HGF-treated neurons.

SB2250002, or with SB328437, an antagonist of CCR3 (that acts as the only receptor of CCL20 and one of the receptors of CCL5), potently inhibited axon outgrowth and branching to values below those of control neurons (**Figure 3**). These results suggest that CXCL2 and CCL20 are secreted upon HGF stimulation and that endogenous CXCL2 and CCL20 signaling plays a role in axon morphogenesis.

HGF REGULATES CXCL2 EXPRESSION THROUGH MET AND β-CATENIN/TCF

We sought to study the pathway regulating chemokine expression downstream of HGF signaling. We demonstrated that HGF stimulation induces PY142 β-catenin phoshorylation and transcriptional regulation by TCF/β-catenin (David et al., 2008). To check if chemokine expression is controlled by TCF and β-catenin downstream of HGF signaling, we followed both a pharmacological and a gene silencing approach. We used SU11274 that inhibits Met activity (Berthou et al., 2004) and FH535, which inhibits TCF/β-catenin by blocking the recruitment of β-catenin to the promoter of target genes (Handeli and Simon, 2008). First, we studied β-catenin transcriptional activation using the TOP reporter plasmid in Hek293 cells, in which HGF/Met signaling is active (Royal and Park, 1995). Although HGF did not stimulate luciferase reporter activity vs. control, treatment with SU11274 significantly reduced β-catenin transcriptional activation (**Figure 4B**). This result suggests that in Hek293 cells, HGF/Met signaling through β-catenin is already active in basal conditions, likely by an autocrine production of HGF. Furthermore, treatment with FH535 significantly reduced luciferase activity, demonstrating that TCF activation is involved in HGF/Met signaling (**Figure 4B**). We used Wnt-3a as a positive control, which produced a clear activation of luciferase activity and was also inhibited by FH535 (**Figure 4B**). These results confirmed FH535 as an effective TCF/β-catenin inhibitor and indicated that HGF/Met signal through TCF/β-catenin in Hek293 cells among other cell systems (Monga et al., 2002).

In hippocampal neurons, both SU11274 and FH535 inhibited the axon outgrowth induced by HGF signaling (**Figure 4A**). As a positive control we also treated neurons with Wnt-3a with or without FH535. As expected, TCF inhibitor blocked the axon outgrowth promoted by Wnt-3a. FH535 treatment also blocked the axon outgrowth promoted by HGF, rendering axon length values below those of control neurons, thus confirming that HGF signaling is dependent on TCF-driven transcription (David et al., 2008).

Next, we investigated whether chemokine expression is regulated through the HGF/Met/TCF/β-catenin pathway. Because

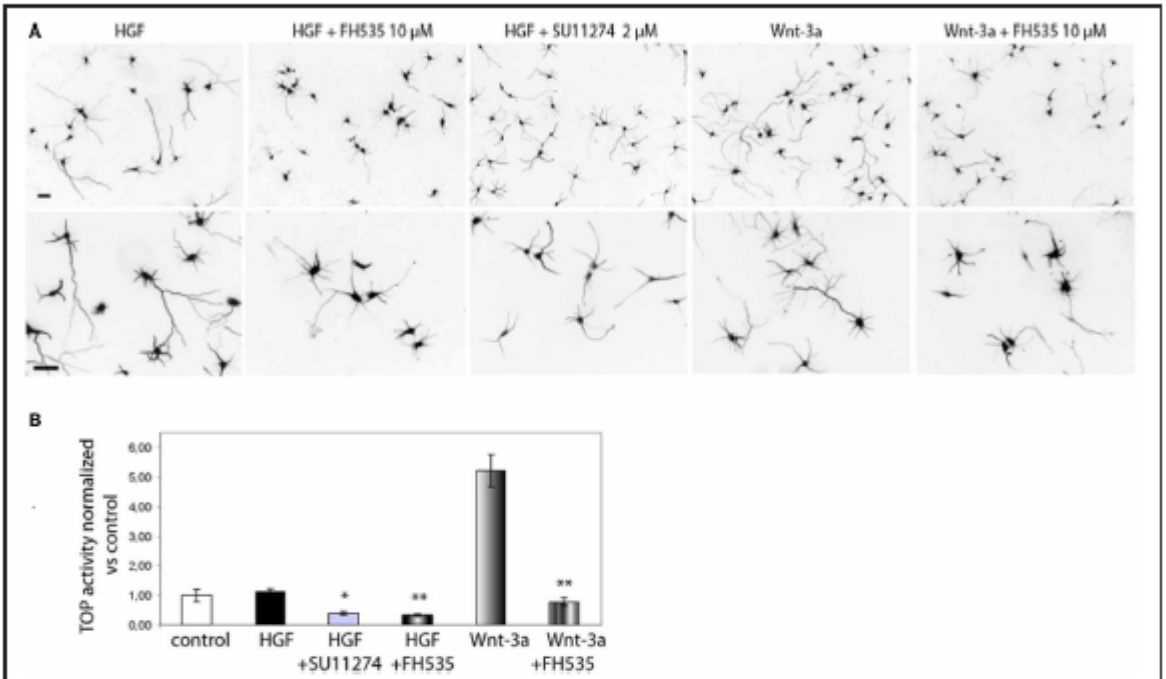

FIGURE 4 | Met and TCF inhibition reduce the axon outgrowth induced by HGF. (A) Hippocampal neurons treated with HGF (50 ng/ml) or Wnt-3a (100 ng/ml) alone or together with FH535 (10 µM) or SU11274 (2 µM) were immunostained for βIII-tubulin to reveal the axon morphology. Top pictures were taken at 10× and bottom at 20×. Bars = 30 µm. **(B)** β-catenin transcriptional activity determined using the TOP reported plasmid in transfected Hek293 cells. Relative luciferase activity was determined at 48 h after transfection. Treatments were applied for the last 24 h (see Materials and Methods for details). $^{*}p \leq 0.05$, $^{**}p \leq 0.01$.

CXCL2 promoted both axon outgrowth and branching, we focused on this chemokine and on CCL5 as a member of the CC family. We analyzed the expression of these chemokines using real time qPCR from control neurons, neurons treated with HGF or treated with HGF together with SU11274 or FH535. Expression of CXCL2 increased by 1.6-fold in HGF-treated neurons compared to untreated neurons, but was reduced to values below those of untreated neurons following the treatment with HGF and SU11274 (**Figure 5A**). Pervanadate (a tyrosine phosphatase inhibitor) was previously used to stabilize the PY142-β-catenin form (David et al., 2008). However, HGF stimulation increased CXCL2 expression in pervanadate-treated neurons in a similar way than in the absence of pervanadate (**Figure 5B**). Furthermore, the increase in CXCL2 expression induced by HGF signaling was lost upon the co-treatment of HGF and FH535 (**Figure 5A**), indicating that it was mediated by TCF/β-catenin. In addition, we analyzed CXCL2 mRNA levels in hippocampal neurons in which β-catenin was silenced following lentiviral-driven shRNA expression. The silencing efficiency obtained with this β-catenin shRNA at four days was around 40–50% as similarly reported (David et al., 2008, 2010). Whereas HGF was able to increase the expression of CXCL2 in neurons expressing scrambled shRNA, this increase was lost in neurons expressing β-catenin shRNA (**Figure 5C**). We also analyzed the expression of CCL5, which increased nearly 2-fold upon HGF stimulation and was significantly reduced in the presence of HGF and FH535 (**Figure 5D**).

DISCUSSION

Aiming to identify the genes regulated by HGF signaling in developing hippocampal neurons during axon morphogenesis, we found the upregulation of several chemokines of the CC and CXC families. Following array experiments, we demonstrated that CCL5, CCL20, and CXCL2 significantly promote axon outgrowth and, in the case of CXCL2, also axon branching. PCR data confirmed that chemokines are upregulated by HGF signaling. By blocking chemokine signaling, we demonstrated that CCL20 and CXCL2 act downstream of HGF signaling in axon outgrowth and branching. During the establishment of the axon arbor morphology, HGF signaling induces β-catenin PY142 and TCF4-dependent transcriptional regulation of target genes (David et al., 2008). We inquired whether chemokines are regulated through TCF/β-catenin downstream of HGF signaling. TCF inhibition or β-catenin silencing reduced CXCL2 and CCL5 expression upon HGF stimulation. We conclude that chemokines are new molecules modulating axon outgrowth in hippocampal neurons, which expression is regulated by HGF through TCF/β-catenin signaling.

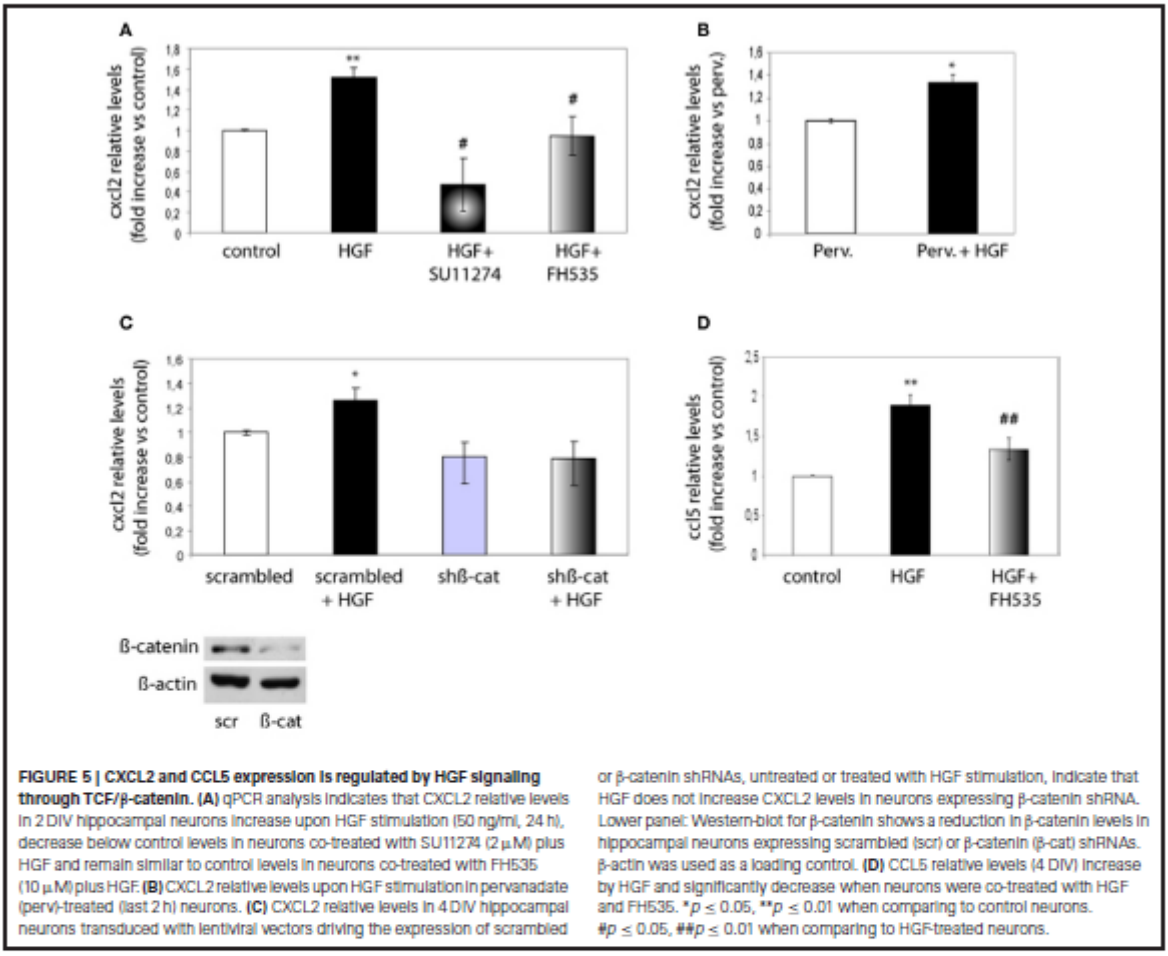

FIGURE 5 | CXCL2 and CCL5 expression is regulated by HGF signaling through TCF/β-catenin. **(A)** qPCR analysis indicates that CXCL2 relative levels in 2 DIV hippocampal neurons increase upon HGF stimulation (50 ng/ml, 24 h), decrease below control levels in neurons co-treated with SU11274 (2 μM) plus HGF and remain similar to control levels in neurons co-treated with FH535 (10 μM) plus HGF. **(B)** CXCL2 relative levels upon HGF stimulation in pervanadate (perv)-treated (last 2 h) neurons. **(C)** CXCL2 relative levels in 4 DIV hippocampal neurons transduced with lentiviral vectors driving the expression of scrambled or β-catenin shRNAs, untreated or treated with HGF stimulation, indicate that HGF does not increase CXCL2 levels in neurons expressing β-catenin shRNA. Lower panel: Western-blot for β-catenin shows a reduction in β-catenin levels in hippocampal neurons expressing scrambled (scr) or β-catenin (β-cat) shRNAs. β-actin was used as a loading control. **(D)** CCL5 relative levels (4 DIV) increase by HGF and significantly decrease when neurons were co-treated with HGF and FH535. $*p \leq 0.05$, $**p \leq 0.01$ when comparing to control neurons. $\#p \leq 0.05$, $\#\#p \leq 0.01$ when comparing to HGF-treated neurons.

CHEMOKINE SIGNALING IN NEURONAL MIGRATION AND AXON OUTGROWTH

Chemokines are well-established chemotactic molecules inducing the migration of leukocytes and hematopoietic progenitors (Rossi and Zlotnik, 2000). In the nervous system, chemokines and chemokine receptor expression are regulated under a variety of conditions, including brain repair (Babcock et al., 2003; Miller et al., 2008; Jaerve et al., 2012). Thus, chemokine signaling has been involved in neuroinflammation, the pathogenesis of chronic pain (White et al., 2005), myelination (Kury et al., 2002) and human immunodeficiency virus-1 (HIV-1)-associated neuropathology (Tran and Miller, 2003). Furthermore, a role for chemokines (in particular for CXCL12/Sdf-1) in regulating axon outgrowth and guidance has also been described during nervous system development (Tran and Miller, 2003). CXCL12-CXCR4 signaling is involved in the guidance of motoneuron's axon (Lieberam et al., 2005) and downstream of Sonic Hedgehog signaling in retinal ganglion cell axon pathfinding (Stacher Horndli and Chien, 2012). CXCL12 can either produce growth cone repulsion or attraction depending on the levels of cGMP (Xiang et al., 2002). Remarkably, CXCL12 signaling regulates the migration of different neuron and neuronal progenitor populations: gonadotropin-releasing hormone-1 neurons emerging from the nasal placode (Casoni et al., 2012), interneurons moving from the medial ganglionic eminence toward the cortical plate (Lopez-Bendito et al., 2008; Lysko et al., 2011), cerebellar progenitors (Zou et al., 1998; Vilz et al., 2005) and sensory neuron progenitors toward dorsal root ganglia (Belmadani et al., 2005). CCL2, CCL7, and their receptors are expressed during midbrain development, promoting the differentiation of dopaminergic neurons and also neuritogenesis (Edman et al., 2008a,b). To our knowledge, this is the first work reporting that CC chemokines induce axon morphogenesis in hippocampal neurons. In agreement with our data, *in situ* hybridization data freely available online reveals the expression of chemokines in the mouse hippocampus during embryonic and adult life (see Genepaint and Allen Brain Atlas webpages). CXCL12 was shown to promote the extension of perforant fibers from the entorhinal cortex to dentate gyrus neurons

and the migration of dentate granule cells during hippocampal development (Bagri et al., 2002; Lu et al., 2002; Ohshima et al., 2008). In addition, CXCL12 reduces axon elongation while promoting axon branching in dissociated hippocampal neurons (Pujol et al., 2005). We found that CXCL2 increases total axon length and axon branching in hippocampal neurons, suggesting that it plays a role *in vivo* during hippocampal development. In line with an effect of chemokines inducing axon development, mice lacking chemokine receptors showed impairments in hippocampal cognitive function and synaptic plasticity (Rogers et al., 2011; Belarbi et al., 2013).

HGF/β-CATENIN SIGNALING AND CHEMOKINES

Our findings establish a relationship between HGF and chemokine signaling in hippocampal neurons. HGF-treated neurons displayed increased expression of chemokines. Antibodies against CCL20 and CXCL2, as well as the CCR3 and CXCR2 antagonists SB22502 and SB328437, inhibited the axon outgrowth and branching promoted by HGF, implying that chemokines are downstream of HGF signaling. Moreover, treatment with SB22502 or SB328437 resulted in axon length and branching values clearly below those of untreated neurons, suggesting that endogenous chemokine production by hippocampal neurons impacts on axon development. Furthermore, affecting CCL20 signaling by anti-CCL20 antibodies or SB328437 treatment reduced axon branching in the presence of HGF. However, CCL20 at the tested concentrations (10–1000 ng/ml) did not promote significant axon branching. These findings suggest that promotion or inhibition of axon branching exhibit different EC50/IC50 values. Alternatively, CCL20 concentrations lower than tested may induce axon branching.

β-catenin is a classical effector of Wnt signaling and a transcriptional coactivator of LEF/TCF. We and others have described the interaction between Met and β-catenin (Monga et al., 2002; Zeng et al., 2006; David et al., 2008), which results in β-catenin phosphorylation at Y142 *in vitro* (David et al., 2008). In hippocampal neurons, HGF signaling increases PY142-β-catenin, which moves to the nucleus and regulates axonal morphogenesis through TCF4-transcriptional activation (David et al., 2008). Here, we confirm that HGF/Met signaling supports axon outgrowth through TCF/β-catenin transcriptional activity. In line with these findings, the phosphorylation of β-catenin and α-catenin downstream of tyrosine kinase receptor and/or src activation is emerging as a Wnt-independent pathway that promotes β-catenin transcriptional activation and migration of cancer cells (Ji et al., 2009; Xi et al., 2012). In this work chemokines were identified as transcriptional targets of HGF in developing in hippocampal neurons. CXCL2 and CCL5 expression analysis confirmed that these chemokines are regulated by HGF signaling. In addition, TCF inhibition and β-catenin silencing blocked the upregulation of CXCL2 by HGF. A previous paper (Halleskog et al., 2011) described the upregulation of cytokines and chemokines—including CXCL2—by Wnt-3a and β-catenin signaling in activated microglia. TCF inhibition also reduced the expression of CCL5 following HGF stimulation (by ~30% compared to HGF-treated neurons), suggesting that CCL5 is at least in part regulated through TCF/β-catenin downstream of HGF signaling. It is possible that HGF signaling activates another pathway, i.e., NFkB pathway that affects CCL5 expression (Chou et al., 2008).

In summary these findings add different chemokines to the growing list of secreted molecules that modulate axon outgrowth, and highlights new developmental roles for signaling molecules known to regulate immune cell biology. As chemokines play a role in post-ischemic brain repair (Wang et al., 2012) and recruiting stem cells after spinal cord injury (Jaerve et al., 2012), it is tempting to speculate that chemokines at the injury site may serve to improve axon regeneration.

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Review Article

β-Catenin Signalling in Glioblastoma Multiforme and Glioma-Initiating Cells

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Glioblastoma multiforme (GBM) is a commonly occurring brain tumor with a poor prognosis. GBM can develop both "de novo" or evolve from a previous astrocytoma and is characterized by high proliferation and infiltration into the surrounding tissue. Following treatment (surgery, radiotherapy, and chemotherapy), tumors often reappear. Glioma-initiating cells (GICs) have been identified in GBM and are thought to be responsible for tumors initiation, their continued growth, and recurrence. β-catenin, a component of the cell-cell adhesion complex and of the canonical Wnt pathway, regulates proliferation, adhesion, and migration in different cell types. β-catenin and components of the Wnt canonical pathway are commonly overexpressed in GBM. Here, we review previous work on the role of Wnt/β-catenin signalling in glioma initiation, proliferation, and invasion. Understanding the molecular mechanisms regulating GIC biology and glioma progression may help in identifying novel therapeutic targets for GBM treatment.

1. Introduction

Gliomas are the most common primary malignancies in the central nervous system, comprising a heterogeneous group of tumors that display some histological similarities to glia (mainly, astrocytes in astrocytomas and oligodendrocytes in oligodendrogliomas). Astrocytomas account for the majority of gliomas, which can be classified into four different grades according to the World Health Organization (WHO) classification system [1, 2]: grade I and grade II astrocytomas are slow-growing less aggressive tumors, whereas grade III and IV gliomas are malignant tumors characterized by high proliferation rate (grade III) and the presence of necrotic tissue and/or angiogenic activity (grade IV). The most malignant form, glioblastoma multiforme (GBM, grade IV), is one of the most aggressive and lethal forms of cancer with an average survival time of 15 months after diagnosis [3, 4]. Standard treatment consists of surgical removal of the tumor, followed by chemotherapy and radiotherapy. Temozolomide, an oral alkylating agent, is the most commonly used chemotherapy treatment [5]. Importantly, a high infiltration capacity of individual cells over long distances, already present in grade II gliomas, hinders complete tumor resection and most likely contributes to recurrence [6].

GBMs can present as primary or secondary. Primary or "de novo" GBMs, representing the majority of GBM cases, arise without any prior evidence of a lower-grade precursor lesion and more commonly affect older patients (mean age of 62 years). Secondary GBMs progress from a lower-grade glioma and typically develop in younger patients (median age of 45 years). Gliomas exhibit a vast array of genetic changes that contribute to the malignant phenotype [6–10]. These include loss of function mutations in the p53 tumor suppressor and hyperactivation of receptor tyrosine kinase (RTK) signalling, such as epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), and the receptor for hepatocyte growth factor c-Met. The signalling cascades downstream of activated RTK often result in activation of Ras and AKT. Mutations in tumor suppressors such as phosphatase and tensin homolog (PTEN) and neurofibromatosis 1 (NF1) that normally control these pathways further contribute to oncogenesis [2, 3, 6–10]. Based on its gene expression profile, GBM can be further classified into proneural, neural, mesenchymal,

and classical types [8, 9]. This classification should allow a molecular stratification of GBM cases with important therapeutic implications.

Here we focus our attention on Wnt/β-catenin signalling, a pathway primarily involved in embryogenesis and displaying important functions in adulthood when aberrant Wnt signalling has been linked to disease and cancer [11–14]. Understanding how β-catenin signalling regulates gliomagenesis and tumor progression may lead to novel therapeutic interventions in GBM. Firstly, therefore, we discuss the role of glioma-initiating cells (GICs), a type of "cancer stem cells," in glioma development and evidence suggesting that Wnt/β-catenin signalling regulates GICs biology. We then review progress in the understanding of the involvement of Wnt/β-catenin signalling in the proliferation and invasion of glioma tumor cells.

2. Glioma-Initiating Cells (GICs) and Signalling Pathways in GICs

The existence of brain tumor stem cells was proposed about a decade ago, following advances in the stem cell field and the discovery that neurogenesis persists in the adult brain [15–18]. Glioma-initiating cells (GICs) share the features of neural stem cells that have been identified in GBM, including the expression of CD133 (prominin), the ability to form neurospheres, and the reproduction of tumors [19, 20]. However, the role of CD133+ and CD133− GIC subpopulations in tumor initiation is not clear. CD133− cells from the C6 glioma cell line showed clonogenic, self-renewal, and tumorigenic capacities [21]. Nonetheless, CD133− GICs isolated from primary GBM were as capable of producing tumors as CD133+ cells [22]. Individual GBM may contain CD133+ and CD133− GICs that represent different stages of differentiation [23]. Furthermore, multipotent CD133+ GICs contain a CD144+ (vascular endothelial cadherin positive) subpopulation that can give rise to tumor endothelial cells [24].

Signalling by several morphogens and cytokines (including leukemia inhibitory factor (LIF), fibroblast growth factor (FGF) and members of the Wnt, transforming growth factor-β (TGF-β)/bone morphogenetic proteins (BMP) families) maintains the self-renewal capacity of embryonic stem cells and supports cancer stem cell growth [25]. Thus, TGF-β signalling through the induction of LIF and the JAK-STAT pathway promotes the self-renewal of patient-derived GICs [26]. Seoane and coworkers recently identified a population of CD44 high/Id1 high GICs in GBM that locates in the perivascular niche. Depletion of this cell population by TGF-β inhibitors prevents tumor initiation and recurrence. This work identifies CD44 and Id1 levels as prognosis markers in GBM and shows that TGF-β signalling is key to maintain this GIC population [27]. Aberrant activation of sonic hedgehog signaling (another morphogen involved in embryogenesis and brain development) in committed cerebellar granule neuron precursors is responsible for aggressive medulloblastoma, a pediatric cerebellar tumor [28–30]. Hedgehogs signal through Gli transcription factors, with Gli1 and Gli2 acting as activators and Gli3 as a repressor factor. Consistent with the isolation of Gli1 from glioma cells, activation of the Hedgehog-Gli1 pathway is reported in GBM, which is required for the clonogenicity and formation of secondary neurospheres of CD133+ GICs [31].

Wnt factors are a family of secreted glycoproteins (19 members exist in humans) that regulate embryonic patterning and play different roles throughout development of the nervous system [32, 33]. Wnts signal through at least three different pathways [11, 33], the best known being the Wnt/β-catenin canonical pathway (Figure 1). In the absence of Wnt, the Ser/Thr kinase glycogen-synthase kinase (Gsk-3β) in the so-called destruction complex (comprising of Gsk-3β, adenomatosis polyposis coli (APC), Axin and β-catenin) phosphorylates β-catenin, which is then targeted for proteasomal degradation. Upon Wnt binding to Frizzled (Fz) (of which there are 11 family members in humans) and low-density-lipoprotein-related protein (LRP)5/6 receptors, the scaffolding protein Dishevelled (Dvl) and LRP5/6 become phosphorylated by Gsk-3 and Casein-Kinase Iγ. Consequently, the destruction complex components are recruited instead to the receptor complex, leading to β-catenin stabilization [34]. The protooncogene Frat/GBP further prevents the phosphorylation and degradation of β-catenin because it competes with Axin to bind Gsk-3 and removes it from the destruction complex. Stabilized β-catenin translocates to the nucleus, where it binds to lymphoid enhancer factor-1 Lef-1/T-cell factor (Tcf) transcription factors and regulates expression of Wnt target genes. In the absence of nuclear β-catenin, Tcf/Lef factors suppress the expression of target genes through their binding to members of the Groucho/transducin-like enhancer of split (TLE) family of transcriptional corepressors. β-catenin does not have a DNA binding domain but it has a potent transcription activation domain. Conversely, Lef/Tcf transcription factors do not have a strong transcription activation domain, but they do have a good DNA binding/bending domain [35]. Thus, when β-catenin binds to a Lef/Tcf protein, a potent transcription regulatory complex is formed. Nuclear translocation of β-catenin converts Tcf proteins into potent transcriptional activators by displacing Groucho/TLE and recruiting an array of coactivator proteins, including CBP, TBP, BRG1, BCL9/PYGO, Legless, Mediator, and Hyrax [36] (Figure 1).

Canonical Wnt/β-catenin signalling is crucially involved in embryonic development and controls stem cell biology, thus inducing self-renewal properties in embryonic stem (ES) cells and regulating adult stem cells [11, 14, 33, 41–45]. Nanog and Oct-4, two of the four transcription factors required to generate the pluripotency and self-renewal of ES cells, are Tcf3 targets [46, 47]. Interestingly, the ES signature characterized by the expression of Nanog, Oct-4, Sox-2, and c-Myc also associates with aggressive tumors, including poorly differentiated GBM [37]. As aberrant activation of the Wnt/β-catenin signalling pathway is a hallmark of many tumors [12, 14], these findings suggest that Wnt-regulated genes may contribute to the stem cell-like phenotypes displayed by brain tumors. Furthermore, the novel protooncogene *PLAG2* is amplified in GBM and promotes GICs proliferation and gliomagenesis. PLAG2 increases

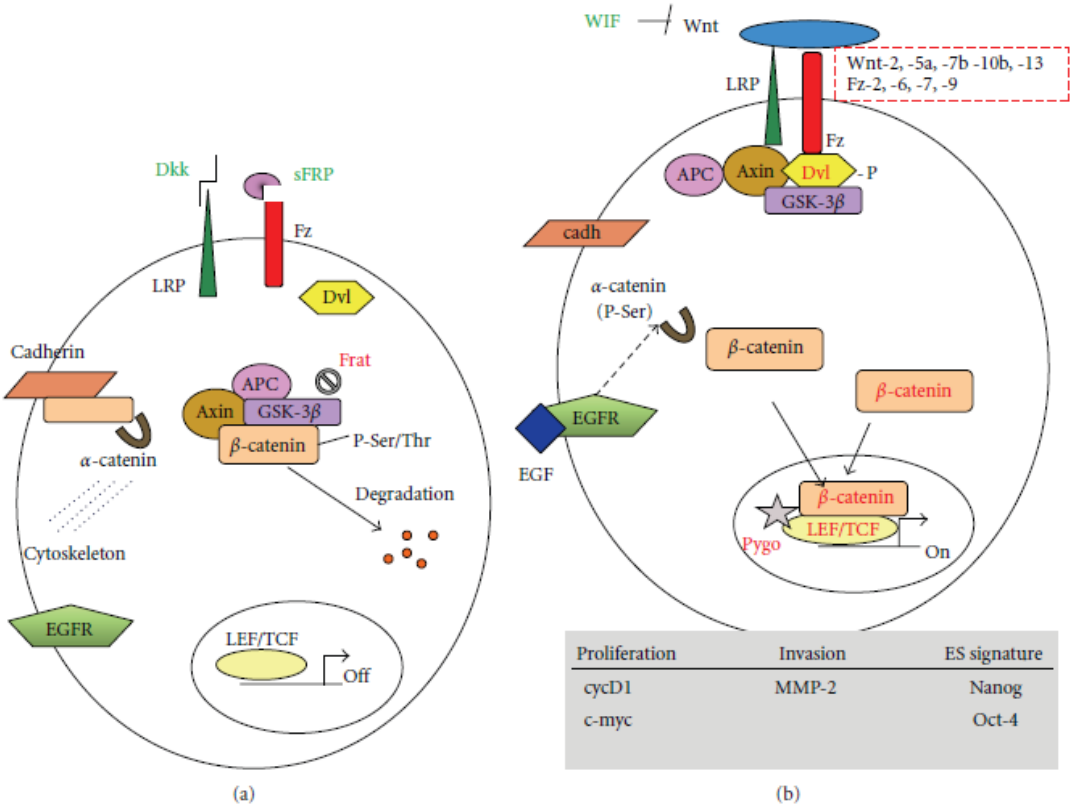

FIGURE 1: Wnt/β-catenin signalling and β-catenin role in adhesion in glioma cells. (a) In the absence of Wnt ligands or in the presence of Wnt antagonists Dkk and sFRP that bind to the respective Wnt receptors Fz and LRP5/6, β-catenin is in a complex together with Axin, APC, and Gsk-3β. Here, β-catenin is phosphorylated by Gsk-3β in key Ser and Thr residues and is thus targeted for proteasomal degradation. Frat prevents the phosphorylation of β-catenin. Transcription by Lef/Tcf is off due to the binding of repressors. In the absence of growth factor signaling, a pool of β-catenin is engaged in the cadherin/β-catenin/α-catenin complex that is linked to the cytoskeleton. (b) Following Wnt binding, the Fz-LRP5/6 complex is formed upon Dvl phosphorylation that recruits Gsk-3β, Axin, and APC to the membrane. This results in free β-catenin that accumulates in the cytosol and translocates to the nucleus, where it binds to Tcf and recruits transcriptional activators (including Pygo). Lef/Tcf transcriptional activation results in the regulation of Wnt target genes. The box shows Wnt target genes implicated in proliferation and invasion of glioma cells or conferring ES cell signature to GICs that might be related to aggressive growth and recurrence [37–39] EGF signalling through EGFR, ERK1/2, and CK2 results in the phosphorylation of α-catenin and promotes β-catenin transactivation [40]. Whether the Wnt-induced and growth factor-induced β-catenin nuclear pools collaborate in glioma cells remains to be studied. Text in red indicates Wnt pathway components that are overexpressed and green indicates Wnt antagonists repressed in high-grade astrocytomas and GBM. Wnt factors and Fz that have been reported to be upregulated in high-grade astrocytomas and GBM are shown (see references in the text).

the expression of Wnt-6, Fz-9, and Fz-2, inhibits differentiation, and increases proliferation of neural progenitors [48]. It is worth noting that *PLAG2* amplification correlates with increased β-catenin levels in GBM samples. These results indicate that PLAG2 imparts stem-cell properties to glioma cells by regulating Wnt signalling. Another gene regulating Wnt signalling in glioma is *PEG3* (paternally expressed gene 3), an imprinted gene with a tumor suppressor activity. Hypermethylation of *PEG3* promoter in glioma decreases *PEG3* mRNA expression and correlates with high-grade gliomas [49]. In turn, low PEG3 expression increases β-catenin that promotes the proliferation of CD133+ GICs [49]. Finally, the interaction between the transcription factor Forkhead box M1 and β-catenin that promotes β-catenin nuclear localization in tumor cells and maintains GIC self-renewal has been recently described [50]. Novel therapeutic interventions for GBM could inhibit Wnt/β-catenin signalling in GICs to decrease GIC proliferation and stop glioma growth, while increasing GICs differentiation.

3. Targeting GIC Chemoresistance and Radioresistance Mechanisms as an Approach to Treat GBM Recurrence

Following surgery, chemotherapy, and radiotherapy GBM recurrence is common. Therefore, a particularly relevant feature of the cancer stem cells is their ability to export drugs and develop resistance mechanisms to cytotoxics and irradiation [17]. Current knowledge suggests that resistance

to temozolomide is promoted by enhanced O-6-methylguanine-DNA-methyltransferase- (MGMT-) mediated DNA repair of mismatches [51]. Thus, *MGMT* promoter methylation status improves the benefits of chemotherapy. According to Liu et al., CD133 expression in tumor tissue is higher in recurrent GBM than in newly diagnosed tumors and CD133+ GICs are chemoresistant to temozolomide [52]. However, these findings are inconsistent with a report indicating that CD133+ GICs are depleted after temozolomide treatment [53]. Consequently, at present it is not clear which glioma cells are responsible for the resistance to temozolomide.

Another mechanism of drug resistance is the expression of the ATP-binding cassette (ABC) transporters by cancer stem cells. BCRP1 and MDR1 ABC transporters allow the exclusion of Hoechst 33342 dye, a feature that defines the pluripotential side population (SP) originally reported in haematopoietic stem cells and now used to identify cancer stem cells. Expression of these ABC transporters accounts for the chemoresistance of some cancers and high drug efflux capacity. CD133+ GICs express higher levels of BCRP1 compared to CD133− cells [52]. Consistent with this, a cancer stem-cells cell line (WJ_2) derived from GBM showed increased expression of BCRP1, CD133 and the neural precursor marker Nestin and at the same time maintained Wnt-1 expression [54]. Interestingly, overexpression of MDR1 downstream of Wnt-1/Fz-1 signalling mediates chemoresistance in neuroblastoma cells [55]. This suggests that a similar mechanism could be operating in the chemoresistance mechanisms of gliomas.

As regards radioresistance, the CD133+ stem cell fraction is enriched after glioma radiation [56]. Furthermore, the CD133+ subpopulation is able to repair radiation-induced DNA damage more efficiently than CD133− tumor cells [57]. These results indicate that the CD133+ tumor cell population confers radioresistance to GBM and most likely accounts for glioma recurrence. Wnt-1 ectopic expression triggers DNA damage response in epithelial mammary cells [58], while activation of Wnt/β-catenin signalling confers radioresistance to mammary progenitors cells through survivin upregulation [59]. In addition, Gsk-3β inhibition enhances DNA repair of double-strand breaks following radiation of hippocampal neurons [60]. Taken together, these findings suggest that Wnt signalling may be involved in the chemo- and radioresistance mechanisms developed by GICs. Expanding our understanding of the molecular mechanisms supporting GICs resistance to conventional glioma treatment will allow the design of novel therapeutic tools to decrease tumor recurrence and improve patient survival.

4. Wnt/β-Catenin Signalling in the Proliferation of Glioma Cells

GICs represent a small percentage of the brain tumor mass, which is thought to contain a heterogeneous mixture of tumor cells with limited proliferation capacity. Molecular analysis on whole tumor samples is expected to mainly represent non-GIC cells. Wnt/β-catenin signalling plays a role in the proliferation of glioma tumor cells and tumor progression. β-catenin has been proposed as a prognostic marker in GBM, as both mRNA and protein levels increase in high-grade astrocytomas and GBM, thus correlating with malignancy [38, 61, 62]. In addition, the expression of other positive regulators of the Wnt pathway (including Dvl-3, FRAT-1, Pygo-2, Tcf4, and Lef-1) [38, 63, 64] and of Wnt target genes (namely the regulators of cell proliferation *Cyclin D1* and *c-myc)* [38, 62] also increases in high-grade astrocytomas and GBM (see Figure 1). Using immunohistological techniques, a nuclear fraction of β-catenin was observed that associates with high-grade astrocytoma and GBM [62]. This result suggests increased cytoplasmic stabilization of β-catenin that escapes proteasomal degradation, in addition to the elevated β-catenin mRNA levels reported in GBM. Silencing β-catenin, Wnt-2, and Pygo-2 expression demonstrated the involvement of Wnt/β-catenin signalling in the proliferation of U251 glioma cell line [63, 65]. Together, these findings point to the activation of nuclear β-catenin signalling as a mediator of Wnt-induced proliferation of glioma cells. Moreover, expression of noncanonical Wnt-5a is also upregulated in high grade gliomas, in which Wnt-5a stimulates cell proliferation [66]. Wnt-5a signalling can inhibit canonical Wnt signalling during development [67, 68]. How Wnt canonical and noncanonical pathways interact in glioma cells remains to be studied.

In contrast to other cancers, no mutations have been found in β-catenin exon 3, a hot spot affecting the GSK-3 phosphorylation sites and β-catenin degradation that renders β-catenin active in glioma samples and cell lines [69, 70]. Truncation of APC, a mechanism causing polyposis and predisposing for Wnt/β-catenin-driven colorectal carcinoma, has not been associated with gliomagenesis (with the exception of Turcot syndrome patients) [71]. These observations suggest the deregulation of the pathway by unbalanced ligand/antagonist expression during tumor initiation and progression. Indeed, in addition to regulation of the expression of Wnt family members, Wnt antagonists often appear repressed in GBM (Figure 1). Expression of the Wnt antagonist and tumor suppressor Wnt inhibitory factor (WIF) decreases with malignancy in astrocytomas, which has been linked to aberrant promoter hypermethylation [72]. Also, hypermethylation of the *secreted-Frizzled-related protein* (*sFRP*) promoters is a significant event in primary "de novo" GBM, whereas hypermethylation of the promoter of the LRP antagonist *Dickkopf* (*Dkk*) associates with secondary GBM [70]. Similar epigenetic modifications are common to other Wnt-driven cancers [73, 74]. In addition, a novel mechanism for β-catenin nuclear localization and transcriptional activation (both constitutive and Wnt-induced) that controls Wnt target gene expression and glioma tumorigenesis has been described, which involves the interaction of β-catenin with FoxM1 [50].

5. β-Catenin and Wnt Signalling in Glioma Invasion

As a component of the cell adhesion complex, β-catenin binds to cadherin, thus regulating cell-cell adhesion. Altering

the binding of β-catenin to cadherin or to α-catenin downregulates cell adhesion, while promoting cell migration and epithelial-mesenchymal transition [75]. However, β-catenin nuclear signalling is not only achieved by Wnt factors in tumor development [76]. Growth factor signalling can induce the phosphorylation of specific tyrosine residues of β-catenin, resulting in increased migration [75, 77–79]. EGFR expression is upregulated in primary GBM correlating with malignancy [15]. EGF/EGFR signalling through extracellular signal-regulated kinases 1/2 (ERK1/2) and casein kinase-2 (CK2) in glioma cells results in the phosphorylation of α-catenin at serine 641, which correlates with glioma malignancy [40]. Interestingly, α-catenin phosphorylation promotes β-catenin transactivation and glioma cell invasion [40]. These results highlight the involvement of β-catenin signalling not only as a mediator of Wnt but also downstream of growth factor signalling in glioma invasion. On the other hand, enhanced expression of the Fz antagonist sFRP2 reduced glioma invasion by decreasing β-catenin tyrosine phosphorylation and downregulating matrix metalloprotease-2 (MMP-2) [39]. However, sFRP2 did not affect β-catenin levels, its cytoplasmic/nuclear distribution, or its serine phosphorylation status [39]. How sFRP2 signalling modulates β-catenin tyrosine phosphorylation requires further investigation.

Noncanonical Wnt-5a, which signals through β-catenin independent pathways (including the planar cell polarity and the calcium pathways [33]), enhances the migration of glioma cells by regulating the expression of MMP-2 [80]. Moreover, silencing the expression of Wnt-2, Wnt-5a, and Fz-2 in the U251 glioma cell line decreases invasion [65, 80]. These findings are consistent with Wnt-5a function in invasion and metastasis in other cancers [11, 81, 82]. Together, these results point to metalloprotease regulation as important downstream targets of β-catenin and Wnt signalling pathways in glioma invasion [39, 80, 83].

We are accumulating knowledge on the signalling pathways responsible for the maintenance of GICs, sustaining the proliferation of bulk tumor cells and dictating the invasive properties of glioma cells. Current and future research should offer novel opportunities for anticancer drug discovery. Undoubtedly, the cancer stem cell hypothesis has provided a promising framework for investigations into an incurable disease. Future combined therapies including cytotoxics, tumor-targeted drugs, and agents that target GICs should be expected to reduce glioma growth and recurrence, raising hopes for glioma patients.

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