UPPSALA
UNIVERSITET

MSc MOL BIOTECH 15 002

Role of Aurora kinase in Medulloblastoma development with correlation to *MYCN* activity

Rashmi Prakash Chowath

Degree project in molecular biotechnology, 2015
Examensarbete i molekylär bioteknik 45 hp till masterexamen, 2015
Biology Education Centre and Department of Immunology, Genetics and Pathology, Uppsala University
Supervisors: Fredrik Swartling and Sanna Hede

Abstract

Brain tumors are abnormal tissue masses found, either malignant or benign in nature. Medulloblastoma is a brain tumor subtype found to arise in the hind region of the brain, which is highly malignant and has poor long term prospects in general. On the basis of the driving force behind the tumor, medulloblastoma is further subgrouped into 4 categories: WNT; SHH; Group 3 and Group 4 tumors. Group 3 tumors show a high expression of N-Myc protein which is seen in certain types of cancerous cells. The cell cycle is regulated at several checkpoints by cyclin/cdk inhibitors. The primary cilium is an organelle found on the cellular surface, which has functions in cell growth, differentiation and neurogenesis. Aurora kinase is a protein kinase involved in the regulation and maintainence of the cilium. Often the cilium gets deleted from the cellular surface in tumors coupled with an increase in the kinase level inside the cells. Hence aurora kinase is found to be a viable target for therapy. Aurora kinase is also involved in stabilizing the *MYCN* gene by protecting it from degradation.

In this project, the primary cilum was studied in neural stem cells and followed by study of its presence on tumor cells in culture. The gene involved in cilium development i.e. *Kif3a* was mutated and its aggressive nature was compared with that of the tumor cells. Aurora kinase was commonly found to be over-expressed in both the tumors and the mutants whereas N-Myc over-expression was seen only in tumors. Experiments suggest that cilia repression in *Kif3a* mutants takes place via an aurora kinase dependent pathway.

Role of Aurora kinase & *MYCN* in medulloblastoma development

Popular Science Summary

Rashmi Prakash Chowath

Human brain formation takes place during early embryo development, continuing to grow after birth. The central nervous system consisting of the brain and the spinal cord is responsible for receiving, synchronizing and relaying information between the various parts of the body. Medulloblastoma is a tumor i.e. an abnormal mass of tissue found to arise in the brain affecting the cerebellar region of the brain. The cerebellum is in control of human balance control, motor coordination as well as speech. Medulloblastoma is observed more frequently in childhood. It can have a very aggressive rate of progression along with long term health drawbacks and a poor prognosis.

Several genes are found to be elevated in medulloblastoma tumors. *MYCN* is one such over-expressed gene which characterizes a particular subtype of brain tumors. Amplification of *MYCN* is found to correlate with elevation in kinase levels of Aurora-A. The primary cilium arises from a non proliferating cell, acting as a mechano-chemical sensor. Aurora-A, a mitotic serine/threonine kinase is involved in maintainence and regulation of the cilium on cellular surface. The *Kif3a* gene is in control of cilium maturation.

In this project, the expression of *MYCN* & Aurora-A was studied on normal cells in culture and compared with tumor cells in culture. The *Kif3a* gene was knocked out on the tumors to test its variation on ciliary expression. The kinase activity of Aurora-A was elevated in both the tumors, as well as in the *Kif3a* mutants whereas in comparison, *MYCN* was elevated only in the tumors. Interestingly, other novel cellular targets also showed an accelerated cleavage in the tumors. The long term goal of the project is to establish a pathway connecting the activity of aurora-A on the cilia repression pathway and couple it with its kinase activity on *MYCN*.

Degree project in Molecular Biotechnology, Master of Science 2015,
Examensarbete i molekylar bioteknik 45 hp till masterexamen, 2015,
Biology Education centre and Department of Immunology, Genetics & Pathology, Uppsala University
Supervisors: Fredrik Swartling and Sanna Hede

Table of Contents

Abbreviations

ARL13B	ADP-ribosylation factor-like protein 13B
AURORA-A	Aurora-A kinase/ serine threonine-protein kinase-6 encoded by *AURKA* gene
BCA	Bicinchonic Assay
BSA	Bovine Serum Albumin
CNTF	Ciliary neurotropic factor
CNPase	2', 3'-Cyclic-nucleotide 3'-phosphodiesterase encoded by *CNP* gene
CSL	CBF1, Suppressor of Hairless, Lag-1 transcription factor
DAPI	diamidino-2-phenylindole
DMSO	Dimethyl Sulphoxide
EGF	Epidermal growth factor
GFAP	Glial fibrillary acidic protein
FBW7	F box/WD repeat containing protein 7 encoded by *FBWX7/FBW7* gene
FGF	Fibroblast growth factor
GNP	Granule Neuron Precursor
GSK3	Glycogen synthase kinase 3
GTML	Glt promoter driven expression of TML (TRE-MycN/Luc) or TRE-LacZ
HDAC6	Histone deacetylase 6
HEF1	Human enhancer of Filamentation 1
HET	Heterozygous genotype
hGFAP	Human Glial Fibrillary Acidic promoter
IFT	Intraflagellar Transport
IGF-1	Insulin-like growth factor
KIF3A	Kinesin like protein, encoded by *Kif3a* gene driving cilia
Kif3a $^{fl/fl}$	*Kif3a* gene knockout
KO	Mutant/Knockout genotype
LCA	Large Cell Anaplasia

MB	Medulloblastoma
NB	NeuroBasal Media
N-Myc	N-Myc protein encoded by *MYCN* oncogene
NSC	Neural stem cell
OLIG2	Oligodendrocyte transcription factor
PBS	Phosphate Buffered Saline
PEST	Penicillin/Streptomycin
T3 Factor	Triiodothyronine factor
TBS-T	Tris Buffered Saline with Tween-20
TCHP	Trichoplein
TRE	Tetracycline Responsive element
TUJ-1	Neuron specific class 3 beta tubulin
RSV-A	Rous Sarcoma Virus-A
RCAS/TVA	Replication-competent avian sarcoma-leukosis virus long terminal repeat with splice acceptor/tumor virus A
SHH	Sonic Hedgehog signaling pathway
SOX9	Sex determining region Y-SRY/Box-9 encoded by *SOX9* gene
WNT	Wingless signaling pathway
WT	Wild type genotype

1. Introduction

A Tumor is an abnormal mass of tissue, commonly termed as a neoplasm. Tumors are found to arise from normal tissue and can be either benign or malignant in nature. Malignant tumors give rise to cancerous cells as well as metastatic tissues which are secondary malignant tumors at different sites in the body. Gene mutations of oncogenes and tumor suppressor genes are the main causative mechanisms in development of cancer (Weineberg 2007). Typically, a tumor cell is dedifferentiated in nature with abnormal cell organelles. Depending on the localization of the tumor, it can be categorized as melanoma, sarcoma, leukemia, lymphoma etc. Glioblastoma and Medulloblastoma (MB) collectively are the tumors arising in the brain. *MYC* gene expression is commonly found to be elevated in several cancer types.

The cell cycle has a network of interacting proteins which receive signals, integrate them and mediate cell fate (Weineberg 2007). The cell cycle includes several checkpoints for regulation along with the synthesis and mitotic phases. Cyclin dependent kinases and its associated inhibitors are in charge of mediating these checkpoints.

1.1. Medulloblastoma and subgroups

MB is a small blue round cell malignant tumor found to arise in the cerebellar region of the brain (Taylor *et al.* 2011). On the basis of a transcriptionally driven classification approach, four molecular subgroups of MB have been identified i.e. WNT, SHH, Group 3 and Group 4 (Table 1). The WNT subgroup, named on the basis of the Wingless Signalling pathway, is the best characterized and has a much better long term prognosis in comparison to the other MB subgroups. The SHH group, named after the Sonic Hedgehog signaling pathway driving tumor initiation in it is found to have an intermediate prognosis. Group 3 tumors have a high *MYCN* gene expression and show the worst prognosis. Currently not much is known about Group 4 type tumors. Tumor types can be identified by transcriptional profiling and immunostaining for gene markers.

MB is the most frequent childhood brain tumor. It is found to be very invasive in nature. MB is found to affect males more often in comparison to females. The major MB subgroups are distinct in every aspect i.e. genetic, transcriptional, demographic and clinical (Kool *et al.* 2008). Each subgroup is characterized by a unique genetic signature or cellular origin. Current treatment for MB includes surgery, chemotherapy and radiotherapy. The side effects of these treatments are serious and include cognitive impairment, growth retardation, endocrine dysfunction and psychiatric disorders. Due to dissemination of the cerebrospinal fluid, the tumor spreads to several regions all over the body with a very aggressive pathological nature and severe metastasis (Swartling *et al.* 2010). The tumor pathology can be classified as classic, desmoplastic and Large Cell Anaplasia (LCA) in nature. LCA tumors have a large cell to nucleus ratio commonly correlating with a poor outcome.

Table 1. Major subgroups of Medulloblastoma: WNT, SHH, Group 3 and 4, adapted from Taylor *et al.* 2011.

	WNT	SHH	Group 3	Group 4
Age group	Child, adults	Infant, child, adults	Infant, child	Infant, child, adults
Male:female ratio	1:1	1:1	2:1	2:1
Histology	Classic; rarely LCA	Desmoplastic; classic; LCA	Classic; LCA	Classic; LCA
Metastasis	Rarely	Uncommonly	Very frequently	Frequently
Prognosis	Very good	Intermediate	Poor	Intermediate

1.2. Primary Cilia in cell cycle progression

Duplication of DNA takes place during the Synthesis i.e. S phase in the chromosomes. Division of this DNA to its daughter cells takes place during Mitosis i.e. M phase. The M and S phases are alternated with gap phases G1/S and G2/M which act as checkpoints for cell proliferation (Goto *et al.* 2013). These cells move into the resting phase/G0 when the cell cycle checkpoint parameters are not met. Primary cilia are present on the cell surface as protrusions, acting as sensors for cell signaling and differentiation (Pugacheva *et al.* 2007). A cilium arises from a basal body, from the centrosome of a non proliferating cell. Nine double microtubules together constitute a ciliary axoneme. In most cells, the cilium disassembly takes place as the cells re-enter the cell cycle.

Aurora-A, a mitotic kinase, regulates ciliary dynamics in proliferating cells. Aurora-A, encoded by the *AURKA* gene belongs to a family of serine/threonine kinases along with Aurora-B and Aurora-C (Goto *et al.* 2013). The functions of Aurora-A include mitotic entry, checkpoint control, centrosome maturation and separation, spindle formation and maintenance. It mediates the ciliary dynamics by enabling deciliation i.e. ciliary resorption at cell cycle re-entry as well as continued inhibition of cilia regeneration during cell cycle proliferation.

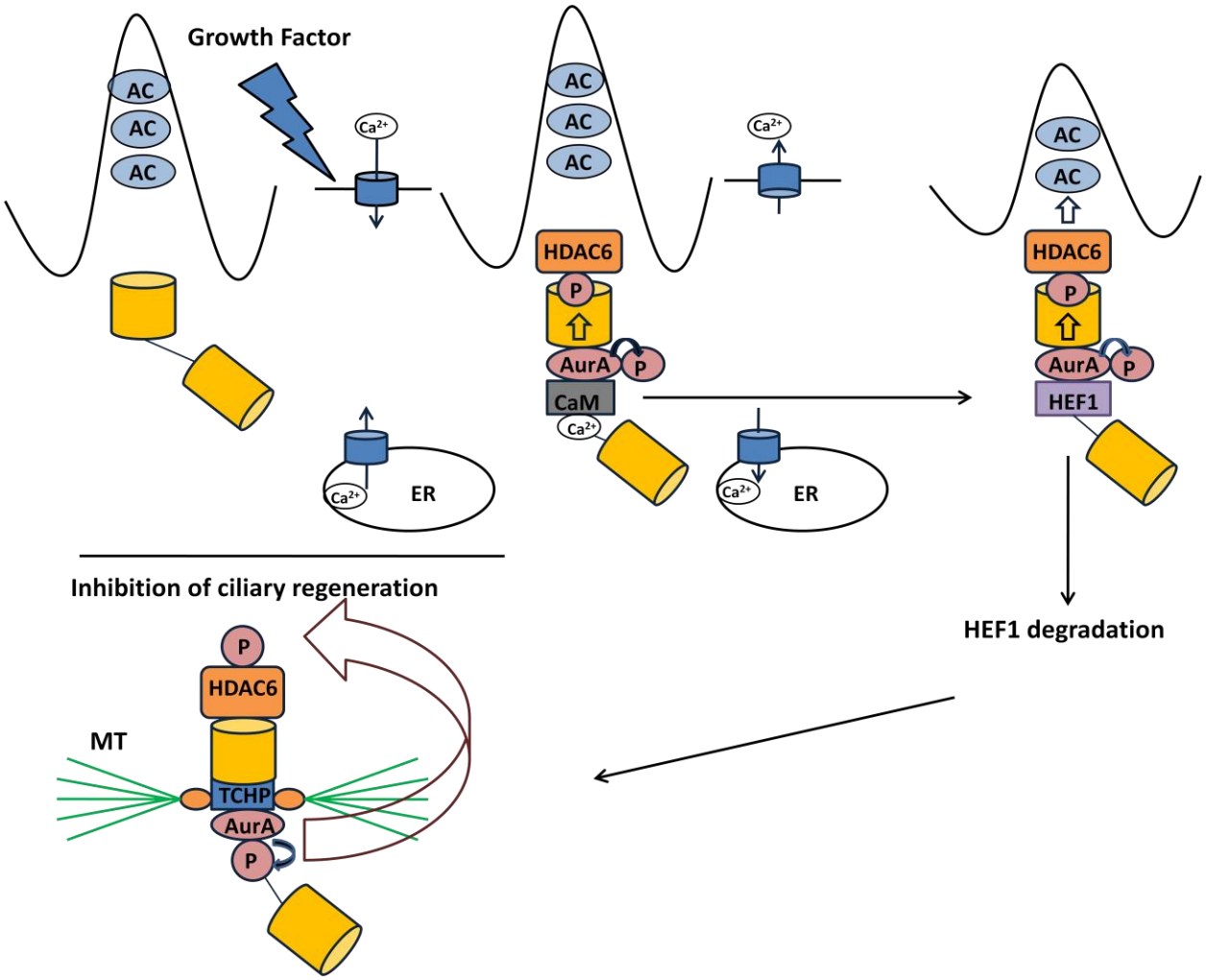


Figure 1. Aurora-A mediated deciliation pathway at cell cycle re-entry, and inhibition of ciliary regeneration in proliferating cells, adapted from Goto *et al.* 2013.

The cilium is anchored to its appendage on the centrosome in the G0/ Resting phase. Growth factor stimulation causes an influx of Calcium ions which activates Aurora-A (Goto *et al.* 2013). This activation takes place by autophosphorylation of Aurora-A at the position Thr288, followed by its binding to Human enhancer of Filamentation 1 (HEF1). Aurora-A then phosphorylates and activates Histone deacetylase 6 (HDAC6), which results in shortening of the ciliary axoneme (Fig 1). As the HEF1 levels decrease, Trichloplein (TCHP), a filament binding protein on the centriole acts as an Aurora-A catalyst to prevent ciliary regeneration during proliferation. As seen, HEF1 and TCHP act as molecular scaffolds to synchronize the cell cycle along with other scaffolding proteins.

It has been discovered that most cancerous cells lack cilia formation, which has been suppressed by the oncogene Aurora-A (Han *et al.* 2009). This makes the procedure of cilium formation a target for therapy. Primary cilia can be either permissive or inhibitory in MB tumor development depending on the initiating oncogenic factor.

1.3. Role of Aurora-A in N-Myc degradation

The N-Myc protein is a DNA binding protein forming a heterodimer with a partner Max protein (Wenzel *et al.* 1991). Amplification of the *MYCN* gene in tumors generally indicates toward a poor prognosis. Conventionally N-Myc is degraded during mitosis by a ubiquitin ligase, Fbw7 in order to enable cell cycle exit. Fbw7 recognizes N-Myc after sequential phosphorylation by cyclin b/ CDK1 and Glycogen synthase kinase 3 (GSK3) and initiates the ubiquitination process. Aurora-A has been identified as a critical gene required in the turnover of N-Myc in amplified neuroblastoma tumor cells (Otto *et al.* 2009). N-Myc has been found to form a complex with Aurora-A to stabilize it during mitosis and maintain a continued proliferative state in tumorigenic cells. Aurora-A enables protection against Fbw7 mediated degradation by inhibition of the ubiquitin ligase.

The formation of the stable Aurora-A/ N-Myc complex is independent of its kinase activity. Aurora-A inhibitors such as MLN8054 and MLN8237 have been implemented to disrupt the Aurora-A/N-Myc complex (Brockmann *et al.* 2013). Usage of these inhibitors has resulted in destabilization of the same complex accompanied by degradation of the protein N-Myc. These results correspond with tumor regression and senescence in *MYCN* driven neuroblastoma. A similar strategy can be applied for therapy against other N-Myc dependent tumors such as Group 3 MBs.

1.4. Primary cilia and hedgehog signaling in Neurogenesis

Granule Neuron Precursors (GNPs) involved in neurogenesis migrate from the hippocampus to the dentate gyrus region, in the sub granular zone and provide neurons throughout life. Primary cilia and SHH signaling via the IFT (Intraflagellar Transport) motors is essential to the growth of GNPs in the brain (Han *et al.* 2008). IFT is a conserved intracellular trafficking process in which Kinesin-2 coded for by *Kif3A* and dynein motors move IFT particles along the ciliary axoneme. Conditional removal of *Kif3a* using *Cre* expressing mice under the Human Glial Fibrillary Acidic promoter (hGFAP::*Cre*) results in the loss of cilia in the dentate gyrus. The ciliary absence can result in a lack of neurons or hypoplasia. This suggests that the *Kif3a* gene is required for proliferation of radial astrocytes in the hippocampus region.

In a normal cerebellum, GNPs proliferate and migrate from the external germinal layer to the internal germinal layer. This cell proliferation is regulated by the SHH ligand binding to the Patched receptor on the GNP (Spassky *et al.* 2008). This SHH binding depends on several transduction proteins including IFT protein complexes. The primary cilium is found to have an important role in early cerebellum development. Loss of either the primary cilia or the Hedgehog pathway components can lead to the formation of a mutant GNP, which eventually leads to MB development (Barakat *et al.* 2013). Depending on the mode of activation of the SHH pathway, ciliary components *Kif3a* and kinesin-2 proteins are found to either promote or suppress MB tumor formation.

1.5.Retroviral RCAS/TVA system

Retroviruses can be efficiently used to introduce genetic information into target cells. The Rous Sarcoma Virus-A (RSV-A) has been adopted as a vector family with a multi-cloning site introduced to insert genes of interest of sizes up to 2.8 kb termed as RCAS (Replication competent avian sarcoma virus) (Werder *et al.* 2012). RCAS vectors express an envelope glycoprotein due to which it can infect cells expressing only corresponding receptor TVA (Tumor virus-A). This TVA receptor is naturally found on avian cells, making chicken fibroblast cell lines an ideal cell line to produce competent RCAS virus titers (Fig 2).

RCAS retroviruses can be used to transduce genetically engineered mammalian cells expressing the TVA receptor (Werder *et al.* 2012). Cre-lox recombination combines the use of a Cre recombinase enzyme with specific LoxP sites to produce Cre-Lox strains to generate gene knockouts or reporter strains (Araki *et al.* 1997). By combining the RCAS-TVA system with Cre-*loxP* recombination, it is possible to study mice models engineered for knock out of tumor suppressor genes and even regulate gene expression in a time controlled manner. The process of transfection of host cell chicken fibroblasts to produce viral titers takes about four weeks.

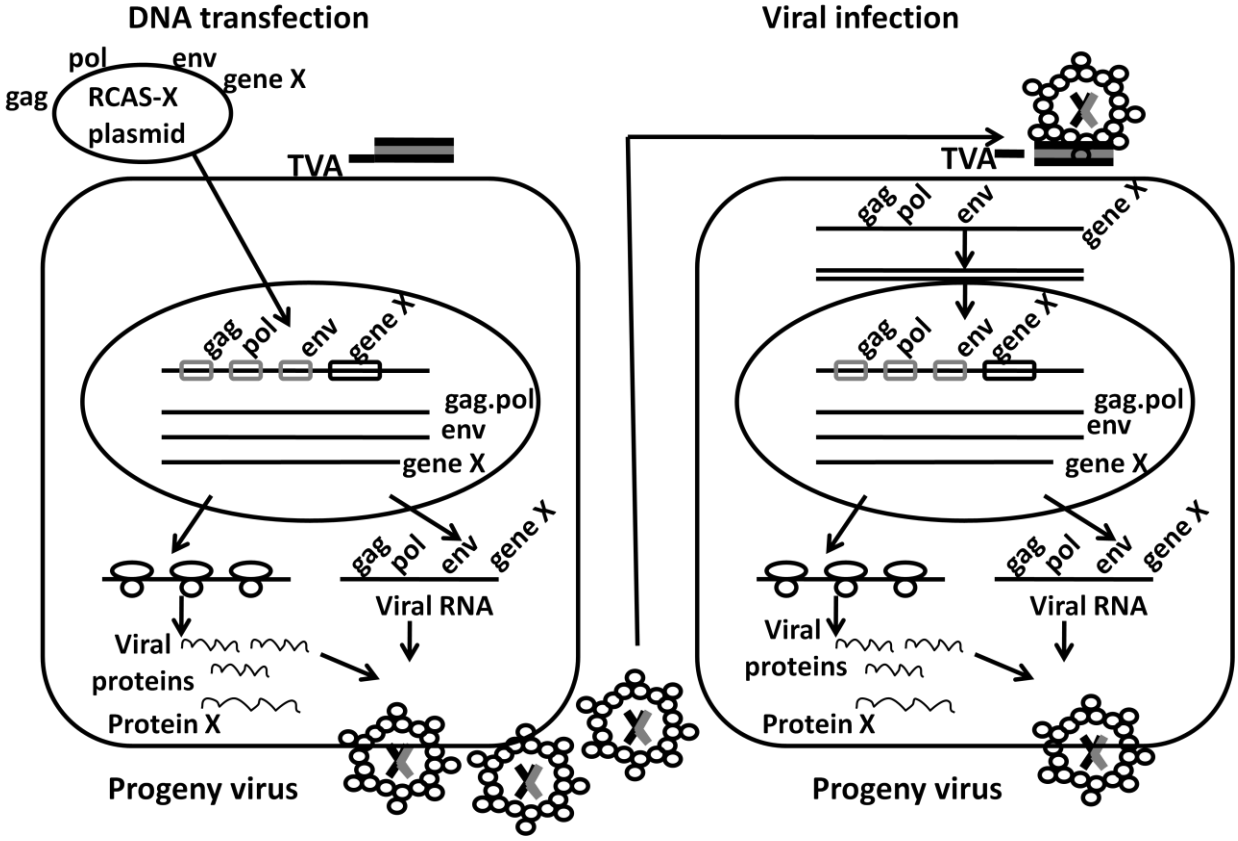


Figure 2. Transfection of chicken fibroblasts with RCAS viruses, to produce viral titer, used to transduce transgenic mice expressing TVA receptors, figure adapted from Fisher *et al.* 1999.

1.6.Mouse models of human *MYCN* driven MB

The *MYCN* gene is found to have a multi-dimensional role in SHH independent MB tumors right from its initiation to the maintenance and metastasis. Such N-Myc amplified tumors show typical classic histology or LCA histology, and are found only in fetal cerebella (Swartling *et al.* 2010). Mice models were generated by Swartling *et al.* to study the N-Myc protein functionality in tumorigenesis. N-Myc expression was targeted in the fetal cerebella using double transgenic mice using a Tetracycline response system (Gossen and Bujard 1992). The transgenic mice were engineered to express Tetracycline (tTA) driven by a Glutamate promoter in the absence of doxycycline (Fig 3). Glt-tTA mice having a bidirectional TRE (Tetracycline Responsive Element) were then crossed with mice expressing either a TML i.e. N-Myc/Luciferase gene or a LacZ gene. The tumors obtained from the fetal cerebella of these GTML (Glt promoter driven TML/TRE-LacZ expression) mice showed an over-expression of N-Myc.

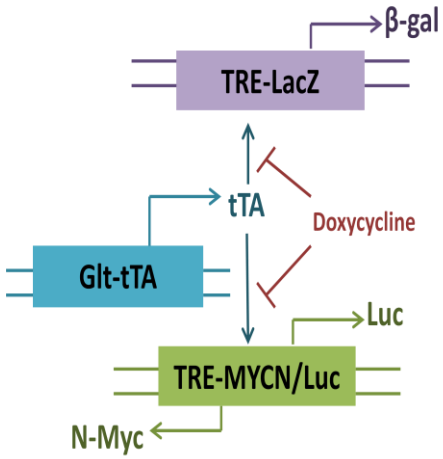


Figure 3. Illustration of model driving GTML mice, Glt promoter driving expression of TML (TRE-*MYCN*/Luc) or TRE-LacZ genes, adapted from Swartling *et al.* 2010

Similar to the GTML model, the *MYCN T58A* mice models were developed using the RCAS technology (Werder *et al.* 2012). RCAS-TVA mice were induced to exhibit an over amplification of N-Myc. N-Myc^{T58A} transduced murine neural stem cells were injected into the cerebellum of Glial fibrillary acidic protein (GFAP) positive mice to generate SHH independent MB tumors (Swartling *et al.* 2012).

2. Aim

The aim of the project was to study and observe the characteristics of the primary cilium *in vitro*. FBW7 mutant cell lines and established medulloblastoma tumor cell lines were compared analytically.

The next focus was to study the protein expression of Aurora kinase for the same cell lines as above and optimize it.

Following it, the goal was to knock out the ciliary gene *Kif3a* using the RCAS-TVA infection method. The main target in this was to investigate the mutant *Kif3a* lines as well as subject it to differentiation.

3. Materials and Methods

3.1. Media, Chemicals, Antibodies and Cell lines used

Media, antibodies and cell lines used in the study are presented in Table 2, 3 and 4, respectively.

Table 2. List of Media, chemicals and buffers used in the experiments.

Media*	Chemicals and Buffers
NeuroBasal Media (NB)	Phosphate Buffered Saline (PBS)
B27 growth factors	Bovine serum albumin (BSA)
L-Glutamine	Goat serum
Penicillin/Streptomycin (PEST)	Triton x-100
Epidermal growth factor (EGF)	TBS-T (Tris Buffered Saline with Tween 20)
Fibroblast growth factor (FGF)	
DMSO (Dimethyl Sulphoxide)	DAP1 (diamidino-2-phenylindole)
CNTF (Ciliary neurotropic factor)	Mowiol
Forskolin	3-N-Morpholino propanesulfonic acid
Triiodothyronine (T3) factor	(MOPS) buffer
Insulin like growth factor (IGF-1)	Xylene
Accutase	Ethanol

*The media and media components including growth factors and serum were sourced from *Life technologies,* aliquoted and used as per the requirement. The buffers used in the experiments were diluted 10 times from the premade stock buffer.

Table 3. Antibodies used for Immunostaining and immunoblotting studies.

Mouse antibodies*/[manufacturer]	Rabbit antibodies/[manufacturer]
Acetylated tubulin [Sigma-Aldrich]	Activated notch [Abcam]
Aurora-A [Abcam]	ADP-ribosylation factor-like protein 13B-(Arl13B) [Abcam]
Fbw7 [Abcam]	
Glutamylated tubulin [Sigma-Aldrich]	Beta-Actin [Santacruz biotechnology]
	GFAP (Glial fibrillary acidic protein)
Nestin [BD Pharmingen]	[Sigma-Aldrich]
N-Myc [Cell signaling technology]	
	KI67 [Abcam]
Proliferating cell nuclear antigen (PCNA) [Abcam]	Olig2 (Oligodendrocyte transcription factor) [Abcam]
Tuj1 (Neuron specific class 3 beta tubulin) [Neuromics]	Phospho-Aurora-A [Cell signaling technology]
	Sox9 [Sigma-Aldrich]

*The antibodies were sourced directly from the manufacturers, and stored at either +4°C or at -20°C, and used individually for each experiment.

Table 4. Cell lines used for immunostaining and immunoblotting studies.

Normal cells*	Primary NSC's (RCAS/TVA)	Induced MB tumor cells	GTML Tumor cells
DF1- chicken fibroblasts	Fbw7 KO (Mutant)	POC2 T2 POC2 T6	2135 (Fbw7 WT)
POC2 Cl4 POC2 GFP	Fbw7 WT (Wild type) Fbw7 HET (heterozygous)	POF2 T6 POF2 T2	2140 (Fbw7 HET)
	Kif3A KO *Kif3A* WT *Kif3A* HET	POF2 T8 POC2 T15	

*The cell lines except for the chicken fibroblast lines were all derived from murine cerebral matter.

3.2. Cell Culturing Conditions

All the cell lines (Table 4) were cultured in NB medium containing 2% B27 growth factor, 1% L-Glutamine, 1% PEST and 0.1% EGF/FGF. The cell lines were grown on standard cell culture dishes. Cell plates were stored in the incubator at 37°C. Cell morphology was checked regularly and split when needed between 48 hours- 96 hours. 2ml Accutase was used to dissociate cells before splitting. Cell maintained in culture were derived from models based on *MYCN* driven MB, Fbw7, GTML (3) or the RCAS/TVA system.

For maintaining a viable cell count 10 µL of trypan blue dye was added to 10 µL of cell culture. 10 µL of the mix was mounted on a slide for counting. Cell counting was done using Burke's chamber or TC-20 Automatic Cell counter. For freezing down cells, cell pellets were re-suspended in 1 ml freezing media i.e. NB media supplemented with 10% DMSO, 1% EGF/FGF. The cell stocks were stored at -80°C until required.

3.3. Immunofluorescence

10000 cells were grown on poly-ornithine and laminin coated 24 well plates with sterile cover slips until 70 percent confluence was achieved as required. The cells were fixed in 4% paraformaldehyde for 15 minutes followed by 2 washes in PBS. The coated cover slips were subjected to blocking buffer made up of 1xPBS with 5% goat serum, 5% BSA, 0.3% triton-X. After blocking for 1 hour at room temperature, the cells were incubated in the appropriate primary antibody diluted in the Blocking buffer overnight at +4°C. The antibodies were used at the flowing dilutions:

Acetylated tubulin at 1:1000; Gamma tubulin at 1:1000; KI67 at 1:100; ARL13b at 1:1000; OLIG-2 at 1:500; Activated notch at 1:500; Aurora-A at 1:1000; TUJ-1 at 1:500.

On the following day, after washing the cover slips in PBS (3 times for 5 minutes each), the secondary antibody was added at a dilution of 1:400 in the blocking buffer. This was covered with aluminium foil and incubated in room temperature for 90 minutes, followed by washes in TBS-T (3 times for 5 min each). The secondary antibodies used were Alexa-antifluor 488/555 [Life technologies]. The cover slips were then incubated in DAPI at a dilution of 1:1000 in TBS-T for 15 minutes in the dark, followed by 3 washes in TBS-T. The cover slips were then mounted

on glass slides in mounting solution Mowiol and labeled appropriately. After the slides were left to dry overnight in the dark, the samples were observed using Axio-vision imager fluorescence microscopy. Specimen sample pictures were captured and documented at 10X, 20X and 40X magnification.

3.4. Genotyping of Mice biopsies

Mice biopsies (ear and tail) obtained from the sample specimen under study were subjected to lysis by a standard reaction mix. The reaction mix contained KAPA express extract enzyme and KAPA express buffer [KAPA Biosystems] made upto 100µl per PCR tube. This mix was subjected to PCR lysis with melting temperature at 75°C for 10 minutes and annealing temperature at 90°C for 5 minutes. This was followed by overnight incubation of the lysed sample biopsies at +4°C. On the following day this sample was subjected to PCR KAPA lysis with appropriate primer pairs. A reaction mix of 10 µl in a tube was prepared depending on the gene being tested.

The genes which were tested included *TML* with a T_m i.e. melting temperature of 57°C, *Kif3a* with a T_m of 57°C, *TreCre* with a T_m of 57°C, *Fbw7* with a T_m of 60°C, *Cre* with a T_m of 60°C, *LacZ* with a T_m of 62°C and *GLT* with a T_m of 62°C. These PCR products were run on a 2% agarose gel against a 1Kb loading marker. The gel was then analyzed via imaging to verify genes present in the sample.

3.5. Immunoblotting

Cell pellets of the appropriate cell line (Table 4) were collected after successive splitting cycles and stored at -20°C until required. While being subjected to tests, these cell pellets were lysed in lysis buffer containing a mix of 1X Protease inhibitor, 1X Phosphatase inhibitor and 10% SDS in Millipore water. After cell lysis of the pellet, its protein concentration was measured by the Bicinchonic Assay (BCA) by comparison of 6 standard dilutions of BSA against the samples.

20 µg of the protein was denatured in a water bath at 72°C for 10 minutes, spun down to concentrate it and then loaded against a standard marker onto a 4%-12% precast NuPAGE BisTris gel [Life technologies] aided by the use of a syringe. The loading chamber contained MOPS buffer with 500 µl of antioxidant added to it. This sample loaded gel was run at 180V for 1.1hrs. The separated protein on the gel was transferred to a nitrocellulose membrane using an ImmBlot apparatus in a 7 min procedure. This membrane was then blocked in the blocking buffer (5% BSA in TBS-T or 2.5% milk in TBS-T) for 1hr with shaking at RT. The membrane was then washed 3 times in TBS-T (10 minutes each) and incubated in the primary antibody overnight at +4°C on a shaker.

On the following day, this membrane was washed 3 times in TBS-T (5 min each), and subjected to labelling with the Horseradish Peroxidase conjugated secondary rabbit antibody/mouse antibody at a dilution of 1:6000. All dilutions were made in the respective blocking buffer of TBS-T. In order to reuse the same membrane for a varied antibody, it was stripped in 0.4M Sodium hydroxide for 20 minutes, and then subjected to the procedure of blocking & labeling again.

For visualization of the gel, a working solution of 2 ml of enhancer with peroxidase (1:1) was prepared. The membrane was incubated in this solution for a few minutes, and then placed in a

plastic pouch inside a cassette. The immune blots were recorded either on a film using a dark room or electronically on a LAS4000 scanner. The time of exposure varied between 15 minutes to overnight depending on the antibody used.

3.6. RCAS *Cre* virus infection

Cerebellar neurospheres were prepared from the mice brain/tumor. The brain was quickly extracted and placed into 20 ml of sterile Hanks (Ca^{2+} /Mg^{2+} free) solution containing 1% PEST and 1% fungizone on ice. After aspiration for a few minutes, the tissue was gently dissociated in 1 ml of papain solution, and left for 20 minutes in the incubator at 37°C. This tissue was dissociated again by pipetting up and down several times, and then washed in PBS, pelleted and re-suspended in NB media followed by culturing on plates.

The RCAS virus containing vials were left on ice for 1.5-2 hrs to thaw. DF-1 chicken fibroblast cell lines in culture were plated onto two 6 well plates (low adhesion). The 1st plate was used for RCAS virus infection, and the 2nd plate was used as a control plate. 1ml of NBE (NB medium with N2 supplement) and 2 ml of the virus media were added to each well on the plate in the 1st round of infection. The control DF-1 cell plate was split as per regular protocol. Both plates were incubated for 48 hrs at 37°C. RCAS infection was tested by the presence of green cells (under the microscope) on the culture plate. If more than half of the cells did not test positive for green, 1 more ml of RCAS virus media was added to each well after taking away 1 ml of the medium present.

After successful transfection, these cells were conventionally cultured in NB media. After generating sufficient RCAS-TVA virus, the cell lines in culture were divided and frozen down, and simultaneously subjected to analytical experiments. Immunostaining and immunoblotting were carried out on these cell lines for comparison with similar experiments on neural stem cells.

3.7. Seeding of cell lines with differentiation factors

The appropriate cell line i.e. the RCAS *Kif3a* $^{fl/fl}$ (knockout) cell line was cultured in NB media on laminin coated 24 well plates, at a concentration of 5000 cells/well. After attachment of the cells onto the plate, the NB medium was taken away and replaced with serum free NB media containing varied differentiation factors. For the purpose of culturing differentiated cells, the serum free media was supplemented with varied differentiation factors. CNTF was added to produce astrocytes. Forskolin was used to give neurons. T3 factor and IGF-1 in a ratio of 1:1 were added to the serum free NB to produce oligodendrocytes.

The differentiation factors were used at the following concentrations:

CNTF at 100 ng/ml; Forskolin at 10 µM; T3 at 60 ng/ml; IGF-1 at 100 ng/ml.

The *Kif3a*$^{fl/fl}$ cell line was subjected to this differentiation medium for a period of 7-10 days, and then analyzed via immunostaining. All experiments were performed in triplicate.

4. Results

4.1. Ciliary study in primary cerebellar stem cells and tumor stem cell lines

4.1.1. Genotyping of mutant FBW7 lines to identify Heterozygous, Homozygous and wild type cell lines

In order to understand cilia morphology, primary neural stem cells i.e. *Fbw7* mutants, Wild type (WT) and Heterozygous (HET) cell lines were cultured and tested for various antibodies via Immunofluorescent staining. For the identification of mutant and heterozygous lines, genotyping by KAPA lysis and PCR was carried out as described in section 3.4 (Fig 4). The mutants were created by the deletion of a specific sequence within the FBW7 gene. So a HET line would yield two separate bands whereas a mutant line would result in a single band of 350 bp, and a WT line would yield a band of 235 bp.

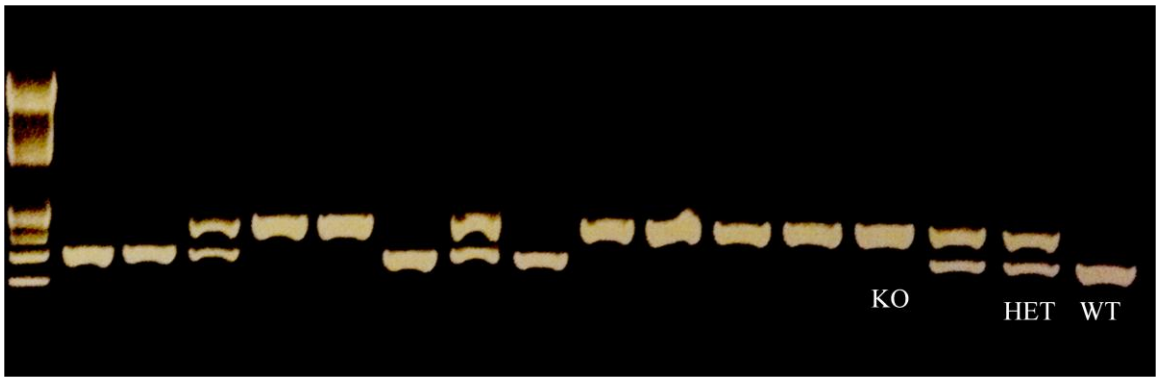


Figure 4. Genotyping of *Fbw7* cell lines identifying KO lines at 350 bp and WT lines at 235 bp.

4.1.2. Immunostaining of *Fbw7* heterozygous and homozygous mutants and comparison with wild type against Acetylated tubulin, Tuj1, N-Myc, Glutamylated tubulin and Notch-1 markers

Microtubules, composed of tubulin, play an important role for the cellular structure. Tubulin undergoes post translational modifications and is present in its acetylated form. Antibodies against acetylated tubulin were used to visualize viable neural stem cells and the primary cilia present on these cells (data not shown). Mutant FBW7 lines were also immunostained for the presence of tubulin and they showed a similar pattern as the WT. TUJ1 is a neuron specific tubulin and immunostaining using anti-tuj1 was used to confirm the identity of neural stem cells (Fig 5a-c). FBW7 HET and KO cell lines also showed the presence of TUJ1 indicating that they retained their neural stem cell identity. The specimen samples were represented at 10X, 20X and 40X magnification.

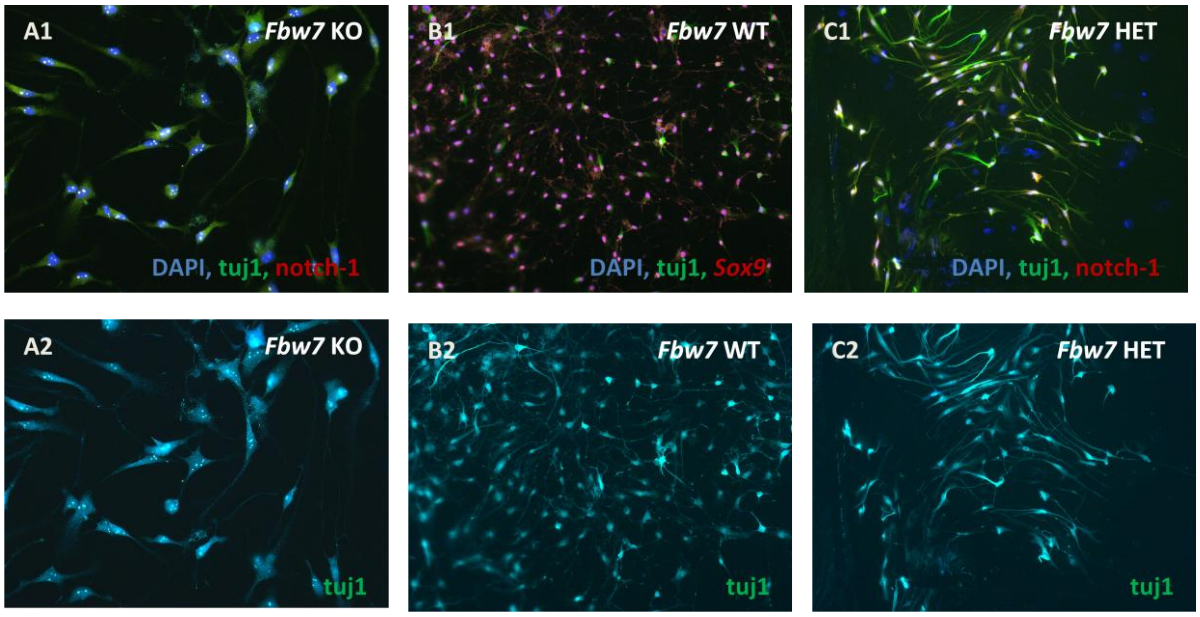


Figure 5. Neural stem cell identity of *Fbw7* cell lines, via immunostaining against Acetylated tubulin and TUJ1, A1-A2 at 40X, B1, B2, C1, C2 at 20X magnification.

In neuroblastoma, the levels of N-Myc have been found to increase as the tumor progresses. In order to characterize the presence of N-Myc in a normal neural stem cell, antibodies against N-Myc were used to immune stain WT type stem cells. High levels of N-Myc were seen in the wild type compared to the FBW7 HET and KO lines. If the FBW7 mutants form tumors, one would expect higher levels of N-Myc but reduced levels indicate the absence of tumorigenic characteristics (Fig 6). Presence of cilia can be visualized by staining for glutamylated tubulin. Wild type neural stem cells showed the presence of glutamylated tubulin on immunostaining whereas FBW7 mutant lines lacked it. However, verification of the presence of cilia on these cell lines was difficult to arrive at (Fig 6).

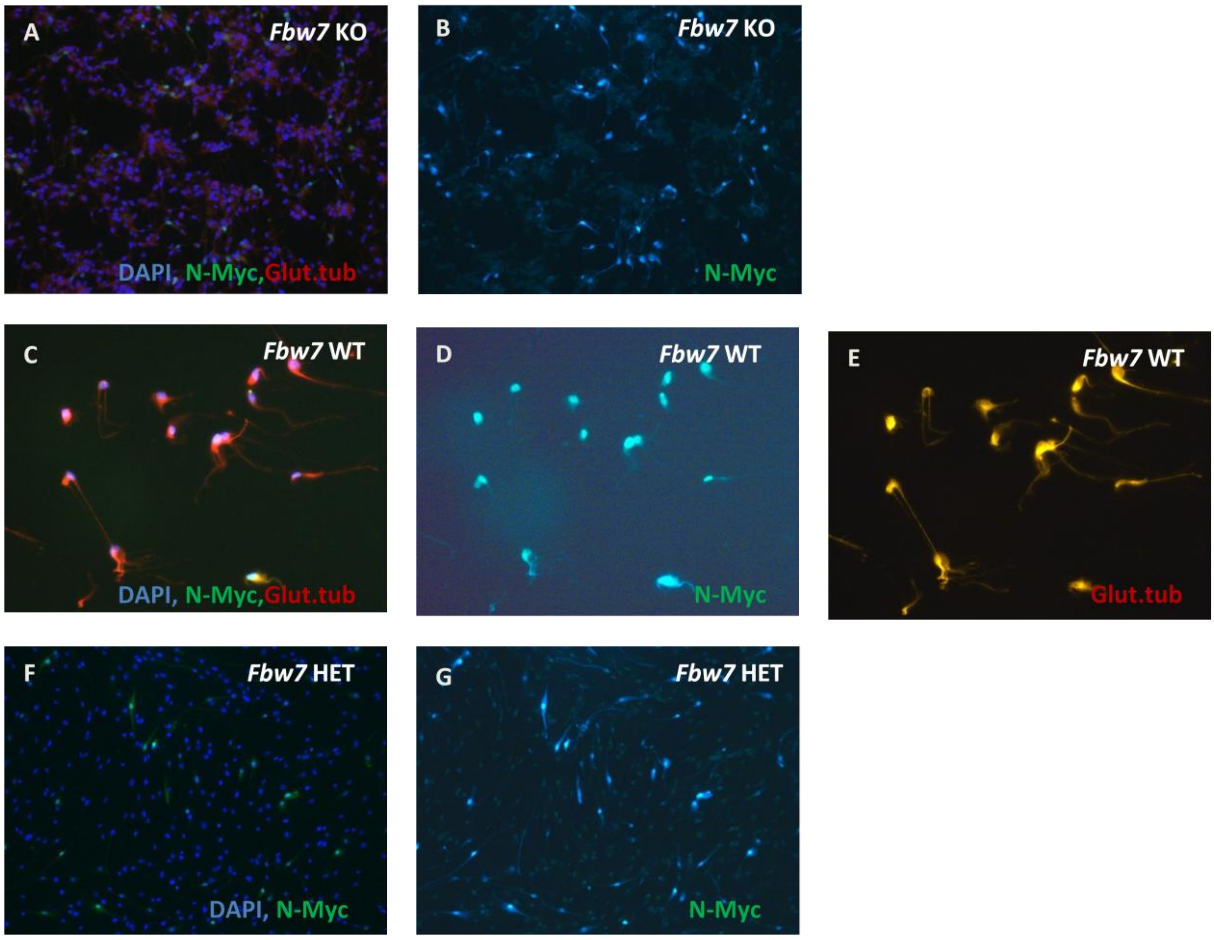


Figure 6. Reduced N-Myc expression in *Fbw7* KO cell lines vs WT, by immunostaining against N-Myc and glutamylated tubulin, A, B, F, G at 10X, C, D, E at 20X magnification.

Activated notch-1 proteins are transmembrane proteins which undergo cleavage as part of a signaling mechanism and enter the nucleus. Immunostaining for activated notch-1 showed that in the FBW7 mutant lines the protein was localized more in the nucleus compared to the wild type (Fig 7a, b). This indicates an accelerated cleavage in the mutant lines which could result from alterations in the notch signaling pathway. Heterozygous FBW7 lines also showed the presence of Notch-1 inside the nucleus like the mutants (Fig 7c).

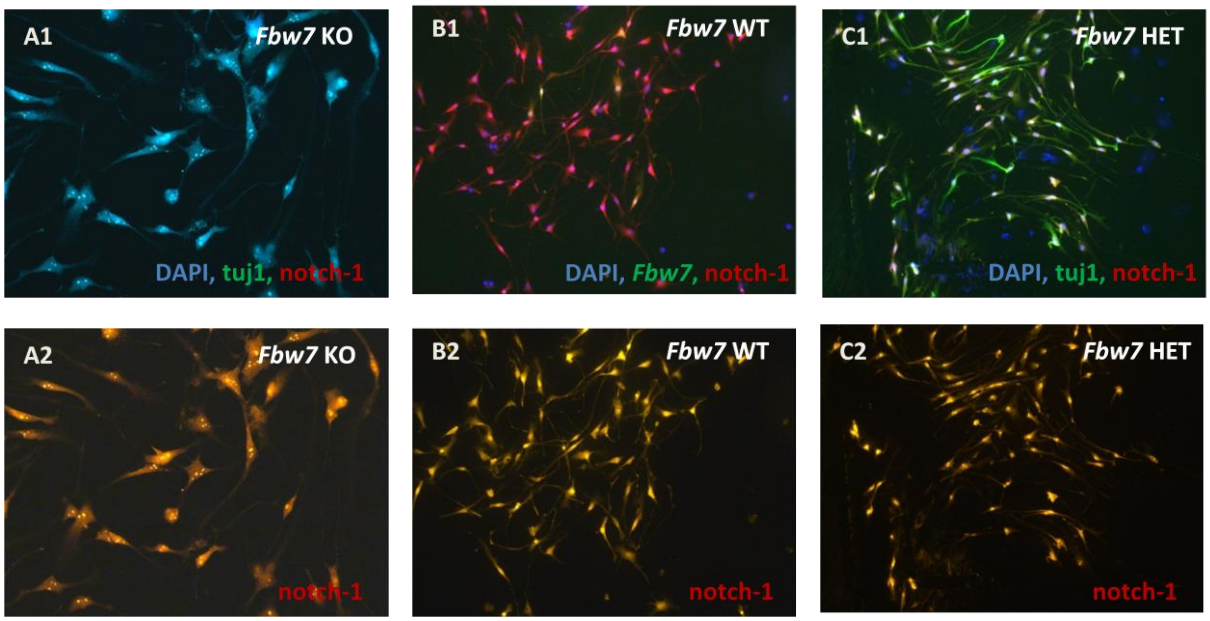


Figure 7. Accelerated cleavage of Notch1 in *Fbw7* KO cell lines vs WT, by immunostaining, A1, A2 at 40X, B1-C2 at 20X magnification.

4.1.3. Immunostaining of tumor cell lines against Acetylated tubulin, KI67, Aurora-A and N-Myc for comparison with *Fbw7* mutant cell lines

In order to identify if the mutant *FBW7* lines showed a similar pattern as tumor cell (T58) lines, immunostaining of tumor cell lines i.e. POF2 T8, POC2 T15, POF2 T6 and POC2 T6 was carried out using similar antibodies. The viability of the tumor cell lines was first confirmed by costaining the two cell lines with acetylated tubulin and KI67 (Fig 8).

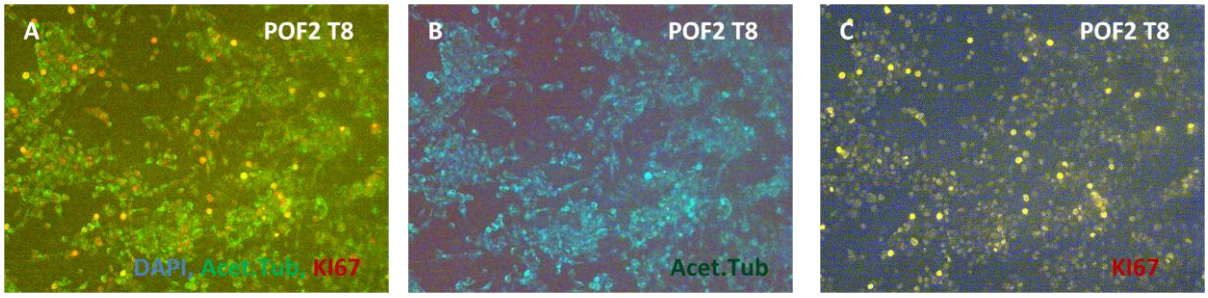


Figure 8. Costaining of POF2 T8 cells in culture with KI67 and acetylated tubulin, at 10X magnification.

As mentioned above, tumor cell lines are expected to show an increased presence of N-Myc. Immunostaining for the presence of the *MYCN* gene in the tumor cell lines revealed that in POC2 T15 cell lines high expression of N-Myc was not seen whereas in POF2 T6 cell lines there was a high expression of N-Myc (Fig 9a,b). Aurora kinase proteins are known to play an important role in the stabilization of N-Myc and preventing it from degradation. Fitting this notion, high levels of N-Myc overlapped with high levels of Aurora-A expression (Fig 9). The unexpected behavior of the POC2 T15 cell lines could be explained by the aging of cells due to several splitting cycles.

KI67 is a commonly used marker for proliferating cells. Tumor cells are characterized by their constantly proliferating nature and are expected to show positive stains when immunostained using KI67 antibodies. Aurora-A is involved in the progression of cell cycle by repression of cilia formation. Proliferating cells are hypothesized to lack cilia. The co-localization of Aurora-A with KI67 in both the tumor cell lines hint at the possibility that tumor progression in these cell lines occur by an Aurora-A dependent cilia repression pathway (Fig 9c,d).

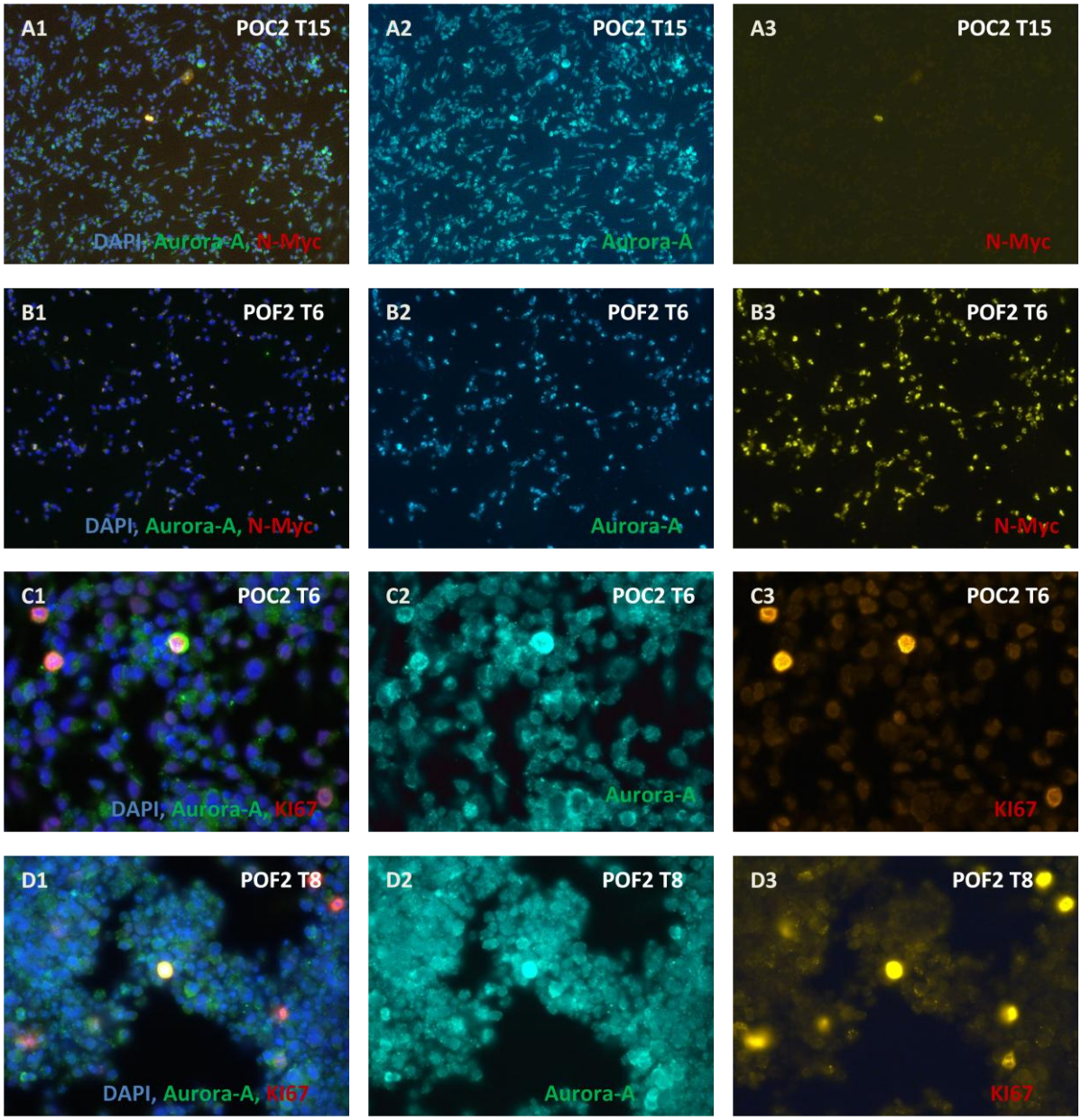


Figure 9. Co-staining of Tumor cell lines with Auroro-A/N-Myc and Aurora-A/KI67 confirming ciliary loss and *MYCN* amplification, A1-B3 at 10X and C1-D3 at 40X magnification.

The comparison of N-Myc immunostaining in the mutant FBW7 and tumor cell lines indicate that the mutant lines are less tumorigenic. Sample specimens were observed and captured at varied magnification levels from 10X-40X. Immunostaining for additional markers of tumor

formation in the KO FBW7 lines would provide a definitive conclusion on the presence of tumor characteristics in these FBW7 lines.

4.2. Expression analysis of Aurora-A and FBW7 on *Fbw7* cell lines

In order to elucidate if the mutant *Fbw7* lines lacked the presence of FBW7 protein, western blotting was carried out. Antibodies against FBW7 were used to visualize the FBW7 protein bands. The wild type and heterozygous lines showed bands at 46kDa indicating the presence of FBW7 protein whereas the mutants lacked it (Fig 10). This confirmed the absence of a functional FBW7 protein in the knockout lines. The presence of FBW7 was analyzed in the tumor cell lines to correlate if the lack of FBW7 led to tumor formation. Most of the tumor cell lines showed lack of FBW7 indicating the possibility of tumor formation in FBW7 mutants. For a more accurate representation of data, the blots against *Fbw7* would have to be standardized and compared against beta-actin (not shown).

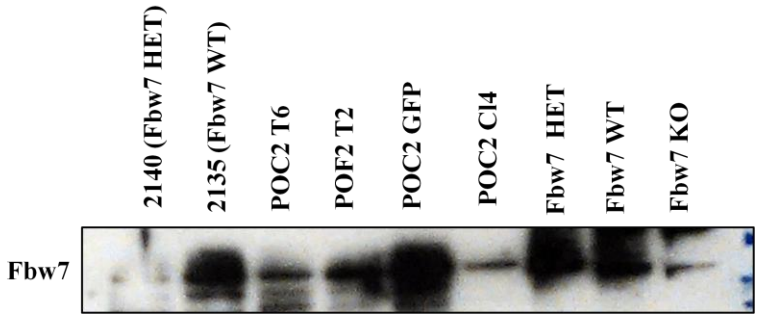


Figure 10. Western blot against *Fbw7* showing its absence in mutant i.e. KO and tumor line i.e. 2140.

Aurora-A, a serine/threonine kinase is found to be over expressed in brain tumors. Aurora-A is localized to the centrosomes and the cytoplasm of mitotically proliferating cells. Aurora-A protein levels increase during the G2/M phase of cell cycle, and decrease during the G1 and S phases. To further explore the presence of tumors in FBW7 mutants, the expression of Aurora-A was studied using western blotting. In the tumor cell lines and the GTML tumor lines, increased Aurora-A expression was seen compared to the expression from the clone which the tumor cell line was derived (Fig 11).

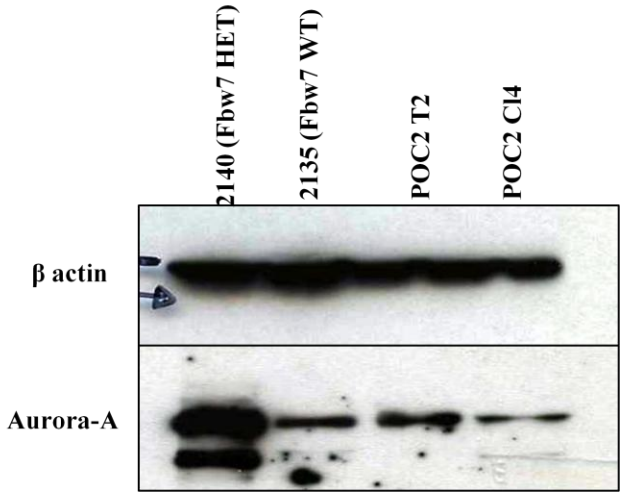


Figure 11. Western Blot for Aurora-A in Tumor cell lines, with higher expression in tumor lines POC2-T2, 2135 and 2140 in comparison to POC2-Cl4.

Further studies on the expression of Aurora-A in mutant *Fbw7* would aid in understanding the role of Aurora-A in tumor formation.

4.3. Comparative analysis of Phospho-aurora-A from immunoblotting

Active Aurora-A is phosphorylated at its catalytic domain at Thr288, causing an increase in its kinase activity. Optimization of western blot for visualization indicated that the efficiency of detection could be improved by using an antibody against phospho-aurora-A. This hinted at the possibility of using anti phospho-aurora-A antibodies which can detect derived Aurora-A levels only when phosphorylated at Thr288. This was used to compare the increase in activation of Aurora-A in the cancer cells compared to non tumor cells (Fig 12). Hence an immunoblot using phospho-aurora-A antibodies to detect Aurora-A in tumor cells was carried out, however this did not result in any marked difference in the overall protein levels in comparison. For a more conclusive representation of data, the blots against phospho-aurora-A would have to be reiterated, standardized and compared against beta actin (not shown).

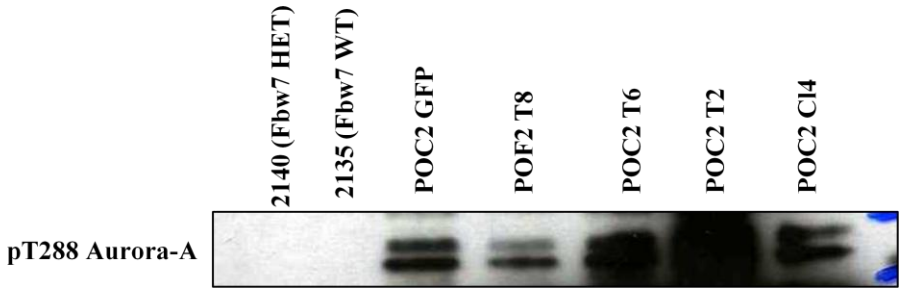


Figure 12. Immunoblotting of GTML lines and N-Myc tumor lines against phospho-aurora-A (Thr 288), in contrast to Aurora-A.

4.4. Comprehensive study of neural stem cells infected with RCAS virus

4.4.1. Genotyping and Immunoblotting of *Kif3a* knockout lines to verify RCAS infection, and compare kinase activity

Mutant *Kif3a* lines were generated by infecting G-tva expressing neural stem cells from *Kif3a* mutant mice with RCAS-Cre retrovirus. The *Cre-loxP* system leads to the deletion of a region of the gene leading to a knockout of the *Kif3a* gene. Genotyping was done by PCR to distinguish between the homozygous mutants, heterozygous and wild type lines (Fig 13).

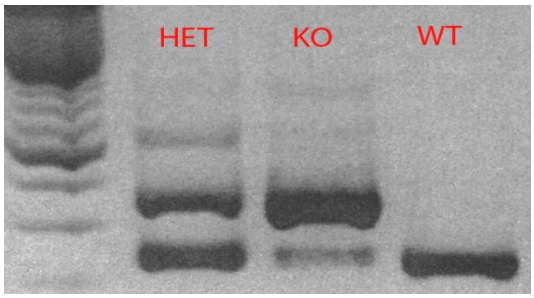


Figure 13. Genotyping of *Kif3a* cell lines via PCR to verify RCAS infection.

Aurora-A regulates cilia resorption. In order to monitor the Aurora-A kinase activity, a western blot was carried out to identify the levels of Aurora-A in *Kif3a* mutants and tumor cell lines. PCNA was used as a marker of proliferation, as ciliary development is dependent on the cell proliferation. It was seen that in the *Kif3a* mutants, higher levels of Aurora-A was present compared to wild type. This indicates to a hypothesis that the tumor formation due to loss of cilia happens via an Aurora-A dependent pathway in which the stability of *MYCN* is increased which is a characteristic of MB. Due to the inherent protein degradation of the GTML sample cell lines used i.e. 2140 and 2135, there was a marked variation in the amount of protein expressed on the blot from Fig 11 (an extra band expressed), Fig 12 (no visible band) and on Fig 14. This was on account of the difference in the time points at which the same lysed pellet was utilized.

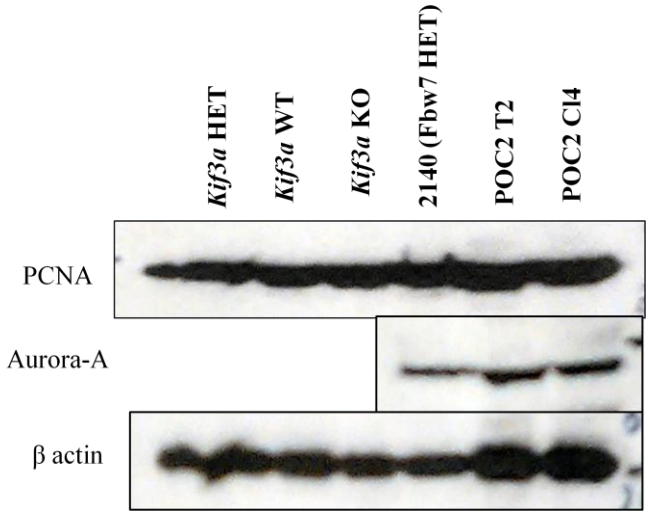


Figure 14. Western blot against Aurora-A to visualize and compare tumor lines 2140 and POC2-T2 with POC2-Cl4.

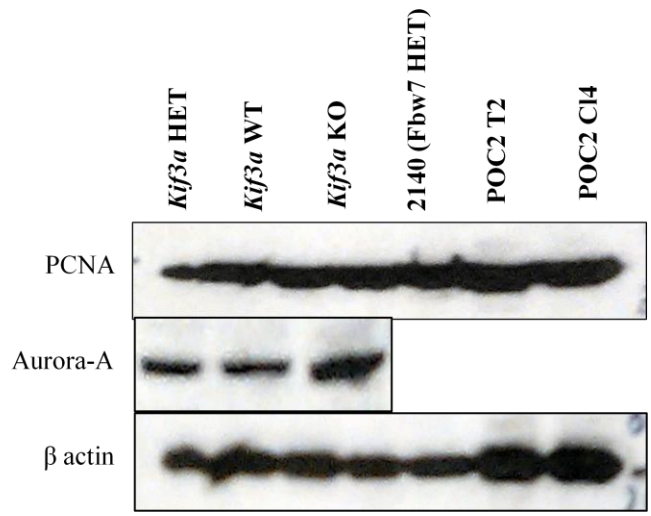
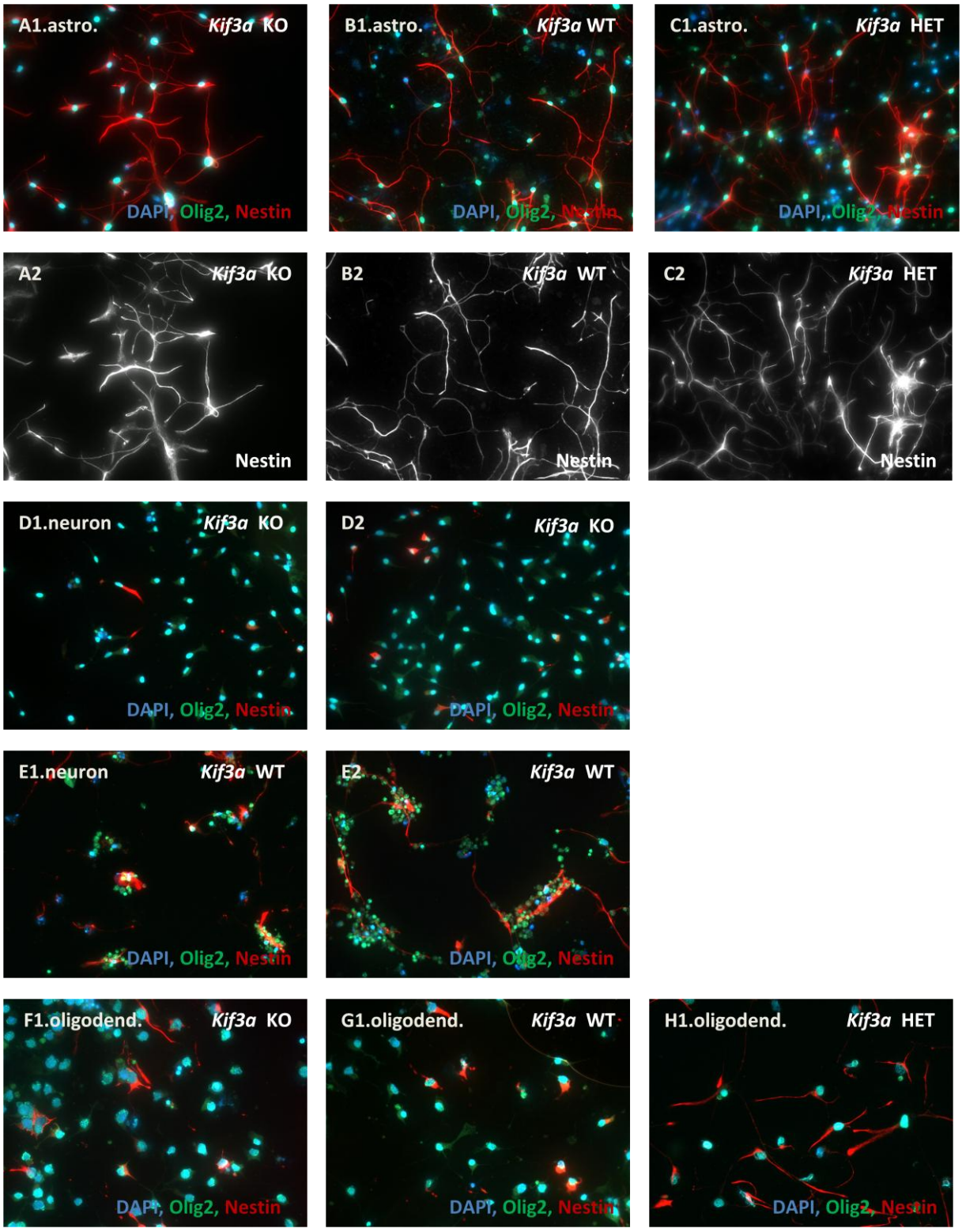


Figure 15. Western blot against Aurora-A to visualize its presence in *Kif3a* mutant lines (KO) and compare it with relative expression in *Kif3a* WT, HET lines.

4.4.2. Effect of a mutant *Kif3a* gene to study its effect on neural stem cell differentiation

Cilia have been implicated to have function in differentiation of stem cells and maintenance of the differentiated state. To identify if the loss of cilia by *Kif3a* $^{fl/fl}$ has an effect on the differentiation of neural stem cells, the mutant *Kif3a* lines were allowed to differentiate into oligodendrocytes, astrocytes and neurons by subjecting them to respective differentiation factors (Fig 16). Differentiated cell lines were stained and recorded at 20X magnification.

Treatment of *Kif3a* KO cell lines with CNTF to facilitate differentiation to astrocytes yielded cells which were morphologically similar to astrocytes. Contradicting this observation was the presence of nestin in differentiated cells which under normal conditions is downregulated on differentiation. *Kif3a* KO cells subjected to neuron differentiation media was tested using different neuron specific markers such as NueN and CNPase to confirm their identity. But the immunostaining with these antibodies gave inconclusive results (data not shown). Induction of oligodendrocyte differentiation yielded cells which showed the presence of an oligodendrocyte specific marker, Olig2 whereas the characteristic morphology of oligodendrocytes was not seen in the WT as well as the *Kif3a* KO lines. These results do not provide concrete evidence as to the role of cilia in differentiation. Further studies on differentiated cells with specific markers could help identify a possible role for cilia in neural stem cell differentiation.


A1.astro. Kif3a KO
DAPI, Olig2, Nestin
B1.astro. Kif3a WT
DAPI, Olig2, Nestin
C1.astro. Kif3a HET
DAPI, Olig2, Nestin
A2 Kif3a KO
Nestin
B2 Kif3a WT
Nestin
C2 Kif3a HET
Nestin
D1.neuron Kif3a KO
DAPI, Olig2, Nestin
D2 Kif3a KO
DAPI, Olig2, Nestin
E1.neuron Kif3a WT
DAPI, Olig2, Nestin
E2 Kif3a WT
DAPI, Olig2, Nestin
F1.oligodend. Kif3a KO
DAPI, Olig2, Nestin
G1.oligodend. Kif3a WT
DAPI, Olig2, Nestin
H1.oligodend. Kif3a HET
DAPI, Olig2, Nestin

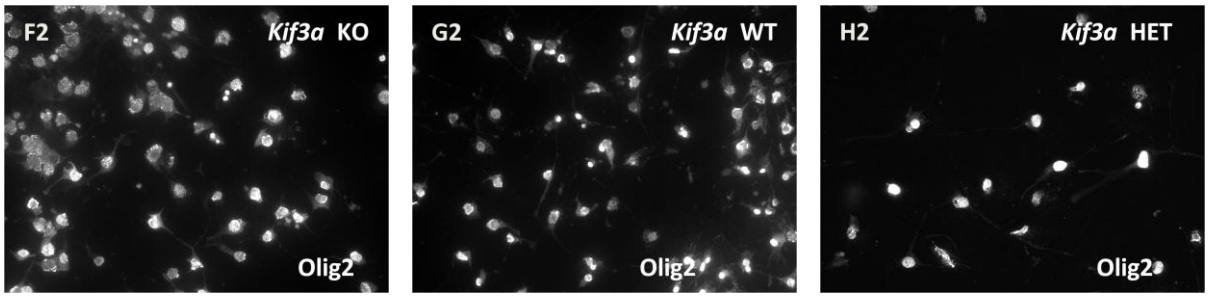


Figure 16. Immunostaining of differentiated RCAS-*Kif3a* cells with varying phenotypes against Nestin and OLIG2 markers for astrocytes, neurons and oligodendrocytes, at 20X magnification.

5. Discussion and Future perspectives

N-Myc is a transcription factor and its direct targets are known to be involved in many growth promoting processes including protein synthesis machinery (Boon *et al.* 2001). FBW7 is a SCF-type ubiquitin ligase and N-Myc has been identified to be one of its substrates. In this study, mutant lines of *Fbw7* were analyzed and compared with tumor cell lines to test the hypothesis if the increase in *MYCN* levels caused by loss of FBW7 function could lead to tumors. To confirm this hypothesis, N-Myc expression levels between WT and *Fbw7* cell lines were compared to reveal no significant change in expression levels. This indicates that the loss of the machinery needed for N-Myc degradation is not sufficient for increasing its levels. This contradicts our hypothesis and hints at the possibility of N-Myc expression being affected at the transcriptional level in tumor cells. Recent studies have shown that loss in cilia plays a role in tumorigenesis.

Dysregulation of notch signaling has been reported in a number of cancer types. Immunostaining against activated notch-1 proteins in *Fbw7* and wild type cell lines showed that in the knockout lines, localization of activated notch1 was inter-nuclear. Activation of notch receptor proteins happens through the cleavage of the protein which leads to the intercellular domain getting localized in the nucleus. The activated notch binds to a transcription factor, CSL (i.e. CBF1, Suppressor of Hairless, Lag-1) and controls the expression of a number of genes (Hamidi *et al.* 2011). Over-expression of activated notch, in other words excessive activation of the notch signaling pathway is known to cause the formation of tumors. The increased activation of notch1 in Fbw7 mutants indicate that FBW7 might be involved in controlling the notch signaling to a proper level so that there is no formation of cancer. Studies indicate that FBW7 SCF ubiquitin ligase mediated degradation of notch-1 is required for maintenance of stem cell characteristics (Matsumato *et al.* 2011). Hence in the *Fbw7* lines, the presence of increased activated notch levels could be a direct consequence of the absence of functional Fbw7 protein.

Immunostaining against acetylated tubulin and TUJ-1 showed the *Fbw7* lines retain their neuronal identity. Immunostaining of the tumor cell lines revealed an increased expression of N-Myc which overlapped with an increased expression of Aurora-A. Aurora-A is known to bind N-Myc and prevent its degradation by FBW7 (Otto *et al.* 2009). Western blot studies on *Fbw7* mutant lines showed that there was an increased expression of Aurora-A (data not shown). Lack of N-Myc overexpression in *Fbw7* mutant lines indicate that the presence of tumor like characteristics do not resemble those of type 3 MBs suggesting a role for Aurora-A in tumorigenesis independent of N-Myc. Another function proposed for Aurora-A is that it represses cilia formation during cell cycle (Goto *et al.* 2013). The increased expression of Aurora-A in the *Fbw7* mutant lines would support this function and indicate that tumor progression in *Fbw7* mutants happens through this pathway.

Phosphorylation of Aurora-A at Thr288 is known to increase its kinase activity. Hence immunostaining with antibodies against phospho-aurora-A would indicate if there is an increased kinase activity in the *Fbw7* mutant lines compared to the WT. In this study, successful immunostaining with phospho-aurora-A antibodies was not achieved. Further optimizations are needed to visualize the phosphorylated forms of aurora-A protein.

Kif3a genes are known to be involved in the formation of cilia hence knockout lines of *Kif* were produced via an RCAS-TVA infection system and analyzed to identify the presence of tumor-like characteristics. Immunoblotting on the *Kif3a* cell lines indicated a higher level of Aurora-A

in the *Kif3a* mutants as compared to the wild type, as similarly seen in tumor cell cultures. This reaffirmed the role of Aurora-A in the pathway involving ciliary loss and tumor formation. Further quantitative studies with N-Myc and pT288 Aurora-A are required to demonstrate a correlation of *MYCN* stabilization simultaneously with this hypothesis.

Studies have shown cilia to have several functions in stem cell development and differentiation. It was important to test if the hypothesis works when reversed i.e. when the ciliary genes were knocked out and the cell cultures were provided with differentiation conditions. Immunostaining showed that differentiation of the mutant *Kif3a* cell lines gave rise to cells having the astrocyte like structures, yet at the same time indicating the presence of nestin. Typically nestin is observed to disappear during cell differentiation (Matsuda *et al.* 2013). This contradicts the accuracy in the differentiation of the *Kif3a* mutant cell lines to astrocytes. Immunostaining for neurons and oligodendrocytes did not give typical cell morphology. The lack of differentiated cell phenotypes indicated the requirement of further studies with improved markers against differentiated cells.

Brockmann *et al.* have indicated that targeting Aurora-A inhibitors has an effect on the kinase activity, and *MYCN* gene expression. It would be beneficial to conduct proliferation studies using these Aurora-A inhibitors to see the difference in cilia expression, and its correlation with N-Myc activity. Currently the antibodies used do not aid microscopic ciliary visibility. Optimization of *Arl13b* or a similar antibody which could aid viewing and measuring cilia length would enable accurate comparison studies between the various MB tumor types in culture. Immunostaining and immunoblotting experiments on human tumors are required as well to examine against the murine tumor cultures. Quantitative analysis of tissue cultures via real-time PCR and gene expression profiling could aid in having more insight into the ciliary role in neural stem cell differentiation. It would be interesting to correlate the ciliary role of Aurora-A with the *Fbw7*-N-Myc substrate activity.

Acknowledgements

I would like to express my gratitude to my supervisor Prof. Fredrik Swartling who gave me the opportunity to be a part of his group and for giving me valuable feedback always.

I am immensely grateful to Sanna Hede for her patience, for teaching me everything that I could possibly learn in the time spent. I will always be thankful to her for always finding time to answer my questions and helping me finish my thesis successfully.

I would also like to thank other members of the group and the NeuroOncology department for being there whenever I required help.

I would like to thank my family for their endless support and encouragement always indispensable in my endeavors and studies.

Last but not the least I would like to thank Uppsala University for making my dream to study here a reality.

References

Araki K, Imaizumi T, Okuyama K, Oike Y, Yamamura KI. (1997) "Efficiency of recombination by Cre transient expression in Embryonic stem cells: comparison of various promoters." *Journal of Biochem.* 122:977-982

Barakat MT, Humke EW, Scott MP. (2013) "Kif3a is necessary for initiation and maintenance of medulloblastoma." *Carcinogenesis* 34.6:1382-1392.

Boon K, Caron HN, Asperen R, Valentijn L, Hermus M-C, Sluis P, Roobeck I, Weis I, Voute PA, Schwab M, Versteg R. (2001) "N-myc enhances the expression of a large set of genes functioning in ribosome biogenesis and protein synthesis" *EMBO Journal* 20,6:1383-1393.

Brockmann M, Poon E, Berry T, Carstensen A, Deubzer HE, Rycak L, Jamin Y, Thway K, Robinson SP, Roels F, Witt O, Fischer M, Chesler L, Eilers M. (2013) "Small molecule inhibitors of Aurora-A induce proteosomal degradation of N-Myc in childhood neuroblastoma" *Cancer Cell* 24:75-89

Fisher GH, Orsulic S, Holland E, Hively WP, LI y, Lewis BC, Williams BO, Varmus HE. (1999) "Development of a flexible and specific gene delivery system for production of murine tumor models." *Oncogene* 18: 5253-5260.

Gossen M, Bujard H. (1992) "Tight control of gene expression in mammalian cells by tetracycline-responsive promoters" *PNAS* 89:5547-5551.

Goto H, Inoko A, Inagaki M. (2013) "Cell cycle progression by the repression of primary cilia formation in proliferating cells." *Cellular and Molecular Life Sciences* 70:3893-3905.

Hamidi H, Gustafason D, Pellegrini M, Gasson J. (2011) "Identification of novel targets of CSL-dependent notch signaling in Hematopoeisis" *PLOS one* 6:1-19.

Han YG, Kim HJ, Dlugosz AA, Ellison DW, Gilbertson RJ, Alvarez-Buylla A. (2009) "Dual and opposing roles of primary cilia in medulloblastoma development." *Nature Medicine* 15:1062-1066.

Han YG, Spassky N, Romaguera-Ros M, Garcia-Verdugo JM, Aguilar A, Schneider-Maunoury S, Alvarez-Buylla A. (2008) "Hedgehog signaling and primary cilia are required for the formation of adult neural stem cells" *Nature Neuroscience* 11.3:277-284.

Kool M, Koster J, Bunt J, Hasselt NE, Lakeman A, Sluis PV, Troost D, Meeteren NS-V, Caron HN, Cloos J, Mrsic A, Ylstra B, Grajkowska W, Hartmann W, Pietsch T, Ellison D, Clifford SC, Versteeg R. (2008) "Integrated genomics identifies five medulloblastoma subtypes with distinct genetic profiles, pathway signatures and clinicopathological features." *PLOS One* 3.3088:1-14.

Matsuda Y, Yoshimura H, Suzuki H, Ishiwata T. (2013) "Nestin: Neural Stem/Progenitor Cell Marker in Brain Tumors." Evolution of the molecular biology of brain tumors and the therapeutic implications, *Intech open* 23:623-638.

Matsumato A, Onoyama I, Sunabori T, Kageyama R, Okano H, Nakayama K. (2011) "Fbxw7-dependent degradation of notch is required for control of stemness and neuronal-glial differentiation in stem cells" *Journal of Biological chemistry* 286.15:13754-13764.

Otto T, Horn S, Brockmann M, Schuttrumpf L, Popov N, Kenney AM, Schulte JH, Beijersbergen R, Christiansen H, Berwanger B, Eilers M. (2009) "Stabilization of N-Myc is a critical function of Aurora A in human neuroblastoma" *Cancer Cell* 15:67-78.

Pugacheva EN, Jablonski SA, Hartman TR, Henske EP, Golemis EA. (2007) "HEF1-Dependent Aurora A activation induces disassembly of the primary cilium." *Cell* 129:1351-1363.

Spassky N, Han YG, Aguilar A, Strehl L, Besse L, Laclef C, Ros MR, Garcia-Verdugo JM, Alvarez-Buylla A. (2008) "Primary cilia are required for cerebellar development and Shh-dependent expansion of progenitor pool" *Developmental Biology* 317:246-259.

Swartling FJ, Grimmer MR, Hackett CS, Northcott PA, Fan Q-W, Goldenberg DD, Lau J, Masic S, Ngyunen K, Yakovenko S, Jenkins RB, Tihan T, Vandenberg SR, James CD, Tanaka K, Taylor MD, Weiss WA, Chessler L. (2010) "Pleiotropic role for *MYCN* in medulloblastoma." *Genes and Development* 24:1059-1072.

Swartling FJ, Savov V, Persson, AI, Chen J, Hackett CS, Northcott PA, Grimmer MR, Lau J, Chesler L, Perry A, Phillips JJ, Taylor MD, Weiss WA. (2012) "Distinct neural stem cell populations give rise to disparate brain tumors in response to N-Myc" *Cancer Cell* 21.5:601-613

Taylor MD, Northcott PA, Korshunov A, Remke M, Cho YJ, Clifford SC, Eberhart CG, Parsons DW, Rutkowski S, Gajjar A, Ellison DW, Lichter P, Gilbertson RJ, Pomeroy SL, Kool M, Pfister SM. (2011) " Molecular subgroups of Medulloblastoma: the current consensus." *Acta Neuropathology* 123:463-472.

Weineberg RA, "The Biology of Cancer", 2nd edition. (2007) *Garland Science.*

Wenzel A, Czeipluch C, Hamann U, Schurmann J, Schwab M. (1991) "The N-Myc oncoprotein is associated *in vivo* with the phosphoprotein Max (p20/22) in human neuroblastoma cells." *EMBO Journal* 10.12:3703-3712.

Werder A, Seidler B, Schmid RM, Schneider G, Saur D. (2012) "Production of avian retroviruses and tissue-specific somatic retroviral gene transfer *in vivo* using the RCAS/TVA system" *Nature Protocol* 7:1167-1183.