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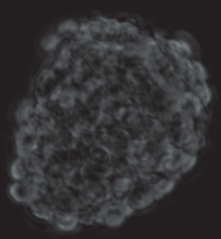

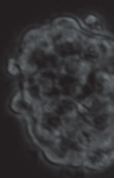

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Isabelle V. Leefa Chong San

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Abstract

Adult neural stem/progenitor cells (NSPs) are multipotent and self-renew over an extensive period of time. Transformation events in such cells, such as genetic or epigenetic alterations can result in brain tumors. One goal of research on brain tumors is to identify which regulators are impaired during tumor initiation, development and maintenance in order to understand the origin of brain tumors and the resistance displayed by tumor cells towards apoptosis, radiation or other aggression. This might ultimately help to target the latter to make them vulnerable to drug treatment. Adult neural stem/progenitor cells therefore represent an essential tool for understanding brain cancer mechanisms.

Article 1 presents our findings as to the role of PODXL in NSPs. Using a gain-of-function approach we investigated the *in vitro* and *in vivo* effects of PODXL in NSPs. We found *Podxl*-overexpressing NSPs to have a higher viable and lower apoptotic cell fraction than control NSPs. We identified concomitant overexpression of ANXA2 at both transcript and protein levels and showed that absence of *Anxa2* in *Podxl*-overexpressing cells reduced cell viability and increases apoptosis. We therefore propose a molecular link between PODXL and ANXA2 where both exert pro-survival effects in NSPs and overexpression of *Podxl* activates the MAP kinase (MAPK) pathway which in turn up-regulates *Anxa2* expression.

In Article 2, we show a mechanism of action of the transcription factor BMI-1, which is up-regulated in several tumors, including brain tumors. We found increased NSP cell proliferation and self-renewal upon *Bmi1* overexpression and identified the tumor-suppressor *EphA7* as a novel target of BMI-1. Absence of *Bmi1* led to de-repression of *EphA7 in vitro*. *In vivo*, while *Bmi1*$^{-/-}$ mice showed severe depletion of dividing cells (neuroblasts and neural progenitor cells), we found that *Bmi1*$^{-/-}$*EphA7*$^{-/-}$ mice showed a partial rescue of their proliferative potential in the dorsolateral corner of the anterior lateral ventricular wall. Lastly, by bisulfite sequencing analysis we demonstrated that silencing of *EphA7* by BMI-1 was accompanied by DNA methylation of the promoter region of *EphA7*.

Article 3 demonstrates our loss-of-function approach with the aims of developing a mouse model of atypical teratoid/rhabdoid tumor (AT/RT) and understanding the role of *Snf5* in brain development. By using the Cre-Lox system, we ablated *Snf5* in Nestin-expressing cells. *Snf5*$^{F/+}$*Nestin-Cre*$^{-}$ animals were normal, compared to *Snf5*$^{F/+}$*Nestin-Cre*$^{+}$ mice that displayed an immature brain with reduced cell density in the cortex and hippocampus. While this model has not shown any sign of AT/RT yet, it represents a starting point in elucidating the function of *Snf5* in early brain development.

In summary, we have reported the involvement of three regulators of NSP behavior in the context of brain tumors. We attempted to elucidate their mechanistic functions in tumor maintenance, tumor-suppressor silencing and brain tumor development.

Key words: neural stem/progenitor cells, regulators, brain tumor, cell of origin, cancer mechanisms

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To Mum, Dad, Mel and Yann

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Abbreviations

AML	Acute myeloid leukemia
ANXA2	Annexin A2
AP-1	Activator protein 1
ATP	Adenosine triphosphate
AT/RT	Atypical teratoid/rhabdoid tumor
BMI-1	B cell-specific Moloney murine leukemia virus integration site 1
CA	Cornu ammonis
CD	Cluster of differentiation
CDK	Cyclin-dependent kinase
ChIP	Chromatin immunoprecipitation
CSS	Coffin-Siris syndrome
DG	Dentate gyrus
DNMT	DNA methyltransferase
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
EPHA7	Ephrin A7
E14.5	Embryonic day 14.5
FGF2	Basic fibroblast growth factor
GFP	Green fluorescent protein
HSC	Hematopoietic stem cell
ICAM-1	Intercellular adhesion molecule 1
KD	Knock-down
KO	Knock-out
LC-MS	Liquid chromatography-mass spectrometry

LV	Lateral ventricle
LVW	Lateral ventricular wall
MAPK	Mitogen-activated protein kinase
MDM2	Mouse double minute 2 (E3 ubiquitin-protein ligase)
MEF	Mouse embryonic fibroblast
MOI	Multiplicity of infection
MRT	Malignant rhabdoid tumor
NFκB	Nuclear factor kappa beta
NHERF1/2	Na^+/H^+ exchange regulatory factor 1/2
NSC	Neural stem cell
NSP	Neural stem/progenitor cell
OB	Olfactory bulb
PcG	Polycomb group
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PNET	Primitive neuroectodermal tumor
PODXL	Podocalyxin
PRC	Polycomb repressive complex
PTEN	Phosphatase and tensin homolog
RMS	Rostral migratory stream
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEZ	Sub-ependymal zone
SGL	Sub-granular layer
SGZ	Sub-granular zone
SHH	Sonic hedgehog
SMARCB1	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily b, member 1 (SNF5)
SVZ	Sub-ventricular zone
SWI/SNF	Switch/sucrose non-fermenting

List of articles included in the thesis

Article 1:
High Podocalyxin levels promote cell viability partially through up-regulation of Annexin A2
Isabelle V. Leefa Chong San, Gaëlle Prost, Jennifer A. Williams, Stephen E. Moss, Ulrike A. Nuber
BBRC (2016), 478(2): 573-579

Article 2:
The putative tumor suppressor gene *EphA7* is a novel BMI-1 target
Gaëlle Prost, Sebastian Braun, Falk Hertwig, Marcus Winkler, Lucas Jagemann, Sara Nolbrant, Isabelle V. Leefa, Nils Offen, Kenichi Miharada, Stefan Lang, Isabella Artner, Ulrike A. Nuber
Oncotarget (2016), Aug 13. doi: 10.18632/oncotarget.11279. [Epub ahead of print]

Article 3:
Monoallelic deletion of *Snf5* exon 1 in Nestin-expressing cells results in a severe developmental brain phenotype
Nanni Schmitt, Alina Filatova, Isabelle V. Leefa Chong San, Ulrike A. Nuber
Manuscript 2016

Summary

Adult neural stem/progenitor cells are multipotent, that is, they can generate different cells of the neural and glial lineage. Additionally, they show long-term self-renewal capacity and hence are liable to accumulate mutations. These properties are relevant to brain cancer because studies on mouse have indicated that many forms of brain tumors result from dysregulation of the intrinsic properties of adult neural stem cells *in vivo*. Adult neural stem cells are therefore a necessary tool for understanding cancer mechanisms.

In study 1, we investigated the role of the protein PODXL in neural stem/progenitor cells and found that *Podxl* overexpression leads to increased cell number and to increased capacity to form spheres. We also found that such cells show a higher viability and lower rate of programmed cell death, compared to control cells. By analysis of the protein content of neural/progenitor stem cells made to overexpress *Podxl*, we identified another protein, ANXA2 to be highly expressed. High gene expression levels of *Podxl* led to high *Anxa2* transcript and protein levels, and similarly reduction in *PODXL* led to lower *ANXA2* transcript and protein levels, showing that PODXL regulates ANXA2. Removal of *Anxa2* in our cells that were already expressing *Podxl* resulted in lower cell viability, suggesting that PODXL effects on cell viability might be partially explained by its regulation of ANXA2. Next, we wanted to find out how PODXL is linked to ANXA2, which could explain the mechanism of action of PODXL. We found that PODXL regulates ANXA2 through MEK1/2 of the MAPK pathway and AP-1.

In study 2, we found that high expression of *Bmi1* leads to increased proliferation of neural stem/progenitor cells *in vitro*. BMI-1 was also found to bind directly to the *EphA7* locus. We thus proposed that *EphA7* is a novel target of BMI-1. Concomitantly, we observed a decrease in *EphA7* expression upon *Bmi1* overexpression *in vitro*. Mice lacking *Bmi1* had less proliferative cells in a postnatal stem cell niche of their brain. Removal of *EphA7* in such mice resulted in a partial restoration of the number of these proliferative cells. Conversely, NSPs isolated from embryos lacking *Bmi1* showed an up-regulation of *EphA7*. Therefore BMI-1 represses *EphA7*. We then established—through analysis of the DNA methylation status of the regulatory region of *EphA7* and the presence of the repressive mark H3K27me3—a possible mechanism through which BMI-1 might lead to repression of *EphA7*.

In study 3, we set out to both understand the function of *Snf5* during brain development and to develop a mouse model of the childhood brain tumor AT/RT. AT/RT is characterized by inactivation of the gene *SNF5*. The World Health Organization classifies AT/RT as an embryonal grade IV neoplasm. AT/RT is often misdiagnosed for other pediatric brain tumors because of overlapping histologic features. The cell of origin of AT/RT is unknown. The rhabdoid tumor cells being histologically located next to areas of primitive neuroectodermal, epithelial or mesenchymal tissue, AT/RT is thought to originate from a primitive cell [1-3]. We therefore deleted *Snf5* specifically in mouse brain cells expressing Nestin, a neural stem/progenitor marker. Even though our model has not developed any tumor so far, having studied it for a limited period of time, our study indicates the essential role of *Snf5* in brain development, as shown by the immature brain, disconnected hemispheres and a missing corpus callosum.

Sammanfattning

Adulta neuronala stamceller är mutipotenta, vilket de kan generera olika celler av neurala och gliala härstamning. Stamcellerna har även förmågan till långsiktig självförnyelse, vilket i sin tur innebär att de har en benägenhet att samla på sig mutationer. Dessa egenskaper är relevanta i studier av hjärncancer, då vetenskapliga försök på möss visar att många hjärntumörer uppkommer då hjärnans stamceller förlorar förmågan att reglera sina inre egenskaper. Hjärnstamceller är således ett nödvändigt verktyg i forskningen på cancer och dess mekanismer.

I studie 1 har jag undersökt ett protein som heter PODXL och dess uttryck i hjärnstamcellerna. Jag har visat att ett för högt uttryck, s.k. överuttryck, av *Podxl* leder till ett ökat cellantal och oförmåga att bilda neuronala sfärer. Mina resultat visar även att dessa celler har en högre livskraft och lägre benägenhet till förprogrammerad celldöd, jämfört med normala hjärnstamceller. Genom att analysera proteininnehållet i de hjärnstamceller som överuttrycker *Podxl*, lyckades jag att identifiera ytterligare ett protein, ANXA2, som uttrycktes i för höga mängder. Höga nivåer av *Podxl* leder alltså till höga nivåer av *Anxa2*. På samma sätt leder låga *PODXL*-nivåer till en lägre *ANXA2*-nivåer, vilket visar att PODXL reglerar uttrycket av ANXA2. Vidare har jag kunnat visa att borttagning av *Anxa2* i de celler som uttrycket *Podxl* resulterar i lägre livskraft för cellerna, vilket innebär att de effekter som PODXL har på livskraft delvis kan förklaras av dess reglering av ANXA2. Jag hade därefter som mål att förstå de bakomliggande mekanismerna i PODXLs koppling till ANXA2 och fann då att PODXL reglerar ANXA2 genom MEK1/2 av MAPK signalvägen och AP-1.

I studie 2 fann jag att höga nivåer av proteinet *Bmi1* leder till ökad celldelning av hjärnstamcellerna in vivo. BMI-1 binder också direkt till *EphA7*-locuset. Min hypotes var därför att *EphA7* interagerar med BMI-1. Reultaten visade att *EphA7*-nivåerna sjunker när *Bmi1* överuttrycks *in vitro*. Möss som saknade *Bmi1*-genen hade lägre celldelning i specifika delar av hjärnan efter födseln. Borttagning av *EphA7* i dessa möss resulterade i delvist återställande av celldelningen. NSPs som isolerats från musfoster som saknar *Bmi1* genen visar på en uppreglering av *EphA7*. Slutsatsen är därmed att BMI-1 undertrycker uttrycker av *EphA7*. Genom att analysera DNA-metyleringsstatusen på den regulatoriska regionen av *EphA7* och observera närvaro av den blockerande markören H3K27me3, fastställde vi en möjlig mekanism genom vilken BMI-1 kan blockera uttrycket av *EphA7*.

I studie 3 avsåg vi att både förstå funktionen av *Snf5* under hjärnutvecklingen och utveckla en musmodell för barndomshjärntumören AT/RT. AT/RT karaktäriseras av inaktivering av genen *SNF5*. Världshälsoorganisationen (WHO) klassifierar AT/RT som en s.k. embryonal nivå IV neoplasm. AT/RT feldiagnostiseras ofta och misstas för andra pediatriska hjärntumörer p.g.a. liknande histologiska egenskaper. Ursprungscellen för AT/RT är dock okänd. De rhabdoida tumörcellerna är histologiskt lokaliserade nära områden för primitivt neuroektoderm, epithelial eller mesenkymal vävnad, varför AT/RT tros uppkomma från en primitive celltyp [1-3] . Med bakgrund av detta, tog vi bort *Snf5*-genen specifikt i hjärnceller som uttrycker proteinet Nestin, en markör för hjärnstamceller. Trots att vår musmodell ännu inte har utvecklar en tumör och att jag enbart har studerat modellen under en begränsad tidsperiod, visar studien på en essentiell roll för *Snf5* i hjärnans utveckling vilket uttrycker sig i form av en outvecklad hjärna, åtskilda hjärnhemisfärer och avsaknad av corpus callosum.

1.0. Introduction

1.1. The adult neural stem cell niche

The initial discovery of adult mammalian neurogenesis was in 1965 by Altman and Das [4]. Injection of H3-thymidine in rat brains labeled granule cells of the dendate gyrus (DG) and ependymal layers of the lateral ventricles. These cells were found to decline from birth and with age, corresponding to a rise in the number of differentiated cells. That neurogenesis occurs in the adult rat DG was confirmed by Kaplan and Hinds [5] who also observed the process in the olfactory bulb (OB) of rats. Adult neurogenesis was later shown in other species by Goldman and Nottebohm [6] who also introduced the concept of activity-dependent regulation of adult regeneration in female songbirds. The H3-thymidine-labeled cells in the ventricular zone were referred to as proliferative neuroblasts. Another basis for adult hippocampal neurogenesis was reported to balance stress-induced cell death [7]. However, despite showing through tritiated thymidine labeling that new neurons were generated in the adult brain, the source of such cells was not defined, given that neurons themselves were known not to divide. Altman hypothesized that precursor cells might exist in the brain. Prior to this, Wilhelm His in 1889, Ramon y Cajal and Camillo Golgi described germinal cells in the developing nervous system of salmons as the precursors to neuroblasts and nerve cells (reviewed in [8]).

The idea of adult (and mammalian) neurogenesis was accepted with the discovery of neural stem cells in the adult mouse brain [9, 10]. Neurogenesis became the process through which neural stem cells or neural progenitor cells generate new neurons. Stem cells, at the top of the neural potency hierarchy, are cells that have the ability to self-renew continuously and differentiate into specialized cell types. Progenitor cells, on the other hand, are proliferative but have a limited capacity for self-renewal. Precursor cells refer to the undifferentiated progeny of stem cells and include both stem cells (daughter cells of the mother stem cells) and progenitor cells.

A population (less than 0.1%) of neural precursors was isolated from the mouse adult brain and could be cultured *in vitro* in the presence of mitogenic factors such as basic fibroblast growth factor and/or epidermal growth factor (making them replication-competent), and could retain their potential to differentiate into neurons and glial cells [9].

Neural progenitor cells in the DG of the adult monkey brain were later shown to migrate via a homologue rostral migratory stream (RMS) and neurogenesis to occur in the OB [11, 12]. Dividing progenitor cells in the human DG and human neurogenesis were finally demonstrated in 1998 by Eriksson *et al.* through the use of BrdU and neuronal markers such as neuron-specific enolase (NSE), calbindin or NeuN [13]. Today, two established niches where mammalian adult neurogenesis occurs under normal physiological conditions are:

1. The sub-granular zone (SGZ) in the DG within the hippocampus.
2. The sub-ependymal zone (SEZ) of the lateral ventricles (LV).

In the mouse brain, radial cells in the SGZ become neuroblasts that either migrate into the adjacent DG granule cell layer or differentiate into excitatory neurons that integrate into the hippocampal network.

Neurons arising in the SEZ migrate along the RMS [14] to reach the OB where they terminally differentiate into interneurons (Figure 1). The polysialylated form of NCAM is required for this tangential migration along the RMS [15].

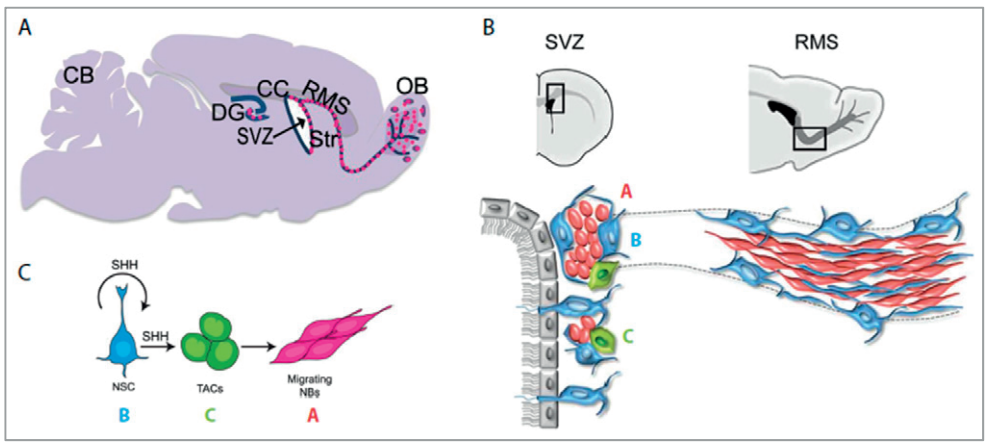

Figure 1. Neural stem cells in the adult mouse brain. A. Sagittal section of murine brain showing the two niches of neurogenesis, the sub-ventricular zone (SVZ) and dentate gyrus (DG). **B.** Migrating neuroblasts (type A cells) ensheathed by astrocytes (type B cells) form chains along the rostral migratory stream (RMS) on their way to the olfactory bulb (OB). **C.** Lineage relationship of SVZ cell types. Modified from [16-18].

Doetsch *et al.* [19] identified astrocytes (type B cells) in the SVZ as the putative neural stem cells in the mouse adult brain. Four cell types make up the SVZ (Table 1):

1. **Ependymal or type E cells** – these cells are ciliated and line the LVs, causing rostral flow of the cerebrospinal fluid. Type E cells interact closely with type B cells. Though they have been found to be Notch-dependent quiescent under normal conditions in the adult mouse [19, 20], ependymal cells show dramatic proliferation in response to stroke to give rise to neuroblasts and astrocytes [20].
2. **Astrocytes or type B cells** – slow-proliferating or quiescent cells expressing astroglial markers such as GFAP. These are considered to represent the endogenous neural stem cells of the brain [19]. Type B cells are in physical contact with rapidly-dividing type A and type C cells. If activated, type B cells divide to generate type C cells that undergo a few rounds of divisions to generate type A cells.
3. **Transit-amplifying progenitor (TAP) or type C** cells are immature cells that are rapidly dividing to generate type A cells.
4. **Neuroblasts or type A cells** – these cells migrate as chains along the RMS by microtubule polymerization/depolymerization. Type A cells are ensheathed by type B cells.

Table 1. Cellular composition of the SVZ as a neural stem cell niche. Table shows marker expression of each cell type and relative abundance according to Doetsch *et al.* [21].

Abundance	SVZ composition	Expression
	Type A	Dcx, PSA-NCAM, Tuj1, Nestin
	Type B, Type E	Vim, GFAP, Nestin
	Type C	Mash1, Nestin, Dlx2

SVZ NSCs respond to EGF and grow as neurospheres *in vitro* [9]. While it was initially reported that the quiescent type B cells give rise to spheres *in vitro* [22], Doestch *et al.* showed that most of the cells that responded to EGF exposure *in vivo* and *in vitro* were actually the actively-dividing type C cells [23]. Hence by forming neurospheres that are multipotent and self-renewing, type C cells retain stem cell competence when under EGF influence.

1.2. Hallmarks of cancer

Cancer is an accumulation of mutations, giving cancer cells a growth advantage over normal cells. Even though each tumor is unique, there exist common traits that all cancer cells share, to facilitate transformation from a normal cell to a cancer cell (and to distinguish a normal from a cancer cell). These traits, referred to as the "Hallmarks of Cancer", were first described by Hanahan and Weinberg in 2000 [24, 25]. The seminal article described common alterations to the cellular processes that must occur before cancer can develop, making tumorigenesis a multi-step process.

Self-sustaining proliferation

Mutations that drive uncontrolled cell cycle progression are requisite events during tumorigenesis. Unlike normal cells that show control over cell growth and division cycles, in cancer cells there is a loss of this control over production and release of growth-promoting signals. Cancer cells can produce their own growth factor ligands (e.g. TGFα, PDGF, TGFβ) and they can also overexpress the corresponding receptors (here, EGFR) on their cell surface [26], such autocrine secretion making them self-sufficient. Additionally, cancer cells have been found to stimulate normal stromal cells to provide the mitogenic factors that they need. Stromal cells (such as inflammatory cells, fibroblasts and endothelial cells) making up the major cellular component of the tumor microenvironment secrete extracellular matrix proteins, growth factors and angiogenic factors, hence aiding in the development and progression of carcinoma (see [27] for a review).

Following the activation of growth factor receptors, tumor cells typically show constitutive activation of signaling such as the MAPK and PI3K pathways. Such constant and over-amplified signaling would be counteracted in normal cells. For instance, PTEN phosphatase would degrade the products of the PI3K pathway. Cancer cells, however, show defects in such negative-feedback mechanisms, for example in the form of PTEN mutations and disrupted Ras GTPase activity.

Insensitivity to growth inhibitors

In healthy cells, tumor-suppressors limit aberrant cell growth and proliferation. Tumor- suppressors can act as caretakers or gatekeepers. **Caretakers**, in the form

of proto-oncogenes (e.g. *P53*) or true (more directly involved in DNA repair) caretaker genes (e.g. *MLH1*, *MSH2*), act through prevention by detecting DNA damage, repairing it and hence preventing or allowing cell-cycle progression, with the aim to prevent proliferation of damaged cells. **Gatekeepers** (e.g. Retinoblastoma protein (Rb) and p53) promote cell death of mutated or stressed cells or senescence of differentiated cells. The majority of human cancers feature loss of p53 function. Additional changes in tumor cells, for instance mutations in the Rb pathway, stabilize mutant p53 to promote tumorigenesis [28], further showing that cancer is indeed an interplay of several dysfunctional molecules or pathways.

Cancer cells also show loss of contact inhibition. As an example, *NF2* which encodes the tumor-suppressor Merlin is inactivated in some forms of tumor, such as neurofibromatosis [29]. In wild type cells, Merlin acts by sequestering cell surface adhesion molecules to receptor tyrosine kinases such as EGFR, thereby preventing constitutive activation of EGFR, hence limiting proliferation [30].

Resistance to apoptosis

Acquired resistance to apoptosis is common to nearly all types of cancers. In order to survive, grow and divide, a cancer cell must hijack normal cellular growth pathways (see above) and evade cellular death pathways.

Apoptosis is a process of controlled cell death. The apoptotic machinery is made up of regulators/sensors and effectors. Regulators monitor the internal and external environment of the cell for any abnormal issues (DNA damage, activation of oncogenes etc). Regulators of apoptosis include anti-apoptotic (e.g. BCL-2, BCL-x_L) and pro-apoptotic proteins (e.g. BAX, BAK, BIM).

Sensors monitor the intra- and extracellular environment. Extracellular signals are sent to effectors of the apoptotic machinery represented by cell surface receptors that bind survival or death factors. For instance, external stimuli go through the FAS ligand/FAS receptor, or the TNFα-TNFR1, activating pro-caspase 8 and subsequent caspase cascade (Figure 2). Intracellular signals such as DNA damage or increased oncogene signaling are received by the BH3-only proteins (pro-apoptotic subgroup of the BCL-2 family), leading to BAX and BAK protein activation, permeabilization of the mitochondrial outer membrane and release of cytochrome C. This cytochrome C release activates the caspase cascade resulting in apoptotic cell death (Figure 2).

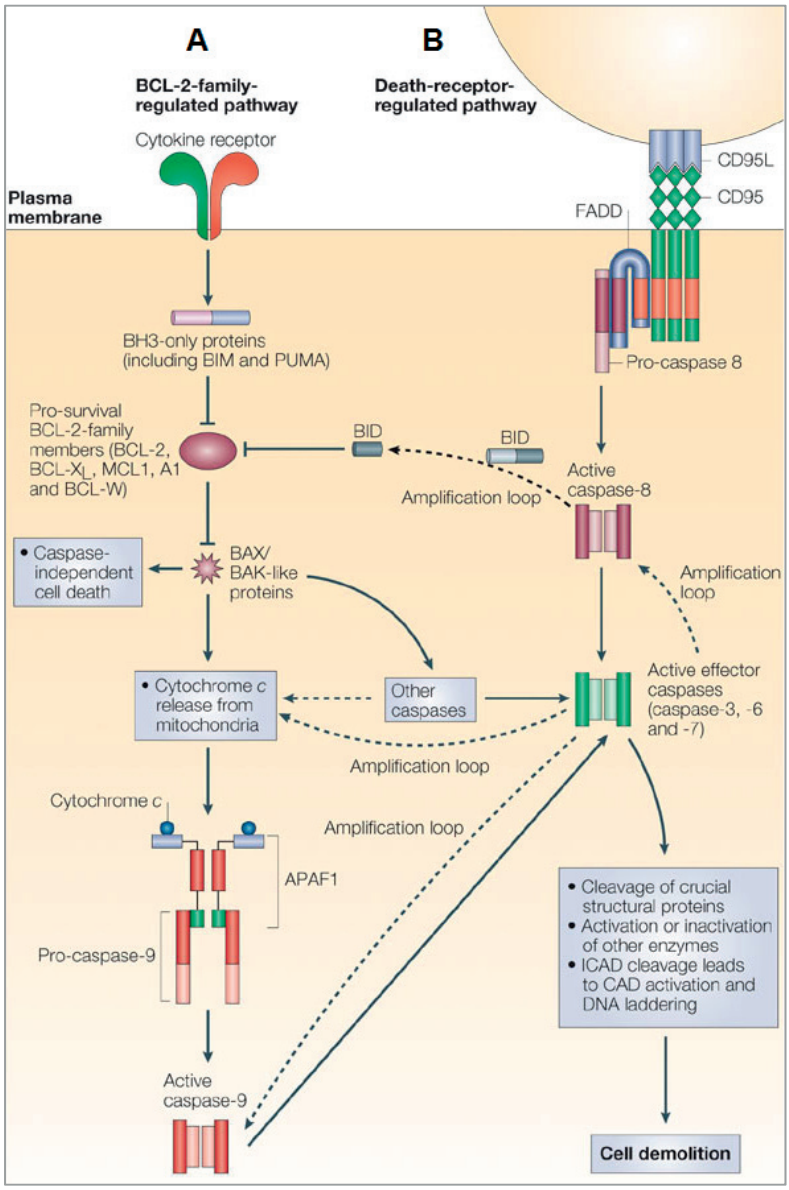

Figure 2. Induction of apoptosis. A. Intracellular or mitochondrial pathway. Mitochondria release cytochrome C that causes adaptor protein Apaf-1 to aggregate. Aggregated Apaf-1 then binds and aggregates pro-capase-9 molecules, leading to their cleavage and initiation of the caspase cascade. **B. Extrinsic activation of apoptosis.** Death ligands such as Fas-L (CD95L) bind and activate Fas (CD95) proteins on the target cell. Adaptor protein FADD binds to the intracellular region of aggregated Fas proteins, causing aggregation of pro-caspase 8 molecules. Aggregation causes cleavage and mutual activation of pro-caspase 8 into caspase-8, initiating the caspase cascade. (Figure taken from [31]).

Cancer cells acquire resistance to apoptosis through abnormal sensors such as the loss of the gatekeeper *p53* tumor-suppressor gene that functions through the BH3 DNA-damage sensor. Indeed, mutations in *p53* are a common feature of most human cancers.

Cancers also commonly show overexpression of the anti-apoptotic genes involved in the regulation of the mitochondrial pathway, such as *BCL-2*, *BCL-x_L*, or down-regulation of pro-apoptotic genes such as *BAX*.

Up-regulation of pathways, such as the PI3K/AKT/mTOR cascade, whose activation inhibit apoptosis and autophagy have been shown in several tumors [32].

Tumor-associated macrophages have also been shown to promote prostate cancer metastasis by production of anti-apoptotic IL-6 that recruits more macrophages to the tumor. The latter produces more TNFα which leads to IL-6 production, and hence through a positive feedback mechanism sustains its own resistance to apoptosis [33].

Immortalization

Replication of cells is limited by apoptosis (see above) and senescence. Healthy cells have a limited proliferative potential due to their shortening telomeres with each replicative cycle, a feature of cellular aging and oxidative stress [34, 35]. Past the apoptotic trigger, remaining surviving cancer cells, on the other hand, show unlimited proliferation resulting from the absence of telomere erosion. The chromosome ends of cancer cells are maintained by expression of telomerase (enzyme adding telomere repeats to the ends of telomere DNA). Thus, cancer cells with non-eroding telomeres become immortalized. Other non-canonical roles of telomerase are in enhanced cell proliferation, apoptotic resistance and DNA-damage repair (see [36] for a review), processes that are all implicated in cancer.

Induction of angiogenesis

Normally, adult vasculature is quiescent or transiently turned on (e.g. following injury). Ongoing angiogenesis is a common trait of tumors [37] that produce a highly disorganized and branched network of blood vessels to support their own growth. VEGF-A and FGF-1/2, two pro-angiogenic regulators, are up-regulated by oncogenic signaling (such as RAS and MYC) and hypoxia, two common conditions in cancers. Other angiogenic inducers include immune inflammatory molecules such as IL-2 and TGFβ [38].

Past the initial step of tumor development, a tumor eventually stimulates its own angiogenesis through the collective contribution of tumor cells and tumor-

associated stromal cells (tumor-associated fibroblasts and resident inflammatory cells) [39]. Tumor vasculature has also been shown to have a loose association of pericytes with the endothelium [40]. This reduced coverage allows escape of tumor cells towards dissemination.

Tissue invasion and metastasis

In order to establish secondary tumor growths—a process referred to as metastasis and responsible for most cancer deaths—a primary tumor has to intravasate, travel via the circulatory system and survive, extravasate and finally colonize other distant tissues/organs. Cancer cell invasion and metastatic spread occur through **(1) The extracellular matrix (ECM)**: Invasion is accompanied by physical changes to the metastatic cells with respect to each other and to the ECM. Invasive and metastatic tumors are therefore associated with loss or reduction of cell-cell adhesion molecules (e.g. E-cadherin) and up-regulation of cell-matrix proteins that are also normally associated with migrating cells (e.g. N-cadherin). **(2) Acquisition of cell motility and migration** by hijacking the epithelial-mesenchymal transition (EMT). EMT is normally an embryonic process. Transcription factors such as the SNAIL, TWIST and ZEB families regulate the changes in gene expression that contribute to repression of the epithelial phenotype and activation of the mesenchymal phenotype [41]. The EMT process leads to loss of E-cadherin, destabilization of adherens junctions, dissolution of tight junctions, change in cell shape, expression of matrix-degrading enzymes such as matrix metalloproteases and migration into surrounding tissues.

Altered energy metabolism

Anaerobic glycolysis is a short and temporary means of energy production used by healthy cells when under limited oxygen supply. Even when in the presence of sufficient oxygen levels, cancer cells utilize glycolysis as an energy supply ("Warburg effect" [42]). Since this process of *aerobic* glycolysis yields only 2 molecules of ATP, compared to the 32 molecules produced if the cancer cells were using normal cellular respiration, tumor cells compensate for this through up-regulation of glucose transporter GLUT-1. This import of glucose into the cytoplasm provides for the high amount of glucose consumed by tumor cells. What might first seem an additional effort or strain for the cancer cells is actually a tactic to not only obtain limited ATP and high glucose, but also to cater for other aspects of tumorigenesis. The hypoxic conditions under which many tumors thrive activate the hypoxia stress response mediated by HIF transcription factors. HIF-1α triggers up-regulation of genes encoding glycolytic enzymes (such as PGK1) and glucose transporters (GLUT-1, GLUT-3) and can divert mitochondrial metabolism, thus limiting cellular respiration [43], to the benefit of tumor cells.

Immune evasion
The host's immune system, through cytotoxic T lymphocytes and natural killer cells can inhibit initial tumor formation and progression. However, established cancer cells are able to evade the body's immune defenses (for a full review, see [44, 45]):

1. Cytotoxic T cells and helper T cells release interferon gamma or cytotoxins to target cancer cells. However, cancer cells can trigger inflammation by producing immune suppressive cytokines such as TGFβ that surpass the immune response, therefore proceeding towards tumor growth and angiogenesis.
2. Cancer cells secrete chemokines attracting regulatory T cells that suppress the immune system.
3. Normal T cells can be converted to regulatory T cells by TGFβ secreted by the tumor cells.
4. Molecules (such as TGFβ, VEGF, IL-10) present within the tumor microenvironment suppress antigen-presenting cells such as dendritic cells. These dysfunctional and immature dendritic cells stimulate T cells to differentiate into regulatory T cells.

Evasion of cancer cells from immune destruction represents a major block in anti-cancer drug targeting. Furthermore, the fact that a tumor is heterogeneous and hence shows antigen heterogeneity implies that it can induce various immune responses to the same trigger. This makes screening of potential therapeutic strategies more complicated and non-specific.

1.3. Brain tumors and stem cells

The initiating cell in brain tumors can be a stem cell [46], restricted progenitor [46, 47] or less frequently a differentiated cell that has undergone neoplastic transformation to de-differentiate ([48, 49], for a review see [50]), thus gaining self-renewal, proliferative and other capabilities making up the hallmarks of cancer. These properties make cancer cells functionally very similar to stem cells. In addition, many regulatory pathways such as EGFR, BMI-1, SHH, and PTEN are common to both stem cells and brain tumor stem cells [51]. Holland *et al.* showed that neural stem/progenitor cells (NSPs) were the initiating cells in astrocytomas when *Ras*- and *Akt*- transformed neural stem/progenitor cells induced astrocytoma formation, but the same did not occur with transformed oligodendrocytes [52]. Several genetic models of mouse brain tumors bear stem/progenitor cells as the cells of origin [53, 54]. Stem/progenitor cells thus represent a useful model to understand the evolution from a normal to a transformed cell.

2.0. Podocalyxin (PODXL)

We found *Podxl* overexpressed in tumorigenic neurospheres [55] and aimed towards understanding the effects of its overexpression in neural stem/progenitor cells.

PODXL has been considered a marker of blood progenitors [56-58] and embryonal carcinoma cells [59, 60]. It is also expressed by embryonic stem cells [61-63]. PODXL is also increasingly recognized as a marker of malignancy (see below). The specific function of PODXL is still unknown, probably because it undergoes tissue- and cell- specific post-translational modifications that alter its functions and it is a member of the closely related CD34 family, comprising of CD34 itself, endoglycan (PODXL2) and PODXL.

First identified as the major sialoprotein on the apical domain of podocytes in the kidney glomeruli [64], PODXL is a 150-165 kDa transmembrane protein. It consists of a serine-, threonine- and proline- rich extracellular mucin domain that is extensively O-glycosylated and sialylated, a cysteine-containing globular domain, a transmembrane region and a short intracellular domain. PODXL is widely expressed on podocytes, vascular endothelial cells, mesothelial cells, platelets and hematopoietic cells and some developing neurons [65-68], suggesting a role in processes of cell development or differentiation. PODXL is essential for kidney development where it disrupts epithelial cell junctions between the developing podocytes in order to form the urinary filter. Knocking out *Podxl* results in kidneys with fewer foot processes and less slit diaphragms [66]. This failure in the formation of foot processes is further proven by the high number of retained junctional complexes, hence making cell-cell junctions impermeable. The podocyte dysfunction observed at the morphological level translates into compromised glomerular filtration or anuria in the newborn *Podxl*$^{-/-}$ mice that die perinatally of renal failure. Under normal conditions, PODXL being highly sialylated and sulfated, this creates charge repulsion required for appropriate spacing of podocyte foot processes. Likewise, *in vitro* studies have shown that over-expression of *Podxl* inhibits cell adhesion via its sialic acid residues [69].

However, conclusive remarks about the role of PODXL in the context of cell adhesion have to be taken with caution, since it has also been shown that PODXL can be pro-adhesive in vascular endothelial cells [70, 71]. Such contradicting observations might be due to different expression levels of *PODXL* [72] or different glycosylation states of PODXL´s extracellular domain [73]. Another

possible explanation might lie in functional redundancy among CD34 family members. For instance, knocking out *Podxl* in mice does not lead to any abnormal cellular vasculature development [66] despite *Podxl* being expressed by vascular endothelial cells. This is because unlike podocytes that express *Podxl* as the only CD34 family member, vascular endothelial cells also express *CD34*. In this case, in the absence of *Podxl*, mouse *Cd34* is up-regulated [66], thereby compensating for the loss of *Podxl*. Likewise, no impairment in hematopoietic development was observed in *Podxl*$^{-/-}$ mice.

Being related to CD34 which is a differentiation marker of hematopoietic cells [74], *Podxl* is highly expressed throughout primitive hematopoiesis by hematopoietic progenitors and erythroblasts in the embryo yolk sac, and later by hematopoietic progenitors and erythroid precursors in the fetal liver. As the embryo develops, expression levels decrease until birth. *Podxl* expression is then reactivated postnatally during homing of hematopoietic progenitors to the spleen and bone marrow. Expression then drops as from four weeks and is restricted to hematopoietic stem cells with long-term reconstituting capacity (developing into myeloid and lymphoid lineages throughout adult life) [57]. PODXL being expressed on vascular endothelial cells and hematopoietic cells, it is also a ligand to the leukocyte adhesion molecule L-selectin [70]. The relevance of PODXL as a pro-adhesive molecule has been proposed to be in the recruitment of leukocytes from the blood via the endothelial vessels to other lymphoid organs.

2.1. PODXL in the brain

PODXL is expressed to variable degrees in most regions of the developing brain. However, expression peaks at prenatal and early postnatal stages [67]. Expression has been shown to be especially high in the embryonic (E16) to adult brain of the mouse and rat, more particularly in the cerebral cortex and cerebellum [75]. *Podxl* transcript and protein were found in the postnatal proliferative ventricular zone, post-mitotic neurons (pyramidal cells in the cortex and hippocampus), granule cells and peri-glomerular cells of the olfactory bulb and the Purkinje and granule cells of the cerebellum. Despite the wide pattern of *Podxl* expression in the proliferative (e.g. SVZ), migratory (RMS) and neuronal regions in the central nervous system, PODXL is not required for cell proliferation or neuronal migration, as analysis of *Podxl*$^{-/-}$ brains showed similar organization and neuronal migration as in wild type brains [67]. However, despite normal formation and distribution, axonal tracts in *Podxl*$^{-/-}$ brains were shorter and had greater branching network. This was further corroborated by explant cultures where axonal growth was diffuse (number of branching points per neuron was higher). Such observations are in contrast to the study by Nowakowski *et al*. [76] where no microscopic difference was found between knock-out and wild type brains except

for enlargement of the ventricles, a higher number of choroidal capillaries lining the ventricular space (resulting in vascular congestion) and an increase in the dimeter of carotid arteries (without any change in blood flow). These observations however did not result in any behavioral phenotype. The functional role of PODXL in the brain therefore remains unclear.

2.2. Downstream effects of PODXL

The small intracellular domain of PODXL contains a conserved PDZ (PSD-95/Dlg/ZO-1)-binding motif that binds to the scaffolding PDZ-family protein and NHERF2 [77, 78] (Figure 3). NHERF (Na^+/H^+ exchange regulatory factor) is a scaffold/adaptor protein that consists of two PDZ-binding domains and an ERM (Ezrin-radixin-moesin)-binding domain. The NHERF2-PODXL membrane complex connects PODXL to the actin cytoskeleton via Ezrin [77, 79], thus functionally keeping the organization of the renal glomerular epithelium [80]. In MDCK cells, PODXL interacts indirectly with Ezrin via NHERF1, leading to phosphorylation of the latter and activation of Ezrin. Activation of Ezrin consequently leads to RhoA activation and reorganization of actin at the junctions [81]. RhoA and Ezrin form a positive feedback loop whereby phosphorylated Ezrin activates RhoA, the latter maintaining Ezrin activation [82]. PODXL can also directly bind to Ezrin through the HQRIS amino acid sequence located in the juxtamembrane region of its cytoplasmic tail [81]; reminiscent of ICAM-1, -2 [83], and -3 [84] membrane proteins that bind directly to Ezrin.

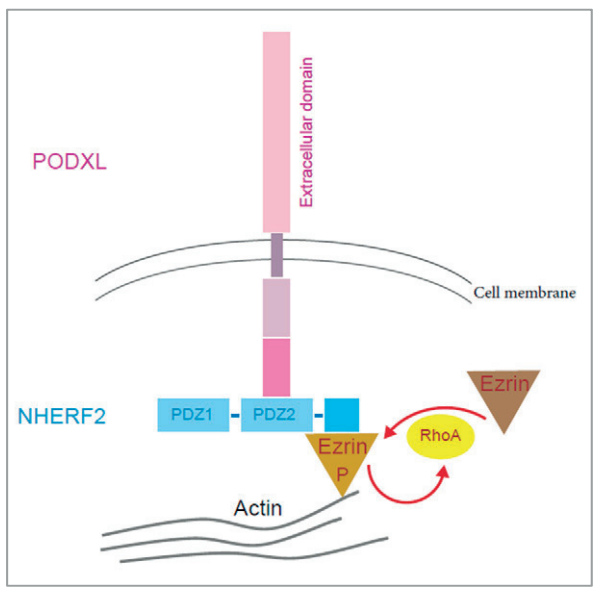

Figure 3. Interaction of PODXL with Ezrin. PODXL interacts with Ezrin indirectly through NHERF2 in podocytes and NHERF1 in MDCK cells. PODXL can also interact directly with Ezrin through its juxtamembrane cytoplasmic domain.

2.3. PODXL in cancer

In recent years, up-regulation of PODXL has been found in several forms of tumors. Often associated with the most aggressive forms of tumors, it has increasingly been regarded as a marker of malignancy. For instance, *PODXL* being overexpressed in leukemic blast cells, it is a recognized marker of monocytic **acute myeloid leukemia** [85, 86]. Schopperle *et al.* reported for the first time the expression of *PODXL* in malignant **testicular** stem cells [60]. The *PODXL* gene was mapped to chromosome 7q32-q33 by Casey *et al.* whose population-based studies revealed that mutations in *PODXL* were associated with the risk and aggressiveness of **prostate** cancer, even though the effect on the function of the protein remains unknown [87]. Tissue microarray studies showed that overexpression of *PODXL* in invasive **breast** carcinoma leads to perturbed cell junctions and cell shedding [88], events that are characteristic of tumor progression. The functional role of PODXL might be in invasion and migration, as indicated by studies on breast and **prostate** cancer cells [89]. These cells showed an increase in migratory and invasive potential, expression of matrix metalloprotease proteins and activities of the MAPK and PI3K pathways. Co-immunoprecipitation experiments showed that PODXL-dependent invasiveness is Ezrin-dependent as formation of the PODXL-Ezrin complex targets PODXL to membrane sites where it prevents cell-cell interaction. Decrease in cell-cell adhesion being one of the features of cancer cells displaying a metastatic phenotype (see earlier section), this therefore suggests a possible role for anti-adhesion molecules such as PODXL in facilitating dissemination of tumor cells. Such invasive and migratory roles of PODXL via the PI3K pathway were consistent with studies on **astrocytomas** [90]. In agreement with these, high expression of *PODXL* has been found at the infiltrating edge (invasive front) of **colorectal** [91] and **pancreatic** [92] tumor cells. This localization of PODXL leads to formation of non-adhesive membrane domains that contribute to the formation of free-floating tumor nodules during the initial stages of metastasis, as observed in studies on **ovarian** carcinoma cells *in vitro* [93]. The few studies investigating the role of PODXL in **brain** tumors are in agreement with the above observations. Overexpression of *PODXL* in two astrocytoma cell lines resulted in increased cell invasion and elevated MMP-9 expression. These events were also accompanied by increased Akt phosphorylation [90]. It is therefore not surprising that up-regulation of *PODXL* transcripts and protein has been associated with higher grades of brain tumors such as anaplastic astrocytoma and glioblastoma [94, 95]. Loss-of-function and gain-of-function experiments *in vitro* have shown

PODXL to be a marker of undifferentiated glioblastoma stem-like cells and to be responsible for their proliferative and oncosphere-forming capacity [95].

During metastasis, a vital step is the interaction of circulating tumor cells with vascular selectins [96]. Selectins recognize siafucosylated oligosaccharides which are expressed by tumor cells and are an indicator of poor prognosis [97]. Siafucosylated PODXL—which is expressed by primary **pancreatic** cancer cells—is a ligand for E- and L- selectin [70, 92] and hence promotes adhesion to the endothelium as a pro-metastatic function.

Malignant cells having high glucose uptake, there are indications that PODXL might also contribute to metastasis by regulating expression of GLUT-3 transporters at the surface of embryonal carcinoma cells [98].

Up-regulation of PODXL is associated with poor prognosis [87, 88, 91]. Despite the mechanistic implications of the de-regulation of PODXL in various malignant tumors being unclear, studies point towards a role in regulating cell adhesion and migration, hence suggesting PODXL as a metastatic facilitator. Despite the critical role that PODXL plays in cancer progression and metastasis, its exact mechanism of action remains unclear, especially due to paucity of *in vivo* studies.

3.0. Polycomb group (PcG) proteins

First discovered in *Drosophila*, PcG proteins comprise a family of evolutionarily conserved proteins. The mechanism of action of PcG proteins is in epigenetic gene silencing by histone modifications [99], resulting in transcriptional repression of developmental genes (e.g. *Hox* gene is one of the classic PcG target genes); hence the involvement of Polycomb Repressive Complex (PRC) proteins in cell-type identity, differentiation and disease. The non-static composition of PcG proteins in different cell types and contexts/developmental stages [100] and the fact that not all genes bound by PcG proteins are permanently silenced (see further below) further demonstrate their role in regulation and fine-tuning of cell fate decisions.

The PcG proteins form two major multiprotein repressive or silencing complexes: Polycomb Repressive Complex 1 (PRC1) and PRC2, which when targeted to specific loci repress gene expression through histone methylation and ubiquitination, resulting in chromatin compaction. Polycomb complexes are recruited/targeted to specific genomic regions at polycomb repressive elements (PREs) in *Drosophila*; so far PREs have not been found in mammalian cells.

Drosophila PRC1 complex is made up of four PcG proteins: Pc, Psc, Ph and dRING1. In humans, the PRC1 complex is made up of additional homologues of the four core *Drosophila* PRC1 proteins ([99] and see below). The major role of PRC1 is in ubiquitination of histone H2A on lysine 119 (H2AK119ub1). Other gene-repressing mechanisms used by PRC1, other than through H2A ubiquitination, have been observed (for a review, see [101]).

Mammalian **PRC2** is made up of four core subunits: EZH1/2 (located in the SET domain of PRC2, EZH2 is its active site in that it has histone methyltransferase activity), EED, SUZ12 and RBAP. The main role of PRC2 is in methyltransferase activity: it adds 3 methyl groups to histone H3 on lysine 27 residue (H3K27me3) (Figures 5, 6).

Different paralogs of each subunit gene exist within PRC1 and PRC2 (only EZH2 of the PRC2 complex has a paralog, EZH1). The fact that some paralogs can have antagonistic effects (e.g. BMI-1 and MEL-18 are two homologues of the PCGF component of PRC1 having opposing effects) means that the composition of each complex determines its specific function.

Canonical PRC1 or PRC2-dependent H2AK119 ubiquitination

The canonical PRC1 complex consists of CBX, PHC, PCGF and RING1a/b subunits. Each subunit has different homologues that exist within different cell types (Figure 4A). PRC2 binds chromatin via its catalytic subunit EZH2. After methylation of H3K27 by EZH2, CBX proteins of canonical PRC1 recognize H3K27me3, thereby aiding in the recruitment of canonical PRC1 to PRC2. Together, the RING1b and PCGF subunits of canonical PRC1 are responsible for the ubiquitination of Histone H2A at lysine 119 (H2AK119ub1), leading to chromatin compaction, pausing of RNA Pol II [102, 103] and repression of genes.

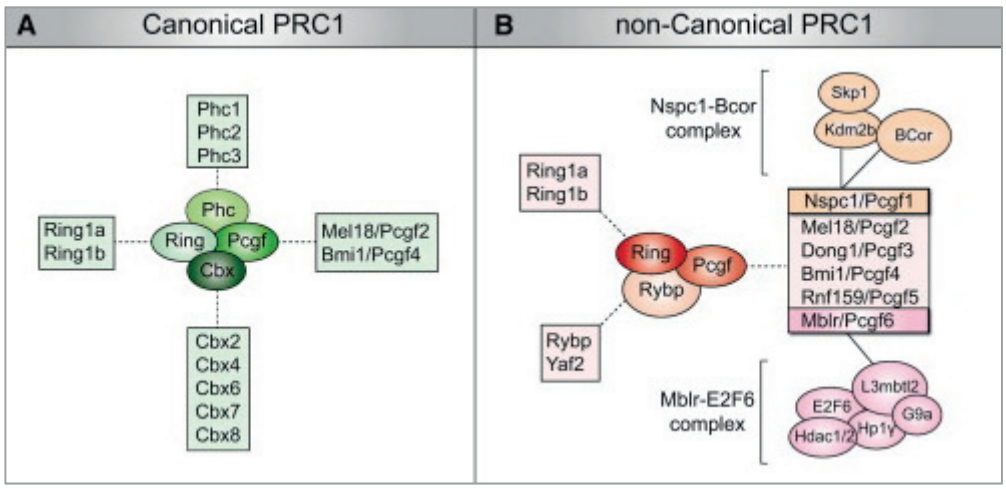

Figure 4. Composition of canonical and non-canonical PRC1 complexes. A. Canonical PRC1 is made up of Cbx, Ring, Pcgf and Phc subunits. **B.** Non-canonical PRC1 is devoid of Cbx and Phc subunits—it instead features a Rybp subunit. Figure taken from [104].

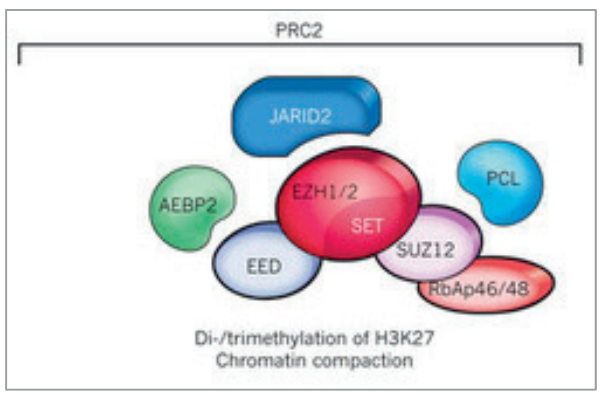

Figure 5. Composition of PRC2 complex. Figure taken from [105].

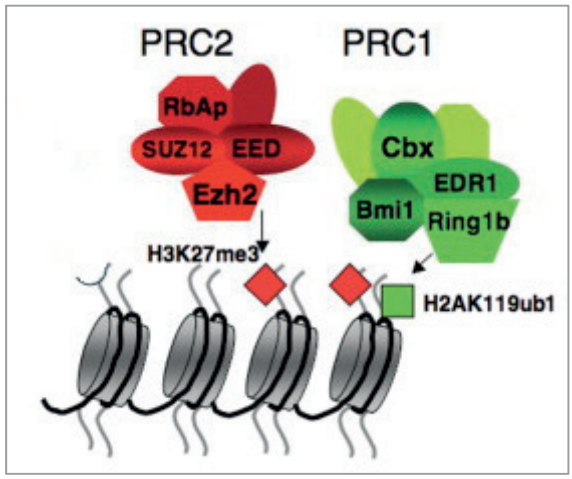

Figure 6. Histone modification by PcG proteins. PRC2 contains EZH2 that trimethylates H3K27. Ring1b of the canonical PRC1 complex has E3 ligase activity that ubiquitinates lysine 119 on histone H2A. Figure taken from [106].

Non-canonical PRC1 complexes (n-PRC1)

The mammalian n-PRC1 complex is made up of RYBP/YAF2, PCGF and RING1a/b subunits (Figure 4B). Unlike canonical PRC1 complex, n-PRC1 complex has no CBX subunit and is thus recruited to chromatin independently of H3K27me3.

Hence, n-PRC1 does not require PRC2 activity or H3K27me3 to mediate H2AK119ub1. Studies *in vitro* have shown that PRC2 can mediate repression of transcription factors even in the absence of its SET domain responsible for methyltransferase activity and by compacting chromatin [107]. Using PRC2-deficient mouse embryonic stem cells (ESCs), Tavares *et al.* demonstrated the recruitment of PRC1 to target genes, independently of H3K27me3 [101]. In this case, ubiquitination of H2A is mediated by the RYBP-PRC1 complex. RYBP stabilizes RING1b and mediates H2AK119ub1. Other n-PRC1 complexes have been described, such as the Kdm2b-PRC1 and the E2F6-PRC1 (reviewed in [108]). The functional relevance of the existence of such diverse n-PRC1 complexes is still unclear, but might indicate that several alternatives are possible in case of mutations during diseases.

The regulation of cell fate and differentiation by PcG proteins

As mentioned above, histone modifications are involved in the differential expression patterns of genes involved in cell fate decisions. Such modifications

(H3K27me3 or H2AK119ub1) are catalyzed by PcG proteins. In ESCs, PcG proteins repress developmental gene expression. However, during differentiation, the repressed genes are reactivated and H3K27me3 and H2K119ub1 levels decrease, while H3K4me3 (trimethylation of histone H3 on lysine 4, associated with an active chromatin state) increases [109]. However, many genes (usually developmental transcription factors) have been shown to carry low levels of both PcG-repressive and active marks in ESCs, making them bivalent or poised loci. By analyzing ESCs and differentiated cell types, bivalent domains were found to be characteristic of pluripotent cells, whereas differentiated cells show a monovalent domain [110] that is an either activated (marked by H3K4me3) or repressed (marked by H3K27me3) domain. Hence in ESCs, bivalent domains silence developmental genes while still keeping them ready or 'poised' until the induction of developmental pathways for lineage specification/differentiation where they resolve to either the activated or the repressed state. Functionally, this mechanism enables ESCs to maintain both their pluripotency and lineage choices.

3.1. BMI-1

BMI-1 (B cell-specific Moloney murine leukemia virus integration site 1) is a member of the PcG proteins that was originally identified to induce lymphoma formation by collaborating with *c-Myc* [111-113]. BMI-1 is a homologue of the PCGF subunit of the PRC1 complex (see previous section on PRC). It is also referred to as PCGF4.

BMI-1 is involved in several cellular processes such as cell cycle, DNA damage response, senescence, stem cell self-renewal and cancer. Located on the short arm of chromosome 10 (10p13), it consists of a RING domain that localizes to DNA breaks, an HTH domain that binds to DNA and other proteins to prevent senescence, a PEST domain that is located on the C-terminal segment and is implicated in its own degradation [114], and two NLS sites (only NLS2 is functional) that are characterized by basic residues (Figure 7).

BMI-1 interacts with each component of the PRC1 complex and is thus essential in maintaining complex integrity. BMI-1 associates with Ring1a/b via its RING domain and has been found to enhance the E3 ligase activity of Ring1a/b in ubiquitination of Histone 2A. Indeed, the physical contacts between the two units have a synergistic effect on the E3 ligase activity. Hence the four-component PRC1 complex is more active in H2A ubiquitination than is the Ring1b subunit alone [115].

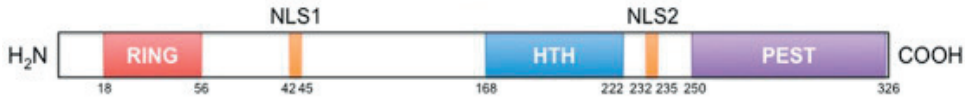

Figure 7: Protein structure of BMI-1. *BMI1* gene encodes a 37kDa protein composed of 326 amino acids. HTH – helix-turn-helix; NLS – nuclear localization signal; PEST – motif rich in proline, glutamic acid, serine and threonine. Figure taken from [116].

BMI-1 is widely distributed amongst organs and expression levels are usually low in most tissues except the brain, lymph nodes, gonads, spinal cord, kidney and lungs [117-119].

3.2. Well-known downstream targets of BMI-1

1. BMI-1 negatively regulates the *Ink4a/Arf* locus, which encodes $p19^{Arf}$ and p16 (Figure 8). These two tumor-suppressor genes are well-known targets of BMI-1 [120]. $p19^{Arf}$ and *p16* are generally not expressed by normal cells *in vivo* in young mice and expression increases with age [121]. In the absence of BMI-1, $p19^{Arf}$ and *p16* become strongly expressed, hence inhibiting CDK4/6, leading to cellular senescence. This would mean that in the adult, BMI-1 suppresses $p19^{Arf}$ and *p16* in order to maintain a stem cell pool throughout adult life.
2. Under normal conditions, $p19^{Arf}$ (mouse homolog of $p14^{Arf}$) sequesters MDM2 and therefore prevents it from degrading p53. Thus $p19^{Arf}$ positively regulates p53-dependent cell death. By repressing $p19^{Arf}$, BMI-1 consequently causes depletion of p53, leading to a drop in cell cycle arrest and apoptosis [120].
3. BMI-1 also affects the stability of p53 by negatively acting through the pRb-p53 pathway.
4. BMI-1 abolishes cell cycle checkpoints p16/p14 in several cell types.
5. By inhibiting *Ink/Arf* tumor-suppressor genes, BMI-1 thus contributes to oncogenic activity.

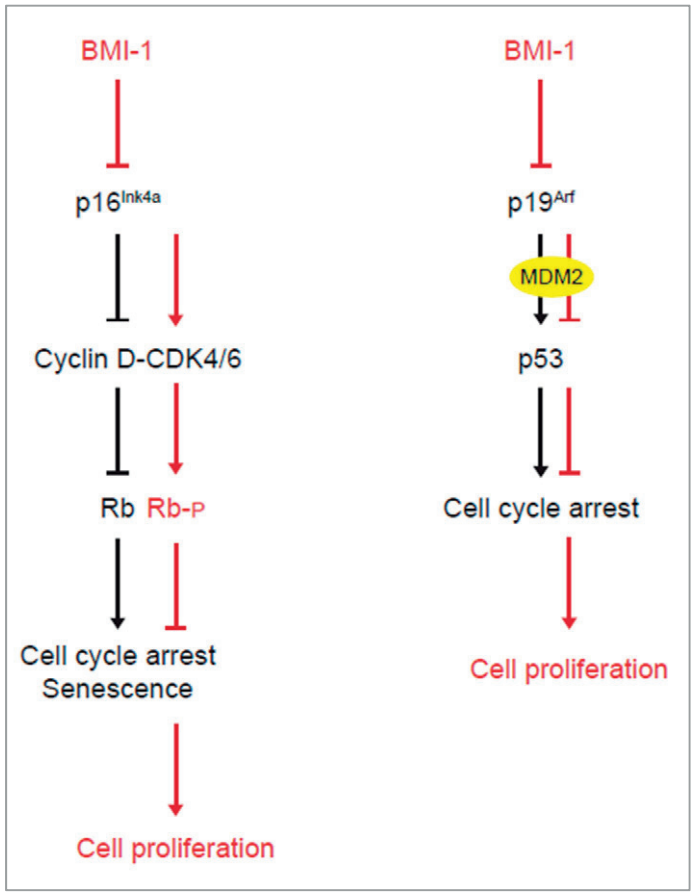

Figure 8: BMI-1 is a negative regulator of *p16*^{Ink4a} and *p19*^{Arf} gene expression. In black, p16^{Ink4a} and p19^{Arf} actions lead to entry into S phase. BMI-1 suppresses p16^{Ink4a}/Rb and p53 pathways, leading to inhibition of cell cycle arrest and hence cell proliferation.

3.3. *Bmi1* knock-out mouse model

The *Bmi1* deficient mouse shows a broad range of phenotypic abnormalities. BMI-1 being required for postnatal maintenance of **hematopoietic** stem cells (HSCs) [117], the *Bmi1*$^{-/-}$ mouse shows progressive and severe depletion of HSCs and subsequent hematopoietic failure of the liver and bone marrow, resulting in a survival rate of only 50% shortly after birth. Those mice that survive exhibit retarded overall growth and show premature death at around week 3 to 20. The *Bmi1*$^{-/-}$ mouse also shows lymphoid abnormalities in the thymus and spleen due to the inability of lymphocytes to proliferate. The mouse also shows severe **skeletal** abnormalities and suffers from ataxic gait, tremors and epileptic-like seizures. The brain of the *Bmi1*$^{-/-}$ mouse, which is smaller in size than wild type brains, shows

reduced cell density in the layers of the **cerebellum** and severe decrease in the thickness of the molecular layer due to loss of neurons, hence accounting for the motor coordination phenotype of the mouse. The *Bmi1*$^{-/-}$ mouse also shows abnormal self-renewal of neural stem cells and brain maintenance [122, 123] (see below).

3.4. BMI-1 in the brain

As mentioned above, deficiency in BMI-1 leads to postnatal growth retardation and neurological defects, including a smaller brain size [117, 123]. Zencak *et al.* found that while BMI-1 is required for self-renewal of NSCs in adult and newborn mice *in vivo* and of embryonic day 14 (E14)- and newborn- isolated NSCs *in vitro*, it is however not required for their survival or differentiation [123]. *Bmi1*$^{-/-}$ NSCs show reduced self-renewal and ultimately account for postnatal depletion [122, 123]. In another cell model, the block in cell proliferation following the absence of *Bmi1* in mouse embryonic fibroblasts was attributed to de-repression of *p16*^{Ink4a} [120]. The same study showed that lack of *Bmi1* also up-regulates p19^{Arf} (but to a lesser extent than p16^{Ink4a}) to inhibit cell proliferation.

While *Bmi1*$^{-/-}$ NSCs showed reduced proliferation *in vivo*, this was not the case for restricted neural progenitors isolated from the gut and forebrain that proliferated normally *in vitro* in the absence of *Bmi1* [122]. Neurosphere formation assays gave an indication of the causes for the above observations: *Bmi1*$^{-/-}$ NSCs isolated from embryonic and postnatal telencephalon formed smaller neurospheres compared to wild type NSCs from the same stages. These neurospheres were also formed at a lower frequency, which indicated fewer NSCs in the embryonic and postnatal *Bmi1*$^{-/-}$ telencephalon. Furthermore, *Bmi1*$^{-/-}$ NSCs from E14 mice formed fewer secondary spheres upon subcloning, showing a defect in their self-renewal capacity. The self-renewal impairment was found to be more important in postnatal versus E14 NSCs and this was exacerbated in adult mice. This diminished self-renewal capacity was due to a decrease in proliferation rather than an increase in cell death. BMI-1 is thus required at the postnatal stage of nervous development to repress *p16*^{Ink4a} so as to enable NSC proliferation. Other pathways downstream of BMI-1, in addition to p16^{Ink4a}, seem to play a part in self-renewal, since *p16*$^{-/-}$*Bmi1*$^{-/-}$ NSCs—despite having greater proliferative capacity than *Bmi1*$^{-/-}$ NSCs—are not to the same proliferative level as *p16*^{wt}*Bmi1*^{wt} NSCs.

While studies consistently show that the proliferation of NSCs is BMI-1-dependent, it might seem that proliferation of committed neural progenitor cells and more differentiated neural cells are BMI-1-independent [122, 123]. BMI-1 loss has been found not to affect astrocyte precursor proliferation *in vivo*, *Bmi1*$^{-/-}$ NSCs generating astrocytes preferentially over neurons both *in vitro* and *in vivo*

[123]. Likewise, HSCs transplanted from *Bmi1*$^{-/-}$ mice fail to generate HSCs but do give rise to more progenitors in the host [124]. These agree with the study by Yadirgi *et al.* who found BMI-1 to be essential for NSC self-renewal and proliferation, and also that *Bmi1* levels should go down for initiation of neuronal (but not glial or oligodendroglial) differentiation (to prevent delay in neuronal differentiation) [125]. Maintaining high *Bmi1* levels would have led to surviving-mediated apoptosis of neuronal-committed progenitors. This BMI-1-mediated apoptosis has never been observed in postnatal NSCs, clearly showing the different effects of BMI-1 on progenitors at different stages of differentiation. Similarly, in the embryonic mouse cerebellum BMI-1 is normally expressed in the external granular layer (EGL); postnatal development sees *Bmi1* expression to increase in the proliferating granule precursors of the EGL [119]. BMI-1 was found to be crucial for the proliferation of E16.5 and early postnatal cerebellar precursor cells, but not of more restricted cells such as the post-mitotic or terminally-differentiated granule cells.

3.5. BMI-1 in cancer

Mostly through tissue microarray studies and Western blot analyses of BMI-1 protein levels and gene expression studies by quantitative PCR, it was shown that BMI-1 is often up-regulated in human cancers (breast cancer [126], medulloblastoma [119], neuroblastoma [127], AML [124, 128], lymphoma [129, 130], ovarian cancer, prostate cancer [126], non-small-cell lung cancer [131, 132], pancreatic cancer [133] and hepatocellular carcinoma [134]) compared to healthy tissue. *BMI1* overexpression correlates with poor prognosis. BMI-1 alone does not seem to induce tumor formation. Focus-formation assays indicated that *Bmi1* cooperates with oncogenic H-*ras* in neoplastic transformation of MEFs [135]. Elevated *BMI1* expression has been noted in medulloblastoma cell lines and primary tumors [127]. The mechanism of action of BMI-1 in these two systems is through inhibition of the apoptotic activity of N- or C- MYC [127, 135]. In contrast, studies of *BMI1* expression in glioblastomas have been inconsistent: Leung *et al.* reported absence of expression of BMI-1 in gliomas [119], whereas He *et al.* and Bruggeman *et al.* observed high expression in human and mouse gliomas respectively, independent of tumor-grade [49, 136]. In the case of breast cancer, *BMI1* is overexpressed and leads to an increase in self-renewal, proliferation and EMT of breast cancer cells *in vitro* [137, 138]. Overexpression of *BMI1* in models of pancreatic cancer leads to increased cell invasion capacity [133]. These studies support the oncogenic role that BMI-1 activation plays in the progression of malignancies.

4.0. SWI/SNF family of nucleosome-remodeling complexes

Initiation of transcription is regulated by DNA accessibility through chromatin structure. This accessibility is controlled by multi-protein complexes. One main class of such complexes is the SWI/SNF family of ATP-dependent nucleosome-remodeling complexes. SWI/SNF complexes control transcription in diverse ways, mostly through transcriptional activation by nucleosome remodeling [139]. Cases where they contribute to transcription repression have however also been reported in yeast [140].

The SWI/SNF complex was first discovered in yeast where mutations in each of *SNF* and *SWI* genes caused defects in expression of their respective target genes (*SUC2* and *HO*) [139]. The SWI/SNF complex is evolutionarily conserved. The mammalian SWI/SNF complex is composed of a set of invariant core subunits (SNF5, BAF155, BAF170), one of the two catalytic ATPase subunits (BRG1 or BRM) and varying subunits. The variable subunits contribute to the heterogeneity of the complex, attributing the complex some tissue/cell- and condition/developmental stage- dependent roles [141, 142].

4.1. Mechanism of nucleosome remodeling by the SWI/SNF complex

During transcriptional regulation, histones are acetylated, displaced and repositioned from promoters. The process involves a series of steps. Some transcription factors that recruit histone modifying enzymes such as HATs, HDACs etc bind to nucleosomes, resulting in nucleosome destabilization. Although still unclear, transcriptional activators seem to be involved in recruitment of ATP-dependent nucleosome remodeling complexes (such as SWI/SNF) to promoters [143]. Hydrolysis of ATP generates enough energy to alter the structure of the chromosome. Upon disruption, DNA around the nucleosome uncoils, thus unwrapping the histone octamer. Two main mechanisms have been put forward ([144]; reviewed in [145]) to explain how the SWI/SNF complex forms such histone-free regions:

1. Trans-displacement: chromatin disruption by the SWI/SNF complex results in displacement or eviction of the histone octamer to another DNA strand [146].

2. Cis-displacement: the movement of the histone octamer along the same DNA strand. This forward movement of the octamer occurs by sliding via a spooling or twisting mechanism along DNA [147].

4.2. The SWI/SNF complex in cancer

The BAF250A subunit of the SWI/SNF complex is the most commonly affected BAF subunit in various tumors. Specific subunits were found to be mutated in specific tumors in a tissue-specific manner; for instance *ARID1B* (coding for BAF250B subunit) is one of the most mutated genes in developmental disorders (for a review see [148]). This highlights the very specific role of each subunit within the complex. Mutations in the genes can function as oncogenes or tumor suppressors—malignant cell lines were found to show loss of the BRG or BRM subunits [149], and loss of BAF47 subunit is found in the rare childhood cancer malignant rhabdoid tumor (MRT) [150]. (Figure 9 and see Article 3).

Haploinsufficiency can also drive tumorigenesis, as demonstrated by *Snf5*$^{+/-}$ and *Brg1*$^{+/-}$ heterozygous mice [151, 152]. This implies that complete absence of a subunit can induce tumor formation, and so does reduced expression of a subunit [151-155].

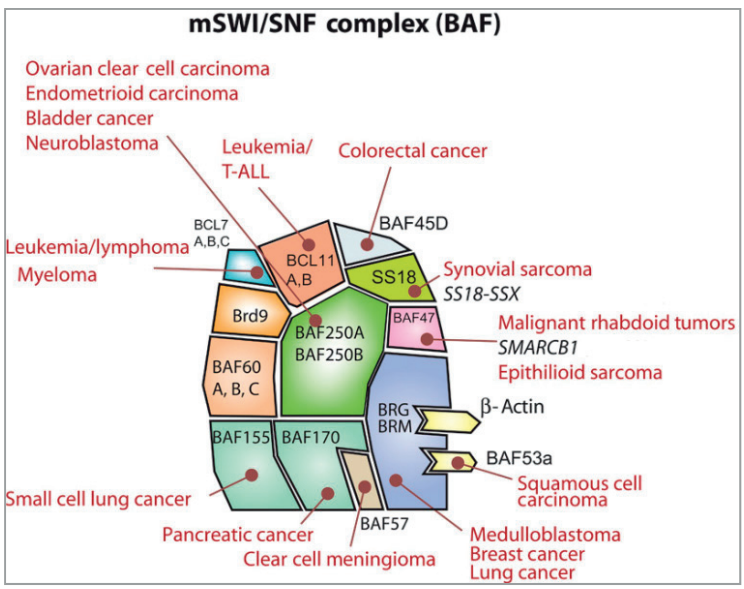

Figure 9: Mutations of BAF subunits in human tumors. Different tumors are associated with mutations in a specific subunit of the complex. Figure taken from [156].

4.3. The SNF5 subunit

One of the core subunits of the SWI/SNF complex is SNF5 (aliases: INI1, SMARCB1). *SNF5* is inactivated in the majority of MRTs by biallelic mutations such as whole-gene deletion, nonsense, missense and frameshift mutations [150].

MRT is a rare and highly malignant childhood tumor with a very poor prognosis, arising primarily in the kidney, brain (in this case it is referred to as AT/RT) or other soft tissues [157]. Loss of *SNF5* has been reported in other pediatric brain tumors such as choroid plexus carcinoma, some PNETs and medulloblastoma [158, 159]. Heterozygous mice show an early onset of nervous system and soft tissue undifferentiated sarcomas with variable rhabdoid features [154]. The fact that these tumors show inactivation of *SNF5* showcases its essential role as a potent tumor-suppressor.

5.0. Experimental procedures

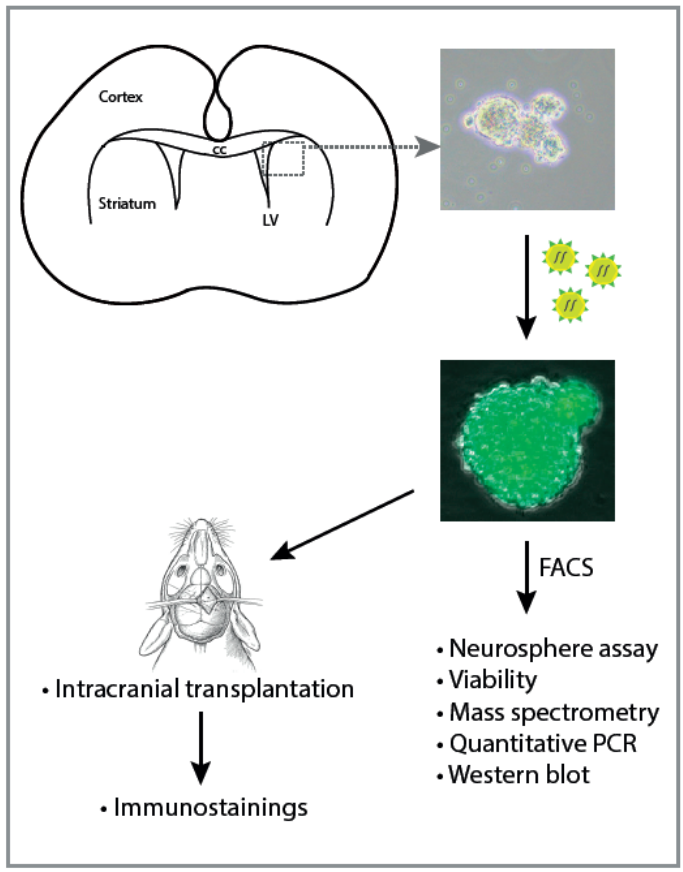

Figure 10: Overview of the experimental procedures used in this thesis. NSPs from postnatal or adult mouse SEZ were isolated, cultured and transduced with retroviruses. A pure population of GFP-positive cells was sorted by flow cytometry and constituted the starting material for following experiments.

5.1. Isolation of postnatal neural stem/progenitor cells (NSPs)

NSPs were isolated from the SEZ of the lateral ventricles of embryonic or postnatal mouse brains (Figure 10). Brains were isolated and a 1-2 mm thick coronal section made 5 mm posterior (for a postnatal brain) to the olfactory bulbs. The SEZ was dissected and cut into small pieces before digestion in Accutase

(PAA Laboratories) at 37 °C for 20 min. After trituration with P200 and P20 tips, the cell suspension was filtered (50 µm filter, BD Biosciences) and plated in NSP medium (DMEM/F12, B27, Penicillin/Streptomycin (all from Life Technologies) and 10 mM HEPES (Sigma)) supplemented with EGF (20 ng/ml, R&D) and FGF2 (20 ng/ml, R&D). Cells were incubated at 37 °C in a humidified 5% CO_2 incubator. Spheres started to appear after approximately 5 days. Cells were passaged every 5-7 days using Accutase and maintained at a density of $1x10^5$ cells/ml.

In our method of isolation, we do not distinguish between sub-ependymal and ependymal layers of the lateral ventricular wall. However, the use of growth factors (EGF and FGF2) selects only the sub-ependymal cells that are known to self-renew and form secondary spheres in the presence of growth factors [160].

5.2. Retroviral production and transduction of NSPs

For production of viral particles, we used Plat-E cells (ATCC) as packaging cell line. One day before transfection, $5x10^6$ cells were plated/75 cm^2 CellBind flask (Corning Inc.). Cells were transfected on the following day with 40 µg of plasmid/flask using the calcium phosphate co-precipitation method according to Hertwig *et al.* [55]. 16 h after transfection, medium was changed. 48 h later, the first viral supernatant was harvested. A second harvest was collected 24 h later. Titration of the viral particles was performed on wild type NSPs by flow cytometry.

Early passage (passage 1 or 2) NSPs in exponential growth phase (day 2 or 3) were collected at 200 g for 5 min at RT. Viral supernatant was added at the desired MOI. Cells were then gently disaggregated and incubated with NSP medium supplemented with 4 µg/ml polybrene (Sigma) to increase the efficiency of retrovirus-mediated gene transfer [161]. Cells were shaken at 900 rpm at 37 °C for 4-5 h, medium was changed without polybrene and the cells were plated in low-attachment plates (Corning Inc.). 24 h later, cells started to express the reporter gene (e.g. *Gfp*). 48 h post-transduction, positive cells could be sorted (FACSAria III, BD Biosciences).

5.3. Flow cytometry

Suspension cultures: Cells were collected at 200 g for 5 min at RT. The pellet was disaggregated with Accutase and incubated at 37 °C for 15 min. Cells were gently dissociated with a P200. The singularized cell suspension was diluted in 2 ml cold FACS buffer (1% BSA (Sigma) in PBS) and centrifuged at 270 g for 7 min at 4 °C. The pellet obtained was stained for flow cytometry analysis (see Article 1) or

re-suspended in cold FACS buffer and kept on ice until sorting. Just before sorting, 7AAD (BD Biosciences) was added (1:100) for exclusion of dead cells.

Adherent cultures: Cells were detached using 0.25% Trypsin-EDTA (Life Technologies). The reaction was stopped with medium containing FBS and filtered (50 µm filter, BD Biosciences). Cells were washed once in cold FACS buffer and centrifuged at 270 g for 5 min at 4 °C. The pellet was stained or re-suspended in cold FACS buffer and kept on ice until sorting.

5.4. Knock-down

We made use of shRNA to knock down genes in cell lines. shRNA vectors were from OriGene (Rockville, MD) and shRNA lentiviral particles from Santa Cruz Biotechnology (Dallas, TX). To produce pseudotyped viruses, Phoenix cells (provided by Beata Lindqvist, Vector Unit, Lund University) were co-transfecfed with pcDNA3-MLV, pMDG-VSVG and the small hairpin vector using the calcium phosphate co-precipitation method. The first viral harvest was done 48 h later. Transduction was performed on cells that were approximately 50% confluent. Selection of transduced cells was with 10 µg/ml puromycin (Sigma). Knock-down was verified by Western blot. Cell lines that achieved at least 80% knock-down were used for experiments.

5.5. SDS-PAGE

Neurospheres were harvested by centrifugation, followed by washing in cold PBS. Cold RIPA buffer (150 mM NaCl, 1% Triton-X, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl pH 8.0, all from Sigma) containing protease inhibitor cocktail (Sigma) (if phosphorylated proteins were assayed, phosphatase inhibitor cocktail (Sigma) was also added) and 2 µM PMSF (Sigma) was added to lyse the cells. Adherent cells were washed in cold PBS and scraped in cold RIPA buffer plus inhibitors. The lysate was left on ice for 1 h with vortexing every 20 min. After centrifugation at 13,000 rpm for 10 min at 4 °C the supernatant was collected and quantified by BCA assay (Thermo Scientific). Proteins were separated on SDS-gels and transferred to nitrocellulose membranes (GE Healthcare). After blocking the membrane in 5% non-fat milk or BSA (Sigma) in TBS-T (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, all from Sigma) for 1 h at RT, primary antibodies were applied overnight at 4 °C. Membranes were washed in TBS-T and incubated with secondary HRP-conjugated antibodies for 1 h at RT. Blots were visualized using ECL (GE Healthcare) on a Bio-Rad imager (Bio-Rad).

5.6. Label-free liquid chromatography-mass spectrometry (LC-MS)

100 µg of protein was denatured in 20 µl Laemmli buffer (Bio-Rad) and 5% β-mercaptoethanol (Sigma). Samples were run on a Criterion TGX gel (Bio-Rad) at 300 V for 3 min. The gel was washed in distilled water and stained with GelCode Blue (Thermo Scientific) for 1 h at RT. After de-staining in water, protein bands were cut and washed in 50% acetonitrile (ACN) and 25 mM ammonium bicarbonate, speed-vacuumed, reduced in 10 mM dithiothreitol at 56 °C for 1 h, alkylated in 55 mM indoacetamide for 45 min (all from Sigma) and dried. The samples were then digested with Trypsin (Promega) overnight at 37 °C. The reaction was stopped with 75% ACN and 5% trifluoroacetic acid (Sigma) and the supernatant collected and dried. The resulting pellet was dissolved in 0.1% formic acid (Sigma) for 30 min at RT and analyzed by LC-MS (Protein Technology, Lund University).

5.7. Immunostainings

Brains were fixed overnight in 4% PFA (Sigma) at 4 °C. Cryoprotection was done in 30% sucrose (Sigma) in PBS at 4 °C before embedding in OCT compound (Tissue-Tek) and snap-freezing on dry ice. 10 µm sections were cut on a cryostat (Microm HM 560) and mounted on Superfrost Plus Adhesion Slides (Thermo Scientific). Sections were permeabilized in 0.1-0.2% Triton-X (Sigma), washed three times in PBS and blocked in 5% normal donkey serum (Dianova). Primary antibodies were diluted in blocking solution and applied overnight at 4 °C. Sections were washed three times in PBS and fluorescently labeled secondary antibodies (Dianova) added (1:300 in blocking solution) for 1 h at RT. Sections were washed in PBS, dehydrated in 100% ethanol and mounted in VectaShield (Vector Laboratories) with 1 µg/ml DAPI (Serva). Stainings were visualized using a Zeiss inverted microscope.

5.8. Quantitative PCR

Total mRNA was extracted from tissue or cells using the Allprep DNA/RNA kit (Qiagen). 2 µg of total RNA was reverse transcribed using random hexamer primers (Thermo Scientific), M-MLV reverse transcriptase (Promega) and RNAse inhibitor (RNasin Plus, Promega). cDNA was diluted 5 times and 5 µl used as template for PCR amplification. PCR was followed using Power SYBR Green PCR master mix (Applied Biosystems) on the StepOne Plus Real Time PCR System (Applied Biosystems). Each sample was run in at least triplicates. Gene expression was normalized to *GAPDH* or *TBP* expression. Statistical significance was calculated by the Student's *t*-test using GraphPad Prism 6 (GraphPad software, San Diego, CA).

6.0. Aims of the thesis

Based on mouse models of brain tumors, NSPs have been proposed as the cells of origin of brain tumors. These normal cells undergo key molecular changes as part of the oncogenic transformation that turns them into tumorigenic cells. The aim of this thesis was to focus on the downstream effects of three regulators (PODXL, BMI-1 and SNF5) of brain tumors on neural stem/progenitor cells in order to understand their mechanism of action in brain tumor development.

Previous studies by our lab [55] revealed an up-regulation of ***Podxl*** in models of mouse brain tumors. PODXL is involved in cell-cell adhesion and junctional permeability during embryonic development of the kidney [66] and is a marker of embryonic stem cells [62]. Up-regulation of PODXL is associated with aggressive forms of tumors. Our aim was thus to investigate the functional role of PODXL in neural stem/progenitor cells.

BMI-1 being essential for the self-renewal of embryonic and postnatal neural stem cells, it is therefore a key player in postnatal brain development [122]. *BMI1* is also overexpressed in several cancers. It promotes cell proliferation and self-renewal by repressing tumor-suppressor genes $p16^{Ink4a}$ and $p19^{Arf}$. As a transcription regulator, BMI-1 binds to several genomic regions of target genes. In this study our objective was to identify novel BMI-1 target genes in an attempt to explain the range of functions displayed by BMI-1.

SNF5, a component of the SWI/SNF complex involved in transcriptional regulation during development, is inactivated in the majority of malignant rhabdoid tumors, including AT/RT. The cell of origin of this childhood brain tumor is not known, despite few studies pointing towards a progenitor cell. Nestin is a known neural stem/progenitor cell marker of the central nervous system. The Nestin-Cre transgene was introduced into mice to ablate *Snf5* specifically in the developing nervous system. Therefore, the aim of our project was to investigate consequences of *Snf5* deletion in Nestin-positive neural stem/progenitor cells.

7.0. Results and discussion

7.1. Article 1: High Podocalyxin levels promote cell viability partially through up-regulation of Annexin A2

PODXL is present on many different cell types (stem cells and non-stem cells) (see earlier text). It is also increasingly considered a marker of malignancy since its expression correlates with aggressiveness of tumors (see Article 1). PODXL is essential during embryonic development of the kidney; without PODXL mice die of anuria within 24 hours of birth [64]. However, the role of PODXL in cancer is not well understood; *in vitro* studies so far have shown its involvement in migration and invasion [69, 89-91, 162] and hence a possible contribution to metastasis. Our microarray analysis on murine tumorigenic NSPs isolated from three mouse models of brain tumors showed up-regulation of *Podxl* in comparison to non-tumorigenic NSPs [55]. We thus wanted to investigate the consequences of such an overexpression in NSPs.

***Podxl*-NSPs have increased cell and sphere numbers**

We stably incorporated *Podxl* or *Empty* control constructs in NSPs and carried out neurosphere assays to determine the self-renewal capacity of our cells. We found three times more *Podxl*-spheres than *Empty* ones and over four times more *Podxl*-cells compared to *Empty* control. These higher numbers in *Podxl* spheres and cells were consistent in independent assays. However, assessment by BrdU did not indicate any proliferative advantage of *Podxl*-overexpressing cells.

Podxl*-NSPs show higher cell viability *in vitro* and *in vivo

To find an explanation for the increased self-renewal in the absence of any increase in proliferation of *Podxl*-NSPs, we labelled cells with Annexin V/PI to analyze apoptotic cells. Independent of plating density or passage number, *Podxl*-NSPs exhibited a significantly higher living cell population, lower early apoptotic population and lower late apoptotic population.

Podxl-NSPs transplanted into the brains of mice and stained with the apoptosis marker cleaved caspase-3 contained fewer positive cells than *Empty*-transplanted cells, indicating that cells overexpressing *Podxl* show lower apoptosis both *in vitro* and *in vivo*.

Overexpression of *Podxl* leads to up-regulation of Annexin A2 at the protein and transcript levels

Proteome analysis by LC-MS showed ANXA2 to be significantly increased in *Podxl*-NSPs. These results were confirmed by Western blotting. We also detected elevated *Anxa2* mRNA levels in *Podxl*-overexpressing cells. Knocking down *PODXL* in the cancer cell line MDA-MB231 also led to a down-regulation of ANXA2 at the protein and transcript levels.

Knock-out of *Anxa2* in *Podxl*-NSPs lowers cell viability

Overexpression of *Podxl* in *Anxa2*$^{-/-}$-NSPs and comparison with *Podxl*-overexpressing *Anxa2*$^{+/+}$-NSPs revealed that the former cells contain a significantly lower viable fraction and a higher early apoptotic fraction.

Regulation of ANXA2 by PODXL occurs through the MAPK pathway

PODXL activates the MAPK pathway [89]. We found the presence of a binding site for AP-1 in the promoter region of *Podxl* and thus hypothesized that the activation of the MAPK pathway by PODXL leads to a higher AP-1 activity. In agreement, we found higher ERK1/2 and phosphorylated c-Jun in *Podxl*-NSPs compared to *Empty*-NSPs. We also thought that blocking of MAPK pathway would lead to down-regulation of ANXA2. Indeed, upon inhibition of MEK1/2, we observed a reduction in protein levels of ANXA2 in *Podxl*-NSPs. Levels of ANXA2 in *Empty*-NSPs also decreased, showing that blocking of MEK1/2 also affects endogenous ANXA2 and that the regulation of ANXA2 occurs through MEK1/2 and AP-1.

To our knowledge, no study has previously shown that cells overexpressing *PODXL* showed a higher viability. Wu *et al.* reported that human astrocytoma cell lines overexpressing *PODXL* have increased survival against the chemotherapeutic agent Temozolomide [90]. However, they and others have also shown that under normal conditions, no difference in viability of *PODXL*-overexpressing or knocked-down tumor cells was observed [163]. In agreement with these, upon knock-down of *PODXL* in the metastatic breast cancer cell line MDA-MB231, we did not observe any change in viability or apoptotic levels. This might be due to compensatory effects of other CD34 family members, such as *CD34* or Endoglycan *(PODXL2)* upon knock-down of *PODXL*, or to other confounding genes that are aberrantly expressed in tumor cell lines.

Overexpression and down-regulation studies have shown that PODXL has an impact on extracellular matrix proteins, the EMT process, metastasis-related cytokines, cell adhesion and invasion (through MMP-9) *in vitro* [89, 90, 164, 165], and more recently in invasion *in vivo* [164]. The MAPK pathway is a recurring

effector in the downstream signaling of PODXL ([89, 165]; our study). So is the PI3K pathway [89, 90]. The mechanisms of how PODXL increases cancer aggressiveness could be by complex formation with Ezrin, a metastatic promoter [79, 89, 166] leading to disruption of cell-cell contacts [89, 162]. We cannot claim the same in our model, but *Podxl*-NSPs are morphologically less compact and are looser (Figure 1a of Article 1). Indeed, interactions of PODXL and MAPK and/or PI3K have been shown to occur via Ezrin, co-immunoprecipitation experiments having shown that PODXL and Ezrin form a complex in several cancer cell lines, and Ezrin being known to influence both these pathways [166, 167]. We found AP-1 as an additional downstream target in the PODXL-mediated effects in NSPs, in agreement with Lin *et al.*'s study on oral squamous cell carcinoma cell lines [165].

The increased invasion occurring as a result of PODXL overexpression, as seen by elevated wound healing capacity, does not appear to be due to proliferation [89, 164], which is consistent with our own observations investigated by neurosphere assays or proliferation assays on *Podxl*-NSPs. Our studies allow us to propose a model whereby PODXL might contribute to cancer progression through cell survival, more specifically that MAPK activation upon PODXL overexpression leads to AP-1 activation and ANXA2 up-regulation and consequently to increased cell survival. The up-regulation of ANXA2 in our *Podxl*-overexpressing NSP cultures is in line with the fact that ANXA2 is also implicated in degradation of the extracellular matrix, increased invasion and angiogenesis and decreased apoptosis [168-170]. One of the mechanisms through which ANXA2 contributes to improved cell survival of our *Podxl*-overexpressing NSPs might be through plasma membrane repair. ANXA2 was shown to be involved in plasma membrane repair in muscle cells by membrane fusion or intracellular vesicle fusion ([171], reviewed in [172]). Additionally, plasma membrane repair proved to be essential for survival of invasive cancer cells [173] which previously were shown to have fragile lysosomal membranes [174] and an unstable and less stiff plasma membrane [175], hence more liable to plasma membrane injuries during invasion. Hence, through up-regulation of repair components such as ANXA2, invasive cancer cells adopting an enhanced plasma membrane repair system are better suited to cope with plasma membrane damage.

Ezrin-mediated survival partially results from MAPK activation [166]. Wu *et al.* have shown that PODXL promotes astrocytoma cell survival against induced apoptosis in a PI3K-dependent manner [90]. We on the other hand show that in NSP cultures and *in vivo*, PODXL could exert its pro-survival advantage to cells through the up-regulation of MEK1/2 and AP-1 activities and subsequent up-regulation of ANXA2.

In conclusion, in addition to the involvement of PODXL in invasion, migration and glucose metabolism of cancer cells [98], we demonstrate that PODXL, through ANXA2, could play a role in cell survival.

7.2. Article 2: The putative tumor suppressor gene *EphA7* is a novel BMI-1 target

Bmi1 was first identified as on oncogene that cooperates with *c-Myc* to generate lymphomas in mouse. It is overexpressed in several malignancies including brain tumors (see previous text). BMI-1 exerts its functional roles by transcriptional repression. Given that brain tumors can arise from NSPs (see previous text), we sought to first search for novel BMI-1 target genes that would help explain how BMI-1 exerts its effects in brain tumors.

Overexpression of *Bmi1* increases self-renewal of NSPs *in vitro*

By neurosphere assays, we compared *Bmi1*-NSPs to *Empty* control NSPs and found that *Bmi1*-NSPs showed a higher self-renewal capacity *in vitro*, both in terms of sphere numbers and sphere sizes.

***EphA7* is a direct target of BMI-1**

Microarray analysis indicated genes that were differentially expressed in *Bmi1*-overexpressing NSPs, compared to control NSPs. To identify direct target genes that were repressed by BMI-1, we selected those genes that were down-regulated in *Bmi1*-overexpressing NSPs. By ChIP experiments, four novel direct BMI-1 target genes were detected. It was then investigated which of these would be de-repressed in the absence of *Bmi1*. qPCR analyses on $Bmi1^{-/-}$-NSPs versus $Bmi1^{+/+}$-NSPs showed *EphA7* to be significantly up-regulated in the absence of *Bmi1*. Similarly, overexpression of *Bmi1* in NSPs and in spleen B cells led to down-regulation of *EphA7*.

Absence of *EphA7* partially rescues the proliferative deficiency in the LVW of *Bmi1*-deficient mice

Since we observed a de-repression of *EphA7* in the absence of *Bmi1* in NSPs *in vitro*, we wondered if this observation would have a visible effect *in vivo*, since $Bmi1^{-/-}$ mice show abnormal self-renewal of NSCs *in vitro* [122]. We generated $Bmi1^{-/-}EphA7^{-/-}$ double knock-out mice and found significantly higher numbers of Ki67 proliferating neuroblasts and progenitor cells in the dorsolateral corner of the brain LVW, compared to $Bmi1^{-/-}EphA7^{+/+}$ brains. This rescue by the absence of *EphA7* was however not enough to reach the higher number of proliferating neuroblasts and neural progenitors in the $Bmi1^{+/+}$ (wild type) brains.

Repression of *EphA7* by methylation can be triggered by *Bmi1* overexpression
As mentioned earlier, *BMI1* is overexpressed in several tumors. The tumor-suppressor *EPHA7* is silenced in a number of lymphomas and colorectal cancers [176, 177]. We thus hypothesized that *Bmi1* overexpression down-regulates *EphA7* through methylation of its promoter-associated CpG island in NSPs. We examined the methylation status of the CpG island around the *EphA7* transcriptional start region in *Bmi1*-overexpressing NSPs. Elevated levels of DNA methylation in *Bmi1*-NSPs compared to *Empty*-NSPs were detected six weeks after exogenous expression of *Bmi1*. In agreement with this observation, blocking methylation in *Bmi1*-NSPs at six weeks restored *EphA7* expression, indicating that methylation does indeed contribute to *EphA7* silencing at this time point.

We also investigated whether any additional repressive modifications were present upon *Bmi1* overexpression. Increased levels of the repressive mark H3K27me3 were observed at both two and six weeks post-transduction. This implies that *EphA7* silencing by BMI-1 already starts as early as two weeks by H3K27 trimethylation (without a change in DNA methylation) and is maintained at six weeks by both H3K27 trimethylation and DNA methylation.

Studies show that the up-regulation of PcG proteins leading to DNA methylation might involve PRC2 complex or both PRC1 and PRC2 complexes. EZH2 of the PRC2 complex has been shown to associate with the activity of DNA methyltransferases DNMT1, DNMT3a and DNMT3b. This would lead to DNA methylation of EZH2-target promoters [178]. Loss of DNMT1 or DNMT3b has been shown to result in lower levels of PRC1 (especially BMI-1) and PRC2 subunits and in loss of PRC1-mediated H3K119ub1 mark [179]. Hence, while EZH2 (PRC2 subunit) acts as a recruitment platform for DNMTs, the latter recruit PRC1 subunits (e.g. BMI-1, MEL-18) for the maintenance of H2AK119 mono-ubiquitination at PcG target genes, thereby leading to chromatin compaction and gene repression.

Our study demonstrates that methylation of both histones and DNA can be mechanisms utilized by BMI-1 to repress *EphA7* in NSPs. Even though total knock-out of *EphA7* does not cause defects in adult neurogenesis, nor does it strongly reverse the phenotype of *Bmi1*$^{-/-}$ neurogenic niches, the partial rescue observed in the LVW indicates that this might be one possible mechanism through which BMI-1 acts as neurogenic control. Furthermore, since EphA7 belongs to a very large family including at least 14 receptors [180], other EphA receptors expressed in the LVW are likely compensating for *EphA7* deficiency, hence explaining the modest proliferative rescue observed in the double knock-out (*Bmi1*$^{-/-}$*EphA7*$^{-/-}$) brain.

7.3. Article 3: Monoallelic deletion of *Snf5* exon 1 in Nestin-expressing cells results in a severe developmental brain phenotype

SNF5 is deleted in the majority of malignant rhabdoid tumors, including AT/RT. AT/RT is believed to originate from a neural progenitor cell [1-3]. Our objective was thus to study the effect of *Snf5* deletion in NSPs. *Snf5* deletion was targeted to Nestin-expressing stem/progenitor cells using the Cre-Lox technology. Interestingly, *Snf5*$^{flox/+}$*/Nestin-Cre*$^{+}$ mice showed some features characteristic of the Coffin-Siris syndrome, a rare congenital disorder where more than 50% of patients carry a mutation in one of the five genes encoding a SWI/SNF complex unit [181].

***Snf5*$^{F/+}$*Nestin-Cre*$^{+}$ mice show a severely immature hippocampus and cortex**

Snf5 heterozygous (*Snf5*$^{F/+}$*Nestin-Cre*$^{+}$) littermates were much smaller in size than *Snf5*$^{F/+}$*Nestin-Cre*$^{-}$ control mice and showed a smaller brain with a thinner cerebral cortex, absence of the corpus callosum and disconnected forebrain hemispheres. This result was unexpected since *Snf5*$^{F/+}$*Nestin-Cre*$^{+}$ mice have recently been generated and found to be normal [182]. This study generated the same mouse model as ours by introduction of the Nestin-Cre transgene in *Snf5*$^{F/F}$ mice. Complete loss of *Snf5* in Nestin-positive cells resulted in embryonic lethality as no live *Snf5*$^{F/F}$ pups were obtained. On the other hand, excision of only one *Snf5* allele in Nestin-expressing cells was not sufficient for AT/RT development as *Snf5*$^{F/+}$*Nestin-Cre*$^{+}$ pups were grossly normal, healthy and fertile and had a lifespan of over a year.

Despite using identical *Nes-Cre* [183] and *Snf5*$^{F/F}$ transgenic animals [151], our two mouse models are strikingly dissimilar. The difference in results might be due to different distribution of Cre recombinase arising probably following recombination events over the years since the first generation of the Nestin-Cre strain. Differences might also be attributed to the different genetic background of the animals used in their study and ours.

The *Snf5*$^{F/+}$*Nestin-Cre*$^{+}$ mouse shows lower neuronal cell density in the hippocampus and cortex

In order to investigate whether the microcephalic brain phenotype of the *Snf5*$^{F/+}$*Nestin-Cre*$^{+}$ mouse was due to altered neuronal density, cresyl violet staining of the two brain areas (the hippocampus and the cortex) was carried out. The *Snf5*$^{F/+}$*Nestin-Cre*$^{+}$ brain showed reduced cell density in these investigated areas. The brain defects could be a consequence of fewer neurons in the hippocampal region (DG and CA) and cortex. Nestin being a marker of the developing CNS neural stem/progenitor cells [184, 185], our study shows that

deletion of *Snf5* severely severs development and/or differentiation of neural progenitors to neurons (and very possibly glial cells too).

Neural specific SWI/SNF complexes, namely neural progenitor BAF (npBAF) and neural BAF (nBAF) exist in mammals where switching from npBAF to nBAF has been found to be essential for neural differentiation [186]. The switch involves exchange of three subunits (BAF53a, BAF45a, SS18) of the npBAF complex as neural progenitors exit the cell cycle, for BAF53b, BAF45b/c and CREST to make up the nBAF complex in post-mitotic neurons (Figure 11) (reviewed in [187]). Another speculative reason why our model shows reduced neural density might be because the haploinsufficiency of *Snf5* produces npBAF and nBAF complexes with lowered expression or altered composition and consequently altered subunit interaction within the complexes, hence resulting in reduced proliferation of neural progenitors and also impaired differentiation of the few progenitors. How the position of the SNF5 subunit (SNF5 subunit being known as BAF47) or its absence within the complex affects the complex as a whole is so far not clear and might be context- and cell- dependent, but in MRTs the loss of SNF5 jeopardizes stability of the complex and prevents a switch from proliferation-promoting to differentiation-inducing SWI/SNF complexes [188].

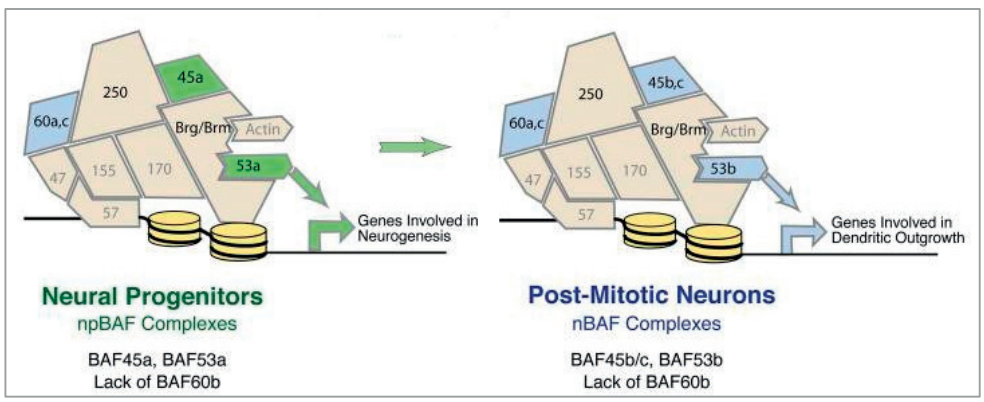

Figure 11. Switch in SWI/SNF complex subunit composition during neural development (Figure taken from [186]).

8.0. Concluding remarks and future perspectives

In **Article 1**, we propose that the regulation of ANXA2 by PODXL occurs via the MAPK pathway. It remains to be established how PODXL and ANXA2 promote cell viability. During execution of the project we speculated that this might occur by stabilization of the plasma membrane, rendering it mechanically resistant. PODXL is the major protein on podocytes that are under constant fluid pressure [64], and mechanical dissociation of our cells during flow cytometry analyses places them under mechanical stress. Our investigations (mechanical wounding using glass beads followed by flow cytometry analyses), though having failed to show this so far, could instead be focused at the lysosomal level [172] since invasive cells have been shown to have fragile lysosomal membranes [174] and improved plasma membrane repair in order to maintain membrane integrity following stresses and injuries [173]. In mammals, repair of wounded plasma membrane can occur through patching of the membrane by fusion of intracellular lysosomes at the injury site (reviewed in [189]. Plasma membrane repair can also occur by removal of the injured membrane by lysosome exocytosis and secretion of acid sphingomyelinase leading to pore endocytosis or shedding of the wounded membrane by ectocytosis [190]. We could measure levels of acid sphingomyelinase secreted by lysosomes during plasma membrane repair, or stain our cells to visualize lysosomes recruitment at the injured membrane. If our hypothesis holds true, following mechanical damage we might observe elevated levels of acid sphingomyelinase and a higher number of lysosomal vesicles at the plasma membrane in *Podxl*-NSPs due to improved plasma membrane repair by ANXA2, compared to control NSPs.

In **Article 2**, we showed that increased levels of the PcG protein BMI-1 promote self-renewal of neural stem/progenitor cells *in vitro*. All reported effects of BMI-1 on processes such as DNA damage response, senescence, stem cell maintenance and cell cycle cannot be explained by known targets and we identified the tumor-suppressor gene *EphA7* as a novel direct target of BMI-1.

As discussed earlier, the up-regulation of PcG proteins such as BMI-1 leading to DNA methylation of targeted genes such as *EPHA7* might involve both PRC1 and PRC2 complexes: EZH2 associates with DNMTs that subsequently recruit PRC1 complex subunits such as BMI-1 for ubiquitination of H2AK119 at *EPHA7*, leading to *EPHA7* repression. The delayed DNA methylation observed *in vitro* at

six weeks in our *Bmi1*-overexpressing NSPs will have to be investigated *in vivo* in order to have an indication whether hypermethylation of *EPHA7* correlates with later stages of tumor development.

In **Article 3**, we showed that *Snf5*$^{F/+}$*Nestin-Cre*$^{+}$ mice exhibit severe microcephaly. Our model demonstrates the lineage-dependent effect of *Snf5* ablation on brain development. We found that the phenotypes of our generated mice are in disagreement with a recent published model using the same mouse crosses, *Snf5*$^{F/F}$ mice and Nestin-Cre mice.

In future studies, we want to investigate why *Snf5*$^{F/+}$*NesCre*$^{+}$ leads to the severely immature brain phenotype we observed. In order to find the cell of origin of AT/RT, one could start *in vitro* by isolating Nestin-expressing cells from the brain of *Snf*$^{-/-}$ and wild type control mouse embryos to observe any change in the cells towards the rhabdoid phenotype.

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