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SOX IN DEVELOPMENT AND DISEASE

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SOX in development and disease

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To my family – past, present and future.

"There is an art, it says, or rather, a knack to flying. The knack lies in learning how to throw yourself at the ground and miss."

Douglas Adams, *Life, the Universe and Everything* (1982)

POPULÄRVETENSKAPLIG SAMMANFATTNING

Kroppen är uppbyggd av tiotals biljoner celler som alla har sitt ursprung i det befruktade ägget. Nya celler bildas under fostertiden via celldelning och cellerna specialiseras under utvecklingens gång för att bilda de ca 200 olika celltyper som kroppen består av. De flesta av cellerna i den vuxna kroppen kan inte dela på sig för att bilda nya celler. Dom är specialiserade och anpassade att utföra en viss uppgift och kan antingen vara långlivade, som hjärnceller, eller kortlivade, som cellerna i mag-tarmkanalen och huden. Celler som är kortlivade, slits och behöver bytas ut. Nya celler bildas av stamceller som finns i de flesta av våra vävnader, men som utgör endast en liten del av dessa. Stamceller har den unika förmågan att kunna dela på sig för att skapa nya specialiserade celler och samtidigt kunna skapa nya stamceller.

Stamceller ser olika ut och deras egenskaper skiljer sig åt i olika vävnader. Till vilken grad stamceller från olika vävnader använder sig av samma mekanismer för att bibehålla stamcellsfunktion och identitet är något som vi har tittat närmare på i denna avhandling. SOX är namnet på en familj av transkriptionsfaktorer dvs. protein som kan binda till arvsmassan (DNA) för att bestämma vilka gener som ska vara aktiva eller inte. Vi har funnit att SOX2, som finns i stamceller från flera olika vävnader, binder till gener som är aktiva gemensamt i dessa celler, men framförallt till gener som är aktiva specifikt i de olika stamcellstyperna. Detta kan verka förvånande eftersom SOX2 binder till samma sekvens (DNA-kod) i arvsmassan i alla stamcelltyper. SOX2-bindning till arvsmassan styrs därför troligtvis med hjälp av cellspecifika partnerfaktorer, vilket också är vad vår analys tyder på. SOX2 som binder till arvsmassan kan i samarbete med andra transkriptionsfaktorer styra aktiviteten av specifika gener i dessa olika stamcellstyper. En gemensam funktion som är beroende av SOX2 i alla olika stamcellstyper, och som vi studerat närmare, är hur ofta stamcellerna delar på sig för att skapa nya celler.

Alla celler som har förmågan att dela på sig i kroppen utgör också en risk för cancerutveckling. Varje gång en cell delar på sig så måste arvsmassan kopieras. Denna process är inte felfri och mutationer, felskrivningar, i arvsmassan kan uppstå vid celldelning, och dessa går i arv till alla nya celler som bildas från den specifika stamcellen som felet uppstod i. Mutationer som uppstår i vissa gener, onkogener eller tumörsuppressorer, är särskilt allvarliga. Dessa kan leda till ökad celldelning, men också till minskad celldöd, som annars är en av kroppens försvarsmekanismer för att bli av med tumörbildande celler. Kroppen har välstuderade försvarsmekanismer mot tumörbildande celler, men hur dessa kan aktiveras efter uppkomsten av tumörframkallande mutationer specifikt i stamceller är fortfarande oklart. Vi har tittat närmare på stamceller i hjärnan och i magsäcken och funnit att medlemmar av SOX-familjen som är aktiva i dessa celler kan reagera på tumörframkallande mutationer och starta försvarsmekanismer för att blockera cancerväxt.

Tillsammans visar våra data på viktiga funktioner för SOX-familjen i regleringen av stamceller under utvecklingen samt för skyddet av stamceller vid ett cancerhot. Vikten av en djupare förståelse för stamceller generellt och för uppkomsten och utvecklingen av cancer är stor för att kunna finna nya sätt och mediciner att behandla denna sjukdom.

ABSTRACT

As stem cells are needed not only to build our bodies during development, but also to maintain tissue function during adulthood, it is of great importance to the organism to maintain their integrity. However, stem cells also pose a threat, as oncogenic mutations can transform them or their progeny to malignant cells giving rise to cancer. Many SOX members have been defined as master regulators of stem cell maintenance, cell fate specification and differentiation. How SOX factors regulate stem cell function across different lineages and also how they can contribute to disease protection following oncogenic insult are key questions in this thesis.

In **Paper I** we investigate how SOX2 can regulate both cell type specific and common features of stem cells from four different tissues of two germ layers. Using ChIP-seq and RNA-seq analysis we find that although SOX2 binds some common targets, SOX2 binding is mostly cell type specific. This specificity coincides with motif enrichment of common or cell type specific transcription factors. We further show that SOX2 can interact with, and functionally regulate gene expression together with these transcription factors. Moreover, we also find that isolated peak regions can act as cis-regulatory modules (CRMs) to activate germ layer specific and common expression in zebrafish.

In **Paper II** we ask how adult stem cells of the brain can evade oncogenic transformation and elicit an appropriate tumor suppressor response. We find that the functionally related SOX5, SOX6 and SOX21 (SOX5/6/21) are induced in neural stem cells after oncogenic expression, and that this upregulation is required for the cells to repress tumor development. We also demonstrate that the expression levels of *SOX5/6/21* are significantly lower in human tissue from highly malignant glioma compared to glioma of lower malignancy grade, and that re-establishing SOX5/6/21 expression in human glioma cells leads to a re-gain in tumor suppressor function and response. We further show that these functional characteristics are at least in part dependent on the ability of SOX21 to stabilize the protein levels of the tumor suppressor p53.

In **Paper III** we expand on the findings demonstrated in Paper II and ask whether stem cells of different origins and with different characteristics use SOX21 in a similar manner to establish protection from oncogenic transformation. Using the stomach as a model system, we find stem cell expression of SOX21 in both mouse and human tissue, and that the *SOX21* mRNA levels are significantly downregulated in human gastric cancer compared to normal tissue. By overexpressing SOX21 in human gastric cancer cell lines, we find that proliferation decreases and apoptosis is induced, but only in cell lines expressing p53. We further show that wt p53 levels are increased after SOX21 expression and we speculate that this could at least in part be responsible for the increase in the anti-tumorigenic response.

Together, the work in this thesis highlights SOX transcription factors as important regulators of the vastly different but connected processes of stem cell maintenance and stem cell protection. Performing these functions SOX proteins use both their well-studied ability to bind and regulate gene expression together with partner factors, but also an ability to bind and affect proteins at a post-translational level.

LIST OF SCIENTIFIC PAPERS

I. Daniel W. Hagey, Susanne Klum*, **Idha Kurtsdotter***, Cécile Zaouter*, Danijal Topcic, Olov Andersson, Maria Bergsland and Jonas Muhr
SOX2 regulates common and specific stem cell features in the CNS and endoderm derived organs
PLOS Genetics 2018 Feb 12;14(2)

II. **Idha Kurtsdotter***, Danijal Topcic*, Alexandra Karlén, Bhumica Singla, Daniel W. Hagey, Maria Bergsland, Peter Siesjö, Monica Nistér, Joseph W. Carlson, Veronique Lefebvre, Johan Holmberg and Jonas Muhr
SOX5/6/21 prevent oncogene-driven transformation of brain stem cells
Cancer Research 2017 Sep 15;77(18):4985-4997

III. **Idha Kurtsdotter**, Maria Bergsland, Daniel W. Hagey, Danijal Topcic, Fredrik Klevebro, Magnus Nilsson and Jonas Muhr
SOX21 induces cell cycle arrest and apoptosis in gastric cancer cells
Manuscript

*These authors contributed equally to this work

CONTENTS

LIST OF ABBREVIATIONS

TF	Transcription Factor
ICM	Inner Cell Mass
TE	Trophoectoderm
ESC	Embyonic Stem Cell
TSC	Trophoblast Stem Cell
iPSC	Induced Pluripotent Stem Cell
NSC	Neural Stem Cell
SVZ	Subventricular Zone
CSC	Cancer Stem Cell
PCD	Programmed Cell Death
MOMP	Mitochondrial Outer Membrane Permeabilization
EMT	Epithelial-Mesenchymal Transition
CTL	Cytotoxic T-Lymphocyte
WHO	World Health Organization
GBM	Glioblastoma Multiforme
TCGA	The Cancer Genome Atlas
CIMP	CpG Island Methylator Phenotype
RTK	Receptor Tyrosine Kinase
CDK	Cyclin Dependent Kinase
OPC	Oligodendrocyte Progenitor Cell
GFAP	Glial Fibrillary Acidic Protein
GSC	Glioma Stem Cell
EBV	Epstein-Barr Virus
MSI	Microsatellite Instability

CIN	Chromosomal Instability
GS	Genomically Stable
MSS	Microsatellite Stable
ChIP-seq	Chromatin Immunoprecipitation sequencing
CRM	Cis-Regulatory Module

1 INTRODUCTION

Stem cells are essential to our lives. From the totipotent stem cells present in the earliest embryo just after the fertilization of the egg, to the pluripotent and multipotent stem cells building the body during embryonic development. As adults, we depend on organ specific stem cells and without them our bodies would deteriorate, some organs even within days, and death would be certain (Arnold et al., 2011). Ageing, characterized by diminishing organ function and declining regeneration (repair), is in part due to a malfunctioning stem cell compartment (Rando, 2006). But stem cells also constitute an inherent threat to our organism. Any actively proliferating cell will incorporate errors during DNA replication that will be passed down to all daughter cells generated. Adult stem cells accumulate mutations at a steady rate, about 36 per cell and year (Blokzijl et al., 2016), and when mutations appear in genes important for cell cycle regulatory functions it can give rise to cells with growth advantages and with the ability to expand at the expense of others. When enough severe mutations have accumulated, hyperplasia and cancer will arise (Curtius et al., 2018).

Because of their importance, the regulation of stem cell function has to be precise. How this is achieved, in stem cells of different origins, and how adult stem cells protect themselves from oncogenic transformation are two questions central to this thesis. We have addressed them in the context of SOX transcription factors as we have examined their roles in development and disease.

1.1 STEM CELLS AND DEVELOPMENT FROM A SOX PERSPECTIVE

Stem cells, as the name suggests, are the stem from which other cell types emerge, and this is one of two defining characteristics. The other characteristic is the maintenance of their own identity and potential after mitosis, known as self-renewal. There are several types of stem cells, with varying potency, ranging from totipotent to unipotent. Cells from the pre-implantation zygote up to the morula stage are totipotent, meaning that they are able to generate all cells within the embryo and in addition all the extraembryonic tissues. Cells from the inner cell mass of the blastocyst are pluripotent and can give rise to all cells within the embryo, but contributes only marginally to the extraembryonic tissues. Finally, further down the developmental cascade multipotent stem cells in different locations give rise to all the specific organs of the animal, and in the adult, both multipotent and unipotent stem cells produce differentiated progeny to maintain organ homeostasis and to repair tissue damage (De Los Angeles et al., 2015).

Multiple transcriptional networks and signaling pathways, including FGF, BMP, WNT, SHH and NOTCH are instrumental for the coordination of the developmental program (Briscoe, 2009; Louvi and Artavanis-Tsakonas, 2006; Merrill, 2012; Thisse and Thisse, 2005; Zhang and Li, 2005). One group of transcription factors (TFs) with important developmental functions, often interacting with theses signaling pathways both genetically and physically, is the SOX family (Sarkar and Hochedlinger, 2013). The SOX (Sry box containing protein) proteins constitute an ancient family of TFs present in all metazoans, with some evidence suggesting that they might even predate multicellularity (Bowles et al., 2000; Guth and Wegner, 2008; Wilson and Dearden, 2008). They bind DNA in a motif specific manner, but rely mainly on co-binding of

partner factors for cell specific DNA binding (Kondoh and Kamachi, 2009), which make them versatile and highly adaptable to influence multiple processes in a wide variety of cell types.

1.1.1 Pluripotent stem cells

The first SOX factor to be expressed during development is SOX2, which is present already in the zygote being translated from maternal mRNA. Early embryonic expression of SOX2 is crucial for the epiblast and the extraembryonic endoderm and targeted embryos die shortly after implantation, whereas zygotes depleted for maternal *Sox2* mRNA arrest already at the morula stage (Avilion et al., 2003; Keramari et al., 2010). After the first lineage specification in the embryo, creating the inner cell mass (ICM) and the outer trophoectoderm (TE) of the blastocyst, the expression of SOX2 becomes mostly restricted to the ICM, being expressed only in a subset of cells of the TE (Cockburn and Rossant, 2010; Keramari et al., 2010). SOX2 binds to the *FGF4* enhancer and induces its expression, which is important for the survival and development of the surrounding TE, which express the receptor FGFR2 (Rossant and Cross, 2001; Yuan et al., 1995).

Pluripotent embryonic stem cells (ESC) can be isolated from the ICM of the blastocyst and be indefinitely propagated *in vitro* as self-renewing undifferentiated cells that retain the potential to differentiate into any cell of the body upon receiving the appropriate cues (De Los Angeles et al., 2015; Martello and Smith, 2014). Central to the transcriptional network maintaining pluripotency are the core transcription factors OCT4 and NANOG together with SOX2. Together they bind enhancers and promoters, including their own, to activate expression causing a feed forward loop stabilizing the expression of pluripotency genes (Boyer et al., 2005). In addition, SOX2, OCT4 and NANOG also cooperatively bind and repress other promoters and enhancers, especially those whose expression induce differentiation along the different germ layers, thus keeping these genes in an inactive, yet ready state (Bernstein et al., 2006; Boyer et al., 2005; Lee et al., 2006). SOX2 by itself is necessary to maintain self-renewal and pluripotency, mainly through the induction of OCT4. ESCs cannot be established from SOX2 deficient ICM and already established ESCs depleted for SOX2 will undergo differentiation towards the TE lineage (Masui et al., 2007). Furthermore, the levels of SOX2 and OCT4 need to be kept within a precise range, as an increase in SOX2 will also lead to differentiation and an increase in SOX21, further pushing the cells down the ectodermal, mesodermal and TE lineage (Mallanna et al., 2010).

Trophoblast stem cells (TSCs) can be isolated from the embryo at a similar stage as ESCs but are derived from the TE rather than the ICM. As a close yet distinct lineage, these cells can also be kept as self-renewing cells *in vitro* and can be differentiated into multiple lineages (Latos and Hemberger, 2014). Whereas SOX2 is also essential for the self-renewal of these cells, it is interesting to note that the DNA binding profile of SOX2 differ extensively between TSCs and ESCs. While SOX2 interacts with OCT4 in ESCs to activate target gene expression, SOX2 interacts and binds together with TFAP2C in TSC to regulate a different set of targets. Thus, although activating a small set of common targets important for self-renewal in both cells types, SOX2 exhibits its functions in a largely cell specific manner, by interacting with cell specific co-factors (Adachi et al., 2013). Apart from SOX2, SOX17 is an important factor establishing TSC,

suppressing the alternative ICM cell fate by counteracting NANOG at SOX2/OCT4/NANOG bound enhancers (Niakan et al., 2010). In addition to SOX2 and SOX17, SOX21 is also expressed in TSCs, and its expression has been found in a subset of TE derived extraembryonic cells. However, its role remains elusive, since although overexpression of SOX21 has an effect on TSC differentiation favoring some fates over others, *Sox21* knock out animals survive the embryonic period with no major phenotypes (Cheung et al., 2017; Kiso et al., 2009; Moretto Zita et al., 2015).

More than ten years ago, the first induced pluripotent stem cells (iPSCs) were produced by transducing mouse embryonic or adult fibroblast with expression constructs for *Oct4*, *Sox2*, *Klf4* and *c-Myc* (OSKM) (Takahashi and Yamanaka, 2006). Reprogramming of differentiated cells had already been done, by transferring nuclear content into oocytes (Wilmut et al., 1997) or fusing cells with ESCs (Cowan et al., 2005) but the possibility to do this by adding only four factors opened up the field of regenerative medicine. It has been shown that upon expression, OSKM bind target genes cooperatively to induce expression and epigenomic changes, where OSK act as pioneering factors binding to inactive chromatin and c-MYC mainly stabilizes OSK binding to make reprogramming more efficient (Soufi et al., 2012). Furthermore, in a single-cell single-molecule study it was shown that SOX2 binding is the first event in which SOX2 searches the DNA for binding sites, and when found, OCT4 joins and the binding is stabilized (Chen et al., 2014).

1.1.2 SOX in tissue specific stem cells

As organogenesis starts, SOX TFs become widely expressed and are involved in the development of most organs, either regulating stem cell identity, or as fate specification and differentiation inducers (Kamachi and Kondoh, 2013). Briefly, stem cell expression can be found in the developing nervous system where it governs stem cell maintenance (Bylund et al., 2003; Scott et al., 2010) and glial specification (Stolt and Wegner, 2010), in neural crest regulating pluripotency, migration and fate choices (Haldin and LaBonne, 2010), in skeletal muscle satellite cells regulating cell cycle progression (Lee et al., 2004), in chondrocytes ensuring proper differentiation during skeletal development (Lefebvre, 2009), in endothelial cells and lymphatic system (Corada et al., 2013; Hosking et al., 2009; Wat et al., 2012) and in mesenchymal progenitor cells important for survival (Bhattaram et al., 2010). Since SOX activity in some organs is of higher relevance to this thesis, the stem cell expression in these cells will be discussed in greater detail.

1.1.2.1 Neural stem cells

At the time of neural induction, just after the formation of the primitive endoderm and concomitant with gastrulation, SOX1 and SOX3 expression is induced in the already SOX2 expressing epiblast, which together specifies the future CNS (Uchikawa et al., 2011). After neural tube formation, most neural stem cells (NSCs) express SOX1/2/3 in a redundant fashion and will continue to do so also in the adult (Favaro et al., 2009; Pevny and Nicolis, 2010), where they maintain the stem cell state and counteract the progression of neurogenesis, partly by blocking the activity of NOTCH signaling induced proneural proteins (Bylund et al., 2003; Holmberg et

al., 2008). This function is counteracted by the expression of the closely related SOX21, which instead promotes cell cycle exit and differentiation (Sandberg et al., 2005).

In addition to blocking differentiation, SOX2 also acts to reduce cell cycle activity by directly binding to and repressing the CyclinD1 (*Ccnd1*) promoter, preserving stem cell characteristics (Hagey and Muhr, 2014). Apart from these important functions, SOX1/2/3 further provides neural specificity to the signal interpretation of SHH and BMP along the dorsoventral axis of the spinal cord. This is achieved by co-binding gene regulatory regions together with the GLI and SMAD effectors of these pathways to activate the expression of target genes in distinct neural precursor cell populations (Oosterveen et al., 2012; 2013; Peterson et al., 2012).

Apart from binding and activating gene expression specific to NSCs, SOX2/3 also bind enhancers of silent genes not expressed in NSCs, but instead in differentiating neurons. While the binding of active genes is associated with the active histone modification H3K4me3, prebinding of silent genes is associated with both active (H3K4me3) and repressive (H3K27me3) histone modifications resulting in a poised state of the enhancers (Bergsland et al., 2011). A similar scenario is seen in ESCs where SOX2 prebinds genes that will later become active in NSCs (Lodato et al., 2013). This has led to a model where SOX prebinding promotes the formation of a permissive chromatin state allowing for rapid activation when the correct cellular context allows, and also where premature activation of these sites is inhibited by the prebinding itself (Bergsland et al., 2011).

In addition to the members of the SOXB family (SOX1/2/3 and SOX21), members of the SOXD (SOX5/6) and SOXE (SOX8/9/10) families are also expressed in NSCs. While SOXE members are mostly studied for their role in glial specification and differentiation, SOXD members have been found to modulate this function, but also to induce cell fate specification of distinct neuronal subtypes in the forebrain (Batista-Brito et al., 2009; Kwan et al., 2008; Lai et al., 2008; Stolt and Wegner, 2010; Stolt et al., 2006). Additionally, SOX5 has also been implicated in cell cycle regulation during development in vertebrate NSCs (Martinez-Morales et al., 2010). Although, most of the SOX TFs expressed in NSCs during development remain in adult NSCs, their functions in these cells have been less studied, except for those of SOX2 (Favaro et al., 2009; Ferri et al., 2004).

1.1.2.2 Endodermal stem cells

The endodermal organs esophagus, lung, stomach, liver, pancreas and intestine are all derived from an embryonic structure called the foregut. The foregut is a tube structure, developing much like the neural tube but in this case by the folding of the definitive endoderm (Sherwood et al., 2009). Patterning signals from the surrounding mesoderm, including WNT, BMP and FGF, induce budding of the foregut, which will create the domains that eventually bud off into the respective organs between E9.5 and E11.5 (Jacobs et al., 2012). In the foregut, SOX2 expression can be found in a patterned manner, with high expression anteriorly and dorsally. The dorsoventral gradient of SOX2, which is opposite to that of NKX2-1, is important for trachea and esophagus separation, and with decreased levels of SOX2 this separation will fail (Que et al., 2007). As the future lungs bud off, the low SOX2 levels will soon increase to become high in the

proximal airways (from trachea to bronchioles) and stay low in the distal airways (terminal bronchioles to alveolar ducts), where instead SOX9 is highly expressed. In the adult lung the SOX2 gradient is maintained and expression can be found in basal progenitors of the proximal airways, which serves important functions during regeneration after tissue damage (Que et al., 2009).

The stomach, similar to the trachea, expresses high levels of SOX2 that ends abruptly at the boundary between the stomach and the small intestine. At this junction, the expression of SOX2 is mutually exclusive with that of CDX2, marking the intestine (Sherwood et al., 2009). The high SOX2 levels during early development will progressively become lower in the glandular, distal stomach, and this coincides with the development from the simple cuboidal epithelium into the stratified squamous epithelia of the forestomach and the columnar gland forming epithelia of the glandular stomach (McCracken and Wells, 2017). SOX2 expression will remain into adulthood marking basal stem cells of the forestomach and gland stem cells in the glandular stomach (Arnold et al., 2011).

In the foregut and its derived organs, SOX2 expression is closely followed by that of SOX21 (Sherwood et al., 2009). Although *Sox21* deleted mice have not revealed any gross abnormalities in these organs, they have not been investigated in detail (Kiso et al., 2009).

1.1.3 Adult stem cells and tissue homeostasis

Adult resident stem cells constitute a minority of an organ and they serve to regenerate the multiple specialized cells that make the organ functional. Adult stem cells have been identified in most organs, but they differ in their abundance, proliferative and productive activity depending on the specific demands of the organ (Batlle and Clevers, 2017). For instance, the digestive tract constantly renews itself. The lifetime of a differentiated cell is between 7-10 days in mouse gastric epithelium, which therefore must have a very active stem cell compartment responding to this demand (Barker et al., 2010). This stem cell compartment express SOX2, but while deletion of these cells drastically affects tissue homeostasis, SOX2 expression by itself seems to be dispensable for stem cell function (Arnold et al., 2011; Sarkar et al., 2016).

In contrast to the epithelial organs with rapid turnover, most cells of the adult brain are never replaced and cell renewal is only rarely seen and then only in specific regions (Frisén, 2016). These neurogenic regions include the subgranular zone in the dentate gyrus of the hippocampus and the subventricular zone (SVZ) lining the lateral ventricles, which in mice give rise to interneurons of the hippocampus and olfactory bulb respectively (Hsieh, 2012). What kind of cells and how many these neurogenic zones give rise to in humans still remains a controversial question (Ernst et al., 2014; Sorrells et al., 2018; Spalding et al., 2013; Wang et al., 2014). Stem cells in these niches express several SOX proteins including SOX1/2/3 (Ellis et al., 2004; Ferri et al., 2004), SOX8/9/10 (Scott et al., 2010), SOX5/6 and SOX21 (Kurtsdotter et al., 2017), but have mostly been studied in the context of SOX2. Adult ablation experiments have revealed the importance of SOX2 for self-renewal and survival of hippocampal stem cells (Favaro et al., 2009), and loss of SOX2 leads to an increase in repressive histone modifications at genes that are normally bound by SOX2 (Amador-Arjona et al., 2015).

1.1.4 Cancer stem cells

The concept of cancer stem cells (CSCs) has been a hot topic in recent years, and the identification of their presence in a large number of different tumor types, mostly based on xenograft studies, have followed in rapid succession since the days of their recognition (Batlle and Clevers, 2017). Their definition has evolved over the years but it is still is based on similarities to normal stem cells, where a limited number of dedicated cells can produce and sustain the tumor mass (Nguyen et al., 2012; Valent et al., 2012). However, although the name might imply it, CSCs should not be confused with normal stem cells as always being the cells of origin for cancer. The definition states nothing about in what cell the first mutation arose or which was the first cell to become malignant. Although mutations and epigenetic alterations could in fact arise in stem cells, they could also arise in progenitor cells or cells further down the differentiation path, which would then acquire stem cell properties (Clarke et al., 2006; Valent et al., 2012).

CSC theory suggests that malignant stem cells produce transit amplifying and differentiated progeny that, at least in the beginning, show hierarchical resemblance to the tissue they arose in (Valent et al., 2012). Although, this seems to be true for many cancers, there are exceptions, and CSCs need not to be rare nor quiescent as was earlier suggested (Batlle and Clevers, 2017). Since CSCs are thought to be the main cause of relapse after treatment, therapies targeting CSCs were in the beginning of the field specifically thought to hold great promise as it was theorized that the ablation of CSCs would lead to the cessation of cancer growth. As it is becoming increasingly clear that the plasticity of normal stem cells seems to be more important than previously thought, remarkable CSC plasticity has also be demonstrated both in cell lines and primary cultures, where transit amplifying, or even fully differentiated cancer cells, can take on CSC features depending more on the niche than on cell intrinsic properties (Batlle and Clevers, 2017; Gupta et al., 2011; Shimokawa et al., 2017). Taken together, although successful treatment most likely will have to include specific targeting of CSCs, these cells can be of multiple identities within a single tumor, residing in several niches and be driven by different molecular programs. Consequently, as the CSC most likely represents a moving target, this constitutes a great challenge and will probably require a multi-targeting approach (Lathia et al., 2015).

SOX expression has been characterized in CSCs from a variety of tissues including brain, lung, colon, skin, and more (Gangemi et al., 2009; Lundberg et al., 2016; Santini et al., 2014; Singh et al., 2012; Vanner et al., 2014). This expression mostly relates to SOX2 and its function as an oncogene in these settings, but SOX9 has also been shown to maintain self-renewal and tumorigenicity of CSCs (Kawai et al., 2016; Larsimont et al., 2015; Matheu et al., 2012; Santos et al., 2016; Sashikawa Kimura et al., 2011). In addition, many more SOX TFs have been found to be differentially expressed in cancer cells compared to normal tissue, where some are correlated with worse prognosis and others with a survival benefit. The relevance of SOX expression in the case of glioma and gastric cancer will be discussed in greater detail in the following chapter.

1.2 CANCER

A beloved family member has during the writing of this thesis passed away in cancer. My dear aunt, who will be greatly missed by a lot of people. In passed years, I have already lost another aunt, an uncle and my grandfather. Two of my other aunts, and my father have been treated for cancer, and many more if I include the extended family and friends. There is no hereditary cancer in my family, this is just life as it is for most people. Cancer affects us all, and during our lifetime, one third of us will at some point receive a cancer diagnosis. The cancer burden is increasing in our society, which can be ascribed to an aging population, better screening and diagnostics, but also to a changed lifestyle with more cancer inducing risk factors (tobacco use, alcohol consumption, obesity and low intake of fruit and vegetables) (Stewart and Wild, 2014). Although the treatment of many cancers has seen great progress and the long term survival for all cancers combined has greatly improved since the 1970's – from 35% 5-year survival in men and 48% in women, to just above 70% for both men and women today – some cancers have proven notoriously difficult to treat and therefore seen little progress, among them brain cancer and stomach cancer (Socialstyrelsen, 2013).

From a historical perspective, the term cancer originates from the words *karkinos* and *karkinoma* coined by Hippocrates (460-360 BC), father of medicine, to describe diseases that produced mass (*onkos*). Cancer has plagued humanity, from prehistoric times to presence, but the earliest solid evidence for cancer comes not from humans, but from dinosaurs that lived ~70 million years ago. Both benign and malignant tumors have been found in fossils from the duck-billed dinosaur *Cretaceous hadrosaur*, indicating that cancer is indeed an old phenomenon (Faguet, 2015). The earliest human, or hominin, cancer found is an osteosacoma in a 1.7 million-year-old fossil from South Africa (Odes et al., 2016). Moreover, there are also descriptions of cancer found in Egyptian papyri written 1500-1600 BC, in which not only the cancer itself is described, but also surgical, pharmacological and magical treatments for the disease (Faguet, 2015).

We can conclude that cancer has been with us as a part of human life and death from the very beginning, and will continue with increasing incidence to burden our society in the future. To answer to this ever increasing health threat, we need to continue to increase our understanding of its origins and mechanisms of progression, to find new treatments, and hopefully cures, to be able to manage this devastating disease.

1.2.1 Prevalence

Cancer is a major cause of death and morbidity all over the world. Although anyone can be afflicted, the incidence and mortality of specific cancers will vary greatly in different areas of the world. There is a difference in genetic predisposition to various cancers, but also in risk factors across the world with some cancers being associated with poverty and some with an industrialized life style. It is estimated that the cancer incidence will increase with more than 70% in the coming two decades, and much of this increase will be seen in low and middle-income countries. These are countries that already struggle with cancers linked to poverty such as infection-related cancers, and which will now see a rise in cancers associated with an

industrialized life style with the increase of obesity, tobacco use and high intake of alcohol and processed foods. (Stewart and Wild, 2014)

In Sweden, we have seen a 40% increase in the number of newly diagnosed cases since 1970, and cancer is expected to continue this increase with approximately 1% per year. The most common cancer in Sweden is prostate cancer, which is closely followed by breast cancer and they account for about 30% of the cancer cases for men and women separately. The following most common cancers are skin cancer, colon cancer, lung cancer and malignant melanoma. The most notable single risk factor for cancer development is tobacco smoking, and its effect on several cancers is well established. In Sweden, about 15% of all cancer cases are estimated to have a link to smoking and this includes cancers of the lung, head and neck, bladder, kidney, stomach, liver and pancreas (Socialstyrelsen, 2013).

1.2.2 Hallmarks of cancer

Cancer is not one disease, but rather a collection of diseases that differ substantially between people, even within the same cancer type. Genetic alterations, phenotype and prognostics vary greatly, not only between people but also dynamically within tumors. Despite this, some fundamental properties are shared between all cancers, and they are what have come to be known as the hallmarks of cancer (Hanahan and Weinberg, 2000). These hallmarks are a set of principles that cancer cells use to thrive (Figure 1). When this idea was first proposed, six hallmarks were recognized as being crucial for cancer growth and they included self-sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis, sustained angiogenesis, limitless replicative potential and tissue invasion and metastasis (Hanahan and Weinberg, 2000). They were later updated to also include two enabling characteristics; tumor promoting inflammation and genome instability and mutation, and two emerging hallmarks; deregulation of cellular energetics and evading immune destruction, (Hanahan and Weinberg, 2011).

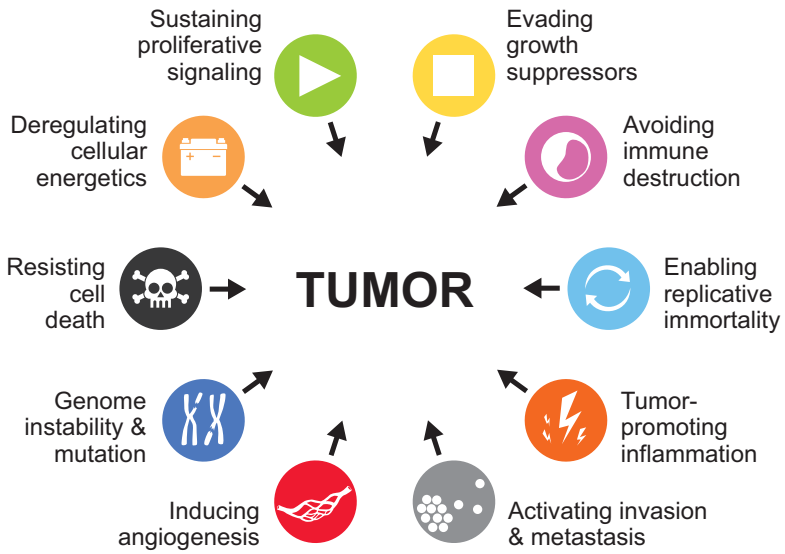

Figure 1. The hallmarks of cancer. Adapted from (Hanahan and Weinberg, 2011)

1.2.2.1 Self-sufficiency in growth signals

While normal cell growth and proliferation are limited by the availability of growth factors, to ensure homeostasis and proper tissue organization, cancer cells have acquired self-sufficiency and are no longer depending on limiting mitogenic signals. There are several ways by which cancer cells can achieve this function and one is by simply producing the growth factor themselves, so that together with the expression of the appropriate receptor, an autocrine loop is established (Patsialou et al., 2009). Alternatively, cancer cells can stimulate surrounding tissue, the cancer stroma, or differentiated cells within the tumor to provide the limiting mitogen (Wang et al., 2018). Furthermore, the growth signal itself can be enhanced by overexpressing the wt receptor or a mutated activated version of it, which is independent of ligand binding for signal transduction (An et al., 2018). Additionally, growth factor independence can also be achieved by mutations deregulating downstream components of the pathway that will ultimately result in increased pathway activation independent of receptor signaling (Hanahan and Weinberg, 2011).

1.2.2.2 Insensitivity to anti-growth signals

The ability to sense when to stop proliferating is essential for tissues in order to prevent hyperplasia or genotoxic effects by DNA damage. The two most important pathways for sensing this are the RB cell cycle pathway and the p53 tumor suppressor pathway. While RB receives and conveys responses from both extracellular signals, including contact inhibition, and intracellular signals such as oncogene activation and hyperproliferation, p53 mainly senses intracellular stresses such as DNA damage. Consequently, activation of the RB pathway will lead to a halt in the cell cycle, and activation of the p53 pathway will lead to cell cycle arrest, transient or permanent (senescence), DNA repair or apoptosis depending on the strength and duration of the signal (Meek, 2009). With these important regulatory functions, it is not surprising to find some of the most frequent cancer mutations in these pathways, both as mutagenic activations or gene amplifications of oncogenes and as homozygous deletions or epigenetic silencing of tumor suppressors (Brennan et al., 2013).

1.2.2.3 Evading cell death

Programmed cell death (PCD), which in contrast to necrosis is a highly regulated and controlled process, can occur by multiple mechanisms, which are all implicated in cancer development. The best studied and probably most relevant of these, apoptosis, can be activated by the extrinsic or the intrinsic pathway, where the former is receiving extracellular death-signals and the latter is sensing various intracellular stresses. Signaling through either the extrinsic or the intrinsic pathway results in the activation of the effector Caspases; Caspase 3, Caspase 6 and Caspase 7 and proteolytic degradation of cytosolic content (Li and Yuan, 2008; Mariño et al., 2014). The intrinsic program, sensing intracellular stressors such as DNA damage, oncogene activation and hyperproliferation, is more widely implicated cancer. It involves the permeabilization of the outer mitochondrial membrane (MOMP) and the release of cytochrome c, serving as a decisive event in determining whether a cell will commit to apoptosis or not (Fuchs and Steller, 2015). Cytosolic cytochrome c will promote the assembly of the apoptosome, a multiprotein complex

to which Caspase 9 is recruited and activated, and ultimately lead to the proteolytic cleavage and activation of the effector Caspases (Li and Yuan, 2008).

The process of MOMP is regulated by the BCL-2 family of proteins, containing both anti-apoptotic and pro-apoptotic members. In healthy cells, the anti-apoptotic BCL-2 and its close relatives BCL-XL, BCL-W, MCL-1, BCL-B and A1, are counteracting pro-apoptotic BAX and BAK of the same family. As stress signals are transmitted, (BCL2 homology domain 3) BH3-only proteins, a third part of the family, are induced either transcriptionally, by p53 activation, or post-translationally. These pro-apoptotic proteins, including BIM, BID, PUMA, BAD and NOXA neutralize the anti-apoptotic proteins and activate BAX and BAK leading to their homo oligomerization in the mitochondrial membrane, MOMP and cytochrome c release (Czabotar et al., 2014). Other modes of PCD include necroptosis and cell death induced by autophagy, where necroptosis is a controlled version of necrosis often induced by the same extracellular stimuli as for apoptosis, but where the downstream pathway is distinct, including several members of the RIPK family (Shan et al., 2018). Autophagy on the other hand, is essentially a cell preserving mechanism by which the stressed cell is trying to recycle its resources by degrading cytoplasmic content in autophagosomes, thereby preventing cell death (Mariño et al., 2014). If these precautions are insufficient, cell death will occur, either directly induced by autophagy, or more commonly, by the induction of apoptosis (Mariño et al., 2014).

Tumor cells find multiple ways to escape PCD, and apoptosis can be avoided by genetic aberrations at several levels. Cancer cells frequently up-regulate the anti-apoptotic BCL-2, BCL-XL or other survival signals, or alternatively downregulate the pro-apoptotic BAX, BIM or PUMA. Additionally, mutations in the p53 apoptosis-inducing pathway, including *TP53* itself, also represent a common mechanism to counteract apoptosis. Autophagy, on the other hand, can act either as a barrier to tumor formation or as an enhancer, depending on the cellular context. Therefore, the benefit for individual cancers of suppressing this pathway may differ depending on the underlying genetic mutations (Hanahan and Weinberg, 2011; Rosenfeldt et al., 2013). Although PCD is actively avoided by many cancers, most cancers are not completely cell death resistant and some tumors are actually more sensitive to apoptosis than normal cells. However, while dying, tumor cells do not disappear in silence, and dying itself, not only necrosis but also PCD, can have a tumor promoting effect by the release of several factors acting as "eat me" and "find me" signals inducing angiogenesis, proliferation and immune modulation (Ichim and Tait, 2016).

1.2.2.4 Sustained angiogenesis

Just like normal cells, tumor cells depend on the delivery of oxygen and nutrients by the blood, and tumor neovascularization is a surprisingly early process during cancer development. Tumor vessels are often highly aberrant, with excessive branching, large vessels, faulty blood flow, microhemorrhage and leakiness. In normal tissues angiogenesis is balanced by the presence of pro-angiogenic and anti-angiogenic signals, a balance that is disrupted in the tumor environment, in part by the presence of inflammatory cells that can help to induce the angiogenic switch. Certain oncogenes themselves can lead to the production of pro-angiogenic factors and

additionally, as previously discussed, signaling by apoptotic cells can lead to a pro-angiogenic environment (Hanahan and Weinberg, 2011).

1.2.2.5 Limitless replicative potential

Most cells in the body can only undergo an already defined number of cell divisions before permanent cell cycle arrest, also known as senescence, will definitively stop proliferation. Senescence is an irreversible, non-proliferative but viable and metabolically active state that is thought to function as a barrier to tumor development (Campisi, 2013; Hanahan and Weinberg, 2011). Replicative senescence is induced by progressively shortened telomeres in the absence of telomerase. This will eventually elicit senescence via the DNA damage response (DDR) signaling through ATM and ATR kinases and the p53 pathway (Kuilman et al., 2010). Unsuccessful execution of this program will lead to the fusion of chromosome ends, and this together with following rounds of mitosis will give rise to genomic instability by cycles of fusion and breakage of chromosomes. This will, under normal circumstances, induce cell crisis and death, unless other genetic perturbations are present. If these genomically instable cells are able to escape senescence or death, they can instead vastly contribute to tumorigenesis (Campisi, 2013).

In addition to telomere function, other signals can also contribute to senescence and thus prevent tumorigenesis, including DNA damage (outside the telomeres), oncogenic signaling, hyperproliferation and epigenetic damage. These signals are mainly transmitted by the master transcriptional regulators p53 and RB, where p53 activation will lead to the transcriptional activation of the downstream effector p21 which together with activated INK4A (also known as p16), an upstream regulator of RB, will halt cell cycle progression (Campisi, 2013).

Similar to PCD, senescent cells are not silent, instead they actively signal to their environment by a process known as Senescence-Associated Secretory Phenotype. This is a function that has been found to have both beneficial and deleterious effects. Beneficial in the way that it helps to establish the tumor suppressing senescent growth arrest, and deleterious in the way that it can stimulate proliferation and angiogenesis (Campisi, 2013). Although senescent cells constitute a frequent find in premalignant lesions, where they can have a beneficial function for the neoplastic community of cells, eventually for malignant progression to occur, tumor cells need to acquire a mechanism to maintain sufficient telomere length to avoid senescence and apoptosis. This is most often achieved by the upregulation of telomerase and less frequently by an alternative mechanism involving recombination based telomere maintenance (Hanahan and Weinberg, 2011).

1.2.2.6 Invasion and metastasis

The capability of a tumor to disseminate from its original mass, enter the blood stream and find new organs to seed metastases in, is largely depending on a process known as epithelial-mesenchymal transition (EMT) (Hanahan and Weinberg, 2011). This is a developmentally conserved process used in multiple stages during embryogenesis such as gastrulation, neural crest formation and heart development, and it involves changes in the adhesion molecule expression, allowing a gain in migratory and invasive behavior (Nieto et al., 2016).

In cancer, EMT TFs, mainly of the SNAIL, TWIST and ZEB families, are expressed and play an important role in all stages of tumor development, as they facilitate tumor growth, invasion, dissemination and metastasis, colonization and therapy resistance (Hanahan and Weinberg, 2011; Nieto et al., 2016). Additionally, EMT TFs have also been shown to maintain stemness properties. During the process of EMT, which is multistep and can also be partial, cells loose their epithelial characteristics, as seen by the downregulation of certain markers such as E-cadherin, Occludins and Cytokeratins. Further down the EMT process mesenchymal markers such as N-CADHERIN and VIMENTIN are up-regulated as the cells become highly migratory and invasive. EMT is not only, as the name might indicate, important for tumors of epithelial origin but is also a common feature of non-epithelial tumors such as glioma, melanoma, sarcoma and even leukemia (Nieto et al., 2016).

1.2.2.7 Genome instability and mutations

As an enabling characteristic, genome instability and mutability creates the functional heterogeneity among premalignant or malignant cells that facilitates the expansion of clones with beneficial traits, thus enabling tumorigenesis to occur or proceed. This can be achieved either by an increased sensitivity to mutagenic agents or by defective genomic maintenance, or by both. In addition, it can also be achieved by a broken surveillance and safety system, where cells with genomic damage should be neutralized, either by senescence or apoptosis, but escape and prevail. Consequently, cells with faulty or even oncogenic genomes are allowed to persist and the mutability can accelerate (Hanahan and Weinberg, 2011).

Although any cancer typically has large number of mutations, a small number of tumors have an elevated mutation rate and an increased mutation burden. This hypermutation phenotype is often found in cancers that evolve in a mutagenic environment such as in the case of melanoma (UV-light) or lung cancer (tobacco smoke), but is also found in many other cancers and can sometimes even be treatment induced and arise after relapse. Mutations in genes important for DNA replication repair or DNA mismatch repair are frequently associated with a hypermutation phenotype (Campbell et al., 2017).

1.2.2.8 Tumor promoting inflammation

Cells from both the innate and adaptive immune system are attracted to the developing tumor, and can be found in virtually all tumors, although with varying density. Despite the fact that these immune cells are probably attempting to clear the lesion, they paradoxically end up promoting tumorigenesis. Macrophages, mast cells, neutrophils and T and B lymphocytes that accumulate at the tumor site have all been shown to have their part in tumor promotion, mainly by secreting growth factors, survival factors, pro-angiogenic factors and extracellular matrix-modifying enzymes. This leads to sustained proliferation, decreased tumor cell death, and a facilitation of vessel formation, invasion and metastasis. Together with chemokines and cytokines that amplify the chronic inflammation, this creates a tumor thriving environment. Additionally, inflammatory cells release reactive oxygen species that increase the mutagenesis in nearby tumor cells thereby accelerating tumor progression (Hanahan and Weinberg, 2011).

1.2.2.9 Deregulation of cellular energetics

Under normal conditions most cells use oxidative phosphorylation as a way to produce energy in the form of ATP. Glycolysis, the first part of glucose catabolism, is mainly used as the dominant source of ATP production in anaerobic or hypoxic conditions and has the benefit of not needing oxygen, but in return produces less ATP. Energy metabolism is rewired in cancer cells and a switch is induced, from oxidative phosphorylation to glycolysis alone, a mechanism known as the Warburg effect. Why tumor cells do this, even in non-hypoxic conditions, is not completely clear, and although this is a common phenomenon, there are still many questions to be answered (Liberti and Locasale, 2016). It has been speculated that this may be a way to increase the production and availability of nucleotides and amino acids required by the tumor cells for the rapid growth, by shunting partially degraded glucose into various biosynthetic pathways (Hanahan and Weinberg, 2011).

1.2.2.10 Avoiding immune destruction

In an immune competent host, the vast majority of tumorigenic cells arising are thought to be eliminated by immune surveillance. This process, also called cancer "immunoediting", is said to have three phases: elimination, equilibrium and escape. By continuously removing immunogenic tumor cells, a selection for cells with reduced immunogenicity occurs, which may eventually lead to immune escape, enabling tumor growth (Fouad and Aanei, 2017; Hanahan and Weinberg, 2011). Interestingly, patients with some types of cancer exhibiting heavy tumor infiltration of Cytotoxic T-lymphocytes (CTL) and Natural Killer cells actually have a better prognosis (Hanahan and Weinberg, 2011).

1.2.3 Glioma

1.2.3.1 Prevalence

The most common source of tumors in the brain is metastases from cancers outside the CNS, which are 5-10 times more common than primary brain cancer (Weller et al., 2015). Primary brain cancer occurs in both children and adults, but differ significantly in frequency, location, tumor type and prognosis. While primary brain cancer is the second most common cancer diagnosis in children (30% of all pediatric cancer) only surpassed by lymphomas together with leukemia, it is relatively uncommon in adults, accounting for only 2.5% of the cancer diagnoses in Sweden and 2% worldwide (Socialstyrelsen, 2013; Stewart and Wild, 2014). However, the impact of brain cancer is dramatic. In children, brain cancer is the leading cause of cancer-related death and in adults, although being rare, malignant brain cancer results in more years of life lost than do any other tumor (Rouse et al., 2016).

Glioma is the most common primary brain tumor type at all ages, but in children, embryonal tumors (mainly medulloblastomas) are almost as frequent. (Ostrom et al., 2017). The incidence rate of glioma increases with age, being the highest in the age group of 75-84 years. The reason for this is unknown, and although several environmental factors have been studied, the only one that has been recognized as causative is ionizing irradiation, where the effects of primary

pediatric brain cancer treatment is seen later in life, in the form of new malignancies of the brain (Weller et al., 2015).

1.2.3.2 WHO Classification

Brain cancer is diagnosed according to the World Health Organization (WHO) classification system that was recently updated, for the first time adding molecular parameters to the diagnosis criteria (Louis et al., 2016). In recent years a large number of sequencing projects have added significantly to our understanding of the molecular differences behind different glioma subtypes, including affected signaling pathways and driver mutations, which in part has led to the update. The classification system also includes grading for malignancy, where grade I is benign and grade IV the most malignant.

Gliomas account for almost 30% of all primary adult brain tumors, and being mostly highly malignant they are also responsible for the majority of deaths within this group. The larger part of glioma is classified under the group "diffuse astrocytic or oligodendroglial tumors", in which oligodendrogliomas are of grade II-III and astrocytomas of grade II-IV (Reifenberger et al., 2017). The most malignant grade IV astrocytoma is called Glioblastoma Multiforme (GBM) and it is sadly also the most common, accounting for more than 50% of all glioma. GBM is incurable and has a 5-year survival rate of only 5.5% (Ostrom et al., 2017). The poor outcome is mainly due to two characteristics of this disease, the first being its infiltrative growth, making complete surgical resection impossible. The second is the presence of cells with a strong resistance to chemo and radiotherapy, causing re-growth of the tumor (Zong et al., 2015).

1.2.3.3 Molecular Classification

In recent years, several large-scale sequencing efforts have rapidly expanded our knowledge and helped to map and classify many of the primary malignancies of the brain, often better than standard histological classification (Brennan et al., 2013; Buczkowicz et al., 2014; Cancer Genome Atlas Research Network, 2008; Cancer Genome Atlas Research Network et al., 2015; Ceccarelli et al., 2016; Jones et al., 2013; Pajtler et al., 2015; Parker et al., 2014; Sturm et al., 2012; Suzuki et al., 2015; Verhaak et al., 2010; Wu et al., 2014; Zhang et al., 2013). Several of these studies are based on material collected by The Cancer Genome Atlas (TCGA) project, which has collected data from more than 11,000 tumors of 33 cancers types, and which also has an Internet platform for easy access of all their data (Hutter and Zenklusen, 2018). Glioma and GBM were among the first tumors to be collected and are probably also the best-studied malignancies in this dataset.

The first molecular classification of GBM was proposed by TCGA to include a Proneural, Neural, Classical and Mesenchymal subtypes. The characteristic genetic events were found to be *IDH* mutation, *PDGFRA* alteration and *TP53* mutation/loss of heterozygosity for the Proneural class, expression of neuron markers for the Neural class, *EGFR* amplification, *TP53* mutation and homozygous *CDKN2A* deletion for the Classical class and deletions and mutations affecting the *NF1* and *PTEN* genes together with expression of mesenchymal markers for the Mesenchymal class (Verhaak et al., 2010). This classification has been mostly confirmed in

several studies, however, the Neural group has been removed, since refined RNA-seq using single cell analysis and pure tumor samples have revealed the tumors of this group as samples with a high degree of non-neoplastic brain cells (Wang et al., 2017). Furthermore, it is becoming increasingly clear, due to single cell analysis and multiple sampling of tumors, that several clones exists within each tumor and that they can often be of different molecular phenotypes (Meyer et al., 2015; Patel et al., 2014; Sottoriva et al., 2013; Wang et al., 2017). Additionally, the GBM subclasses are often not stable and almost half (45%) of tumors switch class after recurrence, which could be explained by differences in therapy resistance among present clones (Meyer et al., 2015; Wang et al., 2017). Altogether this means, that while intertumoral genetic and epigenetic mutation patterns are fairly stereotypical and can be easily categorized, the intratumoral polyclonality and heterogeneity should not be underestimated (Furnari et al., 2015).

Extended work of glioma molecular classification has added three subclasses for lower grade gliomas and two subclasses for pediatric GBM (Cancer Genome Atlas Research Network et al., 2015; Ceccarelli et al., 2016; Sturm et al., 2012). The lower grade clusters are all recognized by mutations in IDH, usually in IDH1, less frequently in IDH2, which lead to increased histone methylation and CpG island hypermethylation, a phenotype known as glioma CpG island methylator phenotype (G-CIMP) (Weller et al., 2015). IDH-mutant tumors can be divided into two clusters based on the presence of 1p/19q co-deletion (codel). IDH-mutant tumors with codel are most often oligodendrogliomas.

Although stratifying patients in this way may seem rational, the molecular classification has so far only added limited diagnostic, prognostic and predictive value, the exception being the recognition of IDH-mutation and 1p/19q codel as diagnostic factors, which has been included in the 2016 WHO classification as biomarkers (Reifenberger et al., 2017). Therefore, although GBM constitutes one of the best genomically characterized cancers, the effect on clinical outcome is yet to come (Lathia et al., 2015).

1.2.3.4 Glioma signaling pathways

Despite the lack of effect on clinical outcome, the extensive molecular mapping of GBM has led to the establishment of the most frequently targeted molecular pathways, and genetic alterations in *RB*, *TP53* and receptor tyrosine kinase (RTK) pathways are now recognized as core events in gliomagenesis. Somatic aberrations within these pathways have been found to be as frequent as 79%, 85% and 90% respectively, and in as much as 74% of tumors, alterations can be found in all three pathways (Brennan et al., 2013).

1.2.3.4.1 Receptor Tyrosine Kinase pathway

RTK signaling, being the most widely affected pathway, is important for mediating cell growth, survival and proliferation through several downstream signaling cascades, most notably through the PI3K/AKT and RAS/MAPK/ERK intracellular pathways (Figure 2) (Pearson and Regad, 2017). In normal cells, growth factors will bind to RTKs and induce their dimerization and autophosphorylation, which in turn will elicit downstream intracellular responses. Upon receptor activation, PI3K will translocate to the plasma membrane and induce the production of the

signaling molecule phosphatidylinositol 3,4,5-triphosphate (PIP_3) from phosphatidylinositol biphosphate (PIP_2), a production that is inhibited by tumor suppressor PTEN. PIP_3 production will lead to the recruitment of AKT to the plasma membrane and its phosphorylation and activation by PDPK1 (Vivanco and Sawyers, 2002). AKT itself is a serine/threonine protein kinase that phosphorylates multiple targets within the cell, including GSK3α and GSK3ß, MDM2, FOXO TFs, p21 (*CDKN1A*) and p27 (*CDKN1B*) to increase survival and induce proliferation (Downward, 2004; O'Donnell et al., 2018).

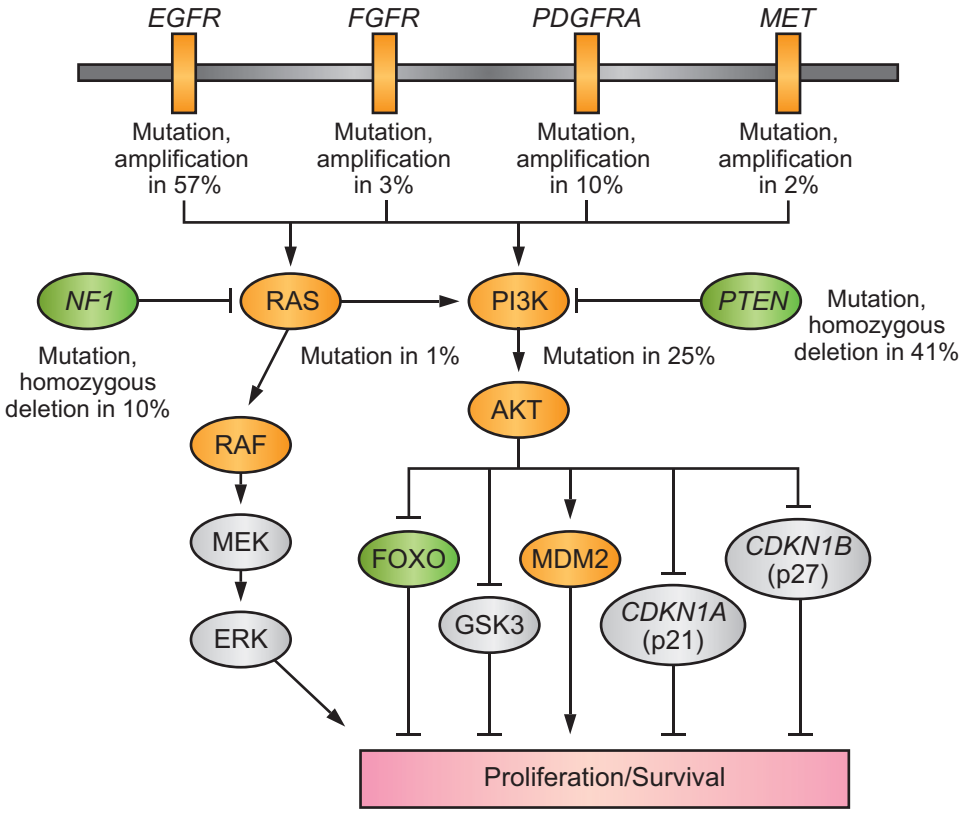

Figure 2. RTK signaling pathway. Oncogenes (yellow) and tumor suppressors (green) frequently targeted in GBM. Adapted from (Brennan et al., 2013) and (Tanaka et al., 2013).

Additionally, RTK signaling will also result in the activation of RAS, which will, in addition to stabilizing PI3K, also lead to its own interaction with RAF, promoting dimerization and activation (McKay and Morrison, 2007; Samatar and Poulikakos, 2014). Activated RAF further phosphorylates the kinase MEK, which in turn phosphorylates and activates the kinase ERK. ERK phosphorylates multiple targets including transcription factors and will end up activating cellular programs promoting cell cycle progression, differentiation, survival and cell growth (Samatar and Poulikakos, 2014). Interestingly, despite the fact that all signaling from RAF and MEK is conveyed by ERK, which it is found activated in more than 85% of all cancers, no ERK mutations have been reported so far and all oncogenic mutations are found higher up in the signaling cascade (Maik-Rachline and Seger, 2016).

Four RTKs; *EGFR*, *PDGFRA, FGFR* and *MET*, are commonly targeted by either mutations or amplifications and among them, *EGFR* by far most frequently (57%) (Brennan et al., 2013). Mutations/amplifications of different RTKs co-occur in a smaller number of tumors, most commonly *EGFR* and *PDGFR*. The most frequent *EGFR* mutation is the aberrant junction

between exons 1-8 which results in an oncogenic receptor, EGFRvIII, which is constitutively active due to the lack of the extracellular ligand binding domain (An et al., 2018). Amplification of the wt *EGFR* gene is also a common event, frequently co-occurring with at least one (71%) or two or more (30%) other aberrant variants of *EGFR* (Francis et al., 2014). The co-occurrence can either be within the same cell or in different subclones of the same GBM tumor adding to tumor heterogeneity. Moreover, downstream of RTK, mutations, deletions and amplifications are found at multiple levels, some co-occurring with each other, others being mutually exclusive, but collectively resulting in the activation of pathway output. Of special importance is two downstream pathway inhibitors that are often lost or mutated in GBM, that is *NF1* (mutation/deletion in 10%) of the RAS/MEK/ERK pathway and *PTEN* (41% mutation/deletion) of the PI3K/AKT pathway (Brennan et al., 2013).

1.2.3.4.2 p53 Tumor suppressor pathway

p53 is probably the most extensively studied tumor suppressor, partly because its gene, *TP53* (*Trp53* in mice), is the most frequently mutated gene in cancer. Its important tumor suppressor function is demonstrated not only by the fact that it is mutated in 50% of all cancer, but also by the increased cancer susceptibility of individuals with Li-Fraumeni syndrome, inheriting a mutant *TP53* allele, and also by the predisposition of *Trp53* null mice to spontaneous tumor development (Bieging et al., 2014). p53 protects the cells from transformation by inducing, transient cell cycle arrest, DNA repair, permanent cell cycle arrest (senescence) and apoptosis in response to a wide range of cellular stresses, including DNA damage, hyperproliferative signals (such as oncogenic stress), hypoxia, oxidative stress, ribonucleotide depletion and nutrient starvation (Figure 3) (Bieging et al., 2014). In unstressed cells, the levels of p53 are kept low due to high p53 instability and turnover mediated by the binding of the MDM2 ubiquitin E3-ligase which leads to the constant break down of p53 protein by the proteasome (Kruse and Gu, 2009). Together with MDM2, the structurally related MDM4 also regulates p53 activity, but with a different non-redundant mechanism. Binding of MDM4 to p53 does not lead to its degradation, rather to its stabilization, but since MDM4 is binding to the activation domain of p53, it efficiently inhibits p53-induced transcription and therefor function. Furthermore, MDM4 can also bind to MDM2 and stabilize it, thus enhancing its function (Kruse and Gu, 2009).

In keeping with being "the guardian of the genome" and the "cellular gatekeeper" p53 receives stress signals from multiple sources in the cell to coordinate the appropriate response, which depends on the intensity and duration of the specific stressor but also on the cell type and genetic background (Meek, 2009). A crucial step in the activation of p53, regardless of the stimulus, is its stabilization by phosphorylations and acetylations that will release it from its inhibitors MDM2 and MDM4 and inhibit degradation (Kruse and Gu, 2009). In the case of DNA damage, ATM is activated by double stranded breaks while ATR is triggered by single stranded breaks and replicative stress, leading to downstream activation of CHK2 or CHK1 respectively. CHK1/2 together with ATM/ATR phosphorylates MDM2/4 and p53 ultimately leading to p53 accumulation and pathway activation (Meek, 2009).

Alternatively, p53 can also be activated by ARF, sensing oncogene activation and hyperproliferation. ARF, which is also known as p14 (p19 in mice), is transcribed from the

CDKN2A locus that also produces another tumor suppressor INK4A (also known as p16), important for the regulation of cell cycle progression by the RB pathway (Sherr, 2006). Aberrant and sustained levels of mitotic signals will induce the transcription of ARF, which is normally expressed at undetectable levels, and ARF will phosphorylate and inhibit the function of MDM2 (Sherr, 2006). p53 tumor suppressor activity depends mostly on its function as a TF, binding to sequence specific motifs as a tetramer most frequently activating target gene transcription. Among the many targets are p21 (*CDKN1A*) and *GADD45A*, whose activity will result in cell cycle arrest, senescence or DNA repair, the apoptosis inducers *BAX*, *FAS*, *NOXA* and *PUMA*, and the DNA repair gene *MGMT*. In addition, p53 also regulates the expression of genes important for autophagy, metabolism control, tumor environment crosstalk, invasion and metastasis and stem cell regulation (Bieging et al., 2014).

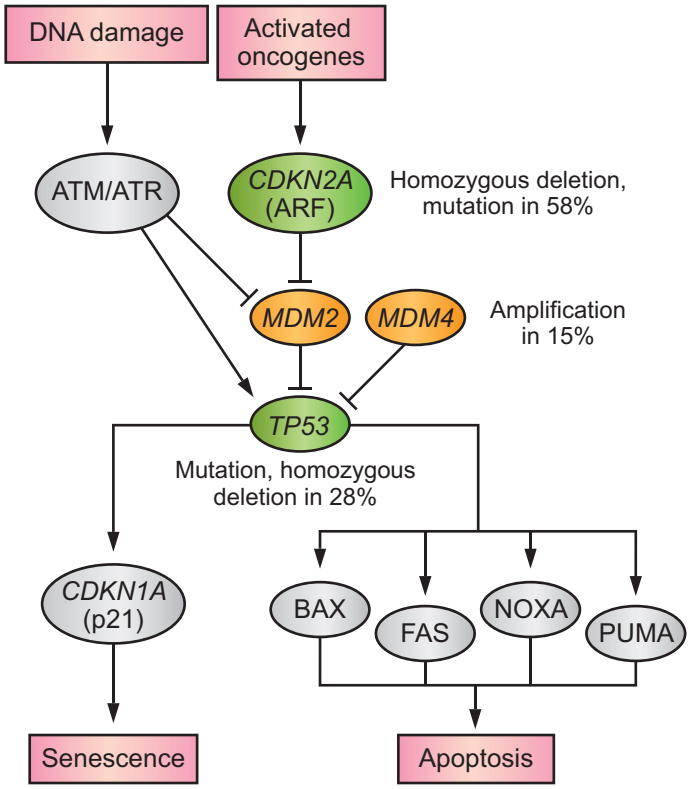

Figure 3. p53 tumor suppressor pathway. Oncogenes (yellow) and tumor suppressors (green) frequently targeted in GBM. Adapted from (Brennan et al., 2013) and (Tanaka et al., 2013).

Furthermore, while wt p53 is critical for tumor suppression, some p53 mutants are actually oncogenic and accumulate in tumor cells, adding an other layer of complexity to p53 and its function in tumor biology (Soussi and Wiman, 2015). In fact, it was actually as an oncoprotein p53 was first discovered and got its name *TP53* – Tumor Protein 53 (Muller and Vousden, 2014). The oncogenic function of mutant p53 is also reflected by the fact that although *TP53* can be found homozygously deleted in cancer, missense mutations are far more frequent, and consistently, patients with germline missense mutations have a much earlier onset of tumor development than patients with mutations that result in loss of p53 protein (Muller and Vousden, 2014). Mutations can been found in all domains of *TP53*, emphasizing the importance of this gene, although most frequent is the targeting of the DNA binding domain where virtually all codons have been found mutated. Six, "hotspot" mutations are found in high frequency in all

cancers and their neomorphic, or gain of function, features have been studied extensively and include enhanced tumorigenesis, metastasis, resistance to therapy and genomic instability (Muller and Vousden, 2014; Soussi and Wiman, 2015).

In the case of GBM, *TP53* is mutated or deleted in 28% of tumors, but together with aberrations in *MDM2* and *MDM4* genes (15%) and the deletion of *CDKN2A* locus (58%), the pathway is de-regulated in 86% of all GBM (Brennan et al., 2013).

1.2.3.4.3 RB Cell cycle regulation

The third process heavily affected in GBM is the cell cycle, more specifically the RB pathway of cell cycle control (Brennan et al., 2013; Cancer Genome Atlas Research Network, 2008). In non-neoplastic cells, the cell cycle progression is tightly regulated by RB binding to the E2F family of transcription factors repressing their transcriptional activation of genes important for S-phase and mitosis. In order to initiate and progress through the cell cycle, RB needs to be phosphorylated by Cyclin dependent kinases (CDK) in order to release its repression and let go of E2F. CDKs themselves are only active when binding to Cyclins, and different combinations of Cyclin/CDK complexes are responsible for initiating and maintaining RB phosphorylation throughout the cell cycle. CyclinD proteins are crucial for initiating the cell cycle in the early G1 phase and their synthesis and assembly with their partners CDK4 or CDK6 are dependent on mitotic signals. Thereafter, the CyclinE/CDK2 complex maintains the RB repression during the remainder of G1, and continuously, CyclinA together with CDK2 or CDK1 take the cell through S-phase and G2 phase, while the CyclinB/CDK1 complex is needed for mitosis (Giacinti and Giordano, 2006). Under normal circumstances, as RB gets hyperphosphorylated it will stay inactivated until phosphatases will relieve it of its inhibitory phosphorylations at the end of mitosis, although there are ways for the cell to stop the cell cycle in an active phase. Members of the INK4 and CIP/KIP families of CDK inhibitors can bind and destabilize Cyclin/CDK complexes thus promoting the reactivation of RB function (Besson et al., 2008). One of the most important CDK inhibitors in the context of tumor suppression is INK4A, transcribed from the *CDKN2A* locus as previously described. Diverse signals such as DNA damage or oncogene activation will increase the normally undetectable levels of INK4A in the cell, which will lead to the binding of CDK4/6 and the displacement of D type Cyclins. This in turn will result in the release of other CDK inhibitors; p21 (*CDKN1A*) and p27 (*CDKN1B*) of the CIP/KIP family, that are normally binding to and stabilizing this complex, and they will further inhibit the activity of CDK2 complexes (Knudsen and Knudsen, 2008). In addition, together with INK4A the other family members INK4B (p15 from the *CDKN2B* gene), INK4C (p18 from the *CDKN2C* gene), INK4D (p19 from the *CDKN2D* gene) will also bind and repress CDK4/6, while and p57 (*CDKN1C*) of the CIP/KIP family will contribute to the regulation of Cyclin/CDK complexes throughout the cell cycle.

In GBM tumors, mutations or deletions of *RB* itself, as well as activating mutations or amplifications of CDKs or Cyclins are common, but the far most frequent pathway alterations are achieved by homozygous deletion of the *CDKN2A* locus or *CDKN2B* locus and less frequently the *CDKN2C* locus (Figure 4) (Brennan et al., 2013).

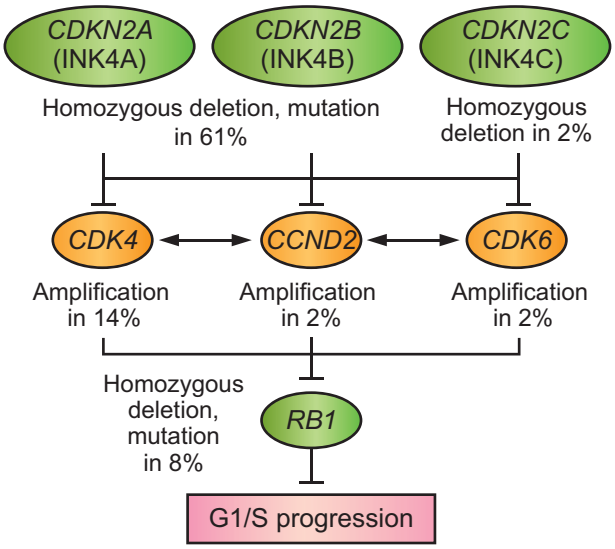

Figure 4. RB cell cycle regulation pathway. Oncogenes (yellow) and tumor suppressors (green) frequently targeted in GBM. Adapted from (Brennan et al., 2013) and (Tanaka et al., 2013).

1.2.3.5 Cell of Origin

As tumorigenesis begins long before the detection and diagnosis of the cancer, and multiple mutations and genetic alterations leading to functional and morphological changes are added during this process, it is inherently difficult to pinpoint the cell of origin. In theory, any cell of the tissue hierarchy with proliferative potential could acquire genetic changes that induce self-renewal and therefore could serve as the cell of origin (Visvader, 2011). Several mouse models have tried to define the cell of origin of glioma and GBM and together they point to several possible sources. Although NSCs, transit amplifying cells, oligodendrocyte progenitor cells (OPCs), differentiated astrocytes and even neurons have all been demonstrated to be susceptible to transformation, the case for NSCs and OPCs is perhaps the strongest.

NSCs expressing NESTIN can give rise to high-grade glioma when forced to express oncogenes *Akt* and *Kras* or when different combinations of the tumor suppressors *Trp53*, *Nf1* and *Pten* are deleted (Alcantara Llaguno et al., 2009; Holland et al., 2000). Consistently, using the NSC and astrocyte specific human glial fibrillary acidic protein (GFAP) promoter to guide tumor suppressor deletion, NSCs but not mature astrocytes can be induced to form tumors (Jacques et al., 2010; Kwon et al., 2008; Wang et al., 2009). Interestingly, although AKT and KRAS overexpression was not sufficient to induce gliomagenesis in GFAP expressing astrocytes (Holland et al., 2000), the activation of these oncogenes on a *Ink4a/Arf* -/- background could induce tumor growth in GFAP expressing cells (Uhrbom et al., 2002). Other studies have also confirmed the ability of differentiated astrocytes to induce tumor growth, but what most of them have in common and what distinguish them from studies which did not find this ability is that they use the expression of activated oncogenes rather than or in combination with tumor suppressor deletion (Bachoo et al., 2002; Friedmann-Morvinski et al., 2012; Marumoto et al., 2009; Uhrbom et al., 2002) or induce neonatal rather than adult astrocytes (Bachoo et al., 2002; Uhrbom et al., 2002). Another study, using inducible hGFAP-CreER to induce the loss of tumor suppressors *Pten/Trp53/Rb* in 50% of adult astrocytes and 1% of adult NSCs, found that despite the fact that most targeted cells were differentiated astrocytes, most tumors (78%) arose in areas

close to the neurogenic niches containing NSCs (Chow et al., 2011). In summary, although both NSCs and astrocytes may undergo transformation, NSCs are probably much more susceptible and might require a lower threshold of oncogenic events to induce gliomagenesis.

Representing the majority of the dividing cells in the adult human brain, OPCs, in addition to NSCs, constitute another likely source of glioma (Geha et al., 2010). In support of this, high grade oligodendrogliomas are generated when an activated allele of *EGFR* (*v-erbB*) is expressed under the control of the human *S100ß* promoter in combination with *Trp53* deletion (Persson et al., 2010). Although S100ß is found expressed in mature astrocytes, ependymal cells and some neurons in addition to OPCs, the authors found that the glioma cells arising are similar to immortalized OPCs in their molecular expression profile and phenotype. Furthermore, the authors could successfully link the localization of human oligodendrogliomas in the brain to white matter regions, rather than the lateral ventricles, as was more common for astrocytomas, thus suggesting a white matter progenitor cell of origin for oligodendroglioma (Persson et al., 2010). Consistently, another study reported similar results using lineage tracing with the Mosaic Analysis with Double Markers system in a mouse model where *Trp53* and *Nf1* were deleted in cells expressing hGFAP or NESTIN. Interestingly, only OPCs were found to aberrantly proliferate in pre-malignant animals, supporting the notion that even though the oncogenic mutations occur in NSCs, it is a progenitor cell that is the cell of origin of the tumor. Additionally, the results were confirmed by directly introducing the mutations in OPCs using OPC specific *NG2* to drive CRE expression. Furthermore, white matter cells stereotactiacally transduced with a viral vector expressing PDGF and CRE causing deletion of *Trp53* and *Pten* in a small subset of cells could faithfully induce GBM like tumors. Hyperproliferating transduced cells expressed OLIG2, again emphasizing the importance of this lineage in glioma development (Lei et al., 2011). Taken together it is clear that OPCs can serve as a cell of origin in malignant glioma and that they are susceptible to transformation by several different mutations commonly associated with human glioma.

Finally, it seems comprehensive that both the transformed cell type and the underlying oncogenic mutations could reflect disease properties, or alternatively that the transforming mutations could confer selectivity targeting cells of differential susceptibility. Indeed, NSCs from different parts of the CNS have been shown to initiate growth of different types of tumors when transformed with the same mutant *MYC* (Swartling et al., 2012). Conversely, in some systems the oncogenic mutations have been found to be superior to the targeted cell type in generating astrocytoma versus oligodendroglioma (Lindberg et al., 2014). This again emphasizes the complexity of the answer to the question of cell of origin, and it is reflected by the notion that glioma probably constitutes a collection of diseases and therefor may have multiple origins (Zong et al., 2015). Although we have begun to understand the different origins of glioma in mouse models we are in need of translation into the human scenario, since progenitor cell types differ between our species.

1.2.3.6 SOX and Glioma

Being developmental regulators, and developmental programs being widely used by cancers to induce malignancy (Suvà et al., 2013), SOX TFs have been implicated in many cancers, not at

least glioma and GBM. As SOX2 is a key regulator of stemness both in ESCs and stem cells of a wide variety of adult organs, it is not surprising to find SOX2 up-regulated in a large panel of cancers (Sarkar and Hochedlinger, 2013). In the case of glioma, SOX2 has been linked to malignancy, therapy resistance, recurrence and core transcriptional regulation of glioma CSCs (GSC) (Favaro et al., 2014; Garros-Regulez et al., 2016; Holmberg et al., 2011; Jeon et al., 2011; Singh et al., 2017; Suvà et al., 2014). SOX2 has been found to be highly expressed in glioma tissue, with increased expression in more malignant samples (Holmberg et al., 2011; Ma et al., 2008), and high expression has also been correlated with aggressiveness and poor outcome (Ben-Porath et al., 2008). The development of cancer share many mechanistic features with reprogramming, and developmental programs are often activated de novo to induce unlimited self-renewal (Suvà et al., 2013). In line with this, the expression of SOX2 often coincides with other ESC TFs, such as OCT4, NANOG and KLF4 in high grade glioma, and the expression of a ESC gene target profile is correlated with increased grade and poor outcome (Ben-Porath et al., 2008; Holmberg et al., 2011). Consistent with its elevated expression in high grade glioma, genetically amplified *SOX2* has been found in GBM cell lines and primary tumors, and promoter CpG island hypomethylation is seen in the majority of GBM (Alonso et al., 2011; Annovazzi et al., 2011). Furthermore, the well recognized characteristics of GSC, self-renewal and in vivo tumor formation (Singh et al., 2004), have in several studies been found to depend on the expression of SOX2 (Alonso et al., 2011; Bulstrode et al., 2017; Gangemi et al., 2009; Ikushima et al., 2009). This seems not only to be the case for human GBM cells but is also seen in a mouse model of high grade oligodendroglioma where the deletion of *SOX2* significantly reduced the tumor growth and increased survival (Favaro et al., 2014). Additionally, SOX2 expression correlates with the proposed GSC marker CD133 (Bao et al., 2006; Ikushima et al., 2009) and is part of a core regulatory program that keeps GSC properties, even in the absence of upstream oncogenic signaling (Singh et al., 2017). This function is further supported by the reprogramming of terminally differentiated GBM cells, which requires SOX2 together with OLIG2, SALL2 and POU3F2 (Suvà et al., 2014). An additional genomic feature important for the gene regulatory network downstream of SOX is the overrepresentation of SOX binding motifs in open chromatin in GBM cells, as well as in enhancers with reduced CpG island methylation in IDH mutant non-codel glioma (Bulstrode et al., 2017; Ceccarelli et al., 2016).

Although there are two more members in the SOXB1 group, with extensively overlapping expression and function in NSC (Bylund et al., 2003; Uchikawa et al., 2011), the focus on SOX2 has been dominating the field, and there are few reports of SOX1 and SOX3 and their relevance for glioma. The expression of SOX1 and SOX3 is overlapping with that of SOX2 in glioma, especially in GBM, and SOX1 has been suggested to serve a similar function to that of SOX2 in glioma cells in that its downregulation cause decreased self-renewal capacity and differentiation (Garcia et al., 2017; Holmberg et al., 2011). Similarly, SOX21 has also been detected to overlap in its expression with SOX2 in glioma tissues and cell lines, but in contrast to SOX2 and SOX1, SOX21 overexpression decrease proliferation and instead induce apoptosis, possibly through the binding to and downregulation of SOX2 protein (Caglayan et al., 2013; Ferletta et al., 2011).

Being expressed both in mature and immature cells of the glial lineage, SOXE expression have been systematically investigated in glioma. As a consequence of the histopathological diagnosis

criteria where the tumors are defined as astrocytic or oligodendroglial, several groups have tried to evaluate the in normal tissue oligodendroglial-specific expression of SOX10 as a marker to distinguish the subtypes. This has proven impossible, since SOX10 is expressed, in varying levels across all glioma pathologies, also in varying degrees together with the other SOXE members SOX8 and SOX9 (Bannykh et al., 2006; Ferletta et al., 2007; Schlierf et al., 2007). The expression of SOX9 overlaps to a great extent with that of SOX2 and high expression has been linked to increased malignancy and poor prognosis (Garros-Regulez et al., 2016; Wang et al., 2012). Functionally, SOX9 is important for self-renewal and survival, which has been demonstrated by knock-down experiments in several glioma cell lines, and mechanistically, SOX9 is acting downstream of SOX2, being transcriptionally regulated by mTOR and translationally by cGKII (Garros-Regulez et al., 2016; Hiraoka et al., 2015; Swartling et al., 2009).

Upstream of SOX2, TGFß stimulation has been found to induce the expression of SOX4, which in turn binds one of the SOX2 enhancers in a complex with OCT4, inducing SOX2 expression. During embryonic development OCT4 normally partners with SOX2 itself to maintain its own expression, a feedback loop that seems disrupted in glioma (Ikushima et al., 2009; 2011). Furthermore, SOX4 expression is increased in glioma compared to normal brain tissue, as is SOX11 of the same group, but while SOX4 is linked to poor survival, high levels of SOX11 provides a beneficial prognosis (Korkolopoulou et al., 2013; Li et al., 2015). While being expressed in glioma, SOX11 is downregulated with disease progression and GSCs often lose their expression in culture (Hide et al., 2009). Consistently, overexpressing SOX11 in mouse or human GSCs decreases tumorigenicity as differentiation increases, whereas knock-down enhances tumorigenicity, as measured by orthotopical transplantation experiments in vivo (Hide et al., 2009).

In line with a beneficial role of some of the SOX TFs, SOX17, a well-established WNT antagonist normally expressed in OPCs as they exit cell cycle during development (Sohn et al., 2006), has been investigated in oligodendroglioma and also been found to confer better prognosis, especially in case of 1p/19q codel (Li et al., 2014). Overexpression of SOX17 in human oligodendroglioma cell lines, reduces proliferation and induces differentiation, through the upregulation of WNT antagonists and the decrease of ß-catenin (Chen et al., 2013). This antagonistic effect on WNT signaling and tumorigenicity has also been seen after the overexpression of SOX7 in glioma cell lines, and glioma patients with high grade disease are more likely to have low levels of SOX7 expression (Zhao et al., 2016).

As SOXE TFs are necessary for glial specification and differentiation, members of the SoxD family have been found to modulate this function to time the expression of terminal differentiation markers (Stolt and Wegner, 2010; Stolt et al., 2006). All SOXD members are expressed in glioma but whether they show increased or decreased expression compared to normal brain tissue remains unclear (Schlierf et al., 2007; Ueda et al., 2004; 2007). Interestingly, a single nucleotide polymorphism (SNP) in the SOX5 gene has been correlated with an increased risk for primary brain tumors (Liu et al., 2012). Moreover, in a mouse model of oligodendroglioma, increased expression of SOX5 suppressed PDGFB-induced tumor

formation through the reduction of activated AKT and increased levels of p27, leading to the induction of senescence (Tchougounova et al., 2009). Furthermore, autoantibodies against SOX5 and SOX6 have been detected in the serum of glioma patients, correlating with prolonged survival (Schlierf et al., 2007; Ueda et al., 2004; 2007). Since SOX proteins are TFs expressed in the nuclei of cells, they have been considered undruggable targets. However, since immunotherapy for glioma has seen cautious progress it is interesting to note that successful DNA vaccination against SOX6 have shown both protective and therapeutic anti-tumor response in mice with glioma, together with an induction of CTLs specific against SOX6-expressing glioma (Reifenberger et al., 2017; Ueda et al., 2008; Weller et al., 2017). The same authors have further shown that in vitro stimulation of peripheral blood mononuclear cells derived from healthy donors and glioma patients could induce the emergence of SOX6 specific CTLs, which were able to lyse several human glioma cell lines (Ueda et al., 2010). This has also been demonstrated for SOX11 derived epitopes that have been able to produce CTLs that can lyse glioma cell lines, suggesting the plausibility immunotherapy against SOX proteins in the case of glioma (Schmitz et al., 2007).

1.2.4 Gastric Cancer

1.2.4.1 Prevalence

Stomach cancer, or gastric cancer as it is also called, is the fifth most common cancer worldwide. It accounts for 7% of all cancer cases, but for 9% of all cancer deaths, making it the third most common cancer related cause of death. Gastric cancer is not evenly distributed worldwide, rather there is a 10-fold difference in the incidence rates, with the highest rates found in Japan, China and East and Central Asia where almost 75% of all the gastric cancer cases are found. Medium incidence rates are found in South America while the lowest rates are found in North America, northern Europe and Africa (Stewart and Wild, 2014). In Sweden, gastric cancer constitutes between 1.2-1.7% of all cancers cases, with the higher incidence rate in men. The incidence rate of gastric cancer has changed tremendously over the past decades both world wide and in Sweden, and it has moved down from being the primary cause of cancer related death globally in 1930, and in Sweden from being the second most common cancer in 1960 to not even be on the top ten list of the most common cancers today (Hayakawa et al., 2016; Socialstyrelsen, 2013)(www.cancerfonden.se). This significant change is mainly due to improved living standards including sanitation, hygiene, water supply, and advances in food preservation (Balakrishnan et al., 2017; Wadhwa et al., 2013). However, gastric cancer is often diagnosed in advanced stages when the prognosis is poor, and the survival rates have not seen the same great improvement over the years and is still only just above 20% (5-year survival) in Sweden (Socialstyrelsen, 2013). This has led to screening programs in high incidence countries such as Japan, which has helped to bring down the death rates, and will probably continue to do so (Balakrishnan et al., 2017; Wadhwa et al., 2013).

1.2.4.2 The gastric epithelium and the development of Gastric Cancer

The human stomach is lined on the inside by a simple columnar epithelium, and is divided into three anatomical regions: the most proximal cardia, the corpus and the distal antrum (Choi et al.,

2014). Rodents have instead of the cardia a large proximal compartment called the forestomach that instead of columnar epithelia consists of a stratified squamous epithelia continuous with the esophagus (Kim and Shivdasani, 2016). The gastric units of the corpus and antrum differ somewhat in the frequency and composition of the main cell types (Figure 5). In a typical corpus unit one can find pit cells located at the top of the gastric unit producing mucous and turning over every 3 days, zymogenic chief cells located at the bottom of the gland producing digestive enzymes such as pepsinogen and turning over every few months, acid-producing parietal cells along the length of the gland and endocrine cells secreting hormones regulating responses to food and starvation. Apart from almost not having any parietal cells, the antral unit instead has specialized cells at the gland base secreting protective acidic mucins (Kim and Shivdasani, 2016).

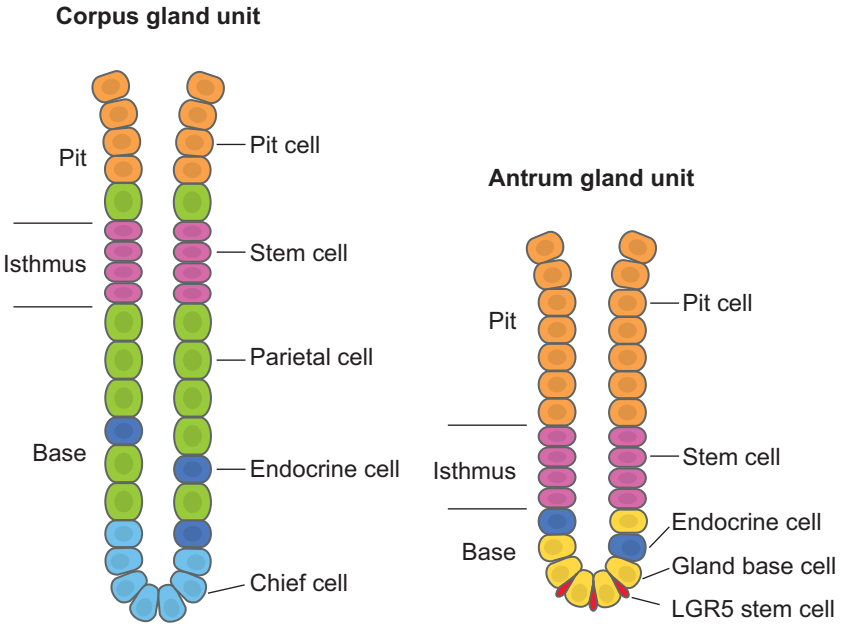

Figure 5. Gastric units of corpus and antrum.

Most stomach cancers (95%) are gastric adenocarcinomas, arising from malignant transformation of the gastric epithelium. Other, much less common, are lymphomas and mesenchymal gastric tumors (Balakrishnan et al., 2017). Most cases are sporadic, however while about 10% seem to be hereditary, only 1-3% of gastric cancer is caused by hereditary syndromes with known mutated genes (Stewart and Wild, 2014). The single biggest risk factor for gastric cancer development is *Helicobacter pylori* infection. It has been estimated to account for 89-95% of all the gastric cancer cases and it is considered to be a Group 1 carcinogen by the International Agency for Research on Cancer (Balakrishnan et al., 2017; Stewart and Wild, 2014).

Cancer development initiated by *H pylori* infection passes several histopathological stages including chronic gastritis, atrophic gastritis, intestinal metaplasia and dysplasia before cancer is established (Correa, 1992). Although this does not have to be the case for all gastric cancer arising, and it is probably more relevant for the development of the intestinal type tumors, this points to the importance of inflammation in disease development (Hayakawa et al., 2017). Inflammation caused by *H pylori* infection induces aberrant DNA methylation patterns, where clones with beneficial phenotypes can expand and a "cancerized field" is created where the level of methylation correlates with cancer risk (Hattori and Ushijima, 2016; Ushijima and Hattori,

2012). Furthermore, aberrant methylation is not only found in pre-malignant lesions and metaplasia, but also in adjacent normal gastric tissue from patients with gastric cancer (Padmanabhan et al., 2017). This, together with the fact that some gastric cancers have a relatively low mutational burden and that many tumor suppressor genes such as *CDKN2A*, *CDH1*, *MLH1* and *RUNX3* are more frequently inactivated by aberrant DNA methylation than mutation, emphasizes methylation as a driving event in gastric tumorigenesis (Hayakawa et al., 2017; Ushijima and Hattori, 2012).

A subset of gastric cancers are associated with Epstein-Barr virus (EBV) infection, which also causes increased DNA methylation although with a different mechanism (Ushijima and Hattori, 2012). Apart from inducing hypermethylation, *H pylori* also interacts with and affects stem cells of the stomach directly. Studies in mice have revealed that soon after infection microcolonies can be detected deep in the gastric glands where they induce increased proliferation of stem cells specifically, resulting in hyperplasia in association with chronic inflammation (Sigal et al., 2015).

Although *H. Pylori* infection is deemed to be necessary, it is not sufficient to cause gastric cancer, and other factors modulate the risk. Diet is the most important of these factors and high intake of salt-preserved and/or smoked foods and pickled vegetables and low intake of fresh fruit vegetables increase the risk (Stewart and Wild, 2014). Smoking and high alcohol consumption are two other well known risk factors (Wadhwa et al., 2013).

1.2.4.3 Molecular Classification

Our molecular understanding of gastric cancer has been limited compared to that of other cancers of similar impact, and this has mainly been due to the lack of knowledge of germ line mutations and tumor drivers for this disease (Wadhwa et al., 2013). Recent whole genome sequencing efforts have tried to confront this, and progress is now seen in this area (Cancer Genome Atlas Research Network, 2014; Cristescu et al., 2015). Based on histology, gastric adenocarcinoma is divided into two subtypes, intestinal and diffuse, according to the Lauren classification (Laurén, 1965). This classification, or an alternative system introduced by WHO, which is also based on histology, provides limited diagnostic or prognostic value to this heterologous disease. In a quest to change this, two larger studies, including Next Generation Sequencing efforts, have analyzed large sets of tumors and classified them according to molecular profiles rather than histology (Katona and Rustgi, 2017).

The first project, by TCGA, has identified four subtypes of gastric adenocarcinoma, based on comprehensive molecular evaluation of RNA, DNA and microRNA whole genome data (Cancer Genome Atlas Research Network, 2014). The four subgroups are EBV positive tumors, tumors with high Micro-Satellite Instability (MSI), Genomically Stable (GS) tumors and tumors with Chromosomal Instability (CIN). EBV positive tumors constitutes the smallest group and they are further characterized by frequent *PIK3CA* mutations and CIMP leading to *CDKN2A* silencing, but rarely to the silencing of the DNA mismatch repair gene *MHL1*. The MSI subtype also exhibits hypermethylation, but in contrast to EBV tumors this also includes the *MHL1* promoter, leading to increased mutation rates and a specific spectrum of mutations. The CIN subgroup has extensive Somatic Copy Number Alterations as apposed to the GS subgroup. The

CIN subgroup displays an over-representation of intestinal type histology tumors and frequent *TP53* mutation and RTK pathway activation, whereas the GS subgroup has an overrepresentation of diffuse histology tumors with frequent *CDH1* mutations.

In a parallel project, lead by the Asian Cancer Research Group consortium (ACRG), expression, SNP and genome wide Copy Number Variation microarray data also led to the similar classification of four subgroups; MSI, Micro-Satellite Stable (MSS)/EMT, MSS/TP53+ with intact p53 activity and MSS/TP53- with loss of p53 function. Although the MSI group in the TCGA and the ACRG classification are relatively similar, the other subgroups while overlapping are different enough not to be considered equivalent (Cristescu et al., 2015). Both classification systems have relevant clinical association with the different subtypes, but only the groups proposed by the ACRG show significant survival differences between them (Cristescu et al., 2015).

1.2.4.4 Gastric Cancer Signaling Pathways

Although the recent sequencing efforts have emphasized the roles of tumor suppressors *TP53* and *CDH1* driver mutations in gastric cancer, it has not lead to the characterization of any dominant pathway heavily mutated as in the case of glioma (Cancer Genome Atlas Research Network, 2008; Katona and Rustgi, 2017). Instead, many pathways can be found altered with lower frequency, again underscoring the heterogeneous nature of this disease. Contributing to the genomic landscape of gastric cancer are pathways governing genome integrity, cell adhesion, cell motility and cytoskeleton, RTK, WNT signaling and chromatin remodeling (Katona and Rustgi, 2017).

Important for genome integrity, the *TP53* gene is mutated in 30-50% of all gastric cancer, and therefor the most common mutation. The *CDH1* gene, coding for the epithelial adhesion molecule E-cadherin, suppress tumorigenesis by maintaining tissue organization (van Roy, 2014), and low levels are strongly associated with diffuse type gastric cancer. It is one of the few genes for which germ line mutations are linked to hereditary gastric cancer (More et al., 2007). RHOA functions in cell cycle motility cytoskeleton remodeling and cell proliferation and is together with *MACF1* significantly mutated in gastric cancer. The most frequent mutations in the RTK pathway are in the *PIK3CA* gene encoding a regulatory subunit of PI3K. These mutations together with *KRAS* mutations, and much less frequent *EGFR* and *HER2* alterations, actively enhance RTK signaling. WNT pathway alterations, generally associated with many tumors and specifically with colorectal tumors, are also significantly found in gastric cancer. In normal cells, pathway signaling is activated by the binding of WNT ligand to their Frizzled receptors and Lrp5/6 co-receptors leading to receptor phosphorylation and GSK3 inactivation. Without WNT signaling GSK3 acts in a complex with APC and AXIN constantly targeting ß-catenin, the transcriptional activator of WNT signaling, for destruction. Activated WNT signaling promotes cell proliferation and stem cell self-renewal through ß-catenin mediated transcription of target genes (Clevers et al., 2014). In gastric cancer, mutations in *APC* and *CTNNB1* (encoding ß-catenin), but also in another negative regulator, *RNF43*, are significant findings. Finally, mutations in chromatin remodelers, most frequently *ARID1A*, a component of the SWI/SNF complex, but also *ARID1B* and members of the MLL family are common.

1.2.4.5 SOX and Gastric Cancer

Although SOX2 is well established to have oncogenic effects when expressed in tumors, often mediating CSC features, SOX2 in gastric cancer has been controversial. There are several studies supporting either an oncogenic role or a tumor suppressor role of SOX2 in gastric cancer (Carrasco-Garcia et al., 2016). In support of an oncogenic role, SOX2 downregulation in gastric cancer cell lines has been shown decrease proliferation and increase apoptosis, both in vivo and in vitro, and high SOX2 expression in human tumor samples has been found to correlate with increased lymph node metastasis and invasion (Hütz et al., 2014; Matsuoka et al., 2012). In contrast, others have found the opposite correlation where decreased SOX2 expression could be correlated with disease progression and increased expression with a survival advantage (Otsubo et al., 2008; Wang et al., 2015). Functionally, SOX2 overexpression in gastric cancer cell lines could induce cell cycle arrest and apoptosis and this via the upregulation of PTEN expression (Otsubo et al., 2008; Wang et al., 2015). Furthermore, in a recent mouse model of gastric adenoma, where *Apc* was homozygously deleted in SOX2 expressing cells, *Sox2* deletion was found to increase the tumor burden. Mechanistically, the SOX2 anti-tumorigenic effect was achieved by counteracting the excessive WNT signaling induced by the loss of APC (Sarkar et al., 2016).

SOX9, in addition to SOX2, is also expressed in normal gastric mucosa but has instead been revealed more clearly as oncogenic and its expression can be widely detected in gastric cancer tissue (Sashikawa Kimura et al., 2011). Its expression has been liked to *H pylori* infection, especially to the more virulent *cagA* and *vacA* expressing strains, and IL-1 expression in mice, as a consequence of *H pylori* infection induces SOX9 expression (Serizawa et al., 2016). In gastric cell lines, SOX9 is also increased by *H pylori* infection and has been found both to be regulated by and to be regulating ß-catenin expression, in addition to being induced by TNFα. The elevation of SOX9 after infection seems to be required for the proliferative and stem cell like properties induced by the bacteria, and inhibition of SOX9 instead leads to reduced proliferation and to apoptosis (Santos et al., 2016).

Other SOX TFs have be been found in gastric cancer tissue, but their roles are more elusive, since most studies are clinical correlations and not functional experimentation. Briefly, SOX4 has been found to be up-regulated in many cancers compared to normal tissue, and so also in gastric cancer (Chen et al., 2015). Conversely, SOX11 has instead been found at lower expression levels in gastric cancer and with high promoter methylation (Qu et al., 2014; Xu et al., 2015). This methylation in cell lines can be removed by treating cells with the DNA methylation inhibitor 5-Aza-dC and SOX11 levels restored, with a decrease in proliferation as a result (Xu et al., 2015). In addition to SOX11, low expression of SOX7 and SOX17 has also been correlated with poor survival and more advanced disease (Cui et al., 2014). Similarly to SOX11, SOX17 has been found heavily methylated, and restoring SOX17 levels decreased colony formation of gastric cancer cell lines (Oishi et al., 2012).

2 AIMS

2.1 PAPER I

All stem cells share the characteristics of self-renewal and the potential to differentiate into one or several specific cell-types. While sharing these features, stem cells of different organs have distinct gene expression profiles and potential when it comes to the production of progeny. However, it is unclear to what extent common features of stem cells from vastly different origins share the same regulatory mechanisms. The aim of **Paper I** was to clarify how SOX2 regulates stem cell properties in different organs. To answer this question, we used ChIP-seq together with RNA-seq to map the binding profiles of SOX2 and the distinct expression patterns in organs of ectodermal origin; cortex and spinal cord, as well as in organs of endodermal origin; lung and stomach.

2.2 PAPER II

Due to their proliferative capacity, stem and progenitor cells are always at risk of incorporating detrimental mutations that will remain in their lineage. Consequently, stem and progenitor cells have an increased susceptibility to oncogenic transformation. Many driver mutations have been defined and it is well established how gain or loss of function in several important genes leads to tumor initiation. Less well characterized are the mechanisms by which stem cells specifically protect themselves from, and react to, oncogenic transformation. The aim of **Paper II** was to investigate how the stem cell expression of SOX5, SOX6 and SOX21 (SOX5/6/21) affects tumor development. To address this question we used a mouse glioma model of brain cancer, and human primary glioblastoma cells, in which we could modulate the levels of SOX5/6/21.

2.3 PAPER III

Adult stem and progenitor cells of different tissues share the defining features of self-renewal and progeny differentiation, but exhibit distinct capacity and competence depending on the specific demands of the organ. While some adult stem cells divide rarely and generate a limited amount of progeny, as in the case of the CNS, others divide rapidly to provide large quantities of differentiated cells to support the function of the organ, such as in the gastrointestinal tract. Since, SOX21 is expressed in both of these stem and progenitor cell types, we asked in **Paper III** whether SOX21 exhibits similar functions in the rapidly proliferating cells of the stomach as in the slowly dividing cells of the brain. To address this we used bioinformatic approaches and *in vitro* based assays of SOX21 overexpression in human gastric cancer cell lines.

3 RESULTS AND DISCUSSION

3.1 PAPER I

To begin to answer our question about how SOX2 regulates stem cell characteristics in distinct cell populations, we first confirmed the stem and progenitor cell expression of SOX2 in mouse cortex, spinal cord, lung and stomach of E11.5 embryos and saw that it overlapped extensively with the proliferation marker Ki67. We next performed *in vivo* SOX2 Chromatin Immunoprecipitation and sequencing (ChIP-seq) on dissected E11.5 tissue from stomach and lung/esophagus, which we compared with E11.5 SOX2 ChIP-seq data from cortex and spinal cord (Hagey et al., 2016). Comparing the overlap of SOX2 binding regions (peaks), we found that although targeting a similar motif in all tissues, SOX2 binding was mostly cell type specific. However, the peaks that did overlap were mostly from tissues of the same germ layer (ectoderm or endoderm). Only 232 peaks were common between both endodermal and ectodermal tissue. Moreover, in a Principal Component Analysis, SOX2 binding in the cortex and spinal cord clustered together with the SOX2 binding pattern of ESC derived NPCs, whereas SOX2 binding in the stomach and lung/esophagus clustered with that of adult stomach. Interestingly, the SOX2 binding pattern of ESCs was equally related to that of ectodermal and endodermal tissues. Together these findings reveal that SOX2 mostly binds cell specific targets and that the binding is more similar in tissues of the same germ layer.

Since SOX transcription factors are known to bind together with partner factors (Hagey et al., 2016; Kondoh and Kamachi, 2009), we next sought to investigate whether this could be a reason for the distinct SOX2 binding patterns in the different tissues. To this end, we searched for the enrichment of binding motifs in neural, endodermal, or common peaks and found several candidates. Within the cortex peaks we found an enrichment of motifs for OTX1, in spinal cord PAX2, in stomach GATA4 and HNF1A and in the lung/esophagus FOXA1 and TEAD4. In peaks common to both neural and endodermal tissue, ZEB1 and ZBTB33 were found to be enriched. Although this does not mean that SOX2 actually co-binds with these factors, all of these transcription factors belong to families that have been shown to bind SOX proteins. Furthermore, they were also found to be appropriately expressed within the different tissues, suggesting that a functional relationship might be possible. To test this possibility, we performed immunoprecipitation of SOX2 together with the neural specific OTX1, the endoderm specific FOXA1 and the common ZEB1, all of which were found to interact with SOX2 in our overexpression assay. Interestingly however, only FOXA1 and ZEB1 seemed to bind SOX2 directly without DNA, since DNaseI treatment completely abrogated the OTX1-SOX2 interaction. While our results are indicative of a physical interaction, we cannot exclude the possibility of other factors binding in complex and thus only mediating a secondary interaction with our suggested partners.

To test functional interaction between SOX2 and our candidate partners, we cloned peak regions, specifically or commonly bound, into luciferase reporter vectors and assayed activation in P19 cells with the different factors. Regions bound by SOX2 specifically in neural tissues could activate the reporter in response to SOX2 and OTX1 expression in an additive fashion.

Furthermore, endodermal specific SOX2 peak regions could activate reporter expression in response to either SOX2 or FOXA1, but did not exhibit any additive effect. In contrast, commonly bound peak regions could activate the reporter in response to SOX2 expression, an activation that was efficiently repressed by ZEB1, in line with its known repressor function (Spaderna et al., 2008). Together this data suggests a physical and functional interaction between SOX2 and TFs specifically in the different cell types, which may contribute to the cell-specific binding of SOX2 within these cells.

In order to address how SOX2 binding affects gene expression profiles in different tissues, we next performed RNA-seq on FACS isolated SOX2-GFP$^+$ cells from mouse E11.5 cortex, spinal cord, stomach and lung/esophagus. Similar to the SOX2 binding profiles, gene expression data showed more extensive overlap between tissues from the same germ layer. Correlating gene expression with SOX2 targeted genes (500 kb within the closest transcriptional start site) revealed that SOX2 binding was specifically enriched around genes expressed within the corresponding tissue. In agreement with this, GO analysis of specifically bound genes showed enrichment for terms appropriate for each tissue, such as *"Pallium development"* for genes bound in cortex, *"Cell differentiation in spinal cord"* for genes bound in spinal cord, *"Embryo digestive tract development"* for genes in stomach and *"Lung alveolus development"* for genes bound in lung/esophagus. Interestingly, genes that were commonly bound by SOX2 in neural and endodermal tissues showed a higher enrichment for GO terms such as *"Regulation of stem cell proliferation"* and *"Regulation of stem cell differentiation"*.

Together this analysis indicated that differential SOX2 binding might be instructive and that SOX2 bound regions could act as cis regulatory modules (CRM) to direct cell specific gene expression. To test this, peak regions were inserted into GFP reporter vectors that were subsequently injected into zebrafish eggs, and screened for GFP expression. Strikingly, neural specific SOX2 bound regions activated GFP expression predominantly in the zebrafish CNS (71% of CRMs), whereas endoderm specific SOX2 bound regions predominantly activated expression in the zebrafish endodermal tissues (57% of CRMs). In contrast, commonly bound SOX2 regions activated GFP expression in both CNS and endodermal tissues (92% of CRMs). Collectively this shows that SOX2 bound regions in mouse neural and endodermal cells can drive gene expression in the corresponding tissue in the developing zebrafish, thus acting as CRMs.

The interesting finding that genes commonly bound by SOX2 between neural and endodermal cells were enriched for genes important for stem cell proliferation resonates well with the notion that stem cell regulation is intimately linked to cell cycle regulation (Dalton, 2015; Gonzales et al., 2015; Pauklin and Vallier, 2013). It has previously been shown that SOX2 regulates the rate of proliferation in mouse embryonic cortex by repressing *Ccnd1* expression and the *Ccnd1* promoter was indeed one of the 32 regions targeted by SOX2 in all four tissues examined (Hagey and Muhr, 2014). To find out whether cell cycle regulation is a common mechanism by which SOX2 regulates stem cells in different tissues, we characterized SOX2 levels and BrdU incorporation in mouse spinal cord and stomach. Cells expressing high levels of SOX2 incorporated significantly less BrdU than did cells of low SOX2 expression. This was in

accordance with previous data from the cortex and true for both spinal cord and stomach (Hagey and Muhr, 2014). Interestingly, in the stomach there was a regional difference in the levels of SOX2 at E15.5 but not at E11.5, which reflected the proliferative rate of the respective regions. Furthermore, SOX2 could act instructively, as chick spinal cord electroporation or *ex vivo* electroporation of mouse stomach decreased BrdU incorporation significantly. On the other hand, downregulation of SOX2 levels, either by dominant negative SOXB1 or by shRNA against SOXB1, increased the incorporation of BrdU significantly. Interestingly, the SOX2 effect on proliferation was dependent on WNT pathway components. This is consistent with what has been shown for cortex, where SOX2 at high levels binds to low affinity sites and interacts with TCF/LEF to recruit the GRO/TLE repressor and where SOX2 at low levels instead binds distinct high affinity motifs in the *Ccnd1* promoter moderating the recruitment of ß-catenin to TCF/LEF bound to this site (Hagey and Muhr, 2014). Since WNT signaling is a common driver of stem cell proliferation in multiple tissues (Clevers et al., 2014), one can speculate that this might be a common target of SOX2 to maintain slow proliferation in several types of stem cells.

3.2 PAPER II

To begin to study the function of SOX5/6/21 in brain tumor development, we first confirmed their expression in the adult mouse SVZ. In this niche, SOX5/6/21 co-labeled with the stem cell markers SOX2 and NESTIN in addition to the proliferation marker Ki67, and it can therefore be concluded that SOX5/6/21 are expressed in actively proliferating stem or progenitor cells. Next we tested the function of SOX5/6/21 by overexpressing SOX5, SOX6 or SOX21 in NSCs isolated from the SVZ. This uniformly led to a decreased rate of proliferation, as determined by EdU incorporation. As high levels of SOX5/6/21 have been shown to counteract tumorigenesis (Ferletta et al., 2011; Tchougounova et al., 2009; Wang et al., 2016; Xie et al., 2012), we wanted to determine SOX5/6/21 levels in the presence of oncogenes. Interestingly, transducing mouse SVZ cells in culture with the activated oncogenes *AKT1* and *H-RAS* increased the protein levels of SOX5/6/21 compared to control transduced cells. In contrast, the protein levels of other stem cell expressed genes such as SOX2 or NESTIN remained unchanged, indicating that the increase in SOX5/6/21 expression was specific, and might serve as an attempt of the SVZ cells to evade oncogenic transformation.

We next sought to verify this possibility by deleting *Sox5/6/21* in mice stereotactically injected with oncogene-expressing viruses. In this mouse glioma model 10-15% of wt mice injected in the SVZ normally develop tumors. In contrast, mice in which a conditional deletion of *Sox5*, *Sox6* or *Sox21,* or a combination of these, was induced at the same time as the viral oncogene injection, an increased tumor size and penetrance was observed, with the more severe phenotype in mice with a deletion of multiple *Sox* genes. Upon histological examination of the tumors, many high-grade glioma features were found including increased cellular density and atypia, hemorrhage and microvascular proliferation. These features were more commonly associated with the combinatorial loss of *Sox5/6/21* compared to the loss of individual *Sox* genes. Thus, SOX5/6/21 can prevent oncogene-driven tumor formation in a partly overlapping fashion. It is however important to note that no hyperproliferative phenotype was found in mice with conditional deletion of *Sox5/6/21* in the absence of oncogenic expression. Consistently,

although loss of function studies have revealed specific roles for SOX5, SOX6 and SOX21 in the specification, differentiation and maturation of neurons and oligodendrocytes (Azim et al., 2009; Lai et al., 2008; Sandberg et al., 2005; Stolt et al., 2006), no general role for these SOX proteins in regulating proliferation has been revealed in the developing or adult CNS. Therefor, it is interesting to note that the ability of SOX5/6/21 to block excessive proliferation is perhaps only revealed under tumorigenic conditions.

To further establish how the loss of *Sox5/6/21* affects oncogene-expressing cells, SVZ cells from mice of different genotypes newly injected with *AKT/H-RAS/Cre* were isolated and grown as spheres in culture. Cells from *Sox5/6, Sox21* or *Sox5/6/21* mutant mice formed significantly more spheres that grew bigger in size compared to cells from wt mice. In line with this result, we also found that *Sox21* and *Sox5/6/21* mutant spheres incorporated EdU at a higher level and were labeled more extensively by Ki67, thus indicating an increased proliferative rate, as compared to wt spheres. Furthermore, GO analysis of RNA-seq data of genes upregulated in *Sox5/6/21* mutant versus wt spheres, revealed significant enrichment of terms for proliferation such as *"Mitotic cell cycle"*, *"Cell cycle"* and *"Cell division"*. This was in contrast to downregulated genes, which instead showed high enrichment for terms associated with differentiation such as *"Neurogenesis"*, *"Gliogenesis"* and *"Axon guidance pathway"*. Moreover, comparing the differentially expressed genes in oncogene-expressing cells with and without *Sox5/6/21* with genes differentially expressed in human GBM versus low grade glioma, a similar pattern emerged where important cell cycle promoting genes were commonly up-regulated and genes important for differentiation and tumor suppression were commonly downregulated. Thus, the same cellular mechanisms that induce more malignant human gliomas are active in the oncogene-induced mouse cells lacking *Sox5/6/21*.

An essential mechanism of cell cycle regulation is the Cyclin/CDK-RB axis in which RB blocks cell cycle progression unless phosphorylated by Cyclin/CDK complexes. An additional regulatory level exists in the CDK inhibitors, which counteract Cyclin/CDK activity, thus inhibiting proliferation. To find out how this pathway was affected, and since most of these factors are regulated at a post-translational level rather than a transcriptional level, we next evaluated their presence in our oncogene-expressing spheres by western blot. Loss of *Sox5/6/21* severely increased the protein levels of several Cyclins. Furthermore, although total RB levels did not change, there was a sharp increase in the levels of the phosphorylated inactive form of RB, which was most prominent in the triple *Sox5/6/21* mutant cells. Moreover, although the levels of the CDK inhibitors p21 and p27 and the tumor suppressor p53 did increase upon expression of the oncogenes in wt cells compared to GFP control, this upregulation was efficiently suppressed when *Sox5/6/21* were deleted.

Since the loss of a p53 and CDK inhibitor response to oncogenes could be an underlying mechanism for the excessive proliferation and the malignant transformation of the mutant cells, we next wanted to test whether we could block the observed phenotypes by increasing their levels. Indeed, restoring the levels of p21, p27 or p53 in spheres or in animals lacking *Sox5/6/21* significantly decreased the proliferative capacity and the tumor forming ability of the oncogenes.

Thus, the anti-tumorigenic response mounted by SOX5/6/21 seems, at least in part, to be achieved by increasing CDK inhibitors and p53 protein levels.

Having established the protective effect of SOX5/6/21 during oncogene expression, we next asked whether SOX5/6/21 could also exhibit an anti-tumorigenic effect in already transformed cells. Lenti-viral overexpression of SOX5/6/21 efficiently decreased proliferation of human primary glioblastoma cells compared to GFP control. Furthermore, intracranial injection of SOX5/6/21 expressing cells into NOD-SCID mice completely blocked their capacity to form tumors compared to GFP-expressing control cells. This striking effect on human primary glioblastoma cells led to the question whether there is a similar negative relationship between *SOX5/6/21* expression and malignancy grade in human glioma. To test this we used publically available RNA expression data from human low grade glioma (grade II and III) and high grade glioma (grade IV) and compared the expression of *SOX5/6/21* in these two sets. Consistently, the expression levels of *SOX5/6/21* normalized against *PCNA*, were all significantly decreased in high versus low grade glioma. Together this shows that SOX5/6/21 have an anti-tumorigenic effect not only during tumor initiation, but also in full-blown disease.

In order to reveal the molecular pathways in malignant cells targeted by SOX5/6/21 upregulation, we next performed RNA-seq on human primary gliomblastoma cells transduced with *SOX5/6/21* or *GFP* control. Thousands of gene were found to be differentially expressed and among the genes significanly downregulated there was an enrichment of GO terms for proliferation such as *"Mitotic cell cycle", "Nuclear division"* and *"RB in cancer"*. In contrast, upregulated genes instead showed significant enrichment for terms associated with tumor suppressor responses, including *"Apoptotic process"*, *"Cellular response to stress", "Direct p53 effector"* and *"Senescence and autophagy"*. Consistantly, the CDK inhibitors and p53 were upregulated by SOX5/6/21 in primary glioblastoma cells at a protein level. Furthermore, assessing the functional tumor suppressor responses apoptosis and senescence revealed a significant increase in both of these processes after SOX5/6/21 expression in human primary glioblastoma cells. Since both apoptosis and senescence can be induced by increased levels of the tumor suppressor p53, we next asked whether the increase in these processes was dependent on p53. Blocking p53 with shRNA completely blocked both apoptosis and senescence induced by SOX21 in human primary glioblastoma cells. Moreover, while the overexpression of SOX21 in human primary glioblastoma cells did not reveal any SOX21 binding in the vincity of the *TP53* gene, as determined by ChIP-seq, or any upregulation of *TP53* expression, as determined by RNA-seq, we could show that it did lead to a decrease in p53 protein turn-over. This stabilization was associated with increased phosphorylation of p53 and decreased protein levels of the negative p53 regulator MDM2. Taken together, these data show that SOX21 can initiate a tumor suppressor response in malignant cells by counteracting p53 protein turn-over.

In summary, we have shown that SOX5/6/21 have partly overlapping activities suppressing oncogene-induced transformation of brain stem and progenitor cells, and that their increased expression in fully malignant cells can re-activate a tumor suppressor response. This is a novel mechanism by which stem and progenitor cells in the brain could potentially evade malignant

transformation driven by oncogenic insult, and thus promote well established tumor suppressor responses.

3.3 PAPER III

As targeted deletion of *Sox21* in **Paper II** revealed a significant tumorigenic phenotype in mouse neural stem cells under oncogenic pressure, we aimed to investigate whether the anti-tumorigenic function of SOX21 could be a more general mechanism of tumor suppression and not only brain stem cell specific. To do this, we started by examining the expression pattern of SOX21 in adult human gastric epithelium, a tissue in which *SOX21* mRNA expression has been reported. In the corpus region, which is the more proximal part of the ventricle, we could find strong immunolabeling for SOX21 in the glandular cells of the gastric pit and the isthmus. In this region cells were also labeled with antibodies against SOX2, and Ki67. Using double immunofluorescence we could see that SOX21 expression overlapped with that of SOX2 in the pit and isthmus region and with Ki67 in the isthmus region. Therefore, SOX21 seems to be expressed in proliferating SOX2 positive cells in the normal human gastric epithelium, in addition to differentiated post-mitotic cells.

As *SOX21* was seen in **Paper II** to be downregulated in high grade glioma compared to lower grade glioma, we wanted to see whether this inverse relationship with malignancy was also true for *SOX21* in gastric cancer. Publically available RNA-seq data from gastric adenocarcinomas and normal gastric tissue samples were compared. We found significantly lower levels of *SOX21* mRNA in the malignant tissue and this reduction was true for all four molecular phenotypes. Interestingly, within this large dataset there were some genetic alterations of the *SOX21* gene, including homozygous deletion, missense mutation and amplification, where the last one was most common (3%). However, regardless of genetic amplification, the mRNA levels of *SOX21* were still lower in these tumor samples compared to normal tissue, indicating that other mechanisms are acting to keep the *SOX21* expression at low levels. Since CpG island methylation is a potent regulator of gene expression, we wanted to explore this by comparing methylation levels of the *SOX21* promoter in malignant versus normal tissue. However, *SOX21* promoter methylation was not significantly changed.

To confirm the human gastric tissue expression of SOX21 in mouse, we performed immunolabeling of SOX21 in mouse embryonic stomach. SOX21 expression follows that of SOX2 and is expressed at high levels in the stomach at E11.5 and E13.5, at lower levels in the esophagus and not at all in the small intestine. RNA-seq of adult mouse gastric epithelium revealed expression of several *Sox* genes, with the highest levels of gastric epithelium and cancer relevant *Sox9* and *Sox2* together with, the in the stomach so far uncharacterized, *Sox21* and *Sox13*. In a more detailed analysis of mouse gastric antrum we found that *Sox21* mRNA located to the isthmus region, while the protein was found in a more widespread manner including most cells from the base to the pit. This protein expression was further seen to overlap extensively with that of, in mouse previously characterized, gastric stem cell marker SOX2, and with proliferation marker Ki67.

So far, no gastric phenotype has been reported after targeted deletion of *Sox21*. Therefore, to specifically assess this, we induced *Sox21* deletion in adult mice using tamoxifen-inducible Cre expression in the *Sox2* locus. One month after tamoxifen induction, no difference in EdU incorporation between targeted glands of *Sox21* homozygous or heterozygous animals could be detected, indicating that *Sox21* expression is dispensable for normal gastric homeostasis.

As the reduced expression of *SOX21* in gastric cancer tissue compared to normal tissue could indicate a loss of anti-tumorigenic activity in these cells, we wanted to find out whether re-establishing SOX21 expression in gastric cancer cells could lead to an induction of an anti-tumorigenic response. Three human gastric cancer cell lines with low expression of *SOX21,* were virally transduced with either *SOX21* or *GFP* control. All three cell lines showed decreased proliferation after transduction with *SOX21* as seen by EdU incorporation. In a subsequent RNA-seq analysis we found thousands of genes to be regulated by SOX21, many of which were implicated in cell cycle regulation as GO terms such as *"Negative regulation of cell proliferation"* was enriched within upregulated genes in the KATOIII cell line, and *"Mitotic cell cycle process"* was enriched within the downregulated genes in the NCI-N87 cell line. Furthermore, enrichment of GO terms including *"Programmed cell death"* were found within the upregulated genes of NCI-N87 and AGS cell lines.

Since apoptosis is a fundamental tumor suppressor response, we next sought to find out whether SOX21 could directly induce this process in the gastric cancer cell lines. Six days after transduction, SOX21 significantly increased the levels of AnnexinV in NCI-N87 and AGS cells, but not in KATOIII cells. As we could show in **Paper II** that the apoptotic phenotype seen in human primary glioblastoma cells was mediated by the stabilization of p53, we then wanted to find out whether this could also be the mechanism by which SOX21 could induce apoptosis in gastric cancer cells. Five days after *SOX21* transduction, a small increase in p53 could be seen in the AGS cell line, but none in the NCI-N87 or in the KATOIII cells. It is interesting to note that while the AGS cell line expresses wild type p53, the NCI-N87 cell line harbors a common *TP53* missense mutation and the KATOIII cell line a homozygous deletion of the *TP53* locus. Nevertheless, although p53 seems to be an important factor for inducing an apoptotic response in AGS cells compared to KATOIII cells, the NCI-N87 cells are still able to mount an apoptotic response without increasing the already high levels of mutant p53. In conclusion, restoring SOX21 levels in human gastric cancer cells can induce an anti-tumorigenic response, which might in part be mediated by increasing wt p53 levels.

4 CONCLUSION AND FUTURE DIRECTIONS

With the work in this thesis we have illustrated how one TF, SOX2, can act in different stem cell populations protecting their status by regulating common features and features specific to each stem cell lineage and their identity, by binding to specific CRMs. In addition, apart from protecting stem cell maintenance, we found that another set of TFs from the same family, SOX5/6/21 also act to protect stem cells, but in a different setting, being revealed only under the stress of oncogenes. As it is becoming increasingly clear that cell cycle control, DNA damage pathways and stem cell regulation are intimately linked (Dalton, 2015; Gonzales et al., 2015; Pauklin and Vallier, 2013), it might not be surprising that TFs previously known to regulate features within stem cells during development could also protect cells against excessive proliferation induced by oncogenes. Interestingly, the regulation of SOX protein levels and the regulatory function achieved by SOX proteins appear not to be controlled at the transcriptional level in the oncogenic setting. Although many SOX TFs are transcriptionally regulated by the activity of specific enhancers (Boyer et al., 2005; Uchikawa, 2008), SOX protein regulation is also well established and many SOX TFs have been found to be regulated by a variety of different post-translational modification (Lim et al., 2017; Liu et al., 2013; Suryo Rahmanto et al., 2016). What requires further investigation is how SOX proteins modulate cell functions in a non-transcriptional manner. The fact that SOX21 seems to function in a similar manner in stem cells of the brain and stomach, justifies why more attention should be paid to the question of how SOX21 could be protecting stem cells from oncogenic transformation. Further studies into the mechanisms activating increased protein levels of SOX21 during stress, and also the direct targets of SOX21, will be interesting paths to pursue.

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