Transcriptomic and functional studies of fusion oncogene-driven salivary gland tumors

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Cover illustration: Circos plot visualizing the *MYB-NFIB* (red line), *NFIB-PLAG1* and *HMGA2-NFIB* (blue lines) fusion oncogenes in salivary gland tumors (by the author).

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To my parents

The knowledge of anything, since all things have causes, is not acquired or complete unless it is known by its causes – Avicenna

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ABSTRACT

Fusion genes are potent oncogenic drivers resulting from exchange of regulatory/coding sequences between two genes. They were originally identified in leukemias but are now recognized as key oncogenic events also in many solid tumors, including salivary gland tumors (SGTs).

Adenoid cystic carcinoma (ACC) is a highly malignant SGT with no effective treatment for patients with recurrent and/or metastatic disease. The *MYB-NFIB* fusion is the main genomic hallmark of ACC and a potential therapeutic target. Here, oncogenic signaling pathways as well as the molecular consequences and regulation of *MYB-NFIB* were assessed in cultured ACC cells and in ACC surgical samples. A combination of molecular and functional assays was used including RNAi, qPCR, western blot, phospho-receptor tyrosine kinase (RTK) arrays, proliferation/apoptosis/sphere assays, and gene expression microarrays. ACC patient-derived xenografts (PDX) were used to study the effects of RTK-inhibition on tumor growth. *MYB-NFIB* was shown to promote proliferation and spherogenesis of ACC cells. The fusion regulated expression of genes involved in DNA replication/repair, cell cycle, and RNA processing, and induced an MYC-like transcriptional program. *MYB-NFIB* was shown to be regulated by IGF1R through IGF2-activated AKT-signaling and pharmacological inhibition of IGF1R partially reversed the transcriptional program induced by MYB-NFIB. Moreover, IGF1R, EGFR, and MET were co-activated in ACC cells. Combined inhibition of these receptors in ACC cells and PDX-models induced differentiation and synergistic growth inhibition. The results provide new insights about the function and regulation of *MYB-NFIB* and are the first to show that a druggable cell surface receptor can regulate a fusion oncogene encoding a transcription factor. Importantly, the results also highlight novel potential treatment strategies for ACC patients.

Pleomorphic adenoma (PA) is the most common SGT. Although it is a benign tumor, treatment may be complicated by recurrence and/or malignant transformation. Previous studies of PA have revealed recurrent chromosomal rearrangements that activate the key oncogenes *PLAG1* and *HMGA2* by gene fusion events. Here, detailed studies of previously uncharacterized subsets of PAs with 8;9- or 9;12-rearrangements revealed breakpoints within or in the proximity of either *PLAG1* or *HMGA2*, and *NFIB*. Further analyses using RNA-seq, RT-PCR, qPCR, and arrayCGH revealed a novel *NFIB-PLAG1* fusion in a PA with an ins(9;8) and *HMGA2-NFIB* fusions in cases with t(9;12). These findings highlight the role of *NFIB* as a fusion partner gene in both benign and malignant SGTs and indicate that *NFIB* can activate both *PLAG1* and *HMGA2* by gene fusion/enhancer hijacking events in PA. Furthermore, RNA-seq based transcriptomic analysis of PAs revealed a high frequency of *PLAG1* and *HMGA2* fusions (≈80% of the cases) and multiple novel fusion partner genes. The findings indicate that gene fusions are more common in PA than previously documented. Global gene expression and pathway analyses revealed several activated oncogenic signaling pathways and showed that the expression profile reflects certain morphological features typical of PA. Finally, the results showed that *PLAG1* and *HMGA2* drive tumorigenesis via shared signaling pathways. The results provide further insights into the pathogenesis of PA and reveal new potential therapeutic targets.

Keywords: fusion oncogene, *MYB*, *NFIB*, *PLAG1*, *HMGA2*, adenoid cystic carcinoma, pleomorphic adenoma, targeted therapy

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SAMMANFATTNING PÅ SVENSKA

Tumörer uppkommer till följd av förändringar i cellers arvsmassa. Exempel på sådana förändringar är mutationer och kromosomförändringar, dvs när delar av olika kromosomer bryts av och sätts samman på felaktigt sätt. De nya gener som då bildas, så kallade fusionsonkogener, bidrar aktivt till tumörutveckling. Fusionsonkogener har framförallt studerats i leukemier där man även tagit fram specifika behandlingar som riktar sig mot dessa gener och deras proteinprodukter. På senare tid har det visat sig att fusionsonkogener är frekventa även i ett flertal andra tumörsjukdomar. Den här avhandlingen fokuserar på studier av fusionsonkogeners roll i spottkörteltumörer, särskilt pleomorft adenom (PA) och adenoidcystisk cancer (ACC).

ACC är en aggressiv spottkörtelcancer där det idag saknas botande behandling för patienter med avancerad sjukdom. Fusionsonkogenen *MYB-NFIB* är specifik för ACC och är därför en viktig måltavla för utveckling av ny behandling. Vi undersökte på molekylär nivå hur aktiviteten hos *MYB-NFIB* fusionsonkogenen regleras i ACC och vilka effekter den har på tumörceller. För att studera genens funktion blockerade vi aktiviteten hos *MYB-NFIB* i ACC-celler med hjälp av RNA-interferens. Vi fann att *MYB-NFIB* stimulerar celldelning hos ACC-celler genom att aktivera en rad tillväxtstyrande gener. Vidare studerade vi receptortyrosinkinaser (RTKer). Dessa är cellyteproteiner som är viktiga för cellsignalering och uppvisar ofta en förändrad aktivitet i tumörceller. Vi studerade aktiviteten hos RTKer i ACC och även effekten av läkemedel som hämmar deras funktion. Läkemedelseffekterna studerades både i cellodling och hos möss som transplanterats med ACC-tumörer från patienter. Vi fann att aktiviteten hos *MYB-NFIB* genen regleras av receptorn IGF1R och att farmakologisk hämning av IGF1R delvis återställer de effekter som inducerats av MYB-NFIB. Vi fann också samaktivering av receptorerna IGF1R, MET och EGFR i ACC-celler och att kombinerad inhibering av dessa receptorer minskar tillväxten av både ACC-celler i cellodling och av tumörer hos möss. Våra resultat ger ny viktig kunskap om funktionen och regleringen av *MYB-NFIB* fusionen och visar på nya potentiella behandlingsstrategier för patienter med ACC.

PA är den vanligaste spottkörteltumören. Det är en i flertalet fall godartad tumör som dock kan återkomma trots behandling och/eller omvandlas till en elakartad tumör. PA uppvisar i hög frekvens kromosomförändringar som leder till aktivering av de tumördrivande generna *PLAG1* och *HMGA2*. I våra studier visade vi att *NFIB* genen tillsammans med både *PLAG1* och *HMGA2* bildar tidigare icke kända fusionsgener i nya subgrupper av PA. Våra resultat antyder också att *NFIB* kan aktivera *PLAG1* och *HMGA2* med hjälp av så kallade förstärkarelement, något man tidigare bl a sett i ACC. Med hjälp av ett flertal molekylärgenetiska metoder kartlade vi även genetiska förändringar i PA. Vi fann att *PLAG1* och *HMGA2* var inblandade i fusionsonkogener i ca 80% av fallen vilket är en högre frekvens än vad man tidigare sett. Vi identifierade också ett flertal nya fusionspartners till *PLAG1* och *HMGA2*. Dessutom studerade vi genuttrycksmönstret i PA och fann att flera viktiga tumördrivande gener och signalvägar var aktiverade. Resultaten ger nya insikter om uppkomstmekanismerna för PA och kan på sikt leda till nya möjligheter att behandla patienter med dessa tumörer.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals:

I. Andersson MK, **Afshari MK**, Andren Y, Wick MJ, Stenman G. Targeting the Oncogenic Transcriptional Regulator MYB in Adenoid Cystic Carcinoma by Inhibition of IGF1R/AKT Signaling. *J Natl Cancer Inst* 2017;109(9).

II. **Afshari MK**, Fehr A, Nevado PT, Andersson MK, Stenman G. Activation of *PLAG1* and *HMGA2* by gene fusions involving the transcriptional regulator gene *NFIB*. *Genes Chromosomes Cancer* 2020;59:652-660.

III. **Afshari MK**, Nevado PT, Fehr A, Stenman G, Andersson MK. Transcriptomic profiling of pleomorphic salivary gland adenomas. *Manuscript*.

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1 INTRODUCTION

Our life depends on an intricate equilibrium of the cells in our tissues and organs formed by millions of years of evolution. Cancer, like many other diseases, is caused by a disruption of this delicate balance, in particular the balance between cell proliferation and cell death. The cause of this disruption lies in the alteration of our genetic material. From a simple concept a century ago, we are now able to detect the underlying causes of cancers at the DNA level. Recent advances in genomic technologies, mainly next generation sequencing, have revolutionized cancer research and accelerated diagnostic, prognostic, and therapeutic developments, leading to an era of precision oncology – targeted treatments based on the genomic profiles of tumors. Yet, as about one in six global deaths is due to cancer (*1*), much work is left to translate this knowledge into clinical practice with the ultimate goal to overcome this devastating disease.

1.1 The genetic basic of cancer

Cancer is characterized by an abnormal and uncontrolled proliferation of cells and their ability to invade adjacent tissues and disseminate to distant organs (*2, 3*). There are several hundreds of cancer subtypes caused by genetic mutations in different cell types. The first insights into the role of the genome in cancer development arose more than a century ago through observations of chromosomal aberrations in tissue sections of malignant tumors (*4, 5*). This led to the hypothesis that tumors are composed of transformed cells with altered genetic material. Indeed, during the last 50 years the concept that chromosome

changes and DNA sequence alterations are the foundation of cancer has been well established. Our knowledge has now expanded to a point where genomics has become an integral part of cancer research and therapy (*6*).

Driver mutations are specific alterations in the DNA sequence that can initiate a cascade of cellular events leading to uncontrolled cell growth (*7*). These mutations disturb the homeostatic regulatory mechanisms in cells and provide mutant cells with a selective advantage over their normal neighbors. Stepwise accumulation of such mutations leads to clonal expansion of mutant cells and ultimately to cancer development (*8-10*). Patients with hereditary cancers, which make up 5-10% of all cases, have driver mutations present in their germ line leading to a significantly increased risk of cancer. These germ line mutations shorten the time of tumor development (*11*). In many cancers, genomic instability is a characteristic feature that accelerates tumor progression. Other characteristic features include certain specific cellular traits designated "the hallmarks of cancer" which are acquired during tumorigenesis (*3*). As a result of driver mutations cancer cells are able to: [1] maintain self-sufficient proliferative signaling, [2] escape external growth suppression, [3] resist apoptosis, [4] gain replicative immortality, [5] promote angiogenesis, [6] initiate invasion and metastasis, [7] avoid immune destruction, and [8] rewire their energy metabolism. The development of these capabilities can be promoted by inflammation and the surrounding microenvironment (*12*).

Mutations in tumor cells are not limited to driver mutations. In fact, the vast majority of mutations in cancer cells are so called "passenger mutations". They have for a long time been considered as random events with no immediate beneficial effect for the tumor or for the clinical outcome of patients (*13, 14*). However, recent studies have shown that passenger mutations can promote therapeutic resistance (*15*). In addition, passenger mutations may encode tumor

neoantigens which have been associated with improved sensitivity to immunotherapy, particularly in the management of malignant melanoma (*16, 17*).

1.2 Cancer genes

To date, there are more than several hundred known cancer genes that can be activated through different mutations and chromosomal rearrangements (*18*). Although mutations in non-coding DNA have recently been linked to tumor development, most driver mutations occur in protein coding genes (*19*). In general, alterations in three types of genes are associated with tumorigenesis: oncogenes, tumor-suppressor genes and DNA repair genes (*7*).

Oncogenes, the most common type of cancer genes, are dominantly acting genes, i.e. an activating mutation in one allele is adequate to render the cell with proliferative and survival advantages. The mechanisms of oncogene activation include gene amplification, point mutation, chromosomal rearrangements, and viral transduction. Chromosomal rearrangements may result in true fusion oncogenes or promoter swapping/enhancer hijacking leading to oncogene activation (discussed in detail below). Examples of well-known oncogenes are *MYC, KRAS, PDGFRA, KIT, EGFR,* and *BRAF* (*2, 7, 20*).

In contrast to activating mutations in oncogenes, mutations in tumor-suppressor genes (TSGs) result in gene inactivation through mechanisms such as point mutation, deletion, or epigenetic silencing. Most often, inactivation of both alleles of a TSG is required for tumor development and they are thus known as recessive cancer genes (*7, 21*). An extensively studied TSG that is

mutated in up to 50% of human cancers is *TP53*, known as the guardian of the genome (*22*). Other well-known examples of TSGs are *RB1*, *CDKN2A*, *APC*, and *PTEN*. The consequence of mutations in oncogenes and TSGs are similar at the cellular level – providing mutant cells with unrestricted proliferative stimuli and/or preventing apoptosis (*7*).

The third group of the genes involved in tumorigenesis are DNA repair genes. Examples of such genes are those involved in mismatch repair, base- and nucleotide-excision repair, as well as those taking part in homologous recombination and chromosome segregation (e.g. *MSH2*, *PARP1*, *XPA*, *BRCA1*, and *BRCA2*). These genes maintain the stability of the genome and prevent genetic changes from becoming permanent. Inactivating mutations in DNA repair genes result in an increased mutational burden, including mutations in oncogenes and TSGs, which may confer a selective growth advantage to the mutant cells. Similar to TSGs, both the maternal and paternal alleles of DNA repair genes must be inactivated in order to contribute to tumor development (*7, 23*).

1.3 Chromosomal rearrangements and fusion oncogenes

Chromosomal rearrangements are a common cause of oncogene activation (*24*). Although chromosomal aberrations were suggested to underlie tumor development already in the early 1900's, it required half a century of science and technical development before the first recurrent tumor-type specific chromosomal aberration was identified in a human malignancy – the Philadelphia chromosome in chronic myeloid leukemia (CML) (*25*). Chromosome banding techniques, introduced in the 1970s, revealed that the

Ph chromosome resulted from a balanced translocation between chromosomes 9 and 22, that is t(9;22)(q34;q11) (*26, 27*). In the following years, several other recurrent balanced chromosomal rearrangements, in particular translocations, were discovered in hematological malignancies including the t(4;11)(q21;q23) in acute lymphoblastic leukemia (*28*), and the t(8;14)(q24;q32) in Burkitt's lymphoma (*29, 30*). Subsequent cytogenetic studies revealed that recurrent chromosomal rearrangements were not limited to hematological malignancies but were also found in malignant mesenchymal tumors and in certain epithelial tumors, such as the t(11;22)(q24;q12) in Ewing sarcoma (*31*) and the t(6;9)(q23;p23) in adenoid cystic carcinoma (ACC) of the salivary glands (*32*). The t(3;8)(p21;q12) in salivary gland pleomorphic adenoma (PA) was the first reciprocal translocation found in a benign tumor (*33*).

Recent advances in whole genome and RNA sequencing have led to the detection of numerous novel genomic rearrangements in cancer (*29*) leading to gene fusions or deregulation of gene expression through promoter swapping or enhancer hijacking as demonstrated in Figure 1. Chromosomal rearrangements are balanced or unbalanced depending on whether the net content of DNA is altered by the rearrangement (*34*). Balanced, or copy number neutral rearrangements, include translocations (i.e. a reciprocal exchange of chromosome material between chromosomes), inversions (a 180-degree rotation of a chromosomal segment), and insertions (relocation of a chromosome segment into the same or another chromosome). However, numerous studies have shown that cytogenetically balanced rearrangements are often in fact unbalanced at the nucleotide level as demonstrated by findings of deletions, insertions, and duplications close to the translocation breakpoints (*35, 36*).

Unbalanced rearrangements, on the other hand, lead to a net gain or loss of genetic material. Examples of unbalanced chromosomal rearrangements are amplifications or deletions of chromosome segments, and ring chromosomes (caused by breaks in both arms of a chromosome followed by fusion of the ends) (*35*).

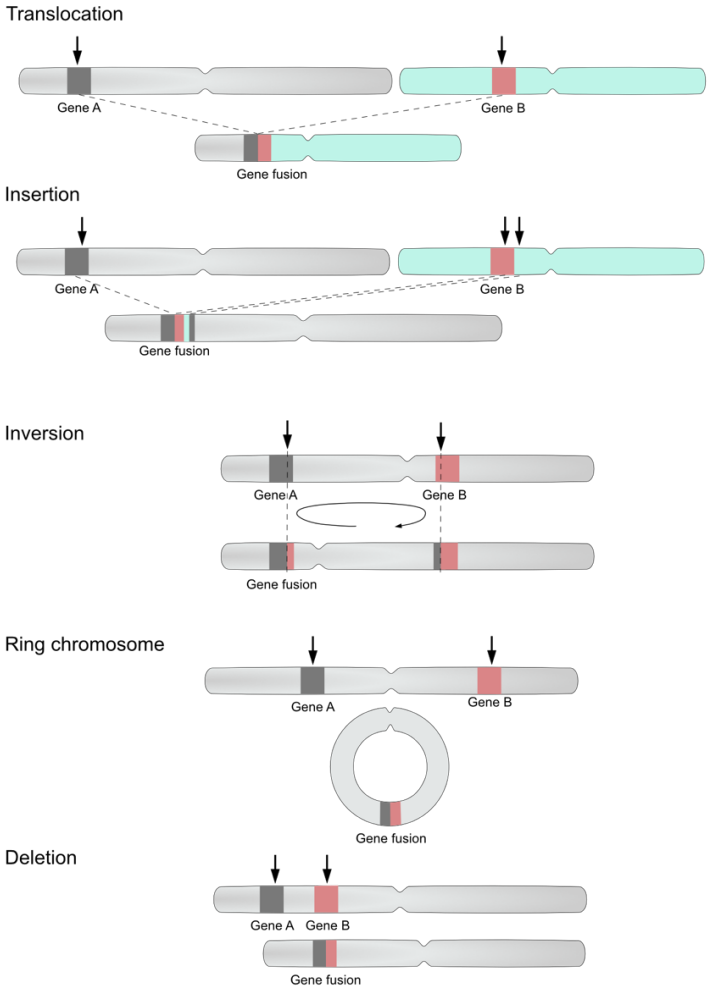

Figure 1. Schematic illustration of chromosomal rearrangements resulting in gene fusions. Chromosomal break points are indicated by arrows.

1.4 Mechanisms of cancer gene deregulation through chromosomal rearrangement

The clinical and pathogenetic importance of chromosomal rearrangements have become evident as the molecular consequences of these aberrations have been elucidated. The main cause of tumor development through these rearrangements are through juxtaposition of two distant chromosomal regions, leading to gene fusions or exchange of regulatory elements (*24*). Figure 2 shows examples of different mechanisms of oncogene activation by chromosomal rearrangements.

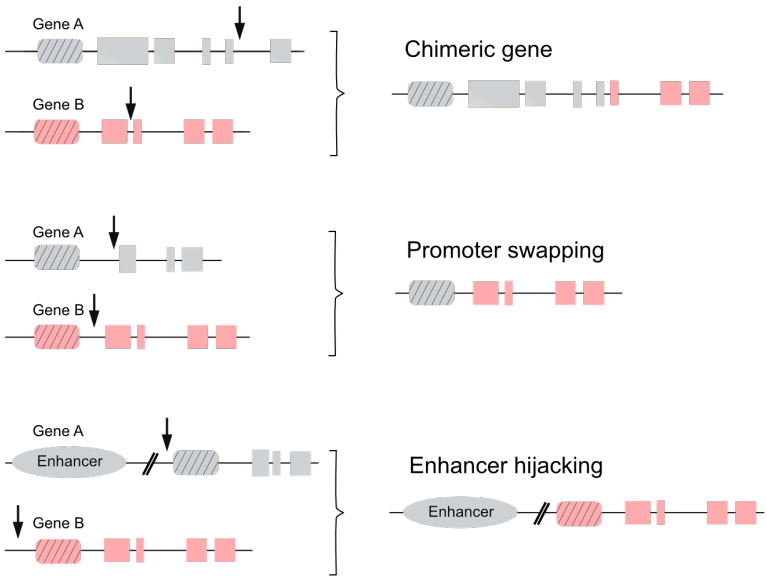

Figure 2. Schematic illustration of different mechanisms of oncogene activation by gene fusion. Promoter regions are indicated by hatched lines and chromosomal break points by arrows.

A prototype gene fusion with a significant impact on patient management and clinical outcome is the *BCR-ABL1* fusion in CML (*37, 38*). The fusion encodes

a chimeric BCR-ABL1 oncoprotein which is crucial for initiation and maintenance of CML. The BCR-ABL1 fusion leads to an increased tyrosine kinase activity of ABL1 and conveys new protein-protein interaction domains to the encoded oncoprotein (such as the SH2-binding site of the growth factor receptor-bound protein 2 (GRB2)) (*39*), resulting in activation of several oncogenic signaling pathways. BCR-ABL1 was the first oncoprotein to be therapeutically targeted with a tyrosine kinase inhibitor (TKI) (*40-42*). Other TKIs have since been used for treatment of malignancies harboring oncogenic gene fusions, for example crizotinib for treatment of *ALK* fusion-positive non-small cell lung cancer (*43*). Additional examples of gene fusions in solid tumors encoding chimeric oncoproteins are *ETV6-NTRK3* in secretory breast and salivary gland carcinomas (*44*), *MYB-NFIB* in ACC (*45*), *EWSR1-FLI1* in Ewing sarcoma (*46*), and *EWSR1-ATF1* in clear cell sarcomas and carcinomas (*47, 48*).

In addition to chimeric fusion gene formation, exchange of transcriptional regulatory elements is an important and recognized consequence of genomic rearrangements (*24, 35*). The activating regulatory elements, promoters and enhancers, are involved in transcriptional initiation responsible for cell type-specific gene expression patterns (*49*). While promoters are immediately proximal to the transcription start site, enhancers can drive transcriptional initiation from long distances (*50*). Chromosomal rearrangements may lead to relocation of regulatory elements and can juxtapose oncogenes in the proximity of an active promoter or enhancer element, leading to aberrant gene expression (*24, 35*). Promoter swapping has been described in several tumor types, for example in PA of the salivary glands (*PLAG1* gene fusions) (*51, 52*), dermatofibrosarcoma protuberans (*COL1A1–PDGFRB*) (*53*), and prostate cancer (*TMPRSS2-ERG*) (*54*). Important examples of oncogene activation through enhancer hijacking are activation of *MYC* and *BCL2* by regulatory

elements in the *IGH* locus in lymphoid malignancies (in Burkitt's lymphoma and follicular lymphoma respectively) (*55*). Recently, oncogene activation through enhancer hijacking have also been described in solid tumors. Examples are activation of *GFI1* and *GFI1B* (of the growth factor independent 1 family) in medulloblastoma, the most common pediatric brain tumor, through juxtaposition of these genes to other loci with active epigenetic states (*56*). Enhancer hijacking has also been described in acinic cell carcinoma of the salivary glands leading to activation of the *NR4A3* transcription factor gene by enhancer elements derived from the secretory Ca-binding phosphoprotein (SCPP) gene cluster at 4q13 (*57*). Moreover, activation of *MYB* through enhancer hijacking has been described in salivary gland ACC (*58*). Loss of negative regulatory elements through fusion events may also lead to altered expression of the affected oncogenes. Examples of this are rearrangements resulting in loss of miRNA binding sites in 3'-UTR of *MYB*, *HMGA2*, and *FGFR3* in ACC, PA, and glioblastoma, respectively (*45, 59-61*).

To date, the total number of gene fusions registered in *the Mitelman Database of chromosome aberrations and gene fusions* amount to more than 32,500 (*29*). Although the absolute majority of these are passenger and non-recurrent mutations (*62*), the importance of gene fusions in the pathogenesis, diagnosis, prognosis, and treatment of human tumors is clear. In addition to being important therapeutic targets, gene fusions have also become an important part of routine molecular pathology. This is due to the tumor-type specificity that many gene fusions show and to the use of the fusions as biomarkers for treatment response (*35*).

1.5 Targeting fusion oncogenes in cancer

Recent studies have shown that most tumors harboring fusion oncogenes have rather stable genomes and comparatively few somatic mutations. This indicates that fusion oncogenes are potent drivers that can promote tumorigenesis alone or in concert with a few other driver events. Due to their tumor-type specificity and central role in tumor development they are potential targets for precision oncology. Genes of mainly two functional classes are dysregulated through fusion events, that is kinases and transcriptional regulators (*63*).

Fusion events affecting kinases lead to constitutive kinase activity and activation of downstream oncogenic signaling pathways. Receptor tyrosine kinases (RTKs) constitute a large family of cell-surface receptors with key roles in regulation of vital cellular processes such as proliferation, differentiation, and cell survival. In addition to gene fusion, they can be activated through other mechanisms such as gene amplification, gain of function mutation, and/or autocrine stimulation (*64*). Kinase inhibitors (KIs) have been widely exploited in cancer treatment. To date, there are over 40 FDA approved KIs, many of which are used as part of standard care for treatment of different cancers (*65*). EGFR, FGFRs, VEGFRs, and PDGFRs are examples of RTKs commonly activated in cancers that can be targeted with KIs. As mentioned above, the prototypic gene fusion *BCR-ABL1* encodes a constitutively active tyrosine kinase that activates key oncogenic pathways in leukemic cells, resulting in increased cell proliferation and survival (*66*). Following the discovery of this fusion, its inhibition with the KI imatinib mesylate has become a paradigm for successful molecularly targeted therapy (*40-42*). Moreover, imatinib has also become an integral part of the treatment

in other fusion oncogene-driven malignancies such as dermatofibrosarcoma protuberans with *COL1A1-PDGFRB* fusion (*67*).

Despite recent advances in the development of targeted therapies, oncogenic transcription factors still remain immensely difficult to target (*68, 69*). Several approaches to target transcriptional regulators are currently under development. For example, the targeting of transcription factor interactions with DNA or co-factors as well as therapeutically-induced degradation of oncogenic transcription factors. However, many challenges remain. For instance, transcription factors usually lack the deep active sites present in kinases, which makes it more difficult to develop small-molecule inhibitors for these. The convex structure of DNA and its highly positive charge at interaction surfaces are other challenges for the development of protein-DNA binding inhibitors (*70*). Thus far, the only successful example of inactivation of an oncogenic transcription factor in the clinical setting is the targeting of the PML-RARA (promyelocytic leukemia protein–retinoic acid receptor-α) fusion protein with all-trans retinoic acid (ATRA) and arsenic trioxide (ATO) in patients with promyelocytic leukemia (*71-74*). Expression of PML-RARA leads to two main consequences: inactivation of the RARA transcriptional program that is central to granulocyte differentiation and disruption of PML nuclear bodies involved in P53 activation. ATRA and ATO bind to the RARA and PML part of the fusion, respectively. ATRA activates RARA target genes leading to cellular differentiation and may also induce PML-RARA degradation at higher doses. ATO treatment leads to degradation of the fusion protein through sumoylation (*74*). An important next step for translational cancer research is to build on these successful results and devise novel strategies to target oncogenic transcription factors also in other malignancies.

1.6 General aspects of salivary gland tumors

Human salivary glands consist of three pairs of major glands, i.e. the parotid, submandibular, and sublingual glands. In addition, there are numerous minor salivary glands located throughout the mucosa of the oral cavity and the upper aerodigestive tract (*75*). The main function of these glands is to produce saliva. The saliva protects the teeth and oropharyngeal mucosa, facilitates articulation and swallowing, provides an optimal environment for microbiota, and initiates the digestive process (*76*).

Salivary gland tumors (SGTs) are a rare and heterogeneous group of benign and malignant tumors with varied clinical behavior. According to the latest *World Health Organization (WHO) Classification of Head and Neck Tumors* there are more than 30 histological subtypes, of which about two thirds are malignant (*77*). SGTs can originate from both major and minor glands. The parotid gland is the most common anatomical site giving rise to 75% of the tumors, of which 25% are malignant. Most tumors originating from the submandibular, sublingual, and minor glands are malignant (*78, 79*). The majority of all SGTs are benign. The benign PA is the most common SGT. Other examples of benign SGTs are myoepithelioma, basal cell adenoma, and Warthin tumor. The malignant SGTs make up less than 10% of head and neck cancers (*75*). The two most common ones, mucoepidermoid carcinoma (MEC) and ACC, constitute about half of the malignant cases (*79, 80*). The rarity of SGTs in combination with their histopathologic diversity make these tumors a diagnostic and therapeutic challenge (*77*).

Current treatment strategies for malignant SGTs are based on surgical resection of the primary tumor and adjuvant radiotherapy in cases with high risk features, including perineural invasion, large tumor size, and high-grade

histology (*81*). There are no standard treatments available for patients with metastatic or recurrent disease. The response rates to chemotherapy and so far tested targeted therapies (e.g., targeting HER2, EGFR, and KIT) are very low or uncertain (*82, 83*). Thus, there is an unmet need for development of new efficient systematic therapies for patients with these malignancies. However, recent efforts in unveiling the molecular underpinnings of SGTs have improved the diagnostic precision and opened new avenues for targeted therapies (*79, 84-87*).

A molecular hallmark of both benign and malignant SGTs is the presence of recurrent chromosomal rearrangements and oncogenic gene fusions (*88*). Examples of gene fusions in SGTs are *CRTC1-MAML2* and *CRTC3-MAML2* in MEC (*89, 90*), *MYB-NFIB* and *MYBL1-NFIB* in ACC (*45, 91, 92*), *ETV6-NTRK3* in secretory carcinoma (*93*), *EWSR1-ATF1* in clear cell carcinoma (*47*) and *PLAG1* and *HMGA2* fusions in PA, carcinoma ex-pleomorphic adenoma (Ca-ex-PA), and myoepithelial carcinoma (*51, 94*). These tumor-type specific aberrations are oncogenic drivers and new potential targets for therapy.

1.7 Adenoid cystic carcinoma

ACC is the second most common malignancy of the salivary glands but also occurs in other organs such as the breast, prostate, lung, and skin (*75, 86*). The most common presentation of ACC is an asymptomatic mass (*77*). However, due to its high propensity for early perineural invasion, pain and cranial neuropathies might also occur (*77, 95, 96*). It is a slow-growing tumor composed of epithelial and myoepithelial cells growing in different often overlapping patterns, including cribriform, tubular, and solid patterns. Tumors

with tubular and cribriform morphology generally have better prognosis than those with a solid component constituting more than one third of the tumor (*75*). Solid tumor histology predicts an aggressive clinical course. The 5-year survival for ACC patients is about 70% but declines to just above 20% at 15 years due to a high frequency of local recurrences and distant metastases (*97*). Patients presenting with distant metastases at diagnosis have significantly shorter survival (*98, 99*). The most common sites for metastasis are the lungs, bone, liver, and brain (*77, 100*). Other predictors of survival include tumor stage, patient age, and tumor site (*77*). The primary treatment of ACC is surgery with or without postoperative radiotherapy (*83*). ACCs are resistant to all systemic treatments tested so far including chemotherapy and targeted therapies (*101, 102*). Thus, there is a need for additional studies to identify new actionable treatment targets for ACC patients.

1.8 Molecular characterization of ACC

ACC was one of the first carcinomas in which a recurrent chromosomal translocation was identified (*32, 103*). In 2009, Persson *et al.* showed that the pathognomonic t(6;9) translocation in ACC leads to a gene fusion between the 5'-part of *MYB* (v-myb avian myeloblastosis viral oncogene homolog) and the 3'-part of *NFIB* (nuclear factor I/B gene) resulting in *MYB* activation (45). The *MYB-NFIB* fusion is the genomic hallmark of ACC and is found in the absolute majority of cases. *MYB* activation is detected also in most fusion-negative ACCs, indicating that there are alternative mechanisms for *MYB* activation (*104-106*). Indeed, Drier *et al.* recently showed that *MYB* can be activated through rearrangements juxtaposing active enhancers located within or near the *NFIB* or *TGFBR3* genes to the vicinity of the *MYB* locus (58). Furthermore,

another member of the MYB family, *MYBL1* (MYB proto-oncogene like 1), was identified as a fusion partner to *NFIB* or *RAD51B* in *MYB-NFIB* fusion-negative cases. Notably, ACCs with *MYBL1* fusions display analogous gene expression patterns as *MYB* activated tumors, indicating that they result in activation of similar downstream oncogenic pathways (*91, 92*).

MYB is a transcription factor with important roles in regulation of cell differentiation and proliferation, primarily in stem and progenitor cells in the bone marrow, colon, and the adult brain. *MYB* is also activated in certain leukemias, as well as in subsets of breast and colon cancers (*107*). In ACC, the fusion between *MYB* and *NFIB* results in a chimeric gene with the DNA-binding and transactivation domains of *MYB* linked to the last coding exon(s) of *NFIB*. The 3'-part of *MYB*, which is lost as a result of the fusion, harbors binding sites for miRNAs that negatively regulate *MYB* expression. The fusion leads to overexpression of *MYB-NFIB* transcripts and the encoded chimeric oncoproteins (45). Our group has recently shown that *MYB* and *MYB-NFIB* drive cell proliferation and spherogenesis in ACC (*84, 85*). Whole exome and genome sequencing studies have revealed that ACC has a relatively quiet genome with a low mutational burden (*105, 106, 108*). These results further emphasize the key role of *MYB* as an oncogenic driver in ACC. Among the mutated genes in ACC are those involved in chromatin regulation, (e.g. *SMARCA2*, *KDM6A*, *KAT6A*, and *CREBBP*), DNA damage response (e.g. *ATM*, *TP53*, *BRCA1*, and *UHRF1*), NOTCH signaling (e.g. *NOTCH1*, *CTNNB1*, *MAML3*, and *FOXP2*), and FGF-IGF-PI3K signaling (e.g. *FGF16*, *PIK3CA*, *FGFR2*, and *INSRR*). Moreover, *TERT* promoter mutations have been reported in a subset of recurrent/metastatic ACCs. *KIT* and *EGFR* are often overexpressed in ACC but rarely amplified or mutated (*101, 105, 106, 109*).

ACC research has been severely hampered by the lack of authentic and carefully validated ACC cell lines and transgenic mouse models (*110, 111*). However, recently published preclinical studies using validated primary or short term cultured *MYB-NFIB* positive ACC cells and PDX-models provide new opportunities for developing improved treatment strategies for ACC patients (*84, 85, 112*). Notably, the so far tested targeted therapies have shown no or very limited response in patients with recurrent or metastatic disease. Clinical studies targeting KIT (Dasatinib), VEGFR/PDGFR (Sorafenib, Axitinib), EGFR (Gefitinib), AKT (MK-2206, Nelfinavir), mTOR (Everolimus), FGFR (Dovitinib), and HDAC (Vorinostat), have induced overall response rates (ORR) varying from 0% to 11% (*81, 113*). Clinical trials targeting NOTCH signaling in ACC patients with NOTCH-activated tumors are ongoing (ClinicalTrials.gov Identifier: NCT03691207, NCT03422679). However, studies with the pan-NOTCH inhibitor crenigacestat (LY3039478) have so far only shown modest effects (*114, 115*). Similarly, two recent phase II studies with the multikinase inhibitor lenvatinib described an ORR of about 15% (*116, 117*). These results emphasize the importance of continued molecular studies of ACC in order to discover new potential therapeutical targets.

1.9 Pleomorphic adenoma

PA is the most common tumor of the salivary glands with an annual incidence of about 2-3.5 per 100 000. It is a benign tumor that makes up about 60% of all neoplasms of the major and minor salivary glands. The parotid gland is the most common site followed by the palate and the submandibular gland (*77*). PAs may occasionally arise also in other anatomical sites, such as the

tracheobronchial tree, breast, and skin (*118-121*). Histopathologically, PAs are characterized by an architectural pleomorphism including epithelial, myoepithelial, myxoid, and chondroid components forming a variety of growth patterns.

PAs are slow-growing tumors, often manifesting as a painless mass. However, symptoms may vary based on tumor size and location (e.g. facial nerve weakness can occur in case of large parotid tumors). Although surgical resection of the tumor is curative in most cases, treatment can be complicated by local recurrences due to rupture of the capsule or to facial nerve dysfunction after parotidectomy. Furthermore, PAs might occasionally undergo malignant transformation, leading to carcinoma-ex-PA (Ca-ex-PA). Ca-ex-PA typically develops in long-standing primary tumors or in recurrent PAs of the parotid gland, presenting with increased growth rate, with or without pain and neuropathy (*75, 77*). The malignant component of Ca-ex-PA may be any subtype of salivary gland carcinoma, for example salivary duct carcinoma, myoepithelial carcinoma, or ACC. Most Ca-ex-PAs harbor *PLAG1* and *HMGA2* gene fusions characteristic for benign PAs (discussed in detail below) (*86*).

1.10 Molecular characterization of PA

PA was the first benign tumor in which a tumor type-specific translocation was identified (*33*). Subsequent comprehensive cytogenetic studies of PAs have shown frequent recurrent chromosomal translocations or intrachromosomal rearrangements involving chromosome bands 8q12 (≈50%) and 12q14-15 (≈10-15%) (*29, 87, 122-125*). Further molecular studies of these

rearrangements revealed that they result in activation of the key oncogenes *PLAG1* and *HMGA2* through gene fusion (*52, 88, 126*).

PLAG1 (pleomorphic adenoma gene 1) maps to 8q12 and encodes a developmentally regulated DNA-binding zinc finger transcription factor that is primarily expressed during embryogenesis. The expression is low or non-detectable in most adult tissues, including the salivary glands (*52, 127*). Chromosomal translocations/rearrangements in PA result in substitution of an inactive *PLAG1* promoter with an active promoter from the fusion partner genes, e.g. *CTNNB1, FGFR1, LIFR, CHCHD7*, or *TCEA1* (*52, 128-131*). This leads to ectopic expression of an intact *PLAG1* gene and activation of its downstream target genes, including *IGF2* (*132, 133*). Notably, *PLAG1* activation through promoter swapping is a key oncogenic event also in a subset of lipoblastomas (*134, 135*).

HMGA2 (High Mobility Group AT-Hook 2), a member of the high mobility group (HMG) gene family, maps to 12q14.3 and encodes an architectural transcription factor that regulates transcription by binding to the minor groove of AT-rich DNA, leading to an altered chromatin architecture. Similar to *PLAG1*, *HMGA2* is mainly expressed in embryonic tissues. The HMGA2 protein contains three DNA-binding domains, a spacer region, and an acidic C-terminal domain (*51, 126, 136, 137*). Rearrangements of 12q14-15 in PA commonly result in the formation of chimeric genes consisting of the 5'-part of *HMGA2*, including the DNA-binding domains, linked to the 3'-part of a fusion partner gene, e.g. *FHIT*, *NFIB*, or *WIF1* (*126, 136, 138*). This leads to loss of miRNA binding sites in the 3'-UTR of *HMGA2*, and dysregulated gene expression (*59, 60*). *HMGA2* is a target of chromosomal rearrangements also in other benign tumor types such as lipomas and uterine leiomyomas (*139, 140*).

2 AIMS OF THE THESIS

The overall aims of the thesis were to (1) further delineate the molecular pathogenesis of ACC and identify novel potential therapeutic targets and (2) characterize the genomic and transcriptomic landscapes of PA.

The specific aims were to:

- study the oncogenic properties and regulation of the *MYB-NFIB* fusion gene – the genomic hallmark of ACC
- identify activated signaling pathways with therapeutic potential in ACC
- discover novel gene fusions in PA
- study the global gene expression profile and activated signaling pathways in PA

3 MATERIALS AND METHODS

Tumor material and normal salivary gland tissue

Fresh frozen material from 15 *MYB-NFIB* positive head and neck ACCs and 7 normal salivary gland (NSG) tissue samples were analyzed in Paper I. In Paper II, 15 previously cytogenetically characterized PAs were included, of which fresh frozen tumor material was available from 10 cases. Tumor material from 38 fresh frozen PAs and 7 NSG samples were studied in Paper III. All tumors were classified based on the *WHO Classification of Head and Neck Tumors* (*77*) and histopathologically re-examined before they were used in the present studies. The studies were approved by the regional ethics committee in Gothenburg, Sweden.

Cultured ACC cells

Lack of validated and authentic ACC cells lines (*110*) and transgenic mouse models have seriously hampered ACC research for many years. In Paper I, we have instead used short-term cultured *MYB-NFIB* positive ACC cells from three cases. ACC cells from one of the cases grew for over 20 passages before they ceased to grow whereas the other two cases stopped growing after about 10 passages. Due to the difficulties in culturing these cells, the *in vitro* experiments were mainly performed on ACC cells from the case that grew up to 20 passages (cf. above). Tumor cells from the other two cases were mainly used to confirm the major findings. The three breast cancer cell lines (MCF-7, T47D, and ZR-75-I) that were used in Paper I were obtained from ATCC and maintained according to the instructions of the supplier.

ACC patient-derived xenograft (PDX) models

Two PDX-models, ACCX5M1 and ACCX6 (*141*), were used to study the effects of TKI-inhibition *in vivo*. Both models were established from lung metastases of head and neck ACCs with *MYB*-activation. The gene expression profiles of the two PDX-models were validated against 13 ACC patient samples using microarray analysis. The expression profiles of the ACC surgical and PDX-samples were indistinguishable (Paper I, supplementary Figure 1B). The studies were performed through the South Texas Accelerated Research Therapeutics (START, San Antonio, TX) program. Animal maintenance, handling, and surgical procedures were performed according to the Institutional Animal Care and Use Committee guidelines. Briefly, female athymic nu/nu mice (Harlan Sprague Dawley, Indianapolis, IN) were transplanted with tumors at 4-6 weeks of age. Tumor-bearing mice were randomized into control and treatment groups with 4-9 mice per group when tumors reached a size of about 200 mm^3. The treatment groups received linsitinib (25 mg/kg, 5 consecutive days a week), and crizotinib and gefitinib (50 mg/kg and 100 mg/kg, respectively, on alternating days with 2 days off per week). Treatment was terminated when the mean tumor volume in the control groups reached 1000 mm^3. Parts of control and drug treated PDX-tumors were formalin-fixed, paraffin-embedded, sectioned, and stained with hematoxylin and eosin for histopathological analysis. Fresh frozen PDX-tumors were analyzed for *MYB* expression with qPCR.

Cell-based assays and molecular analyses

Multiple well-established molecular and cellular methods were used in this thesis and are described in the respective paper. The methods included cell-based assays such as proliferation and apoptosis assays, sphere assays, cell cycle analysis, and siRNA transfection. Protein expression was estimated by western blot and immunohistochemistry, and protein phosphorylation by

phosho-arrays. Genomic and transcriptomic studies were performed by cytogenetic analyses, arrayCGH, RT-PCR, qPCR, and RNA-seq analyses. Bioinformatic analysis of RNA-seq data was done at the UPPMAX (Uppsala Multidisciplinary Center for Advanced Computational Science, Sweden) cluster.

Statistical analyses

Dose-response curves were estimated by nonlinear regression with Prism 6 (GraphPad Software). Evaluation of the effects of combination treatments was based on the definition of drug independence according to Bliss (*142*); if the phenotypic effect (E) of each drug is independent of the presence of another drug, the effects of the two drugs are regarded as additive as in $E_{AB} = E_A E_B$. E_{AB} is the experimentally assessed effect of combined treatment with drugs A and B and $E_A E_B$ is the estimated effect of the combination as calculated from single treatments with each drug. Synergy between two drugs was defined as $E_{AB} > E_A E_B$. Evaluation of triple drug synergy was done with $E_{ABC} > E_A E_B E_C$ (*143*). One-sample t tests were used to evaluate the statistical significance of synergistic drug interactions. Significant differences between other sample groups (except for global gene expression data, see below) were estimated with independent sample t tests, Mann-Whitney, or Kruskal-Wallis tests. Post-hoc *F* tests were used to confirm the assumption of equal variance within groups for parametric tests. All statistical tests were two-sided. A *P*-value of less than 0.05 was considered statistically significant. The DESeq2 R package (v1.22.2) was used to estimate differences between groups in global gene expression data with prior variance stabilizing transformation (VST) of the RNA-seq count data. Differentially expressed genes with an adjusted *P*-value of < 0.01 (Benjamini–Hochberg) and a log2 fold change of >1 were considered significant. Gene ontology and gene set enrichment analyses (GSEA) was done with Nexus Expression 3.0 (BioDiscovery), the ToppGene database

(http://toppgene.cchmc.org), and the GSEA software (v.4.1.0). Upstream regulator analyses were done with the Ingenuity Pathway Analysis portal (Qiagen). Unsupervised hierarchical clustering and principal component analysis was done with the hclust and plotPCA functions in RStudio (v1.1.463). The chi-square test was used to estimate the statistical significance of overlap between global gene expression data sets.

4 RESULTS AND DISCUSSION

4.1 Paper I. Targeting the oncogenic transcriptional regulator MYB in adenoid cystic carcinoma by inhibition of IGF1R/AKT signaling

Previous studies have demonstrated that the key genomic feature of ACC is the *MYB-NFIB* gene fusion found in the majority of ACCs (*45, 104-106*). In Paper I, we have investigated the phenotypic and molecular effects of *MYB-NFIB* inhibition in short-term cultured ACC cells. We have also studied the effects of receptor tyrosine kinase inhibition on tumor growth, differentiation, and gene expression in cultured ACC cells and in two ACC patient-derived xenograft (PDX) models. Our findings were confirmed in primary tumor material from ACC patients.

MYB-NFIB drives ACC cell proliferation and spherogenesis

We studied the biological significance of *MYB-NFIB* in cultured ACC cells through knockdown of the fusion with RNA-interference. Knockdown markedly decreased *MYB-NFIB* mRNA and protein expression and led to G1 arrest, reduced cell proliferation and spherogenesis, but did not increase apoptosis. Our results indicate that MYB-NFIB drives tumor growth in ACC and that the fusion plays an important role in tumor initiation by cancer stem/progenitor cells. These findings are supported by a recent study showing that MYB/MYB-NFIB overexpression can transform human glandular epithelial cells *in vitro* (*84*). Taken together these and previous studies (*45, 105*) show that *MYB* activation is a main oncogenic driver in ACC and thus an interesting therapeutic target.

Co-activation of receptor tyrosine kinases regulates proliferation of ACC cells

To uncover activated signaling pathways with therapeutic potential in ACC, we studied RTK activation in *MYB-NFIB*-positive cells from three ACCs using phospho-arrays. IGF1R, INSR, MET, and EGFR were strongly activated in all three ACCs. Expression analysis by qPCR of these RTK genes in 12 other ACCs revealed overexpression of the genes in all tumors compared with NSG tissue. To further validate the activity of these receptors, we stimulated ACC cells with the corresponding RTK ligands and conversely inhibited the receptors by treating the cells with tyrosine kinase inhibitors (TKIs). Stimulation with the ligands IGF1, insulin, HGF, and EGF increased phosphorylation of their receptors and resulted in downstream activation of the AKT and MAPK pathways. In contrast, TKI treatment with linsitinib (targets IGF1R/INSR), crizotinib (targets MET), and gefitinib (targets EGFR) blocked RTK activation. Our results show that these RTKs are activated and functional in ACC cells. Notably, MET-inhibition with crizotinib increased EGFR phosphorylation, indicating a crosstalk between EGFR and MET in ACC cells.

To study the biological relevance of the activated RTKs, ACC cells were treated with increasing concentrations of linsitinib, crizotinib, and gefitinib. Treatment with each of the TKIs led to a dose-dependent decrease in ACC cell proliferation, but no effects on apoptosis – indicating growth inhibitory rather than cell death-inducing effects. Interestingly, crizotinib and linsitinib also decreased spherogenesis of ACC cells, which implies that these drugs target ACC stem/progenitor cells.

The significance of IGF1R, INSR, MET, and EGFR in ACC pathogenesis is still somewhat unclear. Ho *et al.* previously reported scattered mutations in FGF/IGF/PI3K pathways in a subset of ACCs (*105*). Although overexpression

of *MET* and *EGFR* is documented in ACC (*144, 145*) they are rarely mutated or amplified (*105, 106*). Our findings show that IGF1R/INSR are overexpressed in ACC and that these RTKs, together with MET and EGFR, are important for ACC cell proliferation.

Combined inhibition of IGF1R/INSR, MET, and EGFR synergistically decrease growth of ACC cells in vitro and in vivo

To investigate putative crosstalk between the activated RTK pathways, we studied the effects of co-targeting IGF1R/INSR, MET, and EGFR on ACC cell proliferation. Combined treatment with crizotinib and gefitinib had a synergistic inhibitory effect on ACC cell proliferation, demonstrating a crosstalk between MET and EGFR. A synergistic decrease in cell proliferation was also observed with triple TKI treatment (linsitinib/crizotinib/gefitinib), implying an interdependence between the targeted signaling pathways. We also investigated the effects of TKI treatment on tumor growth *in vivo*, using two ACC PDX models. While single TKI treatment did not affect tumor growth, triple TKI treatment with linsitinib/crizotinib/gefitinib inhibited tumor growth in both PDX models. Both TKI-treated cells and tumors showed morphological signs of differentiation. Moreover, RTK-inhibition decreased *MYB* expression in ACC cells and in PDX tumors. Taken together, combined targeting of RTKs in ACC inhibited cell and tumor growth, downregulated *MYB*, and induced differentiation in ACC cells. Analogously, *MYB*-inhibition has also been shown to induce differentiation in T-ALL cells with *MYB* activation (*146*).

These results reflect the complexity of oncogenic signaling in ACC with activation of multiple pathways. Thus far, single agent targeted therapies have only had modest effects in ACC patients (*83, 101, 113*), which might at least partly be explained by the crosstalk between activated signaling pathways.

Hence, combination treatments may be required for ACC patients with metastatic disease.

IGF1R regulates MYB-NFIB expression through AKT-signaling and is activated by IGF2

To study the potential role of IGF1R/INSR, MET, and EGFR in the regulation of *MYB-NFIB* , we measured the expression of the fusion in ACC cells treated with linsitinib, crizotinib, or gefitinib. Linsitinib treatment targeting IGF1R and INSR reduced *MYB-NFIB* expression but did not affect the expression of the wild-type *MYB* allele in ACC, or in ER+ breast cancer cells. Our results suggest that the fusion creates a dependency on IGF1R-AKT signaling in ACC cells. To gain further insight into the regulation of *MYB-NFIB*, we knocked down *IGF1R* and *INSR* with RNAi. *IGF1R* knockdown led to downregulation of the *MYB-NFIB* fusion both at the mRNA and protein levels and reduced ACC cell proliferation. *INSR* knockdown instead resulted in a slight increase in *MYB-NFIB* mRNA levels, but did not affect MYB-NFIB protein levels.

To further explore the mechanisms behind *MYB-NFIB* regulation through IGF1R, we inhibited the IGF1R downstream pathways with MEK and AKT inhibitors in serum-starved ACC cells re-stimulated with insulin. Insulin-dependent *MYB-NFIB* expression was blocked with the AKT-inhibitor MK-2206 whereas the MEK inhibitor trametinib had no effect on the expression of the fusion. These results indicate that IGF1R regulates *MYB-NFIB* through the AKT signaling pathway.

Next, we investigated the effects of the IGF1R ligands insulin, IGF1, and IGF2 on *MYB-NFIB* expression in ACC cells. Each ligand independently increased *MYB-NFIB* expression both at the mRNA and protein levels. However, *IGF2* was the only ligand overexpressed in an independent set of ACC surgical samples. Moreover, *IGF2* knockdown with RNAi downregulated *MYB-NFIB*

mRNA and protein expression in ACC cells. These findings suggest that IGF2 may promote the expression of the fusion in ACC through an autocrine loop. Figure 3 shows a schematic model of activated RTK signaling pathways in ACC.

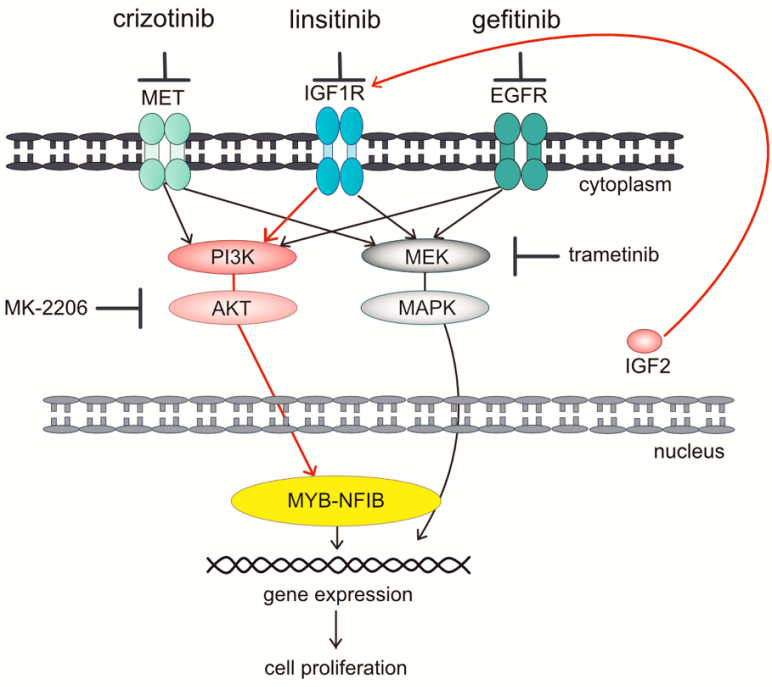

Figure 3. Schematic illustration of activated RTK-signaling in ACC. The RTKs MET, EGFR, and IGF1R are co-activated in ACC. The crosstalk between activated signaling pathways is indicated by arrows. Note that IGF1R regulates MYB-NFIB expression through IGF2-activated AKT-signaling.

Our results demonstrate that the oncogenic transcription factor *MYB-NFIB* can be downregulated by IGF1R/AKT-inhibition. This is an important finding since transcription factors are very difficult to target in the clinic. Although

new approaches are in progress (*68, 69*), there are thus far very few successful examples of targeting transcription factors. The effective targeting of PML-RARA in promyelocytic leukemia is perhaps the most well-known example (cf. Introduction). Our observations thus represent a potential novel approach to target oncogenic transcriptional regulators. Interestingly, targeting *MYB-NFIB* with RTK inhibitors did not affect the wild-type *MYB* allele in ACC cells or other cells. This may prove important in the clinical setting since MYB has key functions in normal hematopoietic and colonic stem cells (*147*).

MYB-NFIB induces an MYC-like transcriptional program that is reversed by pharmacological inhibition of IGF1R

We analyzed the global gene expression in ACC cells after *MYB-NFIB* knockdown to discover potential downstream targets and biological processes affected by the fusion. Microarray gene expression data revealed several hundred downregulated genes, many with key functions, such as cell cycle regulation, DNA replication/repair, and RNA processing. Notably, *IGF2* was amongst the top upregulated genes after knockdown of the fusion, indicating a possible feedback loop between MYB-NFIB and IGF2. Gene set enrichment analysis (GSEA) of the expression data showed that MYB-NFIB induces an MYC-like transcriptional program. Moreover, MYC was identified as a top upstream transcriptional regulator after *MYB-NFIB* knockdown by Ingenuity Pathway Analysis (IPA), indicating that MYB-NFIB and MYC share important oncogenic features. In addition, *MYB-NFIB* knockdown decreased MYC protein expression, implying that MYC protein levels are stabilized by MYB-NFIB leading to activation of MYC target genes. Hence, therapies targeting MYC might be potential treatments also for ACC.

Importantly, gene expression analyses of ACC cells treated with the IGF1R inhibitors linsitinib or BMS-754807 revealed a large overlap with genes

affected by *MYB-NFIB* knockdown, indicating that pharmacological inhibition of IGF1R can reverse the transcriptional program induced by MYB-NFIB. Moreover, the majority of genes downregulated by both IGF1R inhibition and *MYB-NFIB* knockdown were overexpressed in ACC surgical samples, further emphasizing the potential clinical importance of MYB-NFIB/IGF1R inhibition for ACC patients. Although several fusion oncogenes (including *EVT6-NTRK3*, *EWSR1-WT1*, and *CD74-NRG1*) can activate IGF1R signaling (*148-150*), this is the first study to show that IGF1R can regulate an oncogenic transcription factor, entailing new therapeutic opportunities. Efforts in combating ACC has since Paper I was published continued with the identification of new therapeutic targets, including the DNA-damage sensor kinase ATR (a MYB downstream target) (*84*). Moreover, compounds with direct MYB-inhibitory activity in cancers with *MYB* activation, such as acute myeloid leukemia and ACC, have shown promising preclinical results (*112*).

In summary, our results demonstrate that MYB-NFIB is a main oncogenic driver in ACC that promotes cell proliferation and spherogenesis and induces an MYC-like transcriptional program. Moreover, we show that *MYB-NFIB* is regulated by IGF1R through IGF2-activated AKT-signaling. Our findings are of importance to understand the pathogenesis of ACC and reveal a new potential strategy to target oncogenic transcriptional regulators.

4.2 Paper II. Activation of *PLAG1* and *HMGA2* by gene fusions involving the transcriptional regulator gene *NFIB*

PLAG1 and *HMGA2* are key oncogenes that are frequently activated by chromosomal rearrangements in PA. Here, we have identified subsets of PA with 9;12- and 8;9-rearrangements that lead to activation of *PLAG1* and *HMGA2* through fusion with the transcription factor coding gene *NFIB*.

The 9;12- and 8;9-rearrangements in PA result in HMGA2-NFIB and NFIB-PLAG1 gene fusions

Review of 450 cytogenetically characterized PAs (271 published and 179 unpublished from our group) revealed previously unrecognized subsets of tumors with ins(9;8)/t(8;9) (n = 5) and ins(9;12)/t(9;12) (n = 8). These tumors had recurrent breakpoints close to or within the *NFIB* and *PLAG1* loci and the *NFIB* and *HMGA2* loci, respectively. In the majority of cases, these rearrangements were the only cytogenetic aberrations, suggesting that they are pathogenetically important.

Analysis using RT-PCR and Sanger sequencing of cases with t(9;12) translocation revealed that three out of three cases had *HMGA2-NFIB* fusions. The chimeric transcripts in two of the cases consisted of *HMGA2* exon 4 linked to either *NFIB* exon 3 or exon 9. In the third case, *HMGA2* exon 3 was linked to *NFIB* exon 9 (Figure 4A). The index cases with ins(9;12) (*136*), expressed fusion transcripts with *HMGA2* exon 3 or exon 4 linked to the last coding exon of *NFIB* (exon 9). Notably, arrayCGH analysis of one of the cases with ins(9;12) revealed an 142 kb intragenic deletion within the *NFIB* locus as the only copy number alteration (CNA) in this case. We also observed a small

deletion (108 kb) in the 3'-part of *HMGA2* in a case with t(9;12), which is consistent with the *HMGA2-NFIB* fusion in this case.

Our results, which are in line with previously described *HMGA2* fusions, demonstrate recurrent loss of the 3'-UTR of *HMGA2* (*126, 136, 137*). This region contains binding sites for negatively regulating miRNAs and loss of these sites may result in increased *HMGA2* expression (*59, 60*). The *HMGA2* fusions found in the present study also result in loss of the negative regulatory acidic domain (located in the C-terminal) (Figure 4A), which may promote transactivation properties of the HMGA2 protein (*151, 152*).

RNA-seq analysis of tumors with 8;9-rearrangements (n=3) revealed a novel *NFIB-PLAG1* fusion in one tumor with an ins(9;8). This tumor expressed chimeric transcripts with a fusion between *NFIB* exon 4 to *PLAG1* exon 3 (Figure 4B). RT-PCR and Sanger sequencing also confirmed the *NFIB-PLAG1* fusion in this case. The chimeric transcript is expected to encode a truncated NFIB-PLAG1 protein (229 amino acids encoded by *NFIB* and 24 encoded by *PLAG1*) as well as the wild type PLAG1 protein. A similar observation was previously made in in a PA with a *TCEA1-PLAG1* fusion (*131*). In the remaining two cases with t(8;9) no fusion transcripts were detected. Further analysis of these three cases with arrayCGH did not reveal any CNAs.

NFIB encodes a DNA-binding transcription factor with key roles in lung and brain development. It is also involved in the regulation of differentiation in several cell types (*153, 154*). Our results demonstrate that *NFIB* is a recurrent fusion partner gene of *HMGA2* and *PLAG1* in PA. Notably, similar to the *HMGA2-NFIB* fusions in PA, *NFIB* is involved in recurrent gene fusions also in ACC of the salivary glands, of which the absolute majority are *MYB-NFIB* fusions (Figure 4C) (*45, 104, 105, 155*).

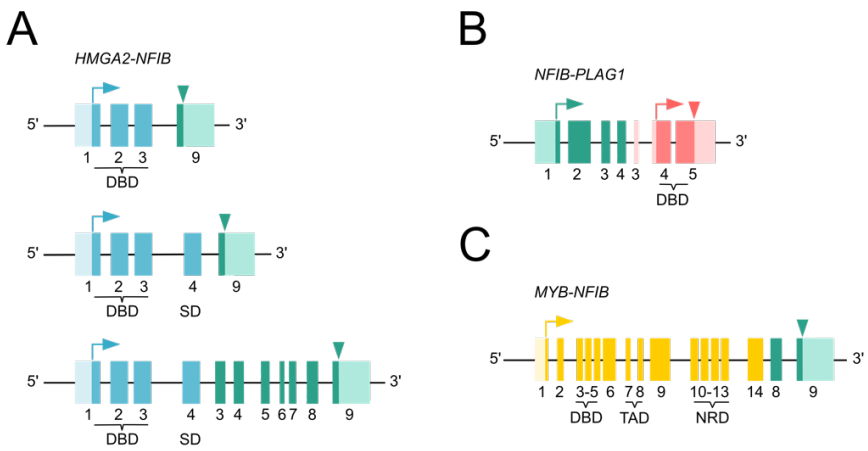

Figure 4. Schematic illustration of gene fusions in PA and ACC. (A) Different HMGA2-NFIB fusions in PA; (B) the NFIB-PLAG1 fusion in PA; and (C) the MYB-NFIB fusion in ACC. HMGA2 is depicted in blue, PLAG1 in pink, NFIB in green, and MYB in yellow. The start and stop codons are indicated by colored arrows and arrowheads, respectively. DBD, DNA binding domain; SD, spacer domain; TAD, transactivation domain; NRD, negative regulatory domain.

The NFIB fusion events lead to overexpression of HMGA2 and PLAG1 in PA

We investigated the molecular consequences of the *NFIB* fusion events on *HMGA2* and *PLAG1* expression in PAs with 9;12- and 8;9-rearrangements. *HMGA2* was upregulated in all tumors with 9;12-rearrangements and *PLAG1* was upregulated in all tumors with 8;9-rearrangements compared to NSG tissue. Moreover, analysis of RNA-seq data from the case with the *NFIB-PLAG1* fusion showed hundreds of reads for each of the last three exons (exons 3-5) and no reads corresponding to exons 1 and 2. This indicates that the *PLAG1* overexpression originates from the rearranged allele and not from the

wild-type allele. *IGF2*, a key downstream target gene of *PLAG1* was also overexpressed in tumors with 8;9-rearrangements. These results show that *NFIB* can activate both *HMGA2* and *PLAG1* through gene fusion.

NFIB is highly expressed in normal salivary gland, breast, and adipose tissues and harbors super-enhancers

Analysis of RNA-seq data available from *the Genotype-Tissue Expression portal* (GTEx) revealed variable *NFIB* expression in 25 normal human tissues. *NFIB* had the highest expression in normal salivary gland, breast, and adipose tissues, indicating an active *NFIB* promoter in these tissues. Notably, gene fusions involving *NFIB* have mainly been detected in tumors originating from these three tissues, including *HMGA2-NFIB* fusions in lipomas (*45, 156-158*).

Nfib is a key regulator in the development of mouse submandibular glands, in particular in the differentiation of terminal tubule cells which later develops into intercalated ducts (*159-162*). Interestingly, both PA and ACC are thought to arise from intercalated duct cells, suggesting a similar histogenetic background (*163-168*).

To explore the potential contribution of enhancer hijacking in the activation of *PLAG1* in the case with the *NFIB-PLAG1* fusion, we searched for super-enhancers located in proximity of the 5'-part of the *NFIB* gene using the *comprehensive human Super-Enhancer database* (SEdb). Regions with high levels of H3K27 acetylation (indicating the presence of super-enhancers) within and adjacent to the 5'-part of *NFIB* were found in several normal human tissues and cell types. These results suggest that, in addition to providing *PLAG1* with an active promoter, *NFIB* might also provide strong enhancer elements leading to increased *PLAG1* expression. The *NFIB-PLAG1* fusion was only detected in one of the three cases with 8;9-rearrangements with similar breakpoints. Notably, *PLAG1* expression was elevated in all three

cases, suggesting that *NFIB* enhancer elements may be juxtaposed to the vicinity of the *PLAG1* locus leading to activation of *PLAG1*. Further analysis with for example whole genome or targeted sequencing are needed to address this question. Interestingly, hijacking of 3'-*NFIB* enhancer elements has been described as a mechanism for *MYB* activation in *MYB-NFIB* fusions in ACC (*58*). Similar mechanisms may thus be behind *HMGA2* activation in PAs with *HMGA2-NFIB* fusions.

Taken together, we have identified recurrent *NFIB* fusions with the key oncogenes in PA, *PLAG1* and *HMGA2*, in subsets of PAs with 8;9- and 9;12-rearrangements. Our results indicate that enhancer hijacking events may contribute to *PLAG1* and *HMGA2* activation in these tumors.

4.3 Paper III. Transcriptomic profiling of pleomorphic salivary gland adenomas

In this paper we have performed the first comprehensive analysis of the global gene expression pattern and fusion gene landscape of PA. We performed RNA-seq of 38 fresh frozen PAs. Analysis using the Star-Fusion and FusionCatcher softwares revealed that about 80% of the cases harbored gene fusions involving either *PLAG1* or *HMGA2*, demonstrating that gene fusions are more common in PA than previously described (*87, 88*). This may partly be explained by the limitations of conventional cytogenetic analysis in detecting cryptic chromosomal rearrangements. Notably, almost one third of the fusions we detected were identified for the first time. In addition, we found three *PLAG1* fusions that were previously described in other salivary gland neoplasms but not in PA. Consistent with previous findings, *PLAG1* fusions resulted in

exchange of the 5'-part of *PLAG1* with the 5' regulatory elements of the partner genes, demonstrating the importance of promoter swapping for *PLAG1* activation in PA.

HMGA2 fusions resulted in replacement of the 3'-part of *HMGA2* with the 3'-part of a fusion partner gene or a non-coding genomic sequence. The fusion points were located either in intron 3 or 4, or in a few cases after the stop codon of *HMGA2*. The latter fusion variants are thus expected to lead to overexpression of a full-length HMGA2 protein. Hence, common to all fusions was the loss of the 3'-UTR of *HMGA2* which harbors multiple binding sites for miRNAs that negatively regulate the expression of the gene. These results are in line with previous findings (*88, 126, 136*) and indicate that loss of 3'-UTR is an important mechanism of *HMGA2* activation.

Unsupervised hierarchical clustering of RNA-seq data from 38 PAs and seven NSG tissue samples showed a clear separation of normal and tumor tissues and also a few subclusters among the PAs. One of the latter contained all but one PA with *HMGA2* fusions and/or *HMGA2* overexpression. Moreover, principal component analysis (PCA) showed a clear separation between PA and NSG and also two subclusters of PAs corresponding to samples with *PLAG1* or *HMGA2* fusion/overexpression.

As expected, analysis of differentially regulated genes between PA and NSG revealed overexpression of *HMGA2*, *PLAG1*, and *IGF2* (a key downstream target of *PLAG1*) in PAs. Multiple genes encoding extracellular matrix (ECM) components were among the upregulated genes in tumors, consistent with previous morphological observations that PAs are rich in ECM (*77*). Other downregulated genes in PA were associated with normal salivary gland functions (such as secretion of saliva), which reflects the loss of normal physiology in transformed salivary gland cells. Further analysis of

differentially expressed genes and pathways between the two PA clusters (*PLAG1* or *HMGA2* activated) revealed a large overlap of dysregulated genes and pathways. These results indicate that *PLAG1* and *HMGA2* promote tumorigenesis through activation of similar oncogenic signaling pathways in PA.

In summary, we describe the transcriptional landscape of PA with activation of multiple oncogenic signaling pathways. The results also show that *PLAG1* and *HMGA2* fusions are more common in PA than previously described and that either of these oncogenes are overexpressed in the majority of the tumors. Our findings further highlight the importance of these key oncogenic transcriptional regulators in PA and indicate that they drive PA tumorigenesis through similar oncogenic pathways.

5 CONCLUSIONS

The main conclusions of this thesis can be summarized as follows:

- The transcriptional master regulator MYB-NFIB is a key oncogenic driver that promotes proliferation and spherogenesis of ACC cells.
- *MYB-NFIB* is regulated by IGF1R through IGF2-activated AKT-signaling and this pathway is a potential therapeutic target in ACC.
- IGF1R is the first druggable cell surface receptor known to regulate a fusion oncogene encoding a transcription factor.
- MYB-NFIB induces an MYC-like transcriptional program in ACC that is partially reversed by pharmacologic inhibition of IGF1R.
- IGF1R, MET, and EGFR are co-activated in ACC and combined targeting of these receptors inhibits tumor growth in ACC PDX models, indicating that targeting of multiple activated RTKs is a potential therapeutic strategy in ACC.
- *NFIB* can activate both *PLAG1* and *HMGA2* by gene fusion/enhancer hijacking events in PA.
- The global expression profile of PA reflects the morphological features typical of these tumors.
- *PLAG1* and *HMGA2* fusions are detected in high frequency in PA and are more common than previously observed.
- *PLAG1* or *HMGA2* are activated in the majority of PAs and drive tumorigenesis via shared signaling pathways.

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