RNA splicing in breast cancer progression

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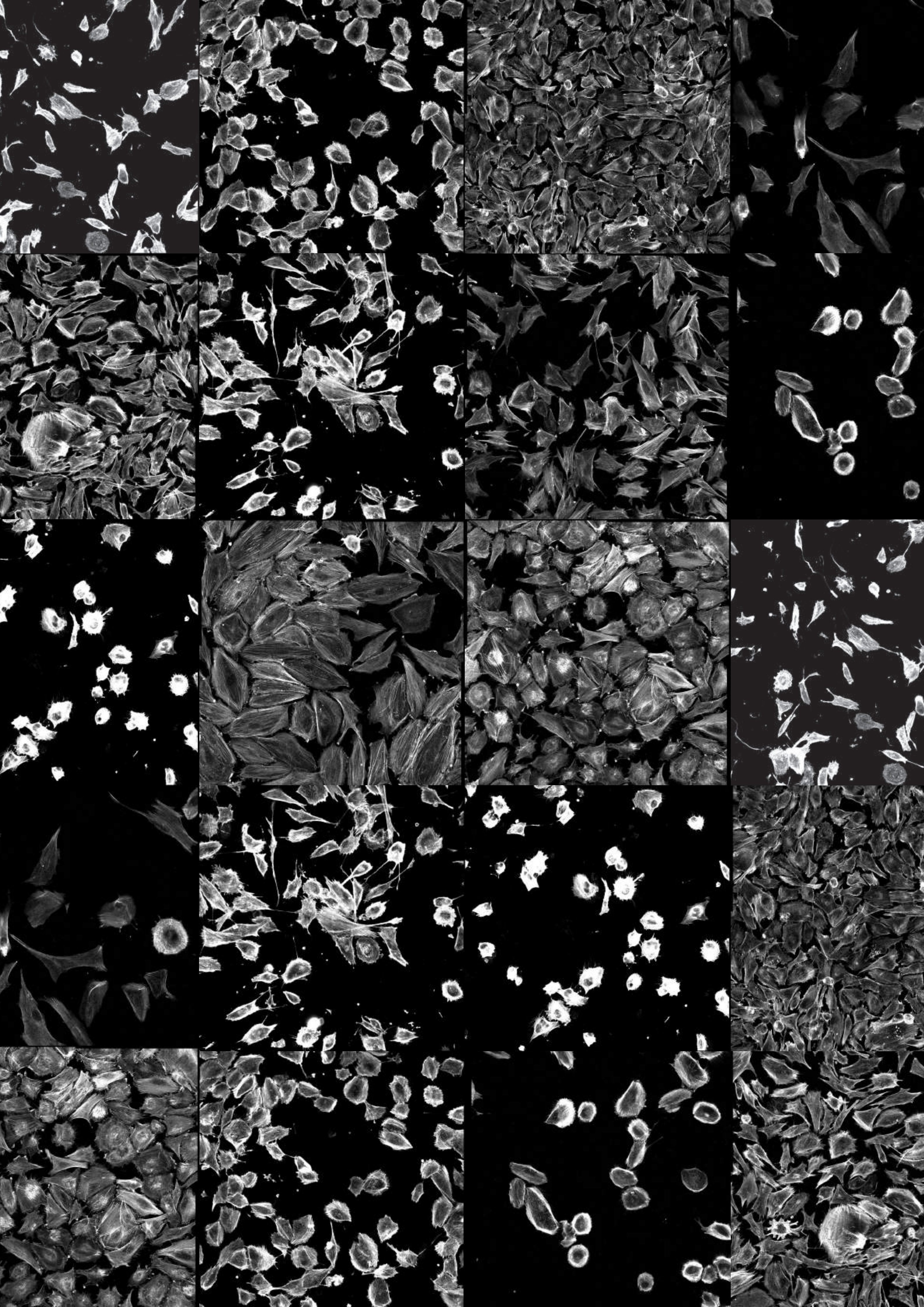

Chapter 8

Discussion and Conclusion

◄ IN THE PICTURE

Microscopic view of the phenotype of triple-negative breast cancer cells after depletion of genes important for cell migration. Cells were stained for the actin cytoskeleton.

◄ IN BEELD

Microscopisch beeld van het uiterlijk van triple-negatieve borstkankercellen na uitputting van genen die belangrijk zijn voor celmigratie. Cellen zijn gekleurd voor het cytoskelet.

Breast cancer has the highest cancer mortality rate in women worldwide[1], primarily caused by metastatic progression of the primary tumor[423]. The triple-negative breast cancer (TNBC) subtype lacking expression of the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) is the most aggressive breast cancer subtype; although it accounts for only 15-20% of all breast cancer cases[14], it is disproportionally responsible for metastatic progression compared to the hormone receptor (HR) positive breast cancer subtypes[15]. Even though an important role for some individual genes in TNBC progression has previously been defined[59–63], there is still yet no targeted therapy for TNBC available. Therefore, this thesis focused on uncovering the signaling landscape driving TNBC progression, with the ultimate goal to identify new targets that can be used to reduce breast cancer related deaths.

The technological approaches

RNAi screening

Understanding the complete signaling pathways driving TNBC metastatic progression is vital to identify potential new drug targets. During the last decades, the development of large RNAi libraries enabled efficient gene knockdown at a genome-wide scale, allowing to investigate many pathways in a single screen. These siRNAs are 20-25 base pair double-stranded RNA molecules directing mRNA breakdown of specific genes by binding via complementary nucleotide sequences. In **chapter 2, chapter 5** and **chapter 6**, we used this genome-wide screening methodology to unravel 1) the signaling landscape driving TNBC migration, 2) the role of splicing factors in TNBC migration and 3) the role of splicing factors in TNBC proliferation, respectively. Although this approach demonstrated to be very effective by us and others[82,129,424], observed effects should be thoroughly validated to exclude off-target effects[425]. More recently, the application of CRISPR/Cas9 to edit the human genome has shown to be a very powerful tool to modify and regulate gene expression levels[426]. Here, the Cas9 nuclease is directed to a specific part of the gene by a single guide RNA (sgRNA)[427] to create a double strand break in the DNA. When this break is repaired via non-homologous end-joining (NHEJ), frameshift mutations can occur that might result in gene knockout. Although the CRISPR/Cas9 technology is superior to RNAi comparing off-target effects[425], the efficiency of CRISPR/Cas9 to edit mammalian genes is still variable ranging from 1 to 79%[428–430]. Therefore, genome-wide screens performed with CRISPR/Cas9 use pooled sgRNAs upon which the cells bearing the phenotype of interest are preferred[431–434]. As a consequence of pooled screening, the range of cellular phenotypes that can be examined is still largely limited to viability, marker expression or expression of a small gene set[435]. In contrast, RNAi screening has a relatively high efficiency to decrease transcript levels in all of the cells allowing assessment of different phenotypes, such

as migratory behavior[436]. Therefore, in this thesis, we used RNAi screening combined with CRISPR Cas9 validation of our major candidates. Nevertheless, future work should involve the optimization of the efficiency of sgRNA-based imaging-based screening using CRISPR/Cas9 technology.

Migration assays

While genome-wide screening for proliferative effects has been frequently performed in the last decades[82,129,424], genome-wide screening for motility often remains challenging because of the dynamic migratory behavior. The majority of previous migratory screens were performed in epithelial or endothelial cells that require cell-cell contacts therefore using wound healing or Boyden Chamber assays[437–440]. In contrast, TNBC cells show increased plasticity and a mesenchymal phenotype associated with a single cell mesenchymal cell migration mode[43]. To identify drivers of TNBC single cell migration using genome-wide screening, we established the phagokinetic track (PKT)[65] and random cell migration (RCM)[64] imaging-based assays, used in **chapter 2** and **chapter 5**. In the PKT assay, cells leave a migratory path after migrating on a bead-coated surface. Analyzing these tracks after fixation allows quantitative migratory assessment of individual cells, but also enables high-throughput screening due to the lack of live cell imaging. The automated RCM assay then allowed candidate validation in a live cell imaging setting.

Cell models

To discover the determinants of TNBC migration, proliferation and metastasis formation, we used a panel of immortalized TNBC cell lines cultured on two-dimensional (2D) tissue culture plastic (**chapter 2**, **chapter 5**, **chapter 6** and **chapter 7**). Advantages of such 2D cultures include equal amounts of nutrients present in the medium resulting in homogenous proliferation, fast growth, low costs and easy maintenance and manipulation[441,442], making them, amongst others, highly efficient models for screening purposes. However, 2D cell cultures lack the three-dimensional (3D) tissue physiology existing in the primary tumor resulting in amongst others differences in cell-cell and cell-extracellular matrix interactions, loss of polarity, changed morphology, loss of heterogeneity and lack of interaction with the tumor micro-environment[443,444]. Of note, since cell-extracellular matrix interactions are of great importance for cancer cell migration[43,445], all migration experiments in this thesis (**chapter 2** and **chapter 5**) were performed on plates coated with fibronectin. To increase clinical translation of in vitro experiments, different 3D culture systems are currently explored, including organoid cultures, patient-derived xenograft models, stem cell reprogramming, tumor-on-a-chip and 3D cultures of immortalized breast cancer cell lines. Although these advanced models better match human patient tumors[381–383], disadvantages include reduced reproducibility[381,384,385], increasing costs,

inconvenient maintenance and difficulties in expanding and genetic modifications[386]. Since transcriptomic analysis can contribute to the understanding of different in vitro models, in **chapter 7** we compared the transcriptomes of 14 breast cancer cell lines (amongst which 8 TNBC cell lines) cultured in conventional 2D tissue culture plates as well as 3D collagen-matrigel environments in which cells spontaneously form spheroid-like structures and experience both cell-cell and cell-matrix interactions. Indeed, we identified a high dependence of the cell phenotype and transcriptome on the culturing conditions; the invasive phenotype of the basal B TNBC cell lines in 3D was associated with increased expression of genes involved in cell-matrix interactions, while these genes were lower expressed in the less invasive basal A TNBC cell lines. Although 3D cultures better resembled human primary tumors based on cell phenotype, transcriptome and growth behavior, sensitivity towards potent anti-cancer drugs such as CDK and aurora kinase inhibitors was not changed. Evidently, we only compared the transcriptomes between 2D and 3D cultures of breast cancer cell lines and did not include any other in vitro models (organoids, patient-derived xenografts, stem cell reprogramming and tumor-on-a-chip[377,420–422] that could be used for drug testing. Future research should systematically compare the transcriptome of these different models and their response to anti-cancer drugs to decide on the best cell model for in vitro screening.

Transcriptomics

During the last decade, the use of 'omics' techniques including genomics, transcriptomics, proteomics and metabolomics increased exponentially and enabled the discovery of new mechanisms and biomarkers in a relatively cheap and high-throughput manner. For example, a public effort from The Cancer Genome Atlas (TCGA) that provides multi-omics data derived from patient tumor material for over 30 cancer types already resulted in more than 5,500 scientific publications. For breast cancer, this dataset resulted for instance in improved classifications, the discovery of new subtype-specific biomarkers, the identification of new loci associated with an increased breast cancer risk and better understanding of the genomic evolution during metastatic progression[19,70,71,446,447]. In **chapter 2,** we used the breast cancer genomics and transcriptomics data derived from TCGA primary breast tumors to validate the role of our in vitro screening candidates in human patients, finally resulting in the discovery of splicing factor PRPF4B as an essential component of metastasis formation. Next, in **chapter 4**, we validated the role of splicing in breast cancer: analysis of transcriptomics data from 1,097 primary breast tumors and 7 metastatic tumors identified two groups of co-regulated splicing factors as drivers of breast cancer progression. In line, the newly identified splicing factor metastatic determinants defined in **chapter 5**, demonstrated a higher expression level in primary tumors compared to normal tissue and TNBC compared to the less aggressive ER positive subtype. Lastly, in **chapter 7**, the transcriptome of primary breast tumors was

correlated to the transcriptome of 14 human breast cancer cell lines in 2D or 3D cultures, respectively. Since the transcriptome correlation was highly pathway dependent, we established a bioinformatics strategy to select the culture model with the best correlation to the patient tumor transcriptome to study the oncogenic pathway of interest thereby supporting clinical translation of in vitro studies. As a next step, integration of other omics platforms in these analyses could 1) add evidence for the selection of the optimal in vitro model and 2) elucidate the suggested interplay between splicing and other omics areas such as metabolomics and epigenomics in breast cancer development and progression[448–450]; **chapter 4**).

Uncovering the signaling landscape driving TNBC progression

To systematically unravel the signaling pathways driving TNBC progression, we performed an imaging-based phenotypic RNAi screen in two highly migratory TNBC breast cancer cell lines (**chapter 2**). We screened ~4,200 individual genes and identified 133 and 113 migration regulators in Hs578T and MDA-MB-231 cell lines, respectively, using previously discussed PKT and RCM assays. Most of these genes were positive regulators of cell migration, which are most interesting for translation into therapeutic targets. Suppressors of cell migration were difficult to validate due to the enhanced migratory behavior of Hs578T and MDA-MB-231 cell lines reaching a physiological ceiling. Additional screening using TNBC cell lines with a low migratory capacity would allow to uncover these negative regulators of TNBC cell migration and potentially metastatic progression. Splicing factors PRPF4B and BUD31 and transcription factor BPTF were essential for TNBC cell migration, often amplified in TNBC primary tumors, associated with metastasis-free survival in breast cancer patients and regulated well-known cancer migratory pathways such as focal adhesion and ECM-receptor interaction. The crucial role for splicing factor BUD31 in TNBC metastatis formation using in vivo mouse models was already shown previously[114]. Here, we demonstrate that also PRPF4B knockdown significantly reduced TNBC metastatic progression in vivo, making it an interesting target for future drug development. Moreover, our selection of validated candidate migratory modulators provide opportunities for future studies in TNBC metastatic progression. For example, we identified 8 ubiquitinase and proteasome components and 16 G-protein coupled receptors (GPCRs). These processes have previously been linked to basal-like TNBC growth and proliferation[129] and were over-represented in ER negative tumors[101], respectively. For other candidates, like BPTF, the amplification status and correlation with metastasis-free survival suggest involvement in TNBC metastatic progression, making it interesting to investigate these candidates in future in vivo studies.

Uncovering the splicing landscape driving TNBC progression

As a result of our screening efforts in **chapter 2**, we identified splicing factors PRPF4B and BUD31 as drivers of TNBC metastatic progression. This, in combination with our previous discovery of splicing factor SRPK1 as a critical determinant in TNBC metastasis formation[66], suggested an essential role for RNA splicing in breast cancer metastatic progression. By specific incorporation of introns and exons (alternative splicing), RNA splicing can regulate isoform expression and mRNA stability, thereby providing an extra layer of genetic control[68,132]. In this thesis, the generic capability of alternative splicing to modulate gene expression was confirmed by the observed intron detention in 3D cultures (**chapter 7**). Intron detention occurs in genes involved in essential pathways such as transcription, DNA damage, cell cycle and splicing resulting in nuclear retention and abrogated translation and is used as a distinct mechanism of transcriptomic regulation[451–453]. Tumor cells can use this flexibility to fulfill their oncogenic needs. For example, alternative splicing has been related to up-regulation of proto-oncogenes, deregulated cell division, increased survival, altered metabolism, onset of angiogenesis, increased invasion and metastatic progression in multiple cancer types including breast cancer (see reviews[67,133–135]). Although the downstream alternative splicing events have been extensively evaluated, the role of all ~250 splicing factors that catalyse the splicing reaction in breast cancer development and progression was not yet known. In **chapter 3**, we summarized the current knowledge about the effects of splicing factors on the acquaintance of oncogenic characteristics during breast cancer development and progression – the so called hallmarks of breast cancer[21,22]. 5 (sustaining proliferation, activation of invasion and metastasis formation, resisting cell death, deregulating cellular energetics and angiogenesis) out of the 10 described hallmarks were shown to be affected by splicing factors in previous studies, while for 4 other hallmarks (enabling replicative immortality, genome instability, avoiding immune destruction and evading growth suppressors) we discovered strongly related splicing factors using publicly available RNA sequencing data. In line, in **chapter 4** we discovered two groups of co-regulated splicing factors that drive breast cancer progression. The classified enhancer splicing factors (21 genes) were associated with aggressive breast cancer phenotypes, enhanced metastatic progression, distinct alternative splicing patterns and high expression of oncogenic pathways such as respiratory electron transport, DNA damage and cell cycle regulation. Since these studies were solely based on co-expression, the causal relation between splicing factor enhancers and cancer signaling network activity could not yet be assigned. Therefore, we conducted two RNAi screens that systematically evaluated the causality between splicing factor expression levels and the two key processes in metastasis formation: 1) tumor cell migration (**chapter 5**) and 2) tumor cell proliferation (**chapter 6**). Similar to PRPF4B and BUD31 (**chapter 2**), depletion of splicing factors SMNDC1, SNRPA1, SNRPG

and SRSF7 resulted in strongly reduced TNBC cell migration via inhibition of extracellular matrix interaction and focal adhesion pathways. These splicing factors regulated these downstream pathways, at least partly, by increased intron retention resulting in reduced total gene expression levels. Differential intron retention patterns have been observed during development and in multiple diseases including cancer[334–336] and, in line with our results, mainly led to reduced protein levels caused by nuclear retention, early degradation and reduced translation efficiency[334]. Next to increased intron usage, SMNDC1 and SRSF7 also regulated alternative exon usage of cell migration modulators such as RABGGTB and ITGB1. Moreover, SRSF7 was functioning in the same complex as previously identified splicing factor migration modulators PRPF4B, BUD31 (**chapter 2**) and SRPK1[66], also partly sharing alternative exon usage events (**chapter 5**). As a next step, specific modulation of these alternative splicing events would be critical to directly link these events to metastasic progression in TNBC patients and identify potential targets for drug treatment.

In **chapter 6**, we discovered 9 splicing factors that upon knockdown inhibited proliferation in TNBC cell lines using a common mechanism implying dysregulation of sister chromatid cohesion (SCC) by increasing sororin intron 1 retention and downregulation of SMC1, MAU2 and ESPL1. This then resulted in double DNA content, stall in G1-S transition and ultimately cell death. Some of these 9 splicing factor candidates have previously been linked to sororin alternative splicing and SCC in the cervical HeLa cancer cell line, suggesting that splicing factors modulate SCC independent of the cancer type[338,358]. Intriguingly, the downregulation of the other SCC factors ESPL1, MAU2 and SMC1 could be TNBC specific, since these events were not observed in HeLa cells[338]. Considering our stringent selection criteria, we suggest other splicing factors to have similar effects on cancer cell proliferation and suggest to use our screening data in future studies to evaluate the therapeutic potential of more splicing factors in cancer cell proliferation.

Targeting splicing as TNBC treatment

Since splicing factors are frequently overexpressed in TNBC and related to TNBC progression (**chapter 2-6**), they might be new promising drug targets to reduce metastasis formation and breast cancer induced mortality. Most of the splicing targeting drugs that have been developed are (derived from) natural compounds and inhibit the SF3B complex: a five polypeptide subcomplex of the U2 snRNP[286]. A major consideration for applying these drugs in a clinical setting could be the appearance of severe side effects caused by abolishing the essential function of splicing factors in normal tissue. Surprisingly, these SF3B inhibitors exhibited cytostatic effects in multiple cancer cell lines in vitro and in vivo without causing general toxicity[261,263,266]. Additionally, these compounds were more effective in cancer cells compared to

normal cells and could even sensitize multidrug-resistant cell lines[264–266]. Interestingly, one of our splicing factor candidates regulating TNBC proliferation was SF3B1, a member of the SF3B complex (**chapter 6**). SF3B1 is known as a breast cancer driver gene[76] and mutated in 20% of uveal melanoma tumors[327]. Also, SF3B1 mutations are associated to adverse clinical outcome in chronic lymphocytic leukemia (CLL)[326,344]. In the last decade, pladienolide B has been discovered as a potent and selective SF3B1 inhibitor that reduced tumor cell growth in CLL and gastric cancer[365,366]. In **chapter 6**, we demonstrated that SF3B1 inhibition by pladienolide B treatment strongly inhibited TNBC proliferation and increased sororin alternative splicing similar to SF3B1 knockdown. As sororin RNA levels were highly correlated to proliferation marker expression levels in breast cancer tumors and significantly higher expressed in TNBC compared to less aggressive hormone receptor positive tumors, sororin downregulation by pladienolide B treatment could be a promising therapy for TNBC patients.
Using our screening approach, we discovered various other splicing factors that are potential targets for drug development (**chapter 2**, **chapter 5** and **chapter 6**). Although new compounds modulating various alternative splicing components have been identified[269], drugs targeting our candidates are not yet available. In particular, we suggest PRPF4B as a promising therapeutic target, since next to the strong inhibition of in vitro TNBC migration, PRPF4B was also essential for metastasis formation in an orthotopic mouse model (**chapter 2**). Therefore, future studies should focus on the development of a specific PRPF4B inhibitor; especially because the x-ray structure of the catalytic domain of PRPF4B suggest this is feasible[128].

Conclusion

Overall, this theses contributes to the understanding of the underlying mechanisms driving TNBC metastatic progression. We established a repository of signaling determinants modulating TNBC migratory behavior and systematically evaluated the vital role of (alternative) splicing in various steps of the metastatic process. Importantly, we identified some splicing factors that are promising therapeutic targets to combat TNBC progression to ultimately reduce breast cancer related deaths.