Disrupting the transcriptional machinery to combat triple-negative breast cancer

Noord, V.E. van der

Chapter 1

Introduction, aim and outline of this thesis

Highlights:

- Triple-negative breast cancer (TNBC) poses a significant challenge due to a poor prognosis and limited effective therapies
- Developing targeted therapies for TNBC is hindered by oncogenic heterogeneity and the emergence of drug resistance
- The transcriptional machinery serves as a central hub connecting various TNBC drivers
- Modulating transcriptional cyclin-dependent kinases offers a promising avenue for disrupting the transcriptional machinery

Triple-negative breast cancer is an aggressive subtype of breast cancer

Breast cancer is the most frequently diagnosed cancer in women, affecting approximately 1 in 8 women during their lifetime[1]. It has recently surpassed lung cancer as the leading cause of cancer-related death in women worldwide. Breast cancer can be classified into distinct subtypes, each with varying prognosis and treatment approaches, based on the expression of human epidermal growth factor receptor 2 (HER2), estrogen receptor (ER), progesterone receptor (PR), and the proliferation marker Ki67[2]. These subtypes include luminal A (ER/PR-positive, HER2-negative and low Ki67), luminal B (ER-positive, HER2-negative, high Ki67), luminal B-like HER2-positive (ER-positive and HER2-positive), HER2-enriched (ER/PR-negative, HER2-positive) and triple-negative (ER/PR/HER2-negative) breast cancer. While the term “triple-negative” denotes the absence of ER, PR, and HER2 receptors it is rather a title of convenience, that does not fully capture the heterogeneity and complexity of this subtype[3]. In fact, triple-negative breast cancer (TNBC) can be further classified into four different subtypes based on transcriptomic features, namely the basal-like 1, basal-like 2, mesenchymal, and luminal androgen receptor subtype[4].

TNBC accounts for approximately 10-20% of all breast cancer cases and stands out as highly aggressive subtype[5]. Compared to other breast cancer subtypes, it occurs more frequently in younger women and is associated with higher invasiveness and metastatic potential to vital organs such as the liver, lungs and brain[5]. Metastasis is the main cause of mortality in breast cancer patients, and this invasive phenotype thus strongly contributes to the poor prognosis of TNBC. Among patients diagnosed with breast cancer, the median 5-year overall survival estimate for TNBC is 77%, compared to 95% for luminal A/B, 91% for luminal B-like HER2-positive, and 86% for HER2-positive breast cancer[6]. However, if TNBC is diagnosed as regionally or distantly metastasized, the median 5-year overall survival declines sharply to 66% and 13%, respectively. This poor prognosis highlights the urgency for effective treatment options.

Breast cancer treatment: an unmet medical need for TNBC

The selection of breast cancer treatment is based on a variety of factors, including breast cancer subtypes and cancer stage[7–9]. Generally, breast cancer treatment includes systemic treatment, surgery, and radiotherapy. Systemic treatment can be administered in the neoadjuvant setting (before surgery, most common for chemotherapy) or adjuvant setting (after surgery). The options for systemic treatment are dependent on the specific breast cancer subtype. For hormone-positive (ER+/PR+) and HER2-enriched breast cancer, targeted therapies are frequently employed together with conventional neo-adjuvant chemotherapy or as an alternative to it, including endocrine (in combination with CDK4/6 inhibitors in metastatic disease) and anti-HER2 therapies[8].

However, due to a lack in HER2, ER and PR expression in the case of TNBC, these therapies are ineffective. Conventional neo-adjuvant chemotherapy, along with

surgery and radiation therapy, has long been the primary option for first-line systemic treatment of most TNBC patients[2]. While TNBC patients generally respond better to neo-adjuvant chemotherapy than non-TNBC, still 50 to 60% of TNBC patients do not achieve a pathological complete response to chemotherapy, which is strongly correlated with a poor prognosis[10]. For patients with germline BRCA1 or BRCA2-mutated metastatic or early high-risk HER2-negative breast cancer, the PARP inhibitor olaparib has been approved recently, offering improved progression-free survival rates and lower side effects compared to standard chemotherapy[11,12]. Similarly, the PD-1 immune-checkpoint inhibitor pembrolizumab, in combination with chemotherapy, has been recently approved for patients with high-risk and early, or metastatic PD-L1-positive TNBC[13,14]. Additionally, sacituzumab govitecan, an antibody-drug conjugate targeted against Trop2 and containing a topoisomerase inhibitor, has been approved as a second-line treatment for metastatic TNBC, which enhances overall survival, but also worsens treatment-related adverse events, compared to chemotherapy[15]. However, as these novel therapeutic options improve median progression-free or overall survival only with a few months, TNBC continues to have a poor prognosis. Moreover, most systemic treatment options for TNBC are aggressive and result in a poor quality of life for these patients. Thus, there remains a significant unmet medical need to find better, targeted therapies that can improve the prognosis of TNBC patients.

Specific drivers of triple-negative breast cancer as alternative targets for new treatments?

TNBC tumors exhibit diverse genomic alterations, which complicates the development of targeted therapy (Figure 1A)[16–18]. Despite this complexity, certain drivers that are frequently altered in TNBC have emerged as potential targets for novel treatments (Figures 1A and 1B)[19]. However, despite tremendous efforts and (pre-) clinical studies, the progress in this area has been limited, with only PARP inhibitors and immune-checkpoint inhibitors receiving approvals for clinical use.

One prevalent characteristic of TNBC is the heightened activation of growth factor signaling pathways, such as the PI3K and MAPK pathway. *PIK3CA* mutations or amplifications and *PTEN* mutations or losses are frequently found in TNBC, which contribute to activation of the PI3K pathway (Figures 1A and 1B)[16,20]. Although activating mutations within the MAPK pathway, as found in ER+ breast cancer, are rare in TNBC, TNBC tumours do exhibit a frequent loss of negative regulators such as *NF1* and *DUSP4* and amplifications of upstream activator *KRAS*[16,21–23]. Moreover, *MYC*, *EGFR* and *FGFR1* are commonly amplified, that are respectively down- and upstream of growth factor signaling pathways, such as the PI3K and MAPK pathway[16]. For these reasons, inhibitors of proteins within these pathways (e.g. EGFR, FGFR, Akt, PI3K, MEK, mTOR) have been studied extensively as TNBC treatment. Yet, despite pre-clinical efficacy, these inhibitors have failed to substantially improve overall survival of TNBC patients[20,24–27]. Also targeting of other signaling pathways that contribute to growth and invasiveness, such as JAK/STAT, MET, VEGFR, and Src

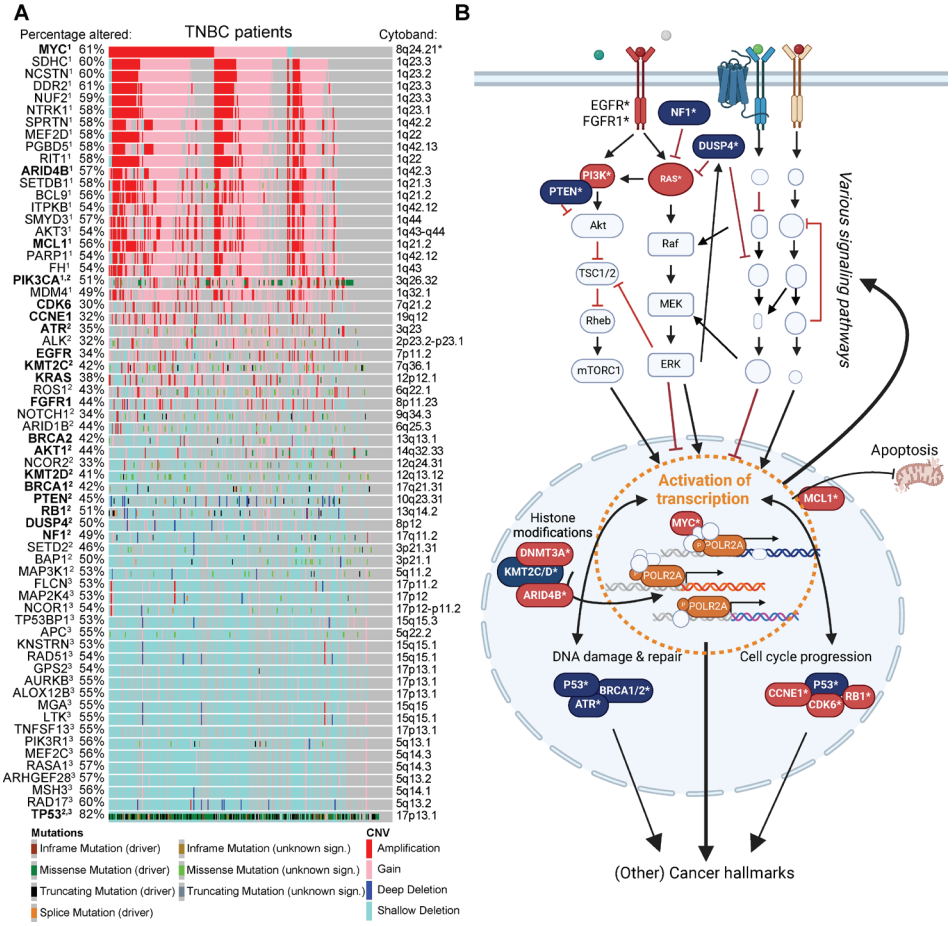

Figure 1. Numerous genomic mutations and copy number variations in TNBC contributing to molecular heterogeneity ultimately relying on transcription. (A) Most frequent copy number alterations and mutations in OncoKB-selected[38] cancer-associated genes in TNBC patients. Top 20 amplified (+2) or gained (+1) copy number variations([1]), top 20 mutated genes([2]), and top 20 hemizygous (-1) or homozygous (-2) deletions([3]), are shown, including key cancer drivers. *MYC co-amplifications (cytoband 8q22-24; *MAL2*, *EXT1*, *RAD21*, *EIF3E*, *AGO2*, *BAALC*, *UBR5*, *RECQL4*, *TONSL*) are not displayed. Data are from the METABRIC[17,18] cohort in cBioportal[39]. (B) Illustrative example of deregulated processes (e.g., MAPK and PI3K pathways, DNA damage repair, cell cycle progression, histone modification, and epigenetic regulation) resulting from these alterations. Transcription, as central hub, integrates multiple of these aberrant cellular signals, eventually activating genes associated with cancer hallmarks. Genes frequently altered in TNBC are highlighted in bold with an asterisk (*), while genes with activating mutations or frequent amplifications are shown in red, and those with (predicted) loss-of-function mutations or frequent copy number losses are in dark blue. This figure was created with BioRender.com.

have not yet led to substantial improved responses in clinical trials[28–32]. The individual reasons behind the limited responses to these targeted therapies in TNBC patients remain incompletely understood, although the development of biomarkers that can better predict response may facilitate the selection of patients that benefit from these

therapies[33].

Approximately 50-70% of TNBC patients exhibit homologous recombination deficiency, similar to *BRCA*-mutated tumours, also known as "BRCAness"[34–36]. This is caused by, amongst others, mutations, copy number alterations or silencing of *BRCA1*, *BRCA2*, *PALB2* and *ATM* genes. While contributing to genomic instability, homologous recombination deficiency creates a vulnerability that can be targeted with PARP inhibitors. Although successful trials have led to the approval of these treatments for patients with germline *BRCA1* or *BRCA2* mutations[11,12], responses in other homologous recombination-deficient TNBC cases vary, and resistance often develops. Ongoing clinical trials are currently investigating whether patients with other mutations, such as germline *PALB2* or somatic *BRCA1/2* mutations could also benefit from this therapy[37]. However, despite initial beneficial responses in patients with *BRCA1/2* germline mutated tumors, the benefit on overall survival using these new treatments still remains limited, highlighting the need for better combination therapies that address mechanisms of resistance, or other therapeutic strategies. Currently, also other DNA damage response inhibitors are under clinical evaluation, such as ATR, and CHK1/2 inhibitors[19].

In TNBC, rapid and uncontrolled cell cycle progression is frequently driven by multiple mutations and copy number alterations affecting genes such as *TP53*, *RB1*, *CCND1*, *CCNE1*, and *CDK6*[16,40]. Additionally, the heightened stimulation of growth factor signaling pathways and MYC further stimulates cell cycle progression. Yet, direct targeting of the cell cycle has not proven effective in TNBC patients. For example, CDK4/6 inhibitors, which have shown strong efficacy in ER+/HER2- breast cancer, have not yielded comparable results in TNBC, possibly due to mutations and losses of *RB1*, which reduces TNBC cells' dependency on CDK4 and 6 activation[41,42]. Nonetheless, CKD4/6 inhibitors are still being studied in combination with antiandrogens in the LAR TNBC subtype[43], which generally lacks strong *RB1* alterations[16]. Various other inhibitors targeting the cell cycle, such as pan-CDK inhibitors (targeting CDK1 and 2, but also other CDK's), aurora kinase inhibitors, and PLK1 inhibitors, have faced challenges and limitations due to off-target effects and dose-limiting toxicities[40].

In addition to these described frequently altered pathways, also other potential new targets are being investigated[44]. Apart from genes altered by copy number variations or mutations, gene expression undergoes further deregulation epigenetically, involving histone modifiers frequently altered or mutated in TNBC (e.g., *KMT2C/D, DNTM3A,* and *ARID4B*)[16–18]. These modifiers can simultaneously alter the transcriptional activity of extensive gene sets. Despite the evaluation of inhibitors targeting enzymes modifying epigenetic marks on DNA and histones, such as DNA methyltransferases (DNMT) or histone deacetylases (HDAC) inhibitors, across various cancer types, clinical success in solid cancers remains elusive[45]. Overall, despite substantial efforts to develop targeted therapies against TNBC drivers, only a few have achieved limited clinical success, questioning whether the targeting of single TNBC drivers are effective approaches, or whether an alternative strategy is needed.

Mechanisms of drug resistance to systemic therapy

To further improve the development of new targeted therapies for TNBC, a thorough understanding of the drug resistance against these previously tested agents is needed. Drug resistance to systemic therapy, including targeted therapy and conventional chemotherapy, can occur either as pre-existing (intrinsic) or drug-induced (acquired) resistance. The genomic instability and intratumor heterogeneity within TNBC play

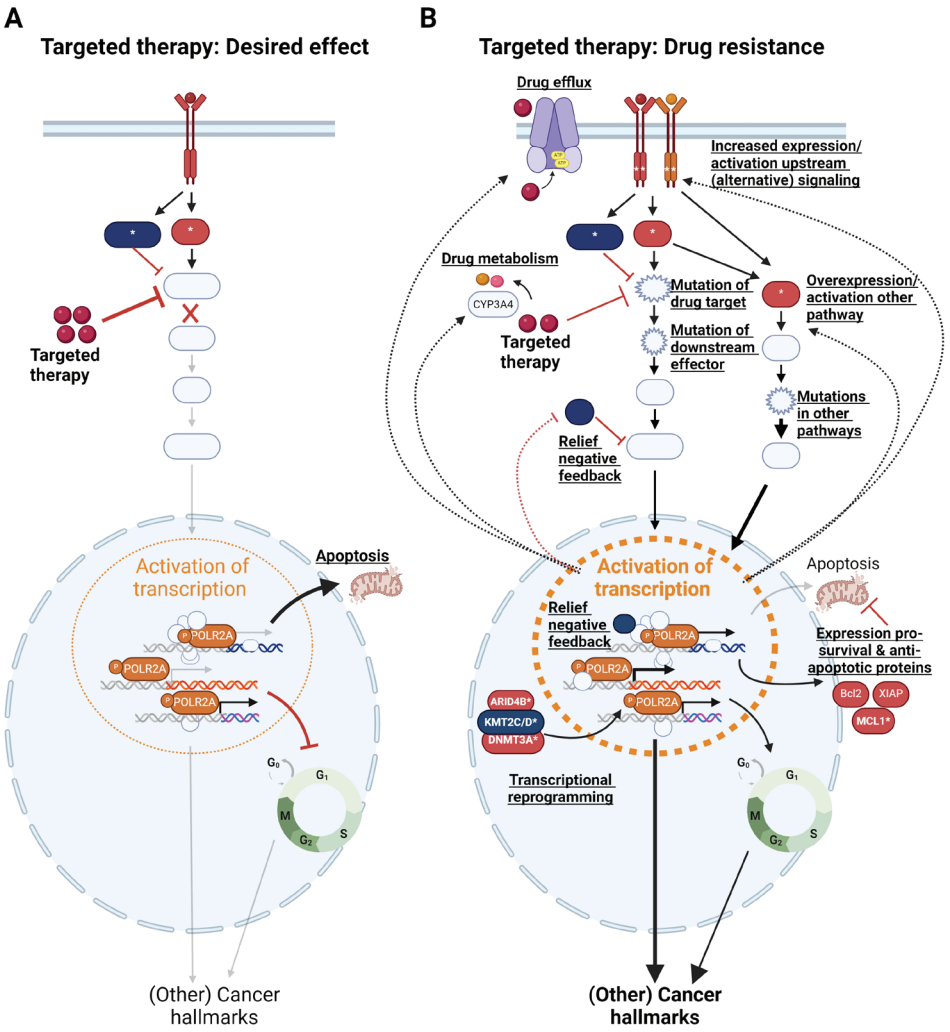

Figure 2. The transcriptional machinery as mediator of drug resistance? (A) Illustration of desired effect of a targeted therapy, mostly involving inhibition of a specific pathway, and subsequently inhibition of transcription of more downstream effectors, that would otherwise contribute to various cancer hallmarks, such as cell proliferation, inhibition of apoptosis and immune evasion. (B) Examples of potential mechanisms of drug resistance against targeted therapy that hamper the desired effect. In addition to mutations causing drug resistance, transcription is a central hub that is frequently deregulated to enable drug resistance. This figure was created with BioRender.com.

significant roles in its drug resistance, contributing to the emergence of resistant subclones[46]. On a molecular level, intrinsic or acquired drug resistance in TNBC can result from a wide variety of causes, including, amongst others, altered drug availability, anti-apoptotic mechanisms, reversal of pathway dependence and redundancy in targeted driver pathways (Figures 2A and 2B)[47,48]. Many of these mechanisms are also employed or activated during epithelial-to-mesenchymal transition and in cancer stem cells, which may contribute to the elevated resistance of these cell types and metastatic TNBC[49–51].

Effective cancer therapy relies on achieving high intracellular drug availability in cancer cells and resistance mechanisms that reduce drug concentrations have therefore been studied extensively. Intracellular cancer cell concentrations can be limited by physical barriers, such as hypoxic environments, or the blood-brain barrier, hindering drug delivery to target sites of solid tumors, and especially brain metastases[52]. Additionally, multi-drug resistance is often attributed to drug efflux by ABC-transporters, including ABCB1, ABCC1, and ABCG2, which increase drug clearance in liver and kidney, reduce drug uptake in the gastrointestinal tract, and facilitate drug export by cancer cells[53]. Overexpression or mutations of these transporters in cancer (stem) cells can confer multi-drug resistance to various anti-cancer drugs, including chemotherapy and kinase inhibitors[53,54]. In addition, alterations in drug metabolism in patients, or tumor cells specifically, such as by cytochrome P450 (CYP3A4), have been associated with drug resistance[55]. Owing to these insights, newly synthesized drugs aim to overcome these problems and have better physicochemical properties, which is, however, not always feasible and anticipated.

Resistance to targeted therapy can also occur through mechanisms that ensure sustained activation of the targeted pathway[52,56]. Mutations in the targeted kinase's ATP binding site can cause lower drug affinity through steric hindrance, and/or may also cause continuous activation of this protein[57]. Therefore, many next-generation kinase inhibitors that are now being developed aim to circumvent this problem, or specifically target the mutated proteins[58–60]. Resistance may also arise from various compensatory activation or relief of negative feedback loops within the same pathway, achieved through upregulation or mutations of proteins up- or downstream of the targeted protein[61–65]. For example, Akt inhibition can be overcome by relieving feedback suppression of receptor tyrosine kinases, which can re-activate Akt and other growth factor signaling pathways[64]. Moreover, re-activation of ERK upon BRAF inhibition is the rationale for combining MEK and BRAF inhibitors[66], which have proven successful in BRAF-mutated melanoma[67,68]. In contrast, in the case of synthetically lethal approaches[69], reversion of the initial trait can lead to drug resistance. For example, restoration homologous recombination proficiency (e.g. by functional restoration of *BRCA1/2* mutations) leads to drug resistance of PARP inhibitors and platinum-based therapy[70–72].

Similarly, deregulation of other (compensatory) pathways is another mechanism of resistance to anti-cancer therapy. For example, overexpression of anti-apoptotic

proteins, such as XIAP and MCL1, has been associated with chemoresistance and resistance to targeted therapy[73–78]. Survival and proliferation signaling can also be maintained indirectly through compensation of other pathways, which can occur through relieve of negative feedback loops, or more comprehensive adaptation, such as (transient) transcriptional reprogramming or plasticity[79–83]. For example, HER2/EGFR inhibitor resistance is caused by increased expression of multiple other receptor tyrosine kinases[84,85]. Similarly, resistance to immune checkpoint inhibitors can be caused by a variety of mechanisms, including disrupted signaling pathways, such as TGF-β or IFN-γ signaling, reduced antigen presentation, expression of other immune checkpoints, and expression of other immunosuppressive cytokines or receptors leading to T cell exhaustion[86–90]. Moreover, transcriptional reprogramming can induce slow-cycling, dormant or senescent cells, that are less sensitive to certain therapies, including chemotherapy[91,92]. Additionally, metabolic reprogramming through induction of oxidative phosphorylation can increase energy required to cope with survival stress and is associated with chemoresistance [75,92,93].

In conclusion, drug resistance in TNBC occurs through diverse and potentially even more still undiscovered mechanisms. The possible redundancy and heterogeneity of oncogenic drivers and genomic instability in TNBC can make it inherently insensitive to many targeted therapies. Moreover, the most critical, yet considered “undruggable” TNBC drivers *MYC* and *TP53* pose significant challenges for drug development.

The transcriptional machinery as central target underlying the expression of multiple TNBC drivers

Transcription, the process of producing mRNA, which can be translated into proteins, also plays a crucial role in tumor development, progression and persistence[94]. Cancer cells employ various mechanisms to selectively boost the transcription of specific genes. These mechanisms include, amongst others, the overexpression of transcription factors (e.g. *MYC* amplifications) and alterations of regulatory domains (e.g. (super-)enhancers and histone modifications)[94]. The mentioned (epi)genetic variations that drive TNBC, and other cancer types, mostly eventually rely on deregulated transcriptional programs (Figure 1B). Moreover, many mechanisms of resistance are sustained by specific transcriptional deregulation (Figure 2B).

This transcriptional addiction could be targeted through inhibition of transcriptional cyclin-dependent kinases (CDKs), for which multiple selective inhibitors are available and have demonstrated pre-clinical anti-cancer efficacy[95]. These transcriptional CDKs, including CDK7, CDK8, CDK9, CDK12 and CDK13, regulate the activity of RNA polymerase II and other transcription factors that govern mRNA transcription (Figure 3). CDK7, in conjunction with cyclin H, is part of the transcription initiation complex, including transcription factor II H (TFIIH), and mediates transcription initiation by phosphorylating serine 5 and 7 residues of the C-terminal domain (CTD) of RNA polymerase II, resulting into promoter escape and recruitment of 5’ capping enzymes[96]. The pre-initiation complex is connected from the transcription start site to distal regulatory regions, such as enhancers, and their associated transcription

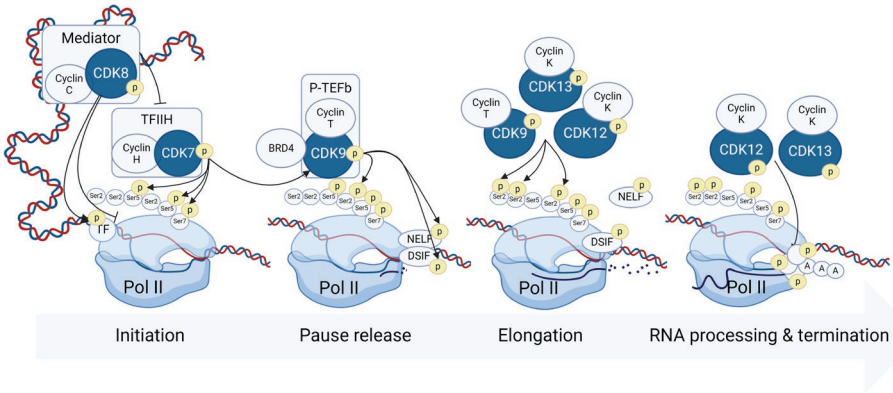

Figure 3. Transcriptional CDKs regulate multiple steps in transcription by phosphorylating RNA polymerase II residues and associated factors. Schematic overview of how transcriptional CDKs 7, 9, 12 and 13 regulate RNA polymerase II (Pol II), and subsequently transcription initiation, pause release, elongation, RNA processing and termination, by phosphorylating, amongst others, serine 7, serine 5 and serine 2 residues on the C-terminal domain of RNA polymerase II and by phosphorylating other transcription factors (e.g. negative elongation factor, NELF; DRB Sensitivity Inducing Factor, DSIF). CDK8 phosphorylates multiple transcription factors associated with RNA polymerase II, regulates phosphorylation of CDK7, and mediates signals from distal regulatory elements, such as enhancers. This figure was created with BioRender.com.

factors, through the Mediator complex. The kinase module of the Mediator complex consists of CDK8 (or its paralog CDK19), along with cyclin C, MED12 and MED13, which can repress CDK7 activity to phosphorylate RNA polymerase II, and can phosphorylate other transcription factors such as STAT1[95–98]. It is both described to stimulate as well as inhibit transcription, depending on the transduced signals[98,99]. After transcription initiation, RNA polymerase II goes into promoter-proximal pausing, due to the binding of the negative elongation factor (NELF) and DRB sensitivity-inducing factor (DSIF)[95,96]. CDK7 contributes to the release of promoter-proximal pausing by phosphorylating CDK9[100]. CDK9, together with cyclin T1 or T2, are part of the positive transcription elongation factor b (P-TEFb) complex, which is negatively regulated by the 7SK small nuclear ribonucleoprotein (snRNP) complex and hexamethylene-bis-acetamide inducible proteins 1/2 (HEXIM1 and/or HEXIM2)[96,101]. P-TEFb is released from the snRNP complex upon recruitment of CDK9 by BRD4, which recognizes histone modifications and can thereby recruit CDK9 to transcription start sites. Upon activation, CDK9 enables pause release by phosphorylating NELF, causing its dissociation, and DSIF, modifying its function from suppressive into supportive of further transcription. CDK9 promotes further transcriptional elongation by phosphorylating serine 2 residues of the CTD of RNA polymerase II. Moreover, CDK12 and CDK13 can phosphorylate serine 2 and 5 residues[101], although these are thought to mainly play a role during later stages of elongation. In addition, mostly CDK12, but also CDK13, has been implicated in mRNA processing, mRNA polyadenylation and transcription termination, for example by co-localizing and phosphorylating RNA processing factors[95,102,103]. While significant process has been made in understanding transcriptional CDKs, their full range of functions in transcription may not yet be fully

elucidated.

In addition to their essential role in general transcription, transcriptional CDKs likely have a more specialized role in preferential gene regulation. The disruption of transcriptional CDKs has been described to result in the deregulation of specific genes, including MYC-driven, pro-survival, cell cycle, and DNA damage response genes[95,101]. Importantly, this regulation of specific genes could make them suitable targets to indirectly regulate multiple TNBC drivers at once, thereby preventing adaptation mechanisms. For example, CDK7 inhibition has shown promise in downregulating MYC and other super-enhancer regulated genes, thereby specifically targeting MYC and super-enhancer driven transcriptional addiction[104,105]. Moreover, CDK12 and 13 inhibition, downregulates primarily long genes through inducing intronic polyadenylation, which are mostly genes involved in DNA damage response[102,103], and could thereby prevent PARP inhibitor resistance[106,107]. However, the gene-specific regulation by these transcriptional CDKs is not yet fully understood in the context of TNBC, and the mechanisms behind it remain unclear. Moreover, determining which of these transcriptional CDKs may be most effective to target and what mechanisms could contribute to resistance to these inhibitors could further potentiate their use for TNBC treatment. Additionally, there is growing interest in combining inhibitors of transcriptional CDKs with other kinase inhibitors, such as EGFR inhibitors[108–111], although the exact mechanisms of these synergistic actions need further investigation.

Aim and outline of this thesis

This thesis aimed to investigate the efficacy, mechanism of action and mechanisms of resistance associated with potential targeted therapies against TNBC, specifically transcriptional CDK inhibitors. In **Chapter 2** we demonstrate the overall poor sensitivity of TNBC cell lines to a wide range of kinase inhibitors, and focus on understanding the mechanisms of intrinsic drug resistance to MEK and Akt inhibitors. A high expression of cell cycle genes was associated with drug resistance to both, and resistant cells were sensitive to pan-CDK inhibitors, that also inhibit transcriptional CDKs. **Chapter 3** provides a comprehensive review of how targeting the transcriptional machinery, including transcriptional CDKs 7, 8, 9, 12, and 13, could indirectly affect TNBC drivers (also including MEK, Akt and cell cycle machinery) and vice versa. This chapter highlights the potential of the transcriptional machinery as a potent target to simultaneously interfere with multiple TNBC drivers. **Chapter 4** focuses on CDK9 inhibitors, including CDKI-73, which effectively synergized with EGFR inhibitors to inhibit TNBC cell proliferation and disrupt transcriptional programs, as demonstrated both in vitro and in vivo. However, toxicity was also observed in vivo, leading to the search for a safer combination therapy, which was further explored in **Chapter 5**. This chapter reveals a broad synergy between more selective transcriptional CDK inhibitors and other tyrosine kinase inhibitors, attributing this effect to the inhibition of ABCG2-mediated drug efflux of these CDK inhibitors. **Chapter 6** delves into understanding how individual transcriptional CDKs are essential for maintaining specific transcriptional programs in TNBC cells, providing further insight into their functions

and their potential as efficient drug targets against TNBC. Finally, **Chapter 7** presents a summary of the key findings and discussion of the research described in the thesis. Overall, this thesis provides valuable insights into the potential of targeting transcriptional CDKs as a promising therapeutic strategy for TNBC.

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