Unravelling epigenetic mechanisms of CAF-chemotherapy resistance in mammary carcinoma

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Affidavit

I hereby declare that the PhD thesis entitled “Unravelling epigenetic mechanisms of CAF-chemotherapy resistance in mammary carcinoma” has been written independently and with no other sources and aids than quoted.

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November, 2019

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Abbreviations

5-FU	5-Fluorouracil
BL1	Basal-like 1
BL2	Basal-like 2
BSA	Bovine serum albumin
°C	Degree Celsius
Ca^{2+}	Calcium ions
CAF	Cyclophosphamide, Doxorubicin (Adriamycin), 5-Fluorouracil
Cat. no.	Catalog number
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
ChIP-Seq	ChIP followed by high-throughput sequencing
CsA	Cyclosporine A
CSC	Cancer stem cell
DAPI	4',6-diamidino-2-phenylindole
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide DNA deoxyribonucleic acid
dNTP	Deoxyribonucleotide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial to mesenchymal transition
EtOH	Ethanol
EZH2	Enhancer of zeste 2
FACS	Fluorescence-activated cell sorting
FDA	Food and Drug Administration
FDR	False discovery rate
GSEA	Gene set enrichment analysis
H&E	Hematoxylin and eosin
H3	Histone 3
HAT	Histone acetyltransferase
HDAC	Histone deacetyltransferase
HDACi	HDAC inhibitor
H3K27ac	Histone 3 acetylated at position lysine 27
H3K27me3	Histone 3 trimethylated at position lysine 27
IAA	Iodacetamide

kDa	kilo Dalton
MEM	Minimum essential media
ML	Mesenchymal-like
MSL	Mesenchymal stem-like
mRNA	messenger RNA
NEM	N-ethylmaleimide
NFAT	Nuclear factor of activated T-cells
NGS	Next generation sequencing
NP-40	Nonidet P40
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with tween-20
PcG	Polycomb group proteins
PCR	Polymerase chain reaction
PRC2	Polycomb repressive complex 2
PTM	Post-translational modification
RBBP7	Retinoblastoma-binding protein 7
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
Rpm	Rounds per minute
RT	Room temperature
RT-PCR/qPCR	Reverse transcription PCR
SDS	Sodium dodecylsulfate
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
siRNA	Small interfering ribonucleic acid
SUZ12	Suppressor of zeste 12 protein homolog
siRNA	Small interfering RNA
TEMED	Tetramethylethylenediamine
TF	Transcription factor
Tris	Tris(hydroxymethyl)aminomethane
TSS	Transcriptional start site
TNBC	Triple negative breast cancer
VIM	Vimentin
WAP	Whey acidic protein
WB	Western blot
Wnt	Wingless and Int-1
WT	Wild type

Abstract

Triple negative breast cancer (TNBC) is one of the most challenging cancers to treat as, despite the initial response to chemotherapy, relapse occurs frequently. In this project, we focused on the mechanisms that allow TNBC cell survival under chemotherapy treatment. Phenotypic changes in TNBC cells such as gain of mesenchymal transition and stemness during genotoxic stress adaptation have been reported. Based on our data, CAF (Cyclophosphamide, Doxorubicin and 5-Fluorouracil)-chemotherapy treatment in TNBC cells leads to downregulation of the Polycomb Repressive Complex containing the methyl transferase EZH2 (PRC2/EZH2) and upregulation of Histone Deacetylases (HDAC4, -7 and -8).

EZH2 is responsible for the gene silencing through H3K27me3 catalysis and is known as an invasiveness marker. It appears that EZH2 can have also anti-tumorigenic function. We identified, among others, Nuclear factor of activated T cells (*NFATc1)*, as a gene regulated by EZH2 loss upon chemotherapy treatment in TNBC cells, indicated epigenetic switch. NFATc1 showed H3K27me3 loss and H3K27ac gain upon chemotherapy, where H3K27ac works antagonistically to the repressive mark, H3K27me3. NFATc1 has been associated with drug resistance and its loss impairs proliferation, migration and mesenchymal properties in TNBC cells *in vitro*. Our results strongly indicate a negative regulation of EZH2 on NFATc1 expression, *in vitro* and *in vivo*. Additionally, in TNBC patient data, we observed that low EZH2 and high NFATc1 expression correlate with poor survival. Therefore, in patients showing low EZH2 expression in cancer, NFATc1 inhibition may represent an alternative treatment option.

Furthermore, our preliminary studies on HDACs suggest that HDAC8 supports TNBC invasiveness, affecting cell growth and modulating EMT. Interestingly, selective HDAC8 inhibition sensitizes TNBC cells to chemotherapy. This provides a potential mechanism linking epigenetic adaptation and cancer state during TNBC chemotherapy resistance.

In summary, this work demonstrates a previously unknown PRC2/EZH2 function in TNBC, where its downregulation contributes to NFAT pathway changes

driving cancer progression. Hence, NFATc1 can be considered as potential PRC2/EZH2–dependent driver of TNBC invasiveness. Additionally, we identified HDAC8 and NFATc1 as an interesting targets for further investigation in potential anti-TNBC therapeutic approach.

1. Introduction

1.1. Breast cancer

According to the World Health Organization, breast cancer is the most common and most frequent diagnosed cancer among women causing a high cancer-related death rate (World Health Organisation, 2018). Breast cancer survival rates vary worldwide, where the highest cancer-related mortality occurs in developing countries (Ferlay et al., 2010). Despite scientific and diagnostic advancements leading to improved patient life quality, breast cancer remains a major cause of mortality (Lukong, 2017; World Health Organisation, 2018). Breast tumors are very complex and heterogeneous with a high diversity in gene expression patterns and pathological features. Based on the high-throughput transcriptomic analysis, breast tumors are classified into major molecular subtypes (Perou et al., 2000). These are Luminal A, Luminal B, human epidermal growth factor receptor 2 (HER2)-positive and triple-negative breast cancer (TNBC) (Figure 1) (Perou et al., 2000; Vallejos et al., 2010). The TNBCs account for 15- 20% of breast tumors. HER2-positive account for 10-15%, Luminal B for 20% and Luminal A for 40% of breast cancer cases (Metzger-Filho et al., 2013). Breast cancer can be further categorized into subset, which based on three common immunohistochemical and targetable breast cancer biomarkers, the hormone receptors: estrogen receptor (ER), progesterone receptor (PR) and HER2.

The treatment of breast cancer varies according to the subtype. TNBC, as its name suggests, does not express any of these receptors. It is not responsive to target therapies against hormone receptors and HER2 receptor, therefore TNBC has the worst prognosis among breast cancer patients. The treatment of breast cancer varies according to the subtype. Local therapies include surgical resection and/or radiation (Matsen & Neumayer, 2013). Patients with non-metastatic, hormone receptor-positive breast cancer (Luminal A/B) receive hormone therapy, also called endocrine therapy (Wong et al., 2012). To minimize the possibility of metastasis, surgery may be followed by adjuvant therapy, as additional to primary treatment (Dhankhar et al., 2010). Patients presenting metastasis are treated with chemotherapy alone or in combination

with targeted therapies (Chacón López-Muñiz et al., 2019; Mayer & Burstein, 2007). Patients with HER2-positive cancers can receive trastuzumab/lapatinib treatment to block HER2 in combination with chemotherapy. Overall, Luminal A/B and HER2-positive present better treatment options. On the other hand, TNBC, as its name suggests, is not responsive to target therapies against hormone receptors and the HER2 receptor, therefore non-resectable TNBC patients are treated with chemotherapy (Prat, Pineda, et al., 2015).

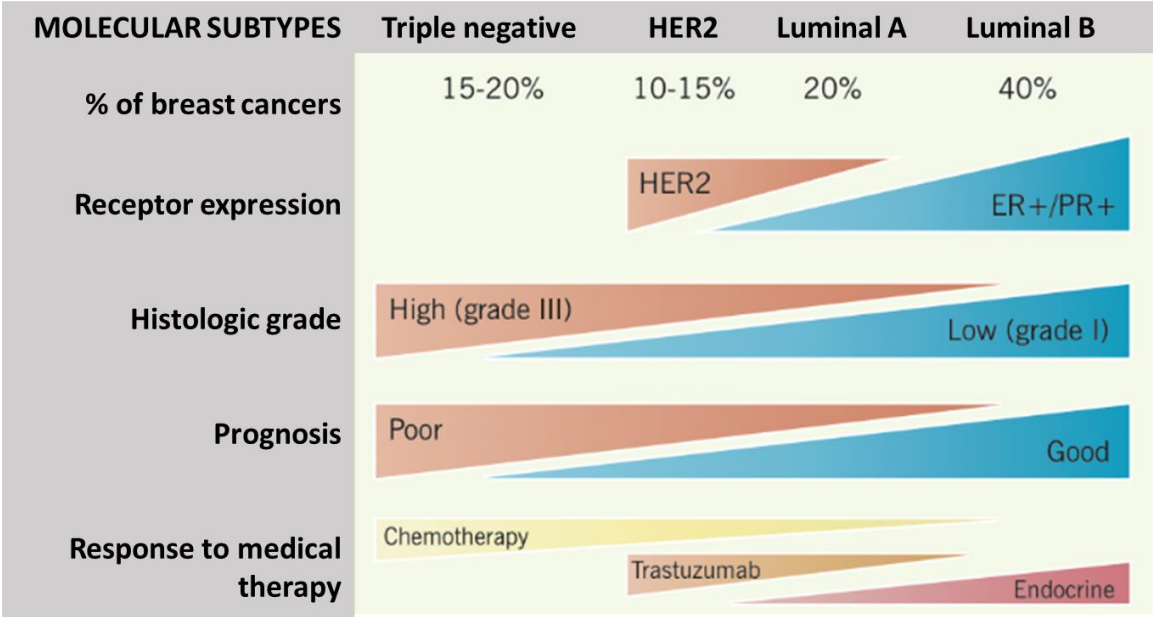


Figure 1: Breast cancer molecular subtypes presenting cancer occurrence, receptor expression, histologic grade (I-III), prognosis indicating chance of patient survival (poor- good) and therapy response indicating treatment option (chemotherapy, trastuzumab, endocrine therapy). (modified from Wong et al., 2012).

1.1.1. Triple-negative breast cancer in patients

TNBC shows high incidence in young (<40 years old) African-American or Hispanic patients. These are often diagnosed at late cancer stage with a tendency to metastasize to lungs and brain (Morris et al., 2007; Sihto et al., 2011; Trivers et al., 2009). TNBC is the most aggressive breast cancer subtype correlating with poorer prognosis and a higher level of recurrence, mitotic rate, grade and tumor size than

non-TNBC subtypes (Figures 1 and 2) (Gonçalves et al., 2018; Hirukawa et al., 2018). Additionally, it was shown that TNBC patients run a higher risk of having early metastasis after neoadjuvant chemotherapy than non-TNBC patients (Liedtke et al., 2008).

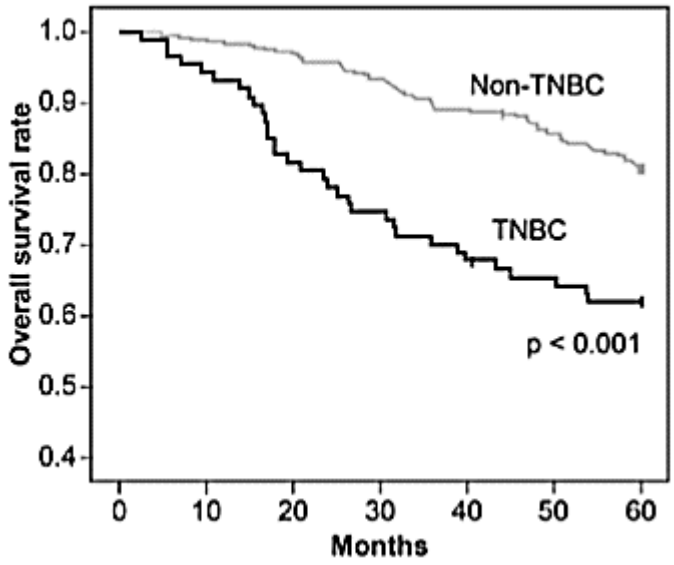


Figure 2: 5-year overall survival in TNBC and non-TNBC patients, 62.1% TNBC, 80.8% for non-TNBC cases ((P <0 .001) (Gonçalves et al., 2018).

1.1.2. Molecular characteristics of TNBC

TNBCs are defined by the lack of ER, PR and HER2, (Perou et al., 2000; Toft & Cryns, 2010). Additionally, 80% of TNBCs harbor *TP53* mutations, a percentage that is much less in other subtypes. Other features of this subtype include loss of *RB1*, *BRCA1* inactivation and high levels of *AKT3* and *MYC* (Koboldt et al., 2012). TNBC is a very heterogeneous disease and is classified into the following subgroups: basal-like 1 and 2 (BL1 and BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal-like (MSL) and luminal androgen receptor-positive (LAR) (Lehmann et al., 2011).

Figure 3 provides an overview about the most enriched pathway signature for each TNBC subtype with potential inhibitors. For instance, BL1 is associated with cell

cycle, proliferation, DNA damage response pathways. Therefore, for instance the inhibitor of Poly (ADP-ribose) polymerase (PARP), which plays a role in DNA damage response, was one of the proposed drugs for this subgroup (Robson, 2011). It is known that *BRCA1* mutation, among others, associates with cancer progression and is highly correlated with the basal-like TNBC subgroup (Haffty et al., 2019). BRCA is responsible for the repair of double-stranded DNA breaks via homologous recombination (HR)**.** *BRCA1* mutations cause DNA repair errors leading to genomic instability and carcinogenesis (Buisson et al., 2010). It was shown that *BRCA1*-deficient tumors seem to be sensitive to PARP inhibitors alone and in combination with cisplatin (Rottenberg et al., 2008). Using PARP inhibitors in *BRCA*-deficient cells leads to DNA damage and cancer cell death (Davar, 2012).

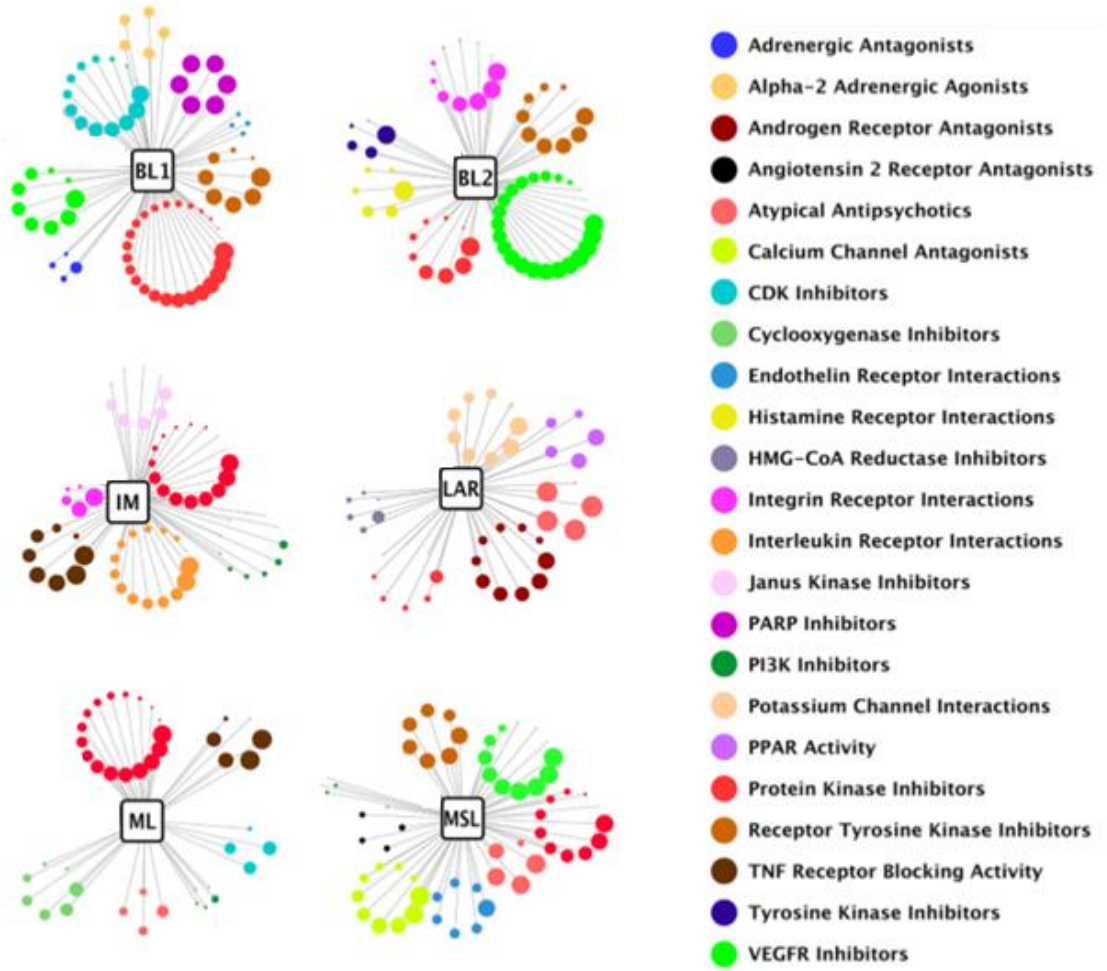


Figure 3: Drug classes in TNBC subtypes such as basal-like 1 and 2 (BL1 and BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal-like (MSL) and luminal androgen receptor (LAR). Each dot represents one drug with its size corresponding to its rank position. Drug classes were derived into FDA New Drug Application (NDA) and GenEx-TNBC drug target profiles (Wathieu et al., 2017).

BL2 is associated with glycolysis, gluconeogenesis, angiogenesis and growth factor signaling pathways like EGF, NGF, MET and Wnt/β-catenin (Shaw et al., 2010). The immunomodulatory subgroup displays a robust immune cell response through immune activation and immunological infiltration. The LAR subgroup partially shares the gene expression profile with TNBC, but also overlaps with HER-2 positive cancer (Wathieu et al., 2017). In the mesenchymal-like and mesenchymal stem-like TNBC subgroups, characteristic pathways correspond to the epithelial to mesenchymal transition (EMT), Wnt/β-catenin, TGF-β and PI3K/AKT/mTOR pathways (Massihnia et al., 2016). Taken together, TNBC subgroups are variable in gene expression signatures and respond differentially to particular chemotherapy agents dosage, combinations and treatment cycles, such that TNBC treatment remains largely unsuccessful (Oleg Gluz et al., 2008).

1.2. Chemotherapy resistance, EMT and stemness

In addition to surgery and radiation, chemotherapy is a common therapeutic option for many cancers. Many TNBC patients are better responders to chemotherapy in comparison to non-TNBC patients. It was reported that patients treated with adjuvant chemotherapy showed a 52% increase of survival than those treated with neoadjuvant chemotherapy (Echeverria et al., 2019). Consequently, apart from surgery, targeted therapy followed by chemotherapy can be a potential strategy to combat TNBC. The most common drugs used for chemotherapy treatments are intercalating agents, anthracyclines (epirubicin or doxorubicin), alkyling agents cross-linking within DNA resulting in mutations (cylophosphamide) and antimetabolites like the pyrimidine antagonist 5-fluorouracil (5-FU) or platinium-based agents (Kashiwagi et al., 2011; Sikov et al., 2015). TNBC resistance to chemotherapy is a major challenge in the clinic

and there is a need to develop targeted treatments in addition to the conventional therapies described (William, 2008).

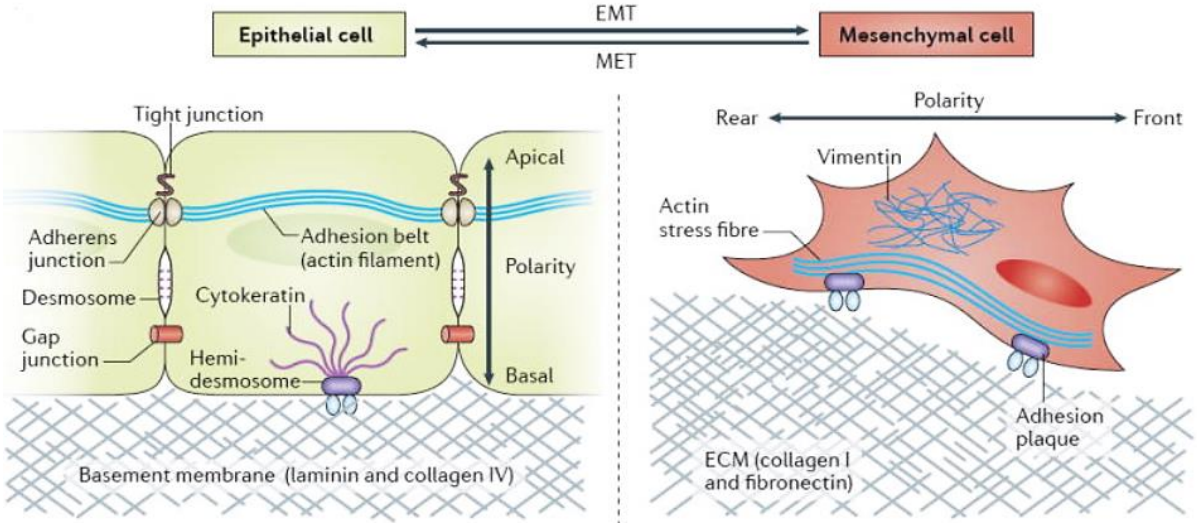
Resistance can emerge due to genetic alterations, where specific mutations can make cells insensitive to drug treatments. For example, loss of function *TP53* allows cells to evade cell death and continue proliferation (Luqmani, 2005). Environment mediated-drug resistance also plays a pivotal role during treatment, fo example, cancer stem cells (CSCs) can scavenge chemotherapy by expressing major ABC transporters (Dean, Fojo, & Bates, 2005).

Cancer stem cells are defined by three features such as their capacity to differentiate according to their hierarchical state, their self-renewal property to maintain stem cell population and their homeostasis between differentiation and self-renewal, according to environmental stimuli (Dalerba, Cho, & Clarke, 2007). The cancer stem cell model suggests that hierarchically organized cells display distinct tumorigenic and metastatic capacities generating tumor cell heterogeneity (Bonnet & Dick, 1997; Shackleton et al., 2009). CSCs display stem-like features, slow growth rate and are involved in primary or acquired chemotherapy or radiotherapy resistance (Lajtha, 1967; Takebe et al., 2015). Additionally, aldehyde dehydrogenase (ALDH) activity is reported as a cell feature strongly correlated with self-renewal capacity (Marcato et al., 2011).

Recent studies on patient-derived xenografts (PDX) with TNBC showed the survival of residual cancer cells upon doxorubicin/cyclophosphamide treatments due to activated signaling pathways protecting cancer cells (Echeverria et al., 2019; Hutchinson et al., 2018). PDX models resistant to neoadjuvant chemotherapy treatment revealed that residual cancer cells displayed unique histological and transcriptomic features in comparison to untreated tumors. Due to the reversible nature of drug-tolerance, tumors can overcome chemotherapy without clonal selection (Echeverria et al., 2019). Cancer cells are able to adapt to a changing environment and stress insults by gene expression reprogramming. One way in which this can occur is that cells undergo epithelial to mesenchymal transition (EMT). This process is reversible and mesenchymal cells have the capacity to transition back to the epithelial state (MET), reflecting the plasticity of cancer cells (Figure 4). Mesenchymal

cells with higher expression of *VIMENTIN (VIM), SNAI1, N-CADHERIN* (*N-CDH*) have weak cell-to-cell contact and a greater invasion and migration potential. In contrast, epithelial cells show prominent expression of *E-CADHERIN* (*E-CDH*) or *EPITHELIAL CELL ADHESION MOLECULE* (*EPCAM*) (Berx et al., 1995). *SNAI1*, a zing-finger protein, is a transcriptional repressor that regulates *E-CDH* in breast cancer, among others (Blanco et al., 2002; Paznekas et al., 1999). During carcinogenesis, *E-CDH* can be repressed via epigenetic modulation or a loss of function mutation (Hennig et al., 1995). EMT can be induced via HIF-1α, Wnt/β-catenin, c-MET and TGF-β (Bladt et al., 1995; Teng et al., 2014; Zhao et al., 2011). EMT was extensively described in the literature, showing that it is not sufficient for spontaneous breast cancer metastasis but is one of the mechanisms supporting cancer progression (Lou et al., 2008; Thiery, 2002). Interestingly, studies on sarcomatoid carcinoma consisting of mixed phenotypes of breast carcinoma and sarcoma, showed that two cancer cell types do not derive from stem cells of epithelial and mesenchymal origin but only from one epithelial cell. It could suggest that EMT was involved in cancer cell plasticity (Thiery, 2002). It was shown that the EMT program is associated with aggressiveness and metastasis in basal-like carcinoma (Ye et al., 2017). The EMT process can induce stem cell differentiation with reduced proliferation and the ability for therapeutic resistance. Standard chemotherapies lead to EMT activation where cancer cells can quickly adapt and overcome hostile environments promoting tumor cell survival and migration (Smith & Bhowmick, 2016).

A.



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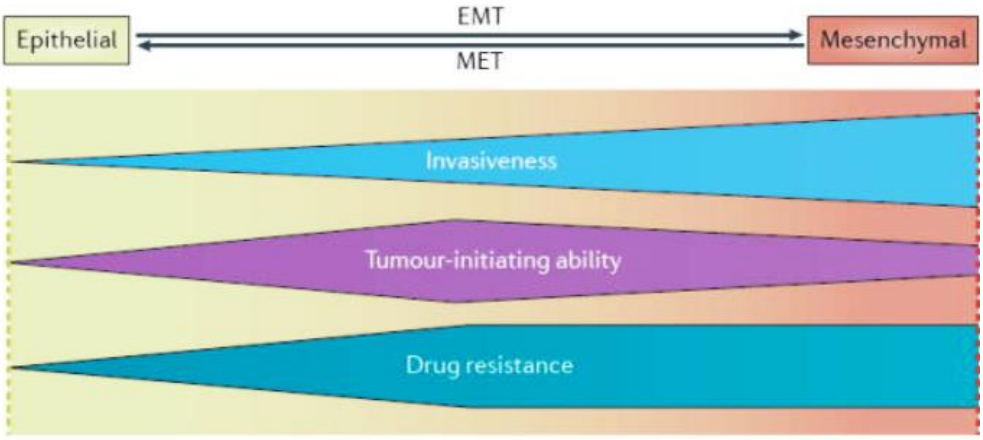

Figure 4: Epithelial to mesenchymal transition (EMT) and reversed mesenchymal to epithelial transition (MET). (A) Scheme of phenotypical and physiological changes during EMT including distinction in cell junction, polarity, cytoskeletal and protein rearrangement. (B) Invasive, tumorigenic and resistance potential within EMT/ MET process (Modified from Shibue & Weinberg, 2017).

1.2.1. The WAP-T mouse model to study resistance to chemotherapy, *in vivo* and *in vitro*.

The WAP-T mouse model was engineered to investigate mammary carcinoma cell properties, (Schulze-Garg et al., 2000). Immune-competent WAP-T mice mimic the clinical situation of basal-like TNBC subtypes in humans (Wegwitz et al., 2010). Mammary carcinogenesis in these mice is driven by the activation of a transgene, the simian virus 40 (SV40), under the control of the whey acidic protein (WAP) promoter. WAP is important for lactation in mammary epithelial cells, thus resulting in the expression of SV40 only in mammary cells (Hennighausen et al., 1990). SV40 large T antigen and small t antigen bind and suppress the tumor suppressors p53 and pRb inducing tumorigenic transformation (Kao et al., 1993).

To investigate the behavior of TNBC cells, a parental G-2 (pG-2) cell line was used. pG-2 (in the publications indicated as G-2 cells) are WAP-T mice-derived cells, displaying high heterogeneity, epithelial-mesenchymal plasticity (EMP) and stem-like features. Hence, this system is considered as a self-proliferating mammary carcinoma system (Maenz et al., 2015; Wegwitz et al., 2010). It was shown that treatment with selected cytotoxic agents like Cyclophosphamide, Doxorubicin (Adriamycin) and 5-Fluorouracil (CAF) also kills the vast majority of these cells. However, a small remaining pool of cells representing cells with a more aggressive and invasive phenotype was observed (Figure 5). This is in accordance with previous findings, where tumors formed from the transplantation of pG-2 cells into WAP-T mice, responded positively to CAF-chemotherapy. However, one round of CAF treatment was not sufficient to eliminate all cancer cells. Further analysis showed a great number of mesenchymal cells among disseminated tumor cells in comparison to untreated mice (Jannasch et al., 2015). Therefore, cancer cell plasticity represents a mechanism that can promote chemotherapy resistance. Interestingly, as described previously, cell plasticity can be mediated by reprogrammed gene transcription, which in part is regulated by epigenetic alterations. The tight regulation of epigenetic and transcriptional processes in the development of therapy resistance is essential and described in more detail in the next sections.

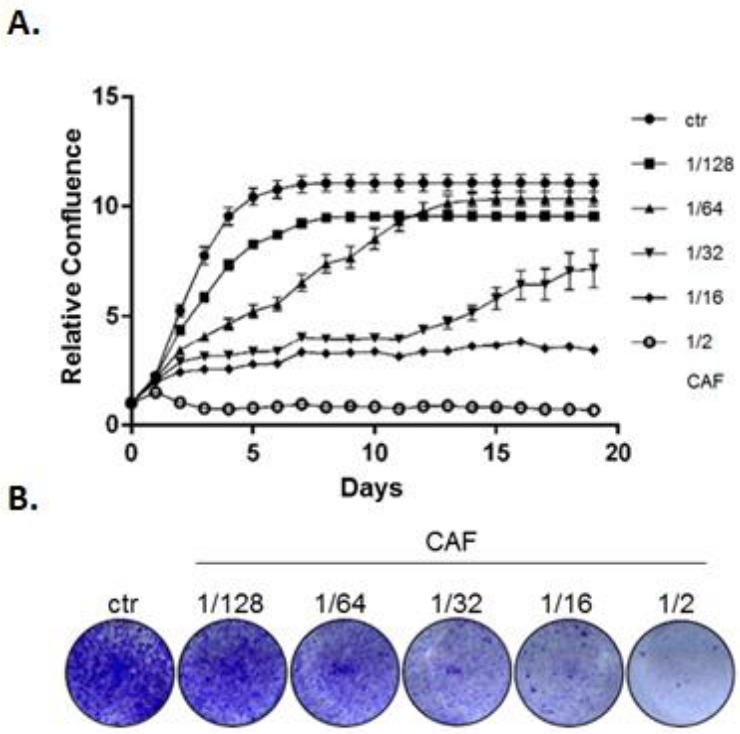

Figure 5: Optimization of CAF-chemotherapy treatment in pG-2 cells. (A) Proliferation curves of pG-2 cells treated with different chemotherapy concentrations: 1/2, 1/16, 1/32, 1/64, 1/128, where 1 represents concentrations 10 µg/ml Cyclophosphamide, 0.5 µg/ml Doxorubicin and 10 µg/ml 5-Fluorouracil (CAF). Cells confluence was measured everyday using the Celigo® cell cytometer and normalized to results at day 0. (B) Crystal violet staining of pG-2 cells at the end of the experiment followed by CAF-chemotherapy treatment (Schmidt, 2016).

1.3. Epigenetic modifications

1.3.1. Chromatin compaction

Within the nucleus of eukaryotic cells, DNA is tightly coiled around histone octamers, composed of H2A, H2B, H3 and H4, resulting in a DNA-nuclear protein complex called chromatin (Arents, 1991; Luger, 1997). Chromatin can be organized in a more open structure, called euchromatin or highly condensed one, called heterochromatin (Figure 6) (Jenuwein & Allis, 2001). Although high DNA compaction allows storing huge amount of genomic information in the nucleus, the access of factors regulating chromatin dynamics is limited due to the compaction. The structure and dynamics of chromatin is associated with gene expression regulation which determines the biological state of a given cell. Epigenetics involves mechanisms altering transcription of genes without changing the DNA sequence, regulating developmental paths initiating tissue-specific gene expression and therefore being crucial in cell fate determination (Reik, 2007; Waddington, 1957).

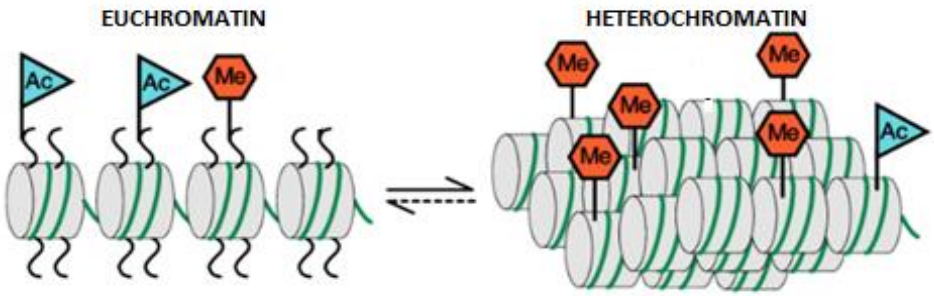

Figure 6: Model of euchromatin and heterochromatin with histone tail modifications; acetylation (Ac) and methylation (Me) (modified from Jenuwein & Allis, 2001).

1.3.2. Histone posttranslational modifications

Histone tails harbor several post-translational modifications (PTMs), such as acetylation, methylation, phosphorylation, sumoylation and ubiquitylation. The N-terminal histone tails harbor a positive charge with a tendency to link to negatively charged DNA. Adding an acetyl group (acetylation) neutralizes the charge resulting in chromatin relaxation (Figure 5) allowing the euchromatin structure to become more accessible for transcription factors inducing transcription. The opposite effect occurs

upon adding a methyl group leading to decreased recruitment of DNA binding proteins that promote transcription (Jenuwein & Allis, 2001).

Proteins known as “writers”, “erasers” and “readers” are responsible for adding, removing and recognizing particular post-translational histone modifications, respectively (Figure 7) (Audia & Campbell, 2016). Epigenetic writers can be histone acetyltransferases (HATs) or histone methyltranferases (HMTs). Protein arginine methyltransferases (PRMTs) and protein lysine methyltransferases (KMTs) have the capacity to transfer a methyl group from the cofactor S-adenosylmethionine, called SAM to the arginine or lysine residues, respectively (Simõ-Riudalbas & Esteller, 2015). Histone acetyltransferase can be reversed via histone deacetylases (HDACs). Histone methylation can be removed through the histone demethylases UTX-1 or JMJD3 (Agger et al., 2007). The bromodomain and extraterminal (BET) family including BRD2, BRD3, BRD4 and BRDT can bind to acetylated lysines on histone tails and further regulate the fate of the chromatin, playing roles in cell growth, chromatin remodeling and DNA damage (Dawson et al., 2011; Simõ-Riudalbas & Esteller, 2015). Histone PTMs can occur on the global or locus-specific level. For instance, H3K27me mark can encompass distal enhancers, proximal promoters (enrichment around the transcription start site (TSS) and gene bodies (Figure 7) (Young et al., 2011). A particular epigenetic mark occupancy can modulate transcription, via fine-tuning RNA polymerase II (RNAPII) recruitment to the promoter and/or enhancer region. In this case, H3K27me3 on enhancers and promoters limits RNAPII progression hindering transcription (Chopra et al., 2011).

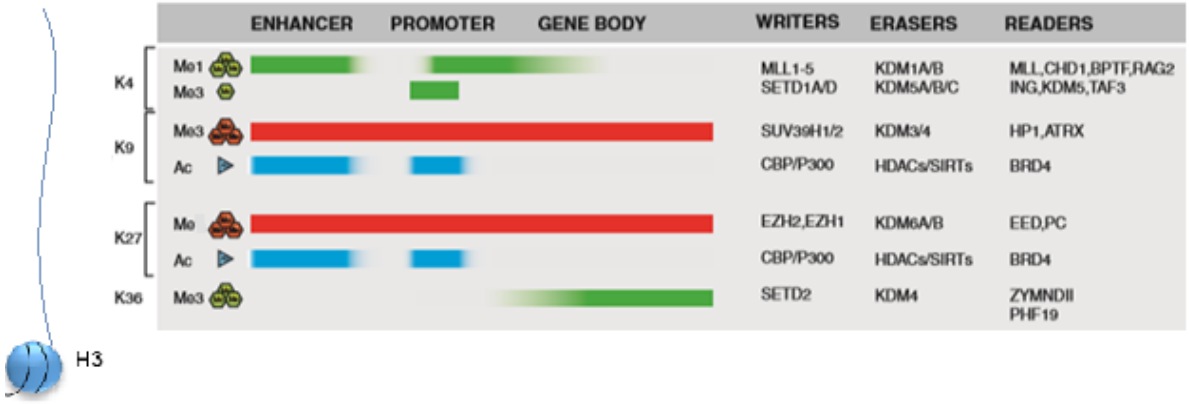

Figure 7: Histone writers, erasers, and readers. Posttranslational modifications (PTMs) on Histone 3 (H3) tail (left) along the gene loci (enhancer, promoter, gene body). Histone marks are indicated in green (methylation) and blue (acetylation) for active genes and in red shading for silent genes. Examples of writers, erasers and readers are indicated on the right side (modified from Audia & Campbell, 2016).

The most studied epigenetic marks with opposing functions are trimethylation of histone 3 at the position of lysine 27 (H3K27me3) and its acetylation (H3K27ac) (Figure 8). H3K27 methylation, in general, is responsible for maintaining the repression of specific target genes, while acetylation promotes chromatin relaxation and transcription activation. Therefore, H3K27me3 and H3K27ac are considered as a repressive and active mark, respectively. These chromatin modifications are involved in the molecular regulation of gene expression patterns and can determine cell fate (Katoh et al., 2018).

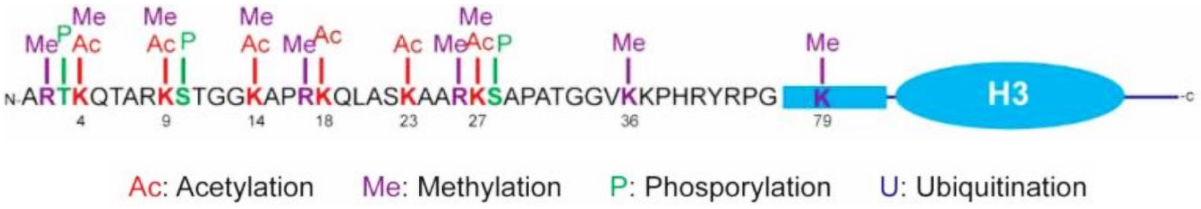

Figure 8: Chromatin modifications on histone 3 tail. Acetylation (Ac), methylation (Me), phosphorylation (P) and ubiquitination (U) occur mostly in N-terminal K and R rich tails (modified from Parsons, 2014).

1.3.1 The Polycomb Repressive Complex 2

One of the most important transcriptional modulators are polycomb group proteins (PcG), playing a role in cell proliferation and differentiation (Antonysamy et al., 2013). Two main PcG complexes have been described, Polycomb repressive complex 1 (PRC1) and 2 (PRC2). PRC2 represses genes by catalyzing the trimethylation of histone 3 at lysine 27. The Chromobox protein (CBX) subunit of PRC1 recognizes H3K27me3 and another member of the PRC1, E3 ubiquitin-protein ligase (RING1A/B), ubiquitinates histone H2A on K119 residue (H2AK119) maintaining a repressed chromatin state (H2AK119ub) (Di Croce & Helin, 2013).

PRC2 takes part in many biological processes, from differentiation to stem-cell plasticity, including repression of numerous developmental regulators in embryonic stem cells (Boyer et al., 2006; Raphaël Margueron & Reinberg, 2011). Apart from EZH1/2, PRC2 consists of Suppressor of zeste 12 protein homolog (SUZ12), Embryonic ectoderm development protein (EED) and Retinoblastoma-binding protein (RbAp46/48, also known as RBBP4/7). Enhancer of zeste 1 and 2 (EZH1/2) are protein homologs forming similar PRC2 complexes (PRC1/EZH1, and PRC2/EZH2). However, the PRC2/EZH1 complex seems to have a lower enzymatic activity and, in contrast to EZH2, loss of EZH1 does not result in a global loss of di- or trimethylation of H3K27 (H3K27me2/3). Previous studies have shown that both EZH1 and EZH2 repress transcription *in vitro* (Raphael Margueron et al., 2008). Additionally, *Ezh1* is ubiquitously expressed, whereas *Ezh2* expression is tightly associated with proliferating cells (Raphael Margueron et al., 2008). EZH2 is an essential PRC2 member, requiring other PRC2 binding partners SUZ12, RBBP7 and EED to be active (Denisenko et al., 1998; Liu et al., 2015; Pasini et al., 2004).

The canonical PRC2/EZH2 pathway leads to gene silencing that can be diverse among organisms and tissues. (Cao & Zhang, 2004; Gan et al., 2018). PRC2 proteins silence target genes upon binding to specific genomic regions called polycomb responsive elements (PREs) (Liu et al., 2015). EZH2 also methylates non-histone proteins such as STAT3, GATA4, RORα, resulting in their regulation (Figure 9) (He et al., 2012; E. Kim et al., 2013; Lee et al., 2012). Interestingly, apart from its transcriptional repressive function, in certain context, EZH2 can also display trans-activating functions positively influencing NF-κB, Wnt/ β-catenin and ERα-driven signaling, in a PRC2-indendependent manner (Jung, H. et al., 2013; K. H. Kim & Roberts, 2016). EZH2 can cooperate with the androgen receptor (AR) and the estrogen receptor (ERα), promoting prostate and breast cancer progression, respectively (Shi et al., 2007; Xu et al., 2012).

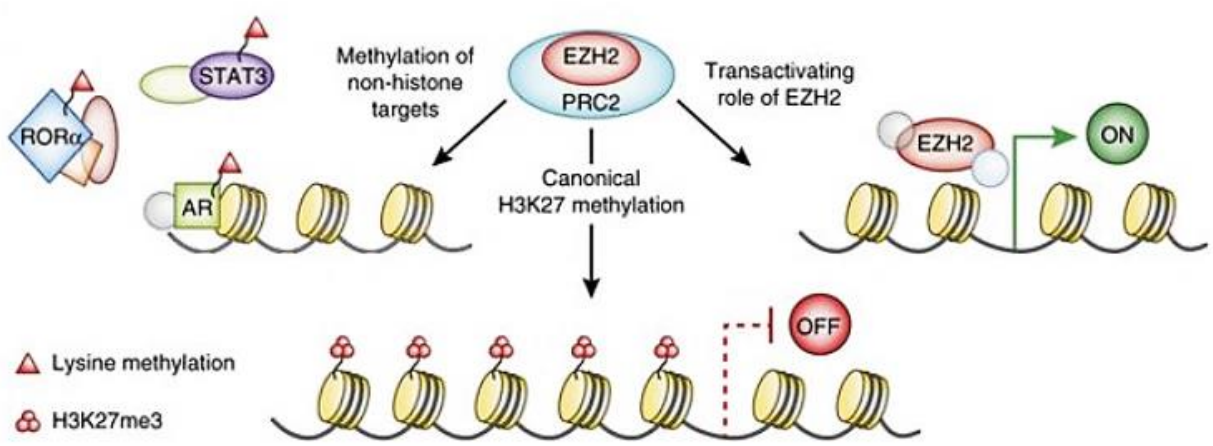

Figure 9: EZH2 transcriptional activity. PRC2/EZH2 gene silencing via H3K27me3, EZH2 methylation of non-histone proteins and PRC2-independent EZH2 transcriptional activation (modified from K. H. Kim & Roberts, 2016).

Aberrant PRC2/EZH2 activity is known to have oncogenic activity in a number of tumors (C. Chang & Hung, 2011). EZH2 is very frequently overexpressed in diverse tumors entities like pancreatic, prostate, breast and bladder cancer (Y. Chen et al., 2010; Kleer et al., 2003; Raman et al., 2005; Varambally et al., 2002). A few studies suggest a pro-tumorigenic EZH2 activity in TNBC. Chien et al. suggest that EZH2 promotes TNBC invasiveness via TIMP2/MMP-2 and -9 regulation (Chien et al., 2018) Another study on MDA-MB-231, a TNBC cell line, revealed that co-treatment with EZH2 and HDAC inhibitors leads to tumor cell death.

However, a growing number of studies reported an anti-tumorigenic function of PRC2/EZH2. In medulloblastoma, it was shown that the inactivation of EZH2 can promotes Myc-driven cancer (Vo et al., 2017). It was also reported that PRC2 loss promotes chemoresistance with reduction of apoptosis in T cell acute lymphoblastic leukemia (Ariës et al., 2018). The role of EZH2 remains elusive in distinct TNBC subgroups. EZH2 could be an oncogene or tumor suppressor in TNBC and can regulate key genes in a context-dependent manner in different cancers.

1.3.2. HDACs

H3K27 can be methylated as mentioned before but also acetylated through histone acetyltransferases (HATs) transfer an acetyl group from a donor molecule, acetyl coenzyme A (acetyl-CoA), to the ε-amino group of lysine residues of the substrate (Vogelauer et al., 2012). Histone acetylation can be reversed by histone deacetylases (HDACs). HDACs can be classified into class I (HDAC1, -2, -3, -8) class IIa (HDAC4, -5, -7, -9), class IIb (HDAC6, -10), class III (sirtuins) and class IV (HDAC11) (Gregoretti et al., 2004).

Aberrant HDAC gene expression is frequently associated with cancer development (reviewed in Y. Li & Seto, 2016). HDACs are overexpressed in various tumors including gastric, breast and prostate cancer, which have higher levels of HDAC1 and colorectal cancer, which upregulate HDAC2 and HDAC3 (Kawai et al., 2003; Mariadason, 2008; Weichert et al., 2008). HDAC inhibitors (HDACi) represent interesting molecules to reverse cancer progression, as they can change the acetylation status of histone and non-histone proteins (Figure 10). HDAC inhibition can alter gene expression inducing apoptosis through Fas, DR5, TRAIL and caspases (Rosato, 2005). These inhibitors have also been shown to impose cell cycle arrest at G0/G1 or G2/M checkpoints and to reduce angiogenesis and metastasis. HDACs are able to target cytoplasmic proteins, such as heat shock protein 90 (HSP90), DNA repair factor Ku70, α-tubulin and β-catenin (Krämer et al., 2014; Ma et al., 2009). Additionally, it was shown that inhibition of HDACs can suppress TGF-β1-induced EMT and chemotherapy resistance through SMAD4 inhibition (T. Sakamoto et al., 2016).

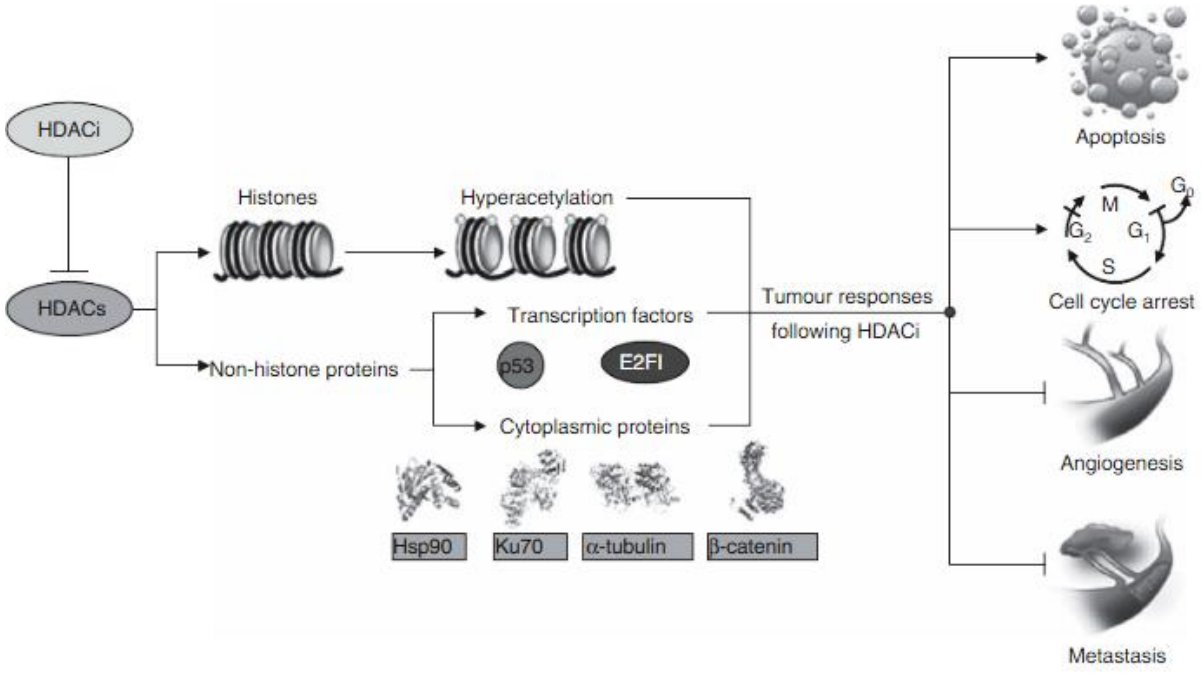

Figure 10: Cellular effects of HDAC inhibitors in cancer. Inhibition of chromatin repression via HDACi can affect apoptotic, cell cycle, angiogenic and metastatic changes in cancer cells through abrogation of acetylation/ deacetylation balance in chromatin and non-histone proteins (modified from Ma et al., 2009)

One of the most well-known and FDA approved drugs in T-cell lymphoma are Vorinostat (SAHA) and Panobinostat (LBH589) which are pan-HDAC inhibitors, targeting class I and II HDACs (Marks & Breslow, 2007; Ververis et al., 2013). In breast cancer, HDAC inhibitors can be used as monotherapy or in combination with other drugs, such as lapatinib (anti-HER2), tamoxifen (anti-ER), olaparib (PARPi) or cisplatin (Hasan et al., 2018; Min et al., 2015; Solomon et al., 2015).

Despite many promising *in vitro* and *in vivo* studies, results from preclinical trials failed when HDACi was used as a single agent (Slingerland et al., 2014). HDAC inhibitors have shown promising results in combination with other anticancer therapies. According to studies on MDA-MB-231, a TNBC cell line, SAHA treatment alone promotes EMT transition via HDAC8/FOXA1 signaling (Oehme et al., 2009). Instead, SAHA in combination with IR radiation or cisplatin can decrease tumor growth, induce apoptosis and cell cycle arrest (Chiu et al., 2013). Furthermore, the

use of a combination of DNA methyltransferases (DNMT) and HDAC inhibitors to dampen TNBC aggressiveness was proposed recently (Su et al., 2018).

1.4. NFAT family

The Nuclear factor of activated T cells (NFAT) family is composed of transcription factors, which induce gene expression during immune responses (Rao et al., 1997). The first report on NFATc2 described it as a DNA-binding protein binding to the interleukin-2 (IL-2) promoter in T cells (Shaw et al., 1988). It is known that NFATs exist also in other immunoregulatory cells such as B cells, NK cells, macrophages, etc. (Rao et al., 1997). Recently, particular research efforts have been made to unravel the function of NFATs factors in cancer (Robbs et al., 2008).

1.4.1. NFAT proteins

The NFAT family consists of five members: NFAT1 (NFATc2), NFAT2 (Nfatc1), NFAT3 (NFATc4), NFAT4 (NFATc3) and NFAT5 (Rao et al., 1997) (Table I 1). NFAT1-4 have a regulatory region called NFAT homology region (NHR) including the transactivation domain (TAD) and a calcineurin docking site (CDS). Moreover, there is a highly conserved DNA-binding domain called Rel-homology domain (RHD) and a carboxy-terminal domain (Luo et al., 1996; Müller & Rao, 2010). Within conserved serine-rich regions (SRR), there are 14 docking sites for phosphorylation of NFATs by CK1, GSK3 and DYRK (Hogan et al., 2003). The N-terminal domain contains two nuclear localization sequences (NLS1, NLS2) and one nucleus export signal (NES) controlling subcellular transport and localization (Beals et al., 1997).

1.4.2. NFAT activation

Except for NFAT5 that is activated by osmotic stress, NFAT1-4 are calcium responsive proteins. (Luo et al., 1996). NFATs become activated after cytosolic Ca^{2+} influx, which is a consequence of G protein coupled receptor signaling or ER stress (Prakriya et al., 2006). Upon physiological stimulation and decrease of Ca^{2+} levels in the ER, the plasma membrane-located CRAC channel is activated to restore calcium ions to normal levels, thus increasing cytosolic calcium levels. Cytoplasmic calcium

binds to and induces conformational changes of the protein calmodulin. The latter can then bind and activate calcineurin, a calcium-dependent phosphatase that is able to dephosphorylate NFATs, rendering them active. The phosphorylation status of NFATs determines their activity and intracellular localization where highly phosphorylated NFATs remain in the cytoplasm and calcineurin-dependent dephosphorylated NFATs are translocated to the nucleus (Hogan et al., 2003).

Table 1. NFATc1 family members and their regulation (adapted from Macian, 2005).

NFAT family member	Alternative names	Regulation	Expression in the immune system
NFAT1	NFATc2 and NFATp	Calcium–calcineurin	Yes
NFAT2	NFATc1 and NFATc	Calcium–calcineurin	Yes
NFAT3	NFATc4	Calcium–calcineurin	No
NFAT4	NFATc3 and NFATx	Calcium–calcineurin	Yes
NFAT5	TonEBP and OREBP	Osmotic stress	Yes

NFAT, nuclear factor of activated T cells; TonEBP, tonicity-responsive enhancer-binding protein.

The NFAT signaling pathway is reported to be related to cancer progression (Mancini & Toker, 2009). The calcineurin inhibitor, cyclosporine A (CsA) can reduce NFAT activity by binding to cyclophlin A and creating a complex that prevents calcineurin from interacting with calmodulin even in the presence of calcium (Flanagan et al., 1991). Studies demonstrate that cyclosporine A is well tolerated by patients in advanced solid malignancies in I/IB clinical trials. They present CsA as calcineurin inhibitor suppressing Wnt/Ca^{2+}/NFAT pathway (Krishnamurthy et al., 2018). CsA could also be combined with chemotherapy treatment of drug-resistant solid tumors (Stiff & Marrow, 1995). Another, more selective NFAT inhibitor is VIVIT and its therapeutic potential was observed in cardiovascular disorders and chronic lymphocytic leukemia resulting in the reduction of disease progression (Le Roy et al., 2012; Yu et al., 2007). The catalytic inhibition of sarcoplasmic reticulum ATPase (SERCA2) by thapsigargin makes it possible to investigate the role of NFATs *in vitro* and *in vivo* by promoting the activation of the latter (Prasad & Inesi, 2009).

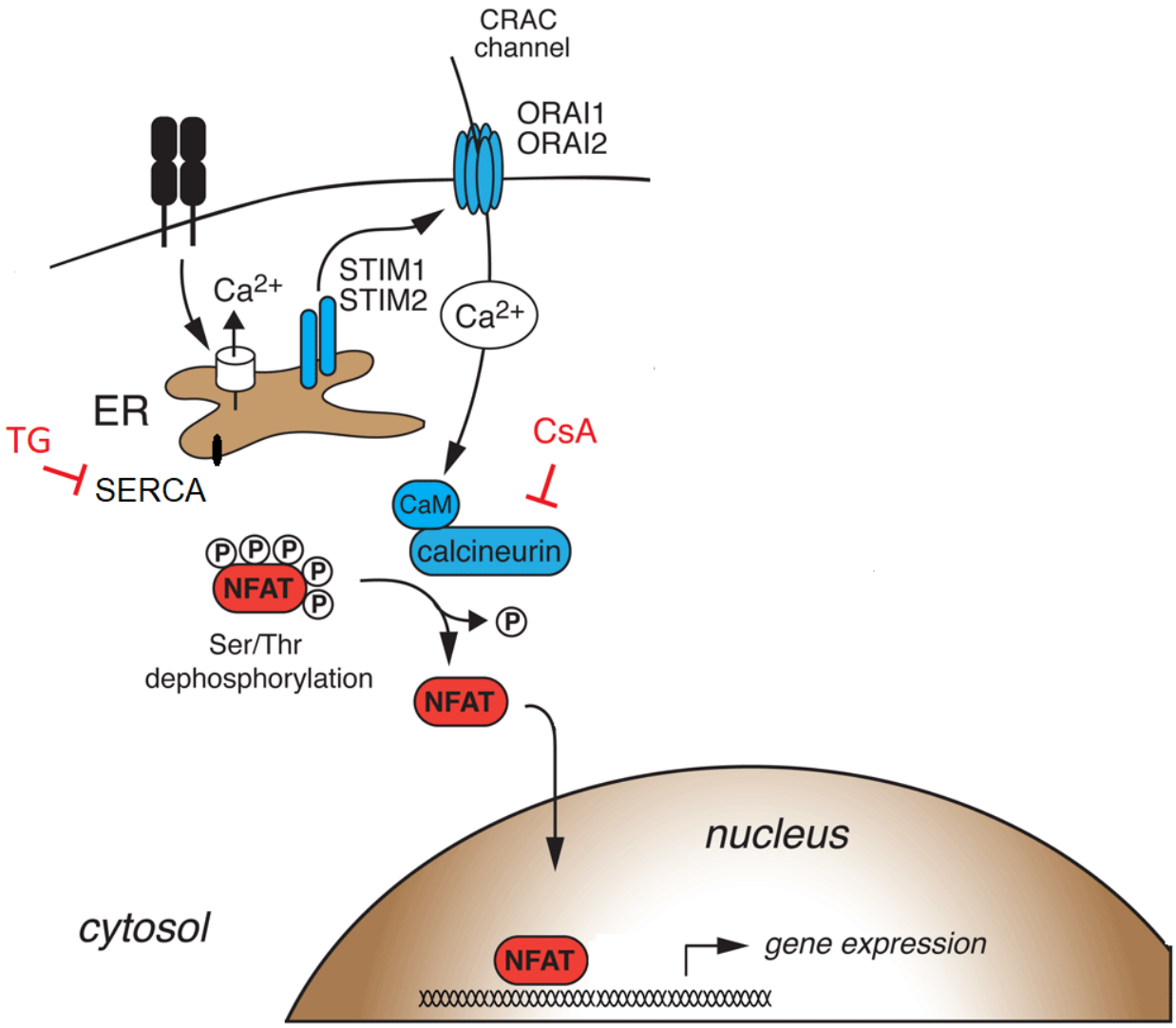

Figure 11: NFAT activation. Calcium/calcineurin, in the presence of Ca^{2+} ions, can dephosphorylate NFATs resulting in their translocation to the nucleus. CsA and Thapsigargin inhibit and activate NFATs, respectively (modified from Vaeth & Feske, 2018).

1.5. Aims of the study

Triple-negative breast cancer (TNBC) is the most challenging breast cancer subtype in the clinic routine. Due to lack of hormone therapy, conventional chemotherapy remains the mainstay in TNBC treatment. However, some cancer cells can be insusceptible to cytotoxic drugs leading to cancer recurrence. Remaining tumor cells adapt to hostile conditions by changing their gene expression and phenotype. Shedding light on the mechanisms driving chemotherapy-resistance is of utmost importance to understand TNBC progression.

Epigenetic regulatory pathways are fast and therefore likely to be implicated in overcoming chemotherapy that is to be associated with switches in gene expression pattern and acquisition of more aggressive features. The initial aim of this study was therefore to identify altered epigenetic mechanisms upon survival of tumor cells to cytotoxic drugs. Genome wide transcriptome (mRNA-seq) and histone mark occupancy (ChIP-seq) analyses were designed to identify up- or down-regulated epigenetic factors and to unravel the potential gene expression programs under control of these epigenetic processes. After subsequent validation of the findings via IHC staining on tumor material and via publically available databases mining, we aimed to assess the potential of interfering with the newly identified epigenetic mechanisms of survival to chemotherapy via *in vitro* functional assays.

Taken together, the present study ultimately aimed to provide new insights about mechanisms underlying chemotherapy resistance that could subsequently serve as a basis for the development of more efficient anti-TNBC therapeutic strategies.

2. Materials

2.1. Equipment

2100 Bioanalyzer	Agilent Technology, Santa Clara, USA
Agarose gel chamber	Harnischmacher Labortechnik, Kassel, Germany
Balance 440-35N	Kern & Sohn GmbH, Balingen, Germany
Biological Safety Cabinet “Safe 2020”	Thermo Fisher Scientific, Waltham, USA
Celigo® S Cell Imaging Cytometer	Nexcelom Bioscience LLC, Lawrence, USA
Centrifuge (Megafuge 1.OR)	Thermo Fisher Scientific, Waltham, USA
Centrifuge (5417R)	Eppendorf, Hamburg, Germany
Centrifuge (Heraeus Fresco 21)	Thermo Fisher Scientific, Waltham, USA
Counting chamber (Neubauer)	Brand GmbH & Co. KG, Wertheim, Germany
DynaMag-2	LifeTechnology, Carlsbad, USA
DynaMag-96 Side Magnet	LifeTechnology, Carlsbad, USA
Eclipse TS100	Nikon, Tokio, Japan
Electrophoresis & Electrotransfer Unit	GE Healthcare Europe GmbH, München, Germany
Freezer -150 °C (MDF-C2156VAN)	Panasonic, Kadoma, Japan
Freezer -20 °C	Liebherr GmbH, Biberach, Germany
Freezer -80 °C “Hera freeze”	Thermo Fisher Scientific, Waltham, USA
Gel iX Imager	Intas Science Imaging GmbH, Göttingen, Germany
HERAcell 150i CO2 Incubator	Thermo Fisher Scientific, Waltham, USA
Imager Western Blot	Bio-Rad Laboratories, Hercules, USA
Inverse Microscope “Axiovert 40 CFL”	Carl Zeiss MicroImaging GmbH, Göttingen, Germany
Isotemp® water bath	Thermo Fisher Scientific, Waltham, USA
Magnet stirrer “MR3001”	Heidolph GmbH & Co. KG, Schwabach, Germany
Microcentrifuge C1413-VWR230	VWR, Radnor, USA
Microscope Axio Scope.A1 with	Carl Zeiss MicroImaging GmbH, Göttingen,

an AxioCam MRc	Germany
Microscope Axiovert 100	Carl Zeiss MicroImaging GmbH, Göttingen, Germany
Microwave	Clatronic International GmbH, Kempen, Germany
Mini Trans-Blot™ Cell	Bio-Rad Laboratories, Hercules, USA
Mini-PROTEAN Tetra Cell	Bio-Rad Laboratories, Hercules, USA
Mr. Frosty® Cryo Freezing Container	Thermo Fisher Scientific, Waltham, USA
Nano Drop® ND-1000	Peqlab Biotechnology GmbH, Erlangen, Germany
Optical Reaction Module CFX96™	Bio-Rad Laboratories, Hercules, USA
pH meter inoLab®	WTW GmbH, Weilheim, Germany
Pipette Aid® portable XP	Drummond Scientific Co., Broomall, USA
Pipettes “Research” Series	Eppendorf, Hamburg, Germany
Power supply Power Pack P25T	Biometra GmbH, Göttingen, Germany
PowerPac™ Basic Power Supply	Bio-Rad Laboratories, Hercules, USA
PowerPac™ HC Power Supply	Bio-Rad Laboratories, Hercules, USA
Qubit® 2.0 Fluorometer	Invitrogen GmbH, Karlsruhe, Germany
Photo Scanner Epson V700	Seiko Epson, Suwa, Japan
Refrigerator	Liebherr GmbH, Biberach, Germany
Shaker “Rocky”	Schütt Labortechnik GmbH, Göttingen, Germany
Test Tube Rotator	Schütt Labortechnik GmbH, Göttingen, Germany
Thermal Cycler T100™	Bio-Rad Laboratories, Hercules, USA
ThermoMixer C	Eppendorf, Wessling-Berzdorf, Germany
Vortex-Genie 2T	Electro Scientific Industr. Inc., Portland, USA

2.2. Consumable materials

96-well Multiplate® PCR plate, white	Bio-Rad Laboratories, Hercules, USA
Cell scraper (16 cm, 25 cm)	Sarstedt AG & Co., Nümbrecht, Germany
Cell culture dishes (10 cm, 14.5 cm)	Greiner Bio-One GmbH, Frickenhausen, Germany
Cellstar 6-,12-well cell culture plates	Greiner Bio-One GmbH, Frickenhausen, Germany
Costar 24- well cell culture plates	Corning Incorporated, New York, USA

2.3. Chemicals and kits

2.3.1 Reagents

Acetic acid	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Agarose	GeneOn GmbH, Ludwigshafen, Germany
Albumin Fraction V (BSA)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Ammonium persulfate (APS)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Ammonium sulfate (NH4)2SO4	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Aprotinin	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Bromophenol blue	Sigma-Aldrich Co., St. Louis, USA
Calcium Chloride ($CaCl_2$)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Colorless co-precipitant	Ambion, Altham, USA
Co-precipitant Pink	Bioline, Luckenwalde, Germany
Crystal violet	Merck Millipore, Darmstadt, Germany
Cyclophosphamide	Pharmacy, University Medicine Göttingen

Cyclosporine A	Biozol, Eching, Germany
Diethylpyrocarbonate (DEPC)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Dimethyl sulfoxide (DMSO)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
di-Sodium hydrogen phosphate	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
DMEM	GlutaMax GIBCO, Invitrogen GmbH, Darmstadt, Germany
dNTPs	Jena Bioscience GmbH, Jena, Germany
Doxorubicin	Pharmacy, University Medicine Göttingen
Ethanol absolute	Merck Millipore, Darmstadt, Germany
EDTA	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
EPZ-6438 (S7128)	Selleckchem, Houston, USA
Fetal Bovine Serum (FBS)	Thermo Scientific HyClone, Logan, USA
Fluorouracil	Pharmacy, University Medicine Göttingen
Formaldehyde	Sigma-Aldrich Co., St. Louis, USA
Gene RulerTM DNA-Ladder	Fermentas GmbH, St. Leon-Rot, Germany
Glycerol	Carl Roth GmbH & Co. KG, Karlsruhe,Germany
Glycine	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Guava EasyCyte plus	Guava Technologies Inc., San Francisco, USA
HD Green® DNA stain	Intas Science Imaging GmbH, Göttingen, Germany
Hydrochloric acid (HCl)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Iodacetamide	Sigma-Aldrich Co., St. Louis, USA

Isopropanol	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Leupeptin	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Lithium chloride (LiCl), 8M	Sigma-Aldrich Co., St. Louis, USA
Magnesium chloride (MgCl2)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Methanol	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
N-ethylmaleimide (NEM)	Sigma-Aldrich Co., St. Louis, USA
Nickel chloride (NiCl2)	Sigma-Aldrich Co., St. Louis, USA
N-Lauryl sarcosine	Sigma-Aldrich Co., St. Louis, USA
Nonidet™ P40 (NP-40)	Sigma-Aldrich Co., St. Louis, USA
Opti-MEM	GIBCO Invitrogen GmbH, Darmstadt, Germany
PageRuler™ Prestained Protein Ladder	Fermentas GmbH, St. Leon-Rot , Germany
PBS tablets	GIBCO Invitrogen GmbH, Darmstadt Germany
Pefabloc SC Protease Inhibitor	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Penicillin-Streptomycin solution	Sigma-Aldrich Co., St. Louis, USA
PMSF	Calbiochem, VWR International GmbH, Darmstadt, Germany
Proteinase K	Invitrogen GmbH, Karlsruhe , Germany
Protein-A Sepharose	CL-4B GE Healthcare, Uppsala, Sweden
Reverse Transcriptase (M-MuLV)	New England Biolabs, Frankfurt am Main, Germany
Roti Phenol/Chloroform/ Isoamyl	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
RNase A	Macherey-Nagel GmbH & Co. KG, Düren, Germany

Rotiphorese Gel 30	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Rotipuran Chloroform	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
RPMI 1640	Life Technologies, Carlsbad, USA
Sepharose^{TM} CL-4B	GE Healthcare, Uppsala, Sweden
Skim milk powder	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium acetate	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium azide	AppliChem GmbH, Darmstadt, Germany
Sodium chloride (NaCl)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium deoxycholate	AppliChem GmbH, Darmstadt, Germany
Sodium dodecylsulfate (SDS)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium fluoride (NaF)	AppliChem GmbH, Darmstadt, Germany
Sodium hydroxide (NaOH)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
β-Glycerolphosphate (BGP)	Sigma-Aldrich Co., St. Louis, USA
SYBR Green	Roche Diagnostics GmbH, Mannheim, Germany
Taq DNA Polymerase	Prime Tech, Minsk, Belarus
TEMED	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
TMP195	Biomol, Hamburg, Germany
TMP269	Biomol, Hamburg, Germany
Tris	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Triton X-100	AppliChem GmbH, Darmstadt, Germany
TRIzol Reagent	Invitrogen GmbH, Karlsruhe, Germany

Trypsin-EDTA (0.05%)	GIBCO, Invitrogen GmbH, Darmstadt, Germany
Tween-20	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
α,α-Trehalose Dihydrate	AppliChem GmbH, Darmstadt, Germany

2.3.2 Kits

Bioanalyzer DNA High sensitivity kit	Agilent Technologies, Santa Clara, USA
Immobilon Western HRP Substrate	Millipore, Billerica, USA
innuPREP RNA Mini Kit 2.0	Analytik Jena AG, Jena, Germany
KAPA Hyper Prep kit	Roche, Pleasanton, USA
NEXTflex™ Rapid Illumina Directional RNA-Seq Library Prep Kit	Bio Scientific Corporation, Austin, USA
Qubit dsDNA HS assay	Invitrogen GmbH, Karlsruhe, Germany
Bioanalyzer DNA High sensitivity kit	Agilent Technologies, Santa Clara, USA
SuperSignal™ West Femto Maximum Sensitivity Substrate	Thermo Fisher Scientific, Waltham, USA

2.4. Nucleic acids

2.4.1. RT-PCR primers

Gene	Primer sequence (5'-3')	Reference
Actin_ChIP	GAGTCTACACGCTAGGCGTAA	F. Wegwitz
	CTCGTGGCTAGTACCTCACTG	F. Wegwitz
E-cadherin	CCTGGCACTGGTATCTCTTCA	E. Lenfert
	AGCCATTGCCAAGTACATCCTC	E. Lenfert
Epcam	GAGTCCGAAGAACCGACAAGG	G.Tolstonog
	CTGATGGTCGTAGGGGCTTTC	G.Tolstonog
Evx1_ChIP	TGGCAGCAGCCTTAAACCTT	This study

	AGCTGCAGTAGACCGTTGAC	This study
Ezh2	TCCATGCAACACCCAACACA	W. Xie
	AACTCCTTAGCTCCCTCCAGAT	W. Xie
Hdac4	CTGTGGAGCTGCTGAATCCT	This study
	TTCCAAGGGCAGTGAGAACT	This study
Hdac7	TCTCTTCCTGGCAGGCTTAC	This study
	AGTTGCCGAAGTTCTTGCTC	This study
Hdac8	ATGACTGTGTCCCTGCACAA	This study
	CTGAATGGGCACATTGACAC	This study
N-cadherin	GCGCAGTCTTACCGAAGGATG	This study
	ATACACCGTGCCGTCCTCGT	This study
Nfatc1	GCCTTTTGCGAGCAGTATCT	E. Hessmann
	GCTGCCTTCCGTCTCATAGT	E. Hessmann
Nfatc2	GAACAACATGAGAGCCACCA	This study
	GTGTTCTTCCTGCCGATGTC	This study
Rplp0	GATTCGGGATATGCTGTTGG	This study
	GCCTGGAAGAAGGAGGTCTT	This study
Snai1	CTGGTGAGAAGCCATTCTCCT	E. Lenfert
	CCTGGCACTGGTATCTCTTCA	E. Lenfert
Suz12	AGCATCAAAAGCTTGTCTGCAC	W. Xie
	ACTTTCACAAGCAGGACTTCCA	W. Xie
Twist2	GGCCGCCAGGTACATAGAC	G.Tolstonog
	GTAGCTGAGACGCTCGTGA	G.Tolstonog
Vimentin	CGGCTGCGAGAGAAATTGC	G.Tolstonog
	CCACTTTCCGTTCAAGGTCAAG	G.Tolstonog
Zeb1	CACCAGAAGCCAGCAGTCAT	This study
	CGTTCTTCTCATGGCGGTACT	This study

2.4.2 siRNAs for transient gene silencing

Gene	Sequence (5'-3')	Cat. no.	Supplier
Ezh2	GGAAAGAACUGAAACCUUA CAGAAGAGCUGAUGAAGUA AGAAAGAUCUAGAGGAUAA GGAGGGAGCUAAGGAGUUU	M-040882-00	Dharmacon
EZH2	GAGGACGGCUUCCCAAUAA GCUGAAGCCUCAAUGUUUA UAACGGUGAUCACAGGAUA GCAAAUUCUCGGUGUCAAA	L-00421800	Dharmacon
Hdac4	GCUCAAGGCUUAAGCAGAA CCAAGAAACUUACCCGUAU GCAGAGGAUCCACCAGUUA GUGGAUAGCGACACCAUAU	M-043626-01	Dharmacon
Hdac7	CCGAAAGGCUUCCCUAGAG	M-040703-01	Dharmacon

	UGACGCAGCAGUUGAUGAA GCUACAGCAACACGGCAAA GAGUGGGACCUAUGGCGAA		
Hdac8	CAUCGAAGGUUAUGACUGU GACGGGAAGUGUAAAGUAG CUACGUGGAUUUGGAUCUA CUGAUUAUGUGCUGGAAAU	M-058613-01	Dharmacon
Nfatc1	GCCAUAACUUUCUGCAAGA GGGCAAGCAUCACGGAGGA CCAACUACUCCUACCCAUA ACGGUUACUUGGAGAAUGA	M-054700-01	Dharmacon
NFATc1		40657	Thermo Fisher
NT5		D-001206-13	Dharmacon
Suz12	GGACCUACAUUACAAUUUA GAUGUAAGUUGUCCAAUAA GCAGGUUCAUCUUCAAUUA GCACAGAACUCUUACUUAC	M-040180-00	Dharmacon

2.5. Proteins

2.5.1. Enzymes

Proteinase-K	Life Technology, Carlsbad, USA
Reverse Transcriptase (M-MuLV)	New England Biolabs, FFM
RNase A	Qiagen GmbH, Hilden
RNase Inhibitor	New England Biolabs, FFM
Taq DNA Polymerase	Prime Tech, Minsk, Belarus

2.5.2. Antibodies

Antibodies were diluted as mentioned below in 5% milk or BSA. Primary antibodies for Western Blot contained 0.01% sodium azide.

Primary antibodies

Antibody	**Cat. no./Clone. no.**	**Source**	**Western blot**	**IHC**	**IF**	**ChIP**	**FACS**

Actin	8224	Abcam	1:2000				
E-cadherin	24E10	Cell Signaling	1:1000				
Ezh2	5246/ D2C9	Cell Signalling	1:1000	1:150	1:100		
FITC-α-EpCAM	118207	Biolegend					1:400
H3	601902	Biolegend	1:500				
H3K27ac	C15410196	Diagenode				2 µg	2 µg
H3K27me3	C15410195	Diagenode				2 µg	2 µg
N-cadherin	D4R1H		1:1000				
Nfatc1	649601	Biolegend	1:500	1:25			
Suz12	D39F6/ 3737	Cell Signalling	1:1000		1:100		
Vimentin	18814	Cell Signaling	1:1000				

Secondary antibodies

Antibody	**Cat. no.**	**Source**	**Western blot**	**IF**
goat anti-mouse (IgG)HRP	Sc-2004	Santa Cruz	1:10000	
goat anti-rabbit (IgG)HRP	Sc-2005	Santa Cruz	1:10000	
donkey anti-goat (IgG)HRP	Sc-2020	Santa Cruz	1:10000	
Alexa Fluor® 488 Goat Anti-Rabbit IgG	A11008	Life Technologies		1:10000
Alexa Fluor® 555 Donkey Anti-Mouse IgG	A31570	Life Technologies		1:10000

2.6. Cell culture

2.6.1. Cell lines

Cell line	**Medium**	**Source**
pG-2	DMEM, GlutaMAX	F. Wegwitz (AG Wegwitz), Göttingen
rG-2	DMEM, GlutaMAX	G. Schmidt (AG Wegwitz), Göttingen
MDA-MB-468	RPMI-1640	ATCC® HTB-132

MDA-MB-231	RPMI-1640	ATCC® HTB-26
HCC1806	RPMI-1640	ATCC® CRL-2335
HCC70	RPMI-1640	ATCC® CRL-2315
HCC1937	RPMI-1640	ATCC® CRL-2336

2.6.2. Media and reagents

Each medium for cell culture contained 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin.

Medium	Supplier
Dulbecco's Modified Eagle Medium (DMEM), high glucose, GutaMAX™	Gibco®, Invitrogen GmbH, Karlsruhe
RPMI-1640	Gibco®, Invitrogen GmbH, Karlsruhe
Fetal Bovine Serum (FBS)	Thermo Scientific HyClone, Logan, USA
Penicillin/streptomycin (P/S)	Sigma-Aldrich Co., St. Louis, USA
0.05% Trypsin-EDTA	Gibco®, Invitrogen GmbH, Karlsruhe
Opti-MEM	Life Technology, Carlsbad, USA

2.7. Buffers

Buffer	Ingredient	
qPCR buffer	Tris/HCl, pH 8.8	75 mM
	$(NH_4)_2SO_4$	20 mM
	Tween 20	0.01%
	$MgCl_2$	3 mM
	dNTPs	0.2 mM
	Triton X-100	0.25%
	Taq polymerase	20 U/ml
	SYBR Green I	1:80 000
	Trehalose	300 mM

Blocking solution	Milk powder	5% (w/v)
	in TBS-T 1x	
CAF stock solution (1)	5-Fluorouracil	77 µM
	Doxorubicin	0.92 µM
	Cyclophosphamide	38 µM
	in cell culture medium	
ChIP wash buffer	SDS	0.01% (w/v)
	Triton X-100	1.1% (v/v)
	EDTA	1.2 mM
	Tris-HCl, pH 8.1	16.7 mM
	NaCl	167 mM
Citric acid buffer, pH 6.0	Citric acid	12 mM
	Tri-sodium citrate	100 mM
Cross-linking buffer	Formaldehyde in PBS	37%
Crystal violet solution	Crystal violet	0.1% (w/v)
	EtOH	20%
DNA loading dye (6x)	Sucrose	40% (w/v)
	Glycerol	10% (v/v)
	Bromophenol blue	0.25% (w/v)
FACS resuspension buffer	FBS	2% (v/v)
	EDTA in PBS	1 mM
IP buffer	NaCl	5 M
	EDTA, pH 8.0	0.5 M
	Tris-HCl, pH 8.0	1 M
	NP-40	10% (v/v)
	Sodium deoxycholate	10% (w/v)
	NaF	0.5 M
	SDS	10 % (w/v)
Lämmli buffer (6x)	DTT	9.3% (w/v)
	Tris, pH 6.8	0.35 M
	Glycerol	30% (v/v)

	SDS	10% (w/v)
	Bromophenol blue	0.02% (w/v)
Nuclear preparation buffer	NaCl	5 M
	EDTA (pH 8.0)	0.5 M
	Tris-HCl (pH 7.5)	1M
	NP-40	10% (v/v)
	Triton-X-100 (v/v)	10% (v/v)
	NaF	0.5 M
PBS for cell culture	PBS tablet	1x
	ddH_2O	500 ml
PBS, pH 7.4	NaCl	137 mM
	$Na_2HPO_4.2H_2O$	4.29 mM
	KCl	2.68 mM
	KH_2PO_4	1.47 mM
PBS-T	Tween-20 in PBS	0.1% (w/v)
RIPA buffer	NP-40	1%
	SDS	0.1%
	Sodium deoxycholate in PBS	0.5%
RNA loading dye	Bromophenol blue	0.1% (w/v)
	DEPC water	49.9%
	Glycerol	50% (w/v)
Running buffer	Glycine	200 mM
	Tris	25 mM
	SDS (w/v)	0.1 %
SDS-PAGE running buffer	Tris	25 mM
	Glycerine	86 mM
	SDS	3.5 mM
SDS-PAGE separating gel	Acrylamide	33% (v/v)
	APS	0.1% (w/v)
	SDS	0.1% (w/v)
	Tris/HCl, pH 8.8	375 mM

	TEMED	0.04% (v/v)
SDS stacking gel	Acrylamide	33% (v/v)
	APS	0.1% (w/v)
	SDS	0.1% (w/v)
	Tris/HCl, ph 6.8	125 mM
	TEMED	0.01% (v/v)
TAE (50x) buffer	Tris	2 M
	Acetic acid	1 M
	EDTA	0.1 M
TBE buffer	Tris	45 mM
	Na_2EDTA	1 mM
	Boric acid	45 mM
TBS, pH 7.4	NaCl	150 mM
	KCl	2.68 mM
	$Na_2HPO_4 \times 2H_2O$	4.29 mM
	KH_2PO_4	1.47 mM
TBS-T	Tween-20 in TBS	0.1% (w/v
TE buffer	EDTA, pH 8.0	0.5 M
	Tris-HCl, pH 8.0	1 M
Transfer buffer	10x western salts	10% (v/v)
	Methanol	20% (v/v)
Western salts (10x), pH 8.3	Glycine	1.92 M
	SDS	0.02% (w/v)
	Tris/HCl	250 mM

2.8 Softwares and online tools

Bio-Rad CFX Manager	Bio-Rad Laboratories, Hercules, USA
DESeq2 package	https://bioconductor.org/packages/release/bioc/html/DESeq2.html

DiffBind package	http://bioconductor.org/packages/release/bioc/html/DiffBind.html
Enrichr	https://amp.pharm.mssm.edu/Enrichr/
Galaxy Deeptools	http://deeptools.ie-freiburg.mpg.de/
Gene Set Enrichment Analysis	http://software.broadinstitute.org/gsea/index.jsp
GIMP 2.10.6	https://www.gimp.org/
GraphPad Prism	https://www.graphpad.com/scientific-software/prism/
GREAT analysis software	http://bejerano.stanford.edu/great/public/html/
Image Lab Version 5.2	Bio-Rad Laboratories, Hercules, USA
Kaplan-Meier plotter	http://kmplot.com/analysis/index.php?p=background
MERAV	http://merav.wi.mit.edu/
NCBI primer-BLAST	www.ncbi.nlm.nih.gov/tools/primer-blast/Primer/designing tool/
R Studio 3.5	https://www.rstudio.com/products/rstudio/
useGalaxy	https://usegalaxy.org/
Zeiss ZEN lite software	http://www.zeiss.com/microscopy/en_de/products/microscope- software/zen-lite.html

3. Methods

3.1. Cell culture

3.1.1. Cell maintenance

pG-2 and rG-2 cells were cultured in DMEM GlutaMAX, whereas MDA-MB-468, MDA-MB-231, HCC1937, HCC1806, HCC70 were maintained in RPMI-1640 at 37°C and 5%

CO_2. All media were supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin. For cell passaging 1x PBS and 0.05% Trypsin-EDTA were used.

3.1.2. Reverse transfection

Cells were reverse transfected with siRNA in 6-well plates using Lipofectamine® RNAiMAX, according to the manufacturer's instructions. 1.5 µl of 20 µM siRNA was mixed gently with 5 µl Lipofectamine® RNAiMAX and 500 µl of Opti-MEM and incubated for 20 minutes at room temperature. After transferring 500 µl of the prepared transfection mixture to the well, 1.5 ml of Opti-MEM with 350,000 cells were added and the plate was kept in the incubator in normal cell culture conditions. After 24 hours, the cells were used for other assays.

3.1.3. Cell proliferation assay

24-well plates with seeded 10.000 cells per well were used to assess cell proliferation. If the cells were already transfected with siRNA, after 48 hours cells were treated with chemotherapy. In the case of inhibition, cells were treated with inhibitor the day after seeding and incubated for 48 hours, for the next 2 days inhibition and chemotherapy treatment was performed or inhibition alone. For the next 48 hours, cells were treated again with inhibitor and afterwards with fresh medium only. Every 2 days cells proliferation was measured using the Celigo® Adherent Cell Cytometer for a total of 1-2 weeks, depending on the treatment and the cell line. On the last day of the experiment, the cells were fixed with 100% methanol and stained using 0.1% crystal violet in EtOH.

3.1.4. Migration assay

Scratch assay. 400.000 cells, previously transfected with siRNA or treated with inhibitor, were seeded on each well. The next day, if the cells were 95% confluent, scratches on monolayer of cells were performed using pipette tips. Immediately, medium was changed for fresh medium with no FBS. Photos were taken after 0 and 12 hours and analyzed via ImageJ.

Boyden chamber assay. Boyden chamber inserts (8.0-µl track-etched membrane cell culture inserts) were equilibrated with serum free medium supplemented with penicillin and streptomycin. Inserts were transferred into 24-well plates containing 500 µl a complete cell culture medium. 50.000 cells in 300 µl serum free medium were seeded into the inserts. After 48 hours, the inserts were washed with PBS and cells on the upper site of the insert were carefully removed. Cells on the lower side of the insert were fixed using 4% PFA for 10 min and stained with 1% crystal violet in 20% EtOH for 20 min. After drying, photos were taken and analyzed using ImageJ.

3.1.5 Colony formation assay

2.000 cells after siRNA transfection or subsequent treatments were seeded per well in a 6-well plate. The colonies were stained with crystal violet and counted manually at the end of the experiment.

3.2. Molecular biology

3.2.1. RNA isolation

From a 6-well plate, the cells were washed with PBS and resuspended in 500 µl of TRIzol®. According to manufacturer's manual, 200 µl chloroform was added and vortexed for 15 seconds. After centrifuging at 4°C, 12.000 g for 15 min, the aqueous phase was taken and vigorously mixed with an equal volume of isopropanol. After minimum 1 hour of precipitation at -80°C, the samples were centrifuged at 4°C, 12,000 g for 15 min. The RNA pellets were washed 2x with cold 70% ethanol in DEPC water. Finally, the supernatant was discarded completely and the RNA pellets were air dried for around 10 minutes and diluted in 40 µl DEPC water. The RNA quantification was performed using the Spectrophotometer, Denovox. Alternatively, the innuPREP RNA Mini Kit 2.0 from Analytik Jena AG was used for RNA isolation and the manufacturer's procedure was followed.

3.2.2. cDNA synthesis

For the cDNA synthesis, a mixture of 0.5-1 µg of RNA, 2 µl 60 µM random primers, 1 µM dNTPs and DEPC water in a total volume of 10 µl was prepared and incubated at 65°C for 5 min and cooled down on ice. 2 µl 10 x M-MuLV buffer, 0.25 µl [10 U] RNase inhibitor, 1 µl M-MuLV reverse transcriptase and DEPC in a volume of 10 µl were added to each reaction mix. The samples were incubated at 25°C for 5 min, 42°C for 1 h and 95°C for 5 min. The cDNA samples were diluted to 5ng/µl and stored at -20°C or -80°C.

3.2.3. Quantitative real-time PCR

To quantify relative gene expression, 1 µl of cDNA was used in master mix containing 14 µl 2x qPCR mix, 9 µl ddH2O and 1 µl primers in one reaction. Firstly, the cDNA was denatured at 95°C for 2 min, then 40 cycles of 95°C for 15 s and 60°C for 30 sec were run. Finally, the melting curve analysis was generated with SYBR green by heating from 60°C to 95°C with one read every 0.5°C. Based on the standard curve, the results were quantified.

3.2.4.1. Crosslinking

For ChIP, pG2 cells cultured with or without chemotherapy treatment for 48 hours on 15 cm plates were used. The cells were crosslinked with 1% formaldehyde in PBS for 20 min, followed by quenching with 125 mM glycine for 5 min. After washing the cells 2x with cold PBS, 1 ml of nuclear preparation buffer containing a proteinase inhibitor cocktail was added. Scraped cells were centrifuged at 12.000 g for 2 min at 4 °C and the nuclear pellet was washed with nuclear preparation buffer.

3.2.4.2. DNA content determination

50µl of the supernatant was transferred from the crosslinking step prior to the last centrifugation. After adding 250µl of sonication buffer-1 and 1µl of Proteinase K (20 mg/ml), the samples were incubated at 65°C overnight, 800 rpm. 250µl of distilled water, 25µl of 8M LiCl and 2µl of colorless co-precipitant were added to each tube.

Upon phenol/chloroform/isoamylic alcohol extraction, samples were vortexed and centrifuged 12,000 g, 2 min, 4°C and the aqueous phase was transferred to a fresh tube. To precipitate, 1 ml of isopropanol was added to each sample, which was then incubated for 1h at -80°C. After 30min of centrifugation at 15.000 g, 4°C, the pellet was washed 2x with 70% EtOH. Afterwards, the final pellets were resuspended in 50 µl Tris 10mM pH 8 with RNase A 100 µg/ml and quantified with the spectrophotometer.

3.2.4.3. Sonication

Based on the DNA concentration, the samples were diluted to 500 µg/ml and incubated at 4°C for 15 min on the wheel. The samples were sonicated using the Bioruptor Pico for 5, 10, 15, 20, 25 and 30 cycles with 30 sec on/off duty time. After centrifuging at 10.000 g, 10 min, 4°C, a fraction of the supernatants was taken for a shearing check.

3.2.4.4. Shearing check

100 µl of sonication buffer-1 and 1 µl of proteinase K were added to each sample, which were then incubated at 65°C for 4h, 800rpm. Next, 100 µl water, 10 µl 8M LiCl and 2 µl PINK precipitant were added. Phenol/chloroform/isoamylic alcohol was added to each sample, followed by 30s vortexing and centrifuging at 2 min, 15 000 g. To the aqueous phase, 1 ml EtOH was added and after 1h of incubation at -80°C, the samples were centrifuged for 30 min, 15 000 g, 4°C. The final pellet was resuspended in 15 µl Tris 10 mM pH 8.0 with RNase A 100 µg/ml (1h, 37°C, 700 g). The samples were mixed with 3 µl of loading dye and run on a 1.5% agarose gel using 1x TAE buffer.

3.2.4.5. Pre-clearing and chromatin immunoprecipitation

For pre-clearing, 100 µl of a 50% sepharose slurry were added and the samples were incubated for 1h, 4°C. After centrifuging at 12 000 g, 4°C, the supernatant was collected and immunoprecipitation and input samples were prepared. Aliquots of chromatin were filled to 500 µl with IP buffer with protease inhibitors and appropriate antibodies (see: Materials) with overnight incubation. On the following day, 30 µl of protein A- sepharose was added and the samples were incubated for 2h at 4°C with rotation. The ChIP complexes washed with IP buffer, wash buffer and TE buffer.

3.2.4.6. DNA isolation

Immunoprecipitated chromatin was treated with 50 µl of 10 µg RNase A diluted in Tris 10 mM pH 8 for 30 min, at 37°C. 50 µl of sonication buffer was added on the beads with 1 µl proteinase K with overnight incubation at 65°C with shaking. After centrifuging (2.000 g, 2 min, at room temperature), the supernatant was taken and 10 µl Tris 10 mM pH 8 was added, mixed and centrifuged, 2 min 15.000 g. The aqueous phase was taken and to precipitate, 1 ml EtOH was used for 2h at -80°C. Next the samples were centrifuged for 30 min, 15.000 g, 4°C and washed with 1 ml 70% EtOH. The DNA was resuspended in 40 µl H_20. 5 µl of each sample was used for ChIP-qPCR.

3.2.4.7. ChIP-seq library preparations

ChIP-seq libraries were prepared using the KAPA Hyper Prep kit according to manufacturer's protocol. The libraries were purified using 1X AMPure® XP beads on a magnetic stand. The quality of the samples was determined using Bioanalyzer with assessment of the fragment length (around 300 bp). A 2 nM pool of ChIP DNA libraries were sequenced by the Transcriptome and Genome Analysis Laboratory in Göttingen (HiSeq 4000).

3.2.4.8. ChIP-seq analysis

For ChIP-seq analysis, the Galaxy server was used. ChIP-seq reads of two biological replicates for each condition in the experiment were used. FASTQ quality check (FastQC) of raw data was followed by Mapping, BamCoverage and Peak Calling. The sequenced reads were aligned to the mouse reference genome (mm9) using Bowtie2 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml). Sequence Alignment Map (SAM) files were converted to Binary Alignment Map (BAM) files using SAMtools (H. Li et al., 2009). After merging replicates (BAM files), Model-based Analysis of ChIP-seq 2 (MACS2) was used for peak calling to identify enriched regions (Zhang et al., 2008). The next step was to generate a coverage file of the reads using bamCoverage with 500bp bin size and reads per kilobase per million (RPKM) normalization. Through

conversion of BAM files to bigwig files, we could visualize ChIP-seq data. Further analysis was performed using computeMatrix, plotHeatmap and plotProfile. Additionally, differential binding analysis was performed for H3K27ac and H3K27me3 binding sites using the R package DiffBind (Stark & Brown, 2011). Enrichr was performed based on significantly enriched genomic regions.

3.3. Protein analysis

3.3.1. Protein harvesting

Cells were washed with PBS and scraped in ice-cold RIPA buffer with a protease inhibitors cocktail: 1 mM Pefabloc, 1 ng/µl Aprotinin/Leupeptin, 10 mM BGP, 1 mM NEM and 8M urea (1/3 of the final volume). The samples were sonicated for 10 cycles at 30 sec on/off duty time using Biorupter Pico sonicator. After centrifugation for 10 min, 12,000 g, 4°C, the protein samples were mixed with Lämmli buffer (Laemmli, 1970) and cooked for 5 min at 95°C. Protein samples with the same concentration, were separated using polyacrylamide gel during SDS-PAGE electrophoresis. Gels were run using running buffer at range of 70-120V.

3.3.2. Western blot

For an electrophoretic protein transfer (Towbin, 1979), nitrocellulose membranes were used. After the transfer, the membranes were blocked in 5% milk in TBS-T for 1h and incubated overnight in primary antibody at 4°C. Following, the membranes were washed with TBS-T and incubated for 1h in secondary antibody diluted. After washing the membranes 3 times washing in TBS-T for 5 minutes each, they were developed using HRP signal and the western blot imager Biorad.

3.4. Stainings

3.4.1. Crystal violet staining

To analyze cell proliferation and colony formation, the cells were stained with 0.1% or 1% crystal violet in 20% EtOH (modified from (Saotome, 1989)). The cells were washed

with PBS, fixed with 100% methanol for 5 min and stained with 0.1% crystal violet for 20 min, room temperature. After drying, the plates were scanned and analyzed.

3.4.2. IHC staining

Tumor sections were deparaffinized after melting for 10 min at 48°C. Following, they were incubated in xylene for 20 min, xylene 1:1 with 100% EtOH, 100% isopropanol, EtOH 100%, 90%, 70% each for 5 min. After washing, the sections were cooked with EDTA or citric acid containing buffer for 10 min. Then, endogenous peroxidase was blocked with 3% superoxide in PBS for 45 min. The samples were washed in PBS and blocked with 3-5% BSA diluted in PBS for 1h, room temperature. Primary antibodies diluted in PBS were applied on top of the sections, overnight at 4°C. To proceed, washing and biotinylated secondary antibodies incubation was carried out. After 1h, sections were washed and treated with avidin 1:1000 in PBS with 90 min incubation and washing afterwards. Development was performed using DAV until strong signal appeared. The sections were counterstained with hematoxylin and eosin. Finally, the samples were incubated in an inverted alcohol series and mounted.

3.4.3. Immunofluorescence staining

Cells were grown on coverslips in 24-well plates and later washed with PBS and fixed using 4% paraformaldehyde for 10 minutes and washed again. 0.1% Triton X-100 was used for 10 min to permeabilize the cells followed by washing. For the blocking step, the cells were incubated in 10% BSA for 30 min followed by primary antibody overnight, 4°C. On the following day, the cells were washed and incubated with the corresponding conjugated secondary antibody for 1h at room temperature. Washing was followed by incubation with DAPI in PBS for 5 min and mounting. Images were taken using the AXIO Scope.A1 microscope.

3.4.4. Flow cytometry

The cells were treated with cyclosporine A or thapsigargin in different concentrations for 48 h on 6-well plates. After trypsinization, the cells were resuspended in 1 ml DMEM with 10% FCS,100 U/ml penicillin and 100 µg/ml streptomycin. 200,000 of filtered cells were collected by centrifugation at 350 g for 5 min and resuspended in 100 µl of a staining solution. To monitor EpCAM expression, the cells were stained with 2.5 µl FITC-anti EpCAM (Biolegend) conjugated antibody in 1 ml MACS buffer. After 20 min of incubation in the dark, the cells were centrifuged for 5 min, 350 g. The samples were resuspended in 500 µl MACS buffer and 200 µl of each sample were loaded in a 96-well plate. FITC intensity was measured using the Guava EasyCyte plus (Guava Technologies) flow cytometer.

3.5. Statistical analysis

To create graphs, GraphPad Prism version 4.03 has been used for in this study. P-values were determined using Student's t-test (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$).

4. Results

Materials and methods (Section number 2 and 3) were prepared together for Chapter I and Chapter II part (Section number 4) of the thesis. The manuscript presented below was prepared based on Clinical Epigenetics Journal guidelines. In Chapter I and Chapter II short discussions are included. The overall discussion (General discussion) of all thesis results was shown below Chapter II. The overall references for all Chapters are indicated at the end of this thesis (Section Bibliography).

4.1. Chapter I Manuscript

Reduction of PRC2/EZH2 activity can promote better survival of TNBC cancer cells in a context-specific manner

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Own contribution:

Planning and conducting the experiments in this project including Fig. I 1B, E-G, Fig. I 2D, F, G, Fig. I 3, Fig. I 4, Fig. I 5A-C, Fig. 6 I A-F, Fig. I S1, Fig. I S2, Fig. I S3, Fig. I S5. Cell culture assays (proliferation, migration, colony formation), treatments, ChIP-seq, western blot, RT-qPCR and stainings. Complete figure layout and writing the manuscript was performed under the supervision of Dr. Florian Wegwitz.

BACKGROUND: Breast cancer is the most common cancer occurring in women. Recent advances in early diagnosis and development of targeted therapies greatly improved the survival rate of breast cancer patients. However, conventional cytotoxic chemotherapies remain often the only treatment option for patients suffering from cancer subtypes where targeted therapies are not viable. Furthermore, the development of resistance is frequent and commonly followed by fatal consequences. In this study, we investigated epigenetic mechanisms underlying tumor cells surviving a combinatory chemotherapy treatment as potential targets to increase cytotoxic efficiency.

METHODS: Murine basal-like WAP-T mammary carcinoma cells and human triple-negative cell lines were utilized to study processes involved in cancer cell survival to a cyclophosphamide/doxorubicin/5-fluorouracil treatment. We performed high throughput RNA- and ChIP-sequencing analyses to assess transcriptome wide gene expression changes and underlying epigenetic regulatory mechanisms in cells surviving chemotherapy. To confirm our findings, we then employed several *in vitro* functional assay and corroborated our results on murine tissues and publicly available patient databases.

RESULTS: Epithelial to mesenchymal transition (EMT) and increased stemness were tightly associated with survival of the cancer cells to chemotherapy. We identified a reduction of Polycomb Repressive Complex 2 (PRC2) activity via downregulation of the *Ezh2*, *Suz12* and *Rbbp7* expression in chemotherapy-treated WAP-T cells. Interestingly, siRNA and small molecule inhibition of PRC2 activity improved the proliferation and survival of murine and human cancer cells to cytotoxic treatment. Mechanistically, loss of PRC2 activity lead to the derepression of a set of genes through a switch from the repressive H3K27me3 to the activating H3K27ac mark at regulatory regions. We identified *Nfatc1* as a gene upregulated by loss of PRC2 activity and directly implicated with the transcriptional changes happening upon survival the chemotherapy. Blocking NFATc1 activation reduced epithelial to mesenchymal transition and aggressiveness of TNBC cells.

CONCLUSIONS: Our data demonstrates a previously unknown function of PRC2 maintaining low *Nfatc1* expression levels and thereby repressing invasiveness of TNBC.

KEYWORDS: PRC2, EZH2, TNBC, chemotherapy, H3K27ac, H3K27me3, NFATc1

Background

Breast cancer (also termed mammary carcinoma) is the most common cancerous disease in women with over 2 million new cases in 2018 worldwide (World Health Organisation, 2018). The mortality of breast cancer patients has significantly decreased over the past decades, mostly because of early diagnosis improvements and the development of several targeted therapies. However, despite intensive efforts to combat the disease, breast cancer remains the first cancer-related cause of death among women. The prognosis of cancer patients is largely determined by the metastatic lesions and recurrent tumor growth. Today, approximately 25% of breast cancer patients still develop distant metastases and ultimately die of their disease (Mathiesen et al., 2012). Even when detected early and treated by standard breast-conserving surgery, breast cancer has a recurrence rate of 5-10% within 10 years (Colzani et al., 2014; Fisher et al., 2002; Harbeck et al., 2019; Veronesi et al., 2002). The high incidence of breast cancer and the high mortality rate of the disease in relapsed patients necessitates the urgent development of improved treatment options.

Because of its highly heterogeneous nature, breast cancer is commonly classified into distinct disease subtypes with specific therapeutic approaches and outcome, based on expression of the receptor molecules ER (estrogen receptor) and PR (progesterone receptor) and HER-2 (human epidermal growth factor receptor 2) (Prat, Pineda, et al., 2015). Despite their great usefulness in the clinic, these histological parameters do not fully reflect the complexity of the disease. Progress in gene expression profiling lead to the definition of at least four different molecular subtypes of breast cancer with very different incidence, prognosis and response to treatments: Luminal A, Luminal B, HER-2 enriched and triple negative breast cancer

(TNBC) (Perou et al., 2000; Prat, Fan, et al., 2015). The possibility to specifically inhibit the activity of ER, PR and/or HER-2 via targeted therapies greatly improved the therapeutic options and prognosis of mammary carcinomas subtypes expressing those receptors. Unfortunately, because of the lack of ER/PR and HER-2 expression, the group of triple negative breast cancers (TNBC), that accounts for approximatively 15% of all mammary carcinomas do not profit from these therapeutic advances. Mammary carcinomas are clinically treated with a combination of surgery, radiation, chemotherapy and/or targeted therapies (if available) depending on the type and stage of the disease. Here, a combination of cyclophosphamide, anthracycline (doxorubicin) and/or 5-fluorouracil (5-FU) (CAF) have been shown to display an increase in pathological response rates in TNBCs compared to the other subtypes (Carey et al., 2007; O. Gluz et al., 2009). Despite a good first response to cytotoxic therapies, a large fraction of TNBC rapidly develop resistance. Consequently, TNBCs show the highest rate of recurrence after treatment and the poorest prognosis among breast cancer diseases (Prat et al. 2015).

Mechanisms allowing a tumor cell to escape conventional chemotherapeutic treatments require fast adaption to hostile conditions. Acquisition of epithelial-mesenchymal plasticity (EMP) and stemness have been identified as potential mechanisms responsible for cancer progression, development of chemotherapy resistance and increased metastatic features (Lu and Kang 2019; Ye and Weinberg 2015). Such alterable properties necessitate rapid reorganization of whole gene expression profiles. Because of the dynamic and reversible nature of epigenetic modifications, epigenetic processes represent very likely mechanisms controlling cellular plasticity. Thus, epigenetic players are attractive targets for the development of the new anti-cancer drugs (Mohammad et al. 2019; Wouters and Delwel 2016). Numerous publications reported the central role of epigenetic factors mediating the function of transcription factors during epithelial to mesenchymal transition (EMT) (Lu and Kang 2019; Wainwright and Scaffidi 2017). In a similar manner, epigenetic mechanisms were shown to be indispensable for the acquisition and maintenance of cancer stem cell (CSC) properties (Skrypek et al., 2017; Wainwright & Scaffidi, 2017). The Polycomb repressive complexes 1 and 2 (PRC1 and PRC2, respectively) are two

well characterized epigenetic factors repressing the expression of specific genes via histone post-translational modification. The canonical PRC2 core complex is composed of four subunits EZH1/EZH2, EED, SUZ12 and RBBP7. Through its catalytic subunit EZH2, the PRC2 catalyzes the di- and trimethylation of histone 3 at lysine 27 (H3K27me2 and H3K27me3, respectively) promoting thereby a compaction of the chromatin, and as a consequence, leading to the silencing of genes located in the given region (Antonysamy et al., 2013; Simon & Kingston, 2013). Interestingly, PRC2 was shown to play an essential role in normal embryonal and adult stem cells homeostasis by maintaining self-renewal and pluripotency through repression of differentiation programs (Raphaël Margueron & Reinberg, 2011; Vizán et al., 2015). In line with these observations, higher EZH2 expression levels were associated with increased cancer stem cell properties and poor prognosis in numerous cancer entities including malignancies of the breast (Wen, Cai, Hou, Huang, & Wang, 2017). Furthermore, the enzymatic activity of the PRC2 complex was shown to actively promote EMT by positively regulating the expression of and cooperating with central EMT-transcription factors (EMT-TFs) like SNAI1 or ZEB1 (Herranz et al., 2008; Martínez-Fernández et al., 2015).

In the past, we developed and characterized the WAP-T mammary carcinoma mouse model to study the biology, progression and metastatic processes of TNBC (Lenfert et al., 2015; Maenz et al., 2015; Otto, Gruner, et al., 2013; Otto, Streichert, et al., 2013; Schulze-Garg et al., 2000; Wegwitz et al., 2010). In a former effort to understand the effects of a CAF therapy on WAP-T mammary carcinomas, we observed that the cytotoxic combination therapy was not able to eradicate the disease *in vivo*. Interestingly, surviving tumor cells displayed a more aggressive mesenchymal-like phenotype with increased stem cell traits and showed a pronounced tendency to dissemination (Jannasch et al., 2015). Because of its good mimicking of the clinical situation, we utilized this model in the present study to get insight into the molecular mechanisms underlying acquisition of EMP and stemness upon chemotherapy treatment and allowing tumor cell survival. We identified here a previously unknown PRC2 function repressing EMT and cancer stem cell program in TNBC cells along a PRC2/NFATc1 axis.

Results

WAP-T cells surviving CAF treatment gain stem cell and EMT properties *in vitro*.

The parental G-2 cell line (pG-2), established from a WAP-T mammary carcinoma (Wegwitz et al., 2010) was utilized to investigate the effects of a CAF combination therapy on TNBC *in vivo* and it was observed that tumor cells surviving the therapy gained stemness and mesenchymal-like characteristics (Jannasch et al., 2015). To get insights into the molecular pathways underlying the survival and the emergence of resistance to the CAF chemotherapy *in vitro*, we optimized in a first step the chemotherapy treatment settings of G-2 cells in the cell culture. Aim here was the identification of treatment conditions eradicating most of the tumor cells but allowing the survival and regrowth of a small tumor cell fraction, mimicking thereby the *in vivo* relapse situation. A combination therapy consisting of 312.5 ng/ml cyclophosphamide, 15,6 ng/ml doxorubicin and 312,5 ng/ml 5-FU, corresponding to the 1/32 dilution of the therapy previously utilized in Jannasch et al *in vivo*, was identified as the best appropriate setting (Fig. I 1A). This treatment was adopted for the rest of the experiments in the present study and will be designated as CAF therapy. Interestingly, pG-2 cells surviving CAF-treatment acquired a more elongated morphology, characteristic for cells undergoing EMT (Fig. I 1B). A chemotherapy resistant variant of the pG-2 cells called rG-2 cells was established through several cycles of CAF treatments (see method section for more details). Strikingly, rG-2 cells harbor in basal growth condition a mesenchymal-like phenotype, supporting the potential implication of EMT mechanisms in resistance to CAF therapy (Fig. I S1A). We performed RNA-sequencing (RNA-seq) and compared the transcriptome of pG-2 cells treated 48 hours with CAF to vehicle treated control cells (ctr). DeSeq2 analyses identified 1021 downregulated and 1448 upregulated genes ($|Log2(Fold\ Change)|>1$, $padj<0.05$) in CAF-treated cells (Fig. I 1C). To get insights into transcriptional program changes occurring during survival to the treatment, we performed Gene Set Enrichment Analyses (GSEA). Strikingly, we observed a strong enrichment of gene sets related to EMT, cancer aggressiveness and stemness (Fig. I 1D). Indeed, the well-known EMT markers Vimentin (*Vim*) and N-cadherin (*Cdh2*) and EMT-TFs *Snai1*, *Twist2* and *Zeb1*

were upregulated in surviving cells whereas the expression of both epithelial marker E-cadherin (*Cdh1*) and *Epcam* were strongly reduced (Fig. I 1E). The regulation of *Vim*, *Twist2*, *Snai1*, *Zeb1* and *Cdh1* was validated using qPCR (Fig. I 1G). In a similar manner, the expression of stem cell specific transcription factors was also found to be increased in CAF-treated cells (Fig. I 1F). Interestingly, rG-2 cells showed increased expression of several EMT and stem cell markers under basal culture conditions (Fig. I S1B). These results support previous *in vivo* studies (Jannasch et al., 2015) and further emphasize the implication of EMT and stem cell properties in therapy survival mechanisms.

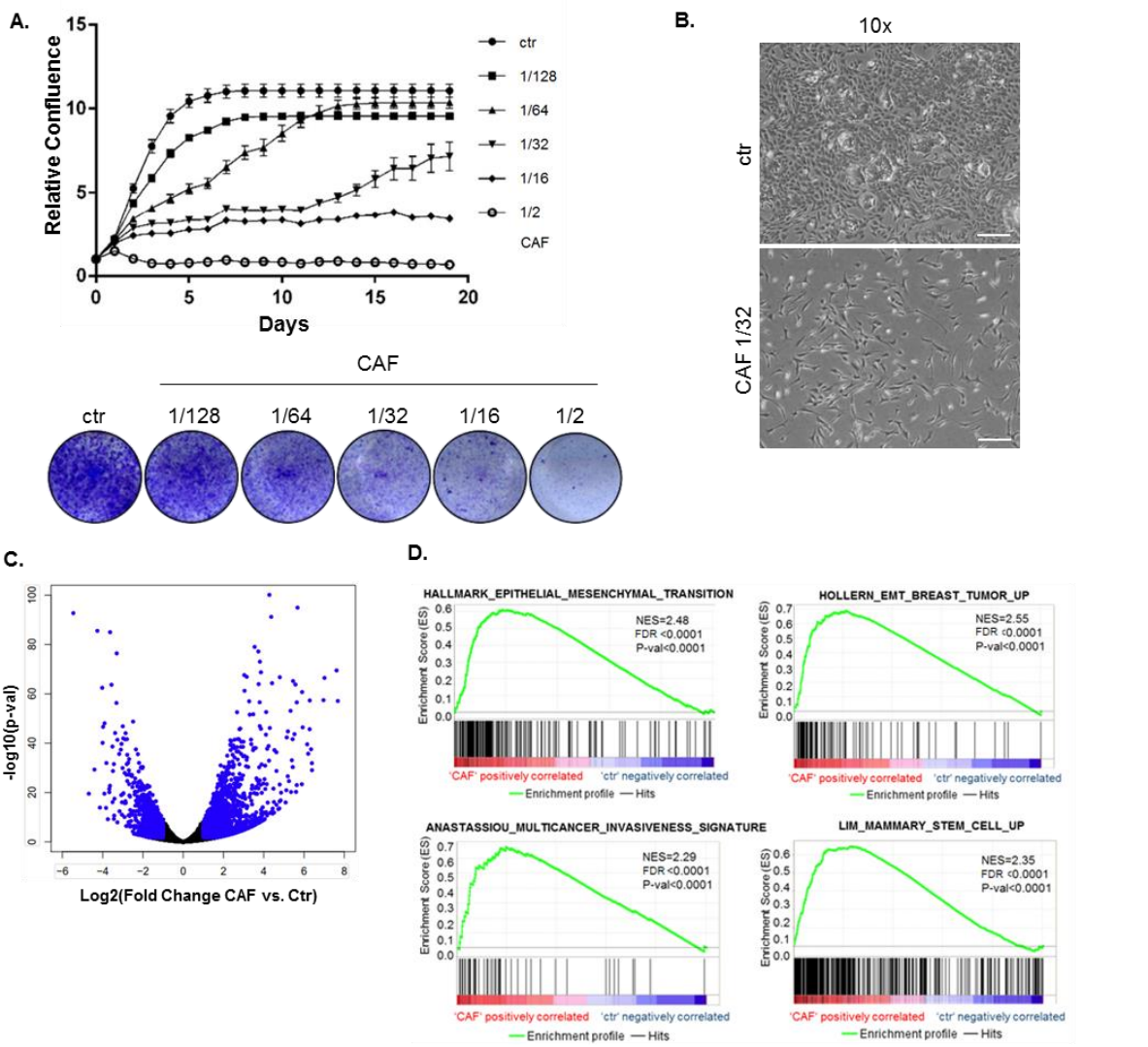
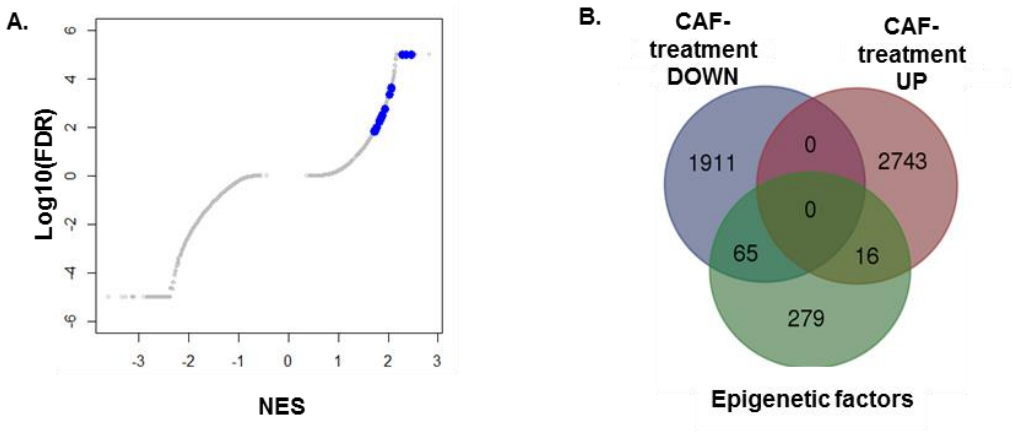

A.
Relative Confluence
Days
ctr
1/128
1/64
1/32
1/16
1/2
CAF
CAF
ctr
1/128
1/64
1/32
1/16
1/2
B.
10x
ctr
CAF 1/32
C.
-log10(p-val)
Log2(Fold Change CAF vs. Ctr)
D.
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION
NES=2.48
FDR <0.0001
P-val<0.0001
HOLLERN_EMT_BREAST_TUMOR_UP
NES=2.55
FDR <0.0001
P-val<0.0001
ANASTASSIOU_MULTICANCER_INVASIVENESS_SIGNATURE
NES=2.29
FDR <0.0001
P-val<0.0001
LIM_MAMMARY_STEM_CELL_UP
NES=2.35
FDR <0.0001
P-val<0.0001
Enrichment Score (ES)
'CAF' positively correlated
'ctr' negatively correlated
Enrichment profile
Hits
E.
ctr
CAF
Row Z-Score
GENE NAME
Log2FC
Vim 1.29
Snai1 0.74
Twist2 1.73
Zeb1 0.95
Cdh2 0.56
Cdh1 -1.66
Epcam -2.35
F.
ctr
CAF
Vim
Cdh2
Cdh1
Actin
G.
Vim
Twist2
Snai1
Zeb1
Cdh1
mRNA relative expression
relative mRNA expression
ctr
CAF

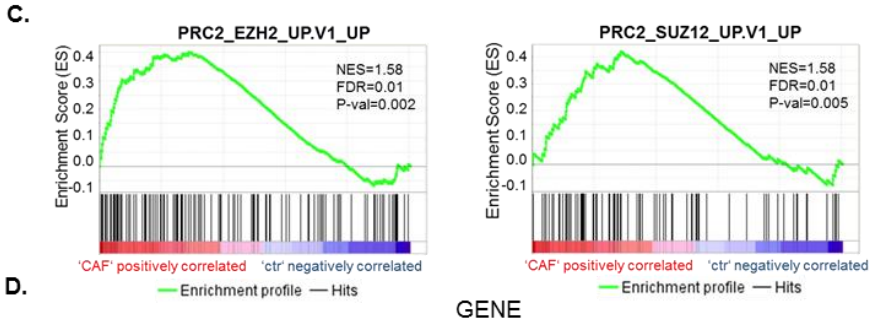
Fig. I 1 WAP-T cells surviving CAF treatment gain stem cell and EMT properties *in vitro*. **A:** Cell proliferation assay of pG-2 cells treated for 48 hours with increasing concentrations of a combinatory CAF chemotherapy. The concentration [1] represents the equivalent of the doses used in previous *in vivo* experiments (10 µg/ml cyclophosphamide, 0.5 µg/ml doxorubicin and 10 µg/ml 5-FU) (Jannasch et al., 2015). Cell confluency was assessed every day using Celigo. For a direct visualization, crystal violet staining was performed at day 2. **B:** Phase contrast images of pG-2 cells after 48 hours CAF-treatment showing a spindle like morphology characteristic for cells that underwent EMT (objective 10x, scale bar = 250 µm). **C:** Volcano plot showing transcriptome wide gene expression changes in pG-2 cells compared to pG-2 cells after 48 hours of CAF-treatment, as measured by RNA-seq (n=3 biological replicates). **D:** Representative GSEA enrichment plots showing a significant enrichment of gene signatures characteristic for EMT-processes, stemness traits and cancer invasiveness in CAF-treated versus control cells. **E:** Heatmap showing the regulation of selected EMT markers identified in the RNA-seq analyses. **F and G**: Validation of EMT-marker regulation on protein level using western blot (**F**) and on mRNA level using qRT-PCR (**G**). qRT-PCR data was normalized to the control condition and normalized to the *Rplp0*. n=3 biological replicates +/- SEM, * p-val ≤ 0.05, ** p-val ≤ 0.01, *** p-val ≤ 0.005.

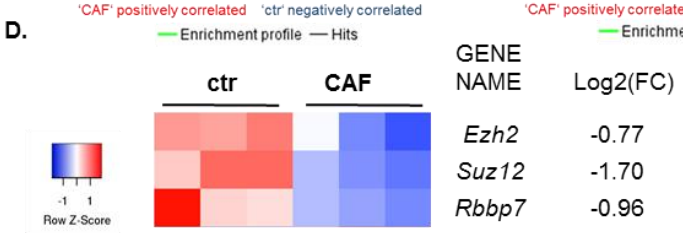
WAP-T tumor cells surviving CAF-treatment downregulate the expression of PRC2 core subunits

We decided to get more insight into the molecular mechanisms allowing tumor cells to activate EMT and stemness transcriptional programs, increasing their aggressiveness and survival to cytotoxic therapies. We therefore returned to our GSEA analyses and interestingly identified an accumulation of gene signatures related to epigenetic regulatory pathways perturbation enriched CAF-treated cells (Fig. I 2A). This was an interesting finding, as several epigenetic mechanisms have been shown to be involved in the processes controlling cellular plasticity (Kiesslich, Pichler, & Neureiter, 2012). Based on the RNA-seq results, we identified 65 down-regulated and 16 up-regulated epigenetic factors (Fig. I 2B, listed in Table I S1). Surprisingly, Gene Set Enrichment Analysis (GSEA) and Enrichr analyses pointed at an enrichment of genes known to be H3K27me3-marked and/or repressed by PRC2 (Fig. I 2C). We therefore checked if changes of PRC2 subunits expression happened upon chemotherapy treatment. Strikingly, the core PRC2 subunits *Ezh2*, *Suz12*, *Rbpp7* were found to be significantly downregulated in cells surviving the CAF treatment (Fig. I 2D). The down-regulation of *Ezh2*, *Suz12* and *Rbbp7* was validated by qPCR (Fig. I 2E). On protein level, Ezh2 and Suz12 were reduced as assessed via western blots and immunofluorescence staining (Fig. I 2F-G). In line with these findings, rG-2 cells grown under normal conditions harbored a constant lower expression of the core PRC2 subunits *Ezh2*, *Suz12* and *Rbbp7* when compared to untreated or treated pG-2 cells.

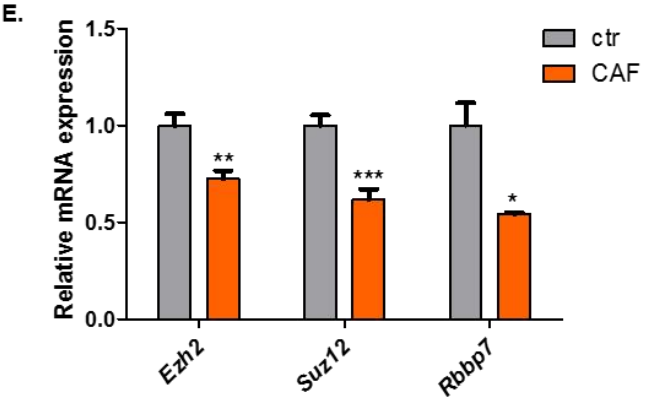
Noticeably, their expression levels were even more reduced upon CAF treatment (Fig. I S2). We concluded that the reduction of PRC2 level was associated with survival to cytotoxic therapies and with increased resistant phenotype.

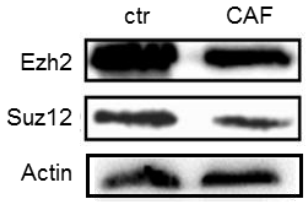






GENE NAME	Log2(FC)
Ezh2	-0.77
Suz12	-1.70
Rbbp7	-0.96





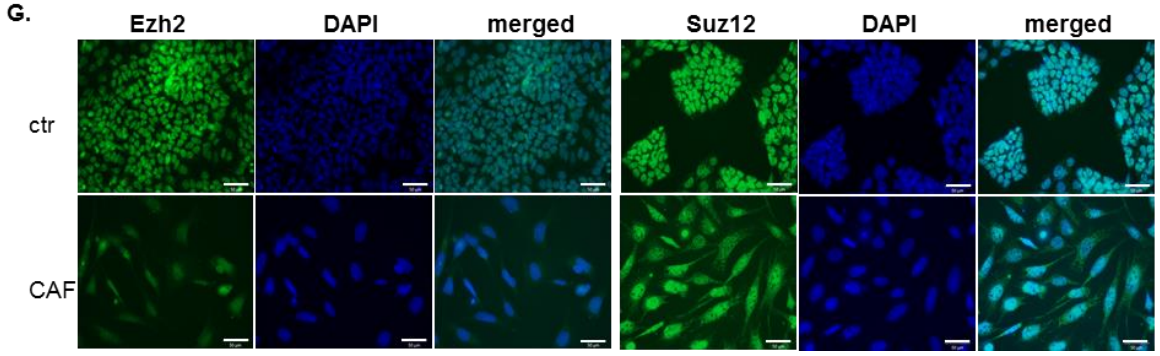


Fig. I 2 WAP-T tumor cells surviving CAF treatment downregulate the expression of PRC2 core subunits. A: GSEA analysis results (MSigDB) plotted as an overview along Normalized Enrichment Score (NES) and log10(FDR). The results show an enrichment of gene signatures associated with epigenetic mechanisms perturbation. Blue dots represent enriched epigenetic pathway. **B:** Identification of differentially regulated epigenetic factors: genes regulated in pG-2 cells upon CAF treatment survival (|Log2(Fold Change)|>0.8, padj<0.05) were intersected with a list of known epigenetic factors. **C:** Representative GSEA enrichment plots showing the enrichment of gene signatures typically repressed by PRC2 in CAF-treated pG-2 cells. **D:** Heatmap showing the downregulation of central PRC2 members upon chemotherapy, as identified in the RNA-seq analyses, padj<0.05. **E:** Validation of *Ezh2*, *Suz12* and *Rbbp7* expression via qRT-PCR. Data was normalized on the control condition and normalized to the *Rplp0* housekeeping gene. n=3 biological replicates +/- SEM, * p-val ≤ 0.05, ** p-val ≤ 0.01, *** p-val ≤ 0.005.**F and G:** Reduction of EZH2 and SUZ12 protein levels upon CAF treatment was assessed via western blot (**F**) and immunofluorescence staining (**G**).

Reduction of EZH2 activity enhances the aggressiveness of TNBC tumor cells

Although the majority of the literature attributes rather tumor promoting functions to the PRC2 complex, a few recent publications have pointed towards a possible tumor suppressive role in ovarian carcinoma (Cardenas et al., 2016). We therefore asked whether the reduction of PRC2 activity could directly mediate WAP-T tumor cell survival to cytotoxic therapies by derepressing aggressive and/or proliferative gene expression programs. To assess the effect of EZH2 activity loss on the proliferation of pG-2 cells, we silenced *Ezh2* using targeted siRNA or treated the cells with a small molecule inhibitor against EZH2 (EPZ-6438) and performed proliferation assays. Interestingly, impairment of EZH2 activity did not reduce proliferation of the tumor cells as it was observed for numerous other BC cell lines in the past (Gonzalez et al., 2009; Song et al., 2016). On contrary, the growth of pG-2 cells was slightly but significantly promoted upon *EZH2* knockdown (Fig. I 3A) and low concentrations of EPZ-6438 (Fig. I 3D). *Ezh2* knockdown efficiency was validated at mRNA level (Fig. I 3B) and loss of H3K27me3 resulting from EPZ-6438 treatment was measured by western blot for different concentrations (Fig. I 3C). Interestingly, colony formation ability of pG-2 cells seeded at limiting dilution was strongly improved upon inhibition of EZH2, suggesting increased tumor initiating properties (Fig. I 3E). Strikingly, this increased colony formation capacity was maintained upon chemotherapy treatment, indicating that the inhibition of the PRC2 complex activity indeed supported cell survival and resistance to the therapy (Fig. I 3E). We asked whether this observation was limited to the murine WAP-T mammary carcinomas or if other human cancer cell lines could also get a growth and survival advantage upon PRC2 activity loss. Interestingly, although certain breast

cancer cell lines showed impaired or unchanged proliferation upon EZH2 inhibition, the MDA-MB-468 TNBC cell line displayed moderate but increased growth properties when treated with siRNA against EZH2 or with EPZ-6438 (Fig. I S3). Interestingly, the proliferation stimulating consequence of an EZH2 inhibition was not limited to breast cancer cell lines, but was also observed in human cancer cell lines of other origins, colorectal and bile duct carcinoma (Fig. I S4). Moreover, the proliferation advantage mediated by *EZH2* knockdown in MDA-MB-468 was even more pronounced in the presence of CAF treatment (Fig. I S3B). Together, inhibition of PRC2 repressive activity increases aggressiveness of cancer cells and increases cytotoxic therapy survival in a context dependent manner.

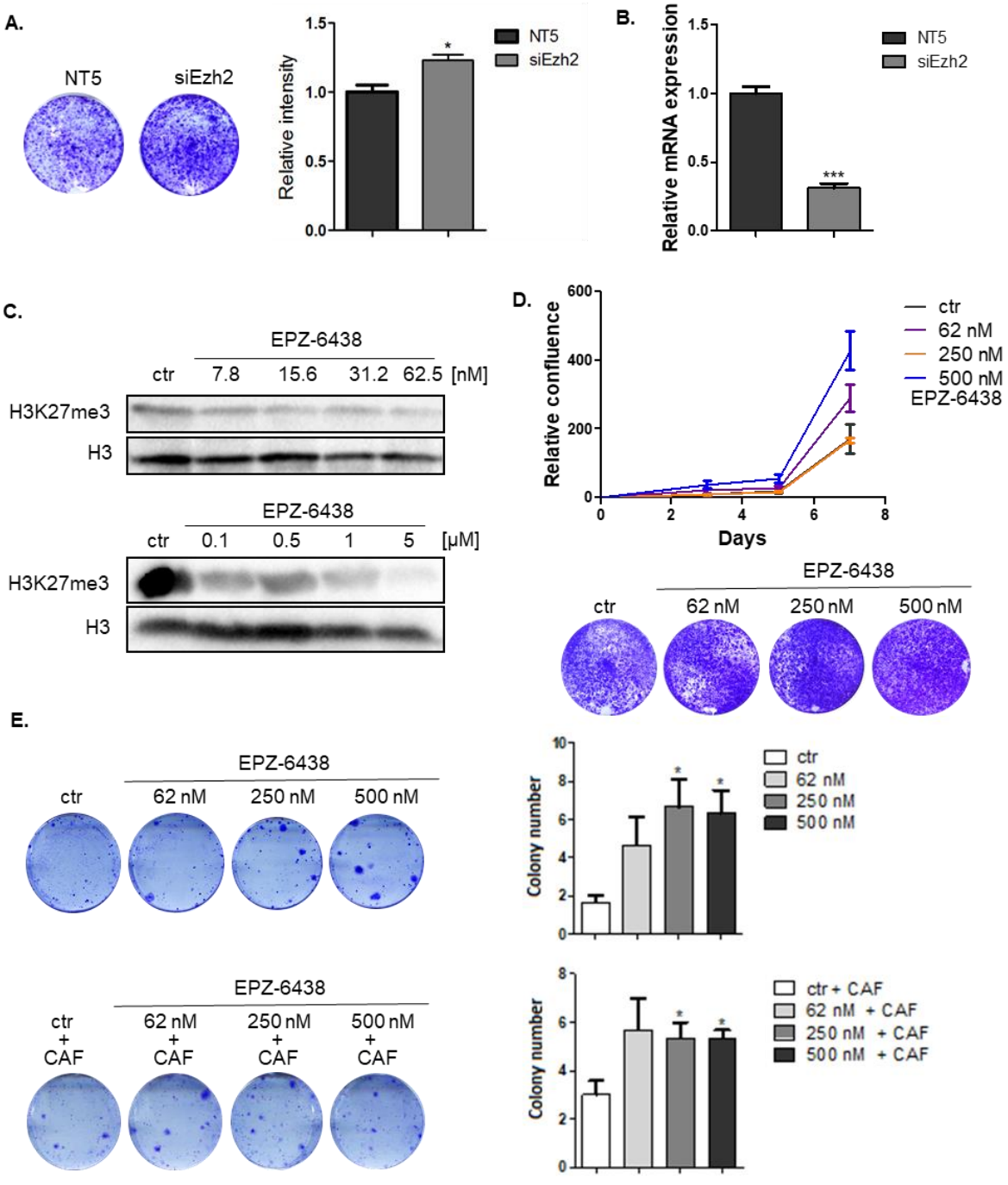


Fig. I 3 Reduction of EZH2 activity enhances the aggressiveness of TNBC tumor cells.
A: Crystal violet staining of pG-2 cells upon *Ezh2* knockdown. The confluency was measured by ImageJ and normalized to the controls. **B:** Validation of *Ezh2* knockdown efficiency using qRT-PCR. Data were calibrated to the control condition and normalized on the *Rplp0*. **C:** Assessment of EZH2 inhibition by H3K27me3 levels upon increasing EPZ-6438 concentration using western blot. **D:** Proliferation assay of EPZ-6438-treated pG-2 cells using Celigo® and crystal violet staining. **E:** Colony formation assay upon treatment of pG-2 cells with EPZ-6438 alone or in combination with CAF. Number of colonies were assessed through ImageJ analysis. n=3 biological replicates +/- SEM, * p-val ≤ 0.05, ** p-val ≤ 0.01, *** p-val ≤ 0.005.

Reduction of PRC2 activity during chemotherapy treatment enables the activation of gene expression programs promoting tumor cell survival.

The trimethylation of H3K27 by PRC2 mediates the silencing of chromosomal regions by promoting chromatin compaction through cooperation with the PRC1 complex (Grossniklaus & Paro, 2014). Furthermore, because of the occupancy of the presence of the methyl groups, H3K27me3 is mutually exclusive with the transcriptional activating mark H3K27ac (Tie et al., 2009). Relying on this knowledge, we hypothesized that loss of PRC2 activity during chemotherapy survival could lead to an epigenetic switch enabling tumor cells to activate translational programs promoting aggressiveness and therapy resistance. To test our hypothesis, we assessed genome wide changes of H3K27me3 and H3K27ac occupancy via ChIP-seq in untreated as well in 48 and 96 hours CAF-treated pG-2 cells. Because we suspected a direct connection between PRC2 repressive activity loss and activation of genes expression programs upon CAF treatment, we decided to investigate the changes of H3K27me3 and H3K27ac at the TSS region of upregulated genes. As presumed, the levels of H3K27me3 at promoter regions of up-regulated genes were significantly reduced already after 48 hours and remained low after 96 hours (Fig. I 4A). This analysis uncovered a switch from H3K27me3 to H3K27ac indicating potential genes activated through PRC2/EZH2 loss. H3K27ac as a mark of active promoters and active gene transcription, we observed its increased level upon chemotherapy. In this analysis, 74 genes showed at the same time a robust up-regulation at the RNA level (Log2FC > 0.8, p-val < 0.05) and a switch from trimethylation to acetylation at H3K27 (Fig. I 4B). Moreover, we identified a number of upregulated genes with subsequent loss of H3K27me3 and H3K27ac gain (Fig. I 4C). We selected few of them with the most relevant H3K27me3/H3K27ac change at promoter regions, such as Nfatc1, Wnt9a, Gli2 and Klf4 (Fig. I 4C, D). Additionally, RNA-seq results presented on the heatmap, shows a characteristic upregulation of the aforementioned genes (Fig. I 4D). To investigate enrichment signatures between CAF-treated (48h) and control cells, we used the online Enrichr tool (Fig. I 4E). One of the most significantly enriched pathways, was calcineurin signaling involving NFATc1. NFATc1 activation was shown to promote EMT and tumor

progression in several tumor entities. Furthermore, Chen et al. reported a context dependent epigenetic regulation of NFATc1 expression by EZH2 in pancreatic tissues (N. M. Chen et al., 2017). Additionally, NFATc1 can be targeted by small molecule inhibitors, some of them being commonly employed in the clinic (e.g. Cyclosporin A, CsA), making this factor very attractive to study in the context of survival and resistance to chemotherapy (Pan, Xiong, & Chen, 2013). Upregulation of *Nfatc1* upon CAF-chemotherapy treatment was confirmed on mRNA (Fig. I 4F) and protein level (Fig. I 4G) indicating its potential role in cancer recurrence.

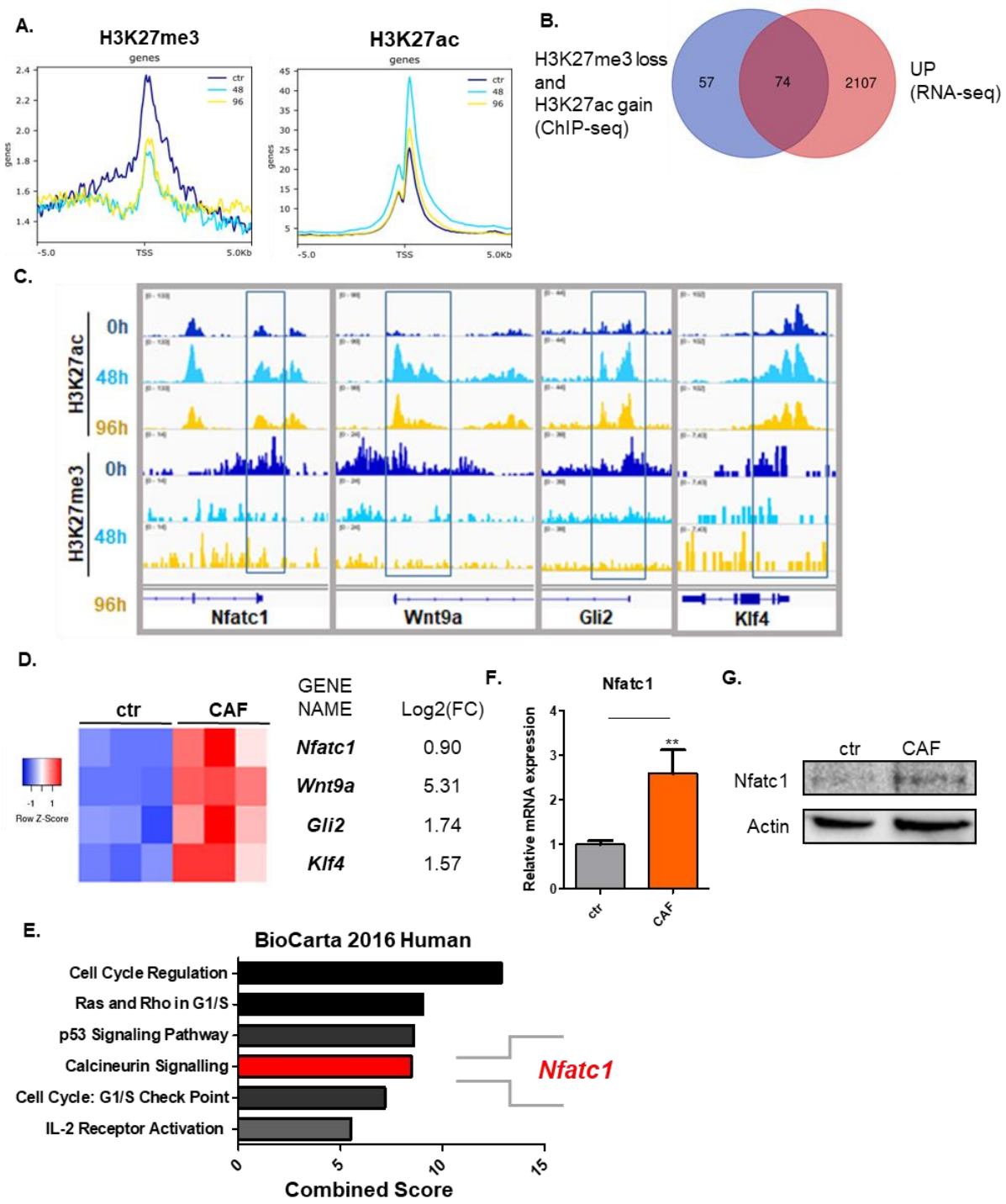


Fig. I 4 CAF-chemotherapy-induced epigenetic regulation in pG-2 cells. A: Aggregate plots of H3K27me3 and H3K27ac ChIP-seq signals at ±5 kb of the TSS) of genes that were upregulated (RNA-seq) upon CAF-chemotherapy treatment for 48 hours (48, light blue line), treated with CAF-chemotherapy for 48 hours and fresh medium for the next 48 hours (96, dark blue line) and untreated (ctr, yellow line). **B:** Venn diagram showing the overlap of the H3K27me3 loss and the H3K27ac gain ChIP-seq peaks with upregulated genes, 0.8≤log2(FC), from RNA-seq data. **C:** H3K27me3 and H3K27ac ChIP-seq tracks at *Nfatc1, Wnt9a, Gli2* and *Klf4* gene loci in CAF-chemotherapy treated (48h,

96h) and control cells (0h). H3K27me loss and H3K27ac gain at promoter region, indicated in blue boxes **D:** RNA-seq heatmap of selected upregulated genes upon 48 hours CAF treatment. **E:** Enrichr analysis, BioCarta for the most significant pathways, on the 74 identified genes from Fig. I 4B. **F:** Expression of Nfatc1 on mRNA, +/- SEM, * p-val ≤ 0.05, ** p-val ≤ 0.01, *** p-val ≤ 0.005 and **G:** protein level in 48 hours CAF-treated pG-2 cells.

EZH2 loss mediates NFATc1-induced cancer progression in TNBC

To investigate whether EZH2 modulates NFATc1 expression, we performed knockdown of *Ezh2* in pG-2 cells. Upon Ezh2 loss, we observed a decrease in H3K27me3 and a Nfatc1 upregulation, meaning that EZH2 activity negatively modulates NFATc1 expression (Fig. I 5A). To further analyze Ezh2 and Nfatc1 expression *in vivo*, we used WAP-T mice tumors followed by CAF treatment (Fig I 5B). IHC staining revealed a loss of EZH2 and an upregulation of NFATc1 in the acute phase of CAF treatment. Whereas during the recovery phase, Ezh2 expression increased and Nfatc1 level came back close to the basal level (control) indicating rapid changes in gene expression upon cytotoxic stimuli. Based on human primary breast tumors, gene expression signature analysis also suggests a frequent negative correlation between *EZH2* and *NFATc1* (Fig. I 5C). Additionally, we used The Cancer Genome Atlas (TCGA) PAM50-based database for human TNBCs where we could observe differential *EZH2*, *NFATc1* expression among patients (Fig. I 5D). Interestingly, survival plots using patients data with low or high expression of *EZH2* (left) and *NFATc1* (right) (Fig. I 5E) suggest that patients survival is positively correlated with EZH2 expression and negatively correlated with NFATc1. These data suggest that NFATc1 can be a potential anticancer target in TNBC.

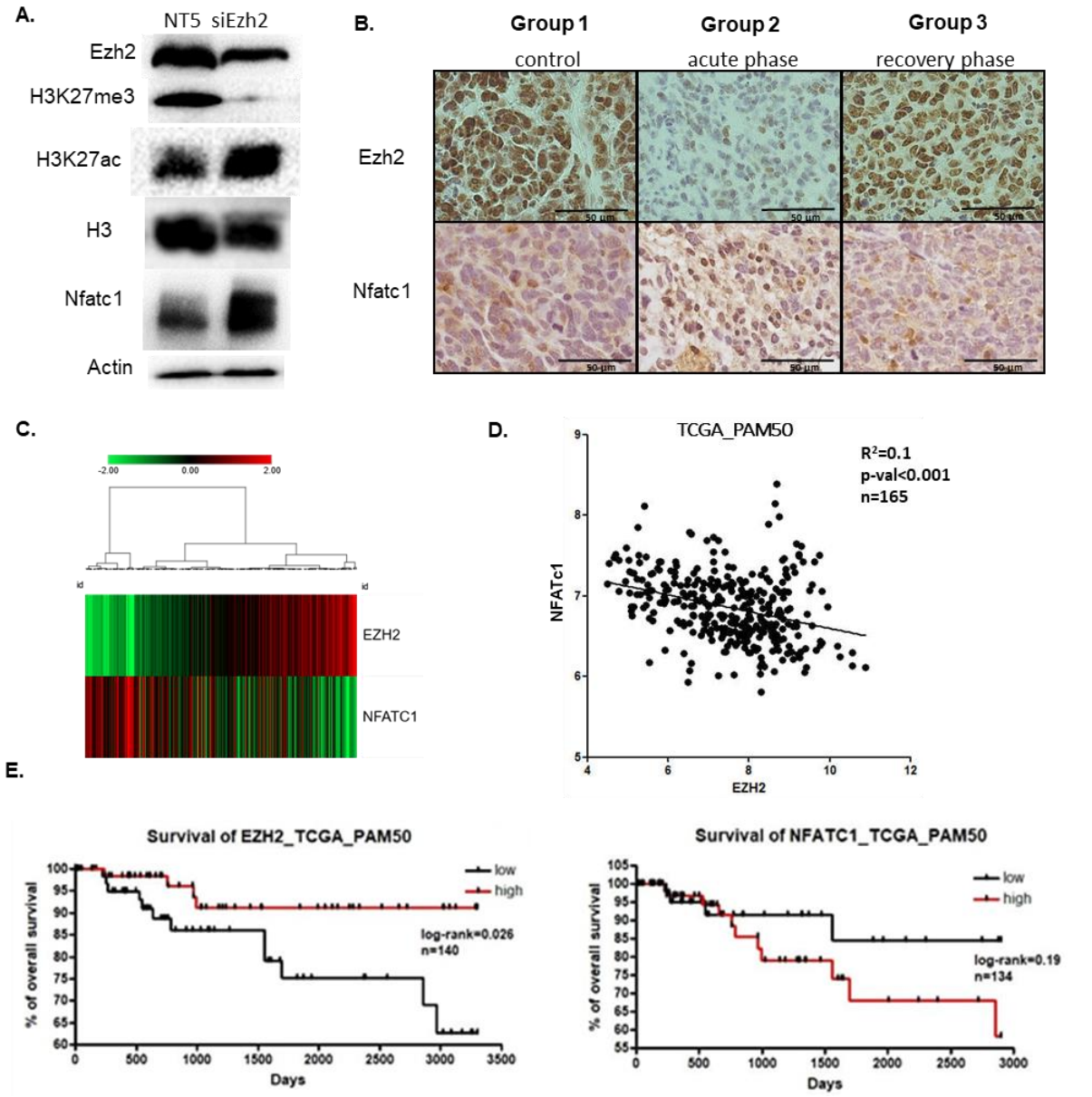


Fig. I 5 Differential expression of Ezh2 and Nfatc1 in TNBC. A: Nfatc1 regulation upon loss of EZH2 in protein level in pG-2 cells. **B:** Representative images of paraffin-embedded tumors from Group 1: dissected when tumors reached 0.5 cm^3, Group 2: CAF treated and dissected after 9 days and Group 3: CAF treated and dissected when reaching initial tumor volume, stained for Ezh2 and Nfatc1. **C:** Gene expression signature of *Ezh2* and *Nfatc1* using MERAV presented with Pearson correlation that is -0.21 in primary breast tumors. **D:** TCGA PAM50 (Xenabrowser)-based scatter plot with linear regression of the genome-wide correlation between *EZH2* and *NFATc1*. **E:** TCGA PAM50-based survival of TNBC patients based on EZH2 and NFATc1 expression level; low or high.

NFATc1 knockdown decreases TNBC cell invasiveness

NFAT proteins have been shown to be involved in EMT processes in breast cancer (Sengupta et al., 2013). In this part of the study we wanted to verify NFATc1 function in TNBC and investigate its involvement in cancer cell motility, growth, EMT and stemness. *Nfatc1* knockdown decreased pG-2 cell growth (Fig. I 6A, B), where Nfatc1 loss efficiency was confirmed on mRNA (Fig. I 6C) and protein level (Fig. I 6D). Additionally, we observed great proliferation impairment upon *NFATc1* depletion alone or in combination with CAF in the human breast cancer cell line, MDA-MB-468 (Fig. I S5). In pG-2 cells, *Nfatc1* loss lead to reduced migratory ability (Fig. I 6E, F). Based on the findings on epithelial-mesenchymal plasticity (EMP) of metastatic breast cancer and our previously shown data (Fig. I 1), we analyzed EMT-related markers in the context of Nfatc1 regulation. Our data demonstrate that upon loss of *Nfatc1,* TNBC cells change to a more epithelial phenotype (Fig. I G, H). In addition, we treated pG-2 cells with cyclosporine A (CsA) and Thapsigargin to inhibit and stimulate Nfat activity, repectively (Fig. I 6H, Fig. I S6). The cells were treated with increasing concentrations of CsA and Thap for 48 hours, stained with FITC conjugated antibody against EpCAM. We observed an increase and a decrease in EpCAM-positive cells upon CsA and Thap treatment respectively, suggesting a key role of Nfatc1 in EMT and indicating its involvement in cancer cell survival.

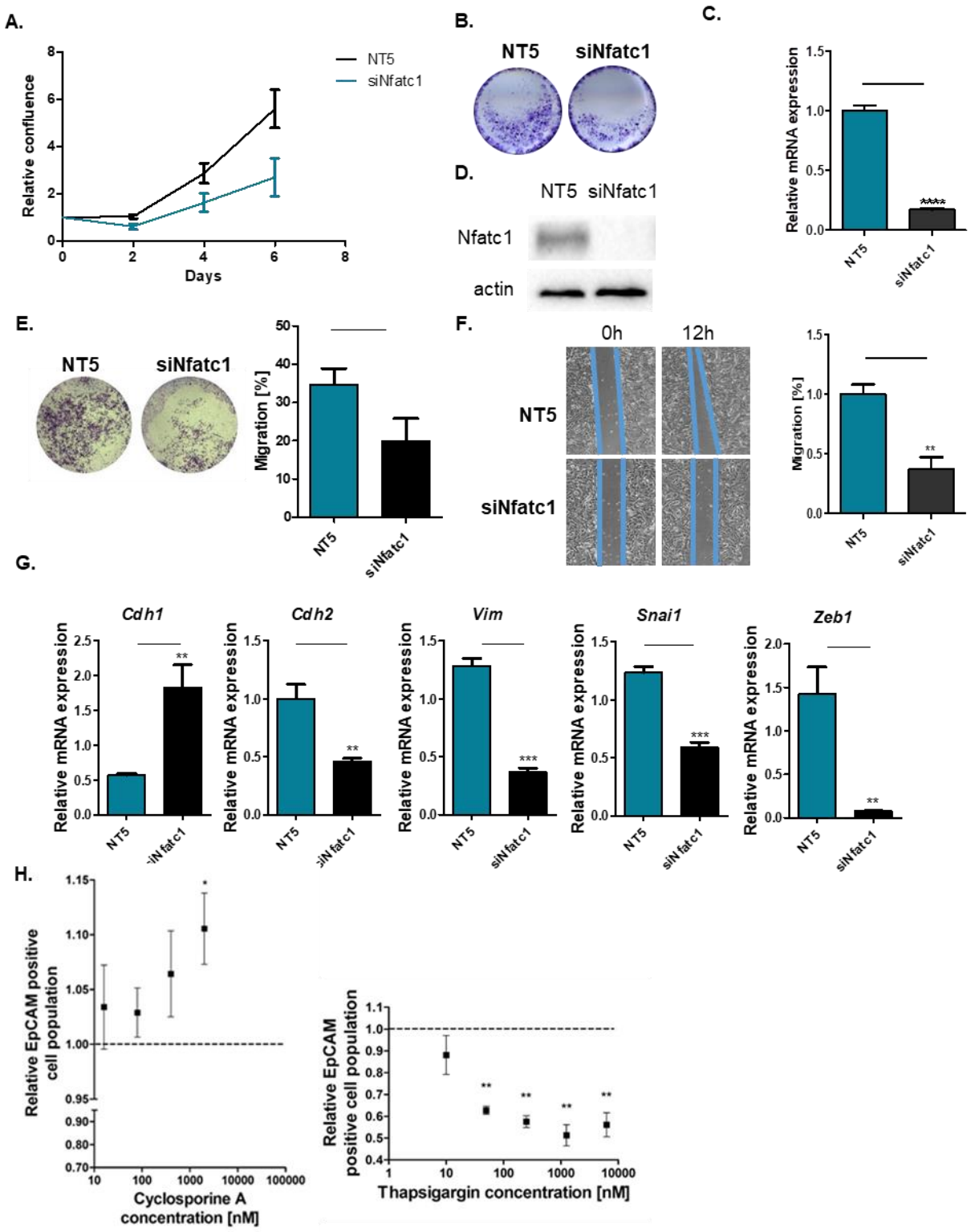


Fig. I 6 Nfatc1 inhibition and knockdown abrogates oncogenic properties of TNBC cells. A: Suppression of cell growth upon *Nfatc1* knockdown determined by Celigo and **B:** crystal violet. **C:** Knockdown of *Nfatc1* was validated on mRNA and **D:** protein level. **E:** Reduced migration capacity upon *Nfatc1* knockdown assesed using Boyden chamber assay and **F:** scratch assay. **G:** Regulation of EMT markers upon *Nfatc1* knockdown on mRNA level,n=3 +/- SEM, * p-val ≤ 0.05, ** p-val ≤ 0.01, *** p-val ≤

0.005. **H:** FACS showing an increase of EpCAM positive cells upon inhibition of Nfatc1 via cyclosporine A treatment and decrease of EpCAM positive cells followed by Nfatc1 activation upon thapsigargin treatment.

Discussion

In contrast to early and locally constrained breast cancer, advanced metastatic disease is often considered as incurable (Harbeck et al., 2019). For this reason, therapies of cancers in advanced stages mainly focus on patient survival and life quality improvement. Because of the lack of specific therapeutic targets, treatment of advanced TNBC almost exclusively relies on the efficiency of cytotoxic therapies, while also being prone to resistance. Hence, a better understanding of the mechanisms leading to chemotherapy resistance represents a crucial step for the development of more efficient therapeutic approaches. In the present study, we utilized the murine WAP-T mammary carcinoma cell system to model and investigate molecular mechanisms underlying TNBC survival to conventional chemotherapy. Our transcriptome wide analyses showed that WAP-T cells activate transcriptional programs characteristic for EMT and cancer stem cells during survival to treatment. Interestingly, gain of epithelial-mesenchymal plasticity was shown to promote tumor cells invasiveness and protect them from pro-apoptotic signals (Kalluri & Weinberg, 2009; Scheel & Weinberg, 2011). Additionally, EMT and CSC properties are tightly linked together and have been frequently shown to positively influence each other (Hennessy et al., 2009; Loret, Denys, Tummers, & Berx, 2019; Mani et al., 2008). Notably, both EMT and CSC properties were implicated in the acquisition of chemotherapy resistant phenotypes by the tumor cells (Izumiya et al., 2012). In order to develop resistance, cell have to rapidly and profoundly reorganize their transcriptional programs. Therefore, because of their very dynamic nature, we expected epigenetic mechanisms to be involved. Combining mRNA-seq and ChIP-seq approaches, we identified a reduction of the PRC2/EZH2 activity occurring during chemotherapy survival in WAP-T cells. Interestingly, the repressive activity of EZH2 on gene expression is mostly known to promote cancer progression and contribute to therapy resistance in various types of cancer (Hirukawa et al., 2018; Hu et al., 2010; Kikuchi et al., 2015; Zhang et al., 2013). Specifically, EZH2 activity was implicated in resistance to programmed cell death in TNBC (J. P. Huang & Ling, 2017; P. Zhang et

al., 2018). Paradoxically, our results unraveled an opposite role of PRC2/EZH2 in TNBC cells, maintaining a more chemotherapy sensitive phenotype via specific repression of EMT and CSC transcriptional programs. Although apparently contradictory at the first glance, our results align with still scarce but growing evidences, that loss of PRC2/EZH2 activity can drive or support initiation and progression of cancers in a context specific manner (Vo et al., 2017; Wassef et al., 2015). In 2015, the group of Raphaël Margueron elegantly demonstrated that reduced EZH2 expression promotes transcriptional instability and is likely to promote breast tumorigenesis (Wassef et al., 2015). Shortly later, Serresi and colleagues showed in two consecutive studies on Non-Small-Cell-Lung Cancer that PRC2 activity can act as a barrier to KRAS-driven inflammation and EMT (Serresi et al., 2016). Our investigations on murine and human TNBC cell lines corroborated these observations and described thereby a new molecular mechanism by which PRC2/EZH2 can exert its repressive function on the EMT transcriptional program. Specifically, loss of PRC2 subunits upon chemotherapy treatment leads to a rapid upregulation of central EMT regulators via a repressive (H3K27me3) to activating (H3K27ac) epigenetic switch. Strikingly, we identified here NFATc1 as one of the major EMT-TF under the immediate epigenetic control of PRC2 in TNBC and upregulated in cells surviving chemotherapy. Interestingly, the group of Hessmann reported that NFATc1 is needed for pancreas during regeneration after injury and is epigenetically silenced by EZH2 activity once regeneration is completed, supporting the mechanism of regulation identified in the present study (N. M. Chen et al., 2017). The pivotal role of NFATc1 in the activation of EMT transcriptional programs in cancer cells and the availability of specific small molecule inhibitors (e.g. cyclosporine A or VIVIT) renders this factor a very interesting potential drug target to increase conventional therapies efficiency (Aramburu et al., 1999; F. Liu et al., 2009). In this study, we observed an increased efficiency of CAF treatment on TNBC cells when co-treated with cyclosporine A or VIVT. These results are in line with former studies on lung cancer, acute myeloid leukemia (AML) and bladder cancer showing that NFATc1 inhibition sensitized cancer cells to cisplatin, sorafenib- and tacrolius-induced apoptosis, respectively (Im et al., 2016; Kawahara et al., 2015; Metzelder et al., 2015).

Conclusions

This study presents the evidence of a context dependent PRC2/EZH2 function in breast cancer that in certain circumstances can sensitize the cells to chemotherapy by epigenetically repressing NFATc1 expression. Our data suggests that targeting NFATc1 signaling in TNBC patients with low EZH2 expression could increase the efficiency of conventional chemotherapeutic treatments and reduce the development of resistance.

Supplementary figures

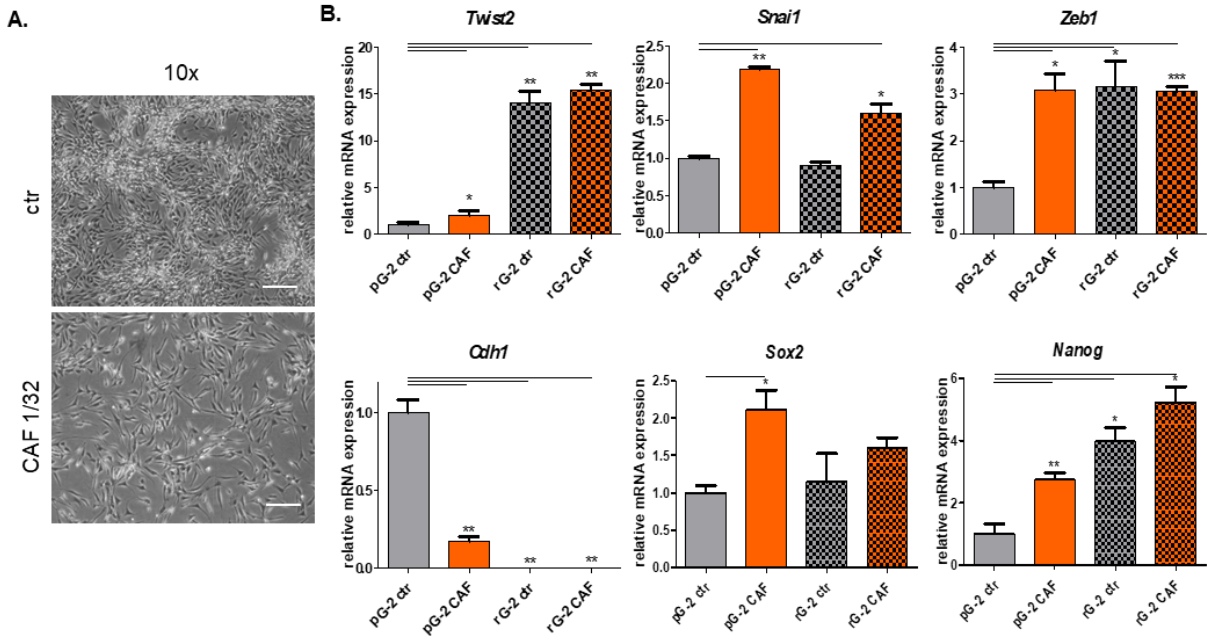


Fig. I S1 WAP-T cells surviving CAF treatment with EMT and stemness changes *in vitro.* A: Phase contrast images of rG-2 cells 48 hours after CAF-treatment show (objective 10x, scale bar = 250 µm). **B:** EMT markers expression on mRNA level in pG-2 and rG-2 cells, n=3, normalized to *Rplp0*, +/- SEM, * p-val ≤ 0.05, ** p-val ≤ 0.01, *** p-val ≤ 0.005.

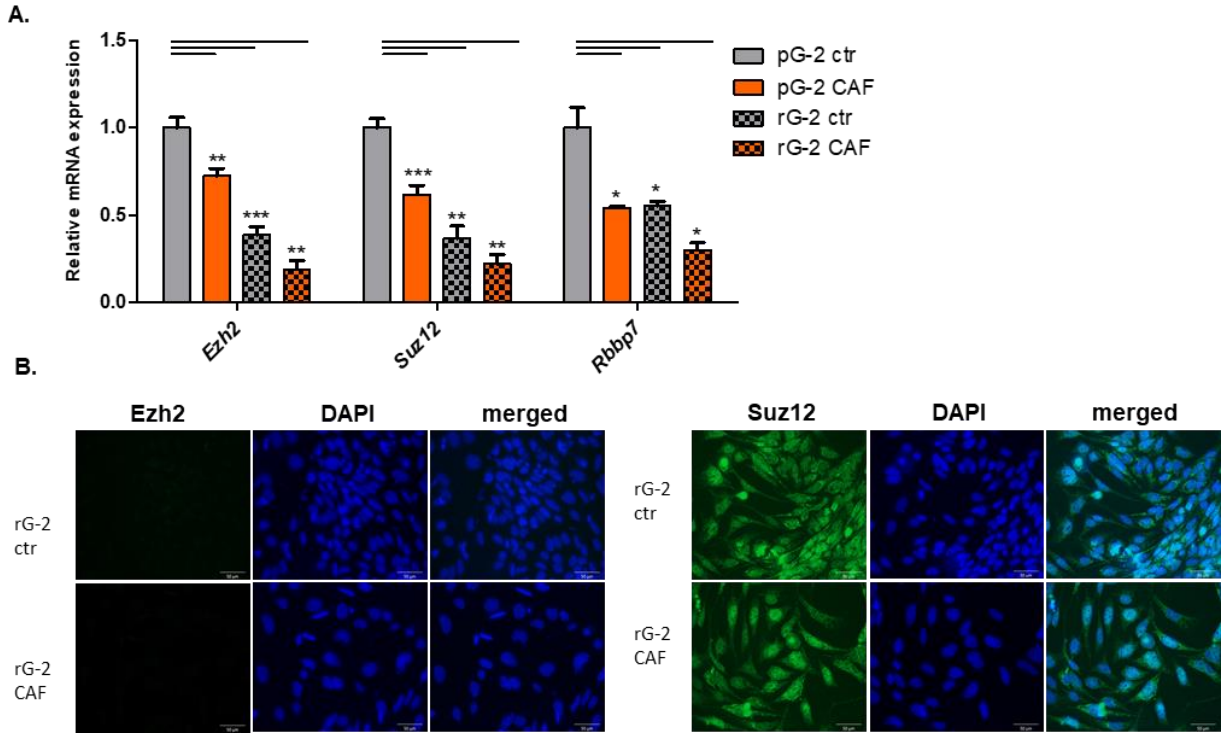


Fig. I S2 Regulation of PRC2 members in pG-2 and rG-2 cells upon 1/32 chemotherapy treatment. A: Relative mRNA expression of *Ezh2*, *Suz12* and *Rbbp7* upon 48 h of CAF treatment, n=3, normalized

to the *Rplp0* housekeeping gene, +/- SEM, * p-val ≤ 0.05, ** p-val ≤ 0.01, *** p-val ≤ 0.005. **B:** Immunofluorescence staining of Ezh2 and Suz12 in CAF-treated rG-2 cells, corresponding to Fig. I 2G.

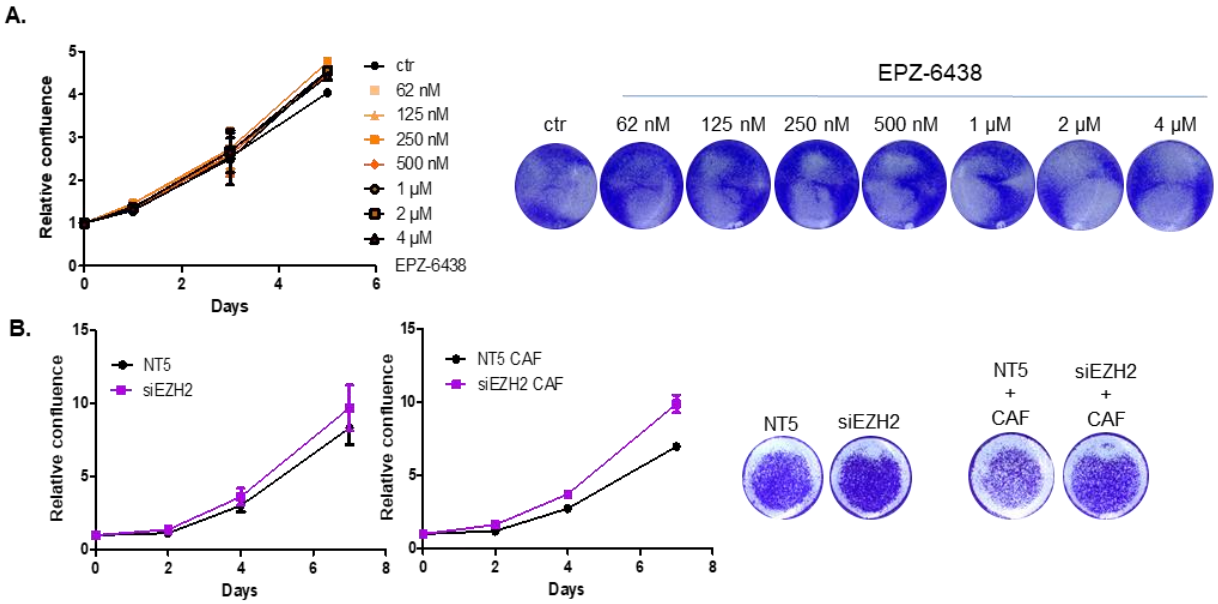


Fig. I S3 EZH2 inhibition and knockdown in MDA-MB-468 cells. A: Proliferation assay of EPZ-6438-treated MDA-MD-468 cells using Celigo (left) and crystal violet staining at day 7 (right). **B:** Proliferation measurement (left) and crystal violet staining (right) of cells upon *EZH2* knockdown with or without 1/32 CAF-chemotherapy treatment.

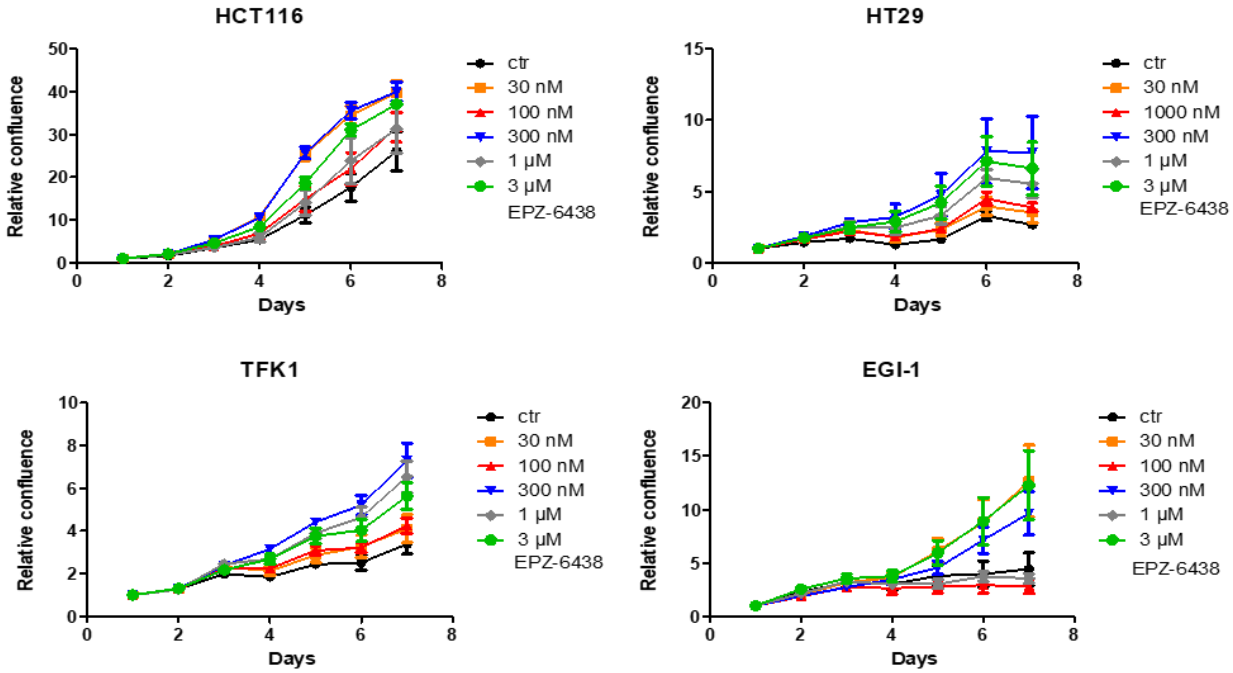


Fig. I S4 EZH2 inhibition in human colorectal and bile duct carcinoma cell lines. Proliferation assay of HCT116 and HT29 (colorectal cancer), TFK1 and EGI-1 (bile duct carcinoma) cells treated with increasing EPZ-6438 concentrations using Celigo (n=3).

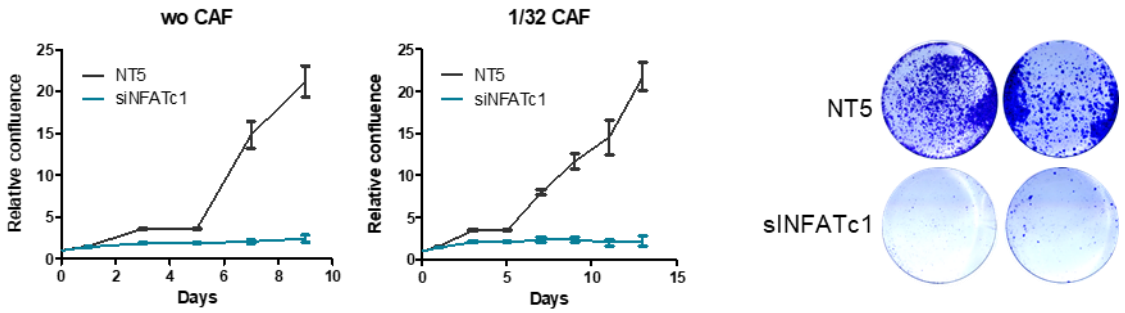


Fig. I S5 *NFATc1* knockdown in MDA-MB-468 cells treated with and without 1/32 CAF-chemotherapy. Proliferation assay of EPZ-6438-treated MDA-MD-468 cells using Celigo (left) and crystal violet staining at day 7 (right).

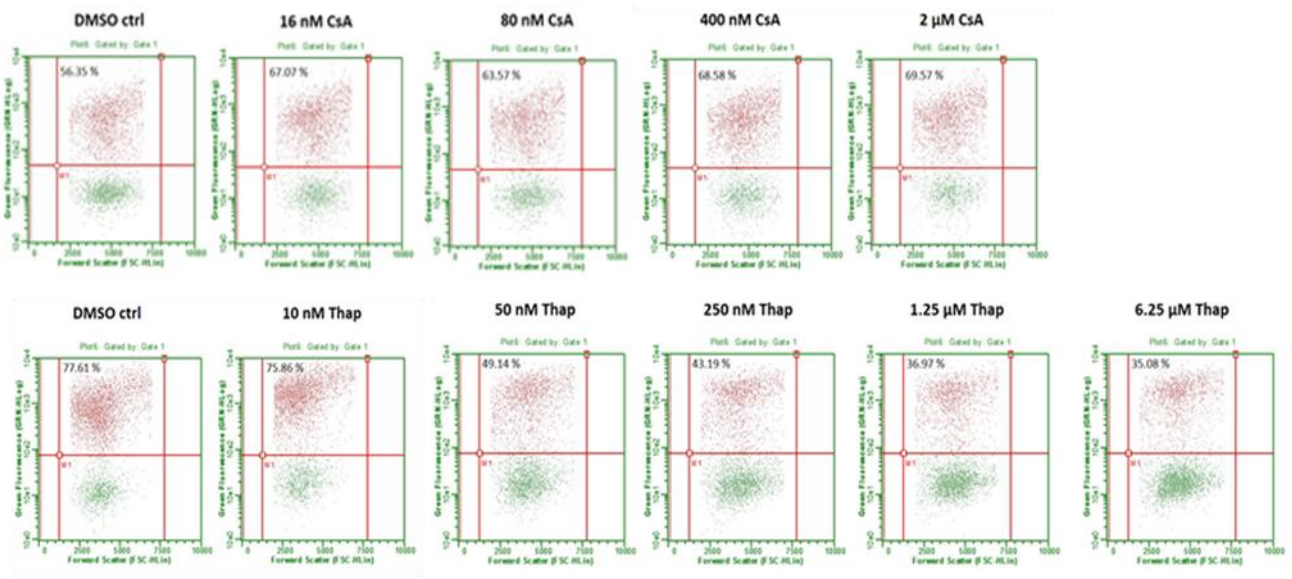


Fig. I S6: Inhibition and activation of NFATc1 leads to epithelial-mesenchymal phenotype changes in pG-2 cells. pG-2 cells were treated for 48 h with different concentrations of cyclosporine A (CsA) or thapsigargin (Thap), harvested and stained with the conjugated antibody FITC-EpCAM, and analyzed by flow cytometry.

Table I S1. Epigenetic regulators list

AIRE	CBX2	DPF2	JARID2	L3MBTL3	NSD1	PRDM 11	SETD1A	SMYD5
AOF2	CBX3	DPF3	JHDM1D	L3MBTL4	OGT	PRDM 12	SETD1B	SP100
ARID1A	CBX4	DPY30	JMJD1C	LEO1	PADI2	PRDM 13	SETD2	SP110
ARID4A	CBX5	DZIP3	JMJD2A	LRWD1	PADI4	PRDM 14	SETD3	SP140
ARID4B	CBX6	EED	JMJD4	MBD1	PAF1	PRDM 15	SETD4	SP140L
ASF1A	CBX7	EHMT1	JMJD6	MBD2	PBRM1	PRDM 16	SETD5	SRCAP
ASF1B	CBX8	EHMT2	JMJD7	MBD3	PCAF	PRDM 2	SETD6	SSRP1

ASH1L	CCDC101	ELP3	JMJD8	MBD4	PCGF1	PRDM4	SETD7	SUPT16H
ASH2L	CDC73	EP300	KAT2A	MBD5	PCGF2	PRDM5	SETD8	SUPT6H
ASXL1	CDY1	EP400	KAT2B	MBD6	PCGF3	PRDM6	SETD9	SUPT7L
ASXL2	CDY1B	EPC1	KAT5	MBTD1	PCGF5	PRDM7	SETDB1	SUV39H1
ASXL3	CDY2A	EPC2	KAT6A	MECP2	PCGF6	PRDM8	SETDB2	SUV39H2
ATAD2	CDY2B	EZH1	KAT6B	MEN1	PHC1	PRDM9	SETMAR	SUV420H1
ATAD2B	CDYL	EZH2	KAT7	MGMT	PHC2	PRMT1	SFMBT1	SUV420H2
ATRX	CDYL2	FANCL	KAT8	MINA	PHC3	PRMT10	SFMBT2	SUZ12
ATXN7	CECR2	FBXO10	KDM1A	MLLT10	PHF1	PRMT2	SHPRH	TADA3L
ATXN7L3	CHAF1A	FBXO11	KDM1B	MLLT6	PHF10	PRMT3	SIN3A	TAF1
BAZ1A	CHAF1B	G2E3	KDM2A	MORC3	PHF11	PRMT5	SIN3B	TAF1L
BAZ1B	CHD1	GADD45A	KDM2B	MORC4	PHF12	PRMT6	SIRT1	TAF3
BAZ2A	CHD1L	HAT1	KDM3A	MORF4L1	PHF13	PRMT7	SIRT2	TAF8
BAZ2B	CHD2	HDAC1	KDM3B	MORF4L2	PHF14	PRMT8	SIRT6	TDRD3
BMI1	CHD3	HDAC10	KDM4A	MPHOSPH8	PHF15	PROM1	SIRT7	TET1
BPTF	CHD4	HDAC11	KDM4B	MSL3L1	PHF16	PYGO1	SKB1	TET2
BRD1	CHD5	HDAC2	KDM4C	MTA1	PHF17	PYGO2	SMARCA1	TET3
BRD2	CHD6	HDAC3	KDM4D	MTA2	PHF19	RAG2	SMARCA2	TRIM24
BRD3	CHD7	HDAC4	KDM4E	MTF2	PHF2	RBBP4	SMARCA4	TRIM28
BRD4	CHD8	HDAC5	KDM5A	MYSM1	PHF20	RBBP7	SMARCA5	TRIM33
BRD7	CHD9	HDAC6	KDM5B	MYST2	PHF20L1	RING1	SMARCAD1	TRIM66
BRD8	CREBBP	HDAC7A	KDM5C	MYST3	PHF21A	RNF168	SMARCAL1	TRRAP
BRD9	CTR9	HDAC8	KDM5D	MYST4	PHF21B	RNF2	SMARCB1	UHRF1
BRDT	CXXC1	HDAC9	KDM6A	NAP1L1	PHF23	RNF20	SMARCC1	UHRF2
BRMS1	DIDO1	HIRA	KDM6B	NAP1L2	PHF3	RNF40	SMARCC2	USP22
BRMS1L	DMAP1	HLTF	KDM8	NAP1L3	PHF5A	RNF8	SMARCD1	UTY
BRPF1	DNAPTP3	HTATIP	KMT2A	NAP1L4	PHF6	RSF1	SMARCD2	WDR82
BRPF3	DNMT1	ING1	KMT2B	NAP1L5	PHF7	RUVBL1	SMARCD3	WDR5

BRWD1	DNMT3A	ING2	KMT2C	NAP1L6	PHF8	RUVBL2	SMARCE1	WHSC1
BRWD3	DNMT3B	ING3	KMT2D	NAT10	PHIP	RYBP	SMYD1	WHSC1L1
C14orf169	DNMT3L	ING4	KMT2E	NCOA1	PHRF1	SCMH1	SMYD2	ZCWPW1
CARM1	DOT1L	ING5	L3MBTL1	NCOA2	PRDM1	SCML2	SMYD3	ZCWPW2
CBX1	DPF1	INTS12	L3MBTL2	NCOA3	PRDM10	SET	SMYD4	ZMYN11
								ZMYND8

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4.2. Chapter II Report

Therapeutical potential of HDACs in chemotherapy resistance in triple-negative breast cancer

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Introduction

The most commonly diagnosed cancers in women are malignancies of the breast (World Health Organisation, 2018). Triple-negative breast cancer (TNBC) is the most aggressive and heterogeneous breast cancer subtype (Perou et al., 2000). Due to lack of estrogen receptor (ER) progesterone receptor (PR) and human epidermal growth factor 2 (HER2) expression, chemotherapy treatment is one of the most common therapy in TNBC. Conventional chemotherapy including platinum agents, such as carboplatin and cisplatin, paclitaxel or 5-fluorouracil before or after surgical resection remain the standard approach in the clinic (Isakoff, 2010; Sirohi et al., 2008).

Unfortunately, cancer relapse occurs quite frequently among TNBC patients followed by chemotherapy (O'Reilly et al., 2015). Epigenetic changes are involved in chemotherapy resistance as many studies and our previous data report (Mieczkowska et al., 2019, unpublished, J. Han et al., 2019; Pineda et al., 2019). Histone deacetylases (HDACs) are a class of epigenetic regulators very frequently altered in cancers including breast cancer (Krusche et al., 2005; K. M. Sakamoto & Aldana-Masangkay, 2011; Shan et al., 2017). HDACs modulate the gene expression by among other controlling the deacetylation/acetylation balance at lysine residues of histones, together

with histone acetyltransferases (HAT), (Grunstein, 1997). The deacetylation of histones leads to chromatin condensation resulting in repression of transcription (Roth et al., 2001). We can distinguish four classes of HDAC family: class I (HDAC1, -2, -3 and -8), class IIa (4, -5, -7 and -9), IIb (HDAC6 and -10), class III (sirtuins) and class IV (HDAC11) (Gregoretti et al., 2004; Seto & Yoshida, 2014). HDAC-triggered abnormality in pivotal gene expression can lead to tumor invasiveness (Richon et al., 2000). As a consequence, anti-cancer potential of therapeutic strategies targeting HDACs have been several times demonstrated *in vitro* and *in vivo*, therefore being very attractive for the development of novel drugs (Bolden et al., 2006). The best studied and FDA-approved pan-HDAC inhibitors are Vorinostat (SAHA) and Panobinostat (LBH-589), both strongly targeting HDACs class I and II (Duvic et al., 2007; San-Miguel et al., 2013; Stahl et al., 2016). HDACs inhibitors are clinically used for cutaneous T-cell lymphoma (CTCLs) (Lopez et al., 2018). Using HDACs inhibitors improved patient outomes with myeloma. However, beneficial therapeutical effects were not obtained in solid tumors (Bolden et al., 2006).

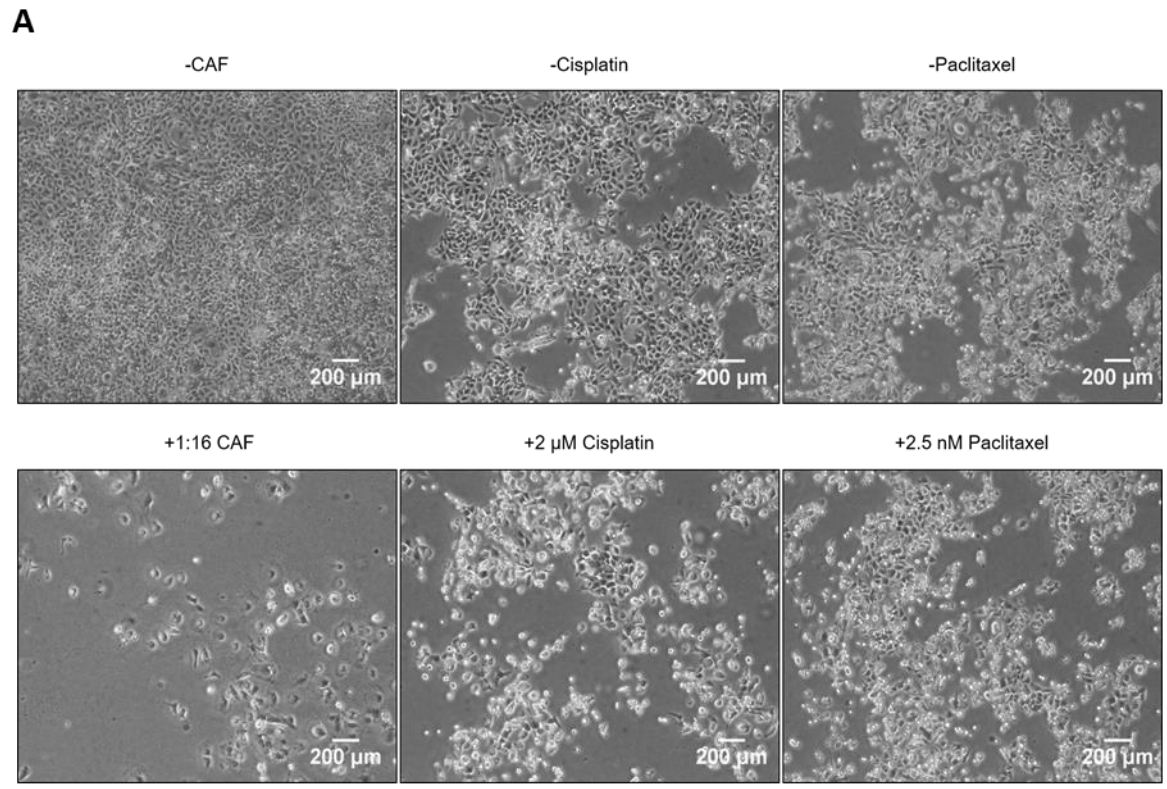
In TNBC, SAHA was reported as the most promising therapeutic treatment, however its efficiency is limited as a single drug (Garmpis et al., 2017). In the present project, we identified an upregulation of *Hdac4*, *Hdac7* and *Hdac8* in murine WAP-T TNBC cells surviving a cytotoxic therapy. We therefore aimed to study the potential of treatments specifically targeting one of these HDACs and to determine possible benefits of a combination with chemotherapy in TNBC.

Results

CAF chemotherapy-induced EMT changes in TNBC

We previously showed that WAP-T cells (pG-2) surviving a CAF (Cyclophosphamide, Doxorubicin/Adriamicin, and 5-Fluorouracil/5-FU) chemotherapy treatment adopted a more mesenchymal phenotype, pointing at an involvement of epithelial-to-mesenchymal transition program in the chemotherapy resistance (Mieczkowska et al., 2019, manuscript under submission). To determine if human TNBC cells undergo similar transcriptional program changes upon survival to cytotoxic

therapies, HCC1806 cells where treated with increasing concentrations of a CAF-chemotherapy as well as cisplatin and paclitaxel. Concentration of 2.5 µM cisplatin, 2.5 nM paclitaxel and 1/16 CAF (30 ng/ml Cyclophosphamide, 0.62 µg/ml Doxorubicin, 0.62 µg/ml 5-FU) showed the optimal results for our purpose (Fig II S1), killing the vast majority of the cells, while allowing the regrowth of more resistant cell phenotypes (Fig II 1A). Strikingly, gene expression analyses of treated HCC1806 cells identified an increased expression of mesenchymal markers (*SNAI1*, *N-CADHERIN*, *VIMENTIN*) in cells surviving the different treatments, pointing at the occurrence of an EMT also in the human context (Fig. II 1B).


A
-CAF
-Cisplatin
-Paclitaxel
200 µm
+1:16 CAF
+2 µM Cisplatin
+2.5 nM Paclitaxel

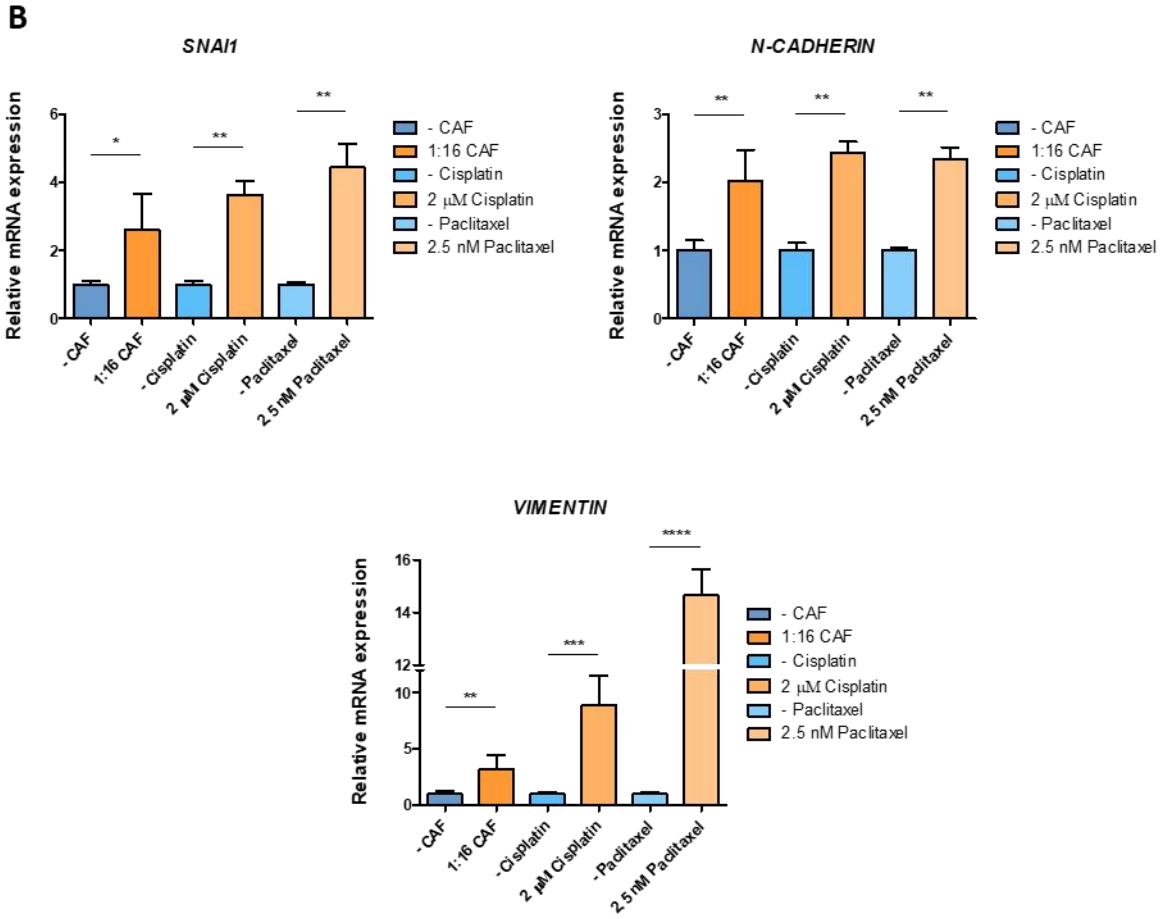

B
SNAI1
N-CADHERIN
VIMENTIN
Relative mRNA expression
- CAF
1:16 CAF
- Cisplatin
2 µM Cisplatin
- Paclitaxel
2.5 nM Paclitaxel

Figure II 1. A: Human TNBC cells undergo EMT upon survival to different cytotoxic treatments. 24 hr after seeding, the cells were treated with either 1/16 CAF (30 ng/ml Cyclophosphamide, 0.62 µg/ml Doxorubicin, 0.62 µg/ml 5-FU), 2 µM cisplatin or 2.5 nM paclitaxel for 48 hours. **A**: bright field pictures of HCC1806 upon various chemotherapy treatments. Microscope: Nikon ECLIPSE TS100-F with 4x objective. **B**: Gene expression analysis of EMT markers in HCC1806 cell line upon treatment, assessed by qRT-PCR. mRNA expression values were normalized to the housekeeping gene expression *Rplp0* and calibrated to the respective untreated controls. Average mRNA expression values are given ± standard deviation (SD). Experiments were conducted in biological duplicates (n = 2) with technical triplicates. *: $p < 0.05$; **: $p < 0.01$.

In our previous work, mRNA-sequencing (mRNA-seq) analyses were performed to study the mechanisms involved in WAP-T cell survival to chemotherapy (Mieczkowska et al., 2019, manuscript under submission). We thereby identified the enrichment of EMT and cancer stem cell (CSC) signatures signature accompanying more resistant WAP-T cancer cell phenotypes. Interestingly, signatures pointing at epigenetic dysregulations were enriched. As epigenetic regulatory pathway are known to control EMT- and CSCs – transcriptional programs (Lu & Kang, 2019) we focused on the regulation of epigenetic factors upon CAF treatment. Here, we observed that the majority of these factors were down-regulated (n=64) and that only a few were upregulated (n=16). We notably observed that *Hdac4*, *Hdac7* (class IIa) and *Hdac8* (class I) figured among the upregulated genes. *Hdac4, -7* and *-8* overexpression was reported to be associated with poor survival and tumor invasiveness (Hsieh et al., 2016; Zeng et al., 2016; Zhu et al., 2011). We performed qRT-PCRs on pG-2 and rG-2 cells, a chemoresistant resistant variant of the parental pG-2 cell line (Mieczkowska et al., 2019, manuscript under submission), to validate our findings. Indeed, CAF treatment of pG-2 cells increased mRNA expression levels of *Hdac4*, and its levels were maintained high in the resistant cells, independent of treatment. *Hdac7* was almost three times upregulated in treated parental cells. Interestingly, rG-2 cells displayed sensibly the same high levels of *Hdac7* expression as treated pG-2 cells and its expression even stronger upon cytotoxic treatment. Finally, levels of *Hdac8* were only moderately upregulated upon CAF treatment of parental cells and its levels were only significantly higher in the treated resistant cells. Taken together, we confirmed the upregulation of *Hdac4*, *7* and *8* upon cytotoxic treatment and observed that high levels of these genes are associated with resistant phenotypes. We therefore asked if

interfering with these factors via siRNA mediated silencing would impact the growth and resistance properties of these cells. We therefore performed crystal violet staining and found that loss of *Hdac4*, *-7* and *-8* slightly reduced the proliferation of pG-2 cells and only marginally the proliferation of rG-2 cells, when cultured under normal conditions. Strikingly, the proliferation of pG-2 and rG-2 was much stronger affected by siRNA treatment when co-treated with low doses of CAF (Fig. II 2C), pointing at a sensitization of the cells to the chemotherapy.

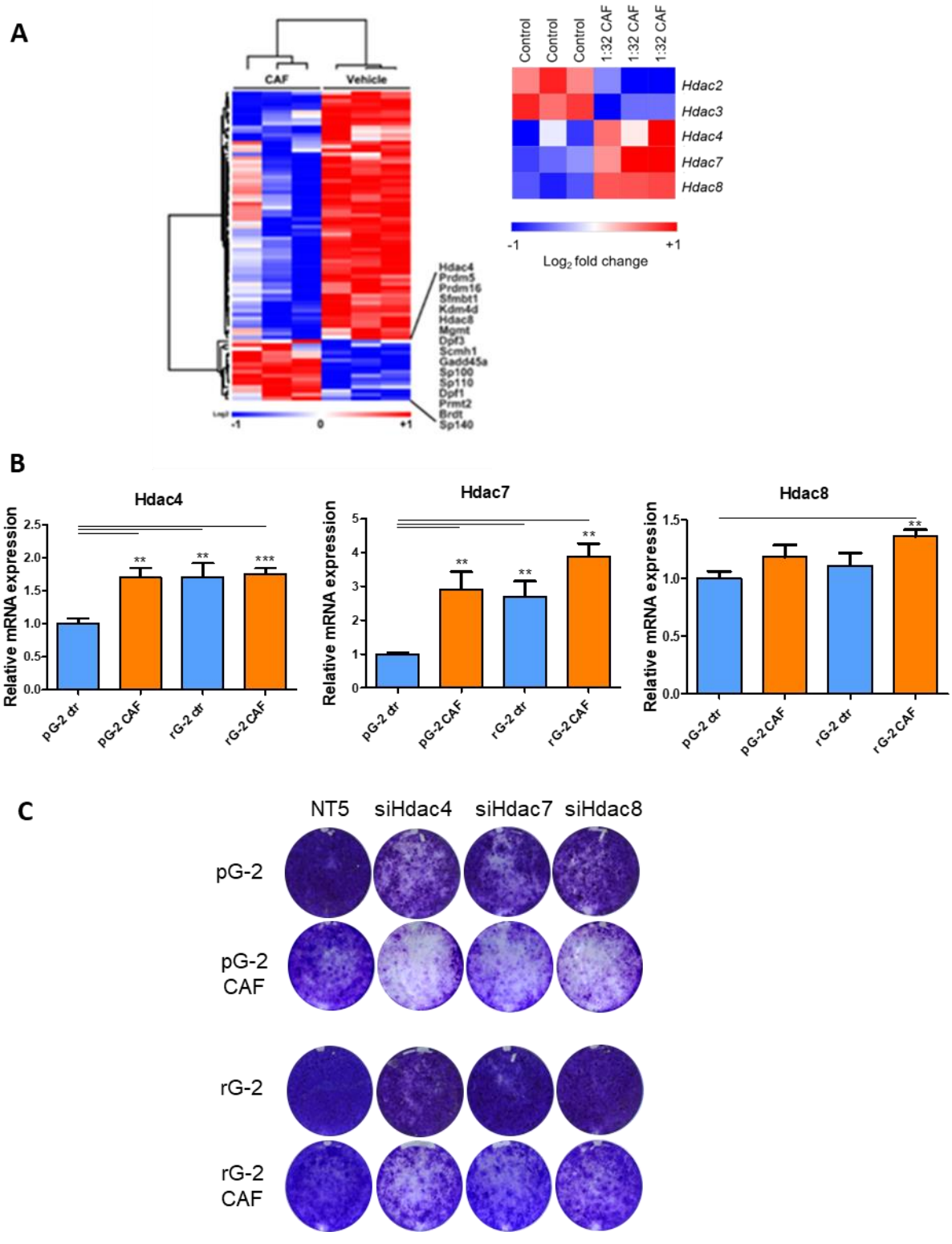


Figure II 2. Upregulation of *Hdac4*, *-7* and *-8* upon CAF-chemotherapy treatment in G-2 cells is necessary for their survival. A: Heatmaps showing epigenetic factors (left panel) and several *Hdac*

genes (right panel) significantly regulated upon 48 hours CAF treatment (312,5 ng/ml cyclophosphamide, 15,6 ng/ml doxorubicin and 312,5 ng/ml 5-FU) in pG-2 cells (*p* adjusted value < 0.05, n = 3 biological replicates). Expression values are displayed as Z-score. **B**: Validation of *Hdac4*, *Hdac7 and Hdac8* gene regulation in pG-2 and rG-2 cells (ctr) upon 48 hours CAF. mRNA expression values were normalized to the housekeeping gene *Rplp0* and to the respective untreated controls. Average mRNA expression values are given ± standard deviation (SD). Experiments were conducted in biological duplicates (n = 3). T-test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. **C**: Proliferation assay using crystal violet staining upon *Hdac4*, *Hdac7 and Hdac8* knockdown in pG-2 and rG-2 cell lines with or without CAF treatment (CAF), n=2 biological replicates.

EMT modulation and TNBC cell survival upon *HDAC4, -7* and *-8* loss

To extend our results to the human situation, we examined the influence of *HDAC4, -7* and *-8* loss in human HCC1806 cells. Decreased cell growth rate under normal conditions was observed upon *HDAC4, -7* and *-8* knockdown (Fig. II 3A). Interestingly, in a similar manner as for murine cells, we observed a clear sensitization of the human TNBC cells to low concentration of CAF treatment (1/256) when combined with *HDAC4, -7* and *-8* knockdown (Fig. II 3B). We next asked whether depletion of HDAC4, 7 or 8 in human cells could also result in an impairment of the EMT transcriptional program. Surprisingly, we observed that solely *HDAC8* loss lead to the downregulation of the mesenchymal markers (*SNAI1, ZEB1, SLUG*) (Fig. II 3C).

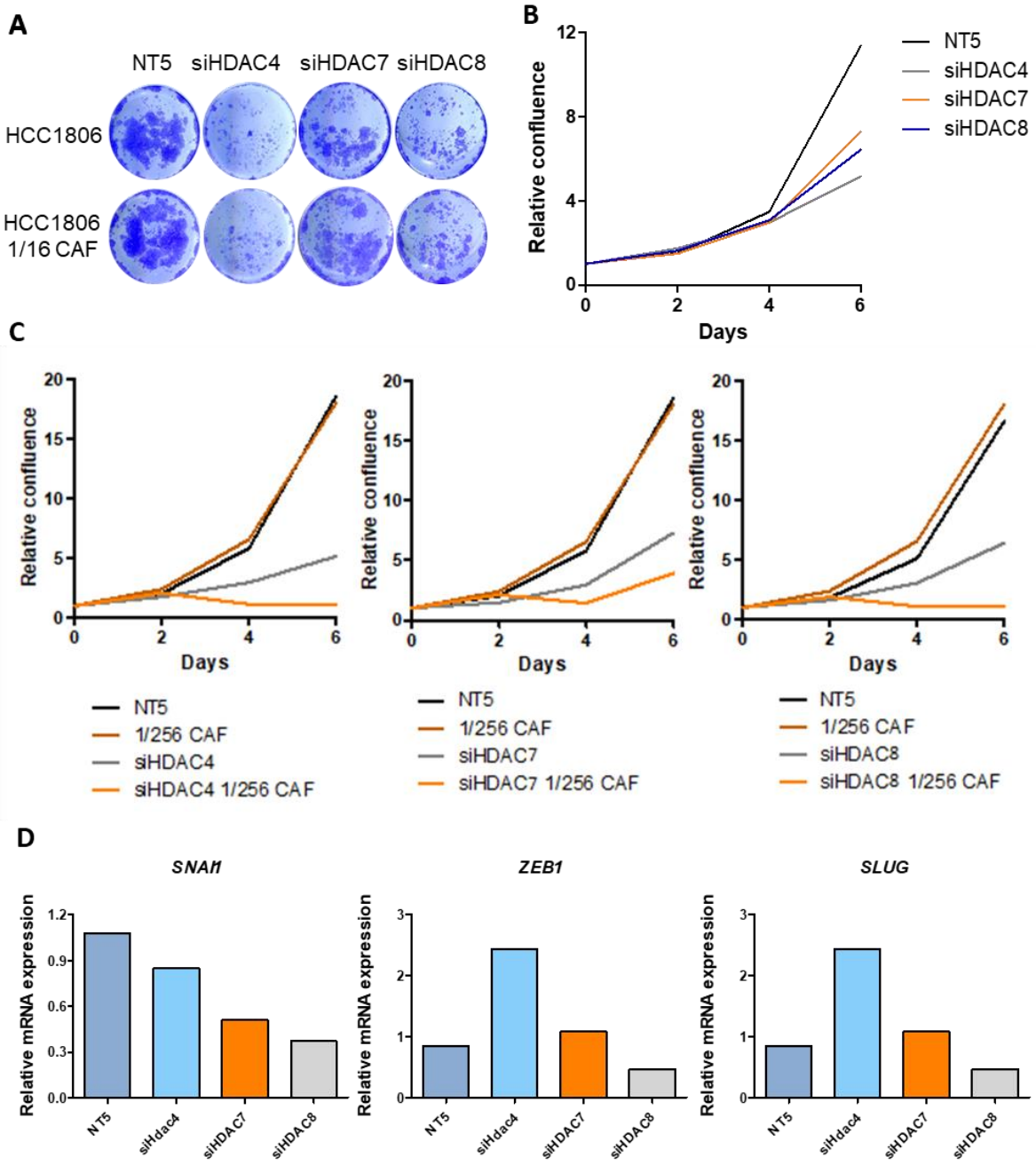


Figure II 3. Impact of *HDAC4*, *-7* and *-8* loss in HCC1806 cells. A and B: Proliferation assays of HCC1806 cells upon *HDAC4*, *-7* and *-8* knockdown with or without CAF-treatment (30 ng/ml Cyclophosphamide, 0.62 µg/ml Doxorubicin, 0.62 µg/ml 5-FU), assessed via crystal violet staining (A) and Celigo cell cytometer measurement**. C:** Proliferation assays of HCC1806 cells upon *HDAC4*, *-7* and *-8* knockdown with or without low dose of CAF-treatment (1/256), assessed via Celigo **D:** Gene expression analysis of EMT markers in HCC1806 cell line using qRT-PCR. mRNA expression values were normalized to *Rplp0* and to the respective untreated controls. Average mRNA expression values are given ± standard deviation (SD), n=1, technical triplicates.

Following, we examined the potential of a co-treatment with chemotherapy and HDAC inhibitors in TNBC. Although silencing of *HDAC4* and *HDAC7* sensitized pG-2 and HCC1806 cells to cytotoxic therapies, the well-established HDAC class II inhibitors TMP269 and TMP195 inhibiting both HDAC4 and -7 could not impair cell viability when combined with CAF, cisplatin or paclitaxel (data not shown). As only *HDAC8* loss reduced EMT induction in HCC1806, we decided to investigate the clinical potential of HDAC8 inhibition using PCI-34051 in combination with cytotoxic drugs in pG-2 (Fig. II 4 A) and rG-2 cells (Fig. II 4B). In pG-2 cells, we could observe a sensitization to the therapy when PCI-34051 and CAF-chemotherapy were combined. Moreover, HDAC8 inhibition alone and in combination with CAF lead to significant cell growth impairment in the resistant rG-2 cells. Together, our findings identified HDAC8 as an attractive target to increase efficiency of cytotoxic therapies in TNBC.

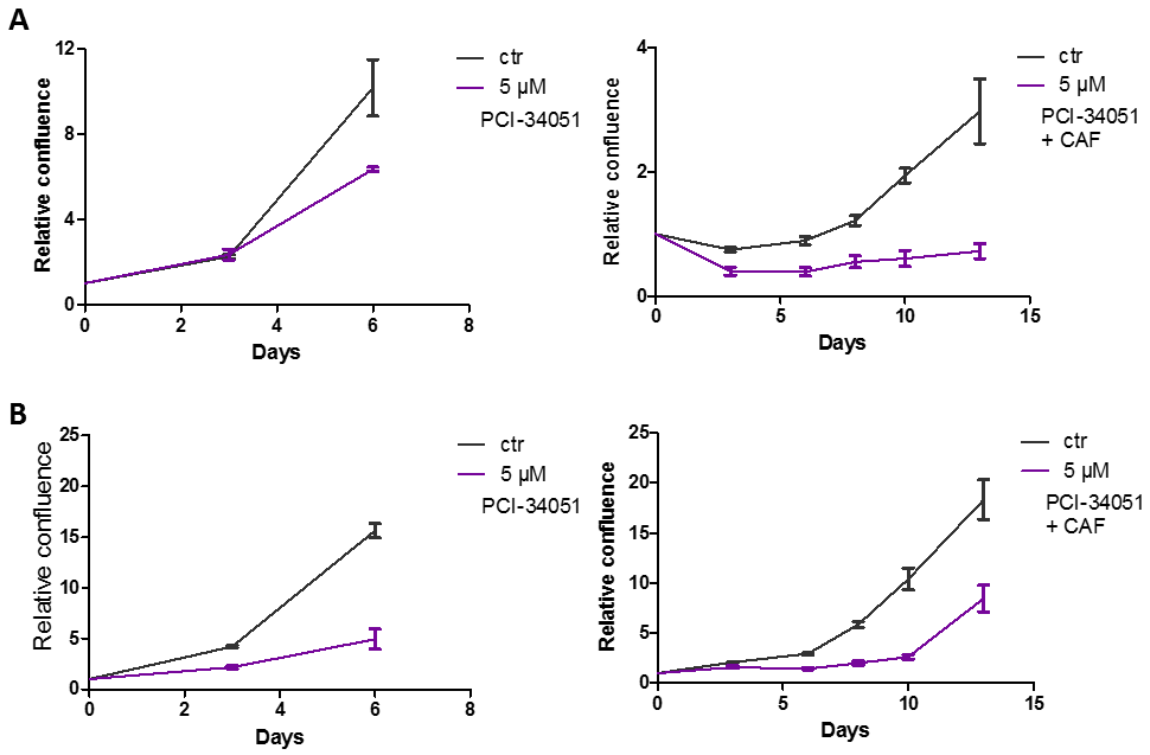


Figure II 4. Suppression of cell growth upon HDAC8 inhibition in murine TNBC cell lines. A and B: Cell proliferation assay in pG-2 (**A**) and rG-2 cells (**B**) upon 5 µM PCI-34051 treatment alone (left panel) or combined with 48 hours CAF-chemotherapy (1/32), followed by fresh medium culturing G-2 cells. Proliferation measurements determined by Celigo, n=3 +/- SEM.

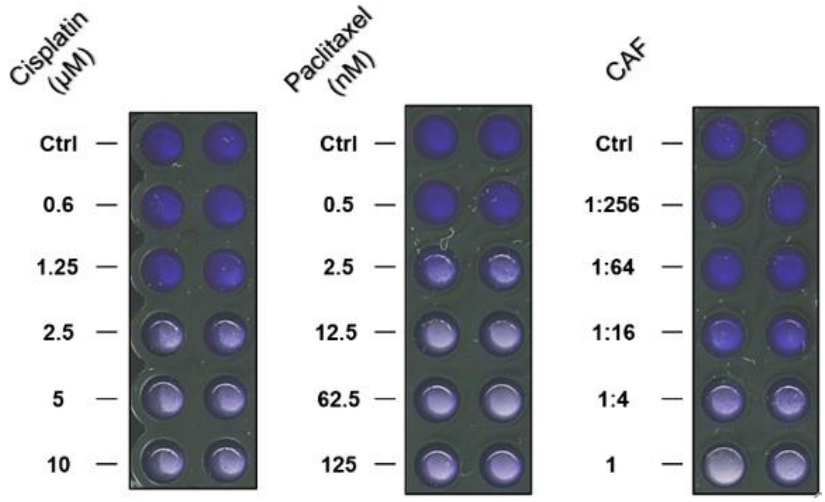


Figure II S1. Proliferation assay using crystal violet staining upon different chemotherapy concentrations in HCC1806 cell line. Cells were treated with chemotherapy 24 hours after seeding and were allowed to grow over 1 week. Concentration [1] for CAF is representing 0.5 µg/ml cyclophosphamide, 10 µg/ml doxorubicin, and 10 µg/ml 5-FU combination.

Discussion

TNBC is one of the most difficult cancers to treat, due to its heterogeneity and high cancer relapse rate (World Health Organisation, 2018). EMT was identified as a mechanism involved in overcoming cytotoxic treatments in TNBC cancer cells. EMT is cellular process modulated through epigenetic modifiers, like HDACs (R. Chang, You, & Zhou, 2013; Lei et al., 2010). Despite first promising results, where all analyzed HDACs (-4, -7, -8) affected proliferation of TNBC cells, *HDAC4* and *-7* loss did not switch cellular phenotype from mesenchymal to epithelial state. Additionally, inhibition of HDAC4 and -7 with or without chemotherapy combination did not synergize. Instead, among HDACs, the most promising anticancer target in TNBC seems to be HDAC8. Loss of HDAC8 activity lead to proliferation impairment in mouse and human TNBC, *in vitro*, that was also shown in neuroblastoma (Rettig et al., 2015). In our project we used PCI-34051 as a commercially available selective HDAC8 inhibitor, that is recommended for T-cell lymphoma and leukemia cells (Balasubramanian et al., 2008). Interestingly it showed cell survival impairment alone and increased sensitivity in

combination with CAF-chemotherapy treatment. It would be intriguing to validate this effect in combination with other chemotherapy agents, like paclitaxel and cisplatin, where EMT regulation was increased even more than in the case of CAF. We could observe that only *HDAC8* loss but not *HDAC4* or *HDAC7* loss regulated EMT state. For further studies one could determine what are the EMT changes upon HDAC8 inhibition in combination with chemotherapy treatment. Nowadays, pan-HDACs inhibitors are the most commonly studied (Singh et al., 2018). Despite the promising *in vitro* and *in vivo* studies, inhibiting most of the HDAC family can bring unwanted side effects among patients (Subramanian et al., 2010). The development of selective HDAC inhibitors is a key issue in the clinic. The results of our study support the hypothesis that HDAC8 inhibitor could represent a promising approach to sensitize or re-sensitize TNBCs to conventional cytotoxic anti-cancer therapies, where therapy options are limited.

5. General discussion

5.1. PRC2/EZH2 in TNBC progression

More than 80% of breast cancer cases can be resected surgically, with 50% chance of cancer relapse among those patients (World Health Organization, 2006). Surgery can be followed by adjuvant chemotherapy to prevent cancer recurrence. Triple-negative breast cancer (TNBC) lacks targeted therapy options, consequently we wanted to elucidate TNBC invasion pathways upon chemotherapy as it is one of the most common therapy in TNBC patients (Wahba & El-Hadaad, 2015). In this project we are focused on TNBC, however similar mechanism of cancer cell escape due to chemotherapy treatment can be found in other cancers. Therefore, we aimed that our results could be applied in other cancer systems.

Cytotoxic stimuli enhances epigenetic alterations to gain self-renewal, mesenchymal and metastatic properties in tumor cells (Easwaran, 2014). In many studies, it was shown that PRC2/EZH2 plays a critical role in cancer progression and metastasis. Unsurprisingly, series of small molecule inhibitors of EZH2 have been developed. The methyltransferase EZH2 with its catalytic SET domain, transfers a methyl group from methyl donor, SAM (S-adenosyl-methionine) to lysine 27 on Histone H3 (H3K27). EZH2 inhibitors, blocking its SET domain, such as EPZ-6438, GSK2816126 and CPI-1205 are used, among others in phase II clinical trials in non-Hodgkin lymphoma (Gulati, 2018). However, EZH2-inhibition-based clinical trials can result in therapeutic response failure (NCT02082977 , 2019), necessitating a greater attention for anti-EZH2 therapeutic approaches, maximizing benefits for cancer patients. In our project, CAF-chemotherapy leads to an epigenetic dysregulation in TNBC cells. To our surprise, we observed unexpected PRC2 components loss favoring better survival of TNBC cells. Growing number of studies confirm our data, resulting in raising concerns about EZH2 as anti-cancer target. (Völkel et al., 2015).

To this date, TNBC patient cases remain a huge challenging clinic hurdle. Many trials on patients failed after EZH2 inhibition therapy. Treatment of some patients with Non-Hodgkin Lymphoma (NHL) during Phase I clinical trial, with EPZ6438 (small molecule EZH2 inhibitor) resulted in cancer recurrence (Italiano et al., 2018). In

malignant myeloid diseases, loss-of-function EZH2 mutations were identified. They determined that loss of EZH2 and reduced H3K27me3 level were associated with neoplastic disorders and leukemia progression (Muto et al., 2013). Interestingly, in hypoxic conditions, hypoxia-inducible factor 1 (HIF1-α) induction leads to PRC2 inactivation. Released EZH2 from the complex, cooperates with Forkhead box M1 (FoxM1) leading to direct MMPs promoters regulation and TNBC invasion (Mahara et al., 2016)

TNBC shares molecular similarities with high-grade serous ovarian tumors (Bell et al., 2011; Wang et al., 2012). Based on TCGA data, both display frequent *p53* mutations, *BRCA1* inactivation, *RB1* loss and *cMYC* amplification correlating with TNBC features. We observed that upon EZH2 inhibition TNBC cancer cells can progress and induce an even worse prognosis than before treatment. In our studies we show similar cell behavior of TNBC (pG-2 and MDA-MB-468) cells such as in colon cancer (HCT116 and HT-29) and bile duct (EGI-1 and TFK-1) carcinoma cell lines, where EZH2 inhibition lead to better cell growth. As we presented, TCGA-based data on TNBC patients indicates low survival rate in cancers with low EZH2 expression. Thus, it seems that the regulation through PRC2/EZH2 could be subtype-specific or context-dependent. Those data suggest a novel strategy in the treatment finding: it is indeed attractive to hypothesize that the same or very similar molecular patterns within cancers or their subtypes, could offer common clinical approach for category of patients with low EZH2 expression in cancer cells (Figure 12).

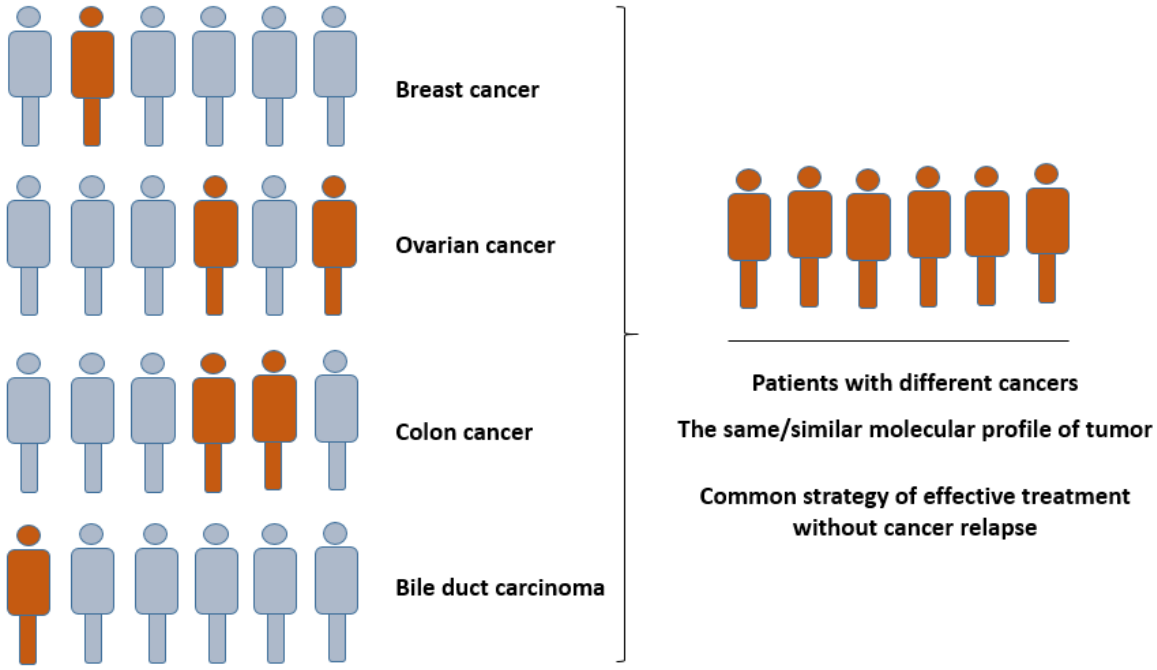


Figure 12: A scheme of the patients with distinct cancer types but similar treatment strategy and response. Orange indicates patient with common anti-cancer therapy strategy, grey reflects the patient with response to different anti-cancer treatment. Selected population of patients with distinct cancers benefited from the same clinical approach (orange, right).

5.2. NFATc1 as a potential PRC2/EZH2-dependent driver of TNBC progression

PRC2/EZH2-dependent activity is an important mechanism to repress gene transcription. Our data shows that PRC2/EZH2 is downregulated upon chemotherapy. Under cytotoxic stimuli, decreased PRC2/EZH2 activity is likely to lead to the activation of previously repressed genes, being crucial for cancer cell fate. Loss of H3K27me3 and gain of H3K27ac on promoter regions can directly activate targeted genes (Grimaldi et al., 2011). As chemotherapy is the most common way to treat TNBC displaying the highest response rate among breast cancer patients, we wanted to explore the mechanism that drive cell survival followed by chemotherapy. We identified Nfatc1, Wnt9a, Gli2 and Klf4 as the most relevant PRC2/EZH2-regulated genes that could be involved in chemotherapy resistance.

A number of studies have shown that NFATc1 favors cancer progression. For instance, overexpression or constitutively active NFATs are commonly linked with

cancer progression involving angiogenesis and migration (Qin et al., 2014). The NFAT/calcineurin pathway is associated with increased invasion of mammary tumor cells (Yoeli-Lerner et al., 2009), as seen also in our TNBC system. Interestingly, breast cancer subtype-based gene expression analysis revealed that NFAT-related pathway is more frequent in TNBC in comparison to non-TNBC patients (Tran Quang et al., 2015). In our study, we showed that EZH2 is negatively correlated with NFATc1 *in vitro* and *in vivo*. *NFATc1* loss lead to impaired cell proliferation and migration in our WAP-T *in vitro* system, indicating the involvement of NFATc1 in TNBC progression. Additionally, cell death in human TNBC cell line upon *NFATc1* depletion suggests that NFATc1 plays essential role in cancer survival. In our data, we observed enrichment for calcineurin pathway in CAF-treated cells involving deregulation of NFATc1.

Intriguingly, NFATc1 was found as a promising anti-leukemia target. For instance, in Chronic Lymphocytic Leukemia (CLL), CsA- and FK-506-mediated NFATc1 inhibition induced apoptosis of CLL cells (Wolf et al., 2014). In Acute Myeloid Leukemia (AML) NFATc1 was proposed as a therapeutic target to overcome resistance (Metzelder et al., 2015). These studies and many other publications regarding blood cancers strongly support our results in TNBC (Pham et al., 2010). As mentioned previously, PRC2/EZH2 loss-of-function was indicated to enhance cancer progression in leukemia and lymphoma. This finding brings a potential into therapy of blood cancers and TNBC, where PRC2/EZH2-mediated NFATc1 regulation could play fundamental role in overcoming cancer progression (Figure 13).

Based on our results, PRC2/EZH2-dependent *NFATc1* regulation can modulate cancer cell behavior through EMT. In previous studies, using the WAP-T model *in vivo* and *in vitro*, TGF-ß pathway activation was shown to increase aggressiveness of WAP-T tumor cells by inducing EMT (Maenz et al., 2015). Moreover, the group of Hessmann demonstrated a synergy between NFATc1 and TGFß1 signaling inducing pancreatic cancer progression by inhibiting apoptosis and growth arrest (Hasselluhn et al., 2019). TGFß1 signaling is also increased upon CAF treatment of G-2 cells (RNA-seq analyses, data not shown). It can be therefore hypothesized that both pathways cooperate with each other to stimulate cancer cell survival. Further studies would be necessary to prove this idea. In our studies, we determined that NFATc1 activity

contributes to the more mesenchymal cell properties. Loss or inhibition of NFATc1 reversed this process, resulting in an increased epithelial cell fraction. These results are in line with former observations that NFATc1 is involved in the regulation of epithelial-to-mesenchymal plasticity and stemness (Gould et al., 2016) It is therefore tempting to speculate that reversal of an invasive phenotype via NFAT inhibition may represent a clinically beneficial approach for TNBC patients.

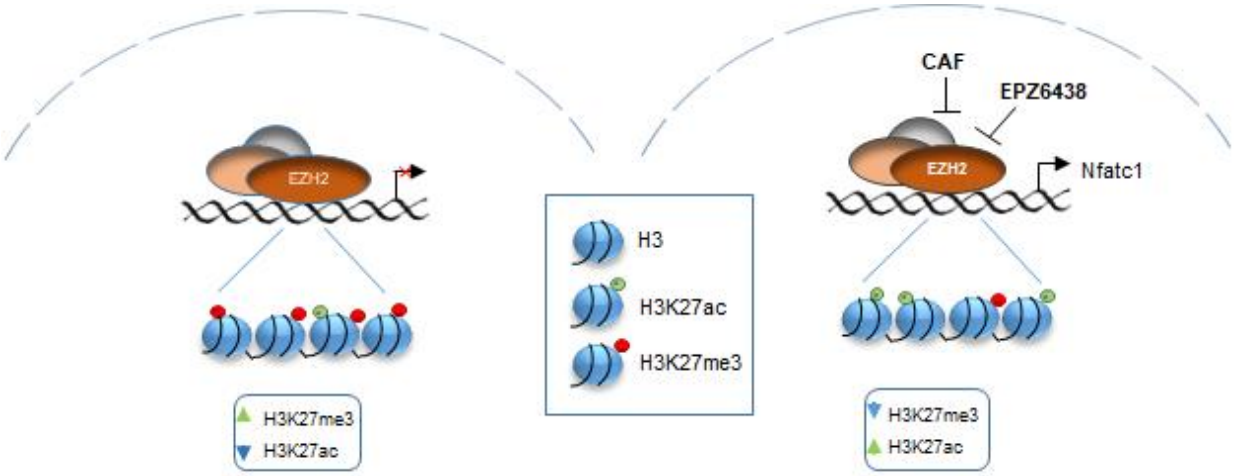


Figure 13: PRC2/EZH2 downregulation upon CAF-chemotherapy or EPZ-6438 treatment leading to loss of H3K27me3 and gain of H3K27ac activating *NFATc1*.

5.3. Gli2, Klf4, Wnt9a in cancer

Apart of NFATc1, other factors were identified as being under control of the PRC2 repressive activity. One of these is Gli2, a transcription factor involved in hedgehog (Hh) pathway (Méthot & Basler, 2001). Many studies support contribution of Hh signaling in cancer progression (Cannonier et al., 2016; D. Huang et al., 2018; N. Li et al., 2018). Gli2 leads to increased invasiveness, migration, angiogenesis and drug resistance in breast cancer (Atwood et al., 2015; Gupta et al. 2015; Han et al., 2016). Interestingly, its the most significant pro-tumorigenic function was reported in TNBC (Habib & O'Shaughnessy, 2016). Gli2 is potential PRC2/EZH2-mediated factor involved in TNBC progression. Hh along with Wnt and TGF-β signaling contributes to

EMT and increased stemness capacity in breast, ovarian and pancreatic cancer (Morel et al., 2008; Noubissi et al., 2018; Scheel et al., 2011). Additionally, the Hh pathway is associated with drug resistance where its enrichment was observed in cancer cells resistant to paclitaxel and doxorubicin (Narita et al., 2008; Zhou et al., 2012). These findings support our results and suggest that PRC2-dependent Gli2 regulation could be additional or cooperating mechanism with PRC2-NFATc1 pathway leading to TNBC cell survival.

Together with hedgehog pathway, Wnt/beta-catenin signaling pathway was reported to promote cell proliferation and stemness maintenance (Mullor et al., 2001; Noubissi et al., 2018). Wnt/beta-catenin can lead to drug resistance and cell immortality through upregulation of MMP7 and hTERT, respectively. Wnt9a was identified as a major player in colon tumorigenesis (Bhattacharyya, Feferman, & Tobacman, 2014). There is no data describing Wnt9a in TNBC progression. However, based on our data Wnt9a could be interesting gene candidate to investigate in chemotherapy resistance in TNBC.

Apart of *NFATc1*, *Gli2* and *Wnt9a*, we identified *Klf4* as a gene regulated upon chemotherapy. Kruppel-like factor 4 (Klf4) plays diverse functions in diseases (Ghaleb & Yang, 2017). Klf4 activation by loss of EZH2 and H3K27me3 upon cytotoxic stimuli, could be one of the players inducing pluripotency of TNBC cells. In Mouse embryonic fibroblasts (MEFs), it was identified that calcineurin can be activated via overexpression of Klf4 (Khodeer & Era, 2017). In TNBC, calcineurin phosphatase induction could potentially dephosphorylate NFATc1 and enhance even more its oncogenic function, additionally to PRC2 regulation.

5.4. NFATc1 and HDAC inhibition in TNBC in the clinic

Our studies indicated NFATc1 (Chapter I) and HDAC8 (Chapter II) as the upregulated factors upon chemotherapy treatment in TNBC cells. Based on our findings, HDAC8 appears to have prominent role in TNBC progression, where its inhibition alone impairs cancer cell growth with sensitization effect upon combination with chemotherapy. NFATc1 and HDAC8 are involved in TNBC cancer progression by

promoting EMT. NFATc1 and HDAC8 loss or inhibition leads to decreased proliferation growth rate. Subsequently, NFATc1 and HDAC8 inhibition could bring therapeutical benefits for TNBC patients. As we showed in our project, HDAC8 inhibition via PCI-34051 have anti-TNBC potential that was also observed in T-cell lymphomas where PCI-34051 treatment induced cell apoptosis (Balasubramanian et al., 2008). Additionally, we propose inhibition of HDAC8 in combination with other drug as an alternative for cytotoxic pan-HDACs inhibition. Despite several promising pan-HDAC inhibitors, FDA-approved or undergoing clinical trials, can give unwanted side effects, strongly affecting patient's life. In contrast, isoform-specific HDAC inhibitor could offer impairment of cancer progression with beneficial effect for patient survival. As epigenetic mechanisms are reversible, anti-tumorigenic effect of HDAC8 inhibition could be used as epigenetic therapy supporting NFATc1 blockade bringing effective therapeutic result. Thus, for further investigation, it would be interesting to check the effect of simultaneous inhibition of NFATc1 and HDAC8 that could synergistically impair cancer progression. This proposed therapy solution should be limited to particular category of patients. As it was discussed previously, in most of the cancers EZH2 was indicated as an oncogenic factor. The downregulated PRC2/EZH2 activity as a novel mechanism in cancer progression, should be further investigated among cancers. This information could imply molecular changes, such as NFAT pathway regulation.

In our project we propose that upregulation of NFATc1 and HDAC8 in TNBC could emerge as essential therapeutic targets. Therefore, selective anticancer drug combination involving NFATc1 and HDAC8 inhibition might be suggested as a novel anti-TNBC approach for further investigation.

In summary, we have investigated the novel landmark in TNBC survival to chemotherapy stimuli. We demonstrated that in chemotherapy-treated TNBC cells, loss of PRC2/EZH2 leads to NFATc1 activation initiating an EMT process and fostering cancer progression (Figure 13). Since many studies have shown critical function of NFATc1 in tumor progression, drug resistance and metastasis, NFATc1 inhibition may represent a potential strategy to overcome chemotherapy resistance in cancer.

Additional investigation on NFATc1 function in TNBC progression will be needed in the future. Moreover, examination of the function of other PRC2/EZH2-dependent factors (Gli2, Wnt9a, Klf4), their importance in chemotherapy resistance and relationship with NFAT pathway could be interesting. Additionally, epigenetic PRC2/EZH2-dependent mechanism could potentially interplay with HDACs function inducing pro-tumorigenic pathways. Our findings suggest NFATc1 and HDAC8 as major regulators of TNBC progression that could be proposed for combinatory therapy, where NFATc1 and HDAC8 inhibition brings novel therapeutical approach.

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