Co-targeting epigenetic and oncogenic enzymes in HER2+ breast cancer

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Co-targeting epigenetic and oncogenic enzymes in HER2+ breast cancer

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Abstract

Breast cancer is the most prevalent cancer worldwide with over 2 million women diagnosed every year, and caused 685,000 deaths globally in 2020. 15-25% of breast cancer patients overexpress the receptor tyrosine kinase (RTK) human epidermal growth factor receptor 2 (HER2). While several agents that target HER2 have been FDA-approved, metastatic HER2+ breast cancer remains incurable with a median overall survival of 57 months. This dissertation describes a promising therapeutic strategy for HER2+ breast cancers that co-targets HER2 along with an epigenetic enzyme, EZH2. Specifically, we show that EZH2 inhibitors not only enhance baseline responses to HER2 kinase inhibitors, but also sensitize tumors that have become resistant to these agents, triggering cell death and dramatic tumor regression.

We further demonstrate that therapeutic efficacy is mediated by the oncoprotein YAP, which dynamically regulates the pro-apoptotic gene BMF in HER2+ breast cancers. Specifically, EZH2 inhibitors trigger the loss of repressive H3K27 methylation marks at proximal BMF-regulatory sequences, which induces the binding of YAP/TEAD complexes. Importantly, in this setting YAP and TEAD repress BMF transcription and protect cells from death. However, subsequent exposure to HER2 kinase inhibitors trigger the release of YAP/TEAD complexes, inducing BMF transcription and apoptosis. Thus, our studies demonstrate that EZH2 and YAP both normally protect HER2+ breast cancers from cell death by buffering the BMF locus through distinct mechanisms, which can be overcome by combining EZH2 and HER2 kinase inhibitors.

In summary, this dissertation describes a promising therapeutic strategy with potential clinical relevance and provides insight into specific vulnerabilities of HER2+ breast cancer, which may ultimately lead to new treatments for this disease.

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Chapter 1 : Introduction

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To the Cichowski grad student who is reading this in a daze as you prepare to write your own thesis: you probably recognize that reading a previous grad student's acknowledgement section won't actually help you write your thesis, but it's a great way of feeling like you're doing something isn't it? We've all been there. =) You're in the home stretch, my friend! You got this! Know that I'm rooting for you from 2021!!

And finally, Mom, this was all for you. I miss you and love you. Happy birthday.

Chapter 1 : Introduction

This dissertation focuses on the development of a promising therapeutic drug combination for patients with advanced HER2+ breast cancer. Breast cancer is the most prevalent cancer worldwide with over 2 million women diagnosed every year (WHO 2021). The receptor tyrosine kinase (RTK) human epidermal growth factor receptor 2 (HER2) is overexpressed in 15-25% of breast cancers and acts as a driver of tumor growth (Rimawi, Schiff, and Osborne 2015; Hung et al. 2006). Over the past 30 years, a variety of FDA-approved HER2-targeting therapies have been utilized with great success, resulting in a very good prognosis for individuals diagnosed with early stage HER2+ breast cancer and a 5 year disease free survival rate of 93-99% (D. Slamon et al. 2011; Fehrenbacher et al. 2014). However, despite these therapeutic advances, metastatic HER2+ breast cancer remains incurable with a median overall survival of 57 months (Bredin, Walshe, and Denduluri 2020). Accordingly, there is a clear unmet need to develop treatments that overcome therapeutic resistance of HER2+ breast cancers. While effective HER2 inhibition will remain an important aspect of these new approaches, additional therapeutic targets must be identified. To this end, my strategy has been to investigate the effects of co-targeting oncogenic and epigenetic pathways to elicit a more potent effect. In this dissertation, I will describe the promising responses to combined HER2 and EZH2 inhibitors that we have observed in various HER2+ breast cancer models and our efforts to deconstruct its mechanism of action.

This introduction sets the stage for my work by outlining the important signaling and epigenetic vulnerabilities in HER2+ breast cancer, some of which have been previously overlooked. I first provide background about HER2+ breast cancer, describing its development and current therapeutic shortcomings. Next, I review the epigenetic landscape of cancer, the role

EZH2 plays in this dysregulated state, and historical efforts to target it. Finally, I provide context and justification for the use of HER2 and EZH2 inhibitors in the treatment of HER2+ breast cancer.

BREAST CANCER

Overview

In the US, 1 in 8 women will be diagnosed with breast cancer during their lifetime and 1 in 39 will die from this disease (ACS 2019). Globally, breast cancer is the most prevalent form of cancer, and women lose more disability-adjusted life years (DALYs) from breast cancer than any other cancer (WHO 2021). With the widespread use of screening mammography and increased performance of breast-self exams, the majority of breast cancers in the US are caught early in disease progression, with 62% confined to the breast at diagnosis, 31% disseminated to regional lymph nodes, and 6% presenting with metastases. In addition to early detection, advances in treatments have contributed to a 5-year relative survival estimate of 99% for patients with localized disease (Waks and Winer 2019). However, this number plummets to 29% for patients with metastatic disease (SEER 2021), highlighting the need for new therapeutic options for these individuals.

Breast Cancer Subtypes

Breast cancer is a highly heterogenous disease that can be separated into four main molecular subtypes based on the presence or absence of estrogen receptor (ER), progesterone

receptor (PR), and/or human epidermal growth factor 2 (HER2). Specifically, these subtypes are referred to as luminal A, luminal B, HER2-enriched (HER2+), and basal-like tumors (Dai et al. 2016; Hon et al. 2016). Subtyping of patient tumors is done largely based on immunohistochemistry (IHC) assays for ER, PR, HER2, and the proliferation marker Ki67, in addition to gene expression patterns determined by PAM50 testing (Raj-Kumar et al. 2019)

Table 1-1. Receptor annotations and characteristics of different breast cancer subtypes
HR: Hormone receptor (estrogen receptor and progesterone receptor), Ki-67: Mark of cell proliferation. Information adapted from (Vuong et al. 2014)

Subtype	HR	HER2	Ki-67	Standard of Care
Luminal A	+	-	Low	Hormone therapy
Luminal B	+	±	High	Hormone therapy + chemotherapy
HER2+	±	+	High	Anti-HER2 therapy + chemotherapy
Basal-like	-	-	High	Chemotherapy

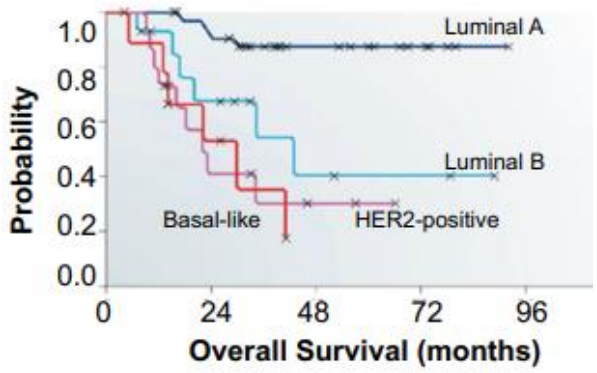


Figure 1-1. Overall survival of breast cancer by subtype
Kaplan-Meier curves depicting overall survival by subtype. Dark blue: Luminal A; Light blue: Luminal B; Pink: HER2+; Red: Basal-like. Data adapted from (Rosen and Vargo-Gogola 2007) by Naomi Olsen

Interestingly, even within these classifications based on receptor status, tumors can be further subdivided based on gene expression profiles (Lehmann et al. 2011). Breast cancer subtypes differ markedly in their progression, prognosis, and outcome, and for this reason different subtypes must be treated as different diseases (Waks and Winer 2019) (Figure 1-1). For

example, though luminal A and luminal B tumors are both hormone receptor positive, luminal B tumors are significantly more aggressive, have higher rates of metastases, and result in a shorter overall survival period, often warranting more intensive treatments (Eroles et al. 2011).

For reasons which will be explained in future chapters, my dissertation touches upon the basal-like subtype, but primarily focuses on HER2+ breast cancer. Thus, I will provide further introduction to both subtypes.

Basal-like Breast Cancer

Basal-like breast cancers are characterized by a lack of ER, PR, or HER2, and therefore are more difficult to treat (Yin et al. 2020; Waks and Winer 2019). Accordingly, the current standard of care is to use non-specific, systemic chemotherapy (Waks and Winer 2019). In the clinic, basal-like tumors are characterized by their larger size, propensity to metastasize, and aggressiveness (Lehmann et al. 2011). Therefore, while it is the least common subtype representing 10-20% of breast cancers, it is the most deadly with a 5 year survival of less than 30% and a mortality rate 3 months after relapse of 75% (Yin et al. 2020; Lehmann et al. 2011). Compared to other subtypes, basal-like is more likely to affect women who are Black or Hispanic and younger (Foulkes, Smith, and Reis-Filho 2010).

Though the terms “basal-like breast cancer” and “triple negative breast cancer (TNBC)” are often used interchangeably, it is important to note that they are not synonymous (Alluri and Newman 2014). The designation “basal-like” is determined by molecular subtyping using gene expression to look at a tumor’s PAM50 gene signature, while the “triple negative” classification

comes from immunohistochemistry for the three aforementioned receptors (Kensler et al. 2019). For the purposes of this dissertation, I will refer to the subtype I worked with as "triple negative breast cancer (TNBC)."

Despite lacking the receptors that are commonly targeted in breast cancer treatments, the mutational profile of TNBC has revealed some actionable genetic alterations. For example, the PI3K pathway is altered in over 29% of TNBC tumors (Kriegsmann et al. 2014). In addition, patients with germline mutations in the DNA repair genes *BRCA1* and/or *BRCA2* are at an increased risk of developing TNBC (Gudmundsdottir and Ashworth 2006; Sørlie et al. 2003). In fact, approximately 20% of primary TNBC tumors carry a germline and/or somatic mutation in *BRCA1* or *BRCA2* (Koboldt et al. 2012). Altogether, these features have generated an interest in combining current chemotherapies with PARP or PI3K/AKT/mTOR pathway inhibitors as therapeutic options (Geenen et al. 2018; Khan et al. 2019) (Table 1-2).

Table 1-2. Highlights of genomic, clinical, and proteomic features of basal-like breast cancer
Data adapted from (Koboldt et al. 2012)

Feature	Rate
TP53 pathway	*TP53* mut (84%); gain of *MDM2* (14%)
PIK3CA/PTEN pathway	PIK3CA mut (7%); PTEN mut/loss (35%); INPP4B loss (30%)
RB1 pathway	*RB1* mut/loss (20%); cyclin E1 amp (9%); high expression of *CDKN2A*; low expression of *RB1*
mRNA expression	Basal signature; high proliferation
Copy number	Most aneuploid; high genomic instability; 1q, 10p gain; 8p, 5q loss; *MYC* focal gain (40%)
DNA mutations	*TP53* (84%); *PIK3CA* (*7%*)
DNA methylation	Hypomethylated
Protein expression	High expression of DNA repair proteins, PTEN and INPP4B loss signature (pAKT)

Finally, the immunotherapy pembrolizumab (Keytruda) has shown promise among TNBC patients, with a phase 3 trial in the US indicating 64.8% of patients treated with pembrolizumab and chemotherapy achieved a pathological complete response compared to 51.2% of patients treated with the current standard of care of chemotherapy alone (Schmid et al. 2020). Pembrolizumab was approved by the FDA for use in TNBC in July 2021, providing an additional therapeutic option for patients whose tumors express PD-L1.

HER2+ BREAST CANCER

Overview

HER2+ breast cancer is characterized by an overexpression of the receptor tyrosine kinase (RTK) human epidermal growth factor 2 (HER2), which drives its growth and leads to an aggressive phenotype and poor prognosis (Prat et al. 2014; Rimawi, Schiff, and Osborne 2015). HER2 is overexpressed in 15-25% of breast cancers, but fortunately, a variety of clinical agents that target this oncogenic protein have been developed (Rimawi, Schiff, and Osborne 2015; Hung et al. 2006). HER2 belongs to the HER family of RTKs, consisting of the four cell-surface receptors HER1 (alternatively known as EGFR), HER2, HER3, and HER4 (Davoli, Hocevar, and Brown 2010). As RTKs, the four members of this family all have a similar structure composed of a cytoplasmic membrane-anchored protein with an extracellular ligand-binding domain, transmembrane domain, and intracellular tyrosine kinase domain (J. L. Hsu and Hung 2016).

The HER family of RTKs is noteworthy due to the members' interdependence on one another and functional complementarity (Hsieh and Moasser 2007). This is especially important

in the case of HER2 and HER3, as HER2 is unique in that it has no recognized ligands and HER3 has no catalytic kinase activity (Yarden and Sliwkowski 2001). While the HER2 ligand is unknown, activation of HER2 occurs via heterodimerization with other family members or spontaneous homodimerization with itself when it is present at high levels, as is the case in HER2+ breast cancer. Interestingly, EGFR, HER3, and HER4 preferentially dimerize with HER2, creating heterodimers that result in longer and stronger signal transduction relative to other dimers that do not contain HER2, illustrating another problematic aspect of HER2 overexpression (Davoli, Hocevar, and Brown 2010).

Activation is similar across the family members in that a ligand will bind to a monomeric RTK (except for HER2), prompting homo- or heterodimerization of the receptors activating the cytoplasmic catalytic function (except for HER3), leading to autophosphorylation on the tyrosine residues that then act as the docking sites for adaptors and enzymes that trigger signaling cascades (Yarden and Sliwkowski 2001) (Figure 1-2). When expressed at normal levels, HER2 acts as a regulator of cell growth, differentiation, and survival. However, when overexpressed, it hyperactivates signaling pathways such as RAS/RAF/MEK/ERK or PI3K/AKT/TOR that lead to aggressive tumor growth and progression (Davoli, Hocevar, and Brown 2010; J. L. Hsu and Hung 2016; Arteaga and Engelman 2014; Yarden and Sliwkowski 2001). (Figure 1-2).

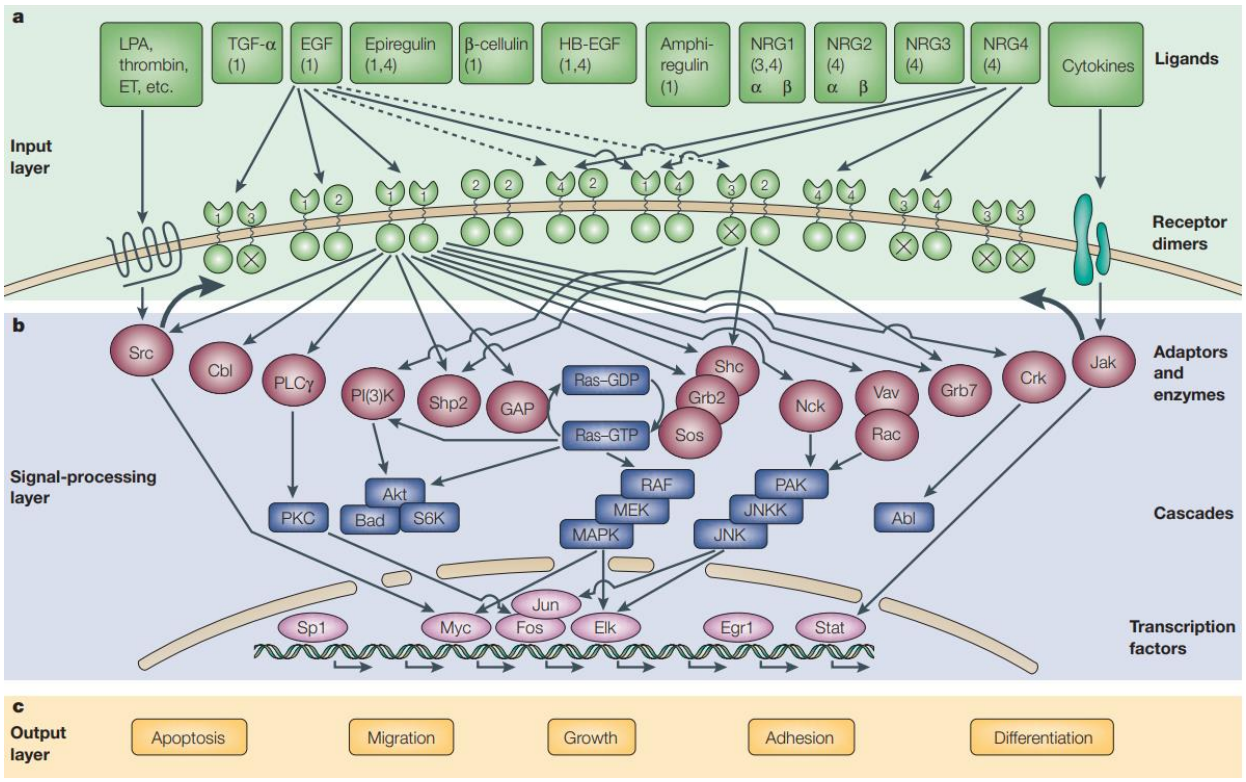


Figure 1-2. HER2 belongs to a family of receptor tyrosine kinases that controls a vast signaling network

Figure from (Yarden and Sliwkowski 2001)

Clinical Outcomes

HER2 overexpression is associated with adverse survival outcomes, early metastasis, and frequent spread to the lymph nodes and central nervous system (Tesch and Gelmon 2020; Rimawi, Schiff, and Osborne 2015). Compared to other breast cancer subtypes, HER2+ tumors have unique epidemiological, clinical, and prognostic differences (Y. Wang et al. 2018). They do not respond well to standard chemotherapy, and individuals with HER2+ breast cancer have shorter overall survival compared to those with the luminal subtypes (Figure 1-1). If detected at an early stage, the tumor can be effectively resected and the patient placed on HER2-targeted therapy to prevent any residual disease from becoming a full-blown recurrence (Rimawi, Schiff,

and Osborne 2015). In fact, the five years disease free survival rate of early stage HER2+ breast cancer patients is 93-99% (D. Slamon et al. 2011; Fehrenbacher et al. 2014). Unfortunately, advanced metastatic HER2+ breast cancer is incurable and invariably fatal, with a median overall survival of 57 months (Bredin, Walshe, and Denduluri 2020).

Molecular Characteristics

The defining characteristic of HER2+ breast cancer is an excess of the receptor tyrosine kinase HER2, generally accomplished through amplification or overexpression of the *HER2* gene. In terms of somatic alterations, TCGA analysis of primary patient samples has further shown that the PIK3CA/PTEN, TP53, and RB1 pathways are also commonly affected (Koboldt et al. 2012) (Table 1-3).

Table 1-3. Highlights of genomic, clinical, and proteomic features of HER2+ breast cancer
Data adapted from (Koboldt et al. 2012)

Feature	Rate
TP53 pathway	*TP53* mut (75%); gain of *MDM2* (30%)
PIK3CA/PTEN pathway	PIK3CA mut (42%); PTEN mut/loss (19%); INPP4B loss (30%)
RB1 pathway	Cyclin D1 amp (38%); *CDK4* gain (24%)
mRNA expression	HER2 amplicon signature; high proliferation
Copy number	Most aneuploid; high genomic instability; 1q, 8q gain; 8p loss; 17q12 focal *ERRB2* amp (71%)
DNA mutations	*TP53* (75%); *PIK3CA* (42%); *PIK3R1* (8%)
Protein expression	High protein and phospho-protein expression of EGFR and HER2

Targeting HER2

The HER2+ breast cancer field has greatly benefited from the identification of HER2 as an oncogenic driver and the development of a variety of HER2-targeted agents (Tesch and Gelmon 2020). Early studies showed that amplification and/or overexpression of HER2 was associated with improved outcomes when anti-HER2 therapies were combined with chemotherapy relative to chemotherapy alone, which led to the standard inclusion of anti-HER2 treatments upon diagnosis (Prat et al. 2014; D. J. Slamon et al. 2001). Advances in HER2-targeted therapies over the past 20 years have led to a vast improvement in patient outcomes with advanced disease, with median survival almost tripling from 20.3 months to 57 months (D. J. Slamon et al. 2001; Bredin, Walshe, and Denduluri 2020). However, despite improvements, treatments for metastatic HER2+ breast cancer are still not curative, demonstrating a need for more effective therapies.

Table 1-4. Food and Drug Administration-approved agents for the treatment of metastatic HER2+ breast cancer

TKI: tyrosine kinase inhibitor, mAb: monoclonal antibody, ADC: antibody-drug conjugate. Information adapted from (Hurvitz and Zhang 2021)

Drug	Type	Line of Therapy	Year Approved	Targets
Lapatinib	TKI	≥ Third	2007	EGFR, HER2
Neratinib	TKI	≥ Third	2020	EGFR, HER2, HER4
Tucatinib	TKI	≥ Third	2020	HER2 selective
Trastuzumab	mAb	First	1998	HER2
Pertuzumab	mAb	First	2012	HER2
Margetuximab	mAb	≥ Third	2020	HER2
Trastuzumab emtansine (T-DM1)	ADC	≥ Second	2013	HER2
Trastuzumab deruxtecan (T-DXd)	ADC	≥ Third	2019	HER2

Since the FDA approval of trastuzumab as the first targeted agent for treatment of metastatic HER2+ breast cancer in 1998, there have been seven more approvals—four of which have taken place in the last two years (Hurvitz and Zhang 2021) (Table 1-4). These agents broadly fall into three classifications as tyrosine kinase inhibitors (TKI), monoclonal antibodies (mAb), or antibody drug conjugates (ADC).

The mechanism of action for the eight HER2-targeted agents differ, and therefore, in many settings patients are placed on more than one concurrently to achieve a deeper response (Choong, Cullen, and O'Sullivan 2020). For example, a study comparing lapatinib monotherapy to a lapatinib and trastuzumab combination in patients with metastatic disease that had progressed during a prior trastuzumab regimen demonstrated that overall survival went from 9.5 months with lapatinib alone to 14 months with the combination (Blackwell et al. 2012). While the antibody drug conjugates TDM-1 is generally used as a monotherapy in the second line setting for metastatic disease, the other agents are generally combined with other HER2-targeting agents or chemotherapy (Choong, Cullen, and O'Sullivan 2020).

The three TKIs lapatinib, neratinib, and tucatinib work by inhibiting kinase activity (as outlined in Table 1-4), thereby suppressing downstream signaling cascades. By contrast, the three mAb partially reduce kinase expression and signaling, but are considered to be immunotherapies, in that they primarily function by triggering immune responses. Though trastuzumab was the first HER2-targeting agent to enter the clinic and has been in use for over three decades, its mechanism of action is still not fully understood. Trastuzumab binds the extracellular juxtamembrane domain of HER2, and was reported to kill tumors via antibody-dependent cell-mediated cytotoxicity (ADCC) (Bang et al. 2017; Choong, Cullen, and O'Sullivan

2020). However, more recent studies have shown that trastuzumab also triggers antibody-dependent cellular phagocytosis (ADCP) (Shi et al. 2015; Tsao et al. 2019; Su et al. 2018; Ravetch et al. 2000). Margetuximab is derived from trastuzumab and thus has the same mechanism of action, but allows for greater binding to the stimulatory receptors on natural killer cells and macrophages that allow for enhanced ADCC to take place (Bang et al. 2017). Pertuzumab recognizes a different portion of HER2, binding to the HER2 dimerization domain to prevent heterodimerization, but also triggers ADCC (Costa and Czerniecki 2020; Ishii, Morii, and Yamashiro 2019). The observation that these therapeutic antibodies function differently than HER2 kinase inhibitors will be relevant to studies described in Chapter 2.

Both ADCs are composed of trastuzumab as the HER2-specific antibody backbone conjugated to a chemotherapeutic agent (Ferraro, Drago, and Modi 2021). T-DM1 consists of trastuzumab conjugated to maytansinoid, a microtubule-disrupting agent, and T-DXd consists of trastuzumab conjugated to deruxtecan, a topoisomerase I inhibitor (Choong, Cullen, and O'Sullivan 2020). ADCs are celebrated due to their multi-pronged effects of trastuzumab's anti-HER2 mechanism, chemotherapy-induced cytotoxicity, and HER2 specific targeting from the antibody-derived nature of the agent (J. Wang and Xu 2019) (Figure 1-3).

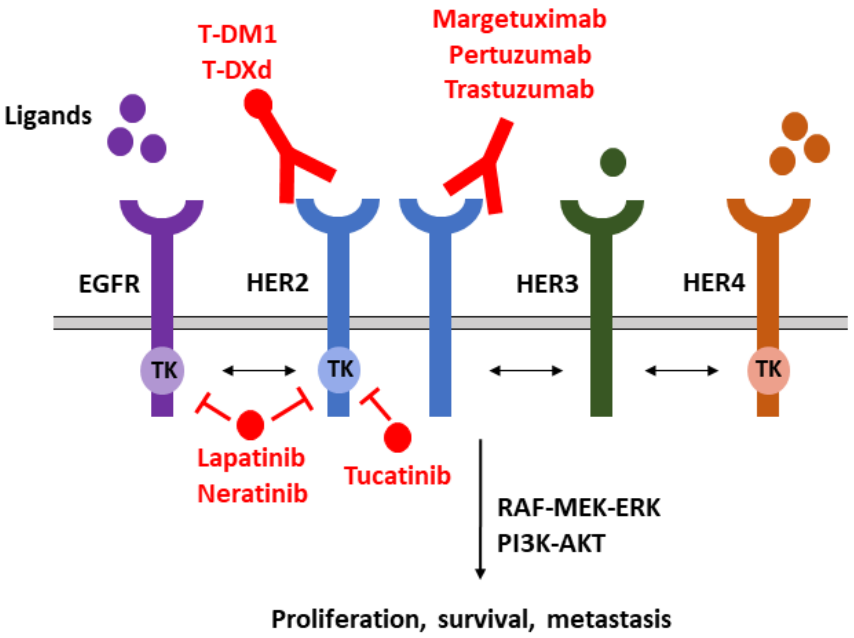


Figure 1-3. Mechanism of action of FDA-approved HER2-directed therapies
Information adapted from (Choong, Cullen, and O'Sullivan 2020) by Naiara Perurena

Through various clinical trials, an accepted optimal sequence of treatment using the FDA-approved HER2-targeting agents in first- and second-line therapy has been established for patients with advanced disease. Unfortunately, response rates vary considerably, with 20-50% and 60-80% of patients unresponsive to these first- and second-line therapies respectively due to *de novo* or acquired resistance (Fehrenbacher et al. 2014; D. Slamon et al. 2011; Bredin, Walshe, and Denduluri 2020; Choong, Cullen, and O'Sullivan 2020) (Figure 1-4). Third-line treatment has yet to be standardized, and metastatic disease is invariably fatal (Tarantino et al. 2021) (Figure 1-4). With the recent influx of FDA-approved agents, patients now have more treatment options than ever, but the lack of a clear standard demonstrates the pressing need for a therapy able to effectively combat HER2 disease and confer a durable effect.

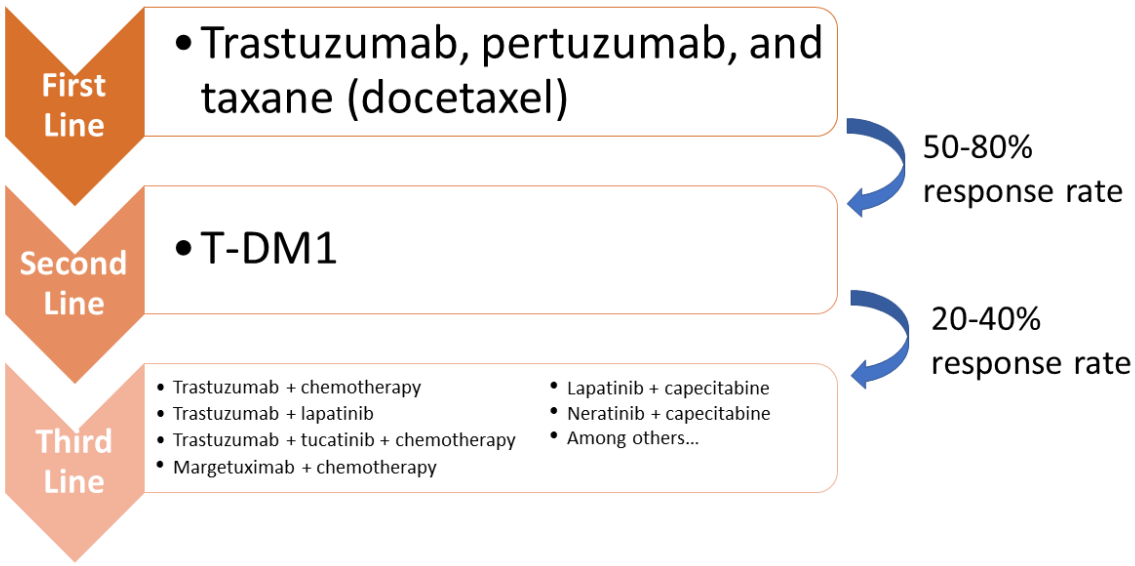


Figure 1-4. Current standard of care for advanced stage HER2+ breast cancer
Information adapted from (Fehrenbacher et al. 2014; D. Slamon et al. 2011; Bredin, Walshe, and Denduluri 2020; Choong, Cullen, and O'Sullivan 2020)

Due to the variety of HER2-targeting therapies and their differential mechanisms of action, multiple resistance mechanisms have emerged (Pernas and Tolaney 2019; Rimawi, Schiff, and Osborne 2015). These broadly fall into two categories (1) reactivation of the HER2 pathway or its downstream signaling using compensatory, redundant, or mutated elements of the pathway or (2) stimulating alternative survival signaling pathways capable of bypassing HER family inhibition (Rimawi, Schiff, and Osborne 2015; Pernas and Tolaney 2019). Here, I will briefly discuss several examples of resistance mechanisms, although more are outlined in Table 1-5.

Table 1-5. Examples of mechanisms of resistance to anti-HER2 drugs
Information adapted from (Vernieri et al. 2019)

Drug	Mechanism of resistance	Factors involved
Trastuzumab	Impaired HER2 binding	Splicing variants (p95HER2; Δ16 HER2)
	Parallel/downstream pathways	PI3KCA mutations, PTEN loss
	Enhanced lipid metabolism	Fatty acid synthase (FASN)
	ER signaling	ER-PR expression
	Cell cycle regulation	Cyclin D1-CDK 4/6 expression
	Escape from ADCC	Poor binding to CD16A antibody receptor
Lapatinib	HER2 signaling	*HER2* mutations
	Cell cycle regulation	Cyclin D1-CDK 4/6 expression
	Parallel/Downstream pathways	PI3K/AKT/mTOR pathway alterations
	ER signaling	ER-PR expression
T-DM1	Impaired HER2 binding	p95HER2; MUC4 expression
	Parallel/downstream signaling	NRG, HER2-HER3, PIK3CA mutations
	T-DM1 internalization/release	SLC46A3, MDR1
Trastuzumab + Lapatinib	Impaired HER2 binding	*HER2* mutations
	FGFR1 signaling	*FGFR1* amplification
	Downstream pathways	*PI3KCA* mutations,
	ER signaling	ER-PR expression
	Cell cycle regulation	Cyclin D1-CDK 4/6 expression
Trastuzumab + Pertuzumab	Altered intracellular pathways	*PIK3CA* mutations

As previously discussed, the HER2 family of RTKs is known for its members' interdependence on one another and functional complementarity (Figure 1-2). This complementarity provides the basis for one resistance mechanism in which HER-targeting agents elicit incomplete inhibition of HER2, and this activates compensatory mechanisms that activate the other HER family members (Pernas and Tolaney 2019). However, in addition to activating other HER family members, HER2+ cells can also activate other RTKs such as insulin-like growth

factor 1 receptor (IGF-1R), an RTK important in signaling cell survival and proliferation (Nahta et al. 2005).

Some resistance mechanisms have a defined genetic or epigenetic cause. Examples of this include splicing variants such as p95HER2 that result in HER2 lacking the trastuzumab-binding epitope and Δ16 HER2, which increase stabilization of HER2 homodimers so they are constitutively activated (Arribas et al. 2011; Castiglioni et al. 2006). In addition, as illustrated by Table 1-3, HER2+ breast cancers often have mutations in critical components of the PIK3CA/PTEN pathway that lead to hyperactivation of the PI3K/AKT/mTOR pathway (Berns et al. 2007; Nagata et al. 2004). This has led to a plethora of clinical trials, some ongoing, using PI3K, AKT, and mTOR inhibitors in combination with HER2-targeted therapy (Table 1-6).

While this section is in no way a complete discussion of resistance mechanisms, its purpose is to introduce the fact that metastatic HER2+ breast cancer is prone to *de novo* and acquired therapeutic resistance, and that the mechanisms are many, varied, and not always fully understood.

Table 1-6. Summary of clinical trials utilizing PI3K/AKT/mTOR pathway inhibitors and HER2-targeting agents

Bevacizumab: monoclonal antibody that binds vascular endothelial growth factor-A (VEGF-A), growth factor that induces the development of tumor-associated blood vessels, LJM716: monoclonal antibody that binds HER3. Information adapted from (Martorana et al. 2021; Akinleye et al. 2013; Paplomata and O'Regan 2014; Yang et al. 2019)

Drug	Target	Combination agents	Clinical phase
Ipatasertib (GDC-0068)	AKT	Trastuzumab + pertuzumab	I
MK-2206	AKT	Trastuzumab + paclitaxel	I
		Lapatinib	I
		Trastuzumab	I
Everolimus	mTOR	Trastuzumab	II
Ridaforolimus	mTOR	Trastuzumab	II
Temsirolimus	mTOR	Neratinib	I/II
Alpelisib (BYL719)	PI3Kα	LJM716 + trastuzumab	I
		T-DM1	I
Buparlisib (BKM120)	PI3Kα/β/γ/δ	Lapatinib	I/II
		Trastuzumab	I/II
		Capecitabine +/− (Trastuzumab + lapatinib)	I
		Trastuzumab + paclitaxel	II
Copanlisib (BAY 80–6946)	PI3Kα/δ	Trastuzumab	I
Pictilisib (GDC-0941)	PI3Kα/β/γ/δ	Paclitaxel +/− bevacizumab or trastuzumab	I
Taselisib (GDC-0032)	PI3Kα/γ/δ	T-DM1 +/− pertuzumab	I
		Pertuzumab + trastuzumab +/− paclitaxel	I
XL-147	PI3Kα/β/γ/δ	Trastuzumab +/− paclitaxel	I/II

THE EPIGENETIC LANDSCAPE OF CANCER

The human body is composed of hundreds of different cell types and tissues that substantially vary in identity and function, yet all contain the same genome. In 1942, the developmental biologist Conrad Waddington coined the word "epigenetics" utilizing the Greek prefix "epi," meaning "in addition to" or "above" to describe the poorly understood phenotypic differences observed in cells during development, without associated changes in genetic

sequence. Though initially studied in the context of development, it is now understood that epigenetics is an omnipresent mechanism of gene regulation (Allis and Jenuwein 2016). To understand epigenetics, one must first understand chromatin structure, starting with histones. The histones H2A, H2B, H3, and H4 form octamers that function together as scaffolding blocks that package and compact DNA into repeating units called nucleosomes, which are in turn packaged and compacted into chromatin. Regulation of any given gene or locus is dependent upon its chromatin organization and how "open" or "closed" it is, allowing access to regulatory factors and transcriptional machinery. The less compacted the chromatin is, the more physically available the genes on that stretch of DNA are for transcription (Allis and Strahl 2000).

Classic examples of epigenetic gene regulation mechanisms are post-translational histone modifications such as acetylation and methylation that occur on histones 3 and 4. As previously described, histones package and compact DNA, and the amount of this compaction directly correlates with how physically accessible a gene is for transcription. Importantly, post-translational modifications of histones have a major effect on gene accessibility and transcription (Allis and Strahl 2000; Allis and Jenuwein 2016). My dissertation work focused on the epigenetic regulator PRC2, and more specifically its catalytic subunit EZH2, which has been shown to be capable of generating global transcriptional changes through the methylation of histone H3.

The PRC2 Complex and EZH2

The polycomb repressive complexes are an integral part of chromatin-based gene regulation and are essential for normal gene regulation and development. Polycomb repressive

complex 1 (PRC1) monoubiquitinates histone H2A at Lys119 (H2AK119ub1), while PRC2 primarily methylates histone H3 at Lys27 (H3K27). The two polycomb repressive complexes were first discovered in the context of *Drosophila* development and their necessity in normal body plan specification. However, subsequent discoveries showed their role in mammalian development (Blackledge and Klose 2021). My dissertation focuses on the PRC2 subunit EZH2, and so I will expand further upon EZH2.

PRC2 is comprised of a core complex containing EZH2, SUZ12, and EED. The histone methyltransferase enhancer of zeste homolog 2 (EZH2) is the catalytic subunit of the PRC2 complex (Yamaguchi and Hung 2014). PRC2 catalyzes a series of methylation reactions at H3K27 that sequentially results in mono-, di-, and tri-methylation at H3K27, resulting in H3K27me1, H3K27me2, and H3K27me3, respectively (J. Tan et al. 2014). This methylation takes place via EZH2's C-terminal SET protein domain to transfer a methyl group from the cofactor S-adenosyl methionine (SAM), which is converted to S-adenosyl homocysteine (SAH) in the process, onto H3K27 (Dockerill, Gregson, and O' Donovan 2021). H3K27me3 mediates gene silencing by repressing transcription at specific sites in the genome (Holm et al. 2012). EZH2's importance in development is demonstrated by the fact that EZH2 knockout mice are early embryonic lethal (Hanaki and Shimada 2021). In addition, EZH2 has been shown to be critical for maintaining stem cell properties of embryonic stem cells where it is found at the promoters of key developmental genes to ensure repression and prevent differentiation (Mendenhall et al. 2010) as well as adult stem cells where it maintains the pluripotent potential of mesenchymal, hematopoietic, and skeletal muscle stem cells (Lifeng Wang et al. 2010; Yu et al. 2011; Chen et al. 2011; Akala and

Clarke 2006; Caretti et al. 2004). However, it has also been proposed to play a role in cell cycle progression, autophagy, apoptosis, and DNA damage repair (Hanaki and Shimada 2021).

EZH2 in Cancer

EZH2 also plays an important role in cancer. While gain of function mutations in EZH2 have been detected in diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), and melanoma, in most solid tumors it is more commonly overexpressed (Chase and Cross 2011; Wassef and Margueron 2017). High levels of EZH2 have been observed in many tumor types including breast, prostate, bladder, gastric, lung, and hepatocellular carcinoma (J. Min et al. 2010; Chase and Cross 2011; Deb, Thakur, and Gupta 2013), and its high expression correlates with disease progression (Kleer et al. 2003; Eskander et al. 2013; Weikert et al. 2005; Zingg et al. 2015; Sudo et al. 2005).

EZH2 has been extensively studied in prostate cancer, and it has been long known that EZH2 is one of the most highly upregulated genes in the metastatic setting (Chinnaiyan et al. 2002). However, the causative, rather than correlative, relationship between EZH2 and prostate cancer was demonstrated when EZH2 was shown to drive tumor development and metastasis in an *in vivo* model (J. Min et al. 2010). Specifically, this work demonstrated that EZH2 overexpression drove prostate cancer growth and metastasis by suppressing the tumor suppressor DAB2IP, in turn activating RAS and NF-κB signaling. Studies related to EZH2 and breast cancer will be described later in this chapter.

Interestingly, while EZH2 is generally known as a repressor of transcription, due to its role as the catalytic subunit of the PRC2 complex, it has been proposed to exhibit PRC2-independent functions. For example in one study, rather than repressing transcription, EZH2 was shown to act as a co-activator for transcription factors such as the androgen receptor (AR) (Xu et al. 2012). This concept will be further addressed in Chapter 2.

Finally, while EZH2 is generally known for its oncogenic role in cancer, it can paradoxically act as a tumor suppressor depending on the context. For example, homozygous EZH2 mutations in myeloid malignancies result in premature chain termination or abrogation of its histone methyltransferase activity, suggesting it normally acts as a tumor suppressor in this particular cancer type (Cross et al. 2010). Loss of function mutations and deletions of SUZ12 and EED are also common events in malignant peripheral nerve sheath tumors (MPNSTs) (De Raedt et al. 2014). The evidence for EZH2/PRC2's opposing roles in cancer suggest that the cellular context is of utmost importance in determining its oncogenic or tumor suppressing functions. However, this thesis will focus exclusively on its oncogenic function in breast cancer.

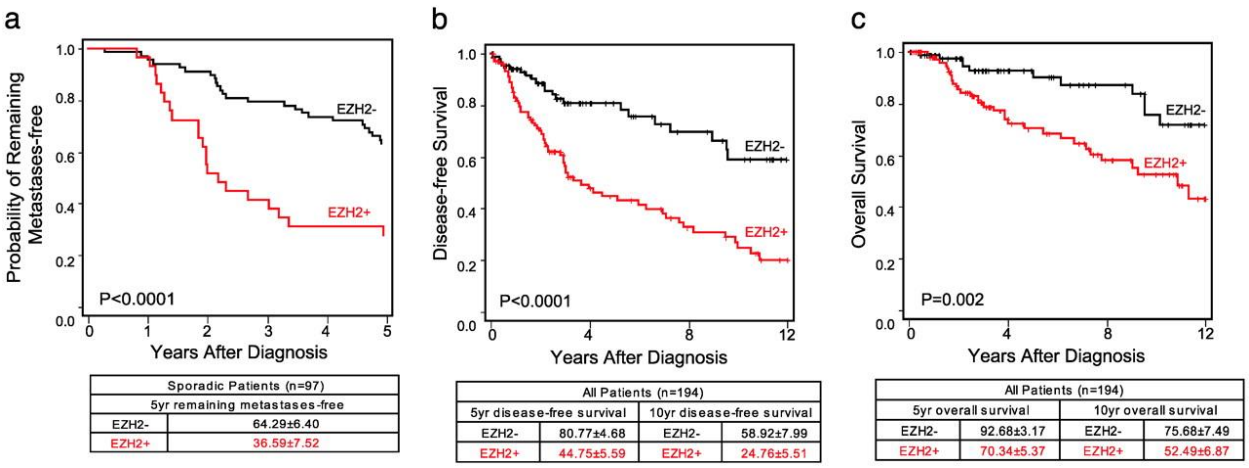
To summarize, a wide variety of EZH2-related perturbations associated with cancer have emerged, including, but not limited to, gain-of-function and loss-of-function mutations, overexpression, mutations in the H3K27 demethylase UTX, and mutations in the SWI/SNF chromatin remodeling complex that partially antagonizes polycomb function (K. H. Kim and Roberts 2016). Despite these complexities EZH2 has proven to be an attractive therapeutic target. Up to 30% of non-Hodgkin lymphomas of follicular and germinal center diffuse large B-cell subtypes have activating somatic heterozygous mutations in EZH2's SET domain resulting in an increase in H3K27me3 at PRC2-regulated loci that promote tumor formation (Souroullas et al.

2016). Because of this, three SAM-competitive EZH2 inhibitors have entered clinical trials for follicular lymphomas and diffuse large B cell lymphoma patients with these gain of function mutations, and one, tazemetostat, has been approved for use in follicular lymphoma (Gulati, Béguelin, and Giulino-Roth 2018). Tazemetostat was also approved for the treatment of locally advanced or metastatic epithelioid sarcomas which harbor defects in SMARCB1 (Gounder et al. 2020; Simeone et al. 2021).

The specific role of EZH2 in breast cancer and the rationale for evaluating EZH2 inhibitors in this disease is discussed below.

EZH2 in Breast Cancer

In breast cancer, high EZH2 expression is correlated with poor clinical outcome and more aggressive and advanced stage disease (Kleer et al. 2003; Sun et al. 2009) (Figure 1-5). These patients tend to be diagnosed at increased tumor size and disease stage and at a younger age (Jang et al. 2016)**.** In addition, women with elevated levels of EZH2 in their normal breast epithelium were found to be at increased risk of breast cancer, suggesting that EZH2 may, at a minimum, be a potential biomarker for breast cancer development and/or more aggressive disease (Beca et al. 2017).



Sporadic Patients (n=97)	
5yr remaining metastases-free	
EZH2-	64.29±6.40
EZH2+	36.59±7.52

All Patients (n=194)			
5yr disease-free survival		10yr disease-free survival	
EZH2-	80.77±4.68	EZH2-	58.92±7.99
EZH2+	44.75±5.59	EZH2+	24.76±5.51

All Patients (n=194)			
5yr overall survival		10yr overall survival	
EZH2-	92.68±3.17	EZH2-	75.68±7.49
EZH2+	70.34±5.37	EZH2+	52.49±6.87

Figure 1-5. High EZH2 levels are correlated with aggressive breast cancer
(a) Kaplan–Meier (KM) analysis of metastasis-free survival based on EZH2 mRNA transcript levels as measured by DNA microarrays by (Veer et al. 2002). KM analysis of (b) disease-free and (c) overall survival based on EZH2 protein levels as established by IHC. Figure from (Kleer et al. 2003)

Overexpression of EZH2 in breast cancer has been associated with high tumor cell proliferation and features of more aggressive diseases such as high nuclear grade and HER2 positivity. Conversely, low-grade breast cancers tend to express low levels of EZH2 (S. Guo et al. 2016). However, as previously discussed, breast cancer is a highly heterogenous disease in which different subtypes must be considered different diseases, and so it is necessary to consider EZH2 in the context of each subtype, rather than as an entire whole, as illustrated in Figure 1-5. Analysis of TCGA primary tumor samples with matched normal tissue broken down by subtype indicate that EZH2 levels are high in the basal, luminal B, and HER2+ subtypes, confirming that high levels of EZH2 correlate with more aggressive disease (Figure 1-6). However, interestingly, even among the generally less aggressive luminal A subtype, patients with high EZH2 expression had a significantly shorter overall survival compared to those with low EZH2 expression, indicating that EZH2's correlative effects for more aggressive disease are applicable across the subtypes (Jang et al. 2016).

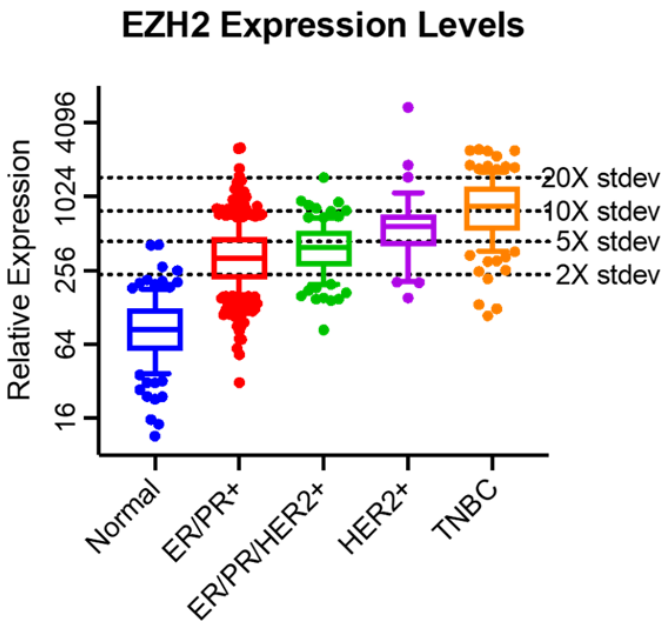


Figure 1-6. EZH2 is overexpressed in the more aggressive breast cancer subtypes
Matched normal breast and tumor tissue data from TCGA Firehouse. ER, PR, and HER2 status were determined by IHC. ER/PR+ samples were not broken into luminal A vs luminal B, so it is likely that the two subgroups are from the EZH2 high luminal B and the EZH2 low luminal A tumors. Figure from Amy Schade

Though the role of EZH2 is less well understood in breast cancer as compared to other cancers such as prostate, gain- and loss-of function studies both *in vitro* and *in vivo* have shown that EZH2 exerts an oncogenic function in breast cancer. For example, EZH2 overexpression in immortalized human mammary epithelial cells induced anchorage-independent growth and cell invasion (Kleer et al. 2003). In addition, mammary-specific EZH2 transgenic mice developed atypical intraductal hyperplasia and exhibited increased cell proliferation compared to control mice, and formed invasive mammary carcinomas significantly earlier (Gonzalez et al. 2014). Genetic suppression of EZH2 in the human TNBC cell line MDA-MB-231 reduced tumor growth, suggesting that EZH2 downregulation may inhibit breast cancer proliferation in some settings (Gonzalez et al. 2009). Finally, EZH2 overexpression in mouse mammary tumor virus-neu (HER2)

mice resulted in accelerated mammary tumor initiation (Gonzalez et al. 2014). Taken together, these results demonstrate that EZH2 plays a functional role in the development and/or progression of breast cancer.

EZH2 Inhibitors as Therapeutic Agents in Cancer

The enzymatic nature of EZH2 makes it an attractive therapeutic target. In fact, there are five small molecule SAM-competitive inhibitors targeting EZH2 already in clinical trials with one approved (Marchesi and Bagella 2016) (Table 1-7). Though we are still in the early days of understanding EZH2 and its functions in cancer, the excitement surrounding the therapeutic potential of targeting EZH2 has led to over 35 clinical trials since 2016 evaluating the efficacy of EZH2i alone or in combination with other agents for a variety of cancers (Dockerill, Gregson, and O' Donovan 2021).

GSK126 was the first commercial EZH2 inhibitor. However, its first clinical trial was terminated early due to only modest anticancer activity at tolerable doses and dosing limitations of the drug resulting from a short half-life that reduced effective exposure (Yap et al. 2019). However, since then, tazemetostat has shown great promise in the clinic with its 2020 approval for follicular lymphoma and epithelioid sarcoma giving hope for the other inhibitors currently in trial (Dockerill, Gregson, and O' Donovan 2021).

Table 1-7. Summary of small molecular SAM-competitive inhibitors targeting EZH1 and/or EZH2 in clinical trials or approved by FDA

ALL: acute lymphoblastic leukemia, AML: acute myeloid lymphoma, CRPC: castration-resistant prostate cancer, DLBCL: diffuse large B cell lymphoma, FL: follicular lymphoma, mCRPC: metastatic castration-resistant prostate cancer, NHL: non-Hodgkin's lymphoma, r/r: relapsed/refractory, SAM S-adenosyl-methionine, SCLC small cell lung cancer. Information adapted from (Duan, Du, and Guo 2020; Rugo et al. 2020)

Drug	Target	Cancer indications in clinical studies	Clinical phase
CPI-1205	EZH2	B cell lymphoma	I
		Advanced solid tumor	I/II
		mCRPC	I/II
CPI-0209	EZH2	Advanced solid tumor	I/II
DS-3201	EZH1/2	AML or ALL	I
		SCLC	I/II
		r/r adult T cell lymphoma	II
Tazemetostat	EZH2	FL	Approved
		Epithelioid sarcoma	Approved
		r/r B cell NHL	II
		Malignant mesothelioma	II
		r/r *INI1*-negative tumors or synovial sarcoma	I
		r/r FL and DLBCL	I
		r/r B cell NHL	I
		B cell NHL, FL, DLBCL, and solid tumors	I/II
PF-06821497	EZH2	SCLC, r/r CRPC, and r/r FL	I
GSK126	EZH2	r/r DLBCL, transformed FL, NHL, solid tumors, and multiple myeloma	Terminated

Recent clinical trials have shown that EZH2 inhibitors as a monotherapy exhibits activity in blood cancers and epithelioid sarcomas, but preclinical studies suggest that it may not be effective as a single agent in other solid tumors, thereby stimulating an interest in evaluating EZH2 inhibitors in combination therapies (C. Li et al. 2021). EZH2 inhibitors have demonstrated cooperative or synergistic results with other drugs in preclinical studies (Table 1-8), lending additional credence to exploring the potential utility of EZH2 inhibitors in breast cancer.

Table 1-8. Summary of therapeutic combinations utilizing EZH2i
Information adapted from (C. Li et al. 2021)

Cancer therapy	Therapeutic method	Cancer types
Immunotherapy	Anti-CTLA-4	Bladder cancer
	Anti-PD-L1	Hepatocellular carcinoma
	Anti-PD-1	Head and neck cancer
	Anti-MDSCs	Colon cancer
Chemotherapy	Docetaxel	Prostate cancer
	Etoposide	Lung cancer
	Cisplatin	Cervical cancer
		Ovarian cancer
		Osteosarcoma
	Temozolomide	Glioblastoma
	Doxorubicin, Melphalan	Multiple myeloma
	Epirubicin, Mitomycin C	Hepatocellular carcinoma
Targeted therapy	PARP inhibitor	Ovarian cancer
		Breast cancer
	HDAC inhibitor	Acute myeloid leukemia
		Lymphomas
		Lung cancer
		Ovarian cancer
Endocrine therapy	Anti-androgen	Prostate cancer
		Prostate cancer
	Anti-estrogen	Breast cancer

Histone Deacetylases

In addition to methylation, another important regulatory histone modification is acetylation, which is associated with open chromatin and active transcription. At the beginning of my thesis work, I also assessed the effects of HDAC inhibitor-based drug combinations. Therefore, I have included a description of these epigenetic enzymes here.

As their name indicates, histone deacetylases (HDACs) remove acetylation from protein substrates such as histone tails. HDACs oppose histone acetyltransferases (HATs), which add acetyl groups, and together, they ensure a steady-state level of acetylation at all times. In short, HDACs remove acetyl groups to compact chromatin, while HATs add acetyl groups to relax chromatin. In this way, the two work in concert to regulate gene expression and maintain order in important processes such as differentiation, angiogenesis, and metabolism (Hontecillas-Prieto et al. 2020). In the context of cancer, there is often a loss of HAT activity that results in widespread gene repression, including at tumor suppressor genes. Furthermore, HDACs are often overexpressed, leading to the removal of activating acetyl marks that results in gene suppression, including tumor suppressor genes (Eich et al. 2020).

HDACs are a diverse family of proteins that are divided into four phylogenetic classes based on their sequence homology to yeast. Class I HDACs (HDAC 1, 2, 3, and 8) are homologous to yeast Rpd3 and class II HDACs (HDAC4, 5, 6, 7, 8, 10) are homologous to yeast protein Hda1. Class III HDACs are also known as Sirtuins and are homologous to yeast Sir2. They include seven members of the Sirtuin HDACs from Sirtuins 1 to 7 (Hontecillas-Prieto et al. 2020). Class IV HDACs (HDAC11) show similarities to both class I and II proteins. Aberrant expression of class I, II, and IV HDACs have been linked to a variety of solid and hematological tumors including neuroblastoma, medulloblastoma, lung, gastric, liver, pancreatic, colorectal, breast and ovarian. High levels of HDACs, regardless of class, are associated with more advanced diseases, poorer outcomes, and poor survival (Y. Li and Seto 2016). A plethora of HDAC inhibitors (HDACi) have been developed, and five have received FDA-approval for use in treating cancer— vorinostat (cutaneous T-cell lymphoma [CTCL]), belinostat (peripheral T-cell lymphoma [PTCL], romidepsin (CTCL, PTCL),

tucidinostat (PTCL), and panobinostat (multiple myeloma) (Bondarev et al. 2021). Though HDAC inhibitors are effective as monotherapies in hematological malignancies, this does not appear to hold true for solid tumors. Interestingly, preclinical and clinical data suggest that HDAC inhibitors have a greater effect and less toxicity when combined with other cancer agents, and these discoveries have spurred advancements in novel therapeutics combining HDAC inhibitors with PARP inhibitors, proteasome inhibitors, immune checkpoint inhibitors, and mTOR inhibitors, among others (Hontecillas-Prieto et al. 2020; A. Min et al. 2015; Laporte et al. 2017; K. Kim et al. 2014; Malone et al. 2017; Booth et al. 2017). In breast cancer, HDAC inhibitors have shown promise in the treatment of luminal, triple negative, and HER2+ breast cancers (Zucchetti et al. 2019). This served as one rationale for assessing the effects of HDACi + EZH2i, which will be discussed in Chapter 3.

APOPTOSIS

Apoptosis is the evolutionarily conserved cell death pathway responsible for culling cells in a controlled manner without harming the surrounding environment (Singh, Letai, and Sarosiek 2019; Martin, Taylor, and Cullen 2008). Apoptosis is necessary for development and normal tissue function, as it is the process of regulated cell death that safely and effectively removes damaged, dysfunctional, or superfluous cells to make way for their replacement with new, necessary, and healthy cells (Singh, Letai, and Sarosiek 2019). The apoptotic process can be initiated by intracellular stimuli such as DNA damage, growth factor deprivation, and/or cytokine deprivation, and extracellular stimuli such as signals from cytotoxic T cells flagging damaged cells

for death (Pfeffer and Singh 2018). These intracellular and extracellular stimuli utilize the intrinsic signaling pathway mediated by mitochondria or an extrinsic signaling pathway mediated by so-called “death receptors,” respectively (Pfeffer and Singh 2018; Singh, Letai, and Sarosiek 2019). Though they respond to different sets of stimuli and initiate different, complex signaling pathways, both end in the activation of the three major effector caspases, caspase-3, caspase-6, and caspase-7 that carry out apoptosis (Martin, Taylor, and Cullen 2008).

In the course of my dissertation research, I investigate how EZH2 and HER2 inhibitors impinge on signals that drive intrinsic apoptosis, and so will provide further background on this pathway.

Intrinsic Apoptosis

As previously mentioned, intrinsic apoptosis is initiated by intracellular perturbations, including, but not limited to, DNA damage, growth factor deprivation, cytokine deprivation, endoplasmic reticulum stress, reactive oxygen species (ROS) overload, replication stress, microtubule alterations, and/or mitotic defects (Reviewed in Galluzzi et al. 2018). The intrinsic pathway depends upon the B-cell lymphoma-2 (BCL-2) family of proteins (Reviewed in Singh, Letai, and Sarosiek 2019). The BCL-2 family members share between one and four BCL-2 homology (BH) domains (numbered BH1, BH2, BH3, and BH4) and are divided into three subfamilies: (1) the anti-apoptotic subfamily, also known as the pro-survival family, whose members contain all four BH domains, with most additionally having transmembrane domains (2) the pro-apoptotic subfamily, whose members all lack a BH4 domain, and includes BAX and

BAK, the proteins that form pores in mitochondrial outer membranes to promote apoptosis and (3) the pro-apoptotic BH3-only subfamily, which consists of eight structurally diverse proteins that have BH3 domains. These BCL-2 family members work together to strike a careful balance that determines whether cells will undergo apoptosis or not (Pentimalli 2018; Martin, Taylor, and Cullen 2008) (Figure 1-7).

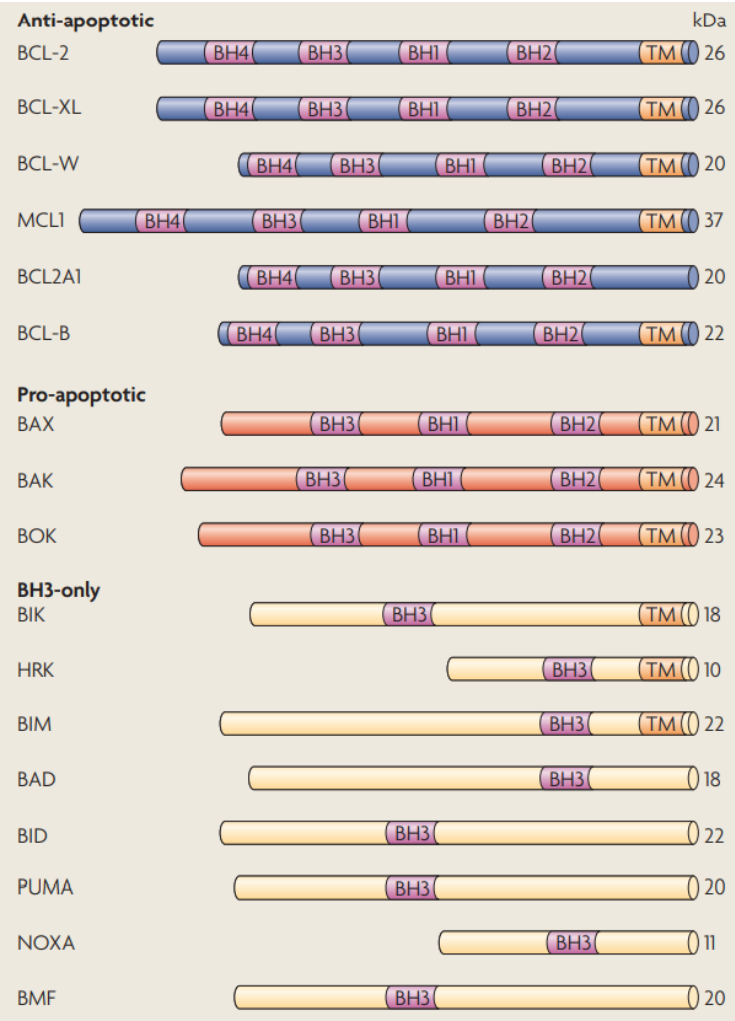


Figure 1-7. The BCL-2 family members and their structures
BH: BCL-2 homology domain, TM: transmembrane domain. Figure from (Martin, Taylor, and Cullen 2008)

The BH3-only family is a grouping designation based on the members' shared BH3 domain, and is further divided into the two subtypes of "activators" and "sensitizers" based on their function. The activators such as BID and BIM can directly activate BAX and BAK to form pores in the mitochondrial outer membrane, while the sensitizers are less efficient at this activation and perform a different function that will be discussed shortly (Reviewed in Giam, Huang, and Bouillet 2008; Singh, Letai, and Sarosiek 2019).

The intrinsic apoptosis pathway can be outlined with the schematic in Figure 1-8. (1) An intracellular insult will (2) stimulate the pro-apoptotic BH3-only activators such as BID and BIM to initiate apoptosis. This is the point that serves as a fork in the road where either apoptosis or survival will take place. First, the activators will (3) bind and be sequestered by the pro-survival family members such as BCL-2, BCL-X, or MCL1. If the activators are few enough that the pro-survival proteins can bind and sequester them all, the cell will continue surviving with no change. If, however, the pro-survival proteins become saturated or are absent, the BH3-only activators will (4) bind to and activate the pro-apoptotic mitochondrial surface pore-forming proteins BAX and BAK. This activation initiates their oligomerization so that BAX and BAK form macropores in the mitochondrial membrane, resulting in mitochondrial outer membrane permeabilization (MOMP) (Reviewed in Singh, Letai, and Sarosiek 2019)

This sequence of events in which the BH3-only activators are first bound and sequestered by the pro-survival proteins rather than directly binding to BAX and BAK is an important and necessary feature that prevents healthy cells from accidentally triggering apoptosis (Giam, Huang, and Bouillet 2008). The positioning of this pro-survival vs pro-apoptosis fork in the road in the intrinsic apoptotic pathway is crucial, as MOMP is generally considered the point of no return

when a cell is definitively damaged beyond repair. MOMP allows for the (5) release of apoptogenic proteins from the mitochondrial intermembrane space such as second mitochondria-derived activator of caspases (SMAC), the serine protease OMI, and cytochrome c. Once in the cytoplasm, cytochrome c can bind to the scaffold protein apoptotic protease-activating factor 1 (APAF1) to form the apoptosome, a protein structure that recruits and activates caspase-9, the initiator caspase. Caspase-9 in turn (6) activates caspases -3 and -7, collectively known as the executioner caspases. In addition, the release of SMAC and OMI as a result of MOMP (7) inhibits X-linked inhibitor of apoptosis protein (XIAP), which normally blocks caspase activation.

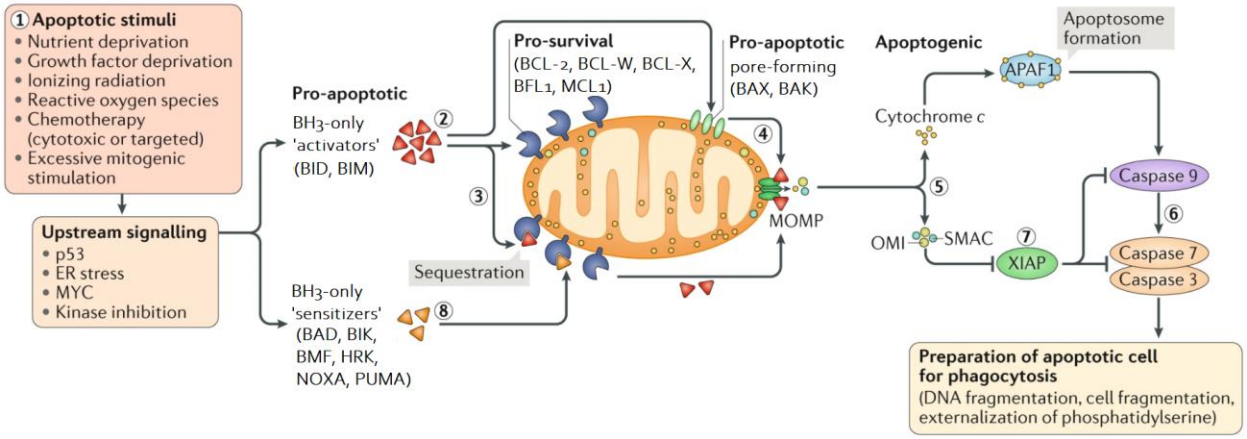


Figure 1-8. The intrinsic apoptotic pathway
Modified from (Singh, Letai, and Sarosiek 2019)

In addition, the aforementioned BH3-only sensitizers that are less efficient at activating BAX and BAK compared to the BH3-only activators play an important role in the intrinsic apoptotic pathway that acts upon the pro-survival/pro-apoptosis junction in the previously described sequence of events. BH3-only sensitizer proteins are not as efficient at binding to BAX and BAK, and as a result, their main function is to inhibit the pro-survival proteins and liberate

any bound activators (Reviewed in Giam, Huang, and Bouillet 2008). As indicated in Figure 1-8 step 8, the activators are then free to bind BAX and BAK to induce MOMP and continue the apoptotic pathway. This lengthy series of events initiated by an intracellular injury culminates in the activation of these caspases that trigger apoptosis (Reviewed in Singh, Letai, and Sarosiek 2019).

While cancer is often thought of as a disease of uncontrolled cell proliferation, it is necessary to note that it is also a disease of dysregulated cell attrition, with apoptosis as the major mechanism of this attrition under normal conditions. In fact, considering apoptosis' function in destroying damaged, dysfunctional, and superfluous cells, preventing cancer is one of its main objectives. Unfortunately, the ability to evade apoptosis is a hallmark of cancer cells, and much work is being conducted on stimulating pro-apoptotic molecules and inhibiting anti-apoptotic molecules as an anti-cancer therapy (Hanahan and Weinberg 2000; Pfeffer and Singh 2018). In fact, BH3 mimetics that inhibit BCL-2, BCL-X and/or MCL-1 are currently in clinical trials against chronic lymphocytic leukemia (CLL), small-cell lung cancer (SCLC), advanced-stage solid tumors, melanoma, non-Hodgkin's lymphoma (NHL), pancreatic cancer, and colorectal cancer (Reviewed in Carneiro and El-Deiry 2020).

In the next section, I will further discuss the pro-apoptotic regulator BMF, as I discovered it plays a crucial role in my combination's synergistic therapeutic effect.

BMF

BCL-2-modifying factor (BMF) is a member of the pro-apoptotic BH3-only subfamily of BCL2 proteins and belongs to the sensitizer subcategory (Certo et al. 2006; Puthalakath et al. 2001) (Figure 1-7). As noted above, BMF is a BH3-only sensitizer, which means its main function is to inhibit anti-apoptotic proteins such as BCL-2 and MCL-1 so that the BH3-only activators BID and BIM can activate BAX and BAK to induce MOMP and ultimately apoptosis (Reviewed in Singh, Letai, and Sarosiek 2019).

In healthy cells, BMF is sequestered in the myosin V motor complex by binding to dynein light chain-2 (DLC2) (Puthalakath et al. 2001). The myosin V motor complex is a protein structure whose function is to attach to organelles and molecules via DLC2 and transport these cargo loads within the cell along actin filaments (Izidoro-Toledo et al. 2013). The first study establishing BMF's interaction with myosin V motor proteins found that BMF is released from this sequestration under certain conditions such as DNA damaging UV radiation or anoikis, apoptosis induced by detachment from the extracellular matrix or neighboring cells that prevents those cells from colonizing elsewhere (Puthalakath et al. 2001). Another study discovered that BMF release was induced by methods of cell stress that repressed the CAP-dependent translation machinery such as serum deprivation, hypoxia, inhibition of the PI3K/AKT pathway or mTOR, or inhibition of the eukaryotic translation initiation factor eIF-4E (Grespi et al. 2010). However, a subsequent study discovered that cell death from these stimuli still takes place in BMF-/- mouse embryonic fibroblasts (MEFs) and gastrointestinal epithelial cells, suggesting there must be redundancy with other BH3-only proteins (Labi et al. 2008). This finding that knockdown of BMF can affect but not

prevent apoptosis in response to specific stimuli was consistent with the categorization of BMF as a pro-apoptotic sensitizer, rather than activator.

In the context of my dissertation centering on breast cancer, an interesting feature of BMF is that along with another BH3-only protein BIM (Figure 1-7, Figure 1-8), it plays an important role in mammary gland development. The mammary gland is a very complex system due to its intricate structure and the fact that it undergoes various stages of development and myriad changes requiring precise control of proliferation and apoptosis throughout a woman's lifetime (Reviewed in Oudenaarden, van de Ven, and Derksen 2018). BIM- and BMF-mediated anoikis keeps the lumen of mammary glands free of any detached luminal epithelial cells during lactation to keep the ducts clear for milk secretion, and also clears epithelial cells during involution after weaning. In both processes, a loss of either of these proteins results in the mammary duct lumens filling with hyperplastic mammary epithelial cells (Mailleux et al. 2007; Schuler et al. 2016). It is important to note that the lumen filling with hyperplastic mammary cells is also a characteristic of ductal carcinoma in situ (DCIS) providing a hint at the way in which BMF and apoptosis may be involved in breast cancer (Pradeep et al. 2012).

BMF in Cancer

As previously discussed, apoptosis is a pathway that allows for the elimination of abnormal cells, and thus acts as an important way to prevent cancer development. Therefore, it is not surprising that evading apoptosis is a hallmark of cancer (Hanahan and Weinberg 2000). This also highlights the potential importance of pro-apoptotic proteins such as BMF to act as

tumor suppressors by coordinating the controlled killing of tumors cells. Indeed, development of irradiation-induced thymic lymphomas is accelerated in BMF knockout mice (Labi et al. 2008) and knocking down BMF in human mammary epithelial cells is sufficient to prevent anoikis and acinar cell death and promote anchorage-independent growth (Schmelzle et al. 2007).

In vivo models of breast cancer have also shown the importance of BMF in metastases. E-cadherin is a vital component of the adherens junctions necessary for cell adhesion and maintaining a cell's epithelial phenotype (Mendonsa, Na, and Gumbiner 2018). In metastatic breast cancer, E-cadherin is inactivated, leading to anoikis resistance and dissemination (Derksen et al. 2006). Expressing BMF in the E-cadherin negative and metastatic mouse invasive lobular carcinoma cell line mILC1 is sufficient to inhibit tumor growth and dissemination, demonstrating loss of E-cadherin and inhibition of BMF-dependent anoikis are factors in malignant tumor progression (Hornsveld et al. 2016).

In addition, constitutive activation of the MEK/ERK or PI3K/AKT pathways represses BMF during anoikis, revealing that these pathways known to be involved in tumorigenesis when deregulated, can clamp down on BMF expression, and with this, the apoptotic machinery (Schmelzle et al. 2007). This fact is especially interesting in the context of my thesis, as HER2 overactivation constitutively activates downstream signaling pathways that include MEK/ERK and PI3K/AKT (Yarden and Sliwkowski 2001) (Figure 1-2).

YAP-TEAD

In this section, I will focus on the YAP/TEAD complex, as it is crucial to the mechanism of action of my combination therapy. I will focus on each component in turn.

The transcriptional regulator yes-associated protein (YAP) is a major effector of the Hippo pathway, an evolutionarily conserved signaling cascade that controls cell growth and fate decision, organ size, and regeneration (Pentimalli 2018). Under conditions that indicate cells should no longer proliferate, such as contact inhibition, the Hippo pathway activates a kinase cascade that phosphorylates and inhibits YAP, causing it to accumulate in the cytoplasm where it is ubiquitinated and degraded by proteasomes (B. Zhao et al. 2007; 2010). Under conditions requiring proliferation, the Hippo pathway is off and YAP is dephosphorylated and translocated into the nucleus where it interacts with transcription factors to induce expression of genes important for cell proliferation, survival, and migration (T. Guo et al. 2013; Koontz et al. 2013).

Though initially studied in the context of development and the Hippo pathway, it is now clear that YAP is an oncogene. In cancer, sustained YAP activation causes aberrant cell proliferation, survival, and reprogramming of non-stem cell tumor cells into cancer stem cells, (Reviewed in Zanconato, Cordenonsi, and Piccolo 2016). Mouse fibroblasts overexpressing YAP continue to proliferate despite reaching confluency when contact inhibition mechanisms would ordinarily prevent cell division (B. Zhao et al. 2007). YAP overexpression in *in* vivo studies utilizing multiple mammary carcinoma cell lines noted an increase in tumor volume as well as metastatic ability and size of metastases. In addition, a benign non-transformed mammary epithelial cell line was rendered highly metastatic upon YAP overexpression (Lamar et al. 2012). Additional studies

using a doxycycline-inducible YAP construct in the liver of mice increased liver size more than 4-fold and eventually caused hepatocellular carcinoma (Camargo et al. 2007). Taken together, these studies illustrate YAP's involvement in cancer.

It is important to note that YAP does not have a DNA binding domain, and so in order to fulfill its role as a transcriptional regulator, it must interact with transcription factors such as members of the TEA domain (TEAD) family (Vassilev et al. 2001). The TEAD family of transcription factors are canonical binding partners of YAP and consist of four members (TEAD1-TEAD4), and it is hypothesized that the TEADs may be key mediators of the growth and tumorigenic potential of YAP. By creating stable pools of human mammary epithelial MCF10A with either constitutively active YAP (YAP-5SA) or a version of YAP unable to bind to TEAD (YAP-S94A), it was demonstrated that as expected, YAP-5SA induced YAP-inducible genes more strongly than the wild-type YAP, while YAP-S94A was, interestingly, severely compromised in its previously established gene regulation functions of promoting cell growth, oncogenic transformation, and epithelial-mesenchymal transition, as determined by microarray (B. Zhao et al. 2008).

The importance of the YAP/TEAD relationship and their dependence on one another to properly carry out YAP's functions as a gene regulator is further confirmed by comparing mice with a TEAD binding deficient YAP construct in the epidermis to those with YAP knockout in the epidermis and seeing that they phenocopy one another (Schlegelmilch et al. 2011). An additional function of YAP/TEAD activity that is of interest in the context of cancer therapeutics and my dissertation is that it has been associated with targeted therapy resistance in NSCLC to EGFR inhibition (Chaib et al. 2017; P.-C. Hsu et al. 2016).

While YAP is canonically considered to be a transcriptional activator, it can also repress transcription. For example, previous studies have indicated that YAP/TEAD suppress expression of differentiation markers to maintain pluripotency in human embryonic stem cells (Beyer et al. 2013), tumor-suppressor genes in human epithelial mammary cells to promote cell proliferation and survival (M. Kim et al. 2015), and the pro-apoptotic protein BMF to suppress apoptosis and induce therapeutic resistance in EGFR-mutant lung cancers (Kurppa et al. 2020). Additionally, YAP acts as a transcriptional repressor when bound to other transcription factors such as the osteoblast-related transcription factor RUNX2 to attenuate skeletal gene expression (Zaidi et al. 2005) and PPARγ, a transcription factor that induces adipocyte differentiation and genes involved in fat deposition, to suppresses adipogenesis (Pan et al. 2018). In Chapter 2, I will discuss the role that YAP plays in regulating the therapeutic response to EZH2 and HER2 inhibitors.

OVERVIEW OF DISSERTATION

Recent therapeutic advancements have improved outcomes for patients with advanced stage HER2+ breast cancer, but it is still an ultimately incurable disease. The lack of an effective therapy demonstrates the clear unmet need for advancements in this field. This dissertation describes a promising new therapeutic combination targeting both epigenetic and oncogenic pathways in the treatment of HER2+ breast cancer.

In Chapter 2, my primary research chapter, I explore the combination of HER2 and EZH2 inhibitors for the treatment of HER2+ breast cancer and elucidate its mechanism of action. By utilizing various *in* vitro and *in vivo* methods, I show that this combination not only overcomes

HER2 inhibitor resistance, but also targets residual disease. In addition, I demonstrate how EZH2 and HER2 inhibitors cooperatively upregulate BMF and trigger apoptosis to cause cell death.

In Chapter 3, I will describe additional preliminary studies that were conducted alongside my initial studies in Chapter 3. This chapter will serve to document other EZH2 inhibitor-based drug combinations that worked or did not work and will explain why we chose to move forward with the HER2/EZH2 inhibitor combination.

Finally, in Chapter 4, I make conclusions and discuss the potential implications of my findings for not just HER2+ breast cancer patients, but other breast cancer subtypes. I review current treatment options for patients and discuss my combination within the context of the clinical landscape. In addition, I discuss the promising results and how they could be utilized to treat individuals with other HER2 overexpressing cancers. Lastly, I will highlight future research questions and avenues of inquiry to pursue in light of the exciting results of my thesis work.

Chapter 2 : EZH2 Inhibitors Create a Dependency on YAP for Survival in HER2+ Breast Cancers

EZH2 Inhibitors Create a Dependency on YAP for Survival in HER2+ Breast Cancers

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ABSTRACT

Human epidermal growth factor receptor positive (HER2+) tumors account for 15-25% of breast cancers. While a variety of HER2-targeted therapies have been developed, tumors can exhibit *de novo* or acquired resistance and metastatic disease remains incurable. Here we show that EZH2 inhibitors shift the epigenetic state of HER2 tumors, dramatically enhancing baseline responses to HER2 kinase inhibitors and re-sensitizing drug resistant tumors *in vitro* and *in vivo*. Specifically, we report that EZH2 normally silences the pro-apoptotic gene, BMF, by methylating H3K27 at the BMF locus. EZH2 inhibitors promote the release of H3K27me3, however this stimulates the binding of repressive YAP/TEAD complexes. Nevertheless, once tumors are exposed to EZH2 inhibitors, genetic or chemical inhibition of YAP/TEAD rapidly induces BMF expression and apoptosis. Importantly, in the presence of EZH2 inhibitors, HER2 kinase inhibitors similarly trigger the dissociation of YAP/TEAD complexes, upregulate BMF, and kill resistant cells. Together these studies show how EZH2 and YAP coordinately insulate the BMF locus and demonstrate that EZH2 inhibitors can be used to reprogram HER2+ tumors, resulting in a dramatic sensitization to HER2 kinase inhibitors and enhanced killing of residual disease.

INTRODUCTION

The human epidermal growth factor receptor 2 (HER2) is overexpressed in 15-25% of breast cancers where it functions as an oncogenic driver (Hung et al. 2006; Rimawi, Schiff, and Osborne 2015). While multiple agents that target HER2 have been FDA-approved, tumors can exhibit *de novo* or acquired resistance. As such, metastatic and/or recurrent HER2+ breast cancer remains incurable with an overall survival of 57 months (Bredin, Walshe, and Denduluri 2020).

Thus, there is a clear need to develop new treatments that overcome therapeutic resistance of HER2+ breast cancers and kill more residual disease.

The current standard of care for metastatic HER2+ breast cancer includes HER2-directed monoclonal antibodies, which are thought to work largely by promoting antibody-dependent cell mediated cytotoxicity (ADCC), followed by the HER2 antibody-drug conjugate T-DM1 in the second line, which brings DM1, a cytotoxic maytansinoid to HER2 expressing cells (Bang et al. 2017; Choong, Cullen, and O'Sullivan 2020). Subsequent treatments typically include HER2 kinase inhibitors; however, there is no standard third-line regimen (Fehrenbacher et al. 2014; D. Slamon et al. 2011; Bredin, Walshe, and Denduluri 2020; Choong, Cullen, and O'Sullivan 2020). Moreover, none of these approaches are curative. Therefore, we set out to develop a strategy that would improve responses to HER2 kinase inhibitors and kill more residual disease. We also reasoned that a promising new kinase inhibitor-based combination, in particular, could be readily translated into a clinical trial in the third-line setting.

When contemplating other targetable oncoproteins in HER2+ breast cancers, we considered the histone methyltransferase enhancer of zeste homolog 2, EZH2. Gain-of-function mutations in EZH2 can occur in some cancers, but EZH2 is more commonly overexpressed in solid tumors (Chase and Cross 2011; Wassef and Margueron 2017). High EZH2 expression levels in breast cancer have been shown to correlate with poor survival (Kleer et al. 2003; Sun et al. 2009), but much remains to be learned about its function generally and in specific breast cancer subtypes.

Here we report that EZH2 inhibitors dramatically sensitize HER2+ breast cancers to HER2 kinase inhibitors. We further show that together these agents kill residual disease via a cell-autonomous mechanism that involves YAP and the pro-apoptotic protein BMF. Importantly, our data suggest that this cooperativity is best conferred by kinase inhibitors, which more effectively suppress HER2, rather than monoclonal antibodies which primarily kill tumors via ADCC. Together, these studies reveal a promising new therapeutic strategy for HER2+ breast cancers and demonstrate how epigenetic enzymes and transcription factors can cooperatively buffer tumors from apoptotic signals.

RESULTS

EZH2 inhibitors dramatically enhance responses to HER2 kinase inhibitors in both HER2 inhibitor-resistant and sensitive models

In breast cancer, high EZH2 expression is correlated with poor clinical outcome and is associated with more aggressive and advanced stage disease (Kleer et al. 2003; Sun et al. 2009). Importantly, matched normal breast and tumor tissue data from TCGA Firehouse demonstrates that EZH2 is overexpressed in 91% of HER2+ breast cancers (>2SD compared to normal breast tissue) (Figure 2-1A). Moreover, EZH2 is overexpressed in HER2+ tumors even at early stages (Figure 2-1B), suggesting that its upregulation occurs early in the development of this breast cancer subtype.

To determine whether EZH2 inhibitors might sensitize cells to HER2 inhibitors, we collected HER2+ breast cancer cell lines that have either been defined as resistant (MDA-MB-453

and HCC202) or sensitive (SKBR3 and BT474) to the HER2/EGFR kinase inhibitor lapatinib (O'Brien et al. 2010). Cells were pre-treated with the EZH2 inhibitor (EZH2i) GSK126 for 5 days and increasing concentrations of lapatinib were added for another 3 days. Notably, the EZH2 inhibitor sensitized both resistant cell lines to lapatinib, resulting in cell death and a dramatic loss of cells over time (Figure 2-1C). Specifically, lapatinib exerted only cytostatic effects in MDA-MB-453 cells, even at high concentrations. However, EZH2 inhibition sensitized these cells to lapatinib, resulting in cell death and a loss of cells (Figure 2-1C, top left). HCC202 cells were slightly more sensitive to lapatinib but again more cells died in the presence of both EZH2 inhibitor and lapatinib at multiple concentrations (Figure 2-1C, top right).

Lower concentrations of lapatinib were evaluated in the lapatinib sensitive cell lines, BT474 and SKBR3. Surprisingly, EZH2 inhibitors also dramatically sensitized these cells to lapatinib. Whereas cell death was only observed at $\geq$300nM lapatinib in SKBR3 cells, in the presence of EZH2 inhibitor, dose-dependent cytotoxic responses were observed beginning at 30nM (Figure 2-1C, bottom left). EZH2 inhibitor similarly shifted the sensitivity of SKBR3 cells, triggering cytotoxic effects at 30nM versus 100nM (Figure 2-1C, bottom right). Notably, EZH2 inhibitors had no effect on the degree of HER2 inhibition conferred by lapatinib in all cell lines, although as might be expected, higher concentrations of lapatinib were required for effective HER2 suppression in resistant lines (Figure 2-1D). Nevertheless, in all instances, effective HER2 and EZH2 target inhibition was observed in cells prior to cell death (Figure 2-1D). These results demonstrate that together EZH2 and HER2 inhibitors kill more residual cells, both in models that are considered to be lapatinib-resistant and sensitive.

The cooperative effects of EZH2 and HER kinase inhibitors and cell death were further confirmed using the FDA-approved EZH2 inhibitor tazemetostat (Figure 2-1E). Importantly, tazemetostat and lapatinib potently synergized in both types of models, as determined by the Gaddum model of non-interaction (Figure 2-1F) (Ianevski et al. 2020). It should be noted that lapatinib was initially selected for these studies because of its routine use in the third-line setting (Waks and Winer 2019; Choong, Cullen, and O'Sullivan 2020). However, a more selective HER2 kinases inhibitor, tucatinib, has more recently been approved (Choong, Cullen, and O'Sullivan 2020). Tazemetostat also cooperated with tucatinib, demonstrating that EZH2 inhibitors may be effectively combined with either of these kinase inhibitors (Figure 2-1G).

EZH2 is the catalytic subunit of polycomb repressive complex 2 (PRC2) (K. H. Kim and Roberts 2016). As a histone methyltransferase, EZH2 trimethylates histone H3 at lysine 27—an epigenetic mark that mediates gene silencing by repressing transcription. However, EZH2 has also been reported to function in a PRC2-independent complex (Xu et al. 2012). To determine whether the suppression of the PRC2 complex could recapitulate the therapeutic effects of EZH2 inhibitors, cells were pre-treated with MAK683, an inhibitor of the obligate PRC2 subunit EED (embryonic ectoderm development protein). MAK683 similarly cooperated with lapatinib to kill these HER2+ cells (Figure 2-1H), demonstrating that suppression of the PRC2 complex is sufficient for these effects and revealing another potential therapeutic combination.

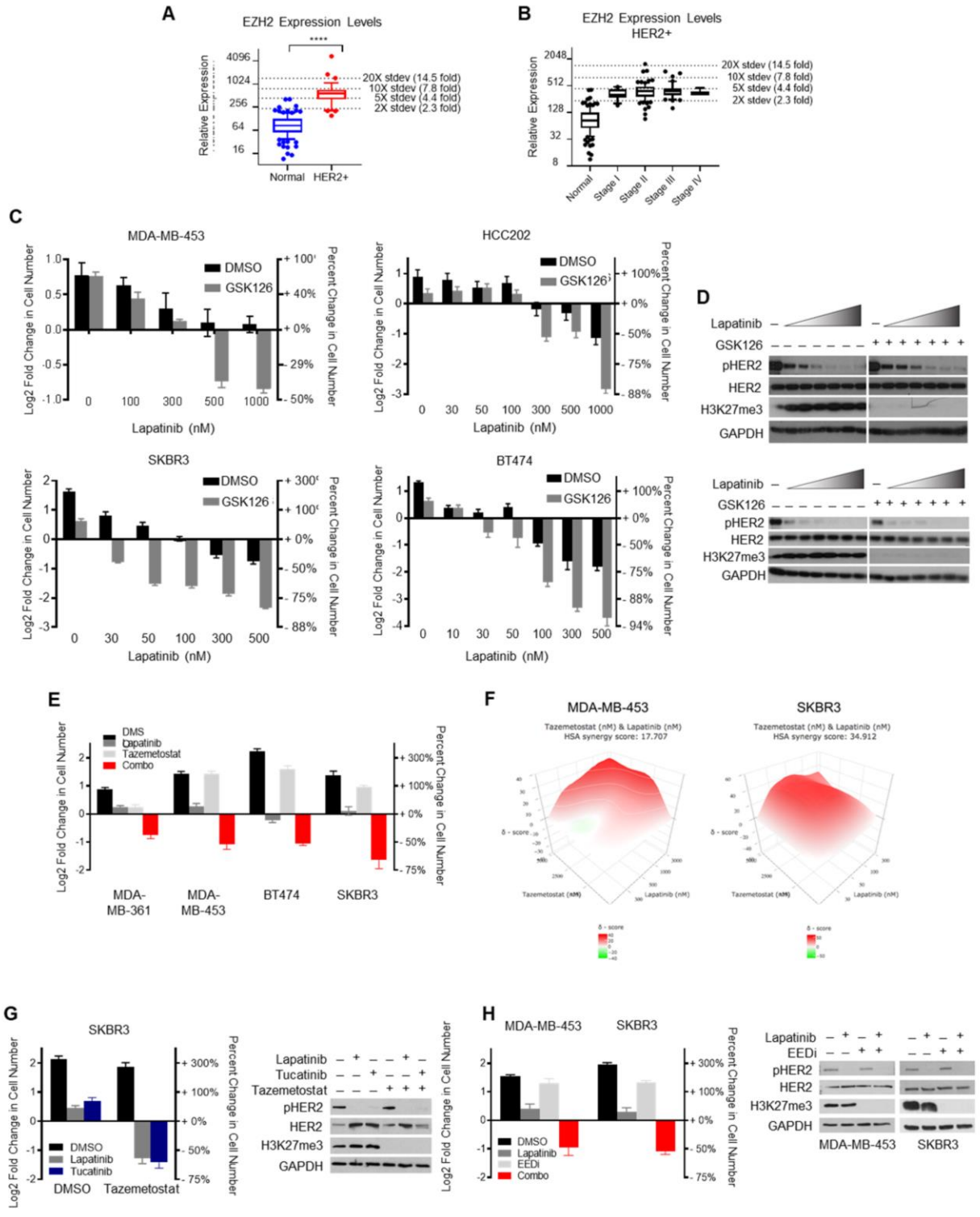


Figure 2-1. HER2 and EZH2 Inhibitor combinations cause cell death in HER2+ breast cancer cell lines

Figure 2-1 (continued)

(A) Relative EZH2 expression in matched normal breast tissue and HER2+ breast cancer tumor samples from TCGA Firehose. HER2 status was determined by IHC.

(B) Relative EZH2 expression in matched normal breast tissue and HER2+ breast cancer tumor samples from TCGA Firehose. HER2 status was determined by IHC.

(C) Response of lapatinib-resistant (MDA-MB-453 and HCC202) and lapatinib-sensitive (SKBR3 and BT474) cells after 3 days of the indicated treatment. After 5 days of pretreatment in 5μM GSK126 or DMSO, indicated concentrations of lapatinib or DMSO was layered on, and cells treated for 3 days. Graphs represent log2 transformation of the fold change in cell number at day 3 versus day 0. Right axis shows percent change in cell number relative to day 0. Negative values represent a decrease in cell number over time and positive values represent an increase in cell number. Bars depict mean change in cell number ± SD from technical triplicates after 3 days of treatment with lapatinib and GSK126 determined by manual counting.

(D) Lapatinib and GSK126 target inhibition was confirmed by immunoblotting.

(E) Response of indicated HER2+ breast cancer cell lines after 5 days of the indicated treatment. After 5 days of pretreatment in 5μM tazemetostat or DMSO, lapatinib (1μM for MDA-MB-361 and MDA-MB-453 and 100nM for BT474 and SKBR3) or DMSO was layered on, and cells treated for 5 days. Graphs represent log2 transformation of the fold change in cell number at day 5 versus day 0. Right axis shows percent change in cell number relative to day 0. Negative values represent a decrease in cell number over time and positive values represent an increase in cell number. Bars depict mean change in cell number ± SD from technical triplicates after 5 days of treatment with lapatinib and tazemetostat determined by manual counting.

(F) Synergy scores for tazemetostat and lapatinib combination for (left) lapatinib-resistant MDA-MB453 and (right) lapatinib-sensitive SKBR3. After 5 days of pretreatment in 5μM tazemetostat or DMSO, lapatinib or DMSO was layered on, and cells treated for 5 days. Strong synergy is indicated in red.

(G) Response of SKBR3 after 5 days of the indicated treatment. After 5 days of pretreatment in 5μM tazemetostat or DMSO, 100nM lapatinib, 10nM tucatinib, or DMSO was layered on, and cells treated for 5 days. Graphs represent log2 transformation of the fold change in cell number at day 5 versus day 0. Right axis shows percent change in cell number relative to day 0. Negative values represent a decrease in cell number over time and positive values represent an increase in cell number. Bars depict mean change in cell number ± SD from technical triplicates after 5 days of treatment with lapatinib and tazemetostat determined by manual counting. HER2 and EZH2 inhibition were confirmed by immunoblotting.

(H) Response of indicated HER2+ breast cancer cell lines after 5 days of the indicated treatment. After 5 days of pretreatment in 5μM MAK683 or DMSO, lapatinib (1μM for MDA-MB-453 and 100nM for SKBR3) or DMSO was layered on, and cells treated for 5 days. Graphs represent log2

Figure 2-1 (continued)
transformation of the fold change in cell number at day 5 versus day 0. Right axis shows percent change in cell number relative to day 0. Negative values represent a decrease in cell number over time and positive values represent an increase in cell number. Bars depict mean change in cell number ± SD from technical triplicates after 5

EZH2 and HER2 kinase inhibitors cooperatively promote tumor regression in drug-resistant and sensitive models

Before dissecting the mechanism of action, we first assessed the *in vivo* efficacy of these agents in lapatinib-resistant MDA-MB-453 and lapatinib-sensitive BT474 orthotopic xenografts. The EZH2 inhibitor GSK126 was used as a tool compound for these studies, so that mice could be treated for a longer period of time, which was not permitted under IACUC guidelines using tazemetostat, due to the requirement for twice daily dosing via oral gavage. Cells were injected into mammary fat pads and animals were randomly enrolled into one of four treatment arms when tumors were $\geq$ 200mm^3: (1) Vehicle (2) EZH2i (3) Lapatinib or (4) EZH2i + lapatinib. All mice were first treated with vehicle or EZH2i for 7 days and then divided into the appropriate experimental arms as described in Experimental Methods and outlined in (Figure 2-2A).

In the resistant MDA-MB-453 model, the addition of lapatinib alone exerted a minimal transient effect on tumor size as depicted by the dark gray line (Figure 2-2B, left). The EZH2 inhibitor alone slightly reduced tumor size, but together EZH2 inhibitor and lapatinib caused tumors to regress on average more than 75% (Figure 2-2B left, red line). As expected, lapatinib treatment caused some tumor regression in the sensitive BT474 model (Figure 2-2C right, dark gray line) and in this setting the EZH2 inhibitor had no effect (light gray line). Nevertheless, the EZH2i + lapatinib combination similarly induced an average tumor regression of 75% (Figure 2-2C,

right, red line). Individual tumor sizes are depicted in waterfall plots below each graph (Figure 2-2B,C). Importantly, this combination had no adverse effects on mouse condition or weight (Supplemental Figure A-1).

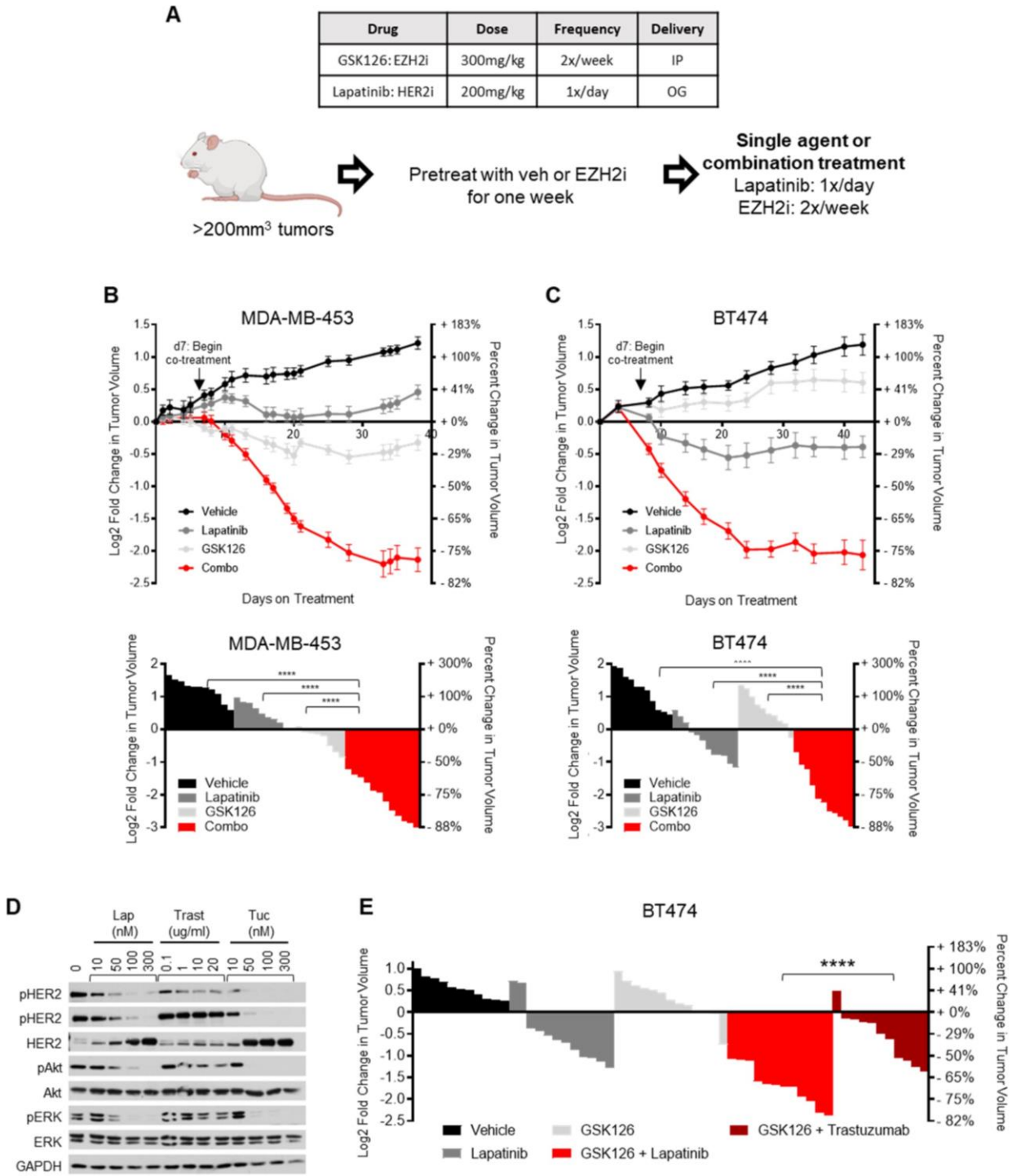


Figure 2-2. HER2 and EZH2 inhibitor combination causes potent tumor regression in HER2+ breast cancer xenografts

(A) Experimental dosages and workflow of *in vivo* orthotopic mammary fat pad xenograft studies.

(B and C) Mean change in tumor size ± SEM of (B) lapatinib-resistant MDA-MB-453 and (C) lapatinib-sensitive BT474 xenografts Waterfall plots depicting size change of MDA-MB-453 tumors at 38 days and BT474 tumors at day 43 ****$p < 0.0001$ (Mann-Whitney test). Each bar depicts one tumor.

(D) Immunoblots on BT474 protein lysates treated with indicated HER2i at indicated concentrations collected after 24 hours of treatment.

(E) BT474 tumors at day 21 ****$p < 0.0001$ (Mann-Whitney test). Each bar depicts one tumor.

A recent study reported that EZH2 inhibitors modestly cooperate with HER2-directed monoclonal antibodies *in vivo* in an immune competent orthotopic model by enhancing interferon-driven innate immune responses (Hirukawa et al. 2019). However, together these agents only attenuated growth, and did not trigger the potent regression observed with combined EZH2 inhibitors and HER2 kinase inhibitors (Figure 2-2B,C). As noted previously, HER2-directed monoclonal antibodies such as trastuzumab modestly suppress HER2 signaling, and instead are thought to function by inducing the activation of antibody-dependent cytotoxicity or phagocytosis of tumor cells (Bang et al. 2017; Choong, Cullen, and O'Sullivan 2020; Shi et al. 2015; Tsao et al. 2019; Su et al. 2018). The relative effects of lapatinib (strong), trastuzumab (weak) and tucatinib (strongest) on the phosphorylation of HER2 and downstream ERK and AKT signals are shown in (Figure 2-2D). Based on the observation that EZH2 and HER2 kinase inhibitors potently kill cells via a cell autonomous mechanism *in vitro*, we would have anticipated that HER2 monoclonal antibodies might exert weaker effects. Indeed, we confirmed that the effects of EZH2i + trastuzumab were less effective than EZH2i + lapatinib, even in the more sensitive

orthotopic model (Figure 2-2E). The observation that EZH2 inhibitors and HER2 kinase inhibitors cause massive tumor regression in two xenografts in the absence of an intact immune system and *in vitro* suggests that these cells are dying via a potent cell-autonomous mechanism of action that relies on the downstream effects of kinase suppression.

EZH2 and HER2 inhibitors cooperatively upregulate BMF and trigger apoptosis

To elucidate the mechanism underlying the cooperativity of EZH2i and HER2i, we first investigated whether these cells were undergoing apoptosis using an Incucyte live-cell imaging system to evaluate caspase activity. In both lapatinib-sensitive and resistant cell lines, EZH2 and HER2 inhibitors triggered much higher levels of apoptosis when combined, as compared to each agent alone (Figure 2-3A). GSEA analysis of transcriptional profiles from drug treated cells confirmed that an apoptotic signature was enriched in cells treated with both HER2 and EZH2 inhibitors (Figure 2-3B). However, a closer inspection of the genes within this signature revealed that BMF, a pro-apoptotic gene highly expressed in the developing mammary gland (Schmelzle et al. 2007), was one of the most differentially expressed genes in the combination-treated cells versus the single treatment arms (Supplemental Figure A-2). The cooperative effect of EZH2 and HER2 inhibitors on BMF mRNA levels was confirmed in both lapatinib-sensitive and resistant cell lines by quantitative PCR (Figure 2-3C). Because high affinity BMF antibodies are not available, we used CRISPR/Cas9 technology to introduce HA-tagged BMF into the endogenous BMF locus in SKBR3 cells. An immunoblot confirmed that both agents cooperatively induced BMF protein levels (Figure 2-3D; Supplemental Figure A-3). Similar to mRNA data, EZH2 inhibitor alone had a

greater effect than lapatinib, suggesting that EZH2 was particularly important for restricting BMF expression.

Pooled siRNAs were then used to investigate whether BMF upregulation was required for cell death. siRNAs effectively reduced BMF expression and prevented cell death in response to tazemetostat and lapatinib in SKBR3 cells, which was confirmed using distinct shRNA sequences (Figure 2-3E). Notably BMF ablation in MDA-MB-453 cells similarly prevented cell death in response to this combination (Figure 2-3F). Taken together, these results demonstrate BMF expression is cooperatively upregulated by EZH2 inhibitors and HER2 kinase inhibitors, and that it is essential for cell death in response to this drug combination.

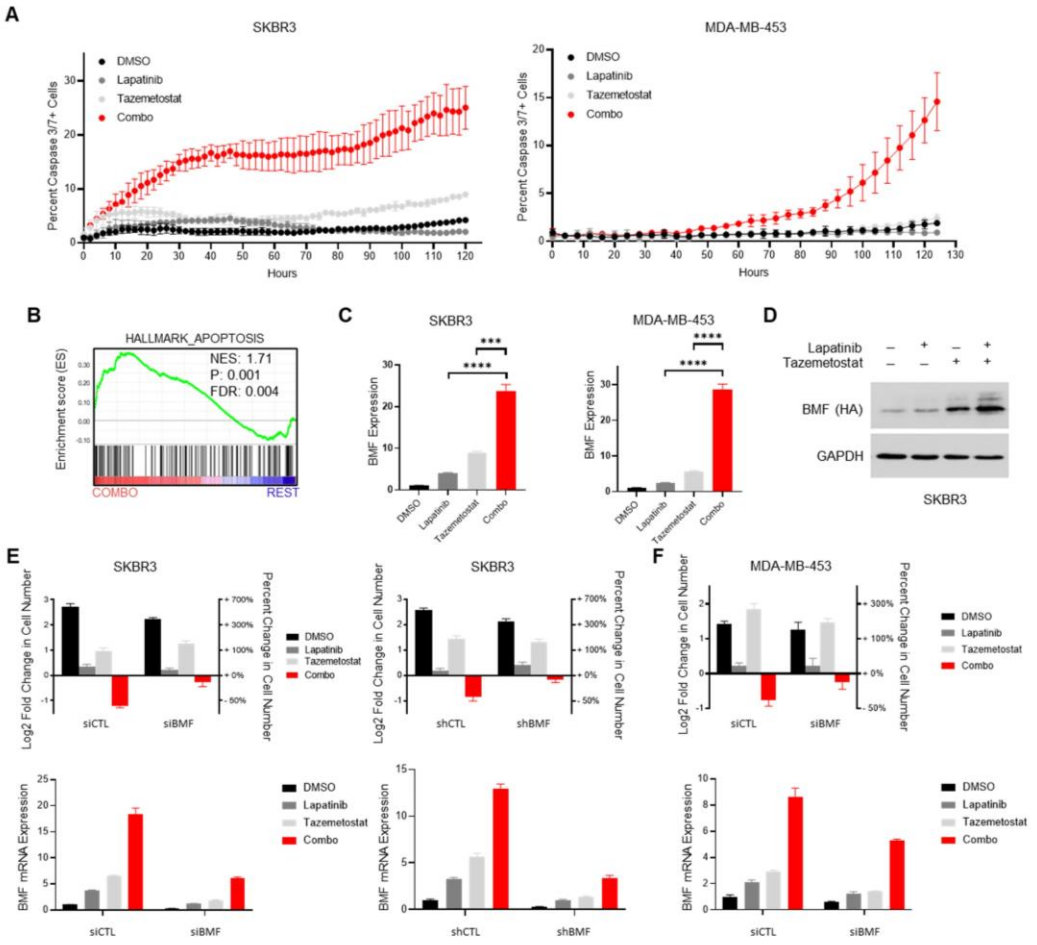


Figure 2-3. EZH2 and HER2 inhibitors cooperatively upregulate BMF and trigger apoptosis
(A) Live-cell imaging depicting mean change in the percent of caspase 3/7+ cells ± SD from technical triplicates over 5 days of indicated treatment. After 5 days of pretreatment in 5μM tazemetostat or DMSO, lapatinib (100nM for SKBR3 and 1μM for MDA-MB-453) or DMSO was layered on, and cells treated for 5 days.

(B) GSEA analysis of RNA-Seq data from SKBR3 cells from technical triplicates collected 24 hours after treatment. After 5 days of pretreatment in 5μM tazemetostat or DMSO, 100nM lapatinib or DMSO was layered on, and cells treated for 24 hours before RNA was extracted.

(C) Fold change ± SD of BMF expression calculated relative to DMSO-treated cells. After 5 days of pretreatment in 5μM tazemetostat or DMSO, lapatinib (100nM for SKBRS or 1μM for MDA-MB-453) or DMSO was layered on, and cells treated for 24 hours before RNA was extracted.

(D) Immunoblots on SKBR3 HA-BMF cells. After 5 days of pretreatment in 5μM tazemetostat or DMSO, lapatinib (100nM for SKBR3 and 1μM for MDA-MB-453) or DMSO was layered on, and cells treated for 24 hours before protein was collected.

(E and F) Mean change in cell number ± SD from technical triplicates determined by manual counting after BMF knockdown in (E) SKBR3 and (F) MDA-MB-453 cells after 5 days of treatment with lapatinib and tazemetostat. After 5 days of pretreatment in 5μM tazemetostat or DMSO, lapatinib (100nM for SKBR3 and 1μM for MDA-MB-453) or DMSO was layered on, and cells treated for 5 days. Graphs represent log2 transformation of the fold change in cell number at day 5 versus day 0. Right axis shows percent change in cell number relative to day 0. Negative values represent a decrease in cell number over time and positive values represent an increase in cell number. BMF knockdown was confirmed by qPCR.

EZH2 and YAP-TEAD complexes coordinately suppress BMF

We next considered mechanisms by which these agents might be promoting BMF expression. Interestingly, BMF has recently been shown to be controlled by the oncoprotein YAP (Yes associated protein) and its binding partner TEAD, which repress its expression in drug-resistant NSCLCs (Kurppa et al. 2020). To determine if and how YAP/TEAD complexes might be regulating BMF expression in this setting, we assessed YAP and TEAD binding to proximal BMF regulatory sequences by ChIP-qPCR. Notably, while lapatinib alone had little effect, EZH2 inhibitor potently induced YAP and TEAD binding (Figure 2-4A). Interestingly, however, the

addition of lapatinib triggered the release of YAP/TEAD complexes from BMF regulatory sequences in EZH2i pretreated cells (Figure 2-4A, red bar). These results demonstrate that EZH2i and HER2i dynamically regulate YAP and TEAD binding to proximal BMF regulatory sequences in HER2+ breast cancer cells.

Given the dynamics of YAP and TEAD binding shown in Figure 2-4A, and the notion that YAP and TEAD repress BMF transcription, we proposed the model shown in Figure 2-4B. First, we hypothesized that high levels of EZH2 were actively suppressing BMF transcription in HER2+ breast cancer by promoting H3K27 methylation at the BMF locus. If that were true, EZH2 inhibitors would trigger H3K27 demethylation, thereby opening chromatin, which then might permit the YAP/TEAD binding observed in Figure 2-4A. Indeed, tazemetostat induced a loss of H3K27me3 at these same regulatory sequences, demonstrated by H3K27me3 ChIP-qPCR (Figure 2-4C). However, while the loss of H3K27me3 alone might permit BMF expression in some cells, EZH2 inhibitor clearly induced YAP/TEAD binding (Figure 2-4A). Therefore, we hypothesized that YAP/TEAD complexes were binding and restricting BMF expression in this setting. Consistent with this model, ablation of YAP with siRNA sequences had no effect on baseline levels of BMF; however, in cells pretreated with EZH2 inhibitor, YAP suppression induced BMF expression (Figure 2-4D). Similarly, while YAP ablation alone did not trigger apoptosis, it was able to do so in cells that were first treated with EZH2 inhibitors (Figure 2-4E). Notably, chemical suppression of TEAD with a covalent inhibitor recapitulated these effects (Figure 2-4F).

Taken together these findings suggest that EZH2 and YAP cooperatively suppress BMF expression. Specifically, high levels of EZH2 maintain the BMF locus in an inactive closed state by promoting H3K27 methylation. While EZH2 inhibitors induce a loss of H3K27 methylation at this

locus, BMF expression becomes restrained by YAP/TEAD binding, which is induced by this epigenetic shift. Accordingly, BMF expression is maximally induced by the combined suppression of EZH2 and YAP. The repressive effects of YAP can be relieved by genetic or chemical inhibition of the YAP/TEAD complex. However, HER2 kinase inhibitors also clearly suppress YAP binding in this context. Specifically, while EZH2 inhibitor induces YAP and TEAD binding to BMF regulatory sequences, the subsequent addition of lapatinib triggers the dissociation of this complex (Figure 2-4A). These findings are further supported by the observation that the cytotoxic effects of the combination require EZH2 inhibitor pretreatment, and do not occur when EZH2 and HER2 inhibitors are administered together (Supplemental Figure A-4). Together, these findings demonstrate how EZH2 and YAP cooperatively regulate the apoptotic machinery and buffer HER2+ breast cancers from cell death.

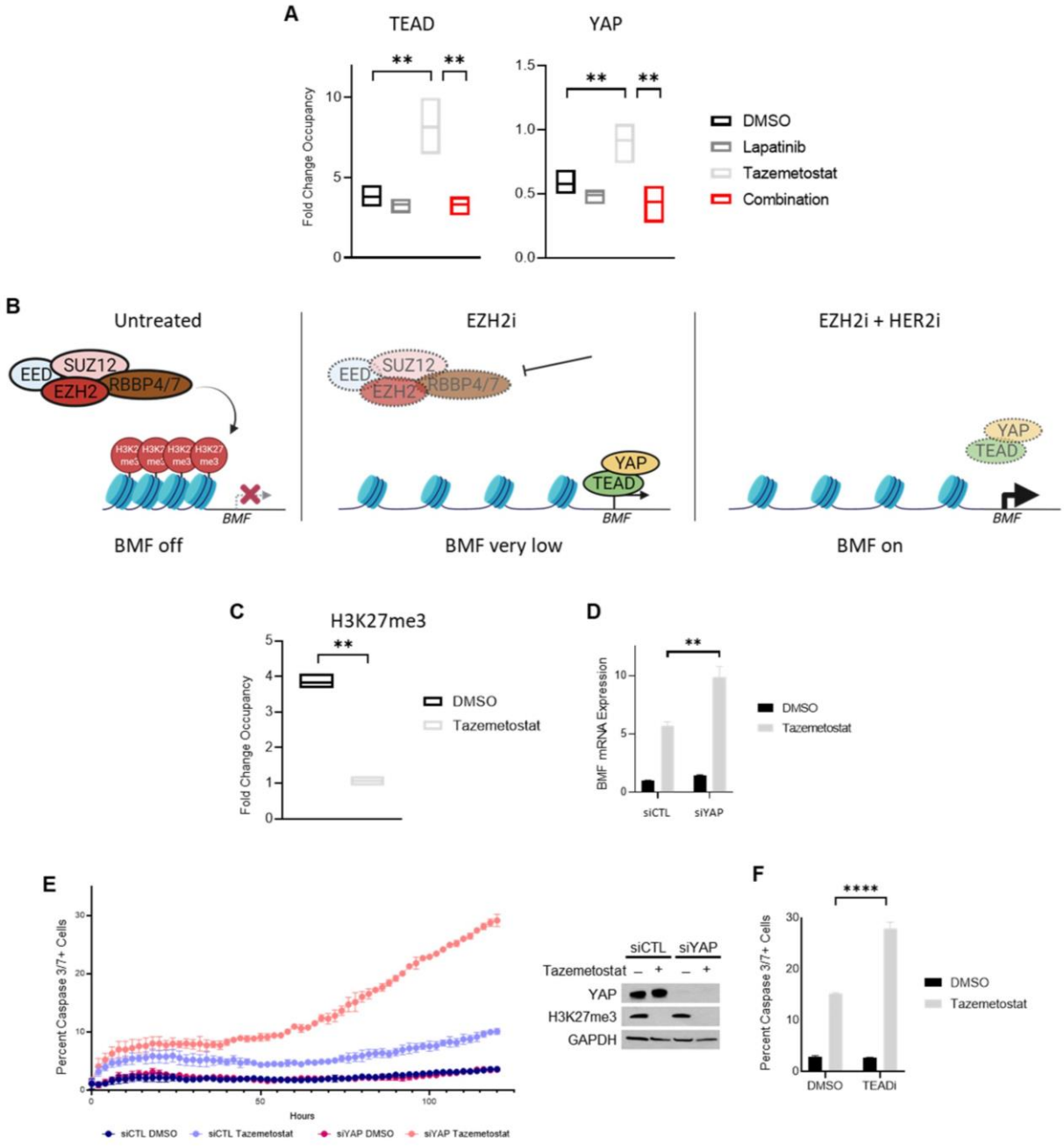


Figure 2-4. YAP-TEAD protects HER2+ breast cancer from cell death in response to EZH2 inhibitor

(A) ChIP-qPCR analysis of TEAD and YAP binding at BMF regulatory region in SKBR3 cells. After 5 days of pretreatment in 5μM tazemetostat or DMSO, 100nM lapatinib or DMSO was layered on, and cells treated for 24 hours before being crosslinked and processed.

(B) The proposed mechanism.

Figure 2-4 (continued)

(C) ChIP-qPCR analysis of H3K27me3 binding at BMF regulatory region in SKBR3 cells. After 5 days of pretreatment in 5μM tazemetostat or DMSO, 100nM lapatinib or DMSO was layered on, and cells treated for 24 hours before being crosslinked and processed.

(D) Fold change ± SD of BMF expression of siCTL and siYAP SKBR3 cells calculated relative to DMSO-treated cells. After 5 days of pretreatment in 5μM tazemetostat or DMSO, media and drugs were refreshed, and cells treated for 24 hours before RNA was extracted.

(E) Live-cell imaging of siCTL and siYAP SKBR3 cells depicting mean change in the percent of caspase 3/7+ cells ± SD from technical triplicates over 5 days of indicated treatment. After 5 days of pretreatment in 5μM tazemetostat or DMSO, media and drugs were refreshed, and cells treated for 5 days. Tazemetostat drug inhibition and YAP knockdown were confirmed by immunoblotting.

(F) Live-cell imaging of SKBR3 cells depicting mean change in the percent of caspase 3/7+ cells ± SD from technical triplicates at 120 hours of indicated treatment. After 5 days of pretreatment in 5μM tazemetostat or DMSO, 10μM of TEAD inhibitor or DMSO was layered on, and cells treated for 5 days.

DISCUSSION

Advances in HER2 targeted therapies have dramatically improved outcomes for HER2+ breast cancer patients. However, the poor prognosis for patients with advanced disease highlight their inadequacies. More durable and ultimately curative responses will require improved strategies for eliminating residual disease. In this study, we describe a combinatorial strategy that dramatically enhances responses to HER2 kinase inhibitors by killing tumor cells that are normally resistant to these agents.

Specifically, we show that EZH2 inhibitors trigger a shift in the epigenetic state of HER2+ breast cancers, which sensitizes them to HER2 kinase inhibitors. This occurs because EZH2, which is frequently overexpressed in HER2+ breast cancers, actively suppresses the expression of the pro-apoptotic protein BMF, by depositing repressive H3K27me3 marks at transcriptional

regulatory sequences. Nevertheless, in most cells EZH2 inhibitors are not sufficient to induce BMF expression because loss of H3K27me3 promotes the binding repressive YAP/TEAD complexes at these regulatory sites. However, repression can be relieved by genetic or chemical inhibition of YAP/TEAD complexes or by HER2 kinase inhibitors which trigger the dissociation of YAP and TEAD from the BMF locus. Together these studies demonstrate how EZH2 and YAP play redundant yet distinct roles in buffering BMF expression and protecting HER2+ breast cancers from apoptotic signals.

Importantly, our data also suggest EZH2 inhibitors should be combined specifically with HER2 kinase inhibitors, because these agents potently induce apoptosis via cooperative cell-autonomous mechanisms. Certainly, the addition of HER2 directed antibodies might further enhance therapeutic responses by promoting ADCC or ADCP, however the full cytotoxic response would not likely be achieved by HER2 antibodies alone. It should be noted that there is currently no standard third-line therapy for HER2+ disease, and some patients cannot or do not wish to endure chemotherapy at this stage. The striking potency of EZH2 and HER2 kinase inhibitors, suggests that these agents may represent an alternative to chemotherapy in this setting, either on their own, or combined with HER2 monoclonal antibodies.

Finally, the observation that EZH2 inhibitors prime tumors to become more responsive to HER2 kinase inhibitors may have important clinical implications for other cancer types. For example, both HER2 and EZH2 are overexpressed in a subset of other advanced solid tumors such as colorectal, non–small cell lung, pancreatic, and ovarian cancers, raising the intriguing possibility of the utility of this combination outside of HER2+ breast cancer (J. Zhao and Xia 2020; Kanayama et al. 2018; Richman et al. 2016; H. Zhang et al. 2016; Bonello, Sims, and Langdon

2018; Jones, Varambally, and Arend 2018; Ougolkov, Bilim, and Billadeau 2008; Shibata et al. 2018). Functional and epigenetic studies must be performed to formally assess this possibility; however, our studies suggest that mechanistic differences between HER2 kinase inhibitors and monoclonal antibodies should be considered when assessing future combinations.

EXPERIMENTAL METHODS

Cell Lines and Reagents

All cell lines were purchased from ATCC, except for HCC202, which were a generous gift from Yannis Zervantonakiss in Joan Brugge's lab. No further authentication of these cell lines was performed. All cell lines were regularly tested for Mycoplasma using the MycoAlert Mycoplasma Detection Kit (Lonza, LT07-318). Cells were used for experiments within 10-15 passages from thawing. HCC202 and BT474 were cultured in RPMI medium supplemented with 10% FBS. MDA-MB-361 and MDA-MB-453 were cultured in DMEM medium supplemented with 10% FBS. SKBR3 were cultured in McCoy's 5A medium supplemented with 10% FBS.

Antibodies for immunoblot were obtained from the following sources: Cell Signaling Technology: GAPDH (2118), H3K27me3 (9733), HER2 (2242), pHER2 (2241), YAP (14074). HA (3724S)

Antibodies for ChIP-qPCR were obtained from the following sources: Cell Signaling Technology: H3K27me3 (9733S), IgG (2729), TEAD (13295), YAP (14074S); Diagenode: H3K27ac (C15410196)

Drugs were obtained from the following sources: Selleck Chem: GSK126 (S7061), lapatinib ditosylate (S1028), MAK683 (S8983), MYF-01-37 (S8950), tazemetostat (S7128), trastuzumab (A2007), tucatinib (S8362); LC Laboratories: Lapatinib, Di-p-Toluenesulfonate Salt (L-4804).

Drug vehicle chemicals were obtained from the following sources: Methocel K15M Premium CR Hydroxypropyl Methylcellulose was a generous gift from Jason Zoeller in Joan Brugge's lab; Ligand Pharmaceuticals: Captisol (RC - 0C7 - K01).

17β-Estradiol 0.36mg 90-day release pellets were obtained from Innovative Research of America (NE-121).

CRISPR/Cas9 HA-BMF N-terminal tagged construct was a gift from Magda Bahcall in Pasi Jänne's lab.

Infections and Transfections

shRNA constructs were prepared and virus was harvested as previously described (McLaughlin et al. 2013). Virus was incubated on target cells for 6 to 16 hours at a 1:2–1:10 dilution with 8-µg/mL polybrene.

Short interfering RNAs (siRNAs) ON-TARGET SMARTpool siRNA were purchased from GE Healthcare/Dharmacon to target BMF and YAP, and transfected with lipofectamine RNAiMAX (Invitrogen, cat. # 13778-075). Cells were transfected approximately 24 hours before proliferation experiments were started. Cells were transfected for 8 hours with 10 mM siRNA constructs using a 1:400 dilution of Lipofectamine RNAiMAX (Invitrogen, cat. # 13778-075) in antibiotic-free media.

Drug Concentrations

For all *in vitro* experiments throughout this manuscript the following drug concentrations were used: EEDi MAK638=5µM, EZH2i GSK126=5µM, EZH2i tazemetostat=5µM, HER2i lapatinib=30-1000nM, HER2i trastuzumab= 0.1-20µg/mL, HER2i tucatinib =10n-300nM, TEADi MYF-01-37=1µM

Cell Proliferation Assays

Cells were pretreated with either EZH2i or DMSO control for 5 days. On day 0 of experiment, 150,000 cells per well were seeded onto 6-well plates in appropriate media. 24 hours later, cells were counted for day 0 timepoint and combination treatment started. Cells were then counted in triplicate on day 3 or 5 to determine the change in cell number compared to day 0. Media was not replaced for day 3 proliferation assays, but was changed at day 3 for day 5 assays. Proliferation experiments that included siRNA knockdown were performed on cells approximately 24 hours after the initial transfection.

Synergy Assays

For the dose-response matrix assay, cells were plated in 96-well plates. 3 replicates were done for each condition. At 24 hours, one plate of cells was flash frozen and stored at -80ºC to represent the time 0 plate. Drugs were added to the remaining plates. After 5 days, each of the plates was frozen. After freezing, the plates (day 0 and 5) were thawed simultaneously and cells were quantified using CellTiter-Glo (Promega) as per manufacturer's instructions. SynergyFinder (Ianevski et al. 2020) was used to analyze drug combination dose-response matrix data. To

determine the combination effects in excess of Loewe additivity, a Synergy Score was calculated to characterize the strength of synergistic interaction.

Incucyte Cell Proliferation and Death Assays

Cells were infected with IncuCyte Nuclight red reagent (Sartorius, cat. #4476) and selected in puromycin to create stable cell lines. Nuclight versions of each cell line were plated at 3,000 cells/well in a 96-well plate. Approximately 24 hours later, the media was removed and media containing 1:1000 green caspase 3/7 apoptosis assay reagent (Sartorius, cat. #4440) and appropriate drug concentrations was added. The 96-well plate was then placed in the Incucyte instrument and images were taken every two to four hours over the course of 5 days. The Incucyte software was then trained to count the cells based on the number of red-expressing nuclei in the field of view. Four images were taken per well and averaged and triplicate wells were counted per condition. Percent caspase 3/7+ cells was assayed by the presence of yellow cells, which contain green signal (caspase reagent) overlapping with red signal (nuclei) and dividing that by the red signal count (nuclei).

Chromatin Immunoprecipitation-qPCR

For YAP and TEAD chromatin immunoprecipitation, chromatin was dually crosslinked using 2 mM DSG (Thermo #20593) for 45 minutes at room temperature and then 1% formaldehyde for 10 minutes at room temperature before quenching with 2.5M glycine. For H3K27me3 chromatin immunoprecipitation, chromatin was crosslinked with 1% formaldehyde only. Chromatin was harvested from cells by lysis in lysis buffer 1 (50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA pH 8, 10% glycerol, 0.0033% NP-40, 0.25% Triton-X100), lysis buffer 2 (10

mM TrisHCl pH 8, 200 mM NaCl), and lysis buffer 3 (10 mM Tris HCl pH 8, 100 mM NaCl, 0.1% sodium deoxycolate, 0.5% sarcosyl). Crosslinked chromatin was digested using micrococcal nuclease (CST 10011) for 10 minutes at 37C and then sonicated for 30 cycles of 30 seconds on, 30 seconds off. Immunoprecipitation was performed using anti-YAP (CST 14074), anti-TEAD (CST 13295), anti-H3K27me3 (CST 9733S), and anti-IgG (CST 2729S) antibodies with 36 ug of chromatin per YAP or TEAD IP and 5 ug of chromatin per H3K27me3 IP. Immunoprecipitate was harvested using ChIP-grade Protein A/G magnetic beads and washed with 3x low salt buffer (0.1% SDS, 1% Triton-X100, 2 mM EDTA, 20mM TrisHCl pH 8, 150 mM NaCl), 3x high salt buffer (0.1% SDS, 1% Triton-X100, 2 mM EDTA, 20mM TrisHCl pH 8, 500 mM NaCl), 3x LiCl wash buffer (0.25M LiCl, 1% NP-40, 1 mM EDTA, 10mM TrisHCl pH 8, 1% sodium deoxycolate) , 1x TE buffer, and eluted in elution buffer (50 mM TrisHCl pH 8, 10 mM EDTA, 1% SDS). Eluted chromatin was reverse crosslinked using proteinase K (CST 10012) overnight at 55C and purified using the Qiagen PCR purification kit (Qiagen 28106). qPCR was performed using SYBR PerfeCTa Master Mix (Quanta 3332S). Fold change enrichment was calculated by normalizing signal to IgG signal for that IP.

In Vivo Drug Studies

Animal procedures were approved by the Center for Animal and Comparative Medicine in Harvard Medical School in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act.

For all *in* vivo experiments, 10 week old female NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) were purchased from The Jackson Laboratory (005557)

For BT474 experiments, mice were first subcutaneously implanted with 17β-Estradiol 0.36mg 90 day pellets, and mammary fat pad injections done 24 hours later. $3x10^6$ cells in 1:1 Matrigel:DMEM/F12 were orthotopically injected into the fourth mammary fat pads of each mouse. Treatment commenced at day 0 when tumors reached an average size of 200 mm^3 approximately 5-6 weeks after initial injection. Mice were randomly assigned to experimental groups. Same procedure was followed with the xenografts of MDA-MB-453, but no estradiol pellets were implanted.

For all mouse experiments, lapatinib was administered at 200 mg/kg daily by oral gavage (vehicle: 0.5% (w/v) hydroxypropyl methylcellulose with 0.1% (v/v) Tween 80). GSK126 was administered at 300 mg/kg intraperitoneally twice weekly (vehicle: 200mg/ml Captisol in water). Trastuzumab was diluted in PBS and administered at 20mg/kg intraperitoneally twice weekly. Compounds given in combination were administered six hours apart. Tumor size was measured every 2-3 days using digital calipers. Tumor volume was calculated using the standard formula $L \times W^2 \times 0.52$.

Appropriate mass of lapatinib was measured onto a weigh paper, and a small amount gently poured into an autoclaved medium-sized mortar. 1mL of 0.5% (w/v) hydroxypropyl methylcellulose with 0.1% (v/v) Tween 80) (lapatinib vehicle) was directly added to lapatinib in mortar and then autoclaved pestle used to grind the lapatinib until a uniform liquid suspension (not a paste) was achieved. This procedure was repeated until all powder was ground. The volume was completed with 0.5% (w/v) hydroxypropyl methylcellulose with 0.1% (v/v) Tween 80) whenever there wasn't enough. The Bioruptor was set to High, pulse on: 30" pulse off 30" and sonicated for 30' at 4C. The mixture was vortexed and made into 1mL aliquots. Lapatinib was

prepared once a week and stored at 4C until 30’ before drug treatment when it was allowed to come to room temperature.

0.5% (w/v) hydroxypropyl methylcellulose with 0.1% (v/v) Tween 80) (lapatinib vehicle) was prepared by first heating ultrapure water to 80-90°C in a sterile 150-250mL plastic bottle. Appropriate volume of HPMC was added and immediately vortexed. Completed the volume and left on shaker at high speed for 30’. Appropriate volume of Tween80 was added and placed on shaker at high speed until Tween80 went into solution. HPMC vehicle was prepared once a month, made into 1mL aliquots, and stored at 4C until 30’ before drug treatment when it was allowed to come to room temperature.

Appropriate masses of GSK126 and Captisol were measured using separate weigh papers. GSK126 was slowly poured over the Captisol, distributing it evenly across the surface. A clean flat edge razor blade was used to thoroughly cut and mix the two powders until no large chunks remained. The powder mix was placed into a sterile 15mL tube and ultra clean water used to bring the volume up to 75% of the final volume. 1N acetic acid was added dropwise while periodically vortexing until the powders went into solution. Completed the volume with ultra clean water. GSK126 was prepared once a week, distributed into 1mL aliquots, and stored at 4C until 30’ before drug treatment when it was allowed to come to room temperature.

To make Captisol vehicle for GSK126, appropriate mass of Captisol powder was massed and placed into a sterile 15mL tube. Ultrapure water was added to complete the volume. Captisol solution was prepared once a week, made into 1mL aliquots, and stored at 4C until 30’ before drug treatment when it was allowed to come to room temperature.

Trastuzumab was made fresh daily by diluting appropriate volume with sterile, USP-grade PBS.

RNA-Seq Expression Analysis

RNA was isolated from MDA-MB-453 and SKBR3 cells 24 hours after treatment with indicated drugs. RNA was isolated using TRIzol, following the manufacturer's protocol and RNA cleanup was performed using the Invitrogen TURBO DNA-free kit. The Molecular Biology Core Facilities at Dana-Farber Cancer Institute constructed cDNA libraries and sequenced samples.

Gene Set Enrichment analysis (GSEA) was performed on RNA-Seq data. Gene lists are from publicly available GSEA lists (MSigDB) of the Broad Institute.

QUANTIFICATION AND STATISTICAL ANALYSIS

For quantitative measurements, graphs represent mean ± SD. Where indicated data are presented as log2 fold change over initial measurements. Changes in tumor volume are presented in a waterfall plot with each bar representing the change in tumor volume of an individual animal in the study. A bar over the zero line indicates tumor growth and a bar under the zero line indicates tumor shrinkage. 2-tailed unpaired t tests and Mann-Whitney U tests were used to compare data sets where indicated, and p values are shown. A p value less than or equal to 0.05 was considered significant. Data were graphed and analyzed using GraphPad Prism. Cell proliferation assays are representative of 3 biological replicates.

Chapter 3 : Identifying Drugs That Cooperate with EZH2 Inhibitors in Breast Cancer Models

PREFACE

This chapter contains unpublished, preliminary data that was chronologically completed before or simultaneous with the earliest experiments shown in Chapter 2. However, after observing the therapeutic potential of co-targeting EZH2 and HER2 in HER2+ breast cancer, I chose to exclusively focus on that project. Nevertheless, this Chapter provides a written record of my studies assessing the effects of other drug combinations in HER2+ and TNBC models and may serve as a launchpad for future investigations.

INTRODUCTION

Breast cancer subtypes vary considerably from one another and must be treated as completely different diseases. Accordingly, a therapy that is effective in one subtype may not work in another. For this reason, I investigated various EZH2 inhibitor-based combination therapies in both HER2+ and TNBC models. Importantly, TNBC and HER2+ breast cancer subtypes overexpress EZH2, suggesting that EZH2 may serve as a potential therapeutic target in these diseases. In addition, these are the two deadliest breast cancer subtypes, and there is an unmet clinical need to develop improved therapies.

Work in our lab and others has shown that EZH2 inhibitors alone are not effective in most solid tumor models. However, we reasoned that EZH2 inhibitors might cooperate with other therapeutic agents. Again, different breast cancer subtypes are treated with different therapeutic agents, and so we chose to determine if layering EZH2 inhibitor onto the current standard of care (SOC) would result in a cooperative effect. For TNBC, the SOC is chemotherapy, but when I began

this project, clinical trials using Poly(ADP-Ribose) Polymerase Inhibitors (PARPi) and histone deacetylase inhibitors (HDACi) were emerging (Schech et al. 2015; Comen and Robson 2010). In addition, unpublished work from our lab showed that combined EZH2i + HDACi could kill castration-resistant prostate cancers, due to the cooperative effects of these agents on H3K27 acetylation, and so we were inspired to assess this combination in the triple negative and HER2+ subtypes. Therefore, in TNBC models we evaluated the effects of EZH2 inhibitors combined with (1) various chemotherapies or (2) HDAC inhibitors. For HER2+ tumors, the standard of care includes HER2-targeted therapies, which led to our assessment of the primary drug combination described in this thesis: combined HER2 and EZH2 inhibitors. However, we also chose to investigate EZH2 and HDAC inhibitors in HER2+ tumors, based on the scientific rationale noted above and the fact that there was an ongoing clinical trial evaluating the HDAC inhibitor entinostat combined with lapatinib and trastuzumab in recurrent or metastatic HER2+ tumors (Lim et al. 2019). Our studies investigating the effects of EZH2 and HER2 inhibitors were extensively discussed in Chapter 2, however the effects of these other drug combinations, both positive and negative, are described here.

RESULTS

EZH2i + chemotherapy do not cooperate in TNBC

As discussed in the introduction and Chapter 2, high EZH2 levels in breast cancer are associated with poor clinical outcome, a more aggressive phenotype, and advanced stage disease (Kleer et al. 2003; Sun et al. 2009) (Figure 1-5). Analysis of TCGA primary tumor samples with

matched normal tissue broken down by subtype indicate that EZH2 levels are high in the basal, luminal B, and HER2+ subtypes (Figure 1-6), confirming that high levels of EZH2 correlate with the subtypes known to have more aggressive disease and worse outcomes (Figure 1-1).

Triple negative tumors are often diagnosed at later stages due to their aggressive nature, and so it is important to determine if the higher expression in these tumors might simply be due to a higher population of later stage tumors that skews the data (Zuo et al. 2017). Comparing TNBC samples to matched normal breast tissue from TCGA Firehouse demonstrated that EZH2 expression was substantially increased in breast cancer tissues irrespective of the relative stage of the tumor (Figure 3-1)**.** EZH2 expression is independent of staging, suggesting that gain of EZH2 expression occurs early in tumor development, and is not simply a result of more advanced stage disease. These elevated levels of EZH2 in an incurable disease led us to consider EZH2 as a potential therapeutic target in TNBC.

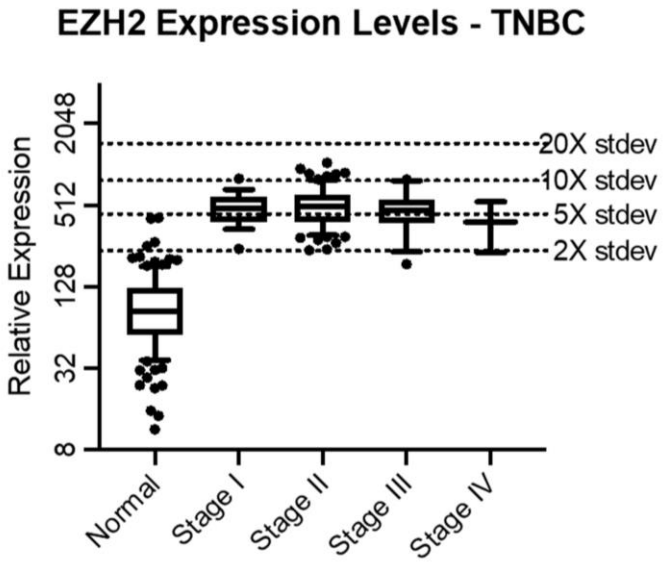


Figure 3-1. EZH2 is overexpressed in TNBC irrespective of staging
Matched normal breast and TNBC tumor tissue data from TCGA Firehouse. ER, PR, and HER2 status were determined IHC. Figure from Amy Schade

The standard of care for TNBC has long been chemotherapy. However, recent insights into the molecular characteristics of TNBC (Table 1-2) have led to clinical trials and FDA-approval of agents that utilize a more targeted approach. For example, patients with BRCA1 or BRCA2 mutations typically have homologous recombination deficiency (HRD), leading to faulty DNA repair that allows for the abnormal cells to survive (Denkert et al. 2017). For these patients, DNA-crosslinking platinum chemotherapies such as cisplatin and carboplatin have been of interest, and have shown promise in clinical trials (Pandy et al. 2019). In addition, olaparib and talazoparib, inhibitors of the poly(ADP-ribose) polymerase (PARP) family of enzymes involved in DNA repair, are theorized to trigger cell death in BRCA1/2-mutant cancers via synthetic lethality and have been FDA-approved for patients with germline BRCA1/2 mutations (Robson et al. 2017). More recently, the 2021 FDA-approvals of the monoclonal antibody pembrolizumab and antibody drug conjugate sacituzumab govitecan illustrate that some TNBCs are sensitive to immunotherapies, providing more treatment options to combat a disease with few effective therapies (Torres and Emens 2021).

Though chemotherapy is the standard of care for TNBC, it is not curative, and patients have a 5 year survival of less than 30% and a mortality rate 3 months after relapse of 75% (Yin et al. 2020; Lehmann et al. 2011). We therefore investigated whether EZH2 inhibitors could enhance the effects of various chemotherapies. Specifically, we evaluated the effects of EZH2 inhibitor combined with 5 individual chemotherapeutic drugs currently in use in the treatment of TNBC—3 FDA approved and 2 not. Because TNBC is treated by different categories of chemotherapies, we selected a panel of drugs that represent these different classifications (Figure 3-2A).

Unfortunately, we did not observe any cooperativity between chemotherapeutic agents and EZH2 inhibition (Figure 3-2B-F and data not shown). Any cytotoxic effects in the combination-treated cells seemed to be largely driven by the chemotherapy drug, and EZH2 inhibitor did not enhance the effects of chemotherapy in non-responsive cells. Given that the combination of chemotherapeutic agents and EZH2 inhibitor did not yield a cooperative effect, we chose to move forward testing new combinations.

A

Drug	FDA-approved for TNBC	Category
Cisplatin	No	Platinum-based compound
Gemcitabine	Yes	Nucleoside antimetabolite
Doxorubicin	Yes	Anthracycline
Topotecan	No	Topoisomerase I inhibitor
Paclitaxel	Yes	Taxane

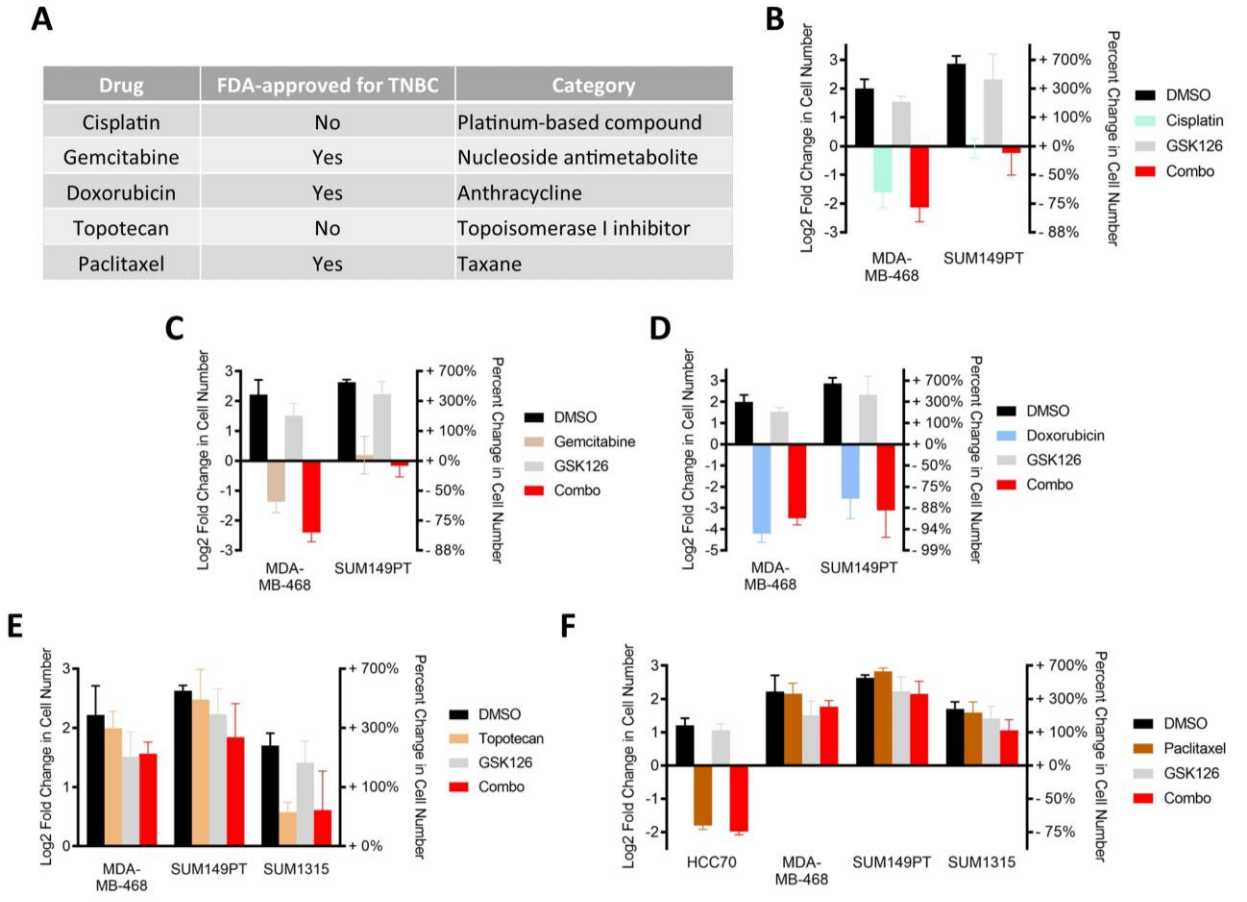


Figure 3-2. Chemotherapeutic agents and EZH2i combinations do not have a cooperative effect

(A) Chemotherapeutic drugs we used for our preliminary panel, whether they have been FDA-approved for use in TNBC or not, and what category of chemotherapy they belong to.

(B) Response of indicated TNBC cells after 3 days of the indicated treatment. After 5 days of pretreatment in 5μM GSK126 or DMSO, 1.56μM cisplatin or DMSO was layered on and cells were treated for 3 days. Graphs represent log2 transformation of the fold change in cell number at day

3 versus day 0. Right axis shows percent change in cell number relative to day 0. Negative values represent a decrease in cell number over time and positive values represent an increase in cell number.

(C) Response of indicated TNBC cells after 3 days of the indicated treatment. After 5 days of pretreatment in 5μM GSK126 or DMSO, 10mM gemcitabine or DMSO was layered on and cells

Figure 3-2 (continued)

were treated for 3 days. Graphs represent log2 transformation of the fold change in cell number at day 3 versus day 0. Right axis shows percent change in cell number relative to day 0. Negative values represent a decrease in cell number over time and positive values represent an increase in cell number.

(D) Response of indicated TNBC cells after 3 days of the indicated treatment. After 5 days of pretreatment in 5μM GSK126 or DMSO, 0.5μM doxorubicin or DMSO was layered on and cells were treated for 3 days. Graphs represent log2 transformation of the fold change in cell number at day 3 versus day 0. Right axis shows percent change in cell number relative to day 0. Negative values represent a decrease in cell number over time and positive values represent an increase in cell number.

(E) Response of indicated TNBC cells after 3 days of the indicated treatment. After 5 days of pretreatment in 5μM GSK126 or DMSO, 1nM topotecan or DMSO was layered on and cells were treated for 3 days. Graphs represent log2 transformation of the fold change in cell number at day 3 versus day 0. Right axis shows percent change in cell number relative to day 0. Negative values represent a decrease in cell number over time and positive values represent an increase in cell number.

(F) Response of indicated TNBC cells after 3 days of the indicated treatment. After 5 days of pretreatment in 5μM GSK126 or DMSO, 1nM paclitaxel or DMSO was layered on and cells were treated for 3 days. Graphs represent log2 transformation of the fold change in cell number at day 3 versus day 0. Right axis shows percent change in cell number relative to day 0. Negative values represent a decrease in cell number over time and positive values represent an increase in cell number.

EZH2i + HDACi cooperate in TNBC

Dysregulation of the epigenome plays an important role in cancer. Notably, HDACs are key epigenetic regulators and are often overexpressed in cancers, including breast cancers (Reviewed in Glozak and Seto 2007). While HDAC inhibitors are effective in some hematopoietic malignancies they do not exhibit activity as single agents in solid tumors (Hontecillas-Prieto et al.

2020). However, promising preclinical studies have fueled an interest in combining HDAC inhibitors with other targeted agents in solid tumors such as, PARP inhibitors, proteasome inhibitors, immune checkpoint inhibitors, and mTOR inhibitors, among others (Hontecillas-Prieto et al. 2020; A. Min et al. 2015; Laporte et al. 2017; K. Kim et al. 2014; Malone et al. 2017; Booth et al. 2017). Clinical trials are also underway testing HDACi with the aromatase inhibitors letrozole (W. W. Tan et al. 2016) and anastrozole, monoclonal antibody atezolizumab (O'Shaughnessy et al. 2020) and taxane paclitaxel (Ramaswamy et al. 2012). This renewed interest in developing clinical trials with HDAC inhibitor-based drug combinations and the potential mechanistic cooperativity between HDAC and EZH2 inhibitors on gene regulation, inspired the following study.

As outlined in the introduction, there are four classes of HDACs that are classified based on their sequence homology to yeast (Hontecillas-Prieto et al. 2020). We tested two HDAC inhibitors: entinostat and panobinostat (Figure 3-3A). We first chose to investigate the more selective HDAC inhibitor, entinostat, which suppresses only a subset of class I HDACs, specifically HDACs1 and 3 (Malone et al. 2017; Hu et al. 2003). Entinostat + EZH2i exerted cooperative effects and potently induced cell death in two of the cell lines tested (Figure 3-3B). The potent but less selective HDAC inhibitor panobinostat, which inhibits Class I, II, and IV HDAC complexes, also killed TNBC cells when combined with an EZH2 inhibitor, although more cell lines should be examined (Figure 3-3C). Nevertheless, the observation that EZH2i + HDACi potently kill TNBC cells suggest that these combinations may be worth further investigation.

A

Drug	HDAC classes inhibited
Entinostat	I (HDAC1 and HDAC4)
Vorinostat	I, II, IV
Panobinostat	I, II, IV

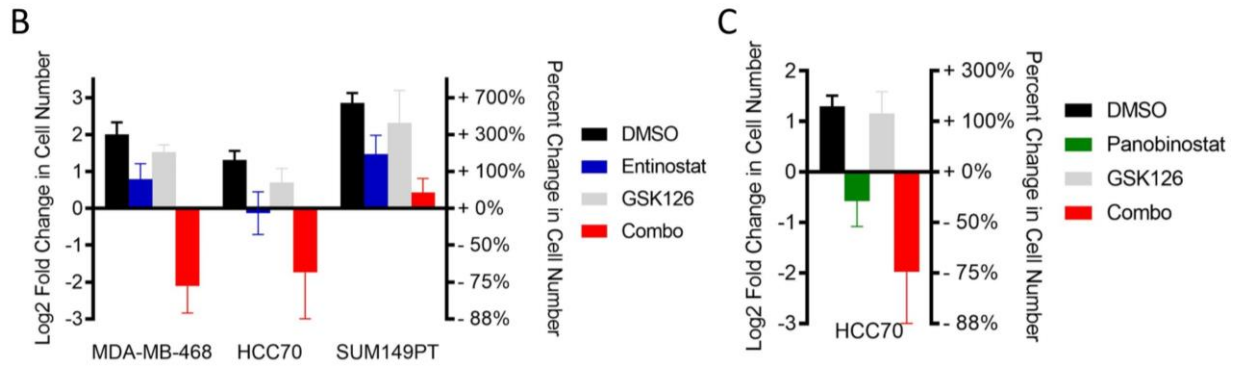


Figure 3-3. Entinostat and panobinostat have cooperative effects with EZH2i in TNBC cell lines
(A) HDAC inhibitors used for our preliminary panel and the HDAC classes they target.

(B) Response of indicated TNBC cells after 3 days of the indicated treatment. After 5 days of pretreatment in 5μM GSK126 or DMSO, 1μM entinostat or DMSO was layered on and cells were treated for 3 days. Graphs represent log2 transformation of the fold change in cell number at day 3 versus day 0. Right axis shows percent change in cell number relative to day 0. Negative values represent a decrease in cell number over time and positive values represent an increase in cell number.

(C) Response of indicated HCC70 after 3 days of the indicated treatment. After 5 days of pretreatment in 5μM GSK126 or DMSO, 10nM panobinostat or DMSO was layered on and cells were treated for 3 days. Graphs represent log2 transformation of the fold change in cell number at day 3 versus day 0. Right axis shows percent change in cell number relative to day 0. Negative values represent a decrease in cell number over time and positive values represent an increase in cell number.

EZH2i + HDACi cooperate in HER2+ breast cancer

Though there are a breadth of FDA-approved HER2-targeting agents for use in HER2+ breast cancer in the clinic, the poor prognosis for metastatic patients demonstrates the need for additional therapeutic targets. HDAC inhibitors were particularly attractive agents to consider as

several known mechanisms of resistance to HER2-targeting agents are thought to be epigenetic in nature (Table 1-5). Indeed, in HER2+ breast cancer cell lines, an entinostat and trastuzumab combination was capable of overcoming trastuzumab-resistance and enhancing its growth inhibition effects and promote apoptosis *in vitro* (Huang et al. 2011). Building off of this observation, another group showed that treating HER2+ breast cancer cell lines that were resistant to single-agent trastuzumab or lapatinib with a lapatinib and entinostat combination, synergistically inhibited cell proliferation, reduced *in vitro* colony formation, and caused *in vivo* tumor shrinking or growth inhibition (Lee et al. 2014). These results spurred a phase Ib clinical trial utilizing entinostat and lapatinib $\pm$ trastuzumab in metastatic HER2+ breast cancer patients who progressed after a trastuzumab-containing regimen. Preliminary results suggested that the combination was well-tolerated with promising clinical benefits for the patients. (Lim et al. 2019). Given the observed cooperativity between HDAC and EZH2 inhibitors in prostate and triple negative breast cancers, and the suggestion that HDACs might be involved in mediating drug resistance in HER2+ breast cancers (Huang et al. 2011), I began evaluating the effects of combined EZH2 and HDAC inhibitors in HER2+ breast cancer.

I began by treating a panel of HER2+ breast cancer cell lines with EZH2 inhibitors and the HDACs 1 and 4 inhibitor entinostat. Notably, EZH2i + entinostat potently cooperated in 7/9 lines, triggering cell death or more cell death than either agent alone (Figure 3-4A). As expected, the potent broader HDAC inhibitor panobinostat exerted similar effects (Figure 3-4B).

Taken altogether with findings discussed in Chapter 2, my results suggest that in TNBC models EZH2 inhibitors do not potentiate the effects of chemotherapy but do cooperate with HDAC inhibitors. In HER2+ tumors, EZH2 inhibitors cooperate with both HER2 kinase inhibitors

and HDAC inhibitors. The rationale for exclusively moving forward with the HER2i + EZH2i combination and the future prospects of other EZH2-inhibitor based combinations are discussed below.

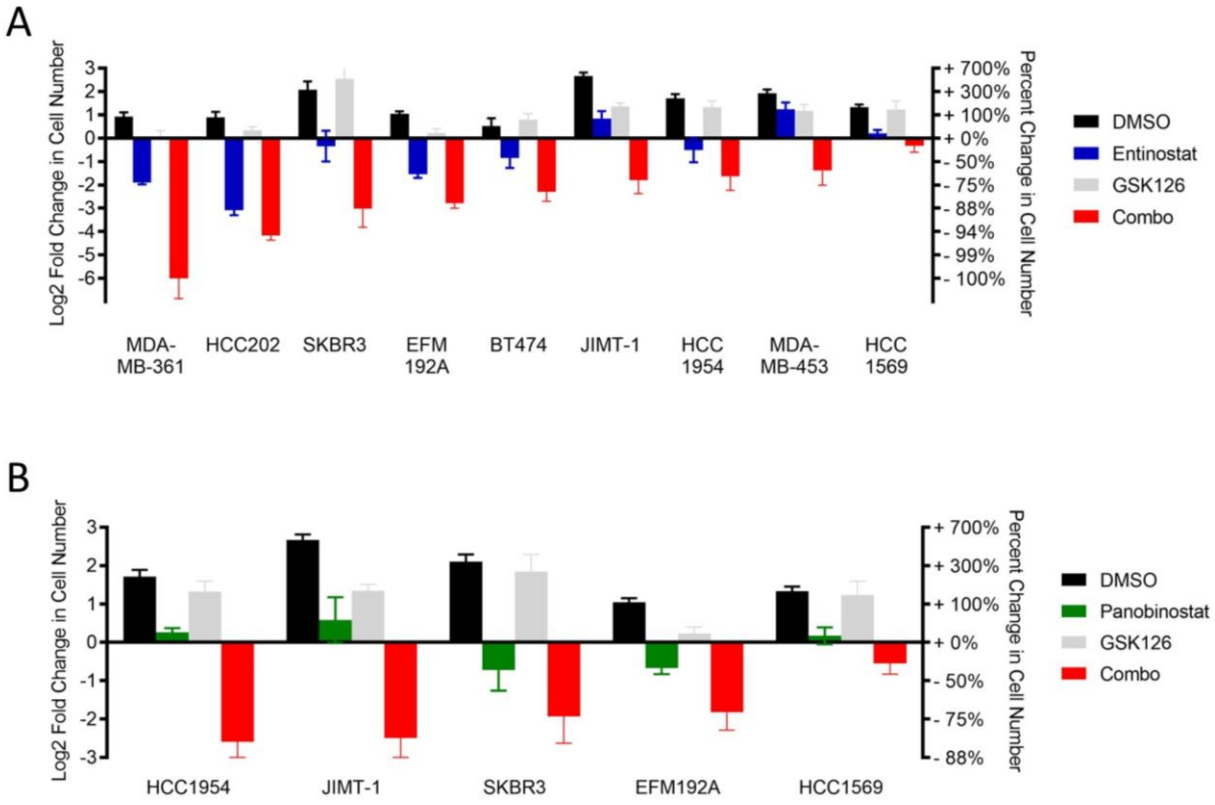


Figure 3-4. Entinostat and panobinostat have cooperative effects with EZH2i in HER2+ breast cancer cell lines

(A) Response of indicated HER2+ breast cancer cells after 3 days of the indicated treatment. After 5 days of pretreatment in 5μM GSK126 or DMSO, 1μM entinostat or DMSO was layered on and cells were treated for 3 days. Graphs represent log2 transformation of the fold change in cell number at day 3 versus day 0. Right axis shows percent change in cell number relative to day 0. Negative values represent a decrease in cell number over time and positive values represent an increase in cell number.

(B) Response of indicated HER2+ breast cancer cells after 3 days of the indicated treatment. After 5 days of pretreatment in 5μM GSK126 or DMSO, 10nM panobinostat or DMSO was layered on and cells were treated for 3 days. Graphs represent log2 transformation of the fold change in cell number at day 3 versus day 0. Right axis shows percent change in cell number relative to day 0. Negative values represent a decrease in cell number over time and positive values represent an increase in cell number.

DISCUSSION

The goal of my thesis work has been to identify promising EZH2 inhibitor-based therapeutic combinations for breast cancer and deconstruct their mechanism of action. The data reported in this Chapter was part of a series of preliminary studies conducted at the beginning of my graduate work. Specifically, I describe the effects of combining EZH2 inhibitors with either chemotherapies or HDAC inhibitors in TNBC and HER2+ models.

In TNBC, we first chose to evaluate the effects of EZH2 inhibitors combined with chemotherapeutic agents because chemotherapies represented the standard of care. Despite selecting a variety of chemotherapeutic agents with very different mechanisms of action, I found that none of the agents appeared to cooperate with EZH2 inhibition. However, this does not necessarily mean that EZH2 inhibitors and chemotherapy never cooperate. It may simply mean that the combinations that I evaluated did not work in the specific cell lines I used, which may not represent all TNBCs. When considering TNBC, it is important to remember that it can be further divided into 6 different subtypes based on their gene expression profiles (Lehmann et al. 2011). Out of the four cell lines that I used, only the BL1 and BL2 subtypes were represented.

However, it is also possible that EZH2 inhibitors may ultimately enhance responses to chemotherapy in patients, due to effects on the immune system, which was not assessed in my *in vitro* studies. For example, EZH2 inhibitors have been shown to upregulate MHC Class I genes and promote immune responses in other diseases (Cañadas et al. 2018; Burr et al. 2019). Accordingly, while chemotherapy would be expected to trigger cell death, EZH2 inhibitors might promote an immune response, resulting in a greater therapeutic response in patients. This could

be further enhanced by the addition of immune checkpoint inhibitors. Indeed, a clinical trial evaluating the effects of tazemetostat + pembrolizumab has been initiated in locally advanced or metastatic urothelial carcinoma (NCT03854474).

I also investigated EZH2i + HDACi combinations in TNBC and HER2+ breast cancers, because (1) HDACi + EZH2i cooperatively enhance H3K27 acetylation and gene expression, (2) various studies have implicated HDACs in breast cancer, (3) we found that these agents exerted synergistic effects in other tumor types, and (4) several ongoing clinical trials were initiated to assess the utility of HDAC inhibitors in TNBC and HER2+ breast cancer.

My data suggest that EZH2 and HDAC inhibitors cooperatively kill triple negative and HER2+ breast cancer cells, although additional *in vitro* and *in vivo* preclinical studies must be performed to strengthen that conclusion. Currently, the standard of care for TNBC is non-specific, systemic chemotherapy due to the lack of defined therapeutic targets (Waks and Winer 2019). The high levels of EZH2 and HDACs would provide two specific and defined druggable targets that might yield therapeutic benefit. In addition, there are already many clinical trials underway utilizing HDAC inhibitors in combination with such agents as the PARP inhibitor olaparib or chemotherapeutic agents paclitaxel, capecitabine, or carboplatin (Reviewed in Wawruszak et al. 2021), suggesting the use of HDAC inhibitors in the treatment of TNBC may be a feasible therapeutic option.

However, the recent failure of several HDAC inhibitor-based trials, in both TNBC and HER2+ tumors may likely dampen enthusiasm for additional clinical trials, whether this belief is warranted or not (Conte et al. 2009; Goldstein et al. 2017; O'Shaughnessy et al. 2020).

Nevertheless, our studies in prostate cancer have shown us that we can still glean information about important biological (protective) pathways and new therapeutic targets by dissecting the mechanism by which these agents kill tumors, which could be assessed in the future.

For both subtypes, the initial set of experiments would parallel the path of my dissertation—first testing a larger panel of cell lines to confirm that the combination is synergistic in a substantial number of cell lines, and then use transcriptional and epigenetic studies to interrogate the mechanism. Interestingly, other soon to be published studies from our laboratory demonstrate that EZH2 and HDAC inhibitors are extremely potent in castration-resistant prostate cancer and that EZH2 and HDACs cooperatively buffer the ATF3 gene, a broad stress sensor that kills cells when its expression is upregulated and sustained. Specifically, in the presence of EZH2 and HDAC inhibitors, H3 histones (bound to the *ATF3* promoter) become progressively demethylated and then acetylated specifically at lysine 27. This potently induced ATF3 expression and killed advanced CRPC, due to the inherent cellular stresses that are present in advanced tumors (e.g. DNA damage, oxidative stress, metabolic stress, etc.) (Kuzmickas 2019). Similar mechanistic studies in TNBC and HER2+ breast cancers could reveal important genes and key vulnerabilities in these tumor types.

METHODS

Cell Lines and Reagents

All cell lines were purchased from ATCC, except for HCC202, which were a generous gift from Yannis Zervantonakiss in Joan Brugge's lab and HCC70, which were a generous gift from Laura Ghisolfi in Alex Toker's lab. No further authentication of these cell lines was performed. All cell lines were regularly tested for Mycoplasma using the MycoAlert Mycoplasma Detection Kit (Lonza, LT07-318). Cells were used for experiments within 10-15 passages from thawing. HCC70, HCC202 and BT474 were cultured in RPMI medium supplemented with 10% FBS. MDA-MB-361 and MDA-MB-453, MDA-MB-468 were cultured in DMEM medium supplemented with 10% FBS. SKBR3 were cultured in McCoy's 5A medium supplemented with 10% FBS. SUM149PT were cultured in Ham's F12 medium supplemented with 5% FBS, 5µg/mL insulin, 1µg/mL hydrocortisone. SUM1315 were cultured in Ham'e F12 medium supplemented with 5% FBS, 5µg/mL insulin, 10ng/mL EGF.

Drugs were obtained from the following sources: Cisplatin was a generous gift from Isaac Harris in Joan Brugge's lab; Selleck Chem: GSK126 (S7061), Topotecan (S1231), Paclitaxel (S1150), Entinostat (S1053), Panobinostat (S1030), Vorinostat (S1047); Tocris: Gemcitabine hydrochloride (3259); Cell Signaling Technology: Doxorubicin (5927)

Drug Concentrations

For all in vitro experiments throughout this chapter, the following drug concentrations were used: GSK126=5µM, cisplatin=1.56 µM, germcitabine=10nM, doxorubicin=0.5µM, topotecan=1nM, paclitaxel=1nM, entinostat=1µM, panobinostat=10nM, vorinostat= 1µM

Cell Proliferation Assays

Cells were pretreated with either GSK126 or DMSO control for 5 days. On day 0 of experiment, 150,000 cells per well were seeded onto 6-well plates in appropriate media. 24 hours later, cells were counted for day 0 timepoint and combination treatment started. Cells were then counted in triplicate on day 3 to determine the change in cell number compared to day 0.

Chapter 4 : Conclusions and Future Directions

SUMMARY

The goal of my thesis work has been to develop new combinatorial therapies for breast cancer by exploiting agents that target the epigenetic enzyme, EZH2. EZH2 has been shown to play a role in breast cancer development and progression, and is highly overexpressed in HER2+ and triple negative breast cancers. It is also known to maintain stem-cell like states. As such, we reasoned that the epigenetic changes conferred by EZH2 inhibitors might sensitize tumors to other therapeutic agents. Despite advances in breast cancer therapies, the current standard of care is not curative for recurrent and/or metastatic disease, underscoring the need for more effective treatments.

In Chapter 2, I found that EZH2 inhibitors dramatically potentiate the effects of HER2 kinase inhibitors in HER2+ breast cancers: both by enhancing baseline responses to these agents and re-sensitizing resistant cells. Importantly, this combination has real potential to be translated into a clinical trial. HER2 kinase inhibitors are already routinely used in the metastatic setting, EZH2 inhibitors are currently in clinical trials for a variety of cancers, and one agent has already been FDA-approved. In the course of my studies, I primarily utilized the HER2 tyrosine kinase inhibitor lapatinib because it is used in the third-line setting for patients with advanced stage disease who have relapsed after treatment with alternative HER2 drugs (monoclonal antibodies and antibody-drug conjugates). However, I also showed that tucatinib, which has been more recently approved, is also effective when combined with EZH2 inhibitors. I also used two EZH2 inhibitors in my studies, tazemetostat and GSK126. GSK126 is no longer being clinically developed but tazemetostat has been FDA approved and therefore could be readily integrated into a clinical trial. Other promising EZH2 inhibitors, such as CPI-0209, could also be evaluated (Table 1-7).

Importantly I found that this drug combination was effective in multiple models of HER2+ breast cancer, both *in vitro* and *in vivo.* By performing transcriptional and epigenetic studies, I showed that EZH2 inhibitors cause an epigenetic shift, triggering the loss of H3K27me3 and inducing the binding of repressive YAP-TEAD complexes to regulatory sequences of the pro-apoptotic protein BMF. While BMF levels remain low, EZH2 inhibitor-treated cells are "poised" to be more sensitive to HER2 kinase inhibitors. Accordingly, layering on HER2 kinase inhibitors stimulates the release of YAP/TEAD complexes and an induction of BMF expression, which triggers apoptosis. Importantly, I found that this combination exerted enhanced and more durable tumor responses in distinct *in vivo* models. Therefore, these studies should support the development of a clinical trial and may ultimately represent a promising therapeutic option for HER2+ patients.

In Chapter 3, I shared my unpublished preliminary studies evaluating other EZH2 inhibitor-based combinations in triple negative and HER2+ breast cancers, by using agents that represent the standard of care or were being clinically investigated in these diseases. I chose these two subtypes due to their high EZH2 expression, and the fact that patients with advanced disease have poor prognoses. For both subtypes, I investigated EZH2i + HDACi and EZH2i + chemotherapy (with HER2i + EZH2i being discussed in Chapter 2).

I conducted cell proliferation assays using a panel of TNBC and HER2+ breast cancer cell lines to systematically determine if these agents had a cooperative effect in either subtype. I found that EZH2 inhibitors did not potentiate the effects of various chemotherapies, at least *in vitro*. However, combined EZH2i + HDACi appeared to be a promising combination for both

subtypes. While I chose to not proceed studying this combination, these observations suggest that it may warrant further study in one or both subtypes, which was discussed in Chapter 3.

FUTURE DIRECTIONS

Clinical outlook for co-targeting HER2 and EZH2 in metastatic HER2+ breast cancer

Despite the many treatment options for metastatic HER2+ breast cancer, the lack of a curative regimen highlights the unmet need for new, more effective combinations. In addition, many current options rely on the use of a HER2 inhibitor combined with some form of chemotherapy, and the side effects from such a systemic, untargeted approach are often debilitating and devastating to a patient's quality of life. Thus, I believe that the combination identified by my thesis work, comprised of two targeted agents, may ultimately be beneficial to patients.

I anticipate that this combination could move forward in a clinical trial in the third-line setting, for patients who have progressed on current first- and second- line treatments: trastuzumab + pertuzumab + taxane and T-DM1, respectively (Figure 1-4). Unlike many of the current third-line therapies that pair a HER2-targeted agent with a chemotherapeutic agent, my combination does not include a systemic and non-specific chemotherapeutic agent, which should result in less toxicity for the patient. Alternatively, because trastuzumab can further enhance the effects of lapatinib, even in the third-line setting (Blackwell et al. 2012), perhaps EZH2 inhibitors could be layered on top of these two agents. While additional preclinical studies investigating the efficacy of a triple combination in HER2+ tumors, using an immunocompetent model, would

further support this approach, my dissertation work sets the ground for two potential therapeutic combinations that do not utilize chemotherapy, and may have real promise in the clinic.

Importantly, my studies have shown that inhibiting EZH2 enhances baseline responses to HER2 kinase inhibitors, but also sensitizes tumors that have become resistant to these agents. This is directly relevant for use in the clinic, as patients need treatment options capable of: (1) Destroying tumors before they can acquire resistance mechanisms and (2) Overcoming acquired and *de novo* resistance mechanisms. The potency of my combination suggests that it may be able to kill more tumor cells up front, reducing the potential for developing acquired resistance. However, my combination may still be effective in tumors that already exhibit resistance to HER2-targeted therapies. For example, ~70% of HER2+ breast cancers are resistant to trastuzumab (Spector and Blackwell 2009), and one resistance mechanism is the accumulation of carboxyl tail fragments (CTFs) collectively known as p95HER2, which can emerge either from proteolytic cleavage of the extracellular domain of the full length HER2 receptor or alternative splicing of the mRNA encoding HER2 (Hart et al. 2020). Of particular note is the p95HER2 611-CTF, which results in HER2 that lacks the trastuzumab-binding epitope (Arribas et al. 2011). Because my combination does not rely on trastuzumab or the trastuzumab-binding epitope, it would easily be able to bypass this resistance mechanism and induce cell death. In addition, the observation that EZH2 inhibitors sensitize tumor cells to lower levels of HER2 inhibition, suggest that this combination may overcome resistance mechanisms in which HER2-targeting agents elicit incomplete inhibition of HER2, allowing for the activation of other HER family members (Pernas and Tolaney 2019). Similarly, this increased sensitivity to HER2-targeted agents may prevent the

development of mutations in the PIK3CA/PTEN pathway, by killing cells before they emerge, or render them less effective (Koboldt et al. 2012).

An exciting additional benefit of my combination is its potential to affect brain metastases, which are an incurable and deadly threat. Up to half of metastatic HER2+ breast cancer patients will develop brain metastases. The current standard of care for these patients depends on the number, location, and size of the tumors, as well as the patient's overall health and disease condition, but the options consist of locally directed therapy with surgical resection, stereotactic radiosurgery, and/or whole-brain radiation therapy (Stavrou, Winer, and Lin 2021). All options are invasive and imprecise, and despite their aggressive nature, the rate of intracranial progression is still unacceptably high (N. U. Lin et al. 2020).

Though trastuzumab and pertuzumab have been shown to delay the onset of brain metastases, they are unable to penetrate the blood brain barrier and cannot be utilized as a therapy for established brain metastases (Duchnowska, Loibl, and Jassem 2018). Interestingly, utilizing trastuzumab as an adjuvant therapy is associated with a significantly increased risk of brain metastases as the first site of recurrence (Olson et al. 2013). It is yet unclear whether this is due to the increased life expectancy of trastuzumab-treated patients that now allows them to live long enough to develop brain metastases, improved diagnostic methods, or micro-metastatic cells utilizing the central nervous system as a "sanctuary" from trastuzumab (Bria et al. 2007).

As a small molecule inhibitor, lapatinib is able to cross the blood-brain barrier and has already shown some efficacy against brain metastases in clinical trials, both alone and with the chemotherapy capecitabine, which is also thought to cross the blood-brain barrier (Cameron et

al. 2008; Metro et al. 2011; Bachelot et al. 2013). These results indicate lapatinib-based combination therapies show promise in the clinic and are worth studying in HER2+ disease, due to its propensity for brain metastases.

It should be noted that though we emphasized lapatinib in our study, we also showed that the HER2 selective tyrosine kinase inhibitor tucatinib was also very effective (Figure 2-1G). Tucatinib's recent FDA-approval is very exciting, as it is also a small molecule tyrosine kinase inhibitor that can cross the blood-brain barrier (Duchnowska, Loibl, and Jassem 2018). Recent clinical trial results showed that patients with brain metastases treated with tucatinib, capecitabine, and trastuzumab had a 1-year progression free survival of 24.9% compared to 0% for the capecitabine and trastuzumab-treated patients (Murthy et al. 2020). Such stark numbers provide grounds for further investigations using tucatinib in combination with EZH2 inhibitors.

Preclinical studies have shown that the FDA-approved EZH2 inhibitor tazemetostat cannot cross the intact blood-brain-barrier, as it interacts with the drug efflux transporters ABCB1 and/or ABCG2. However, the ABCB1/ABCG1 inhibitor elacridar led to greater brain penetration, suggesting potential methods for tazemetostat to bypass the blood-brain barrier (P. Zhang et al. 2015). In addition, brain tumors often compromise the blood-brain barrier, creating their own vasculature system known as the blood-tumor barrier (Arvanitis, Ferraro, and Jain 2020), and it is possible that this may allow tazemetostat to penetrate a brain tumor. Preclinical studies have indicated that EZH2 inhibition may be an effective strategy against H3K27M-mutant pediatric gliomas (Mohammad et al. 2017), and tazemetostat is currently being tested in a phase II clinical trial in a variety of recurrent pediatric gliomas (NCT03155620). As a result, more data regarding tazemetostat's ability to cross the blood-brain barrier may shortly become available (Mueller et

al. 2020). The blood-brain barrier permeability of other clinical EZH2 inhibitors is not yet known, but will also become available.

Regardless of tazemetostat's ability to cross the blood-brain barrier, if the combination of HER2 tyrosine kinase inhibitors and EZH2 inhibitors were to be utilized in the third-line setting for disease that has not yet metastasized to the brain, it may be potent enough to completely ablate systemic disease, preventing brain colonization from occurring.

The combination of HER2i + EZH2i represent a promising clinical option as HER2 inhibitors are already a standard treatment for metastatic HER2+ patients, and EZH2 inhibitors are currently being tested in a variety of clinical trials as part of combination therapies. In addition, the 2020 FDA-approval of tazemetostat demonstrates the widespread acceptance of EZH2 inhibitors in cancer treatment and is indicative of the very real potential for this combination to be developed into a clinical trial.

Future questions about the role of YAP/TEAD as a repressor of BMF

My thesis describes a promising novel therapeutic combination in the treatment of metastatic HER2+ breast cancer. However, several important mechanistic questions warrant further investigation.

The exact function of YAP in breast cancer, much less in HER2+ breast cancer, has yet to be understood. As discussed in the introduction, YAP overexpression in *in vivo* studies utilizing multiple mammary carcinoma cell lines resulted in more aggressive disease and rendered a

benign non-transformed mammary epithelial cell line highly metastatic (Lamar et al. 2012). In addition, studies in human epithelial mammary cells showed that YAP/TEAD can suppress tumor-suppressor genes in order to promote cell proliferation and survival (M. Kim et al. 2015). Taken together, these studies suggest an oncogenic role for YAP in breast cancer.

However, others have demonstrated that YAP expression, as measured by IHC, is lower in patient tumor samples relative to normal breast tissue (Yuan et al. 2008; Jaramillo-Rodríguez et al. 2014) and that its knockdown in BT474 (HER2+), MDA-MB-231 (triple negative), and T47D (luminal A) breast cancer cell lines suppressed anoikis *in vitro*. Furthermore, *in vivo* studies using YAP knockdown MDA-MB-231 cells demonstrated that tumors formed earlier and grew faster than in control mice (Yuan et al. 2008).

Taken together these data suggest that YAP functions in a very context-specific manner. Notably, while genetic knockdown of YAP alone did not have a marked effect on apoptosis in my models (Figure 2-4E), the mechanism by which it buffers BMF to prevent its upregulation upon EZH2 inhibitor-treatment, is more in line with an oncogenic function in the context of my combination.

In addition to an incomplete understanding of YAP's function in breast cancer, its role in HER2+ breast cancer is also not fully understood,. Interestingly however, there is some work suggesting that it may be involved in therapeutic resistance triggered by different signals. For example, one study proposes that the Hippo pathway and YAP are important in sensing a cell's environment and responding to microenvironmental rigidity. Specifically, the authors suggest that YAP acts as a mechanosensitive transcriptional activator that promotes lapatinib resistance

in response to increased extracellular matrix rigidity. (C.-H. Lin et al. 2015). However, the upstream signals and downstream effectors were not identified in this study. In a separate report, lapatinib-resistant HER2+ breast cancer cell lines were found to activate the mevalonate pathway (MVA), a metabolic pathway essential in cell growth and proliferation. The authors suggested that enhanced MVA activated YAP, which promoted mTORC1 activation through an unknown mechanism, and ultimately therapeutic resistance (Sethunath et al. 2019). While I did not observe any effects on the mTOR pathway in my studies, these two reports raise the intriguing possibility that multiple signals converge on YAP to promote resistance to HER2 kinase inhibitors. However, it should be noted that in both settings EZH2 inhibitors would be expected to open up the BMF locus and enhance the cell death signals triggered by lapatinib, perhaps overcoming these resistance signals.

The role of HER2 inhibitors in triggering apoptosis

In Chapter 2, we determined that EZH2 inhibitors induce YAP/TEAD to repress BMF, triggering a protective response that prevents apoptosis. However, HER2 induces YAP/TEAD dissociation from the BMF locus, thus promoting its expression and inducing apoptosis (Figure 2-4B). However, we did not investigate how lapatinib transduces its effects. Specifically, how do HER2 inhibitors suppress YAP in HER2+ breast cancer?

Some potential avenues of investigation are to study kinases that are thought to activate YAP and determine what, if any, is their relationship to HER2. One potential candidate includes Src, the proto-oncogene and nonreceptor tyrosine kinase known to be an activator of YAP and

driver of YAP activity and metastatic potential in a mouse TNBC model (Lamar et al. 2019). Src can bind HER2 and these kinases cooperatively control cell growth and survival (Belsches-Jablonski et al. 2001). In addition, aberrant Src activation has been shown to promote resistance to HER2 inhibitors and Src inhibition can overcome trastuzumab resistance both *in vitro* and *in vivo* (S. Zhang et al. 2011). In fact, the Src inhibitor dasatinib was combined with trastuzumab and paclitaxel in a phase II clinical trial for first line HER2+ metastatic breast cancer patients and showed very promising results (Ocana et al. 2019). Notably, amplification of another Src-family kinase member, YES1, has been shown to promote acquired resistance to neratinib (Takeda et al. 2020), trastuzumab/lapatinib (Takeda et al. 2017), and T-DM1 (Lei Wang et al. 2020). In fact, YAP's full name is "YES-associated protein 1," and YES1 has been shown to regulate YAP's transcriptional activity. All of these studies demonstrate that Src or Src-family tyrosine kinases are critical regulators of YAP activity.

Because Src directly binds HER2-family receptors, and HER2 kinase inhibitors suppress Src binding and its activation, we hypothesize that lapatinib may suppress YAP activity through its effects on Src. One way I can interrogate this possibility would be to monitor YAP/TEAD activity by transfecting a fluorescent YAP/Hippo pathway reporter developed by (Mohseni et al. 2014) into my cells and treat them with the Src-family kinase inhibitor, dasatanib. Using the Incucyte live-cell imaging system, we can track YAP activity over time. If Src suppression mediates YAP suppression in this setting, then dasatanib should suppress YAP activity, but only in the presence of EZH2 inhibitors. Conversely, a constitutively activated Src mutant should prevent YAP inactivation and dissociation from the BMF locus. It would also be expected to prevent cell death in response to EZH2 and HER2 inhibitors.

Targeting other EZH2 and HER2 overexpressing malignancies

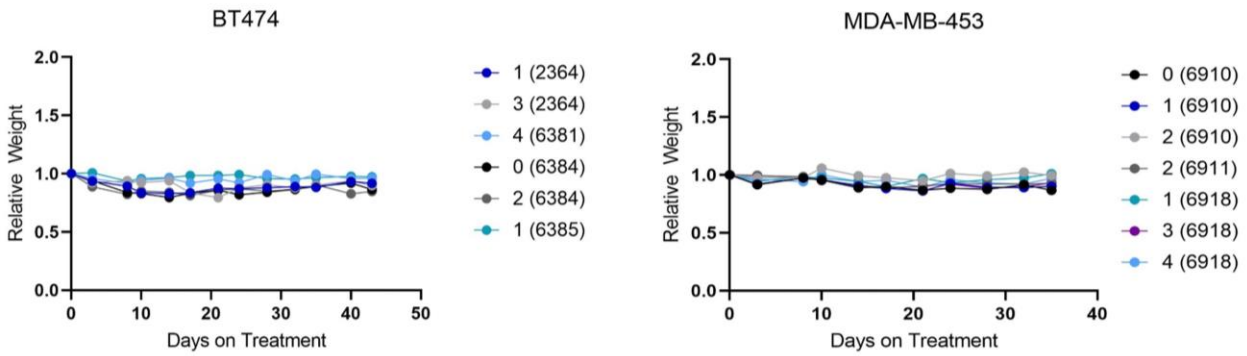
Lastly, it is important to note that EZH2 and HER2 kinase inhibitors may be useful in other cancers. Notably, HER2 is overexpressed in many other solid tumors including, but not limited to, gastric, ovarian, biliary tract, colorectal, and bladder cancers. The frequency of this overexpression can vary from ~2% in NSCLC to >50% of uterine cancers (Reviewed in Oh and Bang 2020). Though HER2 amplification and/or overexpression is observed in 2-3% of solid tumors, HER2-targeted therapies are only FDA-approved for breast, gastric, and gastroesophageal cancers (Meric-Bernstam et al. 2021). However, the therapeutic value for utilizing these agents has become clear with promising preliminary results from HER2 basket trials using pertuzumab + trastuzumab (Meric-Bernstam et al. 2021), T-DM1 (B. T. Li et al. 2018), and neratinib (Hyman et al. 2018). Thus, HER2 is a rational therapeutic target that should be targeted in a variety of cancer types.

Importantly, high levels of EZH2 have been observed in many tumor types including breast, prostate, bladder, gastric, lung, and hepatocellular carcinoma (J. Min et al. 2010; Chase and Cross 2011; Deb, Thakur, and Gupta 2013). While tazemetostat is the only FDA-approved EZH2 inhibitor, and even then only for follicular lymphoma and epithelioid sarcoma, it and other EZH2 inhibitors are in clinical trials for many other cancer types (Duan, Du, and Guo 2020) (Table 1-7). In addition, Epizyme, the manufacturer of tazemetostat has announced plans to initiate basket trials using tazemetostat across heme and solid tumors (Epizyme, Inc. 2021).

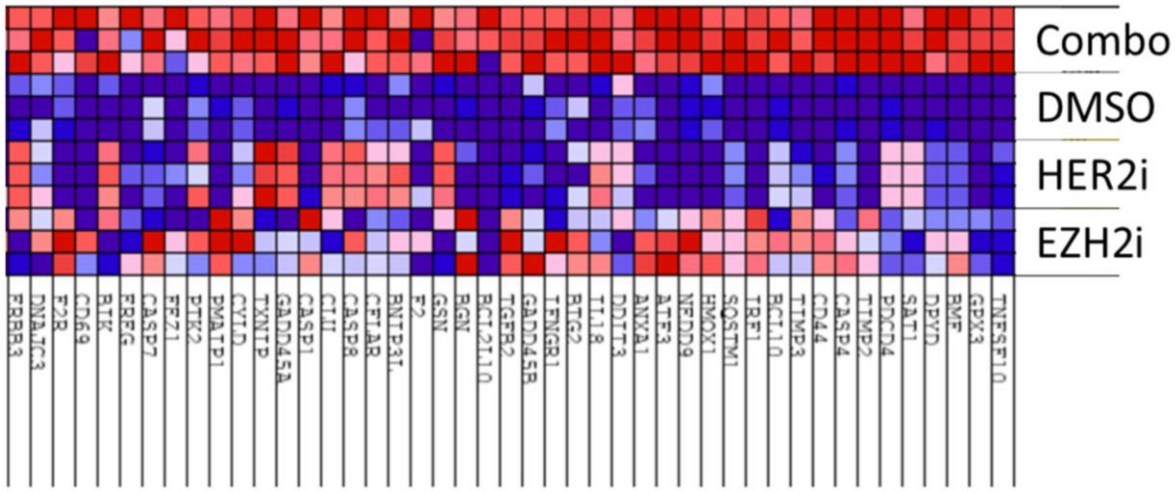
Taken together, these data suggest that HER2 inhibitors and EZH2 inhibitors may be useful across a variety of cancers separately. However, the observation that many of these cancers

express high levels of both EZH2 and HER2 suggest that our combination may have broader utility. Therefore, I plan to test my combination in other cancer types with high levels of EZH2 and HER2 such as colorectal, non–small cell lung, pancreatic, and ovarian.

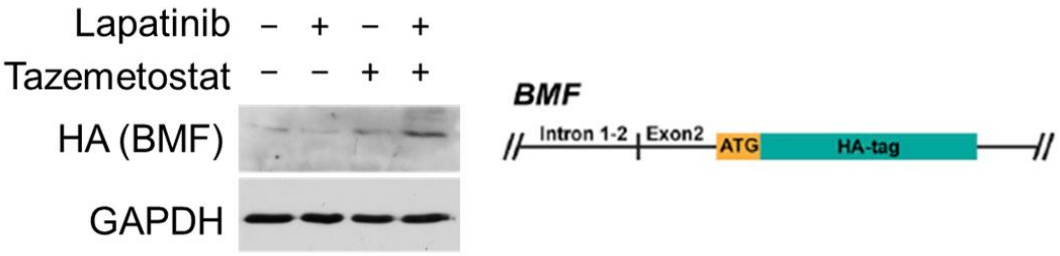
Appendix A: Supplementary Materials for Chapter 2



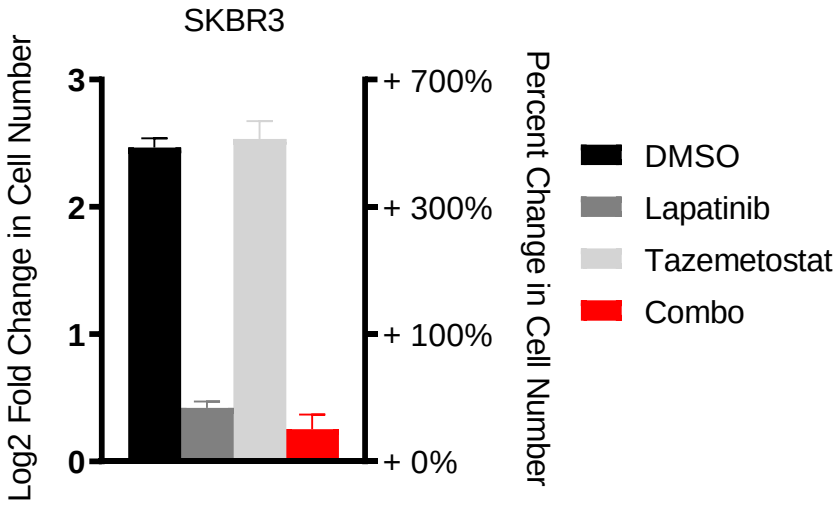
Supplemental Figure A-1 HER2 and EZH2 inhibitor treatment does not affect mouse weight
Weight of mice with (left) BT474 xenografts and (right) MDA-MB-453 xenografts treated with lapatinib and GSK126. X-axis indicates days on treatment. Y-axis indicates relative weight (normalized to day 0 weight of individual animal). Legend at right indicates identifier of each mouse.



Supplemental Figure A-2 Hallmarks of cancer apoptosis signature is enriched in cells treated with both HER2 and EZH2 inhibitors
GSEA analysis of RNA-Seq data from SKBR3 cells from technical triplicates collected 24 hours after treatment. After 5 days of pretreatment in 5μM tazemetostat or DMSO, 100nM lapatinib or DMSO was layered on, and cells treated for 24 hours before RNA was extracted.



Supplemental Figure A-3 EZH2 and HER2 inhibitors cooperatively upregulate BMF
(Left) Immunoblots on MDA-MB-453 HA-BMF cells. After 5 days of pretreatment in 5µM tazemetostat or DMSO, 1µM lapatinib or DMSO was layered on, and cells treated for 24 hours before protein was harvested. (Right) Schematic representation of the endogenous BMF locus



Supplemental Figure A-4 Co-inhibition of EZH2 and HER2 without pre-treatment of EZH2 is not cytotoxic
Response of SKBR3 HER2+ breast cancer cells after 5 days of the indicated treatment. Graphs represent log2 transformation of the fold change in cell number at day 5 versus day 0. Right axis shows percent change in cell number relative to day 0. Negative values represent a decrease in cell number over time and positive values represent an increase in cell number.

References

ACS, Williams. 2019. “Breast Cancer Facts & Figures 2019-2020,” 44.

Akala, Omobolaji O., and Michael F. Clarke. 2006. “Hematopoietic Stem Cell Self-Renewal.” *Current Opinion in Genetics & Development* 16 (5): 496–501. https://doi.org/10.1016/j.gde.2006.08.011.

Akinleye, Akintunde, Parthu Avvaru, Muhammad Furqan, Yongping Song, and Delong Liu. 2013. “Phosphatidylinositol 3-Kinase (PI3K) Inhibitors as Cancer Therapeutics.” *Journal of Hematology & Oncology* 6 (1): 88. https://doi.org/10.1186/1756-8722-6-88.

Allis, C. David, and Thomas Jenuwein. 2016. “The Molecular Hallmarks of Epigenetic Control.” *Nature Reviews. Genetics* 17 (8): 487–500. https://doi.org/10.1038/nrg.2016.59.

Allis, C. David, and Brian D. Strahl. 2000. “The Language of Covalent Histone Modifications.” *Nature (London)* 403 (6765): 41–45. https://doi.org/10.1038/47412.

Alluri, Prasanna, and Lisa A. Newman. 2014. “Basal-Like and Triple-Negative Breast Cancers Searching for Positives Among Many Negatives.” *Surgical Oncology Clinics of North America* 23 (3): 567–77. https://doi.org/10.1016/j.soc.2014.03.003.

Arribas, Joaquín, José Baselga, Kim Pedersen, and Josep Lluís Parra-Palau. 2011. “P95HER2 and Breast Cancer.” *Cancer Research* 71 (5): 1515–19. https://doi.org/10.1158/0008-5472.CAN-10-3795.

Arteaga, Carlos L., and Jeffrey A. Engelman. 2014. “ERBB Receptors: From Oncogene Discovery to Basic Science to Mechanism-Based Cancer Therapeutics.” *Cancer Cell* 25 (3): 282–303. https://doi.org/10.1016/j.ccr.2014.02.025.

Arvanitis, Costas D., Gino B. Ferraro, and Rakesh K. Jain. 2020. “The Blood-Brain Barrier and Blood-Tumour Barrier in Brain Tumours and Metastases.” *Nature Reviews. Cancer* 20 (1): 26–41. https://doi.org/10.1038/s41568-019-0205-x.

Bachelot, Thomas, Gilles Romieu, Mario Campone, Véronique Diéras, Claire Cropet, Florence Dalenc, Marta Jimenez, et al. 2013. “Lapatinib plus Capecitabine in Patients with Previously Untreated Brain Metastases from HER2-Positive Metastatic Breast Cancer (LANDSCAPE): A Single-Group Phase 2 Study.” *The Lancet Oncology* 14 (1): 64–71. https://doi.org/10.1016/S1470-2045(12)70432-1.

Bang, Y. J., G. Giaccone, S. A. Im, D. Y. Oh, T. M. Bauer, J. L. Nordstrom, H. Li, et al. 2017. “First-in-Human Phase 1 Study of Margetuximab (MGAH22), an Fc-Modified Chimeric Monoclonal Antibody, in Patients with HER2-Positive Advanced Solid Tumors.” *Annals of Oncology* 28 (4): 855–61. https://doi.org/10.1093/annonc/mdx002.

Beca, Francisco, Kevin Kensler, Benjamin Glass, Stuart J. Schnitt, Rulla M. Tamimi, and Andrew H. Beck. 2017. “EZH2 Protein Expression in Normal Breast Epithelium and Risk of Breast Cancer: Results from the Nurses’ Health Studies.” *Breast Cancer Research : BCR* 19 (1): 21–21. https://doi.org/10.1186/s13058-017-0817-6.

Belsches-Jablonski, Allison P., Jacqueline S. Biscardi, Dena R. Peavy, David A. Tice, Davis A. Romney, and Sarah J. Parsons. 2001. “Src Family Kinases and HER2 Interactions in Human Breast Cancer Cell Growth and Survival.” *Oncogene* 20 (12): 1465–75. https://doi.org/10.1038/sj.onc.1204205.

Berns, Katrien, Hugo M. Horlings, Bryan T. Hennessy, Mandy Madiredjo, E. Marielle Hijmans, Karin Beelen, Sabine C. Linn, et al. 2007. “A Functional Genetic Approach Identifies the PI3K Pathway as a Major Determinant of Trastuzumab Resistance in Breast Cancer.” *Cancer Cell* 12 (4): 395–402. https://doi.org/10.1016/j.ccr.2007.08.030.

Beyer, Tobias A., Alexander Weiss, Yuliya Khomchuk, Kui Huang, Abiodun A. Ogunjimi, Xaralabos Varelas, and Jeffrey L. Wrana. 2013. “Switch Enhancers Interpret TGF-β and Hippo Signaling to Control Cell Fate in Human Embryonic Stem Cells.” *Cell Reports (Cambridge)* 5 (6): 1611–24. https://doi.org/10.1016/j.celrep.2013.11.021.

Blackledge, Neil P., and Robert J. Klose. 2021. “The Molecular Principles of Gene Regulation by Polycomb Repressive Complexes.” *Nature Reviews. Molecular Cell Biology*. https://doi.org/10.1038/s41580-021-00398-y.

Blackwell, Kimberly L., Harold J. Burstein, Anna Maria Storniolo, Hope S. Rugo, George Sledge, Gursel Aktan, Catherine Ellis, et al. 2012. “Overall Survival Benefit with Lapatinib in Combination with Trastuzumab for Patients with Human Epidermal Growth Factor Receptor 2-Positive Metastatic Breast Cancer: Final Results from the EGF104900 Study.” *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology* 30 (21): 2585–92. https://doi.org/10.1200/JCO.2011.35.6725.

Bondarev, Andrey D., Misty M. Attwood, Jörgen Jonsson, Vladimir N. Chubarev, Vadim V. Tarasov, and Helgi B. Schiöth. 2021. “Recent Developments of HDAC Inhibitors: Emerging Indications and Novel Molecules.” *British Journal of Clinical Pharmacology* n/a (n/a). https://doi.org/10.1111/bcp.14889.

Bonello, Maria, Andrew Harvey Sims, and Simon Peter Langdon. 2018. “Human Epidermal Growth Factor Receptor Targeted Inhibitors for the Treatment of Ovarian Cancer.” *Cancer Biology & Medicine* 15 (4): 375–88. https://doi.org/10.20892/j.issn.2095-3941.2018.0062.

Booth, Laurence, Jane L. Roberts, Andrew Poklepovic, John Kirkwood, and Paul Dent. 2017. “HDAC Inhibitors Enhance the Immunotherapy Response of Melanoma Cells.” *Oncotarget* 8 (47): 83155–70. https://doi.org/10.18632/oncotarget.17950.

Bredin, Philip, Janice M. Walshe, and Neelima Denduluri. 2020. “Systemic Therapy for Metastatic HER2-Positive Breast Cancer.” *Seminars in Oncology* 47 (5): 259–69. https://doi.org/10.1053/j.seminoncol.2020.07.008.

Bria, Emilio, Federica Cuppone, Monica Fornier, Cecilia Nisticò, Paolo Carlini, Michele Milella, Isabella Sperduti, Edmondo Terzoli, Francesco Cognetti, and Diana Giannarelli. 2007. “Cardiotoxicity and Incidence of Brain Metastases after Adjuvant Trastuzumab for Early Breast Cancer: The Dark Side of the Moon? A Meta-Analysis of the Randomized Trials.” *Breast Cancer Research and Treatment* 109 (2): 231–39. https://doi.org/10.1007/s10549-007-9663-z.

Burr, Marian L., Christina E. Sparbier, Kah Lok Chan, Yih-Chih Chan, Ariena Kersbergen, Enid Y. N. Lam, Elizabeth Azidis-Yates, et al. 2019. “An Evolutionarily Conserved Function of Polycomb Silences the MHC Class I Antigen Presentation Pathway and Enables Immune Evasion in Cancer.” *Cancer Cell* 36 (4): 385-401.e8. https://doi.org/10.1016/j.ccell.2019.08.008.

Camargo, Fernando D., Sumita Gokhale, Jonathan B. Johnnidis, Dongdong Fu, George W. Bell, Rudolf Jaenisch, and Thijn R. Brummelkamp. 2007. “YAP1 Increases Organ Size and Expands Undifferentiated Progenitor Cells.” *Current Biology* 17 (23): 2054–60. https://doi.org/10.1016/j.cub.2007.10.039.

Cameron, David, Michelle Casey, Michael Press, Deborah Lindquist, Tadeusz Pienkowski, C. Gilles Romieu, Stephen Chan, et al. 2008. “A Phase III Randomized Comparison of Lapatinib plus Capecitabine versus Capecitabine Alone in Women with Advanced Breast Cancer That Has Progressed on Trastuzumab: Updated Efficacy and Biomarker Analyses.” *Breast Cancer Research and Treatment* 112 (3): 533–43. https://doi.org/10.1007/s10549-007-9885-0.

Cañadas, Israel, Rohit Thummalapalli, Jong Wook Kim, Shunsuke Kitajima, Russell William Jenkins, Camilla Laulund Christensen, Marco Campisi, et al. 2018. “Tumor Innate Immunity Primed by Specific Interferon-Stimulated Endogenous Retroviruses.” *Nature Medicine* 24 (8): 1143–50. https://doi.org/10.1038/s41591-018-0116-5.

Caretti, Giuseppina, Monica Di Padova, Bruce Micales, Gary E. Lyons, and Vittorio Sartorelli. 2004. “The Polycomb Ezh2 Methyltransferase Regulates Muscle Gene Expression and Skeletal Muscle Differentiation.” *Genes & Development* 18 (21): 2627–38. https://doi.org/10.1101/gad.1241904.

Carneiro, Benedito A., and Wafik S. El-Deiry. 2020. “Targeting Apoptosis in Cancer Therapy.” *Nature Reviews. Clinical Oncology* 17 (7): 395–417. https://doi.org/10.1038/s41571-020-0341-y.

Castiglioni, F., E. Tagliabue, M. Campiglio, S. M. Pupa, A. Balsari, and S. Ménard. 2006. “Role of Exon-16-Deleted HER2 in Breast Carcinomas.” *Endocrine-Related Cancer* 13 (1): 221–32. https://doi.org/10.1677/erc.1.01047.

Certo, Michael, Victoria Del Gaizo Moore, Mari Nishino, Guo Wei, Stanley Korsmeyer, Scott A. Armstrong, and Anthony Letai. 2006. “Mitochondria Primed by Death Signals Determine Cellular Addiction to Antiapoptotic BCL-2 Family Members.” *Cancer Cell* 9 (5): 351–65. https://doi.org/10.1016/j.ccr.2006.03.027.

Chaib, Imane, Niki Karachaliou, Sara Pilotto, Jordi Codony Servat, Xueting Cai, Xuefei Li, Ana Drozdowskyj, et al. 2017. “Co-Activation of STAT3 and YES-Associated Protein 1 (YAP1) Pathway in EGFR-Mutant NSCLC.” *Journal of the National Cancer Institute* 109 (9). https://doi.org/10.1093/jnci/djx014.

Chase, Andrew, and Nicholas C. P. Cross. 2011. “Aberrations of EZH2 in Cancer.” *Clinical Cancer Research* 17 (9): 2613–18. https://doi.org/10.1158/1078-0432.CCR-10-2156.

Chen, Ya-Huey, Fang-Ling Yeh, Su-Peng Yeh, Haou-Tzong Ma, Shih-Chieh Hung, Mien-Chie Hung, and Long-Yuan Li. 2011. “Myocyte Enhancer Factor-2 Interacting Transcriptional Repressor (MITR) Is a Switch That Promotes Osteogenesis and Inhibits Adipogenesis of Mesenchymal Stem Cells by Inactivating Peroxisome Proliferator-Activated Receptor Gamma-2.” *The Journal of Biological Chemistry* 286 (12): 10671–80. https://doi.org/10.1074/jbc.M110.199612.

Chinnaiyan, Arul M., Sooryanarayana Varambally, Saravana M. Dhanasekaran, Ming Zhou, Terrence R. Barrette, Chandan Kumar-Sinha, Martin G. Sanda, et al. 2002. “The Polycomb Group Protein EZH2 Is Involved in Progression of Prostate Cancer.” *Nature (London)* 419 (6907): 624–29. https://doi.org/10.1038/nature01075.

Choong, Grace M., Grace D. Cullen, and Ciara C. O’Sullivan. 2020. “Evolving Standards of Care and New Challenges in the Management of HER2-positive Breast Cancer.” *CA: A Cancer Journal for Clinicians* 70 (5): 355–74. https://doi.org/10.3322/caac.21634.

Comen, Elizabeth A., and Mark Robson. 2010. “Poly(ADP-Ribose) Polymerase Inhibitors in Triple-Negative Breast Cancer.” *The Cancer Journal (Sudbury, Mass.)* 16 (1): 48–52. https://doi.org/10.1097/PPO.0b013e3181cf01eb.

Conte, P., M. Campone, P. Pronzato, D. Amadori, R. Frank, F. Schuetz, D. Rea, A. Wardley, C. Britten, and A. Elias. 2009. “Phase I Trial of Panobinostat (LBH589) in Combination with Trastuzumab in Pretreated HER2-Positive Metastatic Breast Cancer (MBC): Preliminary Safety and Tolerability Results.” *Journal of Clinical Oncology* 27 (15_suppl): 1081–1081. https://doi.org/10.1200/jco.2009.27.15_suppl.1081.

Costa, Ricardo L. B., and Brian J. Czerniecki. 2020. “Clinical Development of Immunotherapies for HER2+ Breast Cancer: A Review of HER2-Directed Monoclonal Antibodies and Beyond.” *NPJ Breast Cancer* 6: 10. https://doi.org/10.1038/s41523-020-0153-3.

Dai, Xiaofeng, Liangjian Xiang, Ting Li, and Zhonghu Bai. 2016. “Cancer Hallmarks, Biomarkers and Breast Cancer Molecular Subtypes.” *Journal of Cancer* 7 (10): 1281–94. https://doi.org/10.7150/jca.13141.

Davoli, April, Barbara A. Hocevar, and Thomas L. Brown. 2010. “Progression and Treatment of HER2-Positive Breast Cancer.” *Cancer Chemotherapy and Pharmacology* 65 (4): 611–23. https://doi.org/10.1007/s00280-009-1208-1.

De Raedt, Thomas, Eline Beert, Eric Pasmant, Armelle Luscan, Hilde Brems, Nicolas Ortonne, Kristian Helin, et al. 2014. “PRC2 Loss Amplifies Ras-Driven Transcription and Confers Sensitivity to BRD4-Based Therapies.” *Nature (London)* 514 (7521): 247–51. https://doi.org/10.1038/nature13561.

Deb, Gauri, Vijay S. Thakur, and Sanjay Gupta. 2013. “Multifaceted Role of EZH2 in Breast and Prostate Tumorigenesis: Epigenetics and Beyond.” *Epigenetics* 8 (5): 464–76. https://doi.org/10.4161/epi.24532.

Denkert, Carsten, Cornelia Liedtke, Andrew Tutt, and Gunter von Minckwitz. 2017. “Molecular Alterations in Triple-Negative Breast Cancer—the Road to New Treatment Strategies.” *The Lancet* 389 (10087): 2430–42. https://doi.org/10.1016/S0140-6736(16)32454-0.

Derksen, Patrick W. B., Xiaoling Liu, Francis Saridin, Hanneke van der Gulden, John Zevenhoven, Bastiaan Evers, Judy R. van Beijnum, et al. 2006. “Somatic Inactivation of E-Cadherin and P53 in Mice Leads to Metastatic Lobular Mammary Carcinoma through Induction of Anoikis Resistance and Angiogenesis.” *Cancer Cell* 10 (5): 437–49. https://doi.org/10.1016/j.ccr.2006.09.013.

Dockerill, Milly, Clare Gregson, and Daniel H. O’ Donovan. 2021. “Targeting PRC2 for the Treatment of Cancer: An Updated Patent Review (2016 - 2020).” *Expert Opinion on Therapeutic Patents* 31 (2): 119–35. https://doi.org/10.1080/13543776.2021.1841167.

Duan, Ran, Wenfang Du, and Weijian Guo. 2020. “EZH2: A Novel Target for Cancer Treatment.” *Journal of Hematology and Oncology* 13 (1): 104–12. https://doi.org/10.1186/s13045-020-00937-8.

Duchnowska, Renata, Sibylle Loibl, and Jacek Jassem. 2018. “Tyrosine Kinase Inhibitors for Brain Metastases in HER2-Positive Breast Cancer.” *Cancer Treatment Reviews* 67: 71–77. https://doi.org/10.1016/j.ctrv.2018.05.004.

Eich, Marie-Lisa, Mohammad Athar, James E. Ferguson, and Sooryanarayana Varambally. 2020. “EZH2-Targeted Therapies in Cancer: Hype or a Reality.” *Cancer Research (Chicago, Ill.)* 80 (24): 5449–58. https://doi.org/10.1158/0008-5472.CAN-20-2147.

Epizyme, Inc. 2021. “Epizyme Outlines Clinical Progress, TAZVERIK Development Strategy and Pipeline Priorities During Company’s Strategic Vision Call | Epizyme, Inc.” 2021. https://ir.epizyme.com/news-releases/news-release-details/epizyme-outlines-clinical-progress-tazverik-development-strategy.

Eroles, Pilar, Ana Bosch, J. Alejandro Pérez-Fidalgo, and Ana Lluch. 2011. “Molecular Biology in Breast Cancer: Intrinsic Subtypes and Signaling Pathways.” *Cancer Treatment Reviews* 38 (6): 698–707.

Eskander, Ramez N., Tao Ji, Be Huynh, Rooba Wardeh, Leslie M. Randall, and Bang Hoang. 2013. "Inhibition of Enhancer of Zeste Homolog 2 (EZH2) Expression Is Associated With Decreased Tumor Cell Proliferation, Migration, and Invasion in Endometrial Cancer Cell Lines." *International Journal of Gynecological Cancer* 23 (6): 997–1005. https://doi.org/10.1097/IGC.0b013e318296a265.

Fehrenbacher, Louis, Angela M. Capra, Charles P. Quesenberry, Regan Fulton, Parveen Shiraz, and Laurel A. Habel. 2014. "Distant Invasive Breast Cancer Recurrence Risk in Human Epidermal Growth Factor Receptor 2-Positive T1a and T1b Node-Negative Localized Breast Cancer Diagnosed from 2000 to 2006: A Cohort from an Integrated Health Care Delivery System." *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology* 32 (20): 2151–58. https://doi.org/10.1200/JCO.2013.52.0858.

Ferraro, Emanuela, Joshua Z. Drago, and Shanu Modi. 2021. "Implementing Antibody-Drug Conjugates (ADCs) in HER2-Positive Breast Cancer: State of the Art and Future Directions." *Breast Cancer Research* 23 (1): 84. https://doi.org/10.1186/s13058-021-01459-y.

Foulkes, William D., Ian E. Smith, and Jorge S. Reis-Filho. 2010. "Triple-Negative Breast Cancer." *The New England Journal of Medicine* 363 (20): 1938–48. https://doi.org/10.1056/NEJMra1001389.

Galluzzi, Lorenzo, Ilio Vitale, Stuart A. Aaronson, John M. Abrams, Dieter Adam, Patrizia Agostinis, Emad S. Alnemri, et al. 2018. "Molecular Mechanisms of Cell Death: Recommendations of the Nomenclature Committee on Cell Death 2018." *Cell Death & Differentiation* 25 (3): 486–541. https://doi.org/10.1038/s41418-017-0012-4.

Geenen, Jill J. J., Sabine C. Linn, Jos H. Beijnen, and Jan H. M. Schellens. 2018. "PARP Inhibitors in the Treatment of Triple-Negative Breast Cancer." *Clinical Pharmacokinetics* 57 (4): 427–37. https://doi.org/10.1007/s40262-017-0587-4.

Giam, M., D. C. S. Huang, and P. Bouillet. 2008. "BH3-Only Proteins and Their Roles in Programmed Cell Death." *Oncogene* 27 (1): S128–36. https://doi.org/10.1038/onc.2009.50.

Glozak, M. A., and E. Seto. 2007. "Histone Deacetylases and Cancer." *Oncogene* 26 (37): 5420–32. https://doi.org/10.1038/sj.onc.1210610.

Goldstein, Lori J., Fengmin Zhao, Molin Wang, Ramona F. Swaby, Joseph A. Sparano, Neal J. Meropol, Kapil N. Bhalla, et al. 2017. "A Phase I/II Study of Suberoylanilide Hydroxamic Acid (SAHA) in Combination with Trastuzumab (Herceptin) in Patients with Advanced Metastatic and/or Local Chest Wall Recurrent HER2-Amplified Breast Cancer: A Trial of the ECOG-ACRIN Cancer." *Breast Cancer Research and Treatment* 165 (2): 375–82. https://doi.org/10.1007/s10549-017-4310-9.

Gonzalez, Maria E., Xin Li, Katherine Toy, Matthew DuPrie, Alejandra C. Ventura, Mousumi Banerjee, Mats Ljugman, Sofia D. Merajver, and Celina G. Kleer. 2009. "Down-Regulation of

Enhancer of Zeste-2 Decreases Growth of Estrogen Receptor Negative Invasive Breast Carcinoma and Requires BRCA1." *Oncogene* 28 (6): 843–53. https://doi.org/10.1038/onc.2008.433.

Gonzalez, Maria E., Heather M. Moore, Xin Li, Kathy A. Toy, Wei Huang, Michael S. Sabel, Kelley M. Kidwell, and Celina G. Kleer. 2014. "EZH2 Expands Breast Stem Cells through Activation of NOTCH1 Signaling." *Proceedings of the National Academy of Sciences* 111 (8): 3098–3103. https://doi.org/10.1073/pnas.1308953111.

Gounder, Mrinal, Patrick Schöffski, Robin L. Jones, Mark Agulnik, Gregory M. Cote, Victor M. Villalobos, Steven Attia, et al. 2020. "Tazemetostat in Advanced Epithelioid Sarcoma with Loss of INI1/SMARCB1: An International, Open-Label, Phase 2 Basket Study." *The Lancet. Oncology* 21 (11): 1423–32. https://doi.org/10.1016/S1470-2045(20)30451-4.

Grespi, F., C. Soratroi, G. Krumschnabel, B. Sohm, C. Ploner, S. Geley, L. Hengst, G. Häcker, and A. Villunger. 2010. "BH3-Only Protein Bmf Mediates Apoptosis upon Inhibition of CAP-Dependent Protein Synthesis." *Cell Death & Differentiation* 17 (11): 1672–83. https://doi.org/10.1038/cdd.2010.97.

Gudmundsdottir, K., and A. Ashworth. 2006. "The Roles of BRCA1 and BRCA2 and Associated Proteins in the Maintenance of Genomic Stability." *Oncogene* 25 (43): 5864–74. https://doi.org/10.1038/sj.onc.1209874.

Gulati, Nitya, Wendy Béguelin, and Lisa Giulino-Roth. 2018. "Enhancer of Zeste Homolog 2 (EZH2) Inhibitors." *Leukemia & Lymphoma* 59 (7): 1574–85. https://doi.org/10.1080/10428194.2018.1430795.

Guo, Shuangping, Xia Li, Joseph Rohr, Yingmei Wang, Shirong Ma, Peng Chen, and Zhe Wang. 2016. "EZH2 Overexpression in Different Immunophenotypes of Breast Carcinoma and Association with Clinicopathologic Features." *Diagnostic Pathology* 11 (1): 41–41. https://doi.org/10.1186/s13000-016-0491-5.

Guo, Tong, Yi Lu, Peixue Li, Meng-Xin Yin, Dekang Lv, Wenjing Zhang, Huizhen Wang, et al. 2013. "A Novel Partner of Scalloped Regulates Hippo Signaling via Antagonizing Scalloped-Yorkie Activity." *Cell Research* 23 (10): 1201–14. https://doi.org/10.1038/cr.2013.120.

Hanahan, Douglas, and Robert A Weinberg. 2000. "The Hallmarks of Cancer." *Cell* 100 (1): 57–70. https://doi.org/10.1016/S0092-8674(00)81683-9.

Hanaki, Shunsuke, and Midori Shimada. 2021. "Targeting EZH2 as Cancer Therapy." *Journal of Biochemistry (Tokyo)* 170 (1): 1–4. https://doi.org/10.1093/jb/mvab007.

Hart, Vic, Hannah Gautrey, John Kirby, and Alison Tyson-Capper. 2020. "HER2 Splice Variants in Breast Cancer: Investigating Their Impact on Diagnosis and Treatment Outcomes." *Oncotarget* 11 (46): 4338–57. https://doi.org/10.18632/oncotarget.27789.

Hirukawa, Alison, Salendra Singh, Jarey Wang, Jonathan P. Rennhack, Matthew Swiatnicki, Virginie Sanguin-Gendreau, Dongmei Zuo, et al. 2019. “Reduction of Global H3K27me3 Enhances HER2/ErbB2 Targeted Therapy.” *Cell Reports (Cambridge)* 29 (2): 249-257.e8. https://doi.org/10.1016/j.celrep.2019.08.105.

Holm, Karolina, Dorthe Grabau, Kristina Lövgren, Steina Aradottir, Sofia Gruvberger-Saal, Jillian Howlin, Lao H. Saal, et al. 2012. “Global H3K27 Trimethylation and EZH2 Abundance in Breast Tumor Subtypes.” *Molecular Oncology* 6 (5): 494–506.

Hon, Jane Date C., Baljit Singh, Aysegul Sahin, Gang Du, Jinhua Wang, Vincent Y. Wang, Fang-Ming Deng, David Y. Zhang, Marie E. Monaco, and Peng Lee. 2016. “Breast Cancer Molecular Subtypes: From TNBC to QNBC.” *American Journal of Cancer Research* 6 (9): 1864–72.

Hontecillas-Prieto, Lourdes, Rocío Flores-Campos, Andrew Silver, Enrique de Álava, Nabil Hajji, and Daniel J. García-Domínguez. 2020. “Synergistic Enhancement of Cancer Therapy Using HDAC Inhibitors: Opportunity for Clinical Trials.” *Frontiers in Genetics* 11: 1113. https://doi.org/10.3389/fgene.2020.578011.

Hornsveld, M., M. Tenhagen, R. A. van de Ven, A. M. M. Smits, M. H. van Triest, M. van Amersfoort, D. E. A. Kloet, T. B. Dansen, B. M. Burgering, and P. W. B. Derksen. 2016. “Restraining FOXO3-Dependent Transcriptional BMF Activation Underpins Tumour Growth and Metastasis of E-Cadherin-Negative Breast Cancer.” *Cell Death & Differentiation* 23 (9): 1483–92. https://doi.org/10.1038/cdd.2016.33.

Hsieh, A. C., and M. M. Moasser. 2007. “Targeting HER Proteins in Cancer Therapy and the Role of the Non-Target HER3.” *British Journal of Cancer* 97 (4): 453–57. https://doi.org/10.1038/sj.bjc.6603910.

Hsu, Jennifer L., and Mien-Chie Hung. 2016. “The Role of HER2, EGFR, and Other Receptor Tyrosine Kinases in Breast Cancer.” *Cancer Metastasis Reviews* 35 (4): 575–88. https://doi.org/10.1007/s10555-016-9649-6.

Hsu, Ping-Chih, Bin You, Yi-Lin Yang, Wen-Qian Zhang, Yu-Cheng Wang, Zhidong Xu, Yuyuan Dai, et al. 2016. “YAP Promotes Erlotinib Resistance in Human Non-Small Cell Lung Cancer Cells.” *Oncotarget* 7 (32): 51922–33. https://doi.org/10.18632/oncotarget.10458.

Hu, Erding, Edward Dul, Chiu-Mei Sung, Zunxuan Chen, Robert Kirkpatrick, Gui-Feng Zhang, Kyung Johanson, et al. 2003. “Identification of Novel Isoform-Selective Inhibitors within Class I Histone Deacetylases.” *The Journal of Pharmacology and Experimental Therapeutics* 307 (2): 720–28. https://doi.org/10.1124/jpet.103.055541.

Huang, Xiaoping, Shuiliang Wang, Choon-Kee Lee, XiaoHe Yang, and Bolin Liu. 2011. “HDAC Inhibitor SNDX-275 Enhances Efficacy of Trastuzumab in ErbB2-Overexpressing Breast Cancer Cells and Exhibits Potential to Overcome Trastuzumab Resistance.” *Cancer Letters* 307 (1): 72–79. https://doi.org/10.1016/j.canlet.2011.03.019.

Hung, Mien-Chie, Francisco J. Esteva, Gabriel N. Hortobagyi, Dihua Yu, and Rita Nahta. 2006. “Mechanisms of Disease: Understanding Resistance to HER2-Targeted Therapy in Human Breast Cancer.” *Nature Clinical Practice. Oncology* 3 (5): 269–80. https://doi.org/10.1038/ncponc0509.

Hurvitz, Sara, and Shiliang Zhang. 2021. “Margetuximab-Cmkb: A Novel Agent Overshadowed by an Abundance of Options in HER2-Positive Breast Cancer.” *The ASCO Post*.

Hyman, David M., Sarina A. Piha-Paul, Helen Won, Jordi Rodon, Cristina Saura, Geoffrey I. Shapiro, Dejan Juric, et al. 2018. “HER Kinase Inhibition in Patients with HER2- and HER3-Mutant Cancers.” *Nature* 554 (7691): 189–94. https://doi.org/10.1038/nature25475.

Ianevski, Aleksandr, Liye He, Tero Aittokallio, and Jing Tang. 2020. “SynergyFinder: A Web Application for Analyzing Drug Combination Dose–Response Matrix Data.” *Bioinformatics* 36 (8): 2645–2645. https://doi.org/10.1093/bioinformatics/btaa102.

Ishii, Kei, Nao Morii, and Hiroyasu Yamashiro. 2019. “<p>Pertuzumab in the Treatment of HER2-Positive Breast Cancer: An Evidence-Based Review of Its Safety, Efficacy, and Place in Therapy</P>.” *Core Evidence* 14 (October): 51–70. https://doi.org/10.2147/CE.S217848.

Izidoro-Toledo, T. C., A. C. Borges, D. D. Araújo, D. P. S. Leitão Mazzi, F. O. Nascimento, J. F. Sousa, C. P. Alves, et al. 2013. “A Myosin-Va Tail Fragment Sequesters Dynein Light Chains Leading to Apoptosis in Melanoma Cells.” *Cell Death & Disease* 4 (3): e547–e547. https://doi.org/10.1038/cddis.2013.45.

Jang, Si-Hyong, Jong Eun Lee, Mee-Hye Oh, Ji-Hye Lee, Hyun Deuk Cho, Kyung-Ju Kim, Sung Yong Kim, et al. 2016. “High EZH2 Protein Expression Is Associated with Poor Overall Survival in Patients with Luminal A Breast Cancer.” *Journal of Breast Cancer* 19 (1): 53–60. https://doi.org/10.4048/jbc.2016.19.1.53.

Jaramillo-Rodríguez, Yolanda, Ricardo M. Cerda-Flores, Ruben Ruiz-Ramos, Francisco C. López-Márquez, and Ana Laura Calderón-Garcidueñas. 2014. “YAP Expression in Normal and Neoplastic Breast Tissue: An Immunohistochemical Study.” *Archives of Medical Research* 45 (3): 223–28. https://doi.org/10.1016/j.arcmed.2014.01.010.

Jones, Bayley A., Sooryanarayana Varambally, and Rebecca C. Arend. 2018. “Histone Methyltransferase EZH2: A Therapeutic Target for Ovarian Cancer.” *Molecular Cancer Therapeutics* 17 (3): 591–602. https://doi.org/10.1158/1535-7163.MCT-17-0437.

Kanayama, Kazuki, Hiroshi Imai, Eri Usugi, Taizo Shiraishi, Yoshifumi S. Hirokawa, and Masatoshi Watanabe. 2018. “Association of HER2 Gene Amplification and Tumor Progression in Early Gastric Cancer.” *Virchows Archiv: An International Journal of Pathology* 473 (5): 559–65. https://doi.org/10.1007/s00428-018-2433-y.

Kensler, Kevin H., Venkat N. Sankar, Jun Wang, Xuehong Zhang, Christopher A. Rubadue, Gabrielle M. Baker, Joel S. Parker, et al. 2019. “PAM50 Molecular Intrinsic Subtypes in the

Nurses' Health Study Cohorts." *Cancer Epidemiology, Biomarkers & Prevention* 28 (4): 798–806. https://doi.org/10.1158/1055-9965.EPI-18-0863.

Khan, Mohammad A., Vineet K. Jain, Md Rizwanullah, Javed Ahmad, and Keerti Jain. 2019. "PI3K/AKT/MTOR Pathway Inhibitors in Triple-Negative Breast Cancer: A Review on Drug Discovery and Future Challenges." *Drug Discovery Today* 24 (11): 2181–91. https://doi.org/10.1016/j.drudis.2019.09.001.

Kim, KiBem, Andrew D. Skora, Zhaobo Li, Qiang Liu, Ada J. Tam, Richard L. Blosser, Jr Diaz, et al. 2014. "Eradication of Metastatic Mouse Cancers Resistant to Immune Checkpoint Blockade by Suppression of Myeloid-Derived Cells." *Proceedings of the National Academy of Sciences - PNAS* 111 (32): 11774–79. https://doi.org/10.1073/pnas.1410626111.

Kim, Kimberly H., and Charles W. M. Roberts. 2016. "Targeting EZH2 in Cancer." *Nature Medicine* 22 (2): 128–34. https://doi.org/10.1038/nm.4036.

Kim, Minchul, Taekhoon Kim, Randy L. Johnson, and Dae-Sik Lim. 2015. "Transcriptional Co-Repressor Function of the Hippo Pathway Transducers YAP and TAZ." *Cell Reports* 11 (2): 270–82. https://doi.org/10.1016/j.celrep.2015.03.015.

Kleer, Celina G., Qi Cao, Sooryanarayana Varambally, Ronglai Shen, Ichiro Ota, Scott A. Tomlins, Debashis Ghosh, et al. 2003. "EZH2 Is a Marker of Aggressive Breast Cancer and Promotes Neoplastic Transformation of Breast Epithelial Cells." *Proceedings of the National Academy of Sciences - PNAS* 100 (20): 11606–11. https://doi.org/10.1073/pnas.1933744100.

Koboldt, Daniel C., Robert S. Fulton, Michael D. McLellan, Heather Schmidt, Joelle Kalicki-Veizer, Joshua F. McMichael, Lucinda L. Fulton, et al. 2012. "Comprehensive Molecular Portraits of Human Breast Tumours." *Nature* 490 (7418): 61–70. https://doi.org/10.1038/nature11412.

Koontz, Laura M., Yi Liu-Chittenden, Feng Yin, Yonggang Zheng, Jianzhong Yu, Bo Huang, Qian Chen, Shian Wu, and Duojia Pan. 2013. "The Hippo Effector Yorkie Controls Normal Tissue Growth by Antagonizing Scalloped-Mediated Default Repression." *Developmental Cell* 25 (4): 388–401. https://doi.org/10.1016/j.devcel.2013.04.021.

Kriegsmann, Mark, Volker Endris, Thomas Wolf, Nicole Pfarr, Albrecht Stenzinger, Sibylle Loibl, Carsten Denkert, et al. 2014. "Mutational Profiles in Triple-Negative Breast Cancer Defined by Ultradeep Multigene Sequencing Show High Rates of PI3K Pathway Alterations and Clinically Relevant Entity Subgroup Specific Differences." *Oncotarget* 5 (20): 9952–65. https://doi.org/10.18632/oncotarget.2481.

Kurppa, Kari J., Yao Liu, Ciric To, Tinghu Zhang, Mengyang Fan, Amir Vajdi, Erik H. Knelson, et al. 2020. "Treatment-Induced Tumor Dormancy through YAP-Mediated Transcriptional Reprogramming of the Apoptotic Pathway." *Cancer Cell* 37 (1): 104-122.e12. https://doi.org/10.1016/j.ccell.2019.12.006.

Kuzmickas, Ryan. 2019. “Therapeutic Targeting of Chromatin Modifiers in Advanced Prostate Cancer.” http://nrs.harvard.edu/urn-3:HUL.InstRepos:42029548.

Labi, Verena, Miriam Erlacher, Stephan Kiessling, Claudia Manzl, Anna Frenzel, Lorraine O’Reilly, Andreas Strasser, and Andreas Villunger. 2008. “Loss of the BH3-Only Protein Bmf Impairs B Cell Homeostasis and Accelerates Gamma Irradiation-Induced Thymic Lymphoma Development.” *The Journal of Experimental Medicine* 205 (3): 641–55. https://doi.org/10.1084/jem.20071658.

Lamar, John M., Patrick Stern, Hui Liu, Jeffrey W. Schindler, Zhi-Gang Jiang, and Richard O. Hynes. 2012. “The Hippo Pathway Target, YAP, Promotes Metastasis through Its TEAD-Interaction Domain.” *Proceedings of the National Academy of Sciences* 109 (37): E2441–50. https://doi.org/10.1073/pnas.1212021109.

Lamar, John M., Yuxuan Xiao, Emily Norton, Zhi-Gang Jiang, Genevieve M. Gerhard, Simrin Kooner, Janine S. A. Warren, and Richard O. Hynes. 2019. “SRC Tyrosine Kinase Activates the YAP/TAZ Axis and Thereby Drives Tumor Growth and Metastasis.” *Journal of Biological Chemistry* 294 (7): 2302–17. https://doi.org/10.1074/jbc.RA118.004364.

Laporte, Aimée N., Jared J. Barrott, Ren Jie Yao, Neal M. Poulin, Bertha A. Brodin, Kevin B. Jones, T. Michael Underhill, and Torsten O. Nielsen. 2017. “HDAC and Proteasome Inhibitors Synergize to Activate Pro-Apoptotic Factors in Synovial Sarcoma.” *PLoS ONE* 12 (1): e0169407. https://doi.org/10.1371/journal.pone.0169407.

Lee, Jangsoon, Chandra Bartholomeusz, Oula Mansour, Juliane Humphries, Gabriel N. Hortobagyi, Peter Ordentlich, and Naoto T. Ueno. 2014. “A Class I Histone Deacetylase Inhibitor, Entinostat, Enhances Lapatinib Efficacy in HER2-Overexpressing Breast Cancer Cells through FOXO3-Mediated Bim1 Expression.” *Breast Cancer Research and Treatment* 146 (2): 259–72. https://doi.org/10.1007/s10549-014-3014-7.

Lehmann, Brian D., Joshua A. Bauer, Xi Chen, Melinda E. Sanders, A. Bapsi Chakravarthy, Yu Shyr, and Jennifer A. Pietenpol. 2011. “Identification of Human Triple-Negative Breast Cancer Subtypes and Preclinical Models for Selection of Targeted Therapies.” *The Journal of Clinical Investigation* 121 (7): 2750–67. https://doi.org/10.1172/JCI45014.

Li, Bob T., Ronglai Shen, Darren Buonocore, Zachary T. Olah, Ai Ni, Michelle S. Ginsberg, Gary A. Ulaner, et al. 2018. “Ado-Trastuzumab Emtansine for Patients With HER2-Mutant Lung Cancers: Results From a Phase II Basket Trial.” *Journal of Clinical Oncology* 36 (24): 2532–37. https://doi.org/10.1200/JCO.2018.77.9777.

Li, Chen, Yan Wang, Yueqing Gong, Tengrui Zhang, Jiaqi Huang, Zhen Tan, and Lixiang Xue. 2021. “Finding an Easy Way to Harmonize: A Review of Advances in Clinical Research and Combination Strategies of EZH2 Inhibitors.” *Clinical Epigenetics* 13 (1): 62. https://doi.org/10.1186/s13148-021-01045-1.

Li, Yixuan, and Edward Seto. 2016. “HDACs and HDAC Inhibitors in Cancer Development and Therapy.” *Cold Spring Harbor Perspectives in Medicine* 6 (10): a026831-. https://doi.org/10.1101/cshperspect.a026831.

Lim, Bora, Rashmi K. Murthy, Jangsoon Lee, Summer A. Jackson, Toshiaki Iwase, Darren W. Davis, Jie S. Willey, et al. 2019. “A Phase Ib Study of Entinostat plus Lapatinib with or without Trastuzumab in Patients with HER2-Positive Metastatic Breast Cancer That Progressed during Trastuzumab Treatment.” *British Journal of Cancer* 120 (12): 1105–12. https://doi.org/10.1038/s41416-019-0473-y.

Lin, Chun-Han, Fanny A. Pelissier, Hui Zhang, Jon Lakins, Valerie M. Weaver, Catherine Park, and Mark A. LaBarge. 2015. “Microenvironment Rigidity Modulates Responses to the HER2 Receptor Tyrosine Kinase Inhibitor Lapatinib via YAP and TAZ Transcription Factors.” *Molecular Biology of the Cell* 26 (22): 3946–53. https://doi.org/10.1091/mbc.E15-07-0456.

Lin, Nancy U., Virginia Borges, Carey Anders, Rashmi K. Murthy, Elisavet Paplomata, Erika Hamilton, Sara Hurvitz, et al. 2020. “Intracranial Efficacy and Survival With Tucatinib Plus Trastuzumab and Capecitabine for Previously Treated HER2-Positive Breast Cancer With Brain Metastases in the HER2CLIMB Trial.” *Journal of Clinical Oncology* 38 (23): 2610–19. https://doi.org/10.1200/JCO.20.00775.

Mailleux, Arnaud A., Michael Overholtzer, Tobias Schmelzle, Philippe Bouillet, Andreas Strasser, and Joan S. Brugge. 2007. “BIM Regulates Apoptosis during Mammary Ductal Morphogenesis, and Its Absence Reveals Alternative Cell Death Mechanisms.” *Developmental Cell* 12 (2): 221–34. https://doi.org/10.1016/j.devcel.2006.12.003.

Malone, Clare F., Chloe Emerson, Rachel Ingraham, William Barbosa, Stephanie Guerra, Haejin Yoon, Lin L. Liu, et al. 2017. “MTOR and HDAC Inhibitors Converge on the TXNIP/Thioredoxin Pathway to Cause Catastrophic Oxidative Stress and Regression of RAS-Driven Tumors.” *Cancer Discovery* 7 (12): 1450–63. https://doi.org/10.1158/2159-8290.CD-17-0177.

Marchesi, Irene, and Luigi Bagella. 2016. “Targeting Enhancer of Zeste Homolog 2 as a Promising Strategy for Cancer Treatment.” *World Journal of Clinical Oncology* 7 (2): 135–48. https://doi.org/10.5306/wjco.v7.i2.135.

Martin, Seamus J., Rebecca C. Taylor, and Sean P. Cullen. 2008. “Apoptosis: Controlled Demolition at the Cellular Level.” *Nature Reviews. Molecular Cell Biology* 9 (3): 231–41. https://doi.org/10.1038/nrm2312.

Martorana, Federica, Gianmarco Motta, Giuliana Pavone, Lucia Motta, Stefania Stella, Silvia Rita Vitale, Livia Manzella, and Paolo Vigneri. 2021. “AKT Inhibitors: New Weapons in the Fight Against Breast Cancer?” *Frontiers in Pharmacology* 12: 546. https://doi.org/10.3389/fphar.2021.662232.

McLaughlin, Sara Koenig, Sarah Naomi Olsen, Benjamin Dake, Thomas De Raedt, Elgene Lim, Roderick Terry Bronson, Rameen Beroukhim, et al. 2013. “The RasGAP Gene, RASAL2, Is a Tumor and Metastasis Suppressor.” *Cancer Cell* 24 (3): 365–78. https://doi.org/10.1016/j.ccr.2013.08.004.

Mendenhall, Eric M., Richard P. Koche, Thanh Truong, Vicky W. Zhou, Biju Issac, Andrew S. Chi, Manching Ku, and Bradley E. Bernstein. 2010. “GC-Rich Sequence Elements Recruit PRC2 in Mammalian ES Cells.” *PLoS Genetics* 6 (12): e1001244–e1001244. https://doi.org/10.1371/journal.pgen.1001244.

Mendonsa, Alisha M., Tae-Young Na, and Barry M. Gumbiner. 2018. “E-Cadherin in Contact Inhibition and Cancer.” *Oncogene* 37 (35): 4769–80. https://doi.org/10.1038/s41388-018-0304-2.

Meric-Bernstam, Funda, John Hainsworth, Ron Bose, Howard A. Burris III, Claire Frances Friedman, Razelle Kurzrock, Charles Swanton, et al. 2021. “MyPathway HER2 Basket Study: Pertuzumab (P) + Trastuzumab (H) Treatment of a Large, Tissue-Agnostic Cohort of Patients with HER2-Positive Advanced Solid Tumors.” *Journal of Clinical Oncology* 39 (15_suppl): 3004–3004. https://doi.org/10.1200/JCO.2021.39.15_suppl.3004.

Metro, G., J. Foglietta, M. Russillo, L. Stocchi, A. Vidiri, D. Giannarelli, L. Crinò, et al. 2011. “Clinical Outcome of Patients with Brain Metastases from HER2-Positive Breast Cancer Treated with Lapatinib and Capecitabine.” *Annals of Oncology: Official Journal of the European Society for Medical Oncology* 22 (3): 625–30. https://doi.org/10.1093/annonc/mdq434.

Min, Ahrum, Seock-Ah Im, Debora Keunyoung Kim, Sang-Hyun Song, Hee-Jun Kim, Kyung-Hun Lee, Tae-Yong Kim, et al. 2015. “Histone Deacetylase Inhibitor, Suberoylanilide Hydroxamic Acid (SAHA), Enhances Anti-Tumor Effects of the Poly (ADP-Ribose) Polymerase (PARP) Inhibitor Olaparib in Triple-Negative Breast Cancer Cells.” *Breast Cancer Research: BCR* 17 (March): 33. https://doi.org/10.1186/s13058-015-0534-y.

Min, Junxia, Alexander Zaslavsky, Giuseppe Fedele, Sara K. McLaughlin, Elizabeth E. Reczek, Thomas De Raedt, Isil Guney, et al. 2010. “An Oncogene-Tumor Suppressor Cascade Drives Metastatic Prostate Cancer by Coordinately Activating Ras and Nuclear Factor-[Kappa]B.” *Nature Medicine* 16 (3): 286-. https://doi.org/10.1038/nm.2100.

Mohammad, Faizaan, Simon Weissmann, Benjamin Leblanc, Deo P. Pandey, Jonas W. Højfeldt, Itys Comet, Chunqin Zheng, et al. 2017. “EZH2 Is a Potential Therapeutic Target for H3K27M-Mutant Pediatric Gliomas.” *Nature Medicine* 23 (4): 483–92. https://doi.org/10.1038/nm.4293.

Mohseni, Morvarid, Jianlong Sun, Allison Lau, Stephen Curtis, Jeffrey Goldsmith, Victor L. Fox, Chongjuan Wei, et al. 2014. “A Genetic Screen Identifies an LKB1-MARK Signalling Axis Controlling the Hippo-YAP Pathway.” *Nature Cell Biology* 16 (1): 108–17. https://doi.org/10.1038/ncb2884.

Mueller, Timothy, Ana Sofia Guerreiro Stucklin, Andreas Postlmayr, Sarah Metzger, Nicolas Gerber, Cassie Kline, Michael Grotzer, Javad Nazarian, and Sabine Mueller. 2020. “Advances in Targeted Therapies for Pediatric Brain Tumors.” *Current Treatment Options in Neurology* 22 (12): 43. https://doi.org/10.1007/s11940-020-00651-3.

Mullen, Peter J., Rosemary Yu, Joseph Longo, Michael C. Archer, and Linda Z. Penn. 2016. “The Interplay between Cell Signalling and the Mevalonate Pathway in Cancer.” *Nature Reviews. Cancer* 16 (11): 718–31. https://doi.org/10.1038/nrc.2016.76.

Murthy, Rashmi K., Sherene Loi, Alicia Okines, Elisavet Paplomata, Erika Hamilton, Sara A. Hurvitz, Nancy U. Lin, et al. 2020. “Tucatinib, Trastuzumab, and Capecitabine for HER2-Positive Metastatic Breast Cancer.” *The New England Journal of Medicine* 382 (7): 597–609. https://doi.org/10.1056/NEJMoa1914609.

Nagata, Yoichi, Keng-Hsueh Lan, Xiaoyan Zhou, Ming Tan, Francisco J. Esteva, Aysegul A. Sahin, Kristine S. Klos, et al. 2004. “PTEN Activation Contributes to Tumor Inhibition by Trastuzumab, and Loss of PTEN Predicts Trastuzumab Resistance in Patients.” *Cancer Cell* 6 (2): 117–27. https://doi.org/10.1016/j.ccr.2004.06.022.

Nahta, Rita, Linda X. H. Yuan, Bing Zhang, Ryuji Kobayashi, and Francisco J. Esteva. 2005. “Insulin-like Growth Factor-I Receptor/Human Epidermal Growth Factor Receptor 2 Heterodimerization Contributes to Trastuzumab Resistance of Breast Cancer Cells.” *Cancer Research* 65 (23): 11118–28. https://doi.org/10.1158/0008-5472.CAN-04-3841.

O’Brien, Neil A., Brigid C. Browne, Lucy Chow, Yuhua Wang, Charles Ginther, Jane Arboleda, Michael J. Duffy, John Crown, Norma O’Donovan, and Dennis J. Slamon. 2010. “Activated Phosphoinositide 3-Kinase/AKT Signaling Confers Resistance to Trastuzumab but Not Lapatinib.” *Molecular Cancer Therapeutics* 9 (6): 1489–1502. https://doi.org/10.1158/1535-7163.MCT-09-1171.

Ocana, Alberto, Marta Gil-Martin, Silvia Antolín, María Atienza, Álvaro Montaño, Nuria Ribelles, Ander Urruticoechea, et al. 2019. “Efficacy and Safety of Dasatinib with Trastuzumab and Paclitaxel in First Line HER2-Positive Metastatic Breast Cancer: Results from the Phase II GEICAM/2010-04 Study.” *Breast Cancer Research and Treatment* 174 (3): 693–701. https://doi.org/10.1007/s10549-018-05100-z.

Oh, Do-Youn, and Yung-Jue Bang. 2020. “HER2-Targeted Therapies - a Role beyond Breast Cancer.” *Nature Reviews. Clinical Oncology* 17 (1): 33–48. https://doi.org/10.1038/s41571-019-0268-3.

Olson, E. M., M. Abdel-Rasoul, J. Maly, C. S. Wu, N. U. Lin, and C. L. Shapiro. 2013. “Incidence and Risk of Central Nervous System Metastases as Site of First Recurrence in Patients with HER2-Positive Breast Cancer Treated with Adjuvant Trastuzumab.” *Annals of Oncology* 24 (6): 1526–33. https://doi.org/10.1093/annonc/mdt036.

O'Shaughnessy, Joyce, Rebecca L. Moroose, Sunil Babu, Kakhaber Baramidze, David Chan, Stuart P. Leitner, Gia Nemsadze, et al. 2020. "Results of ENCORE 602 (TRIO025), a Phase II, Randomized, Placebo-Controlled, Double-Blinded, Multicenter Study of Atezolizumab with or without Entinostat in Patients with Advanced Triple-Negative Breast Cancer (ATNBC)." *Journal of Clinical Oncology* 38 (15_suppl): 1014–1014. https://doi.org/10.1200/JCO.2020.38.15_suppl.1014.

Oudenaarden, Clara R. L., Robert A. H. van de Ven, and Patrick W. B. Derksen. 2018. "Re-Inforcing the Cell Death Army in the Fight against Breast Cancer." *Journal of Cell Science* 131 (16). https://doi.org/10.1242/jcs.212563.

Ougolkov, Andrei V., Vladimir N. Bilim, and Daniel D. Billadeau. 2008. "Regulation of Pancreatic Tumor Cell Proliferation and Chemoresistance by the Histone Methyltransferase EZH2." *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research* 14 (21): 6790–96. https://doi.org/10.1158/1078-0432.CCR-08-1013.

Pan, Jin-Xiu, Lei Xiong, Kai Zhao, Peng Zeng, Bo Wang, Fu-Lei Tang, Dong Sun, et al. 2018. "YAP Promotes Osteogenesis and Suppresses Adipogenic Differentiation by Regulating β-Catenin Signaling." *Bone Research* 6 (1): 1–12. https://doi.org/10.1038/s41413-018-0018-7.

Pandy, Jessa Gilda P., Joanmarie C. Balolong-Garcia, Mel Valerie B. Cruz-Ordinario, and Frances Victoria F. Que. 2019. "Triple Negative Breast Cancer and Platinum-Based Systemic Treatment: A Meta-Analysis and Systematic Review." *BMC Cancer* 19 (1): 1065. https://doi.org/10.1186/s12885-019-6253-5.

Paplomata, Elisavet, and Ruth O'Regan. 2014. "The PI3K/AKT/MTOR Pathway in Breast Cancer: Targets, Trials and Biomarkers." *Therapeutic Advances in Medical Oncology* 6 (4): 154–66. https://doi.org/10.1177/1758834014530023.

Pentimalli, Francesca. 2018. "BCL2: A 30-Year Tale of Life, Death and Much More to Come." *Cell Death and Differentiation* 25 (1): 7–9. https://doi.org/10.1038/cdd.2017.189.

Pernas, Sonia, and Sara M. Tolaney. 2019. "HER2-Positive Breast Cancer: New Therapeutic Frontiers and Overcoming Resistance." *Therapeutic Advances in Medical Oncology* 11 (March): 1758835919833519. https://doi.org/10.1177/1758835919833519.

Pfeffer, Claire M., and Amareshwar T. K. Singh. 2018. "Apoptosis: A Target for Anticancer Therapy." *International Journal of Molecular Sciences* 19 (2): 448. https://doi.org/10.3390/ijms19020448.

Pradeep, Chaluvally-Raghavan, Wolfgang J. Köstler, Mattia Lauriola, Roy Granit, Fan Zhang, Jasmine Jacob-Hirsch, Gideon Rechavi, et al. 2012. "Modeling Ductal Carcinoma in Situ: A HER2-Notch3 Collaboration Enables Luminal Filling." *Oncogene* 31 (7): 907–17. https://doi.org/10.1038/onc.2011.279.

Prat, Aleix, Lisa A. Carey, Barbara Adamo, Maria Vidal, Josep Tabernero, Javier Cortes, Joel S. Parker, Charles M. Perou, and Jose Baselga. 2014. “Molecular Features and Survival Outcomes of the Intrinsic Subtypes Within HER2-Positive Breast Cancer.” *JNCI : Journal of the National Cancer Institute* 106 (8). https://doi.org/10.1093/jnci/dju152.

Puthalakath, H., A. Villunger, L. A. O’Reilly, J. G. Beaumont, L. Coultas, R. E. Cheney, D. C. S. Huang, and A. Strasser. 2001. “Bmf: A Proapoptotic BH3-Only Protein Regulated by Interaction with the Myosin V Actin Motor Complex, Activated by Anoikis.” *Science (American Association for the Advancement of Science)* 293 (5536): 1829–32. https://doi.org/10.1126/science.1062257.

Raj-Kumar, Praveen-Kumar, Jianfang Liu, Jeffrey A. Hooke, Albert J. Kovatich, Leonid Kvecher, Craig D. Shriver, and Hai Hu. 2019. “PCA-PAM50 Improves Consistency between Breast Cancer Intrinsic and Clinical Subtyping Reclassifying a Subset of Luminal A Tumors as Luminal B.” *Scientific Reports* 9 (1): 7956. https://doi.org/10.1038/s41598-019-44339-4.

Ramaswamy, B., W. Fiskus, B. Cohen, C. Pellegrino, D. L. Hershman, E. Chuang, Thehang Luu, et al. 2012. “Phase I-II Study of Vorinostat plus Paclitaxel and Bevacizumab in Metastatic Breast Cancer: Evidence for Vorinostat-Induced Tubulin Acetylation and Hsp90 Inhibition in Vivo.” *Breast Cancer Research and Treatment* 132 (3): 1063–72. https://doi.org/10.1007/s10549-011-1928-x.

Ravetch, Jeffrey V., Raphael A. Clynes, Terri L. Towers, and Leonard G. Presta. 2000. “Inhibitory Fc Receptors Modulate in Vivo Cytoxicity against Tumor Targets.” *Nature Medicine* 6 (4): 443–46. https://doi.org/10.1038/74704.

Richman, Susan D, Katie Southward, Philip Chambers, Debra Cross, Jennifer Barrett, Gemma Hemmings, Morag Taylor, et al. 2016. “HER2 Overexpression and Amplification as a Potential Therapeutic Target in Colorectal Cancer: Analysis of 3256 Patients Enrolled in the QUASAR, FOCUS and PICCOLO Colorectal Cancer Trials.” *The Journal of Pathology* 238 (4): 562–70. https://doi.org/10.1002/path.4679.

Rimawi, Mothaffar F., Rachel Schiff, and C. Kent Osborne. 2015. “Targeting HER2 for the Treatment of Breast Cancer.” *Annual Review of Medicine* 66 (1): 111–28. https://doi.org/10.1146/annurev-med-042513-015127.

Robson, Mark, Seock-Ah Im, Elżbieta Senkus, Binghe Xu, Susan M. Domchek, Norikazu Masuda, Suzette Delaloge, et al. 2017. “Olaparib for Metastatic Breast Cancer in Patients with a Germline BRCA Mutation.” *The New England Journal of Medicine* 377 (6): 523–33. https://doi.org/10.1056/NEJMoa1706450.

Rosen, Jeffrey M., and Tracy Vargo-Gogola. 2007. “Modelling Breast Cancer: One Size Does Not Fit All.” *Nature Reviews. Cancer* 7 (9): 659–72. https://doi.org/10.1038/nrc2193.

Rugo, Hope S., Ira Jacobs, Shikhar Sharma, Frank Scappaticci, Thomas A. Paul, Kristen Jensen-Pergakes, and Gabriel G. Malouf. 2020. “The Promise for Histone Methyltransferase Inhibitors for Epigenetic Therapy in Clinical Oncology: A Narrative Review.” *Advances in Therapy* 37 (7): 3059–82. https://doi.org/10.1007/s12325-020-01379-x.

Schech, Amanda, Armina Kazi, Stephen Yu, Preeti Shah, and Gauri Sabnis. 2015. “Histone Deacetylase Inhibitor Entinostat Inhibits Tumor-Initiating Cells in Triple-Negative Breast Cancer Cells.” *Molecular Cancer Therapeutics* 14 (8): 1848–57. https://doi.org/10.1158/1535-7163.MCT-14-0778.

Schlegelmilch, Karin, Morvarid Mohseni, Oktay Kirak, Jan Pruszak, J. Renato Rodriguez, Dawang Zhou, Bridget T. Kreger, et al. 2011. “Yap1 Acts Downstream of α-Catenin to Control Epidermal Proliferation.” *Cell* 144 (5): 782–95. https://doi.org/10.1016/j.cell.2011.02.031.

Schmelzle, Tobias, Arnaud A. Mailleux, Michael Overholtzer, Jason S. Carroll, Nicole L. Solimini, Eric S. Lightcap, Ole P. Veiby, and Joan S. Brugge. 2007. “Functional Role and Oncogene-Regulated Expression of the BH3-Only Factor Bmf in Mammary Epithelial Anoikis and Morphogenesis.” *Proceedings of the National Academy of Sciences - PNAS* 104 (10): 3787–92. https://doi.org/10.1073/pnas.0700115104.

Schmid, Peter, Javier Cortes, Lajos Pusztai, Heather McArthur, Sherko Kümmel, Jonas Bergh, Carsten Denkert, et al. 2020. “Pembrolizumab for Early Triple-Negative Breast Cancer.” *The New England Journal of Medicine* 382 (9): 810–21. https://doi.org/10.1056/NEJMoa1910549.

Schuler, F., F. Baumgartner, V. Klepsch, M. Chamson, E. Müller-Holzner, C. J. Watson, S. Oh, et al. 2016. “The BH3-Only Protein BIM Contributes to Late-Stage Involution in the Mouse Mammary Gland.” *Cell Death & Differentiation* 23 (1): 41–51. https://doi.org/10.1038/cdd.2015.61.

SEER. 2021. “Cancer of the Breast (Female) - Cancer Stat Facts.” SEER. 2021. https://seer.cancer.gov/statfacts/html/breast.html.

Sethunath, Vidyalakshmi, Huizhong Hu, Carmine De Angelis, Jamunarani Veeraraghavan, Lanfang Qin, Nicholas Wang, Lukas M. Simon, et al. 2019. “Targeting the Mevalonate Pathway to Overcome Acquired Anti-HER2 Treatment Resistance in Breast Cancer.” *Molecular Cancer Research* 17 (11): 2318–30. https://doi.org/10.1158/1541-7786.MCR-19-0756.

Shi, Yun, Xuejun Fan, Hui Deng, Randall J. Brezski, Michael Rycyzyn, Robert E. Jordan, William R. Strohl, Quanming Zou, Ningyan Zhang, and Zhiqiang An. 2015. “Trastuzumab Triggers Phagocytic Killing of High HER2 Cancer Cells in Vitro and in Vivo by Interaction with Fcγ Receptors on Macrophages.” *Journal of Immunology (Baltimore, Md.: 1950)* 194 (9): 4379–86. https://doi.org/10.4049/jimmunol.1402891.

Shibata, Wataru, Hiroto Kinoshita, Yohko Hikiba, Takeshi Sato, Yasuaki Ishii, Soichiro Sue, Makoto Sugimori, et al. 2018. “Overexpression of HER2 in the Pancreas Promotes Development

of Intraductal Papillary Mucinous Neoplasms in Mice." *Scientific Reports* 8. https://doi.org/10.1038/s41598-018-24375-2.

Simeone, Noemi, Anna Maria Frezza, Nadia Zaffaroni, and Silvia Stacchiotti. 2021. "Tazemetostat for Advanced Epithelioid Sarcoma: Current Status and Future Perspectives." *Future Oncology (London, England)* 17 (10): 1253–63. https://doi.org/10.2217/fon-2020-0781.

Singh, Rumani, Anthony Letai, and Kristopher Sarosiek. 2019. "Regulation of Apoptosis in Health and Disease: The Balancing Act of BCL-2 Family Proteins." *Nature Reviews. Molecular Cell Biology* 20 (3): 175–93. https://doi.org/10.1038/s41580-018-0089-8.

Slamon, D. J., B. Leyland-Jones, S. Shak, H. Fuchs, V. Paton, A. Bajamonde, T. Fleming, et al. 2001. "Use of Chemotherapy plus a Monoclonal Antibody against HER2 for Metastatic Breast Cancer That Overexpresses HER2." *The New England Journal of Medicine* 344 (11): 783–92. https://doi.org/10.1056/NEJM200103153441101.

Slamon, Dennis, Wolfgang Eiermann, Nicholas Robert, Tadeusz Pienkowski, Miguel Martin, Michael Press, John Mackey, et al. 2011. "Adjuvant Trastuzumab in HER2-Positive Breast Cancer." *New England Journal of Medicine* 365 (14): 1273–83. https://doi.org/10.1056/NEJMoa0910383.

Sørlie, Therese, Robert Tibshirani, Joel Parker, Trevor Hastie, J. S. Marron, Andrew Nobel, Shibing Deng, et al. 2003. "Repeated Observation of Breast Tumor Subtypes in Independent Gene Expression Data Sets." *Proceedings of the National Academy of Sciences* 100 (14): 8418–23. https://doi.org/10.1073/pnas.0932692100.

Souroullas, George P., William R. Jeck, Joel S. Parker, Jeremy M. Simon, Jie-Yu Liu, Joshiawa Paulk, Jessie Xiong, et al. 2016. "An Oncogenic Ezh2 Mutation Induces Tumors through Global Redistribution of Histone 3 Lysine 27 Trimethylation." *Nature Medicine* 22 (6): 632–40. https://doi.org/10.1038/nm.4092.

Spector, Neil L., and Kimberly L. Blackwell. 2009. "Understanding the Mechanisms Behind Trastuzumab Therapy for Human Epidermal Growth Factor Receptor 2–Positive Breast Cancer." *Journal of Clinical Oncology* 27 (34): 5838–47. https://doi.org/10.1200/JCO.2009.22.1507.

Stavrou, E., E. P. Winer, and N. U. Lin. 2021. "How We Treat HER2-Positive Brain Metastases." *ESMO Open* 6 (5): 100256–100256. https://doi.org/10.1016/j.esmoop.2021.100256.

Su, Shicheng, Jinghua Zhao, Yue Xing, Xiaoqian Zhang, Jiang Liu, Qian Ouyang, Jianing Chen, Fengxi Su, Qiang Liu, and Erwei Song. 2018. "Immune Checkpoint Inhibition Overcomes ADCP-Induced Immunosuppression by Macrophages." *Cell* 175 (2): 442-457.e23. https://doi.org/10.1016/j.cell.2018.09.007.

Sudo, T., T. Utsunomiya, K. Mimori, H. Nagahara, K. Ogawa, H. Inoue, S. Wakiyama, H. Fujita, K. Shirouzu, and M. Mori. 2005. "Clinicopathological Significance of EZH2 MRNA Expression in

Patients with Hepatocellular Carcinoma." *British Journal of Cancer* 92 (9): 1754–58. https://doi.org/10.1038/sj.bjc.6602531.

Sun, Feng, Eli Chan, Zhenlong Wu, Xiaojing Yang, Victor E. Marquez, and Qiang Yu. 2009. "Combinatorial Pharmacologic Approaches Target EZH2-Mediated Gene Repression in Breast Cancer Cells." *Molecular Cancer Therapeutics* 8 (12): 3191–3202. https://doi.org/10.1158/1535-7163.MCT-09-0479.

Takeda, Tatsuaki, Hiromasa Yamamoto, Hirotaka Kanzaki, Ken Suzawa, Takahiro Yoshioka, Shuta Tomida, Xiaojiang Cui, et al. 2017. "Yes1 Signaling Mediates the Resistance to Trastuzumab/Lap Atinib in Breast Cancer." *PLOS ONE* 12 (2): e0171356. https://doi.org/10.1371/journal.pone.0171356.

Takeda, Tatsuaki, Hiromasa Yamamoto, Ken Suzawa, Shuta Tomida, Shunsaku Miyauchi, Kota Araki, Kentaro Nakata, et al. 2020. "YES1 Activation Induces Acquired Resistance to Neratinib in HER2-amplified Breast and Lung Cancers." *Cancer Science* 111 (3): 849–56. https://doi.org/10.1111/cas.14289.

Tan, Jin-zhi, Yan Yan, Xiao-xi Wang, Yi Jiang, and H. Eric Xu. 2014. "EZH2: Biology, Disease, and Structure-Based Drug Discovery." *Acta Pharmacologica Sinica* 35 (2): 161–74. https://doi.org/10.1038/aps.2013.161.

Tan, Winston W., Jacob B. Allred, Alvaro Moreno-Aspitia, Donald W. Northfelt, James N. Ingle, Matthew P. Goetz, and Edith A. Perez. 2016. "Phase I Study of Panobinostat (LBH589) and Letrozole in Postmenopausal Metastatic Breast Cancer Patients." *Clinical Breast Cancer* 16 (2): 82–86. https://doi.org/10.1016/j.clbc.2015.11.003.

Tarantino, Paolo, Aleix Prat, Javier Cortes, Fatima Cardoso, and Giuseppe Curigliano. 2021. "Third-Line Treatment of HER2-Positive Advanced Breast Cancer: From No Standard to a Pandora's Box." *Biochimica et Biophysica Acta. Reviews on Cancer* 1875 (1): 188487-. https://doi.org/10.1016/j.bbcan.2020.188487.

Tesch, Megan E., and Karen A. Gelmon. 2020. "Targeting HER2 in Breast Cancer: Latest Developments on Treatment Sequencing and the Introduction of Biosimilars." *Drugs (New York, N.Y.)* 80 (17): 1811–30. https://doi.org/10.1007/s40265-020-01411-y.

Torres, Evanthia T. Roussos, and Leisha A. Emens. 2021. "Emerging Combination Immunotherapy Strategies for Breast Cancer: Dual Immune Checkpoint Modulation, Antibody–Drug Conjugates and Bispecific Antibodies." *Breast Cancer Research and Treatment*. https://doi.org/10.1007/s10549-021-06423-0.

Tsao, Li-Chung, Erika J. Crosby, Timothy N. Trotter, Pankaj Agarwal, Bin-Jin Hwang, Chaitanya Acharya, Casey W. Shuptrine, et al. 2019. "CD47 Blockade Augmentation of Trastuzumab Antitumor Efficacy Dependent on Antibody-Dependent Cellular Phagocytosis." *JCI Insight* 4 (24). https://doi.org/10.1172/jci.insight.131882.

Vassilev, A., K. J. Kaneko, H. Shu, Y. Zhao, and M. L. DePamphilis. 2001. “TEAD/TEF Transcription Factors Utilize the Activation Domain of YAP65, a Src/Yes-Associated Protein Localized in the Cytoplasm.” *Genes & Development* 15 (10): 1229–41. https://doi.org/10.1101/gad.888601.

Veer, LJ van ’t, H. Dai, H. van de Vijver, Y. D. He, A. A. M. Hart, M. Mao, H. L. Peterse, et al. 2002. “Gene Expression Profiling Predicts Clinical Outcome of Breast Cancer.” *Nature (London)* 415 (6871): 530–36. https://doi.org/10.1038/415530a.

Vernieri, Claudio, Monica Milano, Marta Brambilla, Alessia Mennitto, Claudia Maggi, Maria Silvia Cona, Michele Prisciandaro, et al. 2019. “Resistance Mechanisms to Anti-HER2 Therapies in HER2-Positive Breast Cancer: Current Knowledge, New Research Directions and Therapeutic Perspectives.” *Critical Reviews in Oncology/Hematology* 139: 53–66. https://doi.org/10.1016/j.critrevonc.2019.05.001.

Vuong, Darina, Peter T. Simpson, Benjamin Green, Margaret C. Cummings, and Sunil R. Lakhani. 2014. “Molecular Classification of Breast Cancer.” *Virchows Archiv : An International Journal of Pathology* 465 (1): 1–14. https://doi.org/10.1007/s00428-014-1593-7.

Waks, Adrienne G., and Eric P. Winer. 2019. “Breast Cancer Treatment: A Review.” *JAMA : The Journal of the American Medical Association* 321 (3): 288–300. https://doi.org/10.1001/jama.2018.19323.

Wang, Jiani, and Binghe Xu. 2019. “Targeted Therapeutic Options and Future Perspectives for HER2-Positive Breast Cancer.” *Signal Transduction and Targeted Therapy* 4 (1): 1–22. https://doi.org/10.1038/s41392-019-0069-2.

Wang, Lei, Quanren Wang, Piaopiao Xu, Li Fu, Yun Li, Haoyu Fu, Haitian Quan, and Liguang Lou. 2020. “YES1 Amplification Confers Trastuzumab-Emtansine (T-DM1) Resistance in HER2-Positive Cancer.” *British Journal of Cancer* 123 (6): 1000–1011. https://doi.org/10.1038/s41416-020-0952-1.

Wang, Lifeng, Qihuang Jin, Ji-Eun Lee, I.-hsin Su, and Kai Ge. 2010. “Histone H3K27 Methyltransferase Ezh2 Represses Wnt Genes to Facilitate Adipogenesis.” *Proceedings of the National Academy of Sciences of the United States of America* 107 (16): 7317–22. https://doi.org/10.1073/pnas.1000031107.

Wang, Yumeng, Xiaoyan Xu, Dejan Maglic, Michael T. Dill, Kamalika Mojumdar, Patrick Kwok-Shing Ng, Kang Jin Jeong, et al. 2018. “Comprehensive Molecular Characterization of the Hippo Signaling Pathway in Cancer.” *Cell Reports* 25 (5): 1304-1317.e5. https://doi.org/10.1016/j.celrep.2018.10.001.

Wassef, M., and R. Margueron. 2017. “The Multiple Facets of PRC2 Alterations in Cancers.” *Journal of Molecular Biology* 429 (13): 1978–93. https://doi.org/10.1016/j.jmb.2016.10.012.

Wawruszak, Anna, Lidia Borkiewicz, Estera Okon, Wirginia Kukula-Koch, Syeda Afshan, and Marta Halasa. 2021. “Vorinostat (SAHA) and Breast Cancer: An Overview.” *Cancers* 13 (18): 4700-. https://doi.org/10.3390/cancers13184700.

Weikert, Steffen, Frank Christoph, Jens Köllermann, Markus Müller, Mark Schrader, Kurt Miller, and Hans Krause. 2005. “Expression Levels of the EZH2 Polycomb Transcriptional Repressor Correlate with Aggressiveness and Invasive Potential of Bladder Carcinomas.” *International Journal of Molecular Medicine* 16 (2): 349–53. https://doi.org/10.3892/ijmm.16.2.349.

WHO. 2021. “Breast Cancer.” 2021. https://www.who.int/news-room/fact-sheets/detail/breast-cancer.

Xu, Kexin, Zhenhua Jeremy Wu, Anna C. Groner, Housheng Hansen He, Changmeng Cai, Rosina T. Lis, Xiaoqiu Wu, et al. 2012. “EZH2 Oncogenic Activity in Castration-Resistant Prostate Cancer Cells Is Polycomb-Independent.” *Science (New York, N.Y.)* 338 (6113): 1465–69. https://doi.org/10.1126/science.1227604.

Yamaguchi, Hirohito, and Mien-Chie Hung. 2014. “Regulation and Role of EZH2 in Cancer.” *Cancer Research and Treatment* 46 (3): 209–22. https://doi.org/10.4143/crt.2014.46.3.209.

Yang, Jing, Ji Nie, Xuelei Ma, Yuquan Wei, Yong Peng, and Xiawei Wei. 2019. “Targeting PI3K in Cancer: Mechanisms and Advances in Clinical Trials.” *Molecular Cancer* 18 (1): 26. https://doi.org/10.1186/s12943-019-0954-x.

Yap, Timothy A., Jane N. Winter, Lisa Giulino-Roth, Jemma Longley, Juanita Lopez, Jean-Marie Michot, John P. Leonard, et al. 2019. “Phase I Study of the Novel Enhancer of Zeste Homolog 2 (EZH2) Inhibitor GSK2816126 in Patients with Advanced Hematologic and Solid Tumors.” *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research* 25 (24): 7331–39. https://doi.org/10.1158/1078-0432.CCR-18-4121.

Yarden, Yosef, and Mark X. Sliwkowski. 2001. “Untangling the ErbB Signalling Network.” *Nature Reviews. Molecular Cell Biology* 2 (2): 127–37. https://doi.org/10.1038/35052073.

Yin, Li, Jiang-Jie Duan, Xiu-Wu Bian, and Shi-cang Yu. 2020. “Triple-Negative Breast Cancer Molecular Subtyping and Treatment Progress.” *Breast Cancer Research : BCR* 22 (1): 61–61. https://doi.org/10.1186/s13058-020-01296-5.

Yu, Yung-Luen, Ruey-Hwang Chou, Ling-Tzu Chen, Woei-Cherng Shyu, Su-Ching Hsieh, Chen-Shiou Wu, Hong-Jie Zeng, et al. 2011. “EZH2 Regulates Neuronal Differentiation of Mesenchymal Stem Cells through PIP5K1C-Dependent Calcium Signaling.” *The Journal of Biological Chemistry* 286 (11): 9657–67. https://doi.org/10.1074/jbc.M110.185124.

Yuan, M., V. Tomlinson, R. Lara, D. Holliday, C. Chelala, T. Harada, R. Gangeswaran, et al. 2008. “Yes-Associated Protein (YAP) Functions as a Tumor Suppressor in Breast.” *Cell Death & Differentiation* 15 (11): 1752–59. https://doi.org/10.1038/cdd.2008.108.

Zaidi, Sayyed K., Daniel W. Young, Je-Yong Choi, Jitesh Pratap, Amjad Javed, Martin Montecino, Janet L. Stein, Andre J. van Wijnen, Jane B. Lian, and Gary S. Stein. 2005. "The Dynamic Organization of Gene-Regulatory Machinery in Nuclear Microenvironments." *EMBO Reports* 6 (2): 128–33. https://doi.org/10.1038/sj.embor.7400337.

Zanconato, Francesca, Michelangelo Cordenonsi, and Stefano Piccolo. 2016. "YAP/TAZ at the Roots of Cancer." *Cancer Cell* 29 (6): 783–803. https://doi.org/10.1016/j.ccell.2016.05.005.

Zhang, Haikuo, Jun Qi, Jaime M. Reyes, Lewyn Li, Prakash K. Rao, Fugen Li, Charles Y. Lin, et al. 2016. "Oncogenic Deregulation of EZH2 as an Opportunity for Targeted Therapy in Lung Cancer." *Cancer Discovery* 6 (9): 1006–21. https://doi.org/10.1158/2159-8290.CD-16-0164.

Zhang, Ping, Mark C. de Gooijer, Levi C. M. Buil, Jos H. Beijnen, Gang Li, and Olaf van Tellingen. 2015. "ABCB1 and ABCG2 Restrict the Brain Penetration of a Panel of Novel EZH2-Inhibitors." *International Journal of Cancer* 137 (8): 2007–18. https://doi.org/10.1002/ijc.29566.

Zhang, Siyuan, Wen-Chien Huang, Ping Li, Hua Guo, Say-Bee Poh, Samuel W. Brady, Yan Xiong, et al. 2011. "Combating Trastuzumab Resistance by Targeting SRC, a Common Node Downstream of Multiple Resistance Pathways." *Nature Medicine* 17 (4): 461–69. https://doi.org/10.1038/nm.2309.

Zhao, Bin, Li Li, Karen Tumaneng, Cun-Yu Wang, and Kun-Liang Guan. 2010. "A Coordinated Phosphorylation by Lats and CK1 Regulates YAP Stability through SCFβ-TRCP." *Genes & Development* 24 (1): 72–85. https://doi.org/10.1101/gad.1843810.

Zhao, Bin, Xiaomu Wei, Weiquan Li, Ryan S. Udan, Qian Yang, Joungmok Kim, Joe Xie, et al. 2007. "Inactivation of YAP Oncoprotein by the Hippo Pathway Is Involved in Cell Contact Inhibition and Tissue Growth Control." *Genes & Development* 21 (21): 2747–61. https://doi.org/10.1101/gad.1602907.

Zhao, Bin, Xin Ye, Jindan Yu, Li Li, Weiquan Li, Siming Li, Jianjun Yu, et al. 2008. "TEAD Mediates YAP-Dependent Gene Induction and Growth Control." *Genes & Development* 22 (14): 1962–71. https://doi.org/10.1101/gad.1664408.

Zhao, Jing, and Yang Xia. 2020. "Targeting HER2 Alterations in Non–Small-Cell Lung Cancer: A Comprehensive Review." *JCO Precision Oncology*, no. 4 (November): 411–25. https://doi.org/10.1200/PO.19.00333.

Zingg, Daniel, Julien Debbache, Simon M. Schaefer, Eylul Tuncer, Sandra C. Frommel, Phil Cheng, Natalia Arenas-Ramirez, et al. 2015. "The Epigenetic Modifier EZH2 Controls Melanoma Growth and Metastasis through Silencing of Distinct Tumour Suppressors." *Nature Communications* 6 (1): 6051–6051. https://doi.org/10.1038/ncomms7051.

Zucchetti, Bruna, Andrea Kazumi Shimada, Artur Katz, and Giuseppe Curigliano. 2019. "The Role of Histone Deacetylase Inhibitors in Metastatic Breast Cancer." *Breast (Edinburgh)* 43: 130–34. https://doi.org/10.1016/j.breast.2018.12.001.

Zuo, Tingting, Hongmei Zeng, Huichao Li, Shuo Liu, Lei Yang, Changfa Xia, Rongshou Zheng, et al. 2017. “The Influence of Stage at Diagnosis and Molecular Subtype on Breast Cancer Patient Survival: A Hospital-Based Multi-Center Study.” *Chinese Journal of Cancer* 36 (1): 84. https://doi.org/10.1186/s40880-017-0250-3.