SHP2 BLOCKADE SENSITIZES TRIPLE NEGATIVE BREAST CANCERS TO PI3K INHIBITION LEADING TO METASTATIC SHRINKAGE

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“You are the only one responsible for your own wants.” — Isaac Asimov

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2 | SUMMARY

Breast cancer is the most frequent and lethal cancer among women worldwide. A third of breast cancers can progress to metastasis, which remains the major cause of death in patients with solid tumors. Cells are regulated by numerous interconnected pathways, which can be dysregulated and results in uncontrolled proliferation. The phosphatidylinositol 3-kinase (PI3K) pathway is implicated in cellular growth, proliferation and survival, and is aberrantly activated in 70% of breast cancers. Despite the development of specific and effective drugs targeting the PI3K pathway, most clinical trial outcomes have been disappointing. In fact, mechanisms of resistance can short-circuit the efficacy of such inhibitors. Some of these mechanisms are receptor tyrosine kinase (RTK) driven, activating compensatory pathways and dramatically reducing the initial efficacy.

Downstream of various active RTKs, Src-homology 2 domain-containing phosphatase (SHP2), a ubiquitously expressed protein-tyrosine phosphatase (PTP), transduces mitogenic, survival, cell-fate and/or migratory signals. Blockade of SHP2 has been shown to decrease breast tumor growth, progression and metastasis. Given that RTK-driven signaling pathways can overcome the effects of PI3K inhibition, and that SHP2 enhances signaling downstream of these receptors, we studied the effects of targeting PI3K and SHP2 simultaneously.

In this study, we demonstrate a fundamental effect of PI3K/SHP2 dual-inhibition in triple-negative breast cancers (TNBCs), a very aggressive subtype associated with poor prognosis. Dual inhibition targeting PI3K and SHP2 appears to be more effective than single inhibitions by decreasing cell number *in vitro* and tumor volume *in vivo*, as well as increasing cancer cell apoptosis and improving animal survival. Mechanistically, SHP2 inhibition results in activation of the PI3K signaling and dependency on this pathway.

We then assessed the effects of PI3K and/or SHP2 inhibitions on primary tumor growth, animal survival and lung metastases, a major metastatic site in breast cancer. While PI3K inhibition had no effects on primary tumor growth, it resulted in larger lung metastases in the neo-adjuvant setting. SHP2 inhibition decreased primary tumor growth as well as lung metastases. Both PI3K and SHP2 single treatment groups did not improve animal survival. In combination, PI3K/SHP2 dual-inhibition reduced synergistically primary tumor volumes, decreased lung metastases and increased animal survival.

In the adjuvant setting, PI3K and SHP2 single inhibitions, as well as PI3K/SHP2 dual-inhibition, decreased lung metastases and increased animal survival. Despite the lack of lung metastases, concurrent PI3K/SHP2 blockade is not enough for complete metastasis regression. We demonstrated that liver metastases developed in parallel and have revealed to be insensitive to such inhibitions. We highlighted the discrepancy in RTK-dependences with lung metastases being $PDGFR_{\beta}$-dependant, while liver metastases are VEGFRs-dependent. Using a VEGFR/PDGFR inhibitor, we finally indicate that targeting PI3K/SHP2/VEGFR/PDGFR can further improve animal survival.

The observations that pan-PI3K inhibition in the neo-adjuvant setting increases lung metastases in TNBC calls for caution when using such agents in the presence of the primary tumor. We have reported similar results using a dual-PI3K/mTOR inhibitor (Britschgi, Andraos et al. 2012). Our data provide a rationale for using pan-PI3K in combination with SHP2 inhibition to treat metastatic TNBC in the adjuvant setting and support further testing of this possibility. Moreover, we provide evidence that a triple therapy of PI3K, SHP2 and VEGFR/PDGFR inhibitors overcomes niche-dependent resistance and prolongs survival in preclinical models of TNBC.

3 | INTRODUCTION

3.1 Breast cancer

Breast cancer is the most frequent cancer among women representing 25% of all cancers diagnosed worldwide. The World Health Organization (WHO) measured that breast cancer is impacting 2.1 million women per year, and with 627,000 annual death, it represents 15% of all cancer losses among women (Parks, Derks et al. 2018). This heterogeneous disease is characterized by cancer cells that can proliferate, disseminate, survive and form metastases (Weigelt, Peterse et al. 2005). Initial steps of breast cancer take place in the epithelial cells of the mammary gland, progressing through several stages: hyperplasia, atypical ductal hyperplasia, ductal carcinoma *in situ* (DCIS) and invasive ductal carcinoma (**Figure 3-1**). A third of breast cancers progress to metastasis (Early Breast Cancer Trialists' Collaborative 2005), the fatal hallmark of cancer (Nguyen, Bos et al. 2009), and drug resistant metastases remain the cause of death of most breast cancer patients (Klein 2009, Massague and Obenauf 2016).

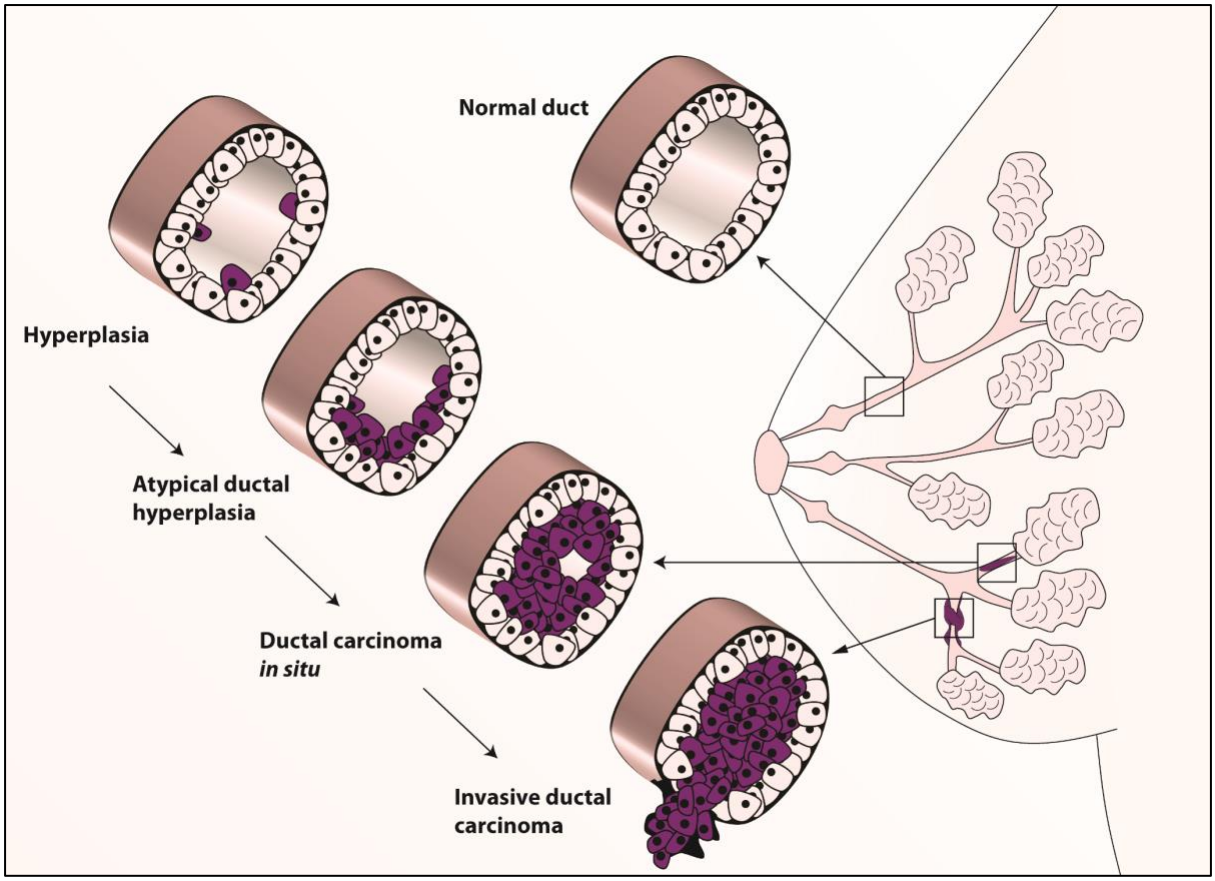

Figure 3-1 | Breast cancer linear progression model

Schematic view of the linear breast cancer progression. Breast cancer arises from epithelial cells of the mammary gland as hyperplasia, which is a benign proliferative breast condition. It can progress into atypical ductal hyperplasia, where the proliferating cells look abnormal, and may evolve into ductal carcinoma *in situ*, a non-invasive stage, in which the duct is filled with confined cancerous cells. The last and potentially lethal step of the metastatic cascade is the progression into invasive ductal carcinoma, in which cancer cells spread from the duct to surrounding tissues, to seed and colonize distant sites.

Breast cancer classification is based on detection of pathological markers like the hormone receptors (HR), estrogen receptor (ER), progesterone receptor (PR), and the tyrosine kinase human epidermal growth factor receptor 2 (HER2/ERBB2), as well as clinical parameters such as age, tumor size, lymph node status and histological grade. However, breast cancer heterogeneity limits this classification. Genome-wide gene-expression profiles revealed 6 different sub-classes: luminal A, luminal B, normal-like, HER2-enriched, basal-like and claudin-low with different clinical prognosis based on their aggressiveness (Perou, Sorlie et al.

2000, Sorlie, Perou et al. 2001, Carey, Perou et al. 2006, Prat, Parker et al. 2010). Then, copy number alterations (CNAs) further defined breast cancer subtypes based on the deoxyribonucleic acid (DNA) CNAs patterns (Hicks, Krasnitz et al. 2006). Further improvements led to integrative clustering using gene expression and DNA CNAs. Such strategy was used in the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) study, where identification of integrative clusters revealed a classification in 10 subtypes. So far, these subtypes represent the most detailed molecular-based taxonomy of breast cancer (Curtis, Shah et al. 2012). In parallel, large DNA sequencing campaigns described the importance of driver-genes and their mutations, showing association between breast cancer classification and genomic drivers (e.g., *TP53, PIK3CA, MYC*) (Cancer Genome Atlas 2012, Shah, Roth et al. 2012, Stephens, Tarpey et al. 2012, Nik-Zainal, Davies et al. 2016, Pereira, Chin et al. 2016)

3.2 Classification of breast cancer

3.2.1 Luminal A and luminal B breast cancers

Across the different subgroups, luminal tumors are characterized by the expression of ER and account for 60% of all breast cancers. Luminal A breast cancer cells mainly co-express ER and PR, while luminal B rarely co-express PR (Sims, Howell et al. 2007). These luminal tumors are hormone dependant and can be targeted by using endocrine therapy to block the effects of estrogen. Patients with luminal A breast tumors have a better response to hormonal therapy and longer survival compared to patients with luminal B breast tumors (Vargo-Gogola and Rosen 2007). Beside the expression of ER and PR, this luminal subtype is also characterized by the overexpression of luminal markers such as the X-box binding protein 1, trefoil factor 3, hepatocyte nuclear factor 3 α, GATA binding protein 3 (GATA3) and estrogen-regulated LIV-1 (Perou, Sorlie et al. 2000, Sorlie, Perou et al. 2001).

Since almost five decades, the ER antagonist tamoxifen has been the gold standard treatment of HR-positive breast cancer (Cole, Jones et al. 1971). It is a selective estrogen receptor modulator (SERM) that blocks the binding of estrogen to its receptor. Another strategy is to prevent estrogen synthesis using aromatase inhibitors, such as letrozole and anastrozole (Aihara, Yokota et al. 2014). This strategy has been shown to be more effective than tamoxifen in post-menopausal women with early-stage breast cancer leading to longer disease-free survival (Arimidex, Forbes et al. 2008). Other strategies include luteinizing hormone-releasing hormone analogs, that blocks hormone production by the ovaries (e.g., goserelin), and fulvestrant, a selective estrogen receptor downregulator (SERD), that degrades ER (Thompson, Katz et al. 1989).

Despite this progress in endocrine therapy, a third of patients develop resistance via compensatory mechanisms that offsets the initial inhibition. It occurs as a result of cross-talk between ERs and RTKs, and with signaling pathways downstream of these receptors such as the PI3K-AKT-mTOR pathway (Meyer and Bentires-Alj 2010, Chen, Hsiao et al. 2017, Keegan, Gleeson et al. 2018). For example, upregulation of HER2, epidermal growth factor receptor (EGFR) or insulin-like growth factor I (IGF-I) (Arpino, Wiechmann et al. 2008, Musgrove and Sutherland 2009). The major consequence of these compensatory mechanisms is a re-expression of ER-dependent genes in absence of the ligand, resulting in the aromatase inhibitors being ineffective.

Several preclinical and clinical studies have resulted in the current standard of care for patients with ER+ breast cancer such as the combination of letrozole with the cyclin-dependent kinase 4/6 (CDK) inhibitor palbociclib. This combination improves progression-free survival compared to single aromatase inhibition (Croxtall and McKeage 2011, Finn, Crown et al. 2015, Finn, Martin et al. 2016, Huang, Yang et al. 2017, Nathan and Schmid 2017).

Premenopausal	**Postmenopausal**	Aromatase inhibitor (Letrozole, anastrozole, exemestane) or
		Selective ER modulators (Tamoxifen) or
		Selective ER down-regulator (Fulvestrant) or
		CDK4/6 inhibitor + aromatase inhibitor or
		CDK4/6 inhibitor + fulvestrant
	+ Ovarian ablation/suppression	

↓ If progression or unacceptable toxicity ↓

Endocrine refractory?	NO	Additional endocrine therapy
	YES	Chemotherapy

↓ If no clinical benefit ↓

Chemotherapy

↓ If no clinical benefit ↓

Consider no further cytotoxic therapy and continue supportive care

Table 3-1 | Treatment recommendations for luminal breast cancers

3.2.2 HER2-positive breast cancer

One of the two most aggressive breast cancer subtypes, representing 15-20% of patients, is characterized by HER2 (or ERBB2) enriched expression. Development of the humanized monoclonal antibody trastuzumab improved considerably the prognosis of such patients (Slamon, Clark et al. 1987, Slamon, Leyland-Jones et al. 2001). Genes in this subtype vary based on the ER status. Many functional mutations of *TP53* are found in HER2+/ER- tumors; while HER2+/ER+ tumors show numerous *GATA3* mutations; and no subgroup prevalence for *PIK3CA* mutations (Pereira, Chin et al. 2016). Overall, two third of this subtype have HER2 overexpression with concomitant gene amplification, while the remaining third has normal HER2 expression, highlighting independent HER2-amplification mechanism like HER2

hyperphosphorylation, truncation or splice variant (Lonardo, Di Marco et al. 1990, Scaltriti, Rojo et al. 2007, Alajati, Sausgruber et al. 2013).

Current standard of care for HER2-positive breast cancer is chemotherapy (e.g., paclitaxel or docetaxel) combined with the trastuzumab (herceptin) targeting the extracellular domain of HER2 (Baselga, Perez et al. 2006). This results in prolonged disease-free survival and improved outcome for early HER2-positive breast cancer patients (Romond, Perez et al. 2005). However, 70% of HER2-positive breast cancer can develop resistance to trastuzumab, and further strategies are used to enhance efficacy. For example, pertuzumab prevents HER2 dimerization, and results in a significantly prolonged overall survival and progression-free survival when combined to trastuzumab (Baselga and Swain 2010, Swain, Baselga et al. 2015). Also, trastuzumab emtansine (trastuzumab-DM1) is an antibody-drug conjugate, joining the HER2 cell specific targeting of trastuzumab combined to the cytotoxic activity of the microtubule-inhibitory agent emtansine (Junttila, Li et al. 2011, Krop, Kim et al. 2014, Krop, Kim et al. 2017). Tyrosine kinase inhibitors (TKIs) are also used in combination with trastuzumab such as lapatinib or afatinib, both targeting EGFR/HER2, or neratinib, a pan-HER inhibitor (Burstein, Sun et al. 2010, Hanusch, Schneeweiss et al. 2015, Xu, Zhang et al. 2017). Furthermore, ongoing clinical trials are evaluating the efficacy of inhibiting the heat shock protein 90 (HSP90), required for the stability of HER2 (Modi, Stopeck et al. 2011, Jhaveri, Wang et al. 2017).

Over the last decade, the outcome of HER2-positive breast cancer patients intensely improved, but many patients still lack effective therapeutic solutions. Different studies are exploring drug combination of anti-HER2 agents with PI3K inhibitors, CDK4/6 inhibitors as well as immunotherapies using anti-programmed death ligand 1 (PD-L1) antibodies (Miller, Hennessy et al. 2010, Loibl and Gianni 2017, Shah and Cristofanilli 2017, Xu, Yu et al. 2017).

Premenopausal	**Postmenopausal**	Pertuzumab + trastuzumab + chemotherapy (taxane) or
		Trastuzumab emtansine (Trastuzumab-DM1) or
		Trastuzumab + chemotherapy or
		Other HER2-targeted therapies
	+ Ovarian ablation/suppression	
↓ If progression or unacceptable toxicity ↓		
Another line of chemotherapy + HER2-targeted therapy		
↓ If no clinical benefit ↓		
Consider no further cytotoxic therapy and continue supportive care		

Table 3-2 │ Treatment recommendations for HER2+ breast cancers

3.2.3 Triple-negative breast cancer

TNBC is named for the lack of expression of ER, PR and HER2 in these tumors and accounts for 10-20% of patients. This subtype includes basal-like and claudin-low breast cancers (Perou 2011). Both share the characteristics of low expression of luminal genes clusters and luminal cytokeratins 9 and 18 (CKs). But, the basal-like subgroup is defined by high expression of the basal cytokeratins 5, 14 and 17 (Elsawaf and Sinn 2011), and the claudin-low subgroup is defined by high immune cells infiltration, stem cell-associated mechanisms and high epithelial-to-mesenchymal transition (EMT) characteristics (Sims, Howell et al. 2007). In fact, these EMT characteristics include loss of E-cadherin, claudin 3, 4 and 7, all cell-to-cell junction proteins (Szasz, Nemeth et al. 2011). Of note, the term basal-like refers to a group of human breast cancers sharing a common signature (Sorlie, Perou et al. 2001). This does not relate to a basal-like tumor arising from myoepithelial cells, nor being the opposite of luminal cells (Gusterson and Eaves 2018).

Beside surgery and radiotherapy, chemotherapy is the only option to treat TNBCs since they do not respond to endocrine, anti-HER2 therapy and poorly to targeted therapy. Despite the aggressiveness of TNBC, 30 to 40 % of patients achieve a pathological complete response (pCR) after chemotherapy. Detection of residual disease after neoadjuvant treatment increases by six the chance of relapse and by twelve the chance to succumb to metastases (Liedtke, Mazouni et al. 2008, Esserman, Berry et al. 2012, Gluck, Ross et al. 2012, Masuda, Baggerly et al. 2013). Recently, poly ADP ribose polymerase (PARP) has been approved for the treatment of patients with HER2-negative metastatic breast cancer and a germline BRCA mutation and brings a significant benefit over the standard therapy (Robson, Im et al. 2017). The increased knowledge of the biology of these tumours over the last 5 years has led to new clinical trials to test the anti-vascular endothelial growth factor (VEGF) monoclonal antibody bevacizumab; multi-target inhibitor like sunitinib targeting VEGF Receptor (VEGFR), platelet-derived growth factor receptor (PDGFR) and c-KIT; as well as inhibitors of EGFR, PI3K and CDK1/2 (Mitri, Karakas et al. 2015, Nakai, Hung et al. 2016, Costa, Han et al. 2018).

<table>
<tr><td rowspan="2">Non-metastatic TNBC</td><td colspan="2" rowspan="2">Cycles of anthracycline-taxane chemotherapy</td><td>1st</td><td>Epirubicin or 5-fluorouracil or cyclophosphamide</td></tr>
<tr><td>2nd</td><td>Paclitaxel or docetaxel</td></tr>
<tr><td rowspan="2">Metastatic TNBC</td><td rowspan="2">Platinum-based chemotherapy</td><td>-</td><td colspan="2">Cisplatin or oxaliplatin or carboplatin</td></tr>
<tr><td>BRCA1/2 mutation</td><td colspan="2">Cisplatin + PARP inhibitors</td></tr>
<tr><td colspan="5">↓ If progression or unacceptable toxicity ↓</td></tr>
<tr><td colspan="5">Another line of chemotherapy</td></tr>
<tr><td colspan="5">↓ If no clinical benefit ↓</td></tr>
<tr><td colspan="5">Consider no further cytotoxic therapy and continue supportive care</td></tr>
</table>

Table 3-3 | Treatment recommendations for TNBC breast cancers

3.3 Dissemination and colonization of metastatic cells

3.3.1 Metastatic cascade

Cancer is a lethal disease that can, from the primary tumor, migrate and grow in secondary distant sites. This process is called metastasis. It remains a major challenge since 90% of deaths are caused by drug resistant metastases in patients with solid tumors (Early Breast Cancer Trialists' Collaborative 2005, Gupta and Massague 2006). Dissemination of cancer cells starts at an early stage of tumor progression, but remains an inefficient process since 99.98% of cells leaving the primary tumor die before forming a metastasis (Valastyan and Weinberg 2011, Massague and Obenauf 2016). In fact, a primary tumor consists of millions of cells, but very few will penetrate the circulatory system, and even fewer will survive in circulation and form distant lesions. Furthermore, cancer cells that successfully penetrated and niched in distant organs can reside as dormant micrometastases that may or may not form macrometastases, termed metastatic latency or metastatic dormancy (Luzzi, MacDonald et al. 1998, Kienast, von Baumgarten et al. 2010, Giancotti 2013).

Dissemination of metastatic cells is a multi-step process. Initial steps are the local invasion and migration of primary tumor cells, promoted by cytoskeletal rearrangements, cell-to-cell interactions, secretion of matrix metalloproteinases (MMPs) and various cathepsins (Kessenbrock, Plaks et al. 2010). Cancer cells have then the capacity to migrate in the extracellular matrix, as single cell or collectively. Surrounding stromal cells can secrete transforming growth factor-β (TGF-β) leading to EMT of cancer cells, resulting in phenotypic changes and enhanced invasiveness (Thiery, Acloque et al. 2009). EMT can promote intravasation of cancer cells (i.e., entering the circulatory system), while reversal of this state can favour extravasation and support colonization. However, the EMT process is not required in all situations, since breast and prostate cancers can invade in an EMT-independent manner (Fischer, Durrans et al. 2015, Zheng, Carstens et al. 2015).

In the blood stream, circulating tumor cells (CTCs) can be found as single cells or clusters. This multi-cell state results in an advantage over single cells to form metastatic lesions (Aceto, Bardia et al. 2014, Szczerba, Castro-Giner et al. 2019). In fact, metastatic lesions are made of multiclonal seeding, suggesting a collection of subclones capable of colonizing metastatic sites (Hoadley, Siegel et al. 2016, Turajlic and Swanton 2016, Turajlic, Xu et al. 2018). Cancer cells need to resist to the immune system, while copping shear forces and oxidative stress (Gay and Felding-Habermann 2011, Le Gal, Ibrahim et al. 2015). Their dissemination relies on blood vessels architecture, capillary wall properties and mechanical constrictions of the visited organs. CTCs trapped in a microvessel can potentially grow and rupture the vessel, or extravasate (Hong, Li et al. 2016). Metastases initiating cells need to survive multiple selective pressure such as the immune system, foreign environment while still retaining capacity to proliferate. This step can last several years and cells survive as disseminated tumor cells (DTCs) (Pantel and Brakenhoff 2004) (**Figure 3-2**). Breast cancer metastases are found in bone, lung, liver and brain (Chiang and Massague 2008). ER+ breast

cancer predominantly form bone metastases; TNBC form metastases in the brain and lungs; and, while anti-HER2 therapy improved HER2+ patient survival, brain metastases remain challenging for this subtype (Chiang and Massague 2008, Dent, Hanna et al. 2009, Kennecke, Yerushalmi et al. 2010).

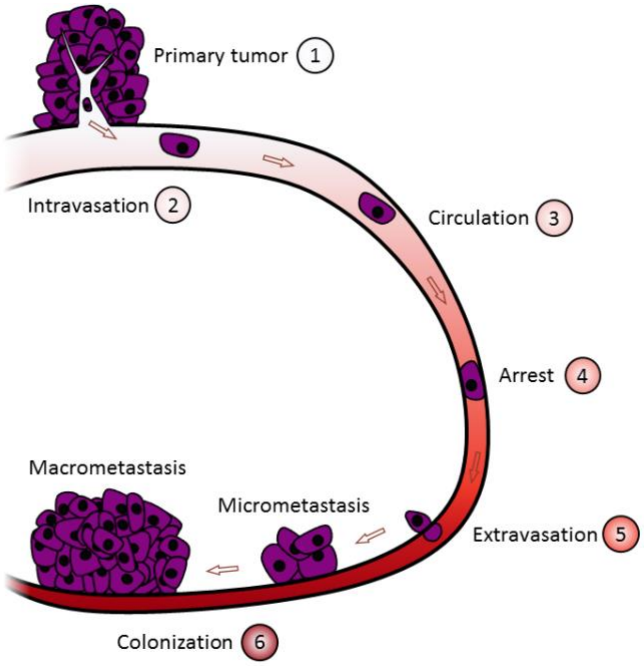

Figure 3-2 | Metastatic progression

Primary tumor cells invade locally (1). The EMT process can support cell intravasation (2) of surrounding tissues or newly formed blood vessels (3). Mechanical constrictions will stop their route in most cases, as single cells or clusters (4), allowing a possible intravasation through the vascular wall (5). If disseminated cells survive in their new environment, they may remain as micrometastases (dormancy) or grow into macrometastases (6).

One commonly admitted model consider cancer cells to arise from cells that conserved their tumor initiating capacity (Kleinsmith and Pierce 1964, Al-Hajj, Wicha et al. 2003). Once these cells reached a secondary site, they might benefit from the niche characteristics that can be in some situation similar to their original niche. Also, they can produce their own niche such as lung-metastatic breast cancer cells producing the extracellular-matrix protein tenascin C, amplifying proliferative pathways such as Notch and Wnt (Oskarsson, Acharyya et al. 2011).

Additionally, recent studies show that metastatic lesions can further generate CTCs and new distant lesions, suggesting a continuous and dynamic dissemination (Gundem, Van Loo et al. 2015).

3.3.2 Disseminated tumor cells

Treatments are administered after surgery to specifically target disseminated cells (adjuvant treatment). DTCs remain very difficult to detect (Chapman, Webster et al. 2013) and can display several cellular states. In fact, it is commonly admitted that metastatic latency is the result of a delayed adaptation to the new microenvironment, worsen by poor neoangiogenesis, immune surveillance and therapeutic pressure (Holmgren, O'Reilly et al. 1995, Eyles, Puaux et al. 2010, Davies, Pan et al. 2013). Adjuvant therapies show high efficacy in ER+ and HER2+ breast cancer, based on five years survival. Beyond this timeframe, the risk of relapse remains important, due to the lack of sensitivity of dormant and non-proliferative DTCs to treatment (Di Cosimo and Baselga 2010, Zhang, Giuliano et al. 2013, Sledge, Mamounas et al. 2014). However, once metastases become clinically manifest, therapeutic solutions vary based on the organ site (e.g., for liver metastases, standard of care can be hepatectomy, liver transplantation or targeted therapy with sorafenib) (Early Breast Cancer Trialists' Collaborative 2015).

3.4 Phosphatases and kinases in cancer

Most cellular processes are regulated by signaling pathways in eukaryotic cells. Protein phosphorylation is a crucial post-translational modification in signal transduction (Hunter 1995). It occurs at specific sites and is mediated by protein kinases, that add a phosphate group, and by protein phosphatases, that remove it.

The phosphorylation process results in conformational changes that influence protein activity with the apparition of docking sites. This enable binding to other proteins and formation of signaling complexes. Phosphorylation of serine (Ser), threonine (Thr) and tyrosine (Tyr) represent 17% of the total-human-proteome, named phospho-proteome (Sharma, D'Souza et al. 2014). Within the 230,000 phospho-sites existing in human proteins, the ratio between serine/threonine/tyrosine has been estimated to be 90/10/0.05 (Hunter and Sefton 1980, Vlastaridis, Kyriakidou et al. 2017). Abnormal tyrosine phosphorylation, despite its low representation, causes a plethora of human diseases including cancer (Gschwind, Fischer et al. 2004, Hunter 2009, Du and Lovly 2018). This led to extensive research on protein tyrosine kinases (PTKs) over the last decades. PTPs were left aside for a substantial time, as they were considered as attenuating effectors with a little selectivity. Nowadays, PTKs and PTPs are both recognized as major actors in phosphorylation of tyrosine residues (**Figure 3-3**).

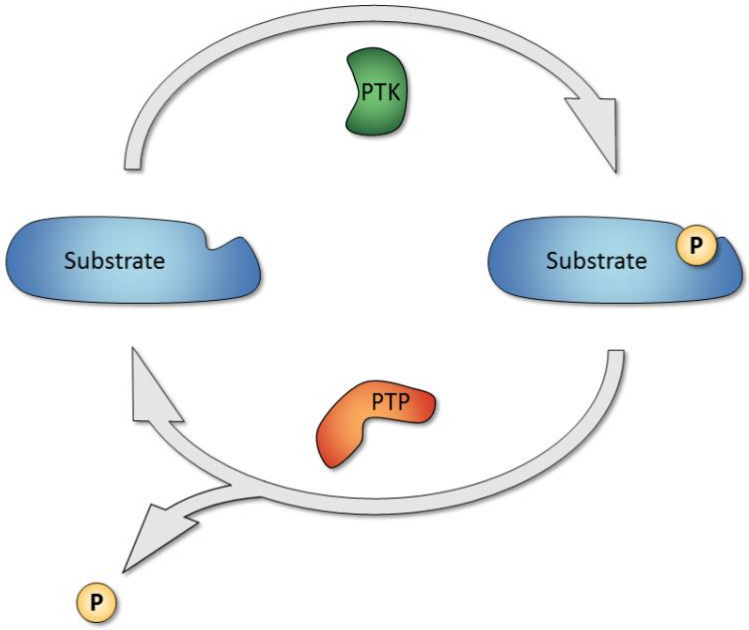

Figure 3-3 │ Schematic of substrate phosphorylation by PTKs and PTPs

Proteins can be phosphorylated on tyrosine residues by PTKs and reversely, can be dephosphorylated by PTPs. Tyrosine phosphorylation is crucial in eukaryotic cells and dysregulations can lead to a plethora of diseases. Furthermore, proteins have several phosphorylation sites, resulting in a multi-modal protein state based on their phosphorylation status (P: phosphate).

3.5 Protein tyrosine phosphatases

Compared to protein kinases, PTPs did not evolve from a common ancestor, but from two distinct families defined by their structures and mechanisms. These families are the classical PTPs dephosphorylating only tyrosine residues; and the dual-specificity PTPs dephosphorylating serine, threonine or tyrosine residues (Tonks 2006). Within the classical PTP family, 37 are encoded in the human genome, sub-divided into receptor-like PTPs and non-transmembrane or intracellular PTPs (Andersen, Mortensen et al. 2001) (**Figure 3-4**).

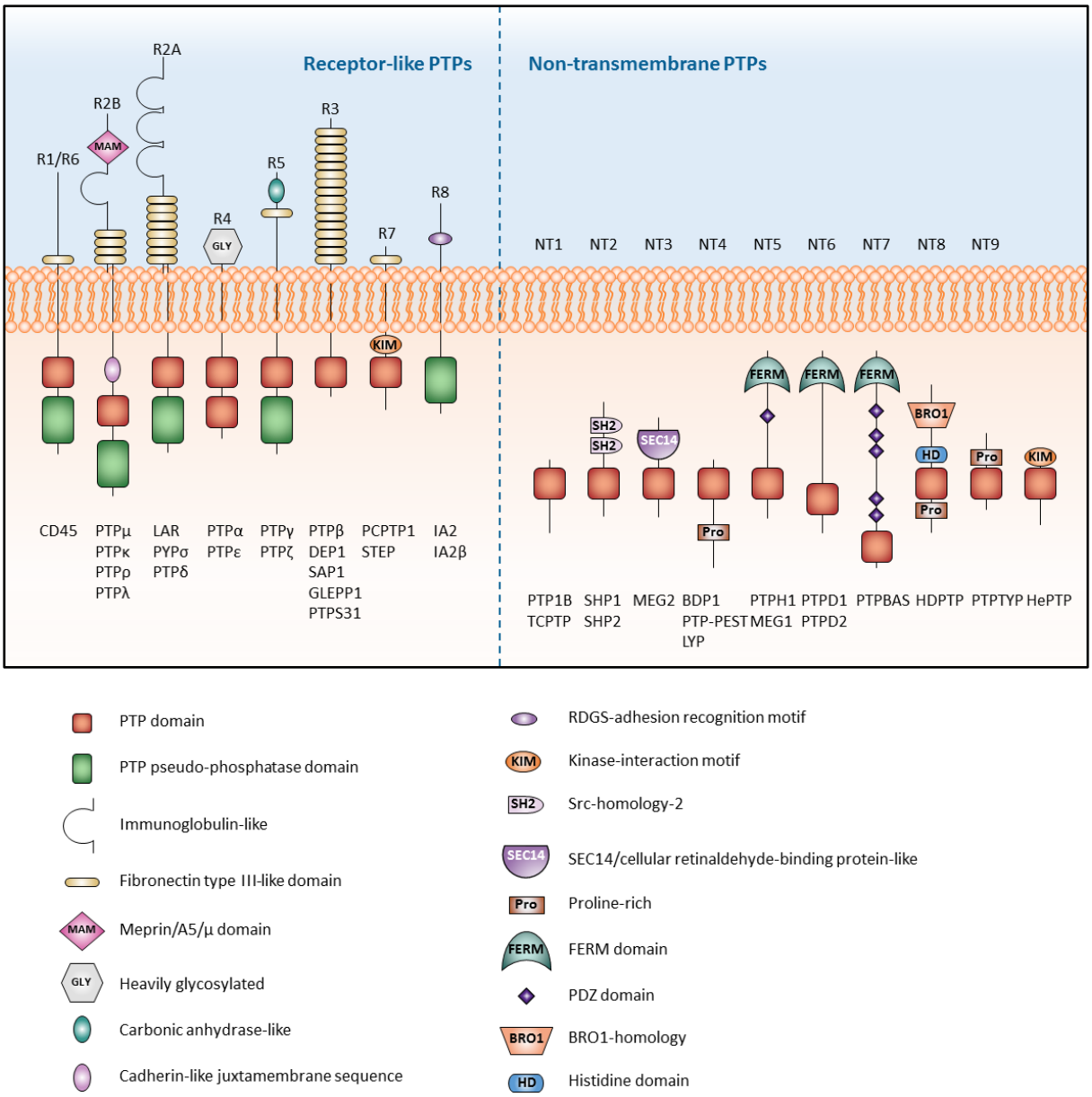

Figure 3-4 │ The classical PTPs family

The classical PTPs family is composed of 37 different members in human, and is sub-divided into receptor-like PTPs (left) and non-transmembrane PTPs (right). The receptor-like PTPs contain an intracellular PTP domain, consisting of the catalytic-site motif $HC(X)_5R$, a transmembrane domain and various types of extracellular domains involved in cell-cell, cell-matrix and cell-ligand interactions. The intracellular PTPs are considerably much diverse in structures with a PTP domain, and domains such as SH2 and FERM targeting them specifically to cellular locations or adapter proteins. Nevertheless, this separation into two families is not absolute since alternative promoters or splicing can result in transmembrane and cytoplasmic forms of some PTPs.

The PTP family dephosphorylates their substrate by a similar mechanism. The active site sequence VHCSXGXGR[T/S]G demarcates the PTP family and is commonly named "PTP loop" or "PTP signature motif". At the basis of the active site cleft, residues of this motif constitute the phosphate-binding loop (Andersen, Mortensen et al. 2001). The cysteine in the PTP loop (Cys459 in SHP2) acts as a nucleophile and accepts phosphate transiently during the catalysis. Firstly, the sulfur atom of the thiolate ion of cysteine attacks the phosphorous atom of the substrate phosphate group. The P-O linking the phosphate group to the tyrosine is protonated by a conserved aspartic acid (Asp425 in SHP2). This aspartic acid resides in the WPD-loop and is brought in close proximity by a conformational change of the protein upon substrate binding. Secondly, hydrolysis of the phosphoenzyme intermediate by a water molecule and the aspartic acid, functioning as a general base, results in the release of the phosphate (**Figure 3-5**).

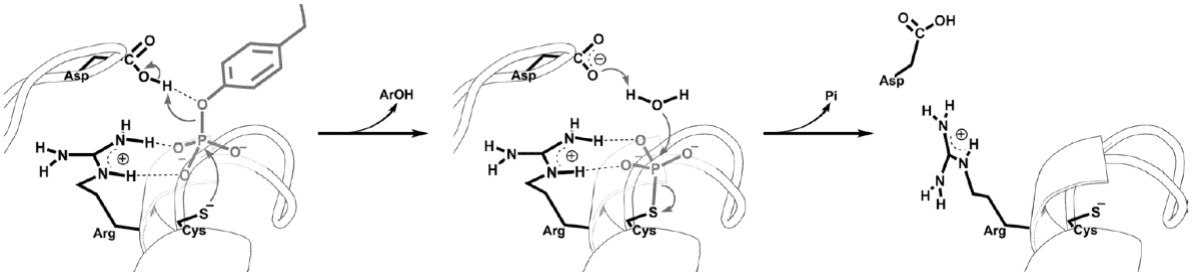

Figure 3-5 | Catalytic mechanism of cysteine-based PTPs

Schematic representation of the general two-step mechanism of the PTP-catalyzed reaction, in which the catalytic cysteine (Cys459 in SHP2) is part of the signature motif $HC(X)_5R$ located in the phosphate-binding loop. The catalytic cysteine attacks the phosphate of the substrate as a nucleophile and is needed to catalyse the hydrolysis of the phosphoester bond via a phosphate-cysteine intermediate. The conserved aspartic acid (Asp425 in SHP2), located in the WPD-loop, functions as the general acid/base during hydrolysis (Brandao, Hengge et al. 2010).

The PTP specificity relies on the active site pocket shape and is determinant for the substrate recognition (Kim and Ryu 2012). This specificity gives the depth between the molecular surface and the phosphate-binding loop (P-loop), which is 9 Å for SHP2, considered

as deep since the catalytic cleft is located at the base of the P-loop. This depth is characteristic for proteins with phospho-tyrosines residues, and non-specific for shorter phospho-serine or phospho-threonine side-chains (Jia, Barford et al. 1995).

3.5.1 The oncogenic tyrosine phosphatase SHP2

The tyrosine phosphatase SHP2, encoded by *PTPN11*, also known as Syp, SH-PTP2, SH-PTP3, PTP1D or PTP2C has been identified in the 1990's (Adachi, Sekiya et al. 1992, Freeman, Plutzky et al. 1992, Ahmad, Banville et al. 1993, Feng, Hui et al. 1993, Vogel, Lammers et al. 1993). This PTP is ubiquitously expressed and can bind several RTKs to transduce mitogenic, pro-survival, cell fate, pro-migratory signals downstream of numerous stimuli such as cytokines, growth factors and extracellular matrix components (Sausgruber, Coissieux et al. 2015, Matalkah, Martin et al. 2016). SHP2 is required for the full activation of the mitogen-activated protein kinase (MAPK)/extracellular signal-related kinase (ERK) pathway (Klinghoffer and Kazlauskas 1995, Montagner, Yart et al. 2005, Aceto, Sausgruber et al. 2012, Bunda, Burrell et al. 2015). It has also been shown that SHP2 can activate or inactivate other pathways such as Janus kinase (JAK)-signal transducer and activator of transcription protein (STAT) and the PI3K pathway, based on the cellular type and receptors involved (Berchtold, Volarevic et al. 1998, Zhang, Tsiaras et al. 2002, Mattoon, Lamothe et al. 2004, Xu and Qu 2008).

The protein phosphatase SHP2 is composed of two SRC homology 2 (SH2) domains (also named N-SH2 and C-SH2), a PTP catalytic domain and a proline-rich motif on the C-terminal tail, with two tyrosyl phosphorylation sites located at tyrosine 542 and tyrosine 580 (Chan, Kalaitzidis et al. 2008) (**Figure 3-6**).

Figure 3-6 │ Schematic representation of the protein tyrosine phosphatase SHP2

The protein tyrosine phosphatase SHP2 is composed of two N-terminal tandem SH2 domains, a catalytic phosphatase domain (PTP), and a C-terminal tail containing a proline-rich motif (not shown) and two tyrosyl residues Y542 and Y580.

SHP2 oscillates between two structural conformations, unveiling/hiding the PTP domain. In the absence of upstream stimulation, the phosphatase SHP2 is in an autoinhibition inactive state due to the binding of the N-SH2 domain with the PTP domain (Hof, Pluskey et al. 1998) (**Figure 3-7**). Upon stimulation, RTKs, cytokine receptors or scaffolding proteins will be phosphorylated on specific tyrosines that can be bound by the SH2 domains of SHP2. These adapter proteins can be the growth factor receptor-bound protein 2 (GRB2), GRB2-associated-binding protein 1 (GAB1) and GRB2-associated-binding protein 2 (GAB2). This binding will change the conformational structure of SHP2, release the PTP domain from its inhibition and result in an active enzyme that can dephosphorylate its substrate (Cunnick, Mei et al. 2001, Chan, Kalaitzidis et al. 2008) (**Figure 3-7**).

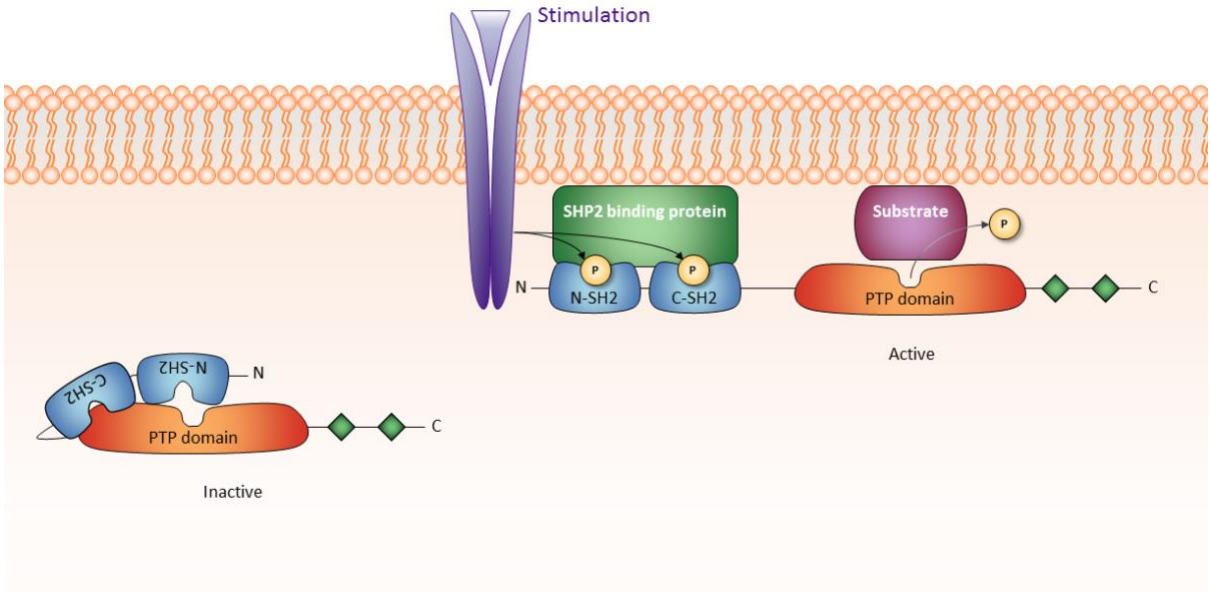

Figure 3-7 │ Schematic of the mechanism of SHP2 activation

The protein tyrosine phosphatase SHP2 can be found in an inactive state, held by the binding of the N-SH2 domain to the PTP domain, in an autoinhibition state (left). Upon stimulation, specific phospho-tyrosines will be bound by the SH2 domains of SHP2, leading to a conformational change and release of the autoinhibition state (right). This results in an open conformation of SHP2 and a PTP domain that can dephosphorylate substrates (P: phosphate).

Mainly all effects of SHP2 have been studied in the context of its PTP catalytic activity, and very few about its PTP-independent properties. For examples, it has been shown that SHP2-phosphatase-dead can activate STAT transcription, SRC tyrosine kinases, the PI3K pathway via IL-3 induction, and prevent p53-mediated apoptosis in the brain and neural crest via its SH2 domains (Berchtold, Volarevic et al. 1998, Walter, Peng et al. 1999, Yu, Hawley et al. 2003, Stewart, Sanda et al. 2010).

3.5.2 Effects of SHP2 in normal tissue and disease

The ubiquitously expressed phosphatase SHP2 is required for development in a large panel of species as shown by loss-of-function (LOF) or loss-of-expression of SHP2 studies in nematode, fly, zebrafish, xenopus and mouse. For example, SHP2 is necessary for gastrulation in mice (Arrandale, Gore-Willse et al. 1996, Saxton, Henkemeyer et al. 1997, Saxton and Pawson 1999). Studies on embryonic stem cells show that lack of SHP2 blocks their differentiation into the three germ layer cell lineages (ectoderm, endoderm and mesoderm) in human and in mice (Wu, Pang et al. 2009). Based on the notion that SHP2 is important for self-renewal, it has been extended to a larger number of progenitors and stem-cells, positioning SHP2 as a key factor in organ morphogenesis (Chan, Cheung et al. 2011, Heuberger, Kosel et al. 2014, Zhou, Deng et al. 2015). Also, SHP2 is expressed in hematopoietic cells and involved in lineages of blood cells. Upon SHP2 inactivation, severe loss of erythroid, myeloid and lymphoid populations occurs (Qu, Nguyen et al. 2001, Chan, Cheung et al. 2011).

Germline missense GOF mutations have been identified in about 40% of patients with Noonan syndrome (NS) (Tartaglia, Mehler et al. 2001). NS is associated with crano-facial dysmorphia, cardiopathies, mental retardation, short stature, increased risk of leukemia and occurs in 1 per 1,000-2,500 live births (Mendez and Opitz 1985). The mutations causing NS, often occur in exon 3 and 8, interrupt the autoinhibition between the PTP domain and the N-SH2, and result in a constitutively active form of SHP2 (Zheng, Alter et al. 2009) (**Figure 3-8**).

The multiple lentigines, electrocardiographic abnormalities, ocular hypertelorism, pulmonary stenosis, abnormal genitalia, retardation of growth, and sensorineural deafness (LEOPARD) or LEOPARD syndrome (LS), named today as Noonan syndrome with multiple lentigines (NS-LM), is also a polymalformative autosomal dominant genetic syndrome, in

which 90% of the patients display a mutation in *PTPN11*. These mutations often occur in exon 7 and 12, coding for the PTP domain, and result in LOF. They give rise to a dominant negative form of SHP2 (Digilio, Conti et al. 2002, Legius, Schrander-Stumpel et al. 2002, Zheng, Alter et al. 2009) (**Figure 3-8**).

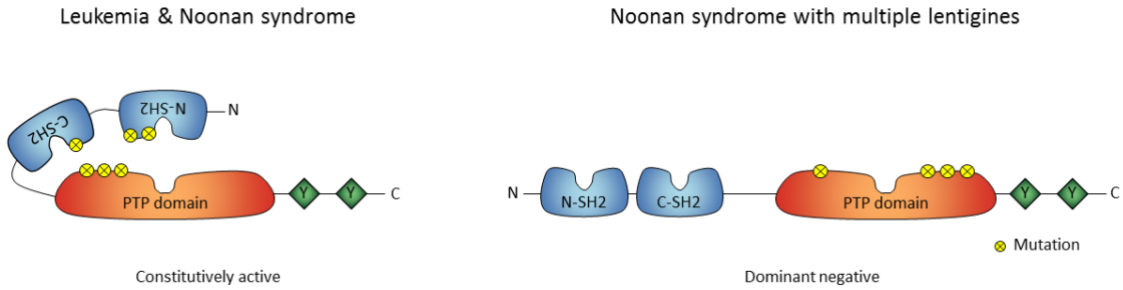

Figure 3-8 │ SHP2 mutations result in human diseases

The protein tyrosine phosphatase SHP2 is subject of mutations leading to diseases. Gain-of-function mutations (left) have been described in leukemia and Noonan syndrome with a constitutively activated form of SHP2, while loss-of-function mutations (right) cause Noonan syndrome with multiple lentigines, due to a dominant negative form of SHP2.

Phenotypically, NS-LM is close to NS, but some symptoms are specific such as cutaneous defects and deafness. The catalytically defective form of SHP2 interferes with signaling. For example, it decreases growth factor-induced activation of the ERK pathway (Kontaridis, Swanson et al. 2006). The exact mechanism leading to NS-LM remain largely debated (Edouard, Montagner et al. 2007, Yart and Edouard 2018). It has been proposed that a preferential recruitment of the catalytically-impaired form of SHP2 prevents the wild-type form for activating the MAPK pathway (Kontaridis, Swanson et al. 2006, Qiu, Wang et al. 2014). Alternatively, the focal adhesion kinase (FAK), substrate of SHP2, remains hyper-phosphorylated and activates the PI3K pathway in response to EGF in hypertrophic heart and insulin-sensitive tissues of mouse models (Ishida, Kogaki et al. 2011, Marin, Keith et al. 2011, Schramm, Fine et al. 2012, Tajan, Batut et al. 2014).

3.5.3 SHP2 in cancer

SHP2 is the first *bona fide* PTP proto-oncogene, and its effects in cancer are essentially non-genetic (Chan and Feng 2007). SHP2 can be activated in cancer by two means: GOF or downstream of oncogenes. In myeloid malignancies, 35% of juvenile myelomonocytic leukemias (JMML) are caused by *PTPN11* somatic GOF point mutations, mostly by disrupting the N-SH2/PTP interaction that results in increased SHP2 activity (Tartaglia, Niemeyer et al. 2003, Keilhack, David et al. 2005, Niihori, Aoki et al. 2005). SHP2 mutations are also found in acute myeloid leukemia (AML), sporadic juvenile myelomonocytic leukemia, myelodysplastic syndrome and acute B lymphoblastic leukemia (Tartaglia, Niemeyer et al. 2003, Loh, Reynolds et al. 2004, Loh, Vattikuti et al. 2004). In rare cases, SHP2 mutations are also found in solid tumors such as neuroblastoma, lung cancer and gastric cancers (Bentires-Alj, Paez et al. 2004).

Since SHP2 is a signal transducer downstream of numerous RTKs, it is central for transformation initiated by RTK mutations or amplifications. SHP2 has been shown to be involved in progression of laryngeal, breast and pancreatic cancers, as well as in malignant glioblastomas (GBM) (Bentires-Alj, Gil et al. 2006, Liu, Feng et al. 2011, Aceto, Sausgruber et al. 2012, Gomes, Connelly et al. 2013, Gu, Han et al. 2014, Hu, Fang et al. 2014, Sausgruber, Coissieux et al. 2015). SHP2 has been shown to mediate signaling downstream of hepatocyte growth factor receptor (HGFR/c-Met), fibroblast growth factor receptor 1 (FGFR1) and EGFR (Matalkah, Martin et al. 2016). Furthermore, the scaffolding adapter GAB2, amplified in 10-15% of human breast tumors, results in the hyperactivation of the SHP2-ERK pathway, increased proliferation and invasiveness (Bentires-Alj, Gil et al. 2006, Banerji, Cibulskis et al. 2012, Stephens, Tarpey et al. 2012).

Inappropriate activation of SHP2 has been described in gastric cancer caused by *Helicobacter pylori*, encoding several determinants including the *CagA* gene. This protein can be phosphorylated by SRC-family kinases on the C-terminal EPIYA motif, leading to the recruitment and activation of SHP2, mimicking a GAB scaffolding adaptor (Higuchi, Tsutsumi et al. 2004). Gastric epithelial cells infected with *H. pylori* or transfected with CagA display a characteristic phenotype termed "hummingbird phenotype", which is reverted by *PTPN11* deletion (Higashi, Tsutsumi et al. 2002, Tsutsumi, Higashi et al. 2003). This phenotype is the result of MAPK pathway hyperactivation, FAK dephosphorylation, and inhibition of the polarity kinase Prader-Willi/Angelman region-1 (PAR1) resulting in cellular polarity defects (Tsutsumi, Takahashi et al. 2006, Saadat, Higashi et al. 2007, Lu, Murata-Kamiya et al. 2009).

Contrary to the above-mentioned proto-oncogenic effects, SHP2 has been proposed as a tumor suppressor in liver. SHP2 deletion promotes inflammatory signaling and hepatic necrosis, leading to tumor development (Bard-Chapeau, Li et al. 2011, Han, Xiang et al. 2015). Similar observations have been shown in cartilage, in which SHP2 deletion leads to metachondromatosis (e.g., exostoses, enchondromas, joint destruction and bony deformities) (Yang, Wang et al. 2013).

3.5.4 SHP2 inhibitors

For years selective inhibitors of SHP2 were lacking and most studies relied on shRNA or knockout mice (Bard-Chapeau, Li et al. 2011, Aceto, Sausgruber et al. 2012). The recent development of selective SHP2 inhibitors facilitated the advancement of the field, with notably the discovery of a potent allosteric SHP2 inhibitor, with high efficacy to block proliferation in several RTK-driven human cancers (Tautz and Mustelin 2007, Zhang, He et al. 2010, Chen, LaMarche et al. 2016, Garcia Fortanet, Chen et al. 2016). A first in human (FIH) clinical trial has been initiated to characterize the tolerability and the safety of the SHP2 inhibitor TNO155

in EGFR mutant NSCLC, esophageal squamous cell cancer (ESCC), head-and-neck squamous cell cancer (HNSCC) on a rat sarcoma/rapidly accelerated fibrosarcoma (RAS/RAF) wild-type background (NCT03114319).

These new molecules facilitated mechanisms of resistance studies by blocking SHP2 simultaneously with other targets. In non-small-cell lung cancer (NSCLC), SHP2 inhibition restored sensitivity to anaplastic lymphoma kinase (ALK) inhibition and to mitogen-activated protein kinase kinase (MEK) inhibition in Kirsten rat sarcoma virus (*KRAS*)-mutant NSCLC (Dardaei, Wang et al. 2018, Fedele, Ran et al. 2018, Mainardi, Mulero-Sanchez et al. 2018, Ruess, Heynen et al. 2018). In TNBC with RAS mutations at G12, combinatorial targeting of SHP2 and components of ERK signaling prevents adaptive resistance (Ahmed, Adamopoulos et al. 2019).

3.6 Protein tyrosine kinases

The proteome is subject to post-translational modifications including glycosylation, nitrosylation, acylation, ubiquitination, lipidation, methylation, proteolysis and phosphorylation (Walsh, Garneau-Tsodikova et al. 2005, Duan and Walther 2015). Kinases conduct the phosphorylation reaction, catalysing the transfer of phosphate groups on ATP to residues of proteins. Various kinases are found in eukaryotic cells such as the serine/threonine kinases, the PTK, and the tyrosine and threonine kinases, also named dual-specificity protein kinases (Cohen 2002, Kennelly 2002, Ubersax and Ferrell 2007, Kannan and Taylor 2008). The human kinome contains 518 genes coding for protein kinases, able to phosphorylate nearly a third of the proteome (Hanks and Hunter 1995, Cohen 2000, Hunter 2000, Ficarro, McCleland et al. 2002, Manning, Whyte et al. 2002).

The PTKs form a large family, with the principal effect is the regulation of numerous cellular aspects such as cell growth signaling, differentiation, proliferation, adhesion, motility

and death (Hunter and Cooper 1985). The human genome analysis shows 90 genes encoding for PTKs. They are subdivided as RTKs (58 genes; e.g., *EGFR*, *PDGFR*, *VEGFR*) and non-RTK (NRTK) (32 genes; e.g., Abelson murine leukemia viral oncogene homolog 1 (*ABL1*), Src kinase family). Dysregulation of this phosphorylation processes lead to a large number of human diseases including cancer, diabetes and congenital syndromes (Blume-Jensen and Hunter 2001, Cohen 2002, Drake, Lee et al. 2014, Jiao, Bi et al. 2018). Four decades of research have considerably increased insights on their structures, functions and regulation, from gene characterization to protein structure.

For 50% of the cases in cancer, proto-oncogene and oncogenes effects result in abnormal PTK activities, driving tumorigenesis (Blume-Jensen and Hunter 2001). Due to the significant implication of PTKs in human diseases, they became a very important target in drug research. The previously cited lapatinib, a reversible dual inhibitor of EGFR and HER2, is approved by the Food and Drug Administration (FDA) since 2007 for the treatment of breast cancer, NSCLC, head and neck cancer and gastric cancer (Hicks, Macrae et al. 2015). Several multi-target kinase inhibitors are approved such as sorafenib, inhibiting PDGFRs/VEGFRs/Raf family kinases and prolongs progression-free survival of patients with kidney cancer (Bolondi, Craxi et al. 2015); or also sunitinib, inhibiting PDGFRs/VEGFRs/c-KIT, approved for inoperable renal cell carcinoma (RCC) (Kalra, Rini et al. 2015).

3.7 Phosphoinositol 3-Kinase signaling

3.7.1 PI3K classification and signaling

Eukaryotic cells have an additional family of kinases phosphorylating small molecules, including lipids. The PI3K is a family of lipid kinases involved in cell growth, proliferation, survival and metabolism. It is composed of regulatory units p85α, p85β, p50α, p55α, p55γ, and catalytic units p110α, p110β, p110γ or p110δ. They are subdivided in three classes based on their structure, substrate affinities, sequence homology, while having distinct cellular effects. The class I PI3Ks preferentially phosphorylate phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3), and are themselves split in two groups based on the upstream mode of activation: the class IA PI3Ks are activated by RTKs; the class IB by G-protein-coupled receptors (GPCRs) (**Figure 3-9**).

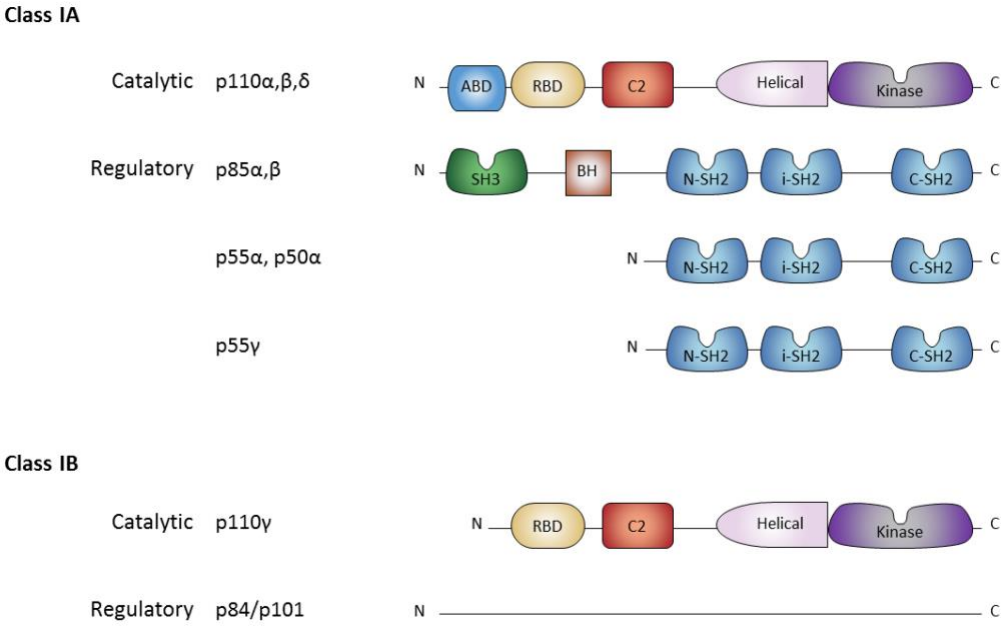

Figure 3-9 │ Schematic overview of class I PI3K catalytic and regulatory subunits

Domain structures of class I PI3K subunits, phosphorylate preferentially PIP2 to PIP3. The class IA are activated by RTKs; the class IB by GPCRs. Among the different possible interactions, p110α and p85α have tight binding between their ABD and i-SH2 domains, conferring stability to p110α. Also, N-SH2 decreases basal activity by interactions to the helical, kinase and C2 domains. These inhibitions are released upon binding to phospho-

tyrosines (ABD: adaptor-binding domain; RBD: Ras-binding domain; BH: breakpoint cluster region homolog).

The catalytic subunit p110 is ubiquitously expressed in human tissues except p110γ and p110δ that are enriched in immune cells. The regulatory subunit p85 interferes with the p110 kinase activity. Upon growth factor binding, RTKs are activated by autophosphorylation, leading to the recruitment of the p85 subunit to the newly activated RTK, or to associate adapter proteins. This binding releases the p110 catalytic subunit which translocates to the nearby plasma membrane, phosphorylates PIP2 to PIP3, resulting in the activation of numerous downstream effectors such as the phosphoinositide-dependent kinase 1 (PDK1), mammalian target of rapamycin complex 2 (mTORC2), AKT (also called protein kinase B), tuberous sclerosis complex (TSC), mammalian target of rapamycin complex 1 (mTORC1) and p70 ribosomal protein S6 kinase (S6K) (Staal 1987, Whitman, Downes et al. 1988, Auger, Serunian et al. 1989, Heitman, Movva et al. 1991, Jones, Jakubowicz et al. 1991, Manning and Toker 2017). PIP3 is transiently induced and is metabolized by lipid phosphatases, notably the phosphatase and tensin homolog (PTEN), that removes the 3'-phosphate from PIP3 and terminates PI3K signaling (Maehama and Dixon 1998) (**Figure 3-10**).

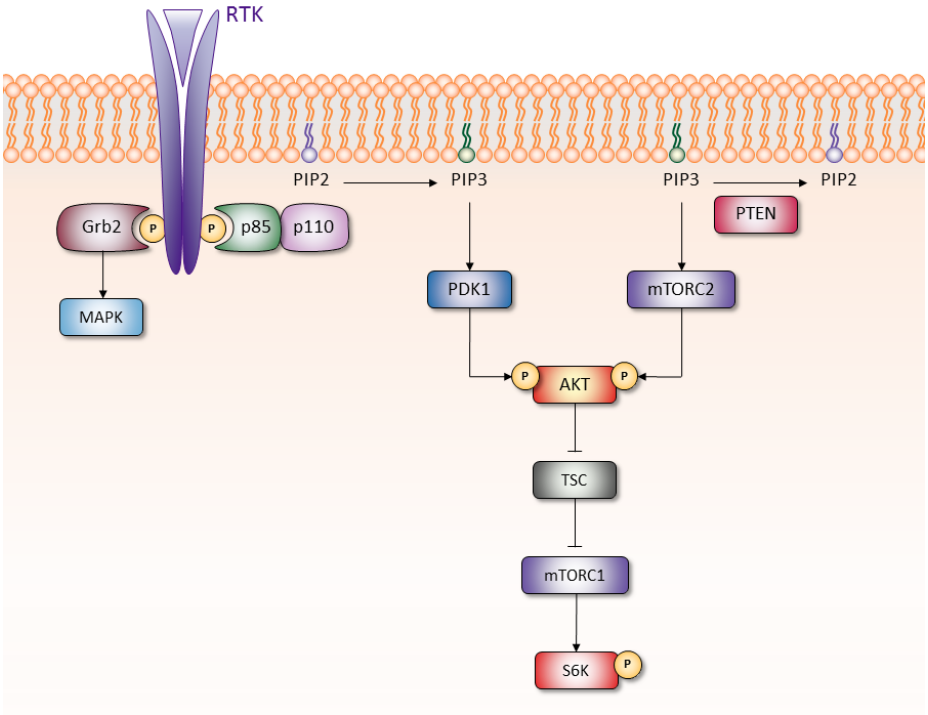

Figure 3-10 | Schematic of the activation mechanism of class IA PI3Ks

Upon ligand binding to a RTK, the phosphorylation of the receptor (or associated adaptors, not shown) creates docking sites for the p85 subunit. Binding of this subunit to a phospho-tyrosine releases the p110 catalytic subunit, leading to its translocation at the plasma membrane, enabling the phosphorylation of PIP2 to PIP3. This will attract downstream effectors such as PDK1, mTORC2, AKT, TSC and S6K, and activate cellular processes involved in proliferation, growth, death and metabolism. Activation of the PI3K signaling is ended by the conversion of PIP3 to PIP2 by the lipid phosphatase PTEN.

Class II PI3Ks can phosphorylate phosphatidylinositol, but also phosphatidylinositol-4-phosphate (PI-4-P) in rarer cases. Finally, class III are essentially involved in membrane trafficking and autophagy, and composed of human vacuolar protein sorting 34 (hVPS34), initially found in yeast as Vps34 (Schu, Takegawa et al. 1993, Wurmser, Gary et al. 1999, Backer 2016).

3.7.2 Dysregulation of PI3K signaling in cancer

The PI3K pathway is among the most frequently activated pathway in human cancer, and is hyperactivated in 70% of breast cancers (Saal, Holm et al. 2005, Stemke-Hale, Gonzalez-Angulo et al. 2008). Often, these dysregulations are within class I PI3Ks, more especially *PIK3CA,* the gene encoding for the p110α subunit. *PIK3CA* is amplified and/or mutated in several human solid tumors like GBM, breast, colorectal and lung cancers (Bachman, Argani et al. 2004, Samuels, Wang et al. 2004, Levine, Bogomolniy et al. 2005, Wu, Xing et al. 2005, Kadota, Sato et al. 2009). In breast cancer, dysregulations of *PIK3CA* is found in 30% of patients at all stages (Bachman, Argani et al. 2004, Samuels, Wang et al. 2004, Saal, Holm et al. 2005, Barbareschi, Buttitta et al. 2007, Miller 2012).

Major mutations are found on two hot spots, located in the helical domain (exon 9, *E542K*, *E545K*) with 33% occurrence, and in the kinase domain (exon 20, *H1047R*) with 47% occurrence, both resulting in a constitutively active form of p110α (Samuels, Wang et al. 2004). *PIK3CA* mutations lead to uncontrolled growth and survival. These mutants of p110α are found in luminal, HER2+ and TNBC breast cancers, while their correlation to pathological parameters is so far debated (Bachman, Argani et al. 2004, Saal, Holm et al. 2005, Mukohara 2015, Sobhani, Roviello et al. 2018). In rare cases, mutations are found in other catalytic subunits like p110β (helical domain, exon 11, *E633K*), important for breast cancer initiation by increased activity of the subunit; or p110γ, found in invasive breast carcinoma (Benistant, Chapuis et al. 2000, Ciraolo, Iezzi et al. 2008, Jia, Liu et al. 2008, Xie, Abel et al. 2013). Very limited studies report alterations of other PI3K family members such as class II and class III (Maffucci, Cooke et al. 2005, Hirsch, Shen et al. 2010, Chikh, Ferro et al. 2016).

Mutations in the p85α regulatory subunit of class I PI3Ks, encoded by *PIK3R1*, have been found in endometrioid endometrial cancers (EEC), GBMs, ovarian, colon and breast

tumors (3%) (Parsons, Jones et al. 2008, Urick, Rudd et al. 2011, Mukohara 2015). Mutations are clustering within the p85α inter-SH2 domain, decreasing the inhibitory effect on the p110α subunit, and promoting phosphorylation of AKT at Ser473 (Jaiswal, Janakiraman et al. 2009, Wu, Shekar et al. 2009, Sun, Hillmann et al. 2010). Other mutations on *PIK3R1* and *PIK3R2* can decreases the stability of the lipid phosphatase PTEN, increasing PI3K pathway activity (Cheung, Hennessy et al. 2011).

PTEN is frequently lost in various cancers, with a chromosome 10q deletion, including PTEN region, found in 24-58% of invasive urothelial carcinomas (Knowles, Platt et al. 2009, Bunney and Katan 2010) and in 30% breast cancers (Stemke-Hale, Gonzalez-Angulo et al. 2008). Other lipid phosphatases are involved in cancer such as SH-2 containing inositol 5' polyphosphatase 1 (SHIP-1), encoded by *INPP5D*, with a correlated reduction in chronic myeloid leukemia (CML), or SHIP-2, encoded by *INPPL1*, with an increased expression in several breast cancer cell line models (Bunney and Katan 2010).

The genes encoding AKT have been found mutated in cancer. For example, *AKT1* (*E17K*) occurs at low frequency in luminal and ER+ breast cancers (1.5%-9%) and leads to a constitutive localization and active state at the membrane, regardless of stimulation inputs (Carpten, Faber et al. 2007). Moreover, *AKT2* mutations (2.8%) correlate with breast cancer aggressiveness and poor prognosis (Bellacosa, de Feo et al. 1995, Cheng, Ruggeri et al. 1996, Saal, Holm et al. 2005).

3.7.3 PI3K inhibitors

The high importance of PI3K-AKT-mTOR pathway in cancer progression led to the development of inhibitors targeting the pathway with various approaches, with nearly 40 compounds reaching different stages of clinical development. The PI3K pathway can be blocked using mTOR, AKT or PI3K inhibitors. Few of them are approved such as mTORC1 inhibitors temsirolimus and everolimus, the pan-PI3K inhibitor copanlisib and the PI3Kδ inhibitor idelalisib (Hudes, Carducci et al. 2007, Motzer, Escudier et al. 2008, Patnaik, Appleman et al. 2016).

Due to the plurality of PI3K subunits, various inhibition strategies have been investigated. Pan-PI3K inhibition targets all four PI3K class I isoforms. This leads to a broad and effective inhibition on tumors with high levels of PIP3. As of today, only copanlisib is approved for the treatment of lymphomas (Dreyling, Santoro et al. 2017). In fact, clinical trials of pan-PI3K inhibitors are often discontinued due to insufficient efficacy, toxicities and lack of biomarkers for clinical activity assessment (Liu, Cheng et al. 2009). In breast cancer, buparlisib has been proven effective in two phase II studies, but has been discontinued for psychiatric adverse events (e.g., suicide attempts), suggesting the drug capacity of crossing the blood brain barrier, supported by the shrinkage of brain metastases (Bendell, Rodon et al. 2012, Baselga, Im et al. 2017, Martin, Chan et al. 2017). Buparlisib combined with fulvestrant is being tested in breast cancer (Di Leo, Johnston et al. 2018).

Isoform-specific PI3K inhibitors have been developed to target cancers specifically dependent on one PI3K isoforms. PI3Kα inhibitors specifically inhibit the p110α subunit, to counter-balance activating mutations of *PIK3CA*. Currently, no PI3Kα inhibitors are approved but several are in clinical trials (e.g., alpelisib, taselisib, TAK-117, ASN003). Compared to pan-PI3K inhibitors, these inhibitors have a favorable toxicity profile, good efficacy on

PIK3CA mutant tumors, and less off-target effects (Janku, Yap et al. 2018). In breast cancer, they are being tested in combination with standard of care hormone treatment (e.g., alpelisib combined to fulvestrant or letrozole) (Juric, Rodon et al. 2018). Of note, other isoform-specific PI3K inhibitors are being investigated: PI3Kβ specific inhibitors (e.g., GSK2636771, AZD8186, SAR260301), particularly effective in PTEN mutant tumors; PI3Kγ, a subunit mainly expressed in leukocyte, with one inhibitor in phase I (IPI-549); and against PI3Kδ-subunit (e.g., duvelisib, AMG319), important mediator of B cell receptor signaling, beside the approved idelalisib (Furman, Sharman et al. 2014).

Another strategy consists in simultaneous blockade of pan-PI3K and mTOR. They have similar toxicity profiles than pan-PI3K inhibitors. Presently, no dual-PI3K/mTOR inhibitors have been approved but several are being tested in clinical trials (e.g., LY3023414, PQR309). The lack of biomarkers remains a major challenge to better stratify patients in clinical trials (Janku, Yap et al. 2018).

3.7.4 Mechanisms of resistance to PI3K inhibition

The importance of PI3K in cancer, and accessibility of druggable targets triggered studies and inhibitors design to interfere with this pathway in cancer (Leroy, Amante et al. 2014). However, results from clinical trials show limited efficacy due to resistance mechanisms.

Resistance to PI3K inhibition can be driven by non-genetic mechanisms. For example, blockade of mTORC1 abrogates the p70 ribosomal protein S6 kinase 1 (S6K1) mediated negative feedback loop, upregulates the insulin receptor substrate 1 (IRS-1), and results in the reactivation of the PI3K pathway. These observations paved the way for the development of dual PI3K/mTOR inhibitors (O'Reilly, Rojo et al. 2006, Thorpe, Yuzugullu et al. 2015). This dual-inhibition is short-circuited in TNBC by the parallel activation of the JAK2/STAT5 signaling pathway (Britschgi, Andraos et al. 2012), or by the rapid increase of expression of

RTK genes such as HER3, EGFR, the insulin receptor (INSR) and insulin like growth factor receptor 1 (IGFR1), driven by the forkhead transcription factors of the O subgroup (FOXO) activity (Chandarlapaty, Sawai et al. 2011, Chakrabarty, Sanchez et al. 2012, Muranen, Selfors et al. 2012).

Acquired resistances to PI3K inhibition also develops. The persistent mTORC1 activity upon BYL719 (alpelisib) inhibition, drove insensitivity of these cells in *PIK3CA* mutant breast cancers. Sensitivity could be restored by simultaneous mTORC1 blockade using RAD001 (everolimus) (Elkabets, Vora et al. 2013). Further, mTORC1 can be activated via PI3K-independent signaling, seen with the serine-threonine kinase PIM1 in luminal A/B and HER2+ breast cancers. Increased PIM1 expression correlates with BYL719 resistance, and has been confirmed in 50% of patient biopsies at time of progression (Le, Antony et al. 2016). In breast cancer, *MYC* copy number and/or c-Myc expression is elevated and drive resistance to PI3K/mTOR inhibition (Ilic, Utermark et al. 2011). Strategies targeting the bromodomain and extra-terminal domain (BET) BRD4 proteins antagonize these resistances by suppressing the super-enhancer-associated genes such as MYC (Liu, Radisky et al. 2012). Also, compensatory mechanisms could be blocked by use of the BET inhibitor JQ1, in *PIK3CA* or *PTEN* mutant cell lines in which PI3K pathway was reactivated by RTKs (Stratikopoulos, Dendy et al. 2015). The MAPK pathway is crucial in these compensation mechanisms to PI3K inhibition, notably upon mTORC1 inhibition (Carracedo, Ma et al. 2008, Leroy, Amante et al. 2014, Ramos and Bentires-Alj 2015).

Furthermore, a cancer is constituted of heterogeneous populations, formed of different clones, which respond differently to treatments. Heterogeneity can be seen in between patients; within patient tumor; and within patient sites such as primary tumor and metastatic lesions (Heppner 1984, McGranahan, Favero et al. 2015, Dagogo-Jack and Shaw 2018). For example, brain metastases have been shown to contain more PI3K activating mutations than primary

tumor, predicting differential site responses to a PI3K inhibition (Brastianos, Carter et al. 2015).

4 | RATIONALE OF THE WORK

Breast cancer is the most frequent cancer and the leading cause of death among women worldwide. Several signaling pathways promote aberrant cancer cell proliferation. Among them, the PI3K pathway is activated in 70% of breast cancers, highlighting the importance of developing strategies to inhibit this pathway. However, PI3K inhibition has shown disappointing results in clinical trials, predominantly due to the occurrence of resistance. Frequently, these mechanisms of resistance are RTK driven, which activate compensatory pathways.

Signaling pathways are composed of several proteins and complexes in which phosphorylation of tyrosine residues is crucial. Abnormal tyrosine phosphorylation causes a wide range of diseases. The reversible phosphorylation of these tyrosine residues is governed by PTKs and PTPs. Whereas PTKs were considered as major drivers, PTPs were only considered as attenuating factors. Previous work in our lab demonstrated that the tyrosine phosphatase SHP2 promotes breast cancer maintenance and progression. Suppression of SHP2 decreases tumor growth, eradicates breast-tumor-initiating cells in xenograft models and reduces metastasis. Furthermore, SHP2 is important for RTK signaling where it facilitates activation of downstream pathways.

We asked whether SHP2 inhibition could improve PI3K inhibition by addressing the following points:

i. What are the effects of pan-PI3K and SHP2 blockade as single- or dual-inhibition on breast cancer progression?
ii. What is the molecular mechanism of action of PI3K/SHP2 dual-inhibition in breast cancer?
iii. What are the effects of PI3K/SHP2 dual-inhibition on metastases?

5 | RESULTS

Research article submitted to *EMBO MOL MED Journal*.

SHP2 Blockade Sensitizes Metastatic Triple Negative Breast Cancer to PI3K Inhibition

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Running title: PI3Ki/SHP2i decrease TNBC metastases

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5.1 Summary

The PI3K pathway is hyperactivated in 70% of breast cancers and numerous inhibitors of this pathway are in clinical trials. Resistance to PI3K inhibition is often driven by activation of receptor tyrosine kinases (RTKs). Given that the protein tyrosine-phosphatase SHP2, encoded by the proto-oncogene *PTPN11*, activates oncogenic pathways downstream of most RTKs, we assessed the effect of co-targeting both PI3K and SHP2 in preclinical models of metastatic triple negative breast cancer (TNBC) a highly aggressive subtype that lacks targeted therapy. Beside the beneficial effects of SHP2 single inhibition, dual PI3K/SHP2 inhibition induced apoptosis, decreased primary tumor growth synergistically, blocked the formation of lung metastases, and increased overall survival in both neoadjuvant and adjuvant settings. Mechanistically, SHP2 inhibition resulted in the activation of PI3K signaling via $PDGFR_{\beta}$ and sensitization of TNBC cells to inhibition of this pathway. The data provide a rationale for co-targeting SHP2 and PI3K in metastatic TNBC.

5.2 Introduction

Breast cancer is the leading cause of cancer death among females, with 1.7 million new cases globally and 600,000 deaths annually (Parks, Derks et al. 2018). A third of breast cancers progress to metastasis (Early Breast Cancer Trialists' Collaborative 2005), the cause of death of most breast cancer patients (Massague and Obenauf 2016). Thus, anti-cancer target identification, drug development and testing should focus on metastatic cells before and/or after they have spread and proliferated in distant organs. This is key to the improvement of current therapies.

The phosphatidylinositol 3-kinase (PI3K) signaling axis is essential for cell proliferation, cell-cycle progression, motility and apoptosis (Fruman, Chiu et al. 2017). Downstream of receptor tyrosine kinases (RTK) and G protein-coupled receptors (GPCR),

class I PI3Ks phosphorylate phosphatidylinositol-4,5-bisphosphate, generating phosphatidylinositol-3,4,5-triphosphate, and lead to the activation of numerous kinases such as PDK1 (phosphoinositide-dependent kinase 1), AKT (also called protein kinase B), and p70 ribosomal protein S6 kinase (S6K) (Manning and Toker 2017).

The PI3K pathway is among the most frequent pathways activated in human cancer, and is hyperactivated in 70% of breast cancers (Saal, Holm et al. 2005, Stemke-Hale, Gonzalez-Angulo et al. 2008). Different classes of inhibitors targeting several key effectors (e.g., PI3K, AKT, mTOR) have been developed over the last two decades and 17 compounds are currently in clinical trials on solid tumors (Janku 2017). Unfortunately, the efficacy of these inhibitors is limited by intrinsic and adaptive (non-genetic) mechanisms: increased transcription of RTK genes, feedback loop disruption, and activation of parallel pathways (Ramos and Bentires-Alj 2015, Fruman, Chiu et al. 2017). These observations pinpoint the crucial need for refined drug combinations to overcome resistance mechanisms.

Downstream of various active RTKs, Src-homology 2 domain-containing phosphatase (SHP2), a ubiquitously expressed protein-tyrosine phosphatase (PTP), transduces mitogenic, survival, cell-fate and/or migratory signals (Bentires-Alj, Gil et al. 2006, Aceto, Sausgruber et al. 2012, Lan, Holland et al. 2015, Sausgruber, Coissieux et al. 2015). SHP2 is fundamental to the activation of the mitogen-activated protein kinase (MAPK)/extracellular signal-related kinase (ERK) pathway (Chan and Feng 2007). Germline-activating mutations of SHP2 cause Noonan syndrome (Tartaglia and Gelb 2005), and somatic gain of function mutations cause several hematological malignancies (Tartaglia, Niemeyer et al. 2003). While *PTPN11* is rarely mutated in solid tumors, SHP2 is activated downstream of several oncogenic signals (Bentires-Alj, Paez et al. 2004, Tsutsumi, Masoudi et al. 2013, Bunda, Burrell et al. 2015). Notably, small hairpin knockdown of SHP2 decreases breast tumor growth and progression (Aceto, Sausgruber et al. 2012, Sausgruber, Coissieux et al. 2015, Matalkah, Martin et al. 2016). A

potent orally active allosteric inhibitor of SHP2 has been reported recently (Chen, LaMarche et al. 2016) but the effect of pharmacological inhibition of SHP2 on breast cancer metastases and overall survival remains ill-defined. Of note, SHP2 inhibition is currently in a phase I trial focusing on advanced solid tumors (NCT03114319).

Given that RTKs often blunt the response to PI3K inhibition and that SHP2 transduces oncogenic signaling downstream of most RTKs, we asked whether co-targeting these pathways would be more effective than single agents in preclinical models of metastatic TNBC. Using *in vitro* models as well as different *in vivo* treatment settings, including neoadjuvant and adjuvant treatments in immunocompetent and immunodeficient models, we compared the efficacy of PI3K and SHP2 alone or as dual-inhibitors, measuring their impact on primary tumor and metastatic growth, as well as on overall survival. Here we provide data that challenge the notions that: a) primary tumors and metastases have the same dependencies, and b) that the quantification of primary tumor response to therapy is a reliable metric for assessing anti-cancer drug efficacy. We also reveal differences in lung- and liver metastases dependency. We provide evidence that PI3K/SHP2 dual-inhibition is necessary and sufficient to overcome lung metastases growth and prolongs survival in preclinical models of metastatic TNBC.

5.3 Results

5.3.1 PI3K/SHP2 dual-inhibition decreases cell number, reduces primary tumor growth and increases overall survival in TNBC models.

We assessed the effects of the pan-PI3K inhibitor CLR457 combined with SHP099, an SHP2 allosteric inhibitor (Chen, LaMarche et al. 2016) on the 4T1 metastatic mouse mammary carcinoma cell line and a representative panel of human breast cancer cell lines. 4T1 cells and all the human TNBC lines but MDA-MB-231 were more sensitive to dual- than to single inhibition, with three lines out of five showing a synergistic effect and two an additive effect (Fig 5-1 A-E). While PI3Ki inhibition alone does not induce cell apoptosis (Chen, Hsiao et al. 2017, Zwang, Jonas et al. 2017), SHP2i induced apoptosis dramatically (Fig EV1A), and PI3K/SHP2 dual-inhibition induced it further (Fig 5-1F).

To elucidate the effect of PI3K/SHP2 dual-inhibition *in vivo*, we used the 4T1 syngeneic mouse model of metastatic breast cancer in a neoadjuvant setting (Fig 5-1G). PI3Ki had no effect on tumor growth, whereas SHP2i decreased average tumor volume by half; dual-inhibition decreased it further in a synergistic manner (Fig 5-1H, Fig EV1B). Quantification of cleaved-Caspase 3 in tumors after treatment indicated increased apoptosis upon SHP2i and PI3K/SHP2 dual-inhibition (Fig EV1C).

After tumor resection, the mice were treated for 5 days in a second round of inhibition and monitored until signs of distress appeared. Only PI3K/SHP2 dual-inhibition enhanced overall survival, with a median survival of 41 days; mice after all other treatments had a median survival of 20-24 days (Fig 5-1I). While the effects on primary tumor growth varied, overall survival was also prolonged by PI3K/SHP2 dual-inhibition in mice transplanted with MDA-MB-436 or MDA-MB-468 cell lines (Fig EV1D-E). Analysis of lungs revealed the presence of larger metastases in the PI3K-treated group than in the control, similar to our previous

observation with TNBC models (Fig EV1F) (Britschgi, Andraos et al. 2012). In contrast, mice after SHP2 single inhibition or combined inhibitors had few or no lung metastases (Fig EV1F). The reduction in lung metastases by SHP2 single inhibition in the absence of increased overall survival may be due to an overshoot of metastasis after cessation of treatment, a phenomenon that is prevented by combined PI3K/SHP2 inhibition.

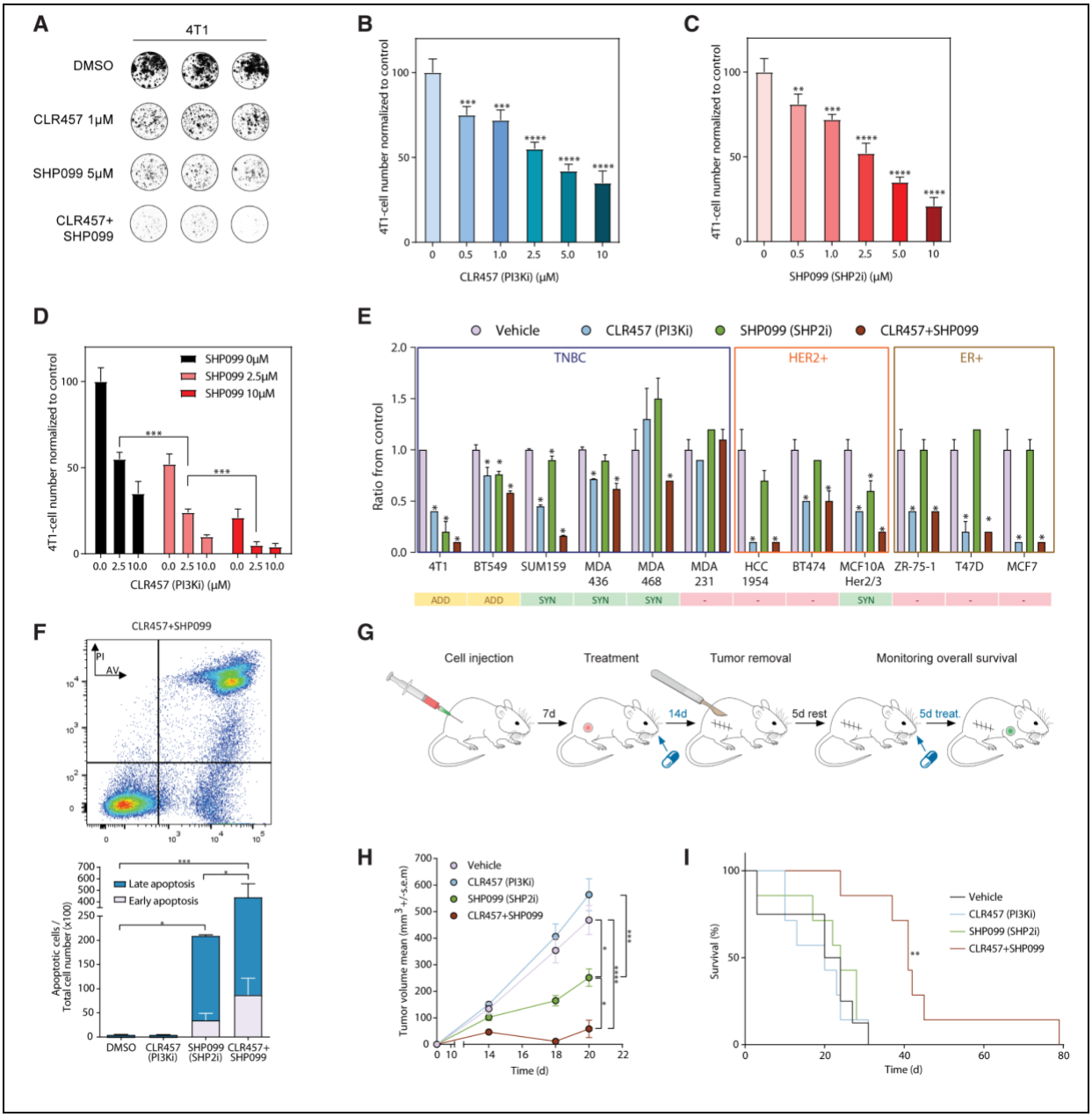

Figure 5-1 | PI3K/SHP2 inhibition reduces cell number and primary tumor volume, and prolongs animal survival.

A Representative images of 4T1 cells treated with CLR457 (PI3Ki) and SHP099 (SHP2i) at the indicated concentrations for 72 h. Each condition is shown in triplicate (horizontal).

B, C, D Bar graph representing cell number of 4T1 cells treated with CLR457 (PI3Ki) or SHP099 (SHP2i) at the indicated concentrations for 72 h. Data shown are mean cell numbers ±STDEV (n=3, **P≤0.01; ***P≤0.001; ****P≤0.0001, One-way ANOVA test).

E Bar graph of the cell numbers of breast cancer lines treated with CLR457 (PI3Ki, 2.5 µM) and/or SHP099 (SHP2i, 5 µM) for 72 h. Dual-inhibition is compared to single inhibition in terms of additive or synergistic (ADD: Additive; SYN: Synergistic). Data shown are mean cell numbers ±STDEV (n=3, *P≤0.05, One-way ANOVA test).

F Representative FACS plots of annexin V (AV) / propidium iodide (PI) apoptosis analysis of 4T1 cells treated with CLR457 and SHP099 for 3 days (top panel). Fresh inhibitors were added after 48 h. Quantification is given as a bar plot (bottom panel). Data shown are means ±STDEV (n=3, *P≤0.05; ***P≤0.001, Two-way ANOVA test).

G Design of treatments in the neo-adjuvant setting. One week after tumor cell injection, treatments were applied for 14 days (d=days). After tumor removal and 5 days of recovery, a second round of treatment of 5 days followed. The overall survival of the animals was recorded.

H Tumor volumes of 4T1-tumor-bearing mice treated with Vehicle, CLR457 and/or SHP099. Data shown are mean tumor volumes ±SEM (n=6-8, *P≤0.05; ***P≤0.001; ****P≤0.0001, One-way ANOVA test).

I Overall survival of 4T1-tumor-bearing mice treated as indicated. An event was scored at the appearance of any sign of distress (n=6-8, **P≤0.0012, Log-rank test).

5.3.2 SHP2 inhibition sensitizes cells to PI3K inhibition.

Immunohistochemistry revealed very low p-AKT levels in 4T1 control tumors (Fig 5-1H, Fig 5-2A, top-panel), with less than 1.6% positive pixel counts in quantified areas, but p-AKT increased significantly (7%) upon prolonged single SHP2i treatment. This increase in p-AKT was blocked by dual-inhibition, which suggested a specific mechanism for the improved response to dual-inhibition on tumor progression (Fig 5-1H). SHP2i blocked p-ERK after 4 days of treatment (Fig EV2A) but cells that survived after 14 days of treatment displayed a level of p-ERK similar to the control and to PI3Ki-treated samples (Fig 5-2A, bottom-panel). Thus, both the PI3K and ERK pathways were reactivated in cells that survived SHP2i, an effect

that was prevented by PI3K/SHP2 dual-inhibition. Furthermore, treatment for 4 days with SHP2i alone had no effect on p-AKT and PI3Ki alone had no effect on p-ERK (Fig EV2A).

To assess the effects of PI3K/SHP2 dual-inhibition on the transcriptome, we performed a RNA sequencing of tumors 3 hours after the above dual-inhibition treatments (Fig 5-2B). After CLR457 treatment, 266 genes were up- or downregulated, 487 genes after SHP099 inhibition, and 1523 after dual-inhibition, consistent with the effects of these treatments on tumor growth (Fig 5-1H, Fig EV2B). Overall gene expression changes were similar within groups, with steady clustering in a PCA plot (Fig 5-2C).

Using the cBioPortal for cancer genomics and the breast cancer METABRIC dataset, network analysis of the 35 most-upregulated genes in the SHP2 inhibition group compared to the vehicle group (Fig 5-2D, Fig EV3A) showed convergence to the Src Homology 2 Domain-Containing 1 (SHC1), PIK3CA and AKT3 (Fig 5-2E). SHC1 is an adaptor protein that facilitates interactions between RTKs and a large number of downstream proteins, including the PI3K subunit p85 that forms an activating complex with p110 (Heldin 2013).

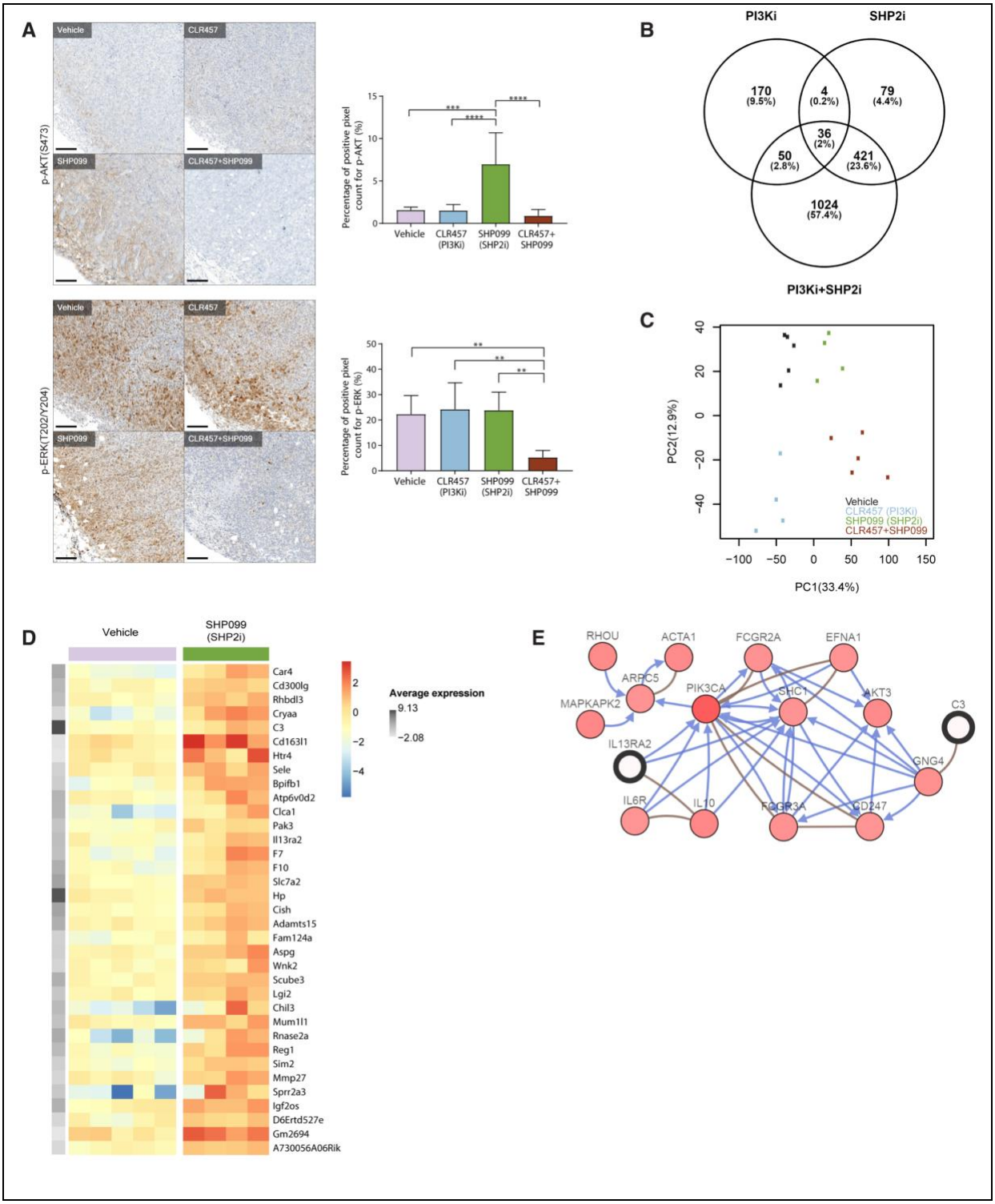
A
Vehicle
CLR457
SHP099
CLR457+SHP099
p-AKT(S473)
Percentage of positive pixel count for p-AKT (%)
Vehicle
CLR457 (PI3Ki)
SHP099 (SHP2i)
CLR457+ SHP099
p-ERK(T202/Y204)
Percentage of positive pixel count for p-ERK (%)
B
PI3Ki
SHP2i
170 (9.5%)
4 (0.2%)
79 (4.4%)
36 (2%)
50 (2.8%)
421 (23.6%)
1024 (57.4%)
PI3Ki+SHP2i
C
PC2(12.9%)
PC1(33.4%)
Vehicle
CLR457 (PI3Ki)
SHP099 (SHP2i)
CLR457+SHP099
D
Vehicle
SHP099 (SHP2i)
Average expression
9.13
−2.08
Car4
Cd300lg
Rhbdl3
Cryaa
C3
Cd163l1
Htr4
Sele
Bpifb1
Atp6v0d2
Clca1
Pak3
Il13ra2
F7
F10
Slc7a2
Hp
Cish
Adamts15
Fam124a
Aspg
Wnk2
Scube3
Lgi2
Chil3
Mum1l1
Rnase2a
Reg1
Sim2
Mmp27
Sprr2a3
Igf2os
D6Ertd527e
Gm2694
A730056A06Rik
E
RHOU
ACTA1
FCGR2A
EFNA1
ARPC5
PIK3CA
SHC1
AKT3
C3
MAPKAPK2
IL13RA2
GNG4
IL6R
IL10
FCGR3A
CD247

Figure 5-2 │ SHP2 blockade reactivates the PI3K pathway.

A Representative images of the immunohistochemistry staining of 4T1 tumors from mice treated for 14 days with Vehicle, CLR457 and/or SHP099. Bar graph shows the quantification of p-AKT (Ser473) (top panel) and p-ERK (Thr202/Tyr204) staining (bottom panel). Tumors were collected as described in Fig 1G. Scale bar 100 µm. Data are means ±STDEV (*n*=7-8, ***P*≤0.01; ****P*≤0.001; *****P*≤0.0001, One-way ANOVA test).

B Venn diagram of transcriptomic variation (up- and down-regulation) in 4T1 tumors from mice treated with CLR457 (PI3Ki) and/or SHP099 (SHP2i) compared to the control group. Data shown are individual values (*n*=4-5, logFC>1 or <-1, FDR<0.05).

C PCA plot of RNA-seq data from 4T1 tumors of mice treated with Vehicle (Vhc), CLR457 and/or SHP099.

D Heat map of the top 35 upregulated genes in 4T1 tumors of mice treated with Vehicle or SHP099. Data shown are individual values (*n*=4-5, logFC>1.5, FDR<0.01).

E Network generated using cBioportal showing the top 35 genes described in Fig 2D and their most frequently altered neighboring genes (filtered, 21%) in the breast cancer METABRIC dataset (Blue: control change of state; brown: in complex).

5.3.3 SHP2 inhibition enhances PDGFR$_{\alpha,\beta}$ signaling.

We next quantified the total tyrosyl-phosphorylation of 39 RTKs in tumor protein lysates from SHP099-treated tumors (Fig 5-1H). Several RTKs were highly phosphorylated after SHP2 inhibition compared to the control (Fig 5-3A, Fig EV3B). We subsequently focused on platelet-derived growth factor receptor (PDGFR) because increased activity of PDGFR signaling has been shown to promote tumor growth, invasion and lung metastasis (Gehmert, Gehmert et al. 2010, Liu, Liao et al. 2011), and PDGFR blockade sensitizes cells to chemotherapeutic agents (Meng, Speyer et al. 2015). Besides the importance of this RTK for tumor progression and metastasis, SHC1 is able to bind autophosphorylation sites of PDGFRs (Heldin, Ostman et al. 1998).

No transcriptomic variation was observed between groups of PDGF receptors and ligands (Fig 5-3B, Fig EV3A). Ligand stimulation of PDGFR$_\alpha$ and PDGFR$_\beta$ has been shown to increase phosphorylation of Y849 and Y857, respectively (Baxter, Secrist et al. 1998). Stimulation of 4T1 cells with the PDGF-BB ligand also increased Y849 and Y857

phosphorylation (Fig 5-3C). Phosphorylation of these sites was found to be enhanced in primary tumors and further enhanced in lung metastases. This correlated with increased p-ERK and p-AKT, suggesting PDGFR-evoked activation of these pathways in lung metastasis (Fig 5-3D, Fig EV4A-B). Similar to its effects on primary tumors, SHP2 inhibition decreased p-ERK and increased p-AKT, whilst PI3K inhibition decreased SHP2i-evoked AKT phosphorylation. SHP2 blockade resulted in increased phosphorylation on SHC1 Y317, which has been shown to include docking sites for other PTB- and SH2- containing proteins and to lead to activation of several pathways, including PI3K/AKT (Ahn, Sabourin et al. 2017). SHP2i also increased PDGFR$_\beta$ Y751 phosphorylation, the docking site for the regulatory subunit of PI3K p85 (Fig 5-3C) (Kazlauskas and Cooper 1990). These observations suggest a mechanism by which SHP2i activates the PI3K/AKT pathway.

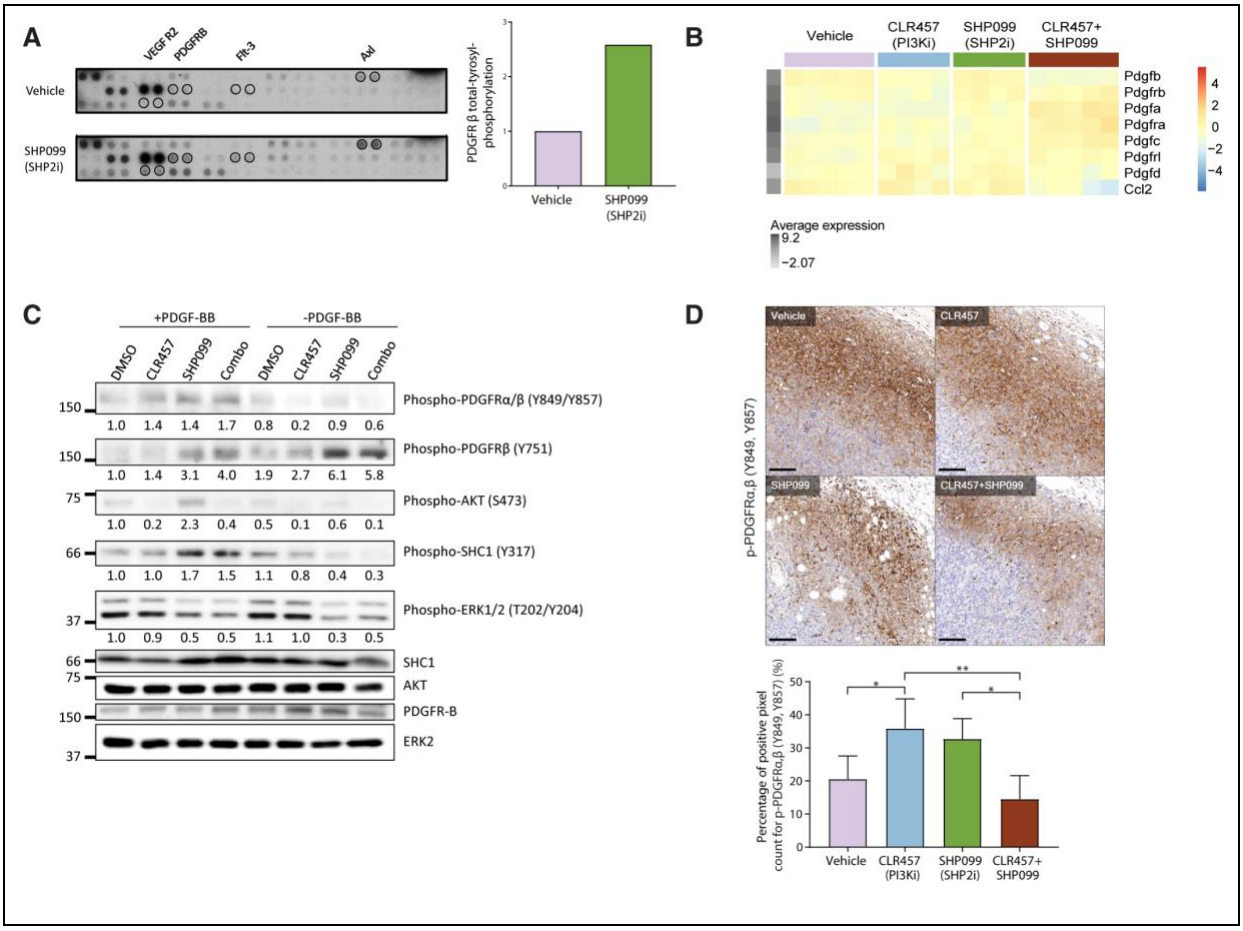

Figure 5-3 | Inhibition by SHP2 activates PDGFR$_{\alpha,\beta}$ signaling and the PI3K pathway.

A Receptor Tyrosine Kinase (RTK) array from 4T1 tumors of mice treated with Vehicle or SHP099 and bar plots (right) of PDGFR$_\beta$ phosphorylation. Data shown are dot quantification by pixel density from RTK-array scans.

B Heat map of PDGF-family-related genes in 4T1 tumors of mice treated with Vehicle, CLR457 and/or SHP099. Data shown are individual values (n=4-5, logFC>4, FDR<0.01).

C Immunoblots of lysates from 4T1 cells that were grown as monolayers and treated with PI3Ki (CLR457, 300 nM) and/or SHP2i (SHP099, 5 µM) and stimulated with PDGF-BB 500 ng/mL for 20 h.

D Representative IHC images of 4T1 tumors from mice treated with Vehicle, CLR457 and/or SHP099. Bar graph showing pixel count from Halo software quantifying PDGF Receptor α (Y849)/PDGF Receptor β (Y857) staining. Tumors were collected as described in Fig 1G. Scale bar 100 µm. Data shown are means ±STDEV (n=6-7, *P≤0.05; **P≤0.01; One-way ANOVA test).

5.3.4 Single PI3K or SHP2 inhibition or dual PI3K/SHP2 inhibition decreases lung metastases in the adjuvant setting.

We assessed the effects of PI3K and/or SHP2 inhibition in the adjuvant setting. 4T1 cells were injected into syngeneic animals and the primary tumors were removed 3 weeks later; animals were then treated as described in Fig 5-4A. After 9 days of treatment, we found numerous metastatic foci in lungs of control mice (an average penetrance of metastasis of 208 arbitrary units) (Fig 5-4B). Surprisingly, the penetrance of metastasis was dramatically lower (15 times) in all treatment groups. Thus, single treatments appear to be as effective as PI3K/SHP2 dual-inhibition in decreasing lung metastases in the adjuvant setting. Both CLR457 and SHP099 potently blocked p-AKT and p-ERK in metastases, respectively (Fig 5-4C).

To test whether the reduction in lung metastases translates to an increase in overall survival, we monitored animals after the adjuvant treatment. All inhibitor treatments extended animal survival, whether treatment was interrupted after 14 days (median survival: vehicle 15 days, treated groups 19-23 days) or was continuous (median survival: vehicle 15 days, treated groups 19-26 days) (Fig 5-4D, Fig EV4C). Similar results were found with a TNBC human

primary-derived xenograft, where animal survival was prolonged in all treated groups in the adjuvant setting (Fig EV4D). In contrast, only PI3K/SHP2 inhibition enhanced overall survival in the neoadjuvant setting (Fig 5-1I).

We assessed the cause of death of mice from the different treatment groups in the adjuvant setting and found lung macro-metastases only in the CLR457 and SHP099 groups. Indeed, the PI3K/SHP2 dual-inhibition group had rare lung metastases despite signs of distress (Fig 5-4E). Because 4T1 cells are highly invasive and can colonize various sites (Pulaski and Ostrand-Rosenberg 1998, Lelekakis, Moseley et al. 1999), we also analyzed livers from these animals. Metastatic liver lesions in the PI3K/SHP2 dual-inhibition group were more frequent than in the other groups (Fig 5-4 F-G), in contrast to the absence of lung macro-metastases. The data suggest that survival and growth of metastases within the lung niche depends on PI3K and SHP2. Within the liver niche, other factors may promote survival and growth of metastases despite dual PI3K/SHP2 inhibition.

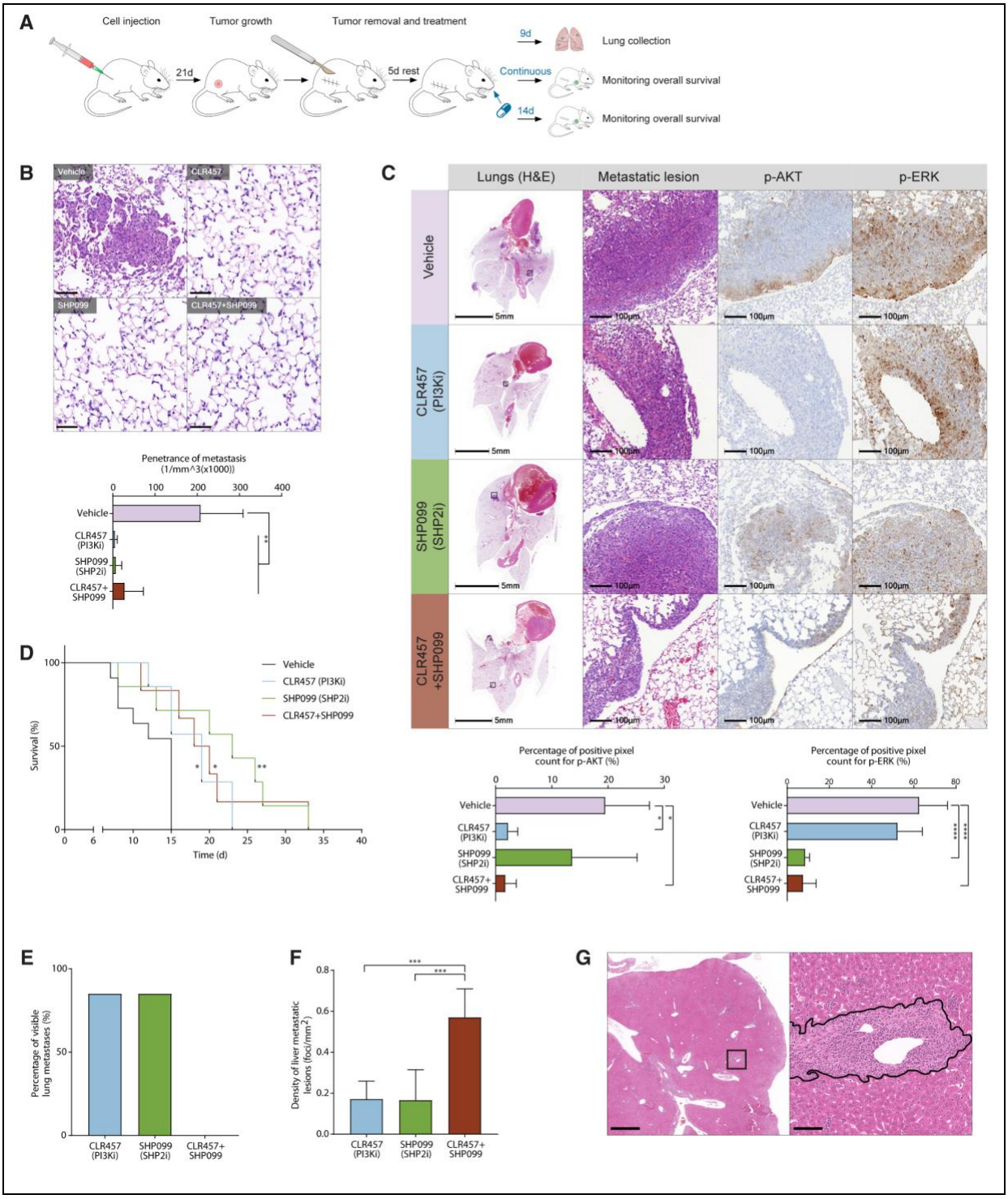
A
Cell injection
Tumor growth
Tumor removal and treatment
21d
5d rest
9d
Lung collection
Continuous
Monitoring overall survival
14d
Monitoring overall survival
B
Vehicle
CLR457
SHP099
CLR457+SHP099
Penetrance of metastasis
(1/mm^3(x1000))
Vehicle
CLR457 (PI3Ki)
SHP099 (SHP2i)
CLR457+ SHP099
C
Lungs (H&E)
Metastatic lesion
p-AKT
p-ERK
Vehicle
CLR457 (PI3Ki)
SHP099 (SHP2i)
CLR457 +SHP099
5mm
100µm
Percentage of positive pixel count for p-AKT (%)
Percentage of positive pixel count for p-ERK (%)
D
Survival (%)
Time (d)
Vehicle
CLR457 (PI3Ki)
SHP099 (SHP2i)
CLR457+SHP099
E
Percentage of visible lung metastases (%)
CLR457 (PI3Ki)
SHP099 (SHP2i)
CLR457+ SHP099
F
Density of liver metastatic lesions (foci/mm²)
G

Figure 5-4 | PI3K and/or SHP2 blockade in the adjuvant setting decreases lung metastases and improves animal survival.

A Experimental design of the adjuvant treatment. Tumors were resected 3 weeks after cancer cell injection. After 5 days of recovery, treatments were applied for 9 days. Lungs were collected continuously until signs of distress developed or for 14 days during which mice were monitored for signs of distress.

B Representative images of H&E staining of lungs from mice treated as indicated for 9 days, together with the associated penetrance of metastasis. Scale bar 100 µm.

C Representative images of H&E, p-ERK (Thr202/Tyr204) and p-AKT (S473) staining of lungs from 4T1-tumor-bearing mice treated as indicated (left). Bar graphs showing quantification of staining (bottom). Data shown are means ±STDEV (n=4-5, *P≤0.05 **P≤0.01; ****P≤0.0001, One-way ANOVA test).

D Kaplan-Meier survival curves of 4T1 tumor-bearing mice treated in the adjuvant setting as indicated. An event was scored when a mouse showed any sign of distress (n=14-16, **P≤0.0012, Log-rank test).

E Bar graphs of the percentage of lungs with macro-metastases from animals treated in the adjuvant setting (Fig 5-4D) as indicated. Lungs were collected at necropsy. Data shown are total counts (n=12-13).

F Bar graphs showing the mean densities of metastatic liver foci from animals treated in the adjuvant setting (Fig 5-4D) as indicated. Livers were collected at necropsy. Data shown are means ±STDEV (n=5-7, ***P≤0.001, One-way ANOVA test).

G Representative image from liver metastases at necropsy from animals treated in the adjuvant setting (Fig 5-4D). Black line delineates metastatic lesion. Scale bars 700 µm (left) and 100 µm (right).

5.4 Expanded view figures

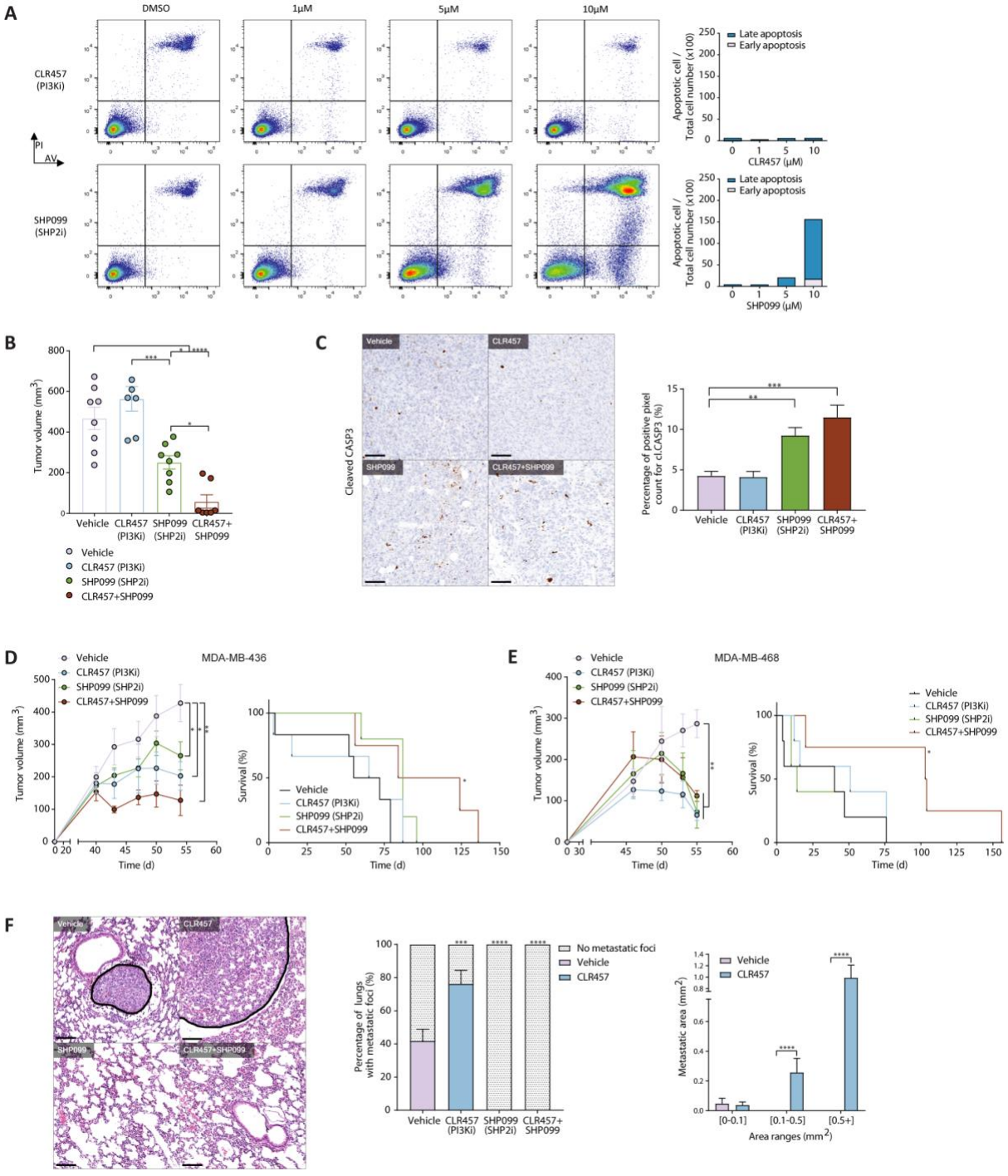

Figure EV1 | SHP2 single inhibition enhances apoptosis and PI3K/SHP2 dual-inhibition prolongs overall survival of TNBC-tumor-bearing mice.

A FACS plot of the apoptosis analysis of 4T1 cells treated for 3 days with CLR457 (top panel) or SHP099 (bottom panel) at the indicated concentrations. Fresh inhibitors were added after 48 h. Quantification is shown as bar plots.

B Bar plot of tumor volumes of 4T1-tumor-bearing mice after 14 days of treatment as indicated. Data shown are mean tumor volumes ±SEM (n=6-8, *P≤0.05; ***P≤0.001; ****P≤0.0001, One-way ANOVA test).

C Representative images of cleaved-Caspase 3 IHC staining of lung metastases from 4T1-tumor-bearing mice treated in the adjuvant setting for 4 days as indicated (left). Bar graph of the quantification using the pixel count algorithm performed with Halo software (right). Scale bar 100 µm. Data shown are means ±STDEV (n=3, **P≤0.01; ***P≤0.001, One-way ANOVA test).

D, E Tumor volume growth and Kaplan-Meier overall survival of MDA-MB-436 and MDA-MB-468 tumor-bearing mice treated as given. Data shown are mean tumor volumes ±SEM (n=4-6, *P≤0.05; **P≤0.01, One-way ANOVA test).

F Representative images of H&E-stained lungs from 4T1-tumor-bearing mice treated as described in Fig. 1G. Black lines delineate metastases. Scale bar 100 µm (left panel). Plot of the percentages of lungs with metastases from Vehicle-, CLR457-, SHP099-, and CLR457+SHP099-treated groups. Data shown are means ±STDEV (n=7-8, ***P≤0.001; ****P≤0.0001, One-way ANOVA test) (middle panel). Bar graph of the quantification of metastatic areas in Vehicle- and CLR457-treated groups (the data for SHP099 and CLR457+SHP099 groups are not shown because no metastatic foci were detected). Small 0-0.1; Medium 0.1-0.5; Large ≥0.5 mm^2. Data shown are means ±STDEV (n=7-8, ****P≤0.0001, t-test) (right panel).

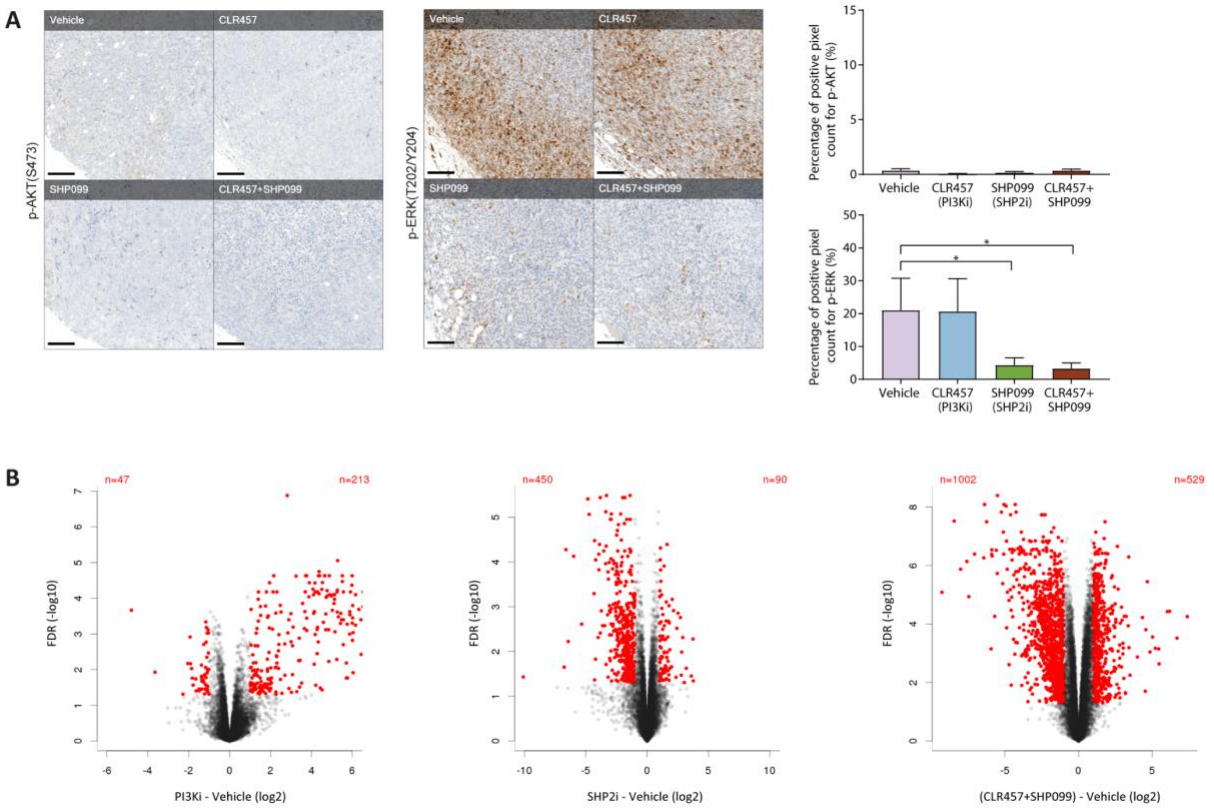

Figure EV2 │ Single inhibition of PI3K or SHP2 *in vivo* blocks activation of the PI3K and the MAPK pathways, respectively, and transcriptomic changes are wider following PI3K/SHP2 dual-inhibition than single inhibitions.

A Representative images of p-AKT (Ser473) (left panel) and p-ERK (Thr202/Tyr204) (middle panel) IHC-stained 4T1 tumors from mice treated for 4 days as indicated. Bar graphs show quantification using the pixel count algorithm performed with Halo software (right panels). Tumors were collected as described in Fig. 1G. Scale bar 100 µm. Data shown are means ±STDEV (n=4-5, **P≤0.01; ***P≤0.001; ****P≤0.0001, One-way ANOVA test).

B Volcano plots showing transcriptomic variation in 4T1 tumors from mice treated as indicated. Data shown are individual values (n=4-5, logFC>1, FDR<0.05).

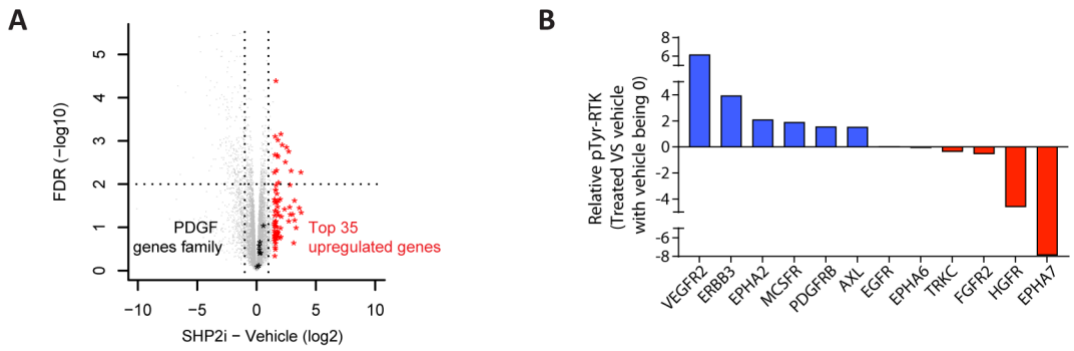

Figure EV3 | Increased tyrosyl-phosphorylation of several RTKs upon SHP2 inhibition without effects on the transcriptome of the PDGF-related gene family.

A Volcano plots of transcriptomic variation of the "PDGF-related gene family" and the "top 35 upregulated genes" in 4T1 tumors from mice treated with SHP099. Data shown are individual values (*n*=4-5, logFC>1.5, FDR<0.01).

B Bar graph of the quantification of the 10 most-enriched phospho-tyrosyl-RTKs in 4T1 tumors from mice treated as indicated. Data shown are dot quantification by pixel density from RTK-array scans.

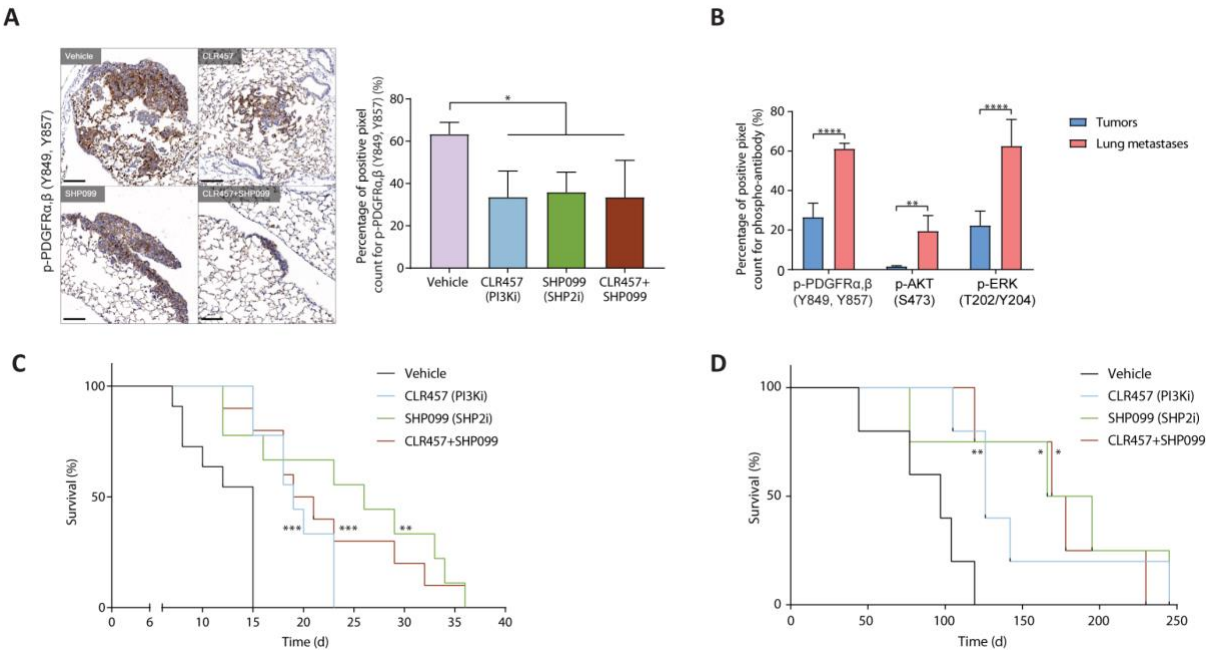

Figure EV4 | PI3K/SHP2 dual-inhibition in the adjuvant setting prolongs overall survival.

A Representative images of PDGF Receptor α (Y849)/PDGF Receptor β (Y857) IHC stained lungs from 4T1-tumor-bearing mice treated as indicated (left). Bar graph shows quantification using the pixel count algorithm performed with Halo software (right). Scale bar 100 µm. Data shown are means ±STDEV (*n*=6-7, **P*≤0.05, One-way ANOVA test).

B Bar plots of the percentages of phosphorylated proteins in primary tumors and lung metastases. Data shown are means ±STDEV (*n*=6-8, ***P*≤0.01; *****P*≤0.0001, One-way ANOVA test).

C Kaplan-Meier survival curves of 4T1-tumor-bearing mice treated continuously in the adjuvant setting as indicated. An event was scored when a mouse showed any sign of distress (*n*=10-16, ***P*≤0.0001, Log-rank test).

D Kaplan-Meier survival curves of HBRX2353 primary-derived xenograft-tumor-bearing mice treated in the adjuvant setting as shown. An event was scored when a mouse showed any sign of distress (*n*=4-5, **P*= 0.0585, Log-rank test).

5.5 Discussion

Targeted therapies in combination with chemo- or hormone therapy have improved the survival of patients at different stages of breast cancer. Approved mechanism-based therapies for breast cancer such as Trastuzumab, Everolimus, Olaparib, Palbociclib, Ribociclib, and Abemaciclib target specific cancer dependencies with consequently fewer side effects. Extensive work on protein 3D structures and detailed inhibitor design have made protein targeting even more specific and effective (Gampenrieder, Rinnerthaler et al. 2013, Garcia Fortanet, Chen et al. 2016). Despite initial successes, these strategies are hindered by the development of resistance, leading to cancer cell insensitivity and restricted therapeutic solutions (Groenendijk and Bernards 2014, Leroy, Amante et al. 2014, Ramos and Bentires-Alj 2015, Dardaei, Wang et al. 2018, Mainardi, Mulero-Sanchez et al. 2018). However, it is anticipated that combining targeted therapies together or with chemotherapy will potentiate the initial inhibition, overcome resistance, and improve patient outcome. In the present study, we found that a dual-inhibition using a pan-PI3K- and an allosteric SHP2-inhibitor significantly improves overall survival of mice with metastatic TNBC.

We compared the effects of PI3K and/or SHP2 inhibition in the neoadjuvant and adjuvant settings by assessing drug response and overall survival of the animals. Neoadjuvant treatment with dual PI3K and SHP2 inhibition synergistically decreased primary tumor volume, led to complete tumor regression in almost all animals, and improved overall survival. While SHP2 but not PI3K inhibition alone decreased primary tumor volume, neither of these single treatments improved overall survival. Protein and transcriptome analyses revealed PI3K pathway activation upon SHP2 blockade in a PDGFR-dependent manner, which was blunted by combined inhibition of PI3K and SHP2.

In contrast, while PI3K inhibition alone had no effect on primary tumor growth, resulting in fact in larger metastases than the control in the neoadjuvant setting, it did decrease metastases and improve overall survival in the adjuvant situation. While, SHP2 inhibition decreased primary tumors and lung metastases but did not affect overall survival in the neoadjuvant treatment, it did decrease lung metastases and increase overall survival in the adjuvant setting. Combined PI3K and SHP2 inhibition decreased lung metastases and improved overall survival in both the neoadjuvant and adjuvant settings. Thus there appears to be differential sensitivity of cancer cells to targeted therapy at the primary site and in the lungs and, therefore, using primary tumor shrinkage alone as a measure of drug efficacy can be misleading. We have shown previously that anti-CCL2 or JAK2 or IL-8 inhibition have no effect on primary tumor growth but decrease lung metastases and increase overall survival (Britschgi, Andraos et al. 2012, Bonapace, Coissieux et al. 2014). Altogether, these findings stress the need for thorough evaluation of drug efficacy not only *in vitro* and on primary tumor growth but also on metastases and overall survival.

Our observation that all mice treated with PI3K and/or SHP2 inhibitors in the adjuvant setting show similar improvement of overall survival may suggest that a single agent is sufficient. However, assessment of lungs from the different groups showed that mice treated with PI3K or SHP2 inhibitors have large metastases, while metastases were rare in mice treated with both agents. Further analysis revealed three times more liver metastases in mice after dual-inhibitor as against single-inhibitor treatment. These data raise the possibility that metastases developing in the lung niche are sensitive to PI3K and to SHP2 inhibition, whereas resistance to this treatment develops in metastatic cells within the liver niche.

The observation that pan-PI3K inhibition in the neoadjuvant setting increases lung metastases in TNBC calls for caution when using such agents in the presence of the primary tumor. We have reported similar results using a dual PI3K/mTOR inhibitor (Britschgi, Andraos

et al. 2012). A pan-PI3K inhibitor shows some efficacy as a single agent in the adjuvant setting but mice still succumb to lung metastases. Our data provide a rationale for using pan-PI3K in combination with SHP2 inhibition to treat metastatic TNBC in the adjuvant setting and support further testing of this possibility.

5.6 Materials and methods

Compounds

CLR457 and SHP099 were obtained from Novartis (Basel, Switzerland and Cambridge, USA). Compounds were prepared as 10 mM stock solutions in DMSO and stored protected from light at –20°C. CLR457 (20 mg/kg) and SHP099 (100 mg/kg) were freshly formulated in methylcellulose/Tween-80 (0.5% / 0.5%) and administered to mice by oral gavage at 5 ml/kg.

Animal Experiments

All *in vivo* experiments were performed in accordance with the Swiss animal welfare ordinance and approved by the cantonal veterinary office of Basel Stadt. Female severe combined NOD-scid IL2rγnull (NSG) and Balb/c animals were maintained in the Friedrich Miescher Institute for Biomedical Research and the University Department of Biomedicine animal facility in accordance with Swiss guidelines on animal experimentation. For orthotopic engraftment of cell lines, 0.3 x 10^6 4T1, 2 x 10^6 MDA-MB-436 and 2 x 10^6 MDA-MB-468 cells were suspended in 50 µL PBS and injected into mammary fat pad number 4 of 8-week-old mice. Tumor-bearing mice were randomized based on tumor volume prior to the initiation of treatment, which started when average tumor volume was at least 80 mm^3. CLR457 was administered twice a day and SHP099 once daily. Tumors were measured every 3–4 days and tumor volumes calculated by the formula 0.5 x (larger diameter) x (smaller diameter)^2. End point tumor sizes were analyzed for synergism using the formula AB/C < A/C x B/C, where C is tumor volume Vehicle, A is tumor volume compound 1, B is tumor volume compound 2,

and AB is tumor volume combination (Clarke 1997). For survival studies, day 0 corresponds to tumor removal, and animals were sacrificed as soon as they showed any sign of distress (e.g., breathing disorders, weight loss, or immobility).

Cells, Cell Culture, Reagents and PDX Models

SUM159 were propagated in Nutrient Mixture F-12 supplemented with 5% fetal calf serum, 0.5 μg/ml hydrocortisone, and 10 μg/ml insulin (all from Sigma), 100 IU/ml penicillin, 100 μg/ml streptomycin and 100 μg/ml Normocin (InvivoGen). Balb/c tumor-derived mammary cancer lines 4T1 were propagated in DMEM, with 10% fetal calf serum (all from Sigma), 100 IU/ml penicillin, 100 μg/ml streptomycin and 100 μg/ml Normocin (InvivoGen). MCF10A-HER2/HER3 (Aceto, Sausgruber et al. 2012) were propagated in DMEM/F12 medium (Invitrogen) supplemented with 5% horse serum (Hyclone), 20 ng/ml EGF (Peprotech), 0.5 μg/ml hydrocortisone, 100 ng/ml cholera toxin, and 10 μg/ml insulin (all from Sigma), 100 IU/ml penicillin, 100 μg/ml streptomycin and 100 μg/ml Normocin (InvivoGen). All other cell lines were obtained from and were cultured according to the protocols of the American Type Culture Collection. Profiling of human cell lines used highly-polymorphic short tandem repeat loci (STRs) (Microsynth). For treatment with inhibitor(s), cells were synchronized with 0.5% serum for 6 h to avoid masking effects of growth factors present under full-serum conditions. Culture medium containing the inhibitor(s) was then added and cells cultured for 20 h. PDX used for this study was described earlier (DeRose, Wang et al. 2011, Gao, Korn et al. 2015).

Cell Number Assay

Cells were cultured overnight in 96-well plates at 1,000 cells/well before culture medium was added containing 0.5 % FCS (or HS) and the inhibitor(s) described above. The culture medium with inhibitor(s) was renewed 48 h after initial treatment and cells were fixed 24 h later. Cell fixation, staining and quantification were performed using the Sulforhodamine B colorimetric assay (Vichai and Kirtikara 2006).

Immunoblotting and Phospho-RTK Arrays

Cells were lysed with RIPA buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with 1x protease inhibitor cocktail (Complete Mini, Roche), 0.2 mM sodium orthovanadate, 20 mM sodium fluoride and 1 mM phenylmethylsulfonyl fluoride. Lysates from xenografts were prepared by lysing kryo-homogenized tumor powder in RIPA buffer. Whole cell lysates (30-80 µg) were subjected to SDS-PAGE, transferred to PVDF membranes (Immobilon-P, Millipore) and blocked for 1 h at room temperature with 5% milk in PBS-0.1% Tween 20. Membranes were then incubated overnight with antibodies as indicated and exposed to secondary HRP-coupled anti-mouse or anti-rabbit antibodies at 1:7,500 for 2 h at room temperature. The following antibodies were used (from Cell Signaling unless stated otherwise): anti-pAKT (Ser473), anti-pERK1/2 (Thr202/Tyr204), anti-pPDGFR$_{\alpha/\beta}$ (Y849/Y857) anti-pPDGFR$_{\beta}$ (Y751, Thermo Fisher), anti-ERK2 (Santa Cruz), anti-pSHC1 (Y317), anti-PDGFR$_{\beta}$, anti-AKT, anti-SHC1. Phospho-RTK arrays on tumor lysates were performed using the Proteome Profiler Mouse Phospho-RTK Array Kit (R&D systems) according to the manufacture's protocol.

Immunohistochemistry

Tumors and livers were fixed in 10% NBF (neutral buffered formalin) for 24 h at 4°C, washed with 70% EtOH, and embedded in paraffin. Sections of 2.5 µm were cut and processed for hematoxylin and eosin (H&E) staining and immunohistochemistry. Prior to fixation, dissected lungs were inflated by injecting 5 mL of PBS through the trachea, then inflated with 5 mL of 10% NBF and gently released into a tube filled with 10% NBF. Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tissue sections using a Bond-maX (Leica) fully automated system for anti-pPDGFR$_{\alpha/\beta}$ (Y849/Y857), and a Discovery XT (Ventana) fully automated system for anti-pAKT (Ser473), anti-pERK1/2 (Thr202/Tyr204), anti-CD31 (Spring Biosciences) and anti-cleaved Caspase-3 (Asp175). Algorithms for quantitative analysis of immunostained positive areas and the areas of lung metastases were designed in Halo software that allowed assessment of the relative fractions of positive areas. To detect and measure angiogenesis, we performed the Aperio Microvessel Analysis Algorithm using CD31 staining. The "penetrance of metastasis" was calculated following the formula (∑ lung metastatic area/lung epithelium area)*(1/tumor volume)*1000.

Apoptosis Assay

Cells were synchronized with DMEM 0.5% serum overnight and then supplemented with medium containing inhibitor(s). Fresh inhibitors were added after 48 h and cells (floating and adherent) were collected 24 h later using trypsin-EDTA, resuspended in growth medium and counted. For Annexin V/propidium iodide staining, cells were washed twice with cold Cell Staining Buffer (Biolegend, #420201) and resuspended in Annexin V Binding Buffer (Biolegend, #422201) at a concentration of 1 x 10^6 cells/mL. Aliquots of Alexa Fluor 647 Annexin V (5 µL) (Biolegend, #640911) and of Propidium iodide (10 µL) (Biolegend, #421301) were added to 100 µL of this suspension, which was then incubated for 15 min at

room temperature in the dark. After addition of 400 µl of Annexin V Binding Buffer to each tube, samples were analyzed by flow cytometry.

Transcriptomic Analysis

Total RNA was extracted from frozen tumors using the RNeasy Plus Mini Kit (Qiagen, #74136) and sample quality controlled on an Agilent 2100 Bioanalyzer system with the RNA6000 Nano kit (Agilent, #5067-1511). mRNA isolation was performed with the NEBNext Poly(A) mRNA magnetic isolation module (NEB, #E7490) and libraries prepared with the NEBNext Ultra II Directional RNA Library Prep kit (NEB, #E7765) according to the manufacturer's recommendations. Samples were individually barcoded during library preparation using NEBNext Multiplex Oligos for Illumina Index Primers Sets 1 and 2 (NEB, #E7335 and #E7500). Library quality control was performed with the DNA1000 kit (Agilent, #5067-1504) on the Agilent 2100 Bioanalyzer system. Finally, libraries were sequenced on an Illumina NextSeq 500 and paired-end 75-bp reads generated. Adaptor trimming was performed using cutadapt (Martin 2011). Trimmed reads were aligned to the GRCm38 genome using the two-pass approach of STAR (Dobin, Davis et al. 2013). A median of 53 million reads (range 44-60) were aligned per sample. qCount from QuasR (McCarthy, Chen et al. 2012) was then used to obtain counts at the gene level. Differential gene expression was performed using edgeR (McCarthy, Chen et al. 2012). A cutoff of log2 fold change > 1 and adjusted to $P < 0.05$ (corrected by the Benjamini–Hochberg algorithm method) was applied to selected genes. Network analysis was performed on the cBioportal website using the breast cancer dataset METABRIC (http://www.cbioportal.org/).

Statistical Analysis

In each of the studies presented, the results shown represent at least three independent experiments. Values are reported as means ± STDEV. Data were tested for normal distribution and ANOVAs tests were applied. GraphPad Prism 7.04 was used for Kaplan-Meier survival analysis and log rank Mantel-Cox tests were applied to test statistical significance (SAS), as well as for all other statistical tests (SAS). The *P* values < 0.05 were considered statistically significant.

Data availability

Transcriptomic data are available upon request.

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Author contributions

R.J.A designed the study, performed all experiments, analyzed the data and wrote the manuscript. D.D.S, M.A., M.-M.C. and B. T.P. supported cell line, biochemical and tumor xenograft studies. A.S., B.H., S.P. and C.K.Y.N. supported gene expression studies. V.R. supported histology studies. V.R., M.M. were involved with the study design. M.B.-A. conceived the study, designed the experiments and interpreted the results. All authors discussed the results and commented on the manuscript.

Disclosure of potential conflicts of interest

M.M. and V.R. are full-time employees of Novartis Pharma AG. D.D.S. is a full-time employee of F. Hoffmann-La Roche AG. The Bentires-Alj lab receives financial support from Novartis.

5.7 Supplementary data figures: Targeting VEGFR-dependent liver metastases in addition to PI3K/SHP2 inhibition further improves animal survival.

Liver metastases shorten the survival of patients with breast cancer and effective treatment strategies are lacking (Cardoso, Costa et al. 2017). VEGFR, NF-kappa B and MAPK have been shown to be active in 4T1 mammary carcinoma liver metastases (Chen, Zheng et al. 2017), and VEGFR inhibitors are being tested in the clinic for their effects on breast cancer liver metastases as single agent (Chien, Lee et al. 2013) or in combination (Xu, Stevens et al. 2014). Livers from mice in the PI3K/SHP2 dual-inhibition group treated in the adjuvant setting were found to have the highest microvessel density, and liver metastases were essentially growing around blood vessels (Fig EV5A).

We next asked whether a triple therapy targeting PI3K/SHP2 as well as angiogenesis would decrease liver metastasis and further improve overall survival of the animals. Several multi-kinase inhibitors have been developed over the last decade, with different affinities for VEGFRs, PDGFRs, c-kit and FGFRs (Meadows and Hurwitz 2012). Since 4T1 lung metastases express PDGFRs (Fig EV4A) and liver metastases were shown to express VEGFRs (Chen, Zheng et al. 2017) but not PDGFRs (Fig EV5B), we selected the FDA-approved drug sunitinib to target VEGFRs, PDGFRs and c-kit. The effect of combining sunitinib with PI3K/SHP2 dual-inhibition was first tested on 4T1 cells in 3D culture. PI3K/SHP2 dual-inhibition decreased cell viability as seen already with primary tumors (Fig 5-1H; Fig EV1B). PI3Ki had no effect on cell viability, compared to SHP2i (70%) and PI3K/SHP2 dual-inhibition (38%) (Fig EV5C). Sunitinib reduced cell viability to 25% and a triple combination of CLR457, SHP099 and sunitinib further reduced this to 7% (Fig. 5-5A).

We then assessed the effects of the triple combination on animal survival. Mice in the adjuvant setting were treated for 16 days by alternating 4 days of treatment with a 2-day break

in order to limit drug toxicity. The sunitinib single-agent and the CLR457/SHP099 dual-inhibition groups showed similar survival, with medians of 20 days and 23 days, respectively (Fig. 5-5B). The survival of the triple-combination group was significantly longer than any other group (29.5 days). Thus, a combination of PI3K, SHP2 and VEGFR/PDGFR inhibitors in the adjuvant setting had a higher therapeutic value than any other combination tested.

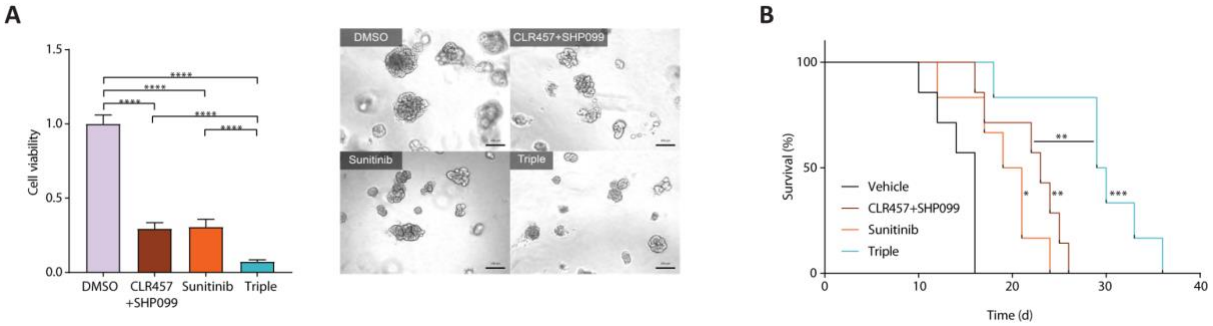

Figure 5-5 │ A triple treatment with PI3K, SHP2 and VEGFR/PDGFR inhibitors further improves overall survival.

A Bar graphs of cell viability of 3D cultures treated as indicated for 5 days; the treatment was refreshed every 2 days (left panel). Representative images of 3D cultures from each condition (right panel). Scale bar 100 µm. Data shown are means ±STDEV (n=4, ****P≤0.0001, One-way ANOVA test).

B Kaplan-Meier survival curves of 4T1-tumor-bearing mice treated in the adjuvant setting as shown. An event was scored when a mouse showed any sign of distress (n=6-7, *P=0.0111 **P=0.0015; ***P=0.0009, Log-rank test).

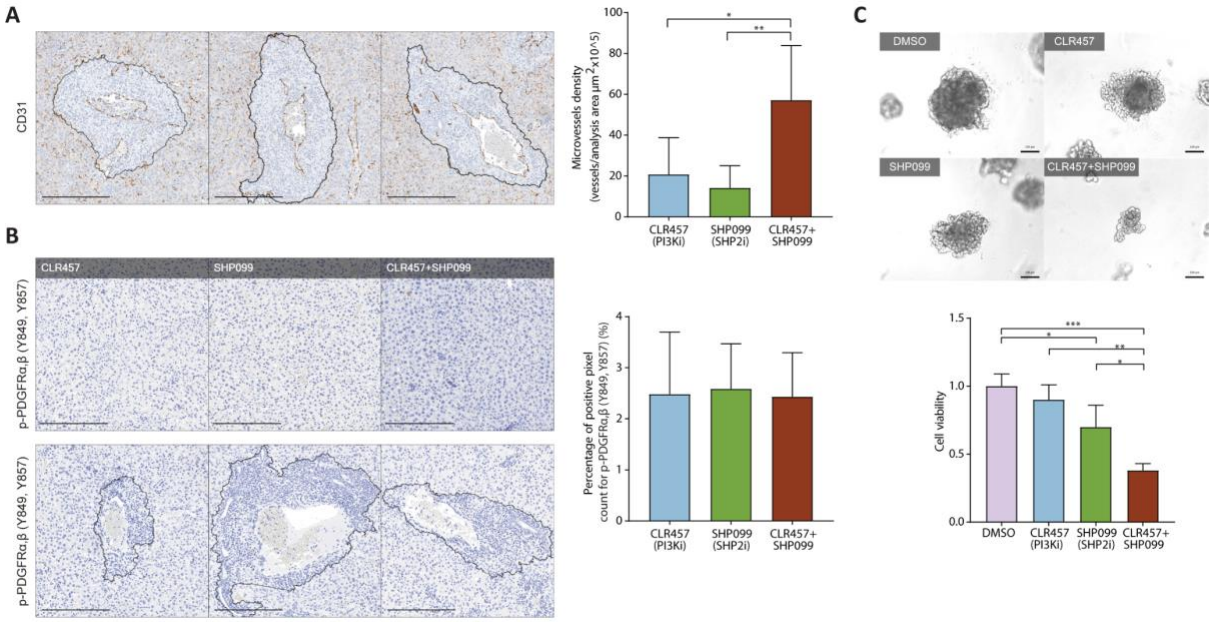

Figure EV5 | The PDGF pathway is not active in liver metastases.

A Representative IHC images (left panel) of individual metastases from the PI3K/SHP2 dual-inhibition group with CD31 staining. Black lines delineate metastases. Scale bar 100 µm. Bar graphs (right panel) of microvessel density in livers from mice treated as indicated in the adjuvant setting (Fig 5-4D). Measurements obtained with the Aperio Microvessel Analysis Algorithm based on CD31 staining. Data shown are means ±STDEV (n=4-7, *P≤0.05, **P≤0.01, One-way ANOVA test).

B Representative images of PDGF Receptor α (Y849)/PDGF Receptor β (Y857) IHC staining of livers from mice treated as indicated in the adjuvant setting. Upper panel: liver cells; lower panel: metastatic lesions delineated by black lines. Bar graphs (right panel) of quantification using the pixel count algorithm performed with Halo software. Scale bar 100 µm. Data shown are means ±STDEV (*n*=5-7, **P*≤0.05, One-way ANOVA test).

C Representative 3D culture images of each condition (top panel). Bar graphs of cell viability of 3D cultures treated for 5 days as indicated, with medium replacement every 2 days (bottom panel). Scale bar 100 µm. Data shown are means ±STDEV (*n*=4, ****$P \leq 0.0001$, One-way ANOVA test).

Supplementary materials and methods

Compounds

Sunitinib (Sutent, Pfizer Inc.) was obtained from LC laboratories (Woburn, Mass., USA). Sunitinib was prepared as 10 mM stock solutions in DMSO and stored protected from light at –20°C. Sunitinib (60 mg/kg) was freshly formulated in methylcellulose/Tween-80 (0.5% / 0.5%) and administered to mice by oral gavage at 5 ml/kg, once daily.

3D Cell Culture

For in vitro drug treatments, 4T1 cells were seeded in DMEM containing 10% FCS and 30% Matrigel Growth Factor Reduced (Corning, 356231) at 300 cells per well in 384-well plates in quadruplicates. After three days, 3D colonies were treated with DMEM containing 0.5% FCS together with CLR457 (1µM), SHP099 (5µM), sunitinib (3.3µM) or combinations. Two days later, 50% of the culture medium was exchanged with medium containing drugs at 200% higher concentrations. Cells were kept under treatment for a further two days. At treatment day 5, the viability of cells was assessed by the CellTiter-Glo 3D Cell Viability Assay (Promega, G9618) according to the manufacturer's instructions. In brief, after removing the culture medium, cells were lysed in 25 µl CellTiter-Glo 3D Reagent. After a 30-min incubation at room temperature on a horizontal shaker, luminescence was recorded for 0.5 s with an ELISA-reader.

Immunohistochemistry

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tissue sections using a Discovery XT (Ventana) fully automated system for anti-CD31 (Spring Biosciences). To detect and measure angiogenesis, we performed the Aperio Microvessel Analysis Algorithm using CD31 staining.

6 | DISCUSSION AND OUTLOOK

We found that dual-inhibition of PI3K and SHP2 decreases synergistically tumor volumes and improves animal survival. Moreover, we demonstrated that the triple combination with the addition of VEGFR/PDGFR inhibition further improved animal survival possibly by blocking niche-specific signals, which previously rendered metastases resistant to PI3K/SHP2 dual-inhibition.

6.1 SHP2 blockade increases PI3K inhibition efficacy

Several decades of research on signaling pathways have revealed their tremendous importance in breast cancer. Among them, the PI3K pathway is hyperactivated in 70% of breast cancer. PI3K inhibition shows limited efficacy in clinical settings. Clinical trials were discontinued due to high toxicity, or occurrence of resistance. Hence, combinatorial strategies to anticipate resistance should be considered. We found that SHP2 inhibition sensitizes breast cancer cells to PI3K inhibition, switching from previously insensitive tumors to full regression in most cases.

Blockade of PI3K/SHP2 *in vitro* in a panel of breast cancer cell lines reduced cell number, with a higher efficacy for the TNBC sub-group. *In vivo*, we used a TNBC cell line where PI3K single inhibition did not affect tumor growth. We observed that PI3K/SHP2 dual-inhibition decreased tumor volumes, with total tumor regression in 70% of mice. This tumor shrinkage was accompanied by an increased tumor cell apoptosis. SHP2 single inhibition displayed an intermediate phenotype. Mechanistically, we demonstrated that SHP2 inhibition increases p-AKT via upregulation of $PDGFR_{\beta}$, and sensitizes cells to PI3K inhibition.

6.2 Effects of PI3K/SHP2 dual-inhibition on metastasis

Primary tumor resection is a very successful procedure to cure breast cancer patients. However, DTC are a major challenge. They remain undetectable and once grown, become difficult lesions to cure. In the neo-adjuvant setting, PI3K/SHP2 dual-inhibition enhances survival compared to control mice, by reducing lung metastases. Moreover, PI3K/SHP2 dual-inhibition in the adjuvant setting led to shrinkage of established lung metastases, and improved animal survival.

6.3 PI3K and SHP2 single blockade: neoadjuvant or adjuvant setting?

In TNBC, we observed that pan-PI3K inhibition increases lung metastases in the neo-adjuvant setting. Equally, we previously published that dual-PI3K/mTOR inhibition in neo-adjuvant setting led to larger lung metastases while having no effects on primary tumors (Britschgi, Andraos et al. 2012). These observations raise the risk of PI3K blockade in the presence of the primary tumor. In contrast, PI3K inhibition in the adjuvant setting improved overall survival of mice. Similarly, we found that SHP2 inhibition did not increase animal survival while decreasing tumor volumes in the neo-adjuvant setting, but increases animal survival in the adjuvant setting. These results highlight the need for careful study design when using such inhibitors, and raise the following questions: Does the initial treatment of primary tumors lead to more aggressive lung metastases due to higher selection pressure? Are lung metastases in adjuvant setting more sensitive to treatments due to unpreceded selection pressure?

Finally, based on the treatment settings (neo-adjuvant vs. adjuvant), we obtained different outcomes that can bias conclusions on drug efficacy, and decisions on pursuing such drugs for development and clinical trials. Our findings emphasize the need for careful preclinical studies (i.e., that relay not only on primary tumor shrinkage, but also on reduced

metastases and enhanced overall survival) before pursuing further development of an anticancer drug.

6.4 From metastatic niche specificities to targeted inhibition design

We found that mice treated with PI3K/SHP2 dual-inhibition, or with single agents, show similar overall survival in the adjuvant setting, arguing that single inhibitions are sufficient. We detected lung and liver metastases in mice from single treatment groups, while mice from PI3K/SHP2 dual-inhibition group had rare lung metastases, but three times more liver metastases. These data suggest a discrepancy between metastatic niches, influencing metastases sensitivity to treatment (Psaila and Lyden 2009, Gundem, Van Loo et al. 2015). Our data show that lung metastases are sensitive to PI3K/SHP2 dual-inhibition, while liver metastases are resistant.

We then demonstrated that lung metastases were PDGFRs positive, whereas liver metastases are negative. Previous reports have shown that liver metastases are mainly VEGFRs dependant (Shaheen, Davis et al. 1999, Chen, Zheng et al. 2017). Thus, we targeted PDGFRs and VEGFRs with the clinically approved drug sunitinib, in combination with PI3K/SHP2 dual-inhibition. We observed a prolonged animal survival, compared to PI3K/SHP2 dual-inhibition, or sunitinib alone. This raises the possibility that macrometastases display different sensitivities based on their niche microenvironment. Additional work is necessary to assess further metastatic sites and their response to this triple therapy.

6.5 SHP2 blockade as a key sensitizer in multiple cancers

In numerous cancers, the tyrosine phosphatase SHP2 is a key activator of the ERK pathway, as well as being a signal enhancer downstream of a large number of RTKs. Recently, SHP2 blockade has been shown to sensitize cells to ALK inhibition in NSCLC, as well as to MEK inhibition in *KRAS*-mutant background (Dardaei, Wang et al. 2018, Fedele, Ran et al. 2018,

Ruess, Heynen et al. 2018, Ahmed, Adamopoulos et al. 2019). Our findings show that SHP2 inhibition further blocks primary tumor growth and metastasis progression in the context of PI3K inhibition.

Taken together, our results suggest a fundamental property of SHP2 blockade, increasing sensitivity of resistant or non-sensitive cancer cells to PI3K inhibition. Knowing that SHP2 promotes self-renewal capacity, breast cancer maintenance, invasion and progression to metastasis, dual-inhibition of SHP2 and potent targets in several cancers could open new therapeutic strategies.

6.6 Concluding remarks and future directions

Unravelling mechanisms of resistance to PI3K inhibitors remains a major clinical challenge. In most cases, compensatory signaling initiated by RTKs short circuit the initial inhibition. SHP2 is a signal enhancer downstream of RTKs. Recent studies have led to the development of SHP2 selective inhibitors, which facilitated the study of the oncogenic properties of SHP2, and provided further mechanistic insights.

In TNBC, SHP2 blockade sensitizes cells to PI3K inhibition. Dual-inhibition decreased tumor volumes synergistically, as well as lung metastases in a PDGFR$_\beta$ dependant manner, and increased animal survival. Further analysis revealed that liver metastases resisted such combination, most likely via activation of VEGFR. These findings encourage the development of new combination strategies to tackle metastatic breast cancers.

These discoveries raise further questions:

i. The observation that SHP2 inhibition sensitizes TNBC cells to PI3K inhibition raises the question whether this is also the case in other breast cancer subtypes (e.g., ER+, HER2) and malignancies. If so, what are the underlying molecular mechanisms?

ii. Treatments of mammary cancer cell lines with the SHP2 inhibitor resulted in resistance both *in vitro* and *in vivo*. What are the mechanisms of resistance to SHP2 inhibition?

iii. We exclusively focused on the cell-autonomous effects of the PI3K/SHP2 dual-inhibition. Does PI3K/SHP2 dual-inhibition also enhances survival by non-cell autonomous mechanisms?

iv. Inhibition of SHP2/PI3K/PDGFR/VEGFR enhances overall survival of mice compared to single or dual combination but did not result in cure of mice. What are the mechanisms of resistance to this combination therapy?

Answering such questions is warranted, eagerly awaited, and further studies are being conducted in our laboratory to address them.

7 | REFERENCES

Aceto, N., A. Bardia, D. T. Miyamoto, M. C. Donaldson, B. S. Wittner, J. A. Spencer, M. Yu, A. Pely, A. Engstrom, H. Zhu, B. W. Brannigan, R. Kapur, S. L. Stott, T. Shioda, S. Ramaswamy, D. T. Ting, C. P. Lin, M. Toner, D. A. Haber and S. Maheswaran (2014). "Circulating tumor cell clusters are oligoclonal precursors of breast cancer metastasis." Cell **158**(5): 1110-1122.

Aceto, N., N. Sausgruber, H. Brinkhaus, D. Gaidatzis, G. Martiny-Baron, G. Mazzarol, S. Confalonieri, M. Quarto, G. Hu, P. J. Balwierz, M. Pachkov, S. J. Elledge, E. van Nimwegen, M. B. Stadler and M. Bentires-Alj (2012). "Tyrosine phosphatase SHP2 promotes breast cancer progression and maintains tumor-initiating cells via activation of key transcription factors and a positive feedback signaling loop." Nat Med **18**(4): 529-537.

Adachi, M., M. Sekiya, T. Miyachi, K. Matsuno, Y. Hinoda, K. Imai and A. Yachi (1992). "Molecular cloning of a novel protein-tyrosine phosphatase SH-PTP3 with sequence similarity to the src-homology region 2." FEBS Lett **314**(3): 335-339.

Ahmad, S., D. Banville, Z. Zhao, E. H. Fischer and S. H. Shen (1993). "A widely expressed human protein-tyrosine phosphatase containing src homology 2 domains." Proc Natl Acad Sci U S A **90**(6): 2197-2201.

Ahmed, T. A., C. Adamopoulos, Z. Karoulia, X. Wu, R. Sachidanandam, S. A. Aaronson and P. I. Poulikakos (2019). "SHP2 Drives Adaptive Resistance to ERK Signaling Inhibition in Molecularly Defined Subsets of ERK-Dependent Tumors." Cell Rep **26**(1): 65-78 e65.

Ahn, R., V. Sabourin, A. M. Bolt, S. Hebert, S. Totten, N. De Jay, M. C. Festa, Y. K. Young, Y. K. Im, T. Pawson, A. E. Koromilas, W. J. Muller, K. K. Mann, C. L. Kleinman and J. Ursini-Siegel (2017). "The Shc1 adaptor simultaneously balances Stat1 and Stat3 activity to promote breast cancer immune suppression." Nat Commun **8**: 14638.

Aihara, T., I. Yokota, Y. Hozumi, K. Aogi, H. Iwata, M. Tamura, A. Fukuuchi, H. Makino, R. Kim, M. Andoh, K. Tsugawa, S. Ohno, T. Yamaguchi, Y. Ohashi, T. Watanabe, Y. Takatsuka and H. Mukai (2014). "Anastrozole versus tamoxifen as adjuvant therapy for Japanese postmenopausal patients with hormone-responsive breast cancer: efficacy results of long-term follow-up data from the N-SAS BC 03 trial." Breast Cancer Res Treat **148**(2): 337-343.

Al-Hajj, M., M. S. Wicha, A. Benito-Hernandez, S. J. Morrison and M. F. Clarke (2003). "Prospective identification of tumorigenic breast cancer cells." Proc Natl Acad Sci U S A **100**(7): 3983-3988.

Alajati, A., N. Sausgruber, N. Aceto, S. Duss, S. Sarret, H. Voshol, D. Bonenfant and M. Bentires-Alj (2013). "Mammary tumor formation and metastasis evoked by a HER2 splice variant." Cancer Res **73**(17): 5320-5327.

Andersen, J. N., O. H. Mortensen, G. H. Peters, P. G. Drake, L. F. Iversen, O. H. Olsen, P. G. Jansen, H. S. Andersen, N. K. Tonks and N. P. Moller (2001). "Structural and evolutionary relationships among protein tyrosine phosphatase domains." Mol Cell Biol **21**(21): 7117-7136.

Arimidex, T. A. o. i. C. T. G., J. F. Forbes, J. Cuzick, A. Buzdar, A. Howell, J. S. Tobias and M. Baum (2008). "Effect of anastrozole and tamoxifen as adjuvant treatment for early-stage breast cancer: 100-month analysis of the ATAC trial." Lancet Oncol **9**(1): 45-53.

Arpino, G., L. Wiechmann, C. K. Osborne and R. Schiff (2008). "Crosstalk between the estrogen receptor and the HER tyrosine kinase receptor family: molecular mechanism and clinical implications for endocrine therapy resistance." Endocr Rev **29**(2): 217-233.

Arrandale, J. M., A. Gore-Willse, S. Rocks, J. M. Ren, J. Zhu, A. Davis, J. N. Livingston and D. U. Rabin (1996). "Insulin signaling in mice expressing reduced levels of Syp." J Biol Chem **271**(35): 21353-21358.

Auger, K. R., L. A. Serunian, S. P. Soltoff, P. Libby and L. C. Cantley (1989). "PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells." Cell **57**(1): 167-175.

Bachman, K. E., P. Argani, Y. Samuels, N. Silliman, J. Ptak, S. Szabo, H. Konishi, B. Karakas, B. G. Blair, C. Lin, B. A. Peters, V. E. Velculescu and B. H. Park (2004). "The PIK3CA gene is mutated with high frequency in human breast cancers." Cancer Biol Ther **3**(8): 772-775.

Backer, J. M. (2016). "The intricate regulation and complex functions of the Class III phosphoinositide 3-kinase Vps34." Biochem J **473**(15): 2251-2271.

Banerji, S., K. Cibulskis, C. Rangel-Escareno, K. K. Brown, S. L. Carter, A. M. Frederick, M. S. Lawrence, A. Y. Sivachenko, C. Sougnez, L. Zou, M. L. Cortes, J. C. Fernandez-Lopez, S. Peng, K. G. Ardlie, D. Auclair, V. Bautista-Pina, F. Duke, J. Francis, J. Jung, A. Maffuz-Aziz, R. C. Onofrio, M. Parkin, N. H. Pho, V. Quintanar-Jurado, A. H. Ramos, R. Rebollar-Vega, S. Rodriguez-Cuevas, S. L. Romero-Cordoba, S. E. Schumacher, N. Stransky, K. M. Thompson, L. Uribe-Figueroa, J. Baselga, R. Beroukhim, K. Polyak, D. C. Sgroi, A. L. Richardson, G. Jimenez-Sanchez, E. S. Lander, S. B. Gabriel, L. A. Garraway, T. R. Golub, J. Melendez-Zajgla, A. Toker, G. Getz, A. Hidalgo-Miranda and M. Meyerson (2012). "Sequence analysis of mutations and translocations across breast cancer subtypes." Nature **486**(7403): 405-409.

Barbareschi, M., F. Buttitta, L. Felicioni, S. Cotrupi, F. Barassi, M. Del Grammastro, A. Ferro, P. Dalla Palma, E. Galligioni and A. Marchetti (2007). "Different prognostic roles of mutations in the helical and kinase domains of the PIK3CA gene in breast carcinomas." Clin Cancer Res **13**(20): 6064-6069.

Bard-Chapeau, E. A., S. Li, J. Ding, S. S. Zhang, H. H. Zhu, F. Princen, D. D. Fang, T. Han, B. Bailly-Maitre, V. Poli, N. M. Varki, H. Wang and G. S. Feng (2011). "Ptpn11/Shp2 acts as a tumor suppressor in hepatocellular carcinogenesis." Cancer Cell **19**(5): 629-639.

Baselga, J., S. A. Im, H. Iwata, J. Cortes, M. De Laurentiis, Z. Jiang, C. L. Arteaga, W. Jonat, M. Clemons, Y. Ito, A. Awada, S. Chia, A. Jagiello-Gruszfeld, B. Pistilli, L. M. Tseng, S. Hurvitz, N. Masuda, M. Takahashi, P. Vuylsteke, S. Hachemi, B. Dharan, E. Di Tomaso, P. Urban, C. Massacesi and M. Campone (2017). "Buparlisib plus fulvestrant versus placebo plus fulvestrant in postmenopausal, hormone receptor-positive, HER2-negative, advanced breast cancer (BELLE-2): a randomised, double-blind, placebo-controlled, phase 3 trial." Lancet Oncol **18**(7): 904-916.

Baselga, J., E. A. Perez, T. Pienkowski and R. Bell (2006). "Adjuvant trastuzumab: a milestone in the treatment of HER-2-positive early breast cancer." Oncologist **11 Suppl 1**: 4-12.

Baselga, J. and S. M. Swain (2010). "CLEOPATRA: a phase III evaluation of pertuzumab and trastuzumab for HER2-positive metastatic breast cancer." Clin Breast Cancer **10**(6): 489-491.

Baxter, R. M., J. P. Secrist, R. R. Vaillancourt and A. Kazlauskas (1998). "Full activation of the platelet-derived growth factor beta-receptor kinase involves multiple events." J Biol Chem **273**(27): 17050-17055.

Bellacosa, A., D. de Feo, A. K. Godwin, D. W. Bell, J. Q. Cheng, D. A. Altomare, M. Wan, L. Dubeau, G. Scambia, V. Masciullo, G. Ferrandina, P. Benedetti Panici, S. Mancuso, G. Neri and J. R. Testa (1995). "Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas." Int J Cancer **64**(4): 280-285.

Bendell, J. C., J. Rodon, H. A. Burris, M. de Jonge, J. Verweij, D. Birle, D. Demanse, S. S. De Buck, Q. C. Ru, M. Peters, M. Goldbrunner and J. Baselga (2012). "Phase I, dose-escalation study of BKM120, an oral pan-Class I PI3K inhibitor, in patients with advanced solid tumors." J Clin Oncol **30**(3): 282-290.

Benistant, C., H. Chapuis and S. Roche (2000). "A specific function for phosphatidylinositol 3-kinase alpha (p85alpha-p110alpha) in cell survival and for phosphatidylinositol 3-kinase beta (p85alpha-p110beta) in de novo DNA synthesis of human colon carcinoma cells." Oncogene **19**(44): 5083-5090.

Bentires-Alj, M., S. G. Gil, R. Chan, Z. C. Wang, Y. Wang, N. Imanaka, L. N. Harris, A. Richardson, B. G. Neel and H. Gu (2006). "A role for the scaffolding adapter GAB2 in breast cancer." Nat Med **12**(1): 114-121.

Bentires-Alj, M., J. G. Paez, F. S. David, H. Keilhack, B. Halmos, K. Naoki, J. M. Maris, A. Richardson, A. Bardelli, D. J. Sugarbaker, W. G. Richards, J. Du, L. Girard, J. D. Minna, M. L. Loh, D. E. Fisher, V. E. Velculescu, B. Vogelstein, M. Meyerson, W. R. Sellers and B. G. Neel (2004). "Activating mutations of the noonan syndrome-associated SHP2/PTPN11 gene in human solid tumors and adult acute myelogenous leukemia." Cancer Res **64**(24): 8816-8820.

Berchtold, S., S. Volarevic, R. Moriggl, M. Mercep and B. Groner (1998). "Dominant negative variants of the SHP-2 tyrosine phosphatase inhibit prolactin activation of Jak2 (janus kinase 2) and induction of Stat5 (signal transducer and activator of transcription 5)-dependent transcription." Mol Endocrinol **12**(4): 556-567.

Blume-Jensen, P. and T. Hunter (2001). "Oncogenic kinase signalling." Nature **411**(6835): 355-365.

Bolondi, L., A. Craxi, F. Trevisani, B. Daniele, G. G. Di Costanzo, S. Fagiuoli, C. Camma, P. Bruzzi, R. Danesi, F. Spandonaro, C. Boni, A. Santoro and M. Colombo (2015). "Refining sorafenib therapy: lessons from clinical practice." Future Oncol **11**(3): 449-465.

Bonapace, L., M. M. Coissieux, J. Wyckoff, K. D. Mertz, Z. Varga, T. Junt and M. Bentires-Alj (2014). "Cessation of CCL2 inhibition accelerates breast cancer metastasis by promoting angiogenesis." Nature **515**(7525): 130-133.

Brandao, T. A., A. C. Hengge and S. J. Johnson (2010). "Insights into the reaction of protein-tyrosine phosphatase 1B: crystal structures for transition state analogs of both catalytic steps." J Biol Chem **285**(21): 15874-15883.

Brastianos, P. K., S. L. Carter, S. Santagata, D. P. Cahill, A. Taylor-Weiner, R. T. Jones, E. M. Van Allen, M. S. Lawrence, P. M. Horowitz, K. Cibulskis, K. L. Ligon, J. Tabernero, J. Seoane, E. Martinez-Saez, W. T. Curry, I. F. Dunn, S. H. Paek, S. H. Park, A. McKenna, A. Chevalier, M. Rosenberg, F. G. Barker, 2nd, C. M. Gill, P. Van Hummelen, A. R. Thorner, B. E. Johnson, M. P. Hoang, T. K. Choueiri, S. Signoretti, C. Sougnez, M. S. Rabin, N. U. Lin, E. P. Winer, A. Stemmer-Rachamimov, M. Meyerson, L. Garraway, S. Gabriel, E. S. Lander, R. Beroukhim, T. T. Batchelor, J. Baselga, D. N. Louis, G. Getz and W. C. Hahn (2015). "Genomic Characterization of Brain Metastases Reveals Branched Evolution and Potential Therapeutic Targets." Cancer Discov **5**(11): 1164-1177.

Britschgi, A., R. Andraos, H. Brinkhaus, I. Klebba, V. Romanet, U. Muller, M. Murakami, T. Radimerski and M. Bentires-Alj (2012). "JAK2/STAT5 inhibition circumvents resistance to PI3K/mTOR blockade: a rationale for cotargeting these pathways in metastatic breast cancer." Cancer Cell **22**(6): 796-811.

Bunda, S., K. Burrell, P. Heir, L. Zeng, A. Alamsahebpour, Y. Kano, B. Raught, Z. Y. Zhang, G. Zadeh and M. Ohh (2015). "Inhibition of SHP2-mediated dephosphorylation of Ras suppresses oncogenesis." Nat Commun **6**: 8859.

Bunney, T. D. and M. Katan (2010). "Phosphoinositide signalling in cancer: beyond PI3K and PTEN." Nat Rev Cancer **10**(5): 342-352.

Burstein, H. J., Y. Sun, L. Y. Dirix, Z. Jiang, R. Paridaens, A. R. Tan, A. Awada, A. Ranade, S. Jiao, G. Schwartz, R. Abbas, C. Powell, K. Turnbull, J. Vermette, C. Zacharchuk and R. Badwe (2010). "Neratinib, an irreversible ErbB receptor tyrosine kinase inhibitor, in patients with advanced ErbB2-positive breast cancer." J Clin Oncol **28**(8): 1301-1307.

Cancer Genome Atlas, N. (2012). "Comprehensive molecular portraits of human breast tumours." Nature **490**(7418): 61-70.

Cardoso, F., A. Costa, E. Senkus, M. Aapro, F. Andre, C. H. Barrios, J. Bergh, G. Bhattacharyya, L. Biganzoli, M. J. Cardoso, L. Carey, D. Corneliussen-James, G. Curigliano, V. Dieras, N. El Saghir, A. Eniu, L. Fallowfield, D. Fenech, P. Francis, K. Gelmon, A. Gennari, N. Harbeck, C. Hudis, B. Kaufman, I. Krop, M. Mayer, H. Meijer, S. Mertz, S. Ohno, O. Pagani, E. Papadopoulos, F. Peccatori, F. Penault-Llorca, M. J. Piccart, J. Y. Pierga, H. Rugo, L. Shockney, G. Sledge, S. Swain, C. Thomssen, A. Tutt, D. Vorobiof, B. Xu, L. Norton and E. Winer (2017). "3rd ESO-ESMO International Consensus Guidelines for Advanced Breast Cancer (ABC 3)." Ann Oncol **28**(1): 16-33.

Carey, L. A., C. M. Perou, C. A. Livasy, L. G. Dressler, D. Cowan, K. Conway, G. Karaca, M. A. Troester, C. K. Tse, S. Edmiston, S. L. Deming, J. Geradts, M. C. Cheang, T. O. Nielsen, P. G. Moorman, H. S. Earp and R. C. Millikan (2006). "Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study." JAMA **295**(21): 2492-2502.

Carpten, J. D., A. L. Faber, C. Horn, G. P. Donoho, S. L. Briggs, C. M. Robbins, G. Hostetter, S. Boguslawski, T. Y. Moses, S. Savage, M. Uhlik, A. Lin, J. Du, Y. W. Qian, D. J. Zeckner, G. Tucker-Kellogg, J. Touchman, K. Patel, S. Mousses, M. Bittner, R. Schevitz, M. H. Lai, K.

L. Blanchard and J. E. Thomas (2007). "A transforming mutation in the pleckstrin homology domain of AKT1 in cancer." Nature **448**(7152): 439-444.

Carracedo, A., L. Ma, J. Teruya-Feldstein, F. Rojo, L. Salmena, A. Alimonti, A. Egia, A. T. Sasaki, G. Thomas, S. C. Kozma, A. Papa, C. Nardella, L. C. Cantley, J. Baselga and P. P. Pandolfi (2008). "Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer." J Clin Invest **118**(9): 3065-3074.

Chakrabarty, A., V. Sanchez, M. G. Kuba, C. Rinehart and C. L. Arteaga (2012). "Feedback upregulation of HER3 (ErbB3) expression and activity attenuates antitumor effect of PI3K inhibitors." Proc Natl Acad Sci U S A **109**(8): 2718-2723.

Chan, G., L. S. Cheung, W. Yang, M. Milyavsky, A. D. Sanders, S. Gu, W. X. Hong, A. X. Liu, X. Wang, M. Barbara, T. Sharma, J. Gavin, J. L. Kutok, N. N. Iscove, K. M. Shannon, J. E. Dick, B. G. Neel and B. S. Braun (2011). "Essential role for Ptpn11 in survival of hematopoietic stem and progenitor cells." Blood **117**(16): 4253-4261.

Chan, G., D. Kalaitzidis and B. G. Neel (2008). "The tyrosine phosphatase Shp2 (PTPN11) in cancer." Cancer Metastasis Rev **27**(2): 179-192.

Chan, R. J. and G. S. Feng (2007). "PTPN11 is the first identified proto-oncogene that encodes a tyrosine phosphatase." Blood **109**(3): 862-867.

Chandarlapaty, S., A. Sawai, M. Scaltriti, V. Rodrik-Outmezguine, O. Grbovic-Huezo, V. Serra, P. K. Majumder, J. Baselga and N. Rosen (2011). "AKT inhibition relieves feedback suppression of receptor tyrosine kinase expression and activity." Cancer Cell **19**(1): 58-71.

Chapman, J. R., A. C. Webster and G. Wong (2013). "Cancer in the transplant recipient." Cold Spring Harb Perspect Med **3**(7).

Chen, I. C., L. P. Hsiao, I. W. Huang, H. C. Yu, L. C. Yeh, C. H. Lin, T. Wei-Wu Chen, A. L. Cheng and Y. S. Lu (2017). "Phosphatidylinositol-3 Kinase Inhibitors, Buparlisib and Alpelisib, Sensitize Estrogen Receptor-positive Breast Cancer Cells to Tamoxifen." Sci Rep **7**(1): 9842.

Chen, X., Z. Zheng, L. Chen and H. Zheng (2017). "MAPK, NFkappaB, and VEGF signaling pathways regulate breast cancer liver metastasis." Oncotarget **8**(60): 101452-101460.

Chen, Y. N., M. J. LaMarche, H. M. Chan, P. Fekkes, J. Garcia-Fortanet, M. G. Acker, B. Antonakos, C. H. Chen, Z. Chen, V. G. Cooke, J. R. Dobson, Z. Deng, F. Fei, B. Firestone, M. Fodor, C. Fridrich, H. Gao, D. Grunenfelder, H. X. Hao, J. Jacob, S. Ho, K. Hsiao, Z. B. Kang, R. Karki, M. Kato, J. Larrow, L. R. La Bonte, F. Lenoir, G. Liu, S. Liu, D. Majumdar, M. J. Meyer, M. Palermo, L. Perez, M. Pu, E. Price, C. Quinn, S. Shakya, M. D. Shultz, J. Slisz, K. Venkatesan, P. Wang, M. Warmuth, S. Williams, G. Yang, J. Yuan, J. H. Zhang, P. Zhu, T. Ramsey, N. J. Keen, W. R. Sellers, T. Stams and P. D. Fortin (2016). "Allosteric inhibition of SHP2 phosphatase inhibits cancers driven by receptor tyrosine kinases." Nature **535**(7610): 148-152.

Cheng, J. Q., B. Ruggeri, W. M. Klein, G. Sonoda, D. A. Altomare, D. K. Watson and J. R. Testa (1996). "Amplification of AKT2 in human pancreatic cells and inhibition of AKT2 expression and tumorigenicity by antisense RNA." Proc Natl Acad Sci U S A **93**(8): 3636-3641.

Cheung, L. W., B. T. Hennessy, J. Li, S. Yu, A. P. Myers, B. Djordjevic, Y. Lu, K. Stemke-Hale, M. D. Dyer, F. Zhang, Z. Ju, L. C. Cantley, S. E. Scherer, H. Liang, K. H. Lu, R. R. Broaddus and G. B. Mills (2011). "High frequency of PIK3R1 and PIK3R2 mutations in endometrial cancer elucidates a novel mechanism for regulation of PTEN protein stability." Cancer Discov **1**(2): 170-185.

Chiang, A. C. and J. Massague (2008). "Molecular basis of metastasis." N Engl J Med **359**(26): 2814-2823.

Chien, M. H., L. M. Lee, M. Hsiao, L. H. Wei, C. H. Chen, T. C. Lai, K. T. Hua, M. W. Chen, C. M. Sun and M. L. Kuo (2013). "Inhibition of Metastatic Potential in Breast Carcinoma In Vivo and In Vitro through Targeting VEGFRs and FGFRs." Evid Based Complement Alternat Med **2013**: 718380.

Chikh, A., R. Ferro, J. J. Abbott, R. Pineiro, R. Buus, M. Iezzi, F. Ricci, D. Bergamaschi, P. Ostano, G. Chiorino, R. Lattanzio, M. Broggini, M. Piantelli, T. Maffucci and M. Falasca (2016). "Class II phosphoinositide 3-kinase C2beta regulates a novel signaling pathway involved in breast cancer progression." Oncotarget **7**(14): 18325-18345.

Ciraolo, E., M. Iezzi, R. Marone, S. Marengo, C. Curcio, C. Costa, O. Azzolino, C. Gonella, C. Rubinetto, H. Wu, W. Dastru, E. L. Martin, L. Silengo, F. Altruda, E. Turco, L. Lanzetti, P. Musiani, T. Ruckle, C. Rommel, J. M. Backer, G. Forni, M. P. Wymann and E. Hirsch (2008). "Phosphoinositide 3-kinase p110beta activity: key role in metabolism and mammary gland cancer but not development." Sci Signal **1**(36): ra3.

Clarke, R. (1997). "Issues in experimental design and endpoint analysis in the study of experimental cytotoxic agents in vivo in breast cancer and other models." Breast Cancer Res Treat **46**(2-3): 255-278.

Cohen, P. (2000). "The regulation of protein function by multisite phosphorylation--a 25 year update." Trends Biochem Sci **25**(12): 596-601.

Cohen, P. (2002). "Protein kinases--the major drug targets of the twenty-first century?" Nat Rev Drug Discov **1**(4): 309-315.

Cole, M. P., C. T. Jones and I. D. Todd (1971). "A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI46474." Br J Cancer **25**(2): 270-275.

Costa, R. L. B., H. S. Han and W. J. Gradishar (2018). "Targeting the PI3K/AKT/mTOR pathway in triple-negative breast cancer: a review." Breast Cancer Res Treat **169**(3): 397-406.

Croxtall, J. D. and K. McKeage (2011). "Fulvestrant: a review of its use in the management of hormone receptor-positive metastatic breast cancer in postmenopausal women." Drugs **71**(3): 363-380.

Cunnick, J. M., L. Mei, C. A. Doupnik and J. Wu (2001). "Phosphotyrosines 627 and 659 of Gab1 constitute a bisphosphoryl tyrosine-based activation motif (BTAM) conferring binding and activation of SHP2." J Biol Chem **276**(26): 24380-24387.

Curtis, C., S. P. Shah, S. F. Chin, G. Turashvili, O. M. Rueda, M. J. Dunning, D. Speed, A. G. Lynch, S. Samarajiwa, Y. Yuan, S. Graf, G. Ha, G. Haffari, A. Bashashati, R. Russell, S. McKinney, M. Group, A. Langerod, A. Green, E. Provenzano, G. Wishart, S. Pinder, P.

Watson, F. Markowetz, L. Murphy, I. Ellis, A. Purushotham, A. L. Borresen-Dale, J. D. Brenton, S. Tavare, C. Caldas and S. Aparicio (2012). "The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups." Nature **486**(7403): 346-352.

Dagogo-Jack, I. and A. T. Shaw (2018). "Tumour heterogeneity and resistance to cancer therapies." Nat Rev Clin Oncol **15**(2): 81-94.

Dardaei, L., H. Q. Wang, M. Singh, P. Fordjour, K. X. Shaw, S. Yoda, G. Kerr, K. Yu, J. Liang, Y. Cao, Y. Chen, M. S. Lawrence, A. Langenbucher, J. F. Gainor, L. Friboulet, I. Dagogo-Jack, D. T. Myers, E. Labrot, D. Ruddy, M. Parks, D. Lee, R. H. DiCecca, S. Moody, H. Hao, M. Mohseni, M. LaMarche, J. Williams, K. Hoffmaster, G. Caponigro, A. T. Shaw, A. N. Hata, C. H. Benes, F. Li and J. A. Engelman (2018). "SHP2 inhibition restores sensitivity in ALK-rearranged non-small-cell lung cancer resistant to ALK inhibitors." Nat Med **24**(4): 512-517.

Davies, C., H. Pan, J. Godwin, R. Gray, R. Arriagada, V. Raina, M. Abraham, V. H. Medeiros Alencar, A. Badran, X. Bonfill, J. Bradbury, M. Clarke, R. Collins, S. R. Davis, A. Delmestri, J. F. Forbes, P. Haddad, M. F. Hou, M. Inbar, H. Khaled, J. Kielanowska, W. H. Kwan, B. S. Mathew, I. Mittra, B. Muller, A. Nicolucci, O. Peralta, F. Pernas, L. Petruzelka, T. Pienkowski, R. Radhika, B. Rajan, M. T. Rubach, S. Tort, G. Urrutia, M. Valentini, Y. Wang, R. Peto and G. Adjuvant Tamoxifen: Longer Against Shorter Collaborative (2013). "Long-term effects of continuing adjuvant tamoxifen to 10 years versus stopping at 5 years after diagnosis of oestrogen receptor-positive breast cancer: ATLAS, a randomised trial." Lancet **381**(9869): 805-816.

Dent, R., W. M. Hanna, M. Trudeau, E. Rawlinson, P. Sun and S. A. Narod (2009). "Pattern of metastatic spread in triple-negative breast cancer." Breast Cancer Res Treat **115**(2): 423-428.

DeRose, Y. S., G. Wang, Y. C. Lin, P. S. Bernard, S. S. Buys, M. T. Ebbert, R. Factor, C. Matsen, B. A. Milash, E. Nelson, L. Neumayer, R. L. Randall, I. J. Stijleman, B. E. Welm and A. L. Welm (2011). "Tumor grafts derived from women with breast cancer authentically reflect tumor pathology, growth, metastasis and disease outcomes." Nat Med **17**(11): 1514-1520.

Di Cosimo, S. and J. Baselga (2010). "Management of breast cancer with targeted agents: importance of heterogeneity. [corrected]." Nat Rev Clin Oncol **7**(3): 139-147.

Di Leo, A., S. Johnston, K. S. Lee, E. Ciruelos, P. E. Lonning, W. Janni, R. O'Regan, M. A. Mouret-Reynier, D. Kalev, D. Egle, T. Csoszi, R. Bordonaro, T. Decker, V. C. G. Tjan-Heijnen, S. Blau, A. Schirone, D. Weber, M. El-Hashimy, B. Dharan, D. Sellami and T. Bachelot (2018). "Buparlisib plus fulvestrant in postmenopausal women with hormone-receptor-positive, HER2-negative, advanced breast cancer progressing on or after mTOR inhibition (BELLE-3): a randomised, double-blind, placebo-controlled, phase 3 trial." Lancet Oncol **19**(1): 87-100.

Digilio, M. C., E. Conti, A. Sarkozy, R. Mingarelli, T. Dottorini, B. Marino, A. Pizzuti and B. Dallapiccola (2002). "Grouping of multiple-lentigines/LEOPARD and Noonan syndromes on the PTPN11 gene." Am J Hum Genet **71**(2): 389-394.

Dobin, A., C. A. Davis, F. Schlesinger, J. Drenkow, C. Zaleski, S. Jha, P. Batut, M. Chaisson and T. R. Gingeras (2013). "STAR: ultrafast universal RNA-seq aligner." Bioinformatics **29**(1): 15-21.

Drake, J. M., J. K. Lee and O. N. Witte (2014). "Clinical targeting of mutated and wild-type protein tyrosine kinases in cancer." Mol Cell Biol **34**(10): 1722-1732.

Dreyling, M., A. Santoro, L. Mollica, S. Leppa, G. A. Follows, G. Lenz, W. S. Kim, A. Nagler, P. Panayiotidis, J. Demeter, M. Ozcan, M. Kosinova, K. Bouabdallah, F. Morschhauser, D. A. Stevens, D. Trevarthen, M. Giurescu, L. Cupit, L. Liu, K. Kochert, H. Seidel, C. Pena, S. Yin, F. Hiemeyer, J. Garcia-Vargas, B. H. Childs and P. L. Zinzani (2017). "Phosphatidylinositol 3-Kinase Inhibition by Copanlisib in Relapsed or Refractory Indolent Lymphoma." J Clin Oncol **35**(35): 3898-3905.

Du, Z. and C. M. Lovly (2018). "Mechanisms of receptor tyrosine kinase activation in cancer." Mol Cancer **17**(1): 58.

Duan, G. and D. Walther (2015). "The roles of post-translational modifications in the context of protein interaction networks." PLoS Comput Biol **11**(2): e1004049.

Early Breast Cancer Trialists' Collaborative, G. (2005). "Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials." Lancet **365**(9472): 1687-1717.

Early Breast Cancer Trialists' Collaborative, G. (2015). "Adjuvant bisphosphonate treatment in early breast cancer: meta-analyses of individual patient data from randomised trials." Lancet **386**(10001): 1353-1361.

Edouard, T., A. Montagner, M. Dance, F. Conte, A. Yart, B. Parfait, M. Tauber, J. P. Salles and P. Raynal (2007). "How do Shp2 mutations that oppositely influence its biochemical activity result in syndromes with overlapping symptoms?" Cell Mol Life Sci **64**(13): 1585-1590.

Elkabets, M., S. Vora, D. Juric, N. Morse, M. Mino-Kenudson, T. Muranen, J. Tao, A. B. Campos, J. Rodon, Y. H. Ibrahim, V. Serra, V. Rodrik-Outmezguine, S. Hazra, S. Singh, P. Kim, C. Quadt, M. Liu, A. Huang, N. Rosen, J. A. Engelman, M. Scaltriti and J. Baselga (2013). "mTORC1 inhibition is required for sensitivity to PI3K p110alpha inhibitors in PIK3CA-mutant breast cancer." Sci Transl Med **5**(196): 196ra199.

Elsawaf, Z. and H. P. Sinn (2011). "Triple-Negative Breast Cancer: Clinical and Histological Correlations." Breast Care (Basel) **6**(4): 273-278.

Esserman, L. J., D. A. Berry, M. C. Cheang, C. Yau, C. M. Perou, L. Carey, A. DeMichele, J. W. Gray, K. Conway-Dorsey, M. E. Lenburg, M. B. Buxton, S. E. Davis, L. J. van't Veer, C. Hudis, K. Chin, D. Wolf, H. Krontiras, L. Montgomery, D. Tripathy, C. Lehman, M. C. Liu, O. I. Olopade, H. S. Rugo, J. T. Carpenter, C. Livasy, L. Dressler, D. Chhieng, B. Singh, C. Mies, J. Rabban, Y. Y. Chen, D. Giri, A. Au, N. Hylton and I. S. T. Investigators (2012). "Chemotherapy response and recurrence-free survival in neoadjuvant breast cancer depends on biomarker profiles: results from the I-SPY 1 TRIAL (CALGB 150007/150012; ACRIN 6657)." Breast Cancer Res Treat **132**(3): 1049-1062.

Eyles, J., A. L. Puaux, X. Wang, B. Toh, C. Prakash, M. Hong, T. G. Tan, L. Zheng, L. C. Ong, Y. Jin, M. Kato, A. Prevost-Blondel, P. Chow, H. Yang and J. P. Abastado (2010). "Tumor cells disseminate early, but immunosurveillance limits metastatic outgrowth, in a mouse model of melanoma." J Clin Invest **120**(6): 2030-2039.

Fedele, C., H. Ran, B. Diskin, W. Wei, J. Jen, M. J. Geer, K. Araki, U. Ozerdem, D. M. Simeone, G. Miller, B. G. Neel and K. H. Tang (2018). "SHP2 Inhibition Prevents Adaptive Resistance to MEK Inhibitors in Multiple Cancer Models." Cancer Discov.

Feng, G. S., C. C. Hui and T. Pawson (1993). "SH2-containing phosphotyrosine phosphatase as a target of protein-tyrosine kinases." Science **259**(5101): 1607-1611.

Ficarro, S. B., M. L. McCleland, P. T. Stukenberg, D. J. Burke, M. M. Ross, J. Shabanowitz, D. F. Hunt and F. M. White (2002). "Phosphoproteome analysis by mass spectrometry and its application to Saccharomyces cerevisiae." Nat Biotechnol **20**(3): 301-305.

Finn, R. S., J. P. Crown, I. Lang, K. Boer, I. M. Bondarenko, S. O. Kulyk, J. Ettl, R. Patel, T. Pinter, M. Schmidt, Y. Shparyk, A. R. Thummala, N. L. Voytko, C. Fowst, X. Huang, S. T. Kim, S. Randolph and D. J. Slamon (2015). "The cyclin-dependent kinase 4/6 inhibitor palbociclib in combination with letrozole versus letrozole alone as first-line treatment of oestrogen receptor-positive, HER2-negative, advanced breast cancer (PALOMA-1/TRIO-18): a randomised phase 2 study." Lancet Oncol **16**(1): 25-35.

Finn, R. S., M. Martin, H. S. Rugo, S. Jones, S. A. Im, K. Gelmon, N. Harbeck, O. N. Lipatov, J. M. Walshe, S. Moulder, E. Gauthier, D. R. Lu, S. Randolph, V. Dieras and D. J. Slamon (2016). "Palbociclib and Letrozole in Advanced Breast Cancer." N Engl J Med **375**(20): 1925-1936.

Fischer, K. R., A. Durrans, S. Lee, J. Sheng, F. Li, S. T. Wong, H. Choi, T. El Rayes, S. Ryu, J. Troeger, R. F. Schwabe, L. T. Vahdat, N. K. Altorki, V. Mittal and D. Gao (2015). "Epithelial-to-mesenchymal transition is not required for lung metastasis but contributes to chemoresistance." Nature **527**(7579): 472-476.

Freeman, R. M., Jr., J. Plutzky and B. G. Neel (1992). "Identification of a human src homology 2-containing protein-tyrosine-phosphatase: a putative homolog of Drosophila corkscrew." Proc Natl Acad Sci U S A **89**(23): 11239-11243.

Fruman, D. A., H. Chiu, B. D. Hopkins, S. Bagrodia, L. C. Cantley and R. T. Abraham (2017). "The PI3K Pathway in Human Disease." Cell **170**(4): 605-635.

Furman, R. R., J. P. Sharman, S. E. Coutre, B. D. Cheson, J. M. Pagel, P. Hillmen, J. C. Barrientos, A. D. Zelenetz, T. J. Kipps, I. Flinn, P. Ghia, H. Eradat, T. Ervin, N. Lamanna, B. Coiffier, A. R. Pettitt, S. Ma, S. Stilgenbauer, P. Cramer, M. Aiello, D. M. Johnson, L. L. Miller, D. Li, T. M. Jahn, R. D. Dansey, M. Hallek and S. M. O'Brien (2014). "Idelalisib and rituximab in relapsed chronic lymphocytic leukemia." N Engl J Med **370**(11): 997-1007.

Gampenrieder, S. P., G. Rinnerthaler and R. Greil (2013). "Neoadjuvant chemotherapy and targeted therapy in breast cancer: past, present, and future." J Oncol **2013**: 732047.

Gao, H., J. M. Korn, S. Ferretti, J. E. Monahan, Y. Wang, M. Singh, C. Zhang, C. Schnell, G. Yang, Y. Zhang, O. A. Balbin, S. Barbe, H. Cai, F. Casey, S. Chatterjee, D. Y. Chiang, S. Chuai, S. M. Cogan, S. D. Collins, E. Dammassa, N. Ebel, M. Embry, J. Green, A. Kauffmann, C. Kowal, R. J. Leary, J. Lehar, Y. Liang, A. Loo, E. Lorenzana, E. Robert McDonald, 3rd, M. E. McLaughlin, J. Merkin, R. Meyer, T. L. Naylor, M. Patawaran, A. Reddy, C. Roelli, D. A. Ruddy, F. Salangsang, F. Santacroce, A. P. Singh, Y. Tang, W. Tinetto, S. Tobler, R. Velazquez, K. Venkatesan, F. Von Arx, H. Q. Wang, Z. Wang, M. Wiesmann, D. Wyss, F. Xu,

H. Bitter, P. Atadja, E. Lees, F. Hofmann, E. Li, N. Keen, R. Cozens, M. R. Jensen, N. K. Pryer, J. A. Williams and W. R. Sellers (2015). "High-throughput screening using patient-derived tumor xenografts to predict clinical trial drug response." Nat Med **21**(11): 1318-1325.

Garcia Fortanet, J., C. H. Chen, Y. N. Chen, Z. Chen, Z. Deng, B. Firestone, P. Fekkes, M. Fodor, P. D. Fortin, C. Fridrich, D. Grunenfelder, S. Ho, Z. B. Kang, R. Karki, M. Kato, N. Keen, L. R. LaBonte, J. Larrow, F. Lenoir, G. Liu, S. Liu, F. Lombardo, D. Majumdar, M. J. Meyer, M. Palermo, L. Perez, M. Pu, T. Ramsey, W. R. Sellers, M. D. Shultz, T. Stams, C. Towler, P. Wang, S. L. Williams, J. H. Zhang and M. J. LaMarche (2016). "Allosteric Inhibition of SHP2: Identification of a Potent, Selective, and Orally Efficacious Phosphatase Inhibitor." J Med Chem **59**(17): 7773-7782.

Gay, L. J. and B. Felding-Habermann (2011). "Contribution of platelets to tumour metastasis." Nat Rev Cancer **11**(2): 123-134.

Gehmert, S., S. Gehmert, L. Prantl, J. Vykoukal, E. Alt and Y. H. Song (2010). "Breast cancer cells attract the migration of adipose tissue-derived stem cells via the PDGF-BB/PDGFR-beta signaling pathway." Biochem Biophys Res Commun **398**(3): 601-605.

Giancotti, F. G. (2013). "Mechanisms governing metastatic dormancy and reactivation." Cell **155**(4): 750-764.

Gluck, S., J. S. Ross, M. Royce, E. F. McKenna, Jr., C. M. Perou, E. Avisar and L. Wu (2012). "TP53 genomics predict higher clinical and pathologic tumor response in operable early-stage breast cancer treated with docetaxel-capecitabine +/- trastuzumab." Breast Cancer Res Treat **132**(3): 781-791.

Gomes, E. G., S. F. Connelly and J. M. Summy (2013). "Targeting the yin and the yang: combined inhibition of the tyrosine kinase c-Src and the tyrosine phosphatase SHP-2 disrupts pancreatic cancer signaling and biology in vitro and tumor formation in vivo." Pancreas **42**(5): 795-806.

Groenendijk, F. H. and R. Bernards (2014). "Drug resistance to targeted therapies: deja vu all over again." Mol Oncol **8**(6): 1067-1083.

Gschwind, A., O. M. Fischer and A. Ullrich (2004). "The discovery of receptor tyrosine kinases: targets for cancer therapy." Nat Rev Cancer **4**(5): 361-370.

Gu, J., T. Han, R. H. Ma, Y. L. Zhu, Y. N. Jia, J. J. Du, Y. Chen, X. J. Jiang, X. D. Xie and X. Guo (2014). "SHP2 promotes laryngeal cancer growth through the Ras/Raf/Mek/Erk pathway and serves as a prognostic indicator for laryngeal cancer." Int J Oncol **44**(2): 481-490.

Gundem, G., P. Van Loo, B. Kremeyer, L. B. Alexandrov, J. M. C. Tubio, E. Papaemmanuil, D. S. Brewer, H. M. L. Kallio, G. Hognas, M. Annala, K. Kivinummi, V. Goody, C. Latimer, S. O'Meara, K. J. Dawson, W. Isaacs, M. R. Emmert-Buck, M. Nykter, C. Foster, Z. Kote-Jarai, D. Easton, H. C. Whitaker, I. P. Group, D. E. Neal, C. S. Cooper, R. A. Eeles, T. Visakorpi, P. J. Campbell, U. McDermott, D. C. Wedge and G. S. Bova (2015). "The evolutionary history of lethal metastatic prostate cancer." Nature **520**(7547): 353-357.

Gupta, G. P. and J. Massague (2006). "Cancer metastasis: building a framework." Cell **127**(4): 679-695.

Gusterson, B. and C. J. Eaves (2018). "Basal-like Breast Cancers: From Pathology to Biology and Back Again." Stem Cell Reports **10**(6): 1676-1686.

Han, T., D. M. Xiang, W. Sun, N. Liu, H. L. Sun, W. Wen, W. F. Shen, R. Y. Wang, C. Chen, X. Wang, Z. Cheng, H. Y. Li, M. C. Wu, W. M. Cong, G. S. Feng, J. Ding and H. Y. Wang (2015). "PTPN11/Shp2 overexpression enhances liver cancer progression and predicts poor prognosis of patients." J Hepatol **63**(3): 651-660.

Hanks, S. K. and T. Hunter (1995). "Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification." FASEB J **9**(8): 576-596.

Hanusch, C., A. Schneeweiss, S. Loibl, M. Untch, S. Paepke, S. Kummel, C. Jackisch, J. Huober, J. Hilfrich, B. Gerber, H. Eidtmann, C. Denkert, S. Costa, J. U. Blohmer, K. Engels, N. Burchardi and G. von Minckwitz (2015). "Dual Blockade with AFatinib and Trastuzumab as NEoadjuvant Treatment for Patients with Locally Advanced or Operable Breast Cancer Receiving Taxane-Anthracycline Containing Chemotherapy-DAFNE (GBG-70)." Clin Cancer Res **21**(13): 2924-2931.

Heitman, J., N. R. Movva and M. N. Hall (1991). "Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast." Science **253**(5022): 905-909.

Heldin, C. H. (2013). "Targeting the PDGF signaling pathway in tumor treatment." Cell Commun Signal **11**: 97.

Heldin, C. H., A. Ostman and L. Ronnstrand (1998). "Signal transduction via platelet-derived growth factor receptors." Biochim Biophys Acta **1378**(1): F79-113.

Heppner, G. H. (1984). "Tumor heterogeneity." Cancer research **44**: 2259-2265.

Heuberger, J., F. Kosel, J. Qi, K. S. Grossmann, K. Rajewsky and W. Birchmeier (2014). "Shp2/MAPK signaling controls goblet/paneth cell fate decisions in the intestine." Proc Natl Acad Sci U S A **111**(9): 3472-3477.

Hicks, J., A. Krasnitz, B. Lakshmi, N. E. Navin, M. Riggs, E. Leibu, D. Esposito, J. Alexander, J. Troge, V. Grubor, S. Yoon, M. Wigler, K. Ye, A. L. Borresen-Dale, B. Naume, E. Schlicting, L. Norton, T. Hagerstrom, L. Skoog, G. Auer, S. Maner, P. Lundin and A. Zetterberg (2006). "Novel patterns of genome rearrangement and their association with survival in breast cancer." Genome Res **16**(12): 1465-1479.

Hicks, M., E. R. Macrae, M. Abdel-Rasoul, R. Layman, S. Friedman, J. Querry, M. Lustberg, B. Ramaswamy, E. Mrozek, C. Shapiro and R. Wesolowski (2015). "Neoadjuvant dual HER2-targeted therapy with lapatinib and trastuzumab improves pathologic complete response in patients with early stage HER2-positive breast cancer: a meta-analysis of randomized prospective clinical trials." Oncologist **20**(4): 337-343.

Higashi, H., R. Tsutsumi, S. Muto, T. Sugiyama, T. Azuma, M. Asaka and M. Hatakeyama (2002). "SHP-2 tyrosine phosphatase as an intracellular target of Helicobacter pylori CagA protein." Science **295**(5555): 683-686.

Higuchi, M., R. Tsutsumi, H. Higashi and M. Hatakeyama (2004). "Conditional gene silencing utilizing the lac repressor reveals a role of SHP-2 in cagA-positive Helicobacter pylori pathogenicity." Cancer Sci **95**(5): 442-447.

Hirsch, D. S., Y. Shen, M. Dokmanovic and W. J. Wu (2010). "pp60c-Src phosphorylates and activates vacuolar protein sorting 34 to mediate cellular transformation." Cancer Res **70**(14): 5974-5983.

Hoadley, K. A., M. B. Siegel, K. L. Kanchi, C. A. Miller, L. Ding, W. Zhao, X. He, J. S. Parker, M. C. Wendl, R. S. Fulton, R. T. Demeter, R. K. Wilson, L. A. Carey, C. M. Perou and E. R. Mardis (2016). "Tumor Evolution in Two Patients with Basal-like Breast Cancer: A Retrospective Genomics Study of Multiple Metastases." PLoS Med **13**(12): e1002174.

Hof, P., S. Pluskey, S. Dhe-Paganon, M. J. Eck and S. E. Shoelson (1998). "Crystal structure of the tyrosine phosphatase SHP-2." Cell **92**(4): 441-450.

Holmgren, L., M. S. O'Reilly and J. Folkman (1995). "Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression." Nat Med **1**(2): 149-153.

Hong, Y., Z. Li and Q. Zhang (2016). "A circulating tumor cell cluster-based model for tumor metastasis (Hypothesis)." Oncol Lett **12**(6): 4891-4895.

Hu, Z., H. Fang, X. Wang, D. Chen, Z. Chen and S. Wang (2014). "Overexpression of SHP2 tyrosine phosphatase promotes the tumorigenesis of breast carcinoma." Oncol Rep **32**(1): 205-212.

Huang, D., F. Yang, Y. Wang and X. Guan (2017). "Mechanisms of resistance to selective estrogen receptor down-regulator in metastatic breast cancer." Biochim Biophys Acta **1868**(1): 148-156.

Hudes, G., M. Carducci, P. Tomczak, J. Dutcher, R. Figlin, A. Kapoor, E. Staroslawska, J. Sosman, D. McDermott, I. Bodrogi, Z. Kovacevic, V. Lesovoy, I. G. Schmidt-Wolf, O. Barbarash, E. Gokmen, T. O'Toole, S. Lustgarten, L. Moore, R. J. Motzer and A. T. Global (2007). "Temsirolimus, interferon alfa, or both for advanced renal-cell carcinoma." N Engl J Med **356**(22): 2271-2281.

Hunter, T. (1995). "Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling." Cell **80**(2): 225-236.

Hunter, T. (2000). "Signaling--2000 and beyond." Cell **100**(1): 113-127.

Hunter, T. (2009). "Tyrosine phosphorylation: thirty years and counting." Curr Opin Cell Biol **21**(2): 140-146.

Hunter, T. and J. A. Cooper (1985). "Protein-tyrosine kinases." Annual review of biochemistry **54**(1): 897-930.

Hunter, T. and B. M. Sefton (1980). "Transforming gene product of Rous sarcoma virus phosphorylates tyrosine." Proc Natl Acad Sci U S A **77**(3): 1311-1315.

Ilic, N., T. Utermark, H. R. Widlund and T. M. Roberts (2011). "PI3K-targeted therapy can be evaded by gene amplification along the MYC-eukaryotic translation initiation factor 4E (eIF4E) axis." Proc Natl Acad Sci U S A **108**(37): E699-708.

Ishida, H., S. Kogaki, J. Narita, H. Ichimori, N. Nawa, Y. Okada, K. Takahashi and K. Ozono (2011). "LEOPARD-type SHP2 mutant Gln510Glu attenuates cardiomyocyte differentiation and promotes cardiac hypertrophy via dysregulation of Akt/GSK-3beta/beta-catenin signaling." Am J Physiol Heart Circ Physiol **301**(4): H1531-1539.

Jaiswal, B. S., V. Janakiraman, N. M. Kljavin, S. Chaudhuri, H. M. Stern, W. Wang, Z. Kan, H. A. Dbouk, B. A. Peters, P. Waring, T. Dela Vega, D. M. Kenski, K. K. Bowman, M. Lorenzo, H. Li, J. Wu, Z. Modrusan, J. Stinson, M. Eby, P. Yue, J. S. Kaminker, F. J. de Sauvage, J. M. Backer and S. Seshagiri (2009). "Somatic mutations in p85alpha promote tumorigenesis through class IA PI3K activation." Cancer Cell **16**(6): 463-474.

Janku, F. (2017). "Phosphoinositide 3-kinase (PI3K) pathway inhibitors in solid tumors: From laboratory to patients." Cancer Treat Rev **59**: 93-101.

Janku, F., T. A. Yap and F. Meric-Bernstam (2018). "Targeting the PI3K pathway in cancer: are we making headway?" Nat Rev Clin Oncol **15**(5): 273-291.

Jhaveri, K., R. Wang, E. Teplinsky, S. Chandarlapaty, D. Solit, K. Cadoo, J. Speyer, G. D'Andrea, S. Adams, S. Patil, S. Haque, T. O'Neill, K. Friedman, F. J. Esteva, C. Hudis and S. Modi (2017). "A phase I trial of ganetespib in combination with paclitaxel and trastuzumab in patients with human epidermal growth factor receptor-2 (HER2)-positive metastatic breast cancer." Breast Cancer Res **19**(1): 89.

Jia, S., Z. Liu, S. Zhang, P. Liu, L. Zhang, S. H. Lee, J. Zhang, S. Signoretti, M. Loda, T. M. Roberts and J. J. Zhao (2008). "Essential roles of PI(3)K-p110beta in cell growth, metabolism and tumorigenesis." Nature **454**(7205): 776-779.

Jia, Z., D. Barford, A. J. Flint and N. K. Tonks (1995). "Structural basis for phosphotyrosine peptide recognition by protein tyrosine phosphatase 1B." Science **268**(5218): 1754-1758.

Jiao, Q., L. Bi, Y. Ren, S. Song, Q. Wang and Y. S. Wang (2018). "Advances in studies of tyrosine kinase inhibitors and their acquired resistance." Mol Cancer **17**(1): 36.

Jones, P. F., T. Jakubowicz, F. J. Pitossi, F. Maurer and B. A. Hemmings (1991). "Molecular cloning and identification of a serine/threonine protein kinase of the second-messenger subfamily." Proc Natl Acad Sci U S A **88**(10): 4171-4175.

Junttila, T. T., G. Li, K. Parsons, G. L. Phillips and M. X. Sliwkowski (2011). "Trastuzumab-DM1 (T-DM1) retains all the mechanisms of action of trastuzumab and efficiently inhibits growth of lapatinib insensitive breast cancer." Breast Cancer Res Treat **128**(2): 347-356.

Juric, D., J. Rodon, J. Tabernero, F. Janku, H. A. Burris, J. H. M. Schellens, M. R. Middleton, J. Berlin, M. Schuler, M. Gil-Martin, H. S. Rugo, R. Seggewiss-Bernhardt, A. Huang, D. Bootle, D. Demanse, L. Blumenstein, C. Coughlin, C. Quadt and J. Baselga (2018). "Phosphatidylinositol 3-Kinase alpha-Selective Inhibition With Alpelisib (BYL719) in PIK3CA-Altered Solid Tumors: Results From the First-in-Human Study." J Clin Oncol **36**(13): 1291-1299.

Kadota, M., M. Sato, B. Duncan, A. Ooshima, H. H. Yang, N. Diaz-Meyer, S. Gere, S. Kageyama, J. Fukuoka, T. Nagata, K. Tsukada, B. K. Dunn, L. M. Wakefield and M. P. Lee (2009). "Identification of novel gene amplifications in breast cancer and coexistence of gene amplification with an activating mutation of PIK3CA." Cancer Res **69**(18): 7357-7365.

Kalra, S., B. I. Rini and E. Jonasch (2015). "Alternate sunitinib schedules in patients with metastatic renal cell carcinoma." Ann Oncol **26**(7): 1300-1304.

Kannan, N. and S. S. Taylor (2008). "Rethinking pseudokinases." Cell **133**(2): 204-205.

Kazlauskas, A. and J. A. Cooper (1990). "Phosphorylation of the PDGF receptor beta subunit creates a tight binding site for phosphatidylinositol 3 kinase." EMBO J **9**(10): 3279-3286.

Keegan, N. M., J. P. Gleeson, B. T. Hennessy and P. G. Morris (2018). "PI3K inhibition to overcome endocrine resistance in breast cancer." Expert Opin Investig Drugs **27**(1): 1-15.

Keilhack, H., F. S. David, M. McGregor, L. C. Cantley and B. G. Neel (2005). "Diverse biochemical properties of Shp2 mutants. Implications for disease phenotypes." J Biol Chem **280**(35): 30984-30993.

Kennecke, H., R. Yerushalmi, R. Woods, M. C. Cheang, D. Voduc, C. H. Speers, T. O. Nielsen and K. Gelmon (2010). "Metastatic behavior of breast cancer subtypes." J Clin Oncol **28**(20): 3271-3277.

Kennelly, P. J. (2002). "Protein kinases and protein phosphatases in prokaryotes: a genomic perspective." FEMS Microbiol Lett **206**(1): 1-8.

Kessenbrock, K., V. Plaks and Z. Werb (2010). "Matrix metalloproteinases: regulators of the tumor microenvironment." Cell **141**(1): 52-67.

Kienast, Y., L. von Baumgarten, M. Fuhrmann, W. E. Klinkert, R. Goldbrunner, J. Herms and F. Winkler (2010). "Real-time imaging reveals the single steps of brain metastasis formation." Nat Med **16**(1): 116-122.

Kim, S. J. and S. E. Ryu (2012). "Structure and catalytic mechanism of human protein tyrosine phosphatome." BMB Rep **45**(12): 693-699.

Klein, C. A. (2009). "Parallel progression of primary tumours and metastases." Nat Rev Cancer **9**(4): 302-312.

Kleinsmith, L. J. and G. B. Pierce, Jr. (1964). "Multipotentiality of Single Embryonal Carcinoma Cells." Cancer Res **24**: 1544-1551.

Klinghoffer, R. A. and A. Kazlauskas (1995). "Identification of a putative Syp substrate, the PDGF beta receptor." J Biol Chem **270**(38): 22208-22217.

Knowles, M. A., F. M. Platt, R. L. Ross and C. D. Hurst (2009). "Phosphatidylinositol 3-kinase (PI3K) pathway activation in bladder cancer." Cancer Metastasis Rev **28**(3-4): 305-316.

Kontaridis, M. I., K. D. Swanson, F. S. David, D. Barford and B. G. Neel (2006). "PTPN11 (Shp2) mutations in LEOPARD syndrome have dominant negative, not activating, effects." J Biol Chem **281**(10): 6785-6792.

Krop, I. E., S. B. Kim, A. Gonzalez-Martin, P. M. LoRusso, J. M. Ferrero, M. Smitt, R. Yu, A. C. Leung, H. Wildiers and T. R. s. collaborators (2014). "Trastuzumab emtansine versus treatment of physician's choice for pretreated HER2-positive advanced breast cancer (TH3RESA): a randomised, open-label, phase 3 trial." Lancet Oncol **15**(7): 689-699.

Krop, I. E., S. B. Kim, A. G. Martin, P. M. LoRusso, J. M. Ferrero, T. Badovinac-Crnjevic, S. Hoersch, M. Smitt and H. Wildiers (2017). "Trastuzumab emtansine versus treatment of physician's choice in patients with previously treated HER2-positive metastatic breast cancer (TH3RESA): final overall survival results from a randomised open-label phase 3 trial." Lancet Oncol **18**(6): 743-754.

Lan, L., J. D. Holland, J. Qi, S. Grosskopf, J. Rademann, R. Vogel, B. Gyorffy, A. Wulf-Goldenberg and W. Birchmeier (2015). "Shp2 signaling suppresses senescence in PyMT-induced mammary gland cancer in mice." EMBO J **34**(11): 1493-1508.

Le Gal, K., M. X. Ibrahim, C. Wiel, V. I. Sayin, M. K. Akula, C. Karlsson, M. G. Dalin, L. M. Akyurek, P. Lindahl, J. Nilsson and M. O. Bergo (2015). "Antioxidants can increase melanoma metastasis in mice." Sci Transl Med **7**(308): 308re308.

Le, X., R. Antony, P. Razavi, D. J. Treacy, F. Luo, M. Ghandi, P. Castel, M. Scaltriti, J. Baselga and L. A. Garraway (2016). "Systematic Functional Characterization of Resistance to PI3K Inhibition in Breast Cancer." Cancer Discov **6**(10): 1134-1147.

Legius, E., C. Schrander-Stumpel, E. Schollen, C. Pulles-Heintzberger, M. Gewillig and J. P. Fryns (2002). "PTPN11 mutations in LEOPARD syndrome." J Med Genet **39**(8): 571-574.

Lelekakis, M., J. M. Moseley, T. J. Martin, D. Hards, E. Williams, P. Ho, D. Lowen, J. Javni, F. R. Miller, J. Slavin and R. L. Anderson (1999). "A novel orthotopic model of breast cancer metastasis to bone." Clin Exp Metastasis **17**(2): 163-170.

Leroy, C., R. J. Amante and M. Bentires-Alj (2014). "Anticipating mechanisms of resistance to PI3K inhibition in breast cancer: a challenge in the era of precision medicine." Biochem Soc Trans **42**(4): 733-741.

Levine, D. A., F. Bogomolniy, C. J. Yee, A. Lash, R. R. Barakat, P. I. Borgen and J. Boyd (2005). "Frequent mutation of the PIK3CA gene in ovarian and breast cancers." Clin Cancer Res **11**(8): 2875-2878.

Liedtke, C., C. Mazouni, K. R. Hess, F. Andre, A. Tordai, J. A. Mejia, W. F. Symmans, A. M. Gonzalez-Angulo, B. Hennessy, M. Green, M. Cristofanilli, G. N. Hortobagyi and L. Pusztai (2008). "Response to neoadjuvant therapy and long-term survival in patients with triple-negative breast cancer." J Clin Oncol **26**(8): 1275-1281.

Liu, H., D. C. Radisky, D. Yang, R. Xu, E. S. Radisky, M. J. Bissell and J. M. Bishop (2012). "MYC suppresses cancer metastasis by direct transcriptional silencing of alphav and beta3 integrin subunits." Nat Cell Biol **14**(6): 567-574.

Liu, J., S. Liao, Y. Huang, R. Samuel, T. Shi, K. Naxerova, P. Huang, W. Kamoun, R. K. Jain, D. Fukumura and L. Xu (2011). "PDGF-D improves drug delivery and efficacy via vascular normalization, but promotes lymphatic metastasis by activating CXCR4 in breast cancer." Clin Cancer Res **17**(11): 3638-3648.

Liu, K. W., H. Feng, R. Bachoo, A. Kazlauskas, E. M. Smith, K. Symes, R. L. Hamilton, M. Nagane, R. Nishikawa, B. Hu and S. Y. Cheng (2011). "SHP-2/PTPN11 mediates gliomagenesis driven by PDGFRA and INK4A/ARF aberrations in mice and humans." J Clin Invest **121**(3): 905-917.

Liu, P., H. Cheng, T. M. Roberts and J. J. Zhao (2009). "Targeting the phosphoinositide 3-kinase pathway in cancer." Nat Rev Drug Discov **8**(8): 627-644.

Loh, M. L., M. G. Reynolds, S. Vattikuti, R. B. Gerbing, T. A. Alonzo, E. Carlson, J. W. Cheng, C. M. Lee, B. J. Lange, S. Meshinchi and G. Children's Cancer (2004). "PTPN11 mutations in pediatric patients with acute myeloid leukemia: results from the Children's Cancer Group." Leukemia **18**(11): 1831-1834.

Loh, M. L., S. Vattikuti, S. Schubbert, M. G. Reynolds, E. Carlson, K. H. Lieuw, J. W. Cheng, C. M. Lee, D. Stokoe, J. M. Bonifas, N. P. Curtiss, J. Gotlib, S. Meshinchi, M. M. Le Beau, P. D. Emanuel and K. M. Shannon (2004). "Mutations in PTPN11 implicate the SHP-2 phosphatase in leukemogenesis." Blood **103**(6): 2325-2331.

Loibl, S. and L. Gianni (2017). "HER2-positive breast cancer." The Lancet **389**(10087): 2415-2429.

Lonardo, F., E. Di Marco, C. R. King, J. H. Pierce, O. Segatto, S. A. Aaronson and P. P. Di Fiore (1990). "The normal erbB-2 product is an atypical receptor-like tyrosine kinase with constitutive activity in the absence of ligand." New Biol **2**(11): 992-1003.

Lu, H., N. Murata-Kamiya, Y. Saito and M. Hatakeyama (2009). "Role of partitioning-defective 1/microtubule affinity-regulating kinases in the morphogenetic activity of Helicobacter pylori CagA." J Biol Chem **284**(34): 23024-23036.

Luzzi, K. J., I. C. MacDonald, E. E. Schmidt, N. Kerkvliet, V. L. Morris, A. F. Chambers and A. C. Groom (1998). "Multistep nature of metastatic inefficiency: dormancy of solitary cells after successful extravasation and limited survival of early micrometastases." Am J Pathol **153**(3): 865-873.

Maehama, T. and J. E. Dixon (1998). "The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate." J Biol Chem **273**(22): 13375-13378.

Maffucci, T., F. T. Cooke, F. M. Foster, C. J. Traer, M. J. Fry and M. Falasca (2005). "Class II phosphoinositide 3-kinase defines a novel signaling pathway in cell migration." J Cell Biol **169**(5): 789-799.

Mainardi, S., A. Mulero-Sanchez, A. Prahallad, G. Germano, A. Bosma, P. Krimpenfort, C. Lieftink, J. D. Steinberg, N. de Wit, S. Goncalves-Ribeiro, E. Nadal, A. Bardelli, A. Villanueva and R. Bernards (2018). "SHP2 is required for growth of KRAS-mutant non-small-cell lung cancer in vivo." Nat Med **24**(7): 961-967.

Manning, B. D. and A. Toker (2017). "AKT/PKB Signaling: Navigating the Network." Cell **169**(3): 381-405.

Manning, G., D. B. Whyte, R. Martinez, T. Hunter and S. Sudarsanam (2002). "The protein kinase complement of the human genome." Science **298**(5600): 1912-1934.

Marin, T. M., K. Keith, B. Davies, D. A. Conner, P. Guha, D. Kalaitzidis, X. Wu, J. Lauriol, B. Wang, M. Bauer, R. Bronson, K. G. Franchini, B. G. Neel and M. I. Kontaridis (2011). "Rapamycin reverses hypertrophic cardiomyopathy in a mouse model of LEOPARD syndrome-associated PTPN11 mutation." J Clin Invest **121**(3): 1026-1043.

Martin, M. (2011). "Cutadapt removes adapter sequences from high-throughput sequencing reads." 2011 **17**(1): 3.

Martin, M., A. Chan, L. Dirix, J. O'Shaughnessy, R. Hegg, A. Manikhas, M. Shtivelband, P. Krivorotko, N. Batista Lopez, M. Campone, M. Ruiz Borrego, Q. J. Khan, J. T. Beck, M. Ramos Vazquez, P. Urban, S. Goteti, E. Di Tomaso, C. Massacesi and S. Delaloge (2017). "A randomized adaptive phase II/III study of buparlisib, a pan-class I PI3K inhibitor, combined with paclitaxel for the treatment of HER2- advanced breast cancer (BELLE-4)." Ann Oncol **28**(2): 313-320.

Massague, J. and A. C. Obenauf (2016). "Metastatic colonization by circulating tumour cells." Nature **529**(7586): 298-306.

Masuda, H., K. A. Baggerly, Y. Wang, Y. Zhang, A. M. Gonzalez-Angulo, F. Meric-Bernstam, V. Valero, B. D. Lehmann, J. A. Pietenpol, G. N. Hortobagyi, W. F. Symmans and N. T. Ueno (2013). "Differential response to neoadjuvant chemotherapy among 7 triple-negative breast cancer molecular subtypes." Clin Cancer Res **19**(19): 5533-5540.

Matalkah, F., E. Martin, H. Zhao and Y. M. Agazie (2016). "SHP2 acts both upstream and downstream of multiple receptor tyrosine kinases to promote basal-like and triple-negative breast cancer." Breast Cancer Res **18**(1): 2.

Mattoon, D. R., B. Lamothe, I. Lax and J. Schlessinger (2004). "The docking protein Gab1 is the primary mediator of EGF-stimulated activation of the PI-3K/Akt cell survival pathway." BMC Biol **2**: 24.

McCarthy, D. J., Y. Chen and G. K. Smyth (2012). "Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation." Nucleic Acids Res **40**(10): 4288-4297.

McGranahan, N., F. Favero, E. C. de Bruin, N. J. Birkbak, Z. Szallasi and C. Swanton (2015). "Clonal status of actionable driver events and the timing of mutational processes in cancer evolution." Sci Transl Med **7**(283): 283ra254.

Meadows, K. L. and H. I. Hurwitz (2012). "Anti-VEGF therapies in the clinic." Cold Spring Harb Perspect Med **2**(10).

Mendez, H. M. and J. M. Opitz (1985). "Noonan syndrome: a review." Am J Med Genet **21**(3): 493-506.

Meng, F., C. L. Speyer, B. Zhang, Y. Zhao, W. Chen, D. H. Gorski, F. R. Miller and G. Wu (2015). "PDGFRalpha and beta play critical roles in mediating Foxq1-driven breast cancer stemness and chemoresistance." Cancer Res **75**(3): 584-593.

Meyer, D. S. and M. Bentires-Alj (2010). "Can phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibition ERase them all?" Breast Cancer Res **12**(5): 315.

Miller, T. W. (2012). "Initiating breast cancer by PIK3CA mutation." Breast Cancer Res **14**(1): 301.

Miller, T. W., B. T. Hennessy, A. M. Gonzalez-Angulo, E. M. Fox, G. B. Mills, H. Chen, C. Higham, C. Garcia-Echeverria, Y. Shyr and C. L. Arteaga (2010). "Hyperactivation of

phosphatidylinositol-3 kinase promotes escape from hormone dependence in estrogen receptor-positive human breast cancer." J Clin Invest **120**(7): 2406-2413.

Mitri, Z., C. Karakas, C. Wei, B. Briones, H. Simmons, N. Ibrahim, R. Alvarez, J. L. Murray, K. Keyomarsi and S. Moulder (2015). "A phase 1 study with dose expansion of the CDK inhibitor dinaciclib (SCH 727965) in combination with epirubicin in patients with metastatic triple negative breast cancer." Invest New Drugs **33**(4): 890-894.

Modi, S., A. Stopeck, H. Linden, D. Solit, S. Chandarlapaty, N. Rosen, G. D'Andrea, M. Dickler, M. E. Moynahan, S. Sugarman, W. Ma, S. Patil, L. Norton, A. L. Hannah and C. Hudis (2011). "HSP90 inhibition is effective in breast cancer: a phase II trial of tanespimycin (17-AAG) plus trastuzumab in patients with HER2-positive metastatic breast cancer progressing on trastuzumab." Clin Cancer Res **17**(15): 5132-5139.

Montagner, A., A. Yart, M. Dance, B. Perret, J. P. Salles and P. Raynal (2005). "A novel role for Gab1 and SHP2 in epidermal growth factor-induced Ras activation." J Biol Chem **280**(7): 5350-5360.

Motzer, R. J., B. Escudier, S. Oudard, T. E. Hutson, C. Porta, S. Bracarda, V. Grunwald, J. A. Thompson, R. A. Figlin, N. Hollaender, G. Urbanowitz, W. J. Berg, A. Kay, D. Lebwohl, A. Ravaud and R.-S. Group (2008). "Efficacy of everolimus in advanced renal cell carcinoma: a double-blind, randomised, placebo-controlled phase III trial." Lancet **372**(9637): 449-456.

Mukohara, T. (2015). "PI3K mutations in breast cancer: prognostic and therapeutic implications." Breast Cancer (Dove Med Press) **7**: 111-123.

Muranen, T., L. M. Selfors, D. T. Worster, M. P. Iwanicki, L. Song, F. C. Morales, S. Gao, G. B. Mills and J. S. Brugge (2012). "Inhibition of PI3K/mTOR leads to adaptive resistance in matrix-attached cancer cells." Cancer Cell **21**(2): 227-239.

Musgrove, E. A. and R. L. Sutherland (2009). "Biological determinants of endocrine resistance in breast cancer." Nat Rev Cancer **9**(9): 631-643.

Nakai, K., M. C. Hung and H. Yamaguchi (2016). "A perspective on anti-EGFR therapies targeting triple-negative breast cancer." Am J Cancer Res **6**(8): 1609-1623.

Nathan, M. R. and P. Schmid (2017). "A Review of Fulvestrant in Breast Cancer." Oncol Ther **5**(1): 17-29.

Nguyen, D. X., P. D. Bos and J. Massague (2009). "Metastasis: from dissemination to organ-specific colonization." Nat Rev Cancer **9**(4): 274-284.

Niihori, T., Y. Aoki, H. Ohashi, K. Kurosawa, T. Kondoh, S. Ishikiriyama, H. Kawame, H. Kamasaki, T. Yamanaka, F. Takada, K. Nishio, M. Sakurai, H. Tamai, T. Nagashima, Y. Suzuki, S. Kure, K. Fujii, M. Imaizumi and Y. Matsubara (2005). "Functional analysis of PTPN11/SHP-2 mutants identified in Noonan syndrome and childhood leukemia." J Hum Genet **50**(4): 192-202.

Nik-Zainal, S., H. Davies, J. Staaf, M. Ramakrishna, D. Glodzik, X. Zou, I. Martincorena, L. B. Alexandrov, S. Martin, D. C. Wedge, P. Van Loo, Y. S. Ju, M. Smid, A. B. Brinkman, S. Morganella, M. R. Aure, O. C. Lingjaerde, A. Langerod, M. Ringner, S. M. Ahn, S. Boyault, J. E. Brock, A. Broeks, A. Butler, C. Desmedt, L. Dirix, S. Dronov, A. Fatima, J. A. Foekens,

M. Gerstung, G. K. Hooijer, S. J. Jang, D. R. Jones, H. Y. Kim, T. A. King, S. Krishnamurthy, H. J. Lee, J. Y. Lee, Y. Li, S. McLaren, A. Menzies, V. Mustonen, S. O'Meara, I. Pauporte, X. Pivot, C. A. Purdie, K. Raine, K. Ramakrishnan, F. G. Rodriguez-Gonzalez, G. Romieu, A. M. Sieuwerts, P. T. Simpson, R. Shepherd, L. Stebbings, O. A. Stefansson, J. Teague, S. Tommasi, I. Treilleux, G. G. Van den Eynden, P. Vermeulen, A. Vincent-Salomon, L. Yates, C. Caldas, L. van't Veer, A. Tutt, S. Knappskog, B. K. Tan, J. Jonkers, A. Borg, N. T. Ueno, C. Sotiriou, A. Viari, P. A. Futreal, P. J. Campbell, P. N. Span, S. Van Laere, S. R. Lakhani, J. E. Eyfjord, A. M. Thompson, E. Birney, H. G. Stunnenberg, M. J. van de Vijver, J. W. Martens, A. L. Borresen-Dale, A. L. Richardson, G. Kong, G. Thomas and M. R. Stratton (2016). "Landscape of somatic mutations in 560 breast cancer whole-genome sequences." Nature **534**(7605): 47-54.

O'Reilly, K. E., F. Rojo, Q. B. She, D. Solit, G. B. Mills, D. Smith, H. Lane, F. Hofmann, D. J. Hicklin, D. L. Ludwig, J. Baselga and N. Rosen (2006). "mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt." Cancer Res **66**(3): 1500-1508.

Oskarsson, T., S. Acharyya, X. H. Zhang, S. Vanharanta, S. F. Tavazoie, P. G. Morris, R. J. Downey, K. Manova-Todorova, E. Brogi and J. Massague (2011). "Breast cancer cells produce tenascin C as a metastatic niche component to colonize the lungs." Nat Med **17**(7): 867-874.

Pantel, K. and R. H. Brakenhoff (2004). "Dissecting the metastatic cascade." Nat Rev Cancer **4**(6): 448-456.

Parks, R. M., M. G. M. Derks, E. Bastiaannet and K. L. Cheung (2018). Breast Cancer Epidemiology. Breast Cancer Management for Surgeons. L. Wyld, C. Markopoulos, M. Leidenius and E. Senkus-Konefka. Cham, Springer International Publishing**:** 19-29.

Parsons, D. W., S. Jones, X. Zhang, J. C. Lin, R. J. Leary, P. Angenendt, P. Mankoo, H. Carter, I. M. Siu, G. L. Gallia, A. Olivi, R. McLendon, B. A. Rasheed, S. Keir, T. Nikolskaya, Y. Nikolsky, D. A. Busam, H. Tekleab, L. A. Diaz, Jr., J. Hartigan, D. R. Smith, R. L. Strausberg, S. K. Marie, S. M. Shinjo, H. Yan, G. J. Riggins, D. D. Bigner, R. Karchin, N. Papadopoulos, G. Parmigiani, B. Vogelstein, V. E. Velculescu and K. W. Kinzler (2008). "An integrated genomic analysis of human glioblastoma multiforme." Science **321**(5897): 1807-1812.

Patnaik, A., L. J. Appleman, A. W. Tolcher, K. P. Papadopoulos, M. Beeram, D. W. Rasco, G. J. Weiss, J. C. Sachdev, M. Chadha, M. Fulk, S. Ejadi, J. M. Mountz, M. T. Lotze, F. G. Toledo, E. Chu, M. Jeffers, C. Pena, C. Xia, S. Reif, I. Genvresse and R. K. Ramanathan (2016). "First-in-human phase I study of copanlisib (BAY 80-6946), an intravenous pan-class I phosphatidylinositol 3-kinase inhibitor, in patients with advanced solid tumors and non-Hodgkin's lymphomas." Ann Oncol **27**(10): 1928-1940.

Pereira, B., S. F. Chin, O. M. Rueda, H. K. Vollan, E. Provenzano, H. A. Bardwell, M. Pugh, L. Jones, R. Russell, S. J. Sammut, D. W. Tsui, B. Liu, S. J. Dawson, J. Abraham, H. Northen, J. F. Peden, A. Mukherjee, G. Turashvili, A. R. Green, S. McKinney, A. Oloumi, S. Shah, N. Rosenfeld, L. Murphy, D. R. Bentley, I. O. Ellis, A. Purushotham, S. E. Pinder, A. L. Borresen-Dale, H. M. Earl, P. D. Pharoah, M. T. Ross, S. Aparicio and C. Caldas (2016). "The somatic mutation profiles of 2,433 breast cancers refines their genomic and transcriptomic landscapes." Nat Commun **7**: 11479.

Perou, C. M. (2011). "Molecular stratification of triple-negative breast cancers." Oncologist **16 Suppl 1**: 61-70.

Perou, C. M., T. Sorlie, M. B. Eisen, M. van de Rijn, S. S. Jeffrey, C. A. Rees, J. R. Pollack, D. T. Ross, H. Johnsen, L. A. Akslen, O. Fluge, A. Pergamenschikov, C. Williams, S. X. Zhu, P. E. Lonning, A. L. Borresen-Dale, P. O. Brown and D. Botstein (2000). "Molecular portraits of human breast tumours." Nature **406**(6797): 747-752.

Prat, A., J. S. Parker, O. Karginova, C. Fan, C. Livasy, J. I. Herschkowitz, X. He and C. M. Perou (2010). "Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer." Breast Cancer Res **12**(5): R68.

Psaila, B. and D. Lyden (2009). "The metastatic niche: adapting the foreign soil." Nat Rev Cancer **9**(4): 285-293.

Pulaski, B. A. and S. Ostrand-Rosenberg (1998). "Reduction of established spontaneous mammary carcinoma metastases following immunotherapy with major histocompatibility complex class II and B7.1 cell-based tumor vaccines." Cancer Res **58**(7): 1486-1493.

Qiu, W., X. Wang, V. Romanov, A. Hutchinson, A. Lin, M. Ruzanov, K. P. Battaile, E. F. Pai, B. G. Neel and N. Y. Chirgadze (2014). "Structural insights into Noonan/LEOPARD syndrome-related mutants of protein-tyrosine phosphatase SHP2 (PTPN11)." BMC Struct Biol **14**: 10.

Qu, C. K., S. Nguyen, J. Chen and G. S. Feng (2001). "Requirement of Shp-2 tyrosine phosphatase in lymphoid and hematopoietic cell development." Blood **97**(4): 911-914.

Ramos, P. and M. Bentires-Alj (2015). "Mechanism-based cancer therapy: resistance to therapy, therapy for resistance." Oncogene **34**(28): 3617-3626.

Robson, M., S. A. Im, E. Senkus, B. Xu, S. M. Domchek, N. Masuda, S. Delaloge, W. Li, N. Tung, A. Armstrong, W. Wu, C. Goessl, S. Runswick and P. Conte (2017). "Olaparib for Metastatic Breast Cancer in Patients with a Germline BRCA Mutation." N Engl J Med **377**(6): 523-533.

Romond, E. H., E. A. Perez, J. Bryant, V. J. Suman, C. E. Geyer, Jr., N. E. Davidson, E. Tan-Chiu, S. Martino, S. Paik, P. A. Kaufman, S. M. Swain, T. M. Pisansky, L. Fehrenbacher, L. A. Kutteh, V. G. Vogel, D. W. Visscher, G. Yothers, R. B. Jenkins, A. M. Brown, S. R. Dakhil, E. P. Mamounas, W. L. Lingle, P. M. Klein, J. N. Ingle and N. Wolmark (2005). "Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer." N Engl J Med **353**(16): 1673-1684.

Ruess, D. A., G. J. Heynen, K. J. Ciecielski, J. Ai, A. Berninger, D. Kabacaoglu, K. Gorgulu, Z. Dantes, S. M. Wormann, K. N. Diakopoulos, A. F. Karpathaki, M. Kowalska, E. Kaya-Aksoy, L. Song, E. A. Z. van der Laan, M. P. Lopez-Alberca, M. Nazare, M. Reichert, D. Saur, M. M. Erkan, U. T. Hopt, B. Sainz, Jr., W. Birchmeier, R. M. Schmid, M. Lesina and H. Algul (2018). "Mutant KRAS-driven cancers depend on PTPN11/SHP2 phosphatase." Nat Med **24**(7): 954-960.

Saadat, I., H. Higashi, C. Obuse, M. Umeda, N. Murata-Kamiya, Y. Saito, H. Lu, N. Ohnishi, T. Azuma, A. Suzuki, S. Ohno and M. Hatakeyama (2007). "Helicobacter pylori CagA targets PAR1/MARK kinase to disrupt epithelial cell polarity." Nature **447**(7142): 330-333.

Saal, L. H., K. Holm, M. Maurer, L. Memeo, T. Su, X. Wang, J. S. Yu, P. O. Malmstrom, M. Mansukhani, J. Enoksson, H. Hibshoosh, A. Borg and R. Parsons (2005). "PIK3CA mutations

correlate with hormone receptors, node metastasis, and ERBB2, and are mutually exclusive with PTEN loss in human breast carcinoma." Cancer Res **65**(7): 2554-2559.

Samuels, Y., Z. Wang, A. Bardelli, N. Silliman, J. Ptak, S. Szabo, H. Yan, A. Gazdar, S. M. Powell, G. J. Riggins, J. K. Willson, S. Markowitz, K. W. Kinzler, B. Vogelstein and V. E. Velculescu (2004). "High frequency of mutations of the PIK3CA gene in human cancers." Science **304**(5670): 554.

Sausgruber, N., M. M. Coissieux, A. Britschgi, J. Wyckoff, N. Aceto, C. Leroy, M. B. Stadler, H. Voshol, D. Bonenfant and M. Bentires-Alj (2015). "Tyrosine phosphatase SHP2 increases cell motility in triple-negative breast cancer through the activation of SRC-family kinases." Oncogene **34**(17): 2272-2278.

Saxton, T. M., M. Henkemeyer, S. Gasca, R. Shen, D. J. Rossi, F. Shalaby, G. S. Feng and T. Pawson (1997). "Abnormal mesoderm patterning in mouse embryos mutant for the SH2 tyrosine phosphatase Shp-2." EMBO J **16**(9): 2352-2364.

Saxton, T. M. and T. Pawson (1999). "Morphogenetic movements at gastrulation require the SH2 tyrosine phosphatase Shp2." Proc Natl Acad Sci U S A **96**(7): 3790-3795.

Scaltriti, M., F. Rojo, A. Ocana, J. Anido, M. Guzman, J. Cortes, S. Di Cosimo, X. Matias-Guiu, S. Ramon y Cajal, J. Arribas and J. Baselga (2007). "Expression of p95HER2, a truncated form of the HER2 receptor, and response to anti-HER2 therapies in breast cancer." J Natl Cancer Inst **99**(8): 628-638.

Schramm, C., D. M. Fine, M. A. Edwards, A. N. Reeb and M. Krenz (2012). "The PTPN11 loss-of-function mutation Q510E-Shp2 causes hypertrophic cardiomyopathy by dysregulating mTOR signaling." Am J Physiol Heart Circ Physiol **302**(1): H231-243.

Schu, P. V., K. Takegawa, M. J. Fry, J. H. Stack, M. D. Waterfield and S. D. Emr (1993). "Phosphatidylinositol 3-kinase encoded by yeast VPS34 gene essential for protein sorting." Science **260**(5104): 88-91.

Shah, A. N. and M. Cristofanilli (2017). "The Growing Role of CDK4/6 Inhibitors in Treating Hormone Receptor-Positive Advanced Breast Cancer." Curr Treat Options Oncol **18**(1): 6.

Shah, S. P., A. Roth, R. Goya, A. Oloumi, G. Ha, Y. Zhao, G. Turashvili, J. Ding, K. Tse, G. Haffari, A. Bashashati, L. M. Prentice, J. Khattra, A. Burleigh, D. Yap, V. Bernard, A. McPherson, K. Shumansky, A. Crisan, R. Giuliany, A. Heravi-Moussavi, J. Rosner, D. Lai, I. Birol, R. Varhol, A. Tam, N. Dhalla, T. Zeng, K. Ma, S. K. Chan, M. Griffith, A. Moradian, S. W. Cheng, G. B. Morin, P. Watson, K. Gelmon, S. Chia, S. F. Chin, C. Curtis, O. M. Rueda, P. D. Pharoah, S. Damaraju, J. Mackey, K. Hoon, T. Harkins, V. Tadigotla, M. Sigaroudinia, P. Gascard, T. Tlsty, J. F. Costello, I. M. Meyer, C. J. Eaves, W. W. Wasserman, S. Jones, D. Huntsman, M. Hirst, C. Caldas, M. A. Marra and S. Aparicio (2012). "The clonal and mutational evolution spectrum of primary triple-negative breast cancers." Nature **486**(7403): 395-399.

Shaheen, R. M., D. W. Davis, W. Liu, B. K. Zebrowski, M. R. Wilson, C. D. Bucana, D. J. McConkey, G. McMahon and L. M. Ellis (1999). "Antiangiogenic therapy targeting the tyrosine kinase receptor for vascular endothelial growth factor receptor inhibits the growth of

colon cancer liver metastasis and induces tumor and endothelial cell apoptosis." Cancer Res **59**(21): 5412-5416.

Sharma, K., R. C. D'Souza, S. Tyanova, C. Schaab, J. R. Wisniewski, J. Cox and M. Mann (2014). "Ultradeep human phosphoproteome reveals a distinct regulatory nature of Tyr and Ser/Thr-based signaling." Cell Rep **8**(5): 1583-1594.

Sims, A. H., A. Howell, S. J. Howell and R. B. Clarke (2007). "Origins of breast cancer subtypes and therapeutic implications." Nat Clin Pract Oncol **4**(9): 516-525.

Slamon, D. J., G. M. Clark, S. G. Wong, W. J. Levin, A. Ullrich and W. L. McGuire (1987). "Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene." Science **235**(4785): 177-182.

Slamon, D. J., B. Leyland-Jones, S. Shak, H. Fuchs, V. Paton, A. Bajamonde, T. Fleming, W. Eiermann, J. Wolter, M. Pegram, J. Baselga and L. Norton (2001). "Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2." N Engl J Med **344**(11): 783-792.

Sledge, G. W., E. P. Mamounas, G. N. Hortobagyi, H. J. Burstein, P. J. Goodwin and A. C. Wolff (2014). "Past, present, and future challenges in breast cancer treatment." J Clin Oncol **32**(19): 1979-1986.

Sobhani, N., G. Roviello, S. P. Corona, M. Scaltriti, A. Ianza, M. Bortul, F. Zanconati and D. Generali (2018). "The prognostic value of PI3K mutational status in breast cancer: A meta-analysis." J Cell Biochem **119**(6): 4287-4292.

Sorlie, T., C. M. Perou, R. Tibshirani, T. Aas, S. Geisler, H. Johnsen, T. Hastie, M. B. Eisen, M. van de Rijn, S. S. Jeffrey, T. Thorsen, H. Quist, J. C. Matese, P. O. Brown, D. Botstein, P. E. Lonning and A. L. Borresen-Dale (2001). "Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications." Proc Natl Acad Sci U S A **98**(19): 10869-10874.

Staal, S. P. (1987). "Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: amplification of AKT1 in a primary human gastric adenocarcinoma." Proc Natl Acad Sci U S A **84**(14): 5034-5037.

Stemke-Hale, K., A. M. Gonzalez-Angulo, A. Lluch, R. M. Neve, W. L. Kuo, M. Davies, M. Carey, Z. Hu, Y. Guan, A. Sahin, W. F. Symmans, L. Pusztai, L. K. Nolden, H. Horlings, K. Berns, M. C. Hung, M. J. van de Vijver, V. Valero, J. W. Gray, R. Bernards, G. B. Mills and B. T. Hennessy (2008). "An integrative genomic and proteomic analysis of PIK3CA, PTEN, and AKT mutations in breast cancer." Cancer Res **68**(15): 6084-6091.

Stephens, P. J., P. S. Tarpey, H. Davies, P. Van Loo, C. Greenman, D. C. Wedge, S. Nik-Zainal, S. Martin, I. Varela, G. R. Bignell, L. R. Yates, E. Papaemmanuil, D. Beare, A. Butler, A. Cheverton, J. Gamble, J. Hinton, M. Jia, A. Jayakumar, D. Jones, C. Latimer, K. W. Lau, S. McLaren, D. J. McBride, A. Menzies, L. Mudie, K. Raine, R. Rad, M. S. Chapman, J. Teague, D. Easton, A. Langerod, C. Oslo Breast Cancer, M. T. Lee, C. Y. Shen, B. T. Tee, B. W. Huimin, A. Broeks, A. C. Vargas, G. Turashvili, J. Martens, A. Fatima, P. Miron, S. F. Chin, G. Thomas, S. Boyault, O. Mariani, S. R. Lakhani, M. van de Vijver, L. van 't Veer, J. Foekens, C. Desmedt, C. Sotiriou, A. Tutt, C. Caldas, J. S. Reis-Filho, S. A. Aparicio, A. V. Salomon,

A. L. Borresen-Dale, A. L. Richardson, P. J. Campbell, P. A. Futreal and M. R. Stratton (2012). "The landscape of cancer genes and mutational processes in breast cancer." Nature **486**(7403): 400-404.

Stewart, R. A., T. Sanda, H. R. Widlund, S. Zhu, K. D. Swanson, A. D. Hurley, M. Bentires-Alj, D. E. Fisher, M. I. Kontaridis, A. T. Look and B. G. Neel (2010). "Phosphatase-dependent and -independent functions of Shp2 in neural crest cells underlie LEOPARD syndrome pathogenesis." Dev Cell **18**(5): 750-762.

Stratikopoulos, E. E., M. Dendy, M. Szabolcs, A. J. Khaykin, C. Lefebvre, M. M. Zhou and R. Parsons (2015). "Kinase and BET Inhibitors Together Clamp Inhibition of PI3K Signaling and Overcome Resistance to Therapy." Cancer Cell **27**(6): 837-851.

Sun, M., P. Hillmann, B. T. Hofmann, J. R. Hart and P. K. Vogt (2010). "Cancer-derived mutations in the regulatory subunit p85alpha of phosphoinositide 3-kinase function through the catalytic subunit p110alpha." Proc Natl Acad Sci U S A **107**(35): 15547-15552.

Swain, S. M., J. Baselga, S. B. Kim, J. Ro, V. Semiglazov, M. Campone, E. Ciruelos, J. M. Ferrero, A. Schneeweiss, S. Heeson, E. Clark, G. Ross, M. C. Benyunes, J. Cortes and C. S. Group (2015). "Pertuzumab, trastuzumab, and docetaxel in HER2-positive metastatic breast cancer." N Engl J Med **372**(8): 724-734.

Szasz, A. M., Z. Nemeth, B. Gyorffy, M. Micsinai, T. Krenacs, Z. Baranyai, L. Harsanyi, A. Kiss, Z. Schaff, A. M. Tokes and J. Kulka (2011). "Identification of a claudin-4 and E-cadherin score to predict prognosis in breast cancer." Cancer Sci **102**(12): 2248-2254.

Szczerba, B. M., F. Castro-Giner, M. Vetter, I. Krol, S. Gkountela, J. Landin, M. C. Scheidmann, C. Donato, R. Scherrer, J. Singer, C. Beisel, C. Kurzeder, V. Heinzelmann-Schwarz, C. Rochlitz, W. P. Weber, N. Beerenwinkel and N. Aceto (2019). "Neutrophils escort circulating tumour cells to enable cell cycle progression." Nature.

Tajan, M., A. Batut, T. Cadoudal, S. Deleruyelle, S. Le Gonidec, C. Saint Laurent, M. Vomscheid, E. Wanecq, K. Treguer, A. De Rocca Serra-Nedelec, C. Vinel, M. A. Marques, J. Pozzo, O. Kunduzova, J. P. Salles, M. Tauber, P. Raynal, H. Cave, T. Edouard, P. Valet and A. Yart (2014). "LEOPARD syndrome-associated SHP2 mutation confers leanness and protection from diet-induced obesity." Proc Natl Acad Sci U S A **111**(42): E4494-4503.

Tartaglia, M. and B. D. Gelb (2005). "Germ-line and somatic PTPN11 mutations in human disease." Eur J Med Genet **48**(2): 81-96.

Tartaglia, M., E. L. Mehler, R. Goldberg, G. Zampino, H. G. Brunner, H. Kremer, I. van der Burgt, A. H. Crosby, A. Ion, S. Jeffery, K. Kalidas, M. A. Patton, R. S. Kucherlapati and B. D. Gelb (2001). "Mutations in PTPN11, encoding the protein tyrosine phosphatase SHP-2, cause Noonan syndrome." Nat Genet **29**(4): 465-468.

Tartaglia, M., C. M. Niemeyer, A. Fragale, X. Song, J. Buechner, A. Jung, K. Hahlen, H. Hasle, J. D. Licht and B. D. Gelb (2003). "Somatic mutations in PTPN11 in juvenile myelomonocytic leukemia, myelodysplastic syndromes and acute myeloid leukemia." Nat Genet **34**(2): 148-150.

Tautz, L. and T. Mustelin (2007). "Strategies for developing protein tyrosine phosphatase inhibitors." Methods **42**(3): 250-260.

Thiery, J. P., H. Acloque, R. Y. Huang and M. A. Nieto (2009). "Epithelial-mesenchymal transitions in development and disease." Cell **139**(5): 871-890.

Thompson, E. W., D. Katz, T. B. Shima, A. E. Wakeling, M. E. Lippman and R. B. Dickson (1989). "ICI 164,384, a pure antagonist of estrogen-stimulated MCF-7 cell proliferation and invasiveness." Cancer Res **49**(24 Pt 1): 6929-6934.

Thorpe, L. M., H. Yuzugullu and J. J. Zhao (2015). "PI3K in cancer: divergent roles of isoforms, modes of activation and therapeutic targeting." Nat Rev Cancer **15**(1): 7-24.

Tonks, N. K. (2006). "Protein tyrosine phosphatases: from genes, to function, to disease." Nat Rev Mol Cell Biol **7**(11): 833-846.

Tsutsumi, R., H. Higashi, M. Higuchi, M. Okada and M. Hatakeyama (2003). "Attenuation of Helicobacter pylori CagA x SHP-2 signaling by interaction between CagA and C-terminal Src kinase." J Biol Chem **278**(6): 3664-3670.

Tsutsumi, R., M. Masoudi, A. Takahashi, Y. Fujii, T. Hayashi, I. Kikuchi, Y. Satou, M. Taira and M. Hatakeyama (2013). "YAP and TAZ, Hippo signaling targets, act as a rheostat for nuclear SHP2 function." Dev Cell **26**(6): 658-665.

Tsutsumi, R., A. Takahashi, T. Azuma, H. Higashi and M. Hatakeyama (2006). "Focal adhesion kinase is a substrate and downstream effector of SHP-2 complexed with Helicobacter pylori CagA." Mol Cell Biol **26**(1): 261-276.

Turajlic, S. and C. Swanton (2016). "Metastasis as an evolutionary process." Science **352**(6282): 169-175.

Turajlic, S., H. Xu, K. Litchfield, A. Rowan, S. Horswell, T. Chambers, T. O'Brien, J. I. Lopez, T. B. K. Watkins, D. Nicol, M. Stares, B. Challacombe, S. Hazell, A. Chandra, T. J. Mitchell, L. Au, C. Eichler-Jonsson, F. Jabbar, A. Soultati, S. Chowdhury, S. Rudman, J. Lynch, A. Fernando, G. Stamp, E. Nye, A. Stewart, W. Xing, J. C. Smith, M. Escudero, A. Huffman, N. Matthews, G. Elgar, B. Phillimore, M. Costa, S. Begum, S. Ward, M. Salm, S. Boeing, R. Fisher, L. Spain, C. Navas, E. Gronroos, S. Hobor, S. Sharma, I. Aurangzeb, S. Lall, A. Polson, M. Varia, C. Horsfield, N. Fotiadis, L. Pickering, R. F. Schwarz, B. Silva, J. Herrero, N. M. Luscombe, M. Jamal-Hanjani, R. Rosenthal, N. J. Birkbak, G. A. Wilson, O. Pipek, D. Ribli, M. Krzystanek, I. Csabai, Z. Szallasi, M. Gore, N. McGranahan, P. Van Loo, P. Campbell, J. Larkin, C. Swanton and T. R. R. Consortium (2018). "Deterministic Evolutionary Trajectories Influence Primary Tumor Growth: TRACERx Renal." Cell **173**(3): 595-610 e511.

Ubersax, J. A. and J. E. Ferrell, Jr. (2007). "Mechanisms of specificity in protein phosphorylation." Nat Rev Mol Cell Biol **8**(7): 530-541.

Urick, M. E., M. L. Rudd, A. K. Godwin, D. Sgroi, M. Merino and D. W. Bell (2011). "PIK3R1 (p85alpha) is somatically mutated at high frequency in primary endometrial cancer." Cancer Res **71**(12): 4061-4067.

Valastyan, S. and R. A. Weinberg (2011). "Tumor metastasis: molecular insights and evolving paradigms." Cell **147**(2): 275-292.

Vargo-Gogola, T. and J. M. Rosen (2007). "Modelling breast cancer: one size does not fit all." Nat Rev Cancer **7**(9): 659-672.

Vichai, V. and K. Kirtikara (2006). "Sulforhodamine B colorimetric assay for cytotoxicity screening." Nat Protoc **1**(3): 1112-1116.

Vlastaridis, P., P. Kyriakidou, A. Chaliotis, Y. Van de Peer, S. G. Oliver and G. D. Amoutzias (2017). "Estimating the total number of phosphoproteins and phosphorylation sites in eukaryotic proteomes." Gigascience **6**(2): 1-11.

Vogel, W., R. Lammers, J. Huang and A. Ullrich (1993). "Activation of a phosphotyrosine phosphatase by tyrosine phosphorylation." Science **259**(5101): 1611-1614.

Walsh, C. T., S. Garneau-Tsodikova and G. J. Gatto, Jr. (2005). "Protein posttranslational modifications: the chemistry of proteome diversifications." Angew Chem Int Ed Engl **44**(45): 7342-7372.

Walter, A. O., Z. Y. Peng and C. A. Cartwright (1999). "The Shp-2 tyrosine phosphatase activates the Src tyrosine kinase by a non-enzymatic mechanism." Oncogene **18**(11): 1911-1920.

Weigelt, B., J. L. Peterse and L. J. van 't Veer (2005). "Breast cancer metastasis: markers and models." Nat Rev Cancer **5**(8): 591-602.

Whitman, M., C. P. Downes, M. Keeler, T. Keller and L. Cantley (1988). "Type I phosphatidylinositol kinase makes a novel inositol phospholipid, phosphatidylinositol-3-phosphate." Nature **332**(6165): 644-646.

Wu, D., Y. Pang, Y. Ke, J. Yu, Z. He, L. Tautz, T. Mustelin, S. Ding, Z. Huang and G. S. Feng (2009). "A conserved mechanism for control of human and mouse embryonic stem cell pluripotency and differentiation by shp2 tyrosine phosphatase." PLoS One **4**(3): e4914.

Wu, G., M. Xing, E. Mambo, X. Huang, J. Liu, Z. Guo, A. Chatterjee, D. Goldenberg, S. M. Gollin, S. Sukumar, B. Trink and D. Sidransky (2005). "Somatic mutation and gain of copy number of PIK3CA in human breast cancer." Breast Cancer Res **7**(5): R609-616.

Wu, H., S. C. Shekar, R. J. Flinn, M. El-Sibai, B. S. Jaiswal, K. I. Sen, V. Janakiraman, S. Seshagiri, G. J. Gerfen, M. E. Girvin and J. M. Backer (2009). "Regulation of Class IA PI 3-kinases: C2 domain-iSH2 domain contacts inhibit p85/p110alpha and are disrupted in oncogenic p85 mutants." Proc Natl Acad Sci U S A **106**(48): 20258-20263.

Wurmser, A. E., J. D. Gary and S. D. Emr (1999). "Phosphoinositide 3-kinases and their FYVE domain-containing effectors as regulators of vacuolar/lysosomal membrane trafficking pathways." J Biol Chem **274**(14): 9129-9132.

Xie, Y., P. W. Abel, J. K. Kirui, C. Deng, P. Sharma, D. W. Wolff, M. L. Toews and Y. Tu (2013). "Identification of upregulated phosphoinositide 3-kinase gamma as a target to suppress breast cancer cell migration and invasion." Biochem Pharmacol **85**(10): 1454-1462.

Xu, D. and C. K. Qu (2008). "Protein tyrosine phosphatases in the JAK/STAT pathway." Front Biosci **13**: 4925-4932.

Xu, H., S. Yu, Q. Liu, X. Yuan, S. Mani, R. G. Pestell and K. Wu (2017). "Recent advances of highly selective CDK4/6 inhibitors in breast cancer." J Hematol Oncol **10**(1): 97.

Xu, L., J. Stevens, M. B. Hilton, S. Seaman, T. P. Conrads, T. D. Veenstra, D. Logsdon, H. Morris, D. A. Swing, N. L. Patel, J. Kalen, D. C. Haines, E. Zudaire and B. St Croix (2014). "COX-2 inhibition potentiates antiangiogenic cancer therapy and prevents metastasis in preclinical models." Sci Transl Med **6**(242): 242ra284.

Xu, Z. Q., Y. Zhang, N. Li, P. J. Liu, L. Gao, X. Gao and X. J. Tie (2017). "Efficacy and safety of lapatinib and trastuzumab for HER2-positive breast cancer: a systematic review and meta-analysis of randomised controlled trials." BMJ Open **7**(3): e013053.

Yang, W., J. Wang, D. C. Moore, H. Liang, M. Dooner, Q. Wu, R. Terek, Q. Chen, M. G. Ehrlich, P. J. Quesenberry and B. G. Neel (2013). "Ptpn11 deletion in a novel progenitor causes metachondromatosis by inducing hedgehog signalling." Nature **499**(7459): 491-495.

Yart, A. and T. Edouard (2018). "Noonan syndrome: an update on growth and development." Curr Opin Endocrinol Diabetes Obes **25**(1): 67-73.

Yu, W. M., T. S. Hawley, R. G. Hawley and C. K. Qu (2003). "Catalytic-dependent and -independent roles of SHP-2 tyrosine phosphatase in interleukin-3 signaling." Oncogene **22**(38): 5995-6004.

Zhang, S. Q., W. G. Tsiaras, T. Araki, G. Wen, L. Minichiello, R. Klein and B. G. Neel (2002). "Receptor-specific regulation of phosphatidylinositol 3'-kinase activation by the protein tyrosine phosphatase Shp2." Mol Cell Biol **22**(12): 4062-4072.

Zhang, X., Y. He, S. Liu, Z. Yu, Z. X. Jiang, Z. Yang, Y. Dong, S. C. Nabinger, L. Wu, A. M. Gunawan, L. Wang, R. J. Chan and Z. Y. Zhang (2010). "Salicylic acid based small molecule inhibitor for the oncogenic Src homology-2 domain containing protein tyrosine phosphatase-2 (SHP2)." J Med Chem **53**(6): 2482-2493.

Zhang, X. H., M. Giuliano, M. V. Trivedi, R. Schiff and C. K. Osborne (2013). "Metastasis dormancy in estrogen receptor-positive breast cancer." Clin Cancer Res **19**(23): 6389-6397.

Zheng, H., S. Alter and C. K. Qu (2009). "SHP-2 tyrosine phosphatase in human diseases." Int J Clin Exp Med **2**(1): 17-25.

Zheng, X., J. L. Carstens, J. Kim, M. Scheible, J. Kaye, H. Sugimoto, C. C. Wu, V. S. LeBleu and R. Kalluri (2015). "Epithelial-to-mesenchymal transition is dispensable for metastasis but induces chemoresistance in pancreatic cancer." Nature **527**(7579): 525-530.

Zhou, R. P., M. T. Deng, L. Y. Chen, N. Fang, C. Du, L. P. Chen, Y. Q. Zou, J. H. Dai, M. L. Zhu, W. Wang, S. J. Lin, R. H. Liu and J. Luo (2015). "Shp2 regulates chlorogenic acid-induced proliferation and adipogenic differentiation of bone marrow-derived mesenchymal stem cells in adipogenesis." Mol Med Rep **11**(6): 4489-4495.

Zwang, Y., O. Jonas, C. Chen, M. L. Rinne, J. G. Doench, F. Piccioni, L. Tan, H. T. Huang, J. Wang, Y. J. Ham, J. O'Connell, P. Bhola, M. Doshi, M. Whitman, M. Cima, A. Letai, D. E. Root, R. S. Langer, N. Gray and W. C. Hahn (2017). "Synergistic interactions with PI3K inhibition that induce apoptosis." Elife **6**.

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9 | APPENDICES

9.1 Abbreviations

ABL1	Abelson murine leukemia viral oncogene homolog 1
AKT	Protein kinase B
ALK	Anaplastic lymphoma kinase
AML	Acute myeloid leukemia
BET	Bromodomain and extra-terminal domain
CDK	Cyclin-dependent kinase
CK	Cytokeratin
CNAs	Copy number alterations
CTC	Circulating tumor cells
Cys	Cysteine (C)
d	Days
DCIS	Ductal carcinoma *in situ*
DNA	Deoxyribonucleic acid
DTC	Disseminated tumor cells
EEC	Endometrioid endometrial cancers
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
ER	Estrogen receptor
ERK	Extracellular signal-related kinase
ESCC	Esophageal squamous cell cancer
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FDA	Food and drug administration
FGFR1	Fibroblast growth factor receptor 1
FIH	First in human
FOXO	Forkhead transcription factors of the O subgroup
GAB1	GRB2-associated-binding protein 1
GAB2	GRB2-associated-binding protein 2
GBM	Glioblastoma
GOF	Gain-of-function
GPCR	G-protein-coupled receptors
GRB2	Growth factor receptor-bound protein 2
h	Hour
HER2	Human epidermal growth factor receptor 2
HGFR	Hepatocyte growth factor receptor (also called c-Met)
HNSCC	Head-and-neck squamous cell cancer
HR	Hormone receptors
HSP90	Heat shock protein 90
hVPS34	Human vacuolar protein sorting 34
IGF-I	Insulin-like growth factor I
IGFR1	Insulin like growth factor receptor 1
INSR	Insulin receptor
IRS-1	Insulin receptor substrate 1
JAK	Janus kinase
JMML	Juvenile myelomonocytic leukemias
KRAS	Kirsten rat sarcoma virus
LEOPARD	Multiple lentigines, electrocardiographic abnormalities, ocular hypertelorism, pulmonary

	stenosis, abnormal genitalia, retardation of growth, and sensorineural deafness
LOF	Loss-of-function
LS	LEOPARD syndrome
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
METABRIC	Molecular Taxonomy of Breast Cancer International Consortium
MMP	Matrix metalloproteinases
mTORC1	Mammalian target of rapamycin complex 1
mTORC2	Mammalian target of rapamycin complex 2
NRTK	Non-receptor tyrosine kinase
NS	Noonan syndrome
NSCLC	Non-small-cell lung cancer
NS-LM	Noonan syndrome with multiple lentigines
PAR1	Prader-Willi/Angelman region-1
PARP	Poly ADP ribose polymerase
pCR	Pathological complete response
PDGFR	Platelet-derived growth factor receptor
PDGFRβ	Platelet-derived growth factor receptor beta
PDK1	Phosphoinositide-dependent kinase 1
PD-L1	Programmed death ligand 1
PDX	Patient-derived xenograft
PI3K	Phosphoinositol 3-kinase
PI3Ki	PI3K inhibition
PI-4-P	Phosphatidylinositol-4-phosphate
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
P-loop	Phosphate-binding loop
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
PTPN11	Protein tyrosine phosphatase non-receptor type 11
RAF	Rapidly accelerated fibrosarcoma
RAS	Rat sarcoma
RCC	Renal cell carcinoma
RTK	Receptor tyrosine kinase
S6K	p70 ribosomal protein S6 kinase
S6K1	p70 ribosomal protein S6 kinase 1
Ser	Serine (S)
SERD	Selective estrogen receptor downregulator
SERM	Selective estrogen receptor modulator
SFK	Src family kinase
SH2	SRC homology 2
SHIP-1	SH-2 containing inositol 5' polyphosphatase 1
SHP2	SRC-homology 2 domain-containing phosphatase
SHP2i	SHP2 inhibition
STAT	Signal transducer and activator of transcription
TGF-β	Transforming growth factor-β
Thr	Threonine (T)

TKI	Tyrosine kinase inhibitor
TNBC	Triple negative breast cancer
TSC	Tuberous sclerosis complex
Tyr	Tyrosine (Y)
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WHO	World Health Organization

9.2 List of figures and tables

Introduction

Results

9.3 Published manuscripts

The ninth ENBDC Weggis meeting: growth and in-depth characterization of normal and neoplastic breast cells.

Wiese KE*, **Amante RJ***, Vivanco MD, Bentires-Alj M, Iggo RD: **Breast cancer research**: BCR 2017, 19(1):96. doi: 10.1186/s13058-017-0891-9. (*equal contribution). Meeting report.

Anticipating mechanisms of resistance to PI3K inhibition in breast cancer: a challenge in the era of precision medicine.

Leroy C*, **Amante RJ***, Bentires-Alj M: **Biochemical Society transactions** 2014, 42(4):733-741. doi: 10.1042/bst20140034. (*equal contribution). Review.

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Breast Cancer Research

MEETING REPORT Open Access

The ninth ENBDC Weggis meeting: growth and in-depth characterisation of normal and neoplastic breast cells

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Abstract

Mammary gland biologists gathered for the ninth annual workshop of the European Network for Breast Development and Cancer (ENBDC) at Weggis on the shores of Lake Luceme in March 2017. The main themes were oestrogen receptor alpha signalling, new techniques for mammary cell culture, CRISPR screening and proteogenomics.

Keywords: Breast cancer, Oestrogen receptor, Proteomics, Organoid culture, CRISPR screen, European Network for Breast Development and Cancer

Main text

The workshop started with a keynote lecture by *Jason Carroll* (CRUK, Cambridge, UK), who shed new light on the relationship between oestrogen and progesterone receptors in breast cancer. ChIP-sequencing studies revealed that activated progesterone receptor (PR) redirects oestrogen receptor alpha (ER) binding to genomic sites that are associated with better survival of patients, leading Carroll to propose that *PGR* is a tumour suppressor gene [1]. To test this idea, Carroll has initiated a window trial of megestrol to activate PR in ER+ breast cancer. Putting hormone sensing (sensor) cells in a broader context, *Tan Ince* (University of Miami, USA) reviewed his classification of normal human breast cells according to ER, androgen receptor (AR) and vitamin D receptor (VDR) expression [2], explaining the logic of phylogenetic and cladistic classification strategies and how to select the best cell-type specific markers (those with bimodal expression).

Preserving ER expression during culture is one of the main challenges in the field. Last year we learnt that the mammary ducts create a special microenvironment that supports the growth of ER+ human breast cancer [3, 4]; this year we learnt how to reproduce this microenvironment in culture. *Lone Ronnov-Jessen* (University of Copenhagen, Denmark) described how her laboratory first identified new cell surface markers for ER+ cells, and then demonstrated that inhibiting TGFβ receptor signalling helps to sustain ER expression in cells expressing those markers [5]. Taking a similar approach to isolating subpopulations of fibroblasts, she showed that normal breast epithelial cells form tubules if plated on a feeder layer of intralobular fibroblasts. *Oded Kopper* (Hubrecht Institute, Utrecht, the Netherlands) presented work from Hans Clevers' laboratory on organoid cultures of normal mammary epithelial cells and breast cancer. The organoid approach starts from the assumption that the best way to grow tumour cells is to mimic physiological conditions. The Clevers laboratory has pioneered the organoid culture technique where cells are embedded in matrigel, allowing them to grow in a self-organising manner in 3D [6]. This format makes it easy to test multiple different growth factors and inhibitors. With a cocktail of growth factors (EGF, R-Spondin 1, FGF7/10 and NRG1) and inhibitors (for BMP, TGFβ, ROCK and p38) that presumably mimics the paracrine environment ("niche"), they were able to establish 101 breast cancer organoids from 151 tumours tested, including all of the major types of breast cancer. A different organoid approach based on technology from OcellO, a Dutch company that performs drug screening in 3D organoid cultures, is already finding a place in clinical studies, as described by *Rebecca Marlow* from Andrew Tutt's laboratory (King's College, London, UK). Although very promising, some aspects of the technology, such as the composition of the hydrogels, are proprietary, which means the Clevers system is likely to sweep the field, at least among basic scientists.

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Traditionally, Weggis always features an "off-the-wall" talk about new technologies from someone outside the field. This year it fell to *Pierre Nassoy* (University of Bordeaux, France) to describe his alginate microencapsulation technology [7]. The capsules enclose cells in a thin skin of alginate that provides physical constraint. It is possible to generate thousands of capsules in a few minutes, making it possible to study huge numbers of individual clones. The technology can also be used with mixtures of different cell types to mimic the interactions between, for example, tumour cells and cancer-associated fibroblasts. The size and thickness of the capsules can be modified to change the physical properties of the microenvironment. In a final flourish, Nassoy showed that it is even possible to generate long tubes of alginate that could perhaps be used to encapsulate entire mammary ducts. *Barbara Szczerba* from the Aceto laboratory (University of Basel, Switzerland) then described a microfluidic-based technology to isolate and grow clusters of circulating tumour cells from blood samples [8, 9]. The culture system is similar to classic mammosphere culture but preserves ER expression in a substantial proportion of cases, perhaps because the cells are already adapted to surviving in suspension.

Alexandra Van Keymeulen from the Blanpain group (Free University of Brussels, Belgium) first addressed the controversy surrounding her landmark 2011 *Nature* paper proposing that the adult gland contains distinct long-lived unipotent luminal and basal stem cells [10]. That work was challenged by a paper from the Visvader laboratory arguing that bipotent stem cells play a major role in normal homeostasis of the adult gland [11]. To resolve this issue, Van Keymeulen has now performed experiments with the Confetti mouse model that support her original conclusions [10]. Arguably, the main interest of the work is the confirmation that long-lived unipotent stem cells exist. Van Keymeulen ended the ER talks on a high note by performing lineage tracing with Esr1-rtTA/tetO-cre/Rosa-lsl-YFP mice. She showed that long-lived unipotent ER+ stem cells exist in the adult gland. They are prime candidates for the cell of origin of classic ER+ human breast cancers. Previous work from the Blanpain and Bentires-Alj laboratories showed that PIK3CA mutations break the lineage restriction of luminal and basal progenitors [12, 13]. It will be fascinating to see whether the same is true of the new ER+ stem cell population.

For aficionados of CRISPR technology, there was a group of talks on pooled library screens. *Reuven Agami* (NKI, Amsterdam, the Netherlands) described genetic screens to identify functional elements in non-coding DNA [14]. He characterised enhancers targeted by p53 and ER, and found sites important not only for binding by the primary factors but also for cooperating transcription factors. This is an extremely elegant approach but the technology is still a long way from being able to serve genome-wide studies. *Björn von Eyss* (Leibniz Institute on Aging, Jena, Germany), winner of the DeOme prize 2017 for best short talk, then described a CRISPR screen for MST/LATS-independent regulators of YAP/TAZ in a human breast cell line. *Ilirjana Bajrami*, from Chris Lord's laboratory (The Institute of Cancer Research, London, UK), then described siRNA screens looking for genes showing synthetic lethality with CRISPR-engineered loss of E-cadherin. The upshot is that loss of ROS1 causes a mild cytokinesis defect that is made far worse by loss of E-cadherin. Drugs like crizotinib that inhibit ROS1 kill the lobular tumour cells in Jos Jonkers' *Cdh1*-null mouse models [15]. A phase II trial is now under way to see whether humans are as obedient as mice.

Novel techniques to better understand the breast cancer proteome and their potential to improve cancer therapy were the focus of the Proteomics Session (chaired by Romain Amante, University of Basel, Switzerland and Katrin Wiese, University of Amsterdam, the Netherlands). *Johanna Wagner* from Bernd Bodenmiller's laboratory (University of Zürich, Switzerland) discussed how proteomics can reveal breast cancer heterogeneity at the single cell level. Using metal-tag barcoding, they simultaneously profiled 38 markers in tumour samples. This information could potentially form the basis for individualised treatment strategies. Next, *Janne Lehtiö* (Karolinska Institute, Stockholm, Sweden) introduced the emerging field of proteogenomics and highlighted the importance of adding proteome information as an additional data layer in cancer studies. The quantitative methods his laboratory is developing can aid biomarker discovery and identify protein signatures associated with drug resistance [16, 17]. Importantly, the data will soon be shared with other researchers in the form of a database. *Simone Lemeer* (Utrecht University, the Netherlands) gave a thorough introduction about the principles of mass spectrometry, and then presented a study that combined different proteomic and metabolic techniques to identify the mechanism of lapatinib resistance in breast cancer [18]. Finally, *Jukka Westermarck* (Centre for Biotechnology, Turku, Finland) stressed the significance of inhibition of PP2A-mediated protein de-phosphorylation for malignant transformation, and discussed PP2A inhibitor protein CIP2A as a promising target for breast cancer therapy.

Two talks described genomic characterisation of tumours to understand specific phenotypes. *Leonie Young* (RCSI, Dublin, Ireland) described studies trying to explain why particular subtypes of breast cancer metastasise to particular sites. *Therese Sorlie* (Oslo University Hospital, Norway) described a set of tumours induced in mice by exposure to MPA and DMBA. Use of DMBA led

to a 7-fold higher mutation rate than is seen in human tumours, but the most common changes included mouse homologues of many known human breast cancer genes, like *TP53*, *NF1*, *ATR*, *KRAS* and *KMT2C*.

The meeting closed with a presentation on chemotherapy-induced tumour dormancy mediated by IRF7-dependent activation of interferon signalling (*Sanam Peyvandi*, Ruegg laboratory, University of Fribourg, Switzerland), and a beautiful imaging study of proliferation in explant cultures of mammary buds from FUCCI mice (*Riitta Lindstrom*, Mikkola laboratory, University of Helsinki, Finland).

Conclusions

This was the year when culture of all subtypes of breast cancer came of age. It will be interesting to see how quickly the ability to study tumours from individual patients moves into clinical practice. Currently, the main barrier to using NGS data from patients is the inability to predict who will respond to genomically guided therapy. Rapid functional testing in the new culture systems presented at the meeting would go a long way towards solving this problem.

The 10th ENBDC meeting is set for 15–17 March 2018 and the meeting will be chaired by Eva Gonzalez Suarez (Bellvitge Institute for Biomedical Research, Barcelona, Spain).

Abbreviations

AR: Androgen receptor; ENBDC: European Network for Breast Development and Cancer; ER: Oestrogen receptor alpha; PR: Progesterone receptor; VDR: Vitamin D receptor

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Authors' information

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Competing interests

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References

1. Mohammed H, Russell IA, Stark R, Rueda OM, Hickey TE, Tarulli GA, Serandour AA, Birrell SN, Bruna A, Saadi A, et al. Progesterone receptor modulates ERalpha action in breast cancer. Nature. 2015;523(7560):313–7.
2. Santagata S, Thakkar A, Ergonul A, Wang B, Woo T, Hu R, Harrell JC, McNamara G, Schwede M, Culhane AC, et al. Taxonomy of breast cancer based on normal cell phenotype predicts outcome. J Clin Invest. 2014; 124(2):859–70.
3. Sflomos G, Dormoy V, Metsalu T, Jeitziner R, Battista L, Scabia V, Raffoul W, Delaloye JF, Treboux A, Fiche M, et al. A preclinical model for ERalpha-positive breast cancer points to the epithelial microenvironment as determinant of luminal phenotype and hormone response. Cancer Cell. 2016;29(3):407–22.
4. Richard E, Grellety T, Velasco V, MacGrogan G, Bonnefoi H, Iggo R. The mammary ducts create a favourable microenvironment for xenografting of luminal and molecular apocrine breast tumours. J Pathol. 2016;240(3):256–61.
5. Fridriksdottir AJ, Kim J, Villadsen R, Klitgaard MC, Hopkinson BM, Petersen OW, Ronnov-Jessen L. Propagation of oestrogen receptor-positive and oestrogen-responsive normal human breast cells in culture. Nat Commun. 2015;6:8786.
6. Sachs N, Clevers H. Organoid cultures for the analysis of cancer phenotypes. Curr Opin Genet Dev. 2014;24:68–73.
7. Alessandri K, Sarangi BR, Gurchenkov VV, Sinha B, Kiessling TR, Fetler L, Rico F, Scheuring S, Lamaze C, Simon A, et al. Cellular capsules as a tool for multicellular spheroid production and for investigating the mechanics of tumor progression in vitro. Proc Natl Acad Sci U S A. 2013;110(37):14843–8.
8. Yu M, Bardia A, Aceto N, Bersani F, Madden MW, Donaldson MC, Desai R, Zhu H, Comaills V, Zheng Z, et al. Cancer therapy. Ex vivo culture of circulating breast tumor cells for individualized testing of drug susceptibility. Science. 2014;345(6193):216–20.
9. Aceto N, Bardia A, Miyamoto DT, Donaldson MC, Wittner BS, Spencer JA, Yu M, Pely A, Engstrom A, Zhu H, et al. Circulating tumor cell clusters are oligoclonal precursors of breast cancer metastasis. Cell. 2014;158(5):1110–22.
10. Van Keymeulen A, Rocha AS, Ousset M, Beck B, Bouvencourt G, Rock J, Sharma N, Dekoninck S, Blanpain C. Distinct stem cells contribute to mammary gland development and maintenance. Nature. 2011;479(7372):189–93.
11. Rios AC, Fu NY, Lindeman GJ, Visvader JE. In situ identification of bipotent stem cells in the mammary gland. Nature. 2014;506(7488):322–7.
12. Van Keymeulen A, Lee MY, Ousset M, Brohee S, Rorive S, Giraddi RR, Wuidart A, Bouvencourt G, Dubois C, Salmon I, et al. Reactivation of multipotency by oncogenic PIK3CA induces breast tumour heterogeneity. Nature. 2015; 525(7567):119–23.
13. Koren S, Reavie L, Couto JP, De Silva D, Stadler MB, Roloff T, Britschgi A, Eichlisberger T, Kohler H, Aina O, et al. PIK3CA(H1047R) induces multipotency and multi-lineage mammary tumours. Nature. 2015;525(7567):114–8.
14. Korkmaz G, Lopes R, Ugalde AP, Nevedomskaya E, Han R, Myacheva K, Zwart W, Elkon R, Agami R. Functional genetic screens for enhancer elements in the human genome using CRISPR-Cas9. Nat Biotechnol. 2016;34(2):192–8.
15. Annunziato S, Kas SM, Nethe M, Yucel H, Del Bravo J, Pritchard C, Bin Ali R, van Gerwen B, Siteur B, Drenth AP, et al. Modeling invasive lobular breast

carcinoma by CRISPR/Cas9-mediated somatic genome editing of the mammary gland. Genes Dev. 2016;30(12):1470–80.
16. Branca RM, Orre LM, Johansson HJ, Granholm V, Huss M, Perez-Bercoff A, Forshed J, Kall L, Lehtio J. HiRIEF LC-MS enables deep proteome coverage and unbiased proteogenomics. Nat Methods. 2014;11(1):59–62.
17. Johansson HJ, Sanchez BC, Forshed J, Stal O, Fohlin H, Lewensohn R, Hall P, Bergh J, Lehtio J, Linderholm BK. Proteomics profiling identify CAPS as a potential predictive marker of tamoxifen resistance in estrogen receptor positive breast cancer. Clin Proteomics. 2015;12(1):8.
18. Ruprecht B, Zaal EA, Zecha J, Wu W, Berkers CR, Kuster B, Lemeer S. Lapatinib resistance in breast cancer cells is accompanied by phosphorylation-mediated reprogramming of glycolysis. Cancer Res. 2017;77(8):1842–53.

Anticipating mechanisms of resistance to PI3K inhibition in breast cancer: a challenge in the era of precision medicine

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Abstract

Frequent subversion of the PI3K (phosphoinositide 3-kinase) pathway during neoplastic transformation contributes to several hallmarks of cancer that result in a competitive advantage for cancer cells. Deregulation of this pathway can be the result of genomic alterations such as *PIK3CA* mutation, PTEN (phosphatase and tensin homologue deleted on chromosome 10) loss or the activation of upstream protein tyrosine kinases. Not surprisingly, the PI3K signalling pathway has become an attractive therapeutic target, and numerous inhibitors are in clinical trials. Unfortunately, current therapies for advanced cancers that target PI3K often lead to the development of resistance and relapse of the disease. It is therefore important to establish the molecular mechanisms of resistance to PI3K-targeted therapy. With the focus on breast cancer, in the present article, we summarize the different ways of targeting PI3K, review potential mechanisms of resistance to PI3K inhibition and discuss the rationale of combination treatments to reach a balance between efficacy and toxicity.

PI3K: an important target in breast cancer

The PI3K pathway

The PI3K (phosphoinositide 3-kinase) signalling cascade is one of the most important pathways that induce hallmarks of cancer [1]. PI3Ks are lipid kinases that phosphorylate phosphoinositides at the D-3 position (inositol ring), leading to the activation of a series of downstream kinases that influence key physiological processes such as metabolism, proliferation, survival and motility. The PI3K pathway can be activated by RTKs (receptor tyrosine kinases), such as the EGFR (epidermal growth factor receptor) family (e.g. ErbB1–3), IGF-1R (insulin-like growth factor 1 receptor) and FGFRs (fibroblast growth factor receptors), as well as by GPCRs (G-protein-coupled receptors) [2]. The PI3K family can be subdivided on the basis of substrate preference, sequence homology and tissue distribution [2]. As it is principally involved in cancer, we focus on class IA of PI3K and its downstream effectors, which comprise regulatory and catalytic subunits [3]. For example, binding to the regulatory subunit p85 maintains the catalytic subunit p110 in a low–activity state. In response to upstream signals, p85 releases p110 after binding phosphorylated tyrosine residues on RTKs or on adapter proteins through its SH2 (Src homology 2) domain [4]. p110 in turn phosphorylates PtdIns(4,5)P_2, generating PtdIns(3,4,5)P_3, and recruits proteins with a PH (pleckstrin homology) domain to the plasma membrane, such as PDK1 (phosphoinositide-dependent kinase 1) and protein kinase B (also called Akt). Akt is phosphorylated by PDK1 at Thr^{308} and at Ser^{473} by mTORC2 (mammalian target of rapamycin complex 2). Fully active Akt phosphorylates and leads directly or indirectly to the activation of numerous downstream proteins involved in processes such as cell cycle progression, metabolism, cell survival and cell motility. For example, Akt phosphorylates the Foxo (forkhead box O) family of transcription factors that regulate the expression of several pro-apoptotic genes [5]. Phosphorylation of Akt also results in the activation of mTORC1, which in turn phosphorylates S6K (ribosomal protein S6 kinase) and 4E-BP1 (eukaryotic translation initiation factor 4E-binding protein 1). This results in the stimulation of 5′cap-dependent protein translation [6]. The phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10) regulates PI3K activity through the dephosphorylation of PtdIns(3,4,5)P_3 and generation of PtdIns(4,5)P_2.

PI3K pathway deregulation in breast cancer

The PI3K signalling pathway is frequently hyperactivated in breast cancer. It is estimated that up to 70 % of breast cancers feature a hyperactive PI3K cascade [7,8]. Four main mechanisms have been identified that abnormally

Key words: breast cancer, drug resistance, phosphoinositide 3-kinase (PI3K), signalling, targeted therapy.

Abbreviations: BRCA, breast cancer early-onset; CXCR1, CXC chemokine receptor 1; 4E-BP1, eukaryotic translation initiation factor 4E-binding protein 1; EGFR, epidermal growth factor receptor; ER, oestrogen receptor; ERK, extracellular-signal-regulated kinase; FGFR, fibroblast growth factor receptor; Foxo, forkhead box O; HER, human epidermal growth factor receptor; IGF-1R, insulin-like growth factor 1 receptor; IL-8, interleukin 8; IRS1, insulin receptor substrate 1; JAK2, Janus kinase 2; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; mTOR, mammalian target of rapamycin; mTORC, mammalian target of rapamycin complex; PARP, poly(ADP-ribose) polymerase; PDK1, phosphoinositide-dependent kinase 1; PI3K, phosphoinositide 3-kinase; PR, progesterone receptor; PTEN, phosphatase and tensin homologue deleted on chromosome 10; RTK, receptor tyrosine kinase; S6K, ribosomal protein S6 kinase.

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activate this pathway in breast cancer. Overexpression and hyperactivation of RTKs (e.g. ErbB2 or EGFR) result in the activation of PI3K as well as other oncogenic cascades such as MAPK (mitogen-activated protein kinase). Amplification and/or mutation of the *PIK3CA* gene that encodes the p110α catalytic subunit of PI3K, as in 20–40 % of breast cancers, increases the activity of the enzyme and induces heterogeneous mammary tumours when expressed in the mouse mammary gland [9–11]. Amplification and/or mutation of Akt isoforms is also found in 4 % of human breast tumours [12]. Loss of function of the tumour suppressor PTEN (found in 30 % of human breast cancers) [13,14] and subsequent PI3K pathway activation occurs by mutation, deletion [15], transcriptional silencing [16,17], or change in protein stability [18].

PI3K inhibitors

Given the key effects of the PI3K pathway in solid cancers, important drug discovery programs have yielded a variety of compounds that efficiently target the PI3K/mTOR (mammalian target of rapamycin) pathway and are currently being evaluated in clinical trials (Table 1). In the present article, we only discuss PI3K inhibitors; inhibitors targeting mTOR alone or Akt have been reviewed elsewhere [19]. PI3K inhibitors can be divided into the three classes of dual PI3K/mTOR inhibitors, inhibitors of all class I PI3K isoforms (called pan-PI3K inhibitors) and isoform-specific PI3K inhibitors.

Dual PI3K/mTOR inhibitors

Structural similarities in the catalytic domains of mTOR and the p110 subunit of PI3K have prompted the development of compounds with dual activity, with the advantage of targeting the pathway at two levels. Most of the dual PI3K/mTOR inhibitors target mTORC1, mTORC2 and p110 α, β, and δ isoforms, although with different efficiencies [20]. Dual PI3K/mTOR inhibition may offer a better therapeutic efficacy by abrogating the feedback activation of PI3K signalling observed with rapalogues (rapamycin analogues) (see below). Dual inhibitors should efficiently block the PI3K/Akt/mTOR signalling pathway in cancers harbouring *PIK3CA* mutations, *PI3KR1* mutations, PTEN loss or RTK-dependent activation of this signalling cascade. Overall, such compounds exhibit anti-proliferative activity in various breast cancer cell lines. They are well tolerated and produce disease stasis and, in some cases, tumour shrinkage, particularly in HER2 (human epidermal growth factor receptor 2)-amplified, *PIK3CA* mutated or PTEN-deficient human breast cancer cell lines grown as xenografts in mice [20,21]. Initial data from Phase I clinical studies suggest that several dual PI3K/mTOR inhibitors (e.g. BEZ235, XL765 and GDC0980) are active in patients, show prolonged disease stabilization and are well tolerated, with manageable side effects such as fatigue, rash, nausea, hyperglycaemia and/or intestinal disorders [22].

Pan-PI3K inhibitors

Pan-PI3K selective inhibitors target all class IA PI3Ks. Specific inhibition of PI3K mostly decreases mTORC1 activity, which, in principle, may evoke fewer side effects than inhibition of both mTORC1 and mTORC2. This class of inhibitors should be efficient in cancers in which PI3K/Akt signalling is the main driver of mTORC1 activity. In cases where *BRAF* or *KRAS* mutations contribute to mTORC1 activation, dual PI3K/mTOR inhibitors will be the inhibitors of choice, and cases of *AKT* mutations or amplifications are likely to be insensitive to pan-PI3K inhibitors. Pan-PI3K inhibitors (e.g. BKM120, GDC0941 and BAY80-6946) have strong anti-proliferative activity in a broad range of breast cancer cell lines *in vitro*, and anti-tumour effects of these compounds have been described in several xenograft models of metastatic HER2$^+$ breast cancer [23,24]. As expected with p110δ inhibition, strong immunosuppressive activity has been described following pan-PI3K inhibition [25]. These inhibitors are currently in several Phase I clinical trials involving patients with ER$^+$/HER2$^-$ (ER is oestrogen receptor) or HER2$^+$ breast cancer. More than 50 % of patients in these trials show stable disease responses with signs of clinical activity. Overall, pan-PI3K inhibitors are well tolerated, and the side effects include hyperglycaemia, rash, nausea and/or decreased appetite [26].

PI3K isoform-specific inhibitors

Whereas p110α and p110β are expressed ubiquitously in mammals, p110γ and p110δ are expressed preferentially in leucocytes. In addition to their effects on cell growth and survival, class IA PI3K isoforms are key regulators of glucose metabolism and immune cell proliferation. Therefore isoform-specific inhibitors have been developed in an attempt to avoid the toxicity (e.g. glucose intolerance or immunosuppression) of pan-PI3K inhibitors.

PI3Kα-specific inhibitors

Specific p110α inhibitors could effectively shut off the PI3K pathway in cancers harbouring *PIK3CA* mutations and/or amplifications. Screening of the p110α inhibitor BYL719 in a panel of cancer cell lines revealed that those with *PIK3CA* mutations or *ERBB2* amplification are hypersensitive to the inhibitor. In contrast, some breast cancer cell lines harbouring *KRAS* mutations responded to BYL719, but others did not, and cancer cells with *BRAF* or *PTEN* mutations were resistant [27]. With regard to toxicity, *ex vivo* results show that p110α inhibitors have minimal effects on B-cell and T-cell functions, and are less immunosuppressive *in vivo* than pan-PI3K inhibitors [25]. In Phase I clinical studies evaluating BYL719 that restricted enrolment to patients with *PIK3CA* mutant solid cancers, preliminary data show signs of clinical activity and prolonged disease stabilization with tumour shrinkage [19]. As expected, BYL719 seems to be well tolerated with manageable side effects such as hyperglycaemia [28], nausea, vomiting, diarrhoea and/or decreased appetite.

Table 1 | Inhibitors of PI3K in clinical trials as single agents or in combination with hormonal therapy or chemotherapy for breast or solid cancer patients (from http://clinicaltrials.gov as of 17 December 2013)

AR, androgen receptor.

Inhibitor	Associated therapy	Current phase of clinical trial	Breast cancer subtype	Clinical trial identifier
Dual PI3K/mTOR				
BEZ235		Phase I/II	Advanced breast cancer	NCT00620594
	Paclitaxel	Phase Ib	HER2$^-$ advanced or metastatic breast cancer	NCT01495247
		Phase II	HER2$^-$ HR$^+$ metastatic breast cancer	NCT01288092
PF04691502	Letrozol	Phase Ib/II	ER$^+$ HER2$^-$ early breast cancer	NCT01430585
	Exemestane	Phase II	Advanced breast cancer	NCT01658176
GSK2126458		Phase I	Solid tumours	NCT00572686
PF05212384		Phase I	Solid tumours	NCT00940498
BGT226		Phase I/II	Advanced breast cancer	NCT00600275
Pan-PI3K				
BKM120	Fulvestrant	Phase I	ER$^+$ stage IV breast cancer	NCT01339442
		Phase II	Triple-negative breast cancer	NCT01629615
	Paclitaxel	Phase II	HER2$^-$ advanced breast cancer	NCT01572727
	Paclitaxel	Phase II	ER$^+$ HER2$^-$ breast cancer	NCT01953445
		Phase II	Solid cancers with *PIK3CA* mutations	NCT01501604
		Phase Ia	Advanced solid tumours	NCT01068483
	Fulvestrant	Phase III	HR$^+$ HER2$^-$ advanced or metastatic breast cancer	NCT01633060
	Fulvestrant	Phase III	Advanced or metastatic triple-negative breast cancer	NCT01610284
		Phase II	Triple-negative breast cancer	NCT01790932
		Phase I	Advanced breast cancer	NCT01626209
GDC0941	Cisplatin	Phase Ib/II	AR$^-$ triple-negative breast cancer	NCT01918306
	Paclitaxel	Phase II	Metastatic breast cancer	NCT01740336
XL147		Phase I	Breast and lung cancer	NCT00704392
BAY806946	Paclitaxel	Phase I	Advanced cancer	NCT01411420
SF1126		Phase I	Solid tumours	NCT00907205
PX-866		Phase I	Advanced solid tumours	NCT00726583
ZSTK474		Phase I	Advanced solid tumours	NCT01280487
		Phase I	Advanced solid tumours	NCT01682473
α-Specific PI3K				
BYL719	Letrozol	Phase Ib	HR$^+$ metastatic breast cancer	NCT01791478
	Fulvestrant	Phase Ia	Advanced solid tumours with *PIK3CA* mutations	NCT01219699
	Letrozol	Phase I	HR$^+$ metastatic breast cancer	NCT01870505
GDC0032	Fulvestrant/Letrozol	Phase I/II	Advanced or metastatic HR$^+$ breast cancer	NCT01296555
	Docetaxel/Paclitaxel	Phase Ib	HER2$^-$ metastatic breast cancer	NCT01862081
MLN1117		Phase I	Advanced solid tumours	NCT01449370
Dual PI3K/mTOR compared with pan-PI3K				
BEZ235/BKM120	Letrozol	Phase Ib	HR$^+$ metastatic breast cancer	NCT01248494
GDC0941/GDC0980	Fulvestrant	Phase II	Advanced or metastatic breast cancer	NCT01437566
XL147/XL765	Letrozol	Phase I/II	HR$^+$ HER2$^-$ breast cancer	NCT01082068
Pan-PI3K compared with α-specific PI3K				
BKM120/BYL719	Letrozol	Phase II	HR$^+$ HER2$^-$ breast cancer	NCT01923168

PI3Kβ-specific inhibitors

Some breast cancers could benefit from PI3Kβ-specific inhibitors. In pre-clinical models, p110β inhibitors have shown efficacy in PTEN-deficient cell lines and xenograft models [29–32]. These inhibitors should have minimal immunosuppressive effects, as illustrated by the modest effects on lymphocyte functions of the dual p110α/p110β inhibitor MLN316 [25]. To date, there are no reports of clinical trials to evaluate p110β inhibition in PTEN-deficient breast cancer. Despite the potential advantages of isoform-specific inhibitors, it is possible that untargeted p110 isoforms could compensate for the lack of activity of the targeted isoform.

Resistance to PI3K pathway inhibition in breast cancer

Although a broad range of PI3K inhibitors are currently in clinical trials (Table 1), clinical responses to PI3K/mTOR inhibitors as single agents are less frequent than expected. The PI3K pathway is part of a complex signalling network and rewiring of cancer cell circuitries can offset the effects of inhibitors, thus such targeted therapies are not indomitable. Therefore, in addition to the crucial importance of patient stratification and appropriate biomarkers for the success of precision medicine, anticipating mechanisms of resistance to PI3K inhibition is a *conditio sine qua non* for the rational design of combination therapies (Table 2).

Studies in *Drosophila* and mammalian systems have revealed a PI3K pathway regulatory mechanism in which S6K activation evokes a negative feedback that inhibits IRS1 (insulin receptor substrate 1) and thus dampens IGF-1R/PI3K signalling [33–35]. Blockade of this feedback loop turned out to be a key mechanism of adaptive resistance to inhibitors of the PI3K pathway. Other important mechanisms of resistance to these inhibitors are acquired genomic alterations and intrinsic resistance. Although some of these mechanisms have only been observed or tested in cell culture and/or animal models, others are also detected in patients. A thorough analysis of human materials from past and ongoing clinical trials evaluating PI3K inhibitors would further address the prevalence and clinical relevance of these experimentally identified mechanisms of resistance.

Adaptive resistance

Adaptive resistance occurs following mTOR, PI3K/mTOR, PI3K or Akt inhibition. Here are some examples.

mTOR inhibitors

The limited efficacy of allosteric mTORC1 inhibition in patients with advanced or metastatic breast cancer reported in early studies [36,37] was most likely to be due to abrogation of the S6K-mediated negative feedback leading to reactivation of PI3K/Akt signalling [38]. Moreover, it was reported for some cellular models that rephosphorylation of 4E-BP1 shortly after an initial inhibition of the mTORC1 substrates S6K and 4E-BP1 leads to resistance [39]. Observation of the recurrence of 4E-BP1 but not S6K phosphorylation in patient samples would suggest that S6 phosphorylation is not an ideal biomarker for inactivation of mTORC1 effectors [40]. Notably, it was reported that activation of MAPK via an RTK/IRS1/PI3K pathway occurs in ~50 % of breast cancer patients treated with the mTORC1 inhibitor everolimus [41], suggesting that co-inhibition of MAPK would enhance efficacy.

PI3K/mTOR inhibitors

Use of a 3D model system using matrix-attached breast and ovarian cancer cells demonstrated that dual PI3K/mTOR inhibition leads to adaptive resistance via the up-regulation of Bcl2, EGFR and IGF-1R, and provides a rationale for combining BEZ235 with BCL2, EGFR or IGF-1R inhibitors [42]. Moreover, JAK2 (Janus kinase 2)/STAT5 (signal transducer and activator of transcription 5) has been found to stifle the action of the dual PI3K/mTOR inhibitor BEZ235 in triple-negative breast cancer experimental models (cell lines and primary derived breast cancer xenografts). JAK2 seems to be activated in two phases, the first involving IGF-1R/insulin receptor/IRS1 as a consequence of the abrogation of the S6K-evoked negative-feedback loop, and a second implicating the chemokine IL-8 (interleukin 8) and its receptor CXCR1 (CXC chemokine receptor 1). Notably, co-targeting PI3K/mTOR and JAK2 increased cancer cell death, reduced tumour growth and metastasis, and increased overall survival of the animals [43]. Thus combined inhibition of the PI3K/mTOR pathway and the IL-8/CXCR1/JAK2 axis may be a useful strategy for treating metastatic triple-negative breast cancer [44]. Testing in patients whether IL-8 levels and JAK2 phosphorylation increase upon inhibition of PI3K and/or mTOR is warranted and may pave the way for trials to evaluate the combination of PI3K/mTOR and IL-8/JAK2 axis inhibition.

Pan-PI3K inhibitors

In HER2-overexpressing breast cancer, PI3K inhibition with the pan-PI3K inhibitor GDC0941 or the dual PI3K/mTOR inhibitor BEZ235 has been shown to increase HER2/3 signalling and lead to ERK1/2 (extracellular-signal-regulated kinase 1/2) activation [45]. Clinical trials testing the combination of PI3K and HER2 inhibitors are ongoing (Table 2).

Akt inhibitors

Allosteric Akt inhibition reactivates several RTKs in a Foxo-dependent manner that attenuates the beneficial effect of the inhibitor [46].

Taken together, these observations underline the importance of adaptive responses to PI3K pathway inhibition and support not only the need for substantial pre-clinical efforts using pathophysiologically relevant models of breast cancer to anticipate mechanisms of resistance, but also access to human tumour samples during and after therapy to validate

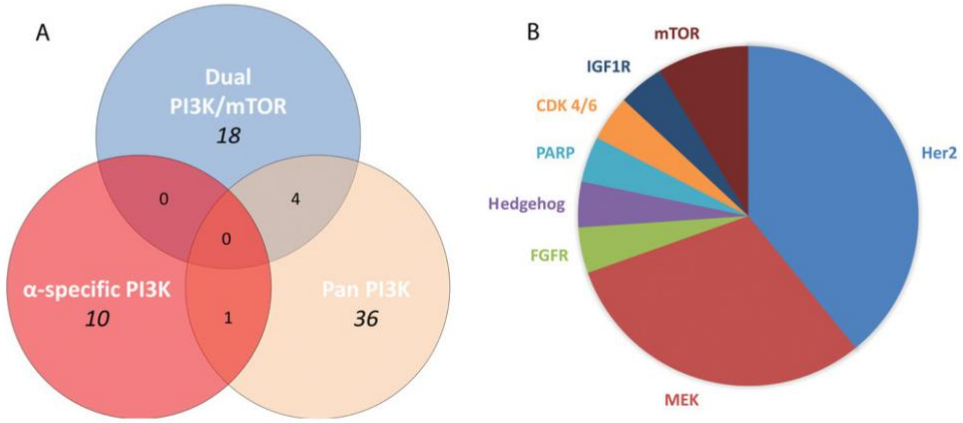
Table 2 | Inhibitors of PI3K in clinical trials in combination with various targeted therapies for breast or solid cancer patients (from http://clinicaltrials.gov as of 17 December 2013)

CDK, cyclin-dependent kinase.

Inhibitor	Combined therapy	Second target	Associated therapy	Current phase of clinical trial	Breast cancer subtype	Clinical trial identifier
Dual PI3K/mTOR						
BEZ235	Trastuzumab	HER2		Phase Ib/II	HER2+ breast cancer	NCT01471847
	MEK162	MEK		Phase Ib	Advanced solid tumours	NCT01337765
	RAD001	mTOR		Phase I	Advanced solid tumours	NCT01482156
GSK2126458	GSK1120212	MEK		Phase I	Advanced solid tumours	NCT01248858
GDC0980	Bevacizumab	HER2	Paclitaxel	Phase Ib	Metastatic breast cancer	NCT01254526
XL765	MSC19363698	MEK		Phase Ib	Advanced or metastatic breast cancer	NCT01390818
Pan-PI3K						
BKM120	Trastuzumab	HER2	Paclitaxel	Phase II	HER2+ breast cancer	NCT01816594
	Lapatinib	HER2		Phase Ib/II	HER2+ *PIK3CA* mutant advanced breast cancer	NCT01589861
	Trastuzumab	HER2		Phase Ib/II	HER2+ breast cancer	NCT01132664
	GSK1120212	MEK		Phase Ib	Advanced solid tumours	NCT01155453
	MEK162	MEK		Phase Ib	Advanced solid tumours	NCT01363232
	LDE225	Hedgehog		Phase Ib	Advanced solid tumours	NCT01576666
	Olaparib	PARP		Phase I	Triple-negative breast cancer	NCT01623349
	Everolimus	mTOR		Phase I	Advanced solid tumours	NCT01470209
GDC0941	Bevacizumab/Trastuzumab	HER2	Paclitaxel	Phase Ib	Metastatic breast cancer	NCT00960960
	Trastuzumab	HER2		Phase Ib	HER2+ metastatic breast cancer	NCT00528330
	GDC-0973	MEK		Phase Ib	Advanced or metastatic solid tumours	NCT00996892
BAY80-6946	BAY86-9766	MEK		Phase Ib	Advanced cancer	NCT01392521
XL147	Trastuzumab	HER2	Paclitaxel	Phase I/II	Metastatic breast cancer	NCT01042925
α-Specific PI3K						
BYL719	BGJ398	FGFR		Phase Ib	Advanced or metastatic solid tumours	NCT01928459
	LEE011	CDK4/6	Letrozol	Phase Ib/II	ER+ breast cancer	NCT01872260
	AMG479	IGF-1R		Phase Ib/II	*PIK3CA* mutant solid tumours	NCT01708161
Dual PI3K/mTOR compared with pan-PI3K						
BEZ235/BKM120	Trastuzumab	HER2	Paclitaxel	Phase Ib	HER2+ metastatic breast cancer	NCT01285466

Figure 1 | PI3K inhibitors in clinical trials

(**A**) Venn diagram of the number of clinical trials currently evaluating the three classes of PI3K inhibitors. An overlap means comparative clinical trials. (**B**) Ongoing combinations of PI3K inhibitors with inhibitors of HER2 (39%), MEK (30%), mTOR (9%), FGFR (4%), Hedgehog (4%), PARP (4%), CDK4/6 (cyclin-dependent kinase 4/6) (4%), and IGF-1R (4%) (from http://clinicaltrials.gov as of 17 December 2013).



these experimental observations and design efficient drug combinations.

Acquired genomic alterations

Resistance can also occur via acquired genetic or, conceivably, epigenetic alterations [44]. Amplification of either *MYC* or *EIF4E* has been shown to be involved in resistance to BEZ235 [47] in various breast cancer cell lines. In addition, use of a chemical genetic screen revealed that resistance to BEZ235 can be caused by Notch and c-Myc activation [48].

Intrinsic resistance

Resistance mechanisms may be present before exposure to inhibitors. For example, mutations in *KRAS* [49] and overexpression of the ribosomal S6 kinases RSK3 and RSK4 [50] have been identified as key mediators of resistance to BEZ235 and the pan-PI3K inhibitor BKM120 in *in vitro* breast cancer cell lines and xenograft models. Moreover, mTORC1/S6K activities endow resistance to the α-specific PI3K inhibitor BYL719 on breast cancer cell lines, mouse models and patient samples with *PIK3CA* mutations, suggesting that combination of BYL719 with an mTOR-specific inhibitor would enhance clinical efficacy [51]. This latter study also reported that S6 can be rephosphorylated upon emergence of acquired resistance in *in vitro* models and in patients with ER^+/PR^+ (PR is progesterone receptor), ER^+/PR^- or $HER2^{+/-}$ breast cancer with mutated *PIK3CA* [51].

From mechanistic studies to combination therapy

Pre-clinical studies and clinical observations show that hyperactivation of the PI3K pathway promotes resistance to anti-oestrogen therapy [20,52,53]. The combination of everolimus (targeting mTORC1) and exemestane (an aromatase inhibitor) is approved for breast cancer in post-menopausal women with advanced ER^+ $HER2^-$ cancer [54]. The clinical effect of direct inhibition of PI3K in combination with anti-oestrogens is being evaluated (Table 1). Further combinations of PI3K pathway inhibitors with other therapies are currently under evaluation in clinical trials on the basis of successful mechanistic preclinical studies (Table 2). These include the combination of PI3K pathway inhibitors with agents targeting HER2, MAPK or PARP [poly(ADP-ribose) polymerase].

Joint therapy of pan-PI3K inhibitors with small-molecule inhibitors or antibodies targeting HER2 in $HER2^+$ breast cancer patients has been the main combination tested so far (Table 2 and Figure 1). Indeed, pre-clinical studies show that breast cancer cells resistant to the HER2-targeted antibody Trastuzumab are sensitive to the pan-PI3K inhibitor XL147 through blockage of the HER2/PI3K/FOXO/Survivin axis [55]. Consistently, $HER2^+$ breast cancer cells resistant to GDC0941 in 3D cultures are sensitive to its combination with either Trastuzumab or the HER2 inhibitor Lapatinib [56]. Moreover, BYL719 has been shown to effectively reverse the resistance of $HER2^+$ breast cancer cells to Lapatinib *in vitro* and *in vivo* [57]. The results of clinical trials testing HER2 and PI3K pathway blockade are eagerly awaited.

The results of pre-clinical studies also provide a rationale for combining PI3K pathway inhibitors with PARP inhibitors. Pan-PI3K inhibition with BKM120 was shown to mediate BRCA1/2 (breast cancer early-onset 1/2) down-regulation, which sensitizes triple-negative breast cancer without BRCA mutations to the PARP inhibitor Olaparib [58]. Consistent with this observation, PI3K and PARP inhibition decreased tumour growth in the MMTV-Cre $Brca1^{f/f}p53^{+/-}$ mouse model [59]. A clinical trial testing BKM120 and Olaparib in triple-negative breast cancer patients is ongoing (Table 2).

Combination therapies targeting the PI3K/mTOR and MEK/ERK (MEK is MAPK/ERK kinase) pathways are currently being tested in the clinic prompted by the importance of these pathways in breast cancer and the results of pre-clinical studies showing that dual inhibition of the PI3K and MEK/ERK pathways with different small kinase inhibitors leads to greater growth inhibition of several breast cancer subtypes than single pathway inhibition [60] (Table 2 and Figure 1). A pooled analysis of Phase I studies indicated enhanced efficacy of dual compared with single inhibition of the PI3K and MAPK pathways, but also higher toxicity [60]. A major challenge now is to find combinations and doses of molecules that maximize the therapeutic effects, but with acceptable toxicity.

Conclusions

Mechanistic understanding of the molecular basis of resistance to PI3K pathway inhibition will increase and should pave the way to rationally designed combination therapies. Putative mechanisms should first be validated in human breast tumour samples and the combination of therapies tested using pathophysiologically relevant mouse models. Ultimately, carefully designed clinical studies (e.g. with patient stratification based on genetic make-up) and the correct choice of biomarkers and study end points will be critical for assessing the efficacy of mechanism-based combination therapies. Just which combination and number of targeted therapies will be needed to overcome resistance to PI3K inhibition and achieve a cure, and whether such therapies will be tolerated by patients, await further investigation, but the evidence to date raises great hopes.

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References

1 Yuan, T.L. and Cantley, L.C. (2008) PI3K pathway alterations in cancer: variations on a theme. Oncogene **27**, 5497–5510 CrossRef PubMed
2 Kumar, A. and Carrera, A.C. (2007) New functions for PI3K in the control of cell division. Cell Cycle **6**, 1696–1698 CrossRef PubMed
3 Chang, H.W., Aoki, M., Fruman, D., Auger, K.R., Bellacosa, A., Tsichlis, P.N., Cantley, L.C., Roberts, T.M. and Vogt, P.K. (1997) Transformation of chicken cells by the gene encoding the catalytic subunit of PI 3-kinase. Science **276**, 1848–1850 CrossRef PubMed
4 Bader, A.G., Kang, S. and Vogt, P.K. (2006) Cancer-specific mutations in *PIK3CA* are oncogenic *in vivo*. Proc. Natl. Acad. Sci. U.S.A. **103**, 1475–1479 CrossRef PubMed
5 Saxena, R., Chandra, V., Manohar, M., Hajela, K., Debnath, U., Prabhakar, Y.S., Saini, K.S., Konwar, R., Kumar, S., Megu, K. et al. (2013) Chemotherapeutic potential of 2-[piperidinoethoxyphenyl]-3-phenyl-2*H*-benzo(*b*)pyran in estrogen receptor-negative breast cancer cells: action via prevention of EGFR activation and combined inhibition of PI-3-K/Akt/FOXO and MEK/Erk/AP-1 pathways. PLoS ONE **8**, e66246 CrossRef PubMed
6 Chen, Y., Wei, H., Liu, F. and Guan, J.L. (2014) Hyper-activation of mTORC1 promotes breast cancer progression through enhancing glucose starvation-induced autophagy and Akt signaling. J. Biol. Chem. **289**, 1164–1173 CrossRef PubMed
7 Saal, L.H., Holm, K., Maurer, M., Memeo, L., Su, T., Wang, X., Yu, J.S., Malmstrom, P.O., Mansukhani, M., Enoksson, J. et al. (2005) *PIK3CA* mutations correlate with hormone receptors, node metastasis, and ERBB2, and are mutually exclusive with PTEN loss in human breast carcinoma. Cancer Res. **65**, 2554–2559 CrossRef PubMed
8 Stemke-Hale, K., Gonzalez-Angulo, A.M., Lluch, A., Neve, R.M., Kuo, W.L., Davies, M., Carey, M., Hu, Z., Guan, Y., Sahin, A. et al. (2008) An integrative genomic and proteomic analysis of *PIK3CA*, *PTEN*, and *AKT* mutations in breast cancer. Cancer Res. **68**, 6084–6091 CrossRef PubMed
9 Meyer, D.S., Brinkhaus, H., Müller, U., Müller, M., Cardiff, R.D. and Bentires-Alj, M. (2011) Luminal expression of PIK3CA mutant H1047R in the mammary gland induces heterogeneous tumors. Cancer Res. **71**, 4344–4351 CrossRef PubMed
10 Koren, S. and Bentires-Alj, M. (2013) Mouse models of PIK3CA mutations: one mutation initiates heterogeneous mammary tumors. FEBS J. **280**, 2758–2765 CrossRef PubMed
11 Meyer, D.S., Aceto, N., Sausgruber, N., Brinkhaus, H., Müller, U., Pallen, C.J. and Bentires-Alj, M. (2014) Tyrosine phosphatase PTPα contributes to HER2-evoked breast tumor initiation and maintenance. Oncogene **16**, 398–402 CrossRef
12 O'Brien, C., Wallin, J.J., Sampath, D., GuhaThakurta, D., Savage, H., Punnoose, E.A., Guan, J., Berry, L., Prior, W.W., Amler, L.C. et al. (2010) Predictive biomarkers of sensitivity to the phosphatidylinositol 3′ kinase inhibitor GDC-0941 in breast cancer preclinical models. Clin. Cancer Res. **16**, 3670–3683 CrossRef PubMed
13 Rhei, E., Kang, L., Bogomolniy, F., Federici, M.G., Borgen, P.I. and Boyd, J. (1997) Mutation analysis of the putative tumor suppressor gene *PTEN/MMAC1* in primary breast carcinomas. Cancer Res. **57**, 3657–3659 PubMed
14 Gonzalez-Angulo, A.M., Ferrer-Lozano, J., Stemke-Hale, K., Sahin, A., Liu, S., Barrera, J.A., Burgues, O., Lluch, A.M., Chen, H., Hortobagyi, G.N. et al. (2011) PI3K pathway mutations and PTEN levels in primary and metastatic breast cancer. Mol. Cancer Ther. **10**, 1093–1101 CrossRef PubMed
15 Poliseno, L., Salmena, L., Zhang, J., Carver, B., Haveman, W.J. and Pandolfi, P.P. (2010) A coding-independent function of gene and pseudogene mRNAs regulates tumour biology. Nature **465**, 1033–1038 CrossRef PubMed
16 Kim, H., Huang, W., Jiang, X., Pennicooke, B., Park, P.J. and Johnson, M.D. (2010) Integrative genome analysis reveals an oncomir/oncogene cluster regulating glioblastoma survivorship. Proc. Natl. Acad. Sci. U.S.A. **107**, 2183–2188 CrossRef PubMed
17 Wiencke, J.K., Zheng, S., Jelluma, N., Tihan, T., Vandenberg, S., Tamguney, T., Baumber, R., Parsons, R., Lamborn, K.R., Berger, M.S. et al. (2007) Methylation of the PTEN promoter defines low-grade gliomas and secondary glioblastoma. Neuro Oncol. **9**, 271–279 CrossRef PubMed
18 Jones, N., Bonnet, F., Sfar, S., Lafitte, M., Lafon, D., Sierankowski, G., Brouste, V., Banneau, G., Tunon de Lara, C., Debled, M. et al. (2013) Comprehensive analysis of *PTEN* status in breast carcinomas. Int. J. Cancer **133**, 323–334 CrossRef PubMed
19 Rodon, J., Dienstmann, R., Serra, V. and Tabernero, J. (2013) Development of PI3K inhibitors: lessons learned from early clinical trials. Nat. Rev. Clin. Oncol. **10**, 143–153 CrossRef PubMed

20 Maira, S.M., Stauffer, F., Brueggen, J., Furet, P., Schnell, C., Fritsch, C., Brachmann, S., Chene, P., De Pover, A., Schoemaker, K. et al. (2008) Identification and characterization of NVP-BEZ235, a new orally available dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor with potent *in vivo* antitumor activity. Mol. Cancer Ther. **7**, 1851–1863 CrossRef PubMed

21 Folkes, A.J., Ahmadi, K., Alderton, W.K., Alix, S., Baker, S.J., Box, G., Chuckowree, I.S., Clarke, P.A., Depledge, P., Eccles, S.A. et al. (2008) The identification of 2-(1*H*-indazol-4-yl)-6-(4-methanesulfonyl-piperazin-1-ylmethyl)-4-morpholin-4-yl-thieno[3,2-*d*]pyrimidine (GDC-0941) as a potent, selective, orally bioavailable inhibitor of class I PI3 kinase for the treatment of cancer. J. Med. Chem. **51**, 5522–5532 CrossRef PubMed

22 Markman, B., Atzori, F., Perez-Garcia, J., Tabernero, J. and Baselga, J. (2010) Status of PI3K inhibition and biomarker development in cancer therapeutics. Ann. Oncol. **21**, 683–691 CrossRef PubMed

23 Maira, S.M., Pecchi, S., Huang, A., Burger, M., Knapp, M., Sterker, D., Schnell, C., Guthy, D., Nagel, T., Wiesmann, M. et al. (2012) Identification and characterization of NVP-BKM120, an orally available pan-class I PI3-kinase inhibitor. Mol. Cancer Ther. **11**, 317–328 CrossRef PubMed

24 Nanni, P., Nicoletti, G., Palladini, A., Croci, S., Murgo, A., Ianzano, M.L., Grosso, V., Stivani, V., Antognoli, A., Lamolinara, A. et al. (2012) Multiorgan metastasis of human HER-2+ breast cancer in $Rag2^{-/-};Il2rg^{-/-}$ mice and treatment with PI3K inhibitor. PLoS ONE **7**, e39626 CrossRef PubMed

25 So, L., Yea, S.S., Oak, J.S., Lu, M., Manmadhan, A., Ke, Q.H., Janes, M.R., Kessler, L.V., Kucharski, J.M., Li, L.S. et al. (2013) Selective inhibition of phosphoinositide 3-kinase p110α preserves lymphocyte function. J. Biol. Chem. **288**, 5718–5731 CrossRef PubMed

26 Martini, M., Ciraolo, E., Gulluni, F. and Hirsch, E. (2013) Targeting PI3K in cancer: any good news? Front. Oncol. **3**, 108 CrossRef PubMed

27 Huang, A., Fritsch, C., Wilson, C., Reddy, A., Liu, M., Lehar, J., Quadt, C., Hofmann, F. and Schlegel, R. (2012), Single agent activity of PIK3CA inhibitor BYL719 in a broad cancer cell line panel. AACR Annual Meeting 2012, Chicago, IL, U.S.A., 31 March–4 April 2012, Abstract 3749

28 Busaidy, N.L., Farooki, A., Dowlati, A., Perentesis, J.P., Dancey, J.E., Doyle, L.A., Brell, J.M. and Siu, L.L. (2012) Management of metabolic effects associated with anticancer agents targeting the PI3K–Akt–mTOR pathway. J. Clin. Oncol. **30**, 2919–2928 CrossRef PubMed

29 Jia, S., Liu, Z., Zhang, S., Liu, P., Zhang, L., Lee, S.H., Zhang, J., Signoretti, S., Loda, M., Roberts, T.M. et al. (2008) Essential roles of PI(3)K-p110β in cell growth, metabolism and tumorigenesis. Nature **454**, 776–779 PubMed

30 Wee, S., Wiederschain, D., Maira, S.M., Loo, A., Miller, C., deBeaumont, R., Stegmeier, F., Yao, Y.M. and Lengauer, C. (2008) PTEN-deficient cancers depend on PIK3CB. Proc. Natl. Acad. Sci. U.S.A. **105**, 13057–13062 CrossRef PubMed

31 Ni, J., Liu, Q., Xie, S., Carlson, C., Von, T., Vogel, K., Riddle, S., Benes, C., Eck, M., Roberts, T. et al. (2012) Functional characterization of an isoform-selective inhibitor of PI3K-p110β as a potential anticancer agent. Cancer Discov. **2**, 425–433 CrossRef PubMed

32 Certal, V., Halley, F., Virone-Oddos, A., Delorme, C., Karlsson, A., Rak, A., Thompson, F., Filoche-Romme, B., El-Ahmad, Y., Carry, J.C. et al. (2012) Discovery and optimization of new benzimidazole- and benzoxazole-pyrimidone selective PI3Kβ inhibitors for the treatment of phosphatase and TENsin homologue (PTEN)-deficient cancers. J. Med. Chem. **55**, 4788–4805 CrossRef PubMed

33 Radimerski, T., Montagne, J., Hemmings-Mieszczak, M. and Thomas, G. (2002) Lethality of *Drosophila* lacking TSC tumor suppressor function rescued by reducing dS6K signaling. Genes Dev. **16**, 2627–2632 CrossRef PubMed

34 Harrington, L.S., Findlay, G.M., Gray, A., Tolkacheva, T., Wigfield, S., Rebholz, H., Barnett, J., Leslie, N.R., Cheng, S., Shepherd, P.R. et al. (2004) The TSC1–2 tumor suppressor controls insulin–PI3K signaling via regulation of IRS proteins. J. Cell Biol. **166**, 213–223 CrossRef PubMed

35 Haruta, T., Uno, T., Kawahara, J., Takano, A., Egawa, K., Sharma, P.M., Olefsky, J.M. and Kobayashi, M. (2000) A rapamycin-sensitive pathway down-regulates insulin signaling via phosphorylation and proteasomal degradation of insulin receptor substrate-1. Mol. Endocrinol. **14**, 783–794 CrossRef PubMed

36 Chan, S., Scheulen, M.E., Johnston, S., Mross, K., Cardoso, F., Dittrich, C., Eiermann, W., Hess, D., Morant, R., Semiglazov, V. et al. (2005) Phase II study of temsirolimus (CCI-779), a novel inhibitor of mTOR, in heavily pretreated patients with locally advanced or metastatic breast cancer. J. Clin. Oncol. **23**, 5314–5322 CrossRef PubMed

37 Wolff, A.C., Lazar, A.A., Bondarenko, I., Garin, A.M., Brincat, S., Chow, L., Sun, Y., Neskovic-Konstantinovic, Z., Guimaraes, R.C., Fumoleau, P. et al. (2013) Randomized phase III placebo-controlled trial of letrozole plus oral temsirolimus as first-line endocrine therapy in postmenopausal women with locally advanced or metastatic breast cancer. J. Clin. Oncol. **31**, 195–202 CrossRef PubMed

38 O'Reilly, K.E., Rojo, F., She, Q.B., Solit, D., Mills, G.B., Smith, D., Lane, H., Hofmann, F., Hicklin, D.J., Ludwig, D.L. et al. (2006) mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. Cancer Res. **66**, 1500–1508 CrossRef PubMed

39 Choo, A.Y., Yoon, S.O., Kim, S.G., Roux, P.P. and Blenis, J. (2008) Rapamycin differentially inhibits S6Ks and 4E-BP1 to mediate cell-type-specific repression of mRNA translation. Proc. Natl. Acad. Sci. U.S.A. **105**, 17414–17419 CrossRef PubMed

40 Klempner, S.J., Myers, A.P. and Cantley, L.C. (2013) What a tangled web we weave: emerging resistance mechanisms to inhibition of the phosphoinositide 3-kinase pathway. Cancer Discov. **3**, 1345–1354 CrossRef PubMed

41 Carracedo, A., Ma, L., Teruya-Feldstein, J., Rojo, F., Salmena, L., Alimonti, A., Egia, A., Sasaki, A.T., Thomas, G., Kozma, S.C. et al. (2008) Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer. J. Clin. Invest. **118**, 3065–3074 PubMed

42 Muranen, T., Selfors, L.M., Worster, D.T., Iwanicki, M.P., Song, L., Morales, F.C., Gao, S., Mills, G.B. and Brugge, J.S. (2012) Inhibition of PI3K/mTOR leads to adaptive resistance in matrix-attached cancer cells. Cancer Cell **21**, 227–239 CrossRef PubMed

43 Britschgi, A., Andraos, R., Brinkhaus, H., Klebba, I., Romanet, V., Müller, U., Murakami, M., Radimerski, T. and Bentires-Alj, M. (2012) JAK2/STAT5 inhibition circumvents resistance to PI3K/mTOR blockade: a rationale for cotargeting these pathways in metastatic breast cancer. Cancer Cell **22**, 796–811 CrossRef PubMed

44 Britschgi, A., Radimerski, T. and Bentires-Alj, M. (2013) Targeting PI3K, HER2 and the IL-8/JAK2 axis in metastatic breast cancer: which combination makes the whole greater than the sum of its parts? Drug Resist. Updat. **16**, 68–72 CrossRef PubMed

45 Serra, V., Scaltriti, M., Prudkin, L., Eichhorn, P.J., Ibrahim, Y.H., Chandarlapaty, S., Markman, B., Rodriguez, O., Guzman, M., Rodriguez, S. et al. (2011) PI3K inhibition results in enhanced HER signaling and acquired ERK dependency in HER2-overexpressing breast cancer. Oncogene **30**, 2547–2557 CrossRef PubMed

46 Chandarlapaty, S., Sawai, A., Scaltriti, M., Rodrik-Outmezguine, V., Grbovic-Huezo, O., Serra, V., Majumder, P.K., Baselga, J. and Rosen, N. (2011) AKT inhibition relieves feedback suppression of receptor tyrosine kinase expression and activity. Cancer Cell **19**, 58–71 CrossRef PubMed

47 Ilic, N., Utermark, T., Widlund, H.R. and Roberts, T.M. (2011) PI3K-targeted therapy can be evaded by gene amplification along the MYC-eukaryotic translation initiation factor 4E (eIF4E) axis. Proc. Natl. Acad. Sci. U.S.A. **108**, E699–E708 CrossRef PubMed

48 Muellner, M.K., Uras, I.Z., Gapp, B.V., Kerzendorfer, C., Smida, M., Lechtermann, H., Craig-Mueller, N., Colinge, J., Duernberger, G. and Nijman, S.M. (2011) A chemical-genetic screen reveals a mechanism of resistance to PI3K inhibitors in cancer. Nat. Chem. Biol. **7**, 787–793 CrossRef PubMed

49 Brachmann, S.M., Hofmann, I., Schnell, C., Fritsch, C., Wee, S., Lane, H., Wang, S., Garcia-Echeverria, C. and Maira, S.M. (2009) Specific apoptosis induction by the dual PI3K/mTor inhibitor NVP-BEZ235 in HER2 amplified and PIK3CA mutant breast cancer cells. Proc. Natl. Acad. Sci. U.S.A. **106**, 22299–22304 CrossRef PubMed

50 Serra, V., Eichhorn, P.J., Garcia-Garcia, C., Ibrahim, Y.H., Prudkin, L., Sanchez, G., Rodriguez, O., Anton, P., Parra, J.L., Marlow, S. et al. (2013) RSK3/4 mediate resistance to PI3K pathway inhibitors in breast cancer. J. Clin. Invest. **123**, 2551–2563 CrossRef PubMed

51 Elkabets, M., Vora, S., Juric, D., Morse, N., Mino-Kenudson, M., Muranen, T., Tao, J., Campos, A.B., Rodon, J., Ibrahim, Y.H. et al. (2013) mTORC1 inhibition is required for sensitivity to PI3K p110α inhibitors in PIK3CA-mutant breast cancer. Sci. Transl. Med. **5**, 196ra199 CrossRef

52 Fox, E.M., Arteaga, C.L. and Miller, T.W. (2012) Abrogating endocrine resistance by targeting ERα and PI3K in breast cancer. Front. Oncol. **2**, 145 CrossRef PubMed

53 Miller, T.W., Hennessy, B.T., Gonzalez-Angulo, A.M., Fox, E.M., Mills, G.B., Chen, H., Higham, C., Garcia-Echeverria, C., Shyr, Y. and Arteaga, C.L. (2010) Hyperactivation of phosphatidylinositol-3 kinase promotes escape from hormone dependence in estrogen receptor-positive human breast cancer. J. Clin. Invest. **120**, 2406–2413 CrossRef PubMed

54 Dhillon, S. (2013) Everolimus in combination with exemestane: a review of its use in the treatment of patients with postmenopausal hormone receptor-positive, HER2-negative advanced breast cancer. Drugs **73**, 475–485 CrossRef PubMed
55 Chakrabarty, A., Bhola, N.E., Sutton, C., Ghosh, R., Kuba, M.G., Dave, B., Chang, J.C. and Arteaga, C.L. (2013) Trastuzumab-resistant cells rely on a HER2–PI3K–FoxO–survivin axis and are sensitive to PI3K inhibitors. Cancer Res. **73**, 1190–1200 CrossRef PubMed
56 Yao, E., Zhou, W., Lee-Hoeflich, S.T., Truong, T., Haverty, P.M., Eastham-Anderson, J., Lewin-Koh, N., Gunter, B., Belvin, M., Murray, L.J. et al. (2009) Suppression of HER2/HER3-mediated growth of breast cancer cells with combinations of GDC-0941 PI3K inhibitor, trastuzumab, and pertuzumab. Clin. Cancer Res. **15**, 4147–4156 CrossRef PubMed
57 Brady, S.W., Zhang, J., Seok, D., Wang, H. and Yu, D. (2014) Enhanced PI3K p110α signaling confers acquired lapatinib resistance which can be effectively reversed by a p110α-selective PI3K inhibitor. Mol. Cancer Ther. **13**, 60–70 CrossRef PubMed
58 Ibrahim, Y.H., Garcia-Garcia, C., Serra, V., He, L., Torres-Lockhart, K., Prat, A., Anton, P., Cozar, P., Guzman, M., Grueso, J. et al. (2012) PI3K inhibition impairs BRCA1/2 expression and sensitizes BRCA-proficient triple-negative breast cancer to PARP inhibition. Cancer Discov. **2**, 1036–1047 CrossRef PubMed
59 Juvekar, A., Burga, L.N., Hu, H., Lunsford, E.P., Ibrahim, Y.H., Balmana, J., Rajendran, A., Papa, A., Spencer, K., Lyssiotis, C.A. et al. (2012) Combining a PI3K inhibitor with a PARP inhibitor provides an effective therapy for BRCA1-related breast cancer. Cancer Discov. **2**, 1048–1063 CrossRef PubMed
60 Saini, K.S., Loi, S., de Azambuja, E., Metzger-Filho, O., Saini, M.L., Ignatiadis, M., Dancey, J.E. and Piccart-Gebhart, M.J. (2013) Targeting the PI3K/AKT/mTOR and Raf/MEK/ERK pathways in the treatment of breast cancer. Cancer Treat. Rev. **39**, 935–946 CrossRef PubMed

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