The role of the scaffolding protein PTPIP51 in signal transduction in Her2 amplified breast cancer cells

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Introduction

The protein tyrosine phosphatase interacting protein 51 (PTPIP51) was initially described as an interaction partner of two non-receptor tyrosine phosphatases, viz. protein tyrosine phosphatase 1B (PTP1B) and T-cell protein tyrosine phosphatase (Stenzinger et al. 2005; Stenzinger et al. 2009). The identification was performed using a yeast two-hybrid screen of a cDNA library constructed from Jurkat cell mRNA (Porsche 2002). As a result of this, a 396-amino acids sequence encoding a novel protein was identified and named protein tyrosine phosphatase interacting protein 51 (PTPIP51; SwissProt accession code Q96SD6). Subsequently, the interaction was proved by the ability of immobilized PTPIP51 to pull down overexpressed PTP1B and the nuclear 45-kDa isoform of TCPTP. At the same time, Ota and coworkers published a more complete cDNA encoding PTPIP51 (Ota et al. 2004). The corresponding protein has a calculated molecular weight of 52,118 Da and consists of 470 amino acids (aa). The gene is located on human chromosome 15 (15q15.1). The gene name of PTPIP51 is summarized as “family with sequence similarity 82 (FAM82)”. This family is composed of three members. The PTPIP51 specific suffix is FAM82A2.

PTPIP51 mRNA and transcription

The mRNA sequence of PTPIP51 is formed by 2251 base pairs (bp) allocated over 13 exons (National Center for Biotechnology Information [NCBI] Reference Sequence database accession code NM_018145). 5′ untranslated region (5′-UTR) of the PTPIP51 mRNA is formed by the Exon 1. The triplet on Exon 13 at position 1596 resembles a stop-codon and terminates protein translation. Protein synthesis is started at an AUG triplet with a specific surrounding sequence. The following consensus sequence has been identified as optimal: GCCRCCAUGG (R represents a purine base).

Interestingly, the first AUG triplet is located on Exon 1, which, as aforementioned, does not encode the PTPIP51 protein. The analysis of the full mRNA sequence of PTPIP51 by an open reading frame (ORF) finder by Brobeil and coworkers identified five ORFs by using AUG as the exclusive start codon (Brobeil et al. 2011b). One ORF was located at position 45 to 47 of the PTPIP51 mRNA. The coding mRNA sequence ends at a stop codon at position 72 to 74. The second ORF from base 186 to base 1598 encodes the coding sequence for the PTPIP51 full-length protein. Brobeil and coworkers postulated that the upstream ORF (upORF) might serve as a regulator for the translation of the

PTPIP51 protein (Brobeil et al. 2011b). The upORF regulation of mRNA translation is usually observed for growth factors and other proto-oncogenes, indicating a crucial function of PTPIP51 in these signaling structures.

PTPIP51 protein, isoforms and structural domains

The complete translation of PTPIP51 mRNA results in a 52kDa protein. Nethertheless, immunoblotting of various tissues revealed several isoforms of PTPIP51 with multiple molecular weights. The precise mechanisms of the synthesis of PTPIP51 isoforms is up to now unknown. Two possible mechanisms were postulated by Brobeil and coworkers (Brobeil et al. 2011b). One possible mechanism is the start of translation at different translation initiation sites (TIS) on PTPIP51 mRNA. Analysis of the coding ORF showed six additional AUG triplets located at exon 3, 4, 5, 6, 9 and 12, respectively. In silico experiments resulted in expected molecular weights of 45 kDa, 38 kDa and 30 kDa. This mode of isoform synthesis is called leaky scanning. Here, a subset of ribosomes does not recognize the start codon and continues scanning downstream until an alternative AUG triplet is detected. The postulated second mechanism of isoform synthesis is alternative splicing. PTPIP51 mRNA consists of 13 exons and is therefore likely to be alternatively spliced. Up to now only in silico experiments regarding the alternative splicing of PTPIP51 mRNA have been performed. Here, theoretical calculations showed 24 alternative splicing variants of the PTPIP51 mRNA, which could contribute to the several isoforms of the PTPIP51 protein (Brobeil et al. 2011b). To further support this hypothesis additional experiments would be needed.

The full length protein form of PTPIP51 encompasses several structural domains. PTPIP51 contains two conserved region spanning from aa 43-48 and aa 146-154. These regions are essential for protein-protein-interaction, e.g. with the 14-3-3 protein family. Interestingly, both of these conserved regions are accompanied by serine and tyrosine residues. Here, Brobeil and coworkers postulated a potential regulatory function of these residues via phosphorylation and dephosphorylation and thus a regulation of protein-protein interactions (Brobeil et al. 2011b; Brobeil et al. 2012a). Up to now, only a regulatory influence of the tyrosine 176 residue on protein-protein interactions was shown (Brobeil et al. 2010; Brobeil et al. 2011a; Brobeil et al. 2013; Brobeil et al. 2017). A transmembrane domain is located at the N-terminus (aa 13-35) (Stenzinger et al. 2009; Lv et al. 2006). This domain is lacking in the 38kDa isoform of PTPIP51.

Thus, implying differing allocations of the several isoforms of PTPIP51. As a member of the regulators of microtubule dynamics, PTPIP51 protein also contains a coiled-coil domain, which mediates the interaction of PTPIP51 with the spindle apparatus and other kinetochore forming proteins, like hNUF2, CGI-99, mitosin, centromere protein F or centromere/kinetochore protein ZW10 (Brobeil et al. 2012b; Brobeil et al. 2011b).

Regulation of PTPIP51 protein expression

The expression of PTPIP51 protein underlies various modulators and exhibits a tissue-specific pattern. Stenzinger and co-workers showed, that the cellular expression of PTPIP51 is regulated by retinoic acid, the epidermal growth factor (EGF), 1,25-dihydroxycholecalciferol and the transforming growth factor β in the HaCaT keratinocyte cell line (Stenzinger et al. 2006). The regulation of PTPIP51 expression by these molecules supports the hypothesis of the involvement of PTPIP51 in cellular differentiation processes. Multiple studies further showed, that especially end-differentiated tissues exhibit high amounts of PTPIP51 protein, as in nervous tissue, photoreceptors of the retina, skeletal muscles and blood cells of the myeloid lineage (Stenzinger et al. 2005; Petri et al. 2015; Koch et al. 2008; Koch et al. 2009a; Koch et al. 2009b; Brobeil et al. 2010; Barop et al. 2009; Bobrich et al. 2011; Maerker et al. 2008; Orieux et al. 2015; Petri et al. 2011). Interestingly, Koch and coworkers found a significant upregulation of the PTPIP51 protein in prostate cancer compared to benign prostate hyperplasia. Here, the analysis of methylation of CpG-rich regions in the PTPIP51 promotor revealed a hypomethylation in prostate cancer. Thus, leading to a less dense chromatin structure, which may causes activation of retrotransposon elements leading to an aberrant overexpression of the PTPIP51 gene (Koch et al. 2009b).

PTPIP51 in signal transduction

PTPIP51 is involved in many different cellular functions ranging from cell growth over differentiation, proliferation to apoptosis. The interaction partners include the Mitogen activated protein kinase (MAPK)-associated proteins (EGFR, Raf1), scaffolding proteins (14.3.3), NFkB signaling proteins (RelA, IkB) and mitosis-associated proteins (CGI-99, Nuf2) (Yu et al. 2008; Brobeil et al. 2015; Brobeil et al. 2012b). PTPIP51 plays a crucial role in the genesis of several cancer types. For example, malignant blasts of acute myeloid leukemia (AML) exhibit PTPIP51 expression, whereas healthy bone marrow cells do not express PTPIP51 protein. Here, the interaction of PTPIP51 with the

MAPK pathway via Raf1 and 14-3-3 proteins is inhibited as a result of its highly phosphorylated Tyr176 residue (Brobeil et al. 2010; Brobeil et al. 2011a). Also, the expression of PTPIP51 correlates with the malignancy of glioblastoma (Petri et al. 2011; Petri et al. 2015). In basal cell and squamous cell carcinoma, the expression pattern of PTPIP51 is altered (Koch et al. 2008).

First, Yu and coworkers linked PTPIP51 to the MAPK pathway via the interaction on the Raf-1 level mediated by the 14-3-3 proteins (Yu et al. 2008). As aforementioned, the interaction of PTPIP51 and 14-3-3 is mediated by two conserved regions. The formation of the PTPIP51/14-3-3/Raf1 complex leads to an activation of the MAPK pathway (Yu et al. 2008; Brobeil et al. 2012a). A well-titrated signal is essential for an optimal cellular function. Therefore, the formation of this complex is tightly regulated by kinases and phosphatases. Here, the tyrosine residue 176 of PTPIP51 depicts an essential regulating site of these interactions. Its phosphorylation leads to a break-up of the PTPIP51/14.3.3/Raf1 complex and thus an inhibition of the PTPIP51 induced MAPK signaling stimulation. The phosphorylation of the Tyr176 residue is controlled by the EGFR and other non-receptor kinases, e.g. the c-Src kinase. Dephosphorylation is performed by PTP1B (Bobrich et al. 2011; Brobeil et al. 2010).

Besides the titration of MAPK signaling, PTPIP51 is an essential part of the communication site between mitochondria and endoplasmic reticulum (ER) (Stoica et al. 2014; Stoica et al. 2016; Vos et al. 2012). These sites are named mitochondrial-associated ER membranes (MAM). Here, the interaction of PTPIP51 and VAPB acts as a mechanical stabilizer of these contact sites. The communication is essential for the calcium homeostasis, apoptosis, and autophagy, which are crucial for the regulation of cell survival and cell death (Dietel et al. 2018a; Galmes et al. 2016; Gomez-Suaga et al. 2017a; Herrera-Cruz und Simmen 2017). The MAMs also represent signaling hubs for mammalian target of rapamycin (mTOR) and Akt signaling (Moore et al. 2011; Rao et al. 2012; Shimobayashi und Hall 2014).

Additionally, Lv and coworkers revealed another connection between PTPIP51 and the mitochondrial apoptotic machinery (Lv et al. 2006). They showed, that an overexpression of PTPIP51 led to cleavage of Caspase-3 and poly(ADP-ribose) polymerase (PARP) as well as the release of cytochrome C from the mitochondria, which represent the hallmarks of apoptosis.

More recent studies of Brobeil and coworkers linked PTPIP51 to the NFκB signaling (Brobeil et al. 2015). The activation of the NFκB signaling is performed via two distinct ways: the canonical and the non-canonical pathway. The canonical pathway is activated by many cellular receptors, which are connected to inflammation, e.g., the T-cell receptor, the B-cell receptor, the toll-like receptors and the tumor necrosis factor (TNF) receptor (Li und Verma 2002; Liu et al. 2012). All of these signaling are subsequently channeled to the IkB kinases (IKKs), which in turn phosphorylate IkBα. This phosphorylation marks IkBα for ubiquitinilation and proteasomal degradation. The degradation of IκB results in a break-up of the RelA/IκB complex. Thus, the nuclear localization sequence of RelA is revealed and RelA can exert its transcription inducing effect after translocation to the nucleus. Recently, Brobeil and coworkers established a link of PTPIP51 to the NF-kB pathway (Brobeil et al. 2015). PTPIP51 is part of the aforementioned complex of RelA and IkB. In the human keratinocyte cell line HaCat, the application of TNFα decreases PTPIP51 mRNA and protein expression. Interestingly, the PTPIP51 gene encompasses a RelA-binding site at its promoter region. Thus, indicating a potential feed-back loop. PTPIP51 interacts with RelA and IkBα as determined by Duolink proximity ligation assay. The interaction of PTPIP51 with either RelA or IkBα is reduced after application of tumor necrosis factor α (TNFα). Brobeil and coworkers hypothesized that PTPIP51 displays a new component of the inhibiting RelA complex. PTPIP51 might cover additional sites of RelA, which inhibit the translocation of RelA and ultimately its transcriptional activity.

PTPIP51 and breast cancer

Breast cancer is the most common cancerous disease in women (Slamon et al. 1987; Tao et al. 2015). Tumors of the breast glands account for about one quarter of all diagnosed tumors. Although early diagnosis and targeted therapies of breast tumors greatly improved the prognosis and overall survival time, breast cancer is still the third most cause of cancer related deaths in the United States. About one third of the breast carcinomas display an amplification of the Her2/ErbB2 receptor, leading to a more aggressive and invasive growth of the cancer cells (Slamon et al. 2011). The overexpression of the Her2/ErbB2 receptor activates mainly two different signaling pathways, the MAPK pathway and the Akt signaling (Moasser 2007b). The activation of these signaling pathways is mediated by the increased formation of homodimers and heterodimers of the Her2 receptor and other members of the Her family, viz. the EGFR,

the Her2, and the Her4 receptor (Moasser 2007a). Subsequently, the signaling is channeled into the ERK signaling, resulting in an enhanced proliferation and growth of the tumor cells. The findings about the overexpression and functions of the Her2 receptor prompted the development of several Her2 targeted therapies including small molecule tyrosine kinase inhibitors and monoclonal antibodies. The introduction of these novel therapies resulted in an enhanced clinical management of Her2 amplified breast cancer (Marty et al. 2005; Geyer et al. 2006; Canonici et al. 2013; Blay et al. 2005; Baselga et al. 1999; Azambuja et al. 2014). Targeting the HER2 receptor with the monoclonal antibody trastuzumab improved the disease-free survival rates at 5 years from 75% to 81–84% in HER2-positive early stage breast cancer (Marty et al. 2005). Despite the improved clinical results of the targeted therapies, the treatment of the upcoming anti Her2 therapy resistances are still challenging. The resistance signaling is mediated by several signaling pathways, e.g., the PI3K-Akt-mTOR signaling, c-MET signaling pathway or an impaired immune response. Another crucial mediator of resistances is depicted by the non-receptor tyrosine kinase c-Src (Mueller et al. 2008; Peiró et al. 2014). c-Src is part of many cellular processes including cell proliferation and cell survival. The functions are exerted via the c-Src mediated phosphorylation of diverse receptor tyrosine kinases and other signaling structures, e.g. Akt signaling and the MAPK pathway (Martin 2001). The activation of c-Src alone can result in trastuzumab resistance. Furthermore, the interaction of c-Src and Her family members is pivotal in Her2 amplified breast cancer cells for the exertion of mitogenesis upon EGF stimulation and the correct transduction of growth promoting effects of heregulin (Zhang et al. 2011).

The activation and deactivation of receptor tyrosine kinases (RTKs) and signaling kinases depends on the phosphorylation levels, which are also under control of phosphatases. The protein tyrosine phosphatase 1B (PTP1B) represents one the best described phosphatases and was crucially involved in the discovery of PTPIP51 (Bobrich et al. 2011). PTP1B exerts a modulating effect on several RTK, e.g., EGFR and Her2 (Tonks und Muthuswamy 2007). The importance of PTP1B in Her 2 amplified breast cancer is depicted in its interaction with the Her2 receptor. Usually, dephosphorylation of RTKs induces a deactivation. On the contrary, the Her2 receptor is activated by PTP1B (Tonks und Muthuswamy 2007). The precise mode of action is up to now unknown. These finding are corroborated by a delayed tumor development of

about 85 days after PTP1B knockdown in a Her2 amplified mouse model (Julien et al. 2007). Moreover, inhibition of PTP1B in breast gland cells resulted in a reduced proliferation. Additionally, it also affects the epithelial-mesenchymal-transition, which depicts a hallmark in the formation of metastasis (Hilmarsdottir et al. 2017; Dietel et al. 2018a).

As mentioned above, PTPIP51 depicts a crucial crossing point of these tumor promoting and resistances inducing signaling structures. While, the function of PTPIP51 in MAPK pathway, Akt signaling and the NFκB signaling is already well described in the human keratinocyte cell line HaCat, up to now no data regarding the expression, distribution, interactions and functional consequences of PTPIP51 in Her2 amplified breast cancer exist. This is the subject of these studies.

Summary of Publications

Dietel E, Brobeil A, Tag C, Gattenloehner S, Wimmer M. Effectiveness of EGFR/HER2-targeted drugs is influenced by the downstream interaction shifts of PTPIP51 in HER2-amplified breast cancer cells. *Oncogenesis*. 2018;7(8):64. Published 2018 Aug 24. doi:10.1038/s41389-018-0075-1

Summary:

This study elucidates the functional relevance of PTPIP51 in the downstream signaling of the Her2 receptor in the Her2 amplified breast cancer cell lines SKBR3 and BT474. The interaction of PTPIP51 with the MAPK pathway and the Akt signaling is differentially regulated upon treatment with EGFR/Her2 targeted small molecule tyrosine kinase inhibitors. We showed, that the knockdown of PTPIP51 via shRNA transfection reduced the activation of Akt signaling under selective Her2 inhibition.

Contribution:

Eric Dietel designed the study, performed the experiments and wrote the manuscript.

Dietel E, Brobeil A, Delventhal L, Tag C, Gattenlöhner S, Wimmer M. Crosstalks of the PTPIP51 interactome revealed in Her2 amplified breast cancer cells by the novel small molecule LDC3/Dynarrestin. *PLoS One*. 2019;14(5):e0216642. Published 2019 May 10. doi:10.1371/journal.pone.0216642

Summary:

This study reveals the PTPIP51 interaction shifts induced by the novel small molecule modulator LDC3/Dynarrestin. LDC3/Dynarrestin stabilizes the interaction of PTPIP51 with 14-3-3 and Raf1 and thereby activates the MAPK pathway. Moreover, LDC3/Dynarrestin abolishes the TNFα induced NFκB signaling activation and inhibits the Akt signaling in Her2 amplified breast cancer cells.

Contribution:

Eric Dietel designed the study, performed the experiments and wrote the manuscript.

Dietel E, Brobeil A, Tag C, Gattenloehner S, Wimmer M. PTPIP51 crosslinks the NFκB signaling and the MAPK pathway in SKBR3 cells. *Future Sci OA*. Published online: 4 Mar 2020. https://doi.org/10.2144/fsoa-2019-0136

Summary:

NFκB signaling plays a crucial role in the metastasis and growth of Her2 amplified breast cancer. This study reveals the interaction shifts of PTPIP51 in NFκB signaling upon PDTC and IKK-16 treatment. Furthermore, the results implicate a potential crosstalk of NFκB signaling and the MAPK pathway via PTPIP51.

Contribution:

Eric Dietel designed the study, performed the experiments and wrote the manuscript.

Publications

Dietel et al. *Oncogenesis* (2018)7:64
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ARTICLE Open Access

Effectiveness of EGFR/HER2-targeted drugs is influenced by the downstream interaction shifts of PTPIP51 in HER2-amplified breast cancer cells

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Abstract

Breast cancer is the most common female cancerous disease and the second most cause of cancer death in women. About 20–30% of these tumors exhibit an amplification of the HER2/ErbB2 receptor, which is coupled to a more aggressive and invasive growth of the cancer cells. Recently developed tyrosine kinase inhibitors and therapeutic antibodies targeting the HER2 receptor improved the overall survival time compared with sole radio- and chemotherapy. Upcoming resistances against the HER2-targeted therapy make a better understanding of the receptor associated downstream pathways an absolute need. In earlier studies, we showed the involvement of Protein Tyrosine Phosphatase Interacting Protein 51 (PTPIP51) in the mitogen-activated protein kinase (MAPK) pathway. The MAPK pathway is one of the most frequently overactivated pathways in HER2-amplified breast cancer cells. This study is aimed to elucidate the effects of four different TKIs on the interactome of PTPIP51, namely with the receptors EGFR and HER2, 14-3-3/Raf1 (MAPK pathway), its regulating enzymes, and the mitochondria-associated interaction partners in HER2 breast cancer cell lines (SK-BR3 and BT474) by using the Duolink proximity ligation assay, immunoblotting and knockdown of PTPIP51. Inhibition of both EGFR and HER2/ErbB2R shifted PTPIP51 into the MAPK pathway, but left the mitochondria-associated interactome of PTPIP51 unattended. Exclusively inhibiting HER2/ErbB2 by Mubritinib did not affect the interaction of PTPIP51 with the MAPK signaling. Selective inhibition of HER2 induced great alterations of mitochondria-associated interactions of PTPIP51, which ultimately led to the most-effective reduction of cell viability of SK-BR3 cells of all tested TKIs. The results clearly reveal the importance of knowing the exact mechanisms of the inhibitors affecting receptor tyrosine kinases in order to develop more efficient anti-HER2-targeted therapies.

Introduction

The identification of targetable signal nodes and protein–protein interactions is of utmost interest for the development of novel drugs for the treatment of cancer and other diseases such as neurodegenerative diseases. The human EGFR-related receptor 2 (HER2) oncogene/oncoprotein represents a perfect example of such a treatable target. The amplification of HER2 in breast cancer leads to severe alterations in growth and proliferation signaling, e.g., mitogen-activated protein kinase (MAPK) signaling, resulting in a more aggressive and invasive growth of the tumor[1,2]. Owing to the development of small molecules and therapeutic antibodies against this target, the treatment of HER2-amplified breast cancer made great progress. The combination of anthracyclin-based and non-anthracyclin-based chemotherapies with trastuzumab, a HER2-targeted therapeutic antibody, led to disease-free survival rates at 5 years of 81–84% compared with 75% without trastuzumab

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in HER2-positive early-stage breast cancer[3]. The already clinically established tyrosine kinase inhibitor Lapatinib, which targets epidermal growth factor receptor (EGFR) and HER2, improved the time to progression from 4.4 months to 8.4 months in a capecetabin vs. capecetabine plus lapatinib setting[4].

HER2, also known as ErbB2 (erythroblastosis homolog B2), is an orphan receptor. It belongs to the Her family like the EGFR. As there is no identified ligand of the HER2 receptor, the downstream signaling is activated by autophosphorylation through the formation of homodimers or heterodimers with other members of the Her family. HER2 signaling is channeled into the MAPK and PI3K/Akt signaling leading to proliferation, growth, and survival of the cell.

In consequence of its upstream position, the blockage of the growth and proliferation signaling on the HER2 level can be bypassed and the effect of the small molecule inhibitor or the therapeutic antibody, respectively, is omitted[5]. In order to develop the most-effective drugs, it is crucial to understand regulatory interactions in MAPK and PI3K/Akt signaling downstream of the receptor.

One of the MAPK pathway regulators is the protein tyrosine phosphatase interacting protein 51 (PTPIP51). PTPIP51 is expressed in many highly differentiated tissues and often deregulated in cancer. It is involved in many diverse cellular functions including cell growth, differentiation, proliferation, and apoptosis. The panel of interaction partners ranges from MAPK-associated proteins (EGFR, Raf1) over scaffolding proteins (14.3.3) to NFkB signaling proteins (RelA, IkB) and mitosis-associated proteins (CGI-99, Nuf2)[6–8].

PTPIP51 plays an essential role in the development of several cancer types. For example, the malignancy of glioblastomas is correlated to the expression of PTPIP51[9]. In basal cell and squamous cell carcinoma, the expression pattern of PTPIP51 is altered[10]. In prostate cancer, hypomethylation of the PTPIP51 promoter region results in an increased expression of the protein[11]. Malignant blasts of acute myeloid leukemia (AML) exhibit PTPIP51 expression in contrast to healthy bone marrow cells. The interaction of PTPIP51 with the MAPK pathway in AML blasts is inhibited as a result of its highly phosphorylated Tyr176 residue[12,13].

PTPIP51 exerts its regulating effect on the MAPK pathway on Raf1 level via the scaffolding protein 14-3-3. The recruitment of PTPIP51 into the MAPK signaling leads to an activation of the MAPK pathway[7]. A well-titrated signal is a prerequisite for an optimal cellular function. Therefore, the formation of the PTPIP51/14-3-3/Raf1 complex is tightly regulated by kinases and phosphatases[12,14,15]. One of the crucial spots for this regulation is the tyrosine residue 176 of PTPIP51. Its phosphorylation results in a break-up of the PTPIP51/14.3.3/Raf1 complex and hence an omission of the MAPK signaling stimulation[14]. The phosphorylation of the Tyr176 residue is under the control of the EGFR and other kinases, such as the. c-Src kinase. Dephosphorylation is mainly performed by PTP1B[15].

PTPIP51 is not only regulator of MAPK signaling, but also essential part of the communication site between mitochondria and endoplasmic reticulum (ER)[16,17]. These sites are called mitochondrial-associated ER membranes (MAM) and are defined by close contacts (10–30 nm) of the organelles. One of the interactions, which stabilizes these contact sites is the interaction of PTPIP51 and VAPB[18]. The communication is essential for the regulation of calcium homeostasis, apoptosis, autophagy, and many more processes, which are crucial for cell survival and cell death[19–21]. The MAMs are also signaling hubs for mammalian target of rapamycin (mTOR) and protein kinase B(Akt) signaling[22]. Alterations of these precisely regulated contact sites immensely affect the fate of the cell.

This study analyzed the effects of four different tyrosine kinase inhibitors (TKI) on the MAPK pathway, Akt signaling, and MAM-related interactome of PTPIP51 and in addition, these effects were correlated to the viability of the TKI-treated cells. We used Gefitinib, a selective EGFR inhibitor, Mubritinib, a selective HER2 inhibitor[23], Lapatinib, an already clinically established EGFR and HER2 inhibitor, and Neratinib, a further developed EGFR and HER2 inhibitor.

Results

Selective inhibition of HER2 strongly affects the mitochondrial metabolism in SK-BR3 cells

To monitor the effects of selective and simultaneous inhibition of EGFR and HER2 on mitochondrial metabolism, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assays were performed. SK-BR3 and BT474 cells were treated over 24 h, 48 h, or 72 h with the indicated concentration of TKIs. All cells were incubated with the same amount of dimethyl sulfoxide (DMSO) in order to exclude potential toxic effects of DMSO. The selective inhibition of HER2 by Mubritinib, especially 10 µM, reduced the mitochondrial metabolic rate of SK-BR3 cells by ~ 40%, when treated for 24 h (Fig. 2c). The effect even increased for longer incubation times (reduction of the mitochondrial metabolic rate of ~ 60% for 10 µM Mubritinib for 48 h or 72 h). BT474 cells showed a likewise behavior, when treated with the selective HER2 inhibitor Mubritinib. Although, the reduction of mitochondrial metabolic rate was not as severe as for the SK-BR3 cells (Fig. 2d).

The dual inhibition of EGFR and HER2 by Lapatinib and Neratinib also resulted in a significantly diminished mitochondrial metabolism but reduced the rel.

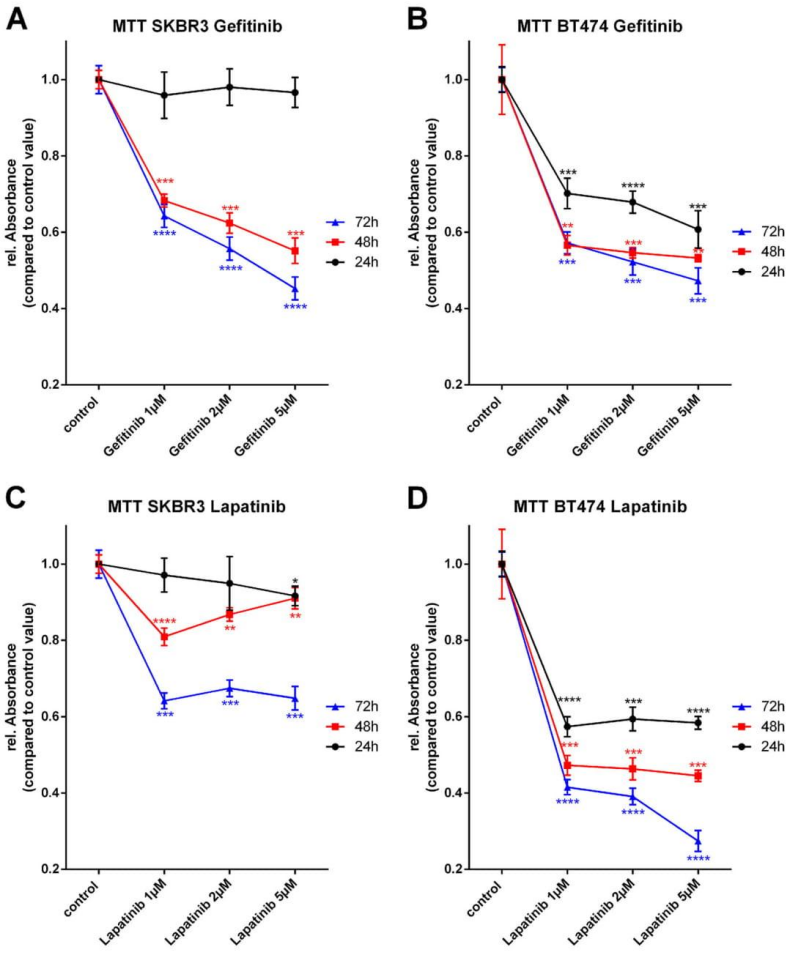

Fig. 1 Mitochondrial metabolic rate under the influence of Gefitinib and Lapatinib. SK-BR3 cells treated with the indicated concentrations of Gefitinib over a time period of 24 h, 48 h, or 72 h (**a**). BT474 cells treated with the indicated concentrations of Gefitinib over a time period of 24 h, 48 h, or 72 h (**b**). SK-BR3 cells treated with the indicated concentrations of Lapatinib over a time period of 24 h, 48, or 72 h (**c**). BT474 cells treated with the indicated concentrations of Lapatinib over a time period of 24 h, 48 h, or 72 h (**d**). ($N = 3$)

Absorbance, which indicates the metabolic activity, only ~ 10–20% for SK-BR3 cells and 24 h incubation time (Figs. 1c and 2a). If incubated for 48 h or 72 h with Lapatinib or Neratinib, the reduction of the mitochondrial metabolic rate in SK-BR3 cells increased for both inhibitors. Noteworthy, application of Neratinib led to stronger reduction of mitochondrial metabolism compared with the application of Lapatinib (5 μM Lapatinib for 72 h, reduction of ~ 40%; 200 nM Neratinib for 72 h, reduction of ~ 55%). Treatment of BT474 cells with dual kinase inhibitors, Lapatinib and Neratinib, induced a reduction of mitochondrial metabolic rate of ~ 50% for the highest applied concentration and incubation time of 72 h (Fig. 1d, Fig. 2b).

The selective inhibition of EGFR by Gefitinib did not alter the mitochondrial metabolism of SK-BR3 cells if

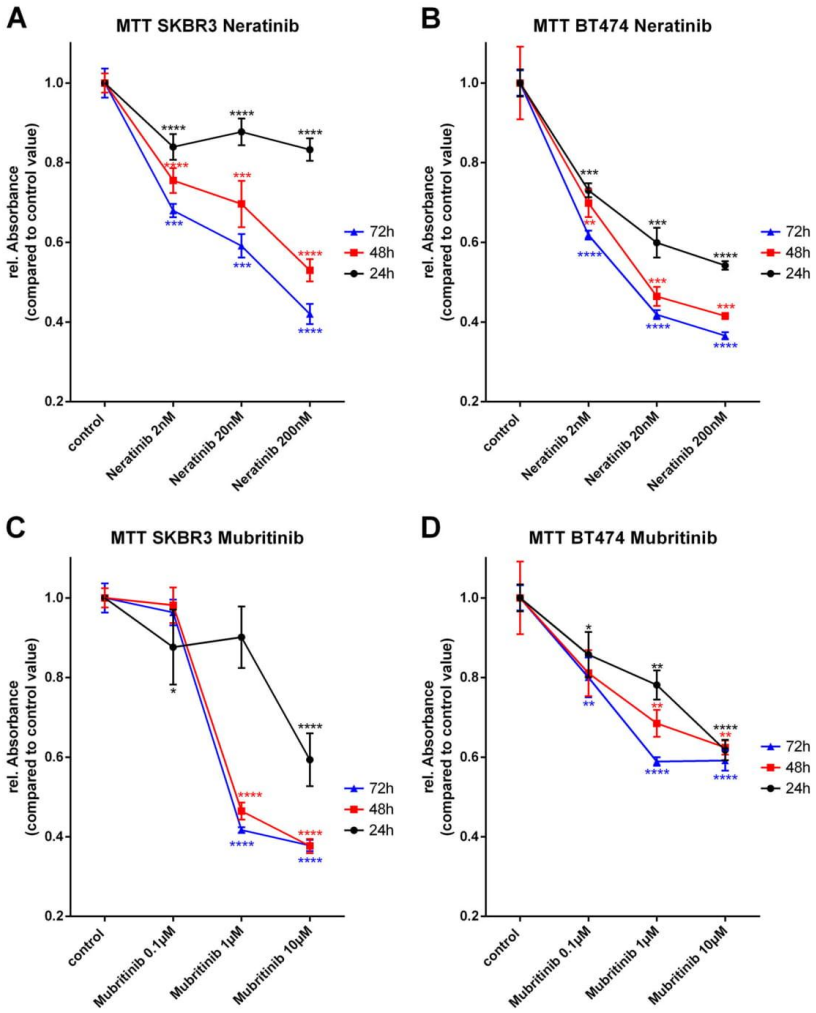

Fig. 2 Mitochondrial metabolic rate under the influence of Neratinib and Mubritinib. SK-BR3 cells treated with the indicated concentrations of Neratinib over a time period of 24 h, 48 h, or 72 h (**a**). BT474 cells treated with the indicated concentrations of Neratinib over a time period of 24 h, 48 h, or 72 h (**b**). SK-BR3 cells treated with the indicated concentrations of Mubritinib over a time period of 24 h, 48 h or 72 h (**c**). BT474 cells treated with the indicated concentrations of Mubritinib over a time period of 24 h, 48 h, or 72 h (**d**). ($N = 3$)

incubated for 24 h (Fig. 1a). Longer incubation times, highly significant, reduced the mitochondrial metabolism for all applied concentrations. Selective inhibition of EGFR with Gefitinib in BT474 cells induced a reduction of mitochondrial metabolism for all concentrations and incubation times (5 μM Gefitinib for 48 h or 72 h, reduction of ~ 50%) (Fig. 1b).

Noteworthy, all applied TKIs displayed a much higher reduction of metabolic activity in BT474 cells compared with SK-BR3 cells when treated for 24 h.

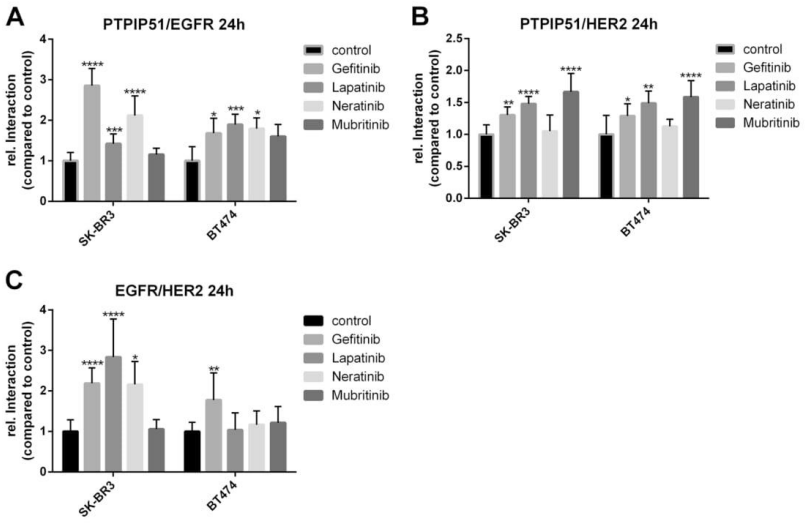

Fig. 3 Interaction of EGFR, HER2, and PTPIP51 under Gefitinib, Lapatinib, Neratinib, and Mubritinib treatment. Interaction of EGFR and PTPIP51 in SK-BR3 and BT474 cells treated with 5 µM Gefitinib, 5 µM Lapatinib, 200 nM Neratinib, or 10 µM Mubritinib for 24 h (**a**). Interaction of HER2 and PTPIP51 in SK-BR3 and BT474 cells treated with 5 µM Gefitinib, 5 µM Lapatinib, 200 nM Neratinib, or 10 µM Mubritinib for 24 h (**b**). Interaction of EGFR and HER2 in SK-BR3 and BT474 cells treated with 5 µM Gefitinib, 5 µM Lapatinib, 200 nM Neratinib, or 10 µM Mubritinib for 24 h (**c**). ($N = 3$)

Selective inhibition of EGFR leads to a formation of EGFR-HER2 dimers

For the examination of the PTPIP51 interactome under the influence of TKIs, we monitored 12 different protein–protein interactions. In order to track down the precise mode of action, we determined the formation of EGFR-HER2 dimers. Cells were treated and fixed as mentioned above. Subsequently, the cells were incubated with primary antibodies raised against EGFR and HER2, followed by Duolink proximity ligation assay (precisely described in Materials and Methods).

The selective inhibition of HER2 by Mubritinib did not affect the numbers of EGFR/HER2 dimers in SK-BR3 cells. Whereas the EGFR-targeted TKIs induced an enhanced formation of EGFR/HER2 dimers (Fig.3c, Supplementary Figure 1). All applied concentrations of Gefitinib induced an augmented formation of EGFR/HER2 dimers (1 µM and 2 µM $p < 0.01$, 5 µM $p < 0.0001$) (Supplementary Figure 1A). The same enhancement was observed for Lapatinib (1 µM, 2 µM, and 5 µM $p < 0.0001$) (Supplementary Figure 1B) and Neratinib (2 nM $p < 0.0001$, 20 nM $p < 0.01$, 200 nM $p < 0.05$) (Supplementary Figure 1D).

Noteworthy, in BT474 cells only the application of Gefitinib led to a significant increase of EGFR/HER2 dimers (5 µM Gefitinib $p < 0.01$) (Fig. 3c). All other applied TKI did not significantly alter the number of EGFR/HER2 dimers.

We also monitored the interactions of PTPIP51 with EGFR and HER2, respectively. The application of EGFR-targeted TKIs, Gefitinib, Lapatinib, and Neratinib, induced an enhanced interaction of PTPIP51 with EGFR and HER2. The selective inhibition of HER2 with Mubritinib only increased the interaction of PTPIP51 and HER2 (0.1 µM $p < 0.05$, 1 µM, and 10 µM $p < 0.0001$) (Supplementary Figure 2G). The interaction of EGFR and PTPIP51 remained unaffected (Supplementary Figure 2A). Likewise results were seen for BT474 cells (Fig. 3a, b)

EGFR-targeted TKIs promote the formation of the PTPIP51–14.3.3-Raf1-complex

To examine the effects on MAPK-related interactions of PTPIP51 under the influence of TKI, we measured the interactions of PTPIP51 with 14.3.3 and Raf1, respectively. The inhibition of EGFR by Gefitinib, Lapatinib, or Neratinib induced the formation of the PTPIP51–14.3.3-Raf1-complex in SK-BR3 cells. Application of 2 µM and 5 µM Gefitinib applied for 24 h highly significant enhanced the interaction of PTPIP51 and 14.3.3 ($p < 0.0001$) (Fig. 4b, Supplementary Figure 3A). The complex formation of PTPIP51/Raf1 was augmented in the same manner (2 µM and 5 µM for 24 h $p < 0.0001$) (Supplementary Figure 3E).

Application of Lapatinib for 24 h induced likewise observations (PTPIP51/14.3.3: 2 µM $p < 0.001$, 5 µM $p < 0.0001$; PTPIP51/Raf1 2 µM and 5 µM $p < 0.0001$) (Supplementary Figure 3B and F). Noteworthy, the complex formation of PTPIP51/14.3.3/Raf1 under Neratinib was measurable for 24 h and 48 h (PTPIP51/14.3.3: 24 h 20 nM $p < 0.001$, 200 nM $p < 0.0001$; 48 h 2 nM $p < 0.05$, 20 nM, and 200 nM $p < 0.0001$; PTPIP51/Raf1: 24 h 200 nM $p < 0.0001$; 48 h 2 nM, 20 nM, and 200 nM $p < 0.0001$) (Supplementary Figure 3D and H). On the contrary, selective inhibition of HER2 did not affect the interaction of PTPIP51 and Raf1 (Fig. 4a, b, Supplementary Figure 3H). These findings are corroborated by the observed changes of PTPIP51 Tyr176 phosphorylation. Dephosphorylation of Tyr176 of PTPIP51 is a prerequisite for the formation of the PTPIP51–14.3.3-Raf1-complex. The application of TKIs targeting EGFR resulted in a reduced phosphorylation level, whereas selective HER2 inhibition even enhanced the Tyr176 phosphorylation status (Fig. 5, Supplementary Figure 7).

The observations of formation of the PTPIP51/Raf1/14.3.3 complex under EGFR and/or HER2 inhibition in BT474 slightly differed. As seen in SK-BR3 cells, all EGFR-targeted TKI (Gefitinib, Lapatinib, and Neratinib) induced an increase of PTPIP51 and Raf1 interaction (5 µM Gefitinib $p < 0.001$, 5 µM Lapatinib, $p < 0.0001$, Neratinib $p < 0.0001$). Also Mubritinib as a selective HER2 inhibitor enhanced the interaction of PTPIP51 and Raf1 (10 µM Mubritinib $p < 0.0001$) (Fig. 4a). In addition, the interaction of PTPIP51 and 14.3.3 in BT474 cells is differently altered compared with the SK-BR3 cells. The application of Gefitinib, Lapatinib, and Mubritinib induced an increase of PTPIP51/14.3.3 interaction in BT474 (5 µM Gefitinib $p < 0.01$, 5 µM Lapatinib $p < 0.001$, 10 µM Mubritinib $p < 0.001$). In contrast to the SK-BR3 cells, Neratinib did not enhance the interaction of PTPIP51 and 14.3.3 in BT474 cells (Fig. 4b).

Besides, EGFR, which phosphorylates the Tyr176 residue, PTPIP51 phosphorylation levels are also equilibrated by the activity of the tyrosine kinase c-Src and the phosphatase PTP1B. All applied TKIs enhanced the interaction of PTPIP51 and c-Src in SK-BR3 cells (Gefitinib: 24 h 5 µM $p < 0.0001$; Lapatinib: 24 h 1 µM $p < 0.05$, 2 µM $p < 0.01$, 5 µM $p < 0.0001$, 48 h 2 µM $p < 0.05$; Neratinib: 24 h 2 nM $p < 0.01$, 20 nM $p < 0.05$, 200 nM $p < 0.0001$, 48 h 2 nM, and 200 nM $p < 0.0001$; Mubritinib: 24 h 1 µM $p < 0.01$, 10 µM $p < 0.0001$, 48 h 1 µM $p < 0.001$, 10 µM $p < 0.0001$) (Fig. 4c, Supplementary Figure 4A, B, C and D). In accordance to the differing alterations of PTPIP51/Raf1 and PTPIP51/14.3.3 interactions seen in BT474 cells, also the interaction of PTPIP51/c-Src and PTPIP51/PTP1B were affected differently. Application of Gefitinib or Mubritinib to BT474 cells reduced the interaction of PTPIP51 and c-Src (5 µM Gefitinib $p < 0.01$, 10 µM Mubritinib $p < 0.01$) (Fig. 4c). Lapatinib and Neratinib did not significantly affect the interaction of PTPIP51/c-Src in BT474 cells (Fig. 4c). The interaction of PTPIP51 with its main phosphatase PTP1B was not affected by the application of Gefitinib, Lapatinib, Neratinib, or Mubritinib in BT474 cells (Fig. 4d).

Yet, effects on the interaction of PTPIP51 with PTP1B in SK-BR3 differed. The application of Gefitinib and Lapatinib induced an increase of interaction after 48 h incubation time (Gefitinib: 1 µM $p < 0.0001$, 2 µM $p < 0.01$, 5 µM $p < 0.001$; Lapatinib: 1 µM, and 2 µM $p < 0.0001$, 5 µM $p < 0.05$) (Fig. 3d, Supplementary Figure 4E and F). On the contrary, Neratinib diminished the PTPIP51-PTP1B interaction (24 h: 20 nM $p < 0.01$, 200 nM $p < 0.0001$; 48 h: 20 nM, and 200 nM $p < 0.001$) (Supplementary Figure 4H). Mubritinib did not affect this interaction at all (Supplementary Figure 4G).

In order to determine the activation status of the MAPK signaling, pMAPK blots were performed. The inhibition of EGFR by Gefitinib, Lapatinib, or Neratinib led to a complete shutdown of MAPK signaling. Mubritinib induced an activation of MAPK signaling (Fig. 5).

Inhibition of the EGFR and/or HER2 affects the tether between ER and mitochondria

The inhibition of EGFR led to an enhanced binding of VAPB and PTPIP51 in both cell lines(Gefitinib: 1 µM and 5 µM $p < 0.01$, 2 µM $p < 0.001$; Lapatinib: 1 µM and 5 µM $p < 0.0001$, 2 µM $p < 0.05$, Neratinib: 2 nM $p < 0.0001$) (Fig. 4e, Supplementary Figure 5A, B and D) in contrast to the selective inhibition of HER2, which led to a reduction of PTPIP51-VAPB interaction in SK-BR3 cells if the highest concentration of Mubritinib was applied (10 µM $p < 0.001$) (Fig. 4e, Supplementary Figure 5C). Treatment of the BT474 cells with Mubritinib did not significantly alter the number of interactions (Fig. 4e). The interaction of PTPIP51 and VAPB is regulated by the GSK3B. Lapatinib and Mubritinib induced a highly significant increase of the PTPIP51-GSK3B interaction in SK-BR3 cells (Lapatinib: 2 µM and 5 µM $p < 0.01$; Mubritinib 0.1 µM $p < 0.05$, 1 µM and 10 µM $p < 0.0001$) (Fig. 4f, Supplementary Figure 5F and G), whereas Gefitinib and Neratinib did not affect the PTPIP51-GSK3B interaction (Fig. 4f, Supplementary Figure 5E and H). In BT474 cells only the application of Mubritinib induced a significant increase of PTPIP51/GSK3B interactions (10 µM Mubritinib $p < 0.05$) (Fig. 4f).

Inhibition of EGFR and HER2 alters the interactions of PTPIP51 with Akt and PKC

We examined the interaction of PTPIP51 with the kinase PKC and Akt. The interaction of PTPIP51 and Akt

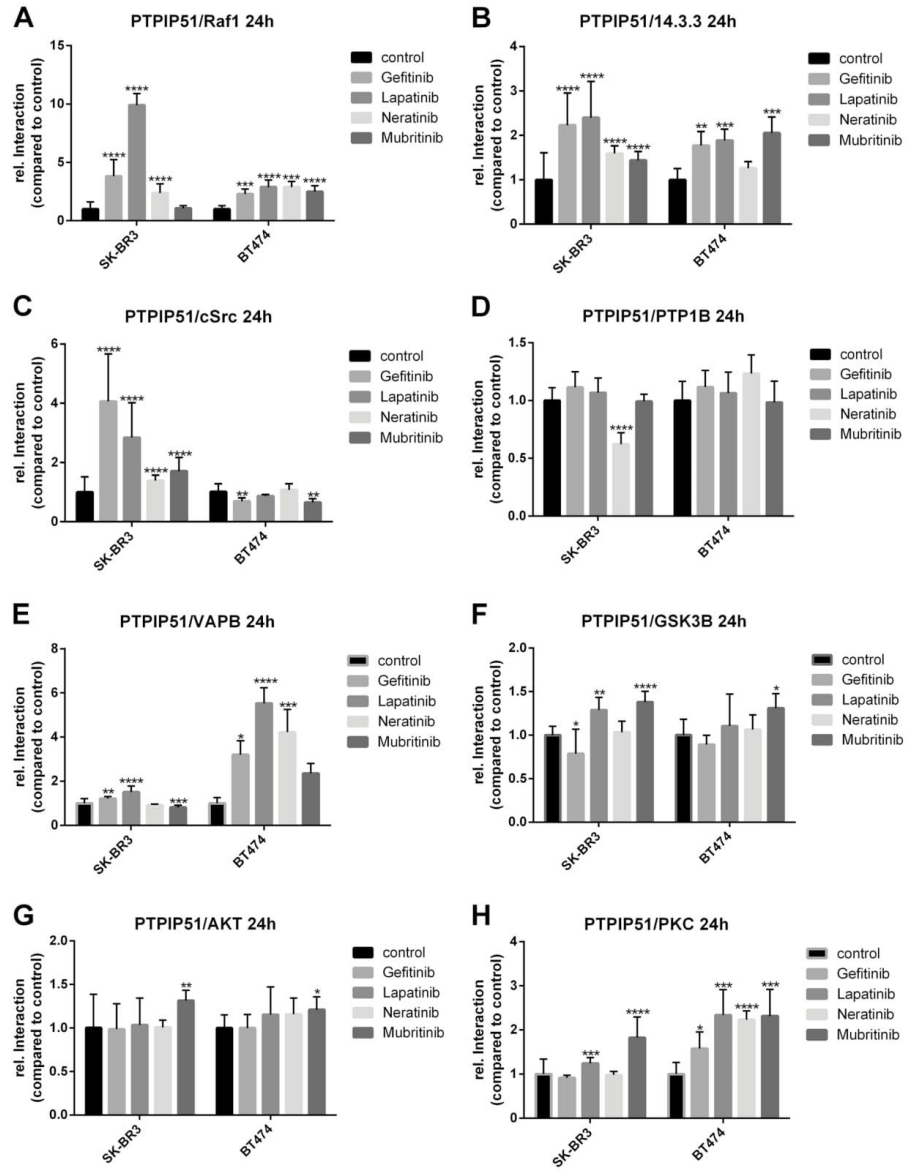

Fig. 4 (See legend on next page.)

Fig. 4 MAPK and MAM-related interactome of PTPIP51 under Gefitinib, Lapatinib, Neratinib, and Mubritinib treatment. Interaction of Raf1 and PTPIP51 in SK-BR3 and BT474 cells treated with 5 µM Gefitinib, 5 µM Lapatinib, 200 nM Neratinib, or 10 µM Mubritinib for 24 h (**a**). Interaction of 14.3.3 and PTPIP51 in SK-BR3 and BT474 cells treated with 5 µM Gefitinib, 5 µM Lapatinib, 200 nM Neratinib, or 10 µM Mubritinib for 24 h (**b**). Interaction of c-Src and PTPIP51 in SK-BR3 and BT474 cells treated with 5 µM Gefitinib, 5 µM Lapatinib, 200 nM Neratinib, or 10 µM Mubritinib for 24 h (**c**). Interaction of PTP1B and PTPIP51 in SK-BR3 and BT474 cells treated with 5 µM Gefitinib, 5 µM Lapatinib, 200 nM Neratinib, or 10 µM Mubritinib for 24 h (**d**). Interaction of VAPB and PTPIP51 in SK-BR3 and BT474 cells treated with 5 µM Gefitinib, 5 µM Lapatinib, 200 nM Neratinib, or 10 µM Mubritinib for 24 h (**e**). Interaction of GSK3B and PTPIP51 in SK-BR3 and BT474 cells treated with 5 µM Gefitinib, 5 µM Lapatinib, 200 nM Neratinib, or 10 µM Mubritinib for 24 h (**f**). Interaction of Akt and PTPIP51 in SK-BR3 and BT474 cells treated with 5 µM Gefitinib, 5 µM Lapatinib, 200 nM Neratinib, or 10 µM Mubritinib for 24 h (**g**). Interaction of PKC and PTPIP51 in SK-BR3 and BT474 cells treated with 5 µM Gefitinib, 5 µM Lapatinib, 200 nM Neratinib, or 10 µM Mubritinib for 24 h (**h**). ($N = 3$)

in both cell lines increased when Mubritinib was added (SK-BR3 10 µM Mubritinib $p < 0.01$; BT474 10 µM Mubritinib $p < 0.05$) (Fig. 4g). All other tested TKI did not significantly alter the interaction of PTPIP51 and Akt (Fig. 4g). The interaction of PKC and PTPIP51 significantly increased in both cell lines if Lapatinib or Mubritinib were applied (SK-BR3 5 µM Lapatinib $p < 0.001$, 10 µM Mubritinib $p < 0.0001$; BT474 5 µM Lapatinib $p < 0.001$, 10 µM Mubritinib $p < 0.001$) (Fig. 4h). Application of Gefitinib or Neratinib did not induce a significant alteration of PTPIP51/PKC interaction in SK-BR3 cell, whereas the same TKI led to an significant increase of the interaction in BT474 cells (5 µM Gefitinib $p < 0.05$, 200 nM Neratinib $p < 0.0001$) (Fig. 4h).

In order to determine the activity of Akt signaling, as an indicator of mTORC2 activity, pSer 473 Akt immunoblots were performed. The inhibition of EGFR led to an almost abolished Akt signaling. Whereas, Mubritinib induced an activation of Akt signaling as determined by its Ser473 phosphorylation (Fig. 5).

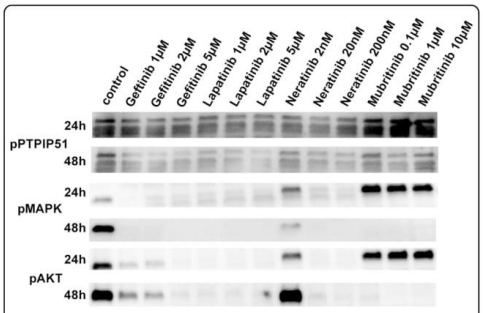

Fig. 5 Phosphorylation status of the Tyr176 residue of PTPIP51, activation status of MAPK signaling and activation status of Akt signaling under Gefitinib, Lapatinib, Neratinib, and Mubritinib treatment. Immunobloting of phospho Tyr176 PTPIP51. Cell were treated with the indicated concentrations of the four different TKIs. The multiple bands are due to the different isoforms of PTPIP51. For evaluation only the 52 kDa isoform of PTPIP51 was used. The immunoblots were normalized to the stain-free blot

PTPIP51 plays an essential part in the mubritinib-induced Akt activation

To precisely determine the importance of PTPIP51 in these complex regulations, we performed a shRNA knockdown experiment of PTPIP51 in SK-BR3 cells. The exact description of the procedure is mentioned in the Materials and Methods section. We used four different constructs for the knockdown of PTPIP51. For evaluation we only used the construct 4 since it showed the most-effective knockdown of PTPIP51 (Fig. 6a, c). The knockdown of PTPIP51 only slightly altered the activation level of the MAPK pathway as indicated by the phosphorylation of ERK1/2 (pMAPK) if no tyrosine kinase inhibitor was applied(Fig. 6a, b). Application of the tested TKI to the PTPIP51 knockdown SK-BR3 cells only slightly altered the activation of MAPK pathway (Fig. 6a, b).

Knockdown of PTPIP51 induced a strongly reduced Akt activation seen under Mubritinib treatment (Fig. 6a, d). All other tested TKI also showed a reduced Akt activation in the PTPIP51 knockdown setting (Fig. 6d).

Selective inhibition of c-Src alters the ternary complex of PTPIP51/HER2/c-Src

For the precise examination of the ternary complex PTPIP51/c-Src/HER2 we used Dasatinib for the selective inhibition of c-Src. Interactions were monitored using the Duolink proximity ligation assay (precisely described in Materials and Methods). Treatment of SK-BR3 cells with Dasatinib did not significantly alter the interaction of PTPIP51 and c-Src (Fig. 7a). Application of Dasatinib induced an significant increase of PTPIP51/HER2 interaction for all applied concentrations (0.1 µM $p < 0.001$, 1 µM $p < 0.05$, 10 µM $p < 0.05$) (Fig. 7b). Interaction of c-Src and HER2 was significantly enhanced if 1 µM or 10 µM Dasatinib were applied (1 µM $p < 0.0001$, 10 µM $p < 0.001$) (Fig. 7c).

Discussion

During the last decades, targeted therapy of malignant diseases experienced a rapid development, e.g., the successful establishment of HER2-targeted therapy using the

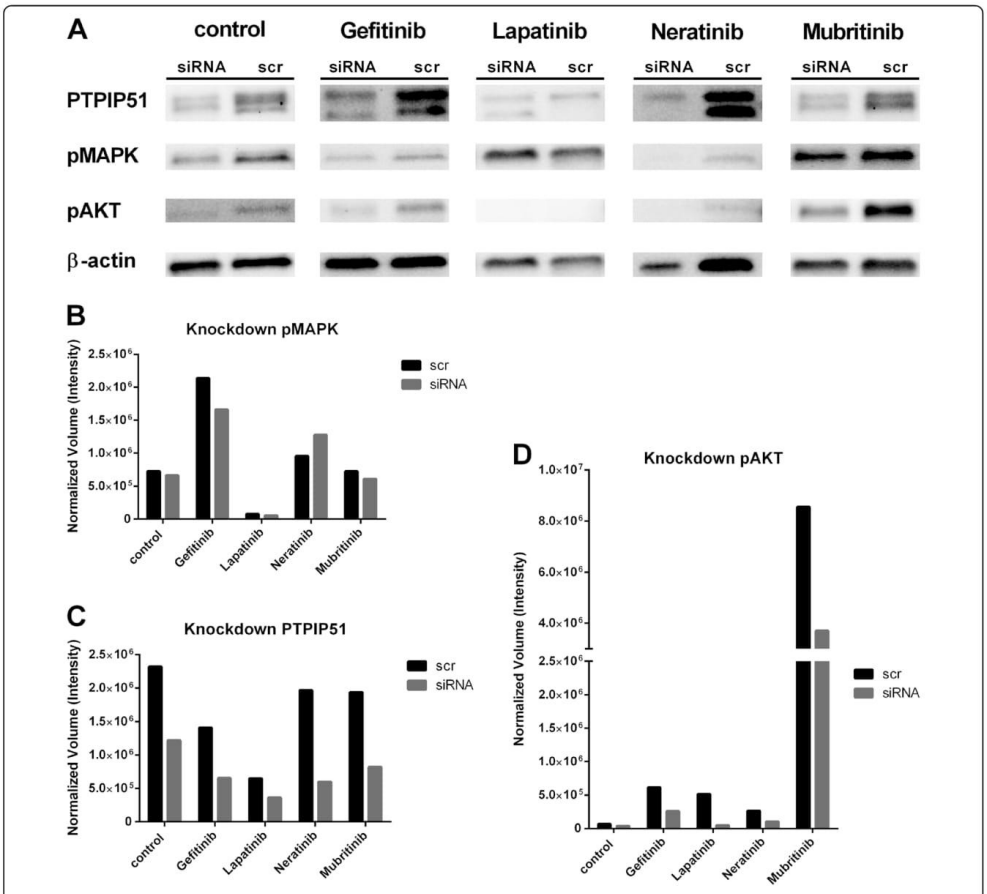

Fig. 6 shRNA knockdown of PTPIP51 in SK-BR3 cells. Immunoblots of PTPIP51, pMAPK, and pAkt of shRNA knockdown of PTPIP51 in SK-BR3 cells treated with 5 µM Gefitinib, 5 µM Lapatinib, 200 nM Neratinib, or 10 µM Mubritinib for 24 h (**a**). Evaluation of pMAPK immunoblots (**b**). Evaluation of pAkt immunoblots (**c**). Evaluation of PTPIP51 protein immunoblots (**d**). The immunoblots were normalized to the β-Actin level

humanized monoclonal antibody Trastuzumab or TKI-like Lapatinib[24,25]. Yet, an increasing number of reports dealing with resistances to the applied targeted therapy were published[26] The application of Trastuzumab results in a downregulation of the HER2 receptor itself, modified heterodimerization, and (de-) activation of associated signaling pathways[27]. In Trastuzumab resistant cancer cells, signaling molecules comprising PI3K and Akt as well as PTEN are dysregulated[26]. The overexpression and activation of the non-membrane tyrosine kinase c-Src was identified as a further cause for the development of resistances against HER2-targeted therapy in breast cancer cells[28]. Interestingly, the same pathways play a critical role in TKI resistance of breast cancer cells[27].

In this context, the precise understanding of the downstream effects of EGFR and HER2 TKI is crucial to identify the affected signaling hubs and protein–protein interactions. Forming homodimers and heterodimers with EGFR and HER3, the receptor signaling of HER2 is coupled for example to the MAPK pathway, to PI3K and Akt signaling[1]. Of note, the investigated PTPIP51 represents a key player in MAPK signaling by interfering on Raf1 level[7,14,15]. The spontaneously immortalized keratinocyte cell line HaCaT displays a specific regulation and interaction pattern of PTPIP51 with the signaling molecules of

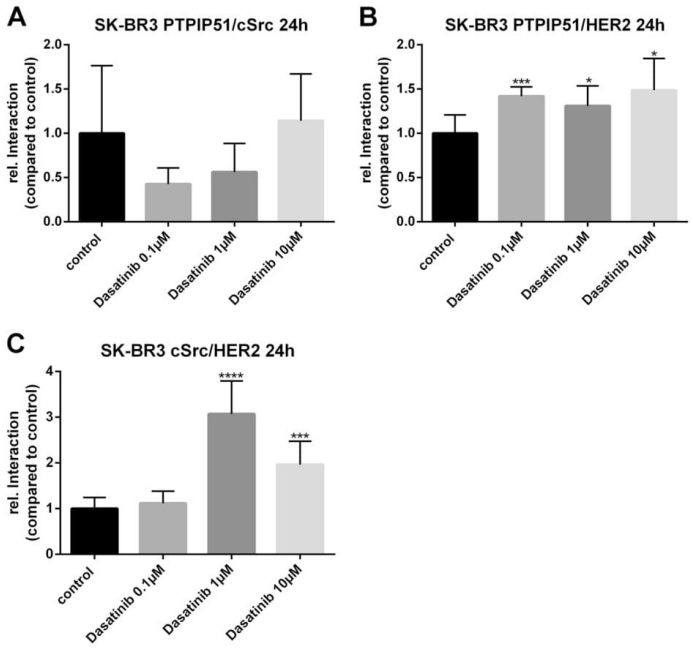

Fig. 7 PTPIP51/HER2/c-Src interactome under selective inhibition of c-Src in SK-BR3 cells. Interaction of PTPIP51 and c-Src in SK-BR3 cells treated with the indicated concentrations of Dasatinib over 24 h (**a**). Interaction of PTPIP51 and HER2 in SK-BR3 cells treated with the indicated concentrations of Dasatinib over 24 h (**b**). Interaction of HER2 and c-Src in SK-BR3 cells treated with the indicated concentrations of Dasatinib over 24 h (**c**)

the MAPK pathway[15] If stimulated by EGF, PTPIP51 interaction with the EGFR is increased accompanied by a higher phosphorylation status of the tyrosine 176. The mechanism prevents PTPIP51 to interact with Raf1 and therefore to over-activate the MAPK pathway[15]. Moreover, PTP1B (dephosphorylates tyrosine 176 residue of PTPIP51), PKA and PKC (phosphorylate serine 212 residue of PTPIP51) interactions are augmented to maintain a basal interaction with and stimulation of Raf1 by PTPIP51[15]. The inhibition of the EGFR by Gefitinib in HaCaT cells induced an increasing interaction of PTPIP51 with 14-3-3 and Raf1 also maintaining a basal level of MAPK activity probably for cell survival[15].

Interestingly, after 48 h incubation the inhibition of the EGFR in SK-BR3 and BT474 cells by Gefitinib, Lapatinib, and Neratinib, regardless whether or not the HER2 receptor was inhibited as well, led to a recruitment of PTPIP51 into MAPK signaling on Raf1 level particularly paralleled by the observed decrease in Tyr176 phosphorylation of PTPIP51 as seen in SK-BR3 immunoblots. In addition, the loss of Tyr176 phosphorylation was accompanied by an increased interaction of PTPIP51 and PTP1B. This interaction also facilitates the interaction of PTPIP51 with Raf1 contributing to the minimal MAPK activity of SK-BR3 cells. Therefore, we conclude that the regulatory function of PTPIP51 on the MAPK pathway is also present in both HER2-positive breast cancer cell lines SK-BR3 and BT474.

However, the tyrosine phosphorylation of PTPIP51 is still observed even though on a very low level. Inhibition of EGFR induced an enhanced interaction of PTPIP51 with the non-receptor tyrosine kinase c-Src in SK-BR3 cells, which partially compensates the inhibited EGFR kinase activity. c-Src plays a pivotal compensatory role in the loss of EGFR kinase activity in breast cancer cells and plays a crucial role in the resistance mechanism against EGFR family targeting TKI[29]. Interestingly, the application of EGFR/HER2-targeted TKI to the BT474 cells did not induce an enhancement of PTPIP51/c-Src interaction. This completely opposes the known regulations of PTPIP51, since the inhibition of EGFR is normally paralleled by an increase in PTPIP51/c-Src interaction.

Furthermore, these findings correlate with a higher sensitivity of BT474 cells to EGFR-targeted TKIs as indicated by the reduction of cell viability after 24 h. There seems to be a connection between the sensitivity of HER2-positive cell lines to EGFR-targeted TKIs and the downstream regulation of the interaction of PTPIP51 and c-Src.

Besides, the potential role of PTPIP51 in the sensitivity of HER2-positive breast cancer cells to EGFR-targeted TKIs, the selective recruitment to the HER2 receptor points to a specific function of PTPIP51 in the changes of cellular signaling induced by selective HER2 inhibition. The selective recruitment is displayed by the unchanged interaction of PTPIP51 and EGFR and the unchanged heterodimer formation of the EGFR with HER2 in SK-BR3 cells. As shown for the EGFR and dual specific TKIs, Mubritinib showed an augmentation of PTPIP51/c-Src interaction in SK-BR3 cells. Of note, the interaction of PTPIP51 and c-Src gradually incremented in the same stoichiometric proportion as the PTPIP51/HER2 interaction. This depicts a potential formation of a ternary complex consisting of PTPIP51, HER2, and c-Src. This ternary interaction was influenceable by the application of the selective c-Src inhibitor Dasatinib. Interestingly, Dasatinib induced the same shift of PTPIP51 toward the HER2 receptor as seen for the application of the EGFR/HER2-targeted TKIs. Furthermore, selective c-Src inhibition also led to an increase in HER/c-Src interaction. All these findings stress an important role of PTPIP51 in the downstream regulations of the EGFR and HER2 signaling.

Beside its function in the MAPK pathway and EGFR signaling, PTPIP51 is also involved in calcium homeostasis by arbitrating the contact of the mitochondrion to the ER by the interaction with the ER bound protein VAPB[17]. The tethering interaction of PTPIP51 and VAPB is precisely regulated by the serine/threonine-kinase GSK3β. The overexpression of GSK3β leads to an impaired interaction of PTPIP51 and VAPB, whereas the inhibition enhances the interaction[18]. Of note, mitochondrial metabolism assayed by a MTT test was significantly impaired under Mubritinib treatment using 10 μM of the inhibitor paralleled by a significant reduction of the PTPIP51/VAPB interaction, indicating the decay of the MAMs in the SK-BR3 cell line. PTPIP51/GSK3β interaction was significantly enhanced under Mubritinib treatment in SK-BR3 and BT474 cells. These findings are not correlated to the observed PTPIP51/VAPB interaction data. We assume, that the PTPIP51/VAPB interaction in SK-BR3 and BT474 cells underlies various regulations and is not only determined by the activity of GSK3β. Selective inhibition of HER2 led to a significantly increased interaction of PTPIP51 with GSK3β, Akt, and PKC in SK-BR3 and BT474 cells.

The group-based prediction system (GPS 3.0; http://gps.biocuckoo.org/) identified PKC, Akt, and GSK3β to phosphorylate PTPIP51 at Ser46, located in its conserved region 1. Phosphorylation of Ser46 is the prerequisite for the PTPIP51/14.3.3. complex formation[7]. Noteworthy, the transmembrane domain of PTPIP51, which is essential for the location to the mitochondrial membrane, lies in direct vicinity to the conserved region 1. The binding of 14.3.3 at conserved region 1 probably caps the transmembrane domain of PTPIP51, thereby preventing a translocation to the mitochondrial membrane[30]. This could be a second potential regulation mechanism for PTPIP51/VAPB interaction preventing mitochondrial membrane translocation of PTPIP51 via 14.3.3 binding. The precise regulation of the PTPIP51/VAPB complex remains enigmatic and needs to be investigated in further studies.

As a functional consequence, the loss of PTPIP51/VAPB is a prerequisite for autophagy. Induction of autophagy in MCF7 breast cancer cells leads to an impaired survival, suggesting a potential tumor supportive effect of autophagy[31]. Here, the PTPIP51/VAPB interaction pattern may be predictive for a more aggressive tumor biology.

As mentioned above, the interaction of PTPIP51 and VAPB forms a physical tether between mitochondrion and ER[17]. These contact sites, namely MAM, represent important signaling hubs, e.g., for mTOR and Akt signaling. The disruption of the MAMs leads to severe alterations in the aforementioned signaling pathways and the regulation of the calcium homeostasis between mitochondrion and the ER[32,33].

As seen here, that Akt signaling is activated under selective HER2 inhibition in SK-BR3 cells as indicated by the elevated S473 phosphorylation of Akt paralleled by a reduced interaction of PTPIP51/VAPB. This activation of Akt signaling is mediated through PTPIP51, as disclosed in the knockdown experiments. The augmented Akt phosphorylation at S473 might indicate an activation of mTORC2 signaling[34]. In addition, activation of mTORC2 signaling leads to an activation of PKC[35]. PKC in turn can activate the MAPK pathway on Raf1 level[36]. This cross-talk to MAPK signaling depicts a potential explanation of MAPK signaling activation under selective HER2 receptor inhibition. Still, the exact mechanism of how the HER2-targeted TKI Mubritinib is capable of altering the PTPIP51/VAPB interaction and the mTORC2 activity remains elusive and needs further investigations.

To sum up, PTPIP51 seems crucial for the downstream regulations of EGFR/HER2-targeted TKIs. We showed, that (1) PTPIP51/c-Src interaction is differently regulated in SK-BR3 and BT474 cells correlating with their particular sensitivity to EGFR-targeted TKIs, (2) selective inhibition of HER2 specifically recruited PTPIP51 to the HER2 receptor and to c-Src reflecting a probable mechanism for resistance, and (3) PTPIP51 is essential for

the activation of Akt signaling under selective HER2 inhibition.

Materials and methods

Cell culture

We obtained the SK-BR3 cell line and the BT474 cell line from Cell Line Service (Eppelheim, Germany). The SK-BR3 cells were cultured in Dulbecco's MEM (Biochrom) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin at 37 °C and 5% CO_2 in a humidified chamber. BT474 cells were cultured in DMEM: Hams F12 (1:1) supplemented with L-glutamine, Insulin and FBS (Cell Lines Service, Eppelheim, Germany). The medium was renewed every 2–3 days. They were cultured until 70–80% confluence. Cell harvesting was performed with Accutase treatment for 10 min in a humidified chamber at 37 °C and 5% CO_2. Subsequently, the cells were rinsed with sterile phosphate-buffered saline (PBS) and counted using a Neubauer counting chamber. The cells were seeded at a density of 30,000–40,000 per well in culture slides (Falcon CultureSlides, Corning Life Science, New York, USA, Cat.# 354108). Mycoplasm infection was excluded with 4′,6-diamidino-2-phenylindole (DAPI) staining for each test.

shRNA experiments

shRNA constructs were obtained from Origene (Rockville, MD, USA). SK-BR3 and BT474 cells were grown in flat-bottomed 24-well tissue culture plates for 24 h with a starting cell number of 100,000 cells per well before transfection. The provided shRNA constructs were initially dissolved in the supplied shRNA dilution buffer. For shRNA experiments the transfection was performed using Viromer Red (Lipocalyx GmbH, Halle, Germany, Cat.# VR-01LB-01) according to the manufacturer's protocol. The shRNA constructs were diluted to a final working dilution of 1 ng/ml.

After transfection, cells were allowed to grow for another 24 h. Transfection rate was controlled by monitoring of expression of GFP via fluorescence microscopy. Transfected cells were incubated for another 24 h with the indicated TKI. The reaction was terminated with NuPAGE LDS Sample Buffer (Thermo Fischer Scientific, Waltham, MA, USA).

Treatment

The cells were allowed to grow for 24 h after seeding. Subsequently, they were treated with different concentrations of Gefitinib (Biaffin, Kassel, Germany, Cat.# PKI-GFTB2-200), Lapatinib (LC Laboratories, Woburn, USA, Cat.# L-4804), Neratinib (LC Laboratories, Woburn, USA, Cat.# L-6404), Mubritinib (Selleckchem, Munich, Germany, Cat.# S2216), or Dasatinib (LC Laboratories, Woburn, USA, Cat.# D-3307) for either 24 h or 48 h.

Immunocytochemistry

The slides were washed in PBS two times for 5 min. After fixation in cold Methanol for 10 min, the slides were washed again in PBS for 8 min. The primary antibodies were diluted in blocking solution to the concentration as reported in Supplementary Table 1. After incubation at room temperature overnight under continuous movement in a humidified chamber, the slides were washed 3 × in PBS for 10 min. The secondary antibodies were diluted (Supplementary Table 1) in PBS and 10% DAPI was added. The samples were incubated for 45 min in a humidified chamber at room temperature in the dark. After washing 3 × in PBS for 10 min the slides were mounted with Mowiol and stored at 4 °C until examination.

Duolink proximity ligation assay

To determine the interactions of proteins the Duolink Proximity ligation assay (PLA probe anti-rabbit minus, Cat.# 92005, PLA probe anti-mouse plus, Cat.# 92001; Detection Kit Orange, Cat.# 92007) was used. The assay is based on the binding of PLA probes to the primary antibodies. If these are closer than 40 nm a signal is generated. After washing the fixed SK-BR3 cells 10 min in PBS the primary antibodies diluted in blocking solution were applied (concentrations in Supplementary Table 1). The slides were allowed to incubate overnight in a humidified chamber under continuous movement. The primary antibodies were tapped off and the slides were washed in PBS 2 × 10 min. PLA probes detecting mouse (Cat# 92001–0100), goat (Cat# 92003–0100), and rabbit antibodies (Cat# 92005–0100) were diluted (1:5) in PBS. Slides were incubated at 37 °C in a humidified chamber for 1 h. The excess amount of PLA probes was tapped off and the samples were washed in Wash-Buffer A 2 × 10 min. Duolink II Ligation stock (1:5) and Duolink Ligase (1:40) were diluted in high purity water and added to the slides. After incubation for 30 min in a humidified chamber at 37 °C, the solution was tapped off and the slides were washed in Wash-Buffer A 2 × 5 min. Duolink Polymerase (1:80) and Duolink Amplification and Detection stock (1:5) were diluted in high purity water and added to the samples. The slides were allowed to incubate for 100 min in a humidified chamber at 37 °C in the dark. Finally, the slides were washed 2 × in Wash-Buffer B for 10 min and 1 × in 0.01 × Wash-Buffer B for 1 min. Nuclear staining was performed using DAPI. After drying for 30 min at room temperature in the dark they were mounted with Mowiol and stored at 4 °C until examination. Leuchowius et al.[37] identified the Duolink proximity ligation assay as an adequate tool for identification of small molecule effectors for protein–protein interactions.

Fluorescence microscopy

The photo documentation was performed with Axioplan 2 fluorescence microscope equipped with Plan-Apochromat objectives (Carl Zeiss Jena, Jena, Germany).

Protein interaction analysis

For quantification, the DuoLink Image Tool (Olink Bioscience, Uppsala, Sweden, v1.0.1.2) was applied. The software identifies DAPI-positive nuclei for the cell count. Cell borders were set according to the software calculated cell shape using a user defined cell diameter preset. Fluorescence dots of the DPLA were counted by the software for each single marked cell.

Western blot

Samples of SK-BR-3 cell lysates were separated on Mini-PROTEAN TGX Stain-Free Precast Gels (Bio-Rad, München, Germany Cat.# 4568085). The Bio-Rad Trans-Blot Turbo Transfer System (Bio-Rad, München, Germany) with the settings for mixed molecular weight proteins was used for transfer to an Immobilon-P membrane (Millipore, Billerica, USA, Cat.#IPVH07850) according to manufacturer's instructions. The membrane was blocked with 1 × Rotiblock for 1 h h at room temperature. Incubation with anti-pMAPK or anti-Akt was done overnight at 4 °C. Horseradish peroxidase-conjugated anti-rabbit immunoglobulins diluted in 1 × Rotiblock were applied for 1 h at room temperature. The reaction was visualized with the ECL prime substrate. The Bio-Rad ChemiDoc Touch Imaging System (Bio-Rad, München, Germany) was used for documentation. Calibration was performed with a molecular weight marker suitable for chemiluminescence (Life Technologies GmbH, Darmstadt, Germany, Cat.# LC5602). The blots were equalized to the obtained stain-free blot for comparison using the Bio-Rad Image Lab (Bio-Rad, München, Germany). Hence, no loading control is required.

MTT assay

The cells were seeded in a 96-well plate at a density of 10,000 cells per well and were allowed to grow for 24 h. The treatment of the cells was carried out as indicated. The MTT solution was added 4 h before the end of the incubation time. After formation of the formazan crystals, the solubilization solution (10% sodium dodecyl sulphate in 0.01 M HCl) was added. The solution was carried out overnight in a 37 °C 5% CO_2 humidified chamber. Evaluation of the assay was performed with Berthold Tech TriStar ELISA Reader (Bad Wildbad, Germany).

Statistical analysis

The data were evaluated using GraphPad Prism 6 software. For variance analysis One way analysis of variance tests were performed. Statistical testing was done using the Dunnett's multiple comparison test in case of Gaussian distribution. Otherwise, Dunn's multiple comparison was used. Results were considered significant with $p < 0.05$. (*($p < 0.05$), **($p < 0.01$), ***($p < 0.001$), ****($p < 0.0001$))

Availability of data and materials

All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Author contributions

Eric Dietel designed the research, performed parts of the experiments, analyzed data, wrote and revised the manuscript. Alexander Brobeil performed parts of the experiments, analyzed data, and revised the manuscript. Claudia Tag performed parts of the experiments. Stefan Gattenlöhner analyzed data and corrected the manuscript. Monika Wimmer designed the research, analyzed data, and wrote and corrected the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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References

1. Moasser, M. M. The oncogene HER2: its signaling and transforming functions and its role in human cancer pathogenesis. *Oncogene* **26**, 6469–6487 (2007).
2. Graus-Porta, D., Beerli, R. R. & Hynes, N. E. Single-chain antibody-mediated intracellular retention of ErbB-2 impairs Neu differentiation factor and epidermal growth factor signaling. *Mol. Cell Biol.* **15**, 1182–1191 (1995).
3. Slamon, D. et al. Adjuvant trastuzumab in HER2-positive breast cancer. *N. Engl. J. Med* **365**, 1273–1283 (2011).
4. Geyer, C. E. et al. Lapatinib plus capecitabine for HER2-positive advanced breast cancer. *N. Engl. J. Med* **355**, 2733–2743 (2006).
5. Gagliato, Dd. M., Jardim, D. L. F., Marchesi, M. S. P. & Hortobagyi, G. N. Mechanisms of resistance and sensitivity to anti-HER2 therapies in HER2 +breast cancer. *Oncotarget* **7**, 64431–64446 (2016).
6. Brobeil, A. et al. PTPIP51-a new relationship with the NFκB signaling pathway. *Biomolecules* **5**, 485–504 (2015).
7. Yu, C. et al. PTPIP51, a novel 14-3-3 binding protein, regulates cell morphology and motility via Raf-ERK pathway. *Cell Signal* **20**, 2208–2220 (2008).
8. Brobeil, A., Graf, M., Eiber, M. & Wimmer, M. Interaction of PTPIP51 with Tubulin, CGI-99 and Nuf2 during cell cycle progression. *Biomolecules* **2**, 122–142 (2012).
9. Petri, M. K. et al. PTPIP51, a positive modulator of the MAPK/Erk pathway, is upregulated in glioblastoma and interacts with 14-3-3β and PTP1B in situ. *Histol. Histopathol.* **26**, 1531–1543 (2011).
10. Koch, P. et al. The novel protein PTPIP51 is expressed in human keratinocyte carcinomas and their surrounding stroma. *J. Cell Mol. Med* **12**, 2083–2095 (2008).
11. Koch, P. et al. PTPIP51 mRNA and protein expression in tissue microarrays and promoter methylation of benign prostate hyperplasia and prostate carcinoma. *Prostate* **69**, 1751–1762 (2009).
12. Brobeil, A. et al. PTPIP51 is phosphorylated by Lyn and c-Src kinases lacking dephosphorylation by PTP1B in acute myeloid leukemia. *Leuk. Res.* **35**, 1367–1375 (2011).

13. Brobeil, A., Graf, M., Oeschger, S., Steger, K. & Wimmer, M. PTPIP51-a myeloid lineage specific protein interacts with PTP1B in neutrophil granulocytes. *Blood Cell Mol. Dis.* **45**, 159–168 (2010).
14. Brobeil, A., Bobrich, M., Tag, C. & Wimmer, M. PTPIP51 in protein interactions: regulation and in situ interacting partners. *Cell Biochem Biophys.* **63**, 211–222 (2012).
15. Brobeil A., Koch P., Eiber M., Tag C., Wimmer M. The known interactome of PTPIP51 in HaCaT cells—inhibition of kinases and receptors. *Int. J. Biochem. Cell Biol.* **46**, 19–31 (2014).
16. Stoica, R. et al. ER-mitochondria associations are regulated by the VAPB-PTPIP51 interaction and are disrupted by ALS/FTD-associated TDP-43. *Nat. Commun.* **5**, 3996 (2014).
17. Vos, K. Jde et al. VAPB interacts with the mitochondrial protein PTPIP51 to regulate calcium homeostasis. *Hum. Mol. Genet* **21**, 1299–1311 (2012).
18. Stoica, R. et al. ALS/FTD-associated FUS activates GSK-3β to disrupt the VAPB-PTPIP51 interaction and ER-mitochondria associations. *EMBO Rep.* **17**, 1326–1342 (2016).
19. Paillusson, S. et al. α-Synuclein binds to the ER-mitochondria tethering protein VAPB to disrupt Ca(2+) homeostasis and mitochondrial ATP production. *Acta Neuropathol.* **134**, 129–149 (2017).
20. Gomez-Suaga, P., Paillusson, S. & Miller, C. C. J. ER-mitochondria signaling regulates autophagy. *Autophagy* **13**, 1250–1251 (2017).
21. Gomez-Suaga, P. et al. The ER-mitochondria tethering complex VAPB-PTPIP51 regulates autophagy. *Curr. Biol.* **27**, 371–385 (2017).
22. Betz, C. et al. Feature article: mTOR complex 2-Akt signaling at mitochondria-associated endoplasmic reticulum membranes (MAM) regulates mitochondrial physiology. *Proc. Natl Acad. Sci. USA* **110**, 12526–12534 (2013).
23. Nagasawa, J. et al. Novel HER2 selective tyrosine kinase inhibitor, TAK-165, inhibits bladder, kidney and androgen-independent prostate cancer in vitro and in vivo. *Int. J. Urol.* **13**, 587–592 (2006).
24. Marty, M. et al. Randomized phase II trial of the efficacy and safety of trastuzumab combined with docetaxel in patients with human epidermal growth factor receptor 2-positive metastatic breast cancer administered as first-line treatment: the M77001 study group. *J. Clin. Oncol.* **23**, 4265–4274 (2005).
25. Azambuja, Ede et al. Lapatinib with trastuzumab for HER2-positive early breast cancer (NeoALTTO): Survival outcomes of a randomised, open-label, multicentre, phase 3 trial and their association with pathological complete response. *Lancet Oncol.* **15**, 1137–1146 (2014).
26. Gajria, D. & Chandarlapaty, S. HER2-amplified breast cancer: mechanisms of trastuzumab resistance and novel targeted therapies. *Expert Rev. Anticancer Ther.* **11**, 263–275 (2011).
27. Moasser, M. M. Targeting the function of the HER2 oncogene in human cancer therapeutics. *Oncogene* **26**, 6577–6592 (2007).
28. Peiró, G. et al. Src, a potential target for overcoming trastuzumab resistance in HER2-positive breast carcinoma. *Br. J. Cancer* **111**, 689–695 (2014).
29. Mueller, K. L., Hunter, L. A., Ethier, S. P. & Boerner, J. L. Met and c-Src cooperate to compensate for loss of epidermal growth factor receptor kinase activity in breast cancer cells. *Cancer Res.* **68**, 3314–3322 (2008).
30. Brobeil, A., Bobrich, M. & Wimmer, M. Protein tyrosine phosphatase interacting protein 51–a jack-of-all-trades protein. *Cell Tissue Res.* **344**, 189–205 (2011).
31. Liang, X. H. et al. Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature* **402**, 672–676 (1999).
32. Shinjo, S. et al. Disruption of the mitochondria-associated ER membrane (MAM) plays a central role in palmitic acid-induced insulin resistance. *Exp. Cell Res.* **359**, 86–93 (2017).
33. Tubbs, E. et al. Disruption of mitochondria-associated endoplasmic reticulum membrane (MAM) integrity contributes to muscle insulin resistance in mice and humans. *Diabetes* **67**, 636–650 (2018).
34. Moore, S. F., Hunter, R. W. & Hers, I. mTORC2 protein complex-mediated Akt (Protein Kinase B) Serine 473 Phosphorylation is not required for Akt1 activity in human platelets corrected. *J. Biol. Chem.* **286**, 24553–24560 (2011).
35. Ikenoue, T., Inoki, K., Yang, Q., Zhou, X. & Guan, K.-L. Essential function of TORC2 in PKC and Akt turn motif phosphorylation, maturation and signalling. *EMBO J.* **27**, 1919–1931 (2008).
36. Schönwasser, D. C., Marais, R. M., Marshall, C. J. & Parker, P. J. Activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by conventional, novel, and atypical protein kinase C isotypes. *Mol. Cell Biol.* **18**, 790–798 (1998).
37. Leuchowius, K.-J. et al. High content screening for inhibitors of protein interactions and post-translational modifications in primary cells by proximity ligation. *Mol. Cell Proteom.* **9**, 178–183 (2010).

PLOS ONE

RESEARCH ARTICLE

Crosstalks of the PTPIP51 interactome revealed in Her2 amplified breast cancer cells by the novel small molecule LDC3/Dynarrestin

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Abstract

LDC3/Dynarrestin, an aminothiazole derivative, is a recently developed small molecule, which binds protein tyrosine phosphatase interacting protein 51 (PTPIP51). PTPIP51 interacts with various proteins regulating different signaling pathways leading to proliferation and migration. Her2 positive breast cancer cells (SKBR3) express high levels of PTPIP51. Therefore, we investigated the effects of LDC3/Dynarrestin on PTPIP51 and its interactome with 12 different proteins of various signal pathways including the interaction with dynein in SKBR3 cells. The localization and semi-quantification of PTPIP51 protein and the Tyr176 phosphorylated PTPIP51 protein were evaluated. Protein-protein-interactions were assessed by Duolink proximity ligation assays. Interactions and the activation of signal transduction hubs were examined with immunoblots. LDC3/Dynarrestin led to an increased PTPIP51 tyrosine 176 phosphorylation status while the overall amount of PTPIP51 remained unaffected. These findings are paralleled by an enhanced interaction of PTPIP51 with its crucial kinase c-Src and a reduced interaction with the counteracting phosphatase PTP1B. Furthermore, the treatment results in a significantly augmented interaction of PTPIP51/14-3-3β and PTPIP51/Raf1, the link to the MAPK pathway. Under the influence of LDC3/Dynarrestin, the activity of the MAPK pathway rose in a concentration-dependent manner as indicated by RTK assays and immunoblots. The novel small molecule stabilizes the RelA/IκB/PTPIP51 interactome and can abolish the effects caused by TNFα stimulation. Moreover, LDC3/Dynarrestin completely blocked the Akt signaling, which is essential for tumor growth. The data were compared to the recently described interactome of PTPIP51 in LDC3/Dynarrestin treated non-cancerous keratinocyte cells (HaCaT). Differences were identified exclusively for the mitochondrial-associated ER-membranes (MAM) interactions and phospho-regulation related interactome of PTPIP51.LDC3/Dynarrestin gives the opportunity/possibility to influence the MAPK signaling, NFkB signaling and probably calcium homeostasis in breast cancer cells by affecting the PTPIP51 interactome.

Introduction

Breast cancer is the most common invasive cancerous disease amongst women. Prognosis of this disease is greatly influenced whether the Her2-oncogene/oncoprotein is amplified. This applies to 20–30% of the tumors [1]. The amplification of Her2 goes hand in hand with severe alterations in growth and proliferation signaling, e.g., mitogen-activated protein kinase (MAPK) signaling, nuclear factor κ B (NFκB) signaling, by deregulation of signal transduction and protein-protein interactions (PPI) [2]. Detection and understanding of these disturbed signal nodes and PPIs are of the utmost interest in order to develop the most suitable drug for each tumor. Up to now different therapeutic antibodies and tyrosine kinase inhibitors (TKI) like Trastuzumab or Lapatinib have been developed to block the altered Her2 signaling by direct attachment to the Her2 receptor [3]. This targeted therapy led to significantly better results than radio- and chemotherapy alone [4,5]. A drawback to these therapeutics is upcoming resistances of some tumors to the TKIs or the antibody blockage of the receptors [3]. One cause is the early position of the Her2 receptor in the signal transduction which gives the tumor many options to bypass the blocked signaling. In order to overcome such resistance, the identification of drugable PPIs and signal nodes downstream of Her2 is of the utmost interest.

Recently, a novel inhibitor of cytoplasmic dynein, namely LDC3/Dynarrestin was described by Höing et al. [6]. The small molecule interferes with the Hedgehog pathway via inhibition of cytoplasmic Dynein and thereby affecting the intraflagellar transport. A disturbed activation of the Hedgehog pathway is linked to medulloblastoma, basal cell carcinoma, and breast cancer. The scaffolding protein-protein tyrosine phosphatase interacting protein 51 (PTPIP51) was identified as a target of a LDC3/Dynarrestin derived probe in a Yeast-3-Hybrid assay (Lead Discovery Center GmbH, Dortmund, Germany, personal communication). LDC3/Dynarrestin displays PTPIP51 dependent effects on cell signaling, as seen by the knockdown experiments of Brobeil et al. The knockdown of PTPIP51 abolishes the MAPK stimulating effect of LDC3/Dynarrestin normally induced by the PTPIP51/14-3-3/Raf1 interactome [7].

Interestingly, a substrate of the Her2 associated protein tyrosine phosphatase 1B (PTP1B) [8], namely Protein tyrosine phosphatase interacting protein 51 (PTPIP51), couples to the aforementioned MAPK pathway on Raf1 level. PTPIP51 can activate the MAPK pathway by its interaction with 14-3-3β on Raf1 level [9–11]. This leads to enhanced downstream signaling and hence results in cell proliferation, which is a hallmark of malignantly transformed cells, e.g., breast cancer cells. Moreover, PTPIP51 is often deregulated in the development of cancer. In basal cell carcinoma and squamous cell carcinoma, an altered expression is found [12]. Glioblastoma display an increasing PTPIP51 expression, which is associated with higher amounts of 14-3-3β, indicating a higher malignancy [13]. In prostate cancer, the expression of PTPIP51 is enhanced by hypomethylation of its promoter region [14].

Physiologically, PTPIP51 exhibits a heterogeneous panel of functions. PTPIP51 is involved in apoptosis, development, differentiation, cell elongation, migration and NFκB signaling [15–19]. Noteworthy, PTPIP51 as well interacts with VAPB in order to regulate the calcium homeostasis and formation of mitochondria-associated endoplasmic reticulum membranes (MAM) [20,21].

Basing on these facts, we initiated the current study to investigate the modulatory effect of LDC3/Dynarrestin on the Her2 positive breast cancer cell line SKBR3. The modification of PTPIP51 related signaling gives the possibility to influence the associated signaling pathways like the MAPK pathway, NFκB signaling and the calcium homeostasis, which if over-activated or deregulated all lead, to enhanced proliferation, growth, and invasiveness of breast cancer. Thus, PTPIP51 may resemble a new drugable, therapeutic target.

Materials and methods

Cell culture

We obtained the SKBR3 cell line from Cell Line Service (Eppelheim, Germany). The cells were cultured in Dulbecco´s MEM (Biochrom) supplemented with 10% fetal calf serum and 1% Penicillin/Streptomycin at 37˚C and 5% CO2 in a humidified chamber. The medium was renewed every 2–3 days. They were cultured until 70–80% confluence. Cell harvesting was performed with Accutase treatment for 10 min in a humidified chamber at 37˚C and 5% CO2. Subsequently, the cells were rinsed with sterile phosphate buffered saline (PBS) and counted using a Neubauer counting chamber. The cells were seeded in a density of 30.000–40.000 per well in culture slides (Falcon CultureSlides, Corning Life Science, New York, USA, Cat.# 354108).

LDC3/Dynarrestin

The aminothiazole derivative LDC3/Dynarrestin was synthesized by the Lead Discovery Center, GmbH, Dortmund, Germany. The stock solution in DMSO was stored at -80˚C. The synthesis and structural formula of LDC3/Dynarrestin was published by Höing et al. [6].

Treatment

The cells were allowed to grow for 24h after seeding. Subsequently, they were treated with different concentrations of LDC3/Dynarrestin (diluted in culture medium) for either 1h, 6h, 24h or 48h.

For the activation of NFκB cells were incubated with TNFα (Recombinant Human TNF-α, Peprotech Germany, Hamburg Germany, Cat.# 300-01A) for 6h in a concentration of 100ng/ml. The NFκB inhibition was performed with Ammonium pyrrolidine dithiocarbamate (Sigma-Aldrich, Cat.# P 8765, Munich, Germany) for 6h in a concentration of 50μM. In the case of dual incubation, both compounds were applied at the same time.

The cells were also treated with Sorafenib (LC Laboratories, Woburn, USA, Cat.# S-8599), Dasatinib (LC Laboratories, Woburn, USA, Cat.#. D-3307), 1B Inhibitor (Calbiochem Cat.# 539741) and Ciliobrevin A (Selleckchem, Cat.# S8249).

Immunocytochemistry

The slides were washed in PBS 2 times for 10 min. The primary antibodies were diluted in blocking solution to the concentration as reported in Table 1. After incubation at room temperature overnight under continuous movement in a humidified chamber, the slides were washed 3 times in PBS for 10 min. The secondary antibodies were diluted (Table 1) in PBS and 10% DAPI was added. The samples were incubated for 45min in a humidified chamber at room temperature in the dark. After washing 3 times in PBS for 10 min, the slides were mounted with Mowiol and stored at 4˚C until examination.

Duolink proximity ligation assay

To determine the interactions of proteins the Olink Duolink Proximity ligation assay (PLA probe anti-rabbit minus, Cat.# 92005, PLA probe anti-mouse plus, Cat.# 92001; Detection Kit Orange, Cat.# 92007) was used. The assay is based on the binding of PLA probes to the primary antibodies. If these are closer than 40nm, a signal is generated. After washing the fixed SKBR3 cells, 10 min in PBS the primary antibodies diluted in blocking solution were applied (concentrations are listed in Table 1). The slides were allowed to incubate overnight in a humidified chamber under continuous movement. The primary antibodies were tapped off,

Table 1. Antibody list.

Name	Immunogen	Antibody source	Clone	Dilution	Manufacturer
PTPIP51(P51ab)	Human recombinant PTPIP51 protein encoding amino acids (aa) 131–470	Rabbit polyclonal		1:500	Prof. HW Hofer, Biochemical Department, University Konstanz, Germany
tyrosine 176 phosphorylated PTPIP51	Purified total IgG fraction KLH-peptide conjugate	Guinea pig polyclonal		1:400	BioLux, Stuttgart, Germany
Raf-1	Mapping the C-terminus of human origin	Mouse monoclonal	E-10	1:100	Santa Cruz Biotechnology Cat.# sc-7267
14-3-3β	Specific for an epitope mapping between aa 220–244 at the C-terminus of 14-3-3β of human origin	Mouse monoclonal	A-6	1:100	Santa Cruz Biotechnology Cat.# sc-25276
PTP1B	epitope mapping at the N-terminus of PTP1B of human origin	Goat Polyclonal	N-19	1:100	Santa Cruz Biotechnology Cat.# sc-1718
c-Src	specific for an epitope mapping between amino acids 1–30 at the N-terminus of c-Src p60 of human origin	Mouse monoclonal	H-12	1:100	Santa Cruz Biotechnology Cat.# sc-5266
GSK-3β	raised against amino acids 345–420 mapping at the C-terminus of GSK-3β of human origin	Mouse monoclonal	E-11	1:100	Santa Cruz Biotechnology Cat.# sc-377213
VAPB	E.coli-derived recombinant human VAP-B Ala2-Pro132	Mouse monoclonal	736904	1:100	R&D systems Cat.# MAB58551
Erβ	raised against ERβ, corresponding to amino acids 256–505 of human origin	Mouse monoclonal	1531	1:100	Santa Cruz Biotechnology Cat.# sc-53494
Her2	ERBB2 (NP_004439, 22aa ~ 121aa) partial recombinant protein with GST tag. MW of the GST tag alone is 26 KDa	Mouse monoclonal	22–121	1:100	Abnova, Taipei, Taiwan Cat.# H0000 2064-M05
RelA	Recognizes an epitope overlapping the nuclear location signal (NLS) of the p65 subunit of the NFkB heterodimer	Mouse monoclonal	12H11	1:100	Merck Millipore, Schwalbach, Germany Cat.# MAB3026
IκBα	Recombinant Human IκB alpha/NFKBIA protein 02	Mouse monoclonal	MM02	1:100	Sino Biological Inc., North Wales, PA, USA Cat.# 12045-H07E
CGI-99	epitope mapping at the N-terminus of CGI-99 of human origin	Goat Polyclonal	N-14	1:100	Santa Cruz Biotechnology Cat.# sc-104834
Nuf-2 (cdcA1)	raised against amino acids 1–300 mapping at the N-terminus of CdcA1 of human origin	Mouse monoclonal	E-6	1:100	Santa Cruz Biotechnology Cat.# sc-271251
Phospho-Akt (Ser473)	a synthetic phosphopeptide corresponding to residues surrounding Ser473 of mouse Akt	Rabbit monoclonal		1:2500	Cell signaling technology #9271
Phospho-p42/p44 MAPK	a synthetic phosphopeptide corresponding to residues surrounding Thr202/Tyr204 of human p44 MAP kinase	Rabbit monoclonal		1:2500	Cell signaling technology #9111
Alexa donkey anti-rabbit Fab fragments		Donkey polyclonal		1:800	Dianova Cat.# 711-166-152
Cy3 donkey anti-guinea pig	IgG (H+L) from guinea pig	Donkey polyclonal		1:800	Dianova Cat.# 706-166-148

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and the slides were washed in PBS 2x10min. PLA probes detecting mouse (Cat# 92001–0100), goat (Cat# 92003–0100) and rabbit antibodies (Cat# 92005–0100) were diluted (1:5) in PBS. Slides were incubated at 37˚C in a humidified chamber for 1h. The excess amount of PLA probes was tapped off, and the samples were washed in Wash-Buffer A 2x10min. Duolink II Ligation stock (1:5) and Duolink Ligase (1:40) were diluted in high purity water and added to the slides. After incubation for 30min in a humidified chamber at, 37˚C the solution was tapped o, and the slides were washed in Wash-Buffer A 2x5 min. Duolink Polymerase (1:80) and Duolink Amplification and Detection stock (1:5) were diluted in high purity water and added to the samples. The slides were allowed to incubate for 100 min in a humidified chamber at 37˚C in the dark. Finally, the slides were washed 2x in Wash-Buffer B for 10 min and 1x in 0,01xWash-Buffer B for 1 min. Nuclear staining was performed using DAPI. After drying for 30 min at room temperature in the dark, they were mounted with Mowiol and stored at

4˚C until examination. Leuchowius and coworkers identified the Duolink proximity ligation assay as an adequate tool for the identification of small molecule effectors for protein-protein interactions [22].

Fluorescence microscopy

The photo documentation was performed with an Axioplan 2 fluorescence microscope equipped with Plan-Apochromat objectives (Carl Zeiss Jena, Jena, Germany). For visualization of the secondary antibody, Alexa Fluor 555 used for the detection of PTPIP51 primary antibody an excitation filter with a spectrum of 530–560 nm and an emission filter with a spectrum 572–647 nm were used. The phosphorylated tyrosine 176 PTPIP51 was visualized by a Cy3 conjugated secondary antibody using the same filter as indicated above.

Semiquantitative analysis

The immune-cytochemical pictures were analyzed by the ImageJ tool in order to investigate the brightness values. For this purpose cell groups were encircled and analyzed. High staining intensity and therefore high grey value levels display high amounts of protein.

Protein interaction analysis

For quantification, the DuoLink Image Tool (Olink Bioscience, Uppsala, Sweden, v1.0.1.2) was applied. The software identifies Dapi positive nuclei for the cell count. Cell borders were set according to the software calculated cell shape using a user-defined cell diameter preset. Fluorescence dots of the DPLA were counted by the software for every single marked cell.

Immunoblot

Samples of SKBR3 cell lysates were separated on Mini-PROTEAN TGX Stain-Free Precast Gels (Bio-Rad, München, Germany Cat.# 4568085). The Bio-Rad Trans-Blot Turbo Transfer System (Bio-Rad, München, Germany) with the settings for mixed molecular weight proteins was used for transfer on an Immobilon P membrane (Millipore, Billerica, USA, Cat. #IPVH07850) according to manufacturer's instructions. The membrane was blocked with 1x Rotiblock for 1h hour at room temperature. Incubation with anti-pMAPK or anti-Akt was done overnight at 4˚C. HRP-conjugated anti-rabbit immunoglobulins diluted in 1x Rotiblock were applied for 1 h at room temperature. The reaction was visualized with the ECL prime substrate. The Bio-Rad ChemiDoc Touch Imaging System (Bio-Rad, München, Germany) was used for documentation. Calibration was performed with a molecular weight marker suitable for chemiluminescence (Life Technologies GmbH, Darmstadt, Germany, Cat.# LC5602). The blots were equalized to the obtained stain-free blot for comparison using the Bio-Rad Image Lab (Bio-Rad, München, Germany). Hence, no loading control is required.

MTT assay

The cells were seeded in a 96-well plate at a density of 10,000 cells per well and were allowed to grow for 24h. The treatment of the cells was carried out as indicated. The MTT solution was added 4h before the end of the incubation time. After formation of the formazan crystals, the solubilization solution (10% SDS in 0,01M HCl) was added. The solution was carried out overnight in a 37˚C 5% CO_2 humidified chamber. Evaluation of the assay was performed with Berthold Tech TriStar ELISA Reader (Bad Wildbad, Germany).

Statistical analysis

Data were evaluated using GraphPad Prism 6 software. Statistical significance was determined using ANOVA followed by the Dunnett´s multiple comparison tests. Results were considered significant with $p<0.05$. ($^{*}(p<0.05)$, $^{**}(p<0,01)$, $^{***}(p<0,001)$, $^{****}(p<0,0001)$). Data in graphs are presented by their mean values and standard deviation.

Results

LDC3/Dynarrestin enhanced the Tyr176 phosphorylation of PTPIP51

SKBR3 cells either untreated (Fig 1A) or treated with 50 μM LDC3/Dynarrestin for 24h (Fig 1C) were immunostained with antibodies to PTPIP51 and in a parallel experiment to its Tyr176 phosphorylated form (Fig 1B and 1D). The stainings were semi-quantitatively analyzed. The amount of PTPIP51 protein in LDC3/Dynarrestin treated SKBR3 cells was not significantly altered compared to the untreated controls (Fig 1E). In contrary investigating the Tyr176 phosphorylated PTPIP51 in LDC3/Dynarrestin treated cells exhibited a concentration-dependent increase in protein (Fig 1F). The values under 5μM, and 50μM LDC3/Dynarrestin treatment were significantly increased above the control values ($p<0.05$ and $p<0.01$, respectively).

Tyrosine 176 phosphorylation of PTPIP51 under LDC3/Dynarrestin influence in combination with c-Src inhibition and PTP1B inhibition

The application of LDC3/Dynarrestin in rising concentrations increased the phosphorylation of Tyr176 of PTPIP51 (Fig 2), thus corroborating the findings of the immunocytochemical stainings. The application of the c-Src inhibitor Dasatinib did not interfere with the LDC3/Dynarrestin induced augmentation of Tyr176 phosphorylation of PTPIP51. The combination of rising concentrations of LDC3/Dynarrestin with the PTP1B inhibitor reverted the increase of Tyr176 phosphorylation and induced a reduced Tyr176 phosphorylation of PTPIP51.

LDC3/Dynarrestin affects the interaction of PTPIP51 and cytoplasmic dynein

LDC3/Dynarrestin inhibits the activity of cytoplasmic Dynein in nanomolar concentrations, through an ATP hydrolysis independent mode of action. Since PTPIP51 is known to interact with cytoplasmic dynein, we monitored the interactional changes of both proteins with proximity ligation assays. If LDC3/Dynarrestin was administrated in nanomolar concentrations (500 nM ($p<0.0001$)) the interaction of PTPIP51 and cytoplasmic Dynein was reduced if applied for 24h. These effects were reversed if LDC3/Dynarrestin was applied in higher micromolar concentrations (10μM ($p<0.0001$) and 50μM ($p<0.0001$)) (Fig 3B). Interestingly, these effects are not seen if LDC3/Dynarrestin was applied for 1h. Application of Ciliobrevin A induced a concentration dependent increase of the PTPIP51/cytoplasmic dynein interaction, but only the highest applied concentration led to a significant increase if applied for 1h. The treatment of SKBR3 cells with Ciliobrevin A for 24h significantly augmented the interaction of PTPIP51 with cytoplasmic dynein, if applied in low concentrations. Of note, the 500μM Ciliobrevin A for 24h severely affected the cell viability, thus preventing a sufficient analysis of protein-protein interactions (Fig 3A).

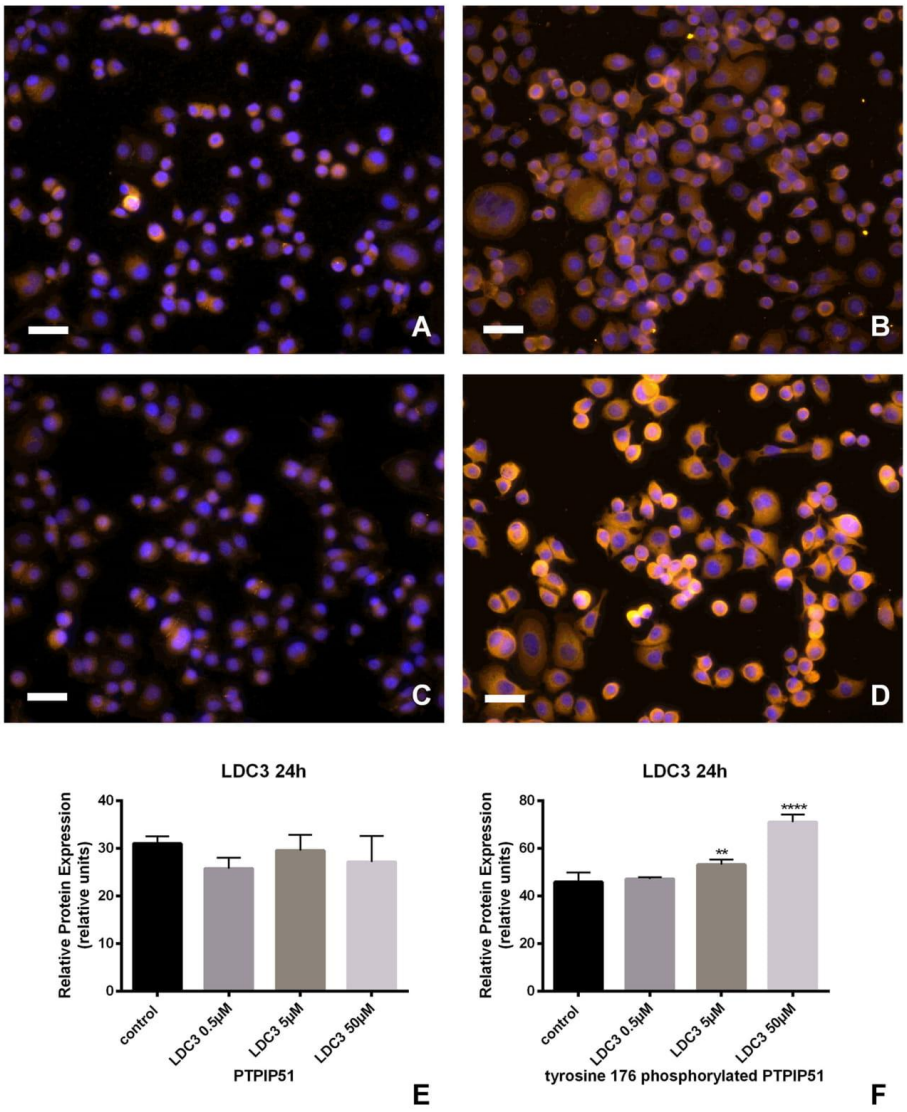

Fig 1. Immunocytochemical staining of PTPIP51 and tyrosine 176 phosphorylated PTPIP51 of SKBR3 cells. **(a)** Immunocytochemical staining of PTPIP51 in untreated cells, **(b)** Immunocytochemical staining of phosphorylated Tyr176 PTPIP51 in untreated cells, **(c)** Immunocytochemical staining of PTPIP51 in 50 μM LDC3/Dynarrestin treated cells (24h), **(d)** Immunocytochemical staining of phosphorylated Tyr176 PTPIP51 in 50 μM LDC3/Dynarrestin treated cells (24h). **(e)** Semiquantitative analysis of the PTPIP51 immunostaining in untreated cell and cell treated with LDC3/Dynarrestin in concentrations of 0.5 μM, 5 μM and 50 μM for 24h, **(f)** Semiquantitative analysis of the phosphorylated Tyr176 PTPIP51 immunostaining in untreated cell and cells treated with LDC3/Dynarrestin in concentrations of 0.5 μM, 5 μM and 50 μM for 24h. Bar = 50μm.

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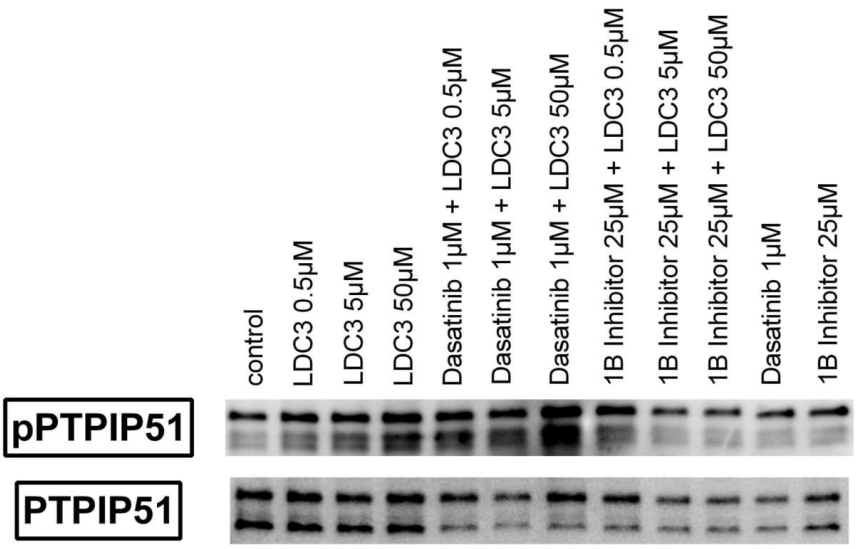

Fig 2. Immunoblots of total PTPIP51protein (PTPIP51) and Tyr176 phosphorylated PTPIP51(pPTPIP51). Cells were treated with LDC3/Dynarrestin, Dasatinib and/or PTP1B inhibitor in the indicated concentration for 1h. Representative blots shown.

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LDC3/Dynarrestin affects the MAPK signaling associated interactome of PTPIP51

Basing on the observed highly significant alterations in the Tyr176 phosphorylation of PTPIP51, we examined the interaction profile of PTPIP51 with its crucial phosphatase PTP1B. Exposure of SKBR3 cells to LDC3/Dynarrestin led to a triphasic interaction pattern with its tyrosine dephosphorylating enzyme PTP1B (Fig 4A). Application of LDC3/Dynarrestin for 1h at 5µM significantly augmented the interaction of PTPIP51 and PTP1B. If exposed for 24h to increasing LDC3/Dynarrestin concentrations the interaction of PTPIP51/PTP1B was significantly increased ($p<0.01$) under the lowest concentration of LDC3/Dynarrestin. Application

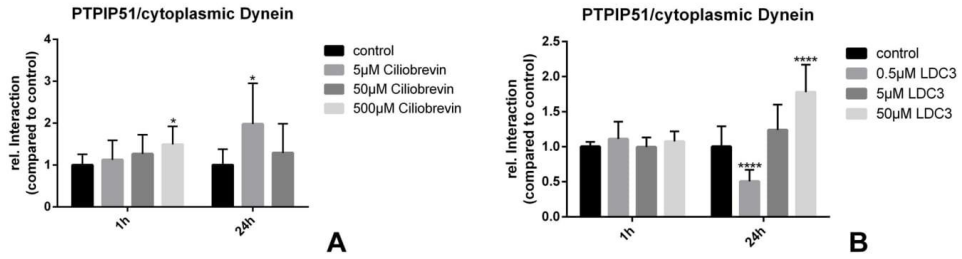

Fig 3. Interactions of PTPIP51 with cytoplasmic dynein in untreated SKBR3 cells and cells treated with LDC3/Dynarrestin (b) or Ciliobrevin A (a) in concentrations ranging from 0.5 µM to 50 µM and 5µM to 500µM, respectively.

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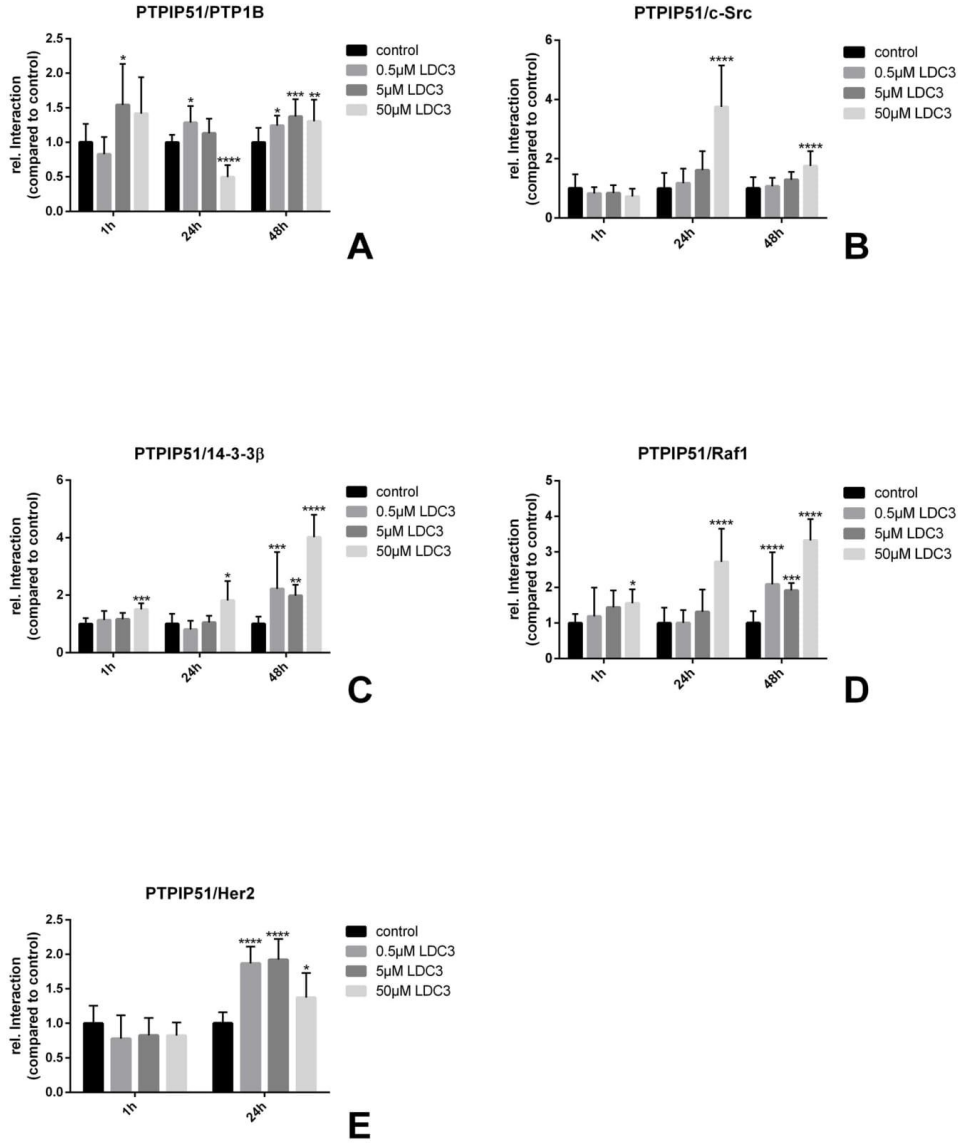

Fig 4. Interactions of PTPIP51 with different partners in untreated SKBR3 cells and cells treated with LDC3/Dynarrestin in concentrations of 0.5 µM, 5 µM, and 50 µM. **(a)** Interaction of PTPIP51 with PTP1B, **(b)** Interaction of PTPIP51 with c-Src, **(c)** Interaction of PTPIP51 with 14.3.3β, **(d)** Interaction of PTPIP51 with Raf1, **(e)** Interaction of PTPIP51 with HER2.

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of 5μM LDC3/Dynarrestin resulted in levels higher than normal, whereas 50μM highly significant reduced the number of interactions ($p<0.0001$). With prolonged incubation times 0.5μM and 5μM significantly surpassed control values (0.5μM $p<0.01$; 5 μM $p<0.001$). Although 50μM led to a slight reduction, the values were still significantly higher than those of the controls ($p<0.05$). Phosphorylation of PTPIP51 at Tyr176 is under control of several tyrosine kinases. One of them is the non-membrane tyrosine kinase c-Src. Interestingly, application of LDC3/Dynarrestin for 1h did not alter the interaction of PTPIP51 and c-Src. The interactions of PTPIP51 and c-Src increased stepwise under LDC3/Dynarrestin treatment both after 24h (5μM $p<0.01$; 50μM $p<0.0001$) and 48h incubation time (50μM: $p<0.01$) (Fig 4B). The observed increase of c-Src/PTPIP51 interaction in combination with the concomitant decrease of PTP1B/PTPIP51 interaction after application of 50μM LDC3/Dynarrestin for 24h explains the increased Tyr176 phosphorylation of PTPIP51. The phosphorylation of Tyr176 is crucial for the interaction of PTPIP51 with 14.3.3β and Raf1, respectively. Treating the cells with LDC3/Dynarrestin changed the interaction of PTPIP51 and 14.3.3β in a concentration and time-dependent manner (Fig 4C). After 24h of exclusive LDC3/Dynarrestin treatment, the highest concentration resulted in a significant increase in the number of interactions ($p<0.01$). However, doubling the exposure time caused a significant enhancement in interactions independent from the applied LDC3/Dynarrestin concentration in comparison to untreated controls (0.5μM $p<0.01$, 5μM $p<0.0001$, 50μM $p<0.0001$). The interaction profile of PTPIP51/Raf1 (Fig 4D) parallels the one seen for the PTPIP51/14.3.3β interactome. After 24h of LDC3/Dynarrestin treatment, significant increases of the interaction profile were only seen for a concentration of 50μM LDC3/Dynarrestin ($p<0.0001$). The 48h incubation led to enhanced interactions for all treated cells independent of the applied concentration of LDC3/Dynarrestin. All higher values differed significantly from the controls (0.5μM $p<0.001$, 5μM $p<0.0001$, 50μM $p<0.0001$). Of note, application of 50μM LDC3/Dynarrestin for 1h significantly increased the interaction of PTPIP51/Raf1 and PTPIP51/14.3.3β (Fig 4C and 4D).

Since the amplification of the HER2 receptor in SKBR3 cells directly affects the MAPK signaling, evaluation of the interaction profile of PTPIP51 and HER2 receptor was indispensable. LDC3/Dynarrestin applied for 1h did not lead to a significantly altered interaction of HER2/PTPIP51. These findings correspond with the observations made for the interaction of PTPIP51 and c-Src under the influence of LDC3/Dynarrestin. Treating SKBR3 cells for 24h with LDC3/Dynarrestin resulted in an enhanced interaction of PTPIP51 and HER2 (Fig 4E). Noteworthy, 0.5μM and 5μM LDC3/Dynarrestin led to a highly significant augmentation ($p<0.0001$), whereas the values are seen after 50μM treatment only differed significantly ($p<0.05$).

ERK1/2 and Akt activation under LDC3/Dynarrestin treatment in combination with Raf1 inhibition or c-Src inhibition

Since LDC3/Dynarrestin enhanced the formation of the Raf1/14.3.3β/PTPIP51 complex, we further examined the influence of LDC3/Dynarrestin on the activation of the MAPK signaling pathway. As an indicator of the MAPK pathway activation the Thr202/Tyr204 phosphorylation of ERK1/2 (pMAPK) was evaluated via immunoblotting. Application of LDC3/Dynarrestin in rising concentrations led to a concentration dependent increase of pMAPK and thus an activation of MAPK signaling. Interestingly, application of the c-Src inhibitor Dasatinib severely reduced the amount of ERK1/2. Nethertheless, the application of LDC3 induced a concentration dependent increase of pMAPK. Likewise, findings were observed for the combination of the Raf1 inhibitor Sorafenib with LDC3/Dynarrestin. The sole inhibition of Raf1via Sorafenib induced an activation of the MAPk pathway compared to the control group. The

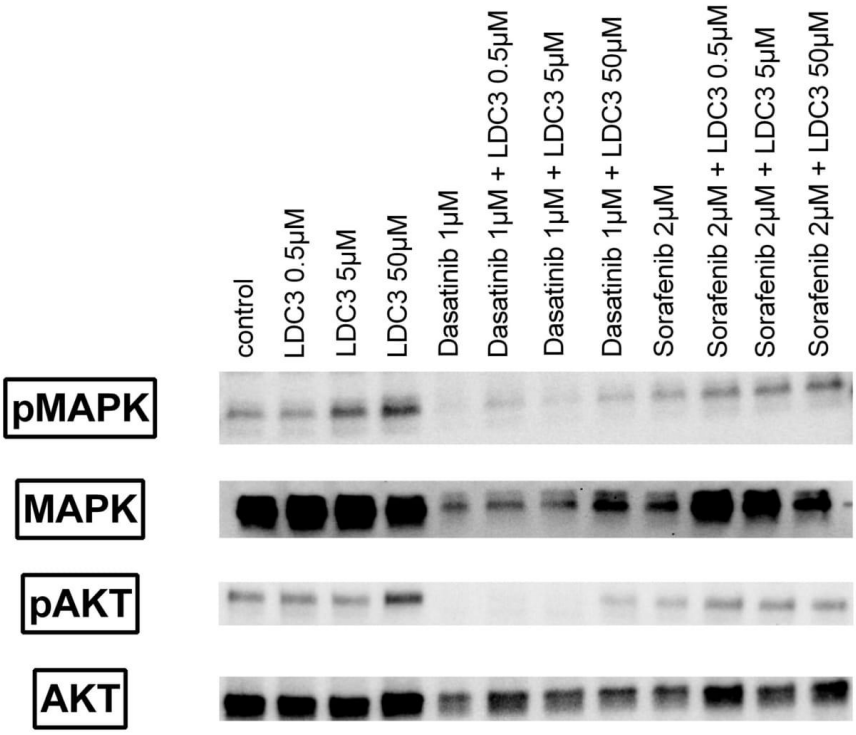

Fig 5. Immunoblots of total ERK1/2 (MAPK), phospho ERK1/2 (pMAPK), panAKT (panAKT) and phospho AKT (pAKT). SKBR3 cell were treated with LDC3/Dynarrestin, Dasatinib and/or Sorafenib in the indicated concentration for 1h. Representative blots shown.

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combination of Raf1 inhibition and low concentrations of LDC3/Dynarrestin slightly reduced the amount of pMAPK. Of note, the combination of Sorafenib with 50μM LDC3/Dynarrestin induced the highest measured amounts of pMAPK (Fig 5).

The Akt signaling represents another central downstream signaling pathway of the HER2 receptor besides the MAPK pathway. In consequence, we also evaluated the phosphorylation of Ser473 of AKT (pAKT) as an indicator of Akt signaling activation. The application of LDC3/Dynarrestin for 1h slightly reduced the amount of pAKT for all applied concentrations. Interestingly, the application of Dasatinib or Sorafenib in combination with LDC3/Dynarrestin reverted the aforementioned reduction of pAkt. The combination of Sorafenib with LDC3/Dynarrestin led to the highest observed pAkt amounts (Fig 5).

NFκB related PTPIP51 interaction under LDC3/Dynarrestin treatment in combination with stimulation and inhibition of NFκB signaling

The amplification of the HER2 receptor is correlated with an activation of the NFκB signaling. To compare the LDC3/Dynarrestin induced alterations in the NFκB signaling interactome with PTPIP51 in SKBR3 cells with the already well evaluated effects in HaCaT cells, examined

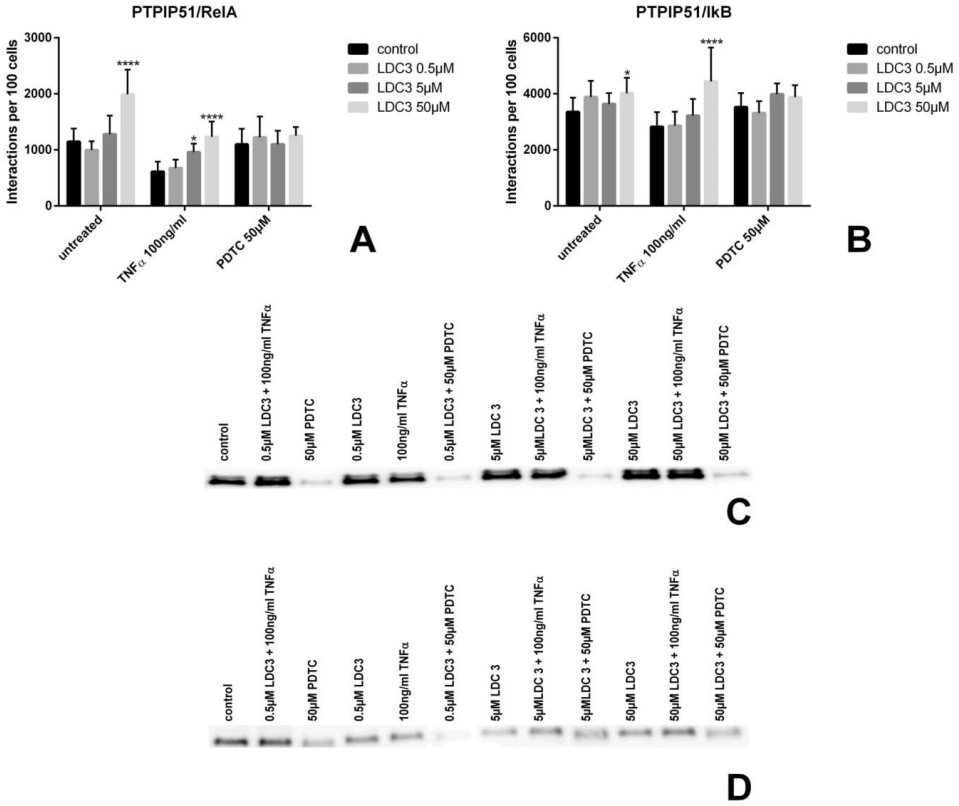

Fig 6. Interactions of PTPIP51 with RelA and IκB in SKBR3 cells either treated with LDC3/Dynarrestin alone or in combination with either TNFα or PDTC in the indicated concentrations for 6h. (a) Interaction of PTPIP51 and RelA, **(b)** Interaction of PTPIP51 and IκB. Immunoblots of SKBR3 cells determining the activity of ERK1/2 and Akt by analysing its phosphorylation of specific sites of the molecules. The cells were treated with rising concentrations of LDC3/Dynarrestin. The control groups of the TNFα respectively PDTC subgroup were treated solely with TNFα or PDTC. Incubation time 6h. **(c)** Activation status of ERK1/2, **(d)** Activation status of Akt. Representative blots shown. Normalization was performed using the total protein amount.

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by Brobeil and coworkers, we selected an incubation time of 6h [19]. SKBR3 cells exposed to increasing LDC3/Dynarrestin concentrations from 0.5μM up to 50μM displayed an increase in PTPIP51/RelA interactions to about 170% of that seen in untreated cells. Stimulation of NFκB signaling by TNFα (100ng/ml) for 6 h halved the number of PTPIP51/RelA interactions compared to untreated controls. The presence of increasing LDC3/Dynarrestin concentrations led to a stepwise augmentation in the number of interactions near to control values. Inhibition of the NFκB signaling by PDTC treatment (50μM) resulted in the abolishment of the LDC3/Dynarrestin effect (Fig 6A). Comparable observations were made for the interaction profile of PTPIP51 with IκB. The exclusive application of LDC3/Dynarrestin (0.5μM, 5μM, and 50μM) did not increase the number of PTPIP51/IκB interactions (Fig 6B). The simultaneous

stimulation of the NFκB signaling pathway by TNFα displayed an insignificant reduction in the number of interactions compared with the untreated control group. The treatment with 50μM LDC3/Dynarrestin and TNFα induced a significant increase of PTPIP51/IκB interactions. PDTC mediated NFκB inhibition resulted in comparable interaction patterns as seen in the untreated controls which correspond to the one observed for PTPIP51/RelA subjected to the same treatments. We also determined the interaction data of PTPIP51 with RelA for a 24h incubation time in order to get better comparability with the interaction profile in HaCaT cells assessed by Brobeil and coworkers [7]. The application of 0.5μM and 5μM LDC3/Dynarrestin highly significant enhanced the interaction of PTPIP51 with RelA (0.5μM $p<0.01$; 5μM $p<0.0001$). No significant difference was seen under 50μM LDC3/Dynarrestin treatment compared to the control group (S1 Fig).

To identify cross talks between these signaling hubs and the role of PTPIP51 in their regulation we performed immunoblots of pERK1/2 and pAkt under LDC3/Dynarrestin treatment in combination with NFκB signaling activation and inhibition. The LDC3/Dynarrestin treatment of TNFα stimulated SKBR3 cells for 6h resulted in the same effects on ERK1/2 activation as seen for the treatment with LDC3/Dynarrestin alone. In contrary, the inhibition of the NFκB pathway by PDTC in combination with LDC3/Dynarrestin treatment led to a sharp drop in ERK1/2 phosphorylation and in consequence in its activation (Fig 6C) abolishing the activation enhancing effect of LDC3/Dynarrestin. Akt Ser473 phosphorylation in TNFα stimulated SKBR3 cells was reduced by about 50% compared to the untreated control cells. Combing TNFα stimulation with increasing LDC3/Dynarrestin concentrations resulted in a slight augmentation of the Akt phosphorylation in comparison to solely LDC3/Dynarrestin exposed cells. Noteworthy, PDTC inhibition of NFκB signaling reduced Akt phosphorylation status by about 60%. Combining PDTC and 0.5μM LDC3/Dynarrestin treatment almost abolished Akt phosphorylation, whereas higher LDC3/Dynarrestin concentrations differed only slightly from cells not inhibited by PTDC. Here, PDTC did not affect the LDC3/Dynarrestin effect (Fig 6D).

LDC3/Dynarrestin induces alterations of the MAM related interactions of PTPIP51 and affects the mitochondrial metabolic rate

The expression of VAPB in SKBR3 cells was confirmed with an immunocytochemistry (S2 Fig). The interaction PTPIP51 with VAPB was not influenced by low LDC3/Dynarrestin concentrations either after 24h or after 48h of treatment. 5μM and 50μM LDC3/Dynarrestin treatment for 24h induced in cells a highly significant increase by a factor of 3.8 and 2.5, respectively. Such drastic differences were no longer seen after 48h LDC3/Dynarrestin exposure. Nevertheless, the values differed significantly from that measured in untreated controls (5μM $p<0.05$, 50μM $p<0.05$) (Fig 7A).

LDC3/Dynarrestin significantly reduced the mitochondrial metabolic rate as assessed by MTT assay if 50μM were applied ($p<0.01$). The treatment of SKBR3 cells with 0.5μM or 5μM LDC3/Dynarrestin did not alter the mitochondrial metabolic rate (Fig 7B).

Mitosis-associated interaction of PTPIP51 remained unaffected by LDC3/Dynarrestin treatment

LDC3/Dynarrestin treatment was unable to affect the interaction profiles of PTPIP51 with two proteins involved in mitosis, namely CGI-99 and Nuf2, which are acknowledged interaction partners of PTPIP51 [23]. Neither the interaction of PTPIP51 and CGI-99 nor the interaction of PTPIP51 and Nuf2 displayed any significant variations after 24h of treatment with concentrations of 0.5μM up to 50μM LDC3/Dynarrestin treatment (S3A and S3B Fig).

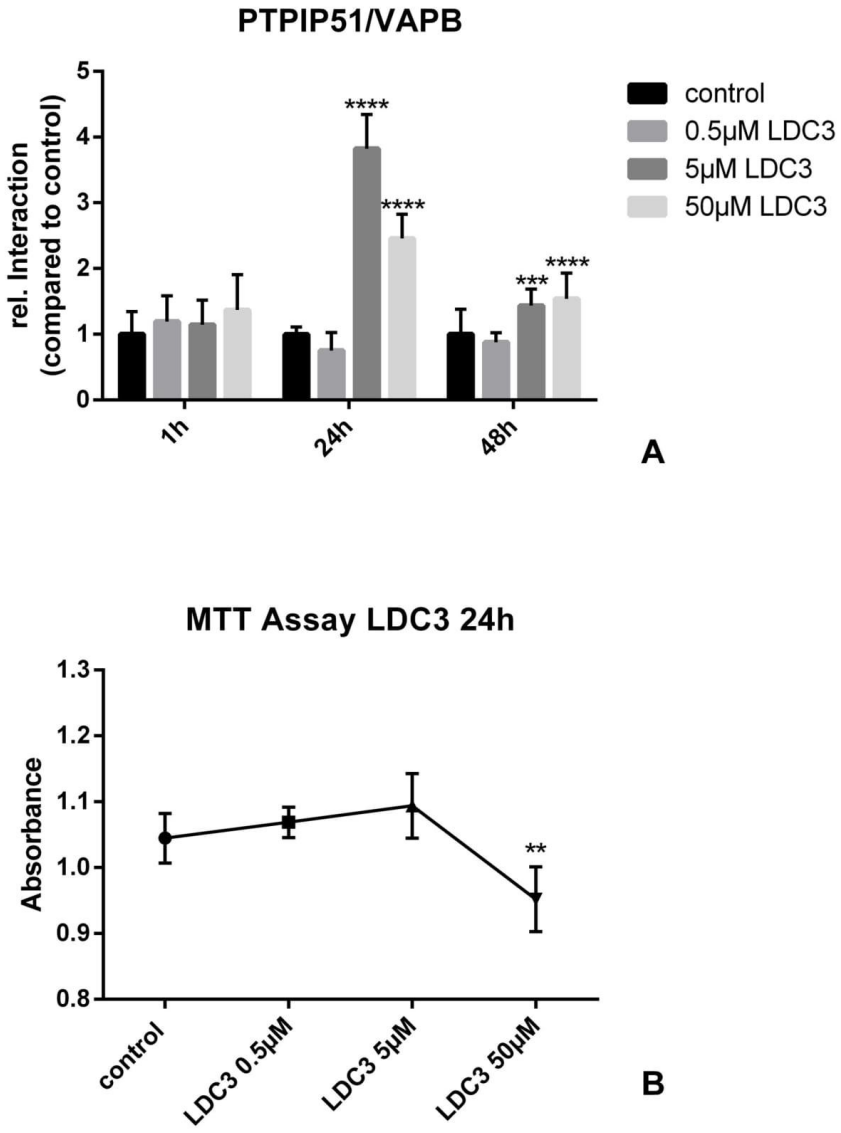

Fig 7. Interactions of PTPIP51 with different partners in untreated SKBR3 cells and cells treated with LDC3/Dynarrestin in concentrations of 0.5 µM, 5 µM, and 50 µM. (a) Interaction of PTPIP51 with VAPB, **(b)** Evaluation of the mitochondrial metabolic rate using the (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT)) assay. SKBR3 cells were treated for 24h with the indicated concentrations of LDC3/Dynarrestin.

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Interaction of PTPIP51 with ERβ

If incubated for 24h with LDC3/Dynarrestin the number of PTPIP51/ERβ interactions was significantly increased (S3C Fig). After an application of LDC3/Dynarrestin for 48h, no changes in the interactions at low concentrations (0.5μM and 5 μM) were observed. 50μM LDC3/Dynarrestin led to a highly significant decrease in the number of PTPIP51/ERβ interactions compared to the untreated controls ($p<0.0001$).

Discussion

Modulating the PTPIP51 interactome in a HER2 amplified breast cancer cell line (SKBR3) with the novel small molecule LDC3/Dynarrestin led to significant alterations of several tumor relevant pathways. We specifically selected the HER2 positive SKBR3 cell line due to the alterations in signaling caused by the amplified HER2 receptor. HER2 affects the MAPK pathway, Akt signaling, NFκB signaling and calcium homeostasis [24–26]. All these different signaling pathways contain signaling proteins which interact with PTPIP51 [9,19–21,23]. Therefore, the SKBR3 cell line represented a perfect model for the investigation of the LDC3/Dynarrestin affected PTPIP51 interactome. Previous work by Brobeil and coworkers characterized the interaction profile of PTPIP51 under LDC3/Dynarrestin treatment in the spontaneously immortalized HaCaT cell line, which represents a physiological model of cell signaling [7]. The comparison of the PTPIP51 interactome variations in the tumor cell line SKBR3 and the non-tumor cell line HaCaT allows a deeper insight into the dysregulated signaling structures in cancer cells.

LDC3/Dynarrestin inhibits cytoplasmic dynein activity in an ATP hydrolysis independent mode of action [6]. Brobeil and coworkers also showed that LDC3/Dynarrestin could directly affect the PTPIP51 related interactome in HaCat cells. Here, LDC3/Dynarrestin exerts its effects through a PTPIP51 dependent modulation of interactions, as shown by PTPIP51 knockdown experiments [7]. Since LDC3/Dynarrestin affects cytoplasmic Dynein and PTPIP51, we monitored the interaction of cytoplasmic Dynein and PTPIP51 via proximity ligation assay. LDC3/Dynarrestin affects the interaction in a dose-dependent manner. Nanomolar concentrations of LDC3/Dynarrestin reduced the interaction of cytoplasmic dynein and PTPIP51, whereas micromolar concentrations induced an enhancement of the interaction. Such biphasic regulations are already known for PTPIP51. Roger et al. described a biphasic expression peak during the treatment of rat retinal explants with ciliary neurotrophic factor [27]. Of note, these interaction changes were only seen after 24h incubation time. When incubated for 1h, LDC3/Dynarrestin did not significantly alter the interaction of PTPIP51 and cytoplasmic Dynein, implying that the regulation of the PTPIP51/cytoplasmic Dynein interaction could be due to secondary mechanisms. Whereas, the application of Ciliobrevin A, an ATP hydrolysis dependent cytoplasmic Dynein inhibitor, immediately exerts an effect on the interaction of PTPIP51 and cytoplasmic Dynein, when applied in high concentrations.

The regulation of PTPIP51 interactions is mediated through the modulation of its phosphorylation [9]. One of the main regulatory phosphorylation sites of PTPIP51 is the tyrosine 176 residue. Its phosphorylation annuls the ability of PTPIP51 to bind to Raf1 through 14.3.3 and thereby its MAPK pathway stimulating effect. LDC3/Dynarrestin treatment of SKBR3 cells led to a high Tyr176 phosphorylation level of PTPIP51. The same effects were observed in the HaCaT cell line.

Despite the augmented phosphorylation level of the Tyr176 residue, we observed recruitment of PTPIP51 into the MAPK signaling as indicated by the enhanced interaction of PTPIP51 with Raf1 and 14.3.3, respectively. Thus, LDC3/Dynarrestin abolishes the known phospho-regulations of PTPIP51 protein-protein-interactions and forces PTPIP51 into

MAPK signaling regardless of its phosphorylation. These observations are paralleled by the MAPK signaling activation under LDC3/Dynarrestin treatment. The findings are in accordance with the data obtained in the HaCaT cell line. Thus, the LDC3/Dynarrestin altered the phosphorylation of the Tyr176 residue and the recruitment of PTPIP51 into MAPK signaling are based on the same modulations both in the breast cancer cell as well as in the physiological system of the HaCaT cell.

Interestingly, inhibition of c-Src or Raf1, two upstream modulators of the MAPK signaling, in combination with low concentrations of LDC3/Dynarrestin reduced the activation of the MAPK signaling compared to control groups. This reduction of MAPK activation is reverted by high concentrations of LDC3/Dynarrestin implying, that LDC3/Dynarrestin is able to activate the MAPK signaling independently of c-Src and Raf1.

The phosphorylation of PTPIP51 is precisely regulated by different kinases and phosphatases. One of the Tyr176 phosphorylating kinases is the non-membrane tyrosine kinase c-Src. C-Src plays a crucial role in the formation of therapy resistance in HER2 amplified breast cancer. Interestingly, LDC3/Dynarrestin enhanced the phosphorylation of Tyr176 of PTPIP51 even under inhibition of c-Src.

PTP1B is mainly responsible for PTPIP51 dephosphorylation. PTP1B is a known positive modulator of the HER2 receptor. Therefore, both PTPIP51 regulating enzymes are potential tumorigenic factors, which presumably exert their effects through modulation of PTPIP51. This thesis is supported by the fact that in LDC3/Dynarrestin treated SKBR3 cells the interaction of PTPIP51 with c-Src and PTP1B is regulated exactly in an opposite way compared to the regulation in HaCaT cells. Interestingly, the inhibition of PTP1B induced results completely opposite of the known regulation. The inhibition of PTP1B in combination with the application with rising concentrations of LDC3/Dynarrestin reduced the phosphorylation of Tyr176 of PTPIP51. This further supports the thesis of a complete disruption of the known phosphoregulation of PTPIP51 by LDC3/Dynarrestin.

Since both PTPIP51 phosphorylation regulating enzymes also modulate the HER2 receptor, we also monitored the interaction of PTPIP51 and HER2. Of note, the application of LDC3/Dynarrestin did not alter the interaction of PTPIP51 and HER2, when applied for 1h. After 24h LDC3/Dynarrestin enhanced the interaction of PTPIP51 and the HER2 receptor. In accordance to the observed interaction changes of PTPIP51/c-Src and PTPIP51/cytoplasmic Dynein, this regulation could be due to secondary mechanisms [28].

TNFα, an activator of the transcription regulator NFκB is highly expressed in breast carcinomas [29]. Moreover, TNFα leads to an augmented expression of prooncogenic factors correlating with poor clinical outcome [30]. Recently, PTPIP51 was identified as a novel partner in NFkB signaling [19]. Strikingly, lower LDC3/Dynarrestin concentrations significantly augmented the interaction of PTPIP51/RelA. The TNFα treatment of SKBR3 cells led to the dissolution of the RelA/IκB/PTPIP51 complex. This effect was already stated for HaCaT cells [19]. The simultaneous application of LDC3/Dynarrestin in rising concentrations completely abolished the TNFα effect. Noteworthy PDTC, an NFκB inhibitor, did not alter the interactions of PTPIP51 neither with RelA nor with IκB but canceled the LDC3/Dynarrestin effect [31]. These results strongly favor the thesis that LDC3/Dynarrestin does not directly influence the RelA/IκB/PTPIP51 interactome but inhibits the activation on the level of IKKs or even upstream. A potential mechanism for the IKK inhibition could be the inhibition of Akt by LDC3/Dynarrestin. Our results showed an inhibition of Akt signaling after 1h and 6h incubation time. Akt is an IKK activator [32]. Therefore, the Akt inhibition might lead to a reduced IKK activity, which ultimately allows the RelA/IκB/PTPIP51 complex to stabilize.

Interestingly, in the SKBR3 cells, LDC3/Dynarrestin induced the same enhancement of PTPIP51/RelA interaction as seen in the HaCaT cells. LDC3/Dynarrestin altered the

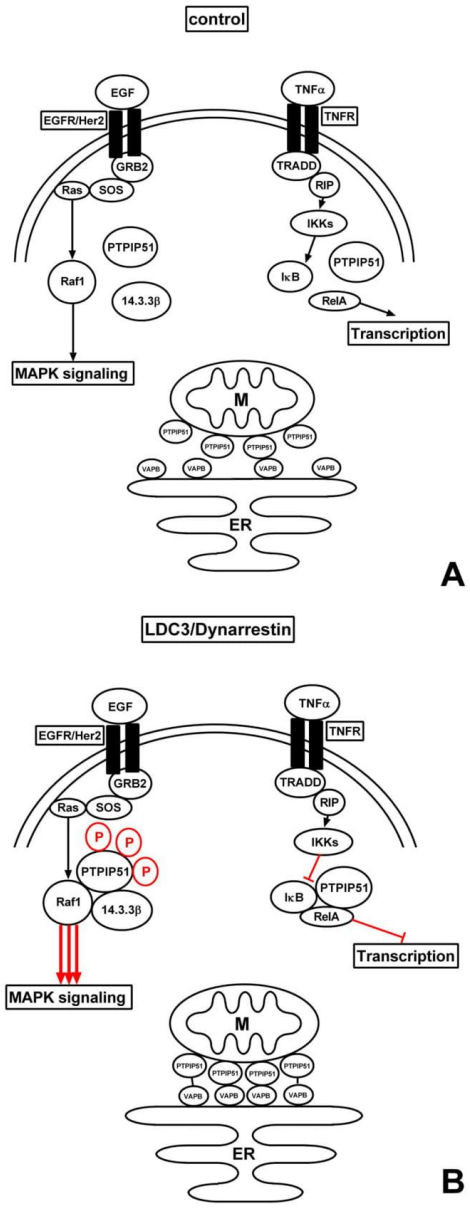

Fig 8. Schematic overview of main signaling pathways and their crosstalks. (a) Signaling without LDC3/Dynarrestin, **(b)** Signaling under the influence of LDC3/Dynarrestin.

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regulation of the Tyr176 residue phosphorylation, the interaction with MAPK signaling and the link to NFκB signaling via RelA in breast cancer cells and in non-tumor HaCat cells all analogously. This might be a hint for the absence of a dysregulated PTPIP51 regulation in this pathway within the breast cancer cell line.

Besides the MAPK pathway and the NFκB signaling, PTPIP51 is also involved in the formation of the MAMs via the interaction with VAPB. VAPB is highly expressed in many breast tumors [33]. Our HER2 overexpressing model cell line (SKBR3) also displayed high expression of VAPB (S2 Fig). The increased interaction of PTPIP51 and VAPB presumably leads to a more stabilized endoplasmatic reticulum-mitochondria association [20,21]. As mentioned above for several interactions, this regulation is only seen for longer incubation times, implying that the enhanced interaction of PTPIP51 and VAPB might be caused by secondary mechanisms and not directly by LDC3/Dynarrestin. The enhanced interaction of PTPIP51 and VAPB might be a counterregulation to the mitochondrial metabolic stress induced by high concentrations of LDC3/Dynarrestin, but further studies would be needed to proof this thesis.

Interestingly, all the PTPIP51/VAPB interactions are is exactly altered contrary to that seen in HaCaT cells. This may indicate a modified regulation of the MAM associated interactome of PTPIP51 in HER2 positive breast cancer cells. MAM related PTPIP51 interactome in neuronal cancer cells seem to underlie similar mechanisms as seen in preliminary results of an ongoing study. The regulation of these organelle communication sites is of utmost interest since recent findings indicate a crucial role of the MAMs in the regulation of tumorigenesis and tumor cell growth [25].

Conclusions

LDC3/Dynarrestin induces alterations in all known PTPIP51 related signaling pathways. (1) LDC3/Dynarrestin enhances the phosphorylation of Tyr176 of PTPIP51. (2) Despite the high Tyr176 phosphorylation, PTPIP51 is forced into the Raf1/14.3.3β/PTPIP51 complex by LDC3/Dynarrestin. (3) LDC3/Dynarrestin leads to an activation of the MAPK pathway independent of c-Src and Raf1 activity. (4) LDC3/Dynarrestin modulates the NFκB related interactions of PTPIP51. (5) LDC3/Dynarrestin affects the MAM-related interaction of PTPIP51 and VAPB (Fig 8). In conclusion, LDC3/Dynarrestin represents a valuable tool compound for the modulation of PTPIP51 related signaling pathways in physiological and pathologic signaling systems.

Supporting information

S1 Fig. Interaction of PTPIP51 with RelA. Cells were treated in the indicated concentrations of LDC3/Dynarrestin for 1h and 24h.
(TIF)

S2 Fig. Immunocytochemical staining of VAPB of SKBR3 cells. (a) VAPB distribution in untreated SKBR3 cells, **(b)** negative control. Bar = 50μm.
(TIF)

S3 Fig. Interactions of PTPIP51 with different partners in untreated SKBR3 cells and cells treated with LDC3/Dynarrestin in concentrations of 0.5 μM, 5 μM, and 50 μM. (a) Interaction of PTPIP51 with CGI-99, (b) Interaction of PTPIP51 with NUF-2, (c) Interaction of PTPIP51 with ERβ.
(TIF)

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Writing – original draft: Eric Dietel.

Writing – review & editing: Alexander Brobeil, Stefan Gattenlöhner, Monika Wimmer.

References

1. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science. 1987; 235: 177–182. PMID: 3798106
2. Moasser MM. The oncogene HER2: its signaling and transforming functions and its role in human cancer pathogenesis. Oncogene. 2007; 26: 6469–6487. https://doi.org/10.1038/sj.onc.1210477 PMID: 17471238
3. dM Gagliato D Jardim DLF, Marchesi MSP Hortobagyi GN. Mechanisms of resistance and sensitivity to anti-HER2 therapies in HER2+ breast cancer. Oncotarget. 2016. https://doi.org/10.18632/oncotarget.7043 PMID: 26824988
4. Baselga J, Tripathy D, Mendelsohn J, Baughman S, Benz CC, Dantis L, et al. Phase II study of weekly intravenous trastuzumab (Herceptin) in patients with HER2/neu-overexpressing metastatic breast cancer. Semin Oncol. 1999; 26: 78–83.
5. Marty M, Cognetti F, Maraninchi D, Snyder R, Mauriac L, Tubiana-Hulin M, et al. Randomized phase II trial of the efficacy and safety of trastuzumab combined with docetaxel in patients with human epidermal growth factor receptor 2-positive metastatic breast cancer administered as first-line treatment: the M77001 study group. J Clin Oncol. 2005; 23: 4265–4274. https://doi.org/10.1200/JCO.2005.04.173 PMID: 15911866
6. Höing S, Yeh T-Y, Baumann M, Martinez NE, Habenberger P, Kremer L, et al. Dynarrestin, a Novel Inhibitor of Cytoplasmic Dynein. Cell Chem Biol. 2018. https://doi.org/10.1016/j.chembiol.2017.12.014 PMID: 29396292
7. Brobeil A, Chehab R, Dietel E, Gattenlöhner S, Wimmer M. Altered Protein Interactions of the Endogenous Interactome of PTPIP51 towards MAPK Signaling. Biomolecules. 2017; 7. https://doi.org/10.3390/biom7030055 PMID: 28754031
8. Tonks NK, Muthuswamy SK. A brake becomes an accelerator: PTP1B—a new therapeutic target for breast cancer. Cancer Cell. 2007; 11: 214–216. https://doi.org/10.1016/j.ccr.2007.02.022 PMID: 17349579
9. Brobeil A, Bobrich M, Tag C, Wimmer M. PTPIP51 in protein interactions: regulation and in situ interacting partners. Cell Biochem Biophys. 2012; 63: 211–222. https://doi.org/10.1007/s12013-012-9357-y PMID: 22544307
10. Brobeil A, Koch P, Eiber M, Tag C, Wimmer M. The known interactome of PTPIP51 in HaCaT cells—inhibition of kinases and receptors. Int J Biochem Cell Biol. 2013. https://doi.org/10.1016/j.biocel.2013.10.007
11. Yu C, Han W, Shi T, Lv B, He Q, Zhang Y, et al. PTPIP51, a novel 14-3-3 binding protein, regulates cell morphology and motility via Raf-ERK pathway. Cell Signal. 2008; 20: 2208–2220. https://doi.org/10.1016/j.cellsig.2008.07.020 PMID: 18771726
12. Koch P, Stenzinger A, Viard M, Marker D, Mayser P, Nilles M, et al. The novel protein PTPIP51 is expressed in human keratinocyte carcinomas and their surrounding stroma. J Cell Mol Med. 2008; 12: 2083–2095. https://doi.org/10.1111/j.1582-4934.2008.00198.x PMID: 19012732
13. Petri MK, Brobeil A, Planz J, Bräuninger A, Gattenlöhner S, Nestler U, et al. PTPIP51 levels in glioblastoma cells depend on inhibition of the EGF-receptor. J Neurooncol. 2015; 123: 15–25. https://doi.org/10.1007/s11060-015-1763-8 PMID: 25862004
14. Koch P, Petri M, Paradowska A, Stenzinger A, Sturm K, Steger K, et al. PTPIP51 mRNA and protein expression in tissue microarrays and promoter methylation of benign prostate hyperplasia and prostate carcinoma. Prostate. 2009; 69: 1751–1762. https://doi.org/10.1002/pros.21025 PMID: 19691131

15. Barop J, Sauer H, Steger K, Wimmer M. Differentiation-dependent PTPIP51 expression in human skeletal muscle cell culture. J Histochem Cytochem. 2009; 57: 425–435. https://doi.org/10.1369/jhc.2008.952846 PMID: 19124842

16. Stenzinger A, Schreiner D, Pfeiffer T, Tag C, Hofer HW, Wimmer M. Epidermal growth factor-, transforming growth factor-beta-, retinoic acid- and 1,25-dihydroxyvitamin D3-regulated expression of the novel protein PTPIP51 in keratinocytes. Cells Tissues Organs. 2006; 184: 76–87. https://doi.org/10.1159/000098949 PMID: 17361080

17. Orieux G, Slembrouck A, Bensaid M, Sahel J-A, Goureau O. The protein tyrosine phosphatase interacting protein 51 (PTPIP51) is required for the differentiation of photoreceptors. Neuroscience. 2015; 300: 276–285. https://doi.org/10.1016/j.neuroscience.2015.05.028 PMID: 25999297

18. Lv BF, Yu CF, Chen YY, Lu Y, Guo JH, Song QS, et al. Protein tyrosine phosphatase interacting protein 51 (PTPIP51) is a novel mitochondria protein with an N-terminal mitochondrial targeting sequence and induces apoptosis. Apoptosis. 2006; 11: 1489–1501. https://doi.org/10.1007/s10495-006-8882-9 PMID: 16820967

19. Brobeil A, Kammerer F, Tag C, Steger K, Gattenlohner S, Wimmer M. PTPIP51-A New RelA-tionship with the NFkappaB Signaling Pathway. Biomolecules. 2015; 5: 485–504. https://doi.org/10.3390/biom5020485 PMID: 25893721

20. de Vos KJ, Morotz GM, Stoica R, Tudor EL, Lau K-F, Ackerley S, et al. VAPB interacts with the mitochondrial protein PTPIP51 to regulate calcium homeostasis. Hum Mol Genet. 2012; 21: 1299–1311. https://doi.org/10.1093/hmg/ddr559 PMID: 22131369

21. Stoica R, de Vos KJ, Paillusson S, Mueller S, Sancho RM, Lau K-F, et al. ER-mitochondria associations are regulated by the VAPB-PTPIP51 interaction and are disrupted by ALS/FTD-associated TDP-43. Nat Commun. 2014; 5: 3996. https://doi.org/10.1038/ncomms4996 PMID: 24893131

22. Leuchowius K-J, Jarvius M, Wickstrom M, Rickardson L, Landegren U, Larsson R, et al. High content screening for inhibitors of protein interactions and post-translational modifications in primary cells by proximity ligation. Mol Cell Proteomics. 2010; 9: 178–183. https://doi.org/10.1074/mcp.M900331-MCP200 PMID: 19864249

23. Brobeil A, Graf M, Eiber M, Wimmer M. Interaction of PTPIP51 with Tubulin, CGI-99 and Nuf2 During Cell Cycle Progression. Biomolecules. 2012; 2: 122–142. https://doi.org/10.3390/biom2010122 PMID: 24970130

24. Graus-Porta D, Beerli RR, Hynes NE. Single-chain antibody-mediated intracellular retention of ErbB-2 impairs Neu differentiation factor and epidermal growth factor signaling. Mol Cell Biol. 1995; 15: 1182–1191. PMID: 7532277

25. Herrera-Cruz MS, Simmen T. Cancer: Untethering Mitochondria from the Endoplasmic Reticulum. Front Oncol. 2017; 7: 105. https://doi.org/10.3389/fonc.2017.00105 PMID: 28603693

26. Merkhofer EC, Cogswell P, Baldwin AS. Her2 activates NF-kappaB and induces invasion through the canonical pathway involving IKKalpha. Oncogene. 2010; 29: 1238–1248. https://doi.org/10.1038/onc.2009.410 PMID: 19946332

27. Roger J, Goureau O, Sahel J-A, Guillonneau X. Use of suppression subtractive hybridization to identify genes regulated by ciliary neurotrophic factor in postnatal retinal explants. Mol Vis. 2007; 13: 206–219. PMID: 17327826

28. Bailey ST, Miron PL, Choi YJ, Kochupurakkal B, Maulik G, Rodig SJ, et al. NF-kappaB activation-induced anti-apoptosis renders HER2-positive cells drug resistant and accelerates tumor growth. Mol Cancer Res. 2014; 12: 408–420. https://doi.org/10.1158/1541-7786.MCR-13-0206-T PMID: 24319068

29. Kamel M, Shouman S, El-Merzebany M, Kilic G, Veenstra T, Saeed M, et al. Effect of tumour necrosis factor-alpha on estrogen metabolic pathways in breast cancer cells. J Cancer. 2012; 3: 310–321. https://doi.org/10.7150/jca.4584 PMID: 22866165

30. Jin L, Wessely O, Marcusson EG, Ivan C, Calin GA, Alahari SK. Prooncogenic factors miR-23b and miR-27b are regulated by Her2/Neu, EGF, and TNF-alpha in breast cancer. Cancer Res. 2013; 73: 2884–2896. https://doi.org/10.1158/0008-5472.CAN-12-2162 PMID: 23338610

31. Liu SF, Ye X, Malik AB. Inhibition of NF-kappaB activation by pyrrolidine dithiocarbamate prevents In vivo expression of proinflammatory genes. Circulation. 1999; 100: 1330–1337. PMID: 10491379

32. Bai D, Ueno L, Vogt PK. Akt-mediated regulation of NFkappaB and the essentialness of NFkappaB for the oncogenicity of PI3K and Akt. Int J Cancer. 2009; 125: 2863–2870. https://doi.org/10.1002/ijc.24748 PMID: 19609947

33. Rao M, Song W, Jiang A, Shyr Y, Lev S, Greenstein D, et al. VAMP-associated protein B (VAPB) promotes breast tumor growth by modulation of Akt activity. PLoS One. 2012; 7: e46281. https://doi.org/10.1371/journal.pone.0046281 PMID: 23049696

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PTPIP51 crosslinks the NFκB signaling and the MAPK pathway in SKBR3 cells

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Aim: PTPIP51 interacts with NFκB signaling at the RelA and IκB level. NFκB signaling is linked to the initiation, progression and metastasis of breast cancer. Her2-amplified breast cancer cells frequently display activation of the NFκB signaling. We aimed to clarify the effects of NFκB inhibition on the NFκB- and MAPK-related interactome of PTPIP51 and cell viability in HaCat cells and SKBR3 cells. **Results:** IKK-16 selectively reduced cell viability in SKBR3 cells. PDTC induced a formation of the Raf1/14-3-3/PTPIP51 complex in SKBR3 cells, indicating a shift of PTPIP51 into MAPK signaling. **Conclusion:** IKK-16 selectively inhibits cell viability of SKBR3 cells. In addition, PTPIP51 might serve as the mediator between NFκB signaling and the MAPK pathway in SKBR3.

Lay abstract: Breast cancer is the most common cancerous disease among women. Prognosis and therapy of breast cancer depends on the expression of hormone and surface receptors such as Her2, which promote tumor growth and invasion via activation of downstream signaling pathways. NFκB signaling represents a downstream signaling pathway that can be activated by Her2. In this study, we demonstrated that inhibition of NFκB signaling with IKK-16 reduces cell viability in breast cancer cells with amplified Her2. Furthermore, we identified PTPIP51 as a potential mediator of crosstalks between the MAPK pathway and NFκB signaling. This signaling pathway could therefore be a target for future drug development.

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Keywords: breast cancer • Her2 • IKK-16 • NFκB • PDTC • protein–protein interactions • PTPIP51

The body of evidence stating the importance of NFκB signaling in the initiation, progression and metastasis of several tumor entities is steadily growing [1–4]. Alterations in NFκB signaling can be the consequence of direct mutations of signaling molecules belonging to the NFκB signaling cascade, stimulation of signaling via the inflammatory tumor microenvironment or crosstalk between NFκB signaling and other dysregulated signaling pathways [5–8].

The amplification and overactivation of the Her2 receptor in breast cancer represents a perfect example of the activation of NFκB signaling via the crosstalk of different signaling pathways [8]. About 20–30% of all breast cancers exhibit amplification of the Her2 receptor, accompanied by more aggressive tumor growth and reduced overall survival [9,10]. The Her2 receptor mainly activates two signaling pathways: the MAPK pathway and Akt signaling [9]. Besides these two pathways, Her2 is also capable of activating IKKs [8]. IKKs are essential for the activation of the NFκB signaling cascade via phosphorylation of IκB. Phosphorylation tags IκB for ubiquitinylation and thus triggers its degradation. After the degradation of IκB, the nuclear localization signal of RelA is exposed. Consequently, RelA can exert its transcriptional activity [11,12]. This Her2-induced NFκB activation contributes to the growth of the tumor, the development of therapy resistance and the epithelial–mesenchymal transition, which represents a hallmark in the formation of metastasis [4,8].

It is noteworthy that the scaffold protein, protein tyrosine phosphatase interacting protein 51 (PTPIP51), interacts with both signaling structures – the Her2 receptor and NFκB signaling [13,14]. The interaction of PTPIP51 with the Her2 receptor seems crucial for the sensitivity of Her2-amplified breast cancer cell lines to EGFR/Her2-targeted

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therapies [14]. Besides the direct interaction with the Her2 receptor, PTPIP51 is involved in the titration of the MAPK signaling [15–17]. Within this pathway, PTPIP51 exerts an activating effect via the binding of Raf1 and 14-3-3 [16]. The formation of the PTPIP51/14-3-3/Raf1 complex induces an activation of ERK1/2, thus an activation of MAPK signaling [15]. The formation of the Raf1/14-3-3/PTPIP51 complex is strictly regulated by the phosphorylation of PTPIP51. Phosphorylation of tyrosine 176 leads to a dissolution of the complex and an omission of the MAPK pathway-stimulating effect. In contrast, the phosphorylation of serine 212 enhances the formation of the ternary complex [15,17,18]. Both phosphorylation sites are under the control of several kinases, including receptor tyrosine kinases (e.g., the EGFR) and nonreceptor kinases (e.g., c-Src) and phosphatases [15,17,18].

The regulation of PTPIP51 in NFκB signaling contradicts the observations made in the MAPK pathway. Here, the formation of the RelA/IκB/PTPIP51 complex inhibits the NFκB signaling [13]. Due to the recency of our knowledge of PTPIP51 function in NFκB signaling, the critical phosphorylation sites, which regulate the binding of PTPIP51 with RelA and IκB, are unknown. Brobei and coworkers showed that stimulation of HaCat cells with TNFα induces a disintegration of the PTPIP51/IκB/RelA complex. Vice versa, inhibition of NFκB signaling led to a formation of the PTPIP51/IκB/RelA complex [13].

Based on these findings, this study aimed to elucidate the interaction shifts of PTPIP51 upon NFκB inhibition in NFκB signaling and their effects on the MAPK pathway using the Duolink proximity ligation assay. NFκB signaling inhibition was performed using pyrrolidine dithiocarbamate (PDTC) and IKK-16, respectively. PDTC was thought to act as an antioxidant and thereby inhibit TNFα-induced NFκB activation. Hayakawa and coworkers showed that PDTC could inhibit ubiquitin ligase activity in a cell-free system, which lacks reactive oxygen species [19]. Thus, the antioxidative properties of PDTC are not needed for the inhibition of NFκB signaling [19,20]. IKK-16 acts as a small molecule inhibitor of IKK1, IKK2 and the IKK complex [21]. Through the inhibition of these serine/threonine kinases, the phosphorylation of IκB is not possible [12] Subsequently, IκB cannot be degraded and RelA cannot exert its transcriptional activity [12]. The impact of the applied agents on cell survival was analyzed by MTT assays. Thus, we were able to describe differential regulations in the Her2-amplified breast cancer cell line SKBR3 and the nontumor keratinocyte cell line HaCat.

Material & methods

Cell culture

SKBR3 cells were purchased from Cell Line Service (Eppelheim, Germany). The cells were cultured in Dulbecco's MEM (Biochrom, Berlin, Germany) containing 10% fetal calf serum and 1% penicillin/streptomycin in a humidified chamber at 37°C and 5% CO_2. The medium renewal was performed every 2–3 days. Cell harvesting was performed at a confluence of 70–80% with Accutase. The SKBR3 cells were seeded in culture slides (30,000 cells per well; Falcon CultureSlides, Corning Life Science, NY, USA, Cat.# 354108) or used as indicated for other experiments.

The HaCaT cells were obtained and handled as described in previous publications of our group [13]. Cells were harvested with Trypsin in a humidified chamber at 37°C and 5% CO_2. Subsequently, the cells were seeded on culture slides (Falcon CultureSlides, Corning Life Science, Cat.# 354108) or 96-well plates (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany, Cat.# CLS3340).

Treatment

The cells were allowed to grow for 24 h after seeding. Subsequently, they were treated with different concentrations of ammonium pyrrolidine dithiocarbamate (PDTC) (Sigma-Aldrich, Cat.# P 8765, Munich, Germany) or IKK-16 (Cat.# S2882, Selleckchem, Munich, Germany) (diluted in culture medium) for either 6 or 24 h. The reaction was stopped by removal of medium and addition of ice-cold phosphate-buffered saline. The fixation was performed with ice-cold methanol for proximity ligation assays. The procedure for the MTT assays is described in the MTT subsection.

Antibodies

All antibodies used are listed in Supplementary Table 1.

Duolink proximity ligation assay

For evaluation of the interactions of proteins, the Olink Duolink proximity ligation assay (PLA probe anti-rabbit minus, Cat.# DUO92005, PLA probe anti-mouse plus, Cat.# DUO92001, anti-goat plus Cat.# DUO92003,

Detection Kit Orange, Cat.# DUO92007, Sigma-Aldrich Chemie GmbH) was used. The assay was carried out according to the manufacturer manual. Leuchowius and coworkers identified the Duolink proximity ligation assay as an adequate tool for the identification of small-molecule effectors for protein–protein interactions [22].

Fluorescence microscopy

The photo documentation was performed with an Axioplan 2 fluorescence microscope equipped with Plan-Apochromat objectives (Carl Zeiss Jena, Jena, Germany).

Protein interaction analysis

Quantification was carried out using the DuoLink Image Tool (Olink Bioscience, Uppsala, Sweden, v1.0.1.2). The software identifies Dapi-positive nuclei and counts fluorescence dots in a user-defined cell diameter preset. For each indicated concentration, at least 100 single cells were analyzed in three independent experiments.

MTT assay

HaCat and SKBR3 cells were seeded at a density of 10,000 cells per well in a 96-well plate. The cells were allowed to grow for 24 h. Cells were treated as indicated. MTT solution was added 4 h before the end of the incubation time. Formazan crystals were solubilized using a solubilization solution (10% SDS in 0.01M HCl). The solution of the crystals was performed overnight in a humidified chamber at 37°C and 5% CO_2. Assays were evaluated with the Berthold Tech TriStar ELISA Reader (Bad Wildbad, Germany). The assays were performed in quintuplicates.

Statistical analysis

Data were evaluated using GraphPad Prism 6 software. Statistical significance was determined using ANOVA, followed by Dunnett's multiple comparison tests. Results were considered significant with $p < 0.05$. (*[$p < 0.05$]; **[$p < 0.01$]; ***[$p < 0.001$]; ****[$p < 0.0001$]).

Results

All experiments were performed with the SKBR3 cell line, an Her2-amplified breast cancer cell line and the spontaneously immortalized keratinocyte HaCat cell line. This setting allows comparison of the effects of NFκB inhibition on the malignantly transformed signaling system in the SKBR3 cell line with the normal signaling in the HaCat cell line.

Inhibition of NFκB signaling with PDTC or IKK-16 leads to differential regulations of the cell viability in SKBR3 cells & HaCat cells

The effects of NFκB inhibition by the application of PDTC and IKK-16, respectively, were monitored using the MTT assay, which measures cell viability through the formation of formazan crystals. The results were equalized/related to the control value equaling 1. Each experiment was performed using the same dilution of DMSO to exclude the cytotoxic effects of DMSO.

Applying PDTC in increasing concentrations to SKBR3 cells resulted in a significant decrease of the cell viability (0.5 μM $p < 0.05$; 5 μM $p < 0.0001$; 50 μM $p < 0.0001$). Comparable results were seen for the inhibition of the NFκB signaling using PDTC in the HaCat cell line (5 μM $p < 0.0001$; 50 μM $p < 0.0001$). The application of IKK-16 induced differing results. The treatment of SKBR3 cells with rising concentrations of IKK-16 resulted in a highly significant decrease in cell viability (5 μM $p < 0.0001$; 50 μM $p < 0.0001$). In contrast, applying IKK-16 to HaCat cells led to a slight but significant increase in cell viability for the application of 5 μM ($p < 0.05$). Increasing the concentration resulted in a highly significant decrease in cell viability ($p < 0.0001$; Figure 1).

NFκB inhibition in SKBR3 cells & HaCat cells induced interaction shifts of the RelA/IκB/PTPIP51 complex

The formation of the RelA/IκB/PTPIP51 complex is essential for the titration of the NFκB signaling [13]. To monitor the interaction shifts of the RelA/IκB/PTPIP51 interactome, Duolink proximity ligation assays were performed. Interestingly, the regulation of the RelA/PTPIP51 interaction varied relative to the applied NFκB inhibitor and the used cell line. Of note, the application of 50 μM IKK-16 to the SKBR3 cell line severely diminished the seeded cell population leaving only cell debris. Thus, an adequate evaluation of the Duolink proximity ligation assays for this setting was not possible.

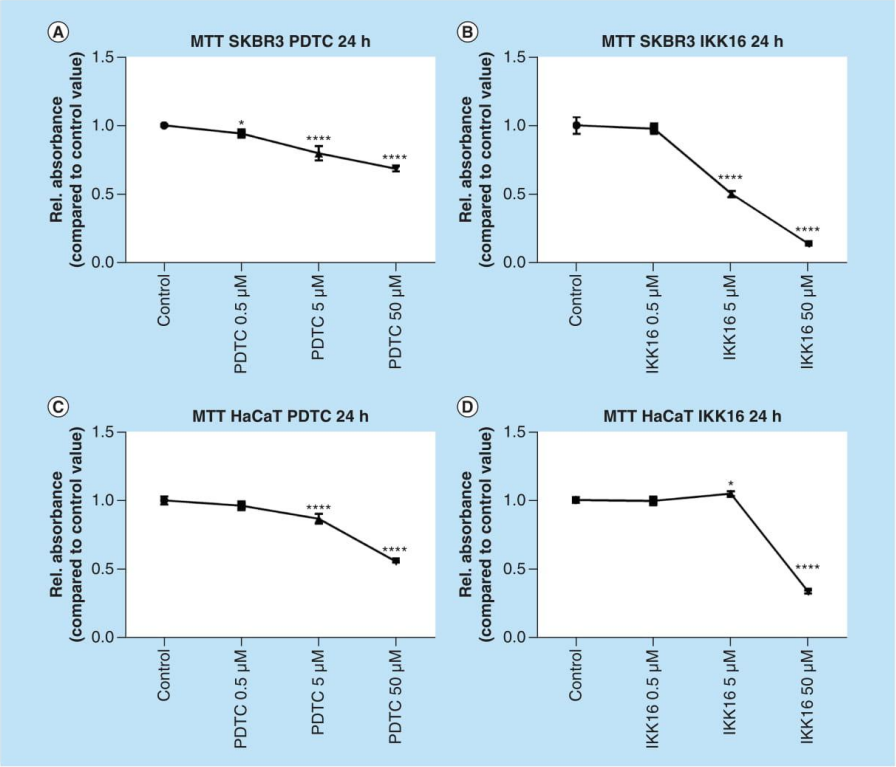

Figure 1. Cell viability of SKBR3 cells and HaCat cells treated with PDTC in concentrations of 0.5, 5 and 50 µM and with IKK-16 in concentrations of 0.5, 5 and 50 µM. (A) SKBR3 cells treated with the indicated concentrations of PDTC for 24 h. **(B)** SKBR3 cells treated with the indicated concentrations of IKK-16 for 24 h. **(C)** HaCat cells treated with the indicated concentrations of PDTC for 24 h. **(D)** HaCat cells treated with the indicated concentrations of IKK-16 for 24 h. The graphs show the mean value and standard deviation.
*$p < 0.05$; ****$p < 0.0001$.

Application of PDTC to SKBR3 cells induced a significant increase of the RelA/PTPIP51 interaction for the lowest and highest concentration used in this study (0.5 µM $p < 0.01$; 50 µM $p < 0.05$). In contrast, HaCat cells submitted to the same agent displayed a highly significant reduction in RelA/PTPIP51 interactions (0.5 µM $p < 0.001$; $p < 0.05$). All applied concentrations of IKK-16 significantly reduced the RelA/PTPIP51 interaction (0.5 µM $p < 0.01$; 5 µM $p < 0.05$) in the breast cancer cell line SKBR3. On the contrary, the application of IKK-16 to HaCat cells enhanced the interaction of RelA and PTPIP51 for the highest tested concentration (50 µM $p < 0.05$). The regulation of the RelA/IκB/PTPIP51 complex was further evaluated by monitoring the interaction of PTPIP51 and IκB in SKBR3 cells. None of the tested PDTC concentrations affected the interaction of PTPIP51 and IκB. In contrast, treatment with IKK-16 led to a significant reduction of PTPIP51/IκB interaction (0.5 µM $p < 0.05$; Figure 2).

Selective IKK inhibition by IKK-16 enhances the interaction of PTPIP51 & the Her2 receptor

The amplified Her2 receptor activates the NFκB signaling via the canonical pathway and the activation of IKKα [8]. PTPIP51 interacts with the Her2 receptor and seems to be crucial for the responsiveness of Her2 amplified breast cancer cells toward Her2 targeted therapies [14]. Thus, we examined the interaction of PTPIP51 and the Her2

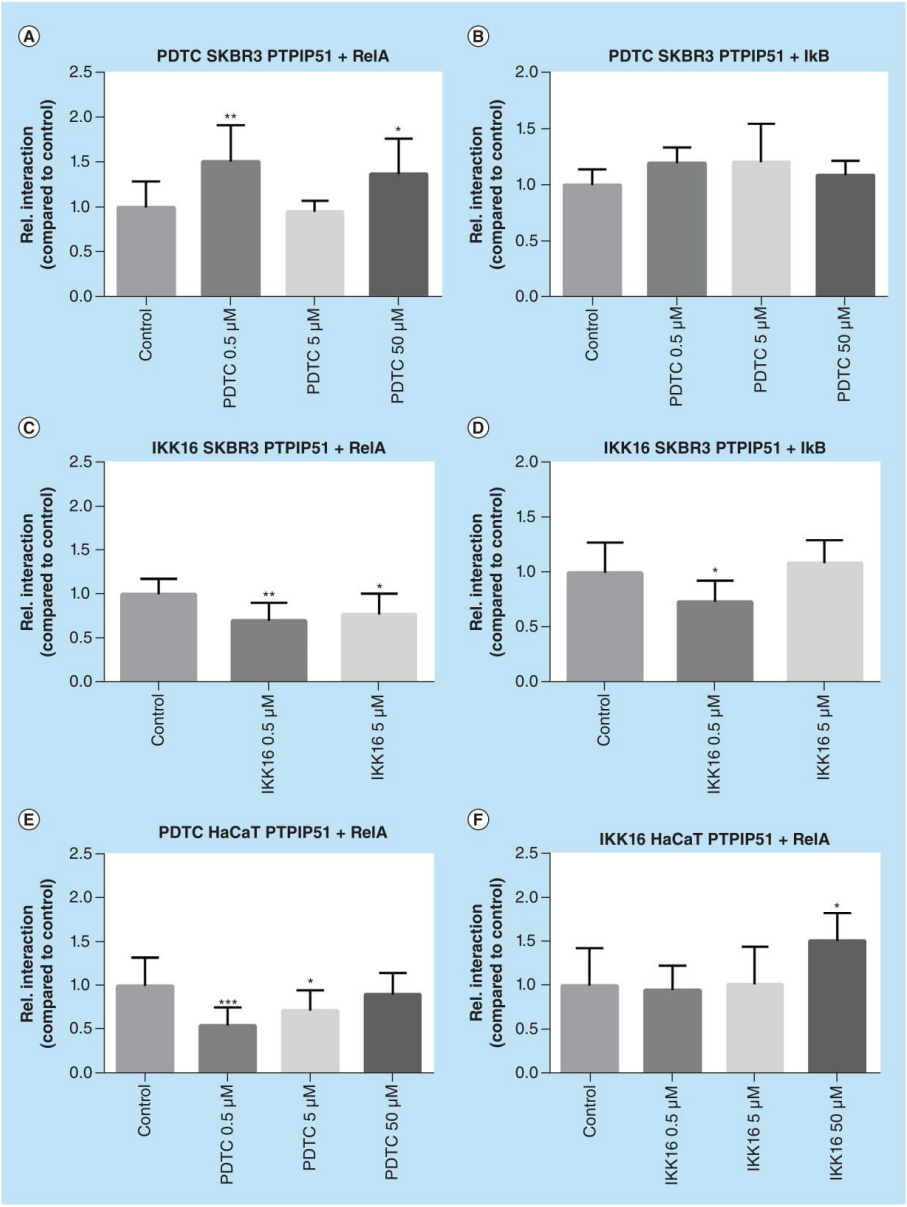

Figure 2. NFκB-related interactome of PTPIP51 in SKBR3 cells and HaCat treated with PDTC in concentrations of 0.5, 5 and 50 µM and with IKK-16 in concentrations of 0.5 and 5 µM. (A) Interaction of PTPIP51 and RelA in SKBR3 cells treated with PDTC in the indicated concentrations for 6 h. **(B)** Interaction of PTPIP51 and IκB in SKBR3 cells treated with PDTC in the indicated concentrations for 6 h. **(C)** Interaction of PTPIP51 and RelA in SKBR3 cells treated with IKK-16 in the indicated concentrations for 6 h. **(D)** Interaction of PTPIP51 and IκB in SKBR3 cells treated with IKK-16 in the indicated concentrations for 6 h. **(E)** Interaction of PTPIP51 and RelA in HaCat cells treated with PDTC in the indicated concentrations for 6 h. **(F)** Interaction of PTPIP51 and RelA in HaCat cells treated with IKK-16 in concentrations of 0.5, 5 and 50 µM for 6 h. The graphs show the mean value and standard deviation.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

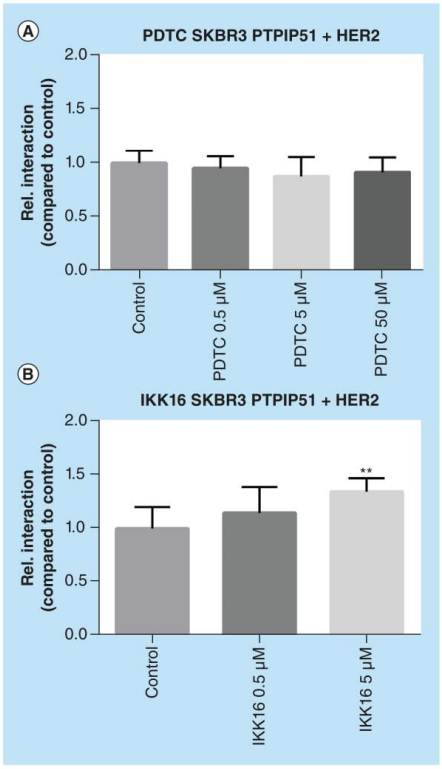

Figure 3. Interaction of PTPIP51 and the Her2 receptor in SKBR3 cells treated with PDTC (0.5, 5 and 50 µM) and IKK-16 (0.5 and 5 µM). (A) Interaction of PTPIP51 and the Her2 receptor in SKBR3 cells treated with PDTC in the indicated concentrations for 6 h. **(B)** Interaction of PTPIP51 and the Her2 receptor in SKBR3 cells treated with IKK-16 in the indicated concentrations for 6 h. The graphs show the mean value and standard deviation. **$p < 0.01$.

receptor under NFκB inhibition. The application of PDTC to SKBR3 cells for 6 h did not affect the interaction of PTPIP51 and Her2. In contrast, the IKK-16 treatment of SKBR3 cells significantly enhanced the interaction of PTPIP51 and the Her2 receptor (5 µM $p < 0.01$; Figure 3).

Inhibition of NFκB signaling induced interaction shifts in the MAPK-related PTPIP51 interactome

Besides the activation of NFκB signaling, activation of the Her2 receptor is mainly channeled to the activation of the MAPK pathway, especially ERK signaling [9]. Furthermore, Brobeil and coworkers identified PTPIP51 as a crosslink between the NFκB signaling and the MAPK pathway [13]. Therefore, we examined the influence of NFκB inhibition on the MAPK-related interactome of PTPIP51. Application of PDTC to SKBR3 cells resulted in a highly significant increase of PTPIP51/14-3-3 and PTPIP51/Raf1 interaction (PTPIP51/14-3-3 5 µM $p < 0.0001$; 50 µM $p < 0.0001$; PTPIP51/Raf1 0.5 µM $p < 0.0001$; 5 µM $p < 0.001$; 50 µM $p < 0.0001$). Of note, inhibition of NFκB signaling using IKK-16 did not affect the interaction of PTPIP51 and Raf1. The PTPIP51/14-3-3 interaction was augmented for the highest applied IKK-16 concentration (5 µM $p < 0.01$). Inhibition of the NFκB signaling in HaCat cells did not severely affect the MAPK-related interactome of PTPIP51. Application of PDTC to HaCat cells reduced the PTPIP51/14-3-3 interaction for the lowest applied concentration (0.5 µM $p < 0.01$) and enhanced the PTPIP51/Raf1 interaction if 5 µM PDTC were applied ($p < 0.01$). Treating HaCat cells with IKK-16 only affected the PTPIP51/Raf1 interaction for the lowest applied concentration (0.5 µM $p < 0.0001$; Figures 4 & 5).

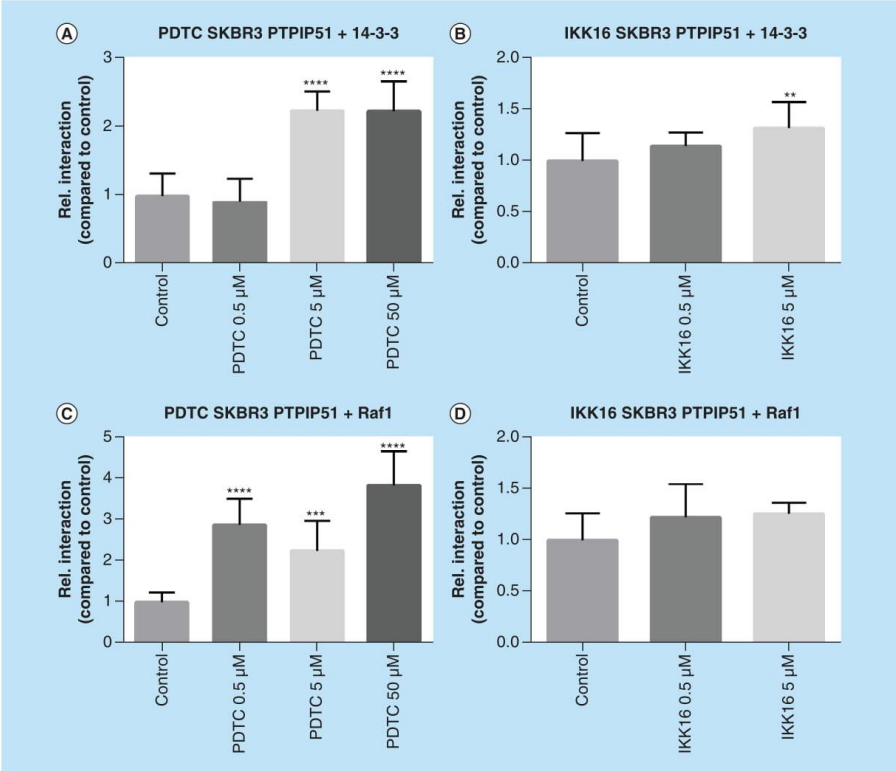

Figure 4. MAPK-related interactome of PTPIP51 in SKBR3 cells treated with PDTC (0.5, 5 and 50 µM) and IKK-16 (0.5 and 5 µM). (A) Interaction of PTPIP51 and 14-3-3 in SKBR3 cells treated with PDTC in the indicated concentrations for 6 h. **(B)** Interaction of PTPIP51 and 14-3-3 in SKBR3 cells treated with IKK-16 in the indicated concentrations for 6 h. **(C)** Interaction of PTPIP51 and Raf1 in SKBR3 cells treated with PDTC in the indicated concentrations for 6 h. **(D)** Interaction of PTPIP51 and Raf1 in SKBR3 cells treated with IKK-16 in the indicated concentrations for 6 h. The graphs show the mean value and standard deviation.
**$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Sensitivity toward IKK-16-induced NFκB inhibition correlates with the interaction changes of PTPIP51 & its crucial phosphatase PTP1B

To exert its scaffold protein properties, the phosphorylation of PTPIP51 is tightly regulated by several kinases and phosphatases. For the interaction of PTPIP51 with 14-3-3 and Raf1, the tyrosine residue 176 and the serine residue 212 are needed. While the phosphorylation of Tyr176 prevents the interaction of PTPIP51 and Raf1, the phosphorylation of Ser212 augments the interaction. The critical phosphorylation sites for the interaction with RelA and IκB, respectively, are up to now not known. Since both the NFκB- and the MAPK-related interactome are affected by the inhibition of NFκB signaling, we examined the interaction of PTPIP51 and its crucial phosphatase PTP1B in SKBR3 and HaCat cells. The application of PDTC to SKBR3 and HaCat cells did not affect the interaction of PTPIP51 and PTP1B. Interestingly, the IKK-16 treatment of SKBR3 and HaCat cells led to divergent results. While, IKK-16 inhibited the interaction of PTPIP51 and PTP1B in SKBR3 cells (0.5 µM $p < 0.0001$; 5 µM $p < 0.0001$), in HaCat cells the interaction was augmented (0.5 µM $p < 0.001$; 5 µM $p < 0.0001$; 50 µM $p < 0.0001$) (Figure 6).

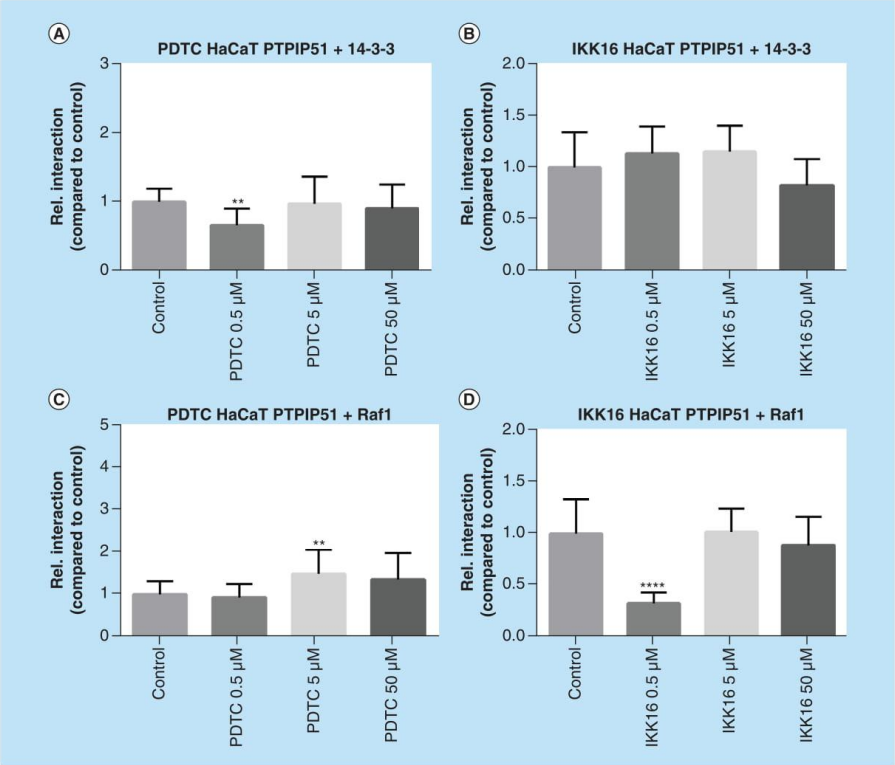

Figure 5. MAPK-related interactome of PTPIP51 in HaCat cells treated with PDTC (0.5, 5 and 50 µM) and IKK-16 (0.5 and 5 µM). (A) Interaction of PTPIP51 and 14-3-3 in HaCat cells treated with PDTC in the indicated concentrations for 6 h. **(B)** Interaction of PTPIP51 and 14-3-3 in HaCat cells treated with IKK-16 in the indicated concentrations for 6 h. **(C)** Interaction of PTPIP51 and Raf1 in HaCat cells treated with PDTC in the indicated concentrations for 6 h. **(D)** Interaction of PTPIP51 and Raf1 in HaCat cells treated with IKK-16 in the indicated concentrations for 6 h. The graphs show the mean value and standard deviation.
**$p < 0.01$; ****$p < 0.0001$.

Discussion

The role in tumor initiation, progression and the formation of metastasis make NFκB signaling a new target for novel therapeutic agents. In this study, we emphasize the importance of choosing the right agent for targeting the NFκB signaling in Her2-amplified breast cancer cells.

The selective inhibition of IKKs using the small molecule inhibitor IKK-16 induced severe impairments in the cell viability of the Her2-amplified breast cancer cell line SKBR3. Of note, the application of 5 µM IKK-16 led to diametrically opposite results in the investigated cell lines. While the HaCat cells displayed an increase in cell viability, the cell viability of SKBR3 cells was highly significantly reduced. This disparity was further analyzed by the evaluation of the RelA/IκB/PTPIP51 complex using the Duolink proximity ligation assay. Here, the results differed not only between the cell lines but also between the applied agent. Brobeil and coworkers stated the stimulation of the NFκB signaling via application of TNFα results in a dissolution of the RelA/IκB/PTPIP51 complex in HaCat cells, indicating an activation of the NFκB signaling [13]. The inhibition of NFκB signaling via IKK-16 in HaCat cells led to results corroborating the theory of a RelA/IκB/PTPIP51 complex formation and disintegration under inhibition and stimulation of NFκB signaling. Comparable observations were made for the application of PDTC

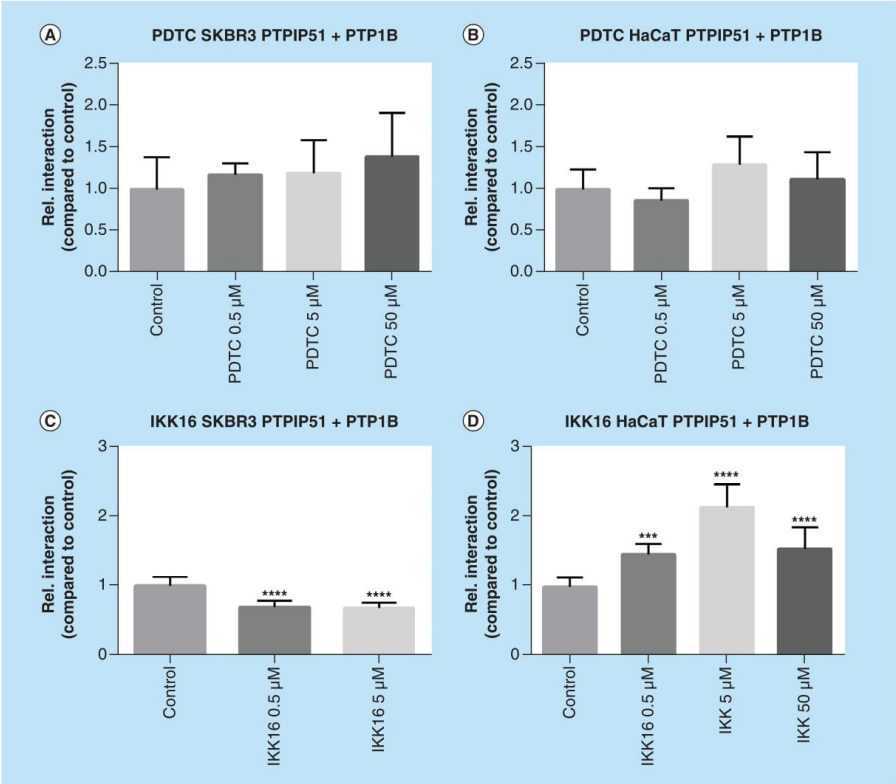

Figure 6. Interaction of PTPIP51 and PTP1B in SKBR3 cells and HaCat cells treated with PDTC (0.5, 5 and 50 µM) and IKK-16 (0.5, 5 and 5 µM). (A) Interaction of PTPIP51 and PTP1B in SKBR3 cells treated with PDTC in the indicated concentrations for 6 h. **(B)** Interaction of PTPIP51 and PTP1B in HaCat cells treated with PDTC in the indicated concentrations for 6 h. **(C)** Interaction of PTPIP51 and PTP1B in SKBR3 cells treated with IKK-16 in the indicated concentrations for 6 h. **(D)** Interaction of PTPIP51 and PTP1B in HaCat cells treated with IKK-16 in the indicated concentrations for 6 h. The graphs show the mean value and standard deviation.
***$p < 0.001$; ****$p < 0.0001$.

to SKBR3 cells. The regulation under NFκB inhibition using IKK-16 in SKBR3 cells entirely opposes the known regulations of PTPIP51 in NFκB signaling. Here, the interaction of PTPIP51 with RelA and IκB, respectively, is reduced, implying an activation of the NFκB signaling. These observations potentially depict an overshooting counter-regulation against the inhibition of IKKs. Up to now, the mechanisms of these regulations are unknown. The evaluation of the NFκB-related interactome of PTPIP51 is not sufficient to explain the different effects on the cell viability by the applied agents in the two cell lines.

The MAPK pathway is one of the essential growth and proliferation promoting pathways in Her2-amplified breast cancer cells [9]. PTPIP51 plays a pivotal role in the titration of the MAPK pathway activation [15–18]. The regulation of the MAPK-related PTPIP51 interactome upon NFκB inhibition significantly differs between the two cell lines. The application of PDTC to SKBR3 cells shifted PTPIP51 into the Raf1/14-3-3/PTPIP51 complex, indicating the activation of MAPK signaling. This shift was not observed under IKK-16 treatment. Thereby, the non-activation of MAPK signaling explains the severe impairment of cell viability in SKBR3 cells under IKK-16 treatment. In the HaCat cell line, neither of the applied agents led to a remarkable shift of PTPIP51 into the MAPK

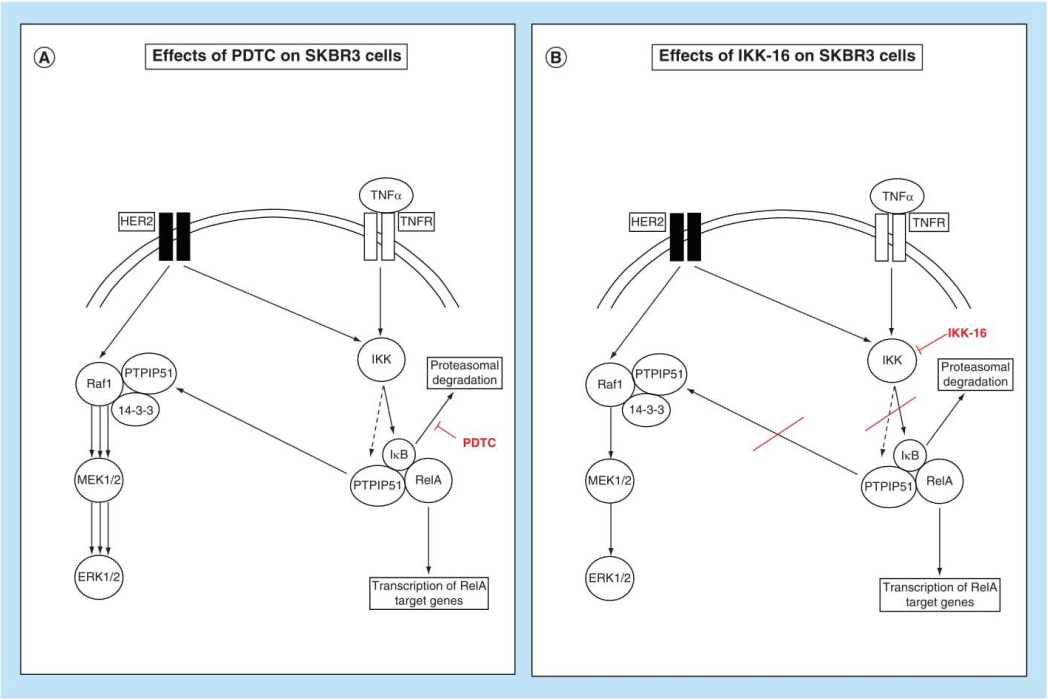

Figure 7. Schematic overview of the interaction shifts in SKBR3 cells after the application of PDTC and IKK-16. (A) Application of PDTC to SKBR3 cells inhibits the IκB ubiquitin ligase, thus inhibiting the degradation of IκB. The activation of IKKs through the Her2 receptor potentially leads to phosphorylation of PTPIP51 at serine 212. This mechanism shifts PTPIP51 into MAPK signaling and induces the formation of the Raf1/14-3-3/PTPIP51 complex. The PTPIP51 induced activation of the MAPK pathway bypasses the blocked NFκB signaling. **(B)** Selective inhibition of IKK1, IKK2 and the IKK complex with IKK-16 inhibits the phosphorylation of IκB. Likewise, IKK is not able to phosphorylate PTPIP51 at serine 212 and, thus, PTPIP51 is not shifted into MAPK signaling. Due to the blockage of the aforementioned bypass mechanism, SKBR3 cell viability is severely reduced by IKK-16.

pathway. These findings depict a potential evasion mechanism of SKBR3 cells against the PDTC mediated NFκB inhibition.

For the precise understanding of these regulations, the exact targets of the applied agents have to be identified. As mentioned in the introduction, PDTC exhibits a NFκB inhibitory property besides its antioxidative effect. IKK-16 is a small molecule inhibitor of the IKK-1, IKK-2 and IKK complex. Through the inhibition of these kinases, phosphorylation of IκB cannot be performed. Subsequently, IκB cannot be degraded and RelA cannot exert its transcriptional activity [12]. Of note, IKK2 is capable of phosphorylating both IκB and PTPIP51. The group-based prediction system (GPS 3.0; http://gps.biocuckoo.org/; [23]) revealed that IKK2 could phosphorylate PTPIP51 at serine 212, which enhances the interaction of PTPIP51 with MAPK signaling on the Raf1 level.

The application of PDTC to the Her2-amplified breast cancer cell line SKBR3 induces inhibition of the IκB ubiquitin ligase resulting in an enhanced interaction of PTPIP51 and RelA. Since IKK activity is not inhibited, but in contrast is even enhanced in the Her2-overactivated setting, IKK2 is still capable of phosphorylating the serine 212 of PTPIP51. The phosphorylation of PTPIP51 at serine 212 forces PTPIP51 into the Raf1/14-3-3/PTPIP51 complex and subsequently leads to a stimulation of MAPK signaling [15,17,18]. Thereby, SKBR3 cells potentially evade the NFκB inhibition via the crosstalk with the MAPK signaling mediated by PTPIP51.

The NFκB inhibition via IKK-16 blocks the phosphorylation of serine 212 of PTPIP51 through IKK2 and thereby the translocation of PTPIP51 into the MAPK signaling. These regulations explain the severe reduction in

SKBR3 cell viability under IKK-16 treatment since the blocked NFκB signaling cannot be bypassed by PTPIP51-induced MAPK stimulation.

The interaction of PTPIP51 with Raf1 and 14-3-3 is not only subjected to the serine 212 phosphorylation of PTPIP51 but also to the tyrosine 176 phosphorylation of PTPIP51 [15,17,18]. A crucial regulator of this phosphorylation site is the PTP1B [15,17,18,24]. Interestingly, the interaction of PTPIP51 and PTP1B depends on the level of NFκB inhibition. The inhibition of IKKs leads to different regulations of the PTPIP51/PTP1B interaction in SKBR3 cells and HaCat cells, respectively. This perfectly correlates with the effects on cell viability. The functional implications of these interaction shifts remain unclear since the observed reduction of PTPIP51/PTP1B interaction in SKBR3 cells implies a reduced interaction with the MAPK pathway due to the enhanced phosphorylation of tyrosine 176 of PTPIP51. In contrast, the interaction of PTPIP51 and 14-3-3 was even enhanced under IKK inhibition. The precise mechanisms of this regulation and the effects on phosphorylation of PTPIP51 need further investigation.

In Her2-amplified breast cancer cells, the activation of IKKs is tightly linked to the overactivation of the Her2 receptor [8]. Recent studies of our group substantiated an interaction of PTPIP51 and the Her2 receptor. Interestingly, selective inhibition of the Her2 receptor using Mubritinib induced a formation of a ternary complex consisting of PTPIP51, c-Src and Her2, which potentially represents a resistance mechanism against Her2-targeted tyrosine kinase inhibitors [14]. The inhibition of IKKs induced a similar enhanced interaction of Her2 and PTPIP51, whereas the PDTC mediated IκB ubiquitin ligase inhibition left the PTPIP51/Her2 interaction unaffected. A schematic overview of the mechanisms above is given in Figure 7. The functional consequences of this interaction shift remain unknown and warrant ongoing studies.

Conclusion & future perspective

Therapy resistance of Her2 amplified breast cancer against Her2 targeted therapies is becoming a relevant issue. Thus, the identification of resistance inducing signaling pathways and alternative therapeutic targets is of the utmost need. This study identifies the NFκB signaling as a possible target for future therapeutics. The crosstalk of NFκB signaling with other relevant signaling pathways, for example, MAPK signaling and Her2 signaling, still need to be identified and precisely described. This should be the subject of future studies.

Summary points

- NFκB inhibition on the IKK level using 5 μM IKK-16 severely affects the cell viability of SKBR3 cells but does not affect HaCat cells.
- PTPIP51 crosslinks the NFκB signaling to the MAPK pathway in SKBR3 cells.
- NFκB inhibition on IκB ubiquitin ligase level is bypassed by translocation of PTPIP51 into the MAPK pathway in SKBR3 cells.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.4155/fsoa-2019-0136

Author contributions

E Dietel designed the research, performed parts of the experiments, analyzed data and wrote the manuscript. A Brobeil performed parts of the experiments, analyzed data and corrected the manuscript. C Tag performed parts of the experiments. S Gattenlöhner analyzed data and corrected the manuscript. M Wimmer designed the research, analyzed data and wrote and corrected the manuscript.

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References

Papers of special note have been highlighted as: • of interest

1. Buchholz TA, Garg AK, Chakravarti N *et al.* The nuclear transcription factor kappaB/bcl-2 pathway correlates with pathologic complete response to doxorubicin-based neoadjuvant chemotherapy in human breast cancer. *Clin. Cancer Res.* 11(23), 8398–8402 (2005).
2. Mann AP, Verma A, Sethi G *et al.* Overexpression of tissue transglutaminase leads to constitutive activation of nuclear factor-kappaB in cancer cells: delineation of a novel pathway. *Cancer Res.* 66(17), 8788–8795 (2006).
3. Wang DJ, Ratnam NM, Byrd JC, Guttridge DC. NF-κB functions in tumor initiation by suppressing the surveillance of both innate and adaptive immune cells. *Cell Rep.* 9(1), 90–103 (2014).
4. Pires BRB, Mencalha AL, Ferreira GM *et al.* NF-kappaB is involved in the regulation of EMT genes in breast cancer cells. *PLoS ONE* 12(1), e0169622 (2017).
5. Gilmore TD, Kalaitzidis D, Liang M-C, Starczynowski DT. The c-Rel transcription factor and B-cell proliferation: a deal with the devil. *Oncogene* 23(13), 2275–2286 (2004).
6. Terzić J, Grivennikov S, Karin E, Karin M. Inflammation and colon cancer. *Gastroenterology* 138(6), 2101–2114.e5 (2010).
7. Xia Y, Shen S, Verma IM. NF-κB, an active player in human cancers. *Cancer Immunol. Res.* 2(9), 823–830 (2014).
8. Merkhofer EC, Cogswell P, Baldwin AS. Her2 activates NF-kappaB and induces invasion through the canonical pathway involving IKKalpha. *Oncogene* 29(8), 1238–1248 (2010).
9. Moasser MM. The oncogene *HER2*: its signaling and transforming functions and its role in human cancer pathogenesis. *Oncogene* 26(45), 6469–6487 (2007).
10. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the *HER-2/neu* oncogene. *Science* 235(4785), 177–182 (1987).
11. Li Q, Verma IM. NF-kappaB regulation in the immune system. *Nat. Rev. Immunol.* 2(10), 725–734 (2002).
12. Liu F, Xia Y, Parker AS, Verma IM. IKK biology. *Immunol. Rev.* 246(1), 239–253 (2012).
13. Brobeil A, Kämmerer F, Tag C, Steger K, Gattenlöhner S, Wimmer M. PTPIP51 – a new RelA-tionship with the NFκB signaling pathway. *Biomolecules* 5(2), 485–504 (2015).

• **Brobeil *et al.* identifies PTPIP51 as an interaction partner of RelA and IκB in HaCat cells.**

14. Dietel E, Brobeil A, Tag C, Gattenloehner S, Wimmer M. Effectiveness of EGFR/HER2-targeted drugs is influenced by the downstream interaction shifts of PTPIP51 in HER2-amplified breast cancer cells. *Oncogenesis* 7(8), 64 (2018).

• **Dietel *et al.* identifies PTPIP51 as an interaction partner of 14-3-3 and Raf1 in Her2 amplified breast cancer cells and shows that PTPIP51 plays a crucial role in the downstream signaling of EGFR and Her2 receptors.**

15. Brobeil A, Bobrich M, Tag C, Wimmer M. PTPIP51 in protein interactions: regulation and *in situ* interacting partners. *Cell Biochem. Biophys.* 63(3), 211–222 (2012).
16. Yu C, Han W, Shi T *et al.* PTPIP51, a novel 14-3-3 binding protein, regulates cell morphology and motility via Raf-ERK pathway. *Cell. Signal.* 20(12), 2208–2220 (2008).
17. Brobeil A, Koch P, Eiber M, Tag C, Wimmer M. The known interactome of PTPIP51 in HaCaT cells – inhibition of kinases and receptors. *Int. J. Biochem. Cell Biol.* 46, 19–31 (2013).
18. Brobeil A, Bobrich M, Wimmer M. Protein tyrosine phosphatase interacting protein 51–a jack-of-all-trades protein. *Cell Tissue Res.* 344(2), 189–205 (2011).
19. Hayakawa M, Miyashita H, Sakamoto I *et al.* Evidence that reactive oxygen species do not mediate NF-kappaB activation. *EMBO J.* 22(13), 3356–3366 (2003).
20. Gupta SC, Sundaram C, Reuter S, Aggarwal BB. Inhibiting NF-κB activation by small molecules as a therapeutic strategy. *Biochim. Biophys. Acta* 1799(10–12), 775–787 (2010).
21. Waelchli R, Bollbuck B, Bruns C *et al.* Design and preparation of 2-benzamido-pyrimidines as inhibitors of IKK. *Bioorg. Med. Chem. Lett.* 16(1), 108–112 (2006).
22. Leuchowius K-J, Jarvius M, Wickström M *et al.* High content screening for inhibitors of protein interactions and post-translational modifications in primary cells by proximity ligation. *Mol. Cell. Proteomics* 9(1), 178–183 (2010).
23. Xue Y, Ren J, Gao X, Jin C, Wen L, Yao X. GPS 2.0, a tool to predict kinase-specific phosphorylation sites in hierarchy. *Mol. Cell. Proteomics* 7(9), 1598–1608 (2008).
24. Brobeil A, Chehab R, Dietel E, Gattenlöhner S, Wimmer M. Altered protein interactions of the endogenous interactome of PTPIP51 towards MAPK signaling. *Biomolecules* 7(3), 55 (2017).

Discussion

The aim of these studies was to describe the expression, distribution, interactions and functional implications of PTPIP51 in Her2 amplified breast cancer cell lines. This is to our best knowledge the first work, which detected PTPIP51 in Her2 amplified breast cancer cells and described the multiple interactions of PTPIP51 with several other signaling and scaffolding proteins under diverse conditions. The basis for this study was laid by previous works from the laboratory of Prof. Wimmer. Stenzinger and coworkers first described a tissue- and cell-specific expression of PTPIP51 in the epidermis, seminiferous epithelium, muscle fibers of the fast-twitching subtype, neurons of the hippocampal region, ganglion cells of the autonomic nervous system, and axons of the peripheral nervous system (Stenzinger et al. 2005). Basing on these findings, Maerker and coworkers also detected PTPIP51 expression during the mouse eye development (Maerker et al. 2008). Koch and coworkers firstly described an expression of PTPIP51 in tumorous tissue, viz. keratinocyte carcinoma (Koch et al. 2008). Shortly after, PTPIP51 was also detected in benign prostate hyperplasia, prostate carcinoma, acute myeloid leukemia blasts, and glioblastoma (Koch et al. 2009b; Brobeil et al. 2011a; Petri et al. 2011). Here, not only the expression of PTPIP51 was evaluated, but also the regulation of PTPIP51 expression and the relevance and regulations of PTPIP51 in the MAPK signaling. As mentioned above, Her2 amplified breast cancer cells are known to display an overactivation of the MAPK pathway, thus the evaluation of PTPIP51 in this context was on hand.

PTPIP51 and the receptor tyrosine kinases EGFR and Her2

Amplification of the Her2 receptor is present in about 30% of all breast tumors (Tao et al. 2015). This overexpression results in an enhanced formation of homo- and heterodimers with other member of the Erb receptor family which is subsequently channeled in an activation of several growth and proliferation signaling pathways, viz. MAPK pathway and Akt signaling, to name some of them (Moasser 2007a, 2007b). We firstly described PTPIP51 as an interaction partner of the EGFR and Her2 receptor in SK-BR3 and BT474 cells (Dietel et al. 2018b). The treatment of these cell lines with EGFR/Her2 targeted small molecule tyrosine kinase inhibitors resulted in target dependent interaction shifts. The sole inhibition of the EGFR or the combined inhibition of EGFR and Her2 receptor led to an enhanced interaction of PTPIP51 with the EGFR and Her2. Interestingly, the sole inhibition of the Her2 receptor using Mubritinib exclusively promote the interaction of PTPIP51 and the Her2 receptor.

These findings stress a target dependent recruitment of PTPIP51 towards the Erb family receptors (Dietel et al. 2018b). Interestingly, inhibition of EGFR resulted in an enhanced interaction of PTPIP51 with c-Src in SK-BR3 cells. The non-receptor tyrosine kinase c-Src plays a crucial compensatory role in the loss of EGFR kinase and depicts a pivotal structure the resistance mechanism against Erb receptor targeting TKIs. Noteworthy, the treatment of BT474 cells with EGFR/HER2-targeted TKI did not enhance the PTPIP51/c-Src interaction. This opposes the known regulations of PTPIP51, since the inhibition of EGFR is normally followed by an enhancement of PTPIP51/c-Src interaction. Moreover, these findings correlate with a higher sensitivity of BT474 cells to EGFR-targeted TKIs. This stresses a connection between the sensitivity of HER2-positive cell lines to EGFR-targeted TKIs and the downstream regulation of the interaction of PTPIP51 and c-Src. The relevance of the interaction of PTPIP51, c-Src, and the Her2 receptor is further mirrored in the interaction shifts induced by Mubritinib. Here, selective inhibition of the Her2 receptor resulted in an augmentation of PTPIP51/c-Src interaction in SK-BR3 cells. Interestingly, the interaction of PTPIP51 and c-Src rose in the same stoichiometric proportion as the PTPIP51/HER2 interaction. This indicates a potential formation of a ternary complex consisting of the Her2 receptor, PTPIP51, and c-Src. Noteworthy, application of the selective c-Src inhibitor Dasatinib induced the same shift of PTPIP51 toward the HER2 receptor as seen for the EGFR/Her2-targeted TKIs. Moreover, the selective inhibition of c-Src led to an enhancement of the Her2 receptor/c-Src interaction (Dietel et al. 2018b). Interestingly, not only the inhibition of tyrosine kinases influences the interaction of PTPIP51 and the Her2 receptor, but also the application of the novel small molecule modulator Dynarrestin/LDC. Here, we were able to show an increase of PTPIP51/Her2 receptor interaction after 24h incubation time. If this mechanism is due to secondary alterations induced by LDC3/Dynarrestin or depicts a direct modulation of the interaction is up to now not clear. Nevertheless, all these findings indicate a pivotal role of PTPIP51 in the regulations of the EGFR and Her2 signaling (Dietel et al. 2020).

MAPK pathway interaction of PTPIP51

The MAPK pathway is one of the best described growth and signaling pathways, consisting of a multilevel structured kinase cascade. Basically, the function of the MAPK pathway is to transduce extracellular signals towards the transcriptional level, to regulate cell growth, differentiation, migration and apoptosis. Due to the multitude of these regulations, the MAPK pathway consist of three distinctive signaling pathways, viz. the extracellular signal-regulated

kinase (ERK) pathway, the p38 kinase pathway and the C-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) pathway (Zhang und Liu 2002). To achieve a sufficient signal strength, all of these pathways are structured in a strictly hierarchically system, defined by a MAPK kinase kinase (MAPKKK), which is superior to a MAPK kinase (MAPKK), which controls a MAPK (Meister et al. 2013). One example for a classical activation of the ERK pathway is represented by the epidermal growth factor receptor (EGFR). Its homo- or heterodimerization with other members of the ERB family results in an autophosphorylation of the receptor (Wee und Wang 2017). Subsequently, a signaling cascade of multiple signaling molecules is activated, namely GRB2 and SOS. Following, Raf kinases, which represent the MAPKKK, are recruited to the cell membrane for activation. Subsequently, Raf kinases activate MEK1/2 (MAPKK) and ERK1/2 (MAPK), which ultimately trigger transcription of target genes (Zhang und Liu 2002). PTPIP51 interferes on the level of Raf1 with the ERK signaling (Yu et al. 2008). PTPIP51 stimulates the MAPK signaling via the complex formation with Raf1 and 14-3-3. A crucial regulating site for this complex formation is represented by the Tyr176 residue of PTPIP51. Here, phosphorylation induces a break-up of the Raf1/14-3-3/PTPIP51 complex und thus an abrogation of the MAPK pathway stimulating effect (Brobeil et al. 2012a; Brobeil et al. 2011b). The inhibition of the EGFR and the Her2 receptor led to a formation of the PTPIP51/14-3-3/Raf1 complex accompanied by a reduced phosphorylation of Tyr176 of PTPIP51 (Dietel et al. 2018b). These findings are in accordance with the previously described mechanisms of MAPK related interactions of PTPIP51. Upon inhibition of the EGFR, the receptor is not able to phosphorylate the Tyr176 residue of PTPIP51, thereby PTPIP51 can shift into MAPK signaling and ensure a minimal MAPK pathway signaling activity. This mechanisms seems intact in the SK-BR3 and BT474 cell line. Interestingly, selective inhibition of the Her2 receptor left the interaction of PTPIP51 and Raf1 unattended in SKBR3 cells. Also, the phosphorylation levels of Tyr176 of PTPIP51 even increased under selective Her2 inhibition. Here, again the aforementioned ternary complex formation of PTPIP51/c-Src/Her2 could be a reason, since c-Src is able to phosphorylate the Tyr176 residue of PTPIP51 (Dietel et al. 2018b; Brobeil et al. 2012a). Nevertheless, the regulation mechanisms of the MAPK pathway related interactions of PTPIP51 are applicable in the Her2 amplified breast cancer cell system. On the contrary, LDC3/Dynarrestin complete abrogates these known mechanisms. Here, the phosphorylation of Tyr176 of PTPIP51 is enhanced and in the same manner the interaction of PTPIP51, 14-3-3 and Raf1 is enhanced. Furthermore, we were also able to show an increase of MAPK pathway activity as measured by the phosphorylation of ERK1/2 upon LDC3/Dynarrestin treatment.

These regulations completely oppose the known mechanisms. How LDC3/Dynarrestin is able to translocate PTPIP51 into MAPK signaling despite its high Tyr176 phosphorylation is up to now not clear. Two potential mechanisms could be the reason. First, LDC3/Dynarrestin induces a conformational change in the protein structure of PTPIP51. Thereby, the Tyr176 residue cannot exert is regulatory effect or second, LDC3/Dynarrestin covers the Tyr176 residue and thus resulting in the same outcome (Dietel et al. 2019).

PTPIP51 in the context of mitochondria associated ER-membranes and Akt signaling

The regulation of growth, migration apoptosis and proliferation is not only dependent on signaling cascades, but also on the precise communication of diverse organelles. One of these crucial sites is represented by the mitochondrial-associated ER membranes, which are defined by the close apposition (10-30 nm) of ER and mitochondrial surfaces (Rowland und Voeltz 2012). Here, calcium homeostasis, lipid trafficking, intracellular trafficking, autophagy, apoptosis and many more functions are regulated (Vos et al. 2012). Dysfunction of these structures can contribute to many neurodegenerative diseases, viz. amyotrophic lateral sclerosis (ALS), Alzheimers disease, Parkinsons disease and fronto-temporal dementia (FTD) (Gomez-Suaga et al. 2017b; Paillusson et al. 2017; Stoica et al. 2014). These sites are not solely for communication between the ER and the mitochondrion, but also harbor signaling hubs for mTor and Akt signaling (Betz et al. 2013). In addition, MAMs also regulate autophagy and calcium-induced apoptosis. Interestingly, many proto-oncogenes and tumor suppressors are located at MAMs, viz. promyelocytic leukemia protein (PML), PTEN and p53 (Patergnani et al. 2015). In summary, only light alteration of these communication sites could contribute to tumorigenesis. Since most of the pathological processes are defined by a disruption of the MAM, the physical tethering of the organelles is of great interest. Up to now, several proteins have been discovered, which tether the ER and the mitochondrion. One of them is PTPIP51. As shown by De Vos and coworkers, the interaction of PTPIP51 and vesicle-associated membrane protein-associated protein B (VAPB) is essential for the physical stability of MAM (Vos et al. 2012). Recent studies also revealed an interaction of PTPIP51 and the oxysterol-binding protein-related proteins 5 and 8 (ORP5/ORP8), which are located at the ER (Galmes et al. 2016). In order to control the diverse functions, the tethering protein tethers have to underlie various types of regulation. Stoica and coworkers showed that the ALS / FTD-associated proteins FUS and TAR DNA-binding protein 43 (TDP-43) induce an activation of GSK-3β, which subsequently hinders the binding of PTPIP51 and VAPB.

Vice versa, inhibition of GSK-3β induces an enhanced interaction and a stabilization of the MAMs, mirrored by a normalized mitochondrial calcium level (Stoica et al. 2014).

The novel small molecule LDC3/Dynarrestin represents another possible way to modulate the interaction of PTPIP51 and VAPB. For long incubation times, LDC3/Dynarrestin led to an enhanced interaction of PTPIP51 with VAPB, indicating a stabilization of MAM in Her2 amplified breast cancer cells. Furthermore, LDC3/Dynarrestin induced a reduction of Akt signaling, which is located at the MAM. But, these regulations are only seen for longer incubation times, hinting that the increased interaction of PTPIP51 and VAPB might be caused by secondary mechanisms and not directly by LDC3/Dynarrestin (Dietel et al. 2019). The enhanced interaction of PTPIP51 and VAPB could depict a counterregulation to the mitochondrial metabolic stress induced by high concentrations of LDC3/Dynarrestin, but further studies would be needed to proof this thesis.

PTPIP51 and the NFkB signaling

Besides the MAPK pathway, the NFκB signaling represents another crucial signaling pathway, which can be activate by the Her2 receptor (Merkhofer et al. 2010). The activation of the NFκB signaling is essential for tumor initiation, progression and the formation of metastasis. Brobeil and coworkers firstly showed the involvement of PTPIP51 in the NFκB signaling via interaction with RelA and IκB. If deactivated, a complex of PTPIP51, RelA and IκB is formed, thereby a translocation of RelA into the nucleus is omitted (Brobeil et al. 2015). Upon activation of the canonical pathway, IκB is phosphorylated by IKKs. Subsequently, IκB is ubiquitinated and degradated, and the interaction of PTPIP51 and RelA is loosened. Thereby, RelA can exert its transcriptional activity after nuclear translocation (Li und Verma 2002; Liu et al. 2012). This interaction is influenceable by the novel small molecule LDC3/Dynarrestin (Dietel et al. 2019). We were able to show, that the application of LDC3/Dynarrestin to SKBR3 cells enhances the interaction of PTPIP51 with RelA and IκB in a concentration dependent manner. Furthermore, the contemporaneous treatment with TNFα and LDC3/Dynarrestin did completely revert the TNFα induced break-up of the PTPIP51/RelA/IκB complex. Noteworthy, these effects are not due to a direct interaction of LDC3/Dynarrestin with the PTPIP51/RelA/IκB complex, since the complex stabilizing properties are completely omitted if PDTC is applied. PDTC represents an NFκB inhibitor, which at least partially exerts its effect via an inhibition of the IκB ubiquitin ligase. Thus, the LDC3/Dynarrestin induced stabilization of the PTPIP51/RelA/IκB complex is highly likely due to up to now unknown secondary mechanisms. One potential mechanisms could be the

LDC3/Dynarrestin induced inhibition of the Akt signaling. Akt is able to activate the NFκB signaling on the level of the IKK kinases. Therefore, the inhibition of Akt signaling results in a reduced IKK activity, which subsequently allows the PTPIP51/RelA/IκB complex to stabilize (Dietel et al. 2019). To further evaluate the relevance of PTPIP51 in the NFκB signaling of Her2 amplified breast cancer cells and the keratinocyte cell line HaCat, we applied two different-level inhibitors of the NFκB signaling, viz. PDTC and IKK-16. The evaluation of cell viability displayed a selective impairment of SKBR3 cells, when treated with 5µM IKK-16. For further evaluation we monitored the PTPIP51/RelA/IκB complex under the two applied agents using the Duolink proximity ligation assay. Here, we were able to prove the known regulatory mechanisms of PTPIP51 in NFκB signaling for PDTC and IKK-16 in HaCat cells and for PDTC in SKBR3 cells, corroborating the theory of a RelA/IκB/PTPIP51 complex formation under inhibition and a disintegration when NFκB signaling is stimulated. Noteworthy, the treatment of SKBR3 cells with IKK-16 entirely opposes the known regulations of PTPIP51 in NFκB signaling. Here, the interaction of PTPIP51 with RelA and IκB, is reduced, hinting at an activation of the NFκB signaling. These observations imply an overshooting counter-regulation against the IKK inhibition. Up to now, the underlying mechanisms are unknown (Dietel et al. 2020).

Of note, as mentioned above PTPIP51 is also a crucial regulator of the MAPK signaling. As we showed the inhibition of the EGFR and the Her2 receptor led to several alterations of the MAPK related interactome of PTPIP51 and the MAPK pathway activity. To further evaluate the mechanistic background of the severe impairment of SKBR3 cell viability under IKK-16 treatment, we monitored the MAPK pathway related PTPIP51 interactions. Interestingly, the regulation of the MAPK-related PTPIP51 interactome upon NFκB inhibition significantly differs between SKBR3 and HaCat cells. The treatment of SKBR3 cells with PDTC shifted PTPIP51 into the Raf1/14-3-3/PTPIP51 complex, indicating an activation of MAPK signaling. This interaction shift was not induced by IKK-16 treatment. Thus, the non-activation of MAPK signaling could be the reason of the severe reduction of cell viability in SKBR3 cells under IKK-16 treatment. Noteworthy, a significant change of the MAPK-related PTPIP51 interactome was not observed in the HaCat cell line for neither of the applied agents (Dietel et al. 2020).

A potential mechanism for these regulations can be found in the targeted molecules of the applied agents. PDTC reduces the NFκB signaling via the inhibition of the IκB ubiquitin ligase (Hayakawa et al. 2003). IKK-16 represents a small molecule inhibitor of the IKK-1,

IKK-2 and IKK complex (Waelchli et al. 2006). As mentioned above the IKK kinases are responsible for the phosphorylation of IκB. If this function is inhibited the ubiquitination of IκB cannot be performed. Subsequently, IκB cannot be degraded and RelA cannot exert its transcriptional activity. Interestingly, in silico experiments revealed, that the Ser212 residue of PTPIP51 can be phosphorylated by IKK2. The Ser212 residue can shift PTPIP51 into the MAPK pathway if phosphorylated and thus exerting its MAPK pathway stimulating effect (Brobeil et al. 2013; Brobeil et al. 2017; Brobeil et al. 2011b). Since IKK activity is not inhibited under PDTC treatment, IKK2 is capable of phosphorylating the serine 212 of PTPIP51. Thus, PTPIP51 can shift into the Raf1/14-3-3/PTPIP51 complex and subsequently stimulate the MAPK signaling. Thereby, SKBR3 cells potentially evade the PDTC induced NFκB inhibition via the crosstalk with the MAPK signaling mediated by PTPIP51. This shift is not possible under IKK-16 treatment, thus explaining the severe cell viability impairment of SKBR3 cells (Dietel et al. 2020).

Conclusion

In summary, protein-protein interactions are the foundation of all signaling events in normal cells as well as in tumor cells. The condensation of the studies displays PTPIP51 as a conductor in the cellular orchestra. We were able to show, that PTPIP51 plays a crucial role in the downstream signaling of the EGFR and the Her2 receptor in Her2 amplified breast cancer cells. Here, PTPIP51 interactions are differentially regulated upon EGFR or Her2 inhibition, providing potential bypass mechanisms. PTPIP51 plays a pivotal role in the Her2 inhibition induced activation of Akt signaling. Furthermore, we highlighted the role of PTPIP51 in the MAPK signaling of Her2 amplified breast cancer cells and found evidence for cross talks towards the NFκB signaling. We also firstly described the protein-protein interaction shifts of PTPIP51 induced by the novel small molecule LDC3/Dynarrestin.

Nevertheless, the precise mechanisms of several interaction shift and pathway cross talks are still unknown and need extensive further investigation. This study represents a starting point of the functional relevance of PTPIP51 in Her2 amplified breast cancer.

Zusammenfassung

Protein-Protein-Interaktionen sind die Grundlage aller Signaltransduktionen in gesunden Zellen sowie in Tumorzellen. Eine Vielzahl von Studien zeigt eine zentrale Rolle von PTPIP51 innerhalb dieser Strukturen. PTPIP51 ist ein Gerüstprotein, welches als Reaktionsmatrix in verschiedenen Signalwegen dient. Wir konnten zeigen, dass PTPIP51 eine entscheidende Funktion bei der Signalübertragung des EGFR- sowie des Her2-Rezeptors in Her2 amplifizierten Brustkrebszellen einnimmt. In diesem Kontext werden die Interaktionen von PTPIP51 durch die Hemmung von EGFR oder Her2 unterschiedlich reguliert, womit sich verschiedene potentielle Bypass-Mechanismen eröffnen. PTPIP51 spielt eine zentrale Rolle bei der durch die Inhibition von Her2 induzierten Akt-Aktivierung. Darüber hinaus nimmt PTPIP51 eine essentielle Funktion im MAPK-Signalweg in Her2 amplifizierten Brustkrebszellen ein. PTPIP51 fungiert als potentieller Mediator im Crosstalk vom MAPK-Signalweg mit dem NFκB-Signalweg. Zudem konnten wir erstmals die durch den small molecule modulator LDC3/Dynarrestin hervorgerufenen Verschiebungen der PTPIP51 abhängigen Protein-Protein-Interaktionen beschreiben. Diese Studien zeigen die funktionelle Relevanz von PTPIP51 in Her2 amplifizierten Brustkrebszellen.

References

Azambuja, Evandro de; Holmes, Andrew P.; Piccart-Gebhart, Martine; Holmes, Eileen; Di Cosimo, Serena; Swaby, Ramona F. et al. (2014): Lapatinib with trastuzumab for HER2-positive early breast cancer (NeoALTTO). Survival outcomes of a randomised, open-label, multicentre, phase 3 trial and their association with pathological complete response. In: *The Lancet Oncology* 15 (10), S. 1137–1146. DOI: 10.1016/S1470-2045(14)70320-1.

Barop, Justus; Sauer, Heinrich; Steger, Klaus; Wimmer, Monika (2009): Differentiation-dependent PTPIP51 expression in human skeletal muscle cell culture. In: *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society* 57 (5), S. 425–435. DOI: 10.1369/jhc.2008.952846].

Baselga, J.; Tripathy, D.; Mendelsohn, J.; Baughman, S.; Benz, C. C.; Dantis, L. et al. (1999): Phase II study of weekly intravenous trastuzumab (Herceptin) in patients with HER2/neu-overexpressing metastatic breast cancer. In: *Seminars in oncology* 26 (4 Suppl 12), S. 78–83.

Betz, Charles; Stracka, Daniele; Prescianotto-Baschong, Cristina; Frieden, Maud; Demaurex, Nicolas; Hall, Michael N. (2013): Feature Article: mTOR complex 2-Akt signaling at mitochondria-associated endoplasmic reticulum membranes (MAM) regulates mitochondrial physiology. In: *Proceedings of the National Academy of Sciences of the United States of America* 110 (31), S. 12526–12534. DOI: 10.1073/pnas.1302455110.

Blay, Jean-Yves; Le Cesne, Axel; Alberti, Laurent; Ray-Coquart, Isabelle (2005): Targeted cancer therapies. In: *Bulletin du cancer* 92 (2), E13-8.

Bobrich, M.; Brobeil, A.; Mooren, F. C.; Krüger, K.; Steger, K.; Tag, C.; Wimmer, M. (2011): PTPIP51 interaction with PTP1B and 14-3-3β in adipose tissue of insulin-resistant mice. In: *International journal of obesity (2005)* 35 (11), S. 1385–1394. DOI: 10.1038/ijo.2010.283.

Brobeil, Alexander; Bobrich, Manuel; Graf, Michaela; Kruchten, Anke; Blau, Wolfgang; Rummel, Mathias et al. (2011a): PTPIP51 is phosphorylated by Lyn and c-Src kinases lacking dephosphorylation by PTP1B in acute myeloid leukemia. In: *Leukemia research* 35 (10), S. 1367–1375. DOI: 10.1016/j.leukres.2011.03.024.

Brobeil, Alexander; Bobrich, Manuel; Tag, Claudia; Wimmer, Monika (2012a): PTPIP51 in protein interactions: regulation and in situ interacting partners. In: *Cell biochemistry and biophysics* 63 (3), S. 211–222. DOI: 10.1007/s12013-012-9357-y.

Brobeil, Alexander; Bobrich, Manuel; Wimmer, Monika (2011b): Protein tyrosine phosphatase interacting protein 51--a jack-of-all-trades protein. In: *Cell and tissue research* 344 (2), S. 189–205. DOI: 10.1007/s00441-011-1146-1.

Brobeil, Alexander; Chehab, Rajaa; Dietel, Eric; Gattenlöhner, Stefan; Wimmer, Monika (2017): Altered Protein Interactions of the Endogenous Interactome of PTPIP51 towards MAPK Signaling. In: *Biomolecules* 7 (3). DOI: 10.3390/biom7030055.

Brobeil, Alexander; Graf, Michaela; Eiber, Moritz; Wimmer, Monika (2012b): Interaction of PTPIP51 with Tubulin, CGI-99 and Nuf2 During Cell Cycle Progression. In: *Biomolecules* 2 (1), S. 122–142. DOI: 10.3390/biom2010122.

Brobeil, Alexander; Graf, Michaela; Oeschger, Sabine; Steger, Klaus; Wimmer, Monika (2010): PTPIP51-a myeloid lineage specific protein interacts with PTP1B in neutrophil granulocytes. In: *Blood cells, molecules & diseases* 45 (2), S. 159–168. DOI: 10.1016/j.bcmd.2010.04.009.

Brobeil, Alexander; Kämmerer, Fabian; Tag, Claudia; Steger, Klaus; Gattenlöhner, Stefan; Wimmer, Monika (2015): PTPIP51-A New RelA-tionship with the NFκB Signaling Pathway. In: *Biomolecules* 5 (2), S. 485–504. DOI: 10.3390/biom5020485.

Brobeil, Alexander; Koch, Philipp; Eiber, Moritz; Tag, Claudia; Wimmer, Monika (2013): The known interactome of PTPIP51 in HaCaT cells - inhibition of kinases and receptors. In: *The international journal of biochemistry & cell biology. DOI:* 10.1016/j.biocel.2013.10.007.

Canonici, Alexandra; Gijsen, Merel; Mullooly, Maeve; Bennett, Ruth; Bouguern, Noujoude; Pedersen, Kasper et al. (2013): Neratinib overcomes trastuzumab resistance in HER2 amplified breast cancer. In: *Oncotarget* 4 (10), S. 1592–1605. DOI: 10.18632/oncotarget.1148.

Dietel, Eric; Brobeil, Alexander; Delventhal, Lucas; Tag, Claudia; Gattenlöhner, Stefan; Wimmer, Monika (2019): Crosstalks of the PTPIP51 interactome revealed in Her2 amplified breast cancer cells by the novel small molecule LDC3/Dynarrestin. In: *PloS one* 14 (5), e0216642. DOI: 10.1371/journal.pone.0216642.

Dietel, Eric; Brobeil, Alexander; Gattenlöhner, Stefan; Wimmer, Monika (2018a): The Importance of the Right Framework. Mitogen-Activated Protein Kinase Pathway and the Scaffolding Protein PTPIP51. In: *International journal of molecular sciences* 19 (10). DOI: 10.3390/ijms19103282.

Dietel, Eric; Brobeil, Alexander; Tag, Claudia; Gattenloehner, Stefan; Wimmer, Monika (2018b): Effectiveness of EGFR/HER2-targeted drugs is influenced by the downstream interaction shifts of PTPIP51 in HER2-amplified breast cancer cells. In: *Oncogenesis* 7 (8), S. 64. DOI: 10.1038/s41389-018-0075-1.

Dietel, Eric; Brobeil, Alexander; Tag, Claudia; Gattenloehner, Stefan; Wimmer, Monika (2020): PTPIP51 crosslinks the NFκB signaling and the MAPK pathway in SKBR3 cells. In: *Future Science OA*, FSO463. DOI: 10.2144/fsoa-2019-0136.

Galmes, Romain; Houcine, Audrey; van Vliet, Alexander R.; Agostinis, Patrizia; Jackson, Catherine L.; Giordano, Francesca (2016): ORP5/ORP8 localize to endoplasmic reticulum-mitochondria contacts and are involved in mitochondrial function. In: *EMBO reports* 17 (6), S. 800–810. DOI: 10.15252/embr.201541108.

Geyer, Charles E.; Forster, John; Lindquist, Deborah; Chan, Stephen; Romieu, C. Gilles; Pienkowski, Tadeusz et al. (2006): Lapatinib plus Capecitabine for HER2-Positive Advanced Breast Cancer. In: *N Engl J Med* 355 (26), S. 2733–2743. DOI: 10.1056/NEJMoa064320.

Gomez-Suaga, Patricia; Paillusson, Sebastien; Miller, Christopher C. J. (2017a): ER-mitochondria signaling regulates autophagy. In: *Autophagy* 13 (7), S. 1250–1251. DOI: 10.1080/15548627.2017.1317913.

Gomez-Suaga, Patricia; Paillusson, Sebastien; Stoica, Radu; Noble, Wendy; Hanger, Diane P.; Miller, Christopher C. J. (2017b): The ER-Mitochondria Tethering Complex VAPB-PTPIP51 Regulates Autophagy. In: *Current biology : CB* 27 (3), S. 371–385. DOI: 10.1016/j.cub.2016.12.038.

Hayakawa, Makio; Miyashita, Hiroshi; Sakamoto, Isao; Kitagawa, Masatoshi; Tanaka, Hirofumi; Yasuda, Hideyo et al. (2003): Evidence that reactive oxygen species do not mediate NF-kappaB activation. In: *The EMBO journal* 22 (13), S. 3356–3366. DOI: 10.1093/emboj/cdg332.

Herrera-Cruz, Maria Sol; Simmen, Thomas (2017): Cancer: Untethering Mitochondria from the Endoplasmic Reticulum? In: *Frontiers in oncology* 7, S. 105. DOI: 10.3389/fonc.2017.00105.

Hilmarsdottir, Bylgja; Briem, Eirikur; Halldorsson, Skarphedinn; Kricker, Jennifer; Ingthorsson, Sævar; Gustafsdottir, Sigrun et al. (2017): Inhibition of PTP1B disrupts cell-cell adhesion and induces anoikis in breast epithelial cells. In: *Cell death & disease* 8 (5), e2769. DOI: 10.1038/cddis.2017.177.

Julien, Sofi G.; Dubé, Nadia; Read, Michelle; Penney, Janice; Paquet, Marilene; Han, Yongxin et al. (2007): Protein tyrosine phosphatase 1B deficiency or inhibition delays ErbB2-induced mammary tumorigenesis and protects from lung metastasis. In: *Nature genetics* 39 (3), S. 338–346. DOI: 10.1038/ng1963.

Koch, P.; Stenzinger, A.; Viard, M.; Marker, D.; Mayser, P.; Nilles, M. et al. (2008): The novel protein PTPIP51 is expressed in human keratinocyte carcinomas and their surrounding stroma. In: *Journal of cellular and molecular medicine* 12 (5B), S. 2083–2095. DOI: 10.1111/j.1582-4934.2008.00198.x.

Koch, P.; Viard, M.; Stenzinger, A.; Brobeil, A.; Tag, C.; Steger, K.; Wimmer, M. (2009a): Expression profile of PTPIP51 in mouse brain. In: *The Journal of comparative neurology* 517 (6), S. 892–905. DOI: 10.1002/cne.22201.

Koch, Philipp; Petri, Meike; Paradowska, Agnieszka; Stenzinger, Albrecht; Sturm, Klaus; Steger, Klaus; Wimmer, Monika (2009b): PTPIP51 mRNA and protein expression in tissue microarrays and promoter methylation of benign prostate hyperplasia and prostate carcinoma. In: *The Prostate* 69 (16), S. 1751–1762. DOI: 10.1002/pros.21025.

Li, Qiutang; Verma, Inder M. (2002): NF-kappaB regulation in the immune system. In: *Nature reviews. Immunology* 2 (10), S. 725–734. DOI: 10.1038/nri910.

Liu, Fei; Xia, Yifeng; Parker, Aaron S.; Verma, Inder M. (2012): IKK biology. In: *Immunological reviews* 246 (1), S. 239–253. DOI: 10.1111/j.1600-065X.2012.01107.x.

Lv, B. F.; Yu, C. F.; Chen, Y. Y.; Lu, Y.; Guo, J. H.; Song, Q. S. et al. (2006): Protein tyrosine phosphatase interacting protein 51 (PTPIP51) is a novel mitochondria protein with an N-terminal mitochondrial targeting sequence and induces apoptosis. In: *Apoptosis : an international journal on programmed cell death* 11 (9), S. 1489–1501. DOI: 10.1007/s10495-006-8882-9.

Maerker, David; Stenzinger, Albrecht; Schreiner, Dietmar; Tag, Claudia; Wimmer, Monika (2008): Expression of PTPIP51 during mouse eye development. In: *Histochemistry and cell biology* 129 (3), S. 345–356. DOI: 10.1007/s00418-007-0361-z.

Martin, G. S. (2001): The hunting of the Src. In: *Nature reviews. Molecular cell biology* 2 (6), S. 467–475. DOI: 10.1038/35073094.

Marty, Michel; Cognetti, Francesco; Maraninchi, Dominique; Snyder, Ray; Mauriac, Louis; Tubiana-Hulin, Michele et al. (2005): Randomized phase II trial of the efficacy and safety of trastuzumab combined with docetaxel in patients with human epidermal growth factor receptor 2-positive metastatic breast cancer administered as first-line treatment: the M77001 study group. In: *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 23 (19), S. 4265–4274. DOI: 10.1200/JCO.2005.04.173.

Meister, Melanie; Tomasovic, Ana; Banning, Antje; Tikkanen, Ritva (2013): Mitogen-Activated Protein (MAP) Kinase Scaffolding Proteins. A Recount. In: *International journal of molecular sciences* 14 (3), S. 4854–4884. DOI: 10.3390/ijms14034854.

Merkhofer, E. C.; Cogswell, P.; Baldwin, A. S. (2010): Her2 activates NF-kappaB and induces invasion through the canonical pathway involving IKKalpha. In: *Oncogene* 29 (8), S. 1238–1248. DOI: 10.1038/onc.2009.410.

Moasser, M. M. (2007a): Targeting the function of the HER2 oncogene in human cancer therapeutics. In: *Oncogene* 26 (46), S. 6577–6592. DOI: 10.1038/sj.onc.1210478.

Moasser, M. M. (2007b): The oncogene HER2: its signaling and transforming functions and its role in human cancer pathogenesis. In: *Oncogene* 26 (45), S. 6469–6487. DOI: 10.1038/sj.onc.1210477.

Moore, Samantha F.; Hunter, Roger W.; Hers, Ingeborg (2011): mTORC2 protein complex-mediated Akt (Protein Kinase B) Serine 473 Phosphorylation is not required for Akt1 activity in human platelets corrected. In: *The Journal of biological chemistry* 286 (28), S. 24553–24560. DOI: 10.1074/jbc.M110.202341.

Mueller, Kelly L.; Hunter, Lauren A.; Ethier, Stephen P.; Boerner, Julie L. (2008): Met and c-Src cooperate to compensate for loss of epidermal growth factor receptor kinase activity in breast cancer cells. In: *Cancer research* 68 (9), S. 3314–3322. DOI: 10.1158/0008-5472.CAN-08-0132.

Orieux, G.; Slembrouck, A.; Bensaid, M.; Sahel, J-A; Goureau, O. (2015): The protein tyrosine phosphatase interacting protein 51 (PTPIP51) is required for the differentiation of photoreceptors. In: *Neuroscience* 300, S. 276–285. DOI: 10.1016/j.neuroscience.2015.05.028.

Ota, Toshio; Suzuki, Yutaka; Nishikawa, Tetsuo; Otsuki, Tetsuji; Sugiyama, Tomoyasu; Irie, Ryotaro et al. (2004): Complete sequencing and characterization of 21,243 full-length human cDNAs. In: *Nature genetics* 36 (1), S. 40–45. DOI: 10.1038/ng1285.

Paillusson, Sébastien; Gomez-Suaga, Patricia; Stoica, Radu; Little, Daniel; Gissen, Paul; Devine, Michael J. et al. (2017): α-Synuclein binds to the ER-mitochondria tethering protein VAPB to disrupt Ca(2+) homeostasis and mitochondrial ATP production. In: *Acta neuropathologica* 134 (1), S. 129–149. DOI: 10.1007/s00401-017-1704-z.

Patergnani, Simone; Missiroli, Sonia; Marchi, Saverio; Giorgi, Carlotta (2015): Mitochondria-Associated Endoplasmic Reticulum Membranes Microenvironment. Targeting Autophagic and Apoptotic Pathways in Cancer Therapy. In: *Frontiers in oncology* 5, S. 173. DOI: 10.3389/fonc.2015.00173.

Peiró, G.; Ortiz-Martínez, F.; Gallardo, A.; Pérez-Balaguer, A.; Sánchez-Payá, J.; Ponce, J. J. et al. (2014): Src, a potential target for overcoming trastuzumab resistance in HER2-positive breast carcinoma. In: *British journal of cancer* 111 (4), S. 689–695. DOI: 10.1038/bjc.2014.327.

Petri, M. K.; Brobeil, A.; Planz, J.; Bräuninger, A.; Gattenlöhner, S.; Nestler, U. et al. (2015): PTPIP51 levels in glioblastoma cells depend on inhibition of the EGF-receptor. In: *Journal of neuro-oncology* 123 (1), S. 15–25. DOI: 10.1007/s11060-015-1763-8.

Petri, M. K.; Koch, P.; Stenzinger, A.; Kuchelmeister, K.; Nestler, U.; Paradowska, A. et al. (2011): PTPIP51, a positive modulator of the MAPK/Erk pathway, is upregulated in glioblastoma and interacts with 14-3-3β and PTP1B in situ. In: *Histology and histopathology* 26 (12), S. 1531–1543. DOI: 10.14670/HH-26.1531.

Porsche, Alexandra (2002): Identifikation von Interaktionspartnern der T-Zell-Protein-Tyrosin-Phosphatase durch das LexA-two-hybrid-System. 1. Aufl. Allensbach: UFO, Atelier für Gestaltung und Verl. (UFO-Dissertation, Bd. 414).

Rao, Meghana; Song, Wenqiang; Jiang, Aixiang; Shyr, Yu; Lev, Sima; Greenstein, David et al. (2012): VAMP-associated protein B (VAPB) promotes breast tumor growth by modulation of Akt activity. In: *PloS one* 7 (10), e46281. DOI: 10.1371/journal.pone.0046281.

Rowland, Ashley A.; Voeltz, Gia K. (2012): Endoplasmic reticulum-mitochondria contacts. Function of the junction. In: *Nature reviews. Molecular cell biology* 13 (10), S. 607–625. DOI: 10.1038/nrm3440.

Shimobayashi, Mitsugu; Hall, Michael N. (2014): Making new contacts: the mTOR network in metabolism and signalling crosstalk. In: *Nature reviews. Molecular cell biology* 15 (3), S. 155–162. DOI: 10.1038/nrm3757.

Slamon, D.; Clark, G.; Wong, S.; Levin, W.; Ullrich, A.; McGuire, W. (1987): Human breast cancer. Correlation of relapse and survival with amplification of the HER-2/neu oncogene. In: *Science* 235 (4785), S. 177–182. DOI: 10.1126/science.3798106.

Slamon, Dennis; Eiermann, Wolfgang; Robert, Nicholas; Pienkowski, Tadeusz; Martin, Miguel; Press, Michael et al. (2011): Adjuvant Trastuzumab in HER2-Positive Breast Cancer. In: *The New England Journal of Medicine* 365 (14), S. 1273–1283. DOI: 10.1056/NEJMoa0910383.

Stenzinger, Albrecht; Kajosch, Tobias; Tag, Claudia; Porsche, Alexandra; Welte, Inka; Hofer, Hans Werner et al. (2005): The novel protein PTPIP51 exhibits tissue- and cell-specific expression. In: *Histochemistry and cell biology* 123 (1), S. 19–28. DOI: 10.1007/s00418-004-0732-7.

Stenzinger, Albrecht; Schreiner, Dietmar; Koch, Philipp; Hofer, Hans-Werner; Wimmer, Monika (2009): Cell and molecular biology of the novel protein tyrosine-phosphatase-interacting protein 51. In: *International review of cell and molecular biology* 275, S. 183–246. DOI: 10.1016/S1937-6448(09)75006-3.

Stenzinger, Albrecht; Schreiner, Dietmar; Pfeiffer, Thorsten; Tag, Claudia; Hofer, Hans Werner; Wimmer, Monika (2006): Epidermal growth factor-, transforming growth factor-beta-, retinoic acid- and 1,25-dihydroxyvitamin D3-regulated expression of the novel protein PTPIP51 in keratinocytes. In: *Cells, tissues, organs* 184 (2), S. 76–87. DOI: 10.1159/000098949.

Stoica, Radu; Paillusson, Sébastien; Gomez-Suaga, Patricia; Mitchell, Jacqueline C.; Lau, Dawn Hw; Gray, Emma H. et al. (2016): ALS/FTD-associated FUS activates GSK-3β to disrupt the VAPB-PTPIP51 interaction and ER-mitochondria associations. In: *EMBO reports* 17 (9), S. 1326–1342. DOI: 10.15252/embr.201541726.

Stoica, Radu; Vos, Kurt J. de; Paillusson, Sebastien; Mueller, Sarah; Sancho, Rosa M.; Lau, Kwok-Fai et al. (2014): ER-mitochondria associations are regulated by the VAPB-PTPIP51 interaction and are disrupted by ALS/FTD-associated TDP-43. In: *Nature communications* 5, S. 3996. DOI: 10.1038/ncomms4996.

Tao, ZiQi; Shi, Aimin; Lu, Cuntao; Song, Tao; Zhang, Zhengguo; Zhao, Jing (2015): Breast Cancer. Epidemiology and Etiology. In: *Cell biochemistry and biophysics* 72 (2), S. 333–338. DOI: 10.1007/s12013-014-0459-6.

Tonks, Nicholas K.; Muthuswamy, Senthil K. (2007): A brake becomes an accelerator: PTP1B--a new therapeutic target for breast cancer. In: *Cancer cell* 11 (3), S. 214–216. DOI: 10.1016/j.ccr.2007.02.022.

Vos, Kurt J. de; Morotz, Gabor M.; Stoica, Radu; Tudor, Elizabeth L.; Lau, Kwok-Fai; Ackerley, Steven et al. (2012): VAPB interacts with the mitochondrial protein PTPIP51 to regulate calcium homeostasis. In: *Human molecular genetics* 21 (6), S. 1299–1311. DOI: 10.1093/hmg/ddr559.

Waelchli, Rudolf; Bollbuck, Birgit; Bruns, Christian; Buhl, Thomas; Eder, Jörg; Feifel, Roland et al. (2006): Design and preparation of 2-benzamido-pyrimidines as inhibitors of IKK. In: *Bioorganic & medicinal chemistry letters* 16 (1), S. 108–112. DOI: 10.1016/j.bmcl.2005.09.035.

Wee, Ping; Wang, Zhixiang (2017): Epidermal Growth Factor Receptor Cell Proliferation Signaling Pathways. In: *Cancers* 9 (5). DOI: 10.3390/cancers9050052.

Yu, Chuanfei; Han, Wenling; Shi, Taiping; Lv, Bingfeng; He, Qihua; Zhang, Yanfei et al. (2008): PTPIP51, a novel 14-3-3 binding protein, regulates cell morphology and motility via Raf-ERK pathway. In: *Cellular signalling* 20 (12), S. 2208–2220. DOI: 10.1016/j.cellsig.2008.07.020.

Zhang, Siyuan; Huang, Wen-Chien; Li, Ping; Guo, Hua; Poh, Say-Bee; Brady, Samuel W. et al. (2011): Combating trastuzumab resistance by targeting SRC, a common node downstream of multiple resistance pathways. In: *Nature medicine* 17 (4), S. 461–469. DOI: 10.1038/nm.2309.

Zhang, Wei; Liu, Hui Tu (2002): MAPK signal pathways in the regulation of cell proliferation in mammalian cells. In: *Cell research* 12 (1), S. 9–18. DOI: 10.1038/sj.cr.7290105.

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List of further Publications

Dietel E, Brobeil A, Gattenlöhner S, Wimmer M. The Importance of the Right Framework: Mitogen-Activated Protein Kinase Pathway and the Scaffolding Protein PTPIP51. *Int J Mol Sci*. 2018;19(10):3282. Published 2018 Oct 22. doi:10.3390/ijms19103282

Brobeil A, Chehab R, **Dietel E**, Gattenlöhner S, Wimmer M. Altered Protein Interactions of the Endogenous Interactome of PTPIP51 towards MAPK Signaling. *Biomolecules*. 2017;7(3):55. Published 2017 Jul 21. doi:10.3390/biom7030055

Brobeil A, **Dietel E**, Gattenlöhner S, Wimmer M. Orchestrating cellular signaling pathways-the cellular "conductor" protein tyrosine phosphatase interacting protein 51 (PTPIP51). *Cell Tissue Res*. 2017;368(3):411–423. doi:10.1007/s00441-016-2508-5

Poster presentation

Dietel E, Brobeil A, Wimmer M (2016). Crosstalks of the PTPIP51 interactome revealed by the novel small molecule LDC3 in Her2 breast cancer cells. 20th Joint Meeting of the Signal Transduction Society, Weimar

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