Mapiranje i funkcionalna karakterizacija porodice receptora tirozin kinaze ErbB2

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Mapping and functional characterization of the ErbB receptor tyrosine kinase family using the Mammalian Membrane Two-Hybrid Assay

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Mapping and functional characterization of the ErbB receptor tyrosine kinase family using the Mammalian Membrane Two-Hybrid Assay

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Around 30% of all proteins in cells are membrane proteins, which have important biological functions. Receptor tyrosine kinases (RTKs) are a family of transmembrane receptors that mediate different cell responses to extracellular stimulus. One of the most studied subfamilies of RTKs, the ErbB family, is related to many human diseases and members are targets for the development of new therapeutics. The ErbB family consists of four members (ErbB1-4) that share similar protein structures. Upon ligand binding to the extracellular domain, two molecules of receptor dimerize, which leads to activation of the intracellular kinase domain and autophosphorylation of the C-terminal tail. The resulting phosphotyrosines are recruitment sites for proteins that typically bind through Src homology-2 (SH2) or phosphotyrosine-binding (PTB) domains and provide specific stimulation of downstream pathways. Molecular mechanisms implicated in disease pathology commonly include aberrant protein-protein interactions (PPIs), which make the understanding of PPIs very significant for developing effective therapies. The aim of this study was to map and characterize interactions between the ErbB2 receptor tyrosine kinase and proteins which contain SH2 and/or PTB domains, using the MaMTH assay, a novel proteomics approach suitable for mapping interactions of membrane proteins. Thirty-six interacting proteins were identified, binding through the SH2 domain was characterised for ErbB2-Sla2 and ErbB2-Crk2, and potential PPIs implicated in aberrant signalling were detected.

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Key words: ErbB2, PPI, SH2 domain, PTB domain, MaMTH

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Mapiranje i funkcionalna karakterizacija porodice receptora tirozinske kinaze ErbB2

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Membranski proteini čine oko 30% svih proteina u stanici i imaju važnu biološku ulogu. Receptori tirozinskih kinaza (RTK) su transmembranski receptori koji sudjeluju u različitim staničnim odgovorima na izvanstanične signale. Porodica ErbB je jedna od najistaživanijih skupina RTK i povezana je s razvojem mnogih ljudskih bolesti. Sastoji se od četiri člana ErbB1-4 koji dijele zajedničku proteinsku strukturu. Vezanjem liganda za izvanstaničnu domenu, dolazi do dimerizacije dvije molekule receptora, aktivacije unutarstanične kinazne domene i autofosforilacije C-terminalnog repa. Fosforilirani tirozinski ostaci su mjesto vezanja mnogih unutarstaničnih proteina i posreduju aktivaciji nizvodnih signalnih puteva. Proteini koji prepoznaju fosforilirane tirozinske ostatke sadrže Src homolognu domenu 2 (SH2) i/ili fosfotirozin veznu domenu (PTB). Protein protein interakcije (PPI) ovise o različitim staničnim uvjetima i često su uključene u patologiju bolesti, što čini istraživanja PPI ključnim za razvoj novih terapija. Cilj ovog istraživanja je bio mapirati i funkcionalno karakterizirati interakcije između receptora ErbB2 i proteina koji sadrže domene SH2 i/ili PTB pomoću *Mammalian Membrane Two-Hybrid* (MaMTH) testa. Tijekom ovog rada detektirano je trideset i šest proteina koji stupaju u interakciju s receptorom ErbB2, ispitano je vezanje proteina Sla2 i Crk2 kroz domenu SH2 i otkriveni su potencijalni preferencijalni interakcijski partneri koji se vežu za onkogene varijante proteina ErbB2.

(71 stranica, 13 slika, 23 tablica, 42 literaturnih navoda, jezik izvornika: engleski)

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Ključne riječi: ErbB2, PPI, SH2 domain, PTB domain, MaMTH

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1. INTRODUCTION

1.1 Receptor tyrosine kinases

Receptor tyrosine kinases (RTKs) are membrane proteins that are involved in important cellular processes, such as proliferation, differentiation, cell migration, cell survival, metabolism and cell cycle control. The human genome harbors 58 known members of the RTK family, which are divided into 20 subfamilies (Figure 1). They all share similar molecular structures and mechanisms of action. They contain a ligand binding region, located in the extracellular domain, a single transmembrane helix, and a cytoplasmic region with the tyrosine kinase domain plus carboxy (C-) terminal and juxtamembrane regulatory regions (Lemmon and Schlessinger, 2010).

Upon ligand binding, self-association of the extracellular domains leads to dimerization of two receptor molecules. This is followed by activation of the tyrosine kinase domains and intracellular autophosphorylation events. In most cases, phosphorylated tyrosine residues are located in the C-terminal tail of the receptor, the juxtamembrane region or the kinase insert region, which are readily accessible to downstream signalling proteins. The resulting phosphotyrosines function as specific sites for the assembly of downstream signalling molecules that are recruited to the receptor (Hubbard and Miller, 2007; Lemmon and Schlessinger, 2010; Regad, 2015). These proteins can be enzymes, regulatory molecules, or adaptors that typically bind through Src homology-2 (SH2) or phosphotyrosine-binding (PTB) domains and will activate downstream signalling cascades (Schlessinger and Lemmon, 2003; Yao *et al.*, 2017).

Numerous human diseases result from genetic changes or abnormalities that alter RTK activity, abundance, cellular distribution or regulation. Constitutively active RTKs cause activation of intracellular signalling pathways that can result in increased cell proliferation, survival, invasion, metastasis and overall aberrant cell function. Mutated RTKs have been connected to cancers, diabetes, inflammation, severe bone disorders, arteriosclerosis and angiogenesis. Aberrant RTK activation in human cancers is mediated by four principal mechanisms: autocrine activation, chromosomal translocations, RTK overexpression, or gain-of-function mutations (Regad, 2015). Understanding the molecular mechanisms of RTK signalling and regulation is critical to establish pathophysiological features of many diseases and to develop new generations of drugs targeting RTKs or components of their signalling pathways (Yao *et al.*, 2017).

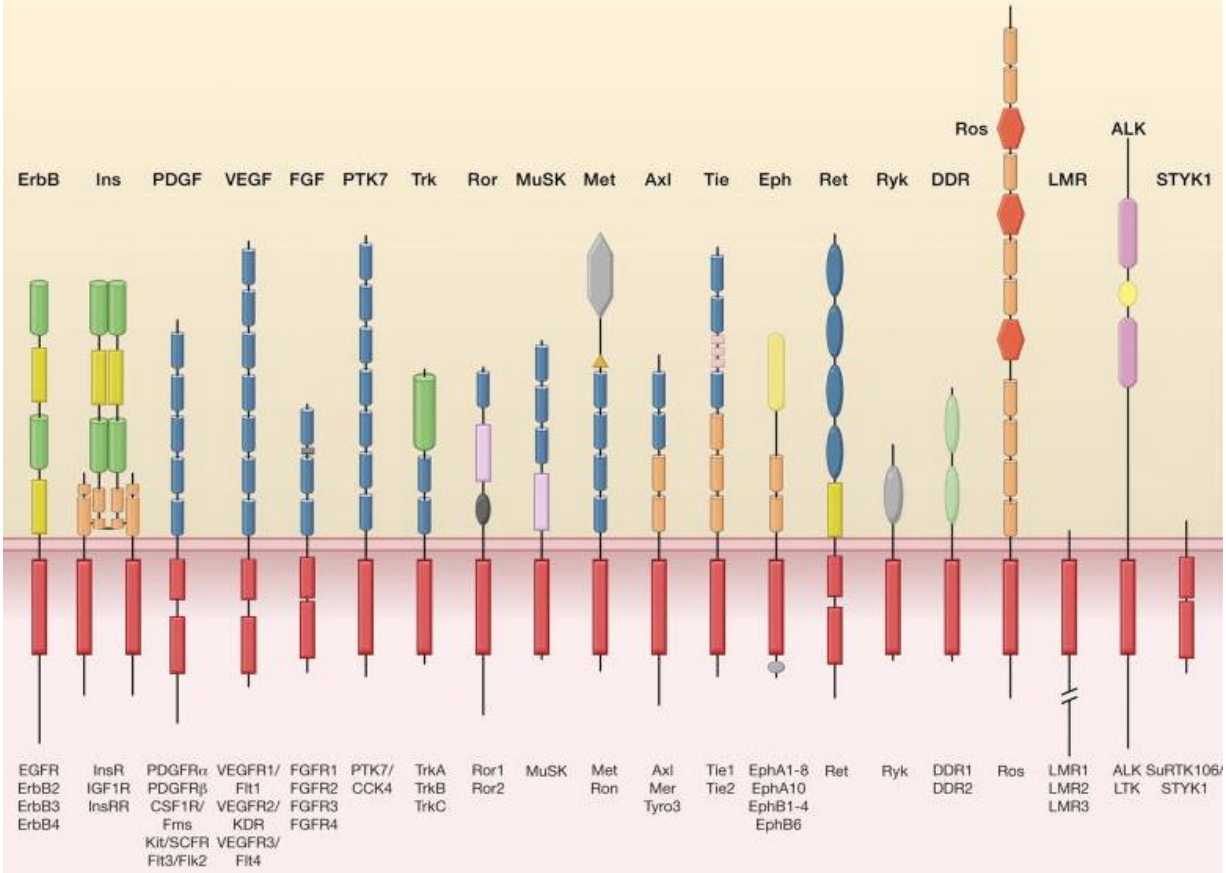


Figure 1. The schematic architecture of receptor tyrosine kinase families. Human receptor tyrosine kinases (RTKs) contain 20 subfamilies with 58 RTKs listed beneath each receptor family (adapted from Lemmon and Schlessinger, 2010).

1.2 The ErbB family of receptor tyrosine kinases

The human epidermal growth factor receptor (ErbB or EGFR) family belongs to large group of receptor tyrosine kinases (RTKs) that control many cellular processes as response to extracellular stimuli. The ErbB family consists of four members: ErbB1 (EGFR, HER1), ErbB2 (HER2, neu in rodents), ErbB3 (HER3) and ErbB4 (HER4). This family of receptors is expressed in epithelial, mesenchymal and neuronal cells and their cellular progenitors. Mice deficient in ErbB family members display a wide range of phenotypes connected with cell differentiation, proliferation, migration and survival, and null mutation of ErbB genes results in embryonic or perinatal lethality. Multiple signal transduction pathways are activated by ErbB family members,

such as the phosphatidylinositol 3-kinase (PI3K)/Akt (PKB) pathway that plays an important role in mediating cell survival, the Ras/Raf/MEK/ERK1/2 and phospholipase C (PLCγ) pathways that participate in cell proliferation, the STAT pathway that has an important role in maintaining epithelial cell polarity and adhesion, and the Par6-atypical PKC pathway important in epithelial organisation. In addition to important signalling roles from the cell membrane, there is also evidence that these receptors are translocated to the nucleus where they influence gene transcription. The ErbB proteins up-regulate many genes including cyclin D1, b-myb, cyclooxygenase-2, and the genes of iNOS/NO pathway (Wieduwilt and Moasser, 2008; Roskoski, 2014).

The ErbB family members are structurally related single chain transmembrane glycoproteins consisting of an extracellular ectodomain, a transmembrane domain, a short juxtamembrane section, a tyrosine kinase domain and a tyrosine-containing C-terminal tail (Figure 2). The general mechanism for activation of the ErbB family is the same as for all RTKs: ligands bind to extracellular domain of two receptors and induce formation of the activated state, whereby the cytoplasmic kinase domains phosphorylate tyrosine residues and creating docking sites for diverse proteins included in downstream signalling. Seven ligands bind to ErbB1, two bind to ErbB3 and seven bind to ErbB4. ErbB2 lacks its own ligand. All the ligands are expressed as cell membrane-anchored proteins that are proteolytically processed to realise soluble molecules. They act over short distances from their sites of generation and may act in an autocrine, juxtacrine or paracrine fashion. An extracellular ligand-binding domain is divided into four parts: domains I and II, which are leucine-rich repeats that participate in ligand binding, and domains III and IV, which have numerous cysteine residues that participle in disulfide bond formation. Ligand binding to the ectodomain of the receptor promotes homo- and heterodimer formation between two receptor molecules, which is crucial for activation of the intracellular tyrosine kinase domain and phosphorylation of the C-terminal tail. Crystal structures of receptors show a “tethered” inactive conformation, in which the beta-hairpin or dimerization loop of domain II interacts with the tethering arm of domain IV. The bivalent ligand binding promotes a conformational change where dimerization loop of one receptor molecule interacts with dimerization loop of second receptor molecule leading to an extended form (Wieduwilt and Moasser, 2008; Lemmon and Schlessinger, 2010; Roskoski, 2014; Purba, Saita and Maruyama, 2017). ErbB1 and ErbB4 can be considered as fully functional receptors with the ability to both bind ligands and autophosphorylate C-terminal

tails through functional intracellular tyrosine kinase activity. ErbB2 does not bind to any growth factor, but it exists in an extended and open conformation, in which the dimerization loop is exposed. Unbound ErbB2 is available for dimerization with other ErbB family members and it is the favored dimerization partner (Wieduwilt and Moasser, 2008; Roskoski, 2014), although active ErbB1 and ErbB4 homodimers can form. ErbB2 homodimers are unlikely, but induced overexpression leads to formation of a functional homodimer. ErbB1/2/4 possess protein kinase activity, while ErbB3 is kinase impaired. ErbB1/2/4 share conserved catalytically important residues within the kinase domain. ErbB3 homodimers fail to stimulate protein kinase activity and downstream signalling, and pseudokinase ErbB3 possesses just 0.1% of the autophosphorylation activity of ErbB1 (Roskoski, 2014).

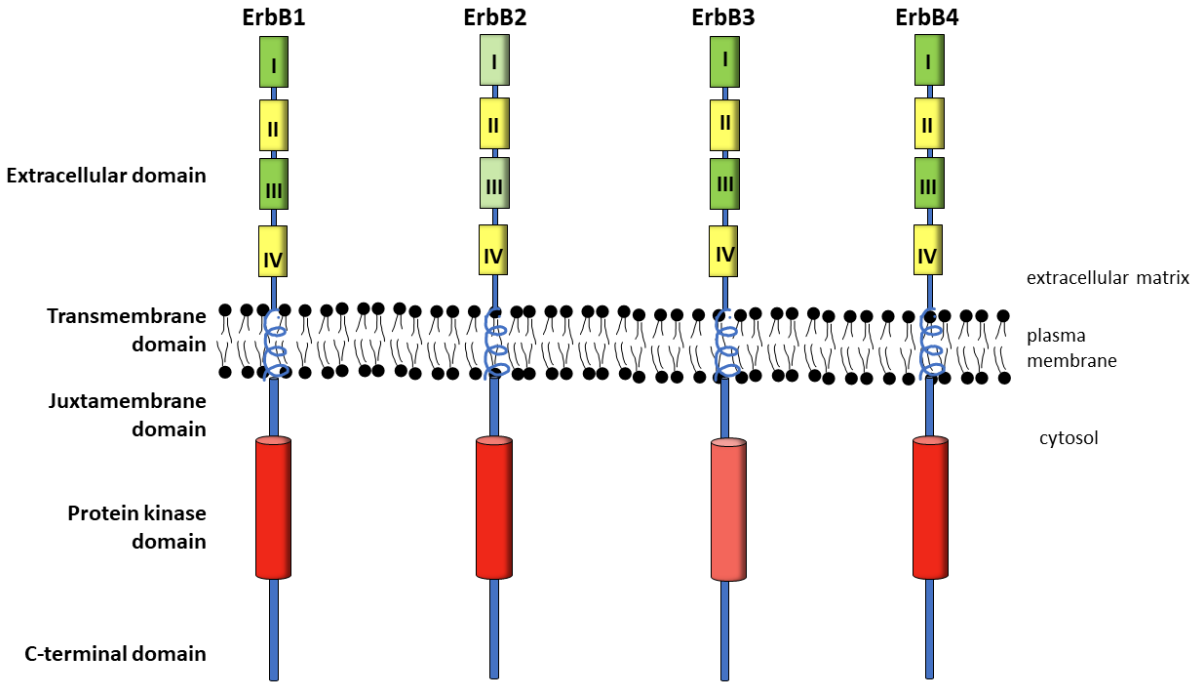


Figure 2. The schematic structure of ErbB receptors. The extracellular domain is structurally divided into domains I and III that directly interact with ligand (except for ErbB2) and domains II and IV which form dimers. Short transmembrane and juxtamembrane domains link the extracellular domain to the tyrosine kinase domain and the C-terminal tail. The kinase domain of ErbB3 is kinase-impaired. The extracellular domain of ErbB2 does not bind ligands.

1.2.1 ErbB family in cancer

Several malignancies are associated with aberrant signalling through the ErbB family of receptors. Overexpression and mutation of ErbB family members have been implicated in different type of cancers including lung, breast, stomach, colon, ovary, prostate, kidney, head, pancreatic and brain cancers. Early evidence for the role of ErbB receptors as protooncogenes came from identification of the rodent homolog *neu* oncogene that induces neuroblastomas in rats (Wieduwilt and Moasser, 2008; Lemmon and Schlessinger, 2010). Principle mechanisms of oncogenic activation of ErbB receptors have been identified; amplification and overexpression, molecular alterations and inhibition of phosphatase activity (Herter-Sprie, Greulich and Wong, 2013), leading to aberrant activation of downstream pathways, thereby promoting cell proliferation, survival, invasion and metastasis. Lung cancer is the most common type of cancer in the wold, and 85% cases are non-small cell lung cancer (NSCLC). ErbB1 has an important role in the pathogenesis of NSCLC where it is amplified in 5-10%, overexpressed in 5-20% and mutated in 10-40% of cases (Wieduwilt and Moasser, 2008; Roskoski, 2014; Regad, 2015). Gene amplification of ErbB2 and receptor overexpression have been reported in 20-30% of breast cancers. Breast cancer is the leading cause of female cancer deaths globally and ErbB2 amplification is associated with decreased survival and shorter relapse time (Moasser, 2007; Wieduwilt and Moasser, 2008; Lemmon and Schlessinger, 2010; Roskoski, 2014; Regad, 2015).

Molecular target therapeutics have been developed and approved by the US Food and Drug Administration (FDA) for threating aberrant ErbB receptors in cancers and other diseases. These drugs are divided into two categories: small molecule inhibitors that target the intracellular tyrosine kinase domain and monoclonal antibodies that target the extracellular domain. Development of drug resistance is a common event in many cases, for example a new activating mutation overcoming the drug-mediated inhibition of receptors (Lemmon and Schlessinger, 2010). Substitution T798M in the kinase domain of ErbB2 is a gatekeeper mutation allowing continued proliferation of breast cancer cells in the presence of the small molecule inhibitor Lapatinib or the monoclonal antibody Trastuzumab (Herter-Sprie, Greulich and Wong, 2013; Rexer *et al.*, 2013). Another interesting oncogenic form of ErbB2 protein is an alternative splice form containing an in-frame deletion of 48 bp of exon 16 in the extracellular domain. This deletion occurs in the same region that is muted in the *neu* oncogene in mice. ErbB2 ex16del appears to be a more aggressive variant compared to wt ErbB2 and this transcript represents about 9% of ErbB2 transcripts in breast

carcinomas. This deletion in the extracellular domain leads to an altered conformation that promotes intermolecular disulfide bonding and active ErbB2 homodimers. ErbB2 ex16del has also been implicated in resistance of HER2 positive tumors to anti-ErbB2 therapies (Castiglioni *et al.*, 2006; Moasser, 2007; Jackson *et al.*, 2013).

1.3 Src homology 2 and phosphotyrosine-binding domains

Upon the activation of RTKs, the autophosphorylated tyrosine residues become platforms for the recruitment of different signalling proteins. The specific interactions of effector proteins that contain phosphotyrosine binding motifs activate signalling pathways, such as the Ras-mitogen activated protein kinase (MAPK), phosphoinositide-3- kinase (PI3K)- Akt, and phospholipase C-γ (PLC-γ) pathways involved in different cell processes (Wagner *et al.*, 2013). Src homology region 2 (SH2) and phosphotyrosine-binding (PTB) domains are identified as small protein modules of 50 to 200 amino acids that recognize phosphorylated tyrosine (pTyr) in RTKs and other signalling proteins. They are found in a diverse set of proteins and provide specific stimulation of downstream pathways. SH2 domain-containing proteins bind to their target according to the specific phosphorylated tyrosine, and most PTB domains proteins bind constitutively (Schlessinger and Lemmon, 2003).

SH2 domains were first defined as conserved sequences that regulate the tyrosine kinase activity of v-FPS and members of the Src kinase family, and they have important roles in mediating cell signalling by RTKs and non-receptor protein tyrosine kinases (Schlessinger and Lemmon, 2003). For some SH2 domains, crystal structures have been solved and typically they consist of three or four β-strands and two α-helices. A positively charged binding pocket on the surface has a highly conserved FLVR motif with a critical arginine residue that binds the pTyr of target ligands. The affinity of an SH2 domain for pTyr depends on the amino acid sequence surrounding the pTyr residue (Wagner *et al.*, 2013). Proteins that contain SH2 domains belong to different protein families according to their function, including enzymes (*e.g.* kinases, phosphatases, guanine nucleotide exchange factors, GTPase activating proteins), adaptors, scaffold proteins, signal regulators and transcription factors (Schlessinger and Lemmon, 2003).

PTB domains appear to share the same folding pattern, termed the pleckstrin homology (PH) domain "superfold". They are composed of two β-strands capped by α-helix that together make up a highly conserved ligand-binding pocket. The NPXY motif is commonly present among PTB

domain substrates and is considered the canonical binding motif. pTyr binding is not required for all PTB domains, and in fact the binding of most PTB domains is independent of pTyr. Because PTB domains are more diverse in sequence than SH2 domains, they have more diverse functions (Schlessinger and Lemmon, 2003; Wagner *et al.*, 2013).

1.4 Methods for analysing protein-protein interaction

Cells are the basic unit of life, composed of multiple diverse biomolecular systems. Proteins are components of these systems with many different and important functions such as molecular machines, sensors, transporters and structural elements (Snider *et al.*, 2015). Many biological processes are sustained by protein-protein interactions and identifying these interactions is very important for understanding cellular function and mechanisms, and for developing therapies and drugs for treatment of human diseases. To date, only around 10% of protein-protein interactions (PPIs) in human cells are known (Kotlyar *et al.*, 2015). For characterization of PPIs and to map interactomes, many methods have been developed. Biochemical, genetic and cell biological approaches have been used for identification of a large number of PPIs, and while numerous methods are available, each of them has advantages and limitations. It is important to select a suitable method for studying interacting partners of the protein of interest, and to validate detected PPIs with another technique.

The classic genetic approach first developed was yeast two-hybrid (Y2H), which is still one of the most popular PPI identification methods. It is a protein complementation assay based on the physical separation of two functional domains of transcription factors that are fused to candidate interacting proteins. Upon interaction, both domains work together as a functional transcription factor leading to expression of a reporter gene. Y2H is used for detection of binary interactions in both large scale screening studies and smaller efforts investigating specific PPIs, but it is not suitable for probing interactions of membrane proteins (Fields and Song, 1989; Snider *et al.*, 2015). Hence, the membrane yeast two-hybrid (MYTH) assay was designed for analysis of the interactions of membrane proteins. It is based on split ubiquitin approach, where the ubiquitin protein is divided into two fragments, each fused to one of the proteins of interest. The C-terminal ubiquitin moiety is also fused to an artificial transcription factor. Interaction of bait and prey protein brings the ubiquitin moieties into close proximity, and allows deubiquitinating enzymes to cleave and release the artificial transcription factor, which activates a reporter system (Stagljar *et al.*, 1998). For both Y2H and MYTH, the biggest disadvantages are problems associated with the

expression, modification and interaction of non-native proteins in the yeast host (Snider *et al.*, 2015).

A widely used biochemical method for detecting PPIs is co-immunoprecipitaion (Co-IP). Cell lysates are generated and the protein complex is precipitated using the antibody against the protein of interest fixed to a solid support. Bound proteins are eluted and analysed (Phizicky and Fields, 1995). Disadvantages include that proteins are not in a native cell environment, and it is hard to isolate membrane proteins. The luminescence-based mammalian interactome mapping (LUMIER) assay is a high-throughput technology based on Co-IP. LUMIER was developed for detecting pathway-specific, posttranslational modification dependent PPIs, constitutive protein interactions and interactions involving transmembrane receptors in mammalian cells. In this method, *Renilla* luciferase enzyme is fused to proteins of interest, which are then coexpressed with individual FLAG-tagged partners in the desired cell line (Barrios-Rodiles *et al.*, 2005). A major disadvantage of the LUMIER method because is based on Co-IP is that it requires cell lysis that can disrupt weak and transient PPIs and introduce potential artefacts (Snider *et al.*, 2015).

An important feature of PPIs is also the subcellular localization of protein complexes in living cells. New technologies have been developed for visualizing the interactions, including non-invasive methods using bioluminescence or fluorescence resonance energy transfer (BRET or FRET) and those centered on protein fragment complementation, such as bimolecular fluorescence complementation (BiFC). The basic principle of RET is transfer of energy from an excited donor to a nearby acceptor molecule. Donors molecules are fluorophores in FRET, whereas in BRET the donor is an enzyme that catalyse a bioluminescent substrate. Bait and prey proteins are fused on donor and acceptor molecules and if two proteins interact, energy transfer occurs, producing a signal. BiFC technique is based on generation of a fluorescent signal from two non-fluorescent fragments of fluorescent protein. The two halves are genetically fused to the proteins under study and upon interaction a fluorescent signal is detectable (Ciruela, 2008).

A popular technology for high-throughput protein interaction mapping is affinity purification-mass spectrometry (AP-MS). Affinity purification refers to capture of biological material via specific enrichment with ligand coupled to a solid support. For detection of PPIs, cells expressing a protein of interest are cultured and harvested allowing purification of the protein by an affinity reagent targeting the protein itself or an epitope tag. Once affinity-purified, captured proteins are digested with protease, resulting peptides are separated by liquid chromatography and identified

by mass spectrometry. AP-MS is a library-independent method and it can be used to analyse the same baits across multiple tissue or cell lines, especially important for studying differences in the interactomes of normal and disease states (Dunham et al., 2012).

Bioinformatics-based approaches are also used for computational PPI prediction. These tools help identify candidate proteins for high-throughput screens prior to experimental work, which may reduce the cost of interactome mapping. For example, FpClass is an *in silico* method to predict high-confidence PPIs proteome-wide. The disadvantage is that the estimated false positive rate is 60% (Kotlyar *et al.*, 2015).

The recently developed Mammalian Membrane Two-Hybrid (MaMTH) assay is a protein-complementation technique for detecting membrane protein interactions in mammalian cells. In comparison to the methods above, MaMTH is suitable for detecting transient and modification-dependent PPIs in response to different cellular conditions (Petschnigg *et al.*, 2014). This method was used for the following work.

1.4.1 The Mammalian Membrane Two-hybrid Assay

The Mammalian Membrane Two-Hybrid (MaMTH) assay was first published by Julia Petschnigg *et al*. in Nature Methods and developed in Igor Stagljar's group as a split ubiquitin two-hybrid approach to study PPIs of full-length integral membrane or membrane-associated proteins in mammalian cells (Petschnigg *et al.*, 2014; Saraon *et al.*, 2017).

MaMTH is based on previously described split ubiquitin approaches (Johnsson and Varshavsky, 1994) and is derived from the membrane yeast two-hybrid (MYTH) assay (Stagljar *et al.*, 1998). A membrane bait protein of interest is tagged with C-terminal moiety of ubiquitin (Cub) followed by a chimeric transcription factor (TF), and cytosolic or membrane-bound prey proteins have the N-terminal unit of ubiquitin (Nub) bound. The proteins can be C- or N-tagged depending on the orientation and nature of the protein, and this has to be decided based on previous work or experimentally. The principle behind MaMTH is described in Figure 3. Upon bait and prey interaction, pseudo-ubiquitin is formed by the association of Nub and Cub. Deubiquitinating enzymes (DUBs) are recruited, leading to a proteolytic cleavage whereby the TF gets released. The reporter signal is activated by the TF which enters the nucleus and binds the Upstream Activating Sequence (UAS) or operator. Different TFs can be used depending on the reporter system, for example *lexA* or *GAL4*-based. Current MaMTH assays are all carried out in HEK 293

cells stably expressing a reporter construct either containing eight *lexA* operator repeats or five *GAL4* UAS repeats, followed by the reporter gene (Petschnigg *et al.*, 2014; Saraon *et al.*, 2017). In the work described in this thesis, the transcription factor used was comprised of amino acids 1-147 of the GAL4 protein (DNA binding domain) fused to amino acids 364-550 of mouse NF-κB.

To express prey and bait in the MaMTH reporter cells there are four possible options. First is transient transfection with both bait and prey. The second variation is stable expression of the bait in the cell line followed by transient transfection of the prey DNA. The third option is to have a stably expressed prey and transiently expressed bait. The last option is stably expressed both bait and prey.

The cells being transfected contain stable integrated region for binding the TF. The output can be green fluorescent protein (GFP) or luciferase, which may be secreted *Gaussia* luciferase or intracellular Firefly luciferase. HEK 293 cells designated B0166 were used in the work described in this thesis, which have stably integrated GAL4 UAS repeats followed by *Gaussia* luciferase gene, and are engineered to stably express bait proteins of interest.

The expression of transiently-expressed prey protein can vary between different transfections. To minimize this influence, the prey construct contains red fluorescent protein (mCherry), which is separated from the prey coding region by a P2A sequence, and which gets translated at a level correlating with the expression of the protein of interest. The RFP levels can therefore be an indication of transfection efficiency.

An important advantage of MaMTH is that full length membrane protein interactions can be analysed directly in their natural cellular membrane context. Additional advantages are low cost, high scalability and ready transferability to virtually any cell line of interest. The assay is highly sensitive, making it suitable for both the measurement of weak/transient interactions and for monitoring dynamic, "condition-dependent" PPIs. Limitations of the assay include the requirement for the bait to be associated with the membrane or other intracellular structures, to prevent non-specific activation of reporter system. Also, the termini of the membranes proteins fused to Cub or Nub must be cytosolic, in order to provide access to the DUBs (Snider *et al.*, 2015; Saraon *et al.*, 2017). The method is best suited for the detection of binary PPIs. The MaMTH assay is a powerful tool for mapping the interaction networks of membrane proteins and characterizing how these interactions respond to different cellular conditions. MaMTH detects interaction changes in response to ligands, starvation conditions, different post translational modifications,

etc. Therefore, the method is suitable for monitoring phosphorylation-dependent interaction, drug-inhibited interactions or interaction patterns of wild type and mutant proteins (Petschnigg *et al.*, 2014; Petschnigg *et al.*, 2017). Mammalian cell-based assays that can identify changes in PPIs conferred by aberrant signalling pathways can help understand disease mechanisms and define new drug targets. The MaMTH assay has been recently modified as a high-throughput, small molecule screening platform. This approach offers a significant advantage, allowing for rapid identification of low toxicity, physiologically active compounds directly in the natural context of the living mammalian cell (Snider and Saraon *et al*., manuscript under review).

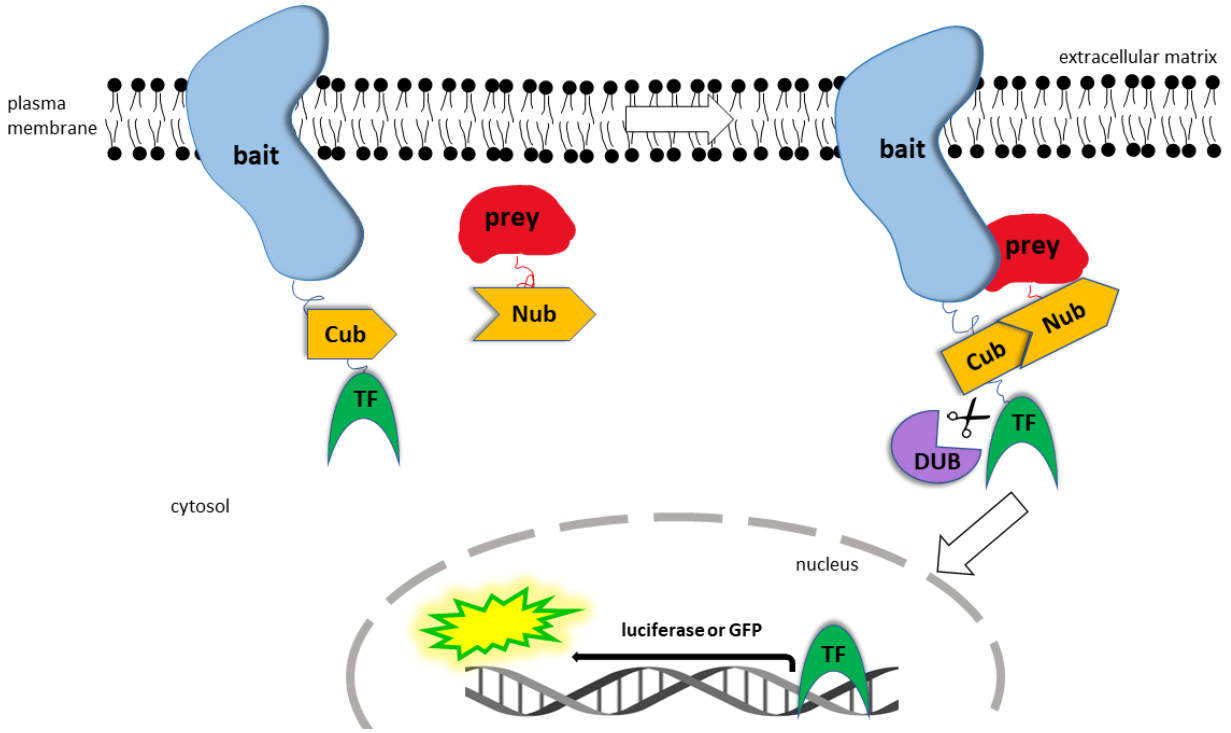


Figure 3. Schematic representation of the MaMTH system. Bait and prey proteins of interest are expressed in MaMTH reporter cells. A membrane bait protein is tagged with the C-terminal half of ubiquitin (Cub) followed by a chimeric transcription factor (TF) and cytosolic or membrane-bound prey protein has the N-terminal unit of ubiquitin (Nub) bound. The ubiquitin fragments remain inactive unless fused to two interacting proteins, whose proximity upon association drives reconstitution of the Cub and Nub into an active pseudo-ubiquitin molecule. This newly reconstituted ubiquitin is then target by human ubiquitin-specific proteases (DUBs) which cleave at the C-terminus of the Cub, releasing an artificial transcription factor (TF), which can then enter the nucleus and activate reporter gene transcription.

2. AIM OF THIS WORK

The aim of this study was to map and characterise interactions between the ErbB2 receptor tyrosine kinase and proteins which contain SH2 and/or PTB domains using the MaMTH assay. First, using site directed mutagenesis and Gateway cloning, mutant versions of ErbB2 receptor (ex16del, T798M and K753A) were generated, and these were used to create MaMTH reporter cell lines stably expressing bait proteins of interest. The tetracycline concentration required to induce expression of the wild type and three mutant baits had to be optimized and the activation level determined through Western blot. All cell lines had to be tested with MaMTH using known positive and negative interactors to validate the system. The interactome was built by a biased screen of a 98 SH2/PTB protein-containing library against ErbB2 wild type. Some interactions were further tested to characterize if the SH2 domain of different interacting proteins mediated the interaction. Interactors were tested using oncogenic mutants ErbB2 ex16del and ErbB2 T798M with constitutive activation, and phosphorylation dependent interactions had to be characterised using inactive mutant ErbB2 K753A.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and reagents

Table 1. List of chemical and company.

Chemical / reagent	Company
10 x TE (Tris HCl, EDTA) Buffer	Tekanova
20% sodium dodecyl sulfate (SDS) solution	BioShop
30% Acrylamide/Bis solution	BioRad
6 x DNA loading dye (LD)	Thermo Fisher Scientific
Acetic acid	BioShop
Agar	BioShop
Agarose	Invitrogen
Albumin	BioShop
Amersham ECL Selected Western Blotting Detection Reagent	GE Healthcare
Ammonium peroxydisulfate (APS)	Thermo Fisher Scientific
Calcium chloride ($CaCl_2$)	BioShop
Coelenterazine	NanoLight Technology
Complete tablets, protein inhibitor cocktail tablets	Roche
Coulter Isoton III Diluent	Beckman Coulter
Dimethyl Sulfoxide (DMSO)	Fisher Bioreagents
Dithiothreitol (DTT)	BioShop
Dpn1 (#R0176S) enzyme	NEB
Dulbecco's Modified Eagle Medium (DMEM)	Gibco
Dulbecco's Phosphate Buffered Saline (PBS)	Sigma Aldrich
Eagles Minimum Essential Media (Opti MEM)	Gibco

Ethanol	Commercial Alcohols
Ethylene glycol- bis (β-aminoethyl ether) - N,N,N',N'- tetraacetic acid (EGTA)	BioShop
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich
Fetal Bovine Serum (FBS)	Gibco
Gateway BP Clonase II enzyme mix	Invitrogen
Gateway LR Clonase II enzyme mix	Invitrogen
Glycerol	BioShop
Glycine	BioShop
Isopropyl alcohol	Colenda
KAPA Hifi PCR Kit	KAPA Biosystems
Lennox Broth (LB)	BioShop
N, N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES)	Sigma Aldrich
PageRuler Plus Prestained Protein Ladder	Thermo Fisher Scientific
Ponceau S	Sigma Aldrich
Proteinase K	Invitrogen
Sodium chloride (NaCl)	BioShop
Sodium orthovanadate	Santa Cruz Biotechonolgy
Super Signal West PICO PLUS Chemiluminescent substrate	Thermo Fisher Scientific
Sybr Safe DNA Gel Stain	Invitrogen
Tetramethylethylenediamine (TEMED)	Invitrogen
Tris	BioShop
TritonX 100	Sigma Aldrich
TrypLE Express	Gibco
Tween 20	Bioshop

Versene	Gibco
X-tremegene 9 DNA transfection reagents	Roche
β-glycerophosphate disodium salt hydrate	Sigma Aldrich

3.1.2 Antibiotics

Table 2. List of antibiotics, working concentration and company.

Antibiotics	**Working Concentration**	**Company**
Blasticidin	15 µg/ml	BioShop
Carbenicillin	100 µg/ml	BioShop
Hygromycin B solution	100 mg/ml	Sigma Aldrich
Penicillin /Streptomycin	(10000 U/ml) / (10000 µg/ml)	Gibco
Spectinomycin	50 µg/ml	BioShop
Tetracycline	1 mg/ml	BioShop

3.1.3 Molecular biological kits

Table 3. Molecular biological kits and company.

Kit	**Company**
GenepHlow Gel/PCR Kit	Geneaid
Pierce BCA Proteins Assay kit	Thermo Fisher Scientific
Presto Mini Plasmid Kit	Geneaid

3.1.4 Antibodies

Table 4. List of antibodies and company.

Antibody	**Company**
FLAG	Sigma Aldrich (#087K6002)

Goat anti-mouse	BioRad (#1721011)
Goat anti-rabbit	BioRad (#1721019)
HER2/ErbB2	Cell Signaling Technology (#2165)
P44/42 MAPK (Erk1/2)	Cell Signaling Technology (#9102S)
Phospho-HER2/ErbB2 (Tyr1221/1222)	Cell Signaling Technology (#2243S)
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	Cell Signaling Technology (#9101S)
pY99	Santa Cruz Biotechnology (#NC9466639)
V5- tag	Cell Signaling Technology (#13202S)
α - tubulin	Santa Cruz Biotechnology (#L1416)

3.1.5 Buffers and solutions

Table 5. Buffers/solution and composition.

Designation	**Composition**
BES (2x)	50 nM BES
	280 mM NaCl
	1.5 mM Na_2HPO_4
	pH 6.96
Cell culture medium	10 % FBS (v/v)
	1 % Penicillin/ streptomycin (v/v)
	DMEM

Cell lysis buffer H	50 mM, pH 7.3 β- glycerophosphate
	1.5 mM EGTA
	1 mM EDTA
	1 mM DTT
	0.1 mM orthovandate
	1 % TritonX 100
LB agar medium (1L)	20 g LB
	20 g agar
LB medium (1L)	20 g LB
Running buffer (10x)	250 mM Tris
	960 mM glycine
	1 % SDS
Sample buffer (4x)	40 % glycerol
	0.2 M Tris/HCl pH 6.8
	bromphenol blue
	8 % SDS
	0.1 M DTT
TBST buffer (1x)	20 mM Tris
	150 mM NaCl
	0.1% Tween20
	pH 7.6
Transfer buffer (10x)	150 mM Tris base
	1.2 M glycine

3.1.6 Laboratory equipment

Table 6. List of laboratory equipment, specification and manufacturer.

Designation	Specification	Manufacturer
Bacteria Incubator		VWR
Bacteria Incubator Shaker	Minitron	Infors HT
Biological Safety Cabinet	CLASS II Type A2	Microzone Corporation
Cell Counter	Coulter Z series	Beckman Coulter
Cell Culture incubator	Hera cell 150i	Thermo Fisher Scientific
Centrifuge	54150	Eppendorf
Centrifuge	Microfuge 18	Beckman Coulter
Centrifuge	5810R	Eppendorf
DNA gel electrophoresis apparatus	Mini-Sub Cell GT Cell	BioRad
Dry Baths	AccuBlock	Labnet International
Luminometer	Luminoskan Ascent	Thermo Labsystems
Microplate Reader	Synergy Neo	BioTek
Microscope	Vista Vision	VWR
Protein gel electrophoresis apparatus	Mini-PROTEAN Tetra Cell	BioRad
Spectrophotometer	NanoDrop ND 1000	Thermo Fisher Scientific
Thermal cycler	Mastercycler epgradient	Eppendorf
Water Bath		VWR
Western blotting apparatus	Criterion™ blotter	BioRad
X-ray developer	SRX-101A	Konica Minolta
X-ray film for immunoblot detection		Mandel Scientific

3.1.7 Cell lines and bacteria strains

Table 7. Bacteria strains.

Bacteria strains	Prepared
Chemically – competent *E. coli* DH5α	Inoue-Method (Sambrook and Green, 2012)

Table 8. List of cell lines and source.

Cell lines	Source
HEK 293 B0166 (MaMTH reporter cell line)	Made by Dr. Jamie Snider, Stagljar lab
HEK 293 B0166 stable expression of ErbB2	Made by Luka Drecun, Stagljar lab
HEK 293 B0166 stable expression of ErbB2 ex16del	Made during this work
HEK 293 B0166 stable expression of ErbB2 T798M	Made during this work
HEK 293 B0166 stable expression of ErbB2 K753A	Made during this work

3.1.8 Vectors

Table 9. List of vectors and source.

Name	Source
pDONR223 entry clone vector	Invitrogen
N-tagged mCherry MaMTH prey vector (A1245)	Built by Dr. Jamie Snider
C-tagged MaMTH bait vector (A1160)	Built by Dr. Jamie Snider
pOG44 Flp-In recombinase expression vector	Thermo Fisher Scientific

Vector maps are shown in Supplementary Figures 1S, 2S and 3S**.**

3.1.9 Software and databanks

Table 10. List of software, company and web address.

Software	Company	Web address
ApE	University of California, Sun	http://biologylabs.utah.edu/jorgensen/wayned/ape/
FpClass	Jurisca Lab, IBM Life Sciences Discovery Centre	http://dcv.uhnres.utoronto.ca/FPCLASS/
NCBI BLAST	National Center for Biotechonology Information	https://blast.ncbi.nlm.nih.gov/Blast.cgi
Primer X	Bioinformatics.Org	http://www.bioinformatics.org/primerx/

3.1.10 Oligonucleotides

Table 11. List of oligonucleotides, sequence and company.

Name	Sequence (5' → 3')	Company
ErbB2 attB site forward	GGGGACAAGTTTGTACAAAAAAGCAGG CTATGGAGCTGGCGGCCTTGTGCCGCT	EUROFINS MWG Operon
ErbB2 attB site reverse	GGGGACCACTTTGTACAAGAAAGCTGG GTGCACTGGCACGTCCAGACCCAGGTA C	EUROFINS MWG Operon
ErbB2 T798M forward	CGGTGCAGCTGGTGATGCAGCTTATGCC CTATG	EUROFINS MWG Operon
ErbB2 T798M reverse	CATAGGGCATAAGCTGCATCACCAGCT GCACCG	EUROFINS MWG Operon

ErbB2 K753A forward	CCAGTGGCCATCGCAGTGTTGAGGG	EUROFINS MWG Operon
ErbB2 K753A reverse	CCCTCAACACTGCGATGGCCACTGG	EUROFINS MWG Operon
SRMS R147A forward	CCTTCCTCATCGCGCCCAGCGAGAG	EUROFINS MWG Operon
SRMS R147A reverse	CTCTCGCTGGGCGCGATGAGGAAGG	EUROFINS MWG Operon
ErbB2 sequencing primer from 1184nt forward	AGTTTCCAGATGAGGAGGGC	EUROFINS MWG Operon

3.2 Methods

3.2.1 Cell culture

During cell culture work, good cell culture practices (GCCP) were followed.

The lab of Dr. Igor Stagljar has generated different HEK 293 reporter cell lines, one subtype is B0166, which has stable integrated *Gaussia princeps* luciferase under the control of a Gal4/UAS system. When expressed, *Gaussia* luciferase is secreted into the media. Stable cell lines of ErbB2 wild type, ErbB2 ex16del, ErbB2 T798M, ErbB2 K753A, were generated through the FLP-In TREx technique in B0166 cell subline as described in "Generation of MaMTH reporter cell lines stably expressing baits". Cell lines are Grip-tite, which means they overexpress the surface protein MSR1 that increases the adherence of the cells to tissue culture plates.

3.2.2 Cell passaging

Cells were cultured at 37 °C under an atmosphere of 5% CO_2 in 10 cm culture dishes and in culture media, DMEM ((+)4.5 g/L D-Glucose, (+) L-Glutamine, (+) 110 mg/L sodium pyruvate) with 10% FBS and 1% penicillin/streptomycin. Cells were passaged when confluent. Old media were aspirated, cells were rinsed once with pre-warmed PBS to remove leftover media and incubated for 5 min at ambient temperature with 1 ml versene, to weaken the attachment of the

cell to surface. The versene was removed and 1 ml of TrypLE was added, plates were incubated for 5 min at 37 °C to detach viable cells. Pre-warmed culture medium was added and TrypLE was inactivated. The cell suspension was transferred to a new culture dish and diluted with culture medium to a final volume of 10 ml. Fresh culture medium was added every three days until cells were ready for splitting.

3.2.3 Cell count

Cell count was determined with the Coulter Z Counter Series cell counter (particle count and size analysers). After treatment with TrypLE and resuspension, 500 µl cell suspension was added to 9.5 ml IsoFlow Sheath Fluid in a CasyCup. The cell count program used for HEK 293 cells is shown in Table 12.

Table 12. Set up of the Cell Counter Coulter Z Series.

Set up	100 µmC
Kd	65.54
Select units	µm
Set Upper Size T_U	30.00 µm
Set Lower Size T_L	10.00 µm
count mode	between

3.2.4 Generation of MaMTH reporter cell lines stably expressing baits

Isogenic MaMTH reporter cell lines stably expressing baits of interest were generated using the Flp-In TREx system (Thermo Fisher Scientific). HEK 293 B0166 cells (MaMTH reporter cells) were grown at 37 °C at 5 % CO_2 in culture media in 2 ml to 50-60 % confluency. Cells were transfected with 900 ng pOG44 and 100 ng of bait construct in A1160 vector using X-tremegene 9 transfection reagent as per manufacturer instructions. After 5 hr, media containing transfection reagent was removed and replaced with fresh culture media. Cells were grown for 48 hr and then split 1 in 2 into a new 6-well plate in 2 ml using culture media with 100 µg/ml Hygromycin and 150 µg/ml Blasticidin until individual foci appeared. Foci were expanded and correct, tetracycline induced bait expression was verified by Western blotting.

3.2.5 MaMTH Assay

Day 1: Cell seeding

Highly confluent (80-100%) cells were washed and treated with TrypLE as above. Cell concentration was measured with Coulter Z Series and 10,000 cells per well were seeded in clear, flat-bottom, tissue-culture 96 well plate and incubated 18 to 24 hr at 37 °C and 5% CO_2.

Day 2: Transfection

BES-$CaCl_2$ transfection was performed in biological triplicates. Per transfection, 4.5 µl H_20, 5 µl 2 X BES (280 mM), 50 ng prey DNA and 0.5 µl 2.5 M $CaCl_2$ were mixed and incubated for 15 min at ambient temperature. 10 µl of transfection mix was transferred into each well of a 96 well plate, and plates were incubated at 37 °C and 5% CO_2 for 5 hr. The media was removed and replaced with 100 µl of fresh media with tetracycline, plates were incubated overnight at 37 °C and 5% CO_2.

Day 3: Luciferase assay

The media were transferred into a new 96 well luminescence plate and diluted in PBS (1:20). The luciferase substrate coelenterazine was prediluted in the PBS (17:10,000) and incubated for 30 mins in the dark. Luciferase activity was measured with Luminoskan Ascent microplate reader (Thermo Labsystems) with optimal settings (Table 13).

Day 4: RFP measurements

After further incubation at 37 °C overnight, the RFP-Signal was measured with SynergyNeo microplate reader (BioTek).

Table 13. Luminometers parameters.

Plate acceleration	10
Settle delay	1,000
Blanking time of integration time	100 %
Dispenser speed settings	
Dispenser 1	30
Dispenser 2	18

Dispenser 3	18
Dispenser and Measurement	20 µl
Integration time	100 ms

3.2.5.1 Data analysis

The relative luciferase signal (RLS) was normalised according to the following formula: $\frac{RLS\,(prey)-RLS\,(LexA)}{RLS\,(GAL4)-RLS\,(LexA)} \cdot 100\%$. From each value background luminesce which corresponds to "bait only" signal (LexA) was subtracted and interaction intensity was shown as percentage of transcription factor GAL4, which has the highest positive signal and determines the maximal activity of the assay.

3.2.6 Site-directed mutagenesis

Site-directed mutagenesis primers were designed with online tool PrimerX (http://www.bioinformatics.org/primerx/) with the specific desired modification for mutation in the centre of oligonucleotide (Table 11). Mutagenesis reactions were run according to the parameters in Tables 14 and 15. After the PCR, 0.5 µl of DpnI enzyme were added to digest the methylated parental DNA and samples were incubated 1 hr at 37 °C. An aliquot of PCR reaction was mixed with 6x Loading Buffer and samples were loaded on 1% agarose gel. Gel electrophoresis was run for 30 min at 100 V, and DNA was visualized with SybrSafe. The DNA was amplified and purified as described in 3.2.7 and 3.2.8, below. DNA sequences were checked by Sanger sequencing.

Table 14. Site directed reaction mix.

Reagent	**Volume (µl)**
KAPA (2x)	12.5
Forward primer (20 mM)	0.5
Reverse primer (20 mM)	0.5
DNA template (100 ng/µl)	0.5
ddH_20	11.5

Table 15. PCR program for SDM.

Temperature	Time
95 °C	30 sec
95 °C	30 sec x 18
65 °C	30 sec x 18
72 °C	3 min x 18
72 °C	7 min
4 °C	∞

3.2.7 Transformation through heat shock method

Highly chemically competent *E. coli* DH5α were transferred to an ice-cold tube with 1 µl of plasmid (or 4 µl of the LR reaction mix). Samples were incubated for 30 min in ice. The tube was placed for 1min at 42 °C , then again on ice for 2 min. Subsequently, 500 µl LB media were added into the tube and cells were incubated for 1 hr at 37 °C and shaking 200 rpm. The cells were centrifuged for 1 min at room temperature at 14,000 *g*. 450 µl of supernatant was removed and the cell pellet was resuspended in the remaining media and plated on LB agar with antibiotic. The plate was incubated at 37 °C overnight.

3.2.8 Purification of the plasmid (Mini Prep)

A single bacteria colony was picked and incubated in 3 ml of LB + antibiotic at 37 °C for 16 hr and shaking 200 rpm. For the purification of the plasmids, the Presto Mini Plasmid Kit from Geneaid was used, according to the manufacturer's instructions. The concentrations of the DNA were determined by NanoDrop spectrophotometry.

3.2.9 DNA sequencing

Sequencing was carried out at The Centre for Applied Genomics, Hospital for Sick Children, Toronto. The results were monitored and analysed using the freeware ApE and NCBI BLAST.

3.2.10 Gateway Cloning

3.2.10.1 Generation of Entry clones

Most cDNAs were originally obtained from human ORFeome collection v 8.1 (Stagljar Lab) and v 9.1 (Taipale Lab). Bacteria glycerol stocks with the desired entry clones were plated on agar plate + antibiotic and left overnight at 37 °C. Plasmids were purified by mini-prep (above). DNA sequences were check by Sanger sequencing.

cDNAs that were not in entry clone vectors were cloned into pDONR223 by PCR and Gateway BP reactions (Life Technologies) as follows. Specific primers with Gateway attB1 and attB2 sequences to the 5' and 3' were designed to amplify specific cDNA fragment of interest (Table 11). A PCR reaction was performed (Table 16 and Table 17), and samples were run on a 1% agarose gel; bands of the right size were extracted with GenepHlow Gel/PCR Kit from Geneaid according to manufacturer's protocol. The DNA concentration was measured with the NanoDrop spectrophotometry. BP Cloning was performed: 0.5 µl of purified cDNA of interest (100 ng/µl), 0.5 µl of pDONR223 entry clone vector (150 ng/µl), 3 µl 1xTE Buffer and 0.5 µl of BP Clonase II Enzyme Mix were mixed and reaction was incubated for 16 hr at room temperature. After incubation, 0.8 µl of Proteinase K was added and incubated 20 min at 37 °C. The DNA was amplified and purified as described above. DNA sequences were confirmed by Sanger sequencing using the M13 forward primer.

Table 16. PCR reaction mix.

Reagent	**Volume (µl)**
KAPA (2x)	25
Forward primer (50 mM)	1
Reverse primer (50 Mm)	1
DNA template (100 ng/µl)	1
DMSO	2.5
ddH_20	19.5

Table 17. PCR program.

Temperature	**Time**
94°C	2 min

94 °C	30 sec x 18
65 °C	30 sec x 18
72 °C	8 min x 18
72 °C	8 min
4 °C	∞

3.2.10.2 Cloning genes of interest into MaMTH expression vectors

Gateway LR Cloning was performed for generation of MaMTH expression vectors. 0.5 µl entry clone (100 ng/µl), 0.5 µl of MaMTH destination vector (A1243 or A1160) (150 ng/µl), 3 µl 1xTE Buffer and 0.5 µl of LR Clonase II Enzyme Mix were mixed and reactions were incubated for 16 hr at ambient temperature. After incubation, 0.8 µl of Proteinase K was added and incubated 20 min at 37 °C. The DNA was amplified and purified as described above. DNA sequences were checked by Sanger sequencing using the CMV forward primer.

3.2.11 Western blot

3.2.11.1 Sample preparation

DMEM media was removed from tissue plates, and cells were washed with ice cold PBS and lysed with cell lysis buffer H plus protease inhibitor. Samples were incubated for 10 min at 4 °C on the shaker and cells were collected with a pipette tip on ice. The cell lysate was centrifuged for 15 min at 14,000 *g* at 4 °C and supernatant was collected. 4x sample buffer was add to the supernatant and boiled for 5min at 95 °C. Samples were stored at -20 °C.

3.2.11.2 Total Protein Assay

The Thermo Fisher Scientific Pierce BCA Protein Assay Kit was used according to the manufacturer's protocol with some modifications. For calibration and preparation of the standard curve**,** 2 µl dilution of standards 0, 25, 125, 250, 500, 750, 1000, 1500 and 2000 ng/µl albumin concentration in ddH_2O were added to a 96-well plate. 2 µl of each sample were added to separate wells. Working reagent was prepared according to the manufacturer's protocol and 100 µl were added to samples and standards. The plate was incubated at 37 °C for 30 minutes. Standards and samples were run in triplicate and absorption at 562 nm was measured with the Synergy Neo microplate reader from BioTek.

3.2.11.3 Western Blots

Equal amounts of protein were loaded onto 10% SDS-PAGE gels and electrophoresis performed. Molecular weights of samples were determined by comparison to PageRuler Prestained Protein Ladder. Gels were run with 1x running buffer for 60-70 min at 150V. Proteins were blotted onto a nitrocellulose membrane with 0.2 μm pore diameter using wet transfer for 90 min at 300 mA with 1x transfer buffer. Membranes were stained in Ponceau S for 1 min, washed for 1 min with 1% acetic acid and then 1 min with water to determine the transfer quality. Prior to blocking, membranes were washed in TBST until all Ponceau S was removed. Membranes were blocked for 1.5 h while shaking in 2% (w/v) BSA. The membranes were incubated in primary antibodies (1:10000 or 1:5000 dilution) in TBST overnight, while shaking at 4 °C. Membranes were then washed 3 x 15 min with TBST while shaking. Secondary antibodies (1:10000 dilution) were incubated with the membranes while shaking at ambient temperature for 1.5 h, then membranes were washed 3 x 15 min with TBST. The membranes were covered with enhanced chemiluminescence (ECL) substrate and film was exposed.

4. RESULTS

4.1 Expression and activation levels of bait proteins

MaMTH reporter cell lines with stable expression of desired baits have to be validated for bait expression after the generation of cell line. The generation of MaMTH expression vectors with four different ErbB2 cDNAs was successful and generation of stable integrated ErbB2 proteins in B0166 with the FLP-In TREx method was accomplished. The generated cell lines ErbB2 wild type (wt), ErbB2 ex16del, ErbB2 T798M and ErbB2 K753A were validated by analysing tetracycline (Tet) inducible bait expression by Western blot. Expression and activation of ErbB2 wt bait protein were tested in absence of Tet and in the presence of different Tet concentrations (0.1, 0.5 and 1.0 µg/ml) **(**Figure 4). Expression and activation of ErbB2 ex16del, ErbB2 T798M and ErbB2 K753A baits were tested with Tet (0.5 µg/ml) and without Tet **(**Figure 5). The antibodies used were anti-V5 and anti-ErbB2 to measure total bait expression, general phosphotyrosine anti-pY99 and anti-pErbB2 (Y1221/Y1222) to measure the phosphorylation state of ErbB2, anti-Erk1/2 and anti-pErk1/2 to determine activation of the downstream components of ErbB2 mediated signalling, and anti-α tubulin as a loading control.

Expression of all bait proteins was detectable only in presence of Tet using anti-V5 and anti-ErbB2 antibodies. Under Tet induction (0.1, 0.5 and 1.0 µg/ml), ErbB2 wt was phosphorylated and increased phosphorylation of pErk1/2 was detected. ErbB2 ex16del and ErbB2 T798M showed increased phosphorylation of ErbB2 and downstream activation of pErk1/2 with 0.5 µg/ml Tet. Phosphorylation of ErbB2 K753A and phosphorylation of Erk1/2 in the same sample were not detectable with or without Tet.

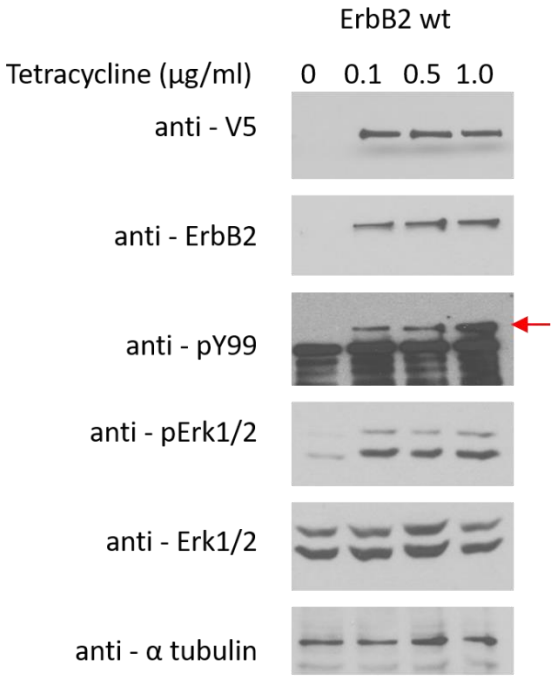


Figure 4. Expression and activation levels of ErbB2 wild type bait protein. HEK293 B0166 cells stably expressing ErbB2 wt bait were cultured with different tetracycline concentrations overnight to induce bait expression, and proteins of interest were evaluated by Western blot with anti-V5 and anti-ErbB2 for bait expression, anti-pY99 to measure phosphorylation state of ErbB2, anti-Erk1/2, anti-pErk1/2 to determine activation of the downstream components and anti-α tubulin as loading control. Red arrow indicates band for ErbB2 protein**.**

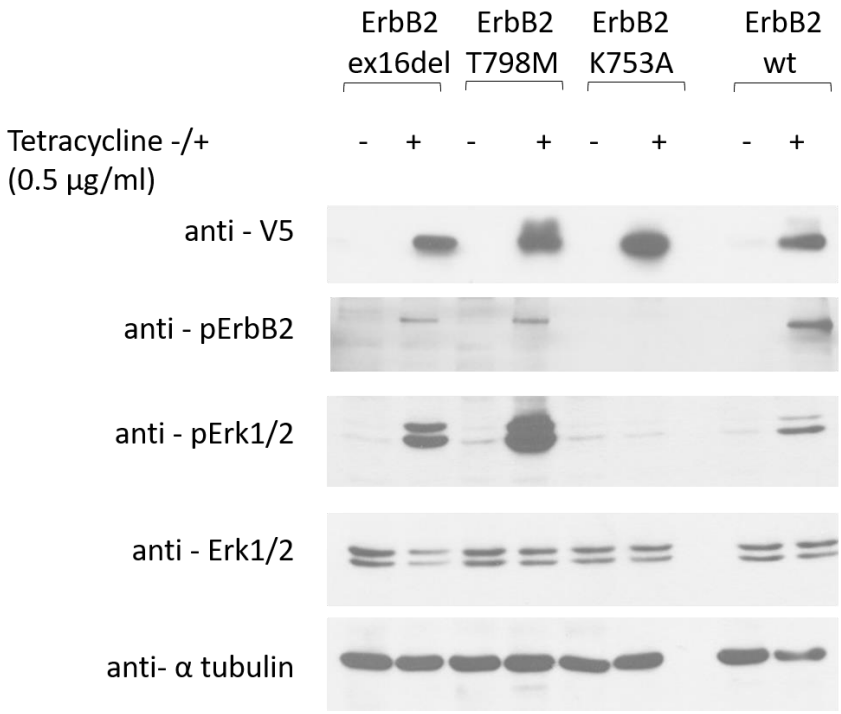


Figure 5. Expression and activation levels of bait proteins were tested by Western blotting. HEK293 B0166 cells stably expressing bait proteins were cultured with 0.5 µg/ml and without tetracycline overnight. Proteins of interest were evaluated with anti-V5 tag for bait expression, anti-pErbB2 (Y1221/Y1222) to measure phosphorylation state of ErbB2, anti-Erk1/2, anti-pErk1/2 to determine activation of the downstream components and anti-α tubulin as loading control for ErbB2 ex16del, ErbB2 T798M and ErbB2 K753A and ErbB2 wt cell lines.

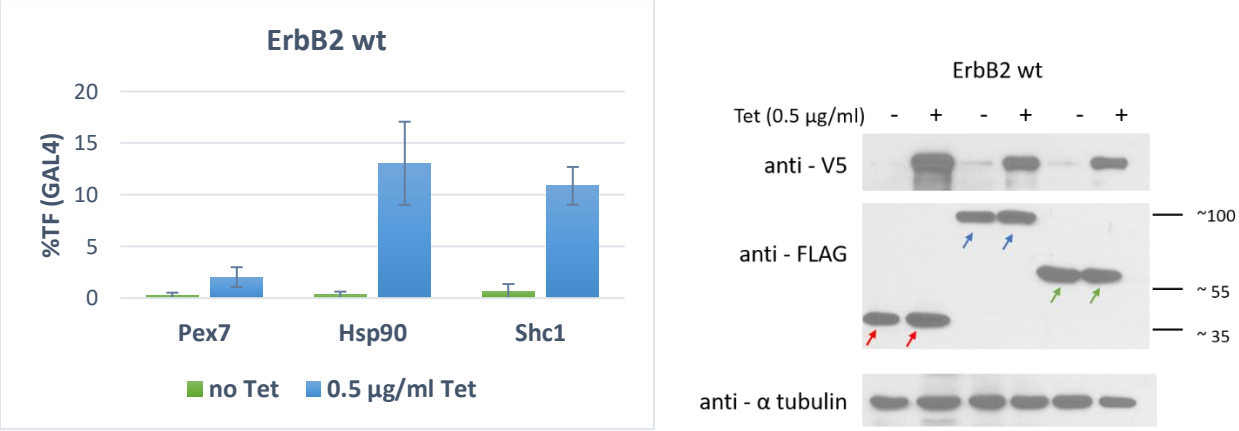
4.2 MaMTH assay using a small set of positive and non-interactors for optimization

For further validation of the MaMTH cell lines with stable expression of bait and to determine whether 0.5 µg/ml Tet is optimal for bait expression for future experiments, the MaMTH assay was performed. Stable cell lines ErbB2 wt, ErbB2 ex16del, ErbB2 T798M and ErbB2 K753A were transfected with prey MaMTH vectors expressing: GAL4 transcription factor, Pex7 as negative control, Hsp90 as positive control, Shc1 as another known positive interactor and transcription factor LexA which is unrelated DNA for "bait only" signal. The interaction levels were measured -/+ tetracycline concentration (0.5 µg/ml). Raw luminescence data was normalised and interaction signals were shown as percentage of transcription factor. After measurement of luciferase activity, Western blot analysis of bait and prey expression was performed to determine if the MaMTH

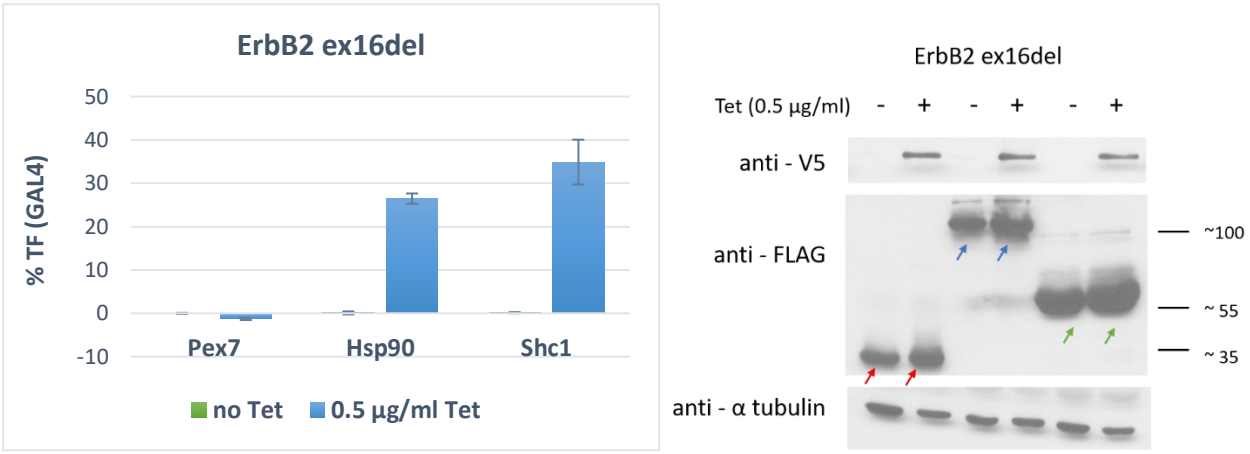
system was working properly. For detection of bait protein anti-V5 was used and for detection of prey proteins anti-FLAG was used, anti-α tubulin was used as loading control. MaMTH and Western blot results are shown in Figure 6.

The MaMTH assay with a small reference set of preys for all four cell lines (ErbB2 wt, ErbB2 ex16del, ErbB2 T798M and ErbB2 K753A) showed higher signal with Hsp90 and Shc1 compared to the negative control, Pex7, at a Tet concentration of 0.5 µg/ml. No strong luminescence signal was detectable without bait expression (no Tet). As shown by Western blot, bait expression was detected only at Tet concentrations of 0.5 µg/ml and prey expression was detected in all samples.

[A]



[B]



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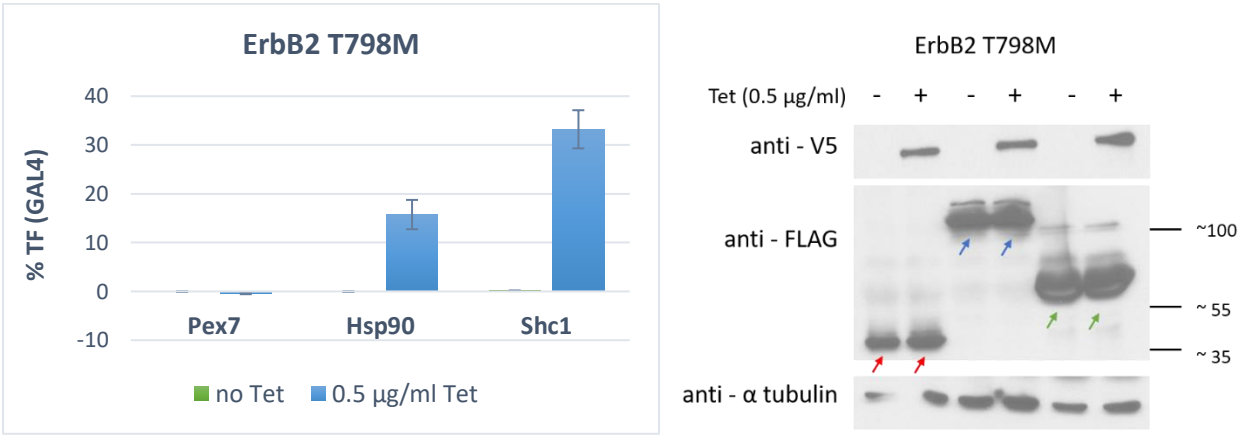

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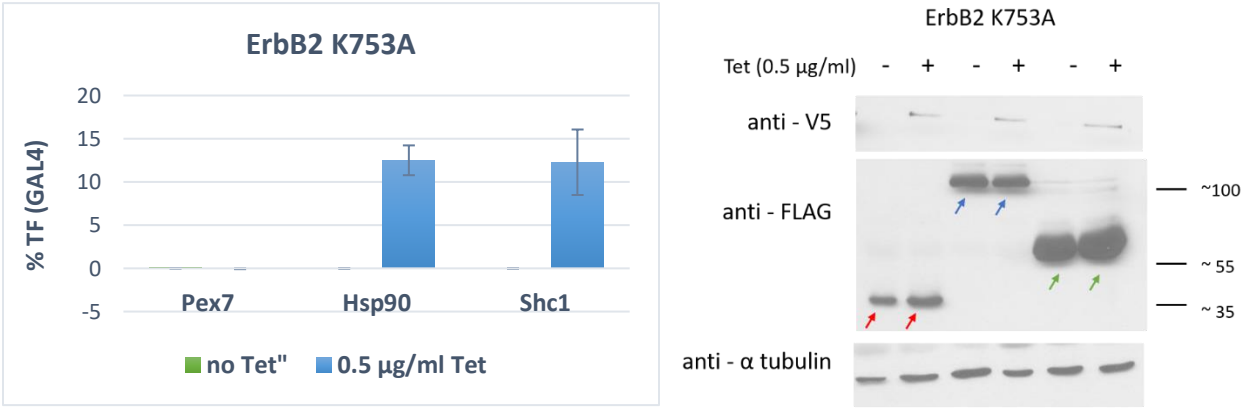


Figure 6. Normalised luminescence data from MaMTH assay using a small set of preys and Western blot analysis of bait and prey expression. MaMTH assay was performed with MaMTH reporter cell lines (HEK293 B0166) stably expressing [A] ErbB2 wild type [B] ErbB2 ex16del,

[C] ErbB2 T798M and [D] ErbB2 K753A baits transfected with Pex7 as negative control, Hsp90 as positive control and Shc1 as known interactor. After MaMTH assay, expression of baits and preys was tested on Western blot using anti-V5 for bait detection, anti-FLAG for prey detection and anti-α tubulin as loading control. Prey proteins are indicated with arrows: Pex7 (red), Hsp90 (blue) and Shc1 (green). Detected prey proteins size is: ~43 kDa for Pex7, ~ 97 kDa for Hsp90 and ~70 kDa for Shc1. Error bars represent standard deviation from 3 biological replicates.

4.3 Screening of the SH2/PTB prey library

The screening of SH2/PTB domain-containing proteins against ErbB2 wt was performed to detect PPIs. For the screen of the SH2/PTB domain-containing proteins, the prey library including 98 proteins was generated by Gateway cloning into the RFP N-tagged MaMTH prey vector (designated A1245) by members of Stagljar Lab (Supplementary Table 1S). HEK 293 B0166 ErbB2 wt cells were transfected with SH2/PTB library prey vectors, with transcription factors GAL4 and LexA for normalization purposes and with positive control Shc1 and negative control Pex7. Expression of bait was induced with 0.5 µg/ml Tet. One day after transfection, luminescence was measured and raw data was normalised as described above (Figure 7). Two days after transfection, RFP fluorescence was measured to determine prey expression. The background fluorescence (results from cells transfected with unrelated DNA, LexA) was subtracted from sample RFP data, and samples with lower signal than background were removed from the screen results as these preys were considered not to be expressed. The list of SH2/PTB library preys and corresponding RFP values is shown in Supplementary Table 2S. Figure 7 shows normalised luminescence data of the 85 expressed proteins from the SH2/PTB prey library, sorted from lowest to the highest value. Table 18 shows 36 detected ErbB2 interactors from the SH2/PTB prey library with score higher than 5,40% of TF, and indicates the 18 that are already known interactors of ErbB2 (information from BioGRID database: (https://thebiogrid.org/). For unknown interactors, FpClass predictions of interactions were calculated and score is indicated (http://dcv.uhnres.utoronto.ca/FPCLASS/).

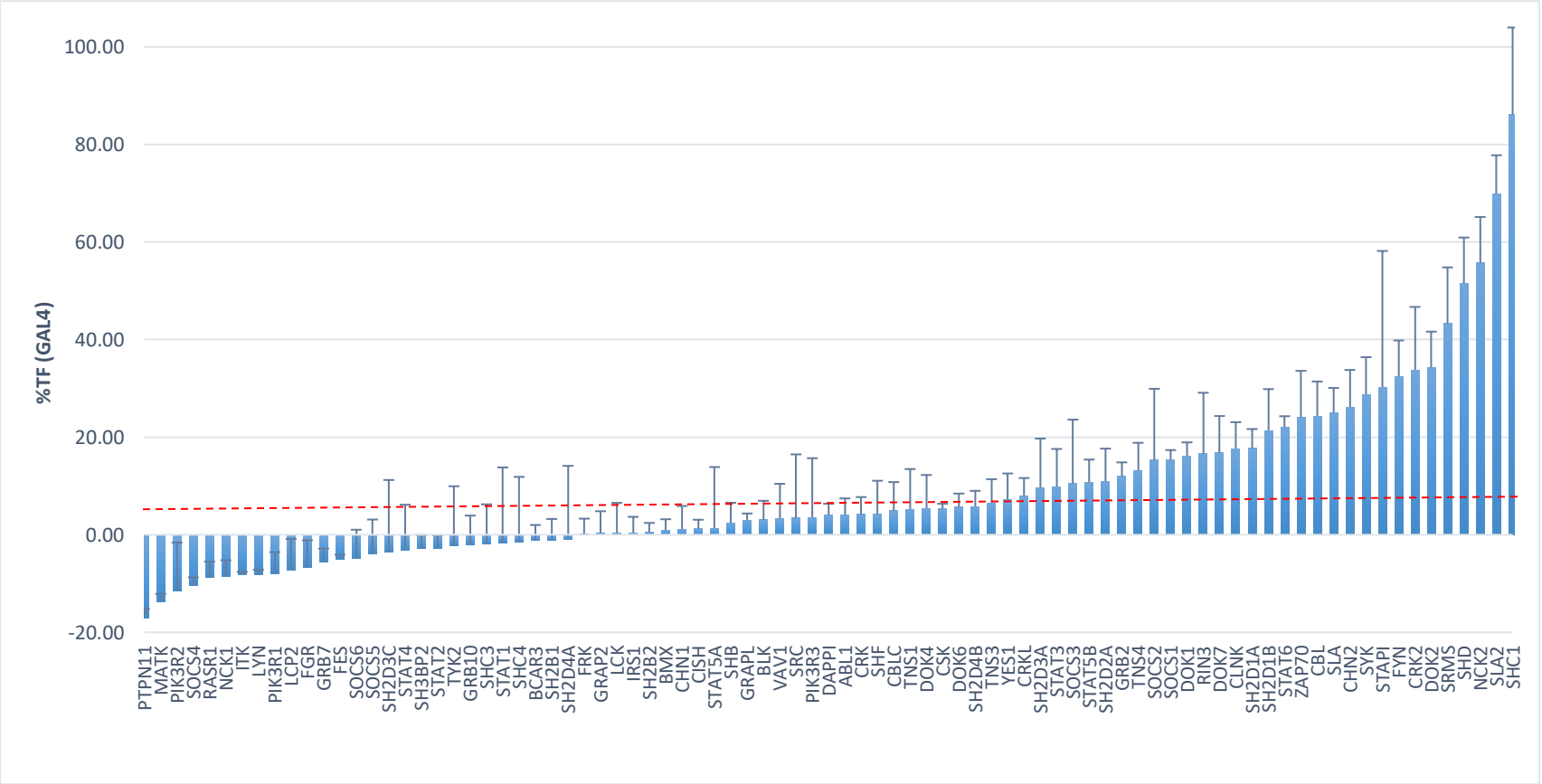


Figure 7. Normalised luminescence data from screen of the SH2/PTB prey library with ErbB2 wt stably expressed in HEK293 B0166. Error bars represent standard deviation across 3 biological replicates. Red line indicates 5,4% cut-off threshold.

Table 18. List of detected ErbB2 interactors from SH2/PTB prey library with corresponding score (% of TF signal). Known interactors from BioGRID database (https://thebiogrid.org/) and FpClass (http://dcv.uhnres.utoronto.ca/FPCLASS/) predicted interactors with score are indicated.

Protein	Score	Known interactor	FpClass score	Protein	Score	Known interactor	FpClass score
CSK	5.4		0.8826	CLNK	17.65	+	
DOK6	5.7	+		SH2D1A	17.72		0.3442
SH2D4B	5.8			SH2D1B	21.39	+	
TNS3	6.55	+		STAT6	22.12		0.3061
YES1	7.24		0.8826	ZAP70	24.04		0.8826
CRKL	7.97	+		CBL	24.32	+	
SH2D3A	9.53	+		SLA	25.05	+	
STAT3	9.89	+		CHN2	26.17		0.4875
SOCS3	10.47		0.8826	SYK	28.79	+	
STAT5B	10.75		0.8826	STAPI	30.26		0.769
SH2D2A	10.97	+		FYN	32.42		0.8826
GRB2	11.96	+		CRK2	33.76	+	
TNS4	13.15		0.7991	DOK2	34.28		0.8826
SOCS2	15.3		0.7376	SRMS	43.35		0.8203
SOCS1	15.36	+		SHD	51.48		0.7991
DOK1	16.18	+		NCK2	55.71	+	
RIN3	16.58		0.7738	SLA2	69.88	+	
DOK7	16.83		0.512	SHC1	86.13	+	

4.4 MaMTH assay using a small set of proteins containing SH2 domain mutation

Predicted interaction of phosphotyrosines with SH2 domains requires a conserved SH2 domain arginine residue. To determine the importance of these residues in interactions with ErbB2, a MaMTH assay was performed. Prior to the MaMTH assay, SHD R265A, SLA2 R143A, Crk2 R38A and SRMS R147A mutant proteins were generated by site-directed mutagenesis and Gateway cloned into the A1245 RFP N-tagged MaMTH prey vector, by members of Stagljar lab. HEK 293 B0166 ErbB2 wt cells were transfected with transcription factors GAL4 and LexA for normalization, Shc1 and Pex7 as positive and negative controls, and SHD, SHD R265A, SLA2, SLA2 R143A, Crk2, Crk2 R38A, SRMS and SRMS R147A preys to test interactions between wt and mutant preys and ErbB2 wt. Expression of bait was induced with 0.5 µg/ml Tet. One day after

transfection, luciferase activity was measured and cell lysates were collected for Western blot analysis of bait and prey expression. Figure 8 shows luminescence signals normalized as described above. P-values were measured compared to corresponding controls with two-tailed unpaired t-test. Western blot analysis of bait and prey expression was performed. For detection of bait protein anti-V5 was used, and for detection of prey proteins anti-FLAG was used, while α tubulin was used as loading control (Figure 9).

The MaMTH assay with a small set of proteins containing SH2 domain mutation with ErbB2 wt showed positive interaction for all preys except Pex7 and SRMS R147A. Western blot analysis showed ErbB2 wt bait expression in all samples and expression of all preys except SRMS R174A.

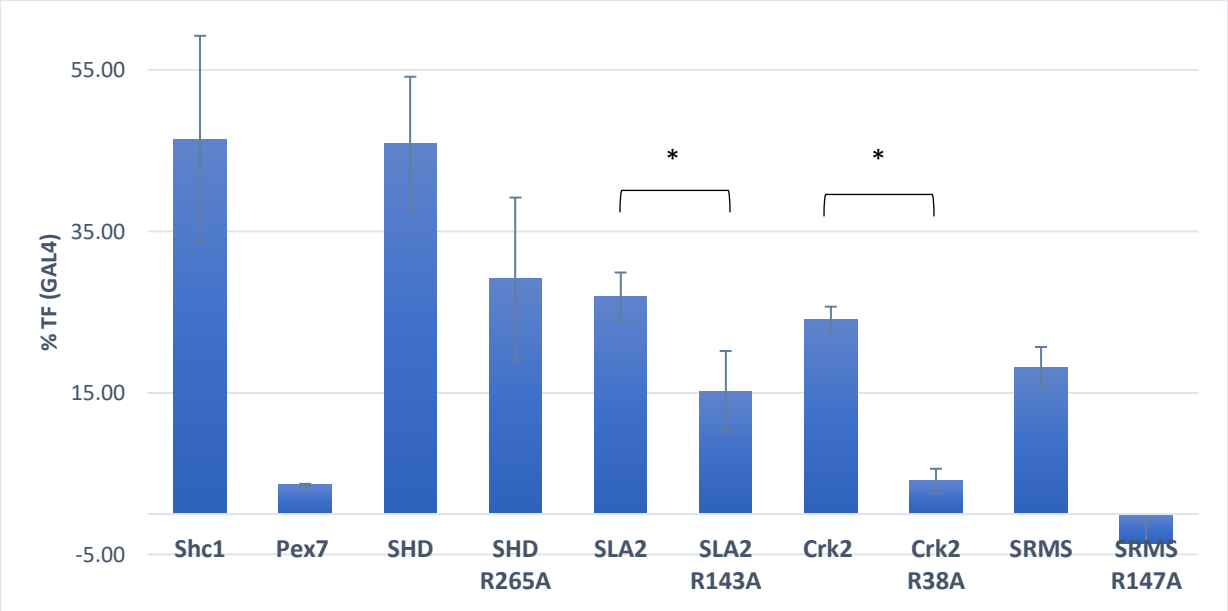


Figure 8. Normalised luminescence data from MaMTH assay using a small set of proteins containing SH2 domain mutation expressed in HEK 293 B0166 ErbB2 wt. Error bars represent standard deviation across 3 biological replicates. *P < 0.05; two-tailed unpaired t-test calculations compared to corresponding controls.

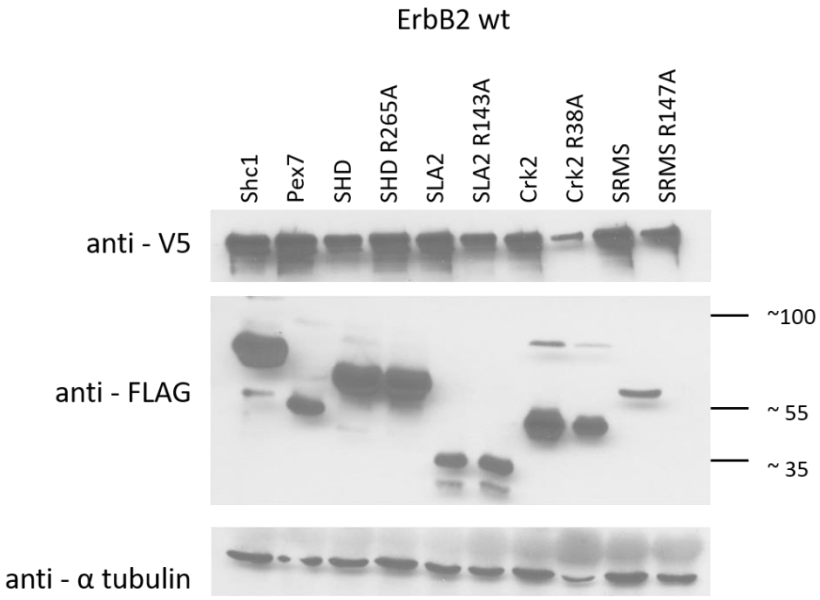


Figure 9. Western blot analysis of bait and prey expression after MaMTH assay using anti-V5 for bait detection, anti-FLAG for prey detection and α tubulin as loading control. Detected prey proteins size is: ~70 kDa for Shc1, ~43 kDA forPex7, ~51 kDa for SHD and SHD R265A, ~35 kDa for SLA2 and SLA2 R143A, ~ 49 kDa for Crk2 and Crk2 R38A and ~63 kDa for SRMS.

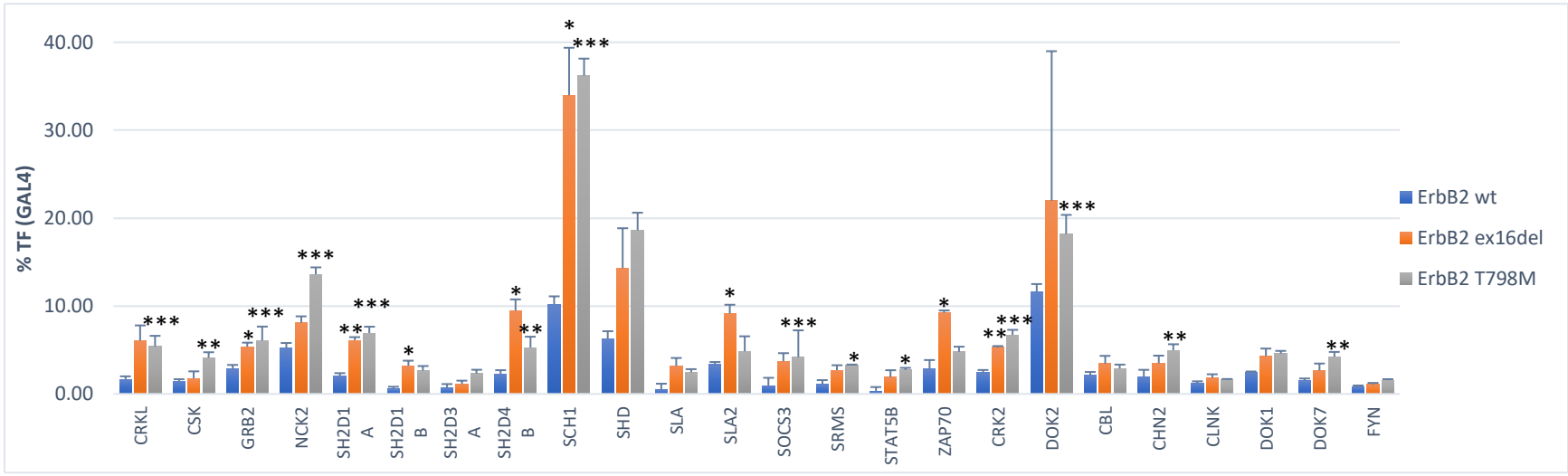
4.5 Screening of the SH2/PTB hits against ErbB2 mutants

The MaMTH assay was performed for functional characterisation of interactions between ErbB2 and detected interactors from SH2/PTB prey library. Twenty-four of 36 detected interacting preys (Supplementary Table 3S) were randomly selected and transfected into HEK 293 B0166 cell lines with stable expression of ErbB2 wt, ErbB2 ex16del, ErbB2 T798M and ErbB2 K753A baits. GAL4 and LexA preys were also transfected for normalization and Pex7 as negative control. Bait expression was induced with 0.5 µg/ml Tet and luminescence was measured. The luminescence signals are presented as percentage of transcription factor (TF) and P-values were measured compared to corresponding wild type controls with two-tailed unpaired t-test (Figures 10).

The MaMTH assay with 24 detected interactors from SH2/PTB prey library and all four cell lines (ErbB2 wt, ErbB2 ex16del, ErbB2 T798M and ErbB2 K753A) showed positive interaction signal for all tested prey proteins. Statistical significance of the differences in interaction signals were calculated and corresponding P values are provided. Eight of 24 tested proteins with ErbB2 ex16del and 15/24 tested proteins with ErbB2 T798M showed significantly

higher interaction signals compared to ErbB2 wt (Table 19). ErbB2 K753A and tested preys did not show different interaction signals compared to ErbB2 wt.

[A]



[B]

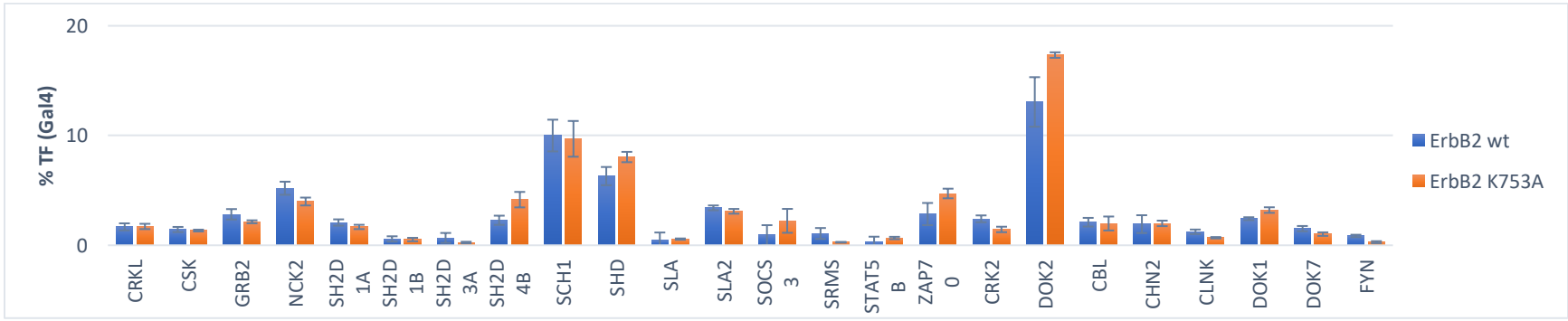


Figure 10. Normalised luminescence data from MaMTH assay with 24 detected ErbB2 hits from SH2/PTB prey library against [A] ErbB2 wt, ErbB2 ex16del and ErbB2 T798M and [B] ErbB2 wt and ErbB2 K753A stably expressed in HEK 293 B0166. Error bars represent standard deviation across 3 biological replicates. *P < 0.05, **P < 0.01, ***P < 0.001 two-tailed unpaired t-test calculations compared to corresponding wild type controls.

Table 19. List of ErbB2 hits from SH2/PTB that showed significantly higher interactions signal with ErbB2 ex16del and ErbB2 T798M compared to ErbB2 wt and corresponding P values.

Protein	ErbB2 ex16del P value	ErbB2 T798M P value
CRKL		< 0.001
CSK		< 0.01
GRB2	< 0.05	< 0.001
NCK2		< 0.001
SH2D1A	< 0.01	< 0.001
SH2D1B	< 0.05	
SH2D4B	< 0.05	< 0.01
SCH1	< 0.05	< 0.001
SHD		< 0.001
SLA2	< 0.05	
SOCS3		< 0.001
SRMS		< 0.05
STAT5B		< 0.05
ZAP70	< 0.05	
CRK2	< 0.01	< 0.001
DOK2		< 0.001
CHN2		< 0.01
DOK7		< 0.01

5. DISCUSSION

5.1 Expression levels of ErbB2 wild type bait with different tetracycline levels

MaMTH reporter cell lines were designed to have tetracycline regulated bait expression, which alows bait expression levels to be better regulated and equalized, making resulting data more comparable. HEK 293 B0166 cell line stably expressing ErbB2 wt protein upon tetracycline induction showed equal bait expression with 0.1, 0.5 and 1.0 µg/ml tetracycline (Figure 4) by Western blot. Determination of optimal expression with ErbB2 in an active state was important for the next experiments of mapping the interactome. ErbB2 activation was shown by increased pY99 signal with 0.1, 0.5 and 1.0 µg/ml tetracycline. The anti-pY88 is a general phospho-tyrosine antibody, and is not specific for the phosphorylation of ErbB2. Phosphorylation was detected for ErbB2 bait with tetracycline and with 1.0 µg/ml it was slightly higher that with 0.1 and 0.5 µg/ml. Because of the ability of anti-pY99 to detect many phosphorylated tyrosines in different proteins, it would be better to use specific pErbB2 antibody to determine if ErbB2 is more active with 1.0 µg/ml tetracycline. The downstream activation of the MAPK-kinase pathway, which is activated through ErbB receptor kinase family (Wieduwilt and Moasser, 2008), was detected with the phosphorylation of Erk1/2 at same level with bait expression at 0.1, 0.5 and 1.0 µg/ml tetracycline. 0.5 µg/ml tetracycline was chosen for future experiments as suitable contraction for bait expression and activation because with all tested tetracycline concentrations the same expression level of bait was detected alongside the same level of Erk1/2 activation.

5.2 Characterization of ErbB2 mutant baits

ErbB2 ex16del is a transcript variant of ErbB2 protein that occurs naturally but rarely, has increased ligand-independent activity and significantly increased tyrosine phosphorylation compared with ErbB2 wt (Moasser, 2007; Jackson *et al.*, 2013; Turpin *et al.*, 2016). ErbB2 T798M is an oncogenic version that has increased affinity for ATP stabilizing an active protein conformation. Compared with ErbB2 wt, ErbB2 T798M has increased autocatalytic activity (Rexer *et al.*, 2013). ErbB2 K753A is expected to have impaired kinase activity. Residue K753 in the ATP binding pocket has been identified as important for ErbB2 activation, through formation of a salt bridge with E770, which is one of the most crucial interactions for kinase activation (Aertgeerts *et al.*, 2011). HEK 293 B0166 cell lines with stable expression of ErbB2 ex16del,

ErbB2 T798M and ErbB2 K753A were tested by Western blot to validate expression and activation levels. Expression of all three mutant ErbB2 baits was induced with 0.5 µg/ml tetracycline, while expression was not detectable without tetracycline (Figure 5). Phosphorylation of baits were tested with anti-pErbB2 antibody that specifically detects phosphorylation of tyrosines at position 1221 and 1222. Phosphorylation was detected for ErbB2 ex16del and ErbB2 T798M and was lower than that of ErbB2 wt. ErbB2 K753A did not show phosphorylation at these particular tyrosine residues. To check downstream activation of signalling pathways, phosphorylation of Erk1/2 was determined. Without tetracycline, endogenous levels of Erk1/2 were detected, and upon tetracycline induction, the ErbB2 baits showed different levels of pErk1/2. ErbB2 ex16del and ErbB2 T798M promote increased Erk1/2 phosphorylation compared to ErbB2 wt, while pErk1/2 level did not increase above background levels upon ErbB2 K753A expression. Expressed ErbB2 ex16del and ErbB2 T798M get phosphorylated which leads to their activation and activation of downstream MAPK-kinase pathway what was expected and has been shown by Castiglioni *et al.* (2006) and Rexer *et al.* (2013). ErbB2 K753A can be considered as less kinase active compared to ErbB2 wt since it was not phosphorylated at Y1221 and Y1222 as it has been shown previously in COS-7 cells (Xu *et al.,* 2001) and increased pErk1/2 was not detectable. These data indicate that 0.5 µg/ml tetracycline was suitable to induce bait expression in all ErbB2 baits.

5.3 Initial MaMTH assay with a small set of positive and negative interactors

To further validate the tetracycline concentration of 0.5 µg/ml for bait expression, a MaMTH assay with a small subset of preys for all HEK 293 B0166 ErbB2 cell lines was performed -/+ tetracycline (0.5 µg/ml). Pex7 was chosen as negative control because has not been reported to interact with the ErbB2. Hsp90 and Shc1 were chosen as positive controls. Hsp90 is molecular chaperone that promotes protein maturation into an active and stable conformation and is involved in maintenance of the mature form of proteins. It has been shown ErbB2 is dependent upon Hsp90 for its stability through the whole life span of the receptor (Citri *et al*., 2004). Shc1 is a signalling adapter protein that binds ErbB2 through its PTB domain (Wagner *et al.,* 2013). ErbB2/Shc1 and ErbB2/Hsp90 interactions have been already shown in MaMTH (Petschnigg *et al.*, 2014; Yao *et al.*, 2017). MaMTH luminescence signal is proportional to interaction between bait and prey (Petschnigg *et al.*, 2014; Saraon *et al.*, 2017). Without tetracycline, interactions were not detected between ErbB2 baits and positive controls. Upon tetracycline induction ErbB2 wt, ErbB2 ex16del,

ErbB2 T798M, ErbB2 K753A showed positive interactions with known interactors Hsp90 and Shc1 and no interactions with Pex7 **(**Figure 6). Additionally, Western blot analysis showed bait expression only with 0.5 µg/ml tetracycline, while comparable levels of prey expression were detectable in the presence and absence of tetracycline, which demonstrates that the luciferase signal is produced upon genuine interactions between bait and prey. By considering the results from the Western blots and the MaMTH data, the concentration of 0.5 µg/ml tetracycline is suitable for induction of bait expression and detection of protein-protein interactions. Therefore, further experiments with all four baits (ErbB2 wt, ErbB2 ex16del, ErbB2 T798M, ErbB2 K753A) were performed with a tetracycline concentration of 0.5 µg/ml.

5.4 Screening of the SH2/PTB prey library against ErbB2 wt

The MaMTH assay is useful method for high throughput screens. A threshold cut-off between positive and negative interactions has to be determined, such that every interaction which shows a higher signal than the threshold can be considered to be a hit. In this screen, the luminescence signal of the negative control/non-interactor Pex7 was lower than 2% of that of the transcription factor. Therefore, a score of 5,40% of the signal of the transcription factor was used as cut-off assuming that interaction signals higher than 5,4% is positive signal. Proteins that meet the criteria are listed in Table 18. There are 36 detected proteins from the SH2/PTB prey library that showed positive interaction signals with ErbB2 wt; 18 are already known ErbB2 interactors according to BioGRID database (https://thebiogrid.org). Fifteen of the known interactors have been reported once using high-throughput approaches based on protein microarrays (Jones *et al.*, 2006), and many ErbB2 interactions have not been functionally characterized. The data presented in this thesis show that the MaMTH assay can be used for validation of previously found interactors. For Cbl, Stat3 and Grb2, function has been established in the context of ErbB2 signalling. Cbl is E3-ubiquitin ligase, a negative regulator that binds to phosphorylated ErbB1 for ubiquitination and lysosomal targeting. Cbl was found to stimulate ErbB2 degradation following its overexpression (Klapper *et al.*, 2000). Stats (signal transducer and activator of transcription) are latent transcription factors which have been found constitutively active in many human tumors. It has been shown that JAK2 (Janus kinase 2) and Stat3 associate with ErbB2 prior to its phosphorylation, and ErbB2 activates Stat3 through JAK2 and Src (Ren and Schaefer, 2002). Grb2 (Growth factor receptor bound protein 2) is a well known adaptor protein, that is recruited to active

ErbB family members to activate MAP kinase and PI3K signalling pathways (Schlessinger and Lemmon, 2003; Roskoski, 2014). ErbB2 interactions with Cbl, Stat3 and Grb2 were confirmed by MaMTH in this experiment. Fifty of 95 total proteins in the SH2/PTB prey library have been previously reported as ErbB2 interactors, and 36% of interactors were confirmed with MaMTH. The fact that all of the previously known interactors were not identified by MaMTH can be explained in part by lack of expression of six of the known interactors (no RFP signal). Another reason could be that all the prey proteins were N-tagged with Nub, and the tag could have had an influence on the protein folding or interrupted the specific interaction. To decrease the possible influence of the N-tag, the C-tagged versions of the preys should also be tested.

Eighteen proteins showed a positive signal with ErbB2 and have not been previously reported as ErbB2 interactors. According to FpClass prediction, 17 of them are predicted interactors (Table 18). For some novel interactors of ErbB2, connections with ErbB1 have previously been reported. For instance, DOK (downstream of tyrosine kinase) proteins are adaptors proteins that regulate tyrosine kinase signalling. DOK2 is phosphorylated after ErbB1 activation and suppresses Src, AKT and ERK phosphorylation after EGF stimulation. DOK2 has been identified as human lung tumor suppressor gene (Berger *et al.*, 2013). Fyn is an effector of oncogenic ErbB1 signalling in glioblastoma that belongs to Src family tyrosine kinase. The active ErbB1 mutant phosphorylates and associates with Src and Fyn, significantly promoting tumour cell growth, migration and invasion (Lu *et al.*, 2009). The roles of DOK2 or Fyn in ErbB2 signalling still remain to be discovered. CHN2, also known as β2-chimaerin, is a GTPase-activating protein that has been linked with breast cancer as tumour suppressor. Studies in breast cancer cells demonstrated that β2-chimaerin inhibits proliferation downstream of the ErbB2 receptors (Casado-Medrano *et al.*, 2016). Direct PPI between ErbB2 and CHN2 has not been established until now. SRMS (Src-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristoylation sites) is nonreceptor tyrosine kinase whose cellular role is unknown. It has been reported that SRMS is overexpressed in the majority of breast cancer cell lines and expressed at a higher level in breast cancers compared to normal mammary tissue (Goel *et al.*, 2013). Since ErbB2 is overexpressed in 20-30% of breast cancers (Roskoski, 2014), additional characterisation of ErbB2 and SRMS interaction may be interesting. The error bars are large in some of the proteins around the cut-off, which suggests the presence of some false positives and negatives. Another control experiment that could be considered in order to eliminate unspecific binding, is the testing of interactions in

the presence of specific tyrosine kinase inhibitors. Tyrosine kinase inhibitors bind to receptors and disable receptor activation and PPIs, therefore the MaMTH interaction signal would decrease for specific interactions. Further confirmation of novel hits should be done with another approach for analysing protein-protein interactions. So far, luminescence-based mammalian interactome (LUMIER) assay, co-immunoprecipitation (IP) and mass spectrometry (MS) have been used for validation of MaMTH-detected interactors in previous studies (Petschnigg *et al.*, 2014, 2017; Yao *et al.*, 2017).

RFP fluorescence was measured to confirm prey expression, because RFP expression has been shown to correlate with protein expression. Although it is possible to normalize the positive interactions to the expression of RFP, to reduce variation in reporter signal resulting from differences in prey expression level, in this case it was not necessary to do it because no exact determination or comparison of interactions was performed.

In addition to the 98 proteins included in the SH2/PTB prey library, 25 proteins that contain SH2 or PTB domains were missing due to availability in ORFeome collections etc.. To fully map the interactome of ErbB2 with SH2/PTB domain containing proteins, the additional proteins should be tested. Fifteen of the missing proteins have been reported as ErbB2 interactors (Supplementary Table 4S). These include Vav2, a guanine nucleotide exchange factor for the Rho family of GTPases, whose recruitment increases metastatic properties and survival of ErbB2 overexpressing cells (Wang *et al.*, 2006), and PLCγ involved in tumour invasion after activation through ErbB2 (Wang *et al.*, 2006). Mapping and characterisation of the interactome of ErbB2-SH2/PTB domain containing proteins can aid understanding of molecular mechanisms of cancer biology. The BioGRID database currently shows 200 unique interactors of ErbB2, and the FpClass algorithm predicts 1056 interactions for ErbB2, so the ultimate goal is to map all ErbB2 interactions of human proteome. To accomplish this, an unbiased screen of total human ORFeome should be considered.

5.5 Validation of SH2 domain binding in MaMTH

SH2 domains have a conserved amino acid sequence, the FLVR motif, with a critical arginine residue that binds the phosphotyrosines of the ErbB proteins and other RTKs (Wagner *et al.*, 2013). Hence, mutating arginine to alanine causes SH2 domain binding ability to phosphotyrosines to

decrease. The MaMTH assay can be used for functional characterisation of PPIs. To examine whether the SH2 domain of SHD, Sla2, Crk2 and SRMS is critical for interaction with ErbB2, a MaMTH assay was performed. Figure 8 shows that significantly lower interaction signals were detected for Sla2 R1431A and Crk2 R38A compared to Sla2 wt and Crk2 wt. Western blot analysis of bait and prey expression showed equal level of bait expression in all samples except in one where Crk2 R38A was expressed. This can be explained that α tubulin showed also lower protein level which is probably caused by lower proteins loading in this particular sample. Expression of preys has remained comparable in all sample as well. These data indicated that ErbB2-Sla2 and ErbB2-Crk2 interactions happen through SH2 domain because the binding of SH2 domain mutants to ErbB2 is significantly lower. To further analyse which phosphotyrosine residues are implicated in these interactions, ErbB2 tyrosine mutants should be generated and tested in MaMTH. Sla2/ Huntingtin Interacting Protein 1, one of the top interactors of ErbB2, is involved in linking the endocytic machinery to the actin cytoskeleton (Engqvist-Goldstein *et al.*, 1999). ErbB2-Sla2 interactions has been reported once (Jones *et al.*, 2006), but no functional studies have been published so far. Crk2 is an adaptor protein that regulates cytoskeletal reorganisation for cell growth and motility (Roskoski, 2014). ErbB2 facilitates cell invasion though assembly of a CAS/Crk2 signalling complex which is necessary for cell migration mediate by ErbB family (Spencer *et al.*, 2000). Of the other two tested proteins, SHD R265A did not show significantly lower interaction signal, and SRMS R147A was not detected by Western blot (Figure 9). It is possible that the SH2 domain is not critical for SHD binding to ErbB2. SRMS binding through the SH2 domain was not validated because of the lack of expression of the SRMS R147A prey. Possible explanations could be that SRMS R147A is not stable, but this is unlikely because expression of this has been reported by Goel *et al.* (2013), therefore the reason could be that the vector or promotor region could have a defect. Overall, the MaMTH assay was shown as a useful approach for characterisation of SH2 domain binding and same approach should be used for testing binding specificity of other detected ErbB2 hits containing SH2 domains.

5.6 MaMTH interaction characterization of ErbB2 interactors from SH2/PTB prey library against ErbB2 mutants

To validate ErbB2 hits from the SH2/PTB prey library, ErbB2 mutants were generated. ErbB2 ex16del and ErbB2 T798M are catalytically more active than wild type, and signal-independent

phosphorylated receptors. Active mutants should provide the same hits from the screen as did the wild type so that the data can be further validated and hits confirmed or refuted. Figure 10 [A] shows that 24 tested hits that were previously detected in the screen showed positive interaction signal with ErbB2 ex16del and ErbB2 T798M, as was expected. Additionally, remaining 12 hits that were detected in the screen should be tested in the same way.

MaMTH data shows differential interaction profiles for eight prey proteins that strongly bind to ErbB2 ex16del and 15 prey proteins that strongly bind to ErbB2 T798M compared with ErbB2 wt (Table 19). These results indicate that mutant receptors preferentially bind proteins that can be involved in aberrant signalling. For instance, ErbB2 ex16del selectively activates Stat3 transcription factor, which leads to upregulation of Stat3 target genes that are important modulators of immune microenvironment (Ren and Schaefer, 2002). Grb2, Shc1 and Crk2 strongly interact with both active mutants, and they can have important roles in promoting oncogenic changes. Grb2 and Shc1 are adaptor proteins involved in MAPK kinase activation which leads to cell proliferation (Roskoski, 2014). Interactions with constantly active receptors may prolong activation of downstream MAP kinase. Previously, it has been shown that Crk2 binds ErbB1 active mutant L858R and it was suggested that Crk2 has a role in cell survival and oncogenic signalling (Petschnigg *et al.*, 2014). Further analysis of these interactions can help in the development of new drug therapies to treat diseases associated with ErbB2 receptor tyrosine kinase.

Another hypothesis was to generate the inactive mutant, which is the kinase dead version of the protein. Disabling the ability for auto-phosphorylation, it is possible to detect proteins which interact independently from the phosphorylation state of the receptor. ErbB2 K753A has been shown in literature to have impaired kinase activity. Testing 24 ErbB2 hits from the SH2/PTB library did not change the MaMTH signal with ErbB2 K753A compared to ErbB2 wt (Figure 10 [B]). It could be that these proteins do not bind in a phosphorylation dependent manner, or that kinase activity of ErbB2 K753A is not completely abolished due to its overexpression in this system. It is also possible that the tested proteins bind ErbB2 before its activation, like Src kinase that does not associate with any of the phosphotyrosine residues in the C terminal tail of ErbB2 (Kim *et al.*, 2005). To find which proteins interact only with phosphorylated receptor, the ErbB2 D845A mutant could be tested. The D845 residue is the catalytic aspartate, one of two key residues required for kinase activity (Aertgeerts *et al.*, 2011).

6. CONCLUSION

This thesis gives insight into the ErbB2-SH2/PTB domain interactome and confirms the power of the MaMTH assay to map and characterize protein-protein interactions. First, the MaMTH reporter cell lines with stable integration of bait proteins ErbB2 wild type, ErbB2 ex16del, ErbB2 T798M and ErbB2 K753A were successfully generated and validated. A suitable tetracycline concentration for induction of bait expression and its activation status were determined. All cell lines were tested by MaMTH assay with a small set of positive and negative interactors. Only protein-protein interaction with positive controls were detected, which led to the conclusion that the system works properly and the MaMTH assay is suitable to study protein-protein interactions in the desired cell lines. Furthermore, the screen of the SH2/PTB prey library with ErbB2 wild type was performed to map the ErbB2-SH2/PTB domain interactome. Thirty-six interaction partners were determined**:** 18 novel interactors and 18 known interactors. SHD, Sla2, Crk2 and SRMS binding through the SH2 domain to ErbB2 was characterized. Results indicate that SH2 domains of Sla2 and Crk2 mediate ErbB2-Sla2 and ErbB2-Crk2 interactions. Twenty-four identified ErbB2 interactors were further validated in cell lines stably expressing ErbB2 ex16del, ErbB2 T798M and ErbB2 K753A, and all of them showed that they interact with mutant ErbB2 proteins. This data confirms the specificity of detected interactions and shows preferential binding of eight proteins to ErbB2 ex16del and 15 proteins to ErbB2 T798M compared to ErbB2 wild type. The presented work is aimed at contributing to a better understanding of protein-protein interactions with the ErbB2 receptor tyrosine kinase. It revealed novel interactors whose functions remain to be characterized and implies the possibility that some protein interactions are involved in aberrant signalling pathways. These findings can be important for future design of new therapeutics that target specific protein-protein interactions associated with development of disease.

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8. SUPPLEMENTARY

8.1 Vectors

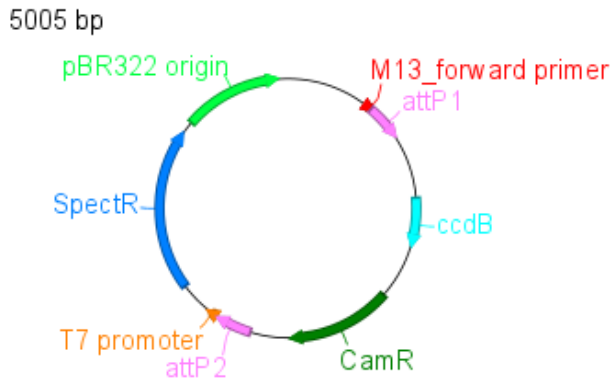

Figure 1S. pDNOR233 vector.

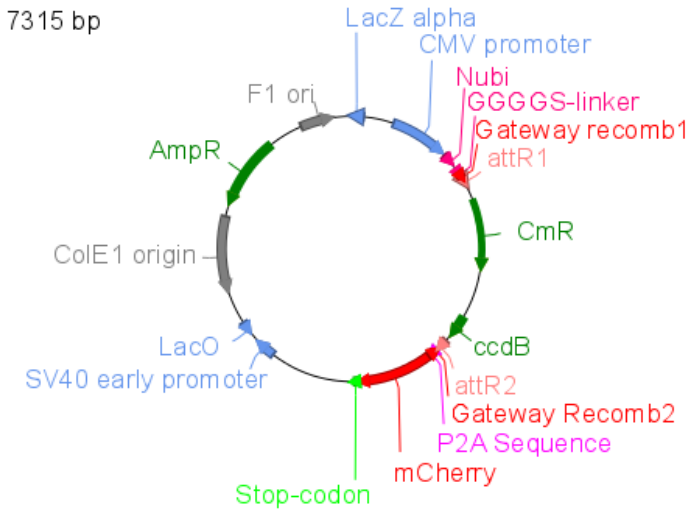

Figure 2S. N-tagged mCherry MaMTH prey vector (A1245).

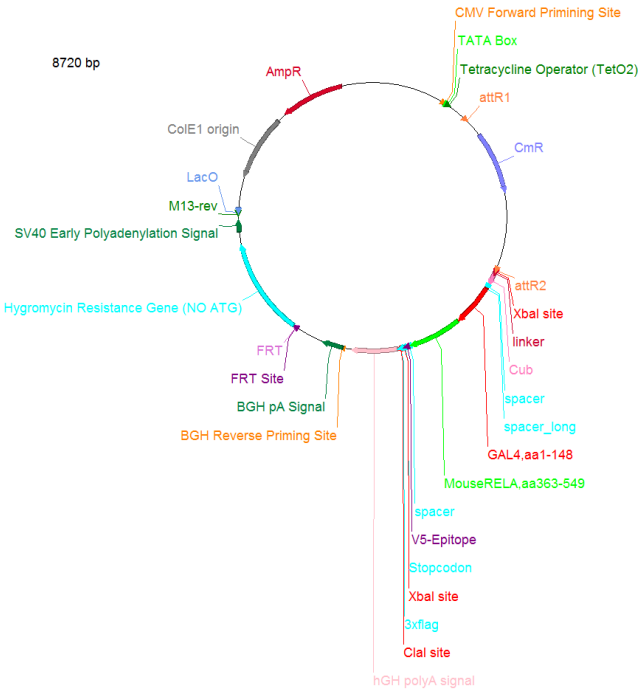

Figure 3S. C-tagged MaMTH bait vector (A1160).

8.2 SH2/PTB Library

Table 1S. List of the whole SH2/PTB prey library with full name, size and source. Information from genecard: http://www.genecards.org/ (accessed January, 14th 2017).

Gene	Full name	Size [kDa]	Source
ABL1	ABL Proto-Oncogene 1, Non-Receptor Tyrosine Kinase	122.9	ol
ABL2	ABL Proto-Oncogene 2, Non-Receptor Tyrosine Kinase	128.9	ol
BCAR3	Breast Cancer Anti-Estrogen Resistance 3	92.6	ol
BLK	BLK Proto-Oncogene, Src Family Tyrosine Kinase	57.7	ol
BLNK	B-Cell Adapter Containing A SH2 Domain Protein	50.5	ol
BMX	BMX Non-Receptor Tyrosine Kinase	78.0	ol
BTK	Bruton Tyrosine Kinase	76.2	ol
CBL	Cbl Proto-Oncogene	99.8	entry clone

CBLB	Cbl Proto-Oncogene B	109.5	ol
CBLC	Cbl Proto-Oncogene C	52.5	ol
CHN1	Rho GTPase-Activating Protein 2/Chimerin 1	53.2	ol
CHN2	Chimerin 2	53.9	hORF 9.1
CISH	Cytokine Inducible SH2 Containing Protein	28.7	ol
CLNK	Cytokine Dependent Hematopoietic Cell Linker	49.6	MCG /PCR
CRK	CRK Proto-Oncogene, Adaptor Protein	33.8	ol
CRK2	CRK Proto-Oncogene, Adaptor Protein 2	42.0	entry clone
CRKL	CRK Like Proto-Oncogene, Adaptor Protein	33.8	ol
CSK	C-Src Tyrosine Kinase	50.7	ol
DAPP1	Dual Adaptor of Phosphotyrosine And 3-Phosphoinositides 1	32.3	ol
DOK1	Docking Protein 1	52.4	hORF 8.1
DOK2	Docking Protein 2	45.4	entry clone
DOK3	Docking Protein 3	53.3	hORF 8.1
DOK4	Docking Protein 4	37	entry clone
DOK6	Docking Protein 6	38.3	entry clone
DOK7	Docking Protein 7	53.1	hORF 9.1
FER	FER Tyrosine Kinase	94.6	ol
FES	FES Proto-Oncogene, Tyrosine Kinase	93.5	ol
FGR	FGR Proto-Oncogene, Src Family Tyrosine Kinase	59.5	ol
FRK	Fyn Related Src Family Tyrosine Kinase	58.3	ol
FYN	FYN Proto-Oncogene, Src Family Tyrosine Kinase	60.8	hORF 9.1
GRAP2	GRB2-Related Adaptor Protein 2	37.9	ol
GRAPL	GRB2 Related Adaptor Protein Like	13.4	ol
GRB10	Growth Factor Receptor Bound Protein 10	67.2	ol

GRB14	Growth Factor Receptor Bound Protein 14	60.9	ol
GRB2	Growth Factor Receptor Bound Protein 2	25.2	ol
GRB7	Growth Factor Receptor Bound Protein 7	59.6	ol
HCK	HCK Proto-Oncogene, Src Family Tyrosine Kinase	59.6	ol
HSH2D	Hematopoietic SH2 Domain Containing	39.0	ol
IRS2	Insulin Receptor Substrate 2	137.3	MCG/PCR
ITK	IL2 Inducible T-Cell Kinase	71.8	ol
LCK	LCK Proto-Oncogene, Src Family Tyrosine Kinase	58	ol
LCP2	Lymphocyte Cytosolic Protein 2	60.2	ol
LYN	LYN Proto-Oncogene, Src Family Tyrosine Kinase	58.6	ol
MATK	Megakaryocyte-Associated Tyrosine Kinase	56.5	ol
NCK1	NCK Adaptor Protein 1	42.9	ol
NCK2	NCK Adaptor Protein 2	42.9	ol
PIK3R1	Phosphoinositide-3-Kinase Regulatory Subunit 1	83.6	ol
PIK3R2	Phosphoinositide-3-Kinase Regulatory Subunit 2	81.5	ol
PIK3R3	Phosphoinositide-3-Kinase Regulatory Subunit 3	54.4	ol
PTPN11	Protein Tyrosine Phosphatase, Non-Receptor Type 11	68.4	ol
PTPN6	Protein Tyrosine Phosphatase, Non-Receptor Type 6	67.6	ol
RASA1	RAS P21 Protein Activator 1	116.4	ol
RIN1	Ras And Rab Interactor 1	84.1	ol
RIN2	Ras And Rab Interactor 2	100.2	hORF 9.1
RIN3	Ras And Rab Interactor 3	107.8	ol
SH2B1	SH2B Adaptor Protein 1	79.4	ol
SH2B2	SH2B Adaptor Protein 2	67.7	ol
SH2B3	SH2B Adaptor Protein 3	63.2	ol
SH2D1A	SH2 Domain Containing 1A	14.2	ol

SH2D1B	SH2 Domain Containing 1B	15.3	ol
SH2D2A	SH2 Domain Containing 2A	42.9	ol
SH2D3A	SH2 Domain Containing 3A	63.1	ol
SH2D3C	SH2 Domain Containing 3C	94.4	ol
SH2D4A	SH2 Domain Containing 4A	52.7	ol
SH2D4B	SH2 Domain Containing 4B	51.2	ol
SH3BP2	SH3 Domain Binding Protein 2	62.2	ol
SHB	SH2 Domain Containing Adaptor Protein B	55.0	ol
SHC	SHC Adaptor Protein 1	62.8	ol
SHC3	SHC Adaptor Protein 3	64.1	ol
SHC4	SHC Adaptor Protein 4	68.8	ol
SHD	Src Homology 2 Domain Containing Transforming Protein D	38.3	ol
SHF	Src Homology 2 Domain Containing F	46.8	ol
SLA	Src-Like-Adaptor	31.2	ol
SLA2	Src Like Adaptor 2	28.6	ol
SOCS1	Suppressor of Cytokine Signaling 1	23.6	ol
SOCS2	Suppressor of Cytokine Signaling 2	22.2	ol
SOCS3	Suppressor of Cytokine Signaling 3	24.8	ol
SOCS4	Suppressor of Cytokine Signaling 4	50.6	ol
SOCS5	Suppressor of Cytokine Signaling 5	61.2	ol
SOCS6	Suppressor of Cytokine Signaling 6	59.5	ol
SRC	SRC Proto-Oncogene, Non-Receptor Tyrosine Kinase	59.8	ol
SRMS	Src-Related Kinase Lacking C-Terminal Regulatory Tyrosine And N-Terminal Myristylation	54.5	ol
STAP1	Signal Transducing Adaptor Family Member 1	34.3	ol

STAT1	Signal Transducer and Activator of Transcription 1	87.3	ol
STAT2	Signal Transducer and Activator of Transcription 2	97.9	ol
STAT3	Signal Transducer and Activator of Transcription 3	88.1	ol
STAT4	Signal Transducer and Activator of Transcription 4	85.9	ol
STAT5A	Signal Transducer and Activator of Transcription 5A	90.6	ol
STAT5B	Signal Transducer and Activator of Transcription 5B	89.9	ol
STAT6	Signal Transducer and Activator of Transcription 6	94.1	ol
SYK	Spleen Associated Tyrosine Kinase	72.1	ol
TNS1	Tensin 1	185.7	ol
TNS3	Tensin 3	155.3	ol
TNS4	Tensin 4	76.8	ol
TYK2	Tyrosine Kinase 2	133.7	hORF 9.1
VAV1	Vav Guanine Nucleotide Exchange Factor 1	98.3	ol
YES1	YES Proto-Oncogene 1, Src Family Tyrosine Kinase	60.8	ol
ZAP70	Zeta Chain of T Cell Receptor Associated Protein Kinase 70	69.9	ol

Table 2S. RFP expression of SH2/PTB prey library after MaMTH assay. The RFP values are shown as average of 3 biological replicates after RFP background was subtracted, proteins that show lower signal than background are indicated in red.

Protein	RFP normalised signal	Protein	RFP normalised signal	Protein	RFP normalised signal
ABL1	259.3	**GRB14**	-136.0	**SHB**	96.0
ABL2	-63.0	**GRB2**	211.7	**SHC**	2497.7
BCAR3	207.3	**GRB7**	709.0	**SHC3**	223.3
BLK	2249.7	**HCK**	-67.0	**SHC4**	1526.7

BLNK	-97.7	**HSHD2**	-144.7	**SHD**	3845.7
BMX	166.7	**IRS1**	625.3	**SHF**	486.0
BTK	-10.0	**ITK**	949.0	**SLA**	133.0
CBL	118.3	**LCK**	1.0	**SLA2**	3005.7
CBLB	-30.0	**LCP2**	41.7	**SOCS1**	365.7
CBLC	241.0	**LYN**	26.7	**SOCS2**	1763.0
CHN1	145.7	**MATK**	51.3	**SOCS3**	139.3
CHN2	2807.0	**NCK1**	177.7	**SOCS4**	214.3
CISH	106.3	**NCK2**	3250.3	**SOCS5**	278.0
CLNK	167.0	**PIK3R1**	18.3	**SOCS6**	546.0
CRK	84.3	**PIK3R2**	2799.0	**SRC**	445.0
CRK2	198.7	**PIK3R3**	1091.3	**SRMS**	179.0
CRKL	648.0	**PTPN11**	9.3	**STAPI**	1620.0
CSK	344.3	**PTPN6**	-92.7	**STAT1**	1042.7
DAPPI	121.0	**RASR1**	958.0	**STAT2**	824.0
DOK1	1564.3	**RIN1**	-187.3	**STAT3**	965.3
DOK2	589.3	**RIN2**	-104.7	**STAT4**	196.0
DOK3	-59.3	**RIN3**	117.0	**STAT5A**	137.3
DOK4	203.0	**SH2B1**	180.7	**STAT5B**	1573.3
DOK6	234.0	**SH2B2**	2437.7	**STAT6**	1501.0
DOK7	79.3	**SH2D1A**	2148.0	**SYK**	1291.3
FER	-98.7	**SH2D1B**	784.3	**TNS1**	198.7
FES	249.3	**SH2D2A**	367.0	**TNS3**	1649.3
FGR	127.7	**SH2D3A**	35.0	**TNS4**	407.3
FRK	332.3	**SH2D3C**	303.7	**TYK2**	844.0
FYN	246.3	**SH2D4A**	945.3	**VAV1**	3581.7
GRAP2	212.7	**SH2D4B**	3595.7	**YES1**	378.7
GRAPL	1282.0	**SH2B3**	-17.7	**ZAP70**	2273.0
GRB10	59.0	**SH3BP2**	1130.7		

Table 3S. List of proteins in SH2/PTB library that are considered as ErbB2 interactors after this study and used in screening against ErbB2 ex16del, ErbB2 T798M and ErbB2 K753A.

Protein	Protein	Protein	Protein
CRKL	SH2D3A	SOCS3	CBL
CSK	SH2D4B	SRMS	CHN2
GRB2	SCH1	STAT5B	CLNK

NCK2	SHD	ZAP70	DOK1
SH2D1A	SLA	CRK2	DOK7
SH2D1B	SLA2	DOK2	FYN

Table 4S. List of missing proteins in SH2/PTB library. Known interactors are indicated according to BioGRID database (https://thebiogrid.org/).

Gene	Full name	Known interactor	Gene	Full name	Known interactor
DOK5	Docking Protein 5		PTK6	Protein Tyrosine Kinase 6	+
FRS1	Fibroblast growth factor Receptor Substrate 1		SH2D5	SH2 Domain Containing 5	+
FRS2	Fibroblast growth factor Receptor Substrate 2		SH2D6	SH2 Domain Containing 6	
FRS3	Fibroblast growth factor Receptor Substrate 3	+	SH2D7	SH2 Domain Containing 7	
GRAP	GRB2-related adapter protein	+	SHC2	SHC Adaptor Protein 2	+
INPP5D	Inositol Polyphosphate-5-Phosphatase D		SHE	Src Homology2 Domain Containing E	
INPPL1	Inositol Polyphosphate Phosphatase Like 1		SOCS7	Suppressor of Cytokine Signaling 7	
IRS4	Insulin Receptor Substrate 4	+	SUPT6H	SPT6 Homolog, Histone Chaperone	

JAK1	Janus Kinase 1	+	TEC	Tec Protein Tyrosine Kinase	+
JAK2	Janus Kinase 1	+	TENC1	Tensin 2	+
JAK3	Janus Kinase 1		TXK	TXK Tyrosine Kinase	+
PLCG1	Phospholipase C Gamma 1	+	VAV2	Vav Guanine Nucleotide Exchange Factor 2	+
PLCG2	Phospholipase C Gamma 2	+	VAV3	Vav Guanine Nucleotide Exchange Factor 3	+

Biography

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2012. – 2015. Undergraduate programme of Molecular Biology, Faculty of Science, University of Zagreb, Zagreb, Croatia

Experience:

March 15th – October 15th, 2017. - International visiting graduate student at Department of Biochemistry, Faculty of Medicine, University of Toronto, Toronto, Canada

- Master thesis research and student internship in Stagljar Lab
- Supervisor Dr. Igor Štagljar, Prof.

October, 2015. – April, 2016. - Laboratory skill training in laboratory for Molecular genetics, Department of Molecular Biology, Faculty of Science, University of Zagreb

- Student internship
- Supervisor Dr. Ivana Ivančić Baće, Assoc. Prof.

November 3rd – 4th, 2016. – Participant in Fourth Meeting of the Croatian Association for Cancer Research, Zagreb, Croatia

November 28th, 2015. – Participant in First Croatian Symposium of Biologist in Health Care, Zagreb, Croatia

March, 2015. – June, 2015. - Teaching assistant for the course Molecular Genetics (40885), Faculty of Science, University of Zagreb

October, 2014. – January, 2015. – Teaching assistant for the course Laboratory Animals in Biological Research (40368), Faculty of Science, University of Zagreb

September, 2014. – December, 2016. – Crew member McDonald's (Globalna hrana d.o.o.), Zagreb, Croatia

- Student part time job

July, 2014. – Field course in Botany and Zoology (40676), Faculty of Science, University of Zagreb

October, 2013. – January, 2014. – Teaching assistant for the course Zoology (35803), Faculty of Science, University of Zagreb

2013., 2014. and 2016. - Organizer and presenter at Night of Biology, Faculty of Science, University of Zagreb