University of Szeged
Albert Szent-Györgyi Medical School
Doctoral School of Multidisciplinary Medical Science

TARG1 affects EGFR signaling through the regulation of RNA metabolism

PhD Thesis

Mihály Mérey

Supervisor(s): Dr. Gyula Timinszky

Szeged,
2025

PUBLICATIONS

1. Publications related to the thesis

I. **Mérey, Mihály**; Fajka-Boja, Roberta; Imre, Gergely; Gudmann, Péter; Török, Zsolt; Mátés, Lajos; Czibula, Ágnes; Timinszky, Gyula. TARG1 affects EGFR signaling through the regulation of RNA metabolism SCIENTIFIC REPORTS 15 : 1 Paper: 23651 (2025)

2. Publications not directly related to the thesis

I. Mamar, Hasan; Fajka-Boja, Roberta*; Mórocz, Mónika; Pinto Jurado, Eva; Zentout, Siham; Mihut, Alexandra; Kopasz, Anna Georgina; **Mérey, Mihály**; Smith, Rebecca; Abhishek, Bharadwaj Sharma et al. The loss of DNA polymerase epsilon accessory subunits POLE3-POLE4 leads to BRCA1-independent PARP inhibitor sensitivity NUCLEIC ACIDS RESEARCH 52 : 12 pp. 6994-7011. , 18 p. (2024)

CONTENTS

ABBREVIATIONS

ARHs	ADP-ribosylhydrolases
ART	ADP-ribosyl transferase
ARTCs	cholera toxin-like ADP-ribosyl transferases
ARTDs	diphtheria toxinlike ADP-ribosyl transferases
ARTs	ADP-ribosyl transferases
BAD	BCL2 Associated Agonist of Cell Death
cDNA	complementary DNA
c-Fos	Fos Proto-Oncogene, AP-1 Transcription Factor Subunit
CNS	central nervous system
CRISPR	clustered regularly interspaced short palindromic repeats
DDR	DNA damage response
DMEM	Dulbecco's Modified Eagle Medium
DraG	dinitrogenase reductase activating glycohydrolase
DNA	deoxyribonucleic acid
DRB	Dichlorobenzimidazole 1-β-D-ribofuranoside
EF-2	Eukaryotic Translation Elongation Factor 2
EGFR	epidermal growth factor receptor
ErbB 2,3,4	Erb-B2 Receptor Tyrosine Kinase 2,3,4
FBS	Fetal Bovine Serum
FGF2	Fibroblast Growth Factor 2
FGFRs	fibroblast growth factor receptors
FOXO	Forkhead Box O
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase

G3BP1	G3BP Stress Granule Assembly Factor 1
HGF	hepatocyte growth factor
HER 2,3,4	Erb-B2 Receptor Tyrosine Kinase 2,3,4
IGF1	Insulin Like Growth Factor 1
IGFRs	insulin-like growth factor receptors
KO	Knock out
MAPK	Mitogen-activated protein kinase
MAR	mono ADP ribose
MEK 1/2	Mitogen-Activated Protein Kinase Kinase 1/2
mRNA	messenger RNA
mTORC1	Mechanistic Target of Rapamycin Kinase
MYC	MYC Proto-Oncogene, BHLH Transcription Factor
NAD+	nicotinamide adenine dinucleotide
NOX	NADPH Oxidase
PAR	Poly ADP-ribose
PARPs	Poly(ADP-ribose) Polymerases
PARG	Poly(ADP-Ribose) Glycohydrolase
PIP2	phosphatidylinositol 4,5-bisphosphate
PI3K	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase
qPCR	quantitative polymerase chain reaction
qRT-PCR	quantitative reverse transcription polymerase chain reaction
rRNA	ribosomal RNA
RAF	RAF Proto-Oncogene Serine/Threonine-Protein Kinase
RANKL	TNF Superfamily Member 11

RAS	RAS Proto-Oncogene, GTPase
RHEB	Ras Homolog, MTORC1 Binding
RPS6	Ribosomal Protein S6
RACK1	Receptor for Activated C Kinase 1
RT	room temperature
RTKs	Receptor Tyrosine Kinases
Sirt4, 6	Sirtuin 4,6
SHC	SHC Adaptor Protein 1
TGF-α	transforming growth factor-alpha
TKI	Tyrosine Kinase inhibitor
TNBCs	Triple negative breast cancers
TSC2	Tuberous sclerosis complex 2
WT	wild type

INTRODUCTION

1. ADP Ribosylation as a biomolecular and post translational modification

ADP-ribosylation is a ubiquitous modification of biomolecules such as nucleic acids and various types of amino acid residues in proteins, which can be found in all kingdoms of life. It was first identified in the 1960's. In this modification process, the responsible enzymes use NAD^+ as a substrate and split it into ADP-ribose and nicotinamide (Suskiewicz et al., 2023). The resulting ADP-ribose molecules are then added to their target sites. These modifications can vary by their length (mono-ADPr modifications, poly chains) as well as in their structure (single or branched chains). The enzymes that covalently attach the ADPr to their targets are called the ARTs and those that cleave off ADPr are called ARHs (Mikolčević et al., 2021).

The formation of polymers or monomers of ADP-ribose is known to be involved in many cellular processes. These include the DNA damage response, as well as the regulation of chromatin structure, transcription, and RNA processing (Lüscher et al., 2018). Here, we focus on the human ADP-ribosylation system, which also consists of "writers," "erasers," and "readers" within this enzyme family. The transferase enzymes are classified based on the homology of their catalytic domains with bacterial toxins into two superfamilies: the ARTCs and the ARTDs. These two classes of enzymes share an evolutionarily conserved protein fold, called ART domain (Palazzo et al., 2019). Three crucial amino acids within the ART domain define their affiliation with the cholera or diphtheria toxin-like superfamilies—the R-S-E and H-Y-E triads. The first two amino acids in the triad are important for the NAD+ binding, while the common glutamate functions in catalysis. ARTCs and ARTDs also differ for their specificity to target distinct amino acids (Barkauskaite et al., 2015). The ARTD group of transferases most commonly modifies acidic residues. The founding member of ARTD family is the diphtheria toxin, an exotoxin secreted by Corynebacterium diphtheriae, which catalyses the modification of the EF-2 at a modified amino acid called diphthamide, thus inhibiting the translation machinery of the host (Van Ness et al., 1980). Seventeen members of the ARTD superfamily have been identified in mammals and are known as PARPs (Cohen & Chang, 2018). PARPs most commonly transfer ADP-ribose onto aspartic/glutamic acids (Asp/ Glu-ADPr), through ester linkages, and on serine (Ser- ADPr) residues through O-glycosylation. Several PARPs can produce chains of ADP-ribose polymers (also called poly-(ADP-ribose), thus abbreviated as PAR), where repeating single ADP-ribose units (up to 200 in length) are linked via unique O-glycosidic ribose-ribose bonds (Crawford et al., 2018). This type of modification is generally named poly-(ADP-ribosylation) (PARylation). Well-characterized PARPs able to generate

PARylation are PARP1, PARP2, Tankyrase-1 and Tankyrase-2. However, the remaining human PARP members are instead only capable of transferring a single ADP-ribose group to their target proteins, thus producing mono-(ADP-ribosylation) (also abbreviated as MARylation) (Kleine et al., 2008).

2. PARP superfamily

Based on the evolutionary timeline, ADP-ribosylation is thought to have emerged in bacteria as part of the rapid expansion of secondary metabolism and conflict- and immunity-related systems during the great oxygenation event. With ADP-ribosylation, the connection with conflict and immunity is well-established and is still visible in the form of numerous extant ADP- ribosylation- based exotoxins, toxin-antitoxin modules, as well as in phage defense and antibiotic-modifying systems.(Aravind et al., 2014; Mikolčević et al., 2021).

PARP Family in Eukaryotes and humans:

In eukaryotes, ADP-ribosylation has multiple roles, both in general maintenance and in the response to various types of danger and stress. In the context of more general pathways, ADP-ribosylation has been implicated in transcription, translation, RNA stability, spindle assembly and cell division, cell signaling, trafficking, and nuclear-cytoplasmic transport (Suskiewicz et al., 2023). Humans are thought to express 17 PARPs identified on the basis of sequence homology to the catalytic domain of PARP1. The PARP family is further grouped into four subfamilies based on the presence of functionally characterized domains in regions outside the PARP domain: DNA-dependent PARPs, initially thought to require DNA binding for enzymatic activity; tankyrases, with protein-binding ankyrin repeats; CCCH zinc finger PARPs that contain CCCH zinc finger domains shown to bind viral RNA; and macro-PARPs, with ADPr-binding macro domains (Karras et al., 2005). Based on the experimental study of a subset of PARPs combined with bioinformatic analysis, each PARP is predicted to exhibit either MAR or PAR synthesis activity or catalytic inactivity. Sequence analysis predicts that DNA-dependent PARPs, tankyrases, and PARP4 generate PAR; PARP9 and 13 are catalytically inactive; and all other PARPs generate MAR. Specific amino acid residues that have been identified as targets of PARP modification include glutamic acid, aspartic acid and lysine residues (Vyas et al., 2013).

3. PARylating and MARylating enzymes

In humans, five enzymes are responsible for PARylation: PARP1, PARP2, PARP3, Tankyrase 1, and Tankyrase 2, as previously mentioned. PARP1, PARP2, and PARP3 are known to catalyze PARylation during the DNA damage response. Among these PARPs, PARP1 is the founding member of the PARP family for the synthesis of PAR chains. Tankyrases, including Tankyrase-1 (PARP5a) and Tankyrase-2 (PARP5b) have been shown to contribute to genomic stability. (Dregalla et al., 2010; M et al., 2012).

The majority of ARTDs are MARTs, which transfer only a single ADP-ribose unit, resulting in the MARylation of targets. These enzymes are PARP3, PARP4, PARP6-12 and PARP14-16 which share a common characterization with no glutamate catalytic activity (Vyas et al., 2014). In humans Sirtuins are a class of deacetylases comprising seven enzymes. Sirt4 and Sirt6 possess MARylation activity (Bheda et al., 2016). Sirtuins cleave acyl groups in an NAD^+-dependent manner and generate O-acyl-ADP-ribose (OAADPr), which is then transferred to target residues such as lysine (Du et al., 2009). Sirt6 is located in the nucleus, while Sirt4 is localized in the cytoplasm, primarily within the mitochondria.

4. Mono ADP ribose hydrolase enzymes and their implications in cancer

As a reversible modification, ADP-ribosylation is tightly regulated by three enzyme families, including the DraG-like ADP-ribosyl hydrolase family (ARHs), macrodomain-containing family, and the nucleoside diphosphate linked to a variable moiety X (Nudix) family (Schuller et al., 2023). In this study, I will only briefly mention the ARH and the Nudix family, leaving more space for the macrodomain family, since TARG1 is one of its members.

Macrodomain-containing enzymes are widely distributed in all domains of life, and share a highly conserved ADP-ribose binding domain, known as macrodomain (Rosenthal et al., 2013). Macrodomains consist of 150–210 amino acids with the core motif harboring a three-layer sandwich architecture, and are able to bind OAADPr, as well as MARylated and PARylated proteins. (Karras et al., 2005). PARG is the only known member with PAR-hydrolyzing activity, although it is unable to remove the terminal ADP-ribose linked to substrates. (Slade et al., 2011). Notably, PARG is also able to reverse MARylation onto the 5’ and 3’ phosphorylated ssDNA and ssRNA (Munnur et al., 2019; Munnur & Ahel, 2017). The functions of PARG are involved in DNA repair, replication forks and recovery from persistent replication stress (Illuzzi et al., 2014). Additionally, the mutation of PARG is clearly associated with neurodegeneration and

accumulation of PAR in the CNS (Hanai et al., 2004). There are also three other human macrodomain-containing family members which have hydrolytic activity towards MARylated substrates: MacroD1, MacroD2, and TARG1. These enzymes were demonstrated to remove the single ADP-ribose unit from modified protein substrates instead of PAR chain. TARG1 also cleaves the ester linkage between glutamate-linked PAR, although its activity is significantly lower than that of PARG. (Sharifi et al., 2013). Importantly, MacroD1, MacroD2, and TARG1 can cleave the single ADP-ribose from the 5' or 3' terminal phosphates of dsDNA and ssRNA to reverse nucleic acid modification (Munnur et al., 2019; Munnur & Ahel, 2017).

TARG1: TARG1 were reported to have hydrolase activity in deacetylating O-acetyl-ADP-ribose (OAADPR) (Chen et al., 2011), removing ADP-ribose from modified proteins (Jankevicius et al., 2013; Rosenthal et al., 2013; Sharifi et al., 2013) and lastly in removing ADP-ribose from MARylated DNA or RNA (Munnur et al., 2019; Munnur & Ahel, 2017). TARG1 also cleaves the ester linkage between glutamate-linked PAR although its activity is much lower compared with PARG (Sharifi et al., 2013). A mutation in TARG1 was found in patients with a severe neurodegenerative phenotype, although the underlying mechanism is unclear. Overexpressed TARG1 resides in both nucleoli and nucleoplasm, compartments between which it can shuttle (Bütepage et al., 2018). A former study characterizing the TARG1 interactome, found that the ribosomal proteins and the proteins associated with rRNA metabolism and RNA binding were the main interaction partners (Bütepage et al., 2018). Furthermore, they stated that TARG1 shuttles continuously between nucleoli and the nucleoplasm and accumulates in transcriptionally active nucleoli under steady-state conditions. Upon DNA damage rapid and reversible relocation into the nucleoplasm occurred, which was dependent on the ADPr binding ability of TARG1. The accumulation in nucleoli and PARylation-dependent relocation to the nucleoplasm are consistent with the ability of TARG1 to bind RNA and PAR in a competitive manner. They concluded that TARG1 may be a nucleolar ribosome biosynthesis quality control factor (Bütepage et al., 2018). Knockdown of TARG1 leads to a decrease in 293T cell proliferation and a slight increase in senescence in U2-OS cells, which are derived from an osteosarcoma (Sharifi et al., 2013). CRISPR-mediated knockdown of TARG1 does not influence HeLa or U2-OS proliferation, which leaves it unclear in which setting TARG1 is required for cell growth. Overexpression does not lead to changes in cell proliferation (Bütepage et al., 2018; Žaja et al., 2020). A recent study demonstrated a therapeutically targetable pathway that controls stress granule assembly and disassembly in ovarian cancer cells through the MARylation of RACK1 protein. This was required for stress

granule formation and promotes the colocalization of RACK1 in stress granules with G3BP1, eIF3η, and 40S ribosomal proteins. TARG1 plays the role to deMARylate RACK1, leading to the dissociation of the stress granules and the restoration of translation (Challa et al., 2023).

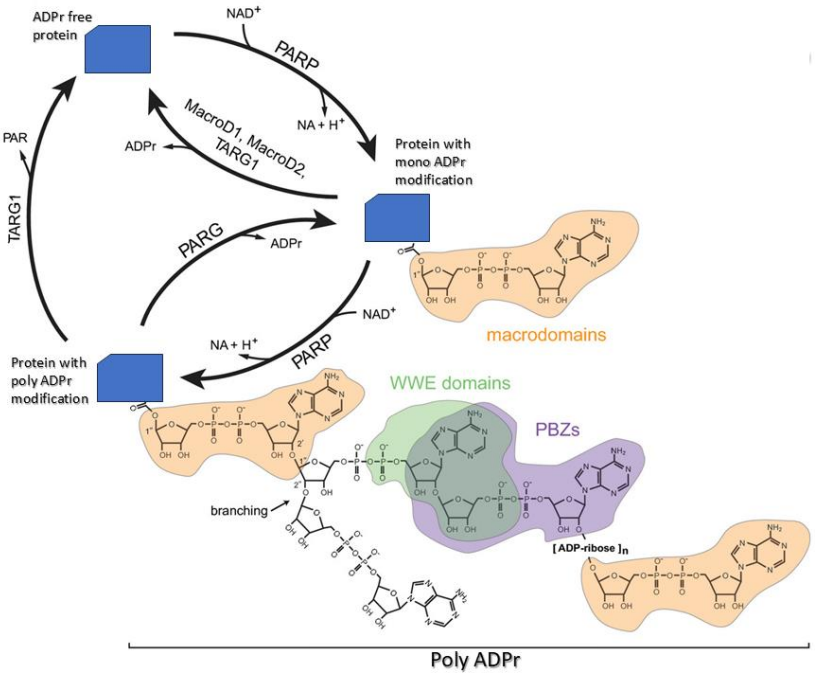

Figure 1. PARP-Dependent ADP-Ribosylation Cycle. Proteins are ADP-ribosylated by PARPs using NAD+ as a co-factor, releasing nicotinamide (NA). Specific PARPs can ADP-ribosylate previous ADP-ribose (ADPr) units, which results in poly(ADP-ribose) formation with occasional branching. Different domains recognize mono-ADP-ribose or poly(ADP-ribose). Macrodomain recognition parts of mono-ADP-ribose and poly(ADP-ribose) are indicated in orange. PAR recognition parts of WWE domain are highlighted in violet and those of PBZs in green. Poly(ADP-ribosyl)ated proteins can be substrates of TARG1, which can remove whole PAR chain from the targets, or of PARG, which degrades PAR, leaving mono-ADP-ribosylated protein. Mono-ADP-ribose can be removed by the action of MacroD1, MacroD2, or TARG1, which complete the ADP-ribosylation cycle.

5. An infamous receptor tyrosine kinase, EGFR signaling pathway

EGFR is a transmembrane glycoprotein belonging to the ErbB family of RTKs which includes ErbB-1 (EGFR), ErbB-2 (HER2/neu), ErbB-3 (HER3), and ErbB-4 (HER4) (Wheeler et al., 2008; Yu et al., 2017). The RTK family mainly consists of ErbBs, FGFRs, IGFRs, VEGFRs, and HGFRs (Lemmon & Schlessinger, 2010). Upon binding with ligands, EGFR is activated and initiates the activation of subsequent intracellular signaling pathways, such as the PI3K/Akt and MAPK, which are involved in the proliferation, differentiation, migration, and apoptosis of

certain cells (Lemmon & Schlessinger, 2010; Yarden & Sliwkowski, 2001). RTKs serve as receptors for various growth factors, cytokines, and hormones. RTKs have a similar molecular structure: an extracellular ligand-binding region, a single hydrophobic transmembrane domain, and a cytoplasmic protein tyrosine kinase region plus additional carboxy terminal and juxtamembrane regulatory regions (Lemmon & Schlessinger, 2010). The binding of ErbB receptors with a specific set of ligands, such as epidermal growth factor (EGF), TGF-α, amphiregulin, betacellulin, or epiregulin, they would form a homodimer by themselves or form a heterodimer with other ErbB family members. Interestingly, no ligand has been identified for the HER2 receptor, which must homodimerize (trough ligand-independent dimerization) or heterodimerize with a ligand-bound EGFR, HER3 or HER4 to induce signaling (Harari & Yarden, 2000). The most potent heterodimer combination for cell growth and transformation is HER2–HER3. HER3 itself has no intrinsic tyrosine kinase activity (Mishra et al., 2018) and must heterodimerize to induce signaling, though it binds the heregulin 1 and 2 ligands. HER4 is capable of homodimerization or heterodimerization and is stimulated by heregulin 1–4, betacellulin, epiregulin, heparin-binding EGF-like ligand and epigen (Karamouzis et al., 2007). Subsequently, the dimerization of EGFR would activate its cytoplasmic tyrosine kinases domain and then trigger a series of signal transduction (Arteaga, 2001; Ellis, 2004). The ligand and its concentration, plus the composition of the receptor dimer determine which intracellular signaling pathways are activated, alongside with the route, that the internalized receptor will follow (Okines et al., 2011). Binding of ligands to EGFR induces translocation of EGFR from the plasma membrane to the intracellular region independently of its phosphorylation-mediated TK activation (Roepstorff et al., 2008). EGFR endocytosis and its endosomal-mediated sorting are thought only to be a cellular mechanism to induce degradation and termination of activated EGFR signaling or as a recycling mechainsm to return to the cell surface for continued signaling (Wiley, 2003). However, some reports show that the intracellular translocation of EGFR regulates the EGFR signaling pathway, consequently affecting cell growth and survival (Demory et al., 2009; S. Y. Lin et al., 2001). In addition to this alternative signaling routes and roles of EGFR, there are well described pathways, that are activated by a wide variety of adaptor proteins attracted to the transphosphorylated intracellular domain of the active receptors. One of these major pathways downstream of EGFR and other RTKs is the MAPK pathway (Hynes & MacDonald, 2009). Two adaptor proteins, GRB2 and SHC, link EGFRs to the ERK-MAPK pathway (Shaul & Seger, 2007). Both engage SOS, which stimulates RAS, and this results in activation of the RAF kinase. RAF proteins comprise the uppermost layer of a cascade of three kinases, which also includes MEK and the terminal MAPK, ERK (Katz et al., 2007). In a similar

manner, EGFR family members recruit a class I phosphatidylinositol 3-kinase (PI3K) (Hay & Sonenberg, 2004). Note, however, that EGFR harbors no direct PI3K docking site. PI3K phosphorylates PIP2 to generate phosphatidylinositol (3,4,5)-trisphosphate (PIP3). PIP3 recruits the AKT kinase to the plasma membrane. When bound to the inner leaflet of the plasma membrane, AKT undergoes activation by both PDK1 and mTORC2 (mTOR complex 2) (Engelman et al., 2006). AKT can inhibit apoptosis by means of phosphorylating BAD and FOXO family transcription factors. AKT can also activate mTOR by means of phosphorylating TSC2. Phosphorylation of TSC2 inhibits its GAP activity towards the GTPase RHEB. Active, GTP-bound RHEB proteins serve as activators of mTORC1, which controls both translation of mRNAs to proteins, and the biosynthesis of cholesterol, which supplies lipids and proteins to growth factor stimulated cells (Laplante & Sabatini, 2012; Tarcic et al., 2012).

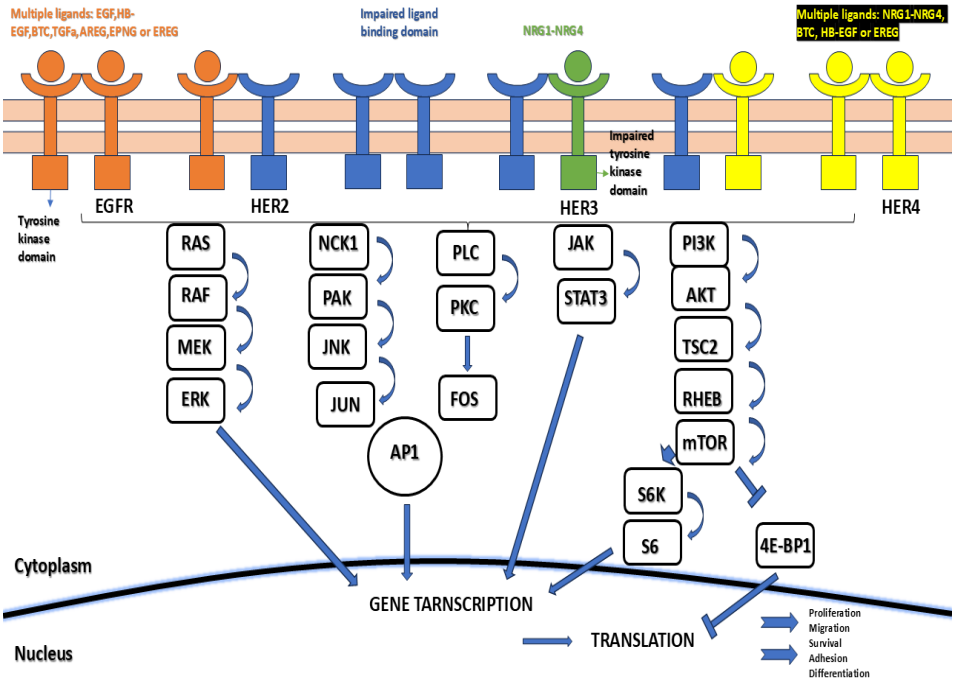

Figure 2. ERBB family members (ERBB1–ERBB4; also known as epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2), HER3 and HER4) are receptor tyrosine kinases (RTKs) harbouring an analogous structure, which comprise an extracellular ligand binding domain, a single hydrophobic transmembrane region and an intracellular tyrosine kinase domain (except for HER3). Among the EGFR ligands, EGF, transforming growth factor-α (TGFα), amphiregulin (AREG) and epigen (EPGN) interact solely with EGFR, whereas epiregulin (EREG), heparin-binding EGF-like growth factor (HB-EGF) and betacellulin (BTC) also bind to and activate HER4. A family of EGF-related ligands, neuregulins (NRGs; composed of NRG1–NRG4) bind to HER3 and HER4. HER2 directly binds to none of these EGF-related ligands. HER3 is considered to have an impaired tyrosine kinase domain and to provide little kinase activity. Therefore, in order to activate and provide signalling of HER2 and HER3, their heterodimerization with other ERBB family members is needed. Downstream signalling pathways activated by ERBB family members overlap and influence each other. In phosphatidylinositol-3 kinase (PI3K)–AKT–mammalian target of rapamycin (mTOR) pathways, AKT phosphorylates and inhibits the tumour suppressor TSC2, thereby activating RHEB, which positively regulates mTOR. mTOR upregulates the canonical mRNA translation through activation of ribosomal protein S6 kinase (S6K) and suppression of eukaryotic translation initiation factor 4E-BP1, and is strongly associated with cell proliferation. RAS–RAF–MEK–ERK contributes to cell survival, proliferation and growth. NCK adaptor protein 1 (NCK1)–p21-activated kinase (PAK)–JNK and phospholipase Cγ (PLCγ)–protein kinase C (PKC) lead to activation of the dimeric activator protein 1 (AP1) transcription factor that promotes tumorigenesis. The Janus kinase 2 (JAK2)–signal transducer and activator of transcription 3 (STAT3) pathway contributes to cell proliferation.

6. EGFR signaling in cancer

EGFR signaling is frequently altered in several human cancers due to EGFR gene amplification and/or protein overexpression, mutations, or in-frame deletions (Roskoski, 2014). Genetic lesions often occur alongside with increased EGFR ligand production due to autocrine or paracrine loops (Wilson et al., 2012). In many cases, EGFR genetic alterations determine abnormal EGFR trafficking, which contributes to increased signaling and tumor development. For instance, the increase in EGFR density at the plasma membrane due to EGFR amplification/overexpression was shown to stimulate receptor homo- and heterodimerization, leading to kinase activation (Chung et al., 2010; Wilson et al., 2009). In particular, heterodimers with the ligand-independent receptor ErbB2 are constitutively active, evade receptor ubiquitination and degradation, and are mostly recycled back to the plasma membrane, thereby producing sustained signaling and cell proliferation (Schneider & Yarden, 2016). Oncogenic EGFR mutations and large genetic rearrangements (as observed in glioblastoma (brain), lung, breast, and ovarian cancers) often cause altered receptor endocytosis, which contributes to increased signaling properties (Yarden & Pines, 2012). In some cases, mutations directly disrupt the recruitment site of the E3 ligase, Cbl, in the intracellular domain of the receptor (i.e., EGFRvIV and EGFRvV mutants), thereby affecting receptor ubiquitination and lysosomal degradation (Roskoski, 2014). In other instances, mutations are located in the extracellular domain (i.e., EGFRvIII), leading to ligand-independent receptor activation (Grandal et al., 2007; Han et al., 2006). Unexpectedly, these mutations also caused reduced phosphorylation of the intracellular tyrosine residue 1045, the direct Cbl-binding site, via an unknown mechanism. In this way, receptor ubiquitination and turnover are affected, resulting in sustained signaling (Grandal et al., 2007; Han et al., 2006; M. H. H. Schmidt et al., 2003).

Overall, overexpression of wild-type (WT) EGFR protein with or without EGFR gene amplification or a kinase-activating mutation further enhances cell proliferation, migration, survival, and antiapoptotic responses through signaling cascades, and these processes are closely related to the occurrence and development of many types of epithelial and also mesenchymal-derived cancer, such as non-small cell lung cancer, breast cancer, and osteosarcoma (OS) (Shi et al., 2022). EGFR has been reported to be involved in cell proliferation, differentiation, migration, and apoptosis of OS (Yuan et al., 2019). Osteosarcoma is the most common type of primary malignant tumors that originate in the bone. Resistance to chemotherapy confers a poor prognosis on OS patients. Dysregulation of EGFR signaling has been reported in sarcomas. However, the functional contribution of EGFR hyperactivation to

the tumor biology and chemoresistance remains largely unexplored in OS. EGFR expression was found to be upregulated in fibroblastic OS cell lines. EGFR knockdown suppressed OS cell proliferation, migration, and invasion in vitro and tumor formation in vivo. Conversely, EGFR overexpression promoted the growth and motility of OS cells. Mechanistically, the levels of phospho-Akt and phospho-ERK were decreased upon EGFR knockdown but increased as a result of EGFR overexpression, implying a possible involvement of PI3K/Akt and ERK pathways in mediating the effects of EGFR on OS cells (S. Wang et al., 2021).

7. EGFR targeting therapeutic implications

EGFR is one of the most prominent pharmacological targets of anti-cancer drugs. There are two types of drugs that are in therapeutic use in clinic: the monoclonal antibodies (mAbs), that bind antagonistically to the ligand binding pocket of the receptor's extracellular domain, and the TKI, that prevents the receptor phosphorylation by crossing the plasma membrane of the cell, and binding to the ATP binding pocket of the receptor on the intracellular domain. Both monoclonal antibodies and TKIs have demonstrated efficacy and acceptable toxicity in large phase III clinical trials (Arteaga & Engelman, 2014; Mitsudomi et al., 2010; Rosell et al., 2012).

However, despite major therapeutic advances, both primary and acquired resistance to these drugs occurs and result in disease recurrence. Notably, while drug resistance arises from evolutionary pressures that select specific clones, resistance to TKIs often associated with the appearance of new on-target mutations, but this mechanism rarely confers resistance to mAbs (Konieczkowski et al., 2018). Besides the secondary mutations there are other mechanisms that can contribute to therapeutic resistance of inhibition. Tumor cells can activate alternative signaling pathways that can bypass EGFR dependence and promote cell survival and proliferation (Q. Liu et al., 2018). For example, activation of the PI3K-Akt pathway may contribute to resistance to EGFR inhibition (Rajendran et al., 2024). Also, cells may upregulate alternative receptor tyrosine kinases, such as HER2 or HER3, which share downstream signaling pathways with EGFR. Amplification of these receptors can compensate for EGFR inhibition and promote tumor growth (Mishra et al., 2018). Additionally, it is worth mentioning that the tumor microenvironment can also undergo dynamic changes in response to therapy, leading to the selection of resistant tumor cell populations. Factors such as hypoxia, inflammation, and stromal interactions can contribute to treatment resistance (Dzobo et al.,

2023). With regard to these secondary mutations and upregulated alternative receptor derived resistances, we distinguish four generations of EGFR-TKI's.

Besides the strategy of developing next-generation inhibitors and identification of predictive biomarkers, the combination therapies targeting multiple signaling pathways also proven to be effective in the ongoing battle against cancer (Huang et al., 2020; Le et al., 2021; S. Lin et al., 2022; Ma et al., 2023; Nowsheen et al., 2012; Tricker et al., 2015).

8. ADP ribosylation in relation with EGFR signaling pathway

Many studies described PARP inhibitors that showed a successful treatment of cancer cells both in vitro and in vivo in interaction with EGFR inhibitors (S. Lin et al., 2022; Nowsheen et al., 2012). Also, cells that overexpressing EGFR showed sensitivity against PARP inhibitors. Many of these studies pointed out a correlation between EGFR and ADP-ribosylation particularly in the context of DNA damage response. One of these studies in 2012 showed contextual synthetic lethality with combined EGFR and PARP inhibition with lapatinib and ABT- 888, respectively, due to a transient DNA double-strand break repair deficit induced by lapatinib and subsequent activation of the intrinsic pathway of apoptosis in TNBCs (Nowsheen et al., 2012). Another study revealed that in TKI resistant, EGFR-mutated lung cancer cells were sensitive to PARP inhibitor treatment. These TKI resistant cells exhibited increased RAC1 activity. RAC1 was shown to regulate ROS production in cells through NOX and that its activity is driven by PARP-mediated ADP-ribosylation. Inhibiting PARP dampened the restrictive activity of RAC1 on NOX (Marcar et al., 2019). Furthermore (Wu et al., 2020) work showed that EGFR-amplified GSCs (glioma sphere-forming cells derived from primary GBM) showed remarkable sensitivity to talazoparib (a highly potent PARP inhibitor) treatment. Besides their separate and also cooperative influence on DDR of ADP-ribosylation and EGFR pathway, it has been also reported before that they regulate the activity of ERK1/2 that is downstream of EGFR signaling and plays a role of regulating cell growth, migration and survival (Boehi et al., 2021). PARP1-mediated ADP-ribosylation was found to promote cell survival by enhancing ERK phosphorylation (p-ERK) in different cellular contexts (Chowdhury et al., 2019; S. Li et al., 2016; Motta et al., 2015). Knockdown and/or inhibition of PARP1 in lung cancer and osteosarcoma cells decreased ERK phosphorylation, which reduced cell proliferation and migration (Chowdhury et al., 2019; S. Li et al., 2016). Interestingly, PARP1 inhibition or knockdown also reduced the expression and phosphorylation of EGFR, an upstream activator

of ERK. This suggests that PARP1-mediated ADP-ribosylation not only reinforces ERK1/2 signaling, but also the expression of its pathway components (Chowdhury et al., 2019).

AIMS OF THE STUDY

EGFR is a famous and well-known therapeutic target in cancer treatment due to its major role in cell growth, migration, survival and also in DDR. Due to its acquired resistance to inhibitors through mutations, and also the complexity of EGFR's signaling pathway, which is full of positive and negative feedback loops, alongside with its cross-signaling with other receptors pathways, keeps EGFR in the spotlight of cancer research. Many studies showed before that PARP inhibition paired with EGFR inhibition has proven to be a promising therapeutic approach against cancer cells that acquired resistance against single chemotherapeutic compound treatments. On the other hand, the involvement of the ADP-ribose hydrolase enzymes, that are responsible for reversing the modifications, synthetized by the PAR/MAR writers, has been less investigated in this context. In this study we aimed to inquire into the possible role of TARG1 in regulating the expression, activity, or associated signaling pathways of EGFR. Among the macrodomain-containing hydrolases, TARG1 was the ideal target for investigation, as it is the only one of the three enzymes (MacroD2, MacroD1, TARG1) capable of reversing both mono- and poly-ADP-ribose modifications from target biomolecules. Also, MacroD1 activity was mostly reported to reside in the mitochondria, while MacroD2 is highly expressed primarily in neuronal tissue. Furthermore, we aimed to determine whether there is a significant correlation between the expression level and the function of EGFR and TARG1, and if so, how relevant this relationship could be in the context of cancer therapy research.

MATERIALS AND METHODS

9. Cell culture

U2-OS wild type and TARG1 knock out (CRISPR/Cas) cell lines have been describe previously (Tromans-Coia et al., 2021) and were cultured in DMEM (LM-D1109 Dulbecco's Modified Eagles High Glucose w/ L-Glutamine w/o Sodium Pyruvate, Biosera Cholet, France), supplemented with 10% FBS (FB-1090/500 Fetal Bovine Serum (South America) Biosera Cholet, France), 1x NEAA (E1154 MEM, Biosera Cholet, France) and Penicillin/Streptomycin (A4118, Biosera Cholet, France) at 37 ℃ in a humidified cell incubator with 5% CO2. The cell lines were routinely tested for mycoplasma contamination using a qPCR-based approach (MQ-50 MycoQuant Mycoplasma Quantification Kit AVIDIN, Szeged, Hungary). For knockdown of TARG1, we used a stable U2-OS cell line constitutively expressing miRNA targeting TARG1. The stable TARG1 knockdown U2-OS cell line was created by genome integration of a transposon-based vector, pNeo-miR constitutively expressing amiR targeting GCCCACTGTATCAGTGAGGATT sequence of TARG1 mRNA. This approach was adapted from the methods described ealier (Kopasz et al., 2022). Briefly, amiR elements were designed following the miR-E backbone structure, and the guide sequences were selected based on their target specificity as previously reported (Dow et al., 2012). The amiR sequences were incorporated into the AgeI/XbaI sites of pNeo-miR. This vector contains Sleeping-beauty (SB) transposon elements for stable integration and a Neomycin expression unit. For the selection of genome-integrated clones, 800 µg/ml G418 (HY-17561, MedChemExpress, Monmouth Junction, NJ, USA) was used for three weeks. For the transient siRNA transfections, ON-TARGETplus, SMARTpool Human OARD1 siRNA (Horizon Discovery; Dharmacon™ Reagents; Catalog ID: L-015886-02-0005) to target TARG1, Ambion™ Silencer™Select Human C20orf133 (s44382, s4480 Ambion, Thermo Fisher Scientific, Waltham, US) for MacroD2 and ON-TARGETplus Non-targeting Control siRNA #1 (Horizon Discovery; Dharmacon™ Reagents; Catalog ID: D-001810-01-20) as control were used.

The cells were transfected with Screenfect siRNA transfection reagent (ScreenFect; Cat#S-4001), following the manufacturer instructions, then 72h following transfection lysates were collected for analysis.

10. Western blot

The cells were seeded at cell numbers to reach 70-80% confluency for the treatments. In case of basal condition blots, the cells were collected right after they reached confluency. For phosphor-EGFR signal detection, FBS was withdrawn for 4h and 100 ng/ml h-EGF (E9644 Sigma Aldrich Saint Louis MO US) containing medium was added back to the cells until the indicated timepoints of sample collection. Cell lysates were collected in 4% SDS lysis buffer (4% SDS, 150 mM NaCl, 5 mM MgCl2, 50 mM HEPES, pH 7.4). The lysates were spun down at 13.000 rpm for 25 minutes and the protein concentration of supernatants was determine using NanoDrop 2000™ spectrophotometer (Thermo Fisher Scientific Inc,). Lysates with equal protein amount were resolved on 9% TRIS/Glycine SDS-PAGE gel and blotted onto nitrocellulose (GE10600004 Amersham Protran Premium 0.2 NC, Cytiva, Boston, MA, USA) or PVDF (GE10600021 Amersham™ Hybond® P, Cytiva, Boston, MA, USA membrane in 10% methanol containing transfer buffer. The blotting efficacy was checked with Ponceau S staining. The membranes were blocked either with 4% gelatin (G7765, Sigma Aldrich Saint Louis MO US) for phospho blots or 5% BSA (A7906 Sigma Aldrich Saint Louis MO US) for 1h in PBST (1x PBS, 0.05% Tween-20). After blocking at RT, the membranes were incubated with the primary antibodies: anti-EGFR [EP38Y] antibody (ab52894 Abcam, Cambridge, UK, 1:000), anti-pEGFR [phospho Y1068] (ab32430, Abcam Cambridge, UK, 1:8000), anti-GAPDH antibody (PA1-16777, Thermo Fisher Scientific Inc., 1:3000) and anti-TARG1 antibody (25249-1-AP, ChromoTek GmbH, Planegg-Martinsried, Germany, 1:2000) overnight at 4 ℃. After washing, the secondary antibody (G-21234 Goat anti-Rabbit IgG (H+L) Secondary Antibody, HRP Thermo Fisher Scientific Inc.1:10.000) was added in blocking buffer for 1h at RT. The protein bands were visualized with enhanced chemiluminescence (ECL) solution (SuperSignal™ West Pico PLUS Chemiluminescent Substrate, 34580 Thermo Fisher Scientific Inc.) using Alliance Q9 Advanced imaging system (Uvitec Cambridge,UK). The intensity of the signals was measured with ImageJ (ImageJ, U.S. National Institutes of Health, Bethesda, MD, USA) and normalized to the loading control signal intensity.

11. Cell migration (wound healing)

One day before the experiment, cells were seeded into a well of micro-insert 4-well system as recommended by the manufacturer [3x105 cells/ml in a total volume of 70µl end volume(*Wound Healing and Migration Assay | Experimental Workflow*, n.d.)], (80469 Culture-Insert 4-Well ibidi GmbH, Gräfelfing, Germany). The inserts were removed, and the cells were

washed with 37 °C DMEM (LM-D1109 Biosera Cholet France) without FBS before being cultured under the indicated conditions: serum-free medium, complete medium, or serum-free medium containing 100 ng/ml h-EGF. Cell migration was monitored at 37 °C using a Zeiss Cell Discoverer 7 fluorescence microscope (Zeiss, Jena, Germany) with CO2 levels regulated at 5%. Images were taken every 30 minutes for 24 hours from the same areas. The closure rate of the gap between the cells was calculated using the following formula: wound closure rate (%)=[(0h - 24h) / 0h] × 100, where "0h" was the cell-free area of the gap at the start of imaging, and "24h" represents the same measurement at the final time point of the experiment. Measurements were performed using ImageJ (ImageJ, U.S. National Institutes of Health, Bethesda, MD, USA).

12. EGFR internalization assay (immunostaining)

Cells were seeded on coverslips and allowed to grow until confluency. Culture medium was changed for 4h to serum-free DMEM then supplemented with 100 ng/ml h-EGF for 30 minutes. After washing with PBS, the cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature. Next, PBS containing 0,2% TritonX-100 was added for 10 min for permeabilization. Following blocking with PBS supplemented with 0,1% Tritonx-100 and 5% FBS for 1h at room temperature, the cells were probed with anti-EGFR [EP38Y] antibody (ab52894 Abcam, Cambridge, UK, 1:000) in blocking buffer overnight at 4 °C. Subsequently, the cells were washed 3 times with PBS 0,1% Triton X-100 for 5 minutes, then probed with Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488, (A11008 Invitrogen, Thermo Fisher Scientific Inc., 1:500) for 1h on room temperature. Following washes, the nuclei of cells were counterstained with Hoechst 33342 (H3570 Thermo Fisher Scientific Inc., 1:10000). After mounting with Prolong™ Glass Antifade Mountant (P36982 Thermo Fisher Scientific Inc.) the images were acquired with Zeiss LSM 800 confocal microscope, Plan-Apochromat, 40X/0.95 NA and 20X/0.8 NA air objective, Fluorescent – LSM, GaAsP (Gallium Arsenide) PMT detector using the Zen 2.6 software.

13. qRT-PCR

To ensure growth restricted condition, cells were serum starved for 24h or serum starved for 24h and further cultured in 10% serum containing DMEM for 5h before RNA preparation. To investigate the effects of transcription and translation inhibition, cells were treated with 75 µM

DRB, D1916 Sigma-Aldrich Saint Louis MO US) or/and 40 µg/ml cycloheximide (CHX, C7698, Sigma-Aldrich Saint Louis MO US) for 12 hours. Total RNA was isolated using NucleoSpin RNA Kit (740955 Macherey-Nagel) following the manufacturer's instructions. RNA concentration was measured using NanoDrop 2000 Spectrophotometer (Themo Fisher Scientific), and cDNA was synthesized from 1 µg of total RNA using the RevertAid First Strand cDNA Synthesis Kit (K16 22 Thermo Fisher Scientific Inc.). Each qPCR reaction contained 400nM of the respective forward and reverse primers, 20 times diluted cDNA in 1x SYBR Select Master Mix for CFX (4472953 Thermo Fisher Scientific Inc.). The used primers were:

EGFR: fwd: 5'- GACTGCTGCCACAACCAGT -3'

rev: 5'- CGTGGCTTCGTCTCGGAAT -3'

MYC: fwd: 5'- AGCGACTCTGAGGAGGAACAA-3

rev: 5'- CTTCAGACCATTCTCCTCCGG-3'

CCND1: fwd: 5'- CCTGTCCTACTACCGCCTCA

rev: 5'- CAGTCCGGGTCACACTTGA

RPL27: fwd: 5'- CGCAAAGCTGTCATCGTG - 3'

rev: 5'- GTCACTTTGCGGGGGTAG - 3'

qPCR was carried out at 95 °C for 2 min, followed by 95 °C for 5 sec, and annealing and extension at 60 °C for 20 sec for 40 cycles in Rotor-Gene Q 2Plex (Qiagen, Hilden, Germany). The Ct values were calculated with the Rotor-Gene Q Series software 2.3.1 version. The relative expression levels were plotted using the equation: dCt = CtRPL27 –CtGOI. Means and error bars were calculated in Microsoft Excel and derive from three independent biological replicates.

14. Total RNA staining

Cells were seeded on coverslips. From following day cells were serum starved for 24h and then reconstituted with 10% serum containing DMEM for 5h or left in serum-depleted DMEM (in the case of 24h samples). Total RNA was visualized with the Cell Navigator Live Cell RNA Imaging Kit (AAT Bioquest Pleasanton, CA, US) according to the manufacturer's instructions. StrandBrite™ RNA Green, used in this kit, exhibits excellent RNA selectivity. DNA was stained with Hoechst 33342 (H3570 Thermo Fisher Scientific Inc.) diluted in PBS (1:10.000) Pictures

were taken with the Zeiss LSM 800 confocal microscope, Plan-Apochromat, 40X/0.95 NA and 20X/0.8 NA air objective and GaAsP (Gallium Arsenide) PMT detector using the Zen 2.6 software. The nucleo-cytoplasmic RNA intensity ratio was measured with the open-source cell image analysis software CellProfiler using a custom pipeline. Briefly, the area of the nucleus was segmented based on the Hoechst channel. Next, the cell outlines were defined by propagation starting from the segmented nuclei using the RNA channel. The cytoplasms were identified as the propagated cytoplasmic areas minus the area of the nucleus. To calculate the nucleo-cytoplasmic RNA intensity ratio the mean intensities of the RNA channel in the cytoplasmic and nuclear areas were measured, and the mean cytoplasmic RNA intensity was divided by the corresponding mean nuclear RNA intensity for each segmented nucleus. The data were plotted, and the statistical tests were done using GraphPad Prism (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com).

15. SUnSET assay (detection of total protein synthesis)

U2-OS wild type, TARG1 knock out and stable TARG1 knockdown cells were cultured in normal culture medium or under serum withdrawal for 24h, and then the indicated samples were serum stimulated for additional 5h. Protein synthesis was detected with SunSET assay (E. K. Schmidt et al., 2009). Briefly, 1 µM puromycin (sc-108071C, Santa Cruz Biotechnology, Dallas, TX, USA) was added to cell cultures and incubated for 30 min. For negative control, the samples were pre-treated with 100 µg/ml cycloheximide (C7698, Sigma-Aldrich Saint Louis MO US) for 10 min prior adding puromycin. After puromycin-treatment the cells were washed with PBS and lysed with 4% SDS lysis buffer and protein concentrations were determine using NanoDrop 2000™ spectrophotometer (Thermo Fisher Scientific Inc,). Equal amounts of protein were separated on 10% SDS-PAGE and transferred to nitrocellulose membrane. The membranes were blocked with 3% gelatin in PBST and incubated with anti-Puromycin mouse monoclonal antibody (MABE343, Sigma-Aldrich Saint Louis MO US 1:20000), followed by HRP-conjugated goat anti-mouse IgG (H+L) (31432, Invitrogen Thermo Fisher Scientific Inc., 1:10000). The protein bands were visualized with ECL solution (SuperSignal™ West Pico PLUS Chemiluminescent Substrate, 34580 Thermo Fisher Scientific Inc.) using Alliance Q9 Advanced imaging system (Uvitec Cambridge,UK). GAPDH was used as loading control.

16. Cell proliferation (resazurin assay)

For the cell proliferation assays cell lines were treated with Rapamycin (37094 Vetranal analytic standard, Merck KGaA, Darmstadt, Germany) and U0126 (9903, Cell Signaling Technology Inc. Danvers MA US).1000 cells were seeded in each well of 96-well plates and the next day 100 ng/ml Rapamycin, 25 μM U0126 or a combination of these were administered in DMEM supplemented with 10% FBS. After 72h the culture medium was changed to a fresh one for an additional 72h. The concentrations of the drugs were kept the same during the experiment (6 days). On the 6th day culture medium was replaced with Gibco™ Leibovitz's L-15 Medium, no phenol red (11540556, Thermo Fisher Scientific Inc.) containing 25ug/ml Resazurin (199303 Sigma Aldrich Saint Louis, MO, US) and incubated for 30 minutes in a CO2 thermostat. The fluorescent metabolic product was measured using a Bio-Tek Synergy H1 (Agilent Technologies Santa Clara, CA US) microplate reader with a 530/590 filter set. The viability of each sample was normalized to the untreated samples of the corresponding genotype.

17. Statistical analysis

Results were expressed as mean ±SEM from at least 3 biological replicates in each assay. Statistical significance determined as it is described in figure legends ($p=0.05$ was taken, as a significant difference in each analysis).

RESULTS

18. Cell migration is impaired in the TARG1 knockouts

To assess if TARG1 deficiency has any significant impact on cell migration, we performed wound healing assay using TARG1 KO and control, WT cell lines. Cells were cultured to confluence in culture wells with Ibidi inserts, which creates a uniform scratch in the monolayer after the removal of the insert. The cells were incubated in serum-free medium to minimize proliferation effects and stimulated with h-EGF to promote migration into the created gap. Wound closure was measured 24 hours after the addition of h-EGF. Quantitative analysis revealed that TARG1 KO cells stimulated with h-EGF exhibited significantly reduced migration compared to WT cells, with a slower rate of wound closure (Fig. 3A). Cells stimulated with 10% FBS-containing medium served as positive control and cells kept under serum-starvation were used as negative control. The positive control yielded results comparable to those obtained with h-EGF stimulation in both cell lines (Fig. 3B), while the negative control showed no significant differences between TARG1 KO and WT indicating that the observed differences are indeed due to migration and not to different proliferative capacity of cell lines (Fig. 3C). These results suggest that TARG1 is required for efficient EGF-stimulated cell migration.

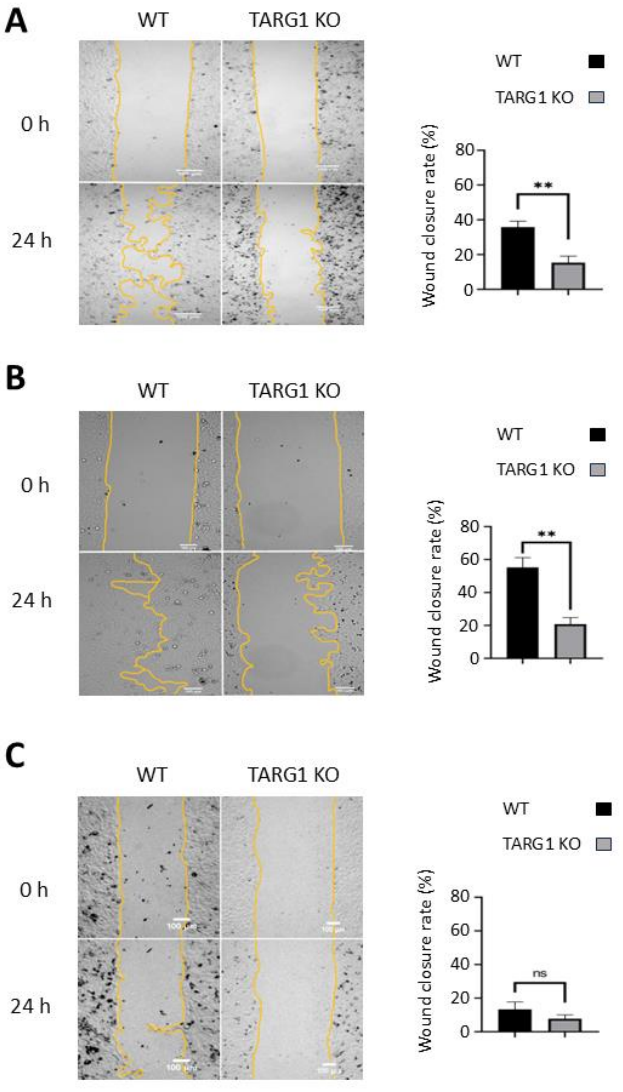

Figure 3. TARG1 loss leads to impaired cell migration. Representative images (left) of wound healing assays with WT and TARG1 KO cells immediately after gap generation (0 h) and 24 hours after it (24 h) in 10% FBS containing medium (A), in the presence of 100 ng/ml h-EGF in serum free medium (B), and in serum free medium (C). Scale bar, 100 μm. Wound closure rate (right) was determined as the percentage of gap closure 24 hours after would generation. Data are mean ± SEM of n ≥ 3 independent experiments. Asterisks indicate *p*-values obtained by multiple t-test Holm-Sidak method, with alpha = 0.05. (ns. Not significant; ** $p<0.01$)

19. No significant difference was observed in the dynamics of EGFR vesicular trafficking between wild-type and TARG1 knockout cells.

In the next step we carried out a receptor internalization assay to see if the TARG1 mutant cell line show any difference in the internalization rate, or the dynamics of the vesicular trafficking of the EGFR receptor (signal accumulation around a specific cell compartment, signal intensity change over time). We stimulated the cells with h-EGF for 30 minutes after serum starvation, then we fixed the samples, and did immunostaining with EGFR intracellular domain epitope specific antibody on both the serum starved (Figure 4A) and the 30 minutes stimulated (Figure

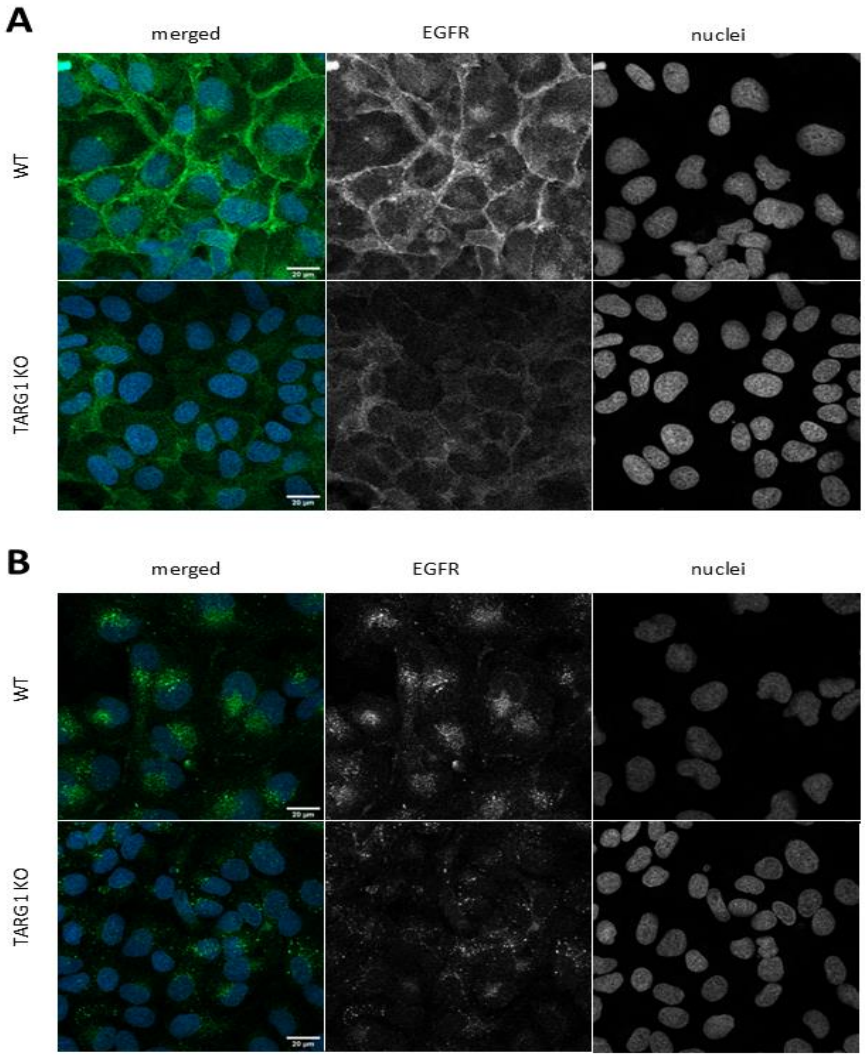

Figure 4. Representative images of immunofluorescence experiments of EGFR in WT and TARG1 KO cells (A, B) 4 hours after serum starvation, where the majority of the EGFR resides on the cell membrane in case of both cell line (A) and after 30min h-EGF (100 ng/ml) stimulation following the 4h serum starvation, a big rate of the receptor activated, internalized, then accumulated around the nuclear area (B). Scale bar, 20 μm.

4B) samples. The wild type and the TARG1 mutant cells did not shown any difference in their internalization dynamics during the time of the experiment, except that the TARG1 knock out cell line showed less signal intensity. This information gave us the deduction, that during the activation and internalization of the EGFR, the vesicular trafficking of the receptor did not suffer any accumulated retention, or accelerated degradation, nor any change in trafficking pathway towards different cell organelles was observable between the WT and the TARG1 knockout mutant.

20. TARG1 KO and silenced cells showed decreased EGFR protein level

Because the only difference we observed during the IF experiments was a lower signal intensity, we made our next step to check on the expression level and the activity state of the receptor with western blot assay. Both total and phospho-EGFR protein levels were quantified from whole cell lysates of WT, TARG1 KO. The results revealed a reduced overall EGFR protein level and decreased receptor phosphorylation in the TARG1 KO cells (Figure 5A, B). However, when normalizing the phospho-EGFR levels to total EGFR, no significant differences were observed between wild-type and TARG1 KO cells (Figure 5C). To confirm the impact of TARG1 on EGFR levels, we performed additional experiments using stable miRNA (TARG1 KD) and transient siRNA transfections to silence TARG1 expression in wild-type cells. Both the stable miRNA-expressing TARG1 KD cell line and the siRNA TARG1 silencing led to reduced EGFR protein levels albeit to a smaller extent than in TARG1 KO (Fig. 5D-F). These finding supported the results of the migration assay we did earlier, since the reduced level of receptor in the TARG1 knock out cell line is able to uptake less signal from the cell extracellular space, thus reducing the signal strength what activates pathways, that regulates the migration ability of the cells.

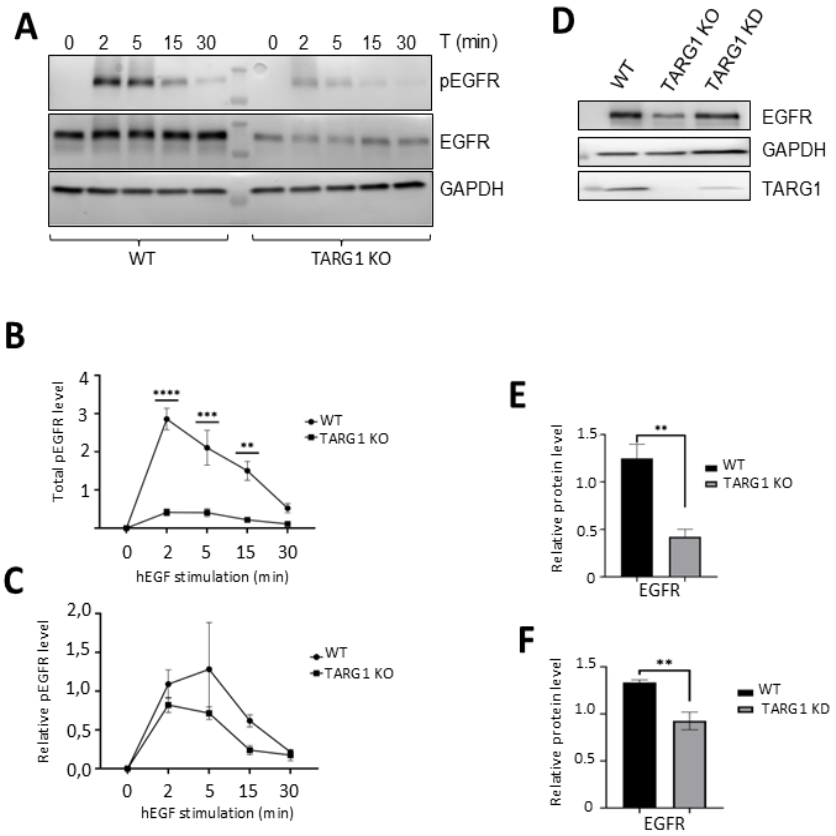

Figure 5. EGFR protein levels are reduced in the TARG1 mutants. (A) Representative Western blot of phosphorylated EGFR (pEGFR), total EGFR (EGFR) and GAPDH at the indicated time points following h-EGF (100 ng/ml) stimulation in WT and TARG1 KO cells. GAPDH served as loading control. (B) Quantification of the phosphorylated EGFR (pEGFR) levels at the indicated time points in WT and TARG1 KO cells; (C) Quantification of the relative phospho-EGFR (pEGFR/EGFR) level in WT and TARG1 KO cells. (D) Representative western blot of the total EGFR level in WT, TARG1 KO and TARG1 knock down (KD) cell lines from whole cell lysate. Quantification of EGFR levels in WT and the TARG1 KO (E) and in WT and TARG1 KD (F). Data in (B, C, E) and (F) are mean ± SEM (n ≥ 3). Asterisks indicate *p*-values obtained by multiple t-test Holm-Sidak method, with alpha = 0.05. (** $p<0.01$; *** $p<0.001$).

21. RNA turnover is increased in TARG1 knockouts

To investigate whether the observed reduction in EGFR protein levels in TARG1 KO cells was accompanied by corresponding changes at the mRNA level, we measured EGFR mRNA expression in WT and TARG1 KO cells. Cells were subjected to 24-hour serum starvation followed by a 5-hour recovery period in medium supplemented with 10% FBS. Quantitative RT-PCR (qRT-PCR) analysis revealed that EGFR mRNA levels were significantly lower in TARG1 KO cells compared to WT cells in both conditions. Notably, while EGFR mRNA levels in WT cells did not change significantly after the 5-hour recovery, a significant increase was

observed in TARG1 KO cells during this period (Fig. 6A) although the serum-induced changes in gene expression are not abrogated the reduced EGFR mRNA level in the TARG1 KO cells.

To assess EGFR signaling at the gene expression level, we measured the changes in mRNA levels of two EGFR targets, MYC and cyclin D1 (CCND1) upon serum stimulation. The transcription of MYC is regulated by EGFR through the MAPK pathway (Bonamy et al., 2018). The expression of CCND1 is modulated by multiple transcription factors that are downstream effectors of the EGFR signaling pathway. These include the MYC proto-oncogene and the AP-1 transcription factor complex, which is composed of Jun and c-Fos proteins (Wee & Wang, 2017). The mRNA levels of MYC significantly increase in both WT and TARG1 KO upon serum stimulation (Fig. 6B). On the other hand, there was a significant increase in the mRNA level of CCND1 in wild-type cells upon serum stimulation, while in the TARG1 KO the increase was not significant (Fig. 6C). It should be noted, however, that the mRNA levels of CCND1 after serum stimulation were very similar in WT and TARG1 KO, and it was the serum-starved condition where the CCND1 mRNA level in TARG1 KO was not reduced to the level observed in WT. Altogether these results suggest that EGFR signaling is not compromised at the level of gene expression in the absence of TARG1 regardless of the reduced EGFR protein levels.

The reduced EGFR mRNA levels observed in TARG1 KO prompted us to further investigate mRNA stability and the potential roles transcription and translation in its regulation. We used Dichlorobenzimidazole 1-β-D-ribofuranoside (DRB) to inhibit RNA polymerase II-mediated transcription and cycloheximide (CHX) to block translation elongation and measured their individual and combined effects on mRNA levels of EGFR, MYC and CCND1. In normal culture medium, the mRNA levels of EGFR were significantly lower in TARG1 KO than in WT (Fig. 6D). This was further corroborated by EGFR mRNA measurements in siRNA transfected TARG1-silenced cells. 12 hours of transcription inhibition decreased EGFR mRNA level both in wild-type and TARG1 KO cells, however the reduction of EGFR mRNA was greater in TARG1 KO than in WT (Fig. 6D). While CCND1 mRNA level was lowered only in TARG1 KO (Fig. 6F). The MYC mRNA levels appear to mildly but not significantly increase in both cell lines when transcription is inhibited revealing intricate feedback between mRNA turnover and transcription. The inhibition of translation with CHX increased MYC mRNA levels in both WT and TARG1 KO (Fig. 6E). Interestingly, the difference between the EGFR mRNA levels of WT and TARG1 KO was abolished when translation is blocked, which might suggest the possibility that TARG1 acts through translational regulation. Yet, when transcription and translation was simultaneously blocked, the mRNA levels of all three tested genes dropped

significantly more in TARG1 KO than in WT when compared to the CHX-only conditions (Fig. 6D-F). Altogether these results suggest that the loss of TARG1 decreased the stability of mRNAs and causes increased mRNA turnover.

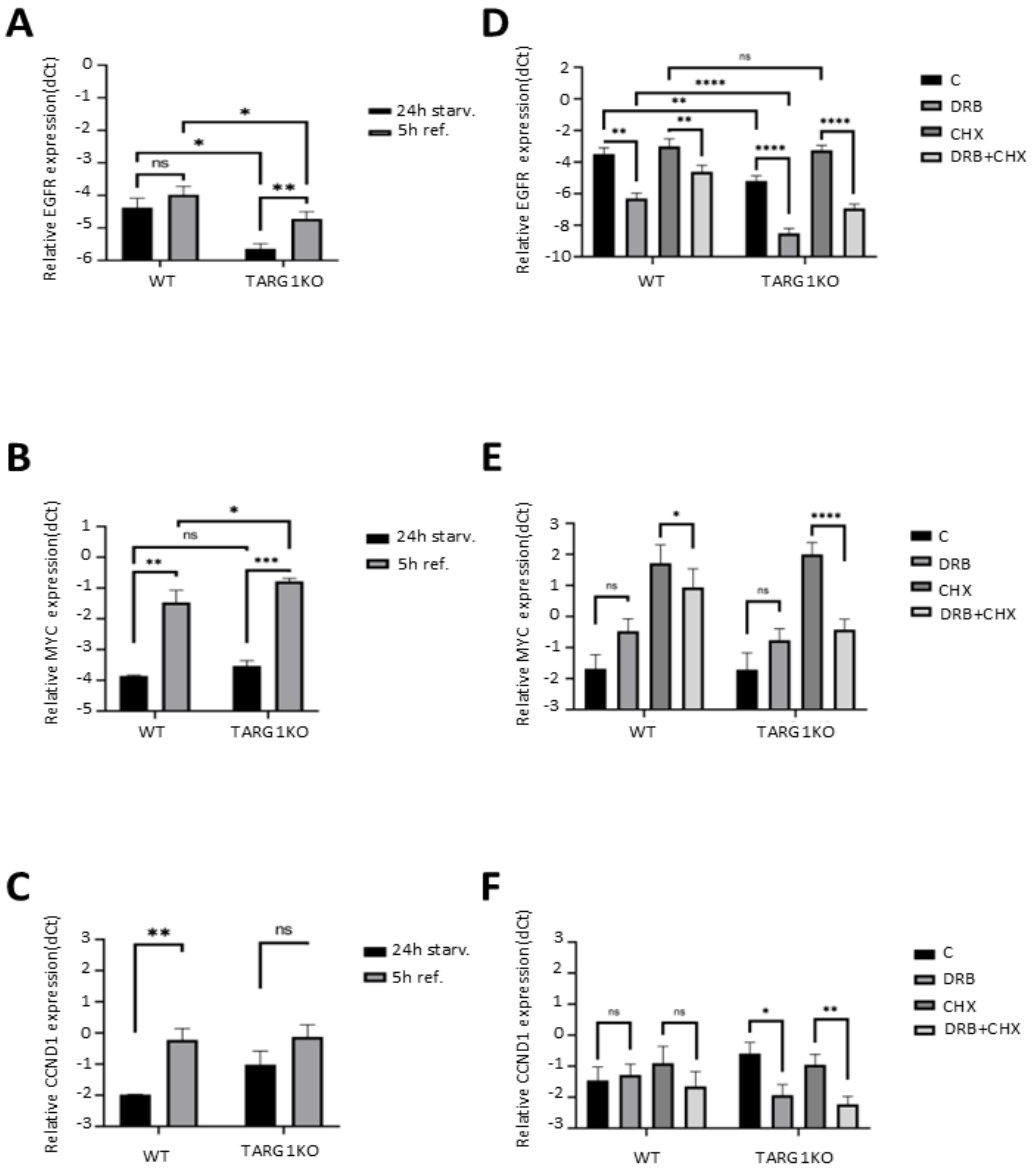

Figure 6. Changes in mRNA levels of EGFR and response genes (MYC, CCDN1) were revealed by qRT-PCR analysis. (A) EGFR, (B) MYC, (C) CCND1 mRNA level in WT and TARG1 KO cells after 24h serum starvation (24h starv.), and after 24 serum starvation followed by 5 hours of 10% serum refeeding (5h ref.). (D) EGFR, (E) MYC, (F) CCND1 mRNA levels in WT and TARG1 KO cells cultured in normal medium (black bars; C), following transcription block (medium grey bars; DRB for 12h), following translation block (dark grey bars; CHX for 12h), and following combined transcription and translation block (light grey bars; DRB+CHX for 12 h). Relative gene expression was calculated by subtracting the Ct value of the gene of interest from the Ct value of RPL27. Data are mean ± SEM ($n \geq 3$). Asterisks indicate p-values obtained by two-sided two-sample unequal variance t-test. (ns. not significant; * $p<0.05$, ** $p<0.01$, **** $p<0.0001$).

22. TARG1-dependent regulation of RNA distribution and translation

To test the hypothesis, that TARG1 has been implicated in RNA metabolism (Žaja et al., 2020), instead of checking on the mRNA level of some specific genes that can be regulated the same pathway, we did a total RNA staining on the cell lines, where we were able to observe the possible changes of total RNA levels fast and easily. The dye we used was specific for RNA without causing a high background staining on the DNA (we also pre-stained the DNA with Hoechst for more relevant results). In our experiments we used the same setup as in the case of qRT-PCR measurements, where we serum starved the cells for 24h and then stimulated them with 10% serum for 5h. We found that in the TARG1 mutant cell lines the total RNA level was significantly higher after 24h serum starvation in the nucleus, and the same trend was observable after 5h serum stimulation. In the cytoplasmatic area after 24h the cell lines didn't show any significant differences, but after the 5h serum stimulation in the wild type cells, the total RNA level was significantly higher than in case of the TARG1 mutant cell lines.

In order to get more relevant information about the total RNA distribution in the nucleus and the cytoplasm between the conditions, the cytoplasmic to nuclear distribution of RNA levels was quantified where the cytoplasmic RNA intensity was divided by the nuclear RNA intensity (Figure 8B). In WT cells, serum stimulation significantly increased the cytoplasmic/nuclear RNA ratio, indicating a redistribution of RNA from the nucleus to the cytoplasm, which may accompany translational restart (Cullen, 2000). (Figure 8A, B). In TARG1 KO cells, we observed a non-significant reduction in the cytoplasmic to nuclear RNA levels after serum

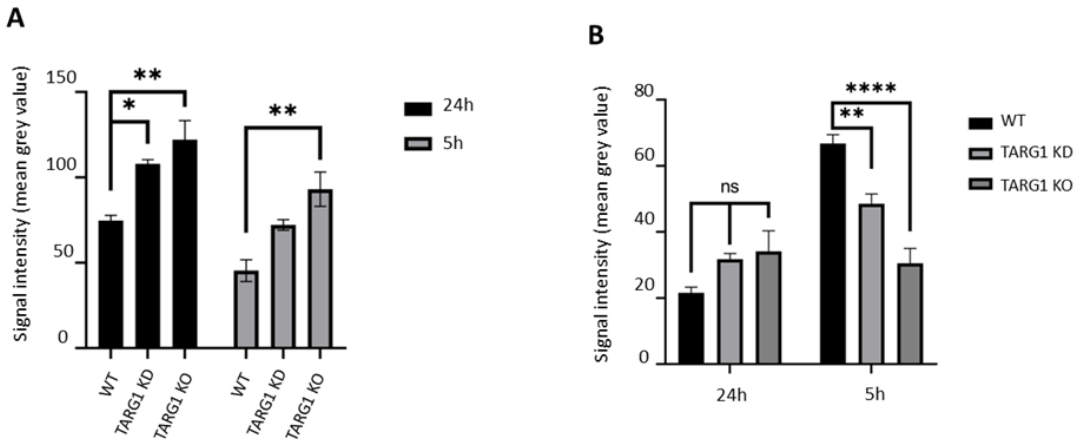

Figure 7. The signal intensity of total RNA level of cell lines (A) in the nucleus, and (B) in the cytoplasm. Data are mean ± SEM (n ≥ 3). Asterisks indicate *p*-values obtained by two-way ANOVA followed by Turkey's multiple comparison (ns. Not significant; ** $p<0.01$; **** $p<0.0001$).

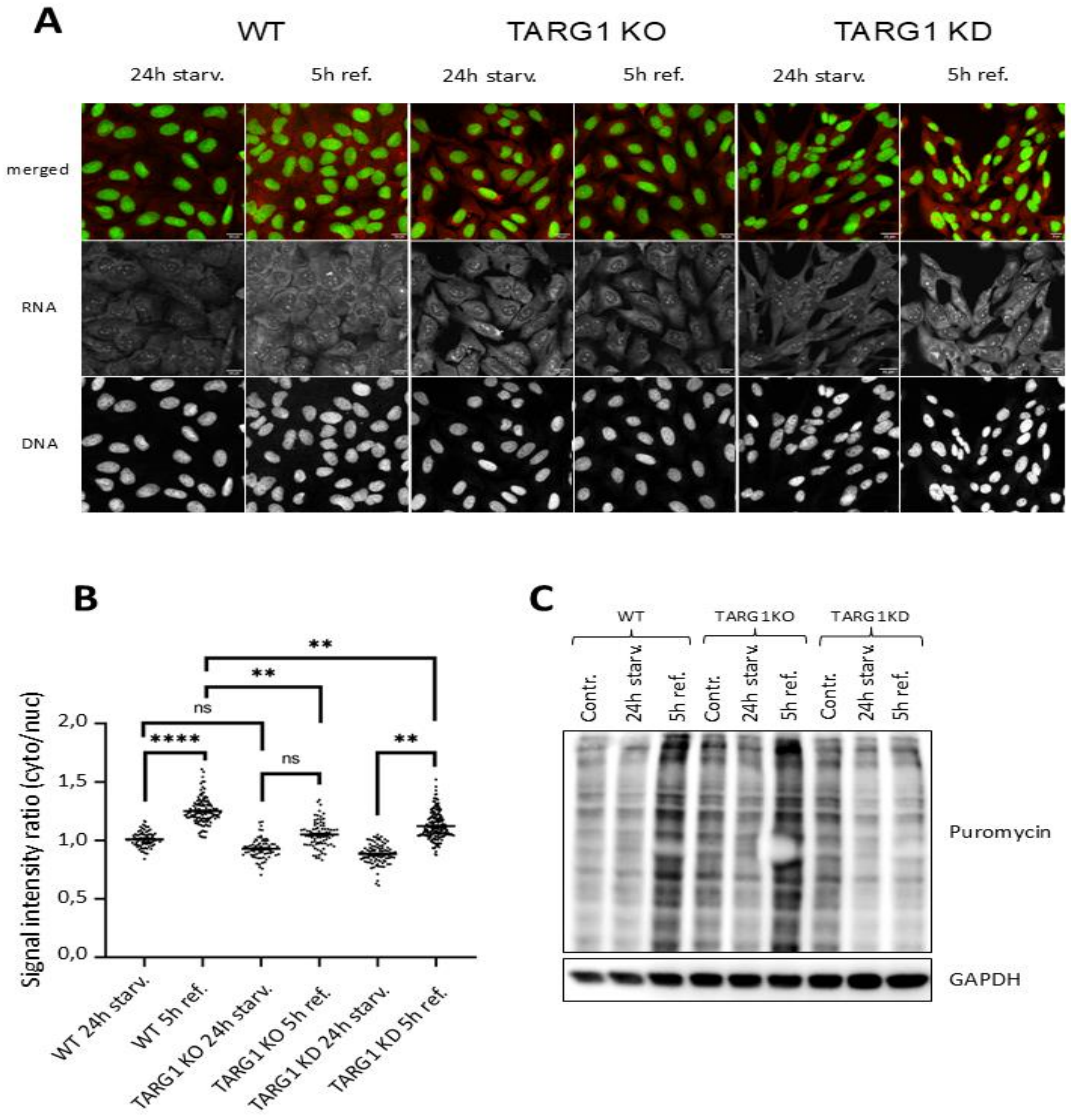

Figure 8. The TARG1 loss altered nuclear-cytoplasmic RNA distribution and translation after serum stimulation. (A) Representative images of total RNA staining in WT, TARG1 KO and TARG1 KD cells after 24h serum starvation (24h starv.) and after 24h serum starvation followed by 5h serum refeeding (5h ref.) Scale bar, 20 μm. (B) Nucleo-cytoplasmic RNA distributions in WT, TARG1 KO and TARG1 KD cell lines after 24h serum starvation (24h starv.) and after 24h serum starvation followed by 5h serum refeeding (5h ref.). Nucleo-cytoplasmic RNA distribution was quantified as the ratio of cytoplasmic to nuclear RNA intensities in individual cells, with cell and nuclear outlines identified using CellProfiler. Data are mean ± SEM ($n \geq 200$). Asterisks indicate p-values obtained by two-way ANOVA followed by Turkey's multiple comparison (ns. Not significant; ** $p<0.01$; **** $p<0.0001$). (C) SUnSET assay showing puromycin incorporation level reflecting translation rate of WT, TARG1 KO and TARG1 KD cells. GAPDH was used as loading control.

starvation as compared to WT cells, which increased only mildly upon the 5-hour serum stimulation (Figure 8A, B). The miRNA-induced TARG1 KD significantly reduced the

redistribution of RNA from the nucleus to the cytoplasm following serum stimulation when compared to WT cells (Figure 8A, B), however, the cytoplasmic to nuclear ratio upon serum stimulation was significantly lower than in WT. These findings suggest that the expression level of TARG1 might play a role in RNA maturation, stability and export. To determine whether these alterations in RNA distribution were linked to changes in translation, we performed the SUnSET assay, which detects newly synthesized proteins by incorporating a brief puromycin pulse followed by anti-puromycin antibody detection (E. K. Schmidt et al., 2009). As control, WT and TARG1 KO cells were treated with puromycin alone or pre-treated with the translational inhibitor CXH, and puromycin incorporation was analyzed by Western blotting. Puromycin efficiently labels newly synthetized proteins, while translational inhibition abrogates puromycin incorporation. Interestingly, the puromycin labeling revealed increased translation in TARG1 KO compared to WT.

We then examined whether serum starvation followed by serum stimulation influenced translation in WT, TARG1 KO and TARG1 KD cell lines. Under normal culture conditions, puromycin labeling was increased in both TARG1 KO and KD cell lines compared to WT. Serum starvation for 24 hours had little effect on translation of WT cells, while translation in both TARG1 KO and KD declined to levels similar to WT. After 5 hours of serum stimulation, translation increased in WT and TARG1 KO, as indicated by elevated puromycin labeling, but this upregulation was not observed in TARG1 KD cells (Fig. 8C). These results showed an elevated level of translation in TARG1 KO and KD compared to the WT further supporting that TARG1 plays a role in translational regulation.

23. TARG1 mutant cell lines showed sensitivity against MEK 1/2 inhibition

Translation and transcription are regulated by two major signaling pathways the PI3K/mTOR and Ras/Raf/MEK/ERK pathways (Hernández et al., 2019). We aimed to investigate whether cell proliferation following treatment with specific pathway inhibitors was affected by altered TARG1 expression.

We treated cells with rapamycin, an mTOR inhibitor and U0126, a MEK1/2 inhibitor alone, or in combination. Rapamycin treatment alone did not reveal significant differences in viability between WT and TARG1 KO cells (Fig. 9A). However, U0126 reduced cell viability to a greater extent in both TARG1 KD and KO cells than in wild-type cells. (Fig. 9B, D). Notably, co-treatment with U0126 and rapamycin increased the sensitivity only in WT cells, thus

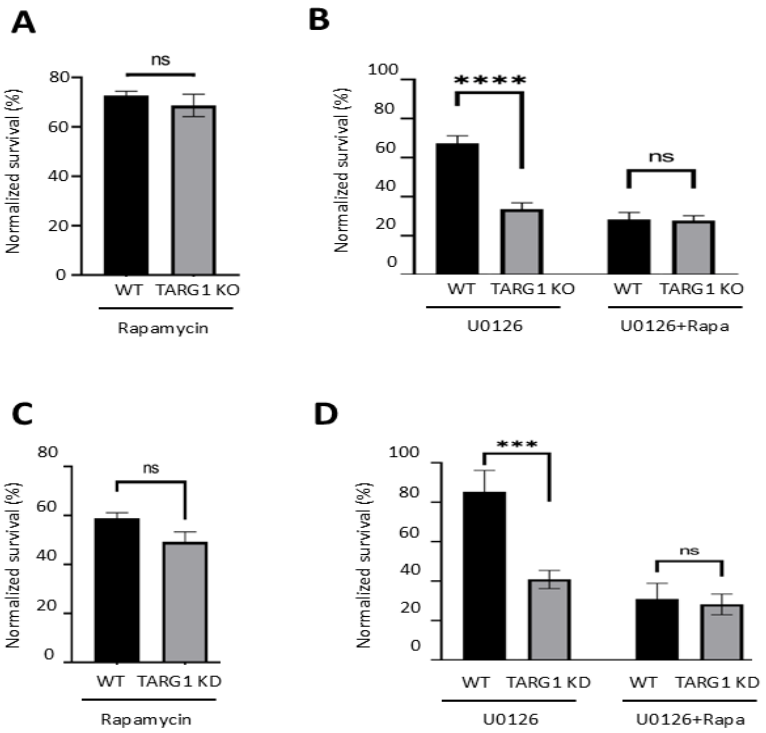

Figure 9. TARG1 KO cells have increased sensitivity to MEK inhibition. Cell viability assay of WT and TARG1 KO (A, B) or TARG1 KD (C, D) cells treated with 100 nM Rapamycin (A, C), and with the MEK1/2 inhibitor, 25 μM U0126 alone (U0126) or in combination with 100 nM Rapamycin (U0126+Rapa) (B, D) for 6 days. The graphs show the relative viability normalized to the untreated samples of each genotype. Data are mean ± SEM of n ≥ 3 independent experiments. Asterisks indicate p-values obtained by multiple t-test Holm-Sidak method, with alpha = 0.05. (ns. Not significant; *** $p<0.001$;**** $p<0.0001$).

eliminating the differential sensitivity of WT and TARG1KO cells to MEK1/2 inhibition (Fig. 9B, D). The sensitivity of TARG1 KD to MEK1/2 and mTOR inhibition was almost identical to that of TARG1 KO (Fig. 9C, D).

These results suggest that TARG1 may influence a regulatory target involved in the crosstalk between the PI3K/mTOR and Ras/MEK/ERK pathways, potentially by modulating mTOR activity.

DISCUSSION

The physiological function of the EGFR is to regulate epithelial tissue development and homeostasis. In pathological settings, mostly in lung and breast cancer, in glioblastoma, and also even in cases of mesenchymal origins like osteosarcoma, the EGFR is a driver of tumorigenesis.

Osteosarcoma is a common primary malignant tumor of the bone, with a peak incidence in adolescents and adults >60 years of age. Although rare, osteosarcoma is the most common primary malignancy of bone, with an incidence in children and adolescents of ~3–4.5 cases per million population per year (Beird et al., 2022). Approximately 80% of patients with osteosarcoma present with radiographically localized disease (Gill & Gorlick, 2021). Those patients with radiographically confirmed non-metastatic osteosarcoma have a 5-year event-free survival of ~60%. In patients who present with a primary lesion and an isolated pulmonary nodule, 5-year event-free survival is generally <40%. For individuals with a primary lesion and multiple pulmonary nodules or radiographically detectable metastatic disease at other sites, 5-year event-free survival is <20% (Gill & Gorlick, 2021). Osteosarcoma can present in any bone in the body, the most common sites are around the knee and the proximal humerus (Bielack et al., 2002). The human osteosarcoma U2OS cell line was derived in 1964 from a moderately differentiated sarcoma of the tibia of a 15-year-old girl. It is one of the first generated cell lines and is used very frequently (Bayani et al., 2003). Compared with other osteosarcoma cell lines, U2-OS cells have the lowest level of chromosomal numerical variations and only 2% of the cells have multipolar mitoses, similar to normal control fibroblasts, probably due to functional p53 and pRb (Isfort et al., 1995). Among the many treatment targeting approaches, cell surface antigens are one such approach that has received much attention. Several cell surface antigens expressed on osteosarcoma cells are also expressed on other adult tumour cells, making it possible to develop approaches that can be used broadly (Beird et al., 2022). Osteosarcoma tumours arise in many cases during puberty, when many progenitor cells undergo differentiation in response to signalling via, for example, FGF2 (Teven et al., 2014), RANKL (Ikebuchi et al., 2018) and IGF1 (Y. Li et al., 2019) and EGFR (X. Liu et al., 2013).

Inappropriate activation of the EGFR in cancer mainly results from amplification of EGFR and point mutations at the genomic locus, but transcriptional upregulation or ligand overproduction due to autocrine/paracrine mechanisms has also been described. Moreover, the EGFR is increasingly recognized as a biomarker of resistance in tumors, as its amplification or secondary

mutations have been found to arise under drug pressure (Sigismund et al., 2018). Some studies connected EGFR and ostosarcoma tumor progression before (H. Liu et al., 2024; Q. Wang et al., 2014; S. Wang et al., 2021). The dynamic regulation of mammalian cell signaling pathways like the EGFR is often modulated by cascades of protein post-translational modifications (PTMs). ADP-ribosylation as a PTM has been previously reported before as an important cell regulator that impacts a plethora of cellular processes, including many intracellular signaling events (Boehi et al., 2021).

In this study, we explored the role of TARG1, a macrodomain-containing (ADP-ribosyl)hydrolase, in regulating EGFR signaling and RNA metabolism. Our findings indicate that TARG1 modulates EGFR expression and mRNA stability, suggesting that ADP-ribosylation may contribute to the regulation of the EGFR signaling pathway. TARG1 loss impaired cell migration, a key process regulated by EGFR signaling. Additionally, TARG1-deficient cells exhibited reduced EGFR mRNA and protein levels, accompanied by lower EGFR phosphorylation, suggesting that TARG1 influences both EGFR expression and activity. However, despite significantly reduced EGFR mRNA levels in TARG1 KO cells, serum-induced gene expression changes were not abrogated, indicating that TARG1 does not influence EGFR signaling at the transcriptional level. Importantly, while EGFR loss likely contributes to the observed defects in proliferation, our findings do not exclude the possibility that TARG1 deficiency disrupts additional, EGFR-independent mechanisms of cell proliferation that may indirectly impair EGFR-driven growth.

One of our findings was the increased mRNA turnover observed in TARG1-deficient cells. Specifically, TARG1 loss led to enhanced degradation of EGFR mRNA and the mRNAs of its downstream targets, MYC and Cyclin D1. These results reveal that TARG1 plays a role in stabilizing mRNA transcripts and preventing their premature degradation. Our data further indicate that TARG1 regulates mRNA stability via translational mechanisms, as inhibiting translation reversed the differences in EGFR mRNA levels between WT and TARG1 KO cells. Additionally, increased puromycin incorporation in TARG1 KO cells suggests enhanced global translation in these cells. Notably, despite this increased translational activity, EGFR protein levels were reduced in TARG1-deficient cells. However, we did not investigate whether this increase in translation resulted in higher protein synthesis overall or led to the production of aberrant proteins. Altogether, these results imply that TARG1 may influence mRNA processing and stabilization by modulating translation efficiency.

These observations align with previous studies identifying numerous TARG1-interacting proteins involved in RNA biogenesis, including enzymes associated with ribosomal maturation, RNA splicing, nuclear export, and translational machinery (Žaja et al., 2020). Given the functional relevance of these proteins in RNA regulation, it is plausible that TARG1, through its (ADP-ribosyl) hydrolase activity, modulates RNA processing, impacting mRNA stability, maturation, and translation. However, it remains unclear whether these effects depend solely on TARG1's catalytic activity, as catalytically inactive TARG1 has also been shown to bind RNA (Bütepage et al., 2018).

Several PARPs have been reported to bind RNA through conserved CCCH RNA-binding domains, (PARP7, PARP12 and PARP13) or RRM motifs (PARP10 and PARP14) (Vyas et al., 2013) highlighting their potential influence on RNA-related processes (Kim et al., 2020).The absence of TARG1 may amplify these effects. Moreover, PARP1 has also been shown to modulate mRNA biogenesis (Eleazer & Fondufe-Mittendorf, 2021). During thermal stress, PARP1 PARylates poly(A) polymerases, causing their dissociation from RNA and leading to a global reduction in polyadenylation. This, in turn, can impair RNA stability, hinder mRNA export, and reduce translation efficiency (Di Giammartino et al., 2013). Additionally, PARP1 depletion has been linked to changes in EGFR expression (Boehi et al., 2021). Beyond nuclear PARP1, an ER transmembrane mono(ADP-ribose)transferase, PARP16, has been shown to MARylate ribosomal proteins essential for polysome assembly, thereby regulating translation initiation (Challa et al., 2021). Interestingly, ERK signaling – known to regulate ribosomal proteins independently of mTOR – targets ribosomal subunits such as RPS6 (Roux et al., 2007), which are also substrates of PARP16 (Challa et al., 2021).

A recent study reported that TARG1 depletion affected the regulation of a ribosome- associated protein, RACK1 MARylation, increasing the translation of certain RNAs while reducing that of others in OVCAR3, an ovarian cancer cell line (Challa et al., 2025). In contrast, a previous study in TARG1-deficient HeLa cells did not report changes in proliferation or translation (Bütepage et al., 2018), suggesting that TARG1's role in RNA metabolism may be cell type-dependent.

Notably, nucleic acids – including both DNA and RNA – can also undergo ADP-ribosylation (Munnur et al., 2019) and MARylation of the 5'-terminal phosphate of RNA has been shown to inhibit translation and affect mRNA stability (Weixler et al., 2022).

Together, these findings underscore the numerous ways in which ADP-ribosylation regulates RNA metabolism – both indirectly through RNA-binding proteins and translation factors, and directly through RNA modifications – highlighting its broad influence on gene expression and cellular signaling.

Our study demonstrated that TARG1 deficiency significantly reduced EGFR expression. EGFR is a key proto-oncogene involved in cancer related processes such as migration, proliferation, and adhesion (Mendelsohn & Baselga, 2006). Many current therapeutic strategies target EGFR by inhibiting its kinase activity or preventing extracellular ligand binding. However, resistance-associated mutations often reduce drug efficacy (Cai et al., 2020; Zubair & Bandyopadhyay, 2023). Our findings suggest a novel regulatory mechanism in which TARG1 modulates EGFR expression at the mRNA level, potentially offering a new therapeutic avenue to circumvent EGFR mutation-driven drug resistance.

Furthermore, the increased sensitivity of TARG1-deficient cells to MEK1/2 inhibition suggests a potential role for TARG1 in signaling pathway regulation. MEK1/2, a key component of the Ras/Raf/MEK/ERK pathway, is critical for cell survival and proliferation (Chang et al., 2003; Shaul & Seger, 2007). This pathway is implicated in approximately one-third of all cancers due to its central role in gene expression, cell proliferation, survival, and apoptosis (Kun et al., 2021). As a result, MEK inhibitors have been extensively studied as potential cancer therapies. However, despite promising results, not all patients respond to these inhibitors, and resistance frequently develops in those who initially do (Kun et al., 2021). Our results suggest that TARG1 may mediate crosstalk between the Ras/MEK/ERK and PI3K/mTOR pathways. The heightened sensitivity of TARG1-deficient cells to MEK1/2 inhibition raises the possibility that TARG1 could be a novel therapeutic target for enhancing the efficacy of MEK inhibitors in cancer therapy.

The role of ADP-ribosylation in RNA metabolism, particularly in mRNA maturation and translation, is an emerging area of investigation. Given the clinical relevance of EGFR signaling in cancer, understanding TARG1's function could have significant therapeutic implications, particularly in tumors reliant on EGFR-driven proliferation and migration. Future studies should aim to elucidate the precise molecular mechanisms by which TARG1 modulates RNA metabolism and signaling pathways and explore its potential as a therapeutic target in cancer treatment.

SUMMARY

Post translational modifications are key elements of regulating enzymatic activities and influencing the stability, dynamics and interactions of other biomolecules as well, in cells. Since in the past few years it has been reported that ADP-ribosylation as a PTM not only modifies amino acid residues of proteins, but nucleic acids as well, has made the research field of the PARP enzyme family more complex, moving them further from the scope of the regulatory role of the DNA damage response elements. Besides the writer enzymes of the family, the ADP-ribose modification eraser hydrolases are also very important, since they have the ability to reverse these modifications, thus terminating a signal (activating or deactivating), or helping a cyclic, continuous signal refeeding on a target site.

In our study, in the recent years, we investigated TARG1, an ADP-ribose hydrolase enzyme role in regulating the expression profile of a well-known important proto-oncogene protein, the EGFR, in the U2-OS osteosarcoma cell line. We showed, using a simple scratch assay, that the cells that were lacking TARG1 had an impaired migration ability. Western blot and RT-qPCR experiments proved that the expression of the EGFR was lower in the TARG1 mutants, both on the protein and mRNA levels as well, and with immunostaining that the endocytosis and the endosomal distribution of the receptor during its activated state was not differed from the wild type. Furthermore, despite the high difference between the amount of phosphorylated (active) receptor during ligand activation (that showed linear correlation with the expression of the amount of the receptor protein, in cell lines), the TARG1 mutants did not showed any impaired transcriptional activity regarding the gene expression of the receptor target genes (CCND1, MYC). On the other hand, blocking transcription and/or translation using specific inhibitors, revealed more instable mRNA levels overtime in the TARG1 KO. This finding further strengthened our conclusion, with RNA-specific dye staining, where the cytoplasmic-to-nuclear ratio of RNA turnover, and with puromycin incorporation assay where the global translation profile showed significant differences between the cell lines, that TARG1 has a regulatory role of RNA biogenesis both on the post transcriptional and translational level as well. Additionally, TARG1 mutants in cell proliferation assay showed sensitivity to MEK inhibitor, but not to mTORC1 inhibitor, suggesting a possible involvement of TARG1 in the RAS-RAF-MEK-ERK/AKT-mTOR crosstalk, which are major regulator enzymatic pathways of RNA biogenesis. Collectively, our data strongly suggest that TARG1 plays a regulatory role in RNA biogenesis, and, through this, in the regulation of EGFR levels in the U2-OS cell line.

ÖSSZEFOGLALÓ

A poszttranszlációs módosítások kulcsfontosságú szabályozói a különböző enzimatikus aktivitásoknak és más biomolekulák stabilitásának, dinamikájának, valamint interakcióinak a sejtekben. Az elmúlt években kimutatták, hogy az ADP-riboziláció, mint poszttranszlációs módosítás, nemcsak a fehérjék különböző aminosav-csoportjait módosítja, hanem nukleinsavakat is, ami még összetettebbé tette a PARP enzimcsalád kutatásának területét, szélesebb körű szerepét reprezentálva a már jól ismert DNS hibajavításban betöltött szerepén kívül. A PARP család polimeráz aktivitású enzimei mellett az ADP-ribóz módosításokat eltávolító hidrolázok is rendkívül fontosak, mivel képesek visszafordítani a polimerázok által végzett módosításokat, ezzel megszüntetve az adott jelet (aktiváló vagy deaktiváló), illetve segítve egy ciklikus vagy folyamatos jel újraírását egy adott célmolekulán.

A közelmúltban végzett kutatásaink során a TARG1 nevű ADP-ribóz-hidroláz enzim szerepét vizsgáltuk egy ismert, proto-onkogén fehérje, az EGFR expressziós profiljának szabályozásában, oszteoszarkóma (U2-OS) sejtvonalban. “Wound healing” kísérlettel kimutattuk, hogy a TARG1 gén kiütött sejteknek gyengébb volt a sejtmigrációs képessége. Western blot és RT-qPCR kísérleteink bizonyították, hogy a TARG1 mutánsokban az EGFR kifejeződése alacsonyabb volt, mind a fehérje, mind az mRNS szinten, valamint immunfestéssel kimutattuk, hogy a receptor endocitózisa és endoszomális eloszlása aktivált állapotában nem különbözött a vad típusú sejtektől. Továbbá, annak ellenére, hogy a ligand általi aktiválásakor a foszforilált (aktív) receptor mennyisége alacsonyabb volt (amely lineáris összefüggést mutatott a receptor fehérje mennyiségének expressziójával a sejtvonalakban), a TARG1 mutánsokban nem volt kimutatható a receptor jelátviteli útvonal célgénjeinek jelentős mértékű transzkripciós szabályozási zavara (CCND1, MYC). Másrészt, specifikus inhibitorokkal végzett transzkripciós és/vagy transzlációs gátlás alatt instabilabb mRNS szinteket mértünk a TARG1 KO sejtekben, az időfüggő vizsgálatok során. Ez az eredmény megerősítette korábbi következtetéseinket, amelyeket RNS-specifikus festéssel végzett jelöléssel (amely az RNS citoplazma és sejtmag közötti forgalmának arányát vizsgálta), valamint puromicin-alapú Western blot analízissel amely a globális transzlációs profilt értékelte támasztottunk alá, és amelyek szignifikáns különbségeket mutattak a sejtvonalak között. Ezekből a kísérletekből arra a következtetésre jutottunk, hogy a TARG1 szabályozó szerepet játszik az RNS biogenezisben mind poszt-transzkripcionális, mind transzlációs szinten. Továbbá, a TARG1 mutánsok sejtosztódás vizsgálatai érzékenységet mutattak a MEK-inhibitoros kezelésre, de nem volt kimutatható különbség mTORC1-inhibítor esetében (RNS

biogenezis fő enzimatikus szabályozó útvonalai), ami arra utal, hogy a TARG1 szerepet játszhat a RAS-RAF-MEK-ERK/AKT-mTOR közös szabályozási útvonalon. Összességében adataink erősen arra utalnak, hogy a TARG1 szabályozó szerepet játszik az RNS biogenezisben, és ezzel együtt az EGFR szintjének szabályozásában az U2-OS sejtvonalakban.

ACKNOWLEDGEMENTS

I wish to express my profound gratitude to my supervisor **Dr. Gyula Timinszky** for the opportunity to get involved in the scientific life of his research group at the beginning of my doctoral studies, so I was able to expand my knowledge of high-quality research. I am grateful for his guidance, advice, and constructive criticism throughout my research. With his humanity, high level of professional knowledge and broad vision, he not only sets an example to follow but also created a truly inspiring environment that encourages us all to develop.

I would also like to thank to all members of our group: **Dr. Ágnes Czibula, Dr. Roberta Fajka-Boja, Dr. László Henn, Hasan Mamar, Anna Kopasz Georgina, Mihut Alexandra Catalina, Pék Ramóna,**

I would like to give my special thanks to **Adrián Koszó** for his support as a lab technician, furthermore for his help and guidance in the laboratory work at the very beginning.

I am grateful to all members of the Department of Genetics for their support and for creating a pleasant working environment during my studies.

REFERENCES

Aravind, L., Burroughs, A. M., Zhang, D., & Iyer, L. M. (2014). Protein and DNA Modifications: Evolutionary Imprints of Bacterial Biochemical Diversification and Geochemistry on the Provenance of Eukaryotic Epigenetics. *Cold Spring Harbor Perspectives in Biology*, *6*(7), a016063. https://doi.org/10.1101/cshperspect.a016063

Arteaga, C. L. (2001). The epidermal growth factor receptor: From mutant oncogene in nonhuman cancers to therapeutic target in human neoplasia. *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology*, *19*(18 Suppl), 32S-40S.

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

Barkauskaite, E., Jankevicius, G., & Ahel, I. (2015). Structures and Mechanisms of Enzymes Employed in the Synthesis and Degradation of PARP-Dependent Protein ADP-Ribosylation. *Molecular Cell*, *58*(6), 935–946. https://doi.org/10.1016/j.molcel.2015.05.007

Bayani, J., Zielenska, M., Pandita, A., Al-Romaih, K., Karaskova, J., Harrison, K., Bridge, J. A., Sorensen, P., Thorner, P., & Squire, J. A. (2003). Spectral karyotyping identifies recurrent complex rearrangements of chromosomes 8, 17, and 20 in osteosarcomas. *Genes, Chromosomes & Cancer*, *36*(1), 7–16. https://doi.org/10.1002/gcc.10132

Beird, H. C., Bielack, S. S., Flanagan, A. M., Gill, J., Heymann, D., Janeway, K. A., Livingston, J. A., Roberts, R. D., Strauss, S. J., & Gorlick, R. (2022). Osteosarcoma. *Nature Reviews Disease Primers*, *8*(1), 1–19. https://doi.org/10.1038/s41572-022-00409-y

Bheda, P., Jing, H., Wolberger, C., & Lin, H. (2016). The Substrate Specificity of Sirtuins. *Annual Review of Biochemistry*, *85*, 405–429. https://doi.org/10.1146/annurev-biochem-060815-014537

Bielack, S. S., Kempf-Bielack, B., Delling, G., Exner, G. U., Flege, S., Helmke, K., Kotz, R., Salzer-Kuntschik, M., Werner, M., Winkelmann, W., Zoubek, A., Jürgens, H., & Winkler, K. (2002). Prognostic Factors in High-Grade Osteosarcoma of the Extremities or Trunk: An Analysis of 1,702 Patients Treated on Neoadjuvant Cooperative Osteosarcoma Study Group Protocols. *Journal of Clinical Oncology*, *20*(3), 776–790. https://doi.org/10.1200/JCO.2002.20.3.776

Boehi, F., Manetsch, P., & Hottiger, M. O. (2021). Interplay between ADP-ribosyltransferases and essential cell signaling pathways controls cellular responses. *Cell Discovery*, *7*(1), 1–22. https://doi.org/10.1038/s41421-021-00323-9

Bonamy, C., Sechet, E., Amiot, A., Alam, A., Mourez, M., Fraisse, L., Sansonetti, P. J., & Sperandio, B. (2018). Expression of the human antimicrobial peptide β-defensin-1 is repressed by the EGFR-ERK-MYC axis in colonic epithelial cells. *Scientific Reports*, *8*(1), 18043. https://doi.org/10.1038/s41598-018-36387-z

Bütepage, M., Preisinger, C., von Kriegsheim, A., Scheufen, A., Lausberg, E., Li, J., Kappes, F., Feederle, R., Ernst, S., Eckei, L., Krieg, S., Müller-Newen, G., Rossetti, G., Feijs, K. L. H., Verheugd, P., & Lüscher, B. (2018). Nucleolar-nucleoplasmic shuttling of TARG1 and its control by DNA damage-induced poly-ADP-ribosylation and by nucleolar transcription. *Scientific Reports*, *8*(1), 6748. https://doi.org/10.1038/s41598-018-25137-w

Cai, W.-Q., Zeng, L.-S., Wang, L.-F., Wang, Y.-Y., Cheng, J.-T., Zhang, Y., Han, Z.-W., Zhou, Y., Huang, S.-L., Wang, X.-W., Peng, X.-C., Xiang, Y., Ma, Z., Cui, S.-Z., & Xin, H.-

W. (2020). The Latest Battles Between EGFR Monoclonal Antibodies and Resistant Tumor Cells. *Frontiers in Oncology*, *10*. https://doi.org/10.3389/fonc.2020.01249

Challa, S., Khulpateea, B. R., Nandu, T., Camacho, C. V., Ryu, K. W., Chen, H., Peng, Y., Lea, J. S., & Kraus, W. L. (2021). Ribosome ADP-ribosylation inhibits translation and maintains proteostasis in cancers. *Cell*, *184*(17), 4531-4546.e26. https://doi.org/10.1016/j.cell.2021.07.005

Challa, S., Nandu, T., Kim, H. B., Gong, A., Renshaw, C. W., Li, W.-C., Tan, X., Camacho, C. V., Chen, J., & Kraus, W. L. (2023). A PARP14/TARG1-Regulated RACK1 MARylation Cycle Drives Stress Granule Dynamics in Ovarian Cancer Cells. *bioRxiv*, 2023.10.13.562273. https://doi.org/10.1101/2023.10.13.562273

Challa, S., Nandu, T., Kim, H. B., Gong, X., Renshaw, C. W., Li, W.-C., Tan, X., Aljardali, M. W., Camacho, C. V., Chen, J., & Kraus, W. L. (2025). RACK1 MARylation regulates translation and stress granules in ovarian cancer cells. *Journal of Cell Biology*, *224*(2), e202401101. https://doi.org/10.1083/jcb.202401101

Chang, F., Steelman, L. S., Lee, J. T., Shelton, J. G., Navolanic, P. M., Blalock, W. L., Franklin, R. A., & McCubrey, J. A. (2003). Signal transduction mediated by the Ras/Raf/MEK/ERK pathway from cytokine receptors to transcription factors: Potential targeting for therapeutic intervention. *Leukemia*, *17*(7), 1263–1293. https://doi.org/10.1038/sj.leu.2402945

Chen, D., Vollmar, M., Rossi, M. N., Phillips, C., Kraehenbuehl, R., Slade, D., Mehrotra, P. V., Delft, F. von, Crosthwaite, S. K., Gileadi, O., Denu, J. M., & Ahel, I. (2011). Identification of Macrodomain Proteins as Novel O-Acetyl-ADP-ribose Deacetylases *. *Journal of Biological Chemistry*, *286*(15), 13261–13271. https://doi.org/10.1074/jbc.M110.206771

Chowdhury, P., Dey, P., Ghosh, S., Sarma, A., & Ghosh, U. (2019). Reduction of metastatic potential by inhibiting EGFR/Akt/p38/ERK signaling pathway and epithelial-mesenchymal transition after carbon ion exposure is potentiated by PARP-1 inhibition in non-small-cell lung cancer. *BMC Cancer*, *19*, 829. https://doi.org/10.1186/s12885-019-6015-4

Chung, I., Akita, R., Vandlen, R., Toomre, D., Schlessinger, J., & Mellman, I. (2010). Spatial control of EGF receptor activation by reversible dimerization on living cells. *Nature*, *464*(7289), 783–787. https://doi.org/10.1038/nature08827

Cohen, M. S., & Chang, P. (2018). Insights into the biogenesis, function, and regulation of ADP-ribosylation. *Nature Chemical Biology*, *14*(3), 236–243. https://doi.org/10.1038/nchembio.2568

Crawford, K., Bonfiglio, J. J., Mikoč, A., Matic, I., & Ahel, I. (2018). Specificity of reversible ADP-ribosylation and regulation of cellular processes. *Critical Reviews in Biochemistry and Molecular Biology*, *53*(1), 64–82. https://doi.org/10.1080/10409238.2017.1394265

Cullen, B. R. (2000). Nuclear RNA Export Pathways. *Molecular and Cellular Biology*, *20*(12), 4181. https://doi.org/10.1128/mcb.20.12.4181-4187.2000

Demory, M. L., Boerner, J. L., Davidson, R., Faust, W., Miyake, T., Lee, I., Hüttemann, M., Douglas, R., Haddad, G., & Parsons, S. J. (2009). Epidermal growth factor receptor translocation to the mitochondria: Regulation and effect. *The Journal of Biological Chemistry*, *284*(52), 36592–36604. https://doi.org/10.1074/jbc.M109.000760

Di Giammartino, D. C., Shi, Y., & Manley, J. L. (2013). PARP1 Represses PAP and Inhibits Polyadenylation during Heat Shock. *Molecular Cell*, *49*(1), 7–17. https://doi.org/10.1016/j.molcel.2012.11.005

Dow, L. E., Premsrirut, P. K., Zuber, J., Fellmann, C., McJunkin, K., Miething, C., Park, Y., Dickins, R. A., Hannon, G. J., & Lowe, S. W. (2012). A pipeline for the generation of shRNA transgenic mice. *Nature Protocols*, *7*(2), 374–393. https://doi.org/10.1038/nprot.2011.446

Dregalla, R. C., Zhou, J., Idate, R. R., Battaglia, C. L. R., Liber, H. L., & Bailey, S. M. (2010). Regulatory roles of tankyrase 1 at telomeres and in DNA repair: Suppression of T-SCE and stabilization of DNA-PKcs. *Aging (Albany NY)*, *2*(10), 691. https://doi.org/10.18632/aging.100210

Du, J., Jiang, H., & Lin, H. (2009). Investigating the ADP-ribosyltransferase activity of sirtuins with NAD analogues and 32P-NAD. *Biochemistry*, *48*(13), 2878–2890. https://doi.org/10.1021/bi802093g

Dzobo, K., Senthebane, D. A., & Dandara, C. (2023). The Tumor Microenvironment in Tumorigenesis and Therapy Resistance Revisited. *Cancers*, *15*(2), Article 2. https://doi.org/10.3390/cancers15020376

Eleazer, R., & Fondufe-Mittendorf, Y. N. (2021). The multifaceted role of PARP1 in RNA biogenesis. *WIREs RNA*, *12*(2), e1617. https://doi.org/10.1002/wrna.1617

Ellis, L. M. (2004). Epidermal growth factor receptor in tumor angiogenesis. *Hematology/Oncology Clinics of North America*, *18*(5), 1007–1021, viii. https://doi.org/10.1016/j.hoc.2004.06.002

Engelman, J. A., Luo, J., & Cantley, L. C. (2006). The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. *Nature Reviews Genetics*, *7*(8), 606–619. https://doi.org/10.1038/nrg1879

Gill, J., & Gorlick, R. (2021). Advancing therapy for osteosarcoma. *Nature Reviews Clinical Oncology*, *18*(10), 609–624. https://doi.org/10.1038/s41571-021-00519-8

Grandal, M. V., Zandi, R., Pedersen, M. W., Willumsen, B. M., van Deurs, B., & Poulsen, H. S. (2007). EGFRvIII escapes down-regulation due to impaired internalization and sorting to lysosomes. *Carcinogenesis*, *28*(7), 1408–1417. https://doi.org/10.1093/carcin/bgm058

Han, W., Zhang, T., Yu, H., Foulke, J. G., & Tang, C. K. (2006). Hypophosphorylation of residue Y1045 leads to defective downregulation of EGFRvIII. *Cancer Biology & Therapy*, *5*(10), 1361–1368. https://doi.org/10.4161/cbt.5.10.3226

Hanai, S., Kanai, M., Ohashi, S., Okamoto, K., Yamada, M., Takahashi, H., & Miwa, M. (2004). Loss of poly(ADP-ribose) glycohydrolase causes progressive neurodegeneration in Drosophila melanogaster. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(1), 82–86. https://doi.org/10.1073/pnas.2237114100

Harari, D., & Yarden, Y. (2000). Molecular mechanisms underlying ErbB2/HER2 action in breast cancer. *Oncogene*, *19*(53), 6102–6114. https://doi.org/10.1038/sj.onc.1203973

Hay, N., & Sonenberg, N. (2004). Upstream and downstream of mTOR. *Genes & Development*, *18*(16), 1926–1945. https://doi.org/10.1101/gad.1212704

Hernández, G., Ramírez, J. L., Pedroza-Torres, A., Herrera, L. A., & Jiménez-Ríos, M. A. (2019). The Secret Life of Translation Initiation in Prostate Cancer. *Frontiers in Genetics*, *10*. https://doi.org/10.3389/fgene.2019.00014

Huang, C., Chen, Z., Yang, C., Chen, L., Lai, C., Zhang, Y., Yuan, W., & Jeong, J.-H. (2020). Combinational inhibition of EGFR and YAP reverses 5-Fu resistance in colorectal cancer. *Journal of Cancer*, *11*(18), 5432–5439. https://doi.org/10.7150/jca.44775

Hynes, N. E., & MacDonald, G. (2009). ErbB receptors and signaling pathways in cancer. *Current Opinion in Cell Biology*, *21*(2), 177–184. https://doi.org/10.1016/j.ceb.2008.12.010

Ikebuchi, Y., Aoki, S., Honma, M., Hayashi, M., Sugamori, Y., Khan, M., Kariya, Y., Kato, G., Tabata, Y., Penninger, J. M., Udagawa, N., Aoki, K., & Suzuki, H. (2018). Coupling of bone resorption and formation by RANKL reverse signalling. *Nature*, *561*(7722), 195–200. https://doi.org/10.1038/s41586-018-0482-7

Illuzzi, G., Fouquerel, E., Amé, J.-C., Noll, A., Rehmet, K., Nasheuer, H.-P., Dantzer, F., & Schreiber, V. (2014). PARG is dispensable for recovery from transient replicative stress but required to prevent detrimental accumulation of poly(ADP-ribose) upon prolonged replicative stress. *Nucleic Acids Research*, *42*(12), 7776–7792. https://doi.org/10.1093/nar/gku505

Isfort, R. J., Cody, D. B., Lovell, G., & Doersen, C. J. (1995). Analysis of oncogenes, tumor suppressor genes, autocrine growth-factor production, and differentiation state of human osteosarcoma cell lines. *Molecular Carcinogenesis*, *14*(3), 170–178. https://doi.org/10.1002/mc.2940140306

Jankevicius, G., Hassler, M., Golia, B., Rybin, V., Zacharias, M., Timinszky, G., & Ladurner, A. G. (2013). A family of macrodomain proteins reverses cellular mono-ADP-ribosylation. *Nature Structural & Molecular Biology*, *20*(4), 508–514. https://doi.org/10.1038/nsmb.2523

Karamouzis, M. V., Badra, F. A., & Papavassiliou, A. G. (2007). Breast cancer: The upgraded role of HER-3 and HER-4. *The International Journal of Biochemistry & Cell Biology*, *39*(5), 851–856. https://doi.org/10.1016/j.biocel.2006.11.017

Karras, G. I., Kustatscher, G., Buhecha, H. R., Allen, M. D., Pugieux, C., Sait, F., Bycroft, M., & Ladurner, A. G. (2005). The macro domain is an ADP-ribose binding module. *The EMBO Journal*, *24*(11), 1911–1920. https://doi.org/10.1038/sj.emboj.7600664

Katz, M., Amit, I., & Yarden, Y. (2007). Regulation of MAPKs by growth factors and receptor tyrosine kinases. *Biochimica Et Biophysica Acta*, *1773*(8), 1161–1176. https://doi.org/10.1016/j.bbamcr.2007.01.002

Kim, D.-S., Challa, S., Jones, A., & Kraus, W. L. (2020). PARPs and ADP-ribosylation in RNA biology: From RNA expression and processing to protein translation and proteostasis. *Genes & Development*, *34*(5–6), 302–320. https://doi.org/10.1101/gad.334433.119

Kleine, H., Poreba, E., Lesniewicz, K., Hassa, P. O., Hottiger, M. O., Litchfield, D. W., Shilton, B. H., & Lüscher, B. (2008). Substrate-assisted catalysis by PARP10 limits its activity to mono-ADP-ribosylation. *Molecular Cell*, *32*(1), 57–69. https://doi.org/10.1016/j.molcel.2008.08.009

Konieczkowski, D. J., Johannessen, C. M., & Garraway, L. A. (2018). A Convergence-Based Framework for Cancer Drug Resistance. *Cancer Cell*, *33*(5), 801–815. https://doi.org/10.1016/j.ccell.2018.03.025

Kopasz, A. G., Pusztai, D. Z., Karkas, R., Hudoba, L., Abdullah, K. S. A., Imre, G., Pankotai-Bodó, G., Migh, E., Nagy, A., Kriston, A., Germán, P., Drubi, A. B., Molnár, A., Fekete, I., Dani, V. É., Ocsovszki, I., Puskás, L. G., Horváth, P., Sükösd, F., & Mátés, L. (2022). A versatile transposon-based technology to generate loss- and gain-of-function phenotypes in the mouse liver. *BMC Biology*, *20*(1), 74. https://doi.org/10.1186/s12915-022-01262-x

Kun, E., Tsang, Y. T. M., Ng, C. W., Gershenson, D. M., & Wong, K. K. (2021). MEK inhibitor resistance mechanisms and recent developments in combination trials. *Cancer Treatment Reviews*, *92*, 102137. https://doi.org/10.1016/j.ctrv.2020.102137

Laplante, M., & Sabatini, D. M. (2012). mTOR Signaling in Growth Control and Disease. *Cell*, *149*(2), 274–293. https://doi.org/10.1016/j.cell.2012.03.017

Le, X., Nilsson, M., Goldman, J., Reck, M., Nakagawa, K., Kato, T., Ares, L. P., Frimodt-Moller, B., Wolff, K., Visseren-Grul, C., Heymach, J. V., & Garon, E. B. (2021). Dual EGFR-VEGF Pathway Inhibition: A Promising Strategy for Patients With *EGFR*-Mutant NSCLC. *Journal of Thoracic Oncology*, *16*(2), 205–215. https://doi.org/10.1016/j.jtho.2020.10.006

Lemmon, M. A., & Schlessinger, J. (2010). Cell signaling by receptor tyrosine kinases. *Cell*, *141*(7), 1117–1134. https://doi.org/10.1016/j.cell.2010.06.011

Li, S., Cui, Z., & Meng, X. (2016). Knockdown of PARP-1 Inhibits Proliferation and ERK Signals, Increasing Drug Sensitivity in Osteosarcoma U2OS Cells. *Oncology Research*, *24*(4), 279–286. https://doi.org/10.3727/096504016X14666990347554

Li, Y., Liu, Q., He, H., & Luo, W. (2019). The possible role of insulin-like growth factor-1 in osteosarcoma. *Current Problems in Cancer*, *43*(3), 228–235. https://doi.org/10.1016/j.currproblcancer.2018.08.008

Lin, S. Y., Makino, K., Xia, W., Matin, A., Wen, Y., Kwong, K. Y., Bourguignon, L., & Hung, M. C. (2001). Nuclear localization of EGF receptor and its potential new role as a transcription factor. *Nature Cell Biology*, *3*(9), 802–808. https://doi.org/10.1038/ncb0901-802

Lin, S., Zhang, X., Yu, Z., Huang, X., Xu, J., Liu, Y., & Wu, L. (2022). Synthesis of novel dual target inhibitors of PARP and EGFR and their antitumor activities in triple negative breast cancers. *Bioorganic & Medicinal Chemistry*, *61*, 116739. https://doi.org/10.1016/j.bmc.2022.116739

Liu, H., Li, Z., Xu, B., Li, Z., Yang, X., & Luo, J. (2024). Curcumin suppresses metastasis, invasion, and proliferation in osteosarcoma cells by regulating the EGFR/Src signaling axis. *International Journal of Immunopathology and Pharmacology*, *38*, 03946320241308082. https://doi.org/10.1177/03946320241308082

Liu, Q., Yu, S., Zhao, W., Qin, S., Chu, Q., & Wu, K. (2018). EGFR-TKIs resistance via EGFR-independent signaling pathways. *Molecular Cancer*, *17*(1), 53. https://doi.org/10.1186/s12943-018-0793-1

Liu, X., Qin, J., Luo, Q., Bi, Y., Zhu, G., Jiang, W., Kim, S. H., Li, M., Su, Y., Nan, G., Cui, J., Zhang, W., Li, R., Chen, X., Kong, Y., Zhang, J., Wang, J., Rogers, M. R., Zhang, H., … Li, M. (2013). Cross-talk between EGF and BMP9 signalling pathways regulates the osteogenic differentiation of mesenchymal stem cells. *Journal of Cellular and Molecular Medicine*, *17*(9), 1160–1172. https://doi.org/10.1111/jcmm.12097

Lüscher, B., Bütepage, M., Eckei, L., Krieg, S., Verheugd, P., & Shilton, B. H. (2018). ADP-Ribosylation, a Multifaceted Posttranslational Modification Involved in the Control of Cell Physiology in Health and Disease. *Chemical Reviews*, *118*(3), 1092–1136. https://doi.org/10.1021/acs.chemrev.7b00122

M, D. V., V, S., & F, D. (2012). The diverse roles and clinical relevance of PARPs in DNA damage repair: Current state of the art. *Biochemical Pharmacology*, *84*(2). https://doi.org/10.1016/j.bcp.2012.03.018

Ma, J., Dong ,Chao, Cao ,Yan-Zhen, & and Ma, B.-L. (2023). Dual Target of EGFR and mTOR Suppresses Triple-Negative Breast Cancer Cell Growth by Regulating the Phosphorylation of mTOR Downstream Proteins. *Breast Cancer: Targets and Therapy*, *15*, 11–24. https://doi.org/10.2147/BCTT.S390017

Marcar, L., Bardhan, K., Gheorghiu, L., Dinkelborg, P., Pfäffle, H., Liu, Q., Wang, M., Piotrowska, Z., Sequist, L. V., Borgmann, K., Settleman, J. E., Engelman, J. A., Hata, A. N., & Willers, H. (2019). Acquired Resistance of EGFR-Mutated Lung Cancer to Tyrosine Kinase Inhibitor Treatment Promotes PARP Inhibitor Sensitivity. *Cell Reports*, *27*(12), 3422-3432.e4. https://doi.org/10.1016/j.celrep.2019.05.058

Mendelsohn, J., & Baselga, J. (2006). Epidermal growth factor receptor targeting in cancer. *Seminars in Oncology*, *33*(4), 369–385. https://doi.org/10.1053/j.seminoncol.2006.04.003

Mikolčević, P., Hloušek-Kasun, A., Ahel, I., & Mikoč, A. (2021). ADP-ribosylation systems in bacteria and viruses. *Computational and Structural Biotechnology Journal*, *19*, 2366–2383. https://doi.org/10.1016/j.csbj.2021.04.023

Mishra, R., Patel, H., Alanazi, S., Yuan, L., & Garrett, J. T. (2018). HER3 signaling and targeted therapy in cancer. *Oncology Reviews*, *12*(1), 355. https://doi.org/10.4081/oncol.2018.355

Mitsudomi, T., Morita, S., Yatabe, Y., Negoro, S., Okamoto, I., Tsurutani, J., Seto, T., Satouchi, M., Tada, H., Hirashima, T., Asami, K., Katakami, N., Takada, M., Yoshioka, H., Shibata, K., Kudoh, S., Shimizu, E., Saito, H., Toyooka, S., … Fukuoka, M. (2010). Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): An open label, randomised phase 3 trial. *The Lancet Oncology*, *11*(2), 121–128. https://doi.org/10.1016/S1470-2045(09)70364-X

Motta, C., D'Angeli, F., Scalia, M., Satriano, C., Barbagallo, D., Naletova, I., Anfuso, C. D., Lupo, G., & Spina-Purrello, V. (2015). PJ-34 inhibits PARP-1 expression and ERK phosphorylation in glioma-conditioned brain microvascular endothelial cells. *European Journal of Pharmacology*, *761*, 55–64. https://doi.org/10.1016/j.ejphar.2015.04.026

Munnur, D., & Ahel, I. (2017). Reversible mono-ADP-ribosylation of DNA breaks. *The FEBS Journal*, *284*(23), 4002–4016. https://doi.org/10.1111/febs.14297

Munnur, D., Bartlett, E., Mikolčević, P., Kirby, I. T., Rack, J. G. M., Mikoč, A., Cohen, M. S., & Ahel, I. (2019). Reversible ADP-ribosylation of RNA. *Nucleic Acids Research*, *47*(11), 5658–5669. https://doi.org/10.1093/nar/gkz305

Nowsheen, S., Cooper, T., Stanley, J. A., & Yang, E. S. (2012). Synthetic Lethal Interactions between EGFR and PARP Inhibition in Human Triple Negative Breast Cancer Cells. *PLOS ONE*, *7*(10), e46614. https://doi.org/10.1371/journal.pone.0046614

Okines, A., Cunningham, D., & Chau, I. (2011). Targeting the human EGFR family in esophagogastric cancer. *Nature Reviews Clinical Oncology*, *8*(8), 492–503. https://doi.org/10.1038/nrclinonc.2011.45

Palazzo, L., Mikolčević, P., Mikoč, A., & Ahel, I. (2019). ADP-ribosylation signalling and human disease. *Open Biology*, *9*(4), 190041. https://doi.org/10.1098/rsob.190041

Rajendran, P., Sekar, R., Dhayasankar, P. S., Ali, E. M., Abdelsalam, S. A., Balaraman, S., Chellappan, B. V., Metwally, A. M., & Abdallah, B. M. (2024). PI3K/AKT Signaling Pathway Mediated Autophagy in Oral Carcinoma—A Comprehensive Review. *International Journal of Medical Sciences*, *21*(6), 1165–1175. https://doi.org/10.7150/ijms.94566

Roepstorff, K., Grøvdal, L., Grandal, M., Lerdrup, M., & van Deurs, B. (2008). Endocytic downregulation of ErbB receptors: Mechanisms and relevance in cancer. *Histochemistry and Cell Biology*, *129*(5), 563–578. https://doi.org/10.1007/s00418-008-0401-3

Rosell, R., Carcereny, E., Gervais, R., Vergnenegre, A., Massuti, B., Felip, E., Palmero, R., Garcia-Gomez, R., Pallares, C., Sanchez, J. M., Porta, R., Cobo, M., Garrido, P., Longo, F., Moran, T., Insa, A., Marinis, F. D., Corre, R., Bover, I., … Paz-Ares, L. (2012). Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer

(EURTAC): A multicentre, open-label, randomised phase 3 trial. *The Lancet Oncology*, *13*(3), 239–246. https://doi.org/10.1016/S1470-2045(11)70393-X

Rosenthal, F., Feijs, K. L. H., Frugier, E., Bonalli, M., Forst, A. H., Imhof, R., Winkler, H. C., Fischer, D., Caflisch, A., Hassa, P. O., Lüscher, B., & Hottiger, M. O. (2013). Macrodomain-containing proteins are new mono-ADP-ribosylhydrolases. *Nature Structural & Molecular Biology*, *20*(4), 502–507. https://doi.org/10.1038/nsmb.2521

Roskoski, R. (2014). The ErbB/HER family of protein-tyrosine kinases and cancer. *Pharmacological Research*, *79*, 34–74. https://doi.org/10.1016/j.phrs.2013.11.002

Roux, P. P., Shahbazian, D., Vu, H., Holz, M. K., Cohen, M. S., Taunton, J., Sonenberg, N., & Blenis, J. (2007). RAS/ERK Signaling Promotes Site-specific Ribosomal Protein S6 Phosphorylation via RSK and Stimulates Cap-dependent Translation *. *Journal of Biological Chemistry*, *282*(19), 14056–14064. https://doi.org/10.1074/jbc.M700906200

Schmidt, E. K., Clavarino, G., Ceppi, M., & Pierre, P. (2009). SUnSET, a nonradioactive method to monitor protein synthesis. *Nature Methods*, *6*(4), 275–277. https://doi.org/10.1038/nmeth.1314

Schmidt, M. H. H., Furnari, F. B., Cavenee, W. K., & Bögler, O. (2003). Epidermal growth factor receptor signaling intensity determines intracellular protein interactions, ubiquitination, and internalization. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(11), 6505–6510. https://doi.org/10.1073/pnas.1031790100

Schneider, M. R., & Yarden, Y. (2016). The EGFR-HER2 module: A stem cell approach to understanding a prime target and driver of solid tumors. *Oncogene*, *35*(23), 2949–2960. https://doi.org/10.1038/onc.2015.372

Schuller, M., Raggiaschi, R., Mikolcevic, P., Rack, J. G. M., Ariza, A., Zhang, Y., Ledermann, R., Tang, C., Mikoc, A., & Ahel, I. (2023). Molecular basis for the reversible ADP-ribosylation of guanosine bases. *Molecular Cell*, *83*(13), 2303-2315.e6. https://doi.org/10.1016/j.molcel.2023.06.013

Sharifi, R., Morra, R., Appel, C. D., Tallis, M., Chioza, B., Jankevicius, G., Simpson, M. A., Matic, I., Ozkan, E., Golia, B., Schellenberg, M. J., Weston, R., Williams, J. G., Rossi, M. N., Galehdari, H., Krahn, J., Wan, A., Trembath, R. C., Crosby, A. H., … Ahel, I. (2013). Deficiency of terminal ADP-ribose protein glycohydrolase TARG1/C6orf130 in neurodegenerative disease. *The EMBO Journal*, *32*(9), 1225–1237. https://doi.org/10.1038/emboj.2013.51

Shaul, Y. D., & Seger, R. (2007). The MEK/ERK cascade: From signaling specificity to diverse functions. *Biochimica Et Biophysica Acta*, *1773*(8), 1213–1226. https://doi.org/10.1016/j.bbamcr.2006.10.005

Shi, K., Wang, G., Pei, J., Zhang, J., Wang, J., Ouyang, L., Wang, Y., & Li, W. (2022). Emerging strategies to overcome resistance to third-generation EGFR inhibitors. *Journal of Hematology & Oncology*, *15*(1), 94. https://doi.org/10.1186/s13045-022-01311-6

Sigismund, S., Avanzato, D., & Lanzetti, L. (2018). Emerging functions of the EGFR in cancer. *Molecular Oncology*, *12*(1), 3–20. https://doi.org/10.1002/1878-0261.12155

Slade, D., Dunstan, M. S., Barkauskaite, E., Weston, R., Lafite, P., Dixon, N., Ahel, M., Leys, D., & Ahel, I. (2011). The structure and catalytic mechanism of a poly(ADP-ribose) glycohydrolase. *Nature*, *477*(7366), 616–620. https://doi.org/10.1038/nature10404

Suskiewicz, M. J., Prokhorova, E., Rack, J. G. M., & Ahel, I. (2023). ADP-ribosylation from molecular mechanisms to therapeutic implications. *Cell*, *186*(21), 4475–4495. https://doi.org/10.1016/j.cell.2023.08.030

Tarcic, G., Avraham, R., Pines, G., Amit, I., Shay, T., Lu, Y., Zwang, Y., Katz, M., Ben-Chetrit, N., Jacob-Hirsch, J., Virgilio, L., Rechavi, G., Mavrothalassitis, G., Mills, G. B., Domany, E., & Yarden, Y. (2012). EGR1 and the ERK-ERF axis drive mammary cell migration in response to EGF. *The FASEB Journal*, *26*(4), 1582–1592. https://doi.org/10.1096/fj.11-194654

Teven, C. M., Farina, E. M., Rivas, J., & Reid, R. R. (2014). Fibroblast growth factor (FGF) signaling in development and skeletal diseases. *Genes & Diseases*, *1*(2), 199–213. https://doi.org/10.1016/j.gendis.2014.09.005

Tricker, E. M., Xu, C., Uddin, S., Capelletti, M., Ercan, D., Ogino, A., Pratilas, C. A., Rosen, N., Gray, N. S., Wong, K.-K., & Jänne, P. A. (2015). Combined EGFR/MEK Inhibition Prevents the Emergence of Resistance in EGFR-Mutant Lung Cancer. *Cancer Discovery*, *5*(9), 960–971. https://doi.org/10.1158/2159-8290.CD-15-0063

Tromans-Coia, C., Sanchi, A., Moeller, G. K., Timinszky, G., Lopes, M., & Ahel, I. (2021). TARG1 protects against toxic DNA ADP-ribosylation. *Nucleic Acids Research*, *49*(18), 10477–10492. https://doi.org/10.1093/nar/gkab771

Van Ness, B. G., Howard, J. B., & Bodley, J. W. (1980). ADP-ribosylation of elongation factor 2 by diphtheria toxin. Isolation and properties of the novel ribosyl-amino acid and its hydrolysis products. *Journal of Biological Chemistry*, *255*(22), 10717–10720.

Vyas, S., Chesarone-Cataldo, M., Todorova, T., Huang, Y.-H., & Chang, P. (2013). A systematic analysis of the PARP protein family identifies new functions critical for cell physiology. *Nature Communications*, *4*, 2240. https://doi.org/10.1038/ncomms3240

Vyas, S., Matic, I., Uchima, L., Rood, J., Zaja, R., Hay, R. T., Ahel, I., & Chang, P. (2014). Family-wide analysis of poly(ADP-ribose) polymerase activity. *Nature Communications*, *5*, 4426. https://doi.org/10.1038/ncomms5426

Wang, Q., Cai, J., Wang, J., Xiong, C., & Zhao, J. (2014). MiR-143 inhibits EGFR-signaling-dependent osteosarcoma invasion. *Tumor Biology*, *35*(12), 12743–12748. https://doi.org/10.1007/s13277-014-2600-y

Wang, S., Wei, H., Huang, Z., Wang, X., Shen, R., Wu, Z., & Lin, J. (2021). Epidermal growth factor receptor promotes tumor progression and contributes to gemcitabine resistance in osteosarcoma. *Acta Biochimica et Biophysica Sinica*, *53*(3), 317–324. https://doi.org/10.1093/abbs/gmaa177

Wee, P., & Wang, Z. (2017). Epidermal Growth Factor Receptor Cell Proliferation Signaling Pathways. *Cancers*, *9*(5), Article 5. https://doi.org/10.3390/cancers9050052

Weixler, L., Feijs, K. L. H., & Zaja, R. (2022). ADP-ribosylation of RNA in mammalian cells is mediated by TRPT1 and multiple PARPs. *Nucleic Acids Research*, *50*(16), 9426–9441. https://doi.org/10.1093/nar/gkac711

Wheeler, D. L., Huang, S., Kruser, T. J., Nechrebecki, M. M., Armstrong, E. A., Benavente, S., Gondi, V., Hsu, K.-T., & Harari, P. M. (2008). Mechanisms of acquired resistance to cetuximab: Role of HER (ErbB) family members. *Oncogene*, *27*(28), 3944–3956. https://doi.org/10.1038/onc.2008.19

Wiley, H. S. (2003). Trafficking of the ErbB receptors and its influence on signaling. *Experimental Cell Research*, *284*(1), 78–88. https://doi.org/10.1016/s0014-4827(03)00002-8

Wilson, K. J., Gilmore, J. L., Foley, J., Lemmon, M. A., & Riese, D. J. (2009). Functional selectivity of EGF family peptide growth factors: Implications for cancer. *Pharmacology & Therapeutics*, *122*(1), 1–8. https://doi.org/10.1016/j.pharmthera.2008.11.008

Wilson, K. J., Mill, C., Lambert, S., Buchman, J., Wilson, T. R., Hernandez-Gordillo, V., Gallo, R. M., Ades, L. M. C., Settleman, J., & Riese, D. J. (2012). EGFR ligands

exhibit functional differences in models of paracrine and autocrine signaling. *Growth Factors (Chur, Switzerland)*, *30*(2), 107–116. https://doi.org/10.3109/08977194.2011.649918

Wound Healing and Migration Assay | Experimental Workflow. (n.d.). Ibidi. Retrieved 14 November 2024, from https://ibidi.com/content/281-experimental-workflow

Wu, S., Gao, F., Zheng, S., Zhang, C., Martinez-Ledesma, E., Ezhilarasan, R., Ding, J., Li, X., Feng, N., Multani, A., Sulman, E. P., Verhaak, R. G., de Groot, J. F., Heffernan, T. P., Yung, W. K. A., & Koul, D. (2020). EGFR Amplification Induces Increased DNA Damage Response and Renders Selective Sensitivity to Talazoparib (PARP Inhibitor) in Glioblastoma. *Clinical Cancer Research*, *26*(6), 1395–1407. https://doi.org/10.1158/1078-0432.CCR-19-2549

Yarden, Y., & Pines, G. (2012). The ERBB network: At last, cancer therapy meets systems biology. *Nature Reviews. Cancer*, *12*(8), 553–563. https://doi.org/10.1038/nrc3309

Yarden, Y., & Sliwkowski, M. X. (2001). Untangling the ErbB signalling network. *Nature Reviews. Molecular Cell Biology*, *2*(2), 127–137. https://doi.org/10.1038/35052073

Yu, S., Li, A., Liu, Q., Yuan, X., Xu, H., Jiao, D., Pestell, R. G., Han, X., & Wu, K. (2017). Recent advances of bispecific antibodies in solid tumors. *Journal of Hematology & Oncology*, *10*(1), 155. https://doi.org/10.1186/s13045-017-0522-z

Yuan, Y., Wang, Y., Liu, Z., Sun, Y., Yao, Y., Yu, W., & Shen, Z. (2019). MAT2B promotes proliferation and inhibits apoptosis in osteosarcoma by targeting epidermal growth factor receptor and proliferating cell nuclear antigen. *International Journal of Oncology*, *54*(6), 2019–2029. https://doi.org/10.3892/ijo.2019.4764

Žaja, R., Aydin, G., Lippok, B. E., Feederle, R., Lüscher, B., & Feijs, K. L. H. (2020). Comparative analysis of MACROD1, MACROD2 and TARG1 expression,

localisation and interactome. *Scientific Reports*, *10*(1), 8286. https://doi.org/10.1038/s41598-020-64623-y

Zubair, T., & Bandyopadhyay, D. (2023). Small Molecule EGFR Inhibitors as Anti-Cancer Agents: Discovery, Mechanisms of Action, and Opportunities. *International Journal of Molecular Sciences*, *24*(3), Article 3. https://doi.org/10.3390/ijms24032651