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)

ABBREVIATIONS

ARHs	ADP-ribosylhydrolases
cDNA	complementary DNA
c-Fos	Fos Proto-Oncogene, AP-1 Transcription Factor Subunit
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
EGFR	epidermal growth factor receptor
ErbB 2,3,4	Erb-B2 Receptor Tyrosine Kinase 2,3,4
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
HER 2,3,4	Erb-B2 Receptor Tyrosine Kinase 2,3,4
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
PARPs	Poly(ADP-ribose) Polymerases

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
RAS	RAS Proto-Oncogene, GTPase
RT	room temperature
RTKs	Receptor Tyrosine Kinases
WT	wild type

INTRODUCTION

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. 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. 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. Macrodomain-containing enzymes are widely distributed in all domains of life, and share a highly conserved ADP-ribose binding domain, known as macrodomain. There are three 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. 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. 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. 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. Furthermore, they stated that TARG1 shuttles continuously between nucleoli and the nucleoplasm and accumulates in transcriptionally active nucleoli under steady-state conditions. It has been concluded that TARG1 may be a nucleolar ribosome biosynthesis quality control factor, and 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. Many of these suggestably TARG1 related cellular processes are mainly regulated by the PI3K/Akt and MAPK, which are involved in the proliferation, differentiation, migration, and apoptosis of certain cells. Upstream of these cellular pathways EGFR is a key receptor, that receives extracellular signals, and upon activation initiates intracellular signal transduction. EGFR is a transmembrane glycoprotein belonging to the ErbB family of RTKs, and is frequently altered in several human cancers due to EGFR gene amplification and/or protein overexpression, mutations, or in-frame deletions. Many studies pointed out a correlation between EGFR and

ADP-ribosylation before, particularly in the context of DNA damage response. Cells that overexpressing EGFR showed sensitivity against PARP inhibitors before. Besides the influence of ADP-ribosylation on DDR, 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. Interestingly, PARP1 (an ADP-ribose polymerase enzyme) inhibition or knockdown also reduced the expression and phosphorylation of EGFR.

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

Cell culture

U2-OS wild type and TARG1 knock out (CRISPR/Cas) cell lines have been describe previously 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 °C 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. 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. 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.

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 °C. 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.

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).

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.

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.

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).

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.

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.

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

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. 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, 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. These results suggest that TARG1 is required for efficient EGF-stimulated cell migration.

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 and the 30 minutes stimulated 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.

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. However, when normalizing the phospho-EGFR levels to total EGFR, no significant differences were observed between wild-type and TARG1 KO cells. 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. 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.

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 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. 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. The mRNA levels of MYC significantly increase in both WT and TARG1 KO upon serum stimulation. 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. 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. 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. While CCND1 mRNA level was lowered only in TARG1 KO. 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. 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. Altogether these results suggest that the loss of TARG1 decreased the stability of mRNAs and causes increased mRNA turnover.

TARG1-dependent regulation of RNA distribution and translation

To test the hypothesis, that TARG1 has been implicated in RNA metabolism, 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. 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. In TARG1 KO cells, we observed a non-significant reduction in the cytoplasmic to nuclear RNA levels after serum starvation as compared to WT cells, which increased only mildly upon the 5-hour serum stimulation. 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, 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. 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. 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.

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. 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. However, U0126 reduced cell viability to a greater extent in both TARG1 KD and KO cells than in wild-type cells. Notably, co-treatment with U0126 and rapamycin increased the sensitivity only in WT cells, thus eliminating the differential sensitivity of WT and TARG1KO cells to MEK1/2 inhibition. The sensitivity of TARG1 KD to MEK1/2 and mTOR inhibition was almost identical to that of TARG1 KO. 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.

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 TARG1's regulatory role in RNA biogenesis, and through that the regulation of EGFR levels in U2-OS cell line.

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.