The diverse functions of mono-ADP-ribosylation from EGFR signaling to DNA damage response – final report of K128239

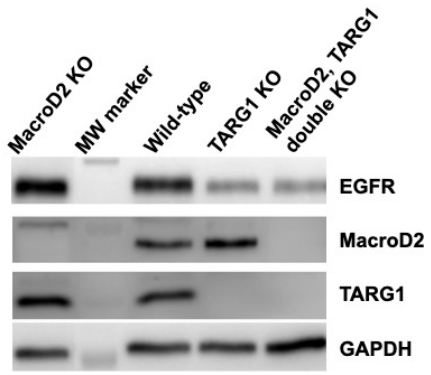
My laboratory's primary research focus is the regulation of ADP-ribosylation and its impact on cellular processes, with a particular emphasis on chromatin regulation during DNA repair (Barkauskaite et al. 2013; Lüscher et al. 2022). ADP-ribosylation is a modification of macromolecules facilitated by the PARP family of enzymes. It is either the attachment of a single ADP-ribose unit, known as mono-ADP-ribosylation, or the addition of multiple ADP-ribose units, referred to as poly-ADP-ribosylation (PARylation). Both of these forms play pivotal roles in regulating a wide array of cellular processes, including chromatin remodeling, epigenetics, transcription, replication, and nucleic acid processing (Palazzo et al. 2019; Huang and Kraus 2022).

EGFR is regulated by ADP-ribosylation at the mRNA level

Mono-ADP-ribosylation is reversed by a family of macrodomain-containing proteins. One of them, MacroD2 is frequently amplified or mutated in a number of cancers (Zhou et al. 2020; Sakthianandeswaren et al. 2018; Mohseni et al. 2014; Feijs, Cooper, and Žaja 2020). When assessing the sensitivity of MacroD2 knockouts to a library of anti-cancer drugs, we found synthetic interactions between MacroD2 knockouts and inhibitors of epidermal growth factor receptor (EGFR) signaling. Consistent with altered EGFR signaling, MacroD2 knockouts have migration defects, and a protein interaction screen identified several cytoskeletal and focal adhesion proteins – many of them regulating EGFR signaling – to bind MacroD2. Moreover, synthetic lethality was found between EGFR and PARP inhibition in human triple negative breast cancer, a particularly aggressive tumor of the breast (Nowsheen et al. 2012; Pfaffle et al. 2013). We aimed to address if ADP-ribosylation was a regulator of EGFR signaling.

EGFR belongs to the receptor tyrosine kinase family and plays a crucial role in various physiological functions of mammalian cells, such as cell proliferation, differentiation, and survival (Brand et al. 2013; Shostak and Chariot 2015; Wee and Wang 2017). A common feature of cancer cells is uncontrolled growth, frequently due to activating mutations of cell surface receptors, such as the EGFR that normally responds to growth promoting stimuli. EGFR, also known as HER1, is a transmembrane receptor with a molecular weight of 175 kDa. It is primarily located on the cell's plasma membrane, existing as a single monomer and comprising extracellular, transmembrane, and cytoplasmic activation domains. The extracellular domain is responsible for receiving signals from the external environment. Upon binding to its ligand, EGFR monomers can form homodimers with other monomers or heterodimers with other members of the receptor family, leading to transphosphorylation on their cytoplasmic C-terminal tail. This phosphorylation initiates a downstream signaling cascade that ultimately reaches the cell nucleus. The regulation of these signaling events is mainly governed by endocytosis and the transport of the activated receptor in vesicles to different cellular compartments, depending on the type and strength of the extracellular signal.

First, we assessed the EGFR receptor protein levels of MacroD2 and TARG1 knockout (KO) cell lines and their wild-type control using Western blotting. The results revealed intriguing differences: the MacroD2 KO cell lines expressed higher levels of EGFR, while the TARG1 KO cells showed lower expression levels compared to their respective wild-type counterparts (**Figure 1**). Notably, we also observed variations in EGFR expression among the wild-type clonal U2OS cell lines themselves. This variation was attributable to the fact that these wild-type cell



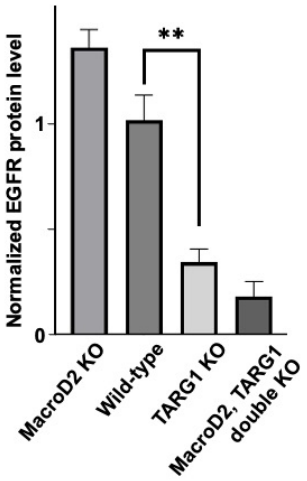


Figure 1. The mono-ADP-ribosylhyrolases MacroD2 and TARG1 have opposite effect on EGFR protein levels. MacroD2 and TARG1 knockouts were generated using CRISPR/Cas9 in the human U2OS cell line. Representative immunoblot of whole cell extracts using the specified antibodies (left panel). GAPDH was used as loading control. Quantification of densitometric measurements of three biological replicates (right panel). Error bars are SEM.
** $p < 0.01$.

lines were derived from a heterogeneous population of the original cell line used to create single cell-derived clones. Significantly, the wild-type cell lines also exhibited differences in MacroD2 expression levels, which appeared to be inversely proportional to their EGFR expression. Moreover, while monitoring the MacroD2 and EGFR expression profiles of cell lines in our laboratory, we observed a similar inverse correlation between MacroD2 and EGFR levels (**Figure 2**). To account for the potential variations arising from the clonal selection of the cell lines, we adopted a siRNA-based gene silencing approach against both MacroD2 and TARG1, which confirmed our initial findings. Cells with elevated receptor levels displayed higher EGFR phosphorylation activity, while those with lower receptor levels exhibited decreased phosphorylation activity indicating that the expressed receptors are functional.

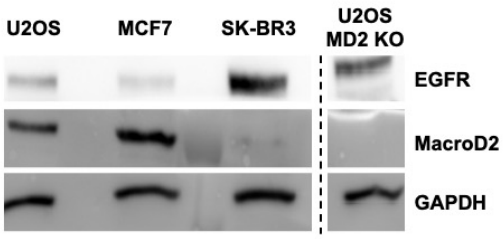


Figure 2. MacroD2 and EGFR protein levels show inverse correlation. Whole cell extracts of human U2OS osteosarcoma (wild-type and MacroD2 KO) and MCF7 and SK-BR3 breast cancer cell lines. Representative immunoblot of whole cell extracts using the specified anti-bodies. GAPDH was used as loading control.

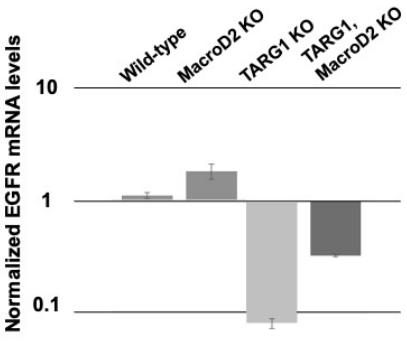


Figure 3. The mono-ADP-ribosyl hyrolases MacroD2 and TARG1 regulate EGFR at the mRNA level. Quantitative RT-PCR analysis of the specified mRNAs from total RNA purifications. EGFR mRNA levels were normalized to Rpl27 mRNA. n = 3, error bars are SEM.
** $p < 0.01$.

Upon observing differences in the receptor protein levels between the mutant and wild-type cells, our next critical step was to investigate whether this phenotype occurred post-translationally or was regulated at the mRNA level. To address this, we performed quantitative RT-PCR to assess the mRNA levels in the different cell lines. The mRNA level analysis yielded the same pattern as what we had observed in the protein profile (**Figure 3**). These results strongly suggest that the expression level of the receptor is transcriptionally regulated by the levels of both MacroD2 and/or TARG1. This finding emphasizes the potential involvement of these proteins in the transcriptional regulation of the receptor, shedding light on the underlying molecular mechanisms at play. We are currently investigating if the regulation of EGFR mRNA levels is achieved at the level of transcription or mRNA turnover.

The mono-ADP-ribosyl hydrolase TARG1 counters toxic DNA ADP-ribosylation

The ADP-ribosylation of nucleic acids and its significance is one of the most exciting discoveries in recent years in the field of ADP-ribosylation (Groslambert, Prokhorova, and Ahel 2021). DarT is present in thermophiles like *Thermus aquaticus* and global pathogens such as *Mycobacterium tuberculosis* and enteropathogenic *Escherichia coli* (Jankevicius et al. 2016). It operates by transferring a single ADP-ribose unit specifically onto thymidine bases within single-stranded DNA (ss-DNA), with no activity towards double-stranded DNA (dsDNA), RNA, or proteins. DarT is part of a toxin:antitoxin system, with DarG serving as the antitoxin. DarG is a macrodomain containing ADP-ribosylhydrolase, which can reverse thymidine-linked ADP-ribosylation. In *Escherichia coli*, DarT's activity has been shown to trigger the DNA damage response. In the absence of DarG, an attenuated DarT mutant can repair thymidine-linked ADP-ribosylation through RecF-mediated homologous recombination in collaboration with NER. Depletion of DarG in *Mycobacterium tuberculosis* also leads to the DDR and bacterial cell death. Remarkably, the catalytic macrodomain of the antitoxin DarG most closely resembles that of the human mono-ADP-ribosylhydrolase TARG1.

Given this structural similarity, we sought to determine if TARG1 could reverse thymidine-linked ADP-ribosylation. Our results revealed that TARG1 can indeed reverse thymidine-linked ADP-ribosylation of DNA and rescue bacterial toxicity induced by DarT, similar to the action of DarG. As TARG1 is a human ADP-ribosylhydrolase, we explored the impact of DarT-mediated DNA ADP-ribosylation in human cells. We developed a system for heterologous expression of DarT to assess the consequences of DNA ADP-ribosylation. Using DarT as a genotoxin, we found that while normal cells can combat these DNA ADP-ribosylating toxins, TARG1-deficient cells cannot. In TARG1-deficient cells, DNA ADP-ribosylation halts replication, ultimately leading to replication catastrophe and cell death (Tromans-Coia et al. 2021). This study highlights the distinct catalytic activity of TARG1 in reversing thymidine-linked ADP-ribosylation and was published in *Nucleic Acids Research*.

Chromatin loosening is important for PARP1 release and efficient DNA repair

The regulation of ADP-ribosylation has clinical relevance. PARP inhibitors are used in medicine to eradicate BRCA-deficient tumors (Noordermeer and van Attikum 2019). The therapeutic effect of PARP inhibitors relies on the presence of PARP1 and its trapping on DNA (Murai et al. 2012). Experiments examining the role of PARP1 in chromatin structure relaxation following DNA damage have also shown that when we block PARP1 activity with specific PARP inhibitors, chromatin structure not only does not relax but becomes more compact. The compaction of chromatin under the influence of PARP inhibitors is not observed in the absence of the PARP1 enzyme (Sellou et al. 2016).

Since the therapeutic and chromatin-regulatory effects of PARP inhibitors operate through the same molecular mechanism, we conducted a full-genome screening using CRISPR gene knockout to investigate which genes are involved in resistance/sensitivity to PARP inhibitors and chromatin structure regulation. ALC1 was one of the genes whose absence increased sensitivity to PARP inhibitors. Our work has shown that the lack of ALC1 greatly increases the sensitivity of both normal and BRCA1/2-deficient cells to PARP inhibitors (Juhász et al. 2020). Moreover, overexpression of ALC1 enhances resistance to PARP inhibitors. ALC1 is an oncogene and is often amplified or overexpressed in malignant tumors (Wu et al. 2014; He et al. 2012; M. Chen et al. 2009; L. Chen et al. 2009; 2010). This suggests that ALC1 inhibitors may have therapeutic significance. It's not surprising that there have been recent indications and

publications characterizing the first ALC1 inhibitors (Abbott et al. 2020; Prigaro et al. 2022). Our study has been published in *Science Advances* in 2020.

Our experiments have also revealed that ALC1 plays an important role in mobilizing PARP1, thus reducing the toxic amount of PARP1 trapped on DNA (Juhász et al. 2020). As mentioned earlier, ALC1 is a chromatin remodeling enzyme activated by PAR. Its C-terminal PAR-binding macrodomain binds PARylated proteins, becomes activated, and its N-terminal SNF2-type ATPase binds DNA, moving it by hydrolyzing ATP (Gottschalk et al. 2009). Both PARP1 and nucleosomes – among other proteins – are PARylated at the site of DNA damage. Therefore, it can move PARylated nucleosomes, for example, by nucleosome sliding. ALC1 may be capable of moving either PARylated PARP1 or PARylated nucleosomes. Our experiments indicate that the latter mechanism also plays a role in mobilizing PARP1, but these experiments do not provide an answer to whether this is the exclusive mechanism. The displaced nucleosome indirectly assists in the removal of PARP1 by, for example, pushing it aside or helping another protein compete for binding with the PARP1 protein. As a result, several proteins involved in DNA repair that we have studied cannot properly bind to the damaged DNA segment (Smith et al. 2019).

We continued this line of research on the role of ADP-ribosylation in chromatin regulation, specifically focusing on the detailed characterization of HPF1. Recent research has revealed the critical role of HPF1 in targeting ADPr chains to specific residues (Bonfiglio et al. 2017; Gibbs-Seymour et al. 2016). HPF1 binds to the C terminus of PARP1, forming a joint catalytic site essential for ADP-ribosylating serines, the primary residues modified by ADPr in the context of the DNA damage response. Consequently, the absence of HPF1 has several implications, including a significant reduction in PARP1 automodification and the inhibition of trans-ADP-ribosylation of histones. Furthermore, *in vitro* studies have shown that HPF1 not only guides ADPr to specific residues but also influences the rate of polymerization, favoring mono-ADPr modifications over poly-ADPr chains. Nevertheless, the precise impact of HPF1 on cellular functions known to be regulated by PARP1 remains somewhat elusive. There are indications of HPF1 playing a role in DNA repair, as its deficiency leads to cellular hypersensitivity to DNA-damaging agents. Our results show that HPF1, and through it, the PARylation of nucleosomes, is essential for chromatin relaxation, and PARP1 PARylation does not play a role in this process (Smith et al. 2023). We found that HPF1 plays a crucial role in controlling both the quantity and length of ADPr chains at damaged sites. We also established that HPF1-dependent histone ADP-ribosylation, rather than PARP1 automodification, significantly contributed to the initial chromatin relaxation at DNA lesions facilitating access to damaged DNA for repair factors. The manuscript presenting our results has been published in *Nature Structural and Molecular Biology* in 2023.

Mono-ADP-ribosylation shapes the DNA damage response

Although recent tools have significantly advanced research on ADP-ribosylation, we still lag behind in our ability to study this elusive post-translational modification compared to more established PTMs like phosphorylation and ubiquitination. These mature PTMs have had decades of tool development, while ADP-ribosylation is still catching up.

We collaborated with the team of Ivan Matic at MPI for Ageing in Köln who use state-of-the-art, recombinant mono-ADP-ribose-binding antibodies combined with mass-spectrometry. We shed light on the role of serine mono-ADP-ribosylation as the second wave of PARP1 signaling in the context of DNA damage. PAR, with its immediate formation and

substantial size, serves as one of the earliest signals produced during the DNA damage response. However, due to its inherent toxicity, PAR is not suitable as a long-lasting signal.

If PAR were the sole signal, the reach of PARP1 signaling would be constrained by the transient nature of PAR. Instead, we proposed that serine mono-ADP-ribose extends the scope of PARP1 signaling, providing a second, enduring post-translational modification (PTM) that regulates biological processes over an extended period (Longarini et al. 2023). The persistence of mono-ADPr and the transient nature of PAR clarify recent puzzling observations where mono-ADP-r appears more abundant than PAR in cells following DNA damage. The dynamic modulation of the PARP1/HPF1 ratio within the chromatin environment serves as the molecular basis for PARP1's dual role as a PAR and mono-ADP-ribose transferase. It acts as a cellular mechanism to regulate levels of chromatin mono-ADP-ribosylation. By identifying the readers of chromatin mono-ADPr, we illustrated how this two-speed signaling pathway operates in recruiting proteins to DNA damage sites. The recruitment of PAR readers is immediate and mostly temporary, whereas the assembly of a mono-ADP-ribose reader, exemplified by RNF114, is gradual and long-lasting. This collaborative study was published in *Molecular Cell* in 2023.

Conclusions

In summary, our investigations into ADP-ribosylation continue to uncover the intricate web of regulatory mechanisms that govern cellular processes. From EGFR regulation and thymidine-linked ADP-ribosylation to the PARP1/HPF1 axis and the role of ALC1 in chromatin structure, our research underscores the pivotal role of ADP-ribosylation in shaping the molecular landscape of cellular physiology and pathology. Through our work, we anticipate uncovering potential new therapeutic avenues, particularly in precision medicine, and a deeper understanding of ADP-ribosylation's impact in gene expression regulation.

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