

Assessing the interplay between the mono-ADP-ribosyltransferase PARP10 and the chikungunya viral macrodomain in modulating the antiviral immune response

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vorgelegt von

Master of Science
Maud Verheirstraeten

Aus Gent

Berichter: Univ.-Prof. Dr. rer. nat. Bernhard Lüscher
Univ.-Prof Dr. med. Mathias Hornef

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Abstract

Chikungunya virus (CHIKV) is a positive single stranded RNA ((+)ssRNA) alphavirus, which causes disease outbreaks worldwide. Currently there is no vaccine or specific treatment for CHIKV infection, in part, due to the lack of understanding of CHIKV replication and host-pathogen interactions. The CHIKV genome encodes four non-structural proteins (nsP1-4). NsP3 contains a macrodomain with mono-ADP-ribosylhydrolase activity capable of removing mono-ADP-ribosylation (MARylation). MARylation is a posttranslational modification (PTM) catalyzed intracellularly by mono-ADP-ribosyltransferases diphtheria toxin-like (mARTDs). Several mARTDs, PARP7/9-12/14-15, are induced by type I interferons (IFN α and IFN β) and pathogen-associated molecular patterns (PAMPs). Our lab previously identified PARP10 and PARP12 as host factors restricting CHIKV replication. Also, PARP14 and PARP15 are linked to affecting viral replication. The hydrolytic activity of nsP3 is essential to CHIKV replication. Without a functional hydrolase, CHIKV is unable to counter IFN-I induced MARylation. Potentially, CHIKV requires the removal of MARylation from host proteins to either promote viral replication or counteract antiviral activity and the innate immune response.

In this dissertation, we aimed at identifying host factors regulated by MARylation during viral infection. To this end, we mapped the interactome of both PARP10 and nsP3 using BioID in combination with mass spectrometry analysis. For both enzymes a diverse number of interactors were successfully identified. An overlap of the 10-fold enriched host factors in both interactomes resulted in the identification of 136 proteins. These host factors are involved in processes such as mRNA processing, cell migration, stress granule assembly, cell cycle and cytoskeleton regulation. Through the combination of *in vitro* ADP-ribosylation assays and cell interaction assays, the specific host proteins will be analyzed as potential interactors and substrates of both PARP10 and nsP3. Besides, their effect on CHIKV replication will indicate them as pro- or antiviral proteins.

One of the identified proteins is Ras-GAP SH3 domain-binding protein (G3BP1). G3BP1 and G3BP2 are essential stress granule proteins, which have been described to interact with nsP3. G3BP dKO is detrimental to CHIKV replication. Interaction with PARP10 has not been reported. Co-immunoprecipitation experiments (co-IPs) confirmed G3BP1 as a common interactor of both nsP3 and PARP10. Furthermore, *in vitro* ADP-ribosylation and hydrolase assays identified G3BP1 as a substrate for the opposing activities of IFN-inducible mARTDs and CHIKV nsP3. Intracellular G3BP1 MARylation could be shown but remains less conclusive. Further, we confirmed that G3BP1-RBD is modified. However, so far there is no clear function for G3BP1-RBD MARylation to innate immunity and CHIKV replication. Through confocal microscopy, we studied G3BP1 and mARTDs localization. Lastly, we started to develop an assay to analyze the effect of PARP expression and CHIKV infection on the G3BP1 interactome.

Previously our lab identified the CHIKV nsP1-3 as substrates for MARylation *in vitro*. In this dissertation, nsP1 was further studied as a substrate of mARTDs. The catalytic domains of PARP10, PARP14 and PARP15 modified nsP1 *in vitro* and the MARylation was efficiently reversed by the hydrolytic activity of nsP3. Even though co-IPs showed interaction between PARP10 and nsP1 in cells, microscopy studies did not reveal co-localization and there was no evidence for in cell MARylation of nsP1. Through homology studies of other alphaviruses, CHIKV nsP1 is believed to catalyze viral RNA capping. We could show CHIKV nsP1 capping activity and membrane interaction. We aim at further investigating CHIKV nsP1 MARylation in cells and defining the consequences of MARylation for CHIKV nsP1's molecular functions specifically and CHIKV replication overall.

Das Chikungunya-Virus (CHIKV) ist ein positives einzelsträngiges RNA ((+)ssRNA) Alphavirus, das weltweit Krankheitsausbrüche verursacht. Derzeit gibt es weder einen Impfstoff noch eine spezifische Behandlung für CHIKV-Infektionen. Das CHIKV-Genom kodiert für vier nicht-strukturelle Proteine (nsP1-4). NsP3 enthält eine Makrodomäne mit Mono-ADP-Ribosylhydrolase-Aktivität, die in der Lage ist, Mono-ADP-Ribosylierung (MARylierung) zu entfernen. Die MARylierung ist eine posttranslationale Modifikation (PTM), die intrazellulär von mono-ADP-ribosyltransferasen Diphtherietoxin-ähnlich (mARTDs), katalysiert wird. Mehrere mARTDs, PARP7/9-12/14-15, werden durch Typ I Interferone (IFN α und IFN β) und pathogen-assoziierte molekulare Muster (PAMPs) induziert. Unser Labor hat bereits PARP10 und PARP12 als Wirtsfaktoren identifiziert, die die CHIKV-Replikation einschränken. Auch PARP14 und PARP15 werden mit einer Beeinträchtigung der viralen Replikation in Verbindung gebracht. Die hydrolytische Aktivität von nsP3 ist für die CHIKV-Replikation unerlässlich und ohne eine funktionsfähige Hydrolase ist CHIKV nicht in der Lage, der IFN-I-induzierten MARylierung entgegenzuwirken. Möglicherweise ist die Entfernung der MARylierung von Wirtsproteinen für CHIKV nötig, um entweder die virale Replikation zu fördern oder der antiviralen Aktivität und der angeborenen Immunantwort entgegenzuwirken. In dieser Dissertation haben wir versucht, Wirtsfaktoren zu identifizieren, die während einer Virusinfektion durch MARylierung reguliert werden. Hierfür haben wir das Interaktom von PARP10 und nsP3 mit Hilfe von BioID in Kombination mit Massenspektrometrieanalysen untersucht. Ein Vergleich der 10-fach angereicherten Wirtsfaktoren in beiden Interaktomen führte zur Identifizierung von 136 Proteinen. Diese Wirtsfaktoren sind an Prozessen wie der mRNA-Prozessierung, der Zellmigration, dem Aufbau von Stressgranula, der Zellzyklus und des Zytoskeletts beteiligt. Durch *in vitro* ADP-Ribosylierungs-Analysen und Co-Immunopräzipitationsstudien (Co-IPs) wurden die Proteinen als potenzielle Interaktoren und Substrate von PARP10 und nsP3 analysiert. Eines der identifizierten Proteine ist das Ras-GAP SH3 Domain-bindende Protein 1 (G3BP1). G3BP1 und G3BP2 sind essentielle Stressgranulaproteine, die mit nsP3 interagieren. Ein Fehlen von G3BP ist schädlich für die CHIKV-Replikation. Bisher wurde keine Interaktion zwischen G3BP und PARP10 veröffentlicht. Co-IPs bestätigten G3BP1 als gemeinsamen Interaktor von nsP3 und PARP10. Darüber hinaus identifizierten *in vitro* ADP-Ribosylierungs- und Hydrolase-Assays G3BP1 als Substrat für die IFN-induzierbaren mARTDs und CHIKV nsP3. Eine intrazelluläre MARylierung von G3BP1 konnte zwar nachgewiesen werden, jedoch war diese weniger eindeutig. Außerdem konnten wir bestätigen, dass die RNA-bindende Domäne (RBD) von G3BP1 modifiziert wird. Bisher gibt es jedoch keine eindeutige Funktion der G3BP1-RBD MARylierung für die angeborene Immunität und die CHIKV-Replikation. Mittels konfokaler Mikroskopie haben wir die Lokalisierung von G3BP1 und mARTDs untersucht. Abschließend begannen wir mit der Entwicklung eines Assays zur Analyse der Auswirkungen der PARP-Expression und der CHIKV-Infektion auf das G3BP1-Interaktom. Unser Labor hat bereits die CHIKV nsP1-3 als Substrate für die MARylierung *in vitro* identifiziert. In dieser Dissertation wurde nsP1 tiefergehend als Substrat von mARTDs untersucht. Die katalytischen Domänen von PARP10, PARP14 und PARP15 modifizierten nsP1 *in vitro* und die MARylierung wurde durch die hydrolytische Aktivität von nsP3 effizient rückgängig gemacht. Obwohl Co-IPs eine Interaktion zwischen PARP10 und nsP1 in Zellen zeigten, ergaben Mikroskopiestudien keine Co-Lokalisierung und es gab keine Hinweise auf eine MARylierung von nsP1 in Zellen. Aufgrund von Homologiestudien anderer Alphaviren wird angenommen, dass CHIKV nsP1 das virale RNA-Capping katalysiert. Wir konnten die Capping-Aktivität von CHIKV nsP1 und seine Interaktion mit der Membran nachweisen. Unser Ziel ist es, die MARylierung von CHIKV nsP1 in Zellen weiter zu untersuchen und die Konsequenzen der MARylierung für CHIKV nsP1 im Speziellen und für die CHIKV-Replikation im Allgemeinen zu definieren.

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1. Introduction

1.1. ADP-ribosylation

Adenosine diphosphate (ADP)-ribosylation is a post-translational modification (PTM), in which an ADP-ribose (ADPr) unit is transferred from nicotinamide adenine dinucleotide (NAD^+) onto a substrate protein with release of nicotinamide (Nam) (**Fig. 1**) (Lüscher et al., 2018, 2022). ADP-ribosylation occurs in two manners, mono-ADP-ribosylation (MARylation) and poly-ADP-ribosylation (PARylation). MARylation is restricted to the transfer of a single ADPr-moiety onto a substrate, while PARylation describes the iterative process in which the initial ADPr-unit is extended into a (linear or branched) poly-ADP-ribose (PAR)-polymer (**Fig. 1**). The two different forms of ADP-ribosylation are associated with different cellular processes. PARylation is the better studied form and known for its role in DNA-damage. PARylation is linked to DNA repair, chromatin organization, telomer maintenance, signaling processes and protein degradation. MARylation is less well-known but in recent years has become the focus in the field. In comparison to PARylation, MARylation is highly associated with innate immunity, stress response, and RNA metabolism (Bütepage et al., 2015; Lüscher et al., 2018, 2022). ADP-ribosylation is mainly catalyzed intracellularly by the ADP-ribosyltransferase Diphtheria toxin-like (ARTDs) family, which includes the PARP and tankyrase (TNKS) proteins. The PTM is fully reversible, and this process is catalyzed by ADP-ribosylhydrolases (Bütepage et al., 2015; Lüscher et al., 2018).

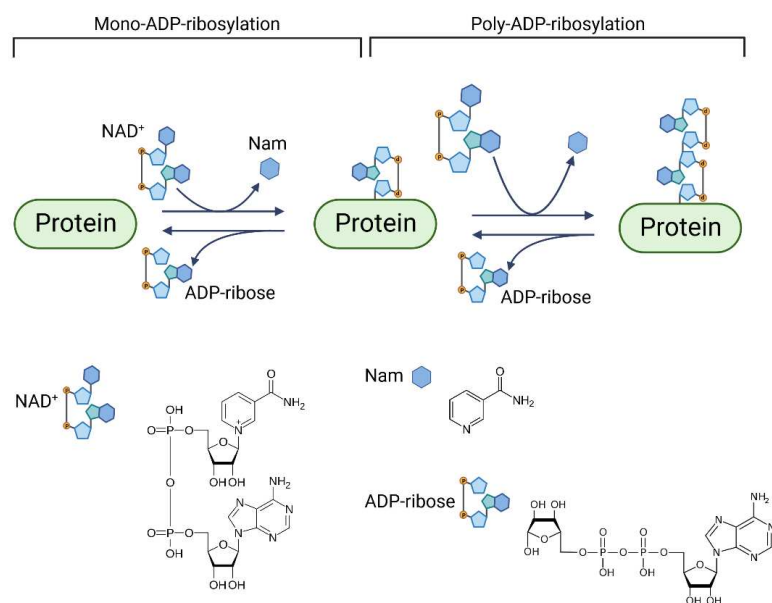


Figure 1. ADP-ribosylation of proteins. Schematic overview of mono-ADP-ribosylation (MARylation) and poly-ADP-ribosylation (PARylation). In MARylation, a single ADP-ribose (ADPr) unit is transferred from nicotinamide adenine dinucleotide (NAD^+) onto a substrate protein with release of nicotinamide (Nam). Subsequently, PARylation is the iterative process in which the initial ADPr-unit is extended into poly-ADP-ribose-polymer. Created with BioRender.com

1.1.1. ADP-ribosylation writers

ADP-ribosylation is a highly conserved PTM. Regulating enzymes have been found in archaea, prokaryotes, eukaryotes, and viruses (Lüscher et al., 2018). In eukaryotes, the two main protein families responsible for ADP-ribosylation are the intracellular ARTDs (**Fig. 2**) and the extracellular ADP-ribosyltransferase Cholera toxin-like ecto-enzymes (ARTCs). The human ADP-ribosyltransferase (ART) superfamily is made-up of 22 proteins. The ARTD-family consists of 17 proteins and the ARTC-family of 4 proteins (Bütepage et al., 2015; Lüscher et al., 2018, 2022). This dissertation focuses on the ARTDs, more specifically mono-ARTDs (mARTDs) capable of MARylation. However, I will shortly discuss poly-ARTDs (pARTDs) too. ARTDs are divided into 3 subclasses. Firstly, enzymes capable of PARylation,

pARTDs: PARP1, PARP2, PARP5/TNKS1 and PARP6/TNKS2 (Lüscher et al., 2018). Secondly, enzymes that catalyze MArlylation, mARTDs: PARP3-4; 7-12; 14-16. Lastly, the third class, classified by inactive enzymes, consists solely of PARP13 (Karlberg et al., 2015). So far, no catalytic activity has been observed for this protein.

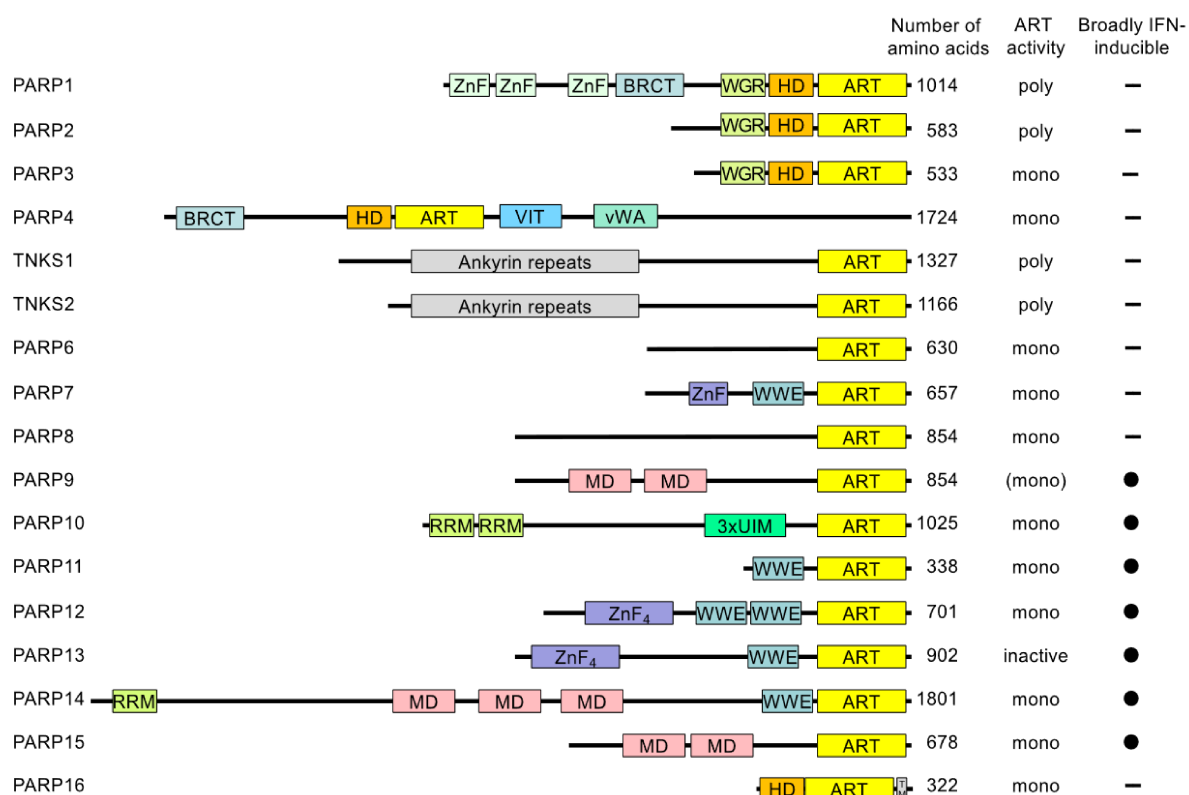


Figure 2. Schematic overview of the eukaryotic, intracellular ADP-ribosyltransferases. ADP-ribosylation is a fully reversible post-translational modification (PTM). The eukaryotic, intracellular ADP-ribosylation writers capable of either mono-ADP-ribosylation or poly-ADP-ribosylation belong to the ADP-ribosyltransferase Diphtheria toxin-like (ARTDs) family. The ARTDs share a highly conserved catalytic ART domain. For every ARTD the domain architecture, function and interferon (IFN) response is listed. ART: ADP-ribosyltransferase domain; BRCT: BRCA1 C-terminus domain; HD: helical domain; MD: macrodomain; RRM: RNA-recognition motif; SAM: sterile alpha motif; TM: transmembrane motif; UIM: ubiquitin-interaction motif; vWA: von Willebrand factor type A domain; VIT: vault protein inter- α -trypsin domain; WGR: conserved Trp-Gly-Arg motif domain; WWE: three conserved residues Trp-Trp-Glu motif domain; ZnF: Zinc finger (adapted from Lüscher et al., 2022).

1.1.1.1. PARylation

The pARTDs are shortly introduced. In this dissertation, the emphasis lies on the role of mARTDs in innate immunity. For this reason, PARylation is not discussed in detail. For further reading, the following reviews are suggested (Fehr et al., 2020; Lüscher et al., 2018).

PARP1 and PARP2 play crucial roles in various cellular processes such as base excision repair (BER), chromatin remodeling, and transcriptional shutdown in response to DNA damage. PARP1 is the best studied enzyme of the ARTD family. It dominated research for many years, before the discovery of other family members. PARP1 contains the three amino acids (AA) making up the catalytic center (H-Y-E). All ARTD members have the conserved ART domain with this AA triad or a close variant depending on the function of the ARTD family members (Lüscher et al., 2018). PARP1 is best known for its role in DNA damage repair. Upon DNA damage, single or double strand breaks (SSBs and DSBs) are bound by PARP1, which results in its activation. PARP1-mediated PARylation recruits DNA damage repair factors and chromatin remodelers (Ahel et al., 2009; Lüscher et al., 2018; Thompson,

2012). PARP1 PARylation also results in DNA decondensation (Lüscher et al., 2018; Shall & de Murcia, 2000; Strickfaden et al., 2016; Tallis et al., 2014). PARP2 cooperates with PARP1 in DNA damage repair and their functions seem to be partially redundant (Lüscher et al., 2018; Ménissier de Murcia et al., 2003).

TNKS1 and TNKS2 are closely related proteins. They are also partially redundant but display some different functions. They localize both in the nucleus and the cytoplasm and have also been described to localize to centrosomes and stress granules (Hsiao & Smith, 2008; Leung et al., 2011; Lüscher et al., 2018). TNKS1 is involved in telomere maintenance and plays a role in metaphase progression (Bhardwaj et al., 2017; Hsiao & Smith, 2008; Slade, 2019). TNKS1-2 have also been linked to ubiquitination and proteasomal degradation (Bhardwaj et al., 2017; Callow et al., 2011; Lüscher et al., 2018).

1.1.1.2. MARYlation in eukaryotes

MARYlation is, in comparison to PARylation, less studied. Even though 12 of the 17 ARTD family members are MARYlating enzymes (**Fig. 2**). Tools to study MARYlation have been weak in the past. However, recently more methods and tools are being developed, our knowledge is increasing and MARYlation is coming forward as an important player in innate immunity and stress responses (Fehr et al., 2020; Lüscher et al., 2018, 2022). Besides, MARYlation has been linked to nuclear processes such as DNA damage repair, transcription and mitosis and cytoplasmic processes such as signal transduction, condensate formation and mRNA metabolism (Lüscher et al., 2018). The catalytic triad of AA looks different between the mARTDs. Both PARP3 and PARP4 have the H-Y-E triad. However, most of the other enzymes possess a variant of the motif, in which the E is replaced by another AA: H-Y-I/L/Y/T (Lüscher et al., 2018; Vyas & Chang, 2014). In PARP9, the H is substituted for a Q and in PARP13, the H for a Y. The H is important for binding of the cofactor NAD⁺, therefore these two enzymes were both classified as inactive (Bütepage et al., 2015; Kleine et al., 2008). However, for PARP9 a study argued for MARYlation activity in complex with the E3 ubiquitin ligase DTX3L (Yang et al., 2017). This is still under discussion, since a more recent study suggested that DTX3L is the active enzyme in this complex (Chatrin et al., 2020). So far, PARP13 has been classified as inactive (Karlberg et al., 2015). Of note, however, is that the long PARP13 isoform, which contains the ART domain, is mainly linked to antiviral activity. So, the possibility for either activity or another role for the ART domain should not be dispelled.

1.1.1.2.1 mARTDs and the immune system

Interferons (IFNs) are known to regulate hundreds of genes, probably many more. For this to work, a precise control is necessary, since a deficiency is linked to a limited or inefficient defence against pathogens, and an over-activation to autoimmune diseases (Coroadinha et al., 2022; J. Zhao et al., 2022). Many interferon-induced genes (ISGs) encode proteins with antiviral activities. Among these IFN-induced genes, several ARTDs reside. Early on, PARP13 expression was found to be stimulated by type I IFNs (Lüscher et al., 2018; Ryman et al., 2005). PARP9 was shown to respond to IFNs just as its co-enzyme DTX3L. PARP9 and DTX3L are regulated by the same promotor. Also, both genes are expressed in cells with a dominant active STAT1 (Zhang et al., 2015). The enzymes were argued to work together to further activate ISGs (Fehr et al., 2020; Lüscher et al., 2022). IFN α treatment of both HeLa and U2OS cells induces PARP10 and PARP14 expression (Eckei et al., 2017). Upon infection of human monocytes with *Borrelia burgdorferi*, spirochetes that stimulate an IFN response, various ISGs are activated. Among these ISGs are PARP10, PARP12, and PARP14 (Salazar et al., 2009). Infection with a murine coronavirus (CoV) or Severe acute respiratory syndrome CoV type 2 (SARS-CoV-2) leads to the increase and upregulation of mARTDs (Grunewald et al., 2019; Heer et al., 2020). Upon infection with Mouse Hepatitis Virus (MHV), transcription of PARP7, PARP9, PARP10, PARP11, PARP12, PARP13, and PARP14 is highly induced in

murine bone marrow-derived macrophages (BMDMs) in an IFN-dependent way (Grunewald et al., 2019). Heer et al. analyzed whether SARS-CoV-2 induces ADP-ribosylation associated genes in different cell types. In Calu-3 cells *PARP7*, *PARP9*, *PARP10*, *PARP12*, and *PARP14* were induced more than 4-fold. *PARP3*, *PARP4*, *PARP5a*, *PARP5b*, and *PARP8* were induced to a lesser extent. In normal human bronchial epithelial cells, transcription of *PARP9*, *PARP12*, and *PARP14* was induced and in A549 cells, *PARP9*, *PARP10*, *PARP12*, *PARP14* and to a smaller degree *PARP7* and *PARP13* were induced (Heer et al., 2020). Infection of organoids resulted in similar findings (Heer et al., 2020). Shaw et al. performed a study in which they compared ISGs between different species (9 mammalian species and chicken) to pinpoint the genes that commonly play a part in innate immunity. Interestingly, they found that many of the *PARPs* are responsive to type I IFN in all or multiple of the studied species. Activated in all the studied species were *PARP9*, *PARP10*, *PARP12*, *PARP13* and *PARP14*, often more than 10-fold. In comparison, *PARP11* and *PARP15* were activated in multiple but not in all species; *PARP3*, *PARP4*, *PARP7*, *PARP8*, and *PARP16* were stimulated less efficiently (Shaw et al., 2017). It is striking that these mARTDs, and *PARP13*, are induced together with known antiviral factors, pathogen-associated molecular patterns (PAMP) sensors and important signaling molecules. The PARylating enzymes *PARP1*, *PARP2*, *TNKS1* and *TNKS2* do not seem to be induced by IFN I treatment. Also, ADP-ribosylhydrolases, capable of reversing ADP-ribosylation were not induced in response to type I IFN. Together pointing to a role specifically for MARYlation in innate immunity.

Besides IFNs, ARTDs have also been reported to be regulated by PAMPs. Lipopolysaccharide (LPS), thus Toll-like receptor 4 (TLR4), promotes the expression of *PARP3*, 4, 7-14 (Caprara et al., 2018; Curina et al., 2017; Ecke et al., 2017). TLR1, 2, 3, and 9 stimulate *PARP14* expression (Caprara et al., 2018). The transcription factor (TF) Interferon Regulatory Factor 1 (IRF1) induces *PARP9*, *PARP10*, *PARP12* and *PARP14* expression (Schoggins et al., 2011). Certain PAMP signals lead to the activation of TF IRF1 (Honda et al., 2006). Both the different *PARPs* and IRF1 expression has been shown to interfere with viral replication (Schoggins et al., 2011). Whether they are mechanistically linked needs further study.

As proven by multiple studies, there is a clear link between innate immune signaling and the expression of MARYlating *PARP* genes (Lüscher et al., 2022). Overall, mARTDs are associated with innate immunity. mARTDs are upregulated upon IFN and PAMPs, while hydrolase enzymes are not affected. This indicates MARYlation as an antiviral function. Below, I will discuss the IFN-induced mARTDs of importance for this study in more detail.

1.1.1.2.2. *PARP10*

PARP10 is 1025 AA, has two RNA recognition motifs (RRM), a glycine-rich region, a glutamate-rich region, nuclear import and export signals, three ubiquitin-interacting motifs (UIMs), a catalytic ART domain and a PCNA interaction peptide box (**Fig. 2**) (Kaufmann et al., 2014; Lüscher et al., 2018; Verheugd et al., 2013; Bjaesch, Knapp et al., 2023). *PARP10* is suggested to modify acidic amino acids (Kleine et al., 2008; Suskiewicz et al., 2021). *PARP10* was identified as an interaction partner of the oncoprotein MYC in the nucleus (Kleine et al., 2008; Yu et al., 2005). By now, *PARP10* has been linked to processes involved in cancer, neurodegenerative disorders, innate immunity, cell cycle and many more (Kaufmann et al., 2014). The enzyme shuttles between the cytosol and the nucleus, nuclear export due to the chromosomal region maintenance 1 (CRM1)-dependent nuclear export sequence and a so far unknown import signal (Kleine et al., 2012). *PARP10* has thus interaction partners and potential substrates in both the nucleus and the cytosol (Feijs et al., 2013; Carter-O'Connell et al., 2016). However, *PARP10* is mostly found in the cytoplasm upon basal conditions (Kleine et al., 2012). In the cytoplasm, *PARP10* forms condensates independent of catalytic activity, although *PARP10* WT condensates seem to be more liquid compared to an inactive mutant such as *PARP10*-GW, which forms aberrant-looking condensates. These *PARP10*

condensates are neither stress granules, P-bodies or any other known condensates but colocalize with ubiquitin, autophagy adapter and ubiquitin receptor p62, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Forst et al., 2013a; Kleine et al., 2012; Mayo et al., 2018). GAPDH is another MAR substrate. In HeLa cells PARP10 inhibits cell proliferation dependent on its catalytic activity, and HeLa cells stably expressing PARP10 go into apoptosis (Herzog et al., 2013; Kleine et al., 2008). PARP10 regulates the nuclear factor 'κ-light-chain-enhancer' of activated B-cells (NF-κB) signaling pathway through its UIMs and catalytic activity. PARP10 UIM1 and UIM2 interact with K63-linked Ubiquitin chains and NF-κB essential modulator (NEMO). NEMO was identified as a PARP10 substrate and is MARYlated. This PTM prevents NEMO poly-ubiquitination, which results in NF-κB translocation inhibition to the nucleus to activate gene expression (Verheugd et al., 2013). Also, PARP10 MARYlates glycogen synthase kinase-3 β (GSK3β) and blocks GSK3β kinase activity in vitro (Feijs et al., 2013). GSK3β plays a pivotal role in various biological processes, including WNT signaling, apoptosis, metabolism, neuronal development, immunity, and tumorigenesis (Souder & Anderson, 2019). Other kinases were also suggested as potential PARP10 substrates, resulting in inhibition of the enzyme's kinase activity, for example protein kinase C delta (PKCδ), Aurora A and polo-like kinase 1 (PLK1) (Feijs et al., 2013; Lu et al., 2019; Zhao et al., 2018). This could indicate a more common mechanism by which MARYlation controls cell signaling. Carter-O'Connell et al. set out to identify protein-protein interactions (PPIs) for multiple PARPs. For PARP10, interactors involved in mRNA regulation and ubiquitination were identified (Carter-O'Connell et al., 2016). Deficiency of PARP10 has been linked to developmental delays and DNA repair defects (Shahrour et al., 2016). Additionally, PARP10 is involved in mitochondrial function. Downregulation of PARP10 has been observed to induce glycolysis and mitochondrial fatty acid oxidation (Márton et al., 2018). Besides, the two macrodomains of murine PARP14 interact with MARYlated PARP10 (Bütepage et al., 2018; Forst et al., 2013) indicating that the mARTDs could function in combination and work together as a network.

PARP10 is (highly) upregulated by IFN (Eckei et al., 2017; Grunewald et al., 2019). Generally, PARP10 seems to play a role in the innate immune system and counteracts viral replication and cellular translation (Atasheva et al., 2014; Eckei et al., 2017). It was shown that PARP10 disrupts Venezuelan equine encephalitis virus (VEEV) replication (Atasheva et al., 2012, 2014). The nonstructural protein 1 (NS1) of avian influenza virus interacts with PARP10 and induces PARP10 translocation from the cytoplasm to the nucleus. A high NS1 expression reduces PARP10 expression (Yu et al., 2011). Our lab showed that PARP10 overexpression restricted Chikungunya virus (CHIKV) replication, while PARP10 knockdown (KD) enhanced CHIKV replication (Krieg et al., 2023). CHIKV nonstructural protein 2 (nsP2) is the protease that processes the polyprotein, as discussed in detail below (**chapter 1.5.4.**). This protease activity is essential for CHIKV replication (Frolov & Frolova, 2018). PARP10 MARYlates nsP2 and the isolated nsP2 protease domain, and this PTM inhibits protease activity (Krieg et al., 2023). Upon incubation with the nsP3 macrodomain, capable of removing this modification, protease activity is active again, indicating that PARP10 inhibits viral activity through MARYlation (Krieg et al., 2023).

1.1.1.2.3. PARP12

PARP12 has multiple domains (**Fig.2**). It has four Zinc finger (ZnF) domains, 2 WWE domains and its catalytic domain. ZnFs are important for RNA-binding, while the WWE motifs can bind to ADP-ribosylation. PARP12 is very similar to PARP13, which is known as an important antiviral protein (Biaesch et al., 2023). PARP12 has been repeatedly linked to antiviral activities (Fehr et al., 2020; Lüscher et al., 2022). It has been shown that PARP12 overexpression slightly inhibits vesicular stomatitis virus (VSV) replication, a negative-strand RNA virus (Liu et al., 2012). Also, VEEV replication was reduced by PARP12, with the latter being dependent on the catalytic domain of PARP12 (Atasheva et al., 2012, 2014). Besides,

PARP12 restricts Sindbis virus (SINV), CHIKV and encephalomyocarditis virus (ECMV) replication (Fehr et al., 2020; Krieg et al., 2023; Lüscher et al., 2022). PARP12 is sufficient for inhibition of Zika virus (ZIKV) replication (Li et al., 2018). The enzyme has been found to interfere with the replication of ZIKV, with its activity leading to the degradation of two ZIKV non-structural proteins, NS1 and NS3 (Li et al., 2018). MARYlation activity was essential for the degradation of the viral proteins, the ZnF or WWE domains of PARP were not. PARP12 induces proteasomal degradation of the viral proteins, which appears to be mediated by PARylation. Since PARP12 has MARYlation activity, a PAR enzyme will probably cooperate in this process (Li et al., 2018). Later, PARP12 was shown to work together in this process with PARP11 (Li et al., 2021). PARP12 also seems to restrict CoV replication. Grunewald et al. studied the effect of PARPs on mouse hepatitis virus (MHV) infection, a murine CoV (Grunewald et al., 2019). Interestingly, only clear effects were obtained with a mutant MHV strain, in which the nsP3 macrodomain was mutated. This MHV mutant strain was sensitive to 3-amino benzamide (3-AB), a broadly acting, low affinity PARP inhibitor (PARPi). Diminished replication of the mutant MHV lacking the active macrodomain, was negated by an additional knockdown of PARP12 (Grunewald et al., 2019). Lastly and of import to this work, our lab has found that PARP12, similar to PARP10, inhibits the replication of CHIKV (Krieg et al., 2023). Together, these findings suggest that PARP12 has antiviral activities. Besides, PARP12 plays a role in controlling microRNA (miRNA) function with PARP13 (Leung et al., 2011).

PARP12 is associated with the stress response and with stress granules (SGs), which are important in controlling translation, mRNA stability, and viral replication. PARP12 has been shown to translocate towards SGs upon cellular stress (Catara et al., 2017; Leung et al., 2011; Welsby et al., 2014). Under normal conditions, PARP12 localizes to the trans-Golgi network (TGN). This translocation is argued to be due to its ZnF domains (Welsby et al., 2014). Mutations of these domains resulted in the suppression of the translocation to SGs (Welsby et al., 2014). Also, the WWE-domains were implicated in SG recruitment. Catara et al. suggested that upon stress, PARP1 acts as a stress sensor and produces PAR. PARP12 binds to this PAR and translocates to SGs. This mechanism is discussed in more detail below (**chapter 1.4.3.**) (Catara et al., 2017).

1.1.1.2.4. PARP14

PARP14 has N-terminally 2 RRM domains, 3 macrodomains (MDs), a WWE domain and a MARYlation domain (**Fig. 2**). So far, PARP14 has not been very well-studied. In BMDM cells treated with LPS, gene expression is activated for several of the PARPs. Upon LPS stimulation, PARP14 translocates from the cytosol to the nucleus (Caprara et al., 2018). Besides, this translocation induces nuclear import of other IFN-induced factors, such as the other mARTDs PARP12 and PARP9/DTX3L (Caprara et al., 2018). It has been suggested that this mechanism results in the MARYlation of multiple nuclear substrates upon PAMP and IFN-signaling (Caprara et al., 2018; Lüscher et al., 2022). In BMDM, PARP14 seems to restrict CoV replication, similarly to PARP12. Again, diminished replication of the MHV strain with an unfunctional hydrolase activity was negated by an additional knockdown of PARP14 (Grunewald et al., 2019). Carter-O'Connell et al. undertook interaction screens to identify PARP14 PPIs (Carter-O'Connell et al., 2016). Multiple RNA binding proteins were identified, among them PARP13.

1.1.1.2.5. PARP15

There is not much known about PARP15. It has 2 isoforms, PARP15iso1 and PARP15iso2 (**Fig. 2**). PARP15iso1 contains an N-terminal domain, 2 MDs and its catalytic fold. Isoform 2 lacks the N-terminal domain and the first MD (Lüscher et al., 2018, 2022). The MDs of the PARP15 enzymes can bind ADP-ribose, but not cleave off the ADPr unit. We therefore refer to them as readers of MARYlation (Lüscher et al., 2018). The MDs of the PARP15 enzymes are

under strong evolutionary selection. The quick adaptation suggests a potential antiviral role (Daugherty et al., 2014). PARP15 is among the genes induced by type I IFN in many different species (Shaw et al., 2017a). PARP15iso2 has been identified in SGs. There is no information on the localization of PARP15iso1 (Leung et al., 2011).

1.1.2. ADP-ribosylation readers

Multiple proteins involved in a diverse number of processes can bind to ADPr. Many different ADPr-binding domains have been identified, which are generally referred to as ADPr-readers. The main protein domains capable of reading ADPr are discussed. Macrodomains are discussed in further detail below, due to their importance to this work (**chapter 1.1.3.2.**).

The first motif discovered to bind PAR is the PAR Binding Motif (PBM) (Bütepage et al., 2015; Grimaldi et al., 2019; Li et al., 2022). It consists of 20 amino acids and is rich in basic and hydrophobic amino acids, capable of binding the negatively charged PAR polymer. The PBM is found in many different proteins involved in processes such as DNA damage and repair, chromatin remodeling and cell cycle (Bütepage et al., 2015; Grimaldi et al., 2019; Li et al., 2022). Another ADPr-binding domain found in DNA damage responses is the poly-ADP-ribose-binding zinc-finger (PBZ) domain (Bütepage et al., 2015; Grimaldi et al., 2019; P. Li et al., 2022). The WWE domain is another important domain. It is present in multiple PARPs. The WWE domain recognizes iso-ADP-ribose in PAR but not the terminal ADPr. Again, a domain involved in recruitment towards DNA damage since many DNA damage factors possess this domain (Bütepage et al., 2015; Grimaldi et al., 2019; Li et al., 2022). As discussed later, PARP12 depends on its WWE domain to translocate to stress granules upon oxidative stress (Catara et al., 2017; Welsby et al., 2014).

Interestingly some RRM recognize PAR as well. This motif is very common in many RNA-binding proteins. Besides RNA, RRMs are able to bind to PAR due to the high similarity of the two molecules. RRM-containing proteins have been reported at DNA damage sites, and during stress granule assembly (Grimaldi et al., 2019; Teloni & Altmeyer, 2016).

The Arginine-Glycine-Glycine motif (GGG) motif is found in proteins associated with diverse processes e.g., DNA damage signaling, transcription and RNA processing. RGG boxes are present in several RNA-binding proteins and mediate protein recruitment to DNA damage sites through PAR interaction. Of interest to this work, this motif has also been found in stress granule factors (Grimaldi et al., 2019; Teloni & Altmeyer, 2016).

Other domains described to bind PAR are the WD40 domain and the Oligonucleotide/Oligosaccharide-binding fold domain (OB fold). Proteins containing WD40 domains are involved in several important cellular functions, such as cell cycle control, intracellular transport, chromatin remodeling, cytoskeletal organization and apoptosis. The OB fold is an abundant motif known to bind DNA or RNA but has also been shown to recognize PAR chains (Grimaldi et al., 2019; Lüscher et al., 2018; Teloni & Altmeyer, 2016).

1.1.3. ADP-ribosylation eraser

ADP-ribosylation is a fully reversible PTM. There are erasers capable of removing either MARYlation or PARYlation (**Fig. 3**). Of note, so far, no hydrolases have been identified that remove MARYlation from cysteines and lysines. Many eukaryotic proteins capable of hydrolysing ADP-ribosylation have been identified that are classified in three main groups: the ADP-ribosylhydrolase (ARH) family (**Fig. 3**), the nucleoside diphosphate linked to a variable moiety X (Nudix) proteins and the macrodomain-containing proteins (**Fig. 3**) (Lüscher et al., 2018). Below we will discuss macrodomains in detail, as these have been identified in some viruses and are of special importance to this thesis.

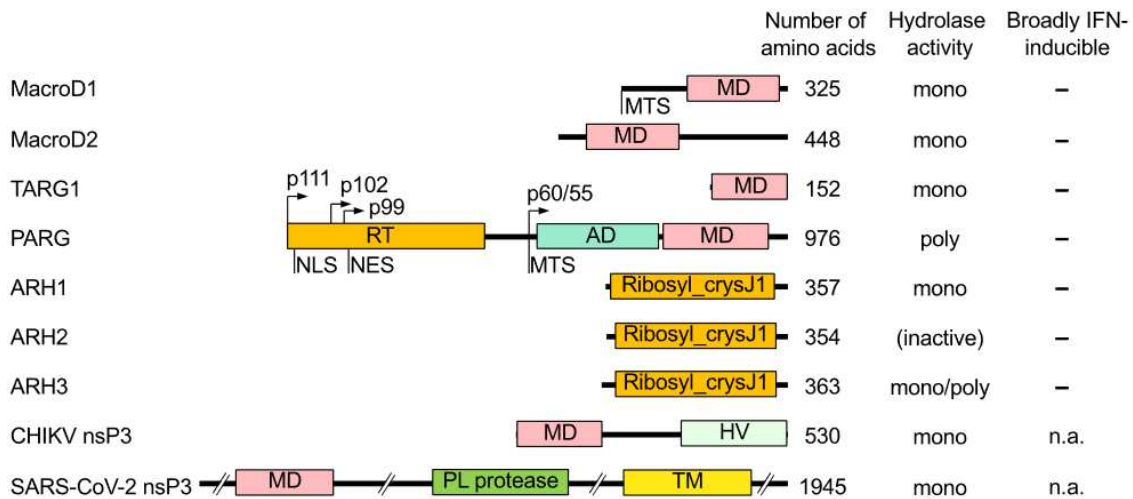


Figure 3. Schematic overview of ADP-ribosylhydrolases. ADP-ribosylation is a fully reversible post-translational modification (PTM). Several proteins with hydrolase activity have been identified. One group contains macrodomains. The eukaryotic, intracellular macrodomains are MacroD1, MacroD2, TARG1 and PARG. The first 3 possess mono-ADP-ribosylhydrolase activity and the latter poly-ADP-ribosylhydrolase activity. Many RNA viruses have been found to possess macrodomains that function as mono-ADP-ribosylhydrolases as well. Respective examples are Chikungunya virus (CHIKV) and severe acute respiratory syndrome coronavirus type 2 (SARS-Cov-2). A second group are the ADP-ribosyl-acceptor hydrolases or ADP-ribosyl-glycohydrolases (ARH1-3) which are characterized by a common Ribosyl_crysJ1 domain. For every ADP-ribosylhydrolases the domain architecture, function and interferon (IFN) response is listed. AD: accessory domain promoting MD function; HV: hypervariable domain; MD: macrodomain; MTS: mitochondrial targeting sequence; NES, nuclear export sequence; NLS, nuclear localization sequence; nsP, non-structural protein; PL protease: papain-like protease domain; Ribosyl_crysJ1: ADP-ribosylation/Crystallin J1 fold; RT: regulatory and targeting domain; TM: transmembrane; 3α: 3-helix bundle (adapted from Lüscher et al., 2022).

1.1.3.1. ARH family and Nudix proteins

One hydrolase group consists of the ADP-ribosyl-acceptor hydrolases or ADP-ribosyl-glycohydrolases (ARH1-3) (Fig. 3). These enzymes are homologous to a selenoprotein family SelJ and share a common Ribosyl_crysJ1 domain. ARH1 was the first identified enzyme capable of removing MARYlation from a protein (Moss et al., 1985). ARH1 specifically hydrolyses N-glycosidic bonds and thus removes arginine-linked MARYlation (Li et al., 2022; Lüscher et al., 2018; Mashimo et al., 2014; O’Sullivan et al., 2019). ARH2, is so far considered to be catalytically inactive (Li et al., 2022; Lüscher et al., 2018, 2022; O’Sullivan et al., 2019). ARH3 specifically hydrolyses the O-glycosidic bonds of ADPr units and thus reduces PAR chains. ARH3 is suggested to only remove the terminal ADPr unit, and thus breaks down PAR chains unit by unit. Also, ARH3 can completely reverse serine-linked ADP-ribosylation (Li et al., 2022; Lüscher et al., 2022; Mueller-Dieckmann et al., 2006; O’Sullivan et al., 2019).

The second class are the Nudix hydrolases, which have also been suggested to process ADP-ribosylation (Li et al., 2022; Lüscher et al., 2018, 2022; O’Sullivan et al., 2019). The Nudix proteins are found in all kingdoms of life and in viruses and have diverse functions. Some have pyrophosphohydrolase activity and target a diverse number of nucleoside diphosphate substrates. Certain Nudix hydrolases may process ADPr or ADP-ribosylated substrates (Li et al., 2022; Lüscher et al., 2018, 2022; O’Sullivan et al., 2019). Nudix hydrolase 5 (NUDT5) has been suggested to process free ADPr and pyrophosphate to generate adenosine triphosphate (ATP). NUDT9, NUDT16 and ectonucleotide pyrophosphatase/phosphodiesterase 1 (EnPP1), another nucleotide pyrophosphatase/phosphodiesterase but without a Nudix domain, target the phosphodiester bond in ADP-ribose. This offers promiscuous activity independent of the type of ribose linkage but does result in a remaining phosphoribose on the substrate. NUDT9 catalyzes

PARylation and NUDT16 and ENPP1 catalyze MAR and PAR (Li et al., 2022; Lüscher et al., 2018, 2022; O'Sullivan et al., 2019).

1.1.3.2. Macrodomains

1.1.3.2.1. Human Macrodomains

Again, one prominent group of hydrolases are macrodomain-containing proteins (**Fig. 3**). Some macrodomains act as readers, but many function as hydrolases. The MD fold is always linked to ADP-ribosylation and is capable of binding ADPr and derivatives (Lüscher et al., 2018; Rack et al., 2016). Some MDs hydrolyze ADP-ribosylation. Their affinity to different ADPr derivatives and thus their specificity differs between MDs (O'Sullivan et al., 2019). This is the only protein fold, identified so far, capable of binding MARylation. The macrodomain is a highly conserved fold that is found in all domains of life and viruses (Lüscher et al., 2018; Rack et al., 2016). MDs were first identified in viruses and were then called X-domains (Rack et al., 2016). Hydrolases have been found in microorganisms such as bacteria and RNA viruses. Many prokaryotic organisms encode proteins associated with ADP-ribosylation. For bacteria, there are the bacterial toxins, from which the ARTD and ARTC nomenclature was derived. The bacterial ADP-ribosylation system was even suggested as the ancestor to the eukaryotic system (Aravind et al., 2015). Rat macroH2A was the first identified mammal MD. Currently, there are twelve human proteins, by 11 genes, with macrodomains known (**Fig. 3**) (Feijs et al., 2013; Lüscher et al., 2018; O'Sullivan et al., 2019). The first identified MD structure was archeon Af1521, from *Archaeoglobus fulgidus* (Allen et al., 2003). The domains remarkable structural conservation was discovered with the study of further MD structures (Feijs et al., 2013; O'Sullivan et al., 2019). MDs are about 130-190 amino acids and consists of a central six-stranded β -sheet flanked by five α -helices. Some MDs vary slightly in the number of β -sheets or α -helices (O'Sullivan et al., 2019; Rack et al., 2016). Dependent on the MD, it functions either as a reader or eraser for either MAR or PAR. The AA sequence is quite diverse between MDs. Some MD folds were only identified after their tertiary structure was resolved, suggesting that future structural analyses may unveil numerous additional MDs (Lüscher et al., 2018; O'Sullivan et al., 2019; Rack et al., 2016). There are 6 MD subclasses: MacroD-, amplified in liver cancer (ALC)-, macroH2A-, macro2-, PARG- and SUD-M-like (Rack et al., 2016). All the MDs have a cleft that binds ADPr or ADPr derivatives and this cleft has certain conserved features. The cleft has an aromatic residue and an aspartate that coordinates the adenine base of ADPr. It also has 2 loops that stabilize the pyrophosphate and the ribose of ADPr. Loop 1 is important for substrate binding and has a conserved glycine that upon mutation abrogates ADPr binding. In the case of the MD hydrolases, other important catalytic residues reside here as well. Loop 2 is also named the diphosphate binding loop (Lüscher et al., 2018; O'Sullivan et al., 2019; Rack et al., 2016).

The human proteins with catalytically inactive MDs but able to bind ADPr or derivatives function as readers. These are macroH2A1.1, CHD1L/ALC1 and PARP9/14/15. The presence of these MDs enables the proteins to recognize the PTM and disseminate information. CHD1L/ALC1 and macroH2A1.1 bind PAR and are involved in the DNA damage response upon PARP1 activation. CHD1L/ALC1 is a chromatin remodelling factor and macroH2A1.1 induces chromatin condensation and gene repression (Feijs et al., 2013; Lüscher et al., 2018; O'Sullivan et al., 2019; Rack et al., 2016).

There are human proteins that contain a MD fold but do not bind to ADPr or derivatives, MacroH2A1.2, macroH2A2 and GDAP2 (Neuvonen & Ahola, 2009). The MacroH2A proteins are encoded by two genes. *H2AFY* encodes the two isoforms macroH2A1.1 and macroH2A1.2, and *H2AFY2* encodes macroH2A2 (Kustatscher et al., 2005). Only macroH2A1.1 can bind PAR. So far, macroH2A1.2 and macroH2A2 have not been shown to bind to ADPr-related molecules (Feijs et al., 2013; Neuvonen & Ahola, 2009). GDAP2 was described to bind poly(A) (Feijs et al., 2013; Neuvonen & Ahola, 2009).

Three PARPs, PARP9/14/15, have multiple MDs. PARP9 and 15 possess two MDs and PARP14 three. PARP9 MDs bind free ADPr, MARYlation and PARylation (Camicia et al., 2013; Karras et al., 2005; Lüscher et al., 2018; Yang et al., 2021). PARP9 macro2 is important for recruitment to DNA damage sites. Also, the PARP9 MDs are required for interaction with STAT1 and STAT2 (Camicia et al., 2013). Lastly, the PARP9 MDs recognize PARP7 mediated MARYlation of the androgen receptor (AR) (Yang et al., 2021). Of note, this illustrates interplay between different PARPs. PARP14 and PARP15 macro1 do not seem to bind ADPr or derivatives. PARP14 macro2-3 and PARP15 macro2 bind the automodified catalytic domains of PARP10, PARP12 and PARP14 *in vitro* (Ekblad et al., 2018; Forst et al., 2013). PARP14 can interact with PARP10 and is recruited to PARP10 condensates dependent on MARYlation activity (Forst et al., 2013). Also, PARP14 macro2 and macro3 interact with PARP10 substrates e.g., Ran and NEMO. Both the human and the murine PARP14 macro2-3 are used to analyse MARYlation of potential substrates (Bütepage et al., 2018; Forst et al., 2013b; Gibson & Kraus, 2017; Verheugd et al., 2013).

Human proteins with catalytically active MDs that function as erasers are MacroD1, MacroD2, TARG1 and PARG. PARG degrades PAR chains (Lüscher et al., 2018; O'Sullivan et al., 2019). In comparison to ARH3, PARG can both remove the terminal ADPr and release longer PAR chains from a substrate. However, it is incapable to cleave between the last ADPr and an amino acid side chain, and thus results in MARYlation (Lüscher et al., 2018; O'Sullivan et al., 2019). PARG has five isoforms of which three possess hydrolase activity, PARG99/102/110 (Lüscher et al., 2018; O'Sullivan et al., 2019). PARG99 and 102 localize to the cytoplasm and localize to stress granules (Jain et al., 2016; Leung et al., 2011). Their overexpression inhibits SG formation, while their KD results in a delayed SGs clearance (Leung et al., 2011). PARG110 localizes to the nucleus and is involved in DNA damage repair mechanism (Lüscher et al., 2018).

MacroD1, MacroD2 and TARG1 are the cellular MAR hydrolases (Feijs et al., 2013; Lüscher et al., 2018a; O'Sullivan et al., 2019). MacroD1 localizes to mitochondria, the nucleus and the cytoplasm and loss of MacroD1 results in abnormal mitochondrial morphology. So far, no specific function has been found (Žaja et al., 2020). In the nucleus, MacroD1 interacts with TFs and is suggested to affect transcription (Ahmed et al., 2015; Bindesbøll et al., 2016; Feijs et al., 2020). MacroD2 is poorly studied. It has been linked to cancer and autism, but no actual function or mechanism are known (Feijs et al., 2020; Lüscher et al., 2018). TARG1 shuttles between the nucleoplasm and nucleoli (Bütepage et al., 2018; Sharifi et al., 2013; Žaja et al., 2020). It hydrolyzes MARYlation and can bind PAR chains. TARG1 is associated with ribosomal proteins and RNA binding proteins. Upon DNA-damage, it interacts with DNA damage response factors through its PAR-binding ability (Bütepage et al., 2018; Lüscher et al., 2018; O'Sullivan et al., 2019).

1.1.3.2.2. Viral Macrodomains

Of interest to this work, multiple RNA viruses, such as alphaviruses and coronaviruses, possess active macrodomains (**Fig. 3**). Interestingly, MDs were even first identified in viruses (Gorbalenya et al., 1991; Li et al., 2022; Lüscher et al., 2022). In RNA viruses they are either essential to viral replication and/or seem to play a role in counteracting innate immunity. The alpha-like supergroup contains the alphaviruses, hepatitis E viruses and rubella virus. These positive-single stranded RNA viruses ((+)ssRNA) encode structurally conserved macrodomains (Egloff et al., 2006; Eriksson et al., 2008; Lüscher et al., 2022; Malet et al., 2009). The viral MDs of alphavirus, orthohopevirus, hepatitis E virus (HEV) and CoV have been shown to be functional as MAR hydrolases, indicating a potential role for counteracting IFN-induced MARYlation (Alhammad et al., 2021; Ecker et al., 2017; Fehr et al., 2015, 2016, 2020; Li et al., 2016; Lüscher et al., 2022; McPherson et al., 2017; Rack et al., 2016). Hydrolase activity was demonstrated for the macrodomains of the alphaviruses Semliki

Forest virus (SFV), SINV, VEEV and CHIKV (Ecke et al., 2017; McPherson et al., 2017; Park & Griffin, 2009). For certain Alphaviruses, the MD activity was shown to be important (Krieg et al., 2023; McPherson et al., 2017). For SINV, MD mutations resulted in reduced viral RNA replication and a decrease in lethality of infected mice. Also, SINV MD mutations mutate back, indicating the necessity of a functional MD (Park & Griffin, 2009). Below, the CHIKV nsP3 and MD are more closely discussed. The CHIKV MD is essential to replication. Mutants of viral MD (vMD) that lack hydrolase activity result in replication deficient virus (Fehr et al., 2020; Krieg et al., 2023; Lüscher et al., 2022; McPherson et al., 2017). For SARS-CoV-2, the MD is also in the nsP3 protein, a more complex multi domain enzyme compared to Alphavirus nsP3. SARS-CoV-2 nsP3 contains three macrodomains, Mac1-3. Mac1 of SARS-CoV-2, SARS-CoV, Middle East respiratory syndrome coronavirus (MERS-CoV) and murine hepatitis virus (MHV) bind ADPr and function as MAR hydrolases (Alhammad et al., 2021; Alhammad & Fehr, 2020; Frick et al., 2020; Michalska et al., 2020; Voth et al., 2021). Mac1 function is important for viral replication. Also, upon mutation of the SARS and HCoV-229E MD, the viruses become more sensitive to IFN signaling (Kuri & Snijder, 2011). Infection of mice with the MHV-A59 strain that has MD mutations resulted in reduced viral titre and a lower production of proinflammatory cytokines. Strikingly, the mice infected with the MHV-A59 strain did not develop liver disease anymore in comparison to the wildtype (WT) strain (Eriksson et al., 2008).

Interestingly, viruses target MARYlation, and not PARYlation (Ecke et al., 2017; Lüscher et al., 2022). Again, showing a role for MARYlation, and not PARYlation, in innate immunity. Taken together, the macrodomain is either essential to viral replication or plays a role in immune modulation and is important to the viral life cycle.

As discussed above, IFN-induced mARTDs are linked to the innate immunity and seem to function in viral infection. The viral hydrolase activity is then in turn important for viral replication, immune modulation and/or to counteract the antiviral response executed by the innate immune system (Lüscher et al., 2022). Targeting the viral MDs could be of interest to block replication, reduce viral load, and support the immune response. For this, further research into their biochemistry and their substrates is required. This would be beneficial for multiple Alphaviruses and Corona viruses, among others. The viruses possessing a functional MD include many animal and human pathogens that cause health crises and economic burdens. Especially, the SARS-CoV2 pandemic demonstrates the urgency (Lüscher et al., 2022).

Efforts have been undertaken to develop therapeutic molecules targeting viral MDs (Lüscher et al., 2022). For example, for CoVs Mac1 there is preliminary data that can lead to the development of specific therapeutics or inhibitors (Babar et al., 2020; Brosey et al., 2021; Dasovich et al., 2022; Fu et al., 2021; Lüscher et al., 2022; Ni et al., 2021; Patel et al., 2022; Schuller et al., 2021; Sowa et al., 2021; Viridi et al., 2020; Yin et al., 2021), but so far no inhibitors have been identified for the viral MD. Given the highly conserved fold, a breakthrough in finding a viral inhibitor would be an initial step towards developing numerous inhibitors for various discussed viruses (Lüscher et al., 2022).

1.2. Cellular condensates

Our cells use organelles to spatially organize different cellular processes. The “classic” idea of organelles is a clearly enclosed system by a distinct lipid layer. For different molecules to enter they need specific signals and mostly active transport systems, illustrating a tight regulation of the respective processes. Overall, compartmentalization ensures processes can occur more efficiently and facilitates their regulation. Allocating and grouping biomolecules related to the same process streamlines reactions. The cytosol takes up 50-60% of a cell and is home to many different processes and reactions. So, here as well, a stringent regulation is required, and process-optimization would be valuable. In recent years, a form of cellular compartmentalization without membranes has come to light, biomolecular condensates. These membrane-less “organelles” are increasingly acknowledged as a common method of cells to regulate processes and reactions by controlling the availability and concentration of molecules (Banani et al., 2017; Niu et al., 2023; Wang et al., 2021).

Biomolecular condensates are liquid-like organelles formed by phase-separation. They do not have a lipid layer and form more freely within their environment. The lack of a defined membrane and the liquid-like form of these condensates favor an interplay with the environment and an easy exchange of molecules with their surroundings. This results in a highly dynamic and distinct setting for molecular processes. Another advantage of the liquid-like and transient nature of condensates is the ability of the cell to quickly react to environmental cues and stressors. By now, a whole new field has opened. The phase-separated condensates are involved in multiple different mechanisms and harbor many different cellular processes within the cytoplasm and the nucleus (Banani et al., 2017; Borodavka & Acker, 2023; Niu et al., 2023; Wang et al., 2021).

Brangwynne et al. were the first to observe condensate formation with the study of the embryonic development of *Caenorhabditis elegans* (Brangwynne et al., 2009). They identified “P-granules”, phase-separated condensates formed by RNA in fertilized worms (Brangwynne et al., 2009). Later, proteins were also found to have the ability to phase separate. By now, condensation is a known common process of different biomolecules, primarily RNA and RNA-binding proteins and there is a multitude of studies showing this general process involved in diverse cellular functions (Banani et al., 2017; Borodavka & Acker, 2023; Niu et al., 2023; Wang et al., 2021).

As mentioned, the transient nature of these condensates offers a huge advantage for the cell to quickly react and adapt to environmental cues. There are many known kinds of biomolecular condensates identified by now, each formed for a specific function (Banani et al., 2017; Borodavka & Acker, 2023; Niu et al., 2023; Wang et al., 2021). Examples in the nucleus are nucleoli, transcription hubs called super-enhancers, condensates associated with transcription factories, or the spliceosome among others. Cytoplasmic examples include stress granules, P bodies and RNA-transport granules (Banani et al., 2017; Borodavka & Acker, 2023; Niu et al., 2023; Wang et al., 2021).

1.2.1. LLPS: the driving force behind condensate formation

Liquid-liquid phase separation (LLPS) drives condensate formation. Condensates are driven by intermolecular interactions, a combination of protein–protein, protein–RNA, and RNA–RNA interactions, resulting in highly concentrated environments (Banani et al., 2017; Borodavka & Acker, 2023; Li et al., 2021; Niu et al., 2023; Wang et al., 2021). RNA and certain proteins can initiate or undergo LLPS depending on their biochemical properties and interactions to other biomolecules. An easy way to depict this process is by envisioning the formation of droplets in mixtures of solutions with different viscosities e.g., oil and water

results in two separate phases due to their biophysical and chemical properties. The liquid de-mixing of a mixture takes place when biomolecules, due to different cues and interactions, surpass a set concentration threshold and so initiate a molecular seeding event. Overall, LLPS is driven by multivalency. It happens through a combination of a multitude of transient and typical low affinity interactions. The seeding event starts with distinct targeted interactions, which ends as the more solid core within the condensates. Once initiated by more targeted interactions, a more transient shell forms around the core. The shell can easily and quickly fuse and diffuse with its surroundings and gives condensates the ability for smooth component exchange. Depending on the function of the condensates and the trigger, their composition is highly variable. However, there are general principles and characteristics of the biomolecules undergoing LLPS, as described below (Li et al., 2021; Wang et al., 2021).

1.2.2. Molecules involved in phase-separation

Proteins initiating and/or undergoing LLPS have some defining characteristics. All together, they make a protein either dynamic or prone for multiple transient interactions, which lies at the basis of LLPS. The fluctuation in components concentrations leads to the induction of LLPS through both intra- and intermolecular interactions (Boeynaems et al., 2018; Li et al., 2021; Niu et al., 2023). The proteins often contain intrinsically disordered stretches (IDRs). So many of the proteins are either intrinsically disordered proteins (IDPs) or structured proteins with intrinsically disordered regions (IDPRs) (Darling et al., 2017; Deiana et al., 2019). This means that big stretches of the protein itself do not have a stable tertiary structure. Also, many of these proteins are RNA-binding proteins (RBPs). Many nucleic acid binding domains are enriched in condensates due to the central role for RNA-protein interactions e.g., motifs such as ZnFs, RRM and RNA-binding domains (RBDs). Another common feature in phase-separating proteins is multivalency. Multivalency lies at the basis of LLPS. Generally, proteins with tandem repeats can drive condensation through multivalent interactions. Each single interaction increases again the affinity between different weaker interactors. Through interactions with other proteins and RNA a multivalent interaction platform is created and thereby phase separation is initiated. Taken together, LLPS-inducing proteins are characterized by structured domains responsible for intermolecular interactions, IDRs and repeated motifs (Boeynaems et al., 2018; Li et al., 2021; Niu et al., 2023).

Nucleic acids are important for phase separation and have been implicated in the formation of different condensates. Both DNA and RNA can function as a binding platform for diverse proteins. As discussed, many nucleic acid binding proteins are enriched in condensates. The presence of RNA or DNA can trigger phase separation by complexing proteins containing IDRs or RNA interaction modules. Our knowledge on the role of RNA in phase separation has progressed in recent years. Different RNA species, RNA structure or inter RNA-RNA interactions seem to have different functions in condensate formation. The delivery of exogenous mRNA and ssDNA in the cytoplasm triggered SGs assembly. This suggests that mRNA release from polysomes could induce SG formation and that SG formation could be driven by protein-nucleic acids interactions (Bounedjah et al., 2014). *In vitro* purified RNA can self-assemble into condensates. Interestingly, mRNAs found in the assemblies in the induced condensates by total yeast RNA largely overlap with the stress granule transcriptome (Van Treeck et al., 2018). Structurally, the secondary structure of mRNA has been suggested to impact the composition of condensates (Langdon et al., 2018). More information can be found in some recent reviews (Garcia-Jove Navarro et al., 2019; Guo et al., 2021; Lin & Fang, 2021; Van Treeck & Parker, 2018).

1.2.3. PTMs in phase-separation

LLPS *in vitro* is induced by changes in salt concentration or pH, a molecular crowder or the molarity of the respective proteins or nucleic acids. How our cells induce LLPS is of course

different. Different condensates form in response to environmental cues and different stressors. The quickest way cells adapt to new impulses is by the direct modulation of the already present macromolecules using PTMs (Darling & Uversky, 2018; Keenan et al., 2021; Li et al., 2022; Luo et al., 2021; Owen & Shewmaker, 2019). With PTMs, molecules can rapidly react to changes in temperature, pH, physiological signals and multiple other stressors or indicators. PTMs change the physicochemical characteristics of proteins or nucleic acids. They can affect conformation, valency, hydrophobicity, charge, activity, subcellular localization, stability or importantly both intra- and intermolecular interactions. Acetylation, phosphorylation, ubiquitination, SUMOylation, arginine methylation, NEDDylation, glycosylation, arginylation and ADP-ribosylation have been linked to condensate assembly and/or disassembly (Darling & Uversky, 2018; Li et al., 2022; Luo et al., 2021; Owen & Shewmaker, 2019).

1.2.4. Stress Granules

Stress granules are among the best-known and -studied biomolecular condensates. They are cytoplasmic RNA-protein condensates that form by LLPS. SGs are highly dynamic and quickly formed upon stress signals such as oxidative stress, heat shock, endoplasmic reticulum (ER) stress, or viral infection as a survival mechanism. During stress, general translation is stopped, and mRNAs dissociate from the polysomes. The increased concentration of free mRNA and its interaction with Ras GTPase-activating protein-binding protein 1 (G3BP1), among other RBPs, or the accumulation of stalled non-translated messenger ribonucleoproteins (mRNPs) initiates the multivalent platform needed for LLPS (Brownsword et al., 2023; McCormick & Khapersky, 2017). Thus, SGs form, harbouring the stalled translation initiation complexes. Upon stress relief, SGs disassemble, the bound mRNAs are released, and the cell can continue the translation process (Brownsword et al., 2023; McCormick & Khapersky, 2017). Their transient nature makes condensates the perfect response to stress. Stress granules have been suggested to have a stable core and a surrounding dynamic shell, in accordance with the condensate model (Jain et al., 2016). Aberrant formation of stress granules and mutations impacting their (dis-)assembly have been associated with neurological diseases and/or viral infection among others (Brownsword et al., 2023; Wlozozin & Ivanov, 2019). Also, different SG proteins have an altered expression in cancer cells (Anderson et al., 2015).

1.2.4.1. Stress Granule formation

Different SG-formation models have been suggested. There are different types of SGs. It seems that dependent on the stressors, a different response is initiated. This includes assembly, disassembly and composition.

Cellular stress can be caused by a variety of triggers. Different stressors are sensed through specific pathways. For example, dsRNA through protein kinase R (PKR), ER stress through PKR-like endoplasmic reticulum kinase (PERK). Detection of these stressors results in the phosphorylation of the eukaryotic translation initiation factor 2 alpha (eIF2 α) (Brownsword et al., 2023; Millar et al., 2022). eIF2 α phosphorylation stalls the translational machinery, causing the release of the mRNPs from polysomes (Brownsword et al., 2023; Millar et al., 2022). SGs are formed through LLPS. There is the general concept of condensate initiation by increase in the local concentration of specific biomolecules. An increase of mRNA in the cytoplasm triggered stress granule assembly, while increasing the concentration of mRNA-binding proteins formed more isolated mRNPs. Upon stress, polysomes disassemble and this increases the local mRNA concentration in the cytoplasm which acts as a seed, then followed by binding of RBPs, and eventually SG formation (Bounedjah et al., 2014). When looking at SG assembly, one more recent and well-studied model is G3BP1-induced SG formation (Guillén-Boixet et al., 2020; Sanders et al., 2020; Yang et al., 2020). This model is

discussed in more detail below. Probably, stress granules are formed by a combination of these events, and dependent on the stressor. The stable core is formed through specific SG proteins and then followed by recruitment of RBPs (Brownsword et al., 2023; Millar et al., 2022).

When discussing SG formation, it is essential to take the different stressors into account. Different stressors activate different pathways resulting in SG formation (Brownsword et al., 2023). Also, it has been shown how some pathways and SG control is completely stressor dependent. Even upon infection with different viruses, different forms of SG formation were observed. Dependent on the virus, SGs were either not formed, stable, transient or even aberrant. Viral infection triggers the cellular stress response, but the response is dependent on the specific virus and they induce distinct responses (McCormick & Khaperskyy, 2017; Ng et al., 2013).

The PTM dependent regulation of SGs appears to be highly context dependent and can differ with regards to the respective condensate and/or the cue inducing condensate assembly or disassembly (Millar et al., 2022). Known PTMs involved in SG dynamics are ADP-ribosylation, phosphorylation, arginine methylation, acetylation, ubiquitination, SUMOylation, NEDDylation, glycosylation, arginylation (Millar et al., 2022). For example, SGs decompose dependent on the exact stressor that induced them in the first place and this regulation is PTM dependent (Gwon et al., 2021). Only heat shock induced SGs seem to rely on ubiquitination of G3BP1, the key component of these condensates, for disassembly (Gwon et al., 2021). This implicates that condensate regulation mechanistically differs dependent on the nature of the stressor and thus different ways for assembly and disassembly are needed. This is quite an important note because most studies addressing SG biology use oxidative stress by arsenite treatment to trigger condensate formation. However, now we know that the statements made with these studies using oxidative stress can not necessarily be extrapolated to other stressors leading to SG induction.

1.2.4.2. Stress Granule composition

SGs composition is widely discussed. Most biomolecules in SGs are translation associated factors and untranslated polyadenylated mRNAs. Especially components of the translation initiation complex are present. The SG proteome includes RNA-binding proteins and proteins linked to mRNA processing and stability, translation, spliceosome assembly and mRNA splicing, and regulation of metabolic processes (Jain et al., 2016; Kedersha et al., 2002, 2005; Markmiller et al., 2018; Youn et al., 2019). When looking at SG cores, RNA-binding proteins, Prion-like domain, ATPase activity and protein modifying enzymes stood out (Jain et al., 2016). Many of these proteins have IDRs (Youn et al., 2019). Proteins with RGG domains, RNA-binding domains, zinc-finger motifs, helicase domains, and of interest to this work the PARP domains are enriched (Youn et al., 2019). Interestingly, SG composition depends on cell type, stress and disease state (Markmiller et al., 2018). Recently, Millar et al. updated the SG proteome information by literature analysis (Millar et al., 2022). Gene ontology analysis of the SG proteome reveals a link to biological processes of RNA stability, translation, transport and splicing, and gene silencing (Millar et al., 2022). SGs regulate cellular reactions by sequestering certain proteins and thus affecting their availability. Many SG proteins are nuclear and move to the cytoplasm and then into SGs under stress (Riggs et al., 2020). How this is facilitated, whether they are recruited or whether their increase in local concentration induces SGs is debated and probably dependent on each component.

1.2.4.3. Stress granules and ADP-ribosylation

Condensate assembly is regulated by PTMs. The same counts for SGs. PTMs have been shown to be essential for both assembly and disassembly (Cao et al., 2020; Guillén-Boixet et

al., 2020; Sahoo et al., 2018; Tourrière et al., 2003; Yang et al., 2020). Our lab specializes in ADP-ribosylation, another PTM that has been linked to LLPS and SGs. ADP-ribose is a bulky modification, has two negative charges and an adenine ring that is capable of both, hydrogen bonding, hydrophobic interactions, and π -stacking, all different characteristics that potentially could help within LLPS. A bulky modification, by either one ADPr (MARylation) or more (PARylation) could induce conformation changes, while the charge and hydrophobicity of the substrate protein is likely also affected. Purely theoretical and based on the nature of the molecule, ADPr has multiple ways to affect LLPS behavior of a protein. PAR has been suggested to be required in the cytoplasm for SG assembly upon various stimuli (Catara et al., 2017; Leung et al., 2011; McGurk et al., 2018). The ART domain is enriched in the SG proteome (Youn et al., 2019). Certain PARPs localize to and/or are associated with SGs; PARP5, PARP12, both isoforms of PARP13, PARP14 and PARP15 (Catara et al., 2017; Isabelle et al., 2012; Jain et al., 2016; Leung et al., 2011). Expression of PARP12 and PARP15 induce SGs (Catara et al., 2017; Leung et al., 2011). This SG induction is independent of eIF2 α phosphorylation (Leung et al., 2011), suggesting either SG induction occurred through a different pathway, or their induction is an overexpression artifact indicating that a heightened presence of these proteins, or ADP-ribosylation, results in SG induction. However, catalytic inactive PARP12 was suggested to induce SGs too, indicating SG formation to be independent of PARP12 MARylation activity. Another group could detect PAR in SGs and PAR accumulation leads to hampered SG disassembly (Catara et al., 2017). PAR was also suggested to induce translocation of certain proteins to SGs. Catara et al. suggested a model in which oxidative stress causes PARP1 activation, which produces PAR molecules and leads to PAR accumulation. Upon release, PAR binds PARP12 through its WWE domain, which results in PARP12 translocation from the Golgi to SGs (Catara et al., 2017). PAR microinjection in HeLa cells resulted in PARP12 release into the cytosol. This translocation requires both a WWE domain, necessary for PAR binding, and the ZnF domains of PARP12. Inhibition of PARP1 activity inhibits PARP12 translocation. Also, upon stress activation and PARP12 relocation, the Golgi becomes fragmented and cellular trafficking is impaired. Whether this is due to absence of PARP12, or part of the overall stress response is not clear. Interestingly, PARP12 localizes to SGs upon oxidative stress and heat shock but not under osmotic stress. Again, this points to stress dependent differences in SG pathways (Catara et al., 2017). Another protein is TAR DNA-binding protein 43 (TDP-43), which upon interaction with PAR/PARylated substrates is transported to SGs. *In vitro*, TDP-43 PAR-binding induces LLPS and in cells PAR is required for TDP-43 SG translocation. TDP-43 binds PAR through its PBM domain and thereby localizes to SGs (Duan et al., 2019; McGurk et al., 2018). TDP-43 translocation prevents hyperphosphorylation, which is associated with disease. Multiple SG-associated proteins are either reported as ADP-ribosylation substrates or capable of binding ADP-ribosylation. These include Argonaute 2 (Ago2), TIA, TDP-43, heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) and G3BPs (Geuens et al., 2016; Leung et al., 2011). The presence of their RBD domain is necessary for ADP-ribosylation (Leung et al., 2011). It is not known whether the RBD domain is the modification site or important for localization or RNA-binding essential to induce ADP-ribosylation. HnRNAP1 is another nuclear protein that translocates to the cytoplasm upon stress (Geuens et al., 2016b). HnRNPA1 was identified as a substrate for ADP-ribosylation and this PTM is required for cytoplasmic trafficking. It also has a PBMs essential for association with PAR and PAR substrates and sorting to SGs. PARP inhibition affects hnRNAP1 SG recruitment and PARG inhibition affects retrieval. *In vitro*, hnRNPA1 induces LLPS in a PAR concentration-dependent manner and the PBM mutant does not induce phase separation. TDP-43 co-phase separates with hnRNPA1 *in vitro*, an effect again dependent on hnRNPA1 PBM (Duan et al., 2019). Isabelle et al. analyzed the pADPr-associated proteome at different time points following DNA damage and found at later time points an enrichment in stress response components, among them G3BP (Isabelle et al., 2012). It was also suggested that SG initiation is ADP-ribosylation-dependent since

different PARP inhibitors delay their assembly (Isabelle et al., 2012). PARP13 was found as well in the G3BP-complexes (Isabelle et al., 2012).

In general, a mechanism is proposed in which PAR polymers function as a signaling molecule during stress responses. It is capable at binding to proteins and leads to their translocation to SG (Altmeyer et al., 2015; Isabelle et al., 2012).

RNA can trigger LLPS through interacting with IDPs and induce a nucleation event. PAR is quite similar to RNA. It is a negatively charged, multivalent molecule capable of acting as a scaffold for multiple proteins. PAR can be both covalently attached to a substrate or be synthesized as a free polymer. Altmeyer et al. could see a significant association between proteins involved in LLPS and proteins in PAR-interactomes. They then demonstrated that, similar to RNA, PAR could trigger condensation and initiate a nucleation event both *in vitro* and *in vivo* (Altmeyer et al., 2015). Upon cellular stress and DNA damage, PAR synthesis is upregulated (Catara et al., 2017). This suggests that cells possess an intrinsic mechanism, by which they can react to environmental cues, and produce a molecular seed, PAR, to induce LLPS. By the presence of PAR, IDPs assemble, and condensation is initiated. For example, upon DNA damage PAR chains are synthesized at the DNA breaks. Here, PAR recruits and assembles the IDPs into condensates. This mechanism was shown for 3 different IDPs: RNA-binding protein FUS, RNA-binding protein Ewing Sarcoma protein (EWS), and TATA-Box Binding Protein Associated Factor 15 (TAF15) (Altmeyer et al., 2015). Upon DNA damage, these 3 proteins localize at the break sites dependent on the presence of PAR. Upon analysis of these proteins, two motifs stood out; an RGG repeat that due to its positive charge can interact with PAR and a Prion-like domain which amplifies LLPS. Together, they offer the necessary tools for the respective proteins to phase-separate at the DNA-damage sites (Altmeyer et al., 2015). In conclusion, upon stressors PAR is synthesized and can serve as a seed to induce LLPS and mediate local phase transitions.

Other ADP-ribosylation associated proteins located to SGs are the PARG isoforms 1 and 2 (Jain et al., 2016; Leung et al., 2011). Upon their overexpression, their adverse effect on SG formation is revealed and SG formation is inhibited. Their KD results in a delayed SGs clearance (Leung et al., 2011). This suggests that ADP-ribosylation has a pro-SG formation function. The PARG isoform PARG110 is nuclear and is involved in DNA damage repair mechanism (Lüscher et al., 2018). It would be interesting to analyze how its presence influences condensates at the sites of DNA-damage. PARG overexpression leads to G3BP nuclear accumulation, and this effect is negated by a catalytic inactive mutant of PARG. This indicates again a role for PAR in SG proteins recruitment and specifically G3BP subcellular localization (Isabelle et al., 2012).

1.3. G3BP1

G3BP1 is an RNA-binding protein, best known for its role in SG assembly. G3BP1 is 466 AA long and has a theoretical weight of 55 kDa and runs around 65 kDa on an SDS-PAGE. This protein localizes primarily to the cytosol. G3BP1 and G3BP2 are paralogs located on chromosomes 5 and 4 respectively. G3BP2 has 2 isoforms, G3BP2a/b. Since its discovery, G3BP1 has been linked to cell proliferation, metastasis, neural development, RNA metabolism and stress response (Alam & Kennedy, 2019b; Kang et al., 2021; Sidibé et al., 2021). Also, it has been found to be an important player during viral replication, both as pro- and antiviral. The majority of our knowledge on G3BP1 is linked to its pivotal role in RNA granules (Alam & Kennedy, 2019; Kang et al., 2021; Sidibé et al., 2021).

G3BP1 was suggested to interact with Ras and play a role in the Ras signalling pathway. However, this has been contradicted later (Annibaldi et al., 2011; Sidibé et al., 2021). G3BP1 ^{-/-} mice are non-viable and die during the late embryonic stage with massive cell death in the central nervous system (CNS) (Zekri et al., 2005). Later, the same research group succeeded in breeding viable G3BP1 ^{-/-} mice via another approach. Again, they observed that G3BP1 ^{-/-} is primarily lethal during fetal development. A small percentage of pups was viable but did not live longer than 2 months and showed neurodegeneration in the CNS, growth retardation, severe motor activity problems, memory deficits and neurocognitive defects, and calcium homeostasis problems comparable to Alzheimer disease (Martin et al., 2013). Since G3BP1 is ubiquitously expressed, it was a quite interesting observation that other organs remained unaffected and the deletion heavily impacted neuronal development in the CNS (Martin et al., 2013; Zekri et al., 2005). This could point to a different role for the G3BPs outside of the CNS. For example, important in stress responses. G3BPs are suggested as the only essential proteins for SG formation (Yang et al., 2020). G3BPs are involved in a variety of processes and implicated in different diseases, e.g., RNA stability, neurodegeneration, cancer, stress response, or viral infections. This dissertation focusses on its role in SGs and viral infection. For further reading, the following reviews are recommended (Alam & Kennedy, 2019; Kang et al., 2021).

1.3.1. G3BP1 domain architecture

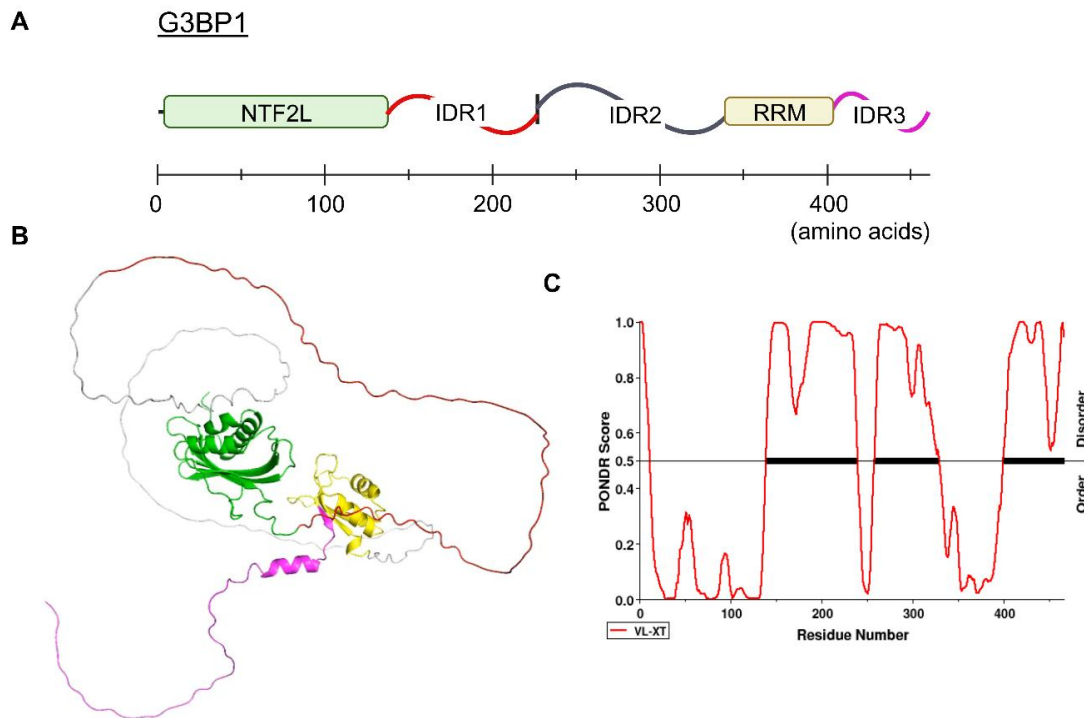


Figure 4. The domain architecture of G3BP1. G3BP1 has a NTF2L-domain, an IDR1, an IDR2 and a RBD that is subdivided in a RRM and an IDR3. AlphaFold analysis of G3BP1 illustrates the folded domains (NTF2L: green; RRM: yellow) and the IDRs (IDR1: red; IDR2: grey; IDR3: pink). PONDR Score analysis indicates the three disordered regions. NTF2L: Nuclear Transport factor 2 Like; IDR: intrinsically disorder region; RBD: RNA-binding domain; RRM: RNA recognition motif.

N-terminally, the highly conserved Nuclear Transport factor 2 Like (NTF2L) domain is located (AA 1-142) (**Fig. 4**). As the name suggests, the domain bears a high similarity to the Nuclear Transport Factor 2, a small protein involved in nuclear transport (Vognsen et al., 2013). This led to the idea that G3BP1 could be involved in nuclear transport. However, there is no significant evidence supporting this claim; G3BPs appear in the nucleus but are mostly cytosolic (Prigent et al., 2000; Vognsen et al., 2013). The NTF2L domain is responsible for dimerization of G3BP1, a function essential to G3BP1's LLPS competence and thus SG assembly (Guillén-Boixet et al., 2020; Sanders et al., 2020; Tourrière et al., 2003; Vognsen et al., 2013; Yang et al., 2020). Deletion of the domain blocks SG assembly in cells and *in vitro* LLPS induction (Guillén-Boixet et al., 2020; Sanders et al., 2020; Yang et al., 2020). It is also responsible for mediating interactions to other SG proteins, independently from its function in LLPS (Kedersha et al., 2016). The NTF2L domain of G3BP1 is required for interaction with SG-associated proteins ubiquitin specific peptidase 10 (USP10), Cell Cycle Associated Protein 1 (CAPRIN1), and ubiquitin associated protein 2 Like (UBAP2L). NTF2L was argued to serve as an interaction platform for other RBPs for the build-up of giant protein-RNA network and thus amplifies the valency of the complex, resulting in LLPS (Sanders et al., 2020).

Central, G3BP1 contains 2 IDRs, IDR1 and IDR2 respectively (**Fig. 4**). IDR1 (AA 143–225) is highly negatively charged and acidic. IDR2 (AA 226–334) is more positively charged and rich in proline residues. These IDR regions are often associated with PPIs. The different members of the G3BP family diverse in the amount of PxxP motifs; G3BP1 has 3, G3BP2a 5 and G3BP2b 6 (Kennedy et al., 2002). Tourrière et al. found firstly that the IDR1 can inhibit SG formation (Tourrière et al., 2003). Recently, it was shown that upon deletion of the IDR1-2 stretch the threshold for G3BP's LLPS propensity lowers. In accordance, reconstituting G3BP1/2 dKO cells with G3BP1-ΔIDR1/2 did restore SG assembly (Guillén-Boixet et al., 2020;

Sanders et al., 2020; Yang et al., 2020). This is quite interesting, since in general IDRs within RBPs mediate LLPS but for G3BPs this stretch is dispensable and the LLPS propensity depends on the conserved NTF2L domain and the RBD domain, further discussed below. However, reconstituting G3BP1/2 dKO cells with G3BP1- Δ IDR1 also restored SG assembly and even increased SG formation, while G3BP1- Δ IDR2 did not (Sanders et al., 2020; Yang et al., 2020). It was suggested that there is a role for the IDR1/2 in G3BP1 dynamics. It was shown that the negative charge of IDR1 inhibits SG formation, and the presence of IDR2 mitigates this inhibition (Guillén-Boixet et al., 2020; Yang et al., 2020). *In vitro*, IDR1 strongly associates with the RBD domain, more specifically IDR3, blocking RNA-interaction and thus LLPS. Also, *in vitro* presence of IDR2 mitigates this interaction (Yang et al., 2020). This is an example of how IDRs can affect LLPS induction.

C-terminally is the RNA-binding domain (RBD) (**Fig. 4**). It consists of an RNA recognition motif (RRM) (AA 335-410) and the IDR3, a glycine-rich box RGG domain (AA 411-466). The latter is positively charged. The RRM domain is build-up of a beta-sheet platform made up of 4 beta-sheets and 2 alpha-helices and is then followed by the unstructured IDR3 (Jumper et al., 2021; "Love That β -Sheet," 1998; Maris et al., 2005; Varadi et al., 2022). Deletion of both the RRM and IDR3 blocks RNA-binding. Δ RRM has reduced RNA-binding, Δ IDR3 has been reported to either block or reduce RNA-binding (Guillén-Boixet et al., 2020; Yang et al., 2020). In accordance, upon deletion of both or either domains of the RBD, RNA-induced LLPS was blocked or severely impaired, respectively (Guillén-Boixet et al., 2020; Sanders et al., 2020; Yang et al., 2020). Reconstitution of G3BP1/2 dKO cells with Δ RBD, Δ RRM or Δ IDR3 resulted in either blocked or severely impaired SG formation, respectively (Sanders et al., 2020; Yang et al., 2020). Similarly, G3BP1-F380/382D, point mutants in the RRM, impairs RNA-binding and SG assembly (Yang et al., 2020). As mentioned above, it was shown how the intramolecular electrostatic interaction between the positively charged RBD-IDR3 and the negatively charged IDR1 regulates G3BP1 phase separation (Guillén-Boixet et al., 2020; Yang et al., 2020). Together proposing a model for G3BP1 RNA-induced phase separation through control of a conformational switch between binding of the RBD to IDR1 or to RNA (Guillén-Boixet et al., 2020; Yang et al., 2020). This switch has been argued to be able to be controlled by PTMs. For example, phosphorylation of IDR1 positively affects the IDR1-RBD interaction due to the electrostatic addition (P. Yang et al., 2020). Besides, IDR3 is necessary for the formation of G3BP1 oligomers, presumably through protein-RNA-protein interactions. G3BP1 forms oligomers upon presence of RNA, however G3BP1- Δ IDR3 is restricted to dimers (Guillén-Boixet et al., 2020).

Interestingly, G3BP binds to pADPr through its IDR3/RGG region (Isabelle et al., 2012). Many RBPs contain RGG domains, and this positively charged motif is necessary for binding of PAR (Gagné et al., 2008; Grimaldi et al., 2019)

1.3.2. G3BP1 in SGs

G3BP1 lies at the basis of SG formation. G3BP1/2 dKO cells do not form SGs in response to eIF2 α phosphorylation or eIF4A inhibition (Kedersha et al., 2016). SG formation due to oxidative stress (sodium arsenite), ER stress (thapsigargin) and clotrimazole is blocked. However, upon hyperosmotic stress (NaCl, sorbitol) or strong heat shock some small foci formed (Kedersha et al., 2016). Another group tested which "essential" SG proteins were indispensable for SG formation by sequential KO of the different genes or combined KO of their paralogues (Yang et al., 2020). Almost all "essential"-gene KO resulted in an aberrant SG assembly but a SG formation none the less. Only the double KO of G3BP1 and G3BP2 resulted in no SGs. This study was done with arsenite induced SGs (Yang et al., 2020). As discussed above, all domains of G3BP1 are fine-tuned for SG-induction through LLPS. Upon stressors, G3BP1 can dimerize and undergoes a conformational switch/change. This is argued to be due to dephosphorylation of S149 and S232 in IDR1. This blocks IDR1

autoinhibitory function by RBD disassociation, resulting in an open conformation (Yang et al., 2020). The RBD domain is now free to interact and bind RNA. RNA lies at the basis of the formation of these granules and without its presence, granules are not induced (Guillén-Boixet et al., 2020; Sanders et al., 2020; Yang et al., 2020). On the other hand, a PPI network is build-up through the G3BP1 NTF2L domain creating all together a multivalent platform which often lies at the basis of LLPS (Sanders et al., 2020). This network can either be enhanced by binding of proteins such as UBAP2L, or inhibited by binding of proteins such as USP10 which is argued to keep G3BP1 soluble and prevent G3BP1 from interacting with other proteins (Kedersha et al., 2016; Sanders et al., 2020). The presence of other known SG partners such as CAPRIN1 and TIA-1 decreases the threshold for RNA induced LLPS (Sanders et al., 2020). Taken together, G3BP1 lies at the basis of SG formation and has been proposed as the central node of the entire network (Guillén-Boixet et al., 2020; Sanders et al., 2020; Yang et al., 2020). Interestingly, by exchanging the different G3BP1 domains for domains of other proteins with parallel functions, foci assembly can be reconstituted in G3BP1/2 dKO cells, showing their exact role within SG assembly (Yang et al., 2020).

1.3.3. G3BP1 and SG composition

Section 1.3.2. discussed SG composition. Whether, and if so how, G3BP affects the protein composition of stress granules is not clearly studied. G3BP1 is argued to be the central node to build-up the foci (Sanders et al., 2020). Dependent on its interactors, different types of foci could be formed. G3BP1 mediates multiple PPIs through its NTF2L-domain, so it does seem intuitive to speculate that it highly influences the SG's proteome (Sanders et al., 2020). In general, SG composition is context dependent.

Markmiller et al. studied the SGs proteome dependent on different stressors and cell types using ascorbate peroxidase (APEX) proximity labeling in combination with mass spectrometry. They found that $\pm 20\%$ of the SG proteome is stress and cell dependent (Markmiller et al., 2018). The mechanism by which this selection occurs remains unclear. One study on alphavirus and SGs does shed some light. SGs are induced upon Alphavirus infection. Alphaviral nsP3 suppresses SG formation at a later stage of infection and replication. The viral protein nsP3 interacts with G3BP1 and sequesters it into condensates. Interestingly, dependent on the macrodomain activity, de-MARYlation, of nsP3 the composition of these condensates is influenced. The new nsP3-G3BP condensates are not sensitive to cycloheximide (CHX) treatment and have different composition (Jayabalan et al., 2021). Thus, the catalytic activity of nsP3 suppresses stress granule formation, releases translation factors and induces the formation of alternative condensates (Jayabalan et al., 2021). NsP3 with an inactive MD results in no release of translation factors. G3BP1 was also suggested as a substrate of nsP3. This would indicate that G3BP1 PTM could influence granule composition (Jayabalan et al., 2021).

1.3.4. G3BP1 in the viral lifecycle

Viral infection induces SG formation (Alam & Kennedy, 2019; Brownsword & Locker, 2023). A diverse number of viruses have been shown to counteract SGs as a response to viral infection (Alam & Kennedy, 2019; Brownsword & Locker, 2023). Many viruses target the SG pathways or important SG proteins, among them G3BPs. Alphaviruses, such as CHIKV, exploit SG components. It seems that depending on the virus, G3BPs, and other SGs components, either act pro- or antiviral (Alam & Kennedy, 2019b; Brownsword & Locker, 2023). Since in general SGs are seen as a cellular safeguard while the cell handles whatever stress induced the SGs to begin with, it is quite interesting to study how viruses bend these condensates toward their own purposes.

1.3.4.1. G3BPs as proviral factors

Early on, G3BP1 was reported as a cellular factor involved in vaccinia virus RNA polymerase activity and transcription. Cellular extracts were necessary for the transcription of viral templates. The relevant proteins were identified to be G3BP and p137 (Katsafanas & Moss, 2004). Hepatitis C virus (HCV) infection induces SGs through IFN and PKR (Garaigorta et al., 2012). G3BP1 was found to bind to the 5' untranslated region (5'-UTR) of HCV using an RNA pull-down assay. Knockdown of G3BP1 resulted in reduced HCV RNA and the viral non-structural protein 5A (NS5A) (Tingting et al., 2006). In another study, G3BP1 was reported as an interactor of HCV NS5B. Again, knockdown of G3BP1 significantly reduced HCV replication (Yi et al., 2006). The interaction of G3BP1 with NS5B and other proteins of the HCV replication complex was confirmed, just as the interaction between G3BP1 and the viral RNA. G3BP1 colocalizes with HCV replication complexes (RCs) in replicon infected cells. G3BP1 knockdown negatively affects HCV replication and results in a reduction of viral particles. These findings led to the hypothesis that G3BP1 is an important RC factor (Yi et al., 2011). Interestingly, G3BP2 knockdown did not affect HCV infection (Yi et al., 2011). TIA-1, TIAR and G3BP1 are needed during early stages of HCV replication. G3BP1 and TIA-1 are also involved in the late stages of HCV infection and HCV proteins further colocalize and interact with these stress granule proteins (Garaigorta et al., 2012). Upon infection, Respiratory Syncytial Virus (RSV) induces SG formation. Upon stable knockdown of G3BP1, SG formation is inhibited, and RSV replication was impaired. Viral RSV RNA was also found to primarily localize to viral cytoplasmic inclusion bodies, but about 2% localized to SGs. Lastly, cells containing SGs have a higher level of RSV protein. Together, it confirms G3BP1 to be a proviral factor for RSV (Lindquist et al., 2010).

1.3.4.2. G3BPs as antiviral factors

Besides proviral, a lot of viruses have developed mechanisms to evade G3BPs and the cellular stress response. In response to viral infection, cells stall translation by SGs (Brownsword & Locker, 2023). This can block viral translation, due to the high dependence of viruses on the host's cellular translation machinery, indicating SGs as an interesting antiviral mechanism. Many viruses have developed ways to target G3BPs, to counteract their role in SGs and translation inhibition (Alam & Kennedy, 2019; Brownsword & Locker, 2023). Here G3BPs are discussed as an antiviral protein and common strategies how viruses disrupt the respective proteins. The G3BPs have been shown to be necessary for efficient translation and involved in translation regulation of ISGs (Alam & Kennedy, 2019; Bidet et al., 2014). G3BPs are suggested to be essential for interferon-induced transmembrane protein 1-3 (IFITM1-3) accumulation and regulating their expression both through regulation of the MEK pathway and through interaction with the 3'-UTRs of the IFITM1-3 transcripts. This mechanism could be more general for ISGs (Alam & Kennedy, 2019).

Picornaviruses are a family of non-enveloped RNA viruses and seem to generally counteract G3BPs by their proteases. Upon poliovirus infection, SGs are formed. To counteract SG formation, the poliovirus 3C proteinase cleaves G3BP. When the cell was reconstituted with a cleavage resistant G3BP (G3BP1 Q325E), SGs were rescued and viral replication inhibited (White et al., 2007). G3BP1 negatively affects EMCV replication. G3BP1 knockdown increases EMCV replication. EMCV also disrupts SG formation by cleavage of G3BP1 at the same site as poliovirus. Overexpression of the cleavage resistant G3BP1 mutant, G3BP1 Q325E, in comparison to G3BP WT results in a significantly lower viral load, stable SGs and an increase in IFN and cytokine induction. In line, endogenous G3BP1 KD lessens IFN- and other cytokine genes expression (Ng et al., 2013). Another example is the Picornavirus foot and mouth disease virus (FMDV). G3BP1 interacts with the (internal ribosome entry site) IRES element and negatively affects FMDV translation. Again, G3BP activity is counteracted by the 3C protease, which cleaves G3BP1 upon FMDV infection, resulting in 2 distinct

fragments (Galan et al., 2017). However, another Picornavirus inhibits SG formation through its L protein and G3BPs are not processed, indicating that G3BP cleavage is not a general mechanism by which Picornaviruses inhibit SGs (Borghese & Michiels, 2011). Besides Picornaviruses, Caliciviruses have been suggested to cleave G3BP1. Feline Calicivirus (FCV) infection hinders SG formation upon different stressors. FCV 3C-like proteinase NS6 Pro cleaves G3BP1 in the RBD at E405/V406 upon infection and this cleavage is responsible for impaired SG formation. The same mechanism applies to G3BP2 (Humoud et al., 2016). Interestingly, another Calicivirus, murine norovirus (MNV) infection does not affect stress induced SG formation or cleaves G3BP1, indicating that the mechanism does not apply generally for Caliciviruses comparable to Picornaviruses (Humoud et al., 2016). Again, it is important to note that each virus deals differently with SG antagonism.

SG proteins, such as G3BP1 and G3BP2, were found to interact with dengue virus 2 (DENV-2) 3' UTR and tend to colocalize with DENV replication sites, although for the latter the available evidence is somewhat unclear (Ward et al., 2011). Knockdown of both G3BPs and CAPRIN1 results in higher viral particle production, while overexpression of G3BP1 resulted in less. The three proteins slightly but significantly affect viral replication. Upon DENV infection, G3BPs have antiviral activity by regulating expression of ISG proteins and contributing to efficient ISG mRNA translation, such as *PKR* and *IFITM2*. To counteract this effect, DENV subgenomic flaviviral RNA (sfRNA) binds to G3BPs and this results in the downregulation of G3BP-induced ISG mRNA expression and translation (Bidet et al., 2014).

Human immunodeficiency virus 1 (HIV-1) was shown to impair SG assembly. The viral Gag polyprotein is capable of disassembling SGs by binding to G3BP1 (Valiente-Echeverría et al., 2014). Another study showed how G3BP1 restricts HIV-1 infection. HIV-1 replication increases upon G3BP1 knockdown. It was shown that G3BP1 interacts with the viral RNA in the cytoplasm, independent of the other SG components. Therefore, it was suggested that G3BP1 sequesters the viral RNA to block translation and/or packaging. This requires further study. Analysing whether the G3BP1 antiviral effect is in early or late stage of infection will clarify at which step G3BP1 blocks replication (Cobos Jiménez et al., 2015). Interestingly this negative effect of G3BP1 on HIV-1 replication was not seen on other retroviruses. Again, indicating the differences between antiviral mechanisms depending on the individual virus (Cobos Jiménez et al., 2015; Valiente-Echeverría et al., 2014).

There seems to be a role preserved for G3BPs during viral infection in many organisms. The nuclear shuttle proteins (NSP) of the plant virus begomo ablutio mosaic virus (AbMV) and the pea necrotic yellow dwarf virus (PNYDV), interact with *Arabidopsis thaliana* G3BP through an FVSF motif (Krapp et al., 2017). Also in insects, Rasputin (G3BP1 homolog) is associated with nsP3 (Gorchakov et al., 2008). However, this is outside of the scope of this thesis. It does illustrate how G3BPs are a common target for diverse viruses, which puts them forward as interesting topics for therapeutic interventions.

So far, G3BPs have been shown to be very involved in the replication of RNA viruses, but it is unclear whether G3BPs play a role in DNA viruses' infection as well.

1.3.4.3. G3BPs and Alphaviruses

This thesis focuses on alphaviruses, and more specifically Chikungunya virus. Dependent on the alphavirus, G3BPs act anti- or proviral. Generally, alphavirus infection causes SG formation, upon which the alphavirus nsP3 suppresses SG formation. For multiple alphaviruses, nsP3 has been shown to interact with the NTF2L domain of G3BPs and their interaction leads to the formation of nsP3-G3BP condensates (Alam & Kennedy, 2019; Cristea et al., 2006, 2010; Frolova et al., 2006; Gorchakov et al., 2008; Panas et al., 2014; Scholte et al., 2015). To identify nsP3 interactors, cells were infected with mutant SINV, in

which nsP3 was tagged with GFP. nsP3 interacts in early and late infection stages with G3BP (Cristea et al., 2006; Frolova et al., 2006). G3BPs are present in all nsP3 condensates, but whether dsRNA, and thus RCs, are present remains unclear. DsRNA was observed in some nsP3-G3BP condensates, but not all (Gorchakov et al., 2008). G3BP was also identified in SINV-nsP2 complexes. Whether this is through the interaction of nsP2 with nsP1 and nsP3, and thus G3BP, is not known (Atasheva et al., 2007). To identify host factors important to SINV replication, the interactome of nsP4, the viral RNA-dependent RNA polymerase, was analyzed. Both in early and late replication, nsP4 was associated with G3BP1 and G3BP2 (Cristea et al., 2010). Silencing G3BPs positively affects SINV polyprotein expression, and slightly affects virion production positively. However, it hardly affects SINV RNA expression (Cristea et al., 2010). So, for SINV, an antiviral effect is argued in this report. This is in contrast to other reports, in which SINV replication is negatively affected by G3BP double knockdown (dKD) (Kim et al., 2016; Scholte et al., 2015). In G3BP dKO cells SINV replication is reduced (Kim et al., 2016).

Also, during SFV infection nsP3 interacts with G3BPs and sequesters it into foci. Panas et al. argue for viral RNA replication complexes since these foci contain dsRNA. Sequestration of G3BP into nsP3 viral condensates, corresponds with SG-disassembly and blocking of further SG formation (Panas et al., 2012). Interestingly, SFV- replicon-induced G3BP-granules seem to lack other typical SG proteins (Panas et al., 2012). Interaction of nsP3 with G3BP1 and subsequent sequestering is dependent on the C-terminal FDGF-motifs of nsP3 (Panas et al., 2012; Panas, Ahola, & Mcinerney, 2014; Panas et al., 2015). The same motif is found in USP10 and responsible for G3BP1-binding (Kedersha et al., 2016; Panas et al., 2015; Sanders et al., 2020).

CHIKV nsP3 interacts with G3BPs, and thus inhibits SG formation by recruiting G3BP into their own condensates. CHIKV nsP3/G3BP condensates differ from classical SGs. Normally, SGs disassemble when treated with CHX. CHIKV-induced G3BP1 condensates do not (Fros et al., 2012; Scholte et al., 2015). Also, when looking at common SG markers, the composition of these condensates appears different (Fros et al., 2012; Jayabalan et al., 2021; Scholte et al., 2015). By G3BP sequestration, nsP3 inhibits SG formation in general (Fros et al., 2012). When analyzing G3BP colocalization with the different CHIKV nsPs and dsRNA, again clear colocalization of nsP3 and nsP2 with G3BPs was observed. There was light colocalization with nsP1, but not with nsP4. DsRNA colocalization with G3BP1 has been reported only once and was then observed during early infection (Scholte et al., 2015), contradictory to another study in which only limited overlap has been observed (Fros et al., 2012). G3BPs would colocalize with dsRNA during early infection and then form clear nsP3-G3BP cytoplasmic condensates. Since all the nsPs are required for replication and the RCs are argued to be membrane-bound, Scholte et al. argue that these nsP3-G3BP condensates are unlikely to be replication hubs. nsP2 and nsP3 have many cellular functions, thus G3BPs could be involved differently (Scholte et al., 2015). Upon knockdown of both G3BPs, replication of a CHIKV reporter virus decreased by $\pm 80\%$. G3BP depletion delayed replication cycle early on. Viral RNA levels, CHIKV protein expression and viral titer were reduced and interestingly the negative-strand RNA levels as well, despite presence of the nsPs. It does not seem to affect polyprotein processing, or the viral entry (Scholte et al., 2015). G3BP dKO is detrimental to CHIKV replication (Kim et al., 2016). In absence of G3BPs or the ability of nsP3 to interact with G3BPs, CHIKV is replication deficient (Kim et al., 2016). DKO G3BP cells reconstituted with G3BP rescues CHIKV replication. Interestingly, reconstitution with most of G3BP1 mutants with domain deletions, does not rescue CHIKV replication (G3BP1 Δ NTF2L; G3BP1 Δ IDR2; G3BP1 Δ RRM; G3BP1 Δ IDR3). Only, reconstitution with G3BP1 Δ IDR1 rescues CHIKV replication (Kim et al., 2016). This domain has an autoinhibitory function on LLPS and SG formation, as discussed extensively above (**chapter 1.4.1.**) (Yang et al., 2020). This begged the question, whether nsP3 needs G3BP for its role in condensation events. However,

nsP3 unable to bind G3BP, still forms condensates. G3BP dKO cells do not obstruct the virion attachment and entry, the RNA uncoating, and primary viral RNA translation. G3BPs absence affects the viral protein count and consequently RNA synthesis. Upon CHIKV infection of the G3BP dKO cells, no dsRNA was detected (Kim et al., 2016). Together this could suggest a role for G3BPs during RNA replication and RC assembly. Interestingly, of the Alphaviruses only CHIKV seems to be completely dependent on the presence of G3BPs (Kim et al., 2016). Pointing out again, the differences even between closely related viruses and the need to study each virus specifically. As mentioned before nsP3-G3BP condensates are different in composition and behaviour. More recently it was shown that nsP3 hydrolase activity is responsible for the different composition of SGs compared to nsP3-G3BP condensates (**Fig. 5**) (Jayabalan et al., 2021). SG form upon viral infection, and at later stages they are suppressed. After 9h, distinct nsP3-G3BP foci form. Mostly, the presence of translation initiation factors is affected, and upon inactive nsP3, these translation initiation factors are retained (Jayabalan et al., 2021). The same study also showed ADP-ribosylation of G3BPs and that nsP3 reverses this PTM (Jayabalan et al., 2021). One big setback of the study is that the studied SGs were induced using arsenite, and thus not virus induced SGs. It would be interesting to look at the effect in either a full virus or replicon-context.

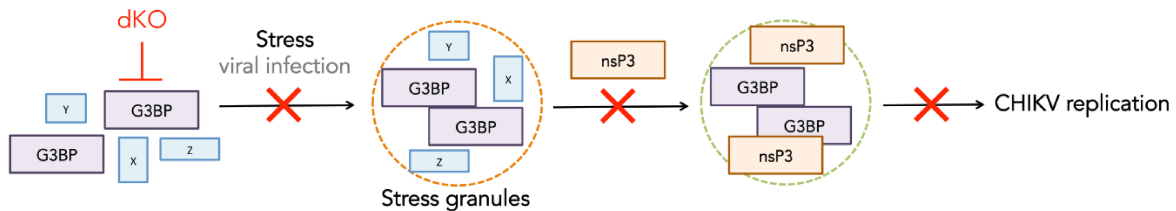


Figure 5. G3BPs are essential for stress granule formation and Chikungunya virus. G3BPs induce stress granule (SG) formation upon viral infection. However, Chikungunya virus suppresses SGs at a later stage during infection. CHIKV nonstructural protein 3 (nsP3) sequesters G3BPs within distinct new viral condensates of which the function is unknown. G3BP double knockout inhibits SG formation and block CHIKV replication. The latter indicates G3BPs as essential proviral host factors for CHIKV replication.

1.4. Alphaviruses

1.4.1. Introduction

Alphaviruses are a diverse group of arthropod-borne, small, enveloped positive-sense RNA viruses causing diseases in a wide host range. Their typical life cycle consists of a hematophagous arthropod as vector, generally mosquitoes, and a vertebrate host, namely birds, rodents, amphibians, reptiles or primates, among which humans. Infection of humans and/or domestic animals generally results in an acute, febrile illness with symptoms such as fever, rash, nausea, arthralgia and encephalitis depending on the species. Overall, disease caused by alphaviruses have a low mortality rate, but symptoms can last from months up to years, all the while seriously affecting the patient's life-quality (Gebhart et al., 2015; Weaver & Lecuit, 2015). However, some members of the genus do not seem to cause any diseases and their transmission cycle is enigmatic, such as Trocara virus and Aura virus (Weaver et al., 2012). Alphaviruses have been found on all continents, with the exception of Antarctica.

Outbreaks in humans and livestock are common and the genus thus forms significant economic and health threats to the population worldwide. For example, VEEV has caused major epidemics in equids and humans in multiple countries and an explosive CHIKV outbreak hit La Reunion Island in 2005 with 300.000 reported cases and multiple recurrent outbreaks since (Borgherini et al., 2007; Taylor & Paessler, 2013; Weaver & Lecuit, 2015). Currently there are no licensed vaccines or antiviral therapies available. More information and a deeper understanding of the alphavirus replication mechanism and the host-pathogen interface would open the way towards treatments. For years, most work has been done on SINV and SFV. Most likely there are general alphavirus mechanisms, which can help us understand other species through homology studies. However, increasingly with evolutionary distance there will be clear differences and variations in viral infection and replication between alphaviruses. Because of these reasons, it is of utmost importance to understand both alphavirus taxonomy and the need to study each species in its own right. More information on other alphaviruses is required.

1.4.2. Taxonomy

Table 1. Alphavirus Taxonomy. Classification of Alphaviruses from realm to species. Relevant viruses are emphasized.

Realm:	Riboviria
Phylum:	incertae sedis
Family:	Togaviridae
Genus:	Alphavirus
Species:	Old World viruses: Bebaru virus, Chikungunya virus , Getah virus, Mayoro virus, O'nyong'nyong virus, Ross River virus , Semliki Forest virus , Una virus, Sindbis virus New World viruses: Aura virus, Cabassou virus, Eastern equine encephalitic virus , Everglades virus, Fort Morgan virus, Highlands J virus, Mucambo virus, Pixuna virus, Rio Negro virus, Tonate virus, Trocara virus, Venezuelan equine encephalitis virus , Western equine encephalitic virus , Whataroa virus Others: Barmah Forest virus, Eilat virus, Madariaga virus, Middelburg virus, Mosso das Pedras virus, Ndumu virus, Salmon Pancreatic disease virus, Southern elephant seal virus,

The genus Alphavirus belongs to the Togaviridae family and includes 31 species of which multiple are important to public health (**Table 1**). The terrestrial alphaviruses are classified based on geographical distribution into Old World (OW) and New World (NW) alphaviruses

(Table 1). OW alphaviruses are generally arthropogenic and include CHIKV, Semliki Forest virus (SFV) and Ross River virus (RRV); NW alphaviruses are generally causing encephalitogenic symptoms and most important members include Venezuelan equine encephalitis virus (VEEV), eastern equine encephalitis virus (EEEV) and western equine encephalitis virus (WEEV). As mentioned before, generally mosquitoes act as the transmitting vector. However, there are two main exceptions: salmon pancreatic disease virus (SPDV), which infects salmon and trout, causes mortality in farmed fish (Weston et al., 1999), and Southern elephant seal virus (SESV) (La Linn et al., 2001). These viruses are transmitted by lice (Forrester et al., 2012; La Linn et al., 2001; Weston et al., 1999). The discovery of alphavirus species infecting fish leads to the assumption that there are still unknown species inhabiting less well-explored niches (Forrester et al., 2012).

Among the OW viruses of the SFV complex we find one of the most important human-disease causing alphaviruses: CHIKV. Chikungunya fever (CHIKF) is a rapid-onset disease, characterized by fever, rash and arthralgia. The disease is mostly located in Africa, Asia and India. However, outbreaks have affected several countries in America and there have been case reports in Southern Europe (Burt et al., 2017; Diop, 2015; Lindh et al., 2019). Among the NW viruses, VEEV is a significant public health threat, mostly located in America. VEEV infection in humans results mostly in mild flu-like syndrome. However, in a small percentage of cases, severe disease develops resulting in encephalitis with high mortality rate. 1–4% of VEEV patients develop neurological symptoms, resulting in lethality or lifelong consequences (Stromberg et al., 2020).

1.5. Chikungunya virus

1.5.1. Introduction

Chikungunya is a mosquito-borne pathogen causing Chikungunya fever. The *Aedes* mosquitoes, mainly *Ae. aegypti* and *Ae. albopictus*, are the main vectors that transmit the virus to humans (Bartholomeeusen et al., 2023; Matusali et al., 2019). CHIKV is a member of the *Togaviridae* family, genus *Alphavirus*. It was first discovered in the 1950 in Tanzania. The name is derived from the Makonde language, an ethnic group from Tanzania, and means “to become contorted”, pointing to its main recognizable symptom, joint pain and lasting arthralgia (Bartholomeeusen et al., 2023; Matusali et al., 2019). While CHIKV used to be more specific to Africa, CHIKV has evolved in an important human health threat in recent years worldwide. By now, CHIKV has been identified in over 110 countries (WHO). Outbreaks have occurred across the globe in Africa, Asia and central and south America and cases in the south of Europe have also been reported (Bartholomeeusen et al., 2023; Matusali et al., 2019).

The emergence of mosquito born viruses is due to multiple reasons: globalisation, travel, viral evolution and spread of the vectors. Concerning the latter, due to climate change the habitat of mosquitoes expands and spreads to previously uninhabitable regions. Also, through urbanisation, there is more stagnant water areas and thus an increase in breeding places. The vectors will keep spreading further and further and more and more outbreaks are expected. The prevention strategies for Arboviruses to limit the mosquito spread include the use of insecticides, mosquito nets and limiting stagnant water sources. However, it is predicted that in many countries, CHIKV will become endemic. CHIKV is categorized as a virus of significant concern and requires further research (Bartholomeeusen et al., 2023; Matusali et al., 2019). Generally, CHIKF is characterized by symptoms such as a sudden onset of high fever, joint pain or swelling, rash, fatigue, headache, nausea and muscle pains. However, CHIKF can be divided into two phases: an acute and a chronic phase. In the acute phase, most patients develop symptoms (85-95%) such as fever, arthralgia, arthritis, myalgia, headache, and rashes (Bartholomeeusen et al., 2023; Suhrbier, 2019). This phase lasts up to 2 weeks. Due to the fact that Dengue and Zika have similar symptoms, CHIKF is often misdiagnosed. So, the number of people affected by chikungunya is underestimated. In severe cases neurological, cardiovascular, respiratory and renal symptoms develop and this can lead to chronic CHIKF (Suhrbier, 2019). Although many infected individuals recover fully from CHIKF, up to 30% of infected people suffer from chronic arthralgia lasting for months up to years (Bartholomeeusen et al., 2023; Suhrbier, 2019). Other severe consequences are encephalitis, cardiac failure, renal failure, multiorgan failure or septic shock that require hospitalization (Suhrbier, 2019). However, death, mortality rates from 0.024-0.7%, or severe lasting symptoms are rare, and if so in older or younger patients and/or patients with underlying health problems. Chronic CHIKF is mainly characterized by chronic arthritis or arthralgia of the joints (Suhrbier, 2019). Although CHIKF does not have high mortality rates, it impacts many patients' quality of life, and poses a social and economic burden (Bartholomeeusen et al., 2023; Suhrbier, 2019).

There is still a lack of understanding of the molecular mechanism and the host-pathogen interface of CHIKV. At the moment there is no cure, vaccine or fitting therapeutic against CHIKV. Treatment is limited to easing symptoms and giving supportive care. However, several candidates for vaccines are in phase I or II clinical trials (Schrauf et al., 2020). One CHIKV vaccine is past phase III clinical trial and looks very promising, VLA1553 of Valneva. The vaccine is live-attenuated and designed by deleting a part of the chikungunya virus genome, at the end of non-structural protein 3 (nsP3). Valneva reports a positive antibody persistence up to a year after vaccination with a single dose of VLA1553 and no other safety

concerns. Currently, it awaits the final stages of approval (Martinon, 2023; Schneider et al., 2023).

Further research on CHIKV pathogenesis and replication is required in the efforts of finding specific therapeutics or a vaccine. Currently, there is still a lack of knowledge on the viral life cycle, the viral proteins and viral immune modulation. This research would help in vaccine and antiviral drug development.

1.5.2. Chikungunya life cycle and replication

The life cycles of the Alphaviruses are closely related. Since this work focuses on CHIKV as a model virus, the life cycle and replication of CHIKV is discussed in detail. When a mosquito bites an infected CHIKV patient, the virus is taken up through the blood. It replicates within the mosquito and ends up in the salivary glands. Upon the next bite, CHIKV is transmitted to the new host (Bartholomeeusen et al., 2023; Frolov & Frolova, 2018; Matusali et al., 2019). Initial replication of CHIKV in humans takes place in keratinocytes and melanocytes. Through the blood stream it spreads and infects multiple cell types, peripheral blood mononuclear cells (PBMCs) including mainly monocytes but also B-cells and dendritic cells. CHIKV travels through the blood to the lymph nodes and spleen, skeletal muscles, joints, and bone and in rare occasions to the nervous system and the kidney or respiratory tract (Bartholomeeusen et al., 2023; Matusali et al., 2019). Main target cells are fibroblasts, monocytes, and macrophages. Firstly, CHIKV binds to a host cell receptor, and enters the cells through receptor-mediated endocytosis. The argued receptor for CHIKV is matrix remodeling associated protein 8 (MXRA8) (Basore et al., 2019; Zhang et al., 2018). MXRA8 would bind to the exposed patches of the E2 glycoprotein of CHIKV (Basore et al., 2019; Zhang et al., 2018). Generally, Alphaviruses are argued to be taken up through clathrin-mediated endocytosis. Upon endocytosis, the virus vesicle fuses with endosomes. The pH change results in conformational changes of the CHIKV glycoproteins and the virus fuses with the endosomal membrane which eventually leads to the release of the nucleocapsid and the 49S RNA genome into the cytoplasm (Bartholomeeusen et al., 2023; Frolov & Frolova, 2018).

The alphavirus genome is a positive sense, single stranded RNA ((+)ssRNA) of ± 12 kb. The (+)ssRNA genome has a 5'(m⁷Gppp)-cap and a 3' poly-A tail (**Fig. 6**). The first nucleotide is not methylated so the alphaviral cap is termed Cap(0). The 5'UTR does not display sequence conservation but is predicted to fold into two distinct stem-loop structures (Frolov & Frolova, 2018). The presence of these structures is said to prevent recognition of the cap(0) by IFIT1, which would block interaction with the cellular translation initiation factor eIF4E. It also has been suggested to hide the viral RNA from detection by the innate immunity sensors e.g., retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated protein 5 (MDA5) or other antiviral defense mechanisms (Frolov & Frolova, 2018).

The viral genome is subdivided into two open reading frames (ORFs) (**Fig. 6**). The first ORF encodes the nonstructural proteins nsP1-nsP4. Alphaviruses replicate in the cytoplasm and upon viral entry the genomic RNA is translated. Translation of ORF1 is done by host cell machinery and results in production of either polyprotein P123 ($\pm 90\%$), or P1234 ($\pm 10\%$), depending on read-through of the opal stopcodon at the nsP3-nsP4 junction (Frolov & Frolova, 2018; Lit & Rice, 1993). P123 and P1234 are post-translationally processed in cis or trans by the viral protease nsP2 into the individual nsPs (nsP1, nsP2, nsP3 and nsP4) (Bartholomeeusen et al., 2023; Frolov & Frolova, 2018). Alphavirus RNA replication requires each non-structural protein both to function in complex, as the viral replication complex, and in their individual forms with separate functions (Bartholomeeusen et al., 2023; Frolov & Frolova, 2018). The RC is associated to the plasma membrane and uses nsPs and host protein activities for viral replication and transcription. P123 in complex with nsP4 is the minus-strand replication complex and capable of (–)strand RNA synthesis from the genomic

RNA (**Fig. 6**). Both P123, lacking RNA synthetic activity, and P1234, with the RNA-dependent RNA polymerase (RdRp) nsP4 not yet released, are incapable of (-)strand synthesis (Frolov & Frolova, 2018). Further processing of P123 by nsP2, in cis or trans, results in cleavage of nsP1 from the polyprotein. This point marks the transition towards (+)strand synthesis (Frolov & Frolova, 2018). The mature RC consisting of all the individual nsPs allows the subsequent synthesis of new genomic 49S RNA and the subgenomic 26S RNA, ORF2, with the (-)strand RNA as a template (**Fig. 6**) (Frolov & Frolova, 2018). nsP1 interacts with membranes and has methyltransferase (MTase) and guanylyltransferase (GTase) activity (Bartholomeeusen et al., 2023; Frolov & Frolova, 2018). nsP2 is the protease required for the processing of P123 and P1234. It also has nucleoside triphosphatase (NTPase) and RNA helicase activities (Bartholomeeusen et al., 2023; Frolov & Frolova, 2018). nsP3 is a hub for protein-protein interactions and has mono-ADP-ribosylhydrolase activity (Bartholomeeusen et al., 2023; Frolov & Frolova, 2018). nsP4 is the viral RNA-dependent RNA polymerase that catalyzes plus- and minus-strand RNA synthesis (Bartholomeeusen et al., 2023; Frolov & Frolova, 2018). Below, all 4 nsPs will be discussed. However, the main focus is on nsP1, due to its relevance in the thesis.

ORF2, expressed from the subgenomic promotor (26S RNA), contains the information for the structural alphaviral proteins. This includes the capsid protein (C-protein), envelope proteins E1 and E2, and two smaller structural proteins not usually found in virions (Bartholomeeusen et al., 2023; Frolov & Frolova, 2018; Rupp et al., 2015). The structural polyprotein is differently processed. First, the structural polyprotein is auto-processed by the C-protein, which leads to its own release. Then the C-protein assembles the icosahedral nucleocapsid with the newly replicated genomic RNA (gRNA) to give rise to the capsids in the cytoplasm (Frolov & Frolova, 2018; Rupp et al., 2015). The rest of the structural polyprotein (pE2-6K-E1) is transported through the ER and processed to the individual proteins, precursor E2 (pE2), 6K and E1 by host signal peptidases (Frolov & Frolova, 2018; Rupp et al., 2015). The respective proteins reach the cell surface through the ER-Golgi secretory machinery. The cellular protease Furin further processes the glycoproteins into their mature forms and the final glycoproteins are deposited at the plasma membrane (Bartholomeeusen et al., 2023). The 6K protein is argued to be involved in this process, but so far, a clear distinct function is unknown (Bartholomeeusen et al., 2023; Ramsey & Mukhopadhyay, 2017). Upon exocytosis, the nucleocapsid is enveloped by the E2-E1 heterodimeric glycoprotein and lipid membrane (Bartholomeeusen et al., 2023).

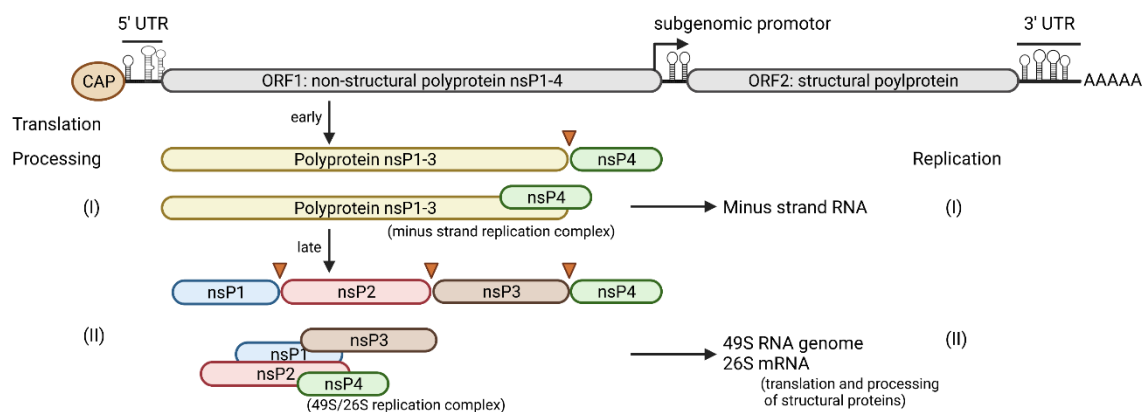


Figure 6. Replication cycle of CHIKV. Schematic representation of the CHIKV genome and replication. The (+)ssRNA genome has a 5'(m⁷Gppp)-cap and a 3' poly-A tail. The 5'UTR is predicted to fold into distinct stem-loop structures. ORF1 is translated into the polyprotein P1234. This polyprotein is proteolytically cleaved into the separate nsPs by nsP2. P123 and nsP4 assemble into the (-)strand replication complex, which forms the template for genomic RNA (gRNA) replication and transcription of the subgenomic (26S) RNA. The polyprotein is further processed into the separate nsPs. The mature RC consists of the individual nsPs1-4 and allows the subsequent synthesis of new genomic 49S RNA and subgenomic 26S RNA which codes for the structural proteins. Figure was designed by P. Korn.

1.5.3. Non-structural Protein 1

1.5.3.1. General

NsP1 is 535 amino acid long protein and has a theoretical molecular weight of 60 kDa. The 3D macromolecular structure of alphavirus nsP1 was analyzed with cryo-electron microscopy (Jones et al., 2020). The protein fulfils multiple functions during replication. One of nsP1's main activities is the capping of the viral genomic and subgenomic RNAs. NsP1 is also responsible for membrane association of the replication complex with cellular membranes (Frolov & Frolova, 2018). These are the two best-studied functions of nsP1. However, these two may not be the only nsP1 specific functions. NsP1 has also been proposed to induce actin rearrangements and to play a role in particle release. All proposed functions are discussed below in more detail. The nsP1s of the different Alphaviruses are closely related and are thus often compared to one another, or mechanisms are extrapolated to Alphaviruses in general. However, it remains of utmost importance to study also each virus in its own right and the functionality of the individual proteins.

NsP1 is built up of three domains identified and predicted through computational analysis and homology studies (**Fig. 7**). First there is an N-terminal domain containing a Rossman-like MTase followed by a GTase (residue 1-170) (Bartholomeeusen et al., 2023; Frolov & Frolova, 2018; Rupp et al., 2015). Next, there is a middle membrane binding domain (MB) involved in nsP1 association to host membranes (residue 171-300). It contains some tandem features and an amphipathic helix (Bartholomeeusen et al., 2023; Frolov & Frolova, 2018; Rupp et al., 2015). Lastly there is a C-terminal domain (301-535 aa) of which the function remains unclear. However, the C-terminus has palmitoylated cysteines, which together with the amphipathic helix, are responsible for membrane association (Bartholomeeusen et al., 2023; Frolov & Frolova, 2018; Rupp et al., 2015). Further, it contains an IDR, spanning amino acids 478-535 (VSL2 Predictor Developed by K. Peng and Z. Obradovic). IDRs are sequence segments containing a low amount of hydrophobic amino acids, which renders them unable to form a unique organized hydrophobic core. This results in a variety of possible conformations in physiological conditions (Romero et al., 2001; Uversky et al., 2013). This IDR can function as a hub for protein interactions (Dunker et al., 2005; Kim et al., 2008).

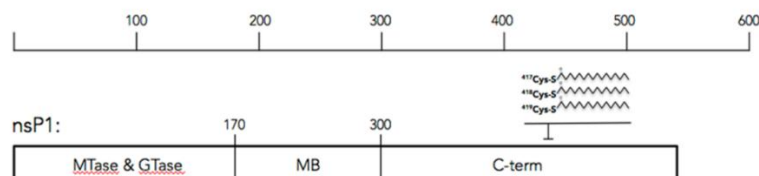


Figure 7. Domain architecture of nsP1. N-terminally nsP1 has a methyltransferase (MTase) domain and a guanylyltransferase domain (GTase). Central it has a membrane binding domain (MB) which contains tandem features and an amphipathic helix. The C-terminal domain has so far, an unknown function, but is also involved in membrane association through conserved cysteines.

1.5.3.2. Alphaviral capping mechanism

The alphavirus capping mechanism takes place in the cytoplasm. It has been described to be in stark contrast to the general eukaryotic capping mechanism, which takes place in the nucleus (Follett et al., 1975; Mizumoto & Kaziro, 1987; Shuman, 1995). Viral enzymes place a Cap(0) (m⁷GpppA)-structure at the 5' end of the viral genomic and subgenomic RNAs (Dubin et al., 1979; Hefti et al., 1976; Vasiljeva et al., 2000). Viral RNA capping is a key step in virus replication for bypassing the innate immune system, avoiding recognition by intracellular receptors, protecting the viral mRNAs from degradation by host 5'-3' exonucleases and enabling efficient translation (Decroly et al., 2012; Kumar et al., 2014; Reynaud et al., 2015). Capping of the genomic (G) and subgenomic (SG) RNA is mainly achieved by the

methyltransferase (MTase) and guanylyltransferase (GTase) enzymatic activities of nsP1 (Ahola et al., 1997; Ahola & Kääriäinen, 1995; Cross, 1983; Laakkonen et al., 1994; Rozanov et al., 1992; Russo et al., 1996; Schluckebier et al., 1995). These activities were first identified 40-50 years ago (Cross, 1983; Mi & Stollar, 1990, 1991; Scheidel & Stollar, 1991). Besides nsP1, alphaviral capping requires the nsP2 triphosphatase activity and S-adenosyl methionine (SAM) and guanosine-5'-triphosphate (GTP) as donors for synthesizing the m⁷GMP-cap (Ahola & Kääriäinen, 1995; Karpe et al., 2011; Vasiljeva et al., 2000). First, the nascent viral RNA is primed by nsP2 triphosphatase activity. NsP2 cleaves the γ - β -phosphate bond at the 5' end of the nascent RNA, leaving a diphosphate group (Karpe et al., 2011; Vasiljeva et al., 2000). Following, the MTase activity of nsP1 uses SAM as methyl donor and forms m⁷GTP and S-adenosyl-homocysteine (SAH). Then the GTase activity of nsP1 forms a covalent bond between the phosphate group of m⁷GTP and its amino acid H37 forming a m⁷GMP-nsP1 intermediate. Lastly, nsP1 transfers the m⁷GMP-cap to the primed RNA (ppRNA) by 5'-5'-linkage through its GTase activity (**Fig. 8**) (Ahola et al., 1997; Ahola & Kääriäinen, 1995; Li et al., 2015; Ortega Granda et al., 2021). More recently, it was shown that a NW alphavirus, namely VEEV, follows the same RNA capping mechanism (Li et al., 2015; Ortega Granda et al., 2021). The MTase activity has been described to be necessary for the GTase activity, as GTase activity is not detected in absence of MTase activity and/or a methyl donor even though m⁷GTP is present (Ahola et al., 1997; Ahola & Kääriäinen, 1995). GTase activity is dependent on the presence of Mg₂ or Mn₂ (Laakkonen et al., 1994; Li et al., 2015; Mi & Stollar, 1991). NsP1-capping mutants lacking MTase or GTase activity of SFV are non-viable (Ahola et al., 1997). Concurrently, it was shown that mutations in conserved amino acid residues abolished SINV infectivity, again pointing to the necessity of viral RNA capping for successful viral replication (Niesterst & Strauss, 1990; Wang et al., 1996). Clearly, capping activity is essential in the Alphavirus replication cycle and mutation of conserved residues important for either nsP1 MTase or GTase harms viral replication. The capping process is thus an interesting antiviral drug target and provides a new avenue for developing inhibitors specific to this process (Kallio et al., 2016; Wang et al., 1996). RNA capping activity seems not to be required for negative-strand synthesis and spherule biogenesis (Kallio et al., 2016). However, SINV and RRV alphavirus genomic RNAs are not ubiquitously capped, making the nsP1-/nsP2-mediated capping reaction not absolute (Lapointe et al., 2018; Sokoloski et al., 2015). The production of non-capped viral genomic RNAs (ncgRNAs) seems to play an important role in establishing and maintaining infection: they are abundantly synthesized early during infection (Sokoloski et al., 2015); attenuated RRV mutants produce less ncgRNAs than WT RRV (Sokoloski et al., 2015; Stoermer Burrack et al., 2014); both, SINV cgRNAs and ncgRNAs are encapsulated into viral particles (Sokoloski et al., 2015); the presence of ncgRNAs correlates with the activation of the cellular innate immune response (Sokoloski et al., 2015). Both the MTase and GTase activity of nsP1 can be both negatively or positively altered through specific point mutations *in vitro* (Ahola et al., 1997; Li et al., 2015; Wang et al., 1996). Later, it was found that decreasing the amount of ncgRNAs, due to changes in capping rate, negatively impacts viral particle production and viral titer (Lapointe et al., 2018). However, the precise mechanism of how ncgRNAs impact particle production remains unclear. As mentioned above, a byproduct of alphavirus capping is SAH (Ahola & Kääriäinen, 1995). The GTase activity is strongly stimulated by the presence of SAH. It has been suggested that SAH induces conformational changes of nsP1 favoring the GTase activity (Ahola et al., 1997; Li et al., 2015). Under normal circumstances, SAH is subsequently hydrolyzed by SAH hydrolase. Inhibition of SAH hydrolase has been described to interfere with the capping process, due to the role of SAH as a feedback inhibitor. The formation of guanylated-nsP1 adduct is regulated by the SAM/SAH balance (De Clercq, 1998; Decroly & Canard, 2017; Li et al., 2015; Turner et al., 2000; Wolfe & Borchardt, 1991). The role of SAH hydrolase as a target for antiviral compounds has long been known and there are multiple inhibitors known as potent antiviral compounds against a wide range of (-)ssRNA viruses, dsRNA viruses, and DNA viruses (De Clercq, 1998; Decroly & Canard, 2017). More recently,

SAH hydrolase inhibitors have been described targeting (+)ssRNA viruses and alphaviruses, among which VEEV and CHIKV (Kovacikova et al., 2020; Li et al., 2015; Yoon et al., 2019).

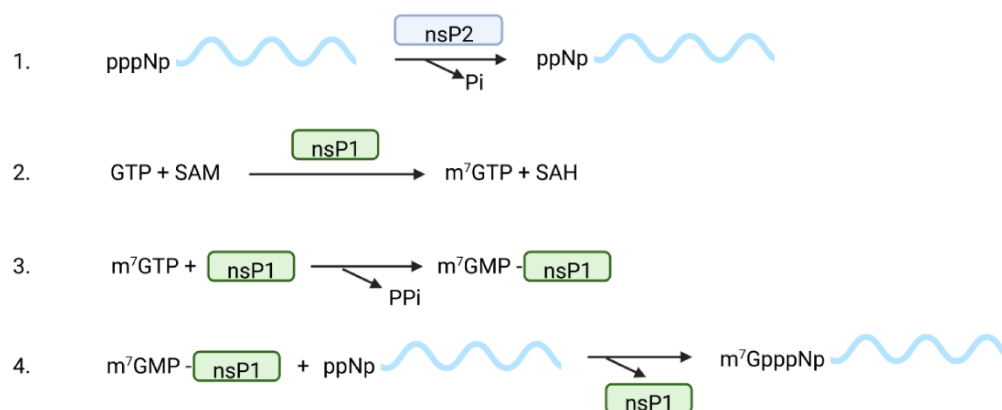


Figure 8. Alphaviral capping mechanism. First, nsP2 primes the viral RNA by cleavage of the terminal phosphate bond at the 5' end of the nascent RNA, leaving a diphosphate group. Secondly, nsP1 methyltransferase (MTase) activity uses S-Adenosyl methionine (SAM) and Guanosine-5'-triphosphate (GTP) to form m^7GTP and S-adenosyl-homocysteine (SAH). Then the nsP1 guanylyltransferase (GTase) activity induces a covalent bond between the phosphate group of m^7GTP and nsP1 H37 forming the m^7GMP -nsP1 intermediate. Lastly, nsP1 transfers the m^7GMP -cap to the primed RNA (ppRNA) through its GTase activity.

1.5.3.3. NsP1 membrane association

The alphavirus superfamily replicates inside spherules, small membrane compartments in the cytoplasm of infected cells. Here, synthesis of both (+)sense and (–)sense RNA occurs by the membrane associated RCs, containing all nsPs and host factors (Bartholomeeusen et al., 2023; Frolov & Frolova, 2018; Rupp et al., 2015). This way, replication can take place safe from detection by the innate immune system. Membrane-associated replication is a common mechanism of (+)ssRNA viruses to evade cellular antiviral processes (Neufeldt et al., 2016; Salonen et al., 2004). Following replication, all components are in close proximity and available for packaging. (Froshauer et al., 1988; Harak & Lohmann, 2015; Kujala et al., 2001; Paul, 2013; Peränen & Kääriäinen, 1991). Another vital function of alphaviral nsP1 is membrane association and nsP1 has been described to be solely responsible for anchoring the RC to host membranes (Salonen et al., 2003). NsP1 or an nsP1-containing-RC anchors to the cellular membranes through an amphipathic helix that interacts with anionic phospholipids and through nsP1 palmitoylation (Laakkonen et al., 1996; Lampio et al., 2000).

The amphipathic helix is formed by the amino acids ± 244 -263 (exact location depends on the alphavirus). Mutating the amphipathic helix, for example R253E and W259A, results in loss of SFV nsP1 membrane interaction as well as in the loss of spherule generation and is detrimental to viral replication in cells (Kallio et al., 2016; Spuul et al., 2007).

Palmitoylation, or S-palmitoylation, is the attachment of palmitic acid, the most common saturated fatty acid found in animals, to a substrate via a thiol group of a cysteine (Hang & Linder, 2011; Resh, 2016). This process is catalyzed by the zinc finger Asp-His-His-Cys (DHHC) domain-containing (ZDHHC) palmitoyl acyltransferase (PAT) family (Gottlieb & Linder, 2017). This PTM increases protein hydrophobicity and induces trafficking of these proteins to cellular membranes (Gottlieb & Linder, 2017). Palmitoylated nsP1 from SFV, SINV and CHIKV localizes to the plasma membrane. Palmitoylation occurs on C418-420 for SFV, on C417-419 for CHIKV and for SINV, which contains only one cysteine residue, on C420 (Ahola et al., 2000; Laakkonen et al., 1996; Zhang et al., 2018). Both SFV and SINV viruses mutated to prevent palmitoylation remained viable, although replicated with a hampered replication time (Ahola et al., 2000; Laakkonen et al., 1996; Žusinaite et al., 2007). Also, the

localization of the other nsPs to host membranes, mediated by nsP1, remained unaltered in these mutants (Ahola et al., 2000). In CHIKV, triple mutation of these cysteines blocked palmitoylation and the infectivity of the mutated virus was strongly diminished compared to the WT CHIKV (Zhang et al., 2018). The results from different alphaviruses seem to be consistent with each other. Palmitoylation of the cysteines does not seem to be essential for nsP1 activity or its membrane binding properties (Ahola et al., 2000; Zhang et al., 2018). The primary mechanism for nsP1-membrane binding is through the amphipathic peptide reinforced by palmitoylation to tighten the association.

Replication and infectivity levels are thus affected by blocking palmitoylation. Also, it should be mentioned that even though the SFV non-palmitoylatable mutants lack a specific phenotype in cellular experiments, they did exhibit diminished pathogenesis in mice (Ahola et al., 2000). This effect could be due to alternative functional roles of nsP1 palmitoylated cysteines. Only palmitoylated nsP1 would be able to induce membrane and cytoskeletal rearrangement, and the development of filopodia-like structures (Gottipati et al., 2020). This in turn could affect cell-to-cell transmission and pathogenesis (Laakkonen et al., 1998). Also, nsP1 palmitoylation could be linked to the structure of nsP1 and its interactions to other RC components, such as nsP4 (Žusinaite et al., 2007).

Recently, Bakhache et al. reported that CHIKV nsP1 interacts with cholesterol-rich plasma membrane microdomains (DRMs), also known as lipid rafts. Palmitoylation of CHIKV nsP1 cysteines was critical for its targeting to the lipid rafts, again showing a new role for nsP1 palmitoylation. Sequestration of cholesterol diminished CHIKV replication. Interestingly, SINV has a low affinity for cholesterol and only colocalizes slightly with DRMs. This again points to differences in the alphavirus genus (Bakhache et al., 2020).

For CHIKV, ZDHHC2 and ZDHHC19 interact with nsP1 and promote nsP1 palmitoylation. Also, the knockdown of ZDHHC2 and ZDHHC19 reduced overall CHIKV replication, indicating their important role as proviral factors (Zhang et al., 2018).

SFV was found to require the presence of lipids for nsP1 activity (Ahola et al., 1999). In contrast, SINV nsP1 is active in absence of lipids (Tomar et al., 2011). Therefore, the need for membrane-association for nsP1 activity seems to depend on the respective alphavirus. The coupling of nsP1 activity and membrane interaction would suggest that nsP1 is adapted to a membranous environment. How the presence of phospholipids affects CHIKV nsP1 activity has not been shown yet. A more recent study suggested that 12 nsP1 subunits assemble in a membrane-associated ring, which functions as the pore to the viral RNA synthesis reactors. This way, nsP1 can control the capping of the viral RNA during the exit from the RC (Jones et al., 2020). It was proposed that the assembly into the ring structure would be necessary for allosteric activation of nsP1 capping activity (Jones et al., 2020).

1.5.3.4. Membrane and cytoskeletal rearrangements

NsP1 has also been described to induce plasma membrane rearrangements and filopodia formation (Bakhache et al., 2020; Laakkonen et al., 1998). Upon infection, alphaviruses induce formation of membrane invaginations, also called spherules, wherein viral replication takes place (Frolov & Frolova, 2018). These invaginations are formed with a constricted neck through which it is connected to the cytoplasm. Salonen et al. postulated that not nsP1 alone but expression of P123 protein was the minimal requirement to induce spherule-like structures in SFV infected cells (Salonen et al., 2003). More recently, Hellstrom et al. showed that the minimal requirement for spherule formation by SFV was the presence of P123 and nsP4 or P23, nsP1 and nsP4 (Hellström et al., 2017). Interestingly, this is the same conformation as required for negative-strand RNA synthesis (Lemm et al., 1994). Both groups showed that presence of unprocessed nsP1, still in P123, induces spherule formation

but needs to interact with other viral and/or cellular proteins to induce spherule formation (Hellström et al., 2017; Salonen et al., 2003). However, it is surprising that individually cleaved nsP4 would be essential for spherule formation. This could be due to its strong interaction with nsP1 and could thus increase membrane recruitment of P123 or P23 in the presence of nsP1. Alternatively, nsP4 interacts with host factors that promote spherule formation (Hellström et al., 2017).

As mentioned before, Gottipati et al. further studied CHIKV nsP1 membrane association. In their study, they also showed that interaction between nsP1 and phospholipids resulted in a change of nsP1 secondary structure. Also, following membrane binding, full-length nsP1, but not the amphipathic helix, induced structural reorganization of the liposomes. Again, suggesting that nsP1 is responsible for the subsequent membrane reorganization upon binding (Gottipati et al., 2020).

SFV or SINV nsP1 expression induces filopodia-like extensions. NsP1 causes disassembly of the actin cytoskeleton and these filopodia-like structures do not contain F actin. NsP1 catalytic activity is not required for their formation. However, only palmitoylated nsP1 induces the filopodia-like structures on the cell surface (Laakkonen et al., 1998). Palmitoylation-defective nsP1 mutants no longer localize to filopodial extensions (Ahola et al., 2000). This could be a reason behind the reduction of viral replication of palmitoylation-deficient nsP1 mutants (Utt et al., 2019; Zhang et al., 2019).

1.5.3.5. NsP1 in the late Replication Complex

As mentioned above, the late replication complex is an organized structure built through interaction between the four nsPs. How each of the nsPs interact and how the late RC complex is built up is discussed here. Interestingly nsP1 was the only nsP to interact with all other nsPs (**Table 2**), indicating its important role in late RC formation and stabilization (Salonen et al., 2003; Shirako et al., 2000; Sreejith et al., 2012; Žusinaite et al., 2007). For SFV, co-IPs showed nsP1 interacting with nsP3 and nsP4 (Salonen et al., 2003). These interactions could be important for assembly and stability of the RC. As mentioned before, mutations preventing nsP1 palmitoylation attenuate viral replication in SFV. Žusinaite et al. found the mutations to affect nsP1-nsP4 interaction. They suggested that nsP1 palmitoylation is not the determining factor for membrane association but may be more important for RC build-up and nsP1 interaction with RC components (Žusinaite et al., 2007). Further research into nsP interactions was done by Sreejith et al. They expressed CHIKV nsPs and used yeast two-hybrid (Y2H), Glutathion-S-Transferase (GST) pull-down and enzyme-linked immunosorbent assays (ELISA) to detect interactions. NsP1 interacted with nsP3 and nsP4 in all three assays; nsP2 interacted with nsP1 in the GST pull-down and ELISA. This way they firstly identified nsP interactions for CHIKV (Sreejith et al., 2012). Sreejith et al. suggested that nsP1, due to its membrane binding property, might be important for keeping the RC intact (Sreejith et al., 2012). A complete nsP interaction and RC organization study followed for CHIKV by Rana et al. They used systems such as pull down, ELISA, and Y2H. For nsP1, it was shown that the MTase domain and the MB domain could interact with the N-terminal helicase domain of nsP2 and the C-terminal domain of nsP4. The MD and Alphaviral unique domain (AUD) of nsP3 interacted with the C-terminal domain of nsP1 (Rana et al., 2014). Following they computationally generated protein structures and with the information of the interaction studies, they created a 3D structure of the spatial arrangement of nsPs in CHIKV late RC (Rana et al., 2014).

More recently, Kumar et al. further characterized the interaction between nsP1 and nsP2 (Rana et al., 2014; Sreejith et al., 2012; Kumar et al., 2018). NsP1 and nsP2 colocalize in the cytoplasm and interact in infected cells through aa 1-95 of nsP2 and aa 170-288 of nsP1 (Kumar et al., 2018). Interestingly, the presence of nsP1 enhanced nsP2 ATPase activity.

Hence, Kumar et al. speculated that nsP2 residues 1-95, mapped outside of the ATPase domain (residues 167-630), could have a regulatory function (Kumar et al., 2018). ATPase activity is important for nsP2 helicase activity, which is crucial for CHIKV replication (Das et al., 2014; Rikonen et al., 1994). Binding of nsP1 could induce conformational changes of nsP2, in turn modulating nsP2 ATPase activity. Also, nsP2 binding to nsP1 residues 171-300, MB region of nsP1, indicate further functions of this region besides membrane binding. Moreover, nsP2 primes the RNA for nsP1-mediated capping, discussed at length above. However, there is no predicted RNA-binding motif in nsP1. So nsP1-nsP2 interaction might also be of importance during capping.

Table 2. The predicted nsP interactions in the late replication complex. √: interaction; X: no interaction; FL: Full-length; MT: Methyltransferase; MB: Membrane-binding; Cterm: C-terminal domain; N-term: N-terminal domain; MD: Macrodomein; AUD: Alphavirus Unique Domain; HVD: Hypervariable domain; RdRp: RNA-dependent RNA polymerase; 1. Salonen et al., 2003; 2. Zusinaite et al., 2007; 3. Sreejith et al., 2012; 4. Rana et al., 2014; 5. Kumar et al., 2018.

	nsP1				nsP2				nsP3				nsP4	
	FL	MT	MB	Cterm	FL	N-term	Helicase	Protease	FL	MD	AUD	HVD	FL	RdRp
nsP1	√ ^{3,4}	√ ⁴	X ⁴	X ⁴	√ ^{3,4,5}	√ ⁵	√ ⁴	X ⁴	√ ^{1,3,4}	√ ⁴	√ ⁴	X ⁴	√ ^{1,2,3,4}	√ ⁴
nsP2	√ ^{3,4,5}	√ ⁴	√ ^{4,5}	X ⁴	X ^{3,4}	X	X ⁴	X ⁴	X ^{3,4}	X ⁴	X ⁴	X ⁴	√ ^{3,4}	√ ⁴
nsP3	√ ^{1,3,4}	X ⁴	X ⁴	√ ⁴	X ^{3,4}	X	X ⁴	X ⁴	X ^{3,4}	X ⁴	X ⁴	X ⁴	X ^{3,4}	X ⁴
nsP4	√ ^{1,2,3,4}	√ ⁴	√ ^{2,4}	X ⁴	√ ^{3,4}	?	√ ⁴	√ ⁴	X ^{3,4}	X ⁴	X ⁴	X ⁴	√ ^{3,4}	√ ⁴

1.5.4. Nsp2

Nsp2 is the best studied CHIKV nsP. It localizes to the nucleus and to the cytoplasm. Depending on the localization it exerts different functions (Frolov & Frolova, 2018; Rupp et al., 2015). It has an N-terminal stretch that can interact with nsP1 (**Table 2**) followed by a domain predicted to have nucleoside triphosphatase activity (NTPase), helicase and 5'-RNA triphosphatase (RTPase) activity (Frolov & Frolova, 2018). ATPase activity was suggested to be boosted by nsP1-nsP2 interaction (Kumar et al., 2018). The helicase activity was suggested to function in coordination with nsP4 to unwind the dsRNA (Rupp et al., 2015). Nsp2 RTPase activity was discussed above (**chapter 1.6.3.2.**). Following there is the protease domain, for which nsP2 is most known. The protease domain is responsible for the essential cleavage of the ns polyprotein. C-terminally there is an MTase-like domain.

CHIKV nsP2 protease activity has been suggested to depend on a catalytic cysteine and a catalytic serine, both need to be mutated for the protein to become inactive (Frolov & Frolova, 2018; Saisawang et al., 2015; Tomar & Aggarwal, 2017). CHIKV nsP2 cleaves either the ns polyprotein in cis- or in trans. It was suggested to cleave nsP3/4 site in cis and nsP1/2 and nsP2/3 sites in trans (Frolov & Frolova, 2018; Rupp et al., 2015; Tomar & Aggarwal, 2017). Initially, there is a low amount of polyprotein, so the first cleavage nsP3/4 happens in cis, leading to the release of nsP4. Later, the presence of more polyprotein promotes trans cleavage (Frolov & Frolova, 2018; Rupp et al., 2015). Besides the protease domain, the MTase-like domain is also suggested as essential to processing for Alphaviruses. Mutations of the MTase-like domain significantly reduces CHIKV cytopathogenicity (Frolov & Frolova, 2018).

Another major function of nsP2 is regulating host cell factors and blocking the antiviral immune response. This function was suggested to be mediated through interaction partners rather than nsP2 catalytic activity (Akhrymuk et al., 2012; Frolov & Frolova, 2018; Fros & Pijlman, 2016; Göertz et al., 2018; Meshram et al., 2019). Nsp2 interacts with proteins involved in transcription, cytoskeletal organization and protein degradation (Bourai et al., 2012). Nsp2 induces a shutoff of host cell transcription (Akhrymuk et al., 2012; Fros &

Pijlman, 2016). Besides, nsP2 decreases the amount of phosphorylated STAT1 (pSTAT1) in the nucleus (Göertz et al., 2018). It was suggested that nsP2 promotes pSTAT1 nuclear export and this diminishes IFN signalling (Frolov & Frolova, 2018; Göertz et al., 2018; Meshram et al., 2019). Lastly, nsP2 has also been linked to translational shutoff of the host cell (Frolov & Frolova, 2018; Fros & Pijlman, 2016).

1.5.5. NsP3

NsP3 has 3 domains. N-terminally there is the macrodomain, next the Alphavirus unique domain (AUD) and C-terminally the unstructured hypervariable domain (HVD). nsP3 is essential for RNA synthesis and polyprotein processing (Frolov & Frolova, 2018; Krieg et al., 2023; Rupp et al., 2015). The MD is capable of reversing MARylation (Lüscher et al., 2022). The viral macrodomain is essential for CHIKV replication. Mutants of vMDs that lack hydrolase activity result in replication deficiency (Krieg et al., 2023; Lüscher et al., 2022). Alphaviruses have a small genome, 10-12 kb, and only 4 nsPs. So, it is remarkable that a prominent domain is dedicated to MAR hydrolase activity, indicating its importance. The AUD is relevant for host protein interactions and identified interactors have been linked to vesicle trafficking and transcription corepressor activities (Ghildiyal & Gabrani, 2021). Besides, the AUD is suggested to be relevant for CHIKV replication especially subgenomic RNA synthesis (Gao et al., 2019). The HVD is an interaction hub for multiple host factors (Frolov & Frolova, 2018). These host factors include known interactors such as the G3BPs, amphiphysin-2 (BIN1), CD2-associated protein (CD2AP) and nucleosome assembly protein 1 like 1 and 4 (NAP1L1 and NAP1L4) (Dominguez et al., 2021; Meshram et al., 2018; Mutso et al., 2018; Tossavainen et al., 2016).

In cells, nsP3 is cytoplasmic and is present in both replication complexes and cytoplasmic condensates, we refer to as nsP3 condensates. This last localization is dependent on its C-terminus. The nsP3 condensates sequester different host factors and the content of these condensates has been attributed to nsP3 MD catalytic activity (Jayabalan et al., 2021). Upon infection, stress granules are induced. However, during a later stage of infection, these are disassembled and nsP3 condensates are formed. This leads to both inhibition of stress granules that may act antivirally and promotes the sequestering of the needed host factors for viral replication (Fros et al., 2012; Jayabalan et al., 2021; Meshram et al., 2018; Scholte et al., 2015). Important to this work are the well-known nsP3 interactors G3BP1 and 2. NsP3 interacts with G3BP1 and G3BP2 via two conserved FGDF motives in its hypervariable domain (Meshram et al., 2018; Panas, Ahola, & McInerney, 2014; Scholte et al., 2015). Mutation of both FGDF motifs inhibits viral replication (Kim et al., 2016; Scholte et al., 2015). This interaction is conserved and likewise essential in mosquito cells, where nsP3 interacts with the mosquito analogue Rasputin (Fros et al., 2015; Meshram et al., 2018). For SFV, another Alphavirus closely related to CHIKV, G3BPs are needed to recruit the translation machinery to viral replication hubs (Götte et al., 2019). Another suggestion was that G3BPs are involved in the transition between polyprotein translation by removing translation factors and preparing for the start of (–)sense RNA synthesis (Scholte et al., 2015).

1.5.6. NsP4

NsP4 is the least abundant nsP. Due to the premature stopcodon behind P123, only in 10% of the cases is the full ORF1 translated and P1234 is produced. NsP4 is the first nsP to be released from the polyprotein P1234 (Frolov & Frolova, 2018; Rupp et al., 2015). The protein is ± 70 kDa and has two distinct domains. It has a highly disordered N-terminal region followed by the RdRp domain (Frolov & Frolova, 2018; Rupp et al., 2015). The N-terminal disordered region is highly specific to Alphaviruses and this stretch is less conserved among the different species. One conserved tyrosine interacts with nsP1 for the minus-strand synthesis (Shirako et al., 2000). Other mutations in the disordered N-terminal region resulted

in defects in (-)-strand or (+)-sense RNA and overall replication. So, it seems that this IDR is implicated in the RNA synthesis activity of nsP4. For example, it could function to assemble the RC and be a hub for PPIs for both viral and host factors (Frolov & Frolova, 2018; Rupp et al., 2015). The RdRp domain within nsP4 was found through alignment studies indicating that the RdRp is similar to the RdRps of other (+)RNA viruses (Frolov & Frolova, 2018; Rubach et al., 2009; Rupp et al., 2015; Tomar et al., 2006). Alignments also uncovered the presence of a GDD active site, which was confirmed by mapping of RNA synthesis-defective mutants (Tomar et al., 2006). However, de novo RNA synthesis requires the presence of the other nsPs *in vitro* (Rubach et al., 2009). So, complete RNA replication requires all four nsPs (Frolov & Frolova, 2018; Rupp et al., 2015). Also as mentioned above, nsP4 is only active upon cleavage by nsP2 and release from the P1234. Then it starts with (-)-strand RNA synthesis and the following G and SG RNAs (Frolov & Frolova, 2018; Rupp et al., 2015). Besides, nsP4 is argued to possess terminal adenylyltransferase (TATase) activity responsible for the synthesis of the poly(A) tail of the genomic and subgenomic vRNAs (Frolov & Frolova, 2018; Rupp et al., 2015; Tomar et al., 2006). However, so far the research on this is inconclusive.

1.6. Aims

Chikungunya virus is an important human health threat. In recent years, outbreaks have occurred worldwide. Due to globalisation, quick viral evolution and the further spread of the vectors, CHIKV is expected to become endemic in many regions. In order to cope with this virus and generate specific therapeutics, we need a better understanding of how CHIKV replicates and circumvents our immune response. Interestingly, CHIKV replication is dependent on the macrodomain of nsP3 which is capable of hydrolyzing MARYlation. However, there is nothing known about the functional relevance of the macrodomain activity. In contrast, host cell mARTDs are capable of MARYlation and their expression has been linked to innate immunity. They are associated with antiviral activities. However, there is still a severe lack of understanding of how MARYlation and the respective enzymes function at the host-virus interface. Elucidating how MARYlation by our host cells and hydrolysis by the viral macrodomain affects viral replication will help our understanding of viral immune evasion strategies and viral replication.

The aim of this work is to understand the interplay between host cell MARYlation and the Chikungunya macrodomain. On one hand, mARTDs have been implicated countless times in innate immunity and antiviral mechanisms. On the other hand, the functional relevance of the CHIKV macrodomain and exactly why its function is so essential to CHIKV replication is unclear. We set out to elucidate the roles of the opposing activities of the host cell mARTDs and the viral macrodomain at the host-pathogen interface. In order to understand the functional relevance of these enzymes, we aim at identifying common substrates of the macrodomain and mARTDs and the implication that (de-)MARYlation has on their respective functions.

Firstly, this thesis focusses on characterizing common cellular substrates of PARP10 and CHIKV nsP3. These proteins may function as anti- or proviral host factors during CHIKV replication potentially controlled by MARYlation. To achieve this, we aim to set up complementary interactome screens using a mass spectrometry approach. Upon identification of common interactors, we assess them as substrates of both PARP10 and nsP3. We aim at characterizing how (de-)MARYlation affects the host factor's function.

Secondly, we aim at studying the role of G3BP1 during CHIKV replication. G3BP1 came forward as an interactor of both PARP10 and nsP3. This protein is essential to CHIKV and is thus a very interesting hit in our proteomics study. We aim to assess how MARYlation by the mARTDs influences G3BP1 and why CHIKV nsP3 reverts this modification and why CHIKV depends on G3BP1.

Lastly, the non-structural proteins nsP1, nsP2 and nsP3 were reported as MARYlation substrates of PARP10 and hydrolase substrates of nsP3 *in vitro*. In this dissertation, MARYlation of nsP1 is further analyzed. Also, the knowledge of the functions of CHIKV nsP1 is largely extrapolated from our knowledge of other Alphaviruses. In this work, CHIKV nsP1 functions are studied within its own right. The eventual goal is to determine how nsP1 MARYlation affects its functions.

Aims of research project

- Identification of both the interactomes of PARP10 and nsP3 followed by the analysis of identified common interactors as substrates of the respective enzymes and how (de-)MAYylation affects their functions.
- Determining the role of G3BP1 in CHIKV replication and the effect of (de-)MAYylation on G3BP1.
- Analysis of CHIKV nsP1 as a (de-)MAYylation substrate of mARTDs and CHIKV nsP3, and setting up functional assays for CHIKV nsP1 to study the effects of this PTM.

2. Results and Discussion

2.1. Identification of host factors that are regulated by MARYlation during Chikungunya infection

MARYlation plays a complex role in the viral life cycle. Especially for CHIKV, and other alphaviruses, as pointed out by the inability of hydrolase-deficient virus to replicate. This could be explained by the incapacity to counter the IFN-I induced MARYlation of specific substrates essential to establish a robust innate immune response. The MARYlation state of the specific proteins could affect their role in the immune response or their function in the viral lifecycle, as anti- vs. proviral. To gain insight into the host-pathogen interface, we aimed at identifying these host factors that are regulated by MARYlation upon CHIKV infection. We set out to determine the interactome of both PARP10 and nsP3 and identify common interactors. These are potential substrates for both proteins, and thus potential host factors regulated by MAR during infection. Since we are interested in both stable and transient interactors, such as enzyme-substrate pairs, we used BioID (**Fig. 9**). BioID allows proximity-dependent biotinylation of stable and transient interactors of the proteins of interest (POIs). For this technique our lab used fusion proteins of nsP3-HA-BirA and PARP10-HA-BirA. Subsequently, the Biotin-tagged proteins can be enriched using streptavidin beads and this technique is combined with subsequent mass spectrometry-based analysis. This analysis should point to host factors that are relevant in the innate immune response to viral infection. Upon identification of this subgroup of proteins potentially regulated by MARYlation, we will continue with the promising hits.

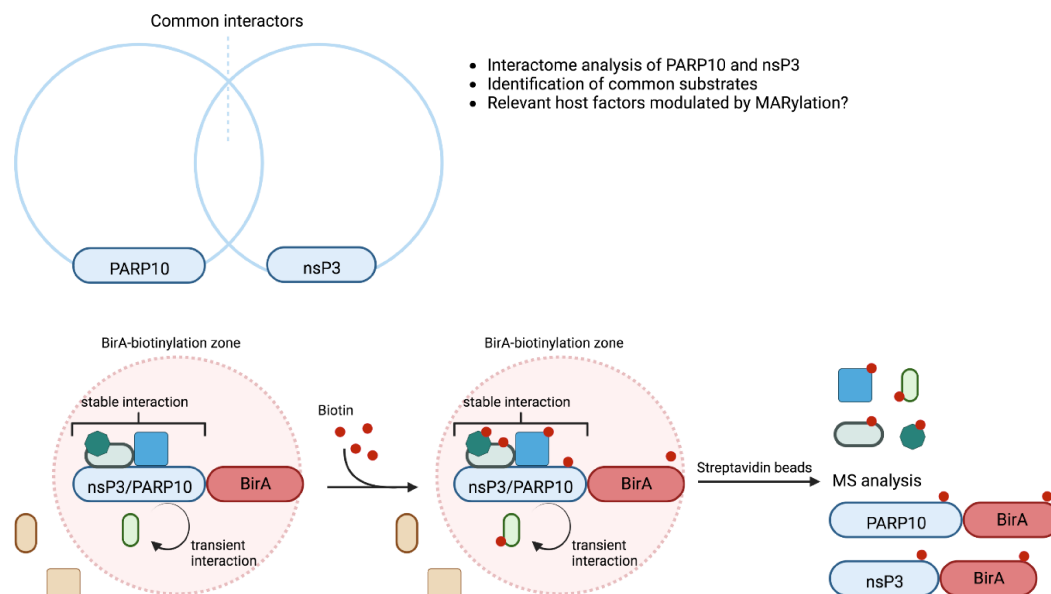


Figure 9. Identification of common interactors of PARP10 and nsP3. Schematic representation of the approach used to identify potential host factors modulated by MARYlation during CHIKV infection. To identify the common substrates an interactome analysis of PARP10 and nsP3 was performed, and the overlap analyzed for overlapping hits. The BioID approach was used for the interactome analysis. BioID is based on C-terminal fusion of a biotin ligase (BirA*) to our protein of interest. Upon treatment with biotin, interactors are biotinylated. Following, biotinylated proteins are immunoprecipitated using streptavidin beads. The interactors are identified by mass spectrometry (MS) analysis.

2.1.1. Establishment of BioID approach

The study of substrate-enzyme pairs, and thus more transient and low-affinity interactions, requires a more robust system than standard interaction experiments such as co-immunoprecipitation (co-IP), in which protein-protein interactions may not be maintained

during cell lysis or the subsequent procedure. Because of this reason, we used BioID, biotin-based proximity labelling, in which the POI, or bait, is fused to a biotin ligase, which results in the covalent biotinylation of both high- and low-affinity interactors in the cell. Subsequently, biotinylated proteins can be enriched using streptavidin-beads, as streptavidin-biotin is one of the strongest known non-covalent interactions (Chivers et al., 2011). In our case, both POIs, PARP10 and nsP3, were fused to the mutant *E. coli* biotin ligase BirA, BirA*R118G (Roux et al., 2012). This mutation results in a lower affinity of the biotin ligase for the bioAMP intermediate, which leads to an early release of the biotin-intermediate and results in promiscuous labeling and thus biotinylation of proteins in close proximity (**Fig. 9**) (Roux et al., 2012). To identify the PPIs of our POIs, we started establishing a robust system and protocol. Since there are many options for streptavidin-based biotin-enrichment, we assessed the working of different potential experimental set-ups. HEK293 cells were transiently transfected with plasmids encoding either C-terminally tagged PARP10-HA-BirA or nsP3-HA-BirA. The plasmid encoding the HA-BirA serves as a negative control for background biotinylation. After transfection, the cells were treated with biotin overnight (ON, 16 h). After cell lysis, the lysates were analyzed using SDS-PAGE and Western blotting (**Fig. 10A**). There is in comparison to the HA-BirA control an enrichment for protein biotinylation in both baits, PARP10 and nsP3. Especially, the nsP3 bait leads to extensive protein biotinylation. Since later on, with MS analysis, both interactomes are individually analyzed, the difference in biotinylation between the 2 POIs is negligible. Next the biotinylated proteins were enriched using either the Dynabeads MyOne Streptavidin T1 (LOT: 01212549) or the Pierce Streptavidin Magnetic Beads (LOT: WC324631) ON. The enriched proteins were eluted from the beads and analyzed using SDS-PAGE and Western blotting (**Fig. 10B**). When analyzing the streptavidin-enriched proteins, we see a clear difference between the different matrices used for enrichment. Comparing the Dynabeads MyOne Streptavidin T1 and the Pierce Streptavidin Magnetic Beads, the latter have a superior performance and capture extensively more biotinylated proteins (**Fig. 10B**).

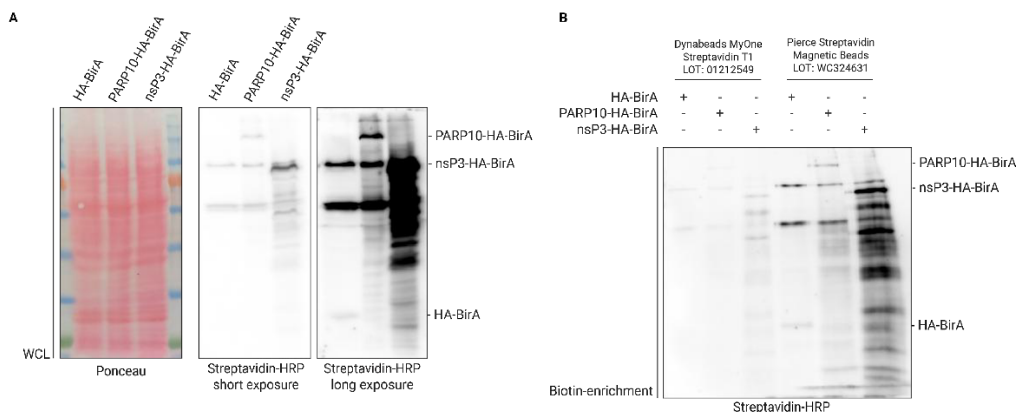


Figure 10. Establishment of the BioID approach: comparison of different matrices for streptavidin-based enrichment. HEK293 cells were transiently transfected with HA-BirA, PARP10-HA-BirA or nsP3-HA-BirA. After transfection, the cells were treated with biotin for 16h overnight (ON). The cells were lysed, and the biotinylated proteins were enriched using either the Dynabeads MyOne Streptavidin T1 (LOT: 01212549) or the Pierce Streptavidin Magnetic Beads (LOT: WC324631) ON. Both the lysates (**A**) and the enriched proteins (**B**) were analyzed using SDS-PAGE and Western blotting (n=1).

In similar fashion, we analyzed different LOT numbers of the Pierce Streptavidin Magnetic Beads (**Fig. 11**). Due to circumstances, we had to shift from LOT:WC324631 to LOT:XD351470. So before proceeding to MS, we compared the recent LOT:XD351470 to the older LOT:WC324631. Set-up was consistent with **Fig. 10**. As expected, there was again a clear enrichment for protein biotinylation in both the PARP10- and nsP3-fusion compared to the HA-BirA control (**Fig. 11A**). Upon streptavidin-enrichment, even though the supernatant (SN) of the enrichment still contains a high concentration of biotinylated proteins, both LOTs of the Pierce Streptavidin Magnetic Beads extensively capture biotinylated proteins (**Fig.**

11B). LOT:XD351470 of the Pierce Streptavidin Magnetic Beads performs well and will be used for subsequent interactome identification using MS analysis.

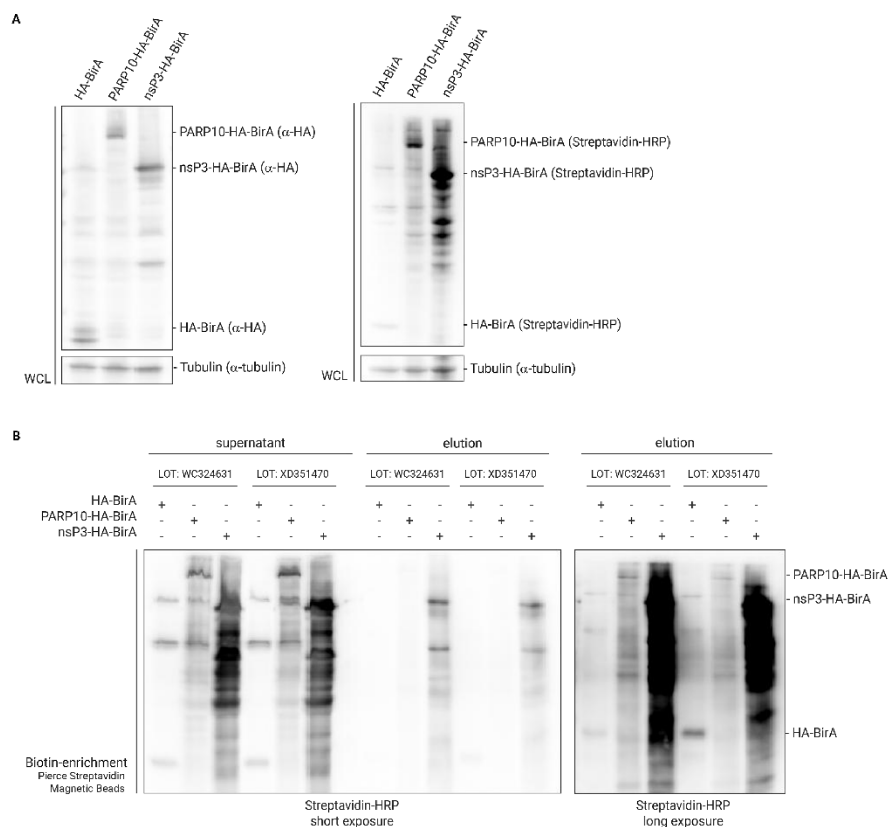


Figure 11. Establishment of the BioID approach: comparison of different LOT numbers of the Pierce Streptavidin Magnetic Beads. HEK293 cells were transiently transfected with HA-BirA, PARP10-HA-BirA or nsP3-HA-BirA. After transfection, the cells were treated with biotin for 16h overnight (ON). The cells were lysed and the biotinylated proteins were enriched using the Pierce Streptavidin Magnetic Beads LOT:WC324631 or LOT:XD351470. Both the lysates (**A**) and the enriched proteins (**B**) were analyzed using SDS-PAGE and Western blotting (n=1).

Important to note, localization of both bait fusions, PARP10-HA-BirA and nsP3-HA-BirA, was analyzed and compared to the WT proteins nsP3 and PARP10 by Sarah Krieg (Krieg, 2022). Fusions localized similarly to their WT counterparts. Also, the enzymatic activities of both fusion proteins were assessed (Krieg, 2022). Taken together, the BirA-tag did not seem to compromise the integrity of either protein and thus we proceeded with the interactome identification.

2.1.2. Identification of common cellular interactors and substrates of PARP10 and CHIKV nsP3

To identify the interactome of both PARP10 and nsP3, HEK293 cells were transiently transfected with plasmids encoding either C-terminally tagged HA-BirA, PARP10-HA-BirA or nsP3-HA-BirA. After transfection, the cells were treated with biotin ON. The cells were lysed the following day and the biotinylated proteins were enriched using Pierce Streptavidin Magnetic Beads (LOT:XD351470). ±95% of the matrix was handed over for LC-MS/MS measurements. This and subsequent analysis of the raw data was performed by Christian Preisinger (head of the IZKF MS Core Facility). ±5% of the enriched protein was eluted from the beads and analyzed using SDS-PAGE and Western blotting (**Fig. 12**). In Fig. 12, we see an enrichment for protein biotinylation for PARP10 and nsP3 in the cell lysates. In the WCL, again nsP3 bait led to more protein biotinylation compared to PARP10 (**Fig. 12A**).

Interestingly, here the elution showed a superior performance for the enrichment of PARP10-HA-BirA-mediated biotinylated proteins (**Fig. 12B**). This could be due to overloading of the system with the nsP3 lysates. However, since there is still a significant enrichment, we proceeded with the analysis. The analysis was done based on 4 independent experiments, of which Fig. 12 is a representative example. Both for PARP10 and nsP3, the intensities of potential hits were analyzed compared to the HA-BirA control. The analyses of the individual and the overlapping interactomes are discussed below.

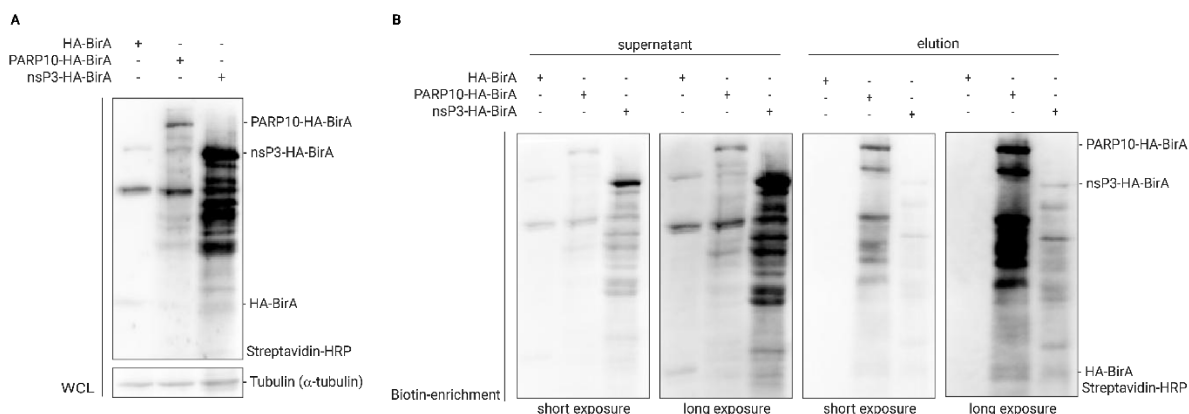


Figure 12. Identification of common cellular interactors and substrates of PARP10 and CHIKV nsP3 using BioID. HEK239 cells were transiently transfected with HA-BirA, PARP10-HA-BirA or nsP3-HA-BirA. After transfection, the cells were treated with biotin for 16h overnight (ON). The cells were lysed, and the biotinylated proteins were enriched using the Pierce Streptavidin Magnetic ON. Both the lysates (**A**), the supernatant of the matrices (**B**) and the enriched proteins (**B**) were analyzed using SDS-PAGE and Western blotting. 4 independent experiments were performed and analyzed by LC-MS/MS (n=4).

2.1.2.1. Identification and analysis of the PARP10 interactome

Currently, there is not a lot known about the function or regulation of PARP10. Information on the PARP10 interactome would shed light on its involvement in different processes and its proposed function in innate immunity in the cell. Also, there is not a lot of data on PARP10 substrate specificity (Feijs et al., 2013). For this as well, PARP10 interactome studies are of importance. To identify and analyze the PARP10 interactome, the 4 independent BioID experiments (**Fig. 12**) were analyzed and examined for overlap and the significant enrichment of proteins (**Fig. 13**). 882 significantly enriched proteins were identified, of which 352 were at least 10-fold enriched compared to the HA-BirA control. Among the enriched proteins, proteins previously identified by our lab as PARP10 interactors (Krieg, 2022), such as G3BP1 (14-fold), CD2 associated protein (CD2AP) (26-fold) and Melanoma-associated antigen D2 (MAGED2) (7-fold), were found. This supports and strengthens our set-up and analysis. In Fig. 13A, we see the enrichment of PARP10 biotinylated proteins in comparison to the HA-BirA control. Significantly enriched proteins are colored yellow, and proteins of interest are marked by name. The proteomics data sets were analyzed by cluster analysis to assess the validity and the quality of the independent datasets (**Fig. 13B**). The 4 PARP10 datasets cluster very well and indicate reproducibility and a robust overall result. The PARP10 interactome is enriched for proteins involved in processes such as cell migration, cytoskeleton, mRNA processing and more (**Table 3**). Table 3 illustrates the top 30 enriched GO terms for the PARP10 dataset. Interestingly, PARP10, often linked to innate immunity and defense against RNA viruses, also seems to be involved in the regulation of the RIG-I signaling pathway and translation. For this dissertation, of particular interest is PARP10 involvement in stress granule assembly.

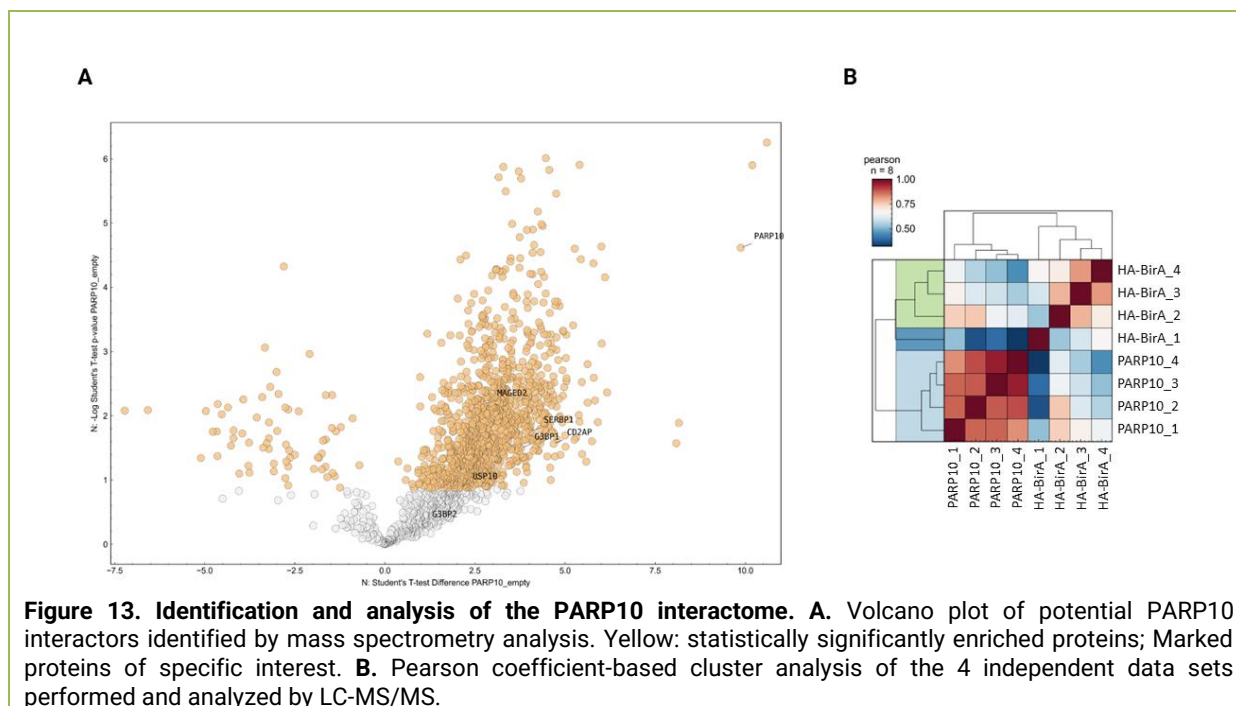


Figure 13. Identification and analysis of the PARP10 interactome. **A.** Volcano plot of potential PARP10 interactors identified by mass spectrometry analysis. Yellow: statistically significantly enriched proteins; Marked proteins of specific interest. **B.** Pearson coefficient-based cluster analysis of the 4 independent data sets performed and analyzed by LC-MS/MS.

Table 3. Gene Ontology analysis of the PARP10 interactome. The Gene ontology analysis of the PARP10 interactome (proteins identified with a p -value ≤ 0.05) was performed using STRING version 11.5, organism: homo sapiens. Top 30 enriched GO terms are shown. Green: processes involving G3BP1.

Biological Process				
term description	observed gene count	background gene count	strength	false discovery rate
Substrate-dependent cell migration, cell extension	5	10	1.45	0.00062
GTP biosynthetic process	4	11	1.31	0.0091
Positive regulation of nuclear-transcribed mRNA poly(a) tail shortening	4	12	1.27	0.0112
Stress granule assembly	5	20	1.15	0.0057
Substrate-dependent cell migration	6	25	1.13	0.0018
Positive regulation of gene silencing by miRNA	5	24	1.07	0.0108
Regulation of RIG-I signaling pathway	4	20	1.05	0.0413
Protein localization to centrosome	4	21	1.03	0.0458
Phosphatidylinositol dephosphorylation	5	27	1.02	0.0152
Regulation of microtubule polymerization	9	53	0.98	0.00021
Positive regulation of microtubule polymerization	5	31	0.96	0.024
Positive regulation of mRNA catabolic process	7	50	0.89	0.0054
Protein depolymerization	5	36	0.89	0.0402
Protein localization to microtubule cytoskeleton	5	37	0.88	0.0431
Regulation of translational initiation	10	75	0.87	0.00037
Ciliary basal body-plasma membrane docking	12	95	0.85	7.90E-05
Positive regulation of organelle assembly	9	71	0.85	0.0015
Cytoplasmic translation	9	72	0.85	0.0016
Gene silencing by miRNA	6	47	0.85	0.0206
Posttranscriptional gene silencing	7	59	0.82	0.0117
Regulation of mitotic sister chromatid separation	7	60	0.82	0.0124
Translational initiation	16	141	0.8	5.29E-06
Regulation of mitotic sister chromatid segregation	8	71	0.8	0.0063
Adherens junction organization	7	62	0.8	0.0143
Regulation of mitotic metaphase/anaphase transition	6	56	0.78	0.0413
Regulation of cilium assembly	6	56	0.78	0.0413
G2/M transition of mitotic cell cycle	14	134	0.77	7.29E-05
Actin filament bundle organization	6	58	0.76	0.046
Centrosome cycle	8	81	0.74	0.0124
Positive regulation of mRNA metabolic process	8	81	0.74	0.0124

2.1.2.2. Identification and analysis of the nsP3 interactome

For nsP3, previous interactome studies have been performed and multiple host factors have been identified as interactors (Frolov & Frolova, 2018; Kim et al., 2016; Krieg, 2022; Meshram et al., 2018). However, why the loss of nsP3 hydrolase activity is detrimental to CHIKV replication is unclear. Knowledge on nsP3 interactome and subsequently potential substrates will help in understanding how the nsP3 hydrolase activity contributes to viral replication. For the identification and analysis of the nsP3 interactome, the 4 independently performed BioID experiments were assessed and examined for overlap in the significant enrichment of proteins (**Fig. 14**). 456 significantly enriched proteins were identified, of which 276 were at least 10-fold enriched compared to the HA-BirA control. Also, in the nsP3 datasets, we found proteins that were previously identified by both our lab (Krieg, 2022) and other labs (Frolov & Frolova, 2018; Kim et al., 2016; Meshram et al., 2018; Mutso et al., 2018; Panas, Ahola, & McInerney, 2014): e.g. G3BP1 (931-fold), G3BP2 (124-fold) CD2AP (234-fold), MAGED2 (13-fold), USP10 (45-fold), UBAP2L (26-fold). This again shows the robustness of our set-up and strengthens our analysis. In Fig. 14A, the enrichment of nsP3 biotinylated proteins in comparison to the HA-BirA control is plotted. Significantly enriched proteins are colored blue and proteins of interest are marked. Again, the proteomics data sets were analyzed by cluster analysis to assess the validity and the quality of the independent datasets (**Fig. 14B**). The 4 nsP3 datasets cluster together and this shows the reproducibility of the data and indicates a robust overall result. Enriched GO terms include cell migration, positive regulation by host of viral genome replication, stress granule assembly, cell cycle and cytoskeletal processes (**Table 4**). Table 4 illustrates the top 30 enriched GO terms for the nsP3 dataset.

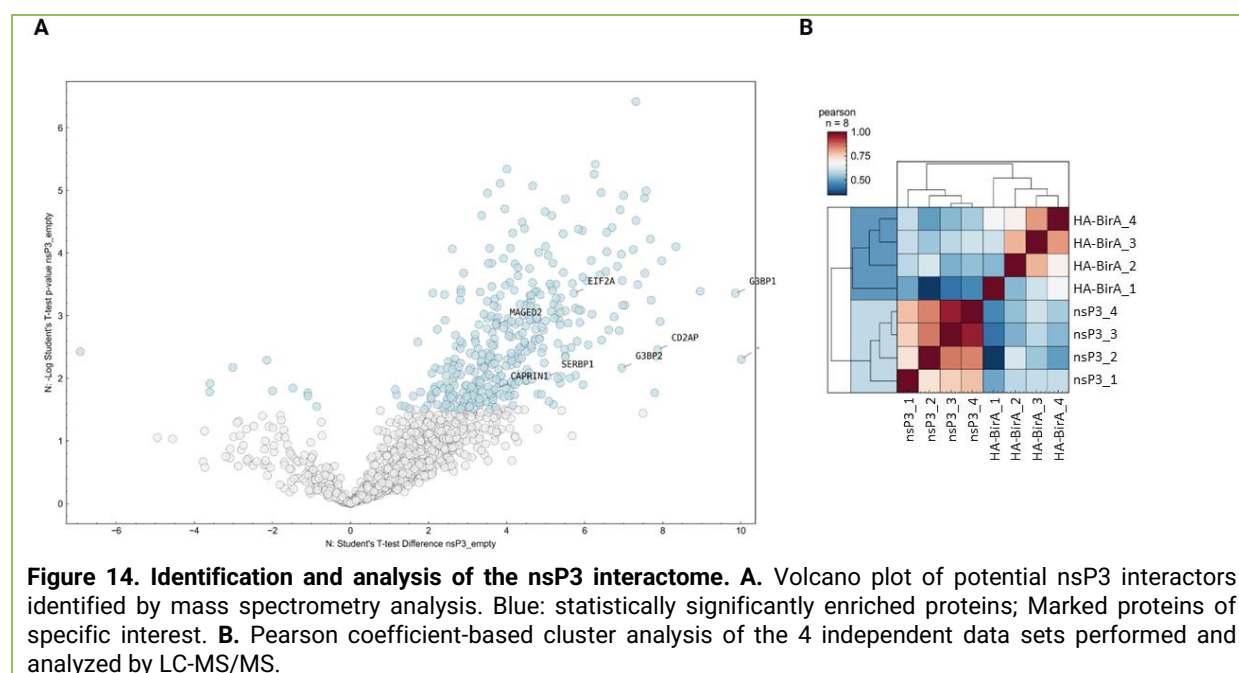


Table 4. Gene Ontology analysis of the nsP3 interactome. The Gene ontology analysis of the nsP3 interactome (proteins identified with a p -value ≤ 0.05) was performed using STRING version 11.5, organism: homo sapiens. Top 30 enriched GO terms are shown. Green: processes involving G3BP1.

Biological Process				
term description	observed gene count	background gene count	strength	false discovery rate
Positive regulation of translation in response to endoplasmic reticulum stress	3	5	1.63	0.0116
Beta-catenin destruction complex assembly	3	5	1.63	0.0116
Substrate-dependent cell migration, cell extension	4	10	1.46	0.0033
Positive regulation by host of viral genome replication	3	8	1.43	0.0274

Dense core granule cytoskeletal transport	3	9	1.38	0.0337
Regulation of attachment of spindle microtubules to kinetochore	4	13	1.34	0.0068
Stress granule assembly	6	20	1.33	0.00018
ESCRT III complex disassembly	3	10	1.33	0.04
Microtubule depolymerization	5	17	1.32	0.0013
Regulation of mitotic spindle assembly	5	18	1.3	0.0016
Attachment of mitotic spindle microtubules to kinetochore	3	11	1.29	0.0475
Mitotic metaphase plate congression	10	44	1.21	7.01E-07
Centriole replication	4	18	1.2	0.0167
Barbed-end actin filament capping	4	18	1.2	0.0167
Midbody abscission	4	18	1.2	0.0167
Metaphase plate congression	11	54	1.16	3.33E-07
Microtubule polymerization or depolymerization	10	51	1.15	2.23E-06
Centrosome duplication	6	30	1.15	0.0011
Ciliary basal body-plasma membrane docking	18	95	1.13	8.53E-12
Protein localization to centrosome	4	21	1.13	0.0253
Regulation of centrosome cycle	11	60	1.12	8.05E-07
Regulation of microtubule depolymerization	6	33	1.11	0.0017
Regulation of centrosome duplication	8	45	1.1	0.0001
Microtubule polymerization	6	34	1.1	0.0019
Positive regulation of microtubule polymerization or depolymerization	6	35	1.09	0.0022
Regulation of mitotic spindle organization	6	35	1.09	0.0022
Negative regulation of microtubule depolymerization	5	29	1.09	0.0087
Protein depolymerization	6	36	1.08	0.0024
G2/M transition of mitotic cell cycle	22	134	1.07	2.38E-13
Establishment of chromosome localization	12	73	1.07	5.03E-07

To verify the nsP3-dataset, selected proteins were analyzed for interaction using Co-IP experiments with EGFP-nsP3 as bait protein (**Fig. 15**). As negative control, EGFP-tag was tested for interaction with prey proteins. In addition, the nsP3 V33E mutant, unable to hydrolase MARYlation, and the nsP3 FA mutant, mutation of the FDGF motifs reported as the interaction site for G3BPs, were tested for interaction with the prey proteins (Panas et al., 2012; Panas, Ahola, & Mcinerney, 2014; Panas et al., 2015). HEK293 cells were transiently transfected with either EGFP or EGFP-nsP3 WT or mutants. The cells were lysed and EGFP and EGFP-nsP3 were immunoprecipitated using GFP-TRAP. EGFP does not interact with any of the prey proteins (**Fig. 15**). NsP3 WT interacts with G3BP1, USP10, CD2AP and MAGED2 (**Fig. 15**). NsP3 V33E also interacts with all 4 proteins, indicating that the hydrolase-activity is not essential for interaction with these specific proteins. NsP3 FA mutant is unable to interact with G3BP1 and USP10 (Panas et al., 2012; Panas, Ahola, & Mcinerney, 2014; Panas et al., 2015). The nsP3-USP10 interaction is likely mediated indirectly through G3BP1. USP10 strongly interacts with G3BP1, so loss of binding of G3BP1 to nsP3 FA could explain the loss of the USP10-nsP3 FA interaction (Kedersha et al., 2016; Panas et al., 2015; Sanders et al., 2020). NsP3 FA mutant interacts with CD2AP and MAGED2. Especially MAGED2 highly interacts with nsP3 FA. This could be due to the increased availability of nsP3. Loss of the G3BP1-USP10 interaction with nsP3 might affect nsP3's availability to other proteins through steric hindrance or localization. Taken together, we could confirm that nsP3 interacts with multiple of the identified proteins (**Fig. 15**). We could verify the MS analysis of G3BP1, CD2AP, MAGED2 and USP10 using Pulldown-experiments. This indicates the validity of the MS dataset.

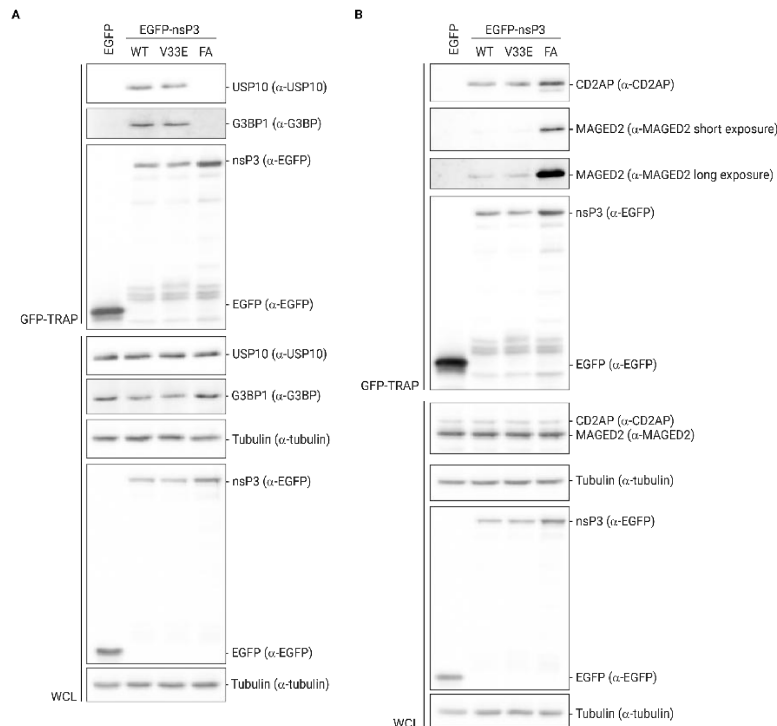


Figure 15. Confirmation of nsP3 interactome. The validation of the nsP3 interactome by selected proteins G3BP1 (A), USP10 (A), MAGED2 (B) and CD2AP (B) using GFP-Trap. HEK293 cells were transiently transfected with plasmids encoding either EGFP, nsP3-EGFP WT, nsP3-EGFP V33E or EGFP-nsP3. The cells were lysed 48h after transfection and EGFP and nsP3-EGFP were immunoprecipitated using GFP-TRAP. Both the lysates and the co-immunoprecipitated proteins were analyzed by western blotting (n = 3).

2.1.2.3. Identification and analysis of common interactors of PARP10 and nsP3

So far, our group could identify both previously known and new interactors for both PARP10 and nsP3. However, as previously stated, our aim is to identify host factors that upon CHIKV infection are regulated by MARYlation through the opposing activities of PARP10 and nsP3. For this, we analyzed the overlap between the 10-fold enriched proteins of both datasets. It is important to keep in mind that through this cut-off already many enriched proteins are eliminated from the screens. However, it gives us an indication of the most important overlapping proteins and thus the host factors to focus on. The results are shown in a Venn-diagram, in which it is shown that there are 136 overlapping proteins between the 10-fold enriched hits of both PARP10 and nsP3 (**Fig. 16A/C**). The 4 Control, PARP10 and nsP3 datasets respectively cluster together. Especially PARP10 shows a high Pearson coefficient. However, all together the data is robust and trustworthy (**Fig. 16B**). Common host factors include G3BP1, CD2AP and SERBP1. Other interesting hits include the tumor suppressor protein TP53, the major 5'-3' exoribonuclease involved in mRNA decay XRN1, the Eukaryotic translation initiation factor 4 gamma 1 (EIF4G1) and many more proteins involved in mRNA processing. Subsequently, to obtain a first idea of the potential processes regulated by the opposing activities of PARP10 and nsP3, also Gene Ontology analysis was performed on the overlapping dataset (**Table 5**). The GO processes were sorted by strength. This measures how large the enrichment effect is dependent on the ratio between the identified proteins from our dataset in a specific process to the number of proteins that would be expected to be annotated in this process for a random dataset. Among the highest enriched processes are cell migration, stress granule assembly, translation, cell cycle regulation, RNA processing and cytoskeleton regulation (**Table 5**). As mentioned, this thesis mainly focuses on the study of G3BP1 and stress granule assembly and thus the processes involving G3BP1 are marked in green.

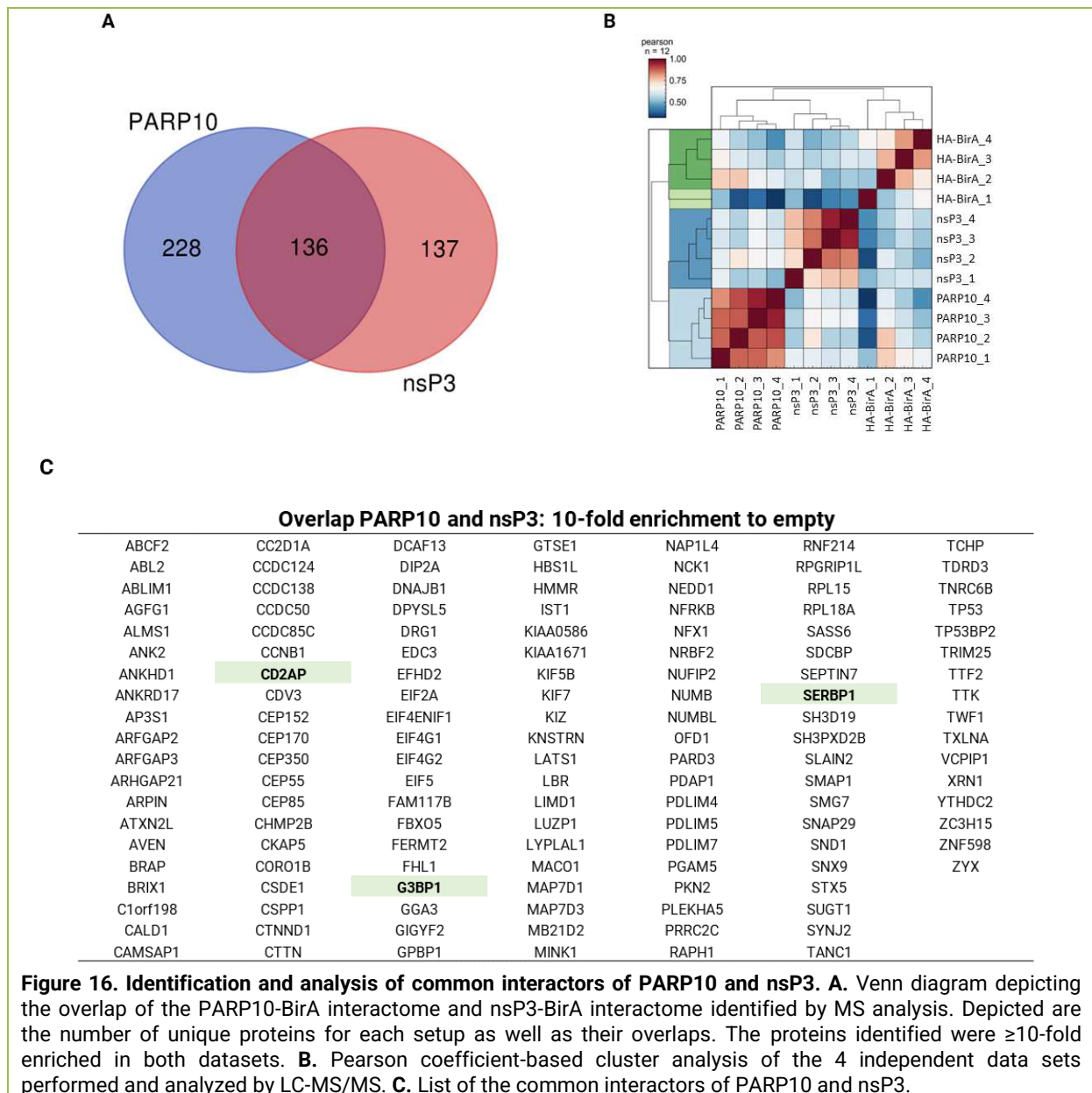


Table 5. Gene Ontology analysis of the common interactors of PARP10 and nsP3. The Gene ontology analysis of the common interactors of PARP10 and nsP3 (proteins identified were ≥ 10 -fold enriched and with a p -value ≤ 0.05 in both datasets) was performed using STRING version 11.5, organism: homo sapiens. Green: processes involving G3BP1.

Biological Process				
term description	observed gene count	background gene count	strength	false discovery rate
Substrate-dependent cell migration, cell extension	4	10	1.76	0.001
Lateral ventricle development	3	12	1.56	0.0347
Stress granule assembly	4	20	1.46	0.0074
Microtubule polymerization	4	34	1.23	0.0343
Cytoplasmic translation	6	72	1.08	0.0063
Ciliary basal body-plasma membrane docking	6	95	0.96	0.0205
Regulation of cell cycle g2/m phase transition	12	215	0.9	5.06E-05
G2/M transition of mitotic cell cycle	7	134	0.88	0.0167
Regulation of g2/m transition of mitotic cell cycle	10	198	0.86	0.00095
Regulation of protein polymerization	11	232	0.83	0.00056
Organelle localization by membrane tethering	8	170	0.83	0.011
Regulation of g1/s transition of mitotic cell cycle	7	156	0.81	0.0347
Regulation of organelle assembly	8	191	0.78	0.0205

Nuclear-transcribed mRNA catabolic process	8	194	0.77	0.0218
Regulation of mitotic cell cycle phase transition	16	416	0.74	4.69E-05
RNA catabolic process	9	247	0.72	0.0205
Negative regulation of mitotic cell cycle phase transition	8	219	0.72	0.0404
Regulation of supramolecular fiber organization	13	368	0.71	0.0011
Cell division	17	493	0.7	6.99E-05
Regulation of mitotic cell cycle	23	676	0.69	1.03E-06
Regulation of actin filament organization	9	273	0.68	0.0369
Organelle assembly	24	735	0.67	1.03E-06
Plasma membrane bounded cell projection assembly	14	433	0.67	0.0011
Regulation of actin cytoskeleton organization	11	348	0.66	0.013
Regulation of cytoskeleton organization	17	546	0.65	0.00021
Regulation of actin filament-based process	12	390	0.65	0.0079
Regulation of cell cycle process	23	760	0.64	5.13E-06
Microtubule cytoskeleton organization	15	492	0.64	0.0011
Cilium assembly	10	339	0.63	0.0373
Regulation of protein-containing complex assembly	13	451	0.62	0.0074
Regulation of cellular amide metabolic process	13	456	0.61	0.0079
Cytoskeleton organization	31	1126	0.6	3.09E-07
Microtubule-based process	20	727	0.6	0.00015
Regulation of translation	11	398	0.6	0.0338
Organelle localization	16	598	0.59	0.0021
Mitotic cell cycle	18	695	0.57	0.001
Mitotic cell cycle process	16	616	0.57	0.003
Supramolecular fiber organization	12	480	0.56	0.0373
Regulation of organelle organization	31	1306	0.53	2.70E-06
Regulation of cell cycle	29	1230	0.53	8.55E-06
Regulation of cellular component biogenesis	23	971	0.53	0.00019
Cell projection organization	27	1170	0.52	4.07E-05
Plasma membrane bounded cell projection organization	25	1122	0.51	0.00018
Cell cycle process	22	976	0.51	0.0007
Posttranscriptional regulation of gene expression	13	574	0.51	0.0473
Cell cycle	29	1313	0.5	3.21E-05
Positive regulation of organelle organization	14	633	0.5	0.0373
Cellular component assembly	44	2359	0.43	1.03E-06
Organelle organization	60	3450	0.4	1.22E-08
Cellular component biogenesis	45	2583	0.4	3.70E-06

Molecular Function				
term description	observed gene count	background gene count	strength	false discovery rate
	32	334	1.14	7.96E-23
Cadherin binding	33	538	0.95	3.06E-18
Cell adhesion molecule binding	10	257	0.75	0.0091
Microtubule binding	11	361	0.64	0.0257
Tubulin binding	39	1649	0.53	8.29E-09
RNA binding	22	973	0.51	0.0008
Cytoskeletal protein binding	21	1216	0.4	0.0463
Protein-containing complex binding	78	7026	0.2	0.00026
Protein binding				

2.2. G3BP1 is a common interactor and substrate of PARP10 and nsP3

2.2.1. G3BP1 interacts with PARP10 and nsP3

One of the most promising hits is G3BP1, which is a known interactor for nsP3, as discussed and shown above (**chapter 1.4.**). G3BP-dKO leads to inhibition of CHIKV replication, showing its necessity for the CHIKV lifecycle (Kim et al., 2016). Besides Chikungunya, G3BP1 plays an important role in the lifecycle of multiple viruses (**chapter 1.4.4.**). However, G3BP1 is a new and previously unknown interactor for PARP10. Since G3BP1 is important in general viral replication and our study focuses on mARTDs role in innate immunity, G3BP1 is a very interesting hit. To validate the MS data and G3BP1 as interactor we proceeded with an analysis of G3BP1 as interactor and potential substrate of PARP10 and nsP3.

To verify PARP10 interaction with G3BP1, HEK293 cells were transiently transfected with either EGFP or mEGFP-G3BP1 alone or in combination with PARP10 WT or PARP10 GW, a catalytically inactive mutant of PARP10. As negative control, EGFP was tested for interaction with PARP10. Cells were lysed and EGFP and mEGFP-G3BP1 was immunoprecipitated using GFP-TRAP (**Fig. 17A**). EGFP does not interact with PARP10. G3BP1 interacts with both PARP10 WT and PARP10 GW, validating the MS identified interaction.

Besides exogenous G3BP1, we also tested whether PARP10 interacts with endogenous G3BP1. HEK293 cells were transiently transfected with PARP10 WT or GW. Cells were lysed and endogenous G3BP1 was immunoprecipitated using α -G3BP. There is a slight interaction between PARP10 WT and G3BP1 but it is difficult to detect (**Fig. 17B**). However, there is a clear interaction between PARP10 GW, the inactive mutant, and G3BP1 (**Fig. 17B**). This could indicate an enzyme-substrate pair, since due to the catalytic inactivity the substrate could be tethered onto the enzyme instead of being released after successful modification.

Taken together, we could confirm the interaction between PARP10 and G3BP1.

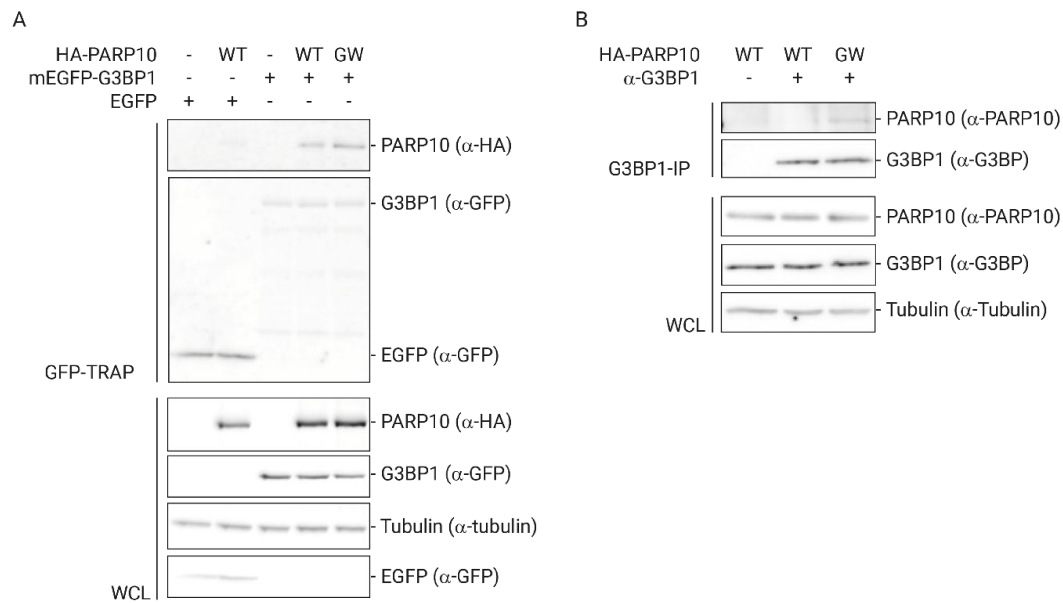


Figure 17. G3BP1 interacts with PARP10. The validation of the PARP10-G3BP1 interaction through co-immunoprecipitation experiments. **A.** HEK293 cells were transiently transfected with plasmids encoding either EGFP or mEGFP-G3BP1 alone or in combination with PARP10 WT or PARP10 GW. EGFP and mEGFP-G3BP1 were immunoprecipitated using GFP-TRAP. The cells were lysed 48h after transfection and EGFP and mEGFP-G3BP1 were immunoprecipitated using GFP-TRAP. Both the lysates and the co-immunoprecipitated proteins were analyzed by means of western blotting (n = 3). **B.** HEK293 cells were transiently transfected with plasmids encoding either PARP10 WT or PARP10 GW. The cells were lysed 48h after transfection and endogenous G3BP1 was immunoprecipitated using α -G3BP antibody. Both the lysates and the co-immunoprecipitated proteins were analyzed by means of western blotting (n = 2).

G3BP1 is a known interactor of nsP3 (Fros et al., 2012, 2015; Gorchakov et al., 2008; Kim et al., 2016). We confirmed the interaction between G3BP1 and nsP3 through co-immunoprecipitation of nsP3-EGFP (**Fig. 15**). Besides, we tested nsP3-mediated proximity labelling of G3BP1 through a G3BP1 IP. HEK293 cells were transiently transfected with either HA-BirA, as negative control, or nsP3-HA-BirA and both conditions were supplemented with biotin (**Fig. 18**). Cells were lysed and endogenous G3BP was immunoprecipitated using α -G3BP. In the lysates, nsP3-HA-BirA shows a high biotinylation signal. Upon the G3BP-IP, G3BP1 is successfully immunoprecipitated in both conditions. However, for the HA-BirA control, G3BP1 is not biotinylated. For the nsP3-HA-BirA condition, G3BP1 interacts with nsP3 and both G3BP1 and nsP3 are biotinylated (**Fig. 18**). Again, showing an nsP3-G3BP1 interaction.

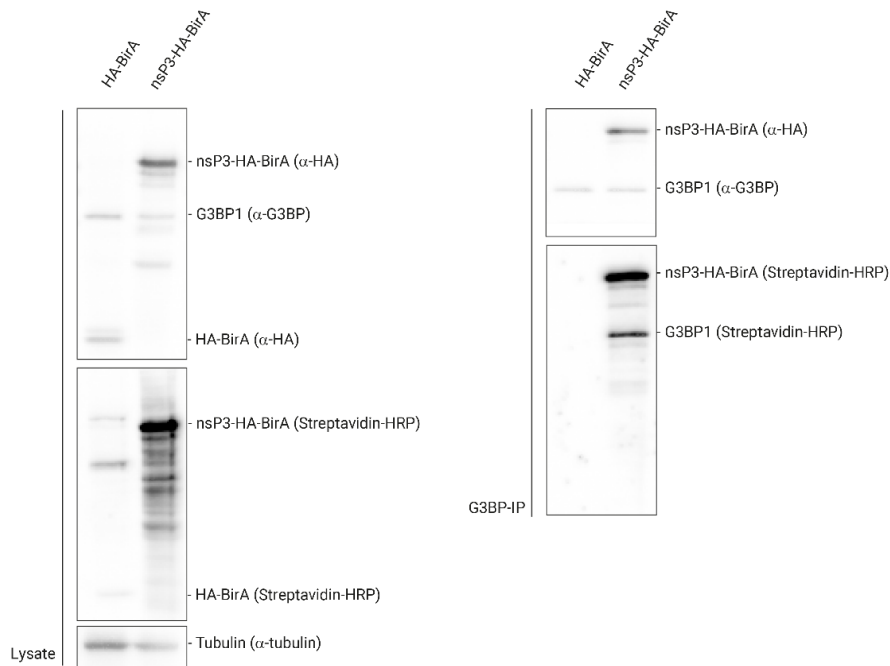


Figure 18. nsP3 interacts with G3BP1. HEK293 cells were transiently transfected with plasmids encoding HA-BirA or nsP3-HA-BirA and both conditions were supplemented with biotin overnight after transfection. The cells were lysed 24h after transfection and endogenous G3BP1 was immunoprecipitated using a-G3BP antibody. Both the lysates and the co-immunoprecipitated proteins were analyzed by means of western blotting (n = 1).

Both nsP3 and PARP10 interact with G3BP1. Our lab set out to test whether nsP3-G3BP1 interaction is affected by PARP10 expression or whether MARYlation has an influence on either interaction. To assess the latter, we used in this experiment both the WT and inactive mutants of PARP10 and nsP3, PARP10 GW and nsP3 V33E, respectively. HEK293 cells were transiently transfected with either EGFP or EGFP-nsP3 (**Fig. 19**). For nsP3, G3BP1-interaction was tested for the WT, the V33E mutant and the FA mutant. The nsP3 fusion proteins were tested alone or in combination with PARP10 WT or PARP10 GW. EGFP and EGFP-nsP3 were immunoprecipitated using GFP-TRAP and assessed for interaction with G3BP1. EGFP does not interact with G3BP1 (**Fig. 19**). NsP3 WT and V33E interacted with G3BP1. NsP3 FA mutant did not interact with G3BP1. Co-expression of PARP10 WT or GW did not affect the nsP3-G3BP1 interaction (**Fig. 19**).

Taken together, MARYlation or hydrolase activity of PARP10 or nsP3 respectively did not affect nsP3-G3BP1 interaction. Also, overexpression of PARP10 did not affect the strong nsP3-G3BP1 interaction.

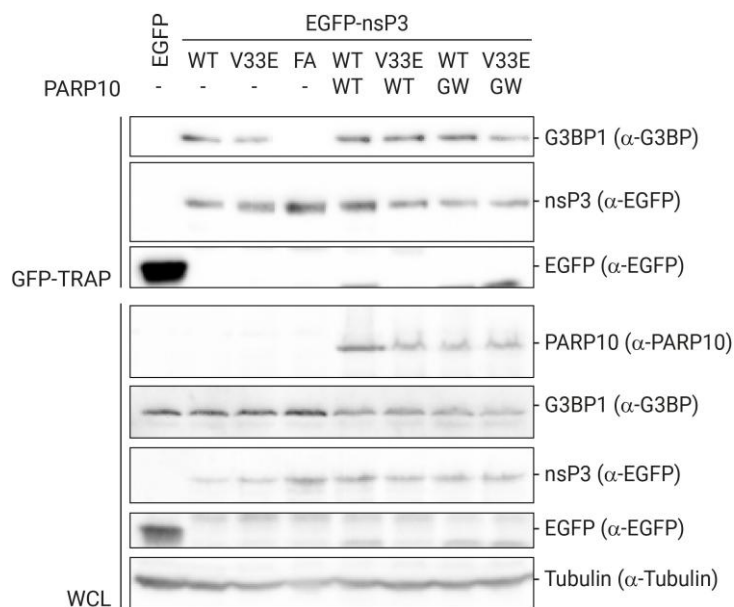


Figure 19. G3BP1 stably interacts with nsP3 independent of the MARYlation-ability or PARP10 expression. HEK293 cells were transiently transfected with plasmids encoding either EGFP, nsP3-EGFP WT/V33E/FA and PARP10 WT/GW as indicated. The cells were lysed 48h after transfection and EGFP and EGFP-nsP3 were immunoprecipitated using GFP-TRAP. Both the lysates and the co-immunoprecipitated proteins were analyzed by western blotting (n = 2).

2.2.2. G3BP1 is a common substrate of mARTDs and CHIKV nsP3

2.2.2.1. G3BP1 is MARYlated by different mARTDs *in vitro*

So far, G3BP1 has been confirmed as a mutual interactor of PARP10 and nsP3. Next, we assessed whether G3BP1 is a substrate of both enzymes. Therefore, His-G3BP1, His-PARP10cat and His-nsP3 were bacterially expressed and purified. G3BP1 was subjected to *in vitro* ADP-ribosylation assays in the presence of radioactively labelled NAD^+ and PARP10cat (**Fig. 20A**). As control, the inactive PARP10cat GW was included. Besides, to also test G3BP1 as a hydrolase-substrate of CHIKV nsP3, nsP3 MD and full-length (FL) nsP3 were included in the assay. Coomassie Blue staining (CB) was used to visualize the proteins and an autoradiograph to visualize the incorporated radioactive label and thus MARYlation. Upon presence of NAD^+ , G3BP1 was MARYlated by PARP10cat. PARP10cat was also automodified, as expected (Kleine et al., 2008). PARP10cat GW did not automodify or MARYlate G3BP1. When nsP3 MD or FL nsP3 was co-incubated, the MARYlation signal was removed (**Fig. 20A**). Unfortunately, nsP3 was only vaguely visible on the CB. This assay showed that G3BP1 is a substrate of both PARP10cat and nsP3.

G3BP1 was confirmed as a substrate of PARP10cat and nsP3. To assess whether G3BP1 is a substrate of IFN-I-inducible mARTDs in general, similar ADP-ribosylation assays were performed with PARP14cat (**Fig. 20B**) and PARP15cat (**Fig. 20C**). These His-tagged fusion proteins were bacterially expressed and purified. As control for PARP15 activity, the inactive PARP15cat HY was included. Besides, for both ADPr-assays, the nsP3 catalytic domain was incorporated. The assays were analyzed by CB-staining and immunoblotting.

G3BP1 is MARYlated by PARP14cat. PARP14cat is also automodified. Upon presence of nsP3 MD or FL nsP3, the MARYlation signal is removed (**Fig. 20B**).

Lastly, we observed that G3BP1 is MARYlated by PARP15cat (**Fig. 20C**). PARP15cat is automodified when incubated alone. There is also an unspecific band that runs under the 17kDa mark of unclear identity. Interestingly, upon presence of G3BP1 and PARP15cat, only G3BP1 is MARYlated by PARP15cat and there is hardly any automodification. It would be interesting to test whether longer incubation results in PARP15cat automodification, or if the

presence of G3BP1 blocks PARP15cat automodification. PARP15cat HY does neither automodify nor MARYlate G3BP1. NsP3 MD removes G3BP1 MARYlation (**Fig. 20C**).

Taken together, G3BP1 is a general substrate for IFN-inducible mARTDs catalytic domains *in vitro*. This PTM is removed by presence of the nsP3 catalytic domain or the full-length protein, indicating, G3BP1 is a substrate of CHIKV nsP3 *in vitro*.

Subsequently, G3BP1 was tested as a substrate for FL mARTDs. For this PARP10 and PARP15iso1 were overexpressed in cells and purified using immunoprecipitation. Subsequently, these purified proteins were used in ADP-ribosylation assays *in vitro* with bacterially expressed G3BP1. Since there is no FL PARP14 available for cellular expression in our lab, this FL protein could not be tested. G3BP1 was incubated with either PARP15iso1 WT/HY or PARP10 WT/GW in combination with radioactively labelled NAD⁺. The assays were analyzed using CB staining and autoradiograph (**Fig. 20D**). G3BP1 is MARYlated *in vitro* by FL PARP15iso1 and FL PARP10 (**Fig. 20D**). There is also automodification of the enzymes visible. Upon presence of nsP3 MD or FL nsP3, G3BP1 MARYlation is removed (**Fig. 20D**).

Taken together, G3BP1 is a general substrate for IFN-I-inducible mARTDs and CHIKV nsP3 *in vitro*.

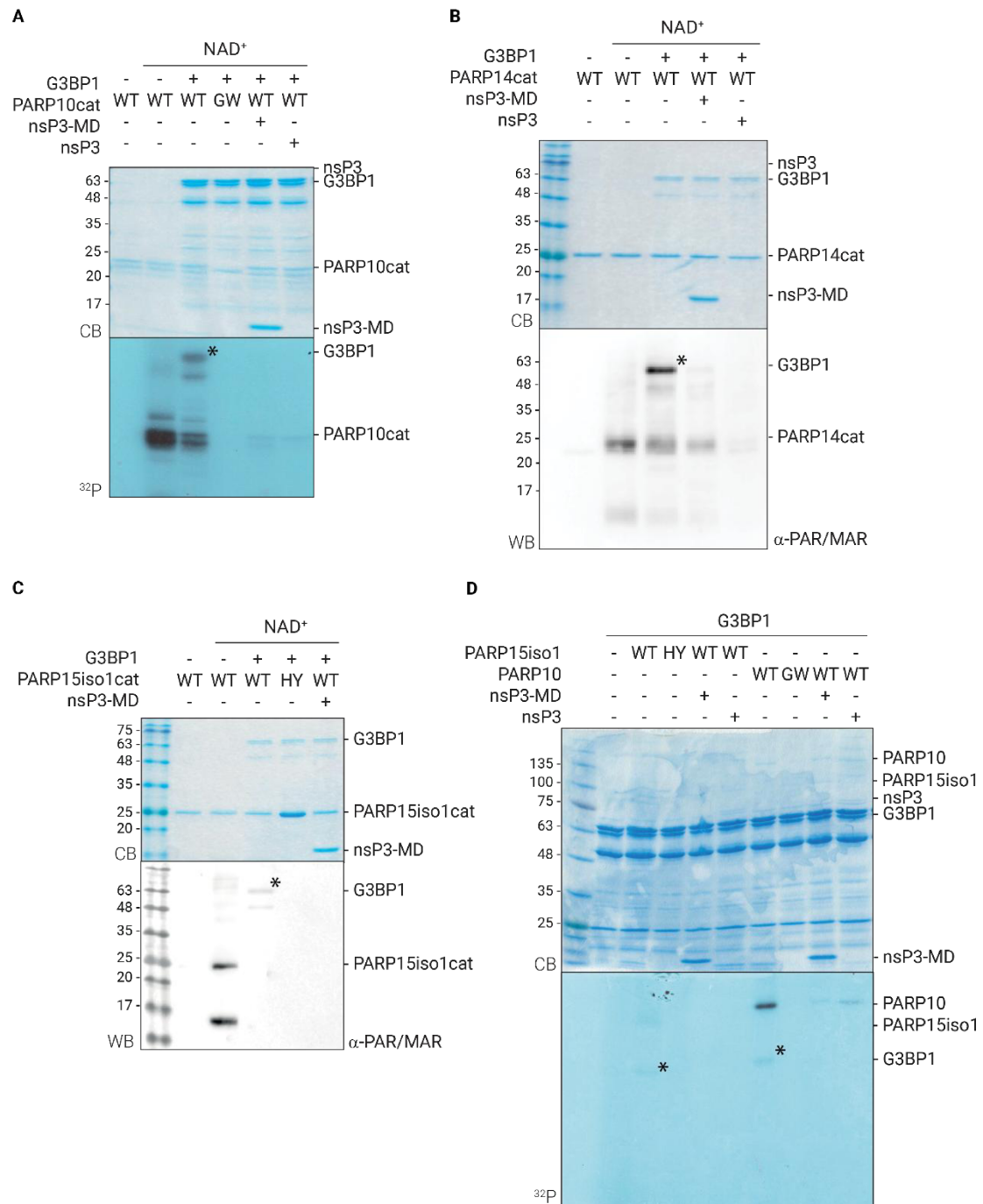


Figure 20. G3BP1 is a substrate of IFN-I-inducible mARTDs and CHIKV nsP3. **A.** Bacterially expressed, His-tagged G3BP1 was incubated with His-tagged PARP10cat WT/GW and full length (FL) nsP3 or nsP3 macrodomain (MD) as indicated in the presence of radioactively labelled ³²P-NAD⁺ for 30 min at 30°C. Total proteins were visualized with Coomassie blue (CB) staining and the incorporated radioactive label was analyzed using autoradiography (³²P) (n=3). **B.** Bacterially expressed, His-tagged G3BP1 was incubated with His-tagged PARP14cat WT and FL nsP3 or nsP3 MD as indicated in the presence of NAD⁺ for 30 min at 30°C. Total proteins were visualized with CB-staining and MARYlation was analyzed by western blotting (WB) (n=3). **C.** Bacterially expressed, His-tagged G3BP1 was incubated with His-tagged PARP15cat WT/HY and nsP3 MD as indicated in the presence of NAD⁺ for 30 min at 30°C. Total proteins were visualized with CB-staining and MARYlation was analyzed by western blotting (WB) (n=3). **D.** Bacterially expressed, His-tagged G3BP1 was incubated with immunoprecipitated FL PARP10 WT/GW and PARP15iso1 WT/HY from HEK293 cells and bacterially expressed, His-tagged FL nsP3 or nsP3 MD as indicated in the presence of radioactively labelled ³²P-NAD⁺ for 30 min at 30°C. Total proteins were visualized with Coomassie blue (CB) staining and the incorporated radioactive label was analyzed using autoradiography (³²P) (n=2). *: G3BP1 MARYlation.

2.2.2.2. G3BP1 is MARYlated in cells

G3BP1 interacts with PARP10 and nsP3 in cells and is a substrate of mARTDs and nsP3 *in vitro*. Consequently, we tested whether G3BP1 is MARYlated in cells.

Since, G3BP1 is essential for SG formation and how SGs are formed is still quite elusive, we wanted to assess whether stress induction resulted in G3BP1 MARYlation (**Fig. 21A**). HEK293 cells were transfected with either EGFP or mEGFP-G3BP1. Two conditions with G3BP1 were treated with Arsenite for 1h. Arsenite treatment is used to induce the oxidative stress response and consequently G3BP1 condensate formation. One of these conditions was then washed with medium to give the cells a recovery time of one hour to allow SG disassembly. EGFP and mEGFP-G3BP1 were immunoprecipitated and analyzed for MARYlation (**Fig. 21A**). G3BP1 was MARYlated in all conditions with no noticeable differences. It seems that G3BP1 is MARYlated on a basal level and unaffected by oxidative stress induction or recovery. Since, other stressors induce different pathways and have different methods of (dis-)assembly, it would be of import to check the other stressors individually.

G3BP1 is MARYlated *in vitro* by PARP10 and PARP15iso1. Next, we wanted to test whether their expression induces G3BP1 MARYlation. HEK293 cells were transfected with mEGFP-G3BP1 and HA-PARP10 WT/GW, HA-PARP15iso1 WT/HY or nsP3 alone or in combinations as indicated (**Fig. 21B/C**). After cell lysis, mEGFP-G3BP1 was immunoprecipitated and analyzed for MARYlation (**Fig. 21B/C**). G3BP1 was MARYlated upon PARP10 WT expression (**Fig. 21B**). PARP10 GW expression did not result in G3BP1 MARYlation. Co-expression of nsP3 removed PARP10-induced G3BP1-MARYlation. G3BP1 was also MARYlated upon PARP15iso1 WT expression (**Fig. 21C**). PARP15iso1 HY expression did not induce G3BP1 MARYlation. NsP3 removes PARP15iso1 WT induced MARYlation.

Expression of both PARP10 and PARP15iso1 resulted in G3BP1 modification, while their inactive mutants did not. Taken together with the *in vitro* assays, we concluded that both enzymes can modify G3BP1 in cells. Similarly, for nsP3 we conclude that G3BP1 is a substrate for hydrolase-activity in cells.

So far, we tested MARYlation of G3BP1 using exogenous G3BP1. Next, we wanted to see whether we could see a difference for endogenous G3BP1. HEK293 cells were transfected with the different mARTDs, PARP10, PARP12 and PARP15iso1 and nsP3 as indicated (**Fig. 22A**) or treated with IFN α to stimulate endogenous mARTDs expression. The cells were lysed and endogenous G3BP1 was immunoprecipitated and analyzed for MARYlation. G3BP1 showed a MARYlation signal in all conditions with no clear difference. Endogenous G3BP1 seems to be MARYlated on a basal level independent of the stimuli used in this experiment (**Fig. 22A**).

Upon analysis of endogenous G3BP1, we see a signal for endogenous G3BP1 in all conditions, despite the expression of different mARTDs (**Fig. 22A**). We wanted to assess if this is MARYlation and not an unspecific signal. HEK293 cells were lysed and G3BP1 immunoprecipitated. Next, we performed a hydrolase assay on the captured G3BP1 either with or without bacterially expressed and purified nsP3 MD. G3BP1 showed a slightly higher MARYlation signal than the MD-treated G3BP1, indicating that endogenous G3BP1 is slightly MARYlated (**Fig. 22B**). Leftover signal could be due to either G3BP1 MARYlation at a different site than targeted by nsP3 MD, G3BP1 PARYlation or unspecific binding of the antibody to G3BP1.

It seems that G3BP1 is indeed MARYlated in cells and this PTM is reversed by CHIKV nsP3. Even though there is no distinct result on endogenous level, this could be due to different reasons. It would be of interest to test different stressors and analysis methods, since it

could be that there is a trigger missing or the detection methods used are not sensitive enough to detect endogenous G3BP1 MARYlation. MARYlation is still poorly studied, and substrates are hard to define due to the lack of tools for MARYlation detection on cellular level.

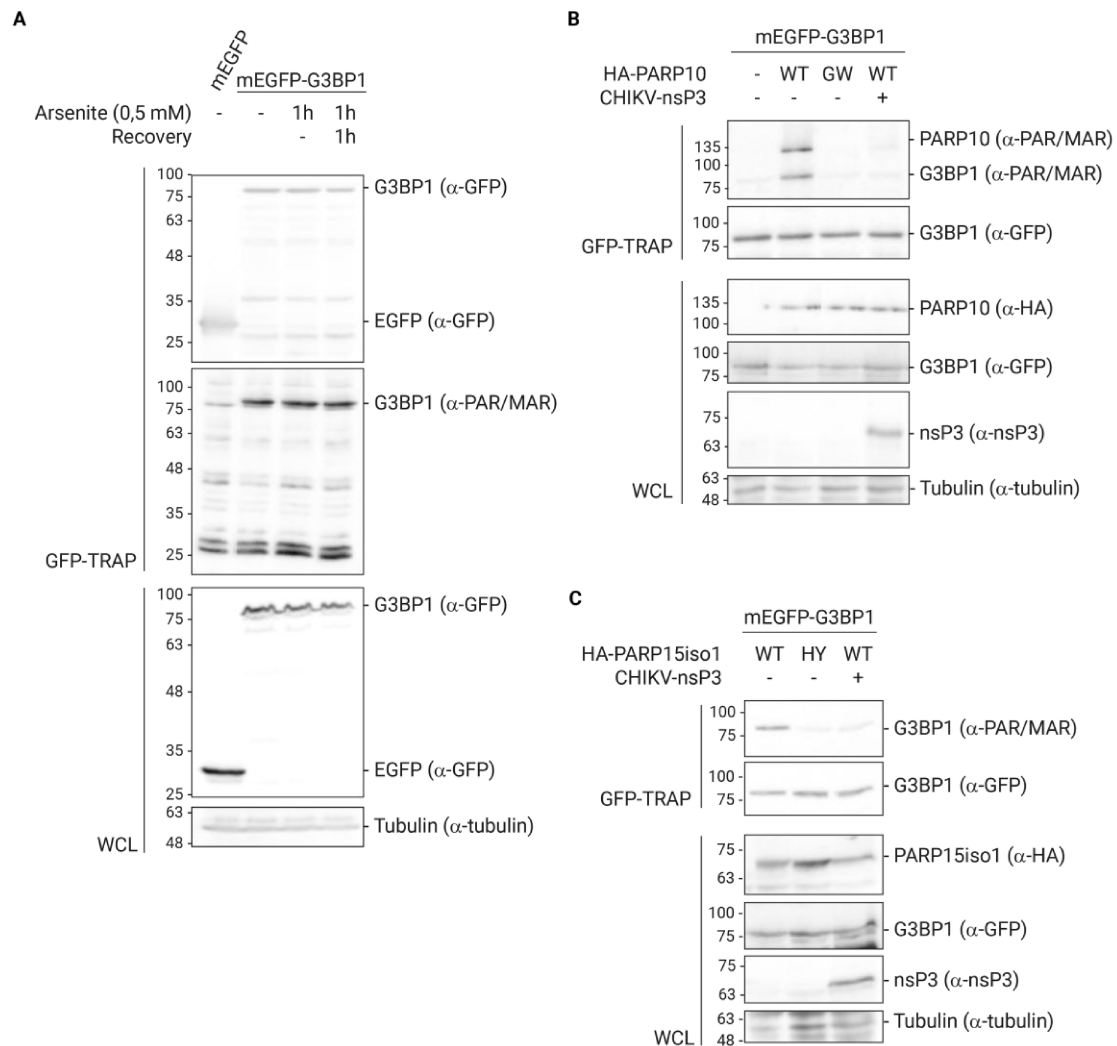


Figure 21. G3BP1 is MARYlated in cells. **A.** HEK293 cells were transiently transfected with plasmids encoding either EGFP or mEGFP-G3BP1. Two conditions were treated with 0,5 mM Arsenite for 1h. One of the respective conditions was afterwards washed with medium and incubated for 1h for stress recovery. The cells were lysed and EGFP and mEGFP-G3BP1 were immunoprecipitated using GFP-TRAP. Both the lysates and the co-immunoprecipitated proteins were analyzed by western blotting (n = 2). **B.** HEK293 cells were transiently transfected with plasmids encoding mEGFP-G3BP1 in combination with PARP10 WT/GW and nsP3 as indicated. The cells were lysed 48h after transfection and mEGFP-G3BP1 was immunoprecipitated using GFP-TRAP. Both the lysates and the co-immunoprecipitated proteins were analyzed by western blotting (n = 3). **C.** HEK293 cells were transiently transfected with plasmids encoding mEGFP-G3BP1 in combination with PARP15iso1 WT/HY and nsP3 as indicated. The cells were lysed 48h after transfection and mEGFP-G3BP1 was immunoprecipitated using GFP-TRAP. Both the lysates and the co-immunoprecipitated proteins were analyzed by western blotting (n = 2).

2.2.3. G3BP1-RBD is MARYlated by mARTDs

We confirmed G3BP1 as a (de-)MARYlation substrate of IFN-induced mARTDs and nsP3 *in vitro* and have strong indication of G3BP1 (de-)MARYlation in cells. Next, we wanted to identify the MARYlation site(s) within the protein. Information on the site will help in determining the exact role of G3BP1 MARYlation and why nsP3 seems to counteract this PTM upon CHIKV infection.

As extensively discussed in the introduction (**chapter 1.4.1.**), G3BP1 consists of the N-terminal NTF2L-domain, IDR1, IDR2 and the RBD, which in turn is build-up of the RRM and the IDR3 (**Fig. 23**). The IDRs are marked when analyzing the protein with PONDR (Predictor of Naturally Disordered Regions – Disorder Protein Predictor). PONDR is a tool that predicts IDRs within sequences based on the specific attributes such as the amino acids distribution, hydropathy, sequence complexity and others. In our case we used the VL-XT predictor algorithm that combines 3 neural networks trained on x-ray crystallographic studies and nuclear magnetic resonance (NMR). G3BP1 was also analyzed using AlphaFold (**Fig. 23C/D**). AlphaFold is an AI system that predicts the three-dimensional (3D) protein structures from their primary sequence. So far, this tool has been shown to be accurate (Jumper et al., 2021). First, the Predicted Aligned Error (PAE) is shown (**Fig. 23C**). PAE shows the expected error at the specific residues, which is used to assess the confidence in whether the respective residue has the predicted position. Basically, the low values indicate clear domains with a well-defined position, while high values indicate an unreliable position and there is no structural relevance to these residues. For G3BP1 it clearly shows a defined domain at the N-terminus (NTF2L) and a smaller defined domain towards the C-terminus (RRM) (**Fig. 23C**). This result is in line with the PONDR disorder prediction (**Fig. 23B**). Also, the AlphaFold predicted structure of G3BP1 shows the same build-up (**Fig. 23D**).

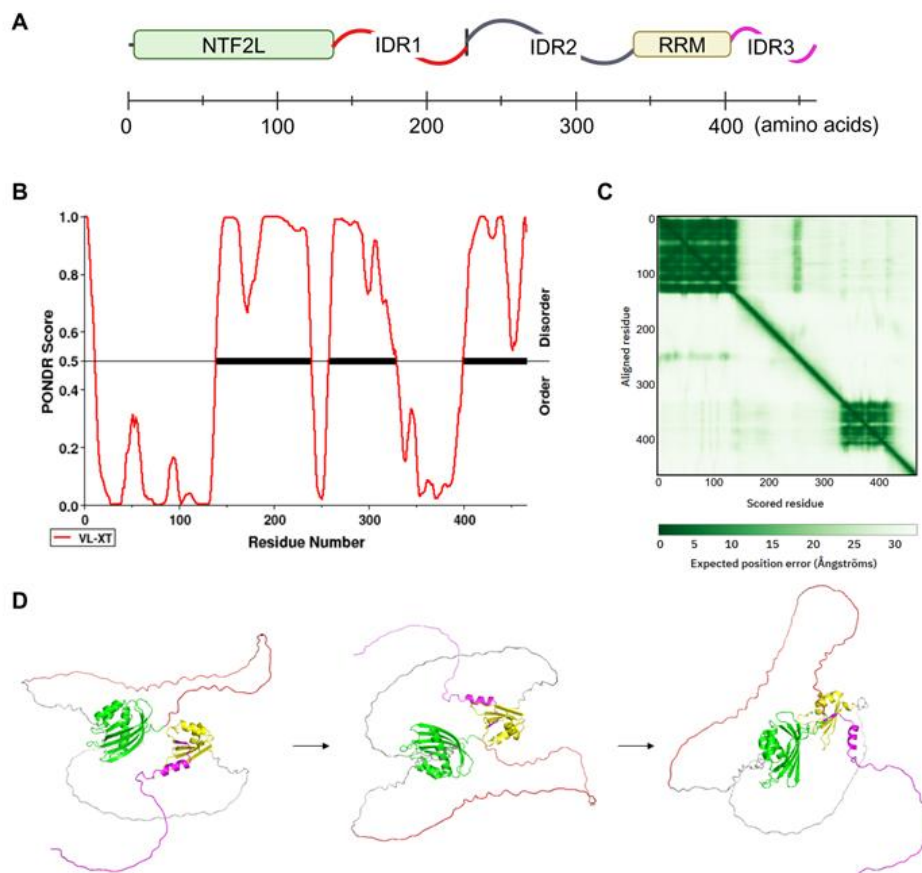


Figure 23. G3BP1 domain architecture. **A.** Schematic overview of G3BP1 domains. Nuclear transport factor 2-like (NTF2L) domain, intrinsically disordered region 1 (IDR1), IDR2 and the RNA-binding domain (RBD), which consists of the RNA recognition motif (RRM) and IDR3. **B.** PONDR VL-XT-based disorder analysis of G3BP1 (red), predicting the IDRs. **C.** Predicted aligned error (PAE) analysis of G3BP1. PAE assesses the confidence in whether the respective residue has the predicted position. Low values (dark green) indicate clear domains with a well-defined position; high values (light green) indicate an unreliable position. **D.** AlphaFold-predicted structure of G3BP1.

2.2.3.1. G3BP1-RBD is MARYlated by mARTDs *in vitro*

The different G3BP1 domains have distinct functions (**chapter 1.4.1.**). The NTF2L-domain is important for dimerization and PPIs (Alam & Kennedy, 2019; Sidibé et al., 2021; Yang et al., 2020). The IDRs have a defined role in LLPS initiation and intramolecular interactions (Sanders et al., 2020; Yang et al., 2020). Lastly, the RBD-domain is important for RNA-binding, as well as LLPS regulation (Alam & Kennedy, 2019; Sidibé et al., 2021; Yang et al., 2020). Knowledge of the G3BP1 site that is MARYlated by IFN-I-induced mARTDs, would thus give an indication on how the PTM affects G3BP1's function(s).

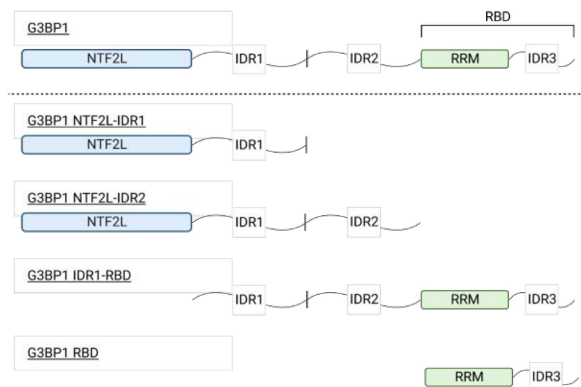
To characterize the G3BP1 modification site(s), we cloned different G3BP1 truncations as His₆ fusion proteins for bacterial expression as visualized in Fig. 24A: G3BP1 NTF2L-IDR1; G3BP1 NTF2L-IDR2 (or G3BP1 Δ RBD); G3BP1 IDR1-RBD; G3BP1 RBD. To test whether the G3BP1 truncations were MARYlated *in vitro*, they were bacterially expressed and purified. The different G3BP1 truncations were then subjected to an *in vitro* MARYlation and hydrolase-assay, using ³²P-NAD⁺ and immunoprecipitated mARTDs from HEK293 cells. Firstly, MARYlation of G3BP1 by PARP10 was checked (**Fig. 24B/E**). As a negative control, G3BP1 was incubated alone with ³²P-NAD⁺. Here, we do not see any MARYlation signal on the autoradiogram. G3BP1 WT and the G3BP1 truncations were incubated with FL PARP10, or with FL PARP10 and nsP3 MD. After 30' the reaction was stopped and subjected to SDS-PAGE. Proteins and the potential MARYlation signal were visualized using CB staining and autoradiography, respectively. G3BP1 WT and G3BP1 RBD are MARYlated by PARP10 *in vitro* and this PTM is removed upon the presence of the nsP3 MD. The remaining truncations do not show a MARYlation signal, indicating the RBD domain as the site of modification by PARP10. For the G3BP1 IDR1-RBD truncation there is a strong signal around 11kDa. It is worth speculating that this is a degradation product harboring a part of the RBD domain fragment. Of note, upon performing these experiments with PARP10cat, all truncations are highly MARYlated by PARP10cat (data not shown). This shows the ambiguity of the catalytic domain in comparison to the FL protein. Because of this reason, all site determination experiments were performed with the FL mARTDs and not the catalytic domains.

To analyze whether the different IFN-I induced mARTDs share a modification site, the G3BP1 truncations were subjected to an *in vitro* MARYlation and hydrolase-assay, using FL PARP12 and FL PARP15iso1. Both enzymes were argued to localize to SGs (Leung et al., 2011). As a negative control, G3BP1 was incubated alone with ³²P-NAD⁺. G3BP1 by itself does not lead to MARYlation (**Fig. 24C-E**). PARP12 MARYlates G3BP1 WT and the G3BP1 RBD domain *in vitro*. The PTM is removed upon co-incubation with nsP3 MD. The other G3BP1 truncations were not MARYlated by PARP12 (**Fig. 24C/E**). When co-incubated with PARP15, G3BP1 WT shows a MARYlation signal, as well as G3BP1 NTF2L-IDR1, G3BP1 NTF2L-IDR2, G3BP1 IDR1-RBD and G3BP1 RBD (**Fig. 24D/E**). This signal is removed upon presence of the nsP3 MD. However, out of all the truncations, the RBD-domain shows the highest MARYlation signal, indicating it as the main site of modification.

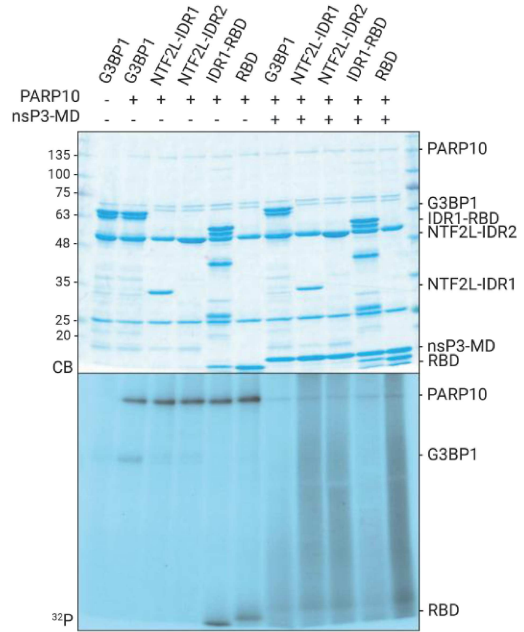
For the different mARTDs tested, PARP10/12/15iso1, the G3BP1 RBD-domain is the main modification site *in vitro*. The different mARTDs do show a difference in G3BP1 MARYlation

signal. PARP10 and PARP12 clearly MARYlate the RBD *in vitro*. PARP15iso1 seems to be able to modify more than the RBD-domain *in vitro*. However, we suggest the RBD as the primary modification site for the IFN-I-inducible mARTDs tested here. Of note, G3BP1 IDR1-RBD is not MARYlated by mARTDs even though it contains the RBD-domain. We speculate that this is due to the interaction between the IDR1 and the RBD (**chapter 1.4.1.**), and thus the unavailability of the modification site (Yang et al., 2020).

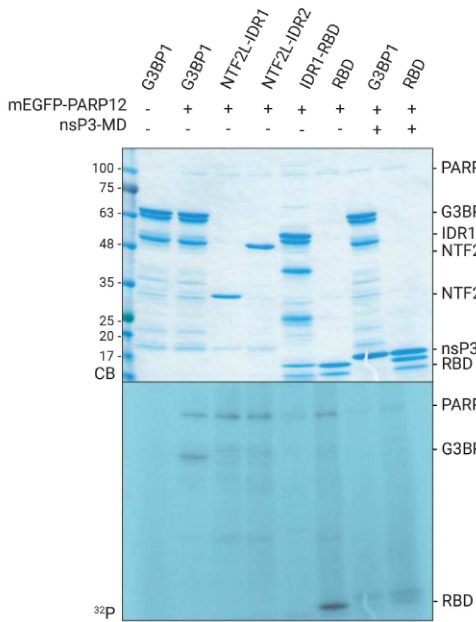
A



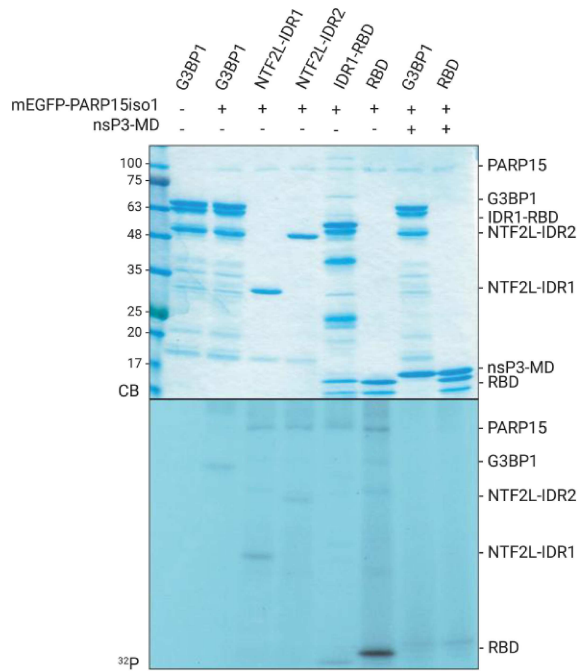
B



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D



E

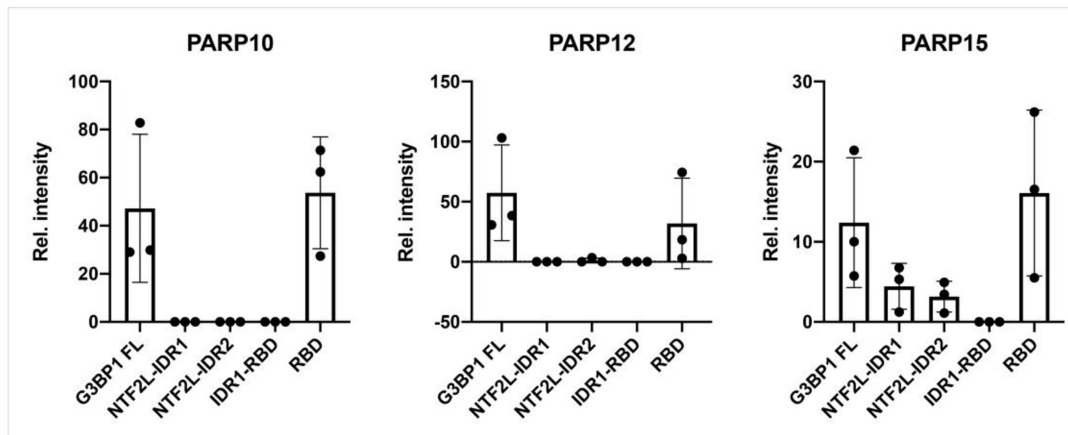


Figure 24. G3BP1-RBD is MARYlated by mARTDs in vitro. **A.** Schematic representation of G3BP1 truncations. **B.** Bacterially expressed, His-tagged G3BP1 or G3BP1 truncations were incubated with FL PARP10 immunoprecipitated from HEK293 cells and bacterially expressed nsP3 macrodomain (MD) as indicated in the presence of radioactively labelled ^{32}P -NAD $^{+}$ for 30 min at 30°C. Total proteins were visualized with Coomassie blue (CB) staining and the incorporated radioactive label was analyzed using autoradiography (^{32}P) (n=3). **C.** Bacterially expressed, His-tagged G3BP1 or G3BP1 truncations were incubated with FL PARP12 immunoprecipitated from HEK293 cells and bacterially expressed nsP3 macrodomain (MD) as indicated in the presence of radioactively labelled ^{32}P -NAD $^{+}$ for 30 min at 30°C. Total proteins were visualized with Coomassie blue (CB) staining and the incorporated radioactive label was analyzed using autoradiography (^{32}P) (n=3). **D.** Bacterially expressed, His-tagged G3BP1 or G3BP1 truncations were incubated with FL PARP15iso1 immunoprecipitated from HEK293 cells and bacterially expressed nsP3 macrodomain (MD) as indicated in the presence of radioactively labelled ^{32}P -NAD $^{+}$ for 30 min at 30°C. Total proteins were visualized with Coomassie blue (CB) staining and the incorporated radioactive label was analyzed using autoradiography (^{32}P) (n=3). **E.** G3BP1 modification was quantified using Fiji and plotted using Graphpad (n=3).

2.2.3.2. G3BP1-RBD is MARYlated by PARP10 in cells

We identified G3BP1-RBD as the main site of modification *in vitro* by PARP10, PARP12 and PARP15iso1. To validate that the RBD is also MARYlated in cells by mARTDs. HEK293 cells were transiently transfected with plasmids encoding either EGFP, EGFP-G3BP1, EGFP-G3BP1-RBD and/or HA-PARP10 and/or FLAG-CHIKV nsP3. The cells were lysed, the EGFP-fusion proteins were immunoprecipitated using GFP-TRAP and the MARYlation signal of the different fusion proteins was analyzed by immunoblotting (**Fig. 25**). EGFP alone was transiently transfected in combination with PARP10. The EGFP-tag is not MARYlated by PARP10. As a negative control, G3BP1 was transfected by itself. G3BP1 or the G3BP1-RBD does not show a MARYlation signal upon the absence of PARP10. Upon presence of PARP10, a clear MARYlation signal is visible for G3BP1 (**Fig. 25**). Also, we saw a signal for PARP10, again showing that it interacts with G3BP1. PARP10 also highly MARYlates G3BP1-RBD (**Fig. 25**). There is also a slight signal for PARP10 after the RBD GFP-TRAP. So, it is worth speculating that PARP10 interacts through the RBD domain with G3BP1. This interaction could be directly or facilitated through their mutual interaction with RNA. Upon presence of nsP3, the FL G3BP1 and the G3BP1-RBD do not show a MARYlation signal, indicating that the PTM is reversed by nsP3 (**Fig. 25**). Taken together, we suggest the G3BP1-RBD as the primary MARYlation site of PARP10, not only *in vitro* but also in cells, which upon presence of nsP3 is reversed. So far, our lab only checked G3BP1-RBD MARYlation by PARP10 in cells. These experiments need to be repeated with other IFN-I-inducible mARTDs.

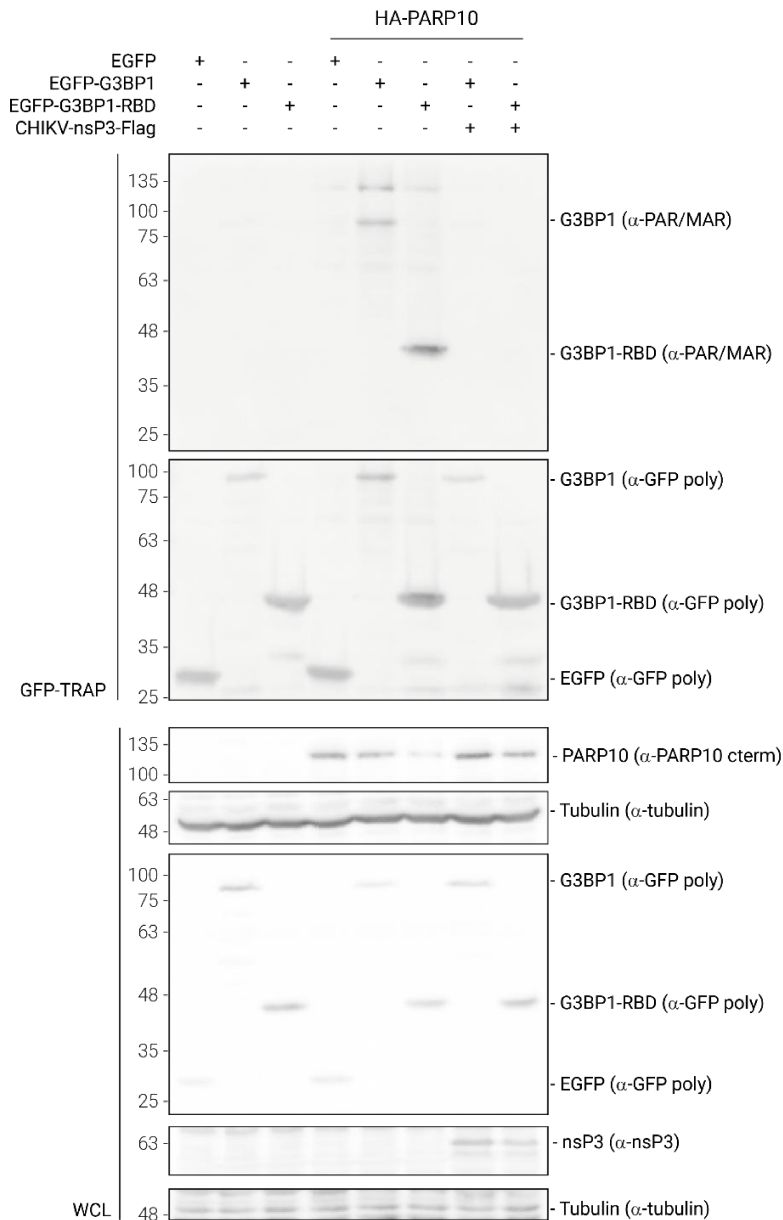


Figure 25. G3BP1-RBD is MARYlated in cells by PARP10. HEK293 cells were transiently transfected with plasmids encoding EGFP, mEGFP-G3BP1 or mEGFP-G3BP1-RBD in combination with PARP10 WT and nsP3 as indicated. The cells were lysed 48h after transfection and the EGFP phusion proteins were immunoprecipitated using GFP-TRAP. Both the lysates and the co-immunoprecipitated proteins were analyzed by western blotting (n = 3).

2.2.3.4. MARYlation of G3BP1-RBD does not influence G3BP1-RNA interaction

The RBD appears to be the primary MARYlation site. Next the functional relevance of MARYlation on G3BP1-activity is addressed. As the name says, the RBD is responsible for G3BP1 RNA-binding ability. As discussed in the introduction, upon deletion of both the RRM and IDR3 RNA-binding is lost while Δ RRM has reduced RNA-binding, and Δ IDR3 blocks or reduces RNA-binding (Guillén-Boixet et al., 2020; Yang et al., 2020). So, by the addition of an ADP-ribose unit the RNA-binding ability of G3BP1 could be affected. The PTM could block RNA binding through its spherical presence or negative charge. On the other hand, it could induce the open confirmation of G3BP1 and promote RNA-binding. To assess this, we set out to establish a method to measure G3BP1-RNA binding. We examined RNA-binding by using electrophoretic mobility shift assays (EMSA) based on the protocol used by Yang et al, 2020 (Yang et al., 2020). EMSA is a rapid method to detect whether proteins interact with nucleic

acids based on the principle that protein-bound RNA migrates slower than free RNA. For the assay, G3BP1 was bacterially expressed and purified. G3BP1 was incubated with Cy3-labeled RNA for 30' at RT. Finally, the samples were analyzed and RNA was detected through the fluorescent label. Free RNA migrates quickly through the gel. However, upon presence of G3BP1, the RNA is bound to G3BP1 and migrates slower (**Fig. 26A**). An increase in protein concentration leads to more bound RNA (**Fig. 26A**). The EMSA shows that purified G3BP1 binds to RNA. To validate that this is a specific interaction, the assay was run with WT G3BP1 and G3BP1 NTF2L-IDR2 (G3BP1 Δ RBD) as control (**Fig. 26B**). Again, we see a clear interaction between RNA and FL G3BP1, while G3BP1 NTF2L-IDR2 does not interact with RNA (**Fig. 26B**).

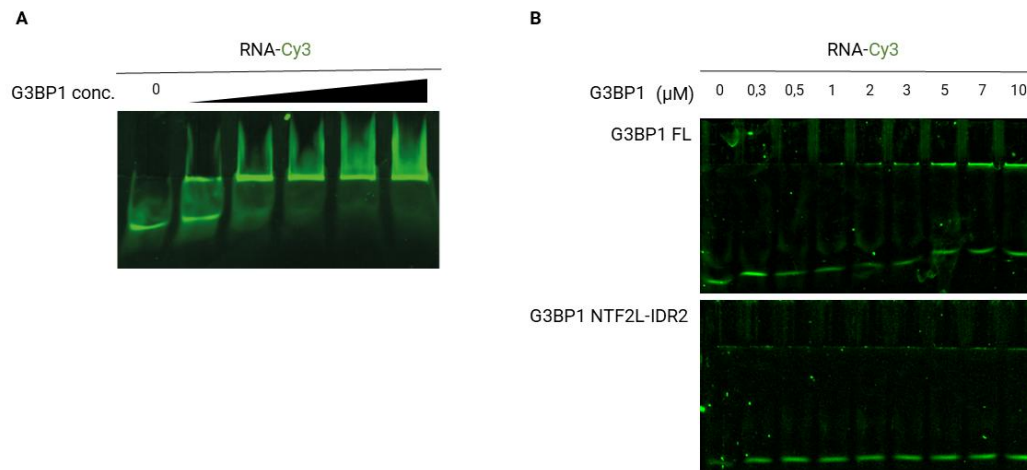


Figure 26. G3BP1 interacts with RNA. **A.** Electrophoretic mobility shift assay (EMSA) of G3BP1 WT in a concentration dependent manner. His-tagged G3BP1 was bacterially expressed and purified. G3BP1 (0; 0.3; 0.8; 1.3; 2.6; 3.9 μ M) was incubated with Cy3-labeled RNA for 30' at RT. G3BP1-RNA interaction was analyzed using SDS-PAGE (n=1). **B.** His-tagged G3BP1 and G3BP1-NTF2L-IDR2 were bacterially expressed and purified. G3BP1 was incubated with Cy3-labeled RNA for 30' at RT. G3BP1-RNA interaction was analyzed using SDS-PAGE (n=1).

After establishing the EMSA to measure G3BP1 RNA-binding, we want to assess whether G3BP1 MARYlation affects this function. For this PARP10 was transiently transfected into HEK293 cells. The cells were lysed and PARP10 was immunoprecipitated using Agarose G beads and an HA-specific antibody. Bacterially expressed and purified G3BP1 was used for an ADP-ribosylation assay and incubated with PARP10-bound beads in absence (unMARYlated G3BP1) or in presence of NAD⁺ (MARYlated G3BP1). The supernatant containing G3BP1 was used for a follow-up EMSA assay and incubated with Cy3-labeled RNA for 30' at RT (**Fig. 27A/B**). Lastly, the different assays were quantified and analyzed together. MARYlation of G3BP1 does not seem to affect RNA-binding. Upon pairwise comparison of the G3BP1 concentrations, unMARYlated vs. MARYlated, there is no significant effect of MARYlation (**Fig. 27A/B**). However, there does seem to be a positive trend of MARYlation on G3BP1 RNA-binding. To assess whether this is valid, either the protocol needs to be further optimized or a more sensitive method than EMSA is required.

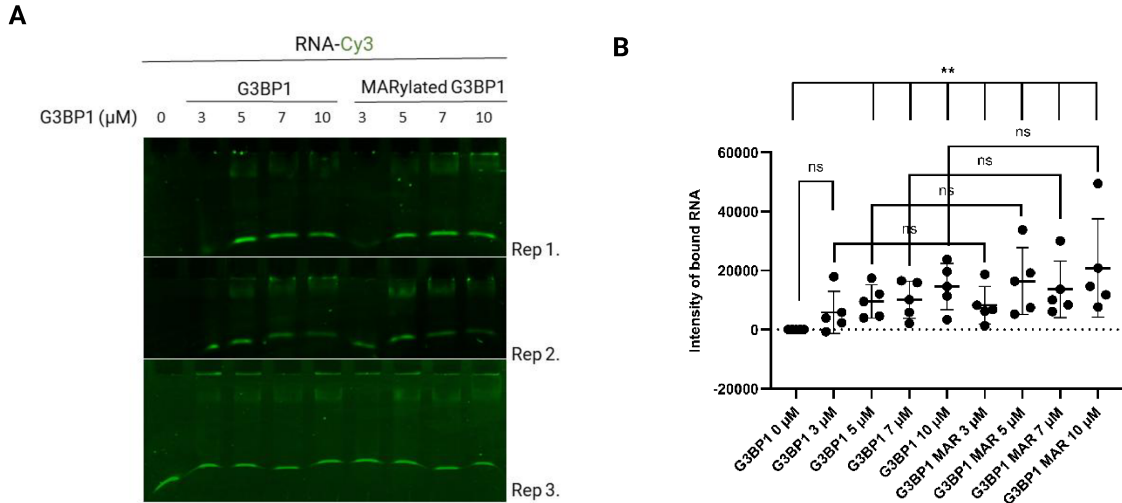


Figure 27. G3BP1 MARYlation does not significantly affect RNA-interaction. **A.** His-tagged G3BP1 was bacterially expressed and purified. G3BP1 was incubated in the presence of NAD^+ with or without FL PARP10 immunoprecipitated from HEK293 for 30 min at 30°C . Following, G3BP1 was incubated with Cy3-labeled RNA for 30' at RT. G3BP1-RNA interaction was analyzed using SDS-PAGE ($n=3$). Three representative assays were shown. **B.** G3BP1-RNA interaction was quantified using Fiji and plotted using Graphpad ($n=5$). Mann-Whitney test was used for pairwise analysis.

2.2.3.5. G3BP1 is not cleaved by CHIKV-nsP2-protease activity

Viruses have developed diverse ways to handle stress granules and SG-proteins, such as G3BP1. Studies have suggested that members of the Caliciviruses and Picornaviruses cleave G3BP1 (**chapter 1.4.4.2.**) (Humoud et al., 2016; White et al., 2007). The viral proteases disrupt SGs by cleavage of G3BP1. In Chikungunya, nsP2 contains the protease activity. As mentioned, our lab has shown that substrate MARYlation inhibits cleavage by nsP2 (Krieg et al., 2023). Also of interest, is that G3BP1 is cleaved by FCV in the RBD-domain (Humoud et al., 2016b). The RBD was pinpointed as the main modification site of G3BP1 by mARTDs. Lastly, when analyzing the sequence (Serial Cloner), we detected a motif within the G3BP1-RBD similar to the nsP2 preferred cleavage site between nsP3 and nsP4 (**Fig. 28A**).

To test G3BP1 as a substrate of nsP2, we ran a protease-assay established in our lab (Krieg et al., 2023). As positive control for nsP2-activity, we used an artificial substrate that is based on the junction between nsP3 and nsP4, which has been described as the best *in vitro* substrate for nsP2 (Rausalu et al., 2016; Vasiljeva et al., 2001) (**Fig. 28B**). During early steps of infection, polyprotein P1234 processing starts with the cleavage of the nsP3-4 junction by the nsP2 protease (Rausalu et al. 2016). The nsP3-4 site is flanked respectively by a GST- and an EGFP-tag (Krieg et al., 2023)(**Fig. 28B**). For the protease assay, all the relevant proteins, G3BP1, nsP2-protease domain (WT and the inactive CASA mutant) and the substrate, were expressed in *E. coli* and purified. A protease assay was run and analyzed by Coomassie blue staining. G3BP1 was neither cleaved by nsP2-protease nor the inactive mutant nsP2-protease-CASA. However, the substrate was successfully cleaved by the nsP2-protease and not by the inactive CASA mutant. These results demonstrate that, even though the nsP2-protease is active, G3BP1 is not cleaved *in vitro* by nsP2 protease activity. Chikungunya protease does not cleave G3BP1, in comparison to Caliciviruses and Picornaviruses G3BP1.

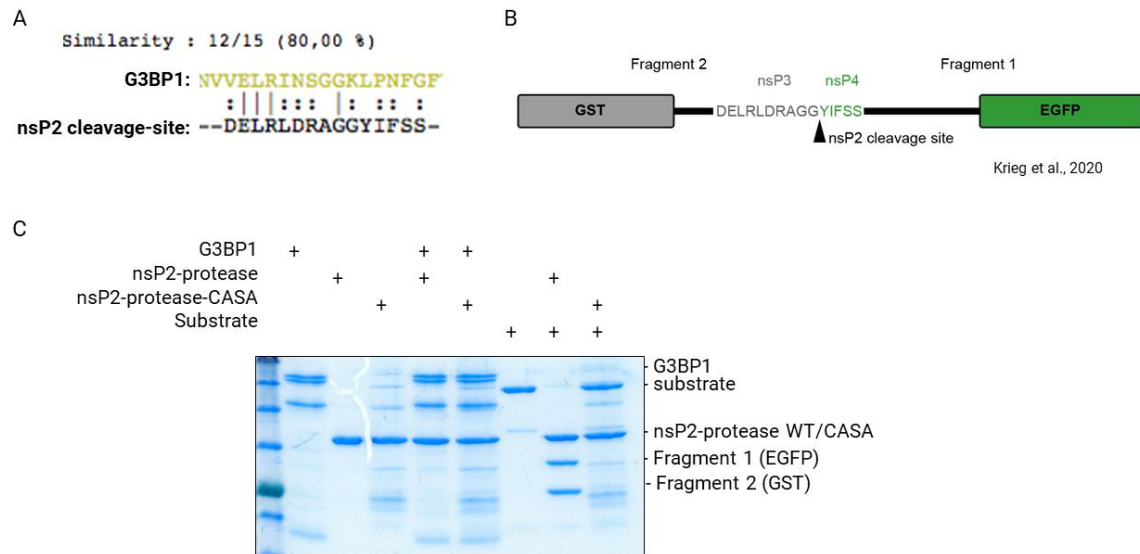


Figure 28. CHIKV-nsP2 does not cleave G3BP1. **A.** Serial Cloner comparison between the nsP2 preferred cleavage site nsP3/nsP4 and G3BP1 identified a similar motif in the G3BP1-RBD. | : identical amino acids; : : Amino acids with similar properties. **B.** Schematic representation of the substrate for the nsP2 protease assay. The peptide consists of the cleavage site between nsP3 and nsP4, flanked by a GST- and an EGFP-tag respectively. **C.** G3BP1, nsP2-protease domain WT/CASA and the Substrate were bacterially expressed and purified. G3BP1 and the Substrate were incubated with nsP2-protease domain WT, or the CASA mutant as indicated for 30min at 30°C. The proteins were analyzed using SDS-PAGE and Coomassie blue (CB) (n = 2).

2.2.4. G3BP1 localization upon mARTDs expression

G3BP1 is a common interactor and substrate of mARTDs and CHIKV nsP3. Next, we assessed whether mARTDs affects G3BP1 localization or SG formation. In a previous report, it was found that CHIKV nsP3 influences G3BP1 and SG formation dependent on its ADP-ribosylhydrolase activity (Jayabalan et al., 2021). We hypothesized that mARTD expression would thus affect G3BP1 and SGs localization and formation dependent on their catalytic activity.

To analyze how mARTD expression affects G3BP1 and SGs, we used U2OS cells that were constructed using CRISPR-Cas9 to express tdTomato-tagged endogenous G3BP1, kindly given to us by the J. Paul Taylor Lab (Yang et al., 2020) (**Fig. 29A**). The endogenous tdTomato-tagged G3BP1 localize similarly to untagged endogenous G3BP1 in these U2OS cells (Yang et al., 2020). Thus, these U2OS tdTomato-G3BP1 form a perfect tool for analyzing how mARTDs expression influence G3BP1 localization and SG formation.

U2OS tdTomato-G3BP1 were analyzed using confocal microscopy (**Fig. 29B**). In unstressed cells, tdTomato-G3BP1 is ubiquitously expressed in the cytoplasm. In stressed cells, tdTomato-G3BP1 localizes to SGs, similarly as untagged G3BP1 in cells. Western blot analysis of these cells shows that the U2OS tdTomato-G3BP1 is a heterozygous mutation (**Fig. 29C**).

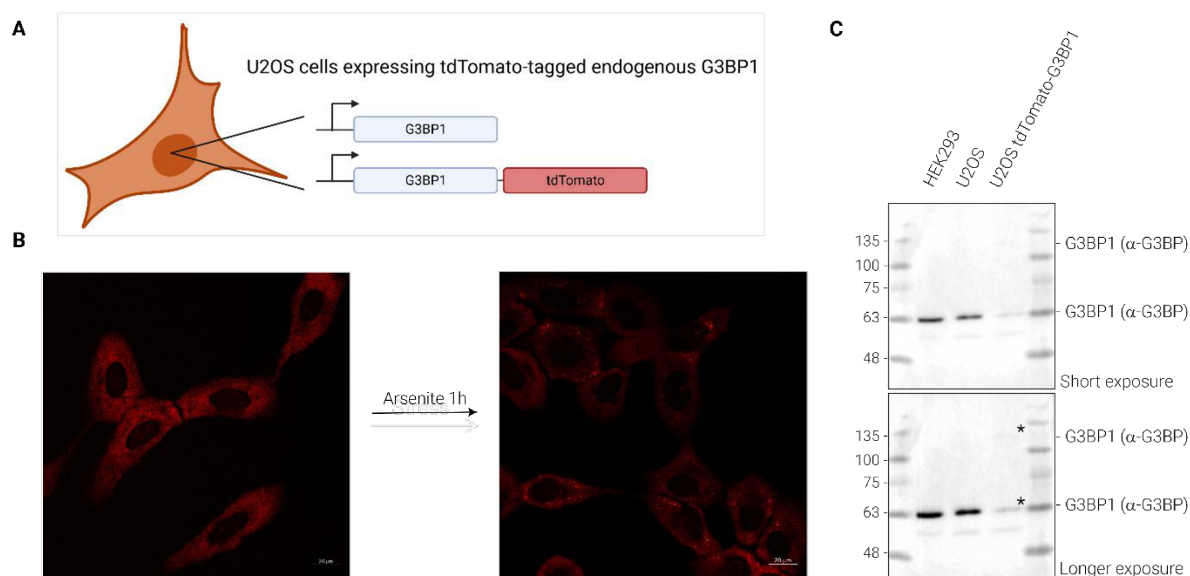


Figure 29. U2OS tdTomato-G3BP1 cells. **A.** Schematic representation of the U2OS cells expressing tdTomato-tagged endogenous G3BP1. **B.** U2OS tdTomato-G3BP1 cells were analyzed using live-cell imaging with confocal microscopy. Cells were treated for 1h with 250μM Arsenite (n=3). Red: tdTomato-G3BP1. **C.** HEK293 cells, U2OS cells and U2OS tdTomato-G3BP1 cells were analyzed for their G3BP1-expression by means of western blotting (n = 1).

To analyze whether the expression of the diverse IFN-I-induced mARTDs influences G3BP1 localization or SG formation, we established multiple cell lines using the U2OS tdTomato-G3BP1 and lentiviral transduction (**Fig. 30A**). This is a common method to create stable cell lines that express a gene of interest. Our lab cloned the genes of interest (PARP10; PARP12; PARP15iso1; PARP15iso2) into the pLIX403 system. This is a lentiviral tetracycline-inducible (TetOn) system of which the vectors contain an EGFP-tag. It contains the reverse tetracycline-controlled transactivator (rtTA), which in presence of doxycycline (a tetracycline derivative), binds to the tetracycline response element (TRE) and induces expression of the gene of interest (**Fig. 30B**). This way, the genes of interest are tetracycline-inducible, creating

a system in which we can analyze the effect of IFN-I-induced mARTDs on G3BP1 localization and SGs. HEK293T cells are transfected with the lentiviral vectors with our genes of interest, a packaging vector and an envelope vector (**Fig. 30A**). Lentiviral particles are produced and released into the cell culture medium and used to transduce the target cells, U2OS tdTomato-G3BP1. The transduced cells are subjected to selection using puromycin. Eventually, the population of transduced cells were enriched. The cell lines were established successfully and analyzed for the mARTDs expression by Ani Sabcheva (data now shown; Sabcheva A., 2022).

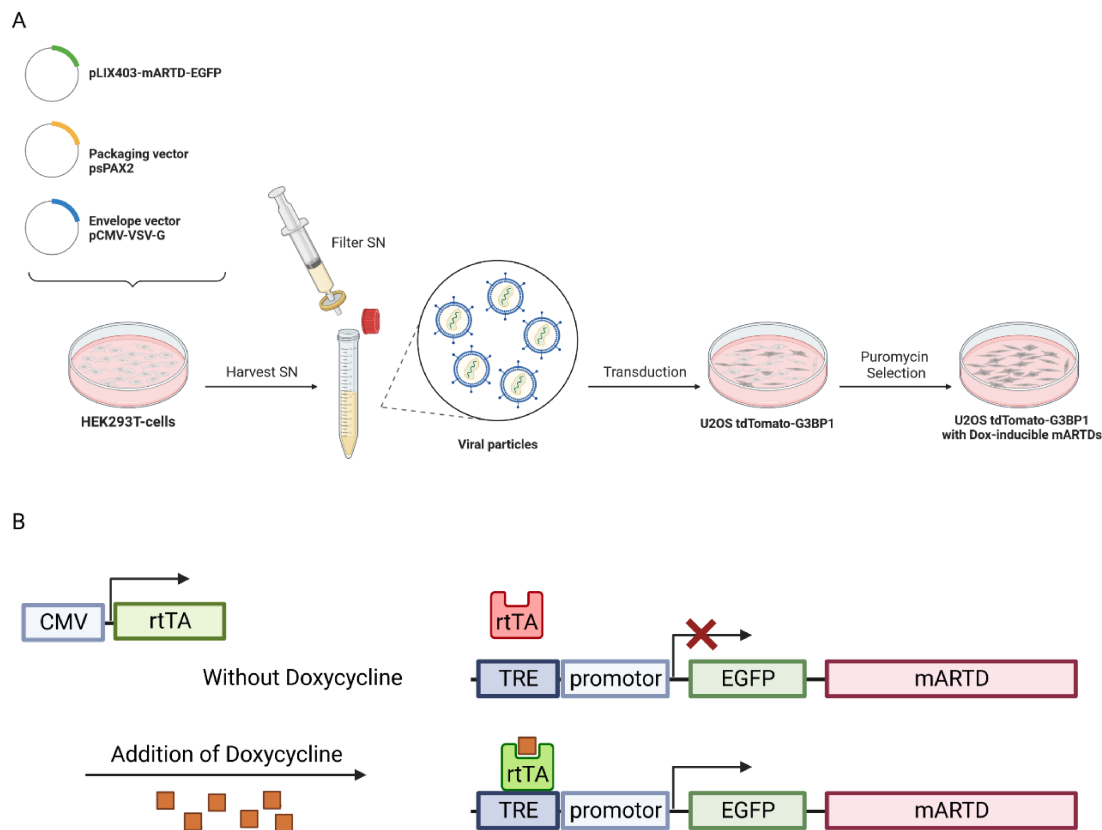


Figure 30. Schematic overview of the generation of the inducible mARTDs U2OS tdTomato-G3BP1 cell lines.

A. HEK293T cells were transfected with the different pLIX403-mEGFP-PARP plasmids in combination with the packaging and envelope vector. 24h after transfection, the viral supernatant is harvested and the U2OS tdTomato-G3BP1 cells are transduced. The transduced cells are selected using puromycin selection. **B.** Schematic representation of the tetracycline-inducible (TetOn) system. It consists of the expression of the tetracycline-controlled transactivator (rtTA), which in presence of doxycycline (a tetracycline derivative), binds to the tetracycline response element (TRE) and induces expression of the gene of interest.

2.2.4.1. PARP10 and stress granules

PARP10 is present both in the cytosol and the nucleus (Kleine et al., 2012). PARP10 has interaction partners and potential substrates in both the nucleus and the cytosol (our data, Feijs et al., 2013). However, under basal conditions, PARP10 is predominantly located in the cytoplasm (Kleine et al., 2012). Cytoplasmic PARP10 is believed to be involved in the innate immune response. Within the cytoplasm, PARP10 has the ability to form condensates regardless of its catalytic activity. However, the condensates formed by PARP10 WT exhibit a more liquid-like state compared to the PARP10 GW condensates. These PARP10 condensates are not SGs or any other recognized condensates (Forst et al., 2013; Kleine et al., 2012; Mayo et al., 2018). Cells overexpressing the PARP10 catalytic domain modify GAPDH, and this leads to GAPDH being recruited into stress granules. In general, there are

many proteins for which ADP-ribosylation is linked to SG trans-localization (Mayo et al., 2018).

To assess the effect of PARP10 expression on SGs and G3BP1, U2OS tdTomato-G3BP1 mEGFP-PARP10 WT cells were treated with different doxycycline concentrations (untreated; 0.125 µg/ml; 0.25 µg/ml; 0.5 µg/ml; 1 µg/ml) for 16h. To induce SGs, cells were treated with 250 µM Arsenite. The cells were fixed with PFA and analyzed with confocal microscopy (**Fig. 31**). G3BP1 is ubiquitously expressed throughout the cytoplasm. Upon Arsenite treatment, SGs are induced. Dox-induction leads to expression of PARP10 WT. PARP10 WT expression is variable from cell to cell, due to the polyclonal cell line. PARP10 is generally uniformly expressed in the cells. In the higher Dox concentration, condensates are induced in some cells. However, PARP10 does not localize to SGs granules and does not seem to alter G3BP1 localization (**Fig. 31**).

The same approach was used for the analysis of the U2OS tdTomato-G3BP1 mEGFP-PARP10 GW cells (**Fig. 32**). Again, G3BP1 is expressed uniformly throughout the cytoplasm and Arsenite treatment results in SG formation. Dox treatment induced PARP10 GW expression. PARP10 GW results in condensates in all different Dox treatments, independent of Arsenite treatment. The inactivity of the enzyme thus results in the formation of these condensates. This would suggest, that for PARP10, at low concentrations MARYlation-ability keeps the enzyme from forming condensates. High overexpression of PARP10 results in condensates (Forst et al., 2013a; Kleine et al., 2012; Mayo et al., 2018). However, PARP10 GW does not localize to SGs and does not seem to alter G3BP1 localization (**Fig. 32**).

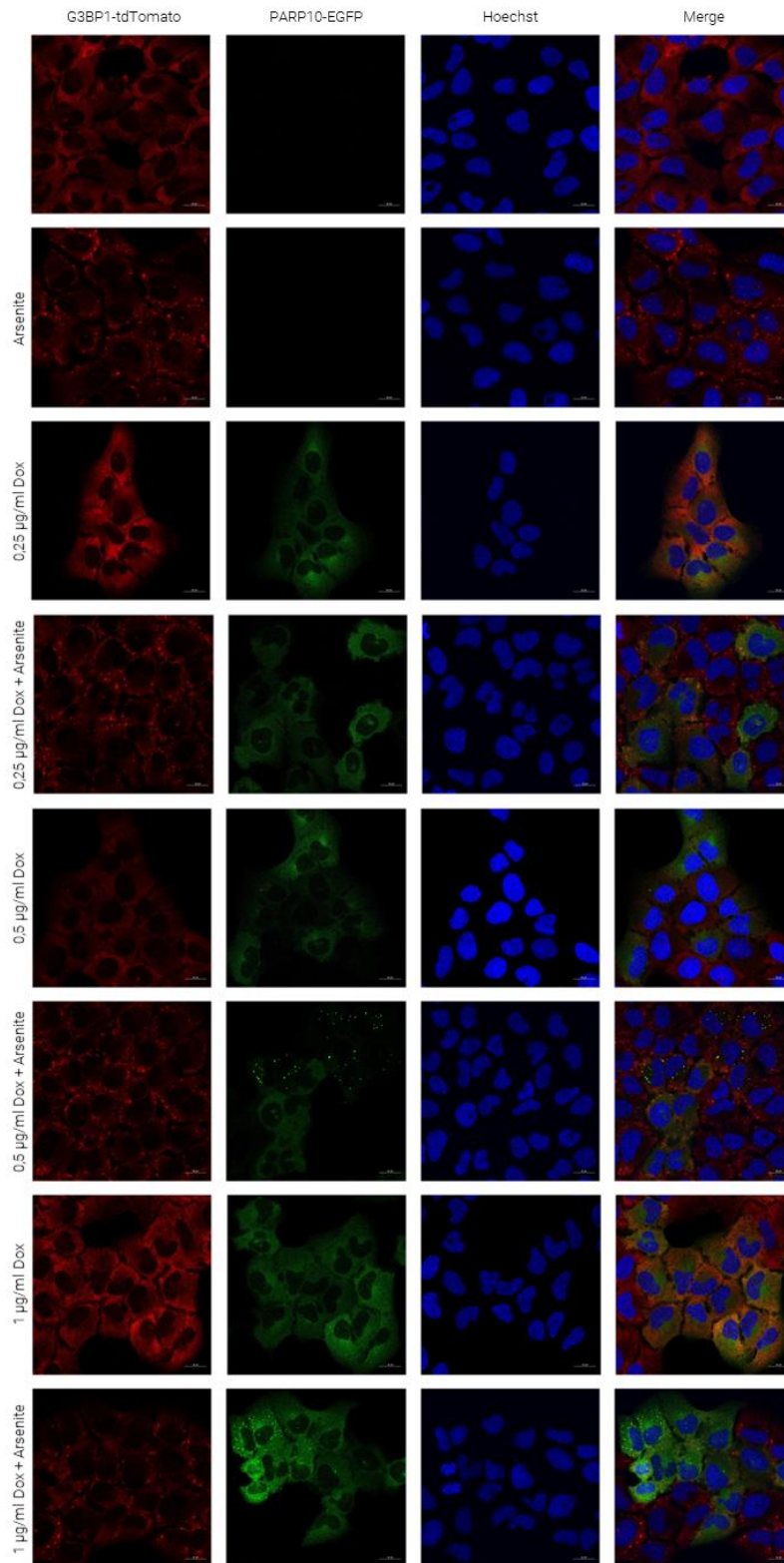


Figure 31. Analysis of the effect of PARP10 WT expression on G3BP1 localization and SG formation. U2OS tdTomato-G3BP1 mEGFP-PARP10 WT cells were treated 48h after seeding with Doxycyclin (untreated; 0.125 µg/ml; 0.25 µg/ml; 0.5 µg/ml; 1 µg/ml) for 16h. To induce SGs, certain conditions were stimulated with 250 µM Arsenite for 1h. Cells were fixated with 3,7% PFA and analyzed using confocal microscopy. Red: G3BP1; Green: PARP10 WT; Blue: Hoechst staining of the Nuclei. Scale bar 10µm (n=2).

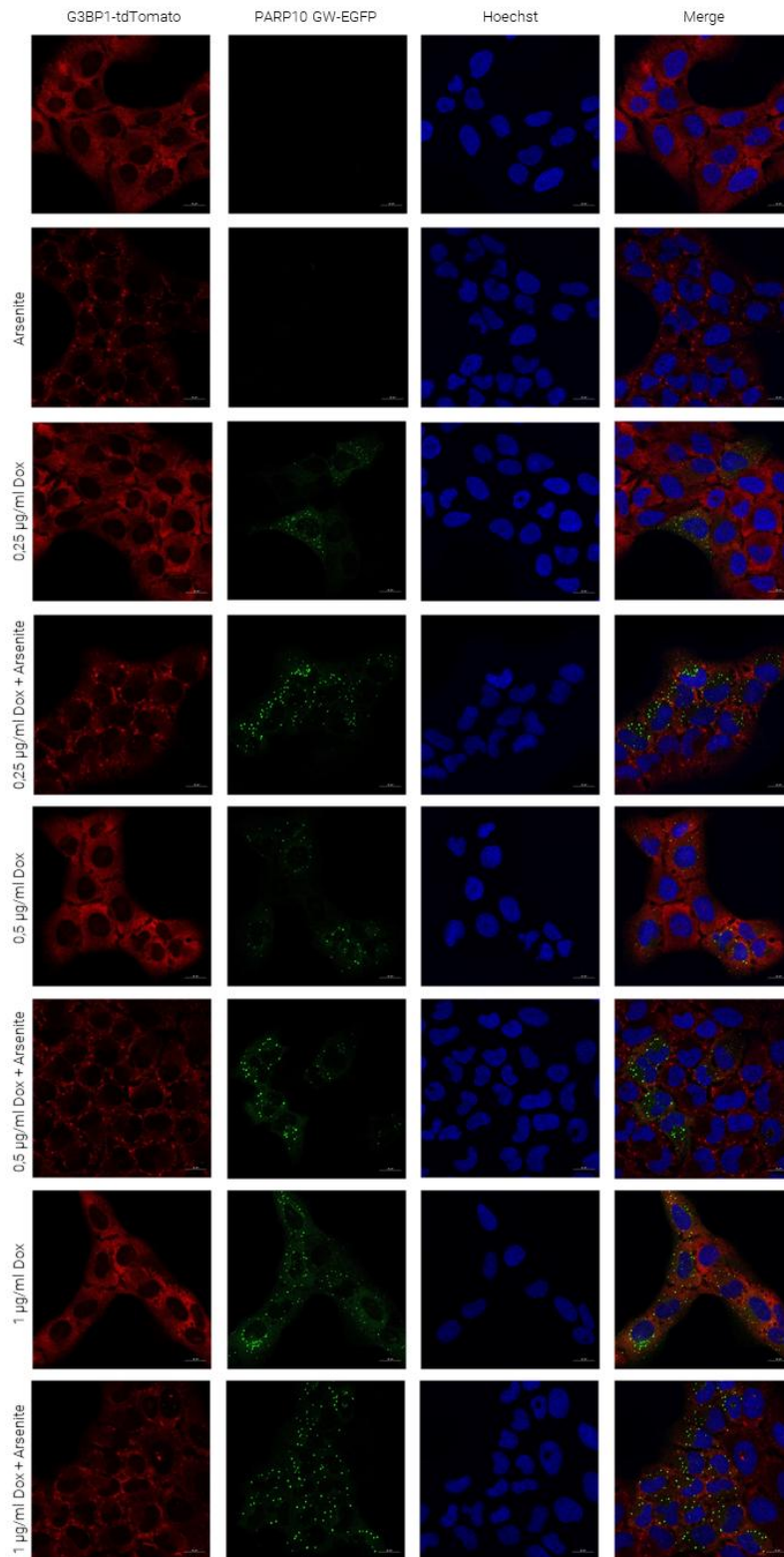


Figure 32. Analysis of the effect of PARP10 GW expression on G3BP1 localization and SG formation. U2OS tdTomato-G3BP1 mEGFP-PARP10 GW cells were treated 48h after seeding with Doxycyclin (untreated; 0.125 $\mu\text{g/ml}$; 0.25 $\mu\text{g/ml}$; 0.5 $\mu\text{g/ml}$; 1 $\mu\text{g/ml}$) for 16h. To induce SGs, certain conditions were stimulated with 250 μM Arsenite for 1h. Cells were fixated with 3,7% PFA and analyzed using confocal microscopy. Red: G3BP1; Green: PARP10 GW; Blue: Hoechst staining of the Nuclei. Scale bar 10 μm (n=2).

2.2.4.2. PARP12 and stress granules

In basal conditions, PARP12 localizes primarily to the cytoplasm, particularly in the perinuclear region and the Golgi apparatus (Welsby et al., 2014). PARP12 has been repeatedly linked to antiviral activities and stress responses (Fehr et al., 2020; Lüscher et al., 2022) and it has been shown to translocate towards SGs upon cellular stress mediated by PARP1 produced PAR (Catara et al., 2017; Leung et al., 2011; Welsby et al., 2014). PARP12 localizes to SGs as a result of oxidative stress and heat shock but not osmotic stress (Sorbitol), indicating the differences between stress responses (Catara et al., 2017). Also, overexpression of PARP12 has been shown to induce SGs (Catara et al., 2017; Leung et al., 2011). Overexpression of catalytically inactive PARP12 induce SGs as well, suggesting that SG formation is independent of PARP12 MARYlation activity (Catara, et al., 2017). The role for PARP12 in stress granules requires further investigation.

To analyze the PARP12 expression in relation to SGs and G3BP1, U2OS tdTomato-G3BP1 mEGFP-PARP12 WT and HY cells were treated with the different doxycycline concentrations (untreated; 0.25 µg/ml; 0.5 µg/ml; 1 µg/ml) for 16h. SGs were induced with 250 µM Arsenite. The cells were fixed with PFA and analyzed with confocal microscopy (**Fig. 33**). G3BP1 is ubiquitously expressed throughout the cytoplasm and SGs are induced in all conditions upon oxidative stress. Low dox induction (0.25 µg/ml) leads to expression of PARP12 WT, which expresses throughout the cytoplasm but particularly to the perinuclear region. Upon Arsenite treatment, PARP12 WT localizes to SGs. Higher expression of PARP12 WT, results in the induction of condensates without external stressors. Here, also G3BP1 localizes to these condensates, indicating that PARP12 WT overexpression results in SG-induction. Our results match previous reports (**Fig. 33**).

PARP12 HY localizes similarly as the WT to cytoplasm, and upon oxidative stress it relocates to SGs (**Fig. 34**). However, upon the higher expression of PARP12 HY, the enzyme stays cytoplasmic in the absence of a stressor. Upon Arsenite treatment, it relocates to SGs. Taken together this indicates that upon an external stressor, PARP12 localizes to SGs, independent of the catalytic activity (**Fig. 34**). This is in accordance with previous reports arguing that the translocation is dependent on the ZnF domains and the WWE-domains (Catara et al., 2017; Welsby et al., 2014). However, PARP12 catalytic activity is capable of inducing condensate formation upon high expression. This would argue that PARP12 MARYlation has an inducing effect upon SG formation. This contrasts with a previous report, where overexpression of the catalytic mutant also induced SGs (Catara, et al., 2017). This could be due to the system used. Overexpression through transfection, as used previously, often results in higher expression compared to the more sensitive transduced cells developed for this work. Overexpression constructs are mainly driven by CMV promoter, which results in high expression levels, whereas for pLIX403, vector used in this study, has a low expressing promoter, TRE promoter. On top, the expression of the genes of interest is adjustable through Dox concentrations.

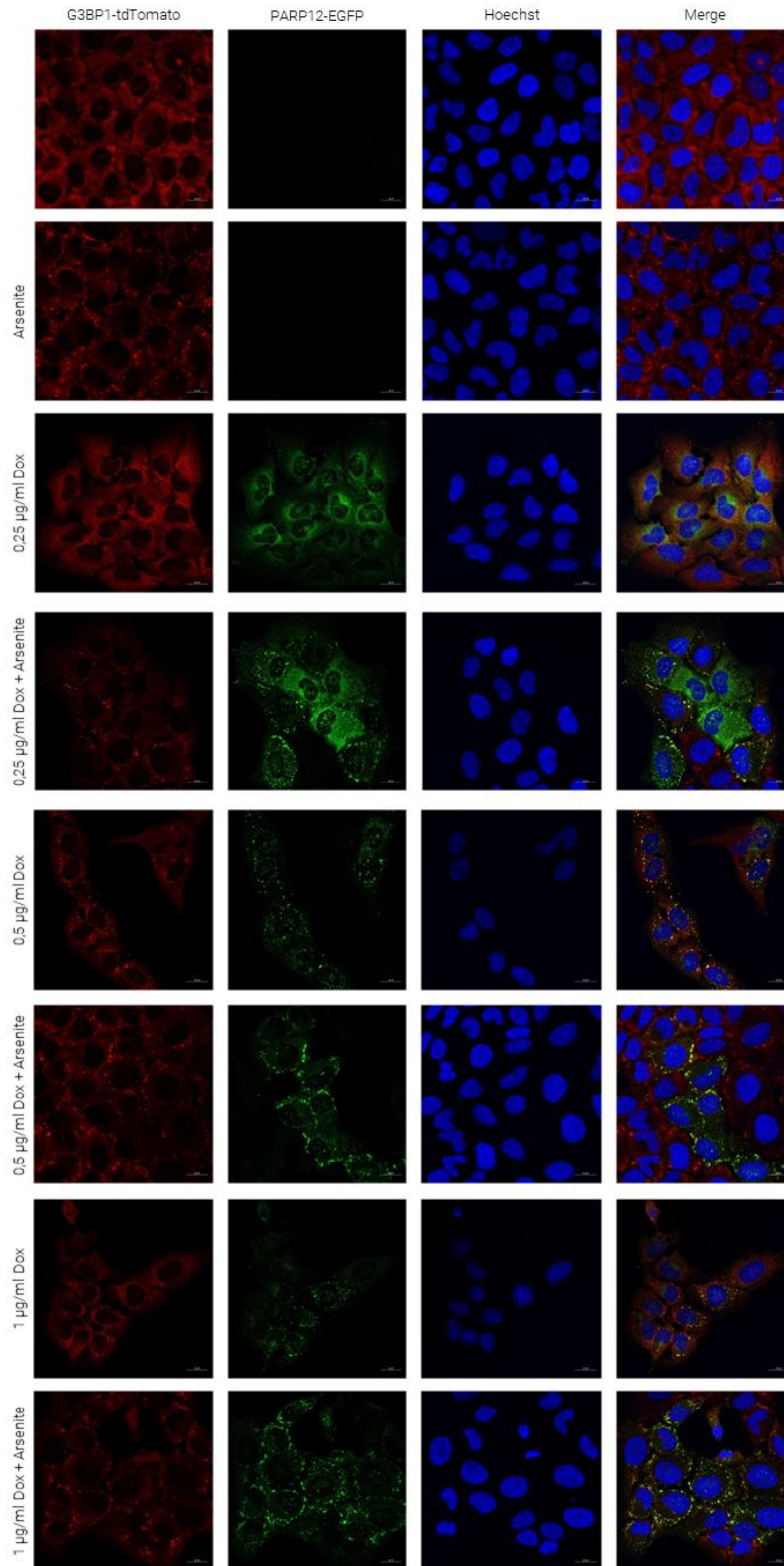


Figure 33. Analysis of the effect of PARP12 WT expression on G3BP1 localization and SG formation. U2OS tdTomato-G3BP1 mEGFP-PARP12 WT cells were treated 48h after seeding with Doxycyclin (untreated; 0.125 µg/ml; 0.25 µg/ml; 0.5 µg/ml; 1 µg/ml) for 16h. To induce SGs, certain conditions were stimulated with 250 µM Arsenite for 1h. Cells were fixated with 3,7% PFA and analyzed using confocal microscopy. Red: G3BP1; Green: PARP12 WT; Blue: Hoechst staining of the Nuclei. Scale bar 10µm (n=2).

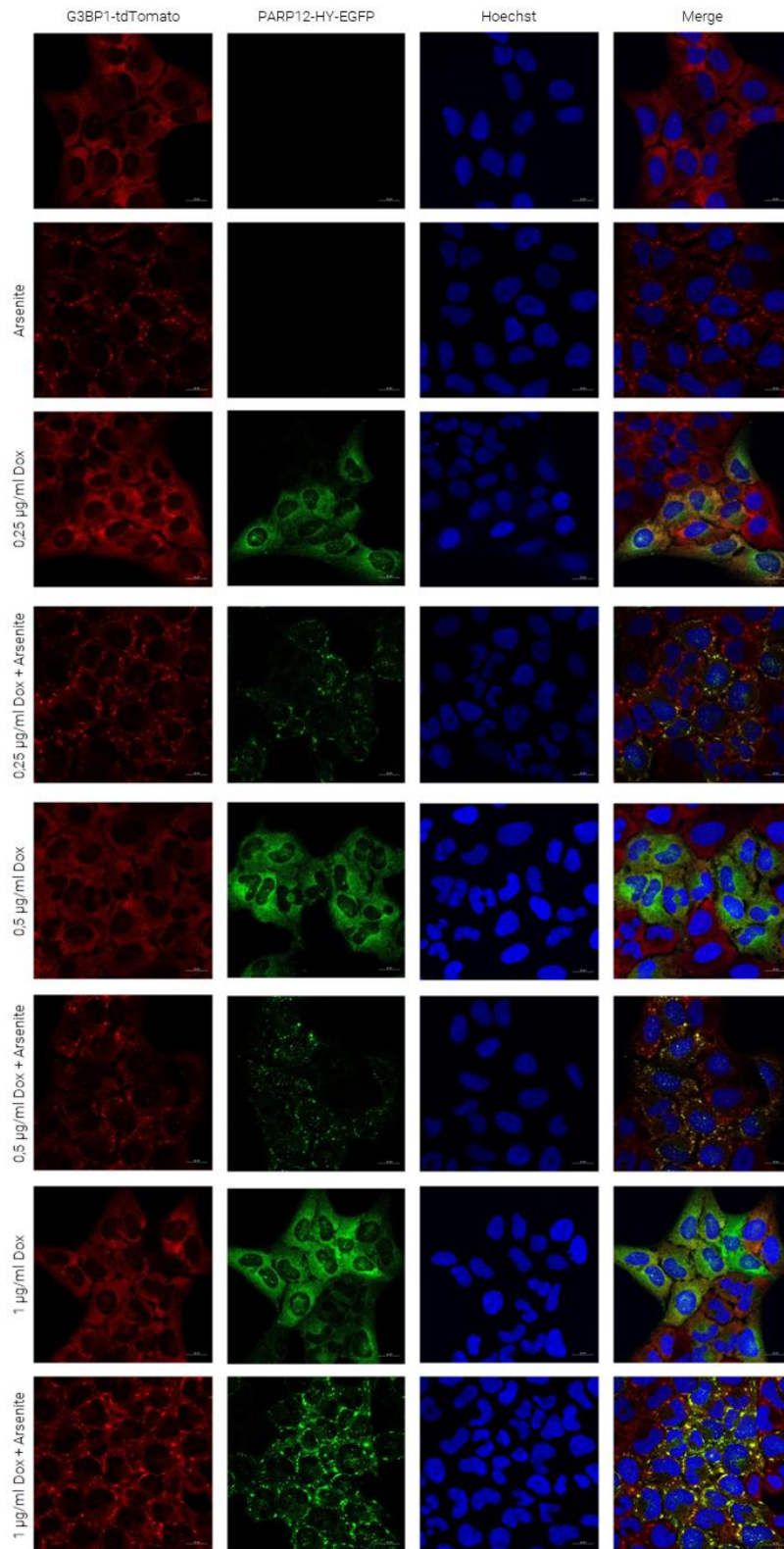


Figure 34. Analysis of the effect of PARP12 HY expression on G3BP1 localization and SG formation. U2OS tdTomato-G3BP1 mEGFP-PARP12 HY cells were treated 48h after seeding with Doxycyclin (untreated; 0.125 µg/ml; 0.25 µg/ml; 0.5 µg/ml; 1 µg/ml) for 16h. To induce SGs, certain conditions were stimulated with 250 µM Arsenite for 1h. Cells were fixated with 3,7% PFA and analyzed using confocal microscopy. Red: G3BP1; Green: PARP12 HY; Blue: Hoechst staining of the Nuclei. Scale bar 10µm (n=2).

2.2.4.3. PARP15 and stress granules

There is not a lot known about PARP15. As stated in the introduction, PARP15 has 2 isoforms, PARP15iso1 and PARP15iso2. Isoform 2 lacks the N-terminal domain and the first MD compared to isoform1 (Lüscher et al., 2018, 2022). PARP15 is IFN-induced (Shaw et al., 2017). PARP15iso2 has been reported to localize to SGs (Leung et al., 2011). On PARP15iso1 there is no information on localization. We used tdTomato-G3BP1 mPARP15iso1 WT U2OS cells and tdTomato-G3BP1 mPARP15iso1 HY U2OS cells to assess the influence of PARP15 expression on G3BP1 localization.

For both mPARP15iso1 WT U2OS cells and tdTomato-G3BP1 mPARP15iso1 HY U2OS cells, G3BP1 localizes to the cytoplasm in the unstimulated cells (**Fig. 35/36**). Upon stimulation, PARP15iso1 WT localizes to nuclear dots. PARP15iso1 expression does not influence G3BP1 localization. Upon oxidative stress, G3BP1 localizes to SGs. These are not affected by PARP15iso1 expression (**Fig. 35**). PARP15iso1 HY shows a diffuse nuclear staining, indicating that the condensates are dependent on ADP-ribosylation (**Fig. 36**). Similarly, to PARP12 activity, condensate formation seems to be induced by mARTDs activity. However, for PARP10, both WT and the catalytically inactive mutant induce speckles, with the GW mutant more prone to condensates. It is worth studying whether these specific speckles of PARP15iso1 are indeed MARYlation dependent and which specific type of condensates they are. Whether they are involved with transcription hubs, PML cages, Cajal bodies, Paraspeckles or something else (Negri et al., 2023; Sabari et al., 2020).

PARP15iso2 localizes to the cytoplasm. In contrast to previous reports, it does not localize to SGs upon oxidative stress (**Fig. 37**) (Leung et al., 2011). PARP15iso2 overexpression also does not result in SG formation. The WT enzyme seems to preferably locate to the perinuclear region (**Fig. 37**). PARP15iso2 HY also does not go into SGs upon oxidative stress and resides primarily in the Golgi apparatus. Lack of MARYlation activity could result in PARP15 retention (**Fig. 38**).

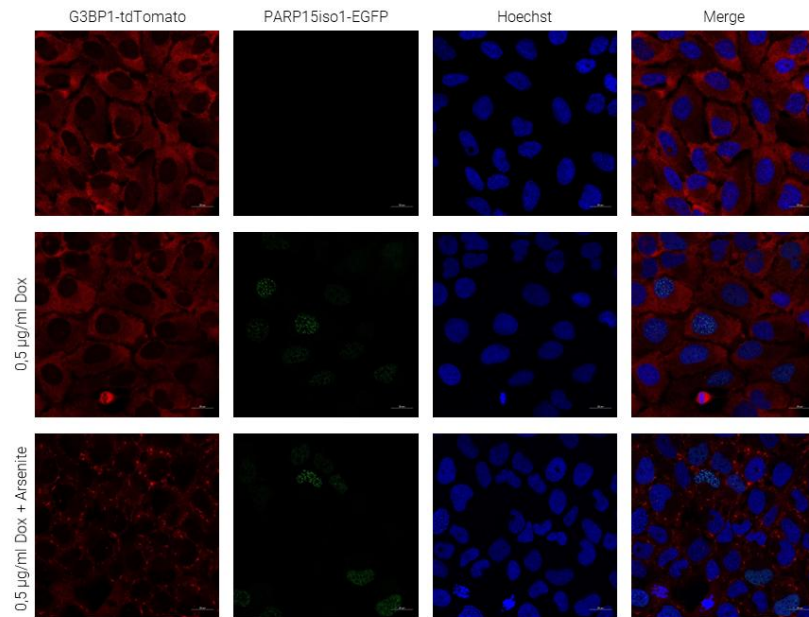


Figure 35. Analysis of the effect of PARP15isoform1 WT expression on G3BP1 localization and SG formation. U2OS tdTomato-G3BP1 mEGFP-PARP15isoform1 WT cells were treated 48h after seeding with Doxycyclin (untreated; 0.5 µg/ml) for 16h. To induce SGs, certain conditions were stimulated with 250 µM Arsenite for 1h. Cells were fixated with 3,7% PFA and analyzed using confocal microscopy. Red: G3BP1; Green: PARP15isoform1 WT; Blue: Hoechst staining of the Nuclei. Scale bar 10µm (n=2).

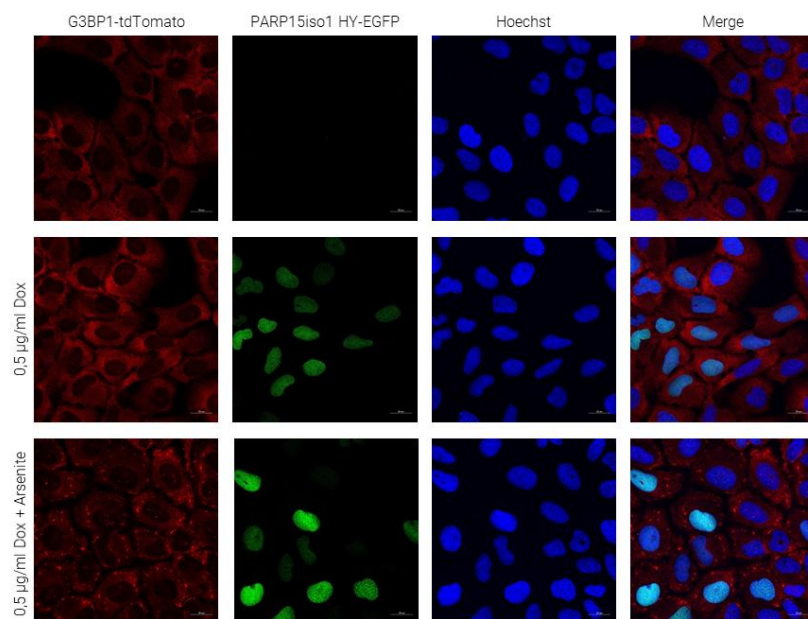


Figure 36. Analysis of the effect of PARP15isoform1 HY expression on G3BP1 localization and SG formation. U2OS tdTomato-G3BP1 mEGFP-PARP15isoform1 HY cells were treated 48h after seeding with Doxycyclin (untreated; 0.5 µg/ml) for 16h. To induce SGs, certain conditions were stimulated with 250 µM Arsenite for 1h. Cells were fixated with 3,7% PFA and analyzed using confocal microscopy. Red: G3BP1; Green: PARP15isoform1 HY; Blue: Hoechst staining of the Nuclei. Scale bar 10µm (n=2).

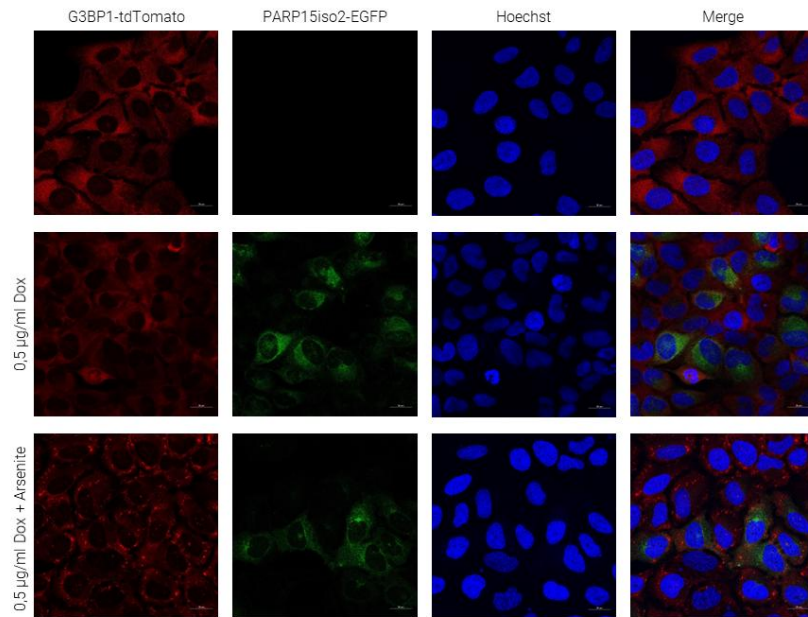


Figure 37. Analysis of the effect of PARP15isoform2 WT expression on G3BP1 localization and SG formation. U2OS tdTomato-G3BP1 mEGFP-PARP15isoform2 WT cells were treated 48h after seeding with Doxycyclin (untreated; 0.5 µg/ml) for 16h. To induce SGs, certain conditions were stimulated with 250 µM Arsenite for 1h. Cells were fixated with 3,7% PFA and analyzed using confocal microscopy. Red: G3BP1; Green: PARP15isoform2 WT; Blue: Hoechst staining of the Nuclei. Scale bar 10µm (n=2).

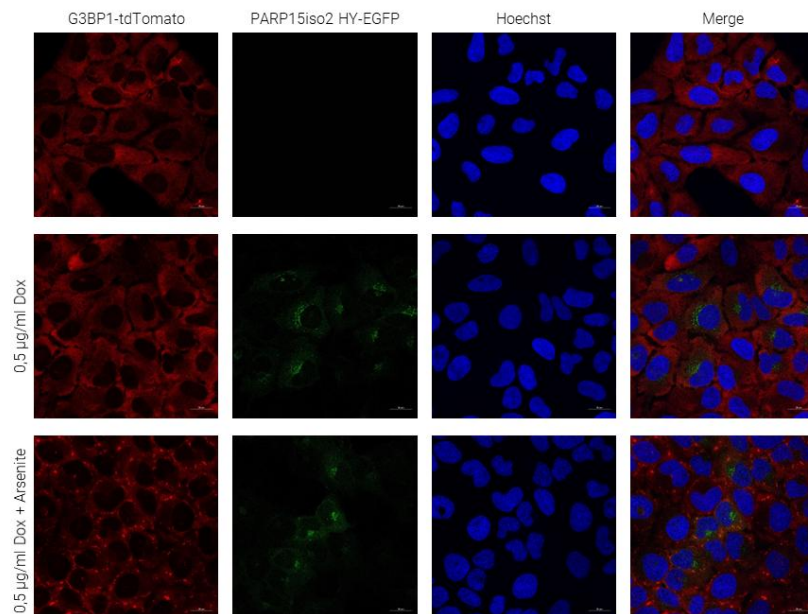


Figure 38. Analysis of the effect of PARP15isoform2 HY expression on G3BP1 localization and SG formation. U2OS tdTomato-G3BP1 mEGFP-PARP15isoform2 HY cells were treated 48h after seeding with Doxycyclin (untreated; 0.5 µg/ml) for 16h. To induce SGs, certain conditions were stimulated with 250 µM Arsenite for 1h. Cells were fixated with 3,7% PFA and analyzed using confocal microscopy. Red: G3BP1; Green: PARP15isoform2 HY; Blue: Hoechst staining of the Nuclei. Scale bar 10µm (n=2).

2.2.5. G3BP1 interactome identification

G3BPs are essential for SG formation. Initially, alphavirus infection induces SG formation in cells. At a later timepoint of infection, alphaviral nsP3 suppresses classical SG formation and sequesters G3BP (Jayabalan et al., 2021). CHIKV nsP3 interacts with G3BPs and binds it into new viral condensates, of which the purpose is unknown. The interaction occurs more specifically through the FDGF motifs within nsP3-HVD and the NTF2L domain of G3BPs. This association is independent of nsP3 ADP-ribosylhydrolase activity. Interestingly, the catalytic activity of the nsP3-macrodomain, capable of reversing MARYlation, affects the composition of these viral condensates (Jayabalan et al., 2021). Jayabalan et al. saw that upon infection, after around 9 hours distinct new foci form, different from SGs (Jayabalan et al., 2021). The nsP3-G3BP condensates are not sensitive to cycloheximide treatment, in comparison to classical SGs, and have a different composition (**Fig. 39**). They argued that the nsP3 catalytic activity suppresses stress granule formation and induces the formation of alternative condensates, marked by the release of translation factors (Jayabalan et al., 2021). Important to note that these studies were done with oxidative stress induced SGs. For a clearer view, this needs to be repeated in the context of viral infection. Altogether, it seems that the MARYlation status of G3BP1 could influence condensates composition. Also, PARP10 MARYlation has been shown to induce GAPDH translocation to SGs. Overexpression of the PARP10 catalytic domain resulted in GAPDH recruitment to SGs (Mayo et al., 2018). The links between ADP-ribosylation and SGs have been extensively discussed in chapter 1.3.3. Overall, mARTD expression might also influence condensate formation. Taken together, this offers another possibility of how MARYlation could influence G3BP1s function.

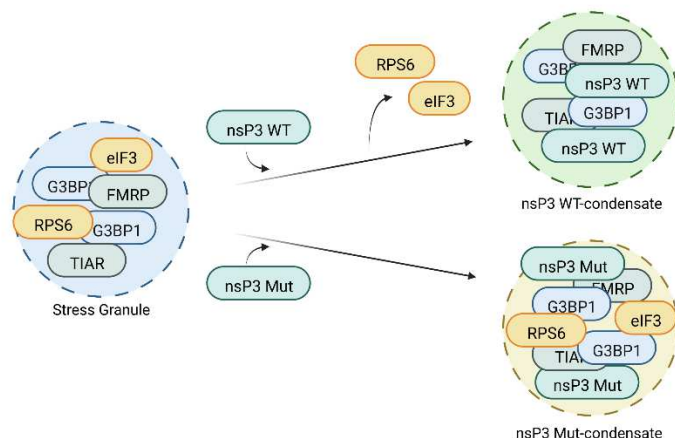


Figure 39. CHIKV ADP-ribosylhydrolase activity regulates the composition of stress granules. Upon viral infection, SGs are formed. Dependent on nsP3 hydrolase activity, CHIKV suppresses stress granule formation and induces the formation of alternative condensates. eIF3: eukaryotic initiation factor 3; G3BP1: Ras GTPase-activating protein-binding protein 1; FMRP: fragile X messenger ribonucleoprotein; RPS6: Ribosomal Protein S6; TIAR: Nucleolysin TIAR; nsP3 WT: non-structural protein 3 wildtype; nsP3 Mut: nsP3 incapable of ADP-ribosylhydrolase activity (adapted from Jayabalan et al., 2021).

To investigate the effect of MARYlation on the G3BP1-interactome, our lab is developing a tool to analyze how the MAR-status of G3BP1 influences its interactome upon viral infection. Again, we will use a biotin-based proximity labelling technique, since this is a robust technique for identifying interactions for different conditions and at different timepoints. Also, this technique has now well been established in our hands (**chapter 2.1.**). Instead of the BirA*-tag used with PARP10 and nsP3, G3BP1 was cloned with the mini-Turbo tag (**Fig. 40A**). This newer version of a biotin ligase has labelling times of 1-2h or less (Samavarchi-Tehrani et al., 2020; Trinkle-Mulcahy, 2019). This makes the system better suited for a spatio-temporal analysis of the SG-composition upon different stressors, such as viral infection, compared to the BirA*-tag which requires overnight treatment with Biotin.

To establish the system, our lab cloned pLix403-Tet-on-G3BP1-miniTurbo-V5. This plasmid will be used later on for lentiviral transduction of G3BP-dKO cells, to establish a Dox-inducible system in which the G3BP1-interactome can be identified (**Fig. 40A; 42A/B**). Firstly,

to test the expression of the fusion protein, HEK293 cells were transiently transfected with either pLix403-Tet-on-miniTurbo-V5, as a negative control, or pLix403-Tet-on-G3BP1-miniTurbo-V5. The cells were either untreated or treated with Doxycycline (ON) and/or Biotin (2h) and/or Arsenite (2h) as indicated (**Fig. 40B**). Samples were analyzed by means of western blotting. Upon Dox-induction, miniTurbo-V5 does not seem to be expressed, however G3BP1-miniTurbo-V5 is expressed well. Addition of biotin results in a higher biotinylation signal, however there is a high unspecific background signal (**Fig. 40B**). Since G3BP1-miniTurbo-V5 shows a clear signal by Streptavidin-HRP upon presence of Biotin (**Fig. 40B**), we concluded that the Tet-ON-G3BP1-miniTurbo-V5 fusion protein is a working system with which it is worth proceeding.

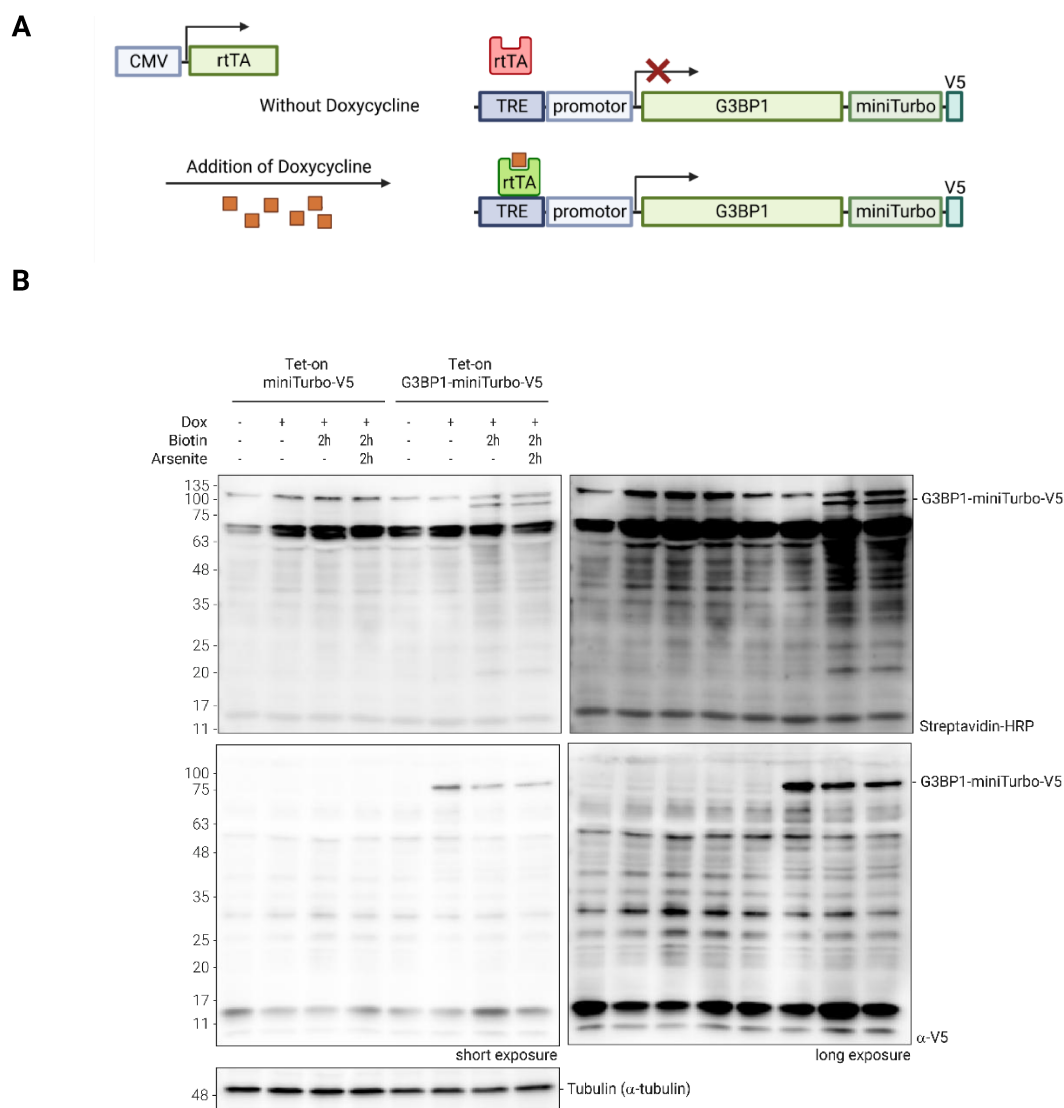


Figure 40. Establishment of the BioID method to identify G3BP1 interactome. A. Schematic representation of the cloned pLix403-Tet-on-G3BP1-miniTurbo-V5 system. Addition of Doxycycline results in its binding to the rtTA and this complex binds the TRE in turn and induces expression of G3BP1-miniTurbo-V5. **B.** HEK293 cells were transiently transfected with either pLix403-Tet-on-miniTurbo-V5 or pLix403-Tet-on-G3BP1-miniTurbo-V5. The cells were treated overnight with Doxycycline (ON), Biotin for 2h and/or Arsenite for 2h as indicated. Cells were lysed and protein expression and biotinylation was analyzed by means of western blotting (n=1).

To further evaluate the Tet-ON-G3BP1-miniTurbo-V5 system, we tested the G3BP1-miniTurbo-V5 specificity using a known and by our own studies confirmed interactor, CHIKV nsP3. HEK293 cells were transiently transfected with either EGFP or nsP3-EGFP alone or in combination with G3BP1-miniTurbo-V5, with or without Biotin treatment (3h). Cells were

lysed and EGFP and nsP3-EGFP were immunoprecipitated using GFP-TRAP. The samples were analyzed using immunoblotting. In the whole cell lysate samples, there is a clear biotinylation by G3BP1-miniTurbo-V5 upon addition of Biotin (**Fig. 41**). EGFP and nsP3-EGFP are successfully immunoprecipitated by GFP-TRAP. Both G3BP1-miniTurbo-V5 and the endogenous G3BP1 interact with nsP3 (**Fig. 41**). Upon presence of Biotin, both G3BP1-miniTurbo and nsP3 are biotinylated. There is also a band visible at the height of endogenous G3BP1. Since G3BPs dimerize and assemble in condensates, also endogenous G3BPs are expected as biotinylation products. However, also EGFP shows a biotinylation signal upon addition of Biotin. Since nsP3-G3BP1 is a well-established interaction, the biotinylation signal of nsP3 is unlikely due to biotinylation of the EGFP-tag. We hypothesize that the unspecific EGFP-biotinylation signal is an overexpression artifact. However, to make sure that EGFP is not the main acceptor for Biotin, but rather the fused protein, namely nsP3, this experiment will be repeated expressing a differently tagged CHIKV nsP3 fusion protein.

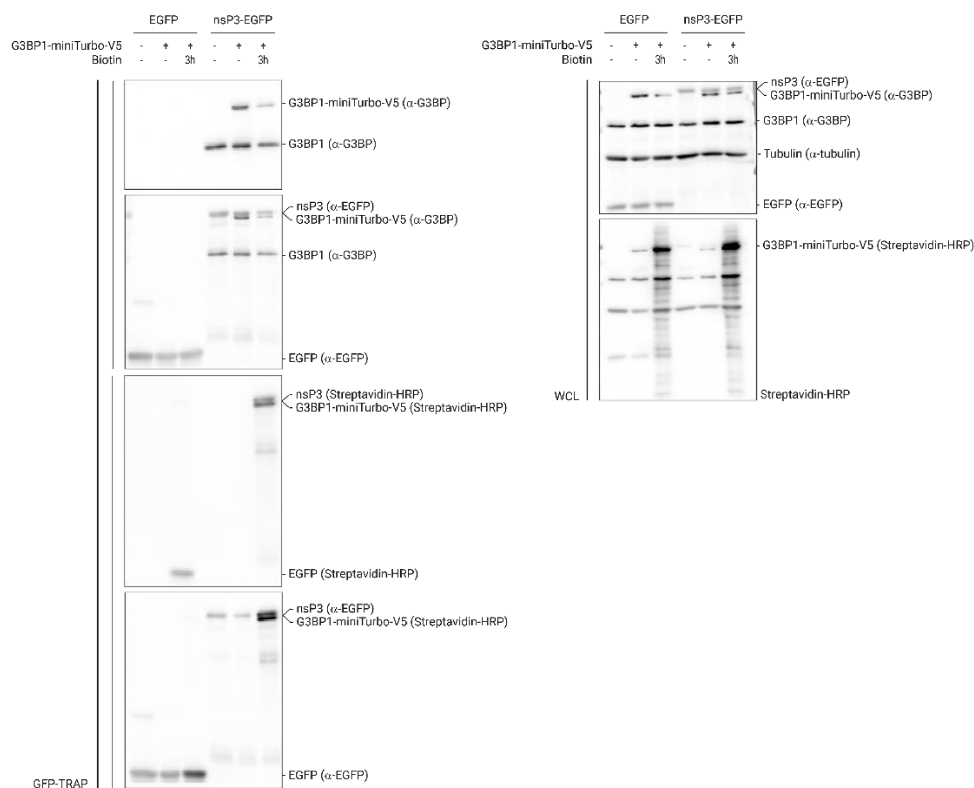


Figure 41. Proof of Principle experiment: G3BP1-miniTurbo biotinylates CHIKV nsP3. HEK293 cells were transiently transfected with either EGFP or nsP3-EGFP alone or in combination with G3BP1-miniTurbo-V5, with or without Biotin treatment (3h) as indicated. Cells were lysed 24h after transfection and EGFP-fusion proteins were immunoprecipitated using GFP-TRAP. The proteins and their biotinylation were analyzed using immunoblotting. For detection, the GFP-TRAP was split over two SDS-PAGES and western blots, as indicated (n=1).

Taken together, we suggest the Tet-ON-G3BP1-miniTurbo-V5 system as a valid system for identifying the G3BP1-interactome upon different conditions and stressors. To proceed pLIX403 Tet-ON-G3BP1-miniTurbo-V5 system will be transduced into G3BP1-dKO-cells (**Fig. 42A**). These cells will be used to determine how MARYlation of G3BP1 potentially affects its interactome and SG composition. The G3BP1-interactome would be assessed between expression of different mARTDs, CHIKV nsP3-expression, CHIKV infection and other stressors (**Fig. 42B**). This falls outside the scope of this thesis.

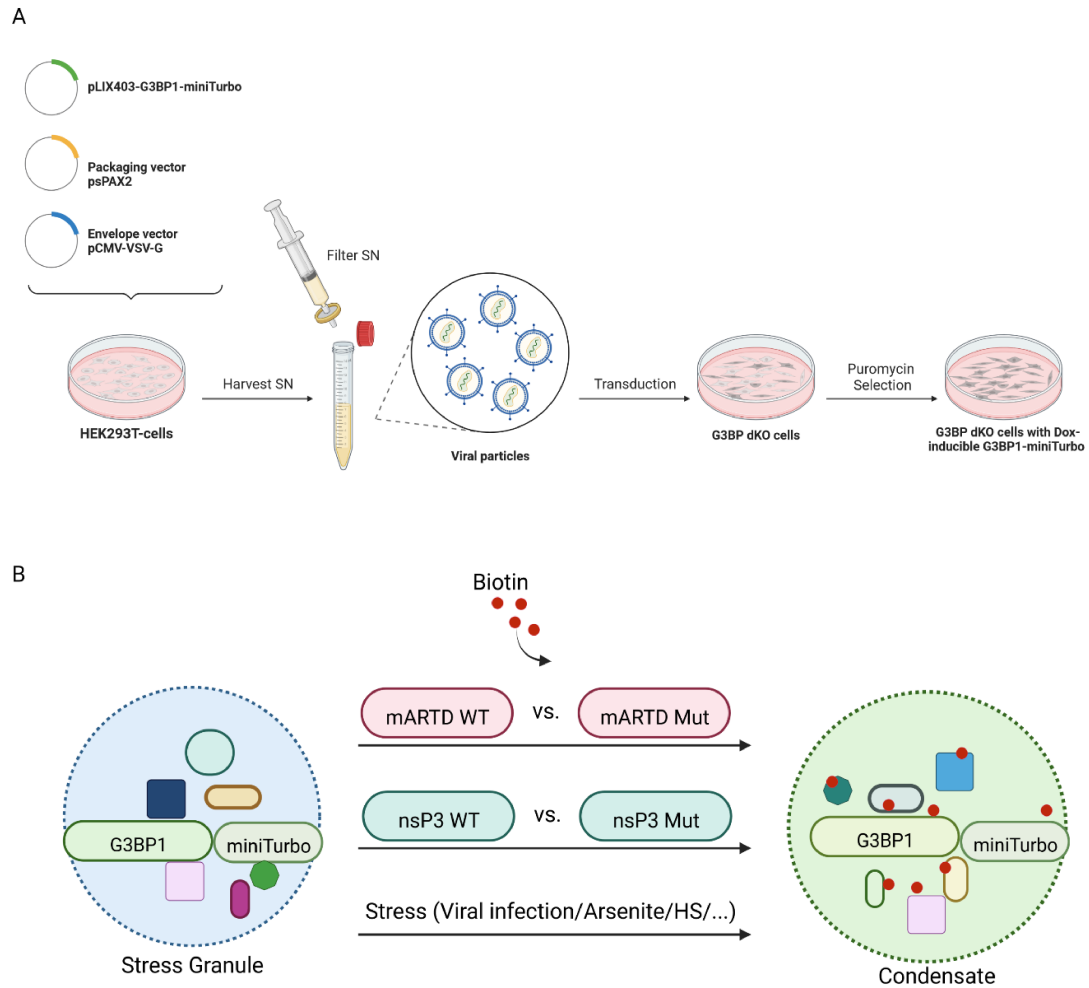


Figure 42. Outlook on the establishment of an inducible system to identify the G3BP1 interactome in different conditions. **A.** The generation of the inducible G3BP1-miniTurbo-V5 cell line. HEK293T cells will be transfected with Tet-ON-G3BP1-miniTurbo-V5 in combination with the packaging and envelope vector. 24h after transfection, the viral supernatant is harvested and the G3BP1-dKO cells are transduced. The transduced cells are selected using puromycin selection. **B.** To study the G3BP1 interactome upon different conditions, G3BP1-miniTurbo-V5 cells will be transfected with proteins affecting G3BP1 MARYlation status or subjected to different stressors. Upon treatment with biotin, the distinct interactors are biotinylated.

2.3. Analysis of CHIKV nsP1 MARYlation and nsP1 functions

Preliminary to this work, the CHIKV nsPs were tested as substrates for MARYlation (Krieg, 2022). The nsPs were MARYlated by PARP10 *in vitro* and this modification was reversible by nsP3. This led to the aim to define the direct impact of MARYlation on the molecular functions of the CHIKV nsPs and consequences for viral replication. For nsP2, our lab found direct implications of MARYlation on the enzymes function (Krieg et al., 2023). During the preliminary work, nsP1 came forward as the highest MARYlated nsP1 *in vitro* (data not shown). NsP1 fulfils multiple functions during replication, such as membrane interaction, viral RNA capping and it functions at the center of the RC (**chapter 1.6.3.**). However, nsP1 still remains the least studied Alphaviral nsP. On top, most Alphaviral nsP1 studies were performed with other viruses such as SFV, VEEV and SINV. These different Alphaviruses are closely related but there remain significant differences (**chapter 1.6.3.**). In the third part of this work, we aimed at analyzing nsP1 as a MARYlation target and the specific functions of CHIKV nsP1.

2.3.1. NsP1 is a substrate for (de-)MARYlation

NsP1 interacts with PARP10 and was confirmed as a substrate of PARP10 and nsP3 (Krieg, 2022). To continue this work, we assessed whether nsP1 is a substrate of IFN-I-inducible mARTDs in general. Therefore, His-nsP1 was bacterially expressed and purified (**Fig. 43A**). Following, we performed nsP1 ADP-ribosylation assays *in vitro*. His-PARP10cat and His-nsP3 were bacterially expressed and purified. NsP1 was incubated with radioactively labelled NAD⁺, PARP10cat WT/GW and nsP3 as indicated (**Fig. 43B**). CB was used to visualize the total protein and autoradiography to visualize MARYlation. PARP10cat WT MARYlates nsP1, while the inactive mutant does not. NsP3 MD removes this modification.

To test nsP1 as a substrate of other mARTDs, similarly an nsP1 ADP-ribosylation assay was run with PARP10cat, PARP14cat and PARP15cat (**Fig. 43C/D**). These His-tagged fusion proteins were bacterially expressed and purified. All three IFN-inducible mARTDs MARYlate nsP1 *in vitro* (**Fig. 43C**). Upon co-incubation of the nsP3 MD, the MARYlation is removed (**Fig. 43D**). Generally, nsP1 is MARYlated by the catalytic domain of IFN-I-inducible mARTDs *in vitro*. NsP1 is also MARYlated by FL PARP10 (Krieg, 2022). This PTM is reversible by nsP3. However, the assays performed in this work need to be repeated with inclusion of the catalytically inactive mutants of PARP14cat and PARP15cat. Next, also nsP1 as a substrate of FL mARTDs needs to be assessed *in vitro*.

Even though *in vitro* ADP-ribosylation assays are very sensitive for analyzing potential substrates and for proving a direct mechanism, nsP1 MARYlation can still be an *in vitro* artifact. In cellular context, the two proteins might not interact with one another, and the modification might not take place in general.

To verify whether PARP10 interacts with CHIKV-nsPs in cells, HEK293 cells were transiently transfected with the individual CHIKV-nsPs (nsP1-3) in combination with PARP10 WT. As negative control, EGFP-tag was tested for interaction with PARP10. Cells were lysed and the nsPs were immunoprecipitated (**Fig. 44**). We see an interaction between PARP10 and all nsPs. This is a further indication that the nsPs could be potential direct substrates for PARP10 in cells.

Detecting MARYlation in cells is a difficult process due to the lack of good tools to detect the intracellular modification. So far, our lab confirmed nsP2 as a substrate for PARP10 in cells (Krieg et al., 2023). To detect intracellular nsP1 modification, we tried many different methods and set-ups. However, so far, we were unable to determine whether nsP1 is

MARylated in cells. One of the applied approaches was successful for nsP2. In this approach, nsP2 modification was triggered by mimicking viral infection using a CHIKV-replicon. This replicon encodes the four nsPs, but the structural proteins are exchanged with Gaussia luciferase, which is used as indicator for viral replication (**Fig. 45A**). The replicon potentially induces IFN signaling and thus mARTDs expression which could result in MARylation of the CHIKV-nsPs. Another hypothesis is the need for the entire RC and viral RNA for mARTDs to start targeting the viral replication. The replicon WT or the replicon V33E, incapable of hydrolyzing MARylation, was transfected into cells with or without the presence of nsP3 MD. Additional transfection of the nsP3 was utilized to augment a potential difference in MARylation signal. Replicon-derived nsP1 was immunoprecipitated and analyzed with western blotting (**Fig. 45B**). NsP1 was successfully immunoprecipitated in all samples. However, no MARylation signal was detected.

So far, there is no indication that nsP1 is MARylated in cells. It would still be worth trying to infect cells with full CHIKV, to completely mimic the environment during CHIKV infection and replication. The set-back of this method is the presence of functional nsP3 hydrolase activity, which is essential to CHIKV replication. This could remove any potential MARylation before we are able to detect the PTM. We propose the use of CHIKV mutants with reduced nsP3 catalytic activity that have slower replication rates but are still active (McPherson et al., 2017).

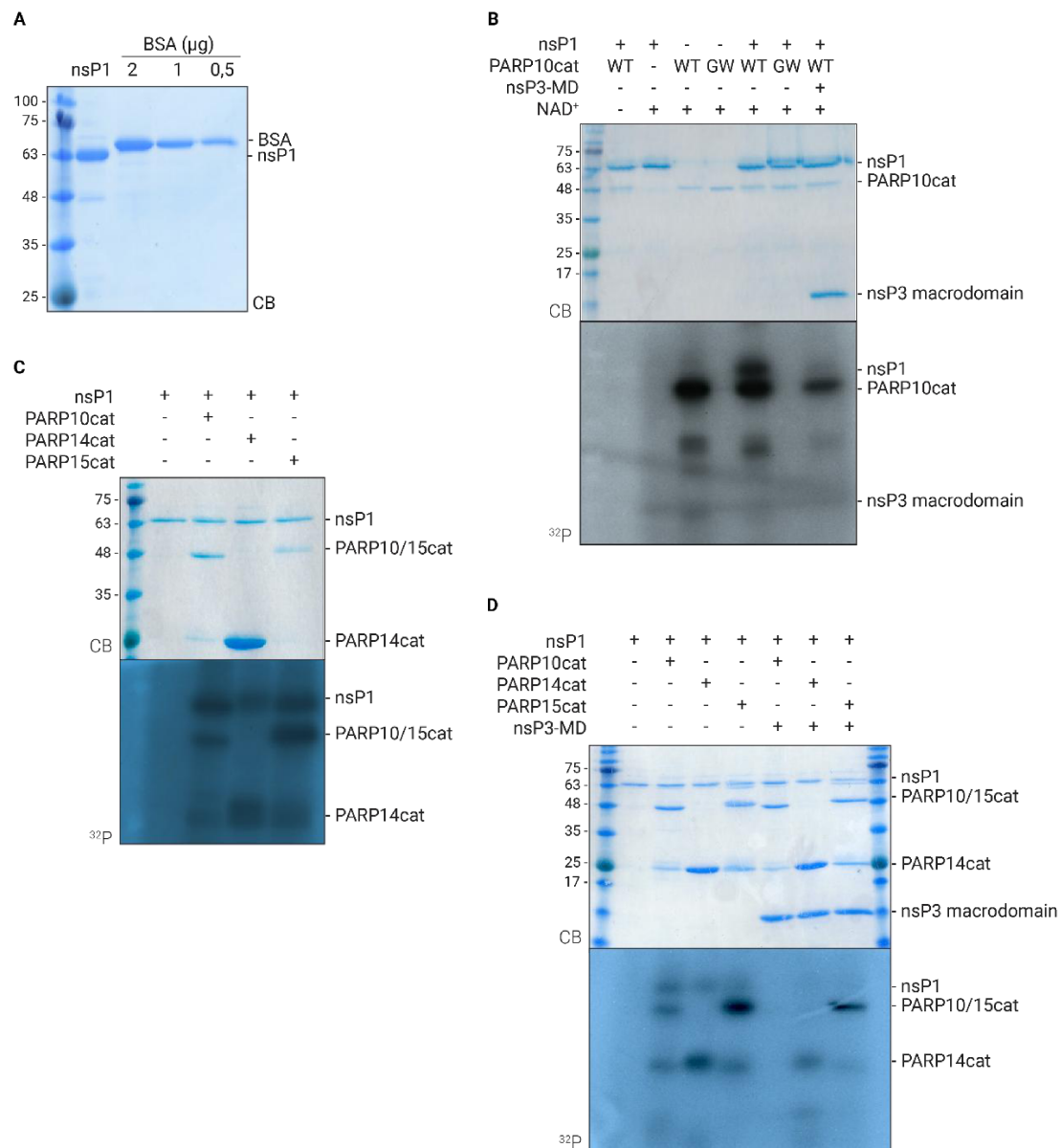


Figure 43. nsP1 is a substrate of IFN-I-inducible mARTDs catalytic domains and CHIKV nsP3-macrodomain.
A. His-tagged nsP1 was bacterially expressed and purified. His-nsP1 was following dialyzed and analyzed using SDS-PAGE and Coomassie blue-staining in comparison to a bovine serum albumin (BSA) standard. **B.** His-nsP1 was incubated with His-tagged PARP10cat WT/GW and nsP3 macrodomain (MD) as indicated in the presence of radioactively labelled ³²P-NAD⁺ for 30 min at 30°C. Total proteins were visualized with CB-staining and the incorporated radioactive label was analyzed using autoradiography (³²P) (n=3). **C.** His-nsP1 was incubated with His-tagged PARP10cat, PARP14cat or PARP15cat as indicated in the presence of NAD⁺ for 30 min at 30°C. Total proteins were visualized with CB-staining and the incorporated radioactive label was analyzed using autoradiography (³²P) (n=1). **D.** His-nsP1 was incubated with His-tagged PARP10cat, PARP14cat or PARP15cat and nsP3 MD as indicated in the presence of NAD⁺ for 30 min at 30°C. Total proteins were visualized with CB-staining and the incorporated radioactive label was analyzed using autoradiography (³²P) (n=1).

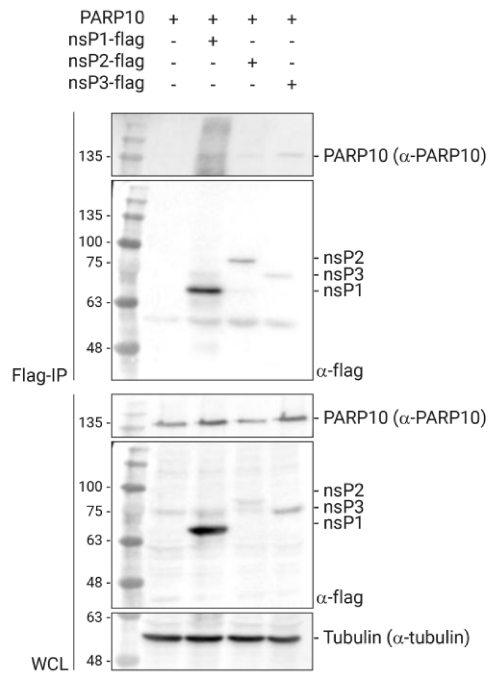


Figure 44. CHIKV nsPs interacts with PARP10. HEK293 cells were transiently transfected with plasmids encoding either PARP10 alone or in combination with Flag-tagged nsP1, nsP2 or nsP3. The cells were lysed 48h after transfection and the Flag-tagged nsPs were immunoprecipitated using Flag beads. Both the lysates and the immunoprecipitated proteins were analyzed by means of western blotting (n = 2).

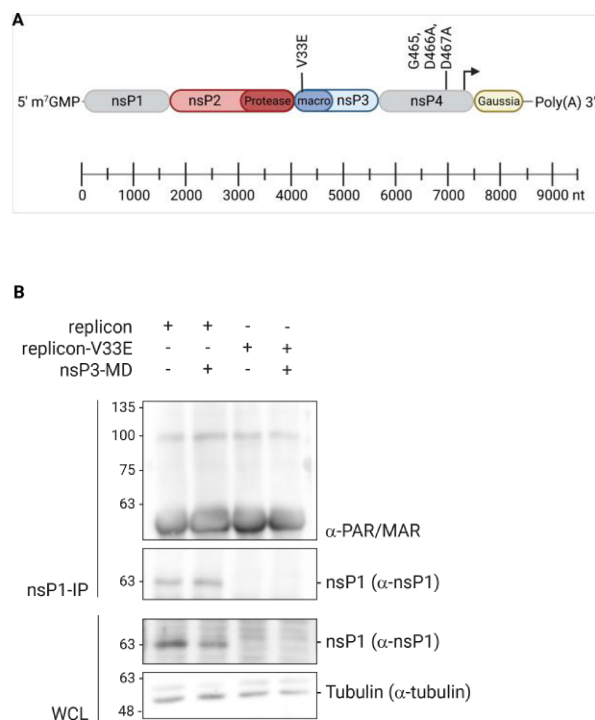


Figure 45. nsP1 is not MARYlated in cells upon CHIKV-replicon expression. **A.** Schematic representation of the CHIKV-replicon constructs. The scale bar indicates the length of the replicons in nucleotides. This figure was created by Patricia Korn. **B.** HEK293 cells were transfected with *in vitro* transcribed replicon RNA WT or the hydrolase inactive V33E mutant in combination with the nsP3-macrodomein (MD) as indicated. 24h after transfection the cells were lysed and nsP1 was immunoprecipitated. The samples were analyzed using immunoblotting (n=1). WCL: whole cell lysate.

2.3.2. NsP1 microscopy analysis

CHIKV nsP1 is primarily located in the cytoplasm of infected cells. After the virus enters a host cell, the viral RNA genome is released and serves as a template for viral replication. Once synthesized, nsP1 interacts with other viral proteins and viral RNA to form the membrane-associated RC. The alphavirus superfamily replicates by the RC inside spherules (Bartholomeeusen et al., 2023; Frolov & Frolova, 2018; Rupp et al., 2015).

To further study nsP1 localization, U2OS cells were transfected with nsP1-mCherry. The cells were analyzed using live-cell imaging. Nsp1 forms dots both cytoplasmic and at the membranes. Interestingly, these dots are spheric and highly dynamic. The condensates fuse and diffuse quickly and are likely formed through LLPS (**Fig. 46**). NsP1 is argued as the main nsP responsible for the RC membrane binding and the RC interaction (Salonen et al., 2003; Shirako et al., 2000; Sreejith et al., 2012; Žusinaite et al., 2007). Here, nsP1 is clearly capable of inducing condensates.

NsP1 and PARP10 seem to interact intracellularly, as indicated by coimmunoprecipitation (**Fig. 44**). Also, both enzymes localize to the cytoplasm and their overexpression induces condensates in cells. Subsequently, potential co-localization of PARP10 and nsP1 was analyzed. U2OS cells were transfected with nsP1-mcherry and PARP10-EGFP and studied using live-cell imaging and confocal microscopy (**Fig. 47**). Both enzymes form their distinct cytoplasmic foci. However, PARP10 and nsP1 condensates do not colocalize. They might interact through their mutual cytoplasmic presence and high mobility, but this is unclear. If the enzyme-substrate interaction is too weak or transient, it may not be detectable with confocal microscopy. Alternatively, it would be of interest to repeat the experiment with CHIKV replicon containing a fluorescently tagged nsP1. NsP1 functions are highly entangled and defined by the presence of the other nsPs. The lack of the rest of the RC might result in a skewed image of nsP1 localization. Besides, the presence of viral RNA might be essential for mARTDs targeting. Several mARTDs have RNA-interaction motifs and the presence viral RNA might be the beacon for mARTDs detection.

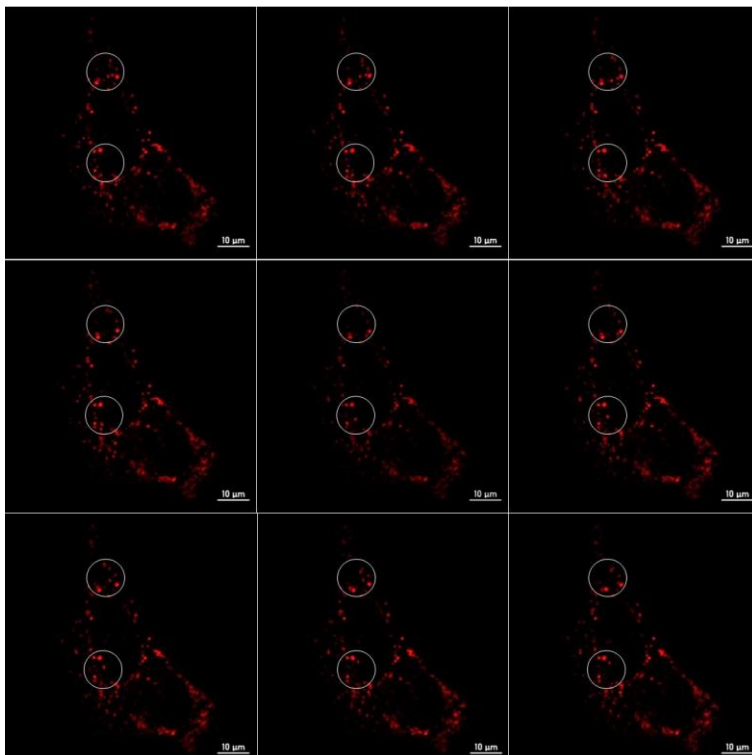


Figure 46. nsP1 localizes to membrane-associated and cytoplasmic condensates. U2OS cells were transfected with a plasmid encoding nsP1-mcherry. 48h after transfection the cells were analyzed using live-cell imaging and confocal microscopy. Representative images were taken at 4 sec intervals. Red: nsP1. Scale bar 10μm (n=2).

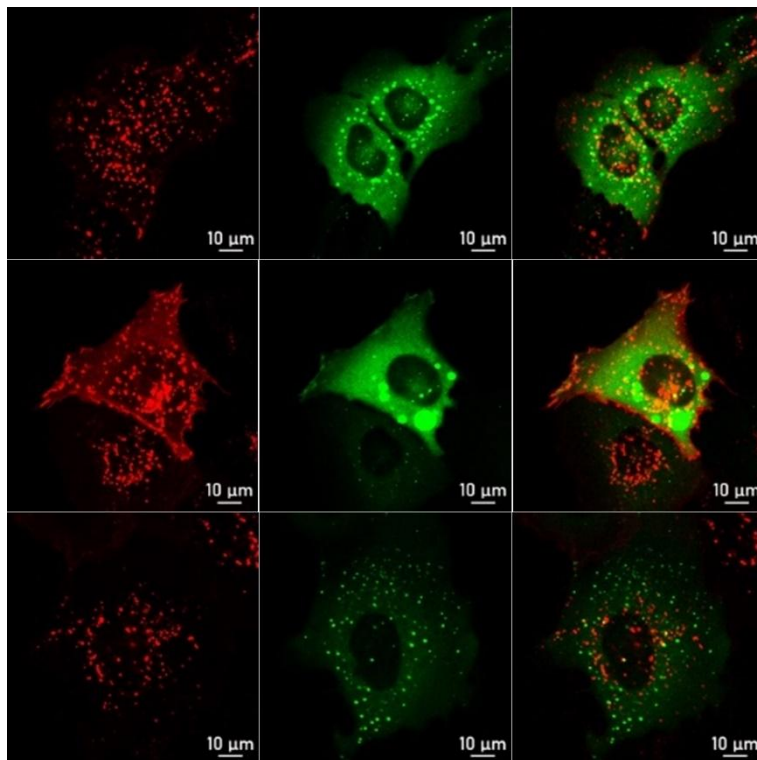


Figure 47. PARP10 does not colocalize with nsP1. U2OS cells were transfected with plasmids encoding nsP1-mcherry and PARP10-EGFP. 48h after transfection the cells were analyzed using live-cell imaging and confocal microscopy. Red: nsP1; Green: PARP10 WT. Scale bar 10μm (n=2).

2.3.3. NsP1 membrane interaction

The alphavirus superfamily replicates inside spherules. NsP1 is solely responsible for the membrane association and anchoring the RC to membranes (Salonen et al., 2003). NsP1 anchors to membranes through an amphipathic helix and is further reinforced by palmitoylation of C417-419 for CHIKV (Ahola et al., 2000; Laakkonen et al., 1996; Zhang et al., 2018). So far, CHIKV nsP1 membrane binding has not been extensively studied. Membrane binding has also been suggested as important for nsP1 capping activity (**Chapters 1.6.3.2, 1.6.3.3 and 2.3.4**). Evaluation of nsP1 membrane association *in vitro* was done in cooperation with Prof. Wolfram Antonin, whose group has the expertise to work with liposome floatation experiments as artificial membrane system.

NsP1 membrane association was studied by liposome floatation experiments, a commonly used technique to study lipid-protein interactions. Liposomes are small artificial spherical vesicles that consist of a lipid bilayer. To mimic a specific membrane composition such as nuclear envelope (NE) or Plasma Membrane (PM), they are generated of a respective lipid mixture consisting of phospholipids, cholesterol, and others. Thus, they can be made to imitate a membrane composition of choice. To study nsP1 affinity for different membranes, bacterially expressed and purified His-nsP1 was incubated with the liposomes mimicking either NE, PM or a general *E. coli* membrane. Following, the liposomes were enriched by sucrose gradient centrifugation and the respective fraction is analyzed for nsP1 binding by means of Western blotting (**Fig. 48A**). The representative experiment is done with liposomes build-up of *E. coli* mix (**Fig. 48B**). As positive control Nucleoporin 153 (Nup153) and as negative control Nup98 was included in the assay. Nup153 possesses an amphipathic helix that binds membranes in liposome floatation assays, Nup98 does not bind to membranes and liposomes (Vollmer et al., 2012, 2015). In a general *E. coli* liposome mixture nsP1 interacts with the liposomes. NsP1 does also bind PM liposomes, but not NE liposomes (**Fig. 48B**). PM liposomes have a higher cholesterol content. CHIKV nsP1 has been shown to prefer to co-segregate with cholesterol-rich membrane domains (Bakhache et al., 2020).

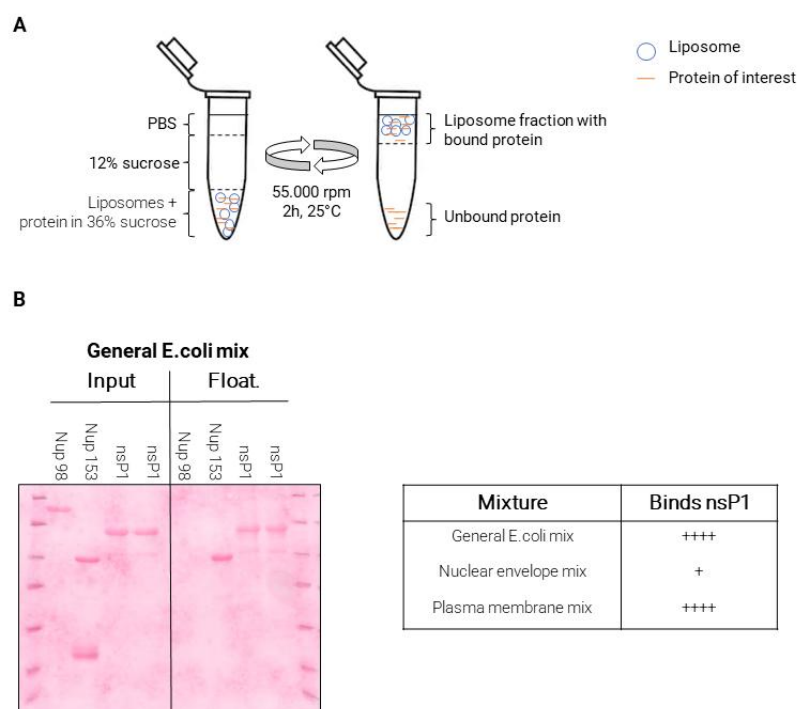


Figure 48. nsP1 interacts with liposomes. **A.** Schematic representation of the liposome floatation assay. The liposomes and protein mixture is equilibrated in 36% sucrose and layered under a 12% sucrose and PBS gradient. The mixture is centrifuged to allow liposome floatation. **B.** Respective experiment showing nsP1 interaction to E. coli liposomes and overview of Liposome experiments. Bacterially expressed and purified His-nsP1 was incubated with either E. coli, Nuclear Envelope or Plasma membrane liposomes. The liposomes were enriched by sucrose gradient centrifugation and the respective fraction is analyzed by means of Western blotting and Ponceau staining. Positive control: Nucleoporin 153 (Nup153); negative control Nup98. (n=2).

2.3.4. NsP1 capping mechanism

NsP1 has MTase and GTase enzymatic activities and is the main nsP responsible for capping of the viral RNA (Rupp et al., 2015). This is described as a general alphaviral mechanism. So far, CHIKV nsP1 mediated capping has not been detected and the study of nsP1 capping has been performed using other alphaviruses. For example, the study of Delang et al. analyzes small inhibitors for CHIKV and traces their activity back to nsP1. However, for follow-up biochemical assays to assess how the compounds affect nsP1 activity, the group turned to VEEV nsP1 (Delang et al., 2016). This indicates that there seem to be difficulties working with CHIKV nsP1. Our lab tried to establish a working set-up to analyze CHIKV nsP1 activity. We postulated that the seemingly low enzymatic activity *in vitro* might be due to the absence of a cofactor, host protein or correct environment. There also seems to be clear differences between the capping mechanisms of the different Alphaviruses. SFV requires the presence of lipids for nsP1-activity in contrast to SINV and VEEV (Ahola et al., 1999; Delang et al., 2016; Tomar et al., 2011). CHIKV is more related to SFV than to SINV and VEEV. Possibly, CHIKV needs membrane-association for nsP1 activity. We showed that CHIKV nsP1 interacts very well with *in vitro* liposomes. Also, another study postulated that nsP1 assembles into a 12 subunits membrane-associated ring and proposes this assembly is necessary for allosteric activation of nsP1 capping activity (Jones et al., 2020). Taken together, nsP1 activity and membrane interaction could be coupled.

NsP1 requires SAM and GTP as cofactors for m⁷GMP-cap (Ahola & Kääriäinen, 1995; Karpe et al., 2011; Vasiljeva et al., 2000). Before capping the viral RNA, the cap is synthesized on nsP1 itself, at position H37. In the next step, the m⁷GMP cap is transferred to the, by nsP2, primed RNA (ppRNA) (Ahola et al., 1997; Ahola & Kääriäinen, 1995; Li et al., 2015; Ortega Granda et al., 2021). This nsP1 intermediate is a useful tool in analyzing nsP1-mediated capping and can be used to measure nsP1 activity without the presence of viral RNA. NsP1 was bacterially expressed and purified. It was incubated with PM liposomes, to imitate the presence of membrane, and SAM and ³²P-GTP for 30' at 30°C. Total protein was analyzed

with SDS-PAGE and CB and cap-formation was analyzed with autoradiography. Experiments were incubated for multiple days at -80°C before analysis of the radioactive label (**Fig 49A**). Only a weak signal for capping activity was detected. In contrast to our hypothesis, the presence of lipids inhibited activity. The experiment was repeated and adapted to longer incubation times and temperature (**Fig 49B**). However, again the presence of PM liposomes inhibited the already low signal of nsP1 mediated capping.

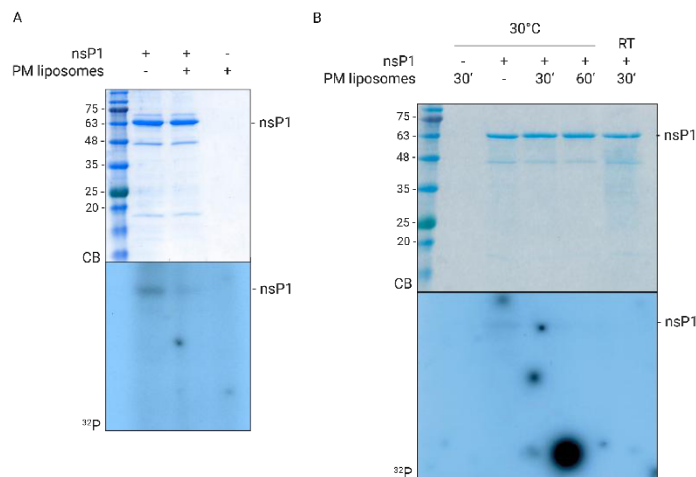


Figure 49. Membrane association has an inhibitory effect on nsP1 capping activity *in vitro*. **A.** Bacterially expressed and purified nsP1 was incubated with liposomes, SAM and ³²P-GTP for 30' at 30°C as indicated. Total protein was analyzed with SDS-PAGE and Coomassie Blue (CB) staining and cap-formation was analyzed by autoradiography (³²P) (n=2). **B.** Bacterially expressed and purified nsP1 was incubated with liposomes, SAM and ³²P-GTP for different timepoints and temperatures as indicated. Total protein was analyzed with SDS-PAGE and CB staining and cap-formation was analyzed with autoradiography (³²P) (n=1).

Taken together, experiments analyzing the *in vitro* activity of CHIKV nsP1 do not result in a clear set-up to analyze nsP1 capping. For CHIKV, presence of lipids does not result in the activation of nsP1 capping activity or there seems to be still a component missing for *in vitro* assays. We postulate that the seemingly low enzymatic activity *in vitro* might be due to the absence of a yet unknown cofactor, host protein or the correct environment.

Next, we wanted to analyze CHIKV nsP1 activity in cells. To study cellular nsP1-activity, two nsP1 mutants were generated by site-directed mutagenesis, H37A and D63A. H37 is the cap attachment site for the nsP1-intermediate and thus H37A stops GTase activity. D63A is unable to bind SAM and blocks the MTase activity (Ahola et al., 1997; Kovacicova et al., 2021). HEK293 cells were transfected with nsP1 WT or mutants, H37A and D63A. Cells were lysed and nsP1 was immunoprecipitated. Following, the immunoprecipitated nsP1 was incubated with SAM and GTP (**Fig. 50A**) or m⁷GTP (**Fig. 50B**) for 30' at 30°C. nsP1-capping was analyzed by immunoblotting (**Fig. 50A/B**). Cellular nsP1 WT has a cap, in contrast to H37A and D63A (**Fig. 50A/B**). Especially, incubation of nsP1 with SAM and GTP result in a clear capping and formation of the m⁷GMP-nsP1 intermediate (**Fig. 50A**). The reaction with m⁷GTP appears less efficient (**Fig. 50B**). Since both SAM and GTP are present in cells, we analyzed whether nsP1 is capped in cells directly. Due to the absence of viral RNA, the nsP1-intermediate should remain stable. HEK293 cells were transfected with nsP1 or the nsP1 mutants nsP1 H37A and D63A. nsP1 was immunoprecipitated and immediately analyzed with immunoblotting (**Fig. 50C**). Intracellular nsP1 is active and seems to result in the m⁷GMP-nsP1 intermediate (**Fig. 50C**). Seemingly, intracellular nsP1 is active in comparison to the *in vitro* assays. Clearly, there is an intracellular component missing. Whether this is the membranous environment, a cofactor or another protein is unclear. It would be interesting to have a CHIKV nsP1 interactome screen. So far, no interaction screen has been performed for nsP1. Bourai et al. performed interactome screens using Yeast Two-Hybrid (Y2H) for all CHIKV-nsPs but could not identify any cellular partner for nsP1 (Bourai et al., 2012). Since nsP1 is a membrane-binding protein, Y2H, which requires translocation to the nucleus, is not a suitable technique.

Capping activity is essential for the Alphavirus replication cycle. Mutation of conserved nsP1 residues important for nsP1 MTase or GTase results in either impaired or completely

blocked viral replication for SFV and SINV (Ahola et al., 1997; Niesterst & Strauss, 1990; Wang et al., 1996). To test whether CHIKV capping is essential for replication, nsP1 mutants H37A and D63A were cloned into the CHIKV replicon (**Fig. 50D**). HEK293 cells were transfected with the WT replicon or the H37A and D63A mutant. NsP1 expression was analyzed by means of immunoblotting. WT replicon replicates in comparison to the nsP1 mutants, indicating that also for CHIKV capping is essential for replication (**Fig. 50D**). The capping mechanism is an interesting antiviral drug target and small molecules targeting nsP1 could be of use in counteracting CHIKV infection.

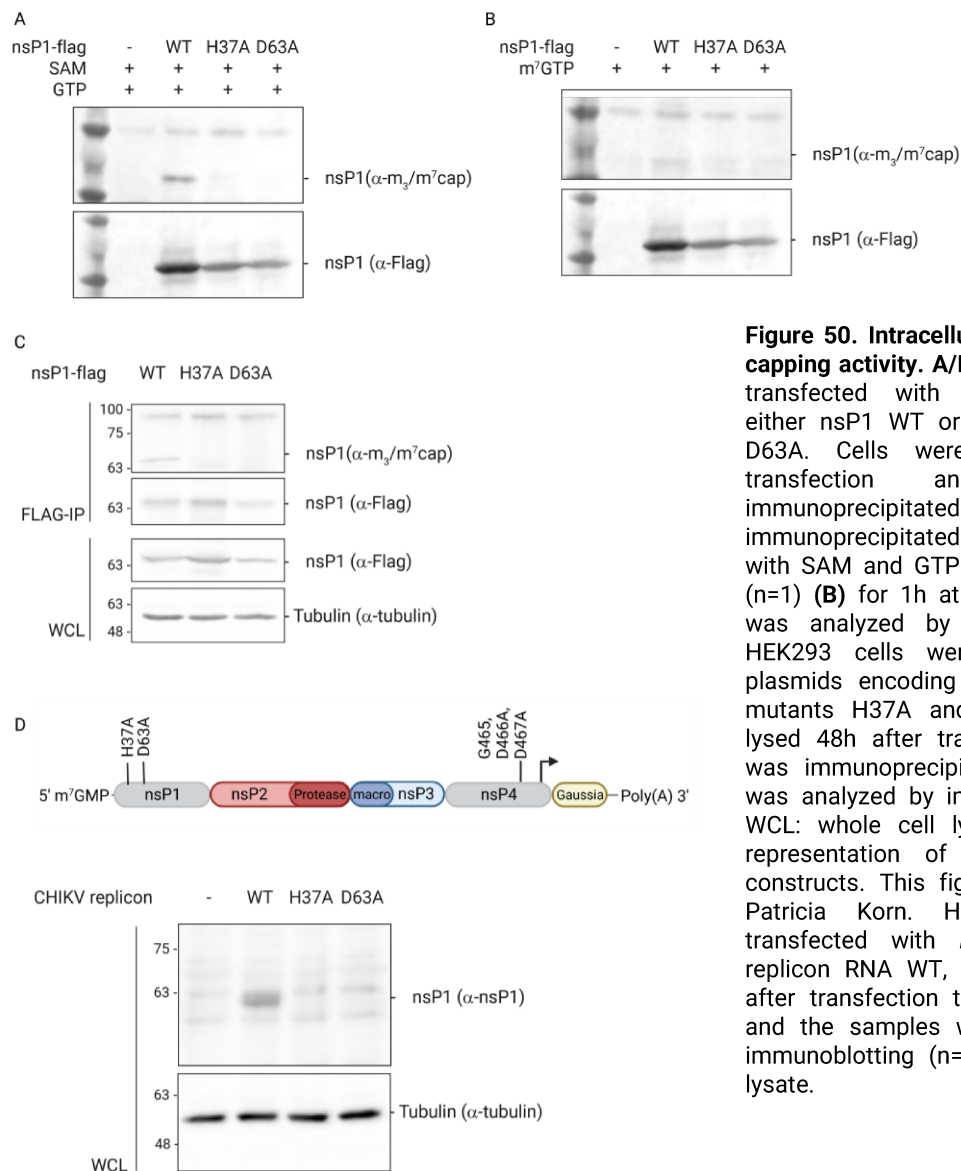


Figure 50. Intracellular CHIKV nsP1 has capping activity. A/B. HEK293 cells were transfected with plasmids encoding either nsP1 WT or mutants H37A and D63A. Cells were lysed 48h after transfection and nsP1 was immunoprecipitated.

The immunoprecipitated nsP1 was incubated with SAM and GTP (n=2) (**A**) or m⁷GTP (n=1) (**B**) for 1h at 30°C. NsP1-capping was analyzed by immunoblotting. **C.** HEK293 cells were transfected with plasmids encoding either nsP1 WT or mutants H37A and D63A. Cells were lysed 48h after transfection and nsP1 was immunoprecipitated. NsP1-capping was analyzed by immunoblotting (n=1). WCL: whole cell lysate. **D.** Schematic representation of the CHIKV-replicon constructs. This figure was created by Patricia Korn. HEK293 cells were transfected with *in vitro* transcribed replicon RNA WT, H37A or D63A. 24h after transfection the cells were lysed, and the samples were analyzed using immunoblotting (n=1). WCL: whole cell lysate.

3. Conclusions and future perspectives

MARylation lies at the interface between the host cell's defense mechanism and RNA viruses. Many mARTDs are interferon inducible or are expressed upon viral infection, suggesting their involvement in the cellular immune response (Lüscher et al., 2022). On the other hand, several RNA viruses, including Alphaviruses and Coronaviruses, encode MDs capable of reversing MARylation. For many of these viruses, the MD is important for virulence and counteracting the immune response (Lüscher et al., 2022). For Chikungunya virus specifically, the MD is essential to viral replication (Krieg et al., 2023; McPherson et al., 2017). The exact mechanism by which mARTDs and MDs respectively promote and counteract the immune response is unknown.

The aim of this dissertation was to elucidate how MARylation is involved at the host-virus interface and the functions of the mARTDs and the viral MDs, respectively PARP10 and CHIKV nsP3 in particular.

3.1. Host factors regulated by MARYlation during CHIKV

CHIKV is dependent on its ability to remove MARYlation. Hydrolase-deficient CHIKV cannot replicate (Krieg et al., 2023; McPherson et al., 2017). So, without functional hydrolase activity, CHIKV seems to be unable to counter the IFN-I induced MARYlation. One explanation could be that CHIKV requires the removal of ADP-ribosylation from certain host proteins either to block the innate immune response or affect their function in the viral lifecycle, as anti- or proviral. To start, we aimed at identifying potential host factors that are regulated by MARYlation upon CHIKV-infection. To do this we set out to characterize the interactome of both PARP10 and nsP3 using BioID. BioID allows proximity-dependent biotinylation of stable and transient interactors and thus enables detection of more dynamic interactions e.g., enzyme-substrate pairings.

Firstly, we established the workflow to identify the interactome of PARP10 and nsP3. BirA-tagged PARP10 and nsP3 expressed well in HEK293 cells and successfully biotinylated many proteins. For enrichment, the Pierce Streptavidin Magnetic Beads had a vastly superior performance to Dynabeads MyOne Streptavidin T1 beads. Consequently, enrichment of biotinylated proteins in all further experiments was performed with Pierce Streptavidin Magnetic Beads. Eventual identification of the enriched biotinylated proteins, and thus PARP10 or nsP3 interactors, was performed using MS analysis. Using this technique, we successfully identified multiple interactors for PARP10 and nsP3, and thus potential host factors regulated by MAR during infection.

Concerning PARP10, 882 significantly enriched proteins were identified. 352 proteins were at least 10-fold enriched. The PARP10 interactome shows involvement in processes such as cell migration, cytoskeleton related functions, stress granule assembly, mRNA processing, RIG-I pathway regulation and translation among others. As stated (**chapter 1.1.2.2.**), PARP10 is linked to processes such as cancer, neurodegenerative disorders, innate immunity, cell cycle and cell proliferation. Abnormalities with cell migration, cell proliferation and cytoskeleton related functions are strongly associated with neurodegeneration and cancer. In turn, regulation of the RIG-I pathway, stress granule assembly and translational processing are linked to innate immunity and host cell defense against viruses. Lower enriched biological processes according to our data (**table 3**) also entail cell cycle functions. Taken together, this shows the robustness of the PARP10 interactome and the validity of our data. The identified PARP10 interactome contained proteins previously identified by our lab, G3BP, CD2AP and MAGED2 (Krieg, 2022). Our lab set out to validate these potential substrates both *in vitro* and in cells through a combination of ADP-ribosylation assays and co-IPs. G3BP1 is discussed further below, as it entailed a detailed chapter in this dissertation. Preliminary data on both CD2AP and MAGED2 indicates that they interact with PARP10 in cells (Data not shown). Our data was further validated upon comparison of the identified PARP10 interactome to an *in vitro* ProtoArray for PARP10 previously performed by our group. A few overlapping proteins were identified: PLK1 (9-fold), MARK2 (7-fold), CDK2 (6-fold), GSK3 β (7-fold) and PRUNE (10-fold) (Feijs et al., 2013). This confirms them as not only interactors in cell, but *in vitro* substrates of PARP10. Overall, the PARP10 screen resulted in a robust dataset of the PARP10 interactome.

For the nsP3 interactome, 456 significantly enriched proteins were identified, of which 276 were at least 10-fold enriched. Enriched GO terms include translation, cell migration, stress granule assembly, cell cycle and cytoskeletal processes (**Table 4**). For CHIKV nsP3 we found proteins that were previously identified by both our lab and others as Alphaviral nsP3 interactors (Bourai et al., 2012; Cristea et al., 2006; Frolov & Frolova, 2018; Frolova et al., 2006; Kim et al., 2016; Krieg, 2022; Meshram et al., 2018; Mutso et al., 2018; Panas, Ahola, & McInerney, 2014), indicating our dataset is reliable and robust. Even though there have been

more specific studies on CHIKV nsP3 interactions to host factors, to our knowledge we performed the first complete interactome study focused solely on CHIKV nsP3 in human cells. Through literature mining, Bouraï et al combined Alphavirus-host interaction data of different groups (Bouraï et al., 2012). For SINV nsP3 two previous interaction studies were performed using recombinant viruses with tagged nsP3 (Cristea et al., 2006, 2010; Frolova et al., 2006). To validate our data further, we analyzed the overlap between their and our CHIKV nsP3 interactome. Cristea et al. identified 33 SINV nsP3 interactors (Cristea et al., 2006, 2010), of which 6 were found for CHIKV nsP3. In turn, Frolova et al. identified 27 SINV nsP3 interactors, of which 3 were found for CHIKV nsP3 (Frolova et al., 2006). Interestingly, only 2 proteins were found in all 3 datasets, G3BP1 and G3BP2 (Bouraï et al., 2012; Cristea et al., 2006, 2010; Frolova et al., 2006). Again, emphasizing their importance in Alphaviral replication.

Examples of some of the interesting identified CHIKV nsP3 interactors are G3BP1, G3BP2, CD2AP, MAGED2, USP10 and UBAP2L. G3BPs, USP10 and UBAP2L are SG associated proteins. G3BPs and UBAP2L are theorized as central nodes capable of building PPI networks, protein-RNA complexes and driving condensation (Sanders et al., 2020). On the other hand, USP10 would function as a “cap” on G3BPs and prevent interaction nodes and thus condensation (Kedersha et al., 2016; Sanders et al., 2020). It is worth speculating that UBAP2L, as known for G3BPs, has a proviral function and USP10 an antiviral function for CHIKV.

Our aim was to identify host factors that are regulated by MARYlation through the opposing activities of PARP10 and nsP3 during CHIKV infection. To characterize the most important host factors, we evaluated only the overlapping proteins between 10-fold enriched proteins of both datasets. This restriction resulted in 136 enriched proteins identified in both interactomes that are at least 10-fold enriched. These host factors are involved in mRNA processing, cell migration, stress granule assembly, cell cycle regulation and cytoskeleton regulation (**Table 5**). RNA related functions are expected to be found upon viral infection. Viruses have a small genome and for replication and translation they hijack the host cell machinery. Concerning the cell migration and cytoskeleton regulation, RNA viruses exploit cell migration proteins to facilitate their replication and dissemination. Through cell-cell contacts, viruses can easily be transmitted. Also, many viruses reshape cellular spaces to replicate their genome e.g., CHIKV in spherules. Lastly, viruses are known to regulate the cell cycle to achieve beneficial cellular conditions. Some viruses induce the cell cycle to increase the pool of deoxynucleotides, others arrest cells in a specific phase favorable for replication and some are known to block the cell cycle in lymphocytes to cripple our immune system (Fan et al., 2018). Stress granule processes are discussed in detail below.

Next our group will focus on analyzing the different identified host factors. This will be done by a combination of *in vitro* ADP-ribosylation assays and in cell interaction studies. The *in vitro* ADP-ribosylation and hydrolase assays illustrate whether the host factor is a direct substrate of mARTDs and nsP3. In cell interaction assays will be a combination of co-immunoprecipitation studies and colocalization analysis using confocal microscopy. Furthermore, the host factors will be tested for either proviral or antiviral activity towards CHIKV. This will be achieved through analyzing viral replication upon either host factor overexpression studies and the respective host factor knockdown or KO experiments. Viral replication will be measured either through the replicon-system or plaque assays with fully infectious CHIKV. The identified common interactors of PARP10 and nsP3 include proteins such as G3BP1, CD2AP, TP53 and XRN1. G3BP1 is discussed further below. CD2AP is a known interactor of nsP3 and binds to the nsP3-HVD through its SH3 domain (Mutso et al., 2018). However, it was unknown as a PARP10 interactor. The protein interacts with actin and multiple cell membrane proteins. It plays a role in actin remodeling and membrane

localization and trafficking (Sayers et al., 2022). It could be important for CHIKV cell-to-cell transmission and spherule organization. TP53 is one of the most known and studied tumor suppressor proteins. It regulates gene expression and is involved in many processes such as senescence, apoptosis, DNA repair, cell cycle and metabolism (Sayers et al., 2022). Viruses can inhibit TP53 to promote survival or block its ability to regulate the IFN I response (Aloni-Grinstein et al., 2018). Lastly, XRN1 is another interesting hit as it is a known cellular exoribonuclease that has been shown to have different roles in viral replication. XRN1 has been shown to limit flaviviral replication by degrading the gRNA and has specifically been suggested as antiviral factor for Dengue (Chapman et al., 2014; Moon et al., 2012; Ng et al., 2020). However, for influenza A virus (IAV), XRN1 is hijacked by the virus and was found as a proviral host factor. XRN1 knockdown decreased replication while XRN1 overexpression boosted replication (Liu et al., 2021). Concerning CHIKV, one group showed that depletion of XRN1 slightly increased the amount of gRNA (Taschuk et al., 2020). However, this needs further research.

It is important to keep in mind that through the 10-fold enrichment cut-off already many interesting, enriched proteins are eliminated from the screens. For example, UBAP2L (8-fold for PARP10; 23-fold for nsP3) and MAGED2 (7-fold for PARP10; 13-fold for nsP3) are omitted. UBAP2L is an interesting target for its involvement in PPI networks and condensation as we discussed above. Especially, considering both PARP10 and nsP3 overexpression results in the formation of condensates. In a very recent report, MAGED2 came forward as an antiviral host factor for corona viruses. The protein is cleaved by the main protease of SARS-CoV, SARS-CoV-2 and MERS-CoV. Clearly, among corona viruses this is an evolutionarily conserved mechanism. During viral replication, MAGED2 interferes with the interaction between SARS-CoV-2 nucleocapsid protein and the viral genome. Corona viruses counteract this by processing MAGED2 (Ju et al., 2023). Since Alphaviruses and Coronaviruses are both enveloped (+)ssRNA viruses, MAGED2 processing could be a conserved mechanism in host cell modulation.

Taken together, we performed a successful interactome screen for both PARP10 and nsP3. The two interactomes of the enzymes have a noteworthy overlap. Further follow-up of the identified host factors will result in relevant information concerning mARTDs role in innate immunity and CHIKV infection.

3.2. G3BP1 as mutual substrate of PARP10 and nsP3

G3BP1 is one of the most promising hits identified in our study. G3BP1 is important in the lifecycle of many RNA viruses, both pro- and antiviral (**chapter 1.4.4.**). More specifically in Alphaviruses it is implicated in SINV, SFV and CHIKV (**chapter 1.4.4.3.**). For SINV there is discussion whether G3BPs are pro- or antiviral (Cristea et al., 2010; Kim et al., 2016; Scholte et al., 2015). However, the more recent reports argue for proviral (Kim et al., 2016; Scholte et al., 2015). This would also be more in line with the data for SFV and CHIKV (Kim et al., 2016; Panas et al., 2012). For CHIKV, G3BPs are of special importance. To our knowledge, it is the only virus shown to be unable to replicate upon G3BP1/2 dKO (Kim et al., 2016). CHIKV nsP3 is known to interact with G3BP1 (Fros et al., 2012; Scholte et al., 2015). Also, G3BP1 has been suggested as an ADP-ribosylation substrate and co-expression of CHIKV nsP3 is able to remove this modification (Jayabalan et al., 2021). I express myself carefully here since this report is not direct evidence for G3BP1 MARYlation. Jayabalan et al. show overall G3BP1 ADP-ribosylation, likely PARYlation. However, they did not use a PARP1/2 inhibitor during cell lysis. In our experience, cell lysis induces PARP1 activation, which leads to massive PARYlation. This could lead to wrong conclusions. Also, they show that co-expression of CHIKV nsP3 reduces G3BP1 ADP-ribosylation (Jayabalan et al., 2021a). Keep in mind, that nsP3 MD is a MAR hydrolase primarily and is inefficient towards PARYlation (Eckei et al., 2017). So, whether this is a direct effect is questionable. Overall, this first study gives an indication of G3BP1 ADP-ribosylation but is not direct evidence. Taken together, everything indicates G3BP1 as an interesting host factor to study at the host-pathogen interface.

Upon identification of G3BP1 as a common interactor of PARP10 and nsP3, we set out to assess whether it is also a common substrate. Firstly, we could confirm it both as an interactor for PARP10 and nsP3 in cells. Exogenous PARP10 and G3BP1 show clear interactions (**Fig. 17**). The interaction between PARP10 and endogenous G3BP1 is more elusive to see (**Fig. 17**). Here, we detect interaction between G3BP1 and PARP10 GW. This could be due to the fact that G3BP1 is tethering on the inactive enzyme because of its inability for modification. While the WT enzyme has a very transient interaction. Also, in general co-IPs are not optimal to detect enzyme substrate pairings, due to their transient nature. Because of this reason, we opted for the BioID system in the first place. Taken together, we argue that G3BP1 interacts with PARP10 in cells. NsP3 interacts strongly with G3BP1 (**Fig. 15**). We also confirmed that nsP3 interacts with G3BP1 through its FDGF motifs. G3BP1-nsP3 interaction is not affected by PARP10 overexpression or nsP3 hydrolase ability (**Fig. 19**).

Next, we assessed G3BP1 as substrate of both PARP10 and nsP3. Since we are interested in the role of mARTDs in innate immunity in general, we expanded our experiment to other IFN-inducible mARTDs: PARP14, PARP15 and later on PARP12. G3BP1 is *in vitro* a substrate of the catalytic domains of PARP10, PARP14 and PARP15 (**Fig. 20**). It is also MARYlated *in vitro* by FL PARP10 and PARP15iso1 (**Fig. 20**). This modification is removed by coincubation with either nsP3 MD or FL nsP3 (**Fig. 20**). Taken together, these *in vitro* experiments show a direct mechanism in which mARTDs are capable of MARYlating G3BP1 and nsP3 can hydrolase this modification. We could also show that G3BP1 is MARYlated in cells by PARP10 and PARP15iso1. Again, this modification is reversed upon expression of nsP3 (**Fig. 21**). The used antibody detects both MARYlation and PARYlation. We argue that G3BP1 is MARYlated. Firstly, due to the mARTDs and nsP3 effect upon the modification. Also, we see a clear, distinct signal for G3BP1 MARYlation, and not a smear as expected for PARYlation. Lastly, due to the direct evidence resulting from the *in vitro* studies. Lastly, we wanted to look into MARYlation of endogenous G3BP1(**Fig. 22.**). Endogenous G3BP1 always shows an ADP-ribosylation signal upon detection, unaffected by the presence of mARTDs or nsP3. To

analyze this signal, we immunoprecipitated G3BP1 and treated it with nsP3 MD in an *in vitro* hydrolase assay. We do see a small reduction in ADP-ribosylation signal upon treatment with nsP3 MD. The assay should be repeated with higher cell count, to visualize the reduction better. Also, more precise methods are needed to study this PTM intracellularly. Tools to study intracellular MARYlation are not robust. However, more and more methods and tools are being developed. For example, the antibody used in this study to detect MARYlation has only been developed and studied a few years ago (Lu et al., 2019).

G3BP1 is a multidomain protein of which each domain has a vastly different role (**chapter 1.4.1.**). NTF2L is responsible for G3BP1 dimerization and PPIs. IDR1 and IDR2 play important roles in G3BP1 LLPS regulation. The RBD is responsible for both RNA interaction and LLPS regulation as well (Sanders et al., 2020; Yang et al., 2020). Pinpointing the target site for MARYlation would give information on how MARYlation influences G3BP1 activity. Whether it affects PPIs, LLPS regulation or RNA-binding. To study the G3BP1 modification site, we successfully cloned different G3BP1 truncations. These were each analyzed using *in vitro* ADP-ribosylation experiments with FL mARTDs PARP10, PARP12 and PARP15iso1. The RBD domain clearly stood out as the main MARYlation site of G3BP1 *in vitro* of PARP10, PARP12 and PARP15iso1 (**Fig. 24**). Also, in cells G3BP1-RBD is MARYlated by PARP10 (**Fig. 25**). Our lab still needs to confirm the G3BP1-RBD as substrate in cells for PARP12 and PARP15iso1. The next step will be to determine the exact G3BP1 MARYlation site through site mutagenesis studies.

The functions of the RBD domain are RNA-binding and LLPS regulation. Deletion of the full RBD blocks RNA-induced LLPS, deletion of only the RRM or the IDR3 severely impairs it (Guillén-Boixet et al., 2020; Sanders et al., 2020; Yang et al., 2020). G3BP1/2 dKO cells reconstituted with Δ RBD results in blocked SG induction, Δ RRM or Δ IDR3 again severely impairs it (Sanders et al., 2020; Yang et al., 2020). The intramolecular electrostatic interaction between the negatively charged IDR1 and the positively charged RBD-IDR3 regulates G3BP1 conformation (Guillén-Boixet et al., 2020; Yang et al., 2020). This conformational switch has been argued to be able to be controlled by PTMs. (Yang et al., 2020). MARYlation is a bulky, negatively charged modification. It could induce G3BP1 conformational changes and charge. Either it could block RNA binding through spheric hindrance or induce RNA binding by inhibiting the IDR1-IDR3 interaction and promoting G3BP1 open conformation. First, we set out to test whether MARYlation of G3BP1 affects RNA binding. To assess this, we used EMSAs. G3BP1 MARYlation did not significantly affect RNA interaction. There was a trend visible for MARYlated G3BP1 to bind more RNA (**Fig. 27**). It is of interest to continue with this study. However, there will be a more sensitive method needed. Before the used EMSA method, G3BP1 was incubated with immunoprecipitated PARP10 on Sepharose beads for an ADP-ribosylation assay. We do not have any knowledge about the stoichiometry of this reaction. So far, we do not know exactly how much G3BP1 is modified by PARP10 in the given amount of time. For example, 15% of G3BP1 MARYlated by PARP10 might be enough to characterize G3BP1 as a PARP10 substrate but might not make a significant difference in the EMSA. We also performed preliminary studies in which we assessed the effect of MARYlation on the IDR1-RBD interaction (data not shown). UnMARYlated RBD seemed to interact slightly better with IDR1, supporting the EMSA trend of MARYlated G3BP1 binding better to RNA. However, this IDR1-IDR3 electrostatic interaction is hard to visualize and needs further investigation. Taken together, we hypothesize that MARYlation of G3BP1 induces the open conformation and RNA-binding. This would be in line with the link of ADP-ribosylation and condensate induction (**chapter 1.3.3.**). Our lab is currently setting up experiments to study G3BP1 LLPS propensity according to previous reports (Sanders et al., 2020; Yang et al., 2020). So far, we have no clear assay to analyze RNA-induced G3BP1 LLPS. This assay would be key in answering the question how G3BP1 MARYlation influences G3BP1 RNA binding and LLPS regulation.

Lastly, we assessed whether CHIKV cleaves G3BP1 such as members of the Caliciviruses and Picornaviruses (**chapter 1.4.4.2.**) (Humoud et al., 2016; White et al., 2007). Their viral proteases inhibit SGs by cleavage of G3BP1. G3BP1 is cleaved by FCV in the RBD-domain (Humoud et al., 2016). CHIKV nsP2 has protease activity, which is responsible for polyprotein processing. G3BP1-RBD contains a motif highly similar to the nsP2 cleavage site between nsP3 and nsP4 (**Fig. 28**). G3BP1 RBD MARYlation could play a role as a defense mechanism by mARTDs against CHIKV infection by blocking G3BP1 processing by CHIKV nsP2. To test this, we ran a protease assay established in our lab. G3BP1 is not cleaved by nsP2 (**Fig. 28**). This is in accordance with cell assays, where we also never perceived a difference in G3BP1 running behavior.

G3BP1 is a substrate of both IFN-inducible mARTDs and CHIKV nsP3. The latter has been reported to affect G3BP1 localization and sequester it from SGs towards viral condensates (Jayabalan et al., 2021). We do not know whether mARTDs affect G3BP1 localization or SG dynamics. ADP-ribosylation of proteins has been shown to result in SG recruitment (**chapter 1.3.3.**). To study how MARYlation affects G3BP1 and SGs, our lab developed inducible U2OS tdTomato-G3BP1 mEGFP-mARTD cell lines for PARP10, PARP12 and PARP15 (**Fig. 30**). PARP10 localizes predominantly to the cytoplasm (Kleine et al., 2012). Overexpression of PARP10 induces condensate formation, regardless of its catalytic activity. The condensates induced by PARP10 WT are more dynamic compared to the PARP10 GW condensates. These PARP10 condensates are not SGs or any other known condensates (Forst et al., 2013; Kleine et al., 2012; Mayo et al., 2018). We found that low expression of PARP10 WT does not result in condensate formation. Higher expression results in PARP10 WT condensates. PARP10 does not localize to SGs. G3BP1 and SG localization seems unaffected by PARP10 WT expression. PARP10 GW induces condensate formation. Again, these are not SGs and G3BP1 and SG localization seems unaffected by PARP GW expression. PARP12 localizes in basal conditions to the cytoplasm and the Golgi apparatus (Welsby et al., 2014). PARP12 is linked to stress responses and translocates towards SGs upon cellular stress and overexpression of PARP12 has been shown to induce SGs, independent of catalytic activity (Fehr et al., 2020; Lüscher et al., 2022; Catara et al., 2017; Leung et al., 2011; Welsby et al., 2014). PARP12 localizes to SGs as a result of oxidative stress and heat shock but not osmotic stress (Sorbitol), indicating the differences between stress responses (Catara et al., 2017). We confirmed that PARP12 WT localizes to SGs upon oxidative stress and higher expression of PARP12 WT resulted in the induction of condensates containing G3BP1 without external stressors. PARP12 HY also localizes to SGs upon oxidative stress, but in contrast to previous reports stays cytoplasmic upon overexpression in absence of stress (Catara, et al., 2017). This argues that upon stress PARP12 localizes to SGs independent of activity. However, PARP12 MARYlation activity has an inducing effect upon SG formation. PARP15 has 2 isoforms, PARP15iso1 and PARP15iso2, of which PARP15iso2 has been reported to localize to SGs (Leung et al., 2011). Isoform 2 lacks the N-terminal domain and the first MD compared to isoform1 (Lüscher et al., 2018, 2022). PARP15iso1 localization has thus far not been studied. PARP15iso1 localizes to the nucleus independent of activity. G3BP1 or SG localization are unaffected by PARP15iso1 expression. Interestingly, PARP15iso1 WT localizes to nuclear dots while PARP15iso1 HY shows a diffuse nuclear staining. This suggests that the condensates are dependent on catalytic activity. Further analysis of these PARP15iso1 WT speckles showed that, when targeted with a MD these speckles diffuse and resemble the PARP15iso1 HY phenotype (data not shown). Clearly, these distinct speckles are formed through MARYlation activity. It is worth studying whether the condensates are transcription hubs, PML cages, Cajal bodies, paraspeckles or other known nuclear bodies. However, this fell outside the scope of this dissertation. PARP15iso2 localizes to the cytoplasm but does not localize to SGs upon oxidative stress and PARP15iso2 overexpression does not result in SG formation. These results contradict previous reports (Leung et al., 2011). So far, initial experiments have been performed to assess the effect of

mARTD expression on G3BP1 and SGs. To truly study how these enzymes affect SGs, our lab will continue with time-point assays and Fluorescence recovery after photobleaching experiments (FRAP) to analyze whether SGs are formed quicker, or diffuse slower, or the other way around. Also, different stressors need to be assessed. For example, PARP12 localizes to SGs as a result of oxidative stress and heat shock but not osmotic stress (Catara et al., 2017). G3BP1 also disassembles SGs differently dependent on the stressor (Gwon et al., 2021). Taken together, we established cell lines, which will serve as useful tools in the further study on mARTDs and stress responses.

G3BP1 has been shown to be essential to SG formation and was suggested as the central node in SGs (Sanders et al., 2020). The IDR and the RBD essential for condensate regulation, while the NTF2L mediates multiple PPIs (Sanders et al., 2020). G3BP1 is at the basis of the SG proteome. Generally, the SG proteome has a similar composition. However, SG composition is also context dependent on stressor and cell type (Markmiller et al., 2018). So far, how this is regulated is unknown. As mentioned, Alphaviral nsP3 suppresses SG formation and sequesters G3BPs to viral condensates. The composition of these viral nsP3 condensates seems dependent on nsP3 catalytic activity (Jayabalan et al., 2021). Since we identified G3BP1 as a substrate of nsP3, the MARYlation status of G3BP1 could influence condensate composition. Also, ADP-ribosylation affects translocation of proteins to SGs, and thus mARTDs expression likely affects SG proteome (**chapter 1.3.3.**). To analyze how MARYlation affects G3BP1 interactions and the SG proteome, we are developing G3BP1/2 dKO cells with inducible G3BP1-miniTurbo-V5. MiniTurbo has an increased activity compared to BioID and a faster biotinylation time that labels in minutes rather than hours (Trinkle-Mulcahy, 2019). This would allow us to assess G3BP1 interactome upon stressors such as oxidative stress, heat shock and viral infection. Also, upon mARTD expression or IFN α stimulation, we could immediately assess how this changes the G3BP1 interactome. Important to note, miniTurbo results in more background biotinylation upon long incubation, as seen in figure 40 (May et al., 2020; Samavarchi-Tehrani et al., 2020; Trinkle-Mulcahy, 2019). We successfully tested the specificity of G3BP1-miniTurbo-V5 on nsP3 (**Fig. 41**). After assessing the G3BP1-interactome, we can analyze our dataset by comparing to different identified SG proteomes (Jain et al., 2016; Markmiller et al., 2018; Millar et al., 2022; Youn et al., 2019). Lastly, upon determination of the G3BP1 MARYlation site, point mutations would be introduced in G3BP1-miniTurbo-V5 which would offer the possibility to exactly determine how G3BP1 MARYlation influences the G3BP1 interactome.

3.3. NsP1 as mutual substrate of PARP10 and nsP3

CHIKV nsP1-3 are MARYlated by PARP10 *in vitro* and this modification is reversible by nsP3. Our lab showed that PARP10-mediated MARYlation inhibits nsP2 proteolytic activity. The hydrolase activity of the viral macrodomain counteracts this. Together, this was the first clear function described for the CHIKV macrodomain activity (Krieg et al., 2023). In this work, we wanted to analyze how MARYlation affects nsP1 and potentially targets its function. nsP1 is MARYlated by PARP10cat, PARP14cat and PARP15cat *in vitro* (**Fig. 43**). This PTM is removed by nsP3 MD *in vitro* (**Fig. 43**). However, so far, we could not prove intracellular MARYlation of nsP1. Using the viral replicon, our lab could show intracellular MARYlation of nsP2 but not nsP1. Upon nsP1 immunoprecipitation from the replicon, nsP1 runs only slightly higher than the heavy chains on the SDS-PAGE (**Fig. 45**). Detecting MARYlation in cells is a sensitive process due to the lack of robust tools. Perhaps, the slight MARYlation modification signal is drowned out by the high signal of the heavy chains during the immunodetection. We propose to insert a tag into the replicon behind nsP1 (mEGFP or FLAG), which will not only allow easy immunoprecipitation and a different running behavior but also the absence of the heavy chains. The experiment could then be repeated and give new information. Another option is to infect cells with full CHIKV, to completely mimic the environment during CHIKV infection and replication. This would be performed by our cooperation partners (Institute of Virology, Campus Charité) who have experience working with CHIKV. To counteract nsP3 hydrolase activity, we propose to use CHIKV mutants with reduced nsP3 catalytic activity that have slower replication rates but are still viable (McPherson et al., 2017).

To analyze how MARYlation could affect nsP1, we set out to study nsP1 functions and set up different functional assays for nsP1. CHIKV nsP1 functions are often extrapolated from our knowledge of other Alphaviruses (**chapter 1.6.3**).

NsP1 induces highly dynamic condensates in cells, likely formed through LLPS (**Fig. 46**). NsP1 is proposed as the nsP at the center of the RC and the main mediator of the spherule build-up (**chapter 1.6.3.5**). Generally, many viruses induce biomolecular condensates to mediate viral replication. Condensates allow the streamlining of processes such as genome replication or virion assembly and can help with evasion of immune detection (Sagan & Weber, 2023). Since nsP1 only has a very small IDR stretch and no modular build-up, we hypothesize that this condensate formation is highly dependent on its PPI network. Unfortunately, there is almost nothing known about the nsP1 interactome. NsP1 interactors could be characterized using a replicon system with Turbo-ID tagged nsP1. This allows for the presence of the full RC but is still an easily manageable system that was well established in this dissertation.

NsP1 anchors the RC to membranes (Salonen et al., 2003). To study nsP1 membrane association *in vitro*, we successfully set up a liposome floatation assay in cooperation with Prof. Wolfram Antonin. CHIKV nsP1 interacts with liposomes *in vitro* (**Fig. 48**). Our results are in line with previous findings (Bakhache et al., 2020). The next step would be to analyze whether MARYlation affects membrane-association. NsP1 would be MARYlated *in vitro* by PARP10 and incubated with liposomes. Binding would be analyzed in comparison to non-MARYlated nsP1.

One of the main activities of Alphaviral nsP1 is the capping of the viral RNA. However, CHIKV nsP1 mediated capping has not been detected and capping mechanisms have been studied for other Alphaviruses. Membrane binding has been suggested as important for nsP1 capping activity (**Chapter 1.6.3.2, 1.6.3.3 and 2.3.4**). To test this, we ran an nsP1 capping assay in the presence of liposomes (**Fig. 49**). However, this did not result in high nsP1

capping activity. On the contrary, it led to inhibition of nsP1 activity. This is opposed to the closely related SFV, which requires the presence of lipids for nsP1-activity, and the hypothesis that membrane association results in the allosteric activation of CHIKV nsP1 (Ahola et al., 1999; Delang et al., 2016; Jones et al., 2020). So far, we could not set up an assay to analyze capping activity of nsP1 *in vitro*. We hypothesize that the low enzymatic activity *in vitro* might be due to the absence of a cofactor, host protein, the viral RNA or the correct environment.

Next, we set up an assay to monitor nsP1 activity in a cellular environment. Indeed, immunoprecipitated nsP1 from HEK293 cells has capping activity (**Fig. 50**). We hypothesize that the immunoprecipitated nsP1 pulls down cofactors leading to enhanced *in vitro* activity. We could also confirm that the mutations H37A and D63A resulted in inactivity. H37 is the cap attachment site for nsP1 and stops GTase activity while D63A cannot bind SAM and thus blocks the MTase activity (Ahola et al., 1997; Kovacicova et al., 2021). This indicates that capping for CHIKV operates in the same manner as other Alphaviruses. Afterwards, we could also show nsP1 activity in cells regardless of a follow-up *in vitro* assay. Taken together, there was likely an intracellular factor missing when performing the *in vitro* assays using bacterially expressed and purified nsP1. Which host factors are responsible for supporting nsP1 activity is unclear. Concerning CHIKV nsP1, the next step is determining the interactome as discussed above. Lastly, we confirmed that capping activity is essential to viral replication (**Fig. 50**), suggesting nsP1 as a promising drug target.

4. Material and Methods

In this chapter the material and methods used in the dissertation are summarized. Reagents and resources are listed and the methods discussed in detail. The materials and methods are described according to standard protocols used in the Institute of Biochemistry and Molecular Biology, RWTH Aachen University, and modified regarding individual differences in experimental procedures.

4.1 Material

Reagents and Resources

The following material and reagents were used in this work. The kits were used according to the manufacturer's instructions if not indicated otherwise in the methods.

Reagent or Resource	Source	Identifier
Antibodies		
HA (WB: 1:1000; IP: 0,6µl per condition)	Covance/Biolegend	16B12
Streptavidin-HRP (WB: 1:5000)	Abcam	ab7403
USP10 (WB: 1:1000)	Cell signaling	8501
G3BP (WB: 1:1000; IP: 1µl per condition)	BD	23/G3BP/611126
GFP (WB: 1:2000)	Rockland	600-101-215
CD2AP (WB: 1:1000)	Cell Signaling	A599/5478
MAGED2 (WB: 1:5000)	Proteintech	15252-1-AP
Tubulin (WB: 1:1000)	Santa Cruz	B-5-1-2/sc-23948
PARP10 AA1-400 (WB 1:1000)	Eurogentec	
PARP10 C-terminal (WB 1:1000)	Eurogentec	1738
PAR/MAR (WB: 1:2000; dependent on LOT nr.)	Cell signaling	E6F6A
nsP3 (WB 1:2000)	Andres Merits	
PARP12 (WB 1:1000)	Sigma	SAB2104087
FLAG (WB 1:5000)	Sigma	F-1804
nsP1 (WB: 1:2000)	Andres Merits	
m ₃ /m ⁷ -cap (WB: 1:1000)	SYSY	201 011
Peroxidase-conjugated goat α-mouse IgG (1:10 000)	Jackson Immunoresearch	115-036-068
Peroxidase-conjugated goat α-rabbit IgG (1:10 000)	Jackson Immunoresearch	111-035-144
Peroxidase-conjugated goat α-rat IgG (1:10 000)	Jackson Immunoresearch	112-035-068
Peroxidase-conjugated rabbit α-goat IgG (1:5000)	Santa Cruz	sc-2768
Bacterial Strains		
<i>E. coli</i> BL21 (DE3) pLysS	Stratagene	
<i>E. coli</i> DH5α	Invitrogen	
<i>E. coli</i> Lemo21 (DE3)	NEB	
<i>E. coli</i> XL10 Gold	Stratagene	
Reagents		
³² P-NAD ⁺	Perkin-Elmer	
ADPr	Sigma	
β-NAD ⁺	Sigma	
Biotin	AppliChem	
GFP-Trap magnetic beads	ChromoTek	
Protein A/G sepharose FastFlow	GE Healthcare Life sciences	
Protein marker VI (10-245) prestained	Applichem	
Dynabeads MyOne Streptavidin T1	ThermoFisher (65601)	

LOT: 01212549			
Pierce Streptavidin Magnetic Beads LOT: WC324631 & LOT:XD351470		ThermoFisher (88817)	
FLAG beads		Sigma (F-3165)	
Hoechst 33258		SigmaAldrich	
Interferon α		Peprotech	
Lipofectamine 2000 Transfection Reagent		Invitrogen	
Opti-MEM™, Reduced Serum Medium		Gibco	
TALON metal affinity resin		BD Bioscience	
SAM		Sigma	
GTP		Sigma Aldrich	
m ⁷ GTP		Jena Bioscience	
³² P α-GTP		Hartmann Analytic	
Olaparib		Selleckchem (S1060)	
Kit			
Gateway™ BP Clonase™ Enzyme Mix		Invitrogen 11789020	
Gateway™ LR Clonase™ Enzyme Mix		Invitrogen 11791020	
mMESSAGE mMACHINE™ SP6 Transcription Kit		Invitrogen AM1340	
Q5® Site-Directed Mutagenesis Kit		NEB E0554	
Zymoclean Gel DNA Recovery Kit		Zymo Research D4001	
NucleoSpin Plasmid EasyPure Mini Kit		Macherey-Nagel 740727	
NucleoBond® Xtra Maxi Kit		Macherey-Nagel 740414	
Cell Lines			
HEK293		ATCC CRL-1573™	
HEK293T		ATCC CRL-3216™	
U2OS		ATCC HTB-96™	
U2OS tdTomato-G3BP1		Yang et al., 2020 N/A	
Insert	mEGFP-PARP10-WT	Sabcheva A. Bachelor Dissertation	N/A
	mEGFP-PARP10-GW		N/A
	mEGFP-PARP12-WT		N/A
	mEGFP-PARP12-HY		N/A
	mEGFP-PARP15iso1-WT	Verheirstraeten M. This work	N/A
	mEGFP-PARP15iso1-HY		N/A
	mEGFP-PARP15iso2-WT		N/A
	mEGFP-PARP15iso2-HY		N/A
Plasmids			
C3X-nsP1		Cloned by classical cloning	
C3X-nsP1-H37A		Cloned by site-directed mutagenesis using Q5 mutagenesis	
C3X-nxP1-D63A		Cloned by site-directed mutagenesis using Q5 mutagenesis	
GW-pDest14-CHIKV nsP3-macro		Gift from B. Coutard, Marseille	
GW-pDest17-ARTD10-818-1025		M. Bütepage	
GW-pDest17-ARTD10-818-1025-GW		B. Lippok	
GW-pDest17-CHIKV-nsP1		S. Krieg	
GW-pDest17-CHIKV-nsP2-459-798		S. Krieg	
GW-pDest17-CHIKV-nsP2-459-798-		S. Krieg	

CASA	
GW-pDest17-CHIKV nsP3	S. Krieg
GW-pDest17-G3BP1	S. Krieg
GW-pDest17-G3BP1trunc-IDR1-RBD	Cloned by site-directed mutagenesis using Phusion polymerase
GW-pDest17-G3BP1trunc-NTF2L-IDR1	Cloned by site-directed mutagenesis using Phusion polymerase
GW-pDest17-G3BP1trunc-NTF2L-IDR2	Cloned by site-directed mutagenesis using Phusion polymerase
GW-pDest17-G3BP1trunc-RBD	Cloned by site-directed mutagenesis using Phusion polymerase
GW-pDest-mCherry-C1	S. Krieg
GW-pEGFP PARP12 wt	A. Forst
GW-pEGFP-ARTD10	N. Herzog
GW-pEGFP-CHIKV nsP3	S. Krieg
GW-pEGFP-CHIKV nsP3-V33E	S. Krieg
GW-pEGFP-G3BP1	S. Krieg
GW-pEGFPm	B. Lippok
GW-pEGFP-m-ARTD7iso1	S. Krieg
GW-pEGFPm-G3BP1-RBD	Cloned by site-directed mutagenesis using Phusion polymerase
GW-pFlag-CHIKV-nsP1	S. Krieg
GW-pFlag-CHIKV-nsP2	S. Krieg
GW-pFlag-CHIKV nsP3	S. Krieg
GW-pFlag-CHIKV nsP3-macro	S. Krieg
GW-pHA-ARTD7iso1	S. Krieg
GW-pHA-ARTD7iso1-H559Y	S. Krieg
GW-pHA-PARP12	A. Forst
GW-pHA-PARP12 H564Y	A. Forst
GW-pLix403-V5-N-mEGFP	A. Sabcheva
pcDNA3.1-ATG-CHIKV nsP3- BirA(R118G)-HA	S. Krieg
pcDNA3-ARTD10-WT-BIRA(R118G)- HA	J. Wozniak
pcDNA3-MCS-BirA(R118G)-HA	K. Feijs
pcDNA5/FRT/TO-H2B-YFP	J. Lüscher-Firzlaff
pCMV-VSV-G	K. Feijs
pDest17-ARTD7cat	P. Korn
pDest17-ARTD7cat-HY	S. Krieg
pDest17-ARTD8cat	L. Ecker
pDest17-ARTD8cat-GW	L. Ecker
pEGFP-CHIKV nsP3-F480A-F498A	F. Kaesler
pEQ176P2	J. Lüscher-Firzlaff
pEVRF0-HA-PARP10	Kleine et al. 2008
pEVRF0-HA-PARP10-GW	Kleine et al. 2008
pGEX4T1-nsP3_4site-polylinker-EGFP	B. Lippok
pLIX403-EGFPm-PARP10	A. Sabcheva
pLIX403-EGFPm-PARP10-GW	A. Sabcheva
pLIX403-EGFPm-PARP12	A. Sabcheva
pLIX403-EGFPm-PARP12HY	A. Sabcheva
pLIX403-EGFPm-PARP15iso 2	A. Sabcheva

pLIX403-EGFPm-PARP15iso1	A. Sabcheva
pLIX403-EGFPm-PARP15iso1 HY	A. Sabcheva
pLIX403-EGFPm-PARP15iso2 HY	A. Sabcheva
pLix403-Tet-on-G3BP1-miniTurbo-V5	Cloned by Gateway cloning
pLix403-Tet-on-miniTurbo-V5	K. Feijs
psPAX2	J. Lüscher-Firzlaff
sp6-CHIKV-replicon-nsP1-D63A-SG-Gluc	Cloned by site-directed mutagenesis
sp6-CHIKV-replicon-nsP1-H37A-SG-Gluc	Cloned by site-directed mutagenesis
SP6-CHIKV-Replicon-SG-GLuc	Gift from B. Kümmerer, Bonn; Glasker et al. 2013
SP6-CHIKV-Replicon-V33E-SG-GLuc	S. Krieg
Mutagenesis primers	
C3X-nsP1-H37A	ACCGAATGACGCAGCTAATGCTAGAGCGTTC GTGACCTGCCTTGTTCC
C3X-nxP1-D63A	AACCATCCTGGCAATCGGCAGTG GAGTCGGGGTCAATTTCC
GW-pDest17-G3BP1trunc-IDR1-RBD	AAAAGCAGGCTTCATGACTGAGCCTCAGGAGG CCTCCTGAGGCTCAGTCATGAAGCCTGCTTTT
GW-pDest17-G3BP1trunc-NTF2L-IDR1	AGAAACTGCCCCTGAGTGAGACCCAGCTTTCT AGAAAGCTGGGTCTCACTCAGGGGCAGTTTCT
GW-pDest17-G3BP1trunc-NTF2L-IDR2	GAACCCCGAAGAATGGTGAGATGAGACCCAG C GCTGGGTCTCATCTCACCATTCTTCGGGGTTC
GW-pDest17-G3BP1trunc-RBD	AAAAGCAGGCTTCATGCACCCTGACAGTCACC GGTGACTGTCAGGGTGCATGAAGCCTGCTTTT
All primers were ordered from IDT (Integrated DNA technologies)	
Software	
PyMol	PyMol
Serial Cloner	SerialBasics
4Peaks	nucleobytes
Fiji	ImageJ
ZEN Microscopy Software	ZEISS
ZEN lite	ZEISS
Graphpad Prism Software	Graphstats
STRING version 11.5	STRING-db
InstantClue	(Nolte et al., 2018)

4.2 Experimental Procedures

4.2.1 Eukaryotic Cell Methods

4.2.1.1. Cell culture

All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, high Glucose, + GlutaMAX™, + Pyruvate (Gibco)) supplemented with 10% FCS (Fetal calve serum, heat inactivated (Gibco)) at 37°C in a humified incubator with 5% CO₂. Cells were kept in 10cm dishes in 10 ml medium. Cells were passaged every ±3 days. For this, cells were washed with 1xPBS (140 mM NaCl; 2.6 mM KCl; 2 mM Na₂HPO₄; 1.45 mM KH₂PO₄), incubated with 1 ml Trypsin/EDTA (0.05 %) (TrypLE™ Express, Gibco) for ±5 minutes, 9 ml medium was added, and 1-2 ml of the cell solution was used to seed in fresh medium. The U2OS tdTomato-G3BP1 mEGFP-PARP cell lines containing inserts were cultivated in DMEM medium with 1 µg/mL puromycin. Expression of the genes of interest were induced with the indicated concentration of Doxycycline (Dox) 16 h prior to transfection, unless otherwise indicated. Oxidative stress was induced in cells by 250 µM Sodium Arsenite (NaAsO₂) treatment for 1h. Biotin treatment was performed using 50µM Biotin at 37°C as indicated.

4.2.1.2 Cryo-conservation of eukaryotic cells

Cryo-conservation medium 90% heat-inactivated FCS, 10% DMSO
For cryo-conservation, all cell lines were grown to ±80% confluency. The cells were detached with 1ml Trypsin/EDTA, incubated for ±5 minutes, 9 ml medium was added, and the cells were centrifuged at 500 rcf and 4°C for 3 min. The medium was removed, and the cell pellet was resuspended in 1 ml of cryo-conservation medium in a cryotube (Nalgene). The cryotubes were cooled down to -80°C in a Styrofoam box for a week and subsequently transferred to -150°C.

To thaw the cells, 1ml of the cell suspension was supplemented with 9 ml pre-warmed DMEM. The cells were centrifuged at 500 rcf and 4°C for 3 min. The supernatant was removed, and the cell pellet was resuspended in 9 ml medium and transferred to a 10cm dish for cultivation.

4.2.1.3. Cell seeding

To seed cells for an experiment, the cells were trypsinized (**chapter 4.2.1.1**) and the concentration of the cell suspension was measured with the CASY cell counter (OMNI Life science). Depending on the experiment, cells were seeded in either cell culture dishes or multi-well plates. The amount of cells seeded was dependent on the cell type and cell culture dish or multi-well plate.

4.2.1.4. Calcium phosphate transfection

For transient overexpression, the cells were transfected with plasmid DNA using the calcium phosphate (CaPO₄) method. Depending on the cell culture dish, DNA was mixed with 1x HBS buffer (17 mM HEPES; 138 mM NaCl; 5 mM KCl; 0.71 mM Na₂HPO₄; pH 6.95) and vortexed. To compare between experiments, amount of DNA was always adjusted to the same end concentration using pEQ176P2. The appropriate volume of CaCl₂ (2.5 M CaCl₂) was added to the DNA-HBS mixture, the mixture was vortexed again, and incubated at room temperature (RT) for 20-30 min. The DNA mixture was added dropwise to the cell culture dish and the cells were incubated under normal cultivation conditions for 6h (HEK293) or 24h (U2OS). Afterwards, the cells were washed with pre-warmed HEPES buffer (1x HEPES buffer: 10 mM HEPES; 142 mM NaCl; 6.7 mM KCl; pH 7.3) and returned to standard cultivation conditions.

Dish format	DNA amount	1xHBS	2,5M CaCl ₂
12 well	2 µg	95 µl	5 µl
6 well	4 µg	190 µl	10 µl
10 cm	20 µg	950 µl	950 µl

4.2.1.5. Preparation of protein lysates

1x PBS	140 mM NaCl; 2.6 mM KCl; 2 mM Na ₂ HPO ₄ ; 1.45 mM KH ₂ PO ₄
RIPA lysis buffer	10 mM Tris pH 7,4; 150 mM NaCl; 1% NP 40; 1% Desoxycholate; 0.1% SDS; bidest ddH ₂ O; PIC 100x
TAP lysis buffer	50 mM Tris base, pH 7.5; 150 mM NaCl; 1 mM EDTA; 10% glycerol; 1% NP-40; 2 mM TCEP

The following procedure takes place on ice and centrifugation was consistently at 4°C. Depending on the experiment, 1 or 2 days after transfection HEK293 cells were flushed of the plate using the current medium and pelleted by centrifugation. Next, the cell pellets were washed with ice-cold 1x PBS. Cells were lysed in 1ml lysis buffer (for a 10cm plate), either RIPA or TAP, and lysates were transferred to a 1,5 ml Eppendorf tube. The suspension was incubated for 5-10 minutes. Dependent on the experiment, samples were sonicated in a water bath sonicator at 4°C, 1 sec/1 sec interval and 70% amplitude for 5 min or were sonicated on ice, 1 sec/1 sec and 10-20% amplitude using the Branson digital sonifier 250D. Subsequently, the lysates were rested on ice for 10 min. The cell lysates were centrifuged at full speed, 4°C for 30 minutes. The SN was taken off and used for analysis and follow-up experiments.

4.2.1.6. (Co-)immunoprecipitation

RIPA lysis buffer	10 mM Tris pH 7,4; 150 mM NaCl; 1% NP 40; 1% Desoxycholate; 0.1% SDS; bidest ddH ₂ O; PIC 100x
TAP lysis buffer	50 mM Tris base, pH 7.5; 150 mM NaCl; 1 mM EDTA; 10% glycerol; 1% NP-40; 2 mM TCEP
2x SB	160 mM Tris-HCl, pH 6.8; 20% (v/v) glycerol; 10% (w/v) SDS; 0.25% bromophenol blue; 100 mM β-mercaptoethanol
Protein-A/G Sepharose beads (Amersham Pharmacia)	
GFP-Trap magnetic agarose beads (Chromotek, gtma)	
FLAG beads (Sigma F-3165)	

Dependent on the experiment, after the preparation of the whole cell lysate, follows (co-)immunoprecipitation. 60 µl of the whole cell lysate is kept for analysis with SDS-PAGE and western blotting. The rest of the whole cell lysates is used for (co-)IP. GFP-tagged proteins were immunoprecipitated using 5 µl of GFP-Trap. FLAG-tagged proteins were immunoprecipitated using 5 µl of FLAG beads. Other proteins were immunoprecipitated using the fitting antibody in combination with 15 µl Protein-A/G Sepharose beads. Beads were washed twice using 1 ml of the appropriate lysis buffer at 4°C, 500 rcf for 3 min. The cell lysates were added to the beads and the samples were incubated on an overhead shaker at 4°C for 1 h. Supernatant was discarded, and the samples were washed three times with 1 ml of the respective lysis buffer. Beads were afterwards resuspended in 20 µl 2x SB and incubated at 95°C for 5 min. Samples were analyzed by SDS-PAGE and Western blot analysis.

4.2.1.7. BioID

1x PBS	140 mM NaCl; 2.6 mM KCl; 2 mM Na ₂ HPO ₄ ; 1.45 mM KH ₂ PO ₄
Lysis buffer	10 mM Tris pH 7,4; 150 mM NaCl; 1% NP 40; 1% Desoxycholate; 0.1% SDS; bidest ddH ₂ O; PIC 100x
Wash buffer 1	10 mM Tris pH 7,4; 150 mM NaCl; 1% NP 40; 1% Desoxycholate; 0.1% SDS; bidest ddH ₂ O
Wash buffer 2	2% SDS in 50 mM Tris-HCl pH 7.4
Elution buffer	30 mM biotin; 100 mM NaCl; 2% SDS; 6 M Urea; 2 M Thiourea in PBS

Dynabeads MyOne Streptavidin T1

Pierce Streptavidin Magnetic Beads

The proteins of interest are tagged with BirA*R118G. Per condition, six 10 cm dishes of HEK293 cells were seeded and transiently transfected using the calcium phosphate method (**chapter 4.2.1.4.**). After 6h the cells were washed according to protocol (**chapter 4.2.1.4.**) and the cells were treated with 50 μ M biotin overnight (16h). The next day, the cells were collected and pooled per condition and were washed three times with 10 ml cold 1 x PBS by centrifugation at 4°C and 500 xg for 3 min. The next steps were performed at RT. The cells were lysed with lysis buffer, 600 μ l per 10 cm plate and thus 3,6 ml per condition. Next, the cells were sonicated for 10 sec, 12% amplitude (Branson digital sonifier 250D) and full pulse on ice. The cells were rested for 5 min on ice and the sonification step was repeated. The cell lysates were centrifuged at 4°C and 16,000 rcf for 30 min. The following steps were performed at 4°C or on ice. In the meantime, per condition either, depending on the experiment, the Dynabeads MyOne Streptavidin T1 or the Pierce Streptavidin Magnetic Beads were washed with lysis buffer at 4°C and 500 rcf for 3 min. Per condition, 60 μ l of the cell lysates was set aside for analysis of the whole cell lysate. The remainder of the lysates were transferred to the Streptavidin beads and incubated on an overhead shaker at 4°C overnight. All subsequent steps were performed at RT. All collecting steps were performed with a magnetic rack allowing binding for 2 min and all washing steps were performed on an overhead shaker for 8 min. The beads were washed once with 500 μ l wash buffer 1. Following, the beads were transferred to a new tube. The beads were washed once with wash buffer 2. Next, the beads were washed twice with wash buffer 1 and three times with PBS. To elute the bound proteins from the beads, 50 μ l of elution buffer was added and incubated at RT for 15 min. 17 μ l of 4x SB was added and the samples were incubated at 95°C for an additional 15 min. The samples were centrifuged at full speed for 5 min and 6 μ l of the elution was analyzed by SDS-PAGE and Western blotting. The rest of the samples were analyzed by MS analysis performed by Stefanie Gostek and Christian Preisinger (IZKF Core facility, Mass spectrometry).

4.2.1.8. Live cell imaging

1x PBS	140 mM NaCl; 2.6 mM KCl; 2 mM Na ₂ HPO ₄ ; 1.45 mM KH ₂ PO ₄
IBIDI μ -Slide 8 Well	IBIDI 80826

U2OS cells were seeded in 10 cm plates ($1,2 \times 10^6$). 48h after seeding, cells were transfected using the Calcium phosphate method. Transfected U2OS cells were washed 24h after transfection with 1x PBS (37°C). Subsequently, cells were trypsinized and seeded into IBIDI-

chambers at 7500 cells per well. After 24h or 48h, cells were analyzed using confocal microscopy.

U2OS tdTomato-G3BP1 cells were seeded directly into IBIDI-chambers and analyzed after 24h using confocal microscopy. According to the experiment, certain conditions were treated for 1h at 37°C with 250 µM Sodium Arsenite to induce oxidative stress.

4.2.1.9. Fixation of adherent cells

1x PBS ⁺⁺	1mM MgCl ₂ ; 0,1 mM CaCl ₂
1x PBST ⁺⁺	0,1 % Triton-X-100 in 1x PBS ⁺⁺
3,7% PFA	3.7% (w/v) paraformaldehyde (PFA) in PBS
Quenching solution	100 mM NH ₄ Cl in 1x PBST ⁺⁺

The U2OS were seeded on coverslips in 12 well plates at 60.000 cells per well. If indicated, cells were transfected using the calcium phosphate method. Cells were cultivated for 48h. Depending on experiment and condition, cells were induced using doxycycline overnight the day before fixation or treated with Arsenite 1h before fixation as indicated.

For fixation, cells were washed twice with 1 ml PBS⁺⁺. Following, cells were fixated with 400 µl 3,7 % PFA for 20 Minuten at RT in the dark. The PBS⁺⁺ washing step is repeated twice. Next, cells were treated with the quenching solution for 5 minutes at RT in the dark. Cells were washed three times with PBST⁺⁺ and three times with ddH₂O. Nuclei were stained for 5 min at RT with 10 µg/ml Hoechst33258 (diluted 1:1000 in ddH₂O). Cells were washed three to five times with ddH₂O. Cells were mounted using Mowiol 4-88 on an object slide. The slides were stored at 4°C in the dark until microscopy.

4.2.1.10. Confocal laser-scanning fluorescence microscopy

Images were captured with a Zeiss LSM710 confocal laser scanning microscope at the IZKF core facility using a LD C-Apochromat 40x/1.1 W Korr M27 objective. The pixel size was 1024 x 1024 px and the pinhole was set to 1 airy unit (AU). Settings were kept equal for all images belonging to a single experiment. The used laser wavelength for excitation and detection for each fluorophore are summarized below. Image adjustments for brightness and contrast were performed in a linear range and the same settings were applied for all images of the same experiment. ZEN lite blue edition software (Zeiss) was used for image analysis and export.

Fluorophore	Laser	Excitation wavelength (nm)	Emission wavelength (nm)	Detection wavelength (nm)
Hoechst33258	405 nm UV diode, 1-2% output	352	453	410 – 496
EGFP	Argon laser, 25 mW, 1-2% output	488	528	494 – 562
tdTomato	561 DPSS laser, 20 mW, 1-2% output	561	629	566 – 691
mCherry	561 DPSS laser, 20 mW, 1-2% output	561	610	562–630

4.2.1.11. Lentiviral transduction of U2OS tdTomato-G3BP1 cells

All following steps were performed under S2 safety conditions. 3×10^6 HEK293T cells were seeded the day before transient transfection in 10 cm plates. 5 µg of the lentiviral transfer plasmid encoding the gene of interest (pLIX403-mARTD-EGFP), 5 µg of the packaging vector (psPAX2), 1 µg of the envelope vector (pCMV-VSV-G) and 9 µg pEQ176P2 were transfected using the Calcium phosphate method. After 48h, the supernatant containing the viral particles was harvested, filtered through a 0.45 µm Pore filter and stored at 4°C. The cells were provided with fresh cell culture medium and after 24h, a second harvesting round of the viral particles was performed.

The U2OS tdtomato-G3BP1 were seeded 24h before lentiviral transduction at 5×10^5 in 10 cm plates per condition. As control for puromycin selection, one condition extra is prepared. Optimal, is a 40% confluency of target cells for lentiviral transduction. The viral supernatant is mixed at a 1:1 ratio with fresh cell culture medium containing polybrene (8 µg/ml) and pipetted onto the target cells. After 6h, the medium is replaced by fresh DMEM medium. Cells were incubated overnight and the next day, lentiviral transduction was repeated. After 24h, 1 µg/µl puromycin is added to the medium. Puromycin treatment is maintained until the puromycin selection control has no more viable cells. The cells were passaged at least four times under S2 safety conditions to ensure absence of infectious viral particles before transferring the resulting cell lines to S1 safety conditions. Expression of the genes of interest was analyzed using western blotting and confocal microscopy.

4.2.2. Prokaryotic Cell Methods

4.2.2.1. Bacterial cultivation

LB low salt medium	1% (w/v) tryptone; 0.5% (w/v) yeast extract; 0.5% (w/v) NaCl 50 µg/ml zeocin
Agar plates (low salt)	LB low salt medium; 1.5% (w/v) bacto agar; 50 µg/ml zeocin
LB medium	1% (w/v) tryptone; 0.5% (w/v) yeast extract; 1% (w/v) NaCl Dependent on the constructs: 100 µg/ml ampicillin or 30 µg/ml kanamycin
Agar plates	LB medium; 1.5% (w/v) bacto agar Dependent on the constructs: 100 µg/ml ampicillin or 30 µg/ml kanamycin

All the bacterial cultures were cultivated with the respective antibiotics, dependent on the antibioticum resistance of the transformed plasmid. For both plasmid preparations and purification of recombinant proteins, agar plates containing plated bacteria were cultivated ON at 37°C. For small scale plasmid preparations, 5 ml bacterial suspension cultures were cultivated ON at 37°C at 130-160 rpm. For large scale plasmid preparations, 300 ml bacterial suspension cultures were cultivated ON at 37°C at 130-160 rpm. For purification of recombinant proteins, 30 ml bacterial suspension pre-cultures were cultivated ON at 37°C at 130-160 rpm. The pre-cultures were used to inoculate 400ml main cultures that were kept incubated at 37°C until the required OD for induction of protein expression was reached.

4.2.2.2. Heat shock transformation

Bacteria were transformed with recombinant DNA using heat shock transformation protocols. The standard procedure is discussed here. Dependent on the bacterial strain, small adjustments were made based on protocols at NEB or other providers. 100µl of

chemically competent bacteria were thawed on ice before addition of 1-5 µl of recombinant DNA. This recombinant DNA – bacterial cell mixture was incubated for 20-30 minutes on ice. Next, the bacteria were heat shocked for 45 sec at 42°C. Immediately after the mixture was chilled on ice for 2 min. 900µl pre-warmed LB medium (37°C) were added and the bacterial suspension was incubated for 1h at 37°C and ±130 rpm. Afterwards, the bacteria were pelleted at 500 rcf for 3 min and 800-900µl SN was discarded. The pellet was resuspended in the residual medium and then plated on agar plates with the appropriate antibiotic. The agar plates were incubated ON at 37°C.

4.2.2.3. Purification of recombinant hexahistidine (His-)tagged fusion proteins

Culture medium 2xYT	1.6% (w/v) tryptone; 1% (w/v) yeast extract; 0.5% (w/v) NaCl 20% (w/v) Glucose solution
1x PBS	140 mM NaCl; 2.6 mM KCl; 2 mM Na ₂ HPO ₄ ; 1.45 mM KH ₂ PO ₄
IMAC lysis buffer	100 mM HEPES pH 8.0; 500 mM NaCl; 10% Glycerol; 10 mM Imidazole; 0.5-2 mM TCEP; Add freshly: 1x PIC; 100 µg/ml lysozyme
IMAC wash buffer	20 mM HEPES, pH 7.5; 500 mM NaCl; 10% Glycerol; 10 mM imidazole; 0.5-2 mM TCEP
IMAC elution buffer	20 mM HEPES, pH 7.5; 500 mM NaCl; 10% Glycerol; 500 mM imidazole; 0.5-2 mM TCEP

TALON metal affinity resin (BD Bioscience)

1 M isopropyl-β-D-thiogalactopyranoside (IPTG)

Recombinant His-tagged proteins were expressed in bacteria and purified from the lysates using affinity chromatography. For His-tagged proteins made possible through the interaction between the His-tag and the TALON metal affinity resin (BD Bioscience). *E. coli* BL21 or Lemo21 were transformed with the plasmid for bacterial expression with N-terminally His-tagged proteins using heat shock. The next day, 30 ml 2xYT medium were inoculated with one colony from the agar plate and incubated ON at 37°C. The next morning, the starter culture was added to 400 ml 2xYT with the appropriate antibiotic and grown at 37°C and 130 rpm until the desired OD₆₀₀ was reached (0,5-1,2). Protein expression was induced with 0,1mM IPTG and the culture was incubated for 4h at 25°C. The bacteria were pelleted at 5000 rpm for 10 min at 4°C and the pellets were resuspended in 15 ml ice-cold IMAC lysis buffer and stored at -80°C until purification. The next day, the bacterial suspension was sonicated for 5 min with a 30b sec/30 sec interval and a 15-25% amplitude on ice using a Branson digital sonifier 250D. The lysate was incubated on ice for 5 min and centrifuged at 16.000 rcf and 4°C for 30 min. In meantime, 250 µl TALON metal affinity resin (BD Bioscience) were washed with 10 ml IMAC lysis buffer by centrifugation at 500 rcf and 4°C for 3 min. The SN of the lysate was incubated with the beads for 1h at 4°C on the overhead shaker. Afterwards the beads were pelleted using centrifugation at 500 rcf and 4°C for 3 min. The beads were washed once using 10ml ice-cold 1xPBS and next with 10 ml IMAC wash buffer. The recombinant proteins were eluted by incubation with 300 µl IMAC elution buffer. This elution step was repeated 3x, resulting in 3 elution fractions. 5 µl of the fractions were used for analysis by SDS-PAGE and subsequent CB staining and the rest was stored at -80°C for *in vitro* assays.

4.2.3. Protein analysis methods

4.2.3.1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

1x Running Buffer	25 mM Tris base, pH 8.3; 250 mM glycine; 0.1% (w/v) SDS
4x SB	320 mM Tris-HCl, pH 6.8; 40% (v/v) glycerol; 20% (w/v) SDS; 0.5% bromophenol blue; 200 mM β -mercaptoethanol

Protein lysates, samples obtained by (co-)immunoprecipitation or *in vitro* samples were diluted in 2x or 4xSB, incubated for 5 min at 95°C and centrifuged for 5 min at full speed. The SDS-PAGE gels consisted of 5% polyacrylamide (PAA) stacking gel and a 10%-15%, depending on the molecular weight of the POIs, PAA concentration separation gel. In case the POIs had highly variable molecular weights, a gradient gel with a PAA concentration ranging from 10%-15% was used. The samples were loaded on the gel and a protein marker (Protein marker VI (10-245) prestained; AppliChem) was used for molecular weight estimation. SDS-PAGE was run using either the Mini-PROTEAN Tetra Cell system (Bio-Rad) at 100-120 V per gel or for ADP-ribosylation and hydrolase assays the Multigel-Long system (Biometra) at 25-30 mA. The gel electrophoresis was stopped when the running front reached the gel bottom. The SDS-PAGE was analyzed following by either CB staining or Western blot.

4.2.3.2. Semidry Western Blot

1x Transfer buffer	25 mM Tris base; 192 mM glycine; 20% (v/v) methanol
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Ponceau S staining solution	0.05% (w/v) Ponceau S; 1% (v/v) acetic acid
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Amersham™ Protran® Western blotting membranes, nitrocellulose, 0.2 μ m pore size (Cytiva)

Whatman® Qualitative Filter Paper, Grade 3

Upon analysis of protein lysates or samples obtained by (co-)immunoprecipitation using Semidry Western blot, the samples were separated using SDS-PAGE gels and following blotted using a Semidry blotting apparatus (PEGASUS model, PHASE GmbH). Before blotting, 6 Whatman papers, the nitrocellulose membrane and the gel were soaked in transfer buffer. Following, in orientation cathode to anode, 3 pieces of Whatman paper, the SDS-PAGE gel, the membrane and another 3 pieces of Whatman paper were stacked. The transfer was performed at 1,5-2 mA/cm² gel for 1 h and 15 min. Transfer efficiency was evaluated with Ponceau S staining solution at RT under gentle agitation. Afterwards, the membrane was washed with TBS-T for a few minutes. The membrane was blocked with TBS-T+ 5% milk at RT for 1h under gentle agitation. For BioID, the membrane was blocked in TBS-T+ 5% BSA. Afterwards, the membrane was washed three times with TBS-T at RT. Next, the membrane was incubated with the primary antibody ON at 4°C or for 1h at RT under gentle agitation. The washing steps were repeated. The membrane was incubated with the secondary antibody for 1h at RT under gentle agitation. The washing steps were repeated. Signal was detected using the Azure c600 (Azure Biosystems).

4.2.3.3. Coomassie Blue (CB) staining

Coomassie brilliant blue G250 staining solution	0.006% (w/v) Coomassie brilliant blue G-250; 10% (v/v) acetic acid
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In case of protein analysis by CB staining, the SDS-PAGE is incubated at RT for 1h under constant gentle agitation. Afterwards to destain the background, the SDS-PAGE is left ON at RT under constant gentle agitation in ddH₂O. In case of long-term storage or subsequent analysis of ADP-ribosylation/hydrolase assays, gels were vacuum-dried on Whatman filter paper.

4.2.4. Molecular cloning and nucleic acid-related methods

4.2.4.1. Plasmid preparation from bacterial cultures

Small scale plasmid preparations were performed according to the manufacturer's instructions using the NucleoSpin Plasmid EasyPure Mini Kit (Macherey-Nagel) and the plasmid DNA was eluted in 50 µl ddH₂O or in 50 µl elution buffer. Large scale plasmid preparations were performed using the NucleoBond® Xtra Maxi Kit (Macherey-Nagel) according to manufacturer's instructions. The resulting DNA concentrations were measured using a NanoDrop™ 1000 (Thermo Fisher Scientific). Plasmid preparations were verified by analytical restriction digest and if necessary, by Sanger sequencing (Microsynth SeqLab).

4.2.4.2. Restriction digestion of DNA

Restriction enzymes and the accompanying buffer (NEB) were used according to the manufacturer's instructions (NEBcloner). Generally, for analytical digests 0.2 µl of enzyme(s), 1 µl of the supplied 10x buffer was used in an overall reaction volume of 10 µl. The reaction was incubated for 1h at 37°C. For preparative restrictions for cloning purposes, 2-5 µg of plasmid was digested in a volume of 50 µl. DNA digests were analyzed using agarose gel electrophoresis.

4.2.4.3. Agarose gel electrophoresis

10x Agarose Loading Buffer (ALP)	50 mM Tris base (pH 8.0); 50 mM EDTA; 50% (v/v); glycerol; 0.25% (w/v) Bromophenol blue; 0.25% (w/v); xylencyanole
TBE buffer	89 mM Tris base (pH 8.0); 89 mM boric acid; 2 mM EDTA
Agarose gel	0.7-2% (w/v) Low EEO (Sigma) in TBE buffer; 0.5 µg/ml ethidium bromide (AppliChem)

DNA restrictions, *in vitro* transcribed RNA and PCR reactions were analyzed using agarose gel electrophoresis. The relevant samples were mixed with 10x ALP, loaded onto 1-2% agarose gels submerged in a chamber with TBE buffer and run at 60-150 V, dependent on the size of the agarose gel for 30-60 min. The samples were compared to a DNA size marker (GeneRuler™ 1 kb Plus DNA Ladder MBI Fermentas). Nucleic acid fragments were visualized on a UV transilluminator (E-BOX VX2, Peqlab; wavelength $\lambda=302$ nm).

4.2.4.4. DNA recovery and purification from an agarose gel

Desired DNA fragments for molecular cloning, from either restriction digests or PCRs, were analyzed using agarose gel electrophoresis and purified from the agarose gel using the ZymoClean™ Gel DNA Recovery Kit (Zymo Research) according to the manufacturer's instructions. The recovered and purified DNA was eluted in 10 µl TE buffer.

4.2.4.5. Sequencing of recombinant DNA

Sequencing of recombinant DNA is performed by Microsynth SeqLab using Sanger sequencing. A volume of 12 µl at a conc. of 0,1 µg/µl plasmid DNA was analysed using the

suitable primers depending on the construct. Results were analyzed using SerialCloner and 4Peaks.

4.2.4.6. Polymerase Chain Reaction (PCR)

DNA inserts for recombinant cloning were amplified using PCR. The desired gene product was amplified using appropriate forward (fw) and reverse (rev) primers depending on cloning method. PCRs were performed using the Phusion® High-Fidelity DNA Polymerase (NEB) according to the manufacturer's instructions. The resulting PCR products were analyzed via agarose gel electrophoresis. Correct gene inserts were recovered from the agarose gel and used for following recombinant cloning.

4.2.4.7. Gateway cloning

For BP reactions, the attB-PCR product was incubated with 150 ng pDONR vector and BP Clonase enzyme (Invitrogen) in BP Clonase Reaction Buffer according to manufacturer's instructions. Reactions were incubated ON at RT. The next day, 1 µl of the Proteinase K solution is added to each reaction and incubated for 10 minutes at 37 °C. 2-5 µl of the BP reaction was transformed into competent bacteria, E. coli DH5α, using heat shock transformation. Next, plasmid preparation of the bacteria took place, and the resulting constructs were analyzed by restriction digest and sequencing.

For the LR reactions, the pDONR Entry vector was incubated with 300 ng of the destination vector of choice and LR Clonase enzyme (Invitrogen) in LR Clonase Reaction Buffer according to manufacturer's instructions. Reactions were incubated ON at RT or for 1h at 37°C. The next day, 1 µl of the Proteinase K solution is added to each reaction and incubated for 10 minutes at 37 °C. 2-5 µl of the LR reaction was transformed into competent bacteria, E. coli DH5α, using heat shock transformation. Next, plasmid preparation of the bacteria took place, and the resulting constructs were analyzed by restriction digest.

4.2.4.8. Site-directed mutagenesis

Plasmids were mutated through site-directed mutagenesis PCR using Phusion polymerase (NEB). Primers were designed using the Quickchange primer design tool from Agilent. PCR was run according to the settings below. Afterwards the template was digested using DpnI (NEB) for 1h at 37°C. Lastly, 2-5 µl of the DNA was transformed into competent bacteria using heat shock transformation. Mutations were checked via restriction digestion and sequencing.

PCR mix	V (µl)	PCR conditions	Time
dsDNA template (50 ng)	2	95°C	1'
Primermix (5uM)	1,25	95°C	30"
dNTP mix	0,5	55°C	1'
5x HF buffer	5	68°C	5'
DMSO	0,6	68°C	7'
ddH2O (to 50 ul)	15,4	4°C	Forever
Phusion polymerase (NEB)	0,25		

Another method used to introduce point mutations was the Q5® Site-Directed Mutagenesis. Forward and reverse primers were designed with the NEBaseChanger tool (NEB). The Q5® Site-Directed Mutagenesis Kit (NEB) was used according to the manufacturer's instructions.

5 µl of the resulting KLD reaction was transformed into competent bacteria using heat shock transformation. Mutations were checked via restriction digestion and sequencing.

4.2.4.9. *In vitro* transcription and transfection of m⁷G-capped replicon RNA

The following procedure was used according to the manufacturer's instructions (mMESSAGE mMACHINE™ SP6 Transcription Kit, Invitrogen). 2 µg plasmid encoding the CHIKV-replicon was linearized using NdeI in a total volume of 30 µl. The DNA was precipitated by addition of 71 µl ddH₂O, 5 µl 0.5 M EDTA, 10 µl 3 M sodium acetate (NaOAc) and 250 µl ice cold ethanol (EtOH) and incubated at -20°C for 30 min. DNA was pelleted by centrifugation at 16000 rcf, 4°C for 15 min. The DNA pellet was dried and resuspended in 4.2 µl RNase free water. Linearization and precipitation of the DNA was analyzed using agarose gel electrophoresis. *In vitro* transcription of the DNA was performed using mMESSAGE mMACHINE™ SP6 Transcriptions Kit (Invitrogen) according to the manufacturer's instructions. The *in vitro* transcription reaction was incubated for 3h at 37°C. The template DNA was digested by addition of TURBO DNase at 37°C for 15 min. The transcribed RNA was precipitated using the lithium chloride (LiCl) precipitation protocol included in the kit. The RNA was dissolved in 20 µl elution buffer High Pure RNA Isolation Kit (Roche). The *in vitro* transcribed RNA was controlled using agarose gel electrophoresis. The concentration and purity of the RNA was analyzed using the NanoDrop™ 1000 (Thermo Fisher Scientific). RNA was stored at -80°C until transfection.

Cells were seeded in 12 well plates 48h before following procedure. For the transfection of the *in vitro* transcribed RNA Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific) was used according to the manufacturer's instructions. Per well, 3 µg RNA were mixed with 100 µl Opti-MEM medium and 5 µl Lipofectamine 2000. RNA mixtures were vortexed and incubated at RT for 5-10 min. The transfection mixture was added dropwise to the cells. 30h after transfection, cells were harvested and analyzed using SDS-PAGE and western blotting.

4.2.5. *In vitro* assays

4.2.5.1. *In vitro* ADP-ribosylation and hydrolase assays

10xADPr buffer	500 mM Tris, pH 8.0; 20 mM TCEP; 40 mM MgCl ₂
1xADPr buffer	50 mM Tris, pH 8.0; 2 mM TCEP; 4 mM MgCl ₂
4x SB	320 mM Tris-HCl, pH 6.8; 40% (v/v) glycerol; 20% (w/v) SDS; 0.5% bromophenol blue; 200 mM β-Mercaptoethanol
TAP lysis buffer	50 mM Tris base, pH 7.5; 150 mM NaCl; 1 mM EDTA; 10% Glycerol; 1% NP-40; 2 mM TCEP
10 mM β-NAD ⁺ (Sigma)	
³² P-NAD ⁺ (Perkin-Elmer)	

Recombinant, bacterially, expressed and purified fusion-proteins, substrates and enzymes, (His-tagged) were used for *in vitro* ADP-ribosylation assays. The appropriate enzymes and substrates were incubated in the presence of 50 µM β-NAD⁺ and 1 µCi of ³²P-NAD⁺ or 500 µM β-NAD⁺ at 30°C and 1000 rpm for 30 min in a total volume of 30 µl. The reactions were stopped by addition of 4x SB and incubation at 95°C for 5 min. The samples were analyzed using SDS-PAGE and CB staining and autoradiography or western blotting.

In the case of *in vitro* ADP-ribosylation assay with immunoprecipitated mARTDs, HA-tagged mARTDs were expressed in HEK293 cells. After 48 h the cells were lysed in TAP lysis buffer and the HA-tagged mARTDs immunoprecipitated in bulk. After immunoprecipitation, the

samples were washed twice with TAP lysis buffer and once with 1x ADPr buffer. The samples were split according to the experimental conditions. The buffer was removed, and the IPs were either stored at -80°C or immediately used for an *in vitro* ADP-ribosylation assay.

4.2.5.2. Electrophoretic Mobility Shift Assay

EMSA buffer	20 mM Hepes pH 7.5; 150 mM NaCl; 10 mM MgCl ₂ ; 1 mM TCEP; 10% Glycerol
Running buffer or 1x TBE	0.13 M tris (pH 7.6); 45 mM boric acid; 2.5 mM EDTA
7% PA gel	40% PA (19:1); TEMED; 20% APS; 1x TBE running buffer

RNA binding of purified G3BP1 WT and G3BP1 NTF2L-IDR2 (G3BP1 Δ RBD) was assessed using EMSAs. Increasing concentrations of G3BP1 were incubated with 10 nM Cy3-labeled RNA (5'P-ssRNA-Cy3; [Phos]-GUU UCG GAU CGA CGC-[Cy3]) for 30 min at RT in EMSA buffer. Complexes were separated using 7% PA gel. Gel was prerun at 100V for 20-30 min. RNA was visualized by fluorescence using the Azure c600 (Azure Biosystems).

4.2.5.3. *In vitro* nsP2 protease assay

ADPr buffer	50 mM Tris, pH 8.0; 2 mM TCEP, 4 mM MgCl ₂
4x SB	320 mM Tris-HCl, pH 6.8; 40% (v/v) glycerol; 20% (w/v) SDS; 0.5% bromophenol blue; 200 mM β -mercaptoethanol

Bacterially purified His-nsP2-459-798 (protease domain) WT or inactive CASA mutant, were incubated with either bacterially purified synthetic substrate or G3BP1 at 30°C for 30 min in a total volume of 15 μ l reaction buffer (50 mM Tris, pH 8.0, 2 mM TCEP, 4 mM MgCl₂). As negative controls either the protease, the substrate or G3BP1 were incubated alone. The reactions were stopped by addition of 4x SB and incubation at 95°C for 5 min. The assay was analyzed by SDS-PAGE and CB staining.

4.2.5.4. Liposome floatation assay

Liposome floatation assays were performed by M. Tatarek-Nossol from the W. Antonin group. Liposome generation has been described before (Kralt et al., 2022; Vollmer et al., 2012, 2015). For liposome flotations, 6 μ M protein of interest were mixed 1:1 with the indicated liposomes mixture (6 mg/mL) and incubated for 30 min at 25°C. 75 μ l of the mixture were set to a 36% sucrose concentration in a total volume of 150 μ l. This was overlaid with 1.7 ml 12% sucrose in PBS buffer, followed by 300 μ l 1x PBS. Samples were centrifuged for 2h at 55000 rpm in a TLS-55 rotor (Beckman) at 25°C through the sucrose gradient. The liposome-containing top layer was collected (450 μ l). Protein fraction was precipitated through the method described by Wessel & Flügge (Wessel & Flügge, 1984). Binding efficiency of the proteins was determined by Western Blot analysis and Ponceau staining.

Liposome compositions. PE: Phosphatidylethanolamine; PC: Phosphatidylcholine; PI: Phosphatidylinositol; SM: Sphingomyelin; PS: phosphatidylserine; PG: phosphatidylglycerol; CA: cholic acid; Cholesterol.

E.coli liposomes		Nuclear Envelope liposomes		Plasma Membrane liposomes	
PE	67%	PC	60%	PC	30%
PG	23.2%	PE	20%	PE	15%
CA	9.8%	PI	10%	PI	5%
		Chol	5%	Chol	25%
		SM	2.5%	SM	15%
		PS	2.5%	PS	10%

4.2.5.5. *In vitro* nsP1 capping assay in presence of liposomes

10X nsP1 reaction buffer 500 mM Tris-HCl, pH 7,8; 50 mM MgCl₂; 100 mM KCl; 5 mM TCEP;

4x SB 320 mM Tris-HCl, pH 6.8; 40% (v/v) glycerol; 20% (w/v) SDS; 0.5% bromophenol blue; 200 mM β-mercaptoethanol

The plasma membrane liposome stock (6,6 µg/µl) was prepared 24h in advance by M. Tatarek-Nossol. 1µg nsP1 were incubated with 50 µg liposome, 100 µM SAM and 3 µCi ³²P α-GTP in a total reaction volume of 30 µl 1x nsP1 reaction buffer. The mixture was incubated for 30 min, unless otherwise indicated, at 30°C at 1000 rpm. The reaction was stopped by addition of 10 µl 4x SB. Capping activity was analyzed by SDS-PAGE and autoradiography.

4.2.5.6. *In vitro* nsP1 capping assay using immunoprecipitated nsP1

10X nsP1 reaction buffer 500 mM Tris-HCl, pH 7,8; 50 mM MgCl₂; 100 mM KCl; 5 mM TCEP;

4x SB 320 mM Tris-HCl, pH 6.8; 40% (v/v) glycerol; 20% (w/v) SDS; 0.5% bromophenol blue; 200 mM β-mercaptoethanol

FLAG-tagged nsP1, and mutants were transfected and expressed in HEK293 cells by the calcium phosphate method. After 48h, nsP1 was immunoprecipitated using FLAG-beads. The enriched nsP1 was incubated with either 100 µM SAM and 100 µM GTP or 100 µM m⁷GTP in a total reaction volume of 30 µl 1x nsP1 reaction buffer. Reactions were incubated for 1h at 30°C. The reactions were stopped by addition of 10 µl 4x SB. Capping activity was analyzed by SDS-PAGE and autoradiography.

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6. Appendix

6.1. Abbreviations

AA	amino acid Amino acids were generally abbreviated using the according three letter or one letter code.
(+)ssRNA	positive-single stranded RNA viruses
(+)ssRNA	positive sense, single stranded RNA
3-AB	3-amino benzamide
3D	three dimensional
5'-UTR	5' untranslated region
AbMV	begomo ablution mosaic virus
ADP	Adenosine diphosphate
ADPr	ADP-ribose
Ago2	Argonaute 2
AR	androgen receptor
ARH	ADP-ribosylhydrolase
ART	ADP-ribosyltransferase
ARTC	ADP-ribosyltransferase Cholera toxin-like
ARTD	ADP-ribosyltransferase Diphtheria toxin-like
ATP	Adenosine triphosphate
AUD	Alphavirus unique domain
BER	base excision repair
BIN1	amphiphysin 2
bioAMP	Biotin-labelled adenosine monophosphate
BioID	Proximity-dependent biotin identification
BMDM	bone-marrow-derived macrophage
Calu-3 cells	Cultured Human Airway Epithelial Cells
CAPRIN1	Cell Cycle Associated Protein 1
CB	Coomassie Blue
CD2AP	CD2-associated protein
cgRNA	capped viral genomic RNA
CHIKF	Chikungunya fever
CHIKV	Chikungunya virus
CHX	cycloheximide
CNS	central nervous system
Co-IP	co-immunoprecipitation
CoV	Corona virus
C-protein	capsid protein
CRM1	chromosomal region maintenance 1
DENV	dengue virus
DNA	Deoxyribonucleic acid
Dox	Doxycycline
DRM	cholesterol-rich plasma membrane microdomains
DSB	double strand breaks
DTX3L	Deltex E3 Ubiquitin Ligase 3L
ECMV	encephalomyocarditis virus

EEEV	eastern equine encephalitis virus
EGFP	enhanced green fluorescent protein
eIF2 α	eukaryotic translation initiation factor 2 α
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assays
EnPP1	ectonucleotide pyrophosphatase/phosphodiesterase 1
ER	endoplasmic reticulum
EWS	RNA-binding protein Ewing Sarcoma protein
FCS	Fetal calve serum
FCV	Feline Calicivirus
Fig.	Figure
FL	full length
FMDV	foot and mouth disease virus
G3BP	Ras GTPase-activating protein-binding protein
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
gRNA	genomic RNA
GSK3 β	glycogen synthase kinase-3 β
GST	Glutathion-S-Transferase
GTase	guanylyltransferase
GTP	Guanosine-5'-triphosphate
HCV	Hepatitis C virus
HEK293 cells	Human Embryonic Kidney 293 cells
HEK293T cells	Human Embryonic Kidney 293 cells with SV40 large T antigen
HeLa cells	Henrietta Lacks cells
HEV	Hepatitis E virus
HIV-1	Human immunodeficiency virus 1
hnRNPA1	Heterogeneous Nuclear Ribonucleoprotein A1
HVD	hypervariable domain
IDR	intrinsically disordered region
IFITM1-3	Interferon-induced transmembrane protein 1
IFN	interferon
IP	immunoprecipitation
IPTG	isopropyl- β -D-thiogalactopyranoside
IRES	internal ribosome entry site
IRF1	Interferon Regulatory Factor 1
ISG	interferon induced gene
KD	knockdown
KO	knockout
LLPS	liquid-liquid phase separation
LPS	lipopolysaccharide
MAGED2	Melanoma-associated antigen D2
mARTD	mono-ADP-ribosyltransferase Diphtheria toxin-like
MARylation	mono-ADP-ribosylation

MB	membrane binding domain
MD	macrodomain
MDA5	melanoma differentiation-associated protein 5
mEGFP	monomeric enhanced green fluorescent protein
MERS-CoV	Middle East respiratory syndrome coronavirus
MHV	Mouse Hepatitis Virus
MHV	murine hepatitis virus
miRNA	microRNA
MNV	murine norovirus
mRNA	messenger RNA
mRNPs	messenger ribonucleoproteins
MS	mass spectrometry
MTase	methyltransferase
Mut	Mutant
MXRA8	matrix remodelling associated protein 8
NAD ⁺	nicotinamide adenine dinucleotide
Nam	nicotinamide
NAP1L	nucleosome assembly protein 1 like
ncgRNA	non-capped viral genomic RNA
NE	Nuclear envelope
NEMO	NF-kappa-B essential modulator
NF-κB	nuclear factor 'k-light-chain-enhancer' of activated B-cells
NHBE cells	Normal Bronchial Epithelial cells
NLS	Nuclear localization signal
NMR	nuclear magnetic resonance
NS1;3	Non-structural protein 1;3 of ZIKV
NS5A/B	non-structural protein 5A/B
nsP	Non-structural protein
NSP	nuclear shuttle proteins
NTF2L	Nuclear Transport factor 2 Like
NTPase	nucleoside triphosphatase
Nudix	nucleoside diphosphate linked to a variable moiety X
NUDT	NUDIX hydrolase
NW	New World
OB fold	Oligonucleotide/Oligosaccharide-binding fold
ON	overnight
ORF	Open reading frame
OW	Old World
PAE	Predicted aligned error
PAMP	Pathogen-Associated Molecular Pattern
PAR	poly-ADP-ribose
PARP1	ADP-ribosyltransferase Diphtheria toxin-like 1
PARP10	ADP-ribosyltransferase Diphtheria toxin-like 10
PARP11	ADP-ribosyltransferase Diphtheria toxin-like 11
PARP12	ADP-ribosyltransferase Diphtheria toxin-like 12

PARP13	ADP-ribosyltransferase Diphtheria toxin-like 13
PARP14	ADP-ribosyltransferase Diphtheria toxin-like 14
PARP15	ADP-ribosyltransferase Diphtheria toxin-like 15
PARP15iso1	PARP15 isoform 1
PARP15iso2	PARP15 isoform 2
PARP16	ADP-ribosyltransferase Diphtheria toxin-like 16
PARP17	ADP-ribosyltransferase Diphtheria toxin-like 17
PARP2	ADP-ribosyltransferase Diphtheria toxin-like 2
PARP3	ADP-ribosyltransferase Diphtheria toxin-like 3
PARP4	ADP-ribosyltransferase Diphtheria toxin-like 4
PARP5	ADP-ribosyltransferase Diphtheria toxin-like 5
PARP6	ADP-ribosyltransferase Diphtheria toxin-like 6
PARP7	ADP-ribosyltransferase Diphtheria toxin-like 7
PARP8	ADP-ribosyltransferase Diphtheria toxin-like 8
PARP9	ADP-ribosyltransferase Diphtheria toxin-like 9
PARPi	PARP inhibitor
pARTD	mono-ADP-ribosyltransferase Diphtheria toxin-like
pARTD	poly-ARTDs
PARylation	poly-ADP-ribosylation
PBM	PAR binding motif
PBMCs	peripheral blood mononuclear cells
PBZ	Poly-ADP-ribose-binding zinc-finger
PCNA	Proliferating cell nuclear antigen
PERK	Protein Kinase R-like ER Kinase
PIP	PCNA interaction peptide box
PKCδ	protein kinase C delta
PKR	protein kinase R
PLK1	polo-like kinase 1
PM	plasma membrane
PNYDV	pea necrotic yellow dwarf virus
POI	protein of interest
PONDR	Predictor of Naturally Disordered Regions
PPI	protein-protein interaction
PTM	post-translational modification
PTM	Post-translational modification
RBP	RNA-binding protein
RGG	Arginine-glycine-glycine motif
RIG-I	retinoic acid-inducible gene I
RNA	ribonucleic acid
RRM	RNA recognition motif
RRV	Ross River virus
RSV	Respiratory Syncytial Virus
RTPase	RNA triphosphatase
rtTA	reverse tetracycline-controlled transactivator
SAH	S-adenosyl-homocysteine

SAM	S-Adenosyl methionine
SARS-CoV-2	Severe acute respiratory syndrome coronavirus type 2
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SESV	Southern elephant seal virus
sfRNA	subgenomic flaviviral RNA
SFV	Semliki Forest virus
SG	stress granule
sgRNA	subgenomic RNA
SINV	Sindbis virus
SN	supernatant
SPDV	salmon pancreatic disease virus
SSB	single strand breaks
STAT1	signal transducer and activator of transcription 1
TAF15	TATA-Box Binding Protein Associated Factor 15
TATase	terminal adenylyltransferase
TDP-43	TAR DNA-binding protein 43
TetOn	tetracyclin inducible
TF	transcription factor
TGN	trans-Golgi network
TLR	Toll-like receptors
TNKS	Tankyrase
TNKS1	Tankyrase 1
TNKS2	Tankyrase 2
TRE	tetracycline response element
U2OS cells	Human Bone Osteosarcoma Epithelial Cells
UBAP2L	Ubiquitin Associated Protein 2 Like
UIM	Ubiquitin Interacting Motif
USP10	ubiquitin specific peptidase 10
VEEV	Venezuelan equine encephalitis virus
vMD	viral macrodomain
VSV	vesicular stomatitis virus
WEEV	western equine encephalitis virus
WT	wildtype
WWE	three conserved residues Trp-Trp-Glu motif domain
Y2H	yeast two-hybrid
ZDHC	zinc finger Asp-His-His-Cys (DHC) domain-containing
ZIKV	Zika virus
ZnF	zinc finger domain

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6.4. Curriculum Vitae



PERSONAL

born on 18 August 1995 | Belgian
+32 (0) 471 245 963
maud.verheirstraeten@gmail.com

LANGUAGES

Dutch (Native) | English (C2)
German (Good) | French (B1)

IT

MES | SAP | LMS Pharma | eLIMS
| MS Office | Graphpad | PyMol |
ZEN

TEACHING EXPERIENCE

Supervision of both Bachelor and
Master students during PhD
Training of new Batch Record
Review team at Legend Biotech
Training and qualification of
Grade A operators

SOFT SKILLS

Teamwork | Communication
Proactive | Interpersonal skills
Flexible | Independent | Creative

OTHER

Qualified in Laboratory Animal
Science (equivalent to FELASA
cat. C)

Diploma in Speech and Dramatic
arts

Diploma as Youth Animator

PERSONAL INTERESTS

Hiking | Running | Bouldering |
Board games | Reading |
Traveling

MAUD VERHEIRSTRAETEN

PROFESSIONAL EXPERIENCE

Legend Biotech | Technologiepark, Gent Belgium

Technical Lead Clean room 07/2024 – present
Coordination & SME Operations

Operations Expert 10/2023 – 06/2024
Grade A operator | SPOC Batch Record Review

Doctoral Student at Universitätsklinikum Aachen, RWTH Aachen

01/2019 - 04/2023 | Institute for Biochemistry and Molecular Biology
Promotor: Prof. Dr. Bernhard Lüscher | Supervisor: Dr. Patricia Korn
"Assessing the interplay between PARP10 and the chikungunya viral
macrodomain in modulating the antiviral immune response"

Master Dissertation at Vlaams Instituut voor Biotechnologie (VIB)

02/2018 - 06/2018 | Molecular Signaling and Cell Death unit
Promotor: Prof. Dr. Wim Declercq | Supervisor: Dr. Michael Devos

EDUCATION

Master of Science in Biochemistry and Biotechnology

09/2016 - 07/2018 | University of Ghent (Belgium)
08/2016 - 01/2017 | Stockholm University (Sweden) | Erasmus+
Graduated **magna cum laude**
Major: Biomedical Biotechnology | Minor: Biochemistry and Structural
Biology

Bachelor of Science in Biochemistry and Biotechnology

09/2013 - 06/2016 | University of Ghent (Belgium)

Diploma secondary school Latin-Mathematiques

09/2007 - 06/2013 | Atheneum Wispelberg (Ghent, Belgium)

PUBLICATIONS & CONFERENCES

Krieg, S., Pott, F., Potthoff, L., **Verheirstraeten, M.**, Bütepage, M.,
Golzmann, A., ... & Korn, P. (2023). Mono-ADP-ribosylation by PARP10
inhibits Chikungunya virus nsP2 proteolytic activity and viral
replication.

Lüscher, B., **Verheirstraeten, M.**, Krieg, S., & Korn, P. (2022).
Intracellular mono-ADP-ribosyltransferases at the host-virus
interphase.

Devos, M., Tanghe, G., Gilbert, B., Dierick, E., **Verheirstraeten, M.**,
Nemegeer, J., ... & Maelfait, J. (2020). Sensing of endogenous nucleic
acids by ZBP1 induces keratinocyte necroptosis and skin
inflammation.

GBM Fall Conference 2022

POSTER

31th Annual Virology Meeting GfV2022

FEBS 2021 – Cellular Stress and ADP-Ribosylation

SPEAKER

30th Annual Virology Meeting GfV2021

SPEAKER

29th Annual Virology Meeting GfV2019

Biomedical Graduate School retreat 2019

POSTER

6.5. Scientific contributions

6.5.1. Publications

Krieg, S., Pott, F., Potthoff, L., **Verheirstraeten, M.**, Bütepage, M., Golzmann, A., ... & Korn, P. (2023). Mono-ADP-ribosylation by PARP10 inhibits Chikungunya virus nsP2 proteolytic activity and viral replication. *Cellular and Molecular Life Sciences*, 80(3), 72.

Lüscher, B., **Verheirstraeten, M.**, Krieg, S., & Korn, P. (2022). Intracellular mono-ADP-ribosyltransferases at the host–virus interphase. *Cellular and Molecular Life Sciences*, 79(6), 288.

Devos, M., Tanghe, G., Gilbert, B., Dierick, E., **Verheirstraeten, M.**, Nemegeer, J., ... & Maelfait, J. (2020). Sensing of endogenous nucleic acids by ZBP1 induces keratinocyte necroptosis and skin inflammation. *Journal of Experimental Medicine*, 217(7).

6.5.2. Conferences and presentations at scientific meetings

POSTER

GBM Fall Conference 2022

MS-based analysis of the PARP10 and CHIKV nsP3 interactome reveals potential host factors regulated by MARYlation

ATTENDEE

31th Annual Virology Meeting GfV2022

SPEAKER & POSTER

FEBS 2021 – Cellular Stress and ADP-Ribosylation

MS-based analysis of the PARP10 and CHIKV nsP3 interactome reveals G3BP1 as a potential host factor regulated by marylation

SPEAKER

30th Annual Virology Meeting GfV2021

G3BP1 is a common substrate and interactor of IFN-I inducible mono-ARTDs and CHIKV nsP3

ATTENDEE

29th Annual Virology Meeting GfV2019

POSTER

Biomedical Graduate School retreat 2019

Assessing the interplay between the mono-adp-ribosyltransferase ARTD10 and the chikungunya viral macrodomain in modulating the antiviral immune response

6.6 Eidesstattliche Erklärung

Maud Verheirstraeten

erklärt hiermit, dass diese Dissertation und die darin dargelegten Inhalte die eigenen sind und selbstständig, als Ergebnis der eigenen originären Forschung, generiert wurden.

Hiermit erkläre ich an Eides statt

1. Diese Arbeit wurde vollständig oder größtenteils in der Phase als Doktorand dieser Fakultät und Universität angefertigt;
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3. Wenn immer andere eigene- oder Veröffentlichungen Dritter herangezogen wurden, wurden diese klar benannt;
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5. Alle wesentlichen Quellen von Unterstützung wurden benannt;
6. Wenn immer ein Teil dieser Dissertation auf der Zusammenarbeit mit anderen basiert, wurde von mir klar gekennzeichnet, was von anderen und was von mir selbst erarbeitet wurde;
7. Kein Teil dieser Arbeit wurde vor deren Einreichung veröffentlicht.

12 May 2025

Maud Verheirstraeten