UNIVERSITY OF CALIFORNIA SAN DIEGO

PARP13 Is Required for IFN-γ-Mediated Inhibition of Sindbis Virus

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by

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The thesis of Xaver Rane Matthew Audhya is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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EPIGRAPH

Through our eyes, the universe is perceiving itself.
Through our ears, the universe is listening to its harmonies.
We are the witnesses through which the universe becomes conscious of its glory, of its magnificence.

Alan Watts

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ABSTRACT OF THE THESIS

PARP13 Is Required for IFN-γ-Mediated Inhibition of Sindbis Virus

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Professor Matthew Daugherty, Chair

Intrinsic antiviral immunity is a critical mediator against viral infection. It is composed of both constitutively expressed antiviral restriction factors and others that are upregulated during infection. Research over the past several years has shown that while type-I and -III IFNs have been traditionally thought to be the primary drivers of the intrinsic antiviral immune response,

type-II IFN has clear antiviral capabilities as well - although many of the details remain shrouded in mystery. Our lab recently showed that PARP13, a robust restriction factor capable of attenuating a wide variety of viruses, has two isoforms with distinctly different functions in type-I IFN signaling. Here, we show that PARP13L, but not PARP13S, is required for IFN-γ-mediated inhibition of Sindbis Virus, and potentially other alphaviruses as well. We additionally show that PARP13L is not upregulated by IFN-γ and that other members of the PARP family are involved, and lastly describe future directions for the preliminary data shown here.

INTRODUCTION

1.1 Intrinsic Antiviral Immunity

Intrinsic antiviral immunity, also known as cell intrinsic immunity or intracellular innate immunity, is the first line of defense and a critical mediator against viral infection. The intrinsic antiviral immune response relies on specific proteins, known as restriction factors, to directly or indirectly interfere with viral replication (Yan & Chen, 2012). These restriction factors are either constitutively expressed or upregulated in response to the recognition of pathogen associated molecular patterns (PAMPs) by cytosolic and membrane-bound pattern recognition receptors (PRRs) during infection (Yan & Chen 2012; Blanco-Melo; Venkatesh & Bieniasz, 2012). Upon recognition, PRRs, such as RIG-I, cGas, and MDA-5, initiate complex signaling cascades that ultimately result in the expression of hundreds of interferon-stimulated genes (ISGs) - many of which code for restriction factors (Schneider et al., 2014).

Although the functions of many ISGs are yet to be identified, several have been well described and serve as examples of the broad mechanisms by which restriction factors work to carry out their antiviral activities. Notable examples include TRIM5α, IFIT1, and PKR, which inhibit replication by interfering with retroviral uncoating, recognizing and sequestering non-self mRNAs, and inducing translational arrest via phosphorylation of eIF2α, respectively (Ganser-Pornillos, et al., 2010; Habjan et al., 2013**;** Lemaire, Anderson, Lary, & Cole, 2008). Some restriction factors are extremely potent and capable of constraining viral replication alone, but most have individually moderate antiviral effects and must be co-expressed with other restriction factors to establish an effective antiviral state (Shneider et al., 2014). Interestingly, however, several ISGs have been co-opted by viruses to promote infection rather than curb it, such as

LY6E, which has been shown to enhance receptor-mediated endocytosis and uncoating for several types of enveloped viruses (Mar et al., 2018).

Interferons (IFNs) are key signaling molecules in host-pathogen interactions. Type-I (α/β) and -III (ƛ) IFNs have been classically thought to be the primary drivers of the intrinsic antiviral immune response. IFN-α/β can be produced and sensed by almost all cells types, while IFN-ƛ can be produced by almost all cell types but only sensed by specific cells (Shneider et al., 2014). After binding to their cell surface receptors, signaling occurs through the JAK-STAT pathway. The JAK-1 and TYK-2 tyrosine kinases become activated and phosphorylate STAT1 and STAT2, which then heterodimerize, form a complex with IRF-9 known as ISGF3, and translocate to the nucleus. Once inside, the complex binds to genomic cis-regulatory interferon-stimulated response elements (ISREs) and activates expression of hundreds of ISGs (Schneider et al, 2014).

1.2 - What Are PARPs?

ADP-ribosylation (ADPr) is a post-translational modification (PTM) found ubiquitously in both the cytoplasmic and nuclear compartments. It is carried out by PARPs (Poly-ADP-Ribose Polymerase), of which there are 17 in the human family. Interestingly, while each member of the human PARP family contains a PARP catalytic domain, there is substantial variation in activity, domain structure, and size across the group (Amé, Spenlehauer, & de Murcia, 2004). PARPs 1-5 catalyze the transfer of ADP-ribose from NAD^+ (nicotinamide adenine dinucleotide) into chains on target proteins and nucleic acids (poly-ADP-ribosylation; PARylation), while the others either transfer a single ADP-ribose (mono-ADP-ribosylation; MARylation) or none at all (Amé et al., 2004; Ryu, Kim, & Kraus, 2015). Like many other enzymes that catalyze PTMs, some PARPs

undergo auto- and catalyze trans-ADPr of other PARPs in addition to nonfamilial targets, which can either enhance or repress activity (Muthurajan et al., 2014; Carter-O'Connell, et al., 2018). Furthermore, a wide variety of secondary structural motifs can be found in the PARP family, such as zinc fingers and WWE domains, which allow for recognition and binding to DNA/RNA and ADP-ribose, respectively (Ali et al., 2012; Melikishvili et al., 2017; Bock et al., 2015; Wang et al., 2012). The smallest PARP, PARP 16, consists of 322 amino acid residues and contains only a PARP catalytic domain, while one of the largest members, PARP 4, consists of more than 1,700 amino acid residues and contains 6 discrete domains (Daugherty et al., 2014).

Although PARPs have been observed in a broad spectrum of vital cellular processes, such as apoptosis, chromatin remodeling, innate immunity, transcriptional regulation, and ubiquitination, most members of the family remain poorly characterized (Los et al., 2002; Simbulan-Rosenthal et al., 1998; Chaudhuri & Nussenzweig, 2017; Fehr et al., 2020; Kraus & Lis, 2003; Vos et al., 2014; Guo et al., 2019). The most well-studied PARP is PARP1, which has numerous physiological functions. Perhaps most importantly, it is a critical enzyme in the DNA damage response that has become an important drug target as PARP1 inhibition triggers synthetic lethality in cells with BRCA1 mutations (Farmer et al., 2005; Bryant et al., 2007). Involvement in such tightly regulated processes suggests that PARPs are likely under tight regulation as well. Indeed, while most PARPs are constitutively expressed at varying levels, some are upregulated in under certain conditions, such as cellular stress (Luo & Kraus, 2012; Juszczynski et al., 2006; Schoggins et al., 2011). Additionally, ADPr can be reversed by several different types of proteins. PARGs (Poly-ADP-Ribose Glycohydrolases) are primarily responsible for PAR degradation, ARHs (ADP-ribosyl hydrolase) reverse MARylation, and Macrodomain proteins are capable of recognizing and disassembling both MAR and PAR

modifications (Davidovic et al., 2001; Min, 2009; Rack et al., 2018; Stevens et al., 2019). Interestingly, PARPs 9, 14, and 15 contain both a PARP catalytic domain and a macrodomain, and have thus appropriately been termed "macro-PARPs" (Amé et al., 2004; Vyas et al., 2013).

1.3 - PARPs in Host-Virus Interactions

A growing body of research suggests that PARPs additionally play expansive and important roles in host-virus interactions (Daugherty et al., 2014; Kuny & Sullivan, 2016; Brady et al., 2018). The first evidence of this came in 2002 with the seminal discovery that PARP13, also called ZAP (Zinc-finger Antiviral Protein), could directly bind to retroviral RNA and promote its degradation (Gao, 2002). Subsequent research revealed several key lines of evidence that suggested other PARPs were involved either directly or indirectly in the antiviral immune response as well. Among them are studies showing that, like many other viral restriction factors, numerous PARPs are under rapid positive selection (PARPs 4, 7, 8, 9 and 13) and are interferon-inducible (PARPs 9, 10, 12, 13, and 14), hallmarks of proteins involved in host-pathogen interactions (Goodier et al., 2015**;** Atasheva et al., 2012; Atasheva et al., 2014; Daugherty et al., 2014). Additionally, members of several viral families, including *Coronaviridae, Hepeviridae, and Togaviridae*, encode macrodomains. Studies show that disruption of these macrodomains, specifically in the ADP-ribose binding pockets, results in diminished viral replication (Li et al., 2016**;** Alhammad & Fehr, 2020**;** Grunewald et al., 2019; Götte et al., 2018). Taken together, these data strongly corroborate a place for PARPs in the intrinsic antiviral response.

Indeed, many members of the human PARP family have been shown to be antiviral, although the mechanisms by which they carry out these effects have not been fully elucidated in many instances. In one example, PARP1 hyperactivity has been shown attenuate Kaposi's

Sarcoma-Associated Herpesvirus (KSHV) proliferation, potentially by binding to KSHV's terminal repeat sequence (TR) of KSHV and ADP-ribosylating a necessary viral replication factor known as LANA (Ohsaki et al., 2004). In another case, PARP12L has been shown to inhibit host translation during infection to limit production of viral proteins, likely by binding to polysomal RNA with its N-terminal domain. PARPs 7 and 10 have also been shown to induce translational arrest, potentially by the same mechanism as PARP12L. Notably, PARPS, 7, 10 and 12L seem to be more robust inhibitors of translation than PKR. (Atasheva et al., 2014; Welsby et al., 2014). PARP9 complexes with the E3 ubiquitin ligase DTX3L, which together interact with STAT1 and modify H2BJ histones to enhance expression of a subset of ISGs - including IFIT1. Furthermore, this complex facilitates the ubiquitination and subsequent degradation of viral 3C proteases, which are necessary for the replication of viruses in *Picornaviridae* family (Zhang et al., 2015; Sun et al., 2016). Additional cases of PARP antiviral activity can be found in the literature, but the examples reviewed here clearly illustrate the diverse means by which PARPs inhibit viral replication.

Interestingly, some PARPs have also been shown to have proviral effects as well. For example, viral core proteins V and VII are ADP-ribosylated during natural adenovirus infection, but mature particle release is substantially reduced in the presence of a PARP1 inhibitor (Déry et al., 1986**)**. PARP1 inhibition shows similar suppressive effects on HSV-1, JC virus, SV40, PRRSV, and Vaccinia Virus (Grady et al., 2012; Nukuzuma et al. 2012; Gordon-Shaag et al., 2003; Liu et al., 2016; Child et al., 1988). Furthermore, HSV-1 (Herpes Simplex Virus 1) replication has been associated with decreased NAD^+, increased PARylation, and degradation of PARG (Grady et al., 2012). Of particular importance given the current SARS-CoV-2 pandemic, the nucleocapsid (N) protein of numerous α- and β-coronaviruses, including SARS-CoV and

MERS-CoV, are ADP-ribosylated during infection - although it is not known whether ADPr facilitates or interferes with coronavirus lifecycles (Grunewald et al., 2018).

1.4 - PARP13: One PARP to Rule Them All

While the repository of data on other PARPs in host-virus interactions is gradually expanding, PARP13 continues to be the most well-described member in this regard. Our lab, in collaboration with Ram Savan's lab at the University of Washington, recently showed that the two major isoforms of PARP13, PARP13L and PARP13S (also known as ZAP-L and ZAP-S, respectively), have two distinct primary functions. The long isoform has 4 CCCH-type zinc fingers towards its N-terminus, a WWE domain, and a catalytically inactive PARP domain at its C-terminus (Guo et al., 2004; Gao, 2002). PARP13L additionally has a CaaX prenylation motif ('C' cysteine, 'a' aliphatic amino acid, 'X' variable amino acid) at its C-terminal end that causes localization of PARP13L to endolysosomal membranes (Schwerk et al., 2019). The PARP domain and CaaX motif are absent from the short isoform as a result of IFN-induced and CSTF2-dependent alternative polyadenylation (Schwerk et al., 2019).

PARP13 is a robust and broadly acting restriction factor that efficiently attenuates replication of numerous viruses, including members of *Filoviridae, Hepadnaviridae, Picornaviridae, Retroviridae,* and *Togaviridae* (Müller et al., 2006; Mao et al., 2013; Li et al., 2015**;** Gao, 2002; Atasheva et al., 2012; Bick et al., 2003**)**. There are several proposed mechanisms by which PARP13L accomplishes this. In one model, PARP13L mediates viral mRNA degradation by non-covalently binding to CG-rich motifs (C[n_7]G[n]CG) on viral mRNAs with its N-terminal zinc fingers and recruiting a variety of machinery, including a poly(a)-specific ribonuclease (PARN), an Xrn1 3'-5' exoribonuclease, and a Dcp1a/Dcp2

decapping complex, to destroy them (Guo et al., 2004; Luo et al., 2020; Guo et al., 2006). Another proposes that before PARP13L can engage in viral mRNA degradation, it must first disrupt the interaction between eIF4A and eIF4G to induce translational arrest and halt viral protein production (Zhu et al., 2012). During stress and infection, PARP13 also localizes to stress granules, which are important for translational regulation during cellular stress and are frequently antagonized or co-opted by viruses (Anderson & Kedersha, 2008; White & Lloyd, 2012; Leung et al. 2011). Among other methods, stress granules utilize microRNAs (miRNAs) to modulate host and viral protein production (Anderson & Kedersha, 2008; Fabian & Sonenberg, 2012). PARP13 activates the necessary miRNA-associated cofactor RISC (RNA-induced Silencing Complex) by facilitating the ADP-ribosylation of its core AGO proteins, which is most likely to be carried out by PARPs 5a and 12 (Leung et al., 2011). PARP13 localization to stress granules has been directly correlated with its antiviral activities (Law et al., 2019). It is possible that all of these pathways contribute additively or synergistically to PARP13L-mediated viral inhibition.

Although PARP13S has the same N-terminal 4 CCCH-type zinc fingers and WWE domain as its longer counterpart and is thus potentially capable of carrying out the same antiviral activities, it has been shown to be dramatically less potent (Kerns et al., 2008). Our recent work posits that the cytoplasmic localization, rather than endolysosomal, of PARP13S, which is due to the absence of the CaaX prenylation motif, is in large part responsible for this disparity in phenotypes between isoforms (Schwerk et al., 2019). Moreover, our collaborators in Ram Savan's lab have observed zinc finger-dependent interaction of PARP13S with AU-rich elements in the 3' UTR of IFN mRNAs and shown that siRNA knockdown of PARP13S, but not PARP13L, results in increased and long-lasting *IFNB*, *IFNL*$_2$, and *IFNL*$_3$ mRNA levels

compared to wild type after poly U/UC RNA treatment (Schwerk et al., 2019). It had been previously reported that PARP13S interacted with RIG-I to amplify type-I IFN production in HEK293T cells, but we were unable to reproduce these results (Hayakawa et al., 2019). Taken together, our findings suggest a completely novel role for PARP13S as a negative regulator of type-I and -III IFNs. Furthermore, unlike PARP13L, which is constitutively expressed and upregulated only moderately after IFN induction, PARP13S expression is IFN-dependent, which may indicate that its function as a negative regulator may be to protect the host from cytotoxicity caused by long-term expression of ISGs and inflammatory cytokines (Schwerk et al., 2019). Although the mechanism by which PARP13S acts to degrade host mRNAs has not yet been fully described, one possibility is that it functions similarly to other PARP13-mediated mRNA degradation pathways, but further investigation is necessary. Additionally, these findings suggest a model wherein PARP13S acts as an evolutionary tripwire. If a virus evolves to disrupt PARP13 function by antagonizing a region shared by both isoforms it may bring about reduced antiviral activity by PARP13L specifically, but also upregulate expression of other interferon-stimulated restriction factors that block replication. As such, viruses targeting PAR13L specifically would have a fitness advantage over those targeting both isoforms. Comparative genomics studies have shown that rapid positive selection is only occurring in the PARP domain of the long isoform, which supports this model (Daugherty et al., 2014).

1.5 - Cofactors in PARP13-Mediated Viral Restriction

Both PARP13 isoforms participate in many protein-protein interactions, many of which have been shown to be necessary and/or synergistic for function. One of these cofactors is p72 (also known as DDX17), a DEAD box RNA helicase involved in transcriptional regulation,

splicing, processing of miRNA and rRNA, and other important cellular processes (Chen et al, 2008; Cordin et al., 2006). p72 has been shown to interact with the Dcp1a/Dcp2 decapping complex in a PARP13-dependent manner to carry out viral mRNA degradation, and knockdown of p72 with shRNA substantially decreases PARP13's antiviral activity (Chen at al., 2008; Zhu et al., 2011). A similar relationship is seen with DHX30, a DEXH-box RNA helicase **(**Ye et al., 2010).

Another example is TRIM25, an E3 ubiquitin ligase that is perhaps best known for its role in the regulation of RIG-I (Mcfadden, 2007). Multiple reports show that knockdown of TRIM25 with sh/siRNA substantially reduces PARP13 antiviral activity, but the mechanism remains unclear (Li et al., 2017**)**. K63-linked TRIM25-mediated polyubiquitination appears to be necessary for PARP13 function, but mutation of PARP13 ubiquitination sites does not reduce PARP13's efficacy as strongly as TRIM25 knockdown - though it does appear to mildly interfere with PARP13's RNA recognition abilities (Zheng et al., 2017**)**. One possibility is that TRIM25 ubiquitinates other proteins in PARP13's interactome necessary for its antiviral activity and/or is involved with viral translational inhibition, but further investigation is necessary (Zheng et al., 2017; Li et al., 2017**)**.

PARP13 interaction with KHNYN is necessary for PARP13-mediated degradation of some, but not all CG-dinucleotide-rich viral mRNAs, although this mechanism, too, remains obscure (Ficarellia et al., 2019). Interestingly, PARP13, KHNYN, and TRIM25 pull down together in co-immunoprecipitation assays. TRIM25 is not required for interaction between PARP13 and KHNYN but is curiously required for KHNYN activity (Ficarellia et al., 2019). While KHNYN had no previously known purpose prior to this finding and the interaction of these three proteins is not yet understood, other work suggests §§NYN-domains may be

regulated by ubiquitination and function as exonucleases in RNA-processing structures (Anantharaman & Aravind, 2006). Of additional note, PARP13 is ADP-ribosylated while in stress granules, most likely by PARPs 12 and 15, and by PARP14 under other circumstances, which may be critical to viral inhibition (Leung et al., 2011; Carter-O'Connell et al., 2018). These are but a few of the PARP13 cofactors discovered, which suggests PARP13-mediated viral inhibition may rely on a tightly regulated proteomic profile.

1.6 - PARP13 and Interferon-γ

While the primary role of Type-II IFN (γ) has been historically thought to be in recruiting and activating specialized immune cells such as macrophages, NK cells, and T-cells during infection, a certain but poorly described role for it in the intrinsic antiviral response has emerged in recent years as well (Guidotti & Chisari, 2001). Although only specialized immune cells can produce IFN-γ, it can be sensed by almost all cell types (Kang, Brown, & Hwang, 2018). After binding to its cell surface receptor, signaling occurs through a signaling cascade similar to the Type-I (α/β) and -III (ƛ) IFN JAK-STAT pathway wherein the JAK-1 and JAK-2 tyrosine kinases become activated and phosphorylate STAT1, which then homodimerizes and translocates to the nucleus. Once inside, the complex binds to gamma activating sequence (GAS) elements and activates expression of ISGs (Schneider & Rice, 2014).

Importantly, there is some degree of cross-talk between the IFN-α/β and -γ signaling pathways that leads to overlapping expression. For example, STAT1 heterodimers can be produced in the α/β signaling cascade and lead to γ-sensitive ISG expression. Conversely, production of IRF-1 and -9, necessary components in α/β-induced ISG expression, is in part driven by IFN-γ (Schroder et al., 2003). Additionally, IFN-γ production of ISGF3 has been

reported, which may allow for the expression of canonical type-I ISG expression upon IFN-γ sensing (Matsumoto et al., 1999). Consequently, the complex interactions between the IFN-α/β and -γ signaling pathways, many of which are likely yet to be discovered, continue to pose a challenge in shedding light on the unique functions of IFN-γ in the intrinsic antiviral response.

Although the direct mechanisms by which IFN-γ exerts its antiviral effects are poorly described relative to IFN-α/β, there are numerous examples of IFN-γ-mediated viral inhibition. For instance, IFN-γ can interfere with viral entry of Hepatitis C Virus by downregulating the expression of receptors necessary for endocytosis (Kang et al, 2018). IFN-γ has also been shown to attenuate Sindbis Virus (SINV) genomic and subgenomic replication, although the downstream JAK-STAT effectors responsible are unknown (Burdeinick-Kerr, & Griffin, 2005; Burdeinick-Kerr et al., 2009). In a third example, IFN-γ regulates the production of nitric oxide synthase (iNOS), which has been shown to block late gene expression of Vaccinia Virus (Kang et al, 2018).

Interestingly, PARP9 and -14 are also upregulated in response to IFN-γ and interact with STAT1, a key regulatory factor in all three IFN signaling pathways, and STAT6, a transcription factor that is activated during viral infection by a non-JAK-STAT pathway and plays a role in recruiting specialized immune cells to the site of infection (Higashi et al., 2019; Iwata et al., 2016; Chen et al., 2011; Mehrotra et al., 2010). These observations suggested to us that other PARPs might play a role in type-II IFN signaling during the antiviral response as well. Specifically, we were curious to know if PARP13 was an antiviral effector in this system. Our work here suggests that PARP13 may be a key regulator of IFN-γ-mediated viral inhibition of alphaviruses in an isoform-specific manner.

METHODS AND MATERIALS

2.1 - Cell Culturing

All Huh7 cells were cultured in DMEM and 10% heat-inactivated fetal bovine serum (FBS) and incubated at 37°C with 5% CO_2. Cells were split 1:4 every 2 days.

2.2 - Generation of Huh7 PARP13 KOs

The PARP13 targeting guide RNA (5'CGAACTATTCGCAGTCCGAG-3') was cloned into the pRRLU6-empty-gRNA-MND-Cas9-t2A Puro vector downstream of the U6 promoter using the In-Fusion enzyme mix from Clonetech. Huh7 cells were transfected with PARP13 gRNA-Cas9-expressing plasmids, with transfection of Cas9-empty-expressing plasmids used as a negative control. Huh7 cells were cultured in a 10cm dish and transfected with 10µg of plasmid using the Invitrogen $CaPO_4$ transfection kit, according to the manufacturer's instructions. After 48 hours, 2µg/mL puromycin to the media for 2 days to select for successfully transfected cells. The selected cells were then passaged and analyzed for the absence of PARP13 protein expression by immunoblot after treatment with IFN-β

2.3 - Infections

Short interfering RNAs (siRNAs) were designed to target either one or both isoforms of PARP13 and were procured from Integrated DNA Technologies. Huh7 cells were cultured in 24-well plates and transfected with siRNA 24 hours prior to infection using Invitrogen Lipofectamine 2000, according to the manufacturer's instructions. Non-targeting "scramble" siRNA was used as a negative control (NC). Cells were then treated with interferon (IFN) 8

hours prior to infection, with opti-MEM alone used as a negative control. Where applicable, cells were treated with drugs at 21 hours pre-infection. Infections were carried out at the indicated MOI, and at 1-hour post infection, the supernatants were aspirated and replenished with new media and equivalent IFN concentrations. SINV and CHIKV infection supernatants were harvested at 24 hours post infection while VSV infection supernatants were harvested 12 hours. Following collection, the samples were titered on BHKs.

2.4 - Immunoblotting

Cells were lysed in a 3:1:0.2 solution of 4X SDS buffer, Milli-Q water, and 2-mercaptoethanol, respectively, and run in a 12% polyacrylamide SDS-PAGE gel. Samples were then transferred to a nitrocellulose membrane and probed for PARP13 in a 5% BSA solution with ZC3HAV1 antibodies from Genetex (#GTX120134) and visualized with a Thermo Scientific West Pico Plus chemiluminescence kit, per the manufacturer's instructions.

2.5 - Semliki Forest Virus (SFV) Reporter Assay

For SFV reporter assays, a pSMART vector was utilized to express the nonstructural proteins of SFV and a subgenomic, promoter-driven β-galactosidase protein. 200ng of pSMART and the indicated amount of pcDNA5/FRT/TO-expressing PARP13 constructs were transfected into Huh7 cells in 24-well plates. To measure β-galactosidase activity, cells were freeze-thawed three times and 4-methylumbelliferyl-β-D-galactopyranoside was added to 200μg/mL after 24 hours of transfection. Fluorescence (excitation/emission wavelength of 365/460nm) was recorded every 20 seconds, and the rate of fluorescence increase was averaged for 5 minutes.

2.6 - Plaque Assay

BHKs monolayers were cultured in 24-well plates. Harvested supernatants from infections were serially diluted and added to approximately 90% confluent BHKs. After 1.5 hours, BHK media was aspirated and replaced with 500mL/well of a half 0.8% carboxymethylcellulose (CMC) and half DMEM with 10% FBS solution. After 48 hours, the CMC overlay was aspirated, and cells were stained with crystal violet (0.1% in 20% ethanol) for 20 minutes and then washed with 20% ethanol. Plaque forming units (PFU) per well of the supernatants added were calculated by multiplying the average number of plaques across replicates by the dilution factor.

2.7 - Co-Transfection

Three short interfering RNAs (siRNAs) were designed to target different regions of PARPs 7, 10, 12, 14, and 15, and were procured from Integrated DNA Technologies. HEK293T cells were cultured in 24-well plates and co-transfected with a plasmid expression a PARP protein fused to an mCherry red fluorescent protein and the siRNA using Invitrogen Lipofectamine 2000, according to the manufacturer's instructions. Non-targeting "scramble" siRNA was used as a negative control (NC). Fluorescence was visualized with a ZOE Fluorescent Cell Imager at 24 hours post-transfection.

RESULTS

3.1 - PARP13 Is Required for IFN-γ-Mediated Inhibition of Sindbis Virus

Sindbis Virus (SINV) is a +ssRNA virus that belongs to *Togaviridae* and is the prototypic member of the alphavirus genus. It induces type-I IFN signaling during infection via PRR sensing and is highly susceptible to the ensuing ISG production (Burke et al., 2009; Akhrymuk et al., 2016; Ryman et al., 2000). IFN-Y has also been shown to attenuate Sindbis Virus (SINV) replication via JAK-STAT signaling, but the downstream restriction factors that directly block its growth are not known (Burdeinick-Kerr et al, 2009; Burdeinick-Kerr et al., 2005). To determine whether PARP13 was involved in IFN-γ-mediated restriction of SINV, we pre-treated WT and PARP13 knock out (ΔPARP13) Huh7 cells with either IFN-α or -γ and then infected them with SINV (MOI = 0.1). Although PARP13 is an important component in IFN-α-mediated restriction of SINV, our results indicate that it is not required for SINV attenuation (Fig. 1A). This finding supports previous studies showing that SINV is sensitive to numerous ISGs and that PARP13 works in synergy with them (Schoggins et al., 2013**;** Zhang et al., 2007; Lenschow et al., 2007; Frolova et al., 2002; Bick et al, 2003; Karki et al., 2012**.**). Remarkably, however, we observed that IFN-γ-mediated restriction of SINV was almost entirely crippled without PARP13 (Fig. 1B).

PARP13 is known to inhibit multiple alphaviruses, so we were curious to know if PARP13 would be required for IFN-γ-mediated inhibition of other alphaviruses as well (Bick et al, 2003). Consequently, we pre-treated WT and ΔPARP13 cells with IFN and challenged them with a vaccine strain of the alphavirus Chikungunya Virus (CHIKV; MOI =1) and a recombinant Semliki Forest Virus (SFV) alphaviral reporter system. Titering showed that IFN-α reduced CHIKV load by approximately 1.5 logs in WT cells and only one log in ΔPARP13 cells, while

IFN-γ was reduced CHIKV by 2 logs in WT cells and less than 1 log in ΔPARP13 cells (Fig. 1C-D). While we did not test the SFV reporter assay against IFN-α, IFN-γ gamma reduced replication by approximately 85% in WT cells, but not at all in ΔPARP13 cells (Fig. 1E).

While PARP13 acts against many types of viruses, it is not a universal restriction factor. To determine whether or not this effect would be seen in non-alphavirus species, we repeated this experiment with Vesicular Stomatitis Virus (VSV), a -ssRNA virus that belongs to *Rhabdoviridae* and is known to not be PARP13-sensitive (Bick et al, 2003; Schwerk et al., 2019**)**. VSV was mildly sensitive to both IFN-α and -γ, but there was no substantial difference between WT and ΔPARP13 conditions (Fig 1F, 1G). Taken together, these results suggest that PARP13 is required for IFN-γ-mediated inhibition of Sindbis virus, and potentially other alphaviruses.

3.2 - PARP13 Is Regulated by IFN-γ In an Isoform-Specific Manner

Our lab's recent work showed that the two major PARP13 isoforms, PARP13L and PARP13S, have different primary functions in type-I and type-III IFN signaling during infection (Schwerk et al., 2019). To determine whether one or both isoforms were required for SINV restriction by IFN-γ, we transfected WT Huh7 cells with siRNA targeting either one or both PARP13 isoforms, treated them with IFN-γ, and then infected them with SINV (MOI = 0.1). 10U of IFN-γ alone reduced SINV titers by over an order of magnitude in WT cells (NC), while concurrent knockdown of both isoforms or PARPL alone resulted in a half order of magnitude decrease in titers or less (Fig 2A). Silencing of PARP13S resulted in viral load similar to WT (Fig 2A).

PARP13 is well-documented as a potent viral restriction factor, so we were curious to know IFN-γ-mediated viral inhibition was merely due to increased PARP13L expression. To answer this question, we treated WT Huh7 cells treated with IFN-γ for 24 hours and probed for PARP13 via immunoblot. Our results indicate that PARP13L is not induced by IFN-γ, but that PARP13S is (Fig 2B). In conjunction, these findings suggest that PARP13 regulates IFN-γ-mediated viral inhibition in an isoform-specific manner that it is not due to IFN-γ-induced upregulation of PAR13L.

3.3 - PARP13 May Require Other PARPs for Regulation by IFN-γ

PARPs are known and sometimes required to work in conjunction with other PARPs. To determine whether PARP13 requires other PARPs to modulate IFN-γ-mediated viral inhibition, we treated either WT or ΔPARP13 Huh7 cells with either DMSO (negative control), Olaparib, a PARP1/2-specific inhibitor, and a broad-spectrum PARP inhibitor (PARPi) called compound 413B, and with IFN-γ, and then infected with SINV (MOI = 1). Viral titers of WT cells after treatment with Olaparib showed similar results to DMSO treatment, while general PARP inhibition moderately rescued SINV infection by ~1 order of magnitude in the presence of IFN- γ (Fig. 3A). There were no observable differences between drug treatment groups in any IFN-γ concentration in ΔPARP13 cells (Fig. 3B).

DISCUSSION

IFN-γ has been shown to attenuate Sindbis Virus (SINV) infection via JAK-STAT signaling, but little is known about the downstream effectors that are directly responsible for blocking viral replication (Burdeinick-Kerr, & Griffin, 2005; Burdeinick-Kerr et al, 2009) Here, our preliminary work shows that PARP13, an important restriction factor against SINV in the Type-I IFN intrinsic antiviral response, is critical to IFN-γ-mediated inhibition of SINV and potentially other alphaviruses as well. Furthermore, our work indicates that IFN-γ-mediated viral inhibition is modulated by PARP13 in an isoform-specific manner.

How exactly PARP13 accomplishes this remains unclear, but we hypothesized several possibilities. Despite being the more potent restriction factor in most cases, PARP13L is upregulated only moderately in comparison to its shorter counterpart in response to type-I IFN signaling (Schwerk et al., 2019; Mao et al., 2013; Seo et al., 2013; Li et al., 2019). One explanation for this may be that because type-I IFN signaling cascade results in the expression of hundreds of restriction factors, it is less dependent on PARP13L specifically for its antiviral activities, and thus does not require meaningful upregulation of PARP13L during infection - an evolutionarily sound setup with regard to the host-pathogen arms race. Conversely, because IFN-γ-mediated restriction of viruses is so strongly dependent on PARP13L, we were curious to know if PARP13L was more strongly IFN-γ-induced either directly or indirectly via cross-talk between type-I and type-II IFN signaling pathways. However, immunoblotting showed no obvious change in PARP13L levels after 24 hours of exposure to various concentrations of IFN-γ.

Because PARP13 interacts and facilitates auto- and trans-ADP-ribosylation by catalytically active PARPs and is itself ADP-ribosylated by other PARPs, we reasoned that other PARPs may be involved or required for IFN-γ modulation of PARP13 (Carter-O'Connell et al.,

2018; Leung et al., 2011). Upon treating WT cells with Olaparib, a PARP1/2 inhibitor, and IFN-γ there was no substantial difference in viral titer relative to the negative control. When we treated cells with a broad-spectrum general PARP inhibitor we saw a rescue of SINV replication by at least one log in both IFN-γ concentrations relative to the negative control. This finding suggests that other PARPs are likely to be involved in PARP13 regulation of viral restriction by IFN-γ, though not PARP1 or 2. While no statistically significant difference was found between the means of DMSO and PARPi treatment groups in the 10 U/mL IFN-γ condition, examination of the data reveals this is due to one extreme value in the PARPi set. Given the dramatic shift in viral titers in different treatment conditions, however, we will be repeating this experiment to substantiate this finding upon reopening of research facilities.

Should successful confirmation of this observation be found in repetition, we will proceed with several additional experiments to further resolve the mechanistic details here. Namely, to determine which other PARP(s) may be responsible for the phenotype observed in PARPi treatment, we will design and target siRNAs against individual members of the family, treat with IFN-γ, and measure infection outcomes via titer, as was done here against PARP13 isoforms. We have already begun testing siRNA knockdown efficiency (Fig. 4). Any implicated PARP(s) (PARPx) will be knocked out via CRISPR/Cas9 technology to confirm involvement. Proper siRNA and CRISPR/Cas9 targeting can be confirmed via immunoblot. From here, we will generate PARPx knockouts in a flip-in cell line and re-integrate a doxycycline-inducible PARPx gene to observe whether or not phenotype rescue is observed (e.g. decrease in viral replication upon integration). We will additionally generate and flip-in PARPx mutants with defects in each domain (e.g. PARP, WWE, zinc fingers, etc…) to determine which regions of the

protein are necessary for regulation of PARP13 by IFN-γ and what that interaction might be (e.g. if WWE domain is mutated, PAR recognition by PARPx may be required).

Similar experiments will be carried out with PARP13 itself to show phenotype rescue upon re-integration, as well as the functional domains required for modulation by IFN-γ. Our recent work showed that PARP13L localization to endosomal membranes is largely responsible for its increased antiviral activity relative to PARP13S, so in addition to modifying the functional domains of PARP13L (e.g. WWE domain, zinc fingers, etc..) we will also flip-in PARP13L with a disrupted C-terminal CaaX motif to determine if localization is important in this regard as well (Schwerk et al., 2019). The results of these experiments together will guide the direction of further examination. For instance, if we find that the catalytically active PARP domain of PAPRx is required, we can flip-in PARP13 with mutations in known ADP-ribosylation sites and monitor the resulting phenotype to determine if it is ADP-ribosylating PARP13. If there is no rescue of viral replication compared to ΔPARP13 viral titers, this would indicate that ADP-riboslyation of PARP13 is required, and is potentially carried out by PARPx. Ideally, we will be able to generate PARP13 isoform-specific knockouts to more easily identify which isoform is responsible for a given phenotype.

In addition to interacting with other members of the PARP family, PARP13 also has a broad nonfamilial protein interactome. Accordingly, these proteins are worth investigating as potential contributing or necessary cofactors for PARP13 regulation of IFN-γ-mediated viral inhibition. Of particular note are TRIM25 and KHNYN, which can be characterized in similar fashion to previously described methods in this discussion (e.g. knock-out/flip-in/mutants). It is also possible that PARP13 interacts with a distinct set of proteins during type-II IFN signaling that it does not interact with during type-I IFN signaling or baseline conditions. One way these

proteins might be found is by treating cells with IFN-γ and performing co-immunoprecipitation with antibodies against PARP13. If the associated protein is unknown, mass-spectrometry and other assays may be used to identify it. Successful identification can be followed up with characterization of these proteins as previously described. It may be additionally worth probing their downstream activities to further delineate mechanistic details.

Though less prevalent in human populations than other types of viruses, some alphaviruses are endemic in many parts of the world and are responsible for numerous outbreaks in both humans in animals with considerable fatality rates. Most clinically important alphaviruses are arboviruses and are transmitted to humans and animals by mosquitos, and as worldwide temperatures continue to rise and mosquito habitation consequently expands, these viruses may pose a considerate threat to global health. These considerations, in addition to recurring outbreaks of other devastating viruses, such as Ebola, illustrate a critical need for additional research to better understand how human endogenous antiviral effectors, like PARP13, block these infections. Furthermore, because some viruses are capable of blocking the production of endogenous type-I IFN, the administration of exogenous type-I IFN has been historically used as a treatment for some infections (Lin et al., 1999; Shiffman et al., 1999; Liaw et al., 1997). However, type-I IFN treatment can be harmful at therapeutic levels (Lane, 1990; Janssen et al., 1994; Fattovich et al., 1996). Conversely, type-II IFN is not as widely used for antiviral therapeutics, although there are some noted clinical benefits and potentially less cytotoxic effects (Miller, Maher, & Young, 2019). The preliminary evidence here reiterates the importance of understanding type-II IFN in the antiviral response, and thus, how it might be utilized in more medical contexts.

FIGURES

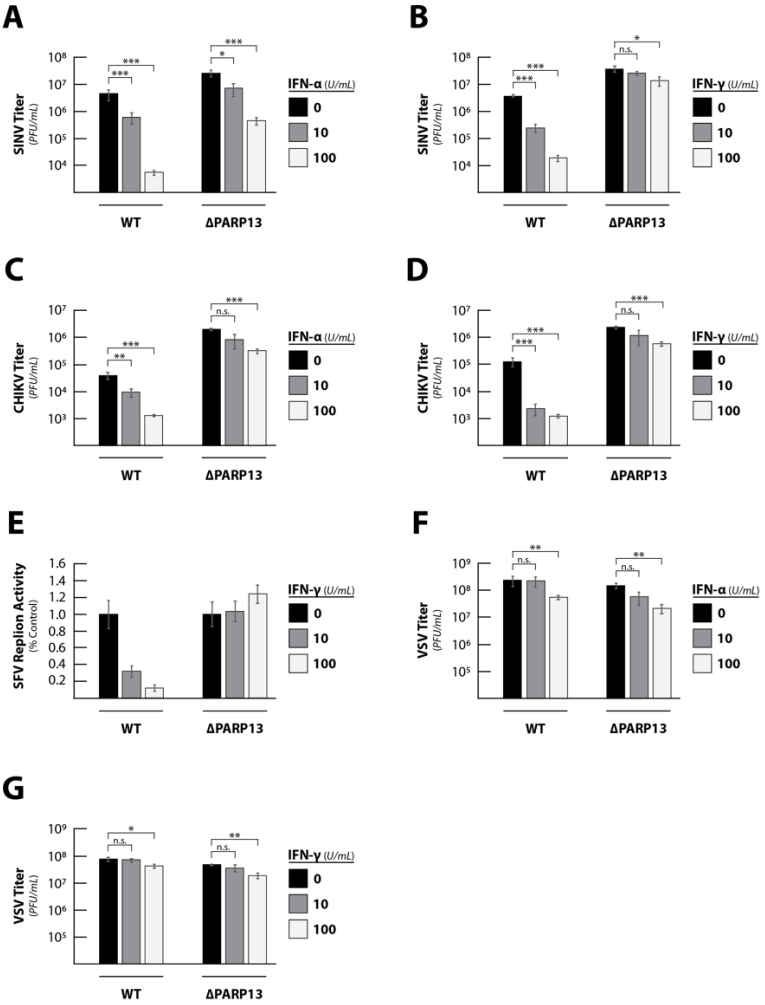

Fig 1. Inhibition of SINV By IFN-γ Is Ineffective Without PARP13. **A**. Viral titer of WT and ΔPARP13 Huh7 cells treated with 0, 10, or 100 units/mL of IFN-α at 20 hpi with SINV (MOI = 0.1). **B.** Viral titer of WT and ΔPARP13 Huh7 cells treated with 0, 10, or 100 units/mL of IFN-γ at 20 hpi with SINV (MOI = 0.1). **C.** Viral titer of WT and ΔPARP13 Huh7 cells treated with 0, 10, or 100 units/mL of IFN-α at 24 hpi with CHIKV (MOI = 1). **D.** Viral titer of WT and ΔPARP13 Huh7 cells treated with 0, 10, or 100 units/mL of IFN-γ at 24 hpi with CHIKV (MOI = 1). **E.** SFV reporter assay of WT and ΔPARP13 Huh7 cells treated with 0, 10, or 100 units/mL of IFN-γ. **F**. Viral titer of WT and ΔPARP13 Huh7 cells treated with 0, 10, or 100 units/mL of IFN-α at 12 hpi with VSV (MOI = 0.01). **G.** Viral titer of WT and ΔPARP13 Huh7 cells treated with 0, 10, or 100 units/mL of IFN-γ at 12 hpi with VSV (MOI = 0.01). **A-G.** Error bars indicate ± 1 s.d. **A-D, F, G.** Data are representative of independently performed experiments with (n=4) replicates and similar results. Data were analyzed with two-way ANOVA with restriction and paired 2-tailed t-tests where appropriate. *p < 0.05, **p < 0.01, ***p < 0.001. Data courtesy of Andrew Ryan. Figure generation and statistics by Xaver Audhya.

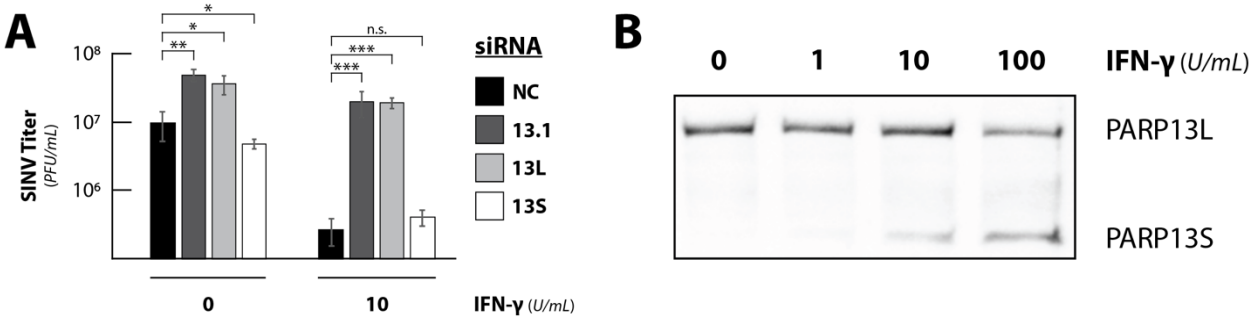

Fig 2. PARP13L Is Modulated By IFN-γ-Mediated Viral Inhibition. **A.** Viral titer of WT Huh7 cells transfected with either a negative control non-targeting siRNA (NC) or siRNA targeting both PARP13 isoforms (13.1), PARP13L (13L), or PARP13S (13S) and treated with 0 or 10 units/mL of IFN-γ at 20 hpi with SINV (MOI = 0.1). Data are representative of independently performed experiments with (n=4) replicates and similar results. Data were analyzed with two-way ANOVA with restriction and paired 2-tailed t-tests where appropriate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. **B.** Immunoblot probing for PARP13L and PARP13S in WT Huh7 cells 24 hours after treatment with 0, 1, 10, or 100 units/mL of IFN-γ. Data courtesy of Andrew Ryan. Figure generation and statistics by Xaver Audhya.

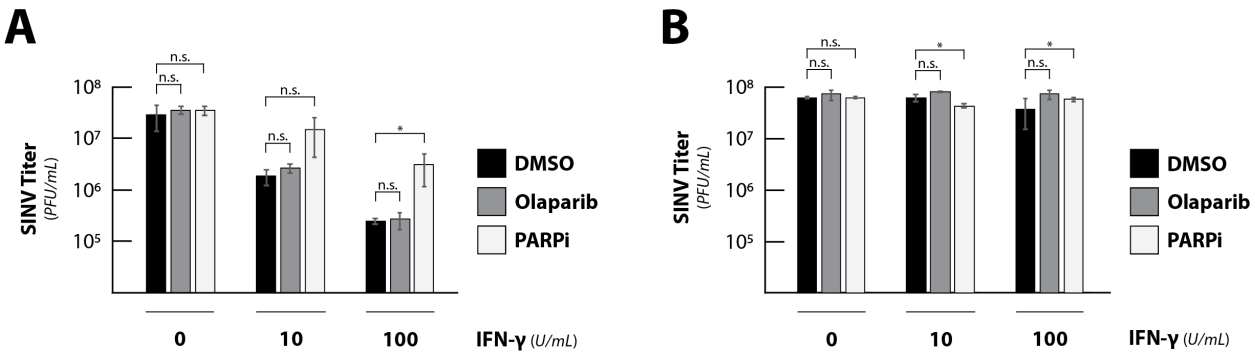

Fig 3. Other PARPs May Be Required for PARP13 Regulation By IFN-γ. **A.** Viral titer of WT Huh7 cells treated with either a negative control (DMSO), Olaparib, or the broad-spectrum PARP inhibitor compound 413B (PARPi) and either 0, 10, or 100 units/mL of IFN-γ at 20 hpi with SINV (MOI = 0.1). **B.** Viral titer of ΔPARP13 Huh7 cells treated with either a negative control (DMSO), Olaparib, or the broad-spectrum PARP inhibitor compound 413B (PARPi) and either 0, 10, or 100 units/mL of IFN-γ at 20 hpi with SINV (MOI = 0.1). **A-B.** Data are representative of independently performed experiments with (n=3) replicates and similar results. Data were analyzed with two-way ANOVA with restriction and paired 2-tailed t-tests where appropriate. Data courtesy of Matthew Daugherty and Andrew Ryan. Figure generation and statistics by Xaver Audhya.

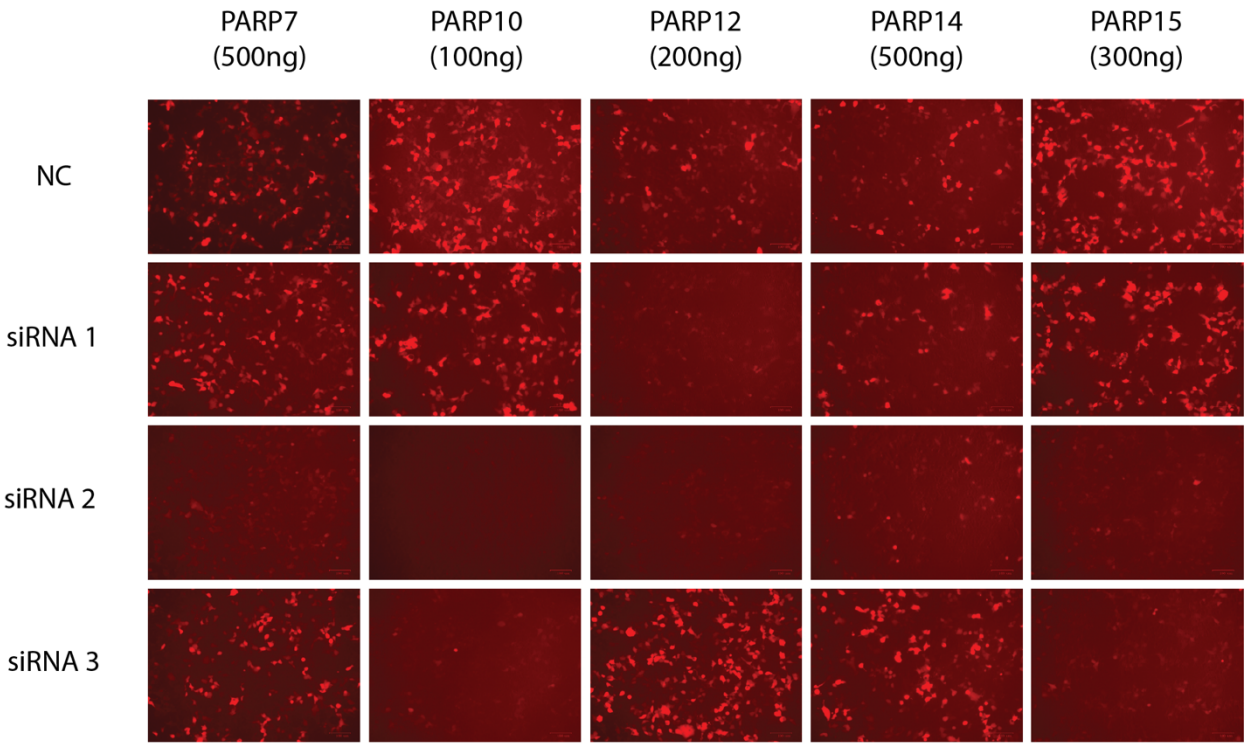

Fig 4. PARP siRNA Candidates. 293T cells were co-transfected with plasmids expressing either PARPs 7, 10, 12, 14, and 15 fused to an mCherry red fluorescent protein and one of three siRNAs targeting different regions of the appropriate PARP transcript. A non-targeting "scramble" siRNA was used as a negative control (NC). Cells were visualized with a ZOE Fluorescent Cell Imager at 24 hours post-transfection and fluorescence was used as a qualitative measure of gene knockdown efficiency by siRNA. Data and figure creation courtesy of Xaver Audhya.

This thesis is co-authored by Xaver Audhya, Andy Ryan, and Matthew Daugherty. The thesis author was primary author of this paper.

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