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Poly(ADP-Ribose) Regulates Stress Responses and MicroRNA Activity in the Cytoplasm

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SUMMARY

Poly(ADP-ribose) is a major regulatory macromolecule in the nucleus, where it regulates transcription, chromosome structure, and DNA damage repair. Functions in the interphase cytoplasm are less understood. Here, we identify a requirement for poly(ADP-ribose) in the assembly of cytoplasmic stress granules, which accumulate RNA-binding proteins that regulate the translation and stability of mRNAs upon stress. We show that poly(ADP-ribose), six specific poly(ADP-ribose) polymerases, and two poly(ADP-ribose) glycohydrolase isoforms are stress granule components. A subset of stress granule proteins, including microRNA-binding Argonaute family members Ago1–4, are modified by poly(ADP-ribose), and such modification increases upon stress, a condition when both microRNA-mediated translational repression and microRNA-directed mRNA cleavage are relieved. Similar relief of repression is also observed upon overexpression of specific poly(ADP-ribose) polymerases or, conversely, upon knockdown of glycohydrolase. We conclude that poly(ADP-ribose) is a key regulator of posttranscriptional gene expression in the cytoplasm.

INTRODUCTION

Poly(ADP-ribose), or pADPr, is a macromolecular polymer and posttranslational modification best known for its functions in the nucleus (Schreiber et al., 2006). These include DNA damage repair, chromatin remodeling, and transcriptional regulation (Krishnakumar and Kraus, 2010). However, increasing evidence suggests that pADPr also functions in the cytoplasm. For example, pADPr is required for the structure and function of the spindle in the mitotic cytoplasm (Chang et al., 2004). Furthermore, the majority of enzymes regulating pADPr synthesis and degradation are localized to the cytoplasm. These include two of the three isoforms of pADPr glycohydrolase (PARG) (Meyer-Ficca et al., 2004) and five pADPr polymerase

(PARP) family members whose cellular localizations are characterized (Juszczynski et al., 2006; Kickhoefer et al., 1999; Liu et al., 2004; Smith and de Lange, 1999; Yu et al., 2005). Seventeen PARPs exist in humans, all defined by a conserved PARP domain, and are classified as pADPr synthesizing, mono-ADP-ribose (mADPr) synthesizing, or enzymatically inactive based primarily on the presence of a triad of histidine, tyrosine, and glutamate (HYE) thought to be required for synthesizing the initial mADPr and/or subsequent pADPr subunits (Hottiger et al., 2010; Kleine et al., 2008).

The ability to synthesize mADPr or pADPr is not a prerequisite for PARP function. For example, PARP-13/ZAP (zinc finger antiviral protein) lacks catalytic activity yet inhibits certain viruses by degrading viral RNAs in the cytoplasm (Gao et al., 2002). PARP-13 proteins either lack a PARP domain (PARP-13.2 isoform) or contain a domain lacking key residues of the HYE motif (PARP-13.1 contains YYV). Such a lack of catalytic activity does not rule out the possibility that PARP-13 function requires ADPr modification by another PARP. Such *trans*-modification is common among PARPs from the same subfamily, including modifications between PARP-1 and PARP-2 (Schreiber et al., 2002) and between PARP-5a and PARP-5b (Sbodio et al., 2002). Consistent with PARP-13's role in regulating RNA in the cytoplasm, pADPr's synthesizing and degrading activities were previously identified in cytoplasmic mRNA-protein complexes (mRNPs) (Elkaim et al., 1983; Thomassin et al., 1985). In this study, we discover two functions of pADPr in the cytoplasm—modulating the expression of microRNA (miRNA) targets and assembling mRNP-rich structures called stress granules (SGs).

miRNAs are ~22 nt short noncoding RNAs that regulate targets via translational inhibition and mRNA degradation (Fabian et al., 2010). Despite the diverse fates of mRNA targets, all processes are mediated through the miRNA-binding protein Argonaute (Ago1–4 in humans). Recent data suggest that the activities of miRNAs can be modulated by multiple mechanisms, including posttranslational modifications of Argonaute (Qi et al., 2008; Rüdell et al., 2011; Zeng et al., 2008), association with other RNA-binding proteins, and relocalization of Argonaute to subcellular locations, such as SGs (reviewed in Leung and Sharp, 2010).

SGs assemble upon stalled translation initiation (Anderson and Kedersha, 2008), which can be triggered via two pathways: (1) phosphorylation of initiation factor eIF2 α , which commonly

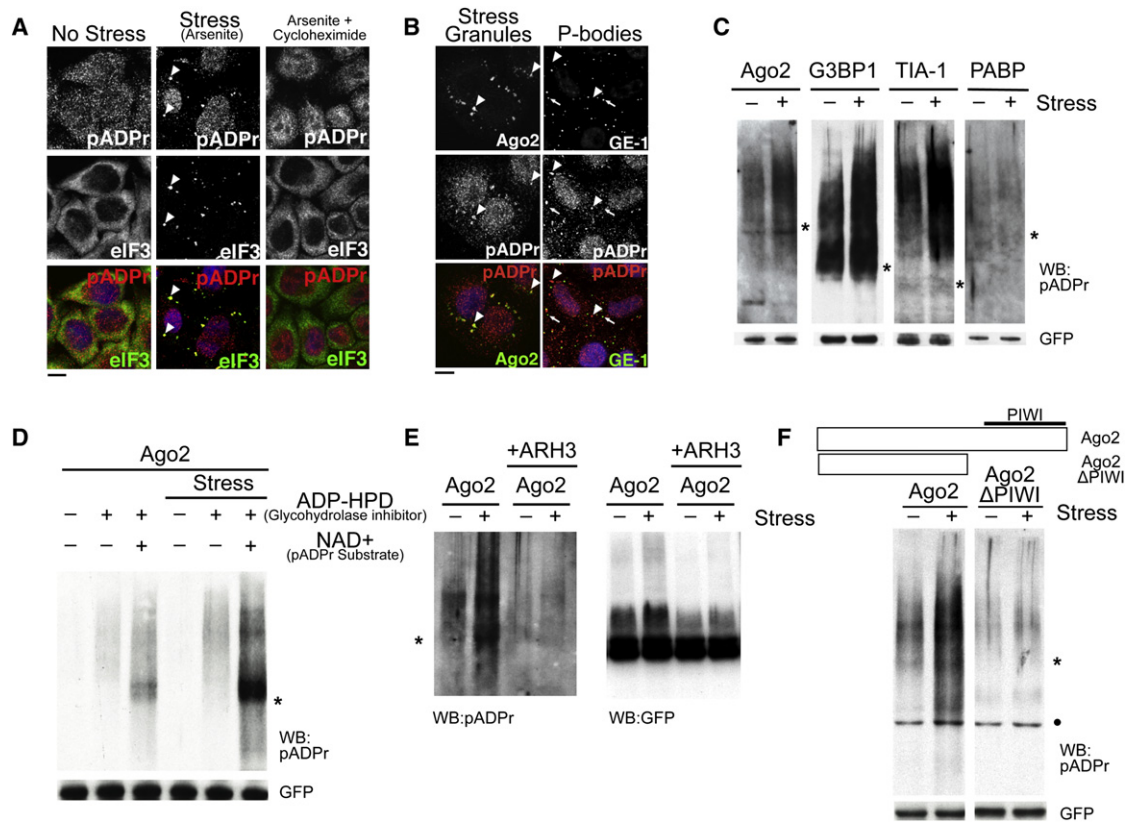


Figure 1. pADPr Is Enriched in SGs upon Multiple Types of Stresses and Modifies Specific Cytoplasmic RNA-Binding Proteins Dependent on RNA-Binding Domain

(A) pADPr staining using LP96-10 antibodies in HeLa cells untreated or treated with 100 μ M arsenite for 60 min or for 30 min followed by 100 μ M arsenite + 100 μ g/ml cycloheximide for 30 min. Arrowheads, SGs; scale bar, 10 μ m.
 (B) HeLa cells treated with 100 μ M arsenite were stained for pADPr, SG component Ago2 (arrowheads), or PB component GE-1 (arrows). DNA was stained with Hoechst 33342 (blue); scale bar, 10 μ m.
 (C) Immunoprecipitates of four GFP-tagged SG-localized RNA-binding proteins from cells treated with or without 20 nM pateamine A were probed for pADPr.
 (D) Immunoprecipitates of GFP-Ago2 from cells treated with or without 20 nM pateamine A were probed for pADPr. The cell extracts either included or excluded 1 μ M ADP-HPD and with or without 1 mM NAD⁺ before immunoprecipitation by anti-GFP.
 (E) pADPr modification of Ago2 from cells treated with or without 20 nM pateamine A was verified by treating the immunoprecipitates with ARH3. The immunoprecipitates were probed for pADPr (left) and GFP (right).
 (F) Immunoprecipitates of wild-type and PIWI mutant of GFP-Ago2 from cells treated with or without 20 nM pateamine A were probed for pADPr. For (C)–(F), cell extracts included 1 μ M ADP-HPD unless stated otherwise; shown are western blots for pADPr (LP96-10) and GFP levels in each immunoprecipitate. Asterisks indicate the position of the corresponding GFP-tagged RNA-binding protein constructs. Black dots indicate nonspecific binding to BSA by LP96-10. See also Figure S1.

occurs during cell stresses, such as heat shock or arsenite-mediated oxidative stress, or (2) by addition of translation initiation inhibitors that do not involve the phosphorylation of eIF2 α . Here, we show that pADPr is important for SG assembly and identify six PARPs including PARP-13 that function in these processes. Interestingly, the majority of these PARPs also bind to Ago2, suggesting that they have overlapping activities in miRNA and SG function.

RESULTS

Poly(ADP-Ribose) Localizes to Stress Granules in the Cytoplasm

Previous work by us and others identified binding interactions between pADPr and cytoplasmic RNA-binding proteins (Chang

et al., 2009; Gagne et al., 2008); included among these are SG components. To determine whether pADPr is present in SGs, cells were stressed with arsenite then stained with antibodies generated against pADPr (LP96-10) and SG marker eIF3. Four human cell lines were analyzed: retinal primary epithelial cells (RPE-1) and three cancer lines, 293T, U2OS, and HeLa (Figures 1A and S1A). In each case, pADPr was enriched in SGs. To confirm, five additional anti-pADPr antibodies were tested (Figure S1B and data not shown). This panel of antibodies includes monoclonal antibody 10H specific for polymers containing ≥ 20 ADP-ribose subunits (Kawamitsu et al., 1984). Each antibody stained the interphase cytoplasm and nucleus in untreated cells and demonstrated a clear colocalization with eIF3-positive puncta in the cytoplasm upon stress, regardless of fixation method (methanol or paraformaldehyde; data not shown).

Moreover, such colocalization was also observed in all examined stress conditions that result in SG assembly, including heat shock, glucose deprivation, treatment with the proteasome inhibitor MG132, or with translation initiation inhibitors (pateamine A and hippuristanol) (Figure S1C and data not shown). Together, these data indicate that pADPr is enriched in SGs upon multiple stresses and contain polymers of at least 20 ADP-ribose subunits.

SGs can be disassembled by cycloheximide, which shifts the equilibrium of mRNA pools from stalled initiation complexes at SGs to polyribosomes (Anderson and Kedersha, 2008). Upon addition of cycloheximide to arsenite-treated cells, pADPr no longer localized as punctate structures (Figure 1A, rightmost panel), suggesting that the pADPr originally at SGs relocated with mRNA and/or their binding proteins. Thus, pADPr colocalization with other mRNA-binding proteins was further analyzed (Anderson and Kedersha, 2008). pADPr colocalized with miRNA-binding protein Ago2, RNA decay factor G3BP1, translational suppressor TIA-1, and poly(A)-binding protein PABP (Figures 1B and S1D). In contrast, no significant pADPr colocalization was observed with GE-1, a marker for P-bodies, another cytoplasmic structure enriched in RNA-binding proteins (Figures 1B and S1E and legends for statistics). Thus, pADPr localization to SGs is not the result of nonspecific binding of pADPr with mRNA-binding proteins.

pADPr Modification of Cytoplasmic RNA-Binding Proteins Requires the Presence of an RNA-Binding Domain

Although pADPr modifications occur primarily on PARP proteins, pADPr also modifies other targets (Schreiber et al., 2006). Because Ago2, G3BP1, TIA-1, and PABP colocalized with pADPr at SGs, we examined their pADPr modification status. Proteins were expressed as GFP-fusions, immunoprecipitated from extracts of either unstressed or stressed cells in the presence of PARG-specific inhibitor ADP-HPD, then immunoblotted for pADPr. If the protein is modified, a slower migrating material stained positively with anti-pADPr is expected and would appear as a “smear” as a result of heterogeneity in the length of polymer attached (Figure 1C). Such pADPr-positive material was observed for Ago2, G3BP1, and TIA-1, but not PABP. Upon stress, Ago2, G3BP1, and TIA-1, but not PABP, exhibited significantly increased pADPr modification (Figure 1C). Similarly, other Argonaute family members—Ago1, Ago3, and Ago4—are modified by pADPr during nonstress conditions, suggesting that all Argonaute proteins are actively regulated by pADPr in unstressed cells and such modification increases upon stress (Figure S1F).

The presence of pADPr modification was further verified for Ago2 in four different ways. First, endogenous Ago2 was immunoprecipitated from unstressed and stressed cells and probed for pADPr (Figure S1G). Similar to GFP-Ago2, endogenous Ago2 exhibited moderate amounts of pADPr modification during nonstress conditions and the modification increased upon stress. Second, the pADPr staining associated with GFP-Ago2 under nonstress and stress conditions decreased when PARG-specific inhibitor ADP-HPD was omitted, and increased upon addition of NAD^+ , a substrate for ADPr synthesis, suggesting

that pADPr modification of Ago2 is actively regulated even in *in vitro* conditions (Figure 1D). Third, treatment of a pADPr glycohydrolase, ARH3, but not RNase A, eliminated the anti-pADPr signal from the immunoprecipitates (Figure 1E and data not shown). These slow-migrating, pADPr-positive materials were also GFP-positive, confirming that the observed signal was derived from GFP-Ago2 (Figure 1E). Finally, the ADP-ribosylating activities in the GFP-Ago2 immunoprecipitates were further examined by incubating with increasing concentrations of NAD^+ containing trace amounts of radioactive NAD^+ . The reactions were then resolved via SDS-PAGE and were visualized by autoradiography (Figure S1H). A heterogeneous mobility of radioactive signal was observed immediately above where GFP-Ago2 migrated (denoted by asterisk). The intensity of this signal was directly dependent on the concentration of NAD^+ added. These data suggest that PARPs associated with Ago2 in the immunoprecipitates can synthesize pADPr. Importantly, addition of the general PARP inhibitor 3-aminobenzamide (3-AB) to the reactions completely eliminated any incorporation of radioactivity in the Ago2 precipitates, suggesting that the signal observed is due to ADP-ribosylating activities (Figure S1H).

Because pADPr modification was observed on mRNA-binding proteins Ago2, G3BP1, and TIA-1, we next tested whether their modification requires mRNA binding. To test this, fragments of each protein were expressed at levels comparable to wild-type, immunoprecipitated from unstressed and stressed cells, and immunoblotted for pADPr. In all cases, pADPr modification required the presence of an mRNA-binding domain, such as Ago2's PIWI domain (Figure 1F) or RRM in G3BP1 and TIA-1 (Figures S1I and S1J) in both nonstress and stress conditions. These data suggest that either the pADPr modification site is within or proximal to the RNA-binding domains of these proteins, or that the modification begins with the activation of PARP(s) that is/are associated via mRNAs.

Specific PARPs and PARG Isoforms Associate with Cytoplasmic mRNP Complexes

We reasoned that PARPs responsible for such modification are likely associated with these cytoplasmic RNA-binding proteins during nonstress conditions and that these associations may be retained in SGs upon stress. Thus, SGs were used as a surrogate to identify such PARPs. We screened a library of GFP fusions (S.V., unpublished data) to 17 of the 18 human PARPs (PARP-13.2 isoform included, but not PARP-14 because the full-length cDNA was unavailable) for colocalization with known SG markers (Figures 2A and S2A and data not shown). Five of 17 PARPs localized to SGs: PARP-5a, -12, -13.1, -13.2, and -15 (Figure S2B). Specific SG localization of these PARPs was confirmed using live-cell imaging. In each case, GFP-tagged PARPs strongly colocalized with RFP-G3BP1 at the assembling SGs (Movie S1). To further rule out overexpression artifacts, we repeated the colocalization screen in HeLa (Figure 2B) and RPE-1 cells (Figure S2C) using a library of affinity purified and characterized antibodies against each PARP (S.V., unpublished data). Antibody staining confirmed the GFP-PARP screen results and also identified PARP-14 as a SG protein (confirmed via two distinct antibodies) (Figures 2B and S2C and data not shown). Consistent with the GFP localization screen, the remaining

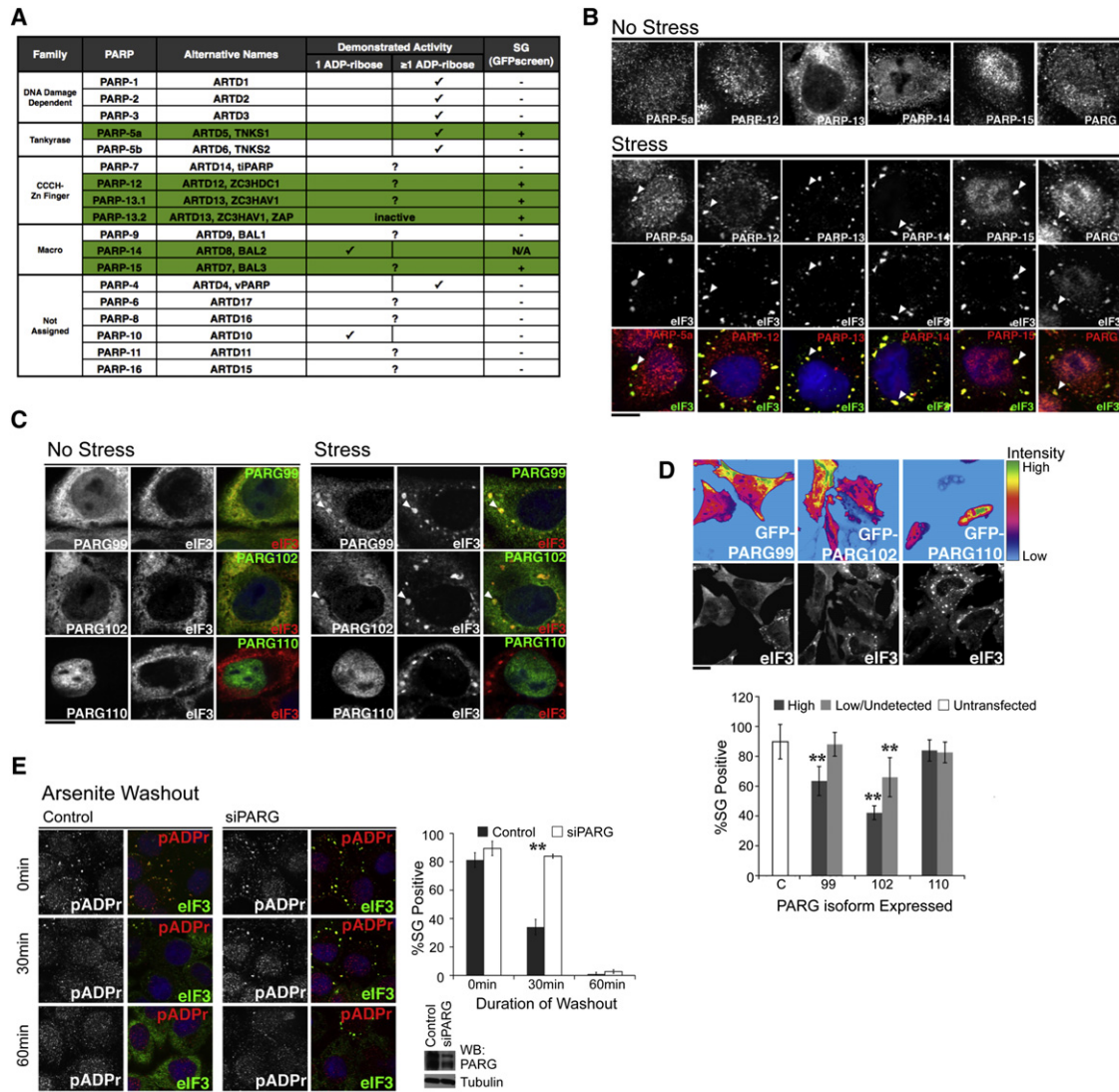


Figure 2. Specific PARPs and PARG Isoforms Localize in the Cytoplasmic SGs and the Level of PARG Modulates the Kinetics of SG Assembly and Disassembly

(A) Summary of SG localization screen of PARP family. Green shading indicates SG-PARPs as determined by GFP-PARP fusions or PARP-specific antibodies. (B) HeLa cells were treated with or without 100 μ M arsenite for 60 min and stained using antibodies against SG-PARPs and PARG. (C) HeLa cells expressing GFP-tagged PARG isoforms were treated with or without 250 μ M arsenite for 30 min. (D) Overexpression of cytoplasmic PARG isoforms inhibits SG assembly. Experiment performed as in (C), but heat map shows level of GFP-PARG isoforms (99, 102, 110) compared with untransfected control (C). Accompanying graph shows quantitation of image data (≥ 200 cells for each condition from at least six independent fields). Cells with GFP intensity above background are classified as “High” whereas cells with intensity indistinguishable from background levels as “Low/Undetected.” Paired t test $p < 0.01$ (**), derived from comparison to untransfected control; error bars indicates SD. (E) pADPr hydrolysis is required for SG disassembly. Shown are representative images taken 0, 30, and 60 min after washout of 30 min 100 μ M arsenite treatment in control and PARG knockdown HeLa cells. Quantitation: ≥ 100 cells for each condition, $n = 3$. Paired t test $p < 0.01$ (**), and error bars indicate SD. Accompanying blot shows level of PARG knockdown with tubulin as loading control. For (B)–(E), pADPr were stained by LP96-10 antibodies, SGs (arrowheads) by anti-eIF3, and DNA by Hoechst 33342 (blue); scale bars = 10 μ m. See also Figure S2 and Movie S1.

12 PARPs did not localize to SGs endogenously, confirming these six PARPs as SG proteins (hereafter referred as SG-PARPs) (Figure S2B).

We next examined the localization of pADPr-hydrolyzing enzyme PARG. Immunostaining suggests that a fraction of endogenous PARG localizes to the cytoplasm (Figure 2B).

Similar to the SG-PARPs, PARG was enriched in SGs upon stress (Figure 2B). Three major PARG isoforms exist: PARG99, PARG102, and PARG110 (Figure S2D). To determine which localize to SGs, GFP fusions of each were screened. Both PARG99 and PARG102 strongly colocalized with SGs upon stress and were dispersed in the cytoplasm during nonstress

conditions. PARG110 was nuclear and unaffected by stress (Figure 2C). Although nuclear PARG110 is known to function in DNA damage repair (Schreiber et al., 2006), PARG99 and PARG102 isoforms are functionally uncharacterized; thus, their identification at SGs uncovers potential function for these cytoplasmic isoforms.

The presence of six PARPs and two PARG isoforms in SGs suggests that the pADPr concentration may be dynamically regulated there. Overexpression of each SG-PARP resulted in de novo assembly of SGs without altering eIF2 α phosphorylation levels (Figure S2E). This finding suggests that these enzymes and/or their product, pADPr, play a structural role in SG function rather than causing a general impairment of translation via eIF2 α regulation. Overexpression of cytoplasmic PARG99 or PARG102 inhibited SG assembly, whereas overexpression of nuclear PARG110 had no effect (Figure 2D). Moreover, three distinct siRNAs targeting the first exon of PARG102 coding region individually delayed SG disassembly (Figures 2E and S2F). No such effect was observed upon knockdown of a nuclear PARG ARH3 (Figure S2G). Interestingly, all tested proteins that can nucleate SGs upon overexpression, including Ago2, TIA-1, and G3BP1, but not nonnucleator PABP (Anderson and Kedersha, 2008), serve as acceptors of pADPr modification (Figure 1C), suggesting a strong correlation between polymer synthesis and SG assembly. Consistent with this, no pADPr modification was identified on mutants of TIA-1 and G3BP1 that are dominant negative in SG formation (Kedersha et al., 1999; Tourriere et al., 2003) (Figures S1I and S1J). Taken together, these data suggest that both assembly and maintenance of SG structure depends on regulating pADPr concentration locally in the cytoplasm.

Stress or Specific PARP Overexpression Alleviates miRNA-Mediated Repression

To identify a possible function for pADPr modification of RNA-binding proteins on their activities, we focused on Argonautes because of their critical function in regulating >60% of all mRNAs (Bartel, 2009). Given that Argonaute localization to SGs is dependent on miRNAs (Leung et al., 2006) and pADPr modification of Ago2 increases upon stress, we examined miRNA activity during stress. miRNA activity was monitored using a luciferase reporter that contains six bulged sites for an siRNA, siCXCR4, in its 3' UTR (Doench et al., 2003). The luciferase protein contains a destabilization signal that reduces its half-life to 20 min so that any change in expression is rapidly monitored. Under nonstress conditions, the luciferase construct was repressed 15-fold by targeting siCXCR4 relative to a control siRNA. Consistent with Bhattacharyya et al. (2006), we observed a relief of miRNA-mediated silencing under stress conditions. Under stress conditions, the relative fold repression is reduced to ~5-fold (Figure 3A). Notably, following these stresses, the translation rate was globally reduced; however, the expression of the luciferase mRNA targeted by siCXCR4 decreased to a lesser extent than the nontargeted reporter.

Because SGs can be assembled via SG-PARP overexpression in the absence of exogenous stress, we next examined whether miRNA activity is correlated with SG induction or specific SG-PARP overexpression (Figure 3B). Interestingly, overexpression of PARP-13.1 or PARP-13.2 each resulted in a ~3-fold

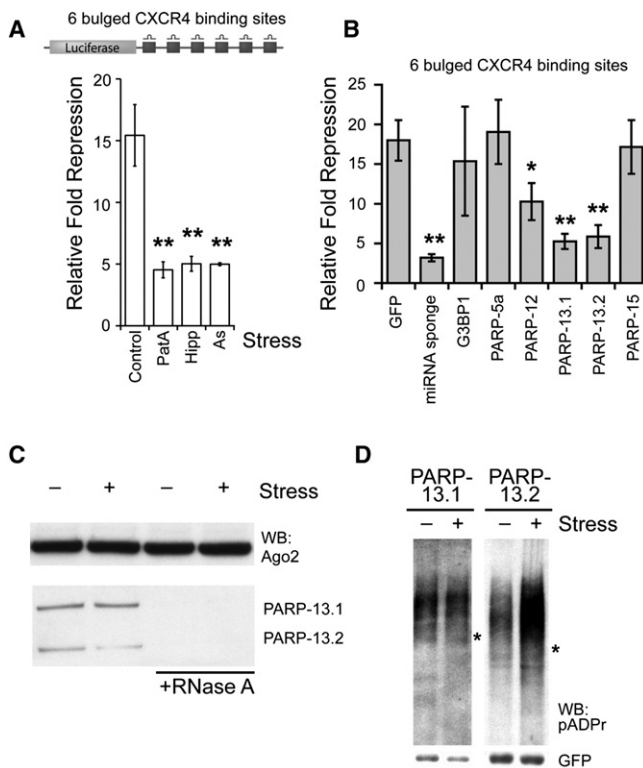


Figure 3. Stress or PARP-13 Overexpression Alleviates miRNA-Mediated Repression

(A) miRNA activity assay in untreated 293T cells or cells treated with 30 nM pateamine A (PatA), 1 μ M hippuristanol (Hipp), or 250 μ M arsenite (As) for 2 hr, where relative fold repression was measured as the activity of luciferase (upper panel) in the presence of the targeting siRNA normalized to a control siRNA; n = 3.

(B) miRNA activity assay upon overexpression of SG-PARPs. Relative fold repression was measured as in (A); n = 4. For (A) and (B), error bars indicate SD; paired t test p < 0.05 (*) and < 0.01 (**).

(C) Antibodies against endogenous Ago2 were used for immunoprecipitation from cytoplasmic extract of HeLa cells treated with or without 250 μ M arsenite for 30 min. On the right, the extract was pretreated with 200 μ g/ml RNase A for 20 min at 25°C.

(D) Immunoprecipitates of GFP-tagged PARP-13.1 or -13.2 from cells treated with or without 20 nM pateamine A for 30 min were probed for pADPr, where cell extracts included 1 μ M ADP-HPD. Asterisks indicate the position of the corresponding GFP-tagged PARPs. See also Figure S3.

decrease in repression of miRNA activity, and overexpression of PARP-12 resulted in a modest ~1.8-fold decrease, whereas overexpression of other SG-PARPs or another SG nucleator G3BP1 had no effect. As controls, overexpression of GFP had no effect, whereas the repression can be relieved by a competitive inhibitor miRNA sponge (Ebert et al., 2007). Thus, the observed decrease in miRNA-mediated repression is not likely due to an overall increase in SG assembly or SG-PARP overexpression, but instead is due to the specific function of PARP-13.1/13.2.

To further explore the role of PARP-13 in miRNA silencing, the association between PARP-13 and Ago2 was investigated (Figure 3C). Immunoprecipitation of endogenous Ago2 identified

PARP-13.1 and PARP-13.2 as binding partners mediated by mRNA. Given that the amount of PARP-13 bound to Ago2 remains unchanged in nonstress and stress conditions, it seems that other properties, such as posttranslational modifications, of PARP-13 are likely altered during stress, resulting in enhanced Ago2 modification.

We therefore examined whether PARP-13 proteins are modified by pADPr. GFP-PARP-13.1 and -PARP-13.2 were immunoprecipitated from unstressed or stressed cells and probed for pADPr. Both isoforms were modified under nonstress conditions. Remarkably, PARP-13.2 exhibited a dramatic increase in modification upon stress, whereas PARP-13.1 modification remained roughly the same (Figure 3D). Similarly, immunoprecipitation of endogenous PARP-13 via a pan-PARP-13 antibody demonstrated an increase in pADPr modification upon stress (Figure S3A). PARP-13 modification was further confirmed by antibody 10H, which recognizes pADPr with ≥ 20 subunits (Figures S3A and S3B), and the pADPr staining can be eliminated by glycohydrolase ARH3 or PARG (Figure S3C) but not RNase A (data not shown). Interestingly, the amount of pADPr modification shifted among the SG-PARPs during stress conditions; similar to PARP-13.1, there was no change in PARP-12 modification, whereas PARP-5a and PARP-15 modifications were reduced (Figure S3D). These results suggest a possible shift in PARP activity from auto-modification to modification of stress targets, including Ago1-4, TIA-1, G3BP1, and PARP-13 family members.

PARP-13 Family Members Are pADPr-Modified by Other SG-PARPs

The pADPr modifications attached to PARP-13 proteins are not due to auto-ADP-ribosylating activities because PARP-13.1's PARP domain is inactive *in vitro* and PARP-13.2 lacks a PARP domain (Kleine et al., 2008). To determine which PARPs modify PARP-13, we examined the catalytic activity of each SG-PARP. GFP-tagged SG-PARPs were purified by immunoprecipitation from either unstressed or stressed cells (PARP-1 serves as a positive control), and the immunoprecipitates were washed twice with buffer containing high salt (450 mM NaCl) and then divided into equal aliquots for ADP-ribosylating activity (Figures 4A and S4A). As expected, PARP-1 and PARP-5a demonstrated auto-poly(ADP-ribosylating) activities, as shown by the mobility shifts above their respective molecular weights (asterisks in Figure 4A). On the other hand, PARP-12 and PARP-15 exhibited mono(ADP-ribosylating) activities, as demonstrated by single radioactive bands at their respective molecular weights. Consistent with their lack of active PARP domains, significant amounts of radioactivity were not associated with PARP-13.1 or PARP-13.2. Interestingly, PARP-12 and PARP-15 samples also contained single bands of radioactivity near the expected mobility of endogenous PARP-13.1 (circle) and PARP-13.2 (triangle). Similar results were observed from stress conditions (data not shown). These results suggest that PARP-12 and PARP-15 could be part of complexes that modify PARP-13 family members even in high salt conditions.

To determine whether SG-PARPs bind to and modify one another, the binding interactions between different PARPs

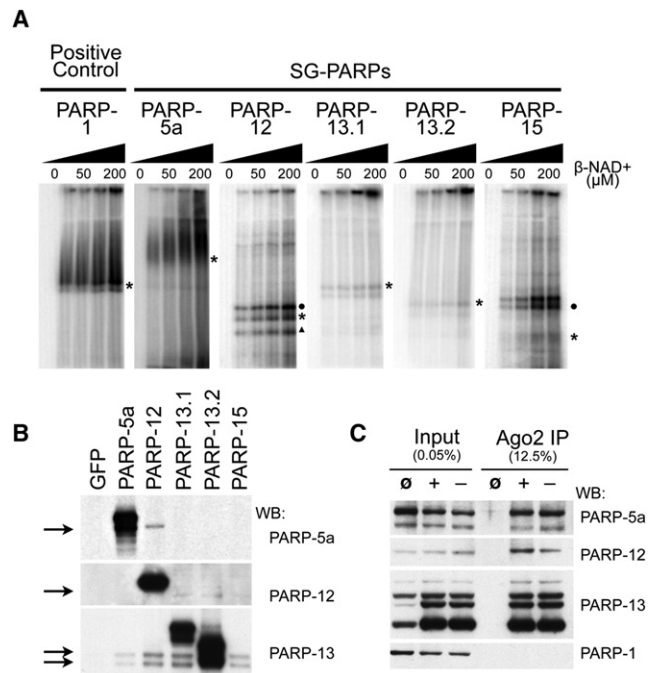


Figure 4. PARP-13 Family Members Are Poly(ADP-Ribosylated) by Other SG-PARPs

(A) *In vitro* ADP-ribosylating assay for PARP-1, -5a, -12, -13.1, -13.2, and -15. HeLa S3 cells were transfected with individual GFP-tagged SG-PARPs, and GFP-PARP immunoprecipitates were washed twice with 450 mM NaCl, then once with 150 mM NaCl. The immunoprecipitates were incubated with 0, 25, 50, 100, or 200 μM NAD^+ (with 1/175-fold of P32-labeled NAD^+) at 16°C for 30 min, separated on a 6% SDS-PAGE gel, and visualized by autoradiography. Asterisks indicate the position of the corresponding GFP-tagged SG-PARPs; circles and triangle indicate the endogenous position of PARP-13.1 and PARP-13.2, respectively.

(B) Western blots of the immunoprecipitates from (A) were probed with PARP-5a, -12, and -13 (antibodies for PARP-15 are not good for detecting endogenous protein). Immunoprecipitates from cells transfected with GFP were used as a negative control.

(C) HeLa S3 cells were transfected with GFP-tagged Ago2 and cells either treated with (+) or without (-) 20 nM pateamine A for 30 min. Untransfected cells treated with 20 nM pateamine A were used as a negative control (ø). The cytoplasmic lysates were immunoprecipitated with anti-GFP and washed thrice with cytoplasmic lysis buffer. The input and immunoprecipitates were probed with antibodies against PARP-1, -5a, -12, and -13. See also Figure S4.

were analyzed in the immunoprecipitates. Samples were analyzed either by immunoblot (Figure 4B) or mass spectrometry (LC-MS/MS) (Figure S4B). Both endogenous PARP-13 isoforms were detected in immunoprecipitates from PARP-12, -13.1, -13.2, and -15 by both methods. The interaction between PARP-13.1 and PARP-13.2 confirms that both isoforms function as a complex (Law et al., 2010). In comparison, the associations of PARP-5a and both PARP-13 isoforms are relatively weak, because they were only detected by immunoblot but not by mass spectrometry. However, the association with PARP-13 is specific with these PARPs because it was not observed with the negative control, GFP (Figure 4B). Interestingly, we also observed an association between PARP-12 and PARP-5a.

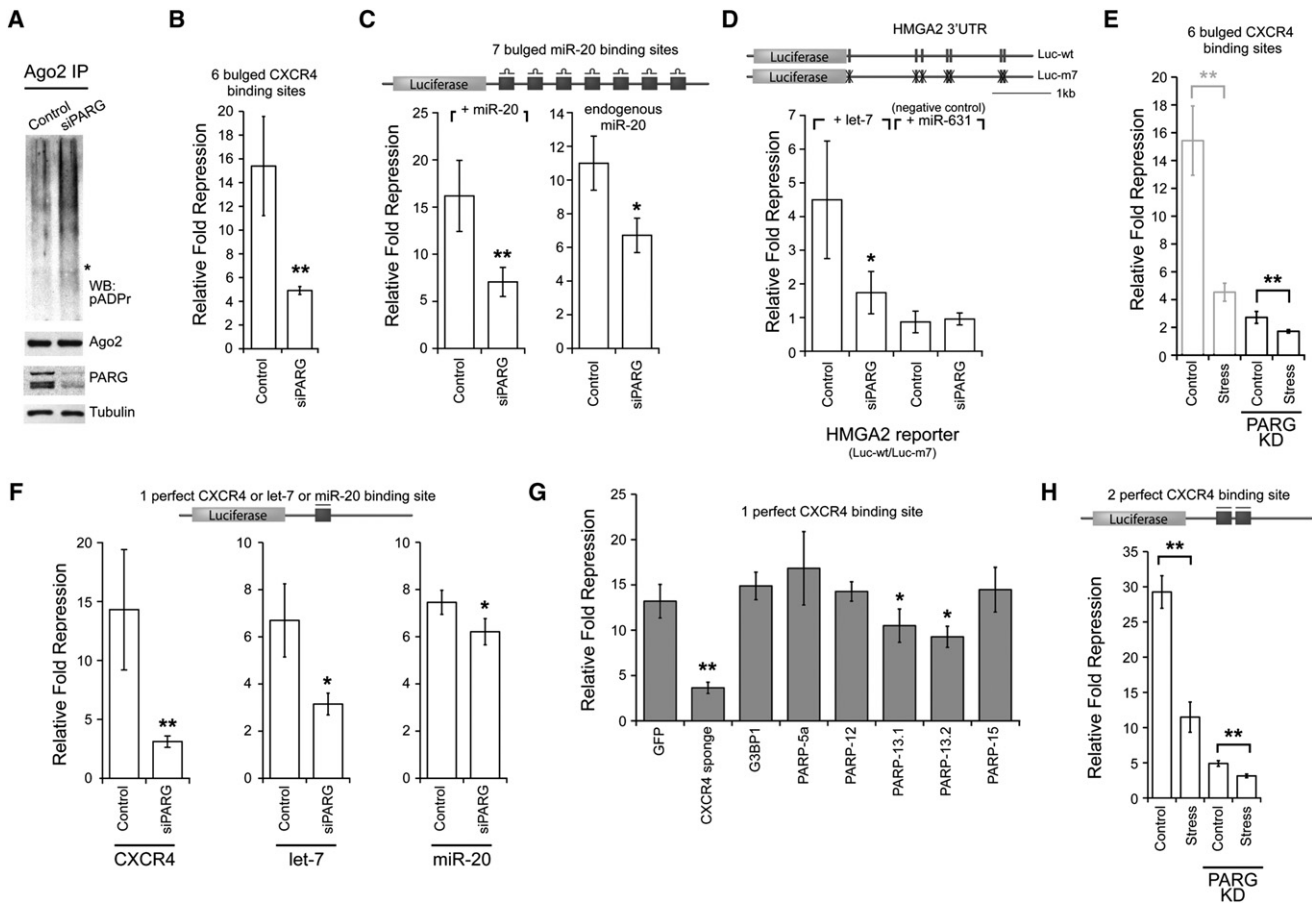


Figure 5. PARG Knockdown Alleviates miRNA-Mediated Repression and miRNA-Directed Cleavage

(A) pADPr modification levels of endogenous Ago2 in HeLa S3 cells transfected with 25 nM control siRNA or siPARG for 48 hr. Asterisk indicates where Ago2 migrated. Shown are western blots for Ago2, PARG, and tubulin.

(B) 293T cells were transfected with 25 nM control siRNA or siPARG for 72 hr. Relative fold repression was measured as in Figure 3A; $n = 3$.

(C) PARG knockdown effect observed in luciferase reporter with seven artificial miR-20 binding sites. The relative fold repression was calculated by the amount of expression of the construct normalized to a construct with all binding sites mutated at their seed positions. The assay was tested with exogenous addition of miR-20 (left) or with endogenous miR-20 (right); $n = 4$.

(D) PARG knockdown effect observed in luciferase reporter with endogenous HMG2 3' UTR. The relative fold repression is calculated by the amount of expression by the wild-type construct (Luc-wt) normalized to the mutant construct Luc-m7; $n = 4$.

(E) siPARG-transfected cells were either treated with or without 30 nM pateramine A for 2 hr (right). As a comparison, part of Figure 3A is reproduced here on the left to show cells transfected with a control siRNA.

(F) The effect of PARG knockdown on miRNA-directed cleavage was examined for luciferase construct with one perfect siCXCR4-, let-7-, or miR-20-binding site; $n = 4$ in each case.

(G) The effect of SG-PARP overexpression on miRNA-directed cleavage assay as in (F); $n = 5$.

(H) The effect of stress on miRNA-directed cleavage was tested with a luciferase reporter with two perfect binding sites for siCXCR4 using the same transfection conditions and drug treatment as in (E); $n = 3$. For (B)–(H), error bars indicate SD; paired t test $p < 0.05$ (*) and < 0.01 (**). See also Figure S5.

Therefore, it seems likely that individual PARPs associate with other PARPs and account for their respective ADP-ribosylating activities in vitro and in cells.

In light of these findings, we re-examined whether Ago2 binds to other SG-PARPs. GFP-Ago2 was immunoprecipitated from either unstressed or stressed cells and probed for individual PARPs (Figure 4C). Immunoblots identified associations between GFP-Ago2 and PARP-5a and PARP-12, in addition to PARP-13, in both unstressed and stressed cells. No such association was observed for the nuclear PARP-1. Such association of Ago2 with catalytically active PARPs is consistent with the

in vitro ADP-ribosylating activities observed in GFP-Ago2 immunoprecipitates (Figure S1H).

Knockdown of Glycohydrolase PARG Alleviates miRNA-Mediated Repression

Because pADPr modification is dynamically regulated by PARG and PARG activities, we next examined the effect of miRNA activity upon knocking down PARG. PARG knockdown results in an increase in pADPr modification on endogenous Ago2 (Figure 5A) and a ~5-fold decrease in miRNA-mediated repression (Figures 5B and S5A). Thus, similar to stress conditions, an

inverse correlation between the pADPr modification of Ago2 and miRNA-mediated repression was observed upon PARG knockdown in nonstress conditions.

The effect of PARG knockdown on miRNA activity in nonstress conditions was further examined with other constructs. Using a luciferase construct with seven miR-20 binding sites, the expression was reduced ~15-fold upon addition of exogenous miR-20, but such repression was reduced by half upon PARG knockdown (Figure 5C). Given that miR-20 is expressed in 293T cells (Landgraf et al., 2007), the repression mediated by endogenous miR-20 was examined. Under this condition, ~11-fold repression was observed, and such repression was reduced 1.8-fold in siPARG-transfected cells (Figure 5C). Similarly, miRNA-mediated repression was examined with a luciferase construct (Luc-wt) fused with the endogenous HMGA2 3' UTR, which contains seven let-7-binding sites (Mayr et al., 2007) (Figure 5D). As a control, a luciferase (Luc-m7) construct with each miRNA-binding site mutated was used to normalize expression. Upon addition of exogenous let-7, the wild-type construct was repressed by 4.5-fold compared to the mutant. On the other hand, there is no such repression upon addition of miR-631, which does not bind anywhere in the HMGA2 3' UTR. Similar to other constructs, the let-7-mediated repression is reduced by half upon PARG knockdown.

Because both PARG knockdown and stress can alleviate miRNA-mediated silencing individually, we asked whether the combination of both results in further relief in miRNA-mediated silencing (Figures 5E and S5B). Indeed, a significant further reduction of miRNA-mediated repression was observed upon stress in siPARG-treated cells, though the magnitude is less than multiplicative. Although stress resulted in a ~3.4-fold repression in control cells, only ~1.6-fold repression was observed upon stress in siPARG-treated cells. Given that stress and PARG knockdown did not synergistically attenuate miRNA silencing, it is likely that the stress pathway involved in modulating the miRNA-mediated silencing is not independent of the pathway that regulates pADPr level via PARG.

PARG Knockdown, PARP-13 Overexpression, or Stress Alleviates miRNA-Directed Cleavage

Apart from inhibiting translation or accelerating mRNA decay, miRNAs can also induce mRNA cleavage when the miRNA binds to its mRNA target in a perfectly complementary manner (e.g., Yekta et al., 2004). The effect of PARG knockdown on the miRNA-directed cleavage was next examined. First, a luciferase construct with a perfectly complementary binding site for siCXCR4 or let-7 was tested (Figures 5F and S5C). Upon addition of siCXCR4 or let-7, expression is reduced 15- or 7-fold, respectively. As in the case of the constructs with bulged configuration for siRNA/miRNA, this repression is relieved upon PARG knockdown (4.5-fold for siCXCR4 and 2-fold for let-7). Such relief in repression upon PARG knockdown was also examined for endogenous miR-20 and the repression was reduced ~1.2-fold upon PARG knockdown (Figure 5F). Because Ago2 is the only member of the Argonaute family that is capable of mediating miRNA-directed cleavage, these statistically significant decreases in repression must reflect inhibition of a complex containing this factor.

Given that the relief of miRNA-mediated translational inhibition/mRNA decay can result from overexpression of specific PARPs or stress, their effects on miRNA-directed cleavage were also examined. In the reporter construct containing one (or two) perfect siCXCR4-binding site(s), overexpression of PARP-13.1 and -13.2 reduced the level of miRNA-mediated directed cleavage by 1.3- (1.6-) and 1.4- (1.7-) fold, respectively (Figures 5G and S5D). Thus, both major miRNA-mediated processes involve PARP-13 family members. Similarly, stress reduced the repression ~2.5-fold using the reporter containing two perfect siCXCR4-binding sites (Figures 5H and S5E). When stress and siPARG treatment were combined, the repression was reduced to ~1.6-fold (Figures 5H and S5F). Thus, similar to miRNA-mediated silencing (Figure 5E), the reduced magnitude observed upon stress in siPARG-treated cells, as compared to untreated cells, is likely because the stress pathway involved in modulating the miRNA-directed cleavage overlaps with the pathway that regulates pADPr level via PARG.

DISCUSSION

pADPr Regulates Posttranscriptional Gene Expression in the Cytoplasm

We report a previously uncharacterized function for pADPr—cytoplasmic posttranscriptional regulation of mRNA. pADPr regulates miRNA silencing and catalyzes the assembly of microscopically visible SG structures. This posttranscriptional regulation occurs in the interphase cytoplasm, further extending the function of pADPr outside the nucleus. Our findings help explain several intriguing observations regarding pADPr function in the cytosol. First, pADPr synthesis and hydrolysis activities are enriched in postnuclear, postmitochondrial fractions and in the free mRNP fractions (Elkaim et al., 1983; Thomassin et al., 1985). Free mRNP fractions are enriched in factors that regulate translation and decay of mRNAs, many of which are SG components. Second, our data indicate potential functions for the two cytoplasmic isoforms of PARG, which together are more active than the single nuclear isoform (Meyer-Ficca et al., 2004). Third, large amounts of RNA-binding proteins were associated with pADPr in our previous analyses and by global proteomic analyses; some of them are SG components, including G3BP1 and PABP (Chang et al., 2009; Gagne et al., 2008).

Here, we report that specific cytoplasmic RNA-binding proteins—Ago2, G3BP1, and TIA-1—are pADPr-modified depending on the presence of their RNA-binding domains. Each protein is increasingly modified by pADPr upon stress and enriched in SGs (though not all components, such as PABP, are modified). Perhaps one general function of pADPr is to recruit RNA-binding proteins to specific locations, such as SGs, thus functioning as a scaffold for protein recruitment. This scaffold function in the cytoplasm is conceptually similar to the role pADPr plays in other complexes or structures, such as the mitotic spindle, where pADPr recruits spindle pole proteins (Chang et al., 2005, 2009); Cajal bodies, a nuclear organelle enriched in nucleic acid-binding proteins that can bind pADPr (Kotova et al., 2009); or at DNA damage sites, pADPr recruits nucleic acid-binding proteins for chromatin remodeling and DNA repair (Ahel et al., 2009). However, in contrast to these examples

involving the activation of a single PARP, we identified multiple PARPs in SGs from distinct subfamilies: Tankyrase PARP-5a; RNA-binding PARP-12 and PARP-13 isoforms; and PARP-14 and PARP-15 that contain pADPr-binding macro-domains. These pADPr-synthesizing activities along with PARG99 and PARG102 isoforms likely regulate the local pADPr concentration that determines the assembly and maintenance of SGs.

pADPr Regulates miRNA Function in the Interphase Cytoplasm

miRNA targets are preferentially expressed relative to total protein synthesis under three conditions: stress, PARP-13 overexpression, and PARG knockdown. Two of these conditions, PARP-13 overexpression and stress, trigger SG assembly. Yet this apparent correlation is paradoxical because SGs are not sites of active translation as they do not contain 60S ribosomes (Anderson and Kedersha, 2008). Therefore, any preferential translation of miRNA targets probably occurs outside SGs in the cytoplasm. Several results suggest that the two phenomena are likely to be coincidental events that are not necessarily mechanistically linked. For example, overexpression of G3BP1, a known inducer of SG assembly, does not result in the relief of miRNA silencing, whereas PARG knockdown results in the relief of miRNA silencing in the presence or absence of SG formation. This is not surprising because the majority of Ago/miRNA complexes are located in the diffuse cytoplasm; only 5% are localized in SGs upon stress and such pool is rapidly exchanging with the cytoplasm (Leung et al., 2006). Thus, the relief of miRNA silencing as a result of poly(ADP-ribosylation) likely occurs in the diffuse cytoplasm.

At what step does pADPr modulate miRNA silencing? Given that the miRNA silencing is alleviated upon increase in pADPr modification level for both endogenous miRNAs and exogenously added siRNA, pADPr likely regulates a step downstream of miRNA processing in the cytoplasm. Consistent with this, the expression levels of nearly all (>99%) miRNAs examined using miRNA microarray remained unchanged upon PARG knockdown (data not shown). In addition, the relief of miRNA silencing was observed in constructs that can be cleaved through perfectly complementary sites and silenced through partially complementary sites. Thus, pADPr likely regulates miRNA function at a step upstream of the direct activity of the Argonaute complex.

Here, we propose that the accessibility of Argonaute/miRNA complex to its target mRNA is affected by an increase in local pADPr modification on multiple proteins that bind to the target, resulting in the relief of miRNA silencing (Figure 6). Those proteins that are modified by pADPr include all Argonaute members and PARP-5a, -12, -13.1, and -13.2, among which Ago1-4 and PARP-13.2 are increasingly modified upon stress when miRNA silencing is relieved. Consistent with the importance of poly(ADP-ribosylation), relief of miRNA silencing was observed upon PARG knockdown when pADPr modification on proteins is generally increased. Such high concentration of negatively charged pADPr modification near the sites of miRNA:mRNA-binding likely disrupts the electrostatic interaction between similarly charged miRNA and mRNA. Alternatively, the sizeable pADPr modification might cause steric hindrance to

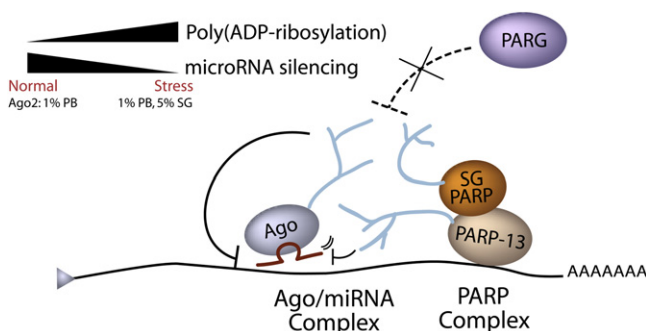


Figure 6. A Working Model: A High Local Concentration of pADPr at miRNA Complex Results in Relief of miRNA Silencing

Upon stress, multiple proteins including all Argonaute family members and PARP-13.1/2 complex are increasingly modified by pADPr. Such increase in poly(ADP-ribosylation) during stress could be due to increase in PARP activity and/or decrease in PARG activity (dotted line). High concentration of pADPr near the Argonaute/miRNA complex might disrupt electrostatic interaction or cause steric hindrance for effective miRNA silencing. Similar relief of miRNA silencing is also observed upon overexpression of PARP-13 or, conversely, upon knockdown of PARG.

prohibit effective miRNA silencing. Recently, it has been shown that in vitro addition of pADPr inhibits the RNA-binding ability of a *Drosophila* heterogeneous nuclear ribonucleoprotein (Ji and Tulin, 2009). Given that pADPr also exhibited binding affinity to RNA-binding proteins (Chang et al., 2009; Gagne et al., 2008), one function of pADPr could be to regulate the binding of RNAs to RNA-binding proteins.

One interesting observation from this study is that overexpression of PARP-13 family members affects both miRNA-mediated repression and miRNA-directed cleavage, yet these PARPs are not catalytically active. Instead, our data suggest that PARP-5a, PARP-12, and PARP-15 are likely the source of pADPr modification, given that (1) PARP-5a has demonstrated poly(ADP-ribosylating) activities and PARP-12 and PARP-15 mono(ADP-ribosylating) activities, (2) PARP-5a and PARP-12, but not PARP-1, associate with Ago2, and (3) all of these PARPs associate with endogenous PARP-13 family members. We note that the association of an inactive PARP with active PARPs resembles the case of the receptor tyrosine kinase erbB-3, which, though itself has no active kinase domain, can mediate signaling through heterodimerization with active EGF family kinases like Her2 (Holbro et al., 2003). Perhaps, because of their ability to bind mRNA, the function of the inactive PARP-13 isoforms is to anchor the activity of the catalytically active PARPs to the mRNP complex. This might partly explain why the mRNA-binding domain is required for pADPr modification of Ago2.

In conclusion, our data point to two functions for pADPr in the cytoplasm. At SGs, pADPr modulates the assembly and maintenance of an mRNP-enriched structure. At submicroscopic miRNP complexes, pADPr relieves miRNA-mediated repression and miRNA-directed cleavage under stress conditions. These cytoplasmic functions are likely mediated through the concerted activities of catalytically inactive and mADPr- and pADPr-synthesizing PARPs. Such cross-subfamily mechanism of pADPr synthesis suggests that pADPr polymerization could be

more complex than previously thought. Given that other post-transcriptional factors such as G3BP1 and TIA-1 are increasingly modified by pADPr upon stress and that these modifications depend on the presence of an RNA-binding domain, it is likely that pADPr is a key regulator of mRNA functions in the interphase cytoplasm.

EXPERIMENTAL PROCEDURES

Immunofluorescence

Following SG induction, coverslips were rinsed twice in PBS and either extracted for 30 s with buffer A, fixed for 15 min in 4% paraformaldehyde in buffer B, or fixed in ice-cold methanol for 5 min, followed by slow rehydration with PBS. Coverslips were incubated with 1° antibodies for 45 min and 2° antibodies for 35 min. See [Supplemental Experimental Procedures](#) for buffer recipes.

Immunoprecipitation

HeLa S3 cells (8×10^7) were transfected with GFP-PARPs or RNA-binding proteins for 48 hr using 293fectin, such that the expression did not induce visible SGs. Cells were either treated with or without 20 nM pateramine A or 250 μ M Arsenite for 30 min. At 10 min before stress, latrunculin B was added to 0.5 μ g/ml. Cells were lysed with cytoplasmic lysis buffer C. Lysate was spun at 18,407 g for 10 min and a final concentration of 10 μ g/ml cytochalasin B and 25 μ M nocodazole was added to the supernatant. The supernatants were incubated with anti-GFP and Protein A beads for 90 min. The immunoprecipitates were washed for 10 min with buffer C, then twice in buffer C containing 300 mM NaCl and again with buffer C. Beads were then eluted with sample buffer and heated for 10 min at 70°C. For endogenous Ago2 modification, anti-Ago2 antibody was preincubated with Protein A beads. The supernatant was made by spinning the lysate at 2,300 g or 18,407 g for 10 min, and the beads were washed 3×5 min with buffer C before eluting with sample buffer. See [Supplemental Experimental Procedures](#) for buffer recipes.

miRNA Reporter Assay

Twenty-four hours after transfection, cells were either untreated or stressed with 30 nM pateramine A, 1 μ M hippuristanol, or 250 μ M arsenite for 2 hr before lysis for luciferase assay. Firefly and Renilla luciferase signals were measured using the Dual Luciferase reporter assay system (Promega). Shown are representative luciferase assay results from 3–5 replicates, as indicated in the figure legend, in each experimental condition, and each condition has been independently examined 2–4 times.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one movie and can be found with this article online at [doi:10.1016/j.molcel.2011.04.015](https://doi.org/10.1016/j.molcel.2011.04.015).

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