PARP16 is a tail-anchored endoplasmic reticulum protein required for the PERK- and IRE1α-mediated unfolded protein response

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Poly(ADP-ribose) polymerases (PARPs; also known as ADP-ribosyl transferase D proteins) modify acceptor proteins with ADP-ribose modifications of varying length (reviewed in refs 1–3). PARPs regulate key stress response pathways, including DNA damage repair and the cytoplasmic stress response2–6. Here, we show that PARPs also regulate the unfolded protein response (UPR) of the endoplasmic reticulum (ER). Human PARP16 (also known as ARTD15) is a tail-anchored ER transmembrane protein required for activation of the functionally related ER stress sensors PERK and IRE1α during the UPR. The third identified ER stress sensor, ATF6, is not regulated by PARP16. As is the case for other PARPs that function during stress, the enzymatic activity of PARP16 is upregulated during ER stress when it ADP-ribosylates itself, PERK and IRE1α. ADP-ribosylation by PARP16 is sufficient for activating PERK and IRE1α in the absence of ER stress, and is required for PERK and IRE1α activation during the UPR. Modification of PERK and IRE1α by PARP16 increases their kinase activities and the endonuclease activity of IRE1α. Interestingly, the carboxy-terminal luminal tail of PARP16 is required for PARP16 function during ER stress, suggesting that it transduces stress signals to the cytoplasmic PARP catalytic domain.

We previously identified a reticular membrane localization for uncharacterized PARP16 in a screen analysing PARP function using the lipophilic dye Dil (S. Vyas, personal communication; Fig. 1a). To identify organelles to which it localizes, HeLa cells (used in all subsequent experiments) were stained with antibodies against PARP16 and markers for membrane-bound organelles—calnexin, lamin A/C, MTCO2, p230 and EEA1. Of these, PARP16 and calnexin localization strongly overlapped, suggesting that PARP16 is an ER protein (Fig. 1a).

On the basis of its primary sequence, PARP16 is predicted to be a tail-anchored protein with a hydrophobic transmembrane domain at amino acids 288–308 (Fig. 1b; UniProtKB; ref. 7). Tail-anchored proteins are single-spanning transmembrane proteins that contain cytoplasmic amino termini, short transmembrane domains (<30 amino acids) and C-terminal domains called C-tails (~10–15 amino acids) positioned within the lumen of target organelles. C-tails target to the ER through net positive charge rather than specific amino-acid composition and are inserted post-translationally into the ER membrane by means of the GET (Golgi–ER trafficking) complex8,9. To determine whether PARP16 is a tail-anchored protein, we performed membrane extraction assays to confirm that PARP16 is a transmembrane protein, protease protection assays to determine whether the N terminus is cytoplasmic, and truncation/mutation assays to determine whether the C terminus acts as a C-tail10. Treatment of purified membrane fractions with 1 M NaCl released the peripherally associated membrane protein lamin B2 but not PARP16, whereas treatment with 1% Triton X-100 resulted in the release of the transmembrane protein lamin B1 and PARP16, identifying PARP16 as a transmembrane protein (Fig. 1c). An N-terminal GFP fusion to PARP16, GFP–PARP16, remained membrane associated in response to digitonin treatment (in contrast to GFP-only controls), and subsequent Proteinase K treatment resulted in loss of the fluorescence signal, suggesting that the N terminus of PARP16 is cytoplasmic (Fig. 1d). Finally, a C-tail truncation (PARP16AC) mutant and a PARP16AA mutant failed to localize to the ER (a small portion of PARP16AA remained ER associated) whereas a cytochrome b5 chimera (PARP16Cb5) with the PARP16 C-tail replaced with the ER-associated cytochrome b5 C-tail retained ER localization even on digitonin treatment, demonstrating that the C terminus of PARP16 functions as a C-tail (Fig. 1e,f).

Human PARP16 ADP-ribosylates itself (S. Vyas, personal communication) and contains histidine and tyrosine residues at amino

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Received 23 July 2012; accepted 3 September 2012; published online 28 October 2012; corrected online 19 November 2012; DOI: 10.1038/ncb2593
ADP-ribosylation activity is cytoplasmic and requires His 152 and Tyr 182. Multiple migrating forms of GFP–PARP16 were detected. In addition, [\(^{32}\)P]NAD\(^{+}\) was incorporated at a higher relative molecular mass, suggesting the presence of binding proteins that are modified by PARP16 (Fig. 2a, asterisk).

Interestingly, prolonged PARP16 overexpression (>28 h) resulted in an abnormal ER morphology with >80% of PARP16-overexpressing cells containing abnormal globular ER structures (Fig. 2c and Supplementary Fig. S2). This phenotype was time and/or protein concentration dependent, as the ER appeared normal at 16 h of expression, and required PARP16 enzymatic activity and an intact C-tail, as only ~5% of cells expressing PARP16\(^{H152Q\ Y182A}\) or PARP16\(^{\Delta}\) at levels similar to wild-type PARP16 contained abnormal ER (Fig. 2c and Supplementary Fig. S2). As GFP–PARP16 and GFP–PARP16\(^{H152Q\ Y182A}\) both localized to the ER, enzymatic activity is not required for ER localization.

The abnormal ER structures resulting from prolonged PARP16 overexpression resemble the ER from stressed cells\(^{14}\), leading us to examine PARP16 function in the UPR (Fig. 2c). The UPR is an ER stress response induced by an increase in the number of unfolded proteins in the ER lumen. In mammals, three transmembrane ER stress sensors, PERK and IRE1\(\alpha\) (kinases with functionally interchangeable luminal domains) and the transcription factor ATF6, regulate separate but interconnected UPR signalling pathways (reviewed in refs 15–19). Under non-stress conditions, each sensor is bound to and inhibited by BiP, an ER specific chaperone. In the presence of stress, the sensors undergo a series of conformational changes that lead to their activation. This results in the activation of the UPR, which can be monitored by the ADP-ribosylation of PARP16 by ATF6. To determine whether PARP16 function is required during the UPR, we examined the effect of PARP16 overexpression on the activation of IRE1\(\alpha\) and ATF6.

Analysis of the PARP16 membrane topology suggests that its catalytic domain is cytoplasmic (Fig. 1f). To determine whether PARP16 ADP-ribosylation activity is cytoplasmic, and to examine its function in the context of ER membrane, we developed an ER microsome assay to monitor NAD\(^{+}\) incorporation, called the ER microsome ADP-ribosylation assay (EMAA). Microsomes were purified from cells expressing GFP–PARP16 or GFP–PARP16\(^{H152Q\ Y182A}\), incubated with [\(^{32}\)P]NAD\(^{+}\), and dissolved to extract and purify GFP–PARP16, and then [\(^{32}\)P]NAD\(^{+}\) incorporation into GFP–PARP16 was assayed using autoradiography\(^{12}\). Microsomes purified for this purpose stained positive for ER tracker, were highly enriched in ER proteins and were intact because they did not contain protein from other cellular compartments (Supplementary Fig. S1a,b). As intact ER microsomes are impermeable to NAD\(^{+}\), any incorporation of [\(^{32}\)P]NAD\(^{+}\) occurs outside the microsome lumen\(^{13}\). Self-modification of GFP–PARP16 was detected at its relative molecular mass in a NAD\(^{+}\)-dose-dependent manner whereas GFP–PARP16\(^{H152Q\ Y182A}\) failed to incorporate NAD\(^{+}\) (Fig. 2b, left), suggesting that PARP16 ADP-ribosylation activity is cytoplasmic and requires His 152 and Tyr 182. Multiple migrating forms of GFP–PARP16 were detected. In addition, [\(^{32}\)P]NAD\(^{+}\) was incorporated at a higher relative molecular mass, suggesting the presence of binding proteins that are modified by PARP16 (Fig. 2a, asterisk).
chaperone. To determine whether PARP16 functions in the UPR, we knocked it down with two short interfering RNAs (siRNAs) generated against distinct sequences, then activated the UPR using tunicamycin, brefeldin A and thapsigargin, and examined the effects. Consistent with perturbed UPR function, PARP16 knockdown rendered cells highly sensitive to ER stress, resulting in an increased level of cell death (Fig. 2d).

Figure 2 ADP-ribosylation activity of PARP16 is required for the ER stress response. (a) [32P]NAD\(^+\) incorporation by recombinant GST–PARP16 and GST–PARP16\(^{H152Q\ Y182A}\). The asterisk marks the relative molecular mass of GST–PARP16. (b) EMMA using GFP–PARP16 or GFP–PARP16\(^{H152Q\ Y182A}\)-containing microsomes. The asterisk marks incorporation of [32P]NAD\(^+\) at a high relative molecular mass. (c) Cells expressing GFP–PARP16, GFP–PARP16\(^{H152Q\ Y182A}\) or GFP–PARP16\(^{Cb5}\) for 16 h or 28 h, or untransfected cells treated with brefeldin A (BFA), stained for PARP16 (green) and calnexin (red). Scale bar, 10\(\mu\)m. (d) Left panels: quantification of trypan blue staining of control or PARP16 knockdowns at the indicated time points after tunicamycin, thapsigargin or brefeldin A treatment. 16.3 and 16.4 are different siRNAs against PARP16 (n = 4 for siRNA 16.3, and 2 for siRNA 16.4). For tunicamycin-, thapsigargin- or brefeldin-A-treated PARP16 knockdown cells, 0.001 < P < 0.05. Right, immunoblots of knockdown cells. Uncropped images of blots are shown in Supplementary Fig. S6.
The high sensitivity to ER stress in PARP16 knockdowns could also be explained by ER dysfunction or a general misregulation of cellular stress responses. However, ER function was intact in PARP16 knockdowns as measured by the intracellular concentration of reactive oxygen species (ROS) and Ca\(^{2+}\). PARP16 and control knockdown cells exhibited similar ROS generation levels, measured using CM-H\(_2\)DCFDA in the presence or absence of H\(_2\)O\(_2\), and similar Ca\(^{2+}\) leakage levels to the cytoplasm assayed using Fura-4F 340/380 nm fluorescence intensity on thapsigargin treatment (Supplementary Fig. S3a,b). Non-UPR-related cellular stress responses, such as DNA damage repair and the cytoplasmic stress response, both known to require PARP activity, were also intact in PARP16 knockdowns. PARP16 and control knockdown cells exhibited a similar response to DNA damage induced by cisplatin, with similar levels of \(\gamma\)H2AX foci formation, and cytoplasmic stress induced by arsenite, although the number of cells positive for TIA-1-staining stress granules was slightly reduced relative to controls (Supplementary Fig. S3c,d).

All known PARP-dependent stress responses result in upregulation of PARP enzymatic activity\(^1\). We examined PARP enzymatic activity during the UPR using EMAA with cells expressing GFP–PARP16 for 16 h, a condition that did not affect ER organization (Fig. 2c). All subsequent EMAAs were performed in this manner. Cells expressing GFP–PARP16 were treated with or without ER-stress-inducing agents, and PARP16 activity was assayed. ER stress resulted in significant increases in the level of GFP–PARP16 self-modification in a NAD\(^+\)-dose-dependent manner (5–8-fold increase at 100 \(\mu\)M NAD\(^+\) and 8–13-fold increase at 200 \(\mu\)M NAD\(^+\) depending on the stressor), and a marked electrophoretic mobility shift of GFP–PARP16 was detected through immunoblotting and autoradiography (Fig. 3a). Additional bands at higher relative molecular masses (\(M_r\)) were also observed on the autoradiogram, migrating at the relative molecular masses of PERK (125,000; 125K) and IRE1\(\alpha\) (130K), but not ATF6 (75K). PERK and IRE1\(\alpha\) were found in these GFP–PARP16 precipitates through immunoblotting under 450 mM NaCl conditions, demonstrating a robust association between PARP16, PERK and IRE1\(\alpha\) (Fig. 3a, right panels). These bands of NAD\(^+\) incorporation at high relative molecular masses could represent ADP-ribosylation of PERK and IRE1\(\alpha\).

To determine whether PARP16 binds to ER stress sensors in the absence of ER stress, GFP fusions to PARP16, PERK, IRE1\(\alpha\), ATF6 or SEC61\(\beta\), a UPR-unrelated ER transmembrane protein, were expressed and co-immunoprecipitation assays were performed. Whereas PERK and IRE1\(\alpha\) were present in GFP–PARP16 precipitates, and PARP16 was identified in GFP–PERK and IRE1\(\alpha\) precipitates, no significant binding was identified between ATF6, SEC61\(\beta\) and PARP16 (Fig. 3b). Thus, PARP16 selectively binds to PERK and IRE1\(\alpha\) but not to ATF6 in the presence and absence of ER stress.

Our data suggested that PERK and IRE1\(\alpha\) could be substrates of PARP16. We examined NAD\(^+\) incorporation onto GFP–PERK or GFP–IRE1\(\alpha\) using EMAA in cells transfected with control or PARP16 siRNA, and treated with or without ER-stress-inducing drugs. Low-level ADP-ribosylation of GFP–PERK and GFP–IRE1\(\alpha\) was detected in control knockdown cells in the absence of drug (Fig. 3c,d), probably due to the previously described UPR induction on PERK or IRE1\(\alpha\) expression\(^2\). ADP-ribosylation of GFP–PERK and GFP–IRE1\(\alpha\) increased under ER stress (5-fold and 4–11-fold, respectively with differences dependent on the stressor). In both cases this increase required PARP16, as modification was markedly reduced in PARP16 knockdowns (Fig. 3c,d). Neither GFP–SEC61\(\beta\) nor GFP–ATF6 was ADP-ribosylated in similar assays (Fig. 3e).

To determine the effects of ADP-ribosylation on PERK and IRE1\(\alpha\) signalling, GFP–PARP16, GFP–PARP16\(^{H152Q\ Y182A}\) or GFP alone was overexpressed at similar concentrations, and activation of PERK and IRE1\(\alpha\) was examined using two standard assays: detection of PERK phosphorylation at Thr981 and phosphorylation of its substrate eIF2\(\alpha\) at Ser51 using phospho-specific antibodies; and monitoring splicing of the IRE1\(\alpha\) substrate XBP-1 messenger RNA. Overexpression of GFP–PARP16, but not GFP–PARP16\(^{H152Q\ Y182A}\) or GFP, resulted in PERK and eIF2\(\alpha\) phosphorylation, and XBP-1 splicing (Fig. 4a,b and Supplementary Fig. S4a), suggesting that ADP-ribosylation by PARP16 is sufficient to activate PERK and IRE1\(\alpha\).

To determine whether PARP16 is required for PERK or IRE1\(\alpha\) activation, we compared activation in PARP16 knockdowns to controls. Control cells treated with brefeldin A or tunicamycin resulted in robust phosphorylation of PERK and eIF2\(\alpha\), and XBP-1 splicing, whereas PARP16 knockdowns similarly treated failed to activate PERK or IRE1\(\alpha\) (Fig. 4a,b and Supplementary Fig. S4a). As PERK and IRE1\(\alpha\) activation results in the time-dependent activation of downstream transcriptional programs regulated by ATF4 and spliced XBP-1, respectively, we analysed PERK and IRE1\(\alpha\) signalling every 4 h over a 12 h period in PARP16 knockdowns and controls treated with tunicamycin. Components of each pathway were analysed using immunoblotting or reverse transcription-quantitative PCR (RT-qPCR) analysis. Whereas IRE1\(\alpha\) activation, detected by phosphorylation of IRE1\(\alpha\), occurred 4 h post treatment in controls, such phosphorylation was barely detectable in PARP16 knockdowns at any time (Fig. 4c, left). At 4 h, spliced XBP-1 protein began to accumulate in controls, but was undetectable in PARP16 knockdowns (Fig. 4c, left). IRE1\(\alpha\)-dependent transcriptional programs were also defective in PARP16 knockdowns; in control cells, the level of unspliced XBP-1 mRNA decreased and that of spliced XBP-1 mRNA increased (Fig. 4c, right), whereas in PARP16 knockdowns, the level of unspliced XBP-1 mRNA increased, owing to ATF6 activation, and spliced XBP-1 mRNA induction was reduced 5-fold at 4 h and 15-fold at 8 h (Fig. 4c, right). P58(IPK) mRNA was reduced in PARP16 knockdowns (Fig. 4c, left). IRE1\(\alpha\) activation, and spliced XBP-1 mRNA induction was reduced at 4 h, 8 h and 12 h in control knockdowns owing to increased transcription of BiP mRNA by spliced XBP1. A minor increase in the level of BiP occurred at 12 h in PARP16 knockdowns (Fig. 4c, left).

Activation of the PERK branch occurred at 8 h in controls as determined by PERK and eIF2\(\alpha\) phosphorylation, and ATF4 synthesis. Such phosphorylation was barely detectable in PARP16 knockdowns at this time point, and PERK-dependent transcriptional programs were defective; whereas ATF3 and ATF4 mRNA began to accumulate at 8 h in controls, the level of accumulation was reduced 5-fold at 8 h and 10-fold at 12 h in PARP16 knockdowns (Fig. 4c). ATF6 activation was also monitored by examining cleavage to its active transcription factor. Cleavage occurred at 4 h in control and PARP16 knockdowns, confirming that ATF6 activation is intact in the PARP16 knockdowns (Fig. 4c, left).

Although our data strongly point to direct effects of PARP16 on PERK and IRE1\(\alpha\) signalling, compromised ERAD (ER-associated degradation) and/or chaperone capacities of the ER in PARP16
Figure 3 PERK and IRE1α are ADP-ribosylated in a PARP16-dependent manner during the UPR. UT, untreated; BFA, brefeldin A treated; TG, thapsagargin treated; TUN, tunicamycin treated. (a) Left, autoradiogram of EMAA showing ADP-ribose incorporation. Right, immunoblots of GFP–PARP16 precipitates. The asterisk marks incorporation of NAD⁺ at a high relative molecular mass. n = 5; 0.01 < P of fold increase < 0.05 for all stressors. (b) ER-microsome-based co-immunoprecipitation assays of GFP-fusion proteins. Shown are immunoblots of precipitated GFP fusions. IN, input. (c,d) EMAA using control or PARP16 knockdowns. Shown are autoradiogram and immunoblots of GFP–PERK (c) or GFP–IRE1α immunoprecipitates (d). For both c and d, n = 4; 0.005 < P of fold increase < 0.05 for all stressors. PARP16 immunoblots of control and PARP16 knockdown lysates are shown. (e) EMAA for SEC61β, ATF6 and PARP16. Shown are autoradiogram and immunoblot of the immunoprecipitated GFP fusions. n = 2. Uncropped images of blots are shown in Supplementary Fig. S6.
knockdowns could also affect UPR activation. ERAD activity in PARP16 knockdowns was examined by measuring the clearance of CD38–YFP, a model substrate of ERAD machinery. CD38–YFP degradation kinetics were similar in PARP16 knockdowns and controls as determined by cycloheximide chase assays. Inhibition of the proteasome by MG132 rescued degradation (Supplementary Fig. S5a), suggesting that ERAD activity is similar in control and PARP16 knockdowns. Cells overexpressing intermediate amounts of mCherry–PARP16 also exhibited similar kinetics of CD38–YFP clearance (Supplementary Fig. S5a), suggesting that overexpression of PARP16 does not perturb ERAD activity. The protein-folding capacity of the ER in PARP16 knockdowns seems to be similar to controls as the protein concentrations of the ER chaperones BiP and calnexin, and the disulphide isomerases PDI and ERP57, were similar (Supplementary Fig. S5b).

The increase in PARP16 enzymatic activity, and ADP-ribosylation of PERK and IRE1α during the UPR, raised the possibility that ADP-ribosylation directly regulates PERK and IRE1α enzymatic activity. We examined PERK and IRE1α kinase activity in response to ADP-ribosylation by PARP16 using self-phosphorylation assays.
ER microsomes purified from GFP–PERK- or GFP–IRE1α-expressing cells were washed with 1 M NaCl to remove bound PARP16, returned to physiological salt buffer, split into duplicate reactions and incubated with unlabelled NAD+ plus either GST–PARP16 or GST–PARP16ΔS45, or [32P]NAD+ plus either recombinant protein. ADP-ribosylated GFP–PERK and GFP–IRE1α were extracted from the microsomes and purified under 1 M NaCl conditions to remove the added recombinant PARP16 proteins. Reactions containing unlabelled NAD+ were incubated with [32P]ATP, and reactions containing [32P]NAD+ were incubated with unlabelled ATP. The level of ADP-ribosylation of GFP–PERK and GFP–IRE1α increased in a GST–PARP16- and NAD+ dose-dependent manner ([32P]NAD+ autoradiograms in Fig. 4d,e and Supplementary Fig. S4c). [32P]NAD+ incorporation at the relative molecular mass of GST–PARP16 was also observed ([32P]NAD+ autoradiograms in Fig. 4d,e and Supplementary Fig. S4c), representing residual binding of GST–PARP16 with GFP–PERK or GFP–IRE1α even after 1 M NaCl washes. As shown in the [32P]ATP autoradiograms in Fig. 4d,e and Supplementary Fig. S4c, increased ADP-ribosylation levels of GFP–PERK or GFP–IRE1α resulted in a dose-dependent increase in kinase activity (for GFP–PERK, a 4–18-fold increase depending on the NAD+ concentration, and for IRE1α, a 2–5-fold increase depending on the NAD+ concentration), suggesting that ADP-ribosylation by PARP16 directly upregulates GFP–PERK and GFP–IRE1α kinase activity. Phosphorylation by PERK at the relative molecular mass of GST–PARP16 was detected in a GST–PARP16- and NAD+ dose-dependent manner, indicating that PARP16 is a probable substrate of PERK. The level of such phosphorylation was markedly reduced (5–10-fold reduction depending on the NAD+ concentration) in GST–PARP16ΔS45ΔY182A samples (Fig. 4d and Supplementary Fig. S4c). Phosphorylation by GFP–IRE1α at the relative molecular mass of GST–PARP16 and GST–PARP16ΔS45ΔY182A was also detected (Fig. 4e), suggesting that PARP16 is an IRE1α substrate. Such phosphorylation does not seem to depend on NAD+ concentration.

Next, we examined the effects of ADP-ribosylation on IRE1α endonuclease activity. GFP–IRE1α purified as in Fig. 4e was incubated with [32P]-labelled mouse XBP-1 mRNA containing the intron flanked by truncated exons. Increased ADP-ribosylation of GFP–IRE1α resulted in a NAD+ dose-dependent cleavage of XBP-1 mRNA, indicated by the appearance of 5’ and 3’ exons (5–12-fold increase depending on NAD+ concentration; Fig. 4f), suggesting that ADP-ribosylation of IRE1α by PARP16 directly upregulates endonuclease activity.

One potential mechanism by which PARP16 regulates PERK and IRE1α is through BiP binding. We examined BiP dissociation from PERK and IRE1α during the UPR to determine whether it is affected in PARP16 knockdowns. ER microsomes were purified from control or PARP16 knockdown cells expressing either GFP–PERK or GFP–IRE1α and treated with tunicamycin. GFP fusions were purified from the microsomes over 4 h for 12 h, and immunoprecipitates were analysed for the presence of BiP. In controls, BiP dissociated from GFP–IRE1α and GFP–PERK at 4 and 8 h, respectively (Fig. 5a). In PARP16 knockdowns, BiP remained bound to GFP–PERK and GFP–IRE1α throughout the time course with a slight reduction in the level of binding, suggesting that BiP dissociation was impaired (Fig. 5a).
As BiP displacement from PERK and IRE1α occurs inside the ER lumen, these data indicate a potential function for the PARP16 C-tail in facilitating BiP dissociation from the luminal domains of PERK and IRE1α.

The requirement of PARP16 for PERK and IRE1α activation suggests that PARP16 could function upstream of PERK and IRE1α and that the C-tail of PARP16 might transduce stress signals from the ER lumen to the cytoplasmic PARP domain. To determine whether this is the case, we expressed GFP–PARP16(365) in cells and treated them with ER-stress-inducing drugs. Cells expressing GFP–PARP16(365) were unable to activate PERK and IRE1α (Fig. S5b,c and Supplementary Fig. S4b), suggesting that the luminal C-tail of PARP16 is necessary for PARP16 function in the UPR, and that PARP16(365) acts as a dominant-negative inhibitor for PARP16 function in the UPR.

We show that the ER-associated tail-anchored protein PARP16 selectively ADP-ribosylates PERK and IRE1α during the UPR, and that such modification is required for activation of PERK and IRE1α at least in part by increasing their kinase and endonuclease activities. While this manuscript was under revision, Di Paola et al. independently showed that PARP16 is ER-localized and is a tail-anchored protein22. Interestingly, Saccharomyces cerevisiae lacks PARP proteins and has only one ER stress sensor, Ire1. In the absence of stress, the peptide-binding pocket of yeast Ire1 is fully open, whereas in humans the peptide-binding pockets of IRE1α and PERK are partially closed (reviewed in ref. 19). Perhaps the C-tail of PARP16 interacts with the peptide-binding pockets of PERK and IRE1α, opening it. As ATF6 lacks a peptide-binding pocket, this could explain the selective activation of PERK and IRE1α by PARP16 (ref. 20). Furthermore, S. cerevisiae Ire1 seems to be activated primarily by direct binding of unfolded protein to the peptide-binding pocket, whereas mammalian IRE1α relies on BiP dissociation for activation (reviewed in ref. 21). PARP16 could facilitate dissociation of BiP from IRE1α and PERK on ER stress as BiP dissociation from IRE1α and PERK was impaired in PARP16 knockdowns.

Our work brings the number of stress responses requiring PARP activity to three—DNA damage, cytoplasmic stress granule assembly and the UPR. The last two stress responses converge on eIF2α phosphorylation, suggesting that PARPs could have evolved functions in regulating translation as a means to respond to cytoplasmic stresses. Cancer cells often exhibit increased protein folding capacities within the ER due to increased protein synthesis. As PARPs have been shown to be highly druggable, PARP16 could be an attractive target for therapeutic inhibition for cancers and protein-folding diseases.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary Information is available in the online version of the paper.

ACKNOWLEDGEMENTS

We thank N. Borgese (University of Milan, Italy) for sharing reagents; T. Sangro for technical assistance; and T. Jacks, H. Lodish, H. Ploegh, U. J. RajBhandary and M. Chesarone-Cataldo for comments on the manuscript. P.C. is a Rita Allen Foundation Scholar, a Kimmel Foundation for Cancer Research Scholar, and was a Howard S. and Linda B. Stern Career Development assistant professor. This work is partially financially supported by Cancer Center Support (core grant P30-CA14051), grant 5R01GM087463-02 from the National Institutes of Health (P.C.), Curt and Kathy Marble, the Jeptha H. and Emily V. Wade Fund (P.C.) and the Ludwig fund for Cancer Research fellowship (M.J.).

AUTHOR CONTRIBUTIONS

P.C. and M.J. designed the experiments and wrote the manuscript. M.J. performed the experiments and data analysis.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at www.nature.com/doi/10.1038/ncb2593

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METHODS

Cell culture and transfection. HeLa and HeLa S3 cells (ATCC) were grown in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) at 37 °C and 3% CO₂. Cells were transfected with DNA and siRNA as described previously. Stealth siRNAs (Invitrogen) directed against the human PARP16 mRNA coding region 5'-GACUGAGCCGUCCGGCUAUAIACA-3' (siRNA 16.3) and 5'-CCCAAGACUACUGGUCAACUAUA-3' (siRNA 16.4) were used to knockdown PARP16. siRNA 16.3 was used for all knockdown experiments, and 16.4 and 16.3 were used for Fig. 2d. Control siRNAs (Qagen AllStars Negative Control siRNA) were used in parallel. All experiments involving overexpression of PARP16 were performed at 16 h post-transfection, except a subset of experiments described in Fig. 2c that were performed at 28 h post-transfection. GFP–PERK, GFP–IRE1α, GFP–ATF6 and GFP–SEC61β were expressed for 24 h. To induce ER stress, tretinoin A (Sigma-Aldrich), tunicamycin (Sigma-Aldrich) and thapsigargin (Sigma-Aldrich) were added to cell cultures at 5 mM MgCl₂, 50 mM K(OAc), 12 mM glycerophosphate, 1 mM MgCl₂, 50 mM NaF, 5 mM β-glycerophosphate and protease inhibitor cocktail; for GFP–IRE1α activity, 20 mM HEPES at pH 7.4, 1 mM dithiothreitol, 2 mM Mg(OAc)₂, 1 mM NaVO₃, 30 mM NaF, 5 mM β-glycerophosphate and protease inhibitor cocktail at 25 °C for 30 min. The β²P-labelled ER microsomes were then lysed by the addition of Triton X-100 at 1%. Proteins were immunoprecipitated, eluted in 1× Laemmli sample buffer by heating at 65 °C for 10 min, and then analysed using autoradiography and immunoblotting.

Cytological, protein and immunological techniques. Immunofluorescence analysis was performed as described previously. ER-Tracker red and a lipophilic dye Dil (Molecular Probes) were used at 1 μM. Trypan blue (Sigma-Aldrich) was used at 0.2%. Immunoprecipitation and immunoblotting were carried out as described previously, with the exception that in some cases proteins were immunoprecipitated from ER microsomes. Fluorescence, biochemical protective assays and membrane extraction assays were performed as described previously. PARP16(OV2012)Δ63, PARP16Δ64 and PARP16Δ66 were generated through PCR-mediated site-directed mutagenesis using psi polymerase. To construct the PARP16Δ63 chimera, the DNA sequence of the C-terminus of cytochrome b₅ (ref. 25) was added to the reverse primer for PCR. All mutations were confirmed by DNA sequencing. XBP1-splcing assays were performed as described previously. The following antibodies were used: PARP16 (Aviva ARJP3751; Cologico Biologicals, custom-made, HM933), calnexin (BD, 610525), lamin B1 (Abcam, ab20396), lamin B2 (Abcam, ab8983), lamin A/C (Abcam, ab8984), tubulin (Abcam, ab1616), GFP (Invitrogen, A11120; Rockland, 600-401-215), red (Chromotek, 5F8), lamin B2 (Abcam, ab8983), lamin A/C (Abcam, ab8984), tubulin (Abcam, ab1616), calnexin (BD, 610523), lamin B1 (Abcam, ab20396), H2AX (Millipore, 05-636), TIA-1 (Santa Cruz, sc-1751). The anti-ATF4, p-p62 and XBP1 antibodies were from Santa Cruz (sc-32577), ab50733, and ab3715, respectively. The anti-H2AX and TIA1 antibodies were from Millipore (05-636) and Santa Cruz (sc-1751), respectively. The antibodies used were 1:50 for immunoprecipitation and 1:100 for immunofluorescence analysis.

NAD⁺ incorporation assays. For ER-microsome-based assays including NAD⁺ incorporation and immunoprecipitation, ER microsomes were fractionated from HeLa cells using an isopycnic flotation method as described previously, and incubated with 100 μM β-NAD⁺ (MP Biomedicals) and 2.5 μCi of [32P]NAD⁺ (Perkin Elmer) in a PARP reaction buffer (50 mM HEPES at pH 7.4, 130 mM NaCl, 1 mM MgCl₂, 10 mM EGTA, 1 mM dithiothreitol, 0.1 mM NaVO₃, 50 mM NaF, 5 mM β-glycerophosphate, 1 μM ADP–RF, and protease inhibitor cocktail) at 25 °C for 30 min. The ratio between unlabelled and labelled NAD⁺ was kept constant on titration of the NAD⁺ concentration. The 32P-labelled ER microsomes were then lysed by the addition of Triton X-100 at 1%. Proteins were immunoprecipitated, eluted in 1× Laemmli sample buffer by boiling at 65 °C for 10 min, and then analysed using autoradiography and immunoblotting. For assays using recombinant proteins, GST–PARP16 wild-type and mutant isoforms were purified from BL21 E. coli cells, following the manufacturer’s protocol (Strategen). NAD⁺ incorporation reactions were performed under the same conditions as described above.

Kinase assays. PERK and IRE1α kinase assays were performed as described previously, with an exception that the kinase activities were measured post NAD⁺ incorporation by PARP16 in the context of ER microsomes. In brief, ER microsomes were subject to NAD⁺ incorporation assays in a PARP buffer as described in the NAD⁺ Incorporation Assays section using 100 μM unlabelled β-NAD⁺ (MP Biomedicals) and GST–PARP16 wild-type or catalytically inactive mutant proteins purified from bacteria. The ADP-ribosylated ER microsomes were incubated with 100 μM ATP (New England BioLabs) and 2.5 μCi of [32P]ATP (Perkin Elmer) in a kinase buffer (for GFP–PERK activity, 20 mM HEPES at pH 7.4, 50 mM KCl, 1.5 mM dithiothreitol, 2 mM Mg(OAc)₂, 2 mM MnCl₂, 0.1 mM NaVO₃, 30 mM NaF, 5 mM β-glycerophosphate and protease inhibitor cocktail; for GFP–IRE1α activity, 20 mM HEPES at pH 7.4, 1 mM dithiothreitol, 10 mM Mg(OAc)₂, 50 mM K(OAc), 0.1 mM NaVO₃, 50 mM NaF, 5 mM β-glycerophosphate and protease inhibitor cocktail) at 25 °C for 30 min. The β²P-labelled ER microsomes were then lysed by the addition of Triton X-100 at 1%. Proteins were immunoprecipitated, eluted in 1× Laemmli sample buffer by heating at 65 °C for 10 min, and then analysed using autoradiography and immunoblotting.

IRE1α endonuclease assays. A 479-base-pair (bp) mouse XBP-1 DNA fragment containing the intron and flanking exons on both sides (263 bp on the 5′ end and 191 bp on the 3′ end) was amplified using PCR using a reverse primer containing the T7 RNA polymerase promoter sequence. In vitro transcription of XBP-1 mRNA and XBP-1 cleavage assays were performed essentially as described previously. A gel-purified transcript equivalent to ~20,000 cpm was incubated with ADP-ribosylated GFP–IRE1α immunoprecipitates in an IRE1α kinase buffer plus 2 μM unlabelled ATP at 25 °C for 30 min. The cleavage products were analysed on 10% TBE–Urea polyacrylamide gels.

ROS generation and Ca²⁺ measurement. ROS generation was evaluated using a cell-permeant carboxyethyl derivative of fluorescent (CM-H₂DCFDA, Invitrogen), following the manufacturer’s protocol (Invitrogen). Cells were loaded with CM-H₂DCFDA at 5 μM for 30 min at 37 °C, and then treated with H₂O₂ at 100 μM for 10 min at 37 °C. The fluorescence intensity of the oxidized ROS probe was measured using a microplate reader (Tecan). To measure the intracellular Ca²⁺ concentration, cells were loaded with a cell-permeant Ca²⁺ probe, Fura-4F (Invitrogen), at 2 μM in HEPES-buffered Krebs-Ringer solution containing 2 mM CaCl₂ for 30 min at 37 °C. The ratiometric fluorescence intensity at 340 nm/380 nm was measured every 2 min for 20 min, using a microplate reader (Tecan). Thapsigargin was added at 1 μM, and EGTA was used at 3 μM to chelate the released Ca²⁺. To induce cytoplasmic stress and DNA damage, arsenite (Sigma-Aldrich) and cisplatin (Sigma-Aldrich) were used at 100 μM and 10 μM, for 30 min and 8 h, respectively. To inhibit translation and the proteasome, cycloheximide (Sigma-Aldrich) and MG132 (Sigma-Aldrich) were used at 100 μg ml⁻¹ and 10 μM, respectively. Total RNA was extracted using RNeasy kits and QIAshredder (Qiagen), and complementary DNA was amplified using the Quantitect reverse transcription kit (Qiagen). RT-qPCR was performed with the QuantiTect SYBR Green PCR kit (Qiagen), using a LightCycler 480 (Roche). mRNA levels were normalized against GAPDH mRNA.

Statistics. All experiments were repeated a minimum of two times and the unpaired Student t-test was used for statistical analysis.

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PARP16 is a tail-anchored endoplasmic reticulum protein required for the PERK- and IRE1α-mediated unfolded protein response

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Nat. Cell Biol. 14, 1223–1230 (2012); published online 28th October 2012; corrected online 15th November 2012

While this manuscript was under consideration, an article by Di Paola et al. reported an ER localization for PARP16. In the version of this Letter initially published online and in print, the reference (PLoS ONE 7, e37352; 2012) was inadvertently omitted from the reference list. On page 8, the following text has replaced the previous text:

We show that the ER-associated tail-anchored protein PARP16 selectively ADP-ribosylates PERK and IRE1α during the UPR, and that such modification is required for activation of PERK and IRE1α at least in part by increasing their kinase and endonuclease activities. While this manuscript was under revision, Di Paola et al. independently showed that PARP16 is ER-localised, and a tail-anchored protein. Interestingly, Saccharomyces cerevisiae lacks PARP proteins and has only one ER stress sensor, Ire1.

The omitted reference has now been added to the reference list:

22. Di Paola, S., Micaroni, M., Di Tullio, G., Buccione, R. & Di Girolamo, M. PARP16/ARTD15 is a novel endoplasmic-reticulum-associated mono-ADP-ribosyltransferase that interacts with, and modifies karyopherin-β1. PLoS ONE 7, e37352 (2012).

References 22–27 have been changed to 23–28, respectively.
Figure S1 Analysis of ER microsomes purified from untransfected HeLa cells. MW (kD) at right or left of blots. a, Purified ER microsomes stained with ER-Tracker Red. RER=Rough ER.  

b, Purified ER microsomes immunoblotted for indicated proteins.  
c, Coomassie-stained gel showing amounts of GST-PARP-16 and GST-PARP-16H152Q,Y182A utilized for reaction shown in Fig. 2a. Asterisk=full-length proteins. Bar, 1 μm.
Figure S2 Prolonged overexpression of PARP-16 causes abnormal ER structures. a, Cells expressing GFP-PARP-16, GFP-PARP-16H152Q Y182A, or GFP-PARP-16C508 for 16h or 28h stained for GFP-PARP-16 (green), Calnexin (red) and DNA (blue). In each condition, expression levels of GFP fusion proteins were monitored via immunoblot (b), and abnormal ER structures quantitated. n=3 (c). Bar, 10 μm.
Figure S3 Effects of PARP-16 knock-down on ROS generation, Ca\(^{2+}\) leakage from the ER, and cellular responses to DNA damage or cytoplasmic stress. 

**a,** ROS generation in control and PARP-16 knock-downs. Shown are values in arbitrary unit (A.U.) for fluorescence intensity of CM-H\(_2\)DCFDA before and after H\(_2\)O\(_2\) treatment. n=2; p > 0.05 after H\(_2\)O\(_2\) treatment.

**b,** Intracellular Ca\(^{2+}\) concentration in control and PARP-16 knock-downs. Shown are values in arbitrary unit (A.U.) for fluorescence intensity ratio 340 nm/380 under basal conditions (time points 1 – 3), during Thapsigargin treatment (time points 4 – 7), and after EGTA addition (time points 8 – 11). Each time points are 2 min apart. n=2; p > 0.1 after Thapsigargin treatment.

**c,** Cells treated with or without Cisplatin were immuno-stained for γ-H2AX (red) and DNA (blue), and γ-H2AX positive cells were counted. n=2; p > 0.1 in PARP-16 knock downs.

**d,** Cells treated with or without Arsenite were immuno-stained for TIA-1 (red) and DNA (blue), and TIA-1 positive cells were counted. n=2; p < 0.05 in PARP-16 knock downs. Bars, 10 μm.
Figure S4 PARP-16 is required for IRE1α-mediated UPR. Full Images of agarose gels shown in Fig. 4b (a) and Fig. 5a (b). Asterisks= 290 and 183 bp fragments originated from unspliced XBP-1 cDNA, upon digestion with PstI restriction enzyme. Triangle represents hybrid amplicons. (OE)= over-expression; (ctrl)= control; (P-16)= PARP-16; (U)= unspliced; (S)= spliced; (UT)= untreated; (BFA)= Brefeldin A treated; (TUN)= Tunicamycin treated. c, ER microsome based NAD⁺ incorporation and kinase assays. ER microsomes containing GFP-PERK were (ADP-ribosyl)ated using either GST-PARP-16 or GST-PARP-16H152Q Y182A in the presence of ³²P-NAD⁺. The duplicate NAD⁺ incorporation reactions were performed under the same conditions using unlabeled NAD⁺ instead, and then subjected to kinase assays using ³²P-ATP.
Figure S5 PARP-16 knock-down does not affect ERAD and chaperoning activities. **a**, Knock-down cells expressing CD3δ-YFP were treated with Cycloheximide in the presence or absence of MG132. CD3δ-YFP clearance, as a measure of ERAD activity, was monitored via immunoblot of lysates. Cells co-expressing CD3δ-YFP and a mCherry fusion to either PARP-16 or PARP-16*H152Q Y182A*, were also analyzed. Immunoblot of PARP-16 and mCherry fusions are shown. **b**, Immunoblots for indicated proteins. (UT)= untreated; (TUN)= Tunicamycin treated; (Ctrl)= control; (P-16)= PARP-16.
Figure S6 Uncropped data.
Figure S6 continued