Structural and functional and analysis of the GADD34:PP1 eIF2α phosphatase

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Supplemental Methods

Cloning and protein expression. PP1α$_{7-300}$ and PP1α$_{7-330}$ were expressed as previously described. Plasmid encoding human GADD34$_{241-674}$(Mikami et al., 2010) was sub-cloned into pTHGT vector that encodes an N-terminal His$_6$-tag followed by glutathione S-transferase (GST) and a TEV (tobacco etch virus) protease cleavage site (pTHGT and pTHMT generously provided by Dr. Cynthia Kinsland, Cornell University). Codon-optimized human GADD34 DNA (552-621, 333-513 and 513-674) was purchased from DNA2.0. GADD34$_{333-463}$ was sub-cloned into pTHMT vector that encodes an N-terminal His$_6$-tag followed by Maltose-Binding Protein (MBP) and a TEV cleavage site. All other GADD34 fragments (513-631, 552-621, 552-602, 552-591 and 333-375) were subcloned from the gene-optimized templates into the RP1B vector that encodes an N-terminal His$_6$-tag followed by a TEV protease cleavage site. The plasmid encoding PKR was obtained from Addgene (Plasmid # 42934) and sub-cloned into RP1B. Expression plasmids of solubility enhanced human eIF2α (pET30-heIF2α$_{4-185}$) were used as previously described(Ito and Wagner, 2004). Mutations in GADD34 were introduced using site directed mutagenesis (Agilent) following the manufacturer's protocol. All constructs were sequence verified.

Expression of GADD34 was carried out either in *E. coli* BL21 (DE3) or BL21-CodonPlus (DE3)-RIPL competent cells (Agilent). Cells were grown in Luria Broth (LB) in the presence of selective antibiotics at 30°C to an OD$_{600}$ of 1.0-1.5. The temperature was lowered to 18°C for an hour and expression induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) into the culture medium. Expression of GADD34$_{552-621}$, GADD34$_{552-602}$ and GADD34$_{552-591}$ was carried out at 37°C for 3-4 h. After induction, cells were harvested by centrifugation at 6000x*g* and the pellets were stored at -80°C until purification.

Purification of PP1α. All PP1 purifications were performed at 4°C. About 5 – 10 g dry weight of cells expressing His$_6$-TEV-PP1α$_{7-300}$ or His$_6$-TEV-PP1α$_{7-330}$ were lysed in PP1 Lysis Buffer (25 mM Tris pH 8.0, 700 mM NaCl, 5 mM imidazole, 1 mM $MnCl_2$, 0.1% Triton X-100) using high-pressure homogenization (Avestin C3 EmulsiFlex) in the presence of EDTA-free protease inhibitor cocktail (Roche). The lysate was clarified by centrifugation at 100,000x*g* and filtered through 0.22 µm polyethersulfone (PES) membrane filter (Millipore) before being loaded onto Ni^{2+}-NTA resin (Qiagen) in a gravity column. Bound His$_6$-TEV-PP1α was washed with 100 mL of PP1 buffer A (25 mM Tris pH 8.0, 700 mM NaCl, 5 mM imidazole, 1 mM $MnCl_2$), followed by more stringent wash with 100 mL stringent wash buffer, consisting of 94% PP1 buffer A and 6% PP1 buffer B (25 mM Tris pH 8.0, 700 mM NaCl, 250 mM imidazole, 1 mM $MnCl_2$). The bound His$_6$-TEV-PP1α was eluted using PP1 buffer B and immediately purified using size exclusion chromatography (SEC, Superdex 75 26/60; 20 mM Tris pH 8.0, 500 mM NaCl, 0.5 mM TCEP). Peak fractions from the SEC were collected and incubated overnight with TEV protease (in-house) at 4°C. The following day, the cleaved PP1α protein was loaded onto Ni^{2+}-NTA resin (Qiagen) to remove the His$_6$-tag and TEV and the flow-through, which consist of PP1α was collected. The cleaved PP1α was further purified using SEC. Pure PP1α was pooled and immediately used.

Purification of GADD34. GADD34$_{552-621}$, GADD34$_{552-602}$ and GADD34$_{552-591}$ were purified using Denaturing Buffer (100 mM NaH_2PO_4, 10 mM Tris, pH 8.0, 8 M urea). Cells were lysed using high-pressure homogenization (Avestin C3 EmulsiFlex). The lysate was clarified by centrifugation at 100,000x*g*, filtered through 0.44 µm PES membrane filter (Millipore) and loaded onto Ni^{2+}-NTA

resin (Qiagen) in a gravity column. The column was successively washed in the denaturing buffers that differed only in their pH, decreasing from pH 8.0, 6.3 to 5.9. GADD34 was eluted at pH 4.5. Prior to TEV cleavage, the eluted GADD34 was dialyzed against GADD34 dialysis buffer (50 mM NaOAc pH 5.0, 0.5 mM TCEP) overnight at 4°C to remove the urea. TEV was then added and the TEV-GADD34 sample incubated at 4°C until the cleavage was completed. Since $GADD34_{552-621}$, $GADD34_{552-602}$ and $GADD34_{552-591}$ weakly associate with the Ni-NTA beads, urea was added back into the protein samples to perform a subtraction purification to remove the cleaved His_6-tag and TEV. Purified GADD34 was then dialyzed against GADD34 dialysis buffer overnight at 4°C and subsequently concentrated to ~1 mM using a 3 kDa cut off concentrator (Amicon, Millipore). Heat purification (80°C, 15 min) was used to further purify the protein and to efficiently inactivate and remove all proteases. The GADD34 proteins were flash frozen as 100 µL aliquots and stored at -80°C until used.

For $GADD34_{241-674}$ and $GADD34_{513-631}$, $GADD34_{333-375}$ and $GADD34_{333-463}$, proteins were purified using native conditions. Lysate was prepared as described but instead of denaturing buffer, cells were lysed in lysis buffer (25 mM Tris pH 8.0, 500 mM NaCl, 5 mM imidazole, 0.1% Triton X-100) using high-pressure homogenization (Avestin C3 EmulsiFlex) in the presence of EDTA-free protease inhibitor cocktail (Roche). The lysate was clarified by centrifugation at 50,000x*g* and loaded onto a HisTrap HP column (GE Healthcare). Elution was carried out in a 50 mM Tris pH 8.0, 500 mM NaCl buffer using a 5 – 500 mM imidazole gradient. Peak fractions were pooled and dialyzed overnight at 4°C (20 mM Tris pH 8.0, 150 mM NaCl, 0.5 mM TCEP for $GADD34_{513-631}$ and $GADD34_{241-674}$; 50 mM NaPO4 pH 7.0, 350 mM NaCl, 0.5 mM TCEP for $GADD34_{333-375}$ and $GADD34_{333-463}$) with TEV protease for His_6-tag cleavage. After dialysis, subtraction purification was performed to remove the TEV and His-tag. Proteins were further purified by SEC or anion exchange (HiTrap Q Sepharose FF, GE Healthcare). Finally, the proteins were concentrated to 1 mM, subjected to heat purification (80°C, 15 min) before being flash frozen as 100 µL aliquots and stored at -80°C.

Crystallization of the GADD34:PP1 holoenzyme. The GADD34:PP1 complex used for crystallization trials was formed as follows (the stability of the GADD34:PP1 complex is sensitive to salt concentration and significant, ≥50%, dissociation of the complex is observed at ionic strengths above 150 mM NaCl; **Fig. S2h,i**). Immediately following the TEV cleavage, PP1 was purified using SEC (25 mM HEPES pH 6.8, 500 mM NaCl, 0.5 mM TCEP). Pooled $PP1\alpha_{7-300}$ fractions were then combined with purified GADD34 at 1:3 molar ratio in buffer with a reduced NaCl concentration (20 mM HEPES, pH 6.8, 250 mM NaCl, 0.5 mM TCEP) that allowed for complex formation, while still being high enough to stabilize free PP1. GADD34:PP1 was then concentrated to 8 mg/mL (Amicon Ultra-15, Millipore) and immediately used for crystallization trials.

Crystals of $GADD34_{552-591}$:$PP1\alpha_{7-300}$ holoenzyme were grown using sitting drop (200 nl) vapor diffusion in 0.2 M ammonium phosphate dibasic, 20% w/v polyethylene glycol 3350. For X-ray diffraction, crystals were cryo-protected by soaking briefly in mother liquor supplemented with 30% (v/v) glycerol and immediately flash frozen in liquid nitrogen. X-ray data to 2.29 Å were collected at the beamline 12.2 Stanford Synchrotron Radiation Lightsource (SSRL) at 100 K and a wavelength of 0.98 Å using a Pilatus 6M PAD detector. The $GADD34_{552-591}$:$PP1\alpha_{7-300}$ structure was solved by molecular replacement using Phaser as implemented in Phenix, using $PP1_{7-300}$ (PDBID: 3E7A) as the search model (Kelker et al., 2009). A solution was obtained in space group $P2_12_12_1$; clear electron density for the bound GADD34 was visible in the initial maps. The initial models of the $GADD34_{552-591}$:$PP1\alpha_{7-300}$ were built using Phenix.AutoBuild, followed by iterative rounds of refinement in PHENIX and manual building using Coot. No residues are in the disallowed region of the Ramachandran diagram, as determined using MolProbity (Davis et al., 2004). To confirm that the $GADD34_{552-591}$ used in the crystallization of GADD34:PP1 was not

proteolytically degraded prior to crystal formation, 20 crystals were washed 5-times in 10 μL of mother liquor at 4°C. SDS loading buffer was added into the final droplet to solubilize the crystals. The sample was transferred into a 1.7 ml tube, heated to 95°C for 5 min and resolved using SDS-PAGE.

NMR spectroscopy. For NMR measurements, expression of uniformly ^{15}N- or ^{15}N/^{13}C-labeled GADD34$_{513-631}$, GADD34$_{552-621}$ and eIF2α$_{4-185}$ was achieved by growing the bacteria in M9 minimal media containing 1 g/L ^{15}NH4Cl and/or 4 g/L [^{13}C]-D-glucose as the sole nitrogen and carbon sources, respectively.

NMR measurements were performed at 298 K in NMR buffers (^{15}N-GADD34$_{513-631}$ and GADD34$_{552-621}$: 20 mM Na-phosphate pH 5.5, 0.5 mM TCEP, 10% D_2O; ^{15}N-labeled eIF2α$_{4-185}$: 50 mM Na-phosphate, pH 7.0, 350 mM NaCl, 0.5 mM TCEP, 10% D_2O). The sequence-specific backbone assignment for GADD34$_{552-621}$ was obtained from the following experiments performed on a Bruker Avance 500 MHz spectrometer with a HCN TCI z-gradient cryoprobe: 2D [^{1}H,^{15}N] HSQC, 3D HNCA, 3D HNCACB, 3D CBCA(CO)NH and 3D (H)CC(CO)NH (τ_m= 12 ms). The NMR spectra were processed and analyzed using Topspin 3.1 (Bruker) and CARA software package (www.cara.nmr.ch). The hetNOE measurement and secondary structure propensity calculation were performed as previously described.

Isothermal titration calorimetry. GADD34$_{552-567}$ (NH2-KARKVRFSEKVTVHFLA-COOH) peptide for ITC was purchased from Bio-Synthesis, Lewisville, Texas. The peptide was dissolved in ITC buffer (20 mM Tris pH 8.0, 500 mM NaCl, 0.5 mM TCEP). GADD34$_{552-567}$ was titrated into PP1α$_{7-330}$ using a VP-ITC micro-calorimeter at 25ºC (Microcal, Inc.). Data collected was analyzed using NITPIC and fitted using SEDPHAT (Keller et al., 2012).

Fluorescence anisotropy. GADD34$_{552-621}$ was labeled with the fluorescent dye DyLight 488 (Life Technologies) at the single cysteine (residue Cys$_{606}$) according to the manufacturer protocol. Labeling of GADD34$_{552-621}$ was confirmed by SDS-PAGE and fluorescent detection using a Typhoon 9410 laser scanner (excitation 488 nm/emission 532 nm). The binding of DyLight 488-GADD34$_{552-621}$ to PP1α$_{7-330}$ was assessed by measuring the increase of fluorescence anisotropy upon binding to PP1α$_{7-330}$ in assay buffer (20 mM Tris pH 8.0, 150 mM NaCl, 0.5 mM TCEP, 1 mM $MnCl_2$). Briefly, 1 μM of DyLight 488-GADD34$_{552-621}$ was added to increasing concentrations of PP1α$_{7-330}$. The mixtures were incubated for 20 min (RT) in the dark, so the binding reached equilibrium. The reaction tubes were centrifuged (14,000xg; 5 min) and the supernatants were collected for measurements. Fluorescence anisotropy was measured in triplicate using a FluoroMax-4 spectrofluorometer (Horiba; 200 μL MicroQuartz fluorometer cell; Starna Cells). For the competitive displacement assays, 1 μM of DyLight 488-GADD34$_{552-621}$ and varying concentrations of unlabeled GADD34$_{552-621}$ or GADD34$_{552-567}$ were added to ~1 μM PP1α$_{7-330}$ in assay buffer. The mixtures were incubated for 30 min (RT) in the dark. Competitive displacement of the labeled peptide by unlabeled peptides was detected as a decrease in fluorescence anisotropy. The measurements were collected in triplicate and all experiments were repeated twice.

Immuno-precipitation and pull-down assays. Flag-tagged GADD34 WT and KARA variant were over-expressed in HEK293T cells as previously reported (Brush et al., 2003). Cells were lysed in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% (v/v) NP-40, 0.5% (v/v) Na-deoxycholate, 0.1% SDS) supplemented with protease inhibitor tablet (Roche). Clarified (14,000x*g*, 10 min) cell lysate was incubated with anti-Flag M2 affinity beads (Sigma) for an hour at 4°C. Beads were washed again with RIPA buffer before re-suspending in SDS loading buffer. Sample was resolved using SDS-PAGE and transferred to PVDF membrane. Membrane with

transferred proteins was blocked with 2% BSA in TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween-20) before incubation with specific primary antibodies overnight at 4°C. Horseradish peroxidase conjugated secondary antibodies were used to detect the bound primary antibody. The membrane was thoroughly washed before chemiluminescent detection using Amersham ECL detection reagent (GE Healthcare) and X-ray film (Kodak). For pull down assays, recombinant proteins His-GADD34$_{241-674}$ and His-GADD34$_{513-631}$ were incubated with clarified HEK293T cell lysate prepared according to method described above but EDTA-free protease inhibitor tablets were used instead. Ni-NTA beads (Qiagen) were used for the pull down of His-tagged proteins. The target proteins, PP1α and eIF2α were detected using Western blot (Brush et al., 2003).

Fluorescence microscopy and BiFC assay. GFP-tagged GADD34 and neurabin-1 were cloned into pEGFP (Clontech) as previously described (Brush et al., 2003). COS-7 cells (ATCC) were grown using glass bottom Lab-Tek™ Chamber Slide™ (Nunc) in DMEM medium supplemented with 10% Fetal Bovine Serum (FBS) and penicillin-streptomycin and maintained in a 5% CO2 incubator at 37°C. Transient transfections were performed using low cytotoxicity FuGENE 6 transfection reagent (Promega) according to the manufacturer's instructions. Briefly, cells were grown to 60-70% confluence. Prior to transfection, serum containing cell culture medium was changed to low serum OPTI-MEM (Life Technologies) medium. Plasmid DNA (1 μg) encoding proteins of interest was mixed with 100 µL OPTI-MEM and 4.5 µL of FuGENE 6. The mixtures were incubated at room temperature for 30 min before added drop wise into the cells. Imaging was carried out the following day. Prior to imaging, culture medium was changed to imaging buffer (DMEM without phenol red, supplemented with 20 mM HEPES buffer and 10% FBS). All imaging experiments were carried out in a temperature controlled and CO_2 delivery chamber and were completed within an hour after buffer changed.

For BiFC assays, plasmid DNAs encode the yellow fluorescence protein (YFP) pair (YN-PP1α & YC-eIF2α), together with the scaffolding proteins GADD34 and neurabin-1 were transfected into 60 - 70% confluence COS-7 cells grown in glass bottom Lab-Tek™ Chamber Slide™ (Nunc) using FuGENE 6. A vector control (Cyan Fluorescence Protein, CFP) was used to evaluate the transfection efficiency, and also to normalize and quantify the BiFC signal. To minimize non-specific BiFC signal, lower plasmid concentrations were used (250 ng for the BiFC pairs and scaffolding protein; 50 ng for the CFP vector control). Transfected cells were grown overnight in a 37°C incubator with 5% CO_2. Two hours before imaging, cells were transferred to an incubator pre-set at 30°C for the maturation of the complemented YFP. The live cell images were captured using Nikon confocal microscope with motorized stage with environment chamber for temperature control and CO_2 delivery. Image analysis was performed using MetaMorph (Molecular Devices).

Phosphatase assays. eIF2α$_{4-185}$ was purified as previously described(Ito and Wagner, 2004). Briefly, cells expressing eIF2α-His$_6$ were lysed in lysis buffer (25 mM Tris pH 8.0, 500 mM NaCl, 5 mM imidazole, 0.1% Triton X-100) with EDTA-free protease inhibitor cocktail (Roche) using high-pressure homogenizer (Avestin C3 EmulsiFlex). The cell lysate was clarified by centrifugation (50,000x*g*, 4°C), filtered through 0.22 µm PES membrane filter (Millipore) and loaded onto a HisTrap HP column (GE Healthcare). The eluted eIF2α was collected, concentrated and further purified using SEC (Superdex 75 26/60) in SEC buffer (50 mM Na-phosphate pH 7.0, 350 mM NaCl, 0.5 mM TCEP). The peak fractions were collected and concentrated up to 12 mg/mL, flash frozen in 100 μL aliquots and stored at -80°C until used. PKR was purified according to methods previously described (Xu et al., 2004). Phosphorylation of eIF2α by PKR was carried out in kinase buffer (15 mM Tris pH 7.5, 0.5 mM $MgCl_2$/ATP at 30°C for 30 min). Phosphorylated eIF2α was purified from PKR by SEC (50 mM Na-phoshate pH 7.0, 350 mM NaCl, 0.5 mM TCEP).

Dephosphorylation of pNPP was initiated by the addition of 5 μM PP1$\alpha_{7\text{-}330}$, GADD34$_{513\text{-}631}$:PP1$\alpha_{7\text{-}330}$ (3:1 molar ratio) or GADD34$_{552\text{-}591}$:PP1$\alpha_{7\text{-}330}$ (3:1 molar ratio) to reaction mixtures containing 4 mM pNPP in assay buffer (150 mM Bis-Tris pH 6.5, 150 mM NaCl). The reaction kinetics were measured at 30°C using a temperature controlled SpectraMax M5 microplate reader. Absorbance readings at 405 nm were taken every 2 min for 90 min. The PP1 inhibitor microcystin-LR (100 nM final; stock in DMSO) was used as a negative control for the assay.

Similarly, dephosphorylation of PKR-phosphorylated eIF2$\alpha_{4\text{-}185}$ was carried out in similar buffer conditions at 30°C for 30 min. The reaction was stopped by adding SDS-loading buffer and the samples were boiled for 5 min. eIF2$\alpha_{4\text{-}185}$ was resolved using SDS-PAGE. Gel was fixed and then stained with Pro-Q Diamond phosphoprotein gel stain (Life Technologies) according to manufacturer's instruction. After destaining, the gel was scanned using a Typhoon 9410 laser scanner (GE Healthcare) and the level of phosphorylation was quantified using ImageQuant (GE Healthcare). For total protein, the same gel was stained with SYPRO Ruby protein stain (Life Technologies) overnight and scanned with a Typhoon 9410 laser scanner (GE Healthcare).

Table S1 Crystallographic data collection and refinement statistics, Related to Figure 2.

	GADD34:PP1[s]
Data Collection	
Space group	$P\ 2_1 2_1 2_1$
Cell dimensions	
a,b,c (Å)	64.31, 112.85, 125.01
α, β, γ (°)	90, 90, 90
Resolution (Å)	50.0 – 2.29 (2.37 – 2.29)*
R_{merge}	0.11 (0.75)
I/σI	14.64 (2.08)
Completeness (%)	97.31 (83.25)
Redundancy	6.4 (6.3)
Refinement	
Resolution (Å)	31.43 – 2.29
No. reflections	40,789
$R_{work}{}^{d}/R_{free}{}^{e}$	0.161/0.205
No. atoms	
Protein	4925
Water	223
Mn^{2+}	4
B factor	
Protein	58.9
Water	58.2
Ions	38.1
R.m.s. deviations	
Bond length (Å)	0.011
Bond angle (°)	1.007
PDB Code	4XPN

[s]1 crystal

*highest resolution shell is shown in parentheses

Supplementary Figures

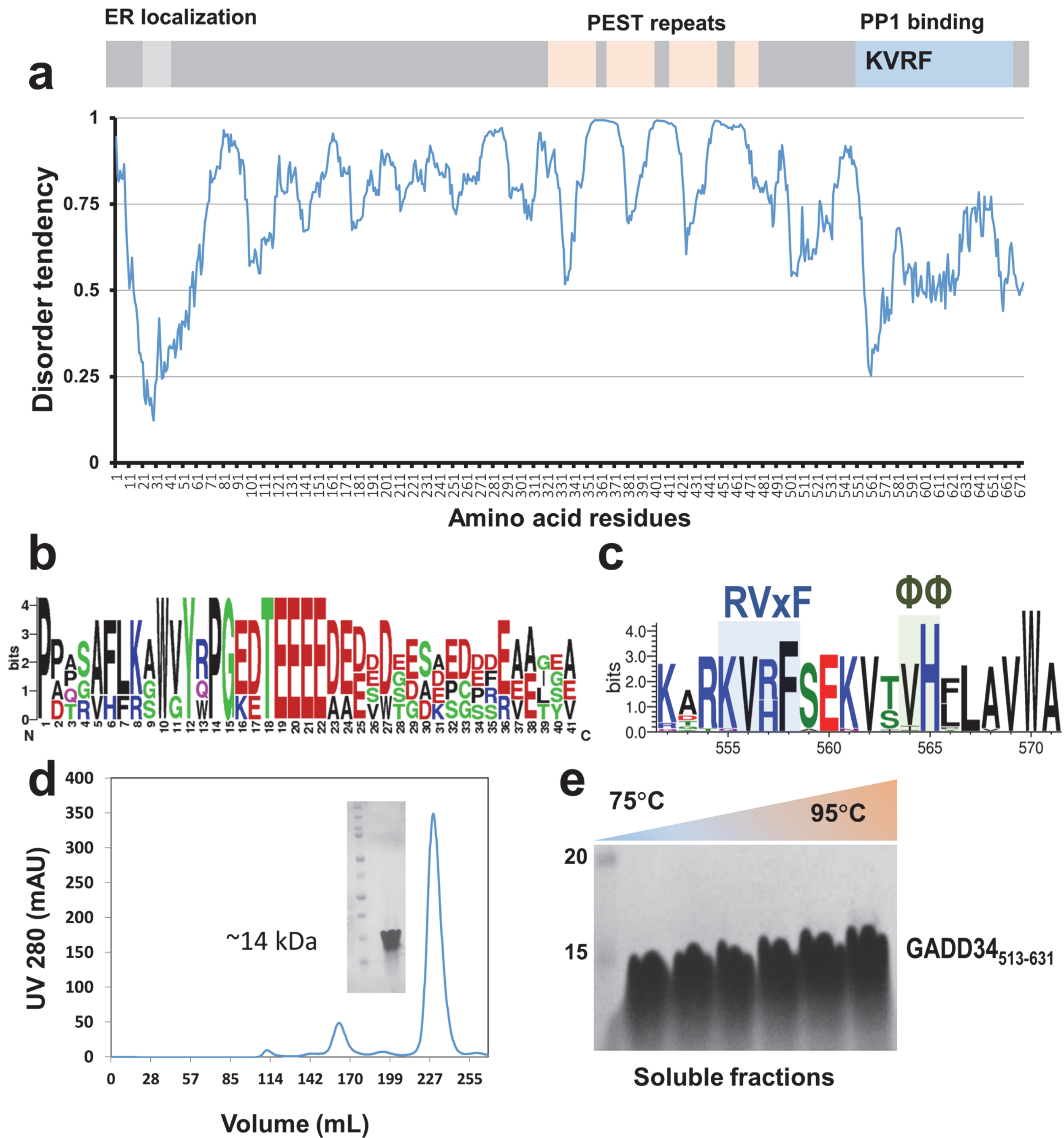
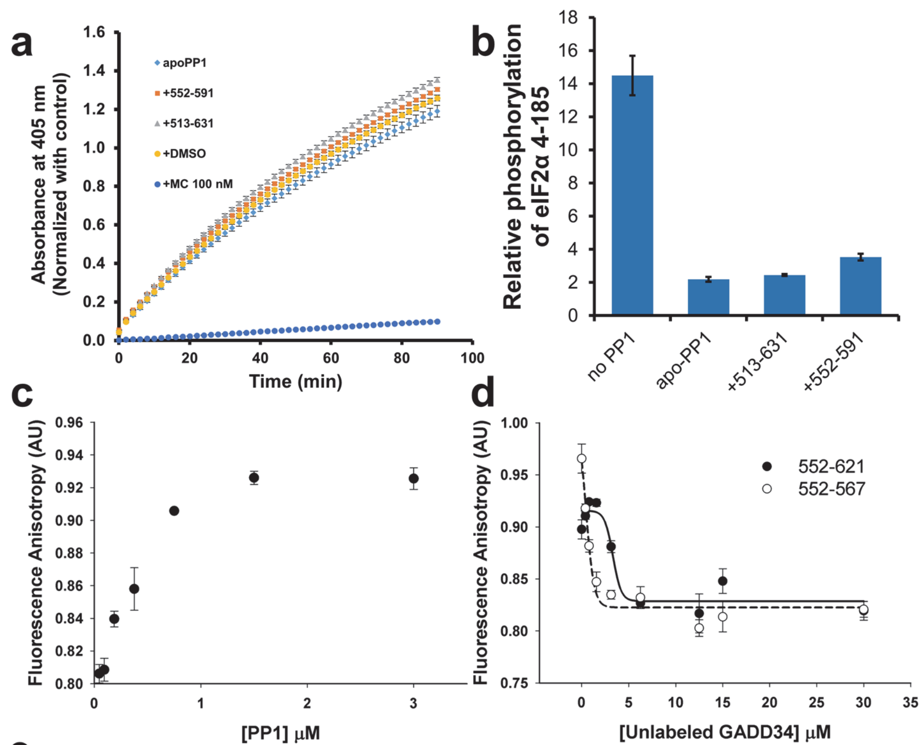


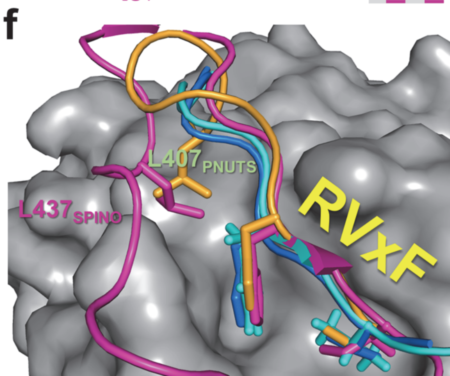
Figure S1 | Disorder Analysis of GADD34, Related to Figure 1. (**a**) Prediction of Intrinsically Disorder Proteins (IUPred) analysis of GADD34. GADD34 is predicted to be unstructured with most of the disordered tendency scores greater than 0.5. The ER localization domain (green) has the lowest disorder tendency score, corresponding to the amphiphatic helix that is responsible for ER binding. PEST repeats (pink) are in highly unstructured regions interrupted by three sharp decreases of disorder tendency scores, corresponding to residues with multiple hydrophobic and aromatic residues. The GADD34 PP1 binding region is shown as blue with the KVRF residues

(RVxF PP1-binding motif) highlighted. (**b**) Sequence logo of the first three PEST repeats of human GADD34. (**c**) Sequence Logo of the GADD34 PP1-binding domain from human, rhesus monkey, marmoset, lemur, gibbon, gorilla, mouse, hamster, bat, Tasmanian devil, rabbit, giant panda, dog, cow, *Xenopus*, puffer fish, stickleback and *Drosophila*, highlights the conservation of residues in this region. (**d**) Size exclusion chromatography (SEC) of $GADD34_{513\text{-}631}$ (Superdex 75 26/60). $GADD34_{513\text{-}631}$ elutes at the molecular weight of a ~14 kDa protein; insert: SDS-PAGE of the main peak fraction. (**e**) Purified $GADD34_{513\text{-}631}$ was subjected to heat treatment (75°C - 95°C for 30 min). Proteins were spun at 14,000x*g* for 10 min to remove any precipitate resulting from heat treatment. Soluble fractions were resolved using SDS-PAGE and stained with coomassie for visualization; $GADD34_{513\text{-}631}$ remained soluble.

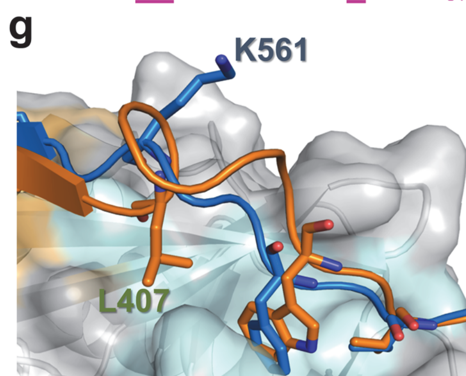


a
Absorbance at 405 nm (Normalized with control)
apoPP1
+552-591
+513-631
+DMSO
+MC 100 nM
Time (min)
b
Relative phosphorylation of eIF2α 4-185
no PP1
apo-PP1
+513-631
+552-591
c
Fluorescence Anisotropy (AU)
[PP1] μM
d
552-621
552-567
[Unlabeled GADD34] μM

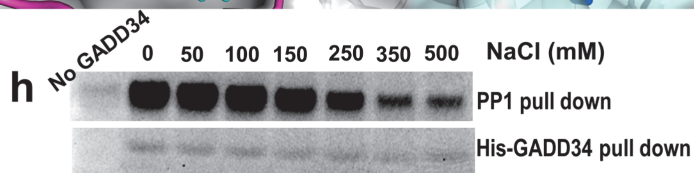
e
RVxF
ΦΦ
Arg
GADD34: 554RKVRFSE---KVTVHFLA567
NIPP1: 199SRVTFSE---DDEIINPED214
PNUTS: 397KTVTWPEEGKLREYFYFELDET-ERVNVN424
SPINO: 437LSEEEDPAPSRKIHFST–APIQVFSTYSNEDYDRRNEDV474



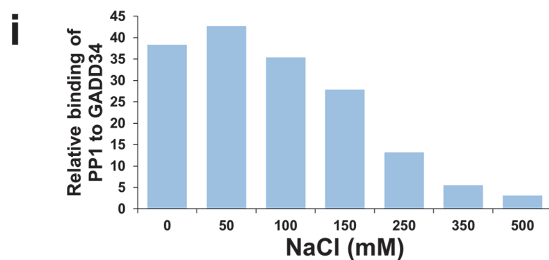
f
L407PNUTS
L437SPINO
RVxF



g
K561
L407



h
No GADD34
0 50 100 150 250 350 500 NaCl (mM)
PP1 pull down
His-GADD34 pull down



i
Relative binding of PP1 to GADD34
NaCl (mM)

Figure S2 | The GADD34:PP1 holoenzyme, Related to Figure 2. (**a**) PP1α$_{7\text{-}330}$, GADD34$_{513\text{-}631}$:PP1α$_{7\text{-}300}$ and GADD34$_{552\text{-}591}$:PP1α$_{7\text{-}330}$ dephosphorylate p-nitrophenyl phosphate (pNPP). 100 nM microcystin-LR (MC), a potent peptidic PP1 inhibitor, was used as a control. DMSO was used as the control for MC. (**b**) GADD34$_{513\text{-}631}$:PP1α$_{7\text{-}330}$ and GADD34$_{552\text{-}591}$:PP1α$_{7\text{-}330}$ dephosphorylate the PKR phosphorylated substrate phospho-eIF2α$_{4\text{-}185}$. Phospho-eIF2α$_{4\text{-}185}$ was stained using pro-Q Diamond phospho-protein stain (Life Technologies) and scanned using a Typhoon 9410 laser scanner (GE Healthcare). Densitometry of phospho-eIF2α was normalized against total eIF2α, obtained by staining the same gel with SYPRO Ruby (Life Technologies) protein gel stain. (**c**) Binding of fluorescent labeled DyLight 488-GADD34$_{552\text{-}621}$ to PP1α$_{7\text{-}330}$ (K_d ~500 nM). (**d**) Competitive displacement of DyLight 488-GADD34$_{552\text{-}621}$ bound to PP1α$_{7\text{-}330}$ by either unlabeled GADD34$_{552\text{-}621}$ or GADD34$_{552\text{-}567}$. Error bars correspond to the standard deviation of triplicate experiments. (**e**) Structure-based sequence alignment of GADD34:PP1 (PDBID: 4XPN), NIPP1:PP1 (PDBID: 3V4Y), PNUTS:PP1 (PDBID: 4MOY) and spinophilin:PP1 (PDBID: 3EGG). RVxF-, ΦΦ- and Arg-motifs are annotated. The RVxF-motif lid residues in PNUTS (Leu407) and Spinophilin (Leu437) are highlighted in black. (**f**) Cartoon representation of the lid RVxF-binding pocket of PNUTS (orange; Leu407) and spinophilin (magenta; Leu437). No lid residue is seen in GADD34 (blue) or NIPP1 (cyan). PP1α (gray; surface representation). (**g**) A "zoom-in" comparison of PNUTS with RVxF-Leu407 (orange) and GADD34 (blue). Instead of a hydrophobic residue, GADD34 has a lysine residue (Lys561) at this position whose positively charged side chain points away from the hydrophobic pocket on PP1. (**h**) Pull down experiments were performed using His$_6$-GADD34$_{513\text{-}631}$ as bait bound to Ni-NTA beads. Excess PP1α$_{7\text{-}330}$ was added to the bound GADD34$_{513\text{-}631}$ in interaction buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 0.5 mM TCEP) before washing twice (15 min each) with the interaction buffer containing increasing concentrations of NaCl (0 – 500 mM). Following the final wash, the beads were boiled in SDS sample buffer and resolved on SDS-PAGE. The gel was stained using SYPRO Ruby protein gel stain and scanned using a Typhoon 9410 laser scanner. (**i**) Densitometry shows relative binding of PP1 to GADD34 of (h). No GADD34 was added in the control.

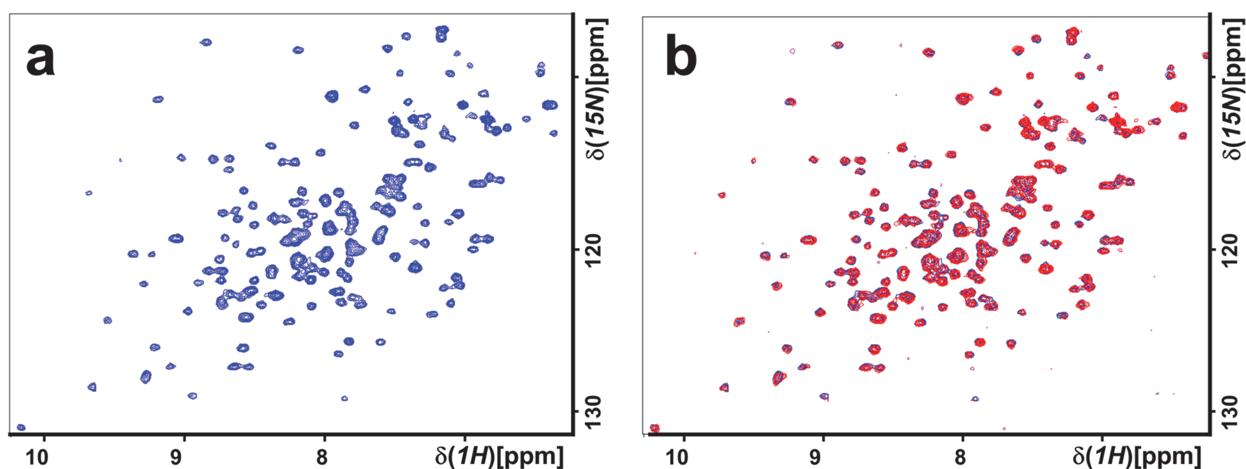


Figure S3 | eIF2α:GADD34 interaction, Related to Figure 3. (**a**) 2D [^{1}H,^{15}N] HSQC spectrum of ^{15}N-labeled eIF2α$_{4\text{-}185}$ (50 mM Na-PO_4 pH 7.0, 350 mM NaCl, 0.5 mM TCEP, 10% D_2O, 298 K; 11.7 T NMR spectrometer) and (**b**) overlay with of ^{15}N-labeled eIF2α$_{4\text{-}185}$ with 5-fold molar excess of GADD34$_{513\text{-}631}$. No significant chemical shift perturbations are observed.

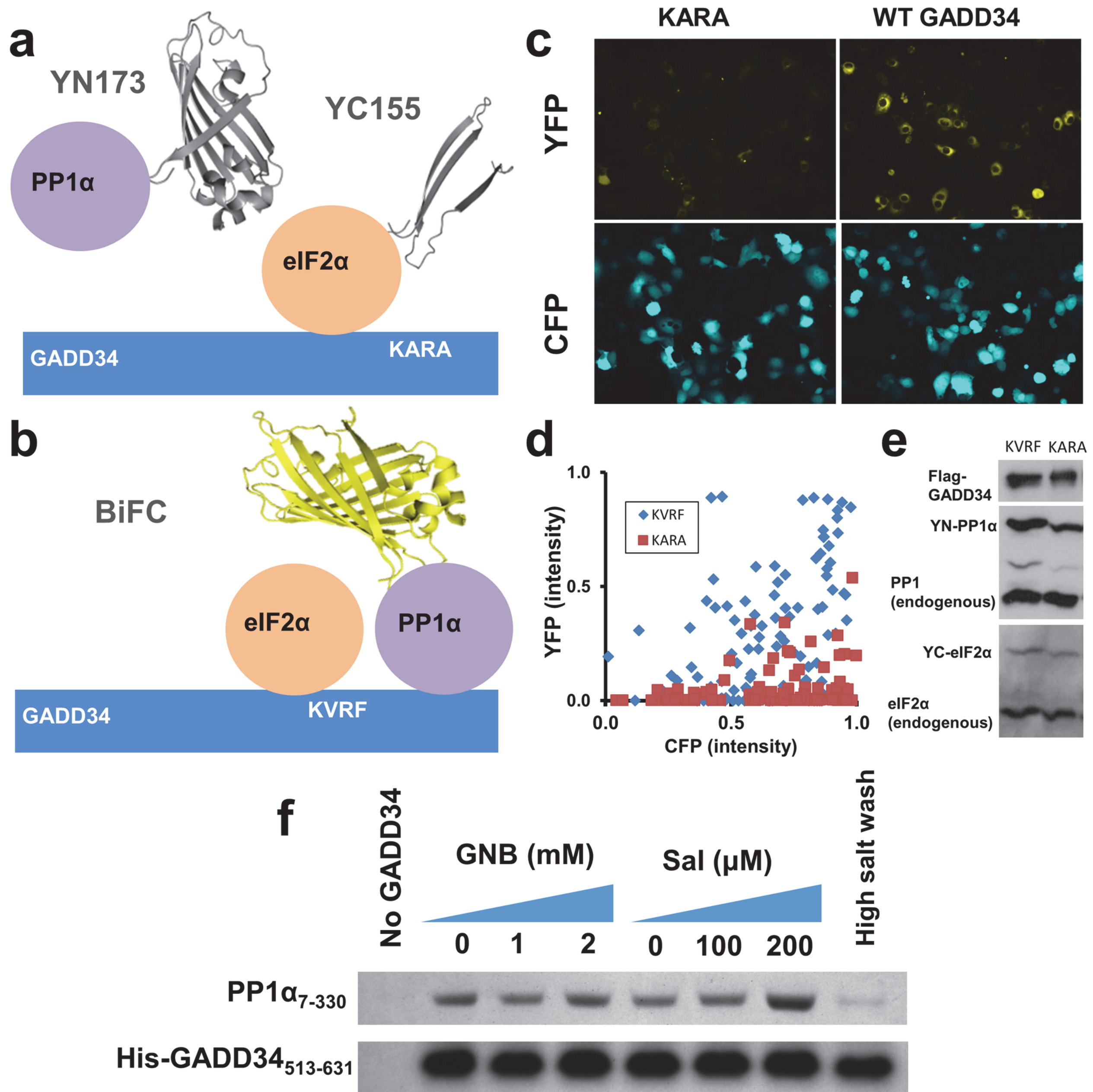


Figure S4 | Validating the BiFC assay using the KARA, non-PP1 binding mutant of GADD34 and small molecule drugs Guanabenz (GNB) and Salubrinal (Sal) to GADD34:PP1, Related to Figure 4. (**a,b**) Diagrams showing BiFC assay comparing the GADD34 WT- and the non-PP1 binding mutant, KARA. Only when WT GADD34 was used that the YN-PP1α and YC-eIF2α reconstituted YFP fluorescence demonstrating BiFC. (**c**) The BiFC complex is seen as YFP fluorescence using live-cell imaging. The GADD34 KARA mutant shows a significantly reduced YFP signal. Vector expressing CFP was used as transfection and expression control in cells. (**d**) Scatter plot showing the intensity of YFP and CFP signals in individual cells. Cells with more BiFC complexes show higher YFP intensity values. (**e**) Western blot showing comparable protein expression of all components used in the BiFC assay, as well as endogenous levels of PP1α and eIF2α. (**f**) The GADD34:PP1 complex was formed under buffer conditions that optimally stabilized

the complex (20 mM Tris pH 8.0, 150 mM NaCl, 0.5 mM TCEP). The ‘no GADD34’ control confirmed that no unspecific binding of PP1α occurred to the Ni-NTA beads. Increasing concentrations of GNB and Sal in the same buffer were used to wash the bound complex. No dissociation of the GADD34:PP1 complex was observed, as evidenced by the similar amount of PP1 bound compared to the control. High salt buffer (20 mM Tris pH 8.0, 500 mM NaCl, 0.5 mM TCEP) provided a positive control for the dissociation of the GADD34:PP1 complex.

Supplemental References

Brush, M.H., Weiser, D.C., and Shenolikar, S. (2003). Growth arrest and DNA damage-inducible protein GADD34 targets protein phosphatase 1 alpha to the endoplasmic reticulum and promotes dephosphorylation of the alpha subunit of eukaryotic translation initiation factor 2. Mol Cell Biol *23*, 1292-1303.

Davis, I.W., Murray, L.W., Richardson, J.S., and Richardson, D.C. (2004). MOLPROBITY: structure validation and all-atom contact analysis for nucleic acids and their complexes. Nucleic Acids Res *32*, W615-619.

Ito, T., and Wagner, G. (2004). Using codon optimization, chaperone co-expression, and rational mutagenesis for production and NMR assignments of human eIF2 alpha. J Biomol NMR *28*, 357-367.

Kelker, M.S., Page, R., and Peti, W. (2009). Crystal structures of protein phosphatase-1 bound to nodularin-R and tautomycin: a novel scaffold for structure-based drug design of serine/threonine phosphatase inhibitors. J Mol Biol *385*, 11-21.

Keller, S., Vargas, C., Zhao, H., Piszczek, G., Brautigam, C.A., and Schuck, P. (2012). High-precision isothermal titration calorimetry with automated peak-shape analysis. Analytical chemistry *84*, 5066-5073.

Mikami, S., Kobayashi, T., Machida, K., Masutani, M., Yokoyama, S., and Imataka, H. (2010). N-terminally truncated GADD34 proteins are convenient translation enhancers in a human cell-derived in vitro protein synthesis system. Biotechnology letters *32*, 897-902.

Xu, Z., Wang, D., Lee, X., and Williams, B.R. (2004). Biochemical analyses of multiple fractions of PKR purified from Escherichia coli. Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research *24*, 522-535.