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## Structural and functional analysis of the GADD34:PP1 eIF2 $\alpha$ phosphatase

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### Summary

The attenuation of protein synthesis via the phosphorylation of eIF2 $\alpha$  is a major stress response of all eukaryotic cells. The Growth Arrest and DNA-damaged protein 34 (GADD34) bound to the serine/threonine protein phosphatase 1 (PP1) is the necessary eIF2 $\alpha$  phosphatase complex that returns mammalian cells to normal protein synthesis following stress. The molecular basis by which GADD34 recruits PP1 and its substrate eIF2 $\alpha$  are not fully understood, hindering our understanding of the remarkable selectivity of the GADD34:PP1 phosphatase for eIF2 $\alpha$ . Here we report detailed structural and functional analyses of the GADD34:PP1 holoenzyme and its recruitment of eIF2 $\alpha$ . The data highlight independent interactions of PP1 and eIF2 $\alpha$  with GADD34, demonstrating that GADD34 functions as a scaffold both in vitro and in cells. This work greatly enhances our molecular understanding of a major cellular eIF2 $\alpha$  phosphatase and establishes the foundation for future translational work.

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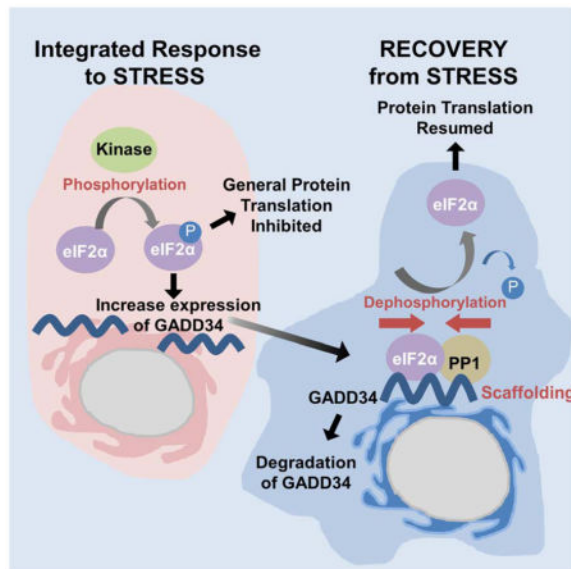
Supplemental Information. Supplemental Information includes detailed Experimental Procedures, 4 figures, and refinement table and can be found with this article online at XX.

### Author Contributions

M.C. designed and performed experiments reported in Figs. 1, 2, 4, S1–S4. P.Y., I.L. and C.G. designed and performed experiments reported in Fig. 3. J.N. designed and performed experiments reported in Fig. S2. W.P., S.S. and R.P. designed the study and wrote the manuscript.

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## Graphical abstract



## Keywords

protein phosphatase 1; GADD34; mRNA translation; eukaryotic translation initiation factor-2; X-ray structure; NMR spectroscopy

## Introduction

Protein synthesis in eukaryotes is actively controlled via the reversible phosphorylation of numerous translation initiation and elongation factors. In this context, the phosphorylation of the  $\alpha$ -subunit of eIF2 (eukaryotic Initiation Factor 2), a trimeric complex composed  $\alpha$ ,  $\beta$  and  $\gamma$  subunits, is a pivotal down-regulator of translation initiation following changes in the cellular environment or stress (Walton and Gill, 1976). Under non-stressed conditions, eIF2 $\alpha$  is largely unphosphorylated. In the presence of GTP, the eIF2 complex recruits Met-tRNA and ribosomal subunits to the translation start site to initiate mRNA translation. However, stresses, such as nutrient deprivation, heat shock, among other factors, activate one or more of four protein kinases, namely GCN2, PERK, PKR and HRI to phosphorylate eIF2 $\alpha$  on a single serine, temporarily repressing general protein synthesis (Harding et al., 2000).

Some mRNAs are, however, preferentially translated following eIF2 $\alpha$  phosphorylation. These include mRNAs encoding the transcription factors, ATF4 and CHOP, which together induce the stress response gene that encodes Growth Arrest and DNA-damaged protein 34 (GADD34; protein phosphatase 1 regulatory subunit 15A, PPP1R15A). As the stress declines, the GADD34 protein, by recruiting the ser/thr protein phosphatase 1 (PP1), facilitates the dephosphorylation of phospho-eIF2 $\alpha$ , restoring normal protein synthesis (Connor et al., 2001; Novoa et al., 2001). The structural basis by which the GADD34:PP1 complex functions as an eIF2 $\alpha$  phosphatase remains unclear.

GADD34 (674 residues, 73.5 kDa) possesses an endoplasmic reticulum (ER) targeting helix, four central PEST repeats and a C-terminal PP1-binding domain (Fig. 1A). Bioinformatics analyses suggest that GADD34 is largely unstructured (Fig. S1), making it impossible to assign function(s) simply by analysis of its primary structure. Phylogenetic analyses point to the existence of GADD34-like proteins in many multicellular organisms, from *Caenorhabditis elegans* to humans (Ceulemans et al., 2002). While some lower eukaryotes, like *Drosophila*, possess a single gene encoding dGADD34, mammals possess two genes encoding GADD34 and CReP (PPP1R15B) that share sequence homology solely in the C-terminal PP1-binding domain (Chen et al., 2015; Jousse et al., 2003). Finally, viral infection often results in PKR-mediated eIF2 $\alpha$  phosphorylation, which inhibits protein synthesis and viral replication in the infected host cells. However, the genomes of selected poxviruses encode polypeptides, like ICP34.5 (also known as  $\gamma$ 34.5), with sequence homology to the PP1-binding domain of GADD34 (He et al., 1997). By recruiting PP1, ICP34.5 promotes eIF2 $\alpha$  dephosphorylation allowing these viruses to evade this host cell surveillance mechanism. Together, these data argued that the approximately 100 amino acids encompassing the conserved PP1-binding domain among distinct eIF2 $\alpha$  phosphatases contain the critical determinants that direct eIF2 $\alpha$  dephosphorylation.

Recent discoveries of small molecular inhibitors, namely Salubrinal (Boyce et al., 2005) and Guanabenz (Tsaytler et al., 2011), which target eIF2 $\alpha$  phosphatases showed that they protected cells from cytotoxic cell death resulting from protein misfolding and aggregation (known as proteotoxicity). Indeed both compounds improved symptoms and prolonged life in animal models of human neurodegenerative diseases (Jiang et al., 2014; Saxena et al., 2009). Interestingly, Salubrinal inhibited the mammalian GADD34 and CReP-containing eIF2 $\alpha$  phosphatases as well as the ICP34.5-containing viral phosphatase. By contrast, Guanabenz showed greater selectivity for the GADD34-containing eIF2 $\alpha$  phosphatases. On the other hand, Guanabenz prevented prion protein aggregation and toxicity in both yeast and mammalian cells (Tribouillard-Tanvier et al., 2008). This is particularly remarkable as *Saccharomyces cerevisiae* does not express a GADD34 ortholog. Instead, the yeast eIF2 $\gamma$  subunit contains an N-terminal extension containing a PP1-binding RVxF motif that directly recruits PP1 (Glc7p) and catalyzes eIF2 $\alpha$  dephosphorylation (Rojas et al., 2014). Thus, there is an urgent need for a better understanding of the structure of mammalian eIF2 $\alpha$  phosphatases and the mode of action of inhibitors to facilitate the future development of therapies against human diseases associated with proteotoxicity.

Here, we utilized NMR spectroscopy, X-ray crystallography, biochemistry and cell biology to elucidate how GADD34 binds PP1 and recruits its substrate, eIF2 $\alpha$ . The crystal structure of the GADD34:PP1 holoenzyme (the complex between PP1 and the PP1-binding domain of GADD34) shows that GADD34 uses both the RVxF and  $\Phi\Phi$  motifs to bind PP1. We also show that the selectivity for eIF2 $\alpha$  as substrate is greatly enhanced by its binding to one or more of the central PEST domains in GADD34. The data establish that GADD34 functions as a scaffold, utilizing distinct domains to recruit PP1 and eIF2 $\alpha$  in vitro and in living cells. Together, this work significantly advances our understanding of a protein phosphatase complex that is present at high levels in stressed or diseased cells but not detected in healthy cells, providing unique opportunities for future drug development.

## Results

### The GADD34 PP1-binding domain is intrinsically disordered

Previous studies identified the RVxF (<sup>555</sup>KVRF<sup>558</sup>) motif that is required for PP1 binding in the C-terminal domain of GADD34 (Brush et al., 2003) (Fig. 1A). To examine the interaction of GADD34 with PP1, we expressed GADD34<sub>513-631</sub>, a construct previously used in the analysis of the interaction of GADD34 with PP1 (Brush et al., 2003). Our studies showed that GADD34<sub>513-631</sub> is heat stable (80°C, 15 min; Fig. S1) and that its 2D [<sup>1</sup>H,<sup>15</sup>N] HSQC spectrum lacked chemical shift dispersion in the <sup>1</sup>H<sup>N</sup> dimension, confirming that the GADD34 PP1-binding domain is an intrinsically disordered protein (IDP).

As ~1/3 of the N-terminal 38 residues in GADD34<sub>513-631</sub> are prolines and there was no experimental evidence that GADD34 residues 513-551 contribute to PP1-binding, we compared the 2D [<sup>1</sup>H,<sup>15</sup>N] HSQC spectrum of GADD34<sub>513-631</sub> with that of GADD34<sub>552-621</sub>. The 2D [<sup>1</sup>H,<sup>15</sup>N] HSQC spectra of the two GADD34 polypeptides overlapped well but the quality of the data for GADD34<sub>552-621</sub> was significantly better. Thus, we used GADD34<sub>552-621</sub> for detailed NMR analysis. We achieved a ~95% sequence specific backbone assignment (4 prolines, Fig. 1B). Secondary structure propensity (SSP) analysis (Marsh et al., 2006) showed two regions with preferred helical secondary structure (helix  $\alpha$ 1, <sup>582</sup>WEQLARDS<sup>590</sup>, ~40% populated; helix  $\alpha$ 2, <sup>610</sup>AARARAWARLRN<sup>621</sup>, ~70% populated; Fig. 1C). Consistently, helix  $\alpha$ 1 and  $\alpha$ 2 also showed reduced fast timescale motions in heteronuclear [<sup>15</sup>N]-NOE experiments (hetNOE), while the region surrounding the RVxF site was highly dynamic (Fig. 1D). Preferred secondary structure elements often contribute to protein:protein interactions. Thus, we used X-ray crystallography to determine the 3-dimensional structure of the GADD34:PP1 holoenzyme to evaluate the roles of helices  $\alpha$ 1 and  $\alpha$ 2 in PP1 binding and to reveal if and how this region of GADD34 dictates substrate selection by bound PP1.

### Crystal structure of GADD34:PP1 holoenzyme

GADD34<sub>552-621</sub>:PP1 (GADD34<sub>552-621</sub> was used in NMR studies) did not produce crystals. Additional GADD34 peptides (e.g. GADD34<sub>552-602</sub>; GADD34<sub>552-591</sub>; constructs based on the NMR analysis) were also screened for crystal formation, ultimately yielding crystals for GADD34<sub>552-602</sub>:PP1 and GADD34<sub>552-591</sub>:PP1, allowing for complete structure determination of the GADD34<sub>552-591</sub>:PP1 complex (Table S1; 2.29 Å). Clear electron density was observed for 16 GADD34 residues with the sequence <sup>553</sup>ARKVRFSEKVTVHFLA<sup>568</sup> (Fig. 2A, 2B). The complex buried ~1,500 Å<sup>2</sup> of solvent accessible surface area. To ensure that GADD34<sub>552-591</sub> was not proteolytically cleaved during crystallization, ~20 GADD34<sub>552-591</sub>:PP1 crystals were washed and subjected to SDS-PAGE. The molecular size, determined by the migration of the free GADD34<sub>552-591</sub> (Fig. 2C), confirmed that GADD34<sub>552-591</sub> was not degraded. Activity of the GADD34:PP1 holoenzyme was confirmed by the dephosphorylation of a model substrate, p-nitro-phenyl phosphate (pNPP) (Fig. S2).

Isothermal titration calorimetry (ITC) of PP1 $\alpha$ <sub>7-330</sub> and GADD34<sub>552-567</sub> confirmed a 1-to-1 binding ratio with a K<sub>d</sub> of 62 ± 14 nM (Fig. 2D). The K<sub>d</sub> of GADD34<sub>552-567</sub> for PP1 was

~6.5-fold weaker than that determined for some PP1 regulators, namely PNUTS and spinophilin (Choy et al., 2014; Ragusa et al., 2010) (8.7 nM and 9.3 nM, respectively), but close to that for NIPP1 ( $K_d$  of 73 nM) (O'Connell et al., 2012). Competitive fluorescence anisotropy experiments with a longer GADD34 construct confirmed that GADD34<sup>552-567</sup> constitutes the complete PP1 binding domain (Fig. S2).

### The GADD34:PP1 holoenzyme

GADD34 bound PP1 via two recognized PP1-binding motifs (Bollen et al., 2010; Choy et al., 2014). First, the residues Val<sub>556</sub> and Phe<sub>558</sub>, which constitute the RVxF-motif in GADD34, bind the hydrophobic RVxF-binding pocket on the surface of PP1 (Fig. 2B). Substituting Val<sub>556</sub> and Phe<sub>558</sub> with alanines results in complete loss of GADD34 binding by PP1 (Brush et al., 2003). Recently, we identified an RVxF motif 'lid' residue (Leu<sub>407</sub> in PNUTS; Leu<sub>437</sub> in spinophilin) that further stabilizes the RVxF interaction (Choy et al., 2014). However, as also observed for PP1 regulator NIPP1, no lid residue is present in the GADD34 (Fig. S2). Second, GADD34 binds PP1 via the  $\Phi\Phi$ -motif, a motif recently identified in several PP1 regulators, including spinophilin (Ragusa et al., 2010), NIPP1 (O'Connell et al., 2012) and PNUTS (Choy et al., 2014). The  $\Phi\Phi$ -motif of GADD34 is formed by Val<sub>564</sub> and His<sub>565</sub>, where His<sub>565</sub> in GADD34 forms a  $\pi$ -stacking interaction with Tyr<sub>78</sub> in PP1 (Choy et al., 2014) (Fig. 2B). Interestingly, the partially populated GADD34 helices  $\alpha 1$  and  $\alpha 2$  do not contribute to the direct interaction with PP1; instead, they likely allow for the recruitment of additional proteins to GADD34 (Brush et al., 2003).

As previously shown, PP1 interaction motifs in regulatory proteins can contribute to PP1 binding and/or PP1 regulation (Choy et al., 2014; O'Connell et al., 2012; Ragusa et al., 2010). So far, both the RVxF- and  $\Phi\Phi$ -motifs have been ascribed roles primarily in PP1 binding (Choy et al., 2014). Indeed, phosphatase assays using the GADD34:PP1 specific substrate, eIF2 $\alpha$ , showed that the complex readily dephosphorylated phospho-eIF2 $\alpha$  (phosphorylated by PKR). Similar levels of dephosphorylation were observed regardless of the GADD34 peptide (GADD34<sub>513-631</sub> or GADD34<sub>552-591</sub>) used for holoenzyme formation suggesting that residues adjacent to the core GADD34 PP1-interaction domain do not contribute to the activity of this heterodimeric complex (Fig. S2). However, this does not preclude the possibility that association of eIF2 $\alpha$  with other regions of GADD34 restrict the substrate specificity and/or enhance the activity of GADD34-associated phosphatase.

### GADD34 recruitment of the substrate eIF2 $\alpha$ is mediated by the GADD34 PEST domain

The GADD34:PP1 holoenzyme structure suggested that residues outside the minimal GADD34 PP1 binding domain recruit eIF2 $\alpha$ . To test if PP1 is required for eIF2 $\alpha$  recruitment by GADD34, we performed immunoprecipitation experiments (IP) using flag-tagged full-length GADD34 (WT and a PP1-binding deficient variant where the GADD34<sup>555</sup>KVRF<sup>558</sup> motif was mutated to KARA). eIF2 $\alpha$  was sedimented to the same extent by both WT and KARA GADD34 (Fig. 3A), confirming that the eIF2 $\alpha$  association with GADD34 is independent of PP1 binding.

We then used NMR spectroscopy to test for a direct interaction between the GADD34 PP1-binding domain and eIF2 $\alpha$ . NMR-active <sup>15</sup>N-labeled eIF2 $\alpha$ <sub>4-184</sub> (N-terminal domain) was

produced and tested for chemical shift perturbations (CSPs) following the addition of unlabeled GADD34<sub>513-631</sub>. CSPs could result from direct interaction between the two proteins or from changes in the conformation of eIF2 $\alpha$  resulting from eIF2 $\alpha$ :GADD34 interactions. No CSPs were detected suggesting that the GADD34 PP1-binding domain does not bind eIF2 $\alpha$  (Fig. S3).

To identify the GADD34 domain(s) that mediates eIF2 $\alpha$  recruitment, we analyzed eIF2 $\alpha$  binding to a GADD34 polypeptide that includes both the PEST repeats and the PP1-binding domain (His-GADD34<sub>241-674</sub>) and then compared it to a GADD34 polypeptide that includes only the PP1-binding domain (His-GADD34<sub>513-631</sub>). Only GADD34<sub>241-674</sub> sedimented eIF2 $\alpha$  from HEK293T cell lysates, establishing that GADD34 residues 241-513 are required for stable eIF2 $\alpha$  recruitment (Fig. 3B). Further deletion analyses established that a peptide encompassing only the PEST repeats (GADD34<sub>241-513</sub>) bound eIF2 $\alpha$ , with the PEST repeats 1, 2 and 3 (GADD34<sub>323-463</sub>) being prominent contributors to eIF2 $\alpha$  recruitment (Fig. 3C).

### GADD34 recruits both PP1 and eIF2 $\alpha$

To study the association of GADD34 with both PP1 and eIF2 $\alpha$  in cells, we used a bimolecular fluorescence complementation (BiFC) assay (Hu et al., 2002). Here, PP1 $\alpha$  and eIF2 $\alpha$ , each fused to distinct non-fluorescent halves of Yellow Fluorescent Protein (YFP), were transfected in COS-7 cells and the cells analyzed for the reconstitution of YFP fluorescence using confocal microscopy. When WT GADD34 was co-transfected in COS-7 cells containing the N-YFP-PP1 and C-YFP-eIF2 $\alpha$ , YFP fluorescence was greatly enhanced, indicating the formation of the heterotrimeric complex compared to cells not expressing GADD34 (vector control) or cells expressing another PP1 regulator, neurabin-1, which binds PP1 but not eIF2 $\alpha$  (Fig. 4A). As anticipated, BiFC fluorescence was localized primarily at the ER, similar to that seen for GFP-GADD34 (Fig. 4B). A similar experiment, performed using the PP1-binding deficient variant of GADD34 (KARA), showed little or no YFP signal establishing that both eIF2 $\alpha$  and PP1 must be recruited to GADD34 to reconstitute YFP fluorescence. These studies demonstrated that GADD34 functions as a protein scaffold to bring PP1 in proximity to its substrate, eIF2 $\alpha$  (Fig. 4C, Fig. S4).

## Discussion

Mammals possess two eIF2 $\alpha$  phosphatases. The CReP:PP1 holoenzyme is constitutively expressed and likely controls basal eIF2 $\alpha$  dephosphorylation to ensure continued protein synthesis in the absence of stress. However, under conditions of stress, increased phosphorylation of eIF2 $\alpha$  results in significant repression of general protein synthesis allowing cells to conserve or redirect their energy sources to overcome the stress. The GADD34 mRNA is one of a handful of genes actively translated in the presence of phospho-eIF2 $\alpha$  and functions in a feed-back loop to restore general protein synthesis and promote cell recovery from stress. Gene disruption studies suggested that inhibiting the CReP:PP1 complex may be associated with toxicity as CReP null mice are smaller and do not thrive after birth (Harding et al., 2009). By contrast, the GADD34:PP1 complex is only present in stressed or diseased cells and GADD34 null mice are largely normal (Harding et al., 2009; Kojima et al., 2003; Patterson et al., 2006). Thus, the major focus of drug discovery



programs targeting eIF2 $\alpha$  phosphatases to protect cells from protein misfolding disorders is to selectively target the GADD34:PP1 complex. However, structural information on the GADD34:PP1 complex that might guide the development of GADD34 specific drugs has been lacking.

Our work shows that GADD34 has little 3-dimensional structure and belongs to the family of IDPs (Fig. 1). This might, in part, explain the short half-life of the GADD34 protein in the cells, where it is degraded by the proteasome with a half-life of 1 hour. Common with many PP1 regulators, which also show disordered structure particularly within the PP1-binding domain, GADD34 readily and specifically interacts with PP1 to form a functional phosphatase holoenzyme (Fig. 2). To do this, GADD34 uses two well-established PP1-anchoring motifs: the canonical RVxF-motif found in 85% of PP1 regulators as well as the  $\Phi\Phi$ -motif, a newly identified motif predicted to be present in ~20% of PP1 regulators (Choy et al., 2014; Peti et al., 2013) (Fig. 2). While these motifs provide for tight binding of PP1 by GADD34 sufficient they do not account for the unique specificity of the GADD34:PP1 complex as an eIF2 $\alpha$  phosphatase.

Here we showed that GADD34 interacts with its substrate eIF2 $\alpha$  via one or more of the central PEST repeats (Fig. 3). Earlier studies established that the PEST sequences not only drive protein turnover but also mediate the association with regulatory proteins (Molinari et al., 1995). In that context, our earlier studies showed that PEST repeats in GADD34 did not determine the stability of the GADD34 protein in cells (Brush and Shenolikar, 2008), leading us to hypothesize that they represented sites of protein-protein interactions. Our cellular experiments in particular highlighted the necessity for GADD34-mediated scaffolding of both PP1 and eIF2 $\alpha$  for efficient and selective dephosphorylation of phospho-eIF2 $\alpha$ . The central PEST domain includes three repeats of ~15 hydrophobic and charged amino acids followed by long stretches of negatively charged poly-Glu and Asp residues. Notably, this domain is only present in GADD34; it is not present in CReP or the viral protein, ICP34.5, which share homology with GADD34 only in the PP1-binding domain. Yet, CReP and ICP34.5 can form active eIF2 $\alpha$  phosphatases. Moreover, BiFC studies of ICP34.5 demonstrated the ability of the viral protein to recruit both PP1 and eIF2 $\alpha$  and reconstitute YFP fluorescence (Brush and Shenolikar, 2008) albeit less efficiently than the fluorescence signal obtained with GADD34 in the current studies. Nevertheless, these studies hinted at a potential eIF2 $\alpha$ -binding site within the PP1-binding domain of ICP34.5. However, our cellular and biochemical studies showed that the isolated PP1-binding domain, GADD34<sub>513-674</sub>, was unable to recruit eIF2 $\alpha$  as effectively as WT GADD34 and identified the PEST domain as the primary, high affinity eIF2 $\alpha$ -docking site in GADD34. We speculate that while the PP1-binding domain of GADD34 together with PP1 may bind eIF2 $\alpha$  transiently to allow for eIF2 $\alpha$  dephosphorylation, the evolution of the additional eIF2 $\alpha$  association domain uniquely present in GADD34 may provide for more efficient reversal of the much higher levels of phospho-eIF2 $\alpha$  seen in stressed cells. Indeed, earlier studies that compared a truncated GADD34 containing the central PEST repeats and the C-terminal PP1-binding domain suggested that this polypeptide generated a more active eIF2 $\alpha$  phosphatase than the full-length GADD34 (Novoa et al., 2001), indicating the presence of both positive and negative structural elements that dictate the activity of GADD34-containing eIF2 $\alpha$  phosphatase.

In recent years, there has been an increased interest in the development of small molecule inhibitors of the GADD34:PP1 holoenzyme, exploiting the cytoprotective effects of attenuating global protein synthesis in diseased cells and tissues experiencing chronic protein misfolding. These efforts have yielded two structurally unrelated compounds, Salubrinal (Boyce et al., 2005) and Guanabenz (Tsaytler et al., 2011). Unlike compounds that target the active site of PP1 and consequently modulate the covalent modifications on a large number of cellular phosphoproteins, the narrower specificity of Salubrinal and Guanabenz to selectively alter cellular eIF2 $\alpha$  phosphorylation was interpreted as arising from their ability to dissociate the GADD34:PP1 complex (Boyce et al., 2005; Tsaytler et al., 2011). Indeed, the disruption of GADD34:PP1 was noted in the presence of millimolar concentrations of Guanabenz. Due to our ability to reconstitute a purified GADD34:PP1 holoenzyme in vitro, we examined the ability of both Guanabenz and Salubrinal to dissociate the GADD34:PP1 complex. No discernable dissociation of the complex was observed. Quite the contrary, high micromolar concentrations of Salubrinal may even enhance the interaction of GADD34 with PP1 (Fig. S4). Furthermore, we performed crystal trials of the GADD34:PP1 complex in the presence of 5 mM Guanabenz, but obtained no evidence for the disruption of the GADD34:PP1 holoenzyme by this drug, i.e. the GADD34:PP1 holoenzyme was intact and no electron density for Guanabenz was identified. Similarly, we have obtained no direct evidence for impaired PP1 or eIF2 $\alpha$  binding by GADD34 in the presence of either Guanabenz or Salubrinal. Taken together, these data highlight the need for more work aimed at defining the mode of action of these drugs. The availability of structural information on the key contact points required to assemble GADD34:PP1 complex may also yield new strategies for the identification of small molecules that selectively disrupt this eIF2 $\alpha$  phosphatase complex and provide a new avenue for drug development.

## Methods

Briefly, proteins were expressed in *E. coli* BL21 (DE3) or BL21-CodonPlus (DE3)-RIPL competent cells (Agilent). Purification of PP1 was performed as previously described (Choy et al., 2014). GADD34 was purified using heat purification (80°C, 15 min).

### Crystallization of the GADD34:PP1 holoenzyme

Crystals of GADD34<sub>552-591</sub>:PP1 $\alpha$ <sub>7-300</sub> holoenzyme were grown using sitting drop (200 nl) vapor diffusion in 0.2 M ammonium phosphate dibasic, 20% w/v polyethylene glycol 3350. 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<sub>552-591</sub>:PP1 $\alpha$ <sub>7-300</sub> structure was solved by molecular replacement using Phaser as implemented in Phenix, using PP1<sub>7-300</sub> (PDBID: 3E7A) as the search model (Kelker et al., 2009). A solution was obtained in space group P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub>; clear electron density for the bound GADD34 was visible in the initial maps. The initial models of the GADD34<sub>552-591</sub>:PP1 $\alpha$ <sub>7-300</sub> were built using Phenix.AutoBuild, followed by iterative rounds of refinement in PHENIX and manual building using Coot.



## NMR Spectroscopy

NMR measurements were performed at 298 K on a Bruker Avance 500 MHz spectrometer with a HCN TCI z-gradient cryoprobe. The NMR spectra were processed and analyzed using Topspin 3.1 (Bruker) and CARA software package ([www.cara.nmr.ch](http://www.cara.nmr.ch)). The hetNOE measurement and secondary structure propensity calculation were performed as previously described.

## BiFC assay

For BiFC assays, plasmid DNAs encode the yellow fluorescence protein (YFP) pair (YN-PP1 $\alpha$  & YC-eIF2 $\alpha$ ), together with the scaffolding proteins GADD34 and neurabin-1 were transfected into 60 – 70% confluence COS-7 cells grown in glass bottom Lab-Tek<sup>TM</sup> Chamber Slide<sup>TM</sup> (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<sub>2</sub>. 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<sub>2</sub> delivery. Image analysis was performed using MetaMorph (Molecular Devices).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

<b>IDP</b>	intrinsically disordered protein
<b>NMR</b>	nuclear magnetic resonance
<b>PP1</b>	protein phosphatase 1

**GADD34** protein encoded by growth arrest and DNA damage-inducible transcript 34

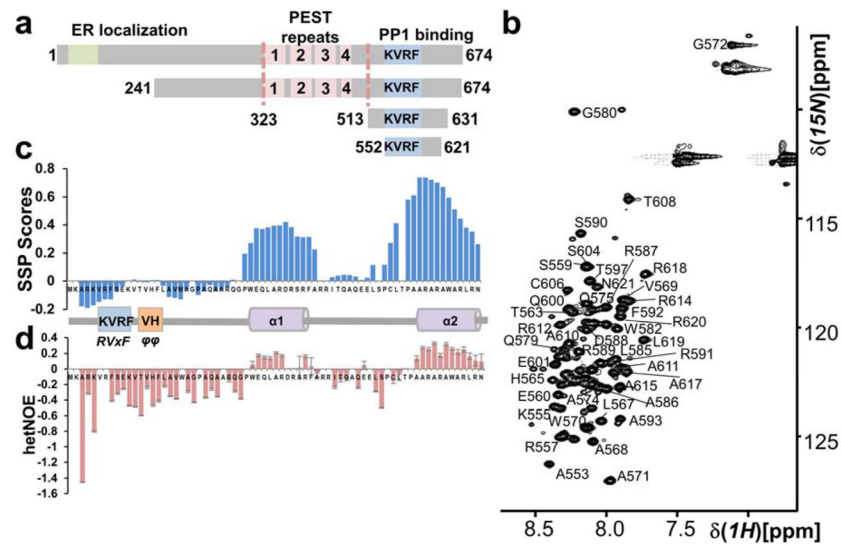
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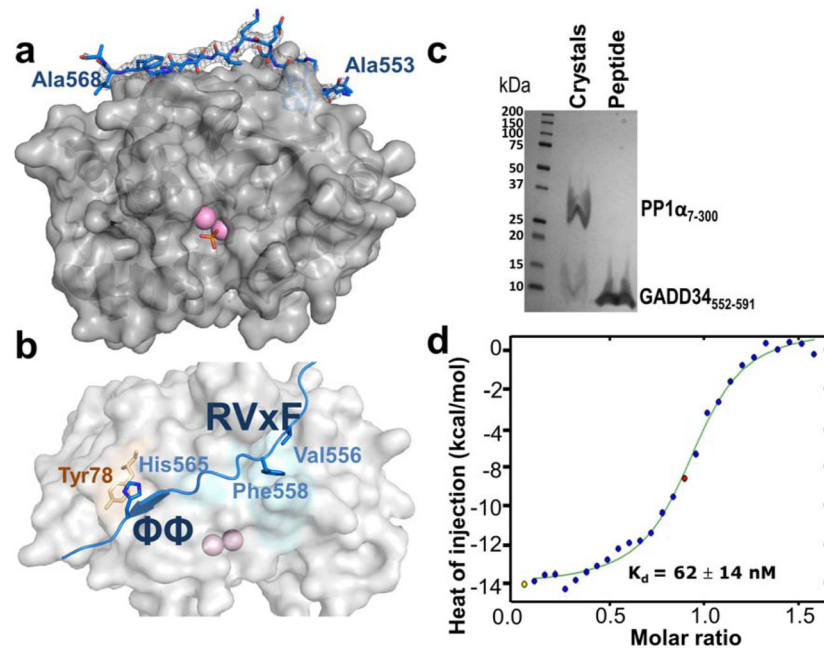
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**Highlights**

- Distinct domains in GADD34 are utilized to scaffold PP1 and eIF2 $\alpha$
- GADD34 is an intrinsically disordered protein that binds PP1 via RVxF and  $\phi\phi$  motifs
- PEST repeats in GADD34 represent an independent site for eIF2 $\alpha$  binding

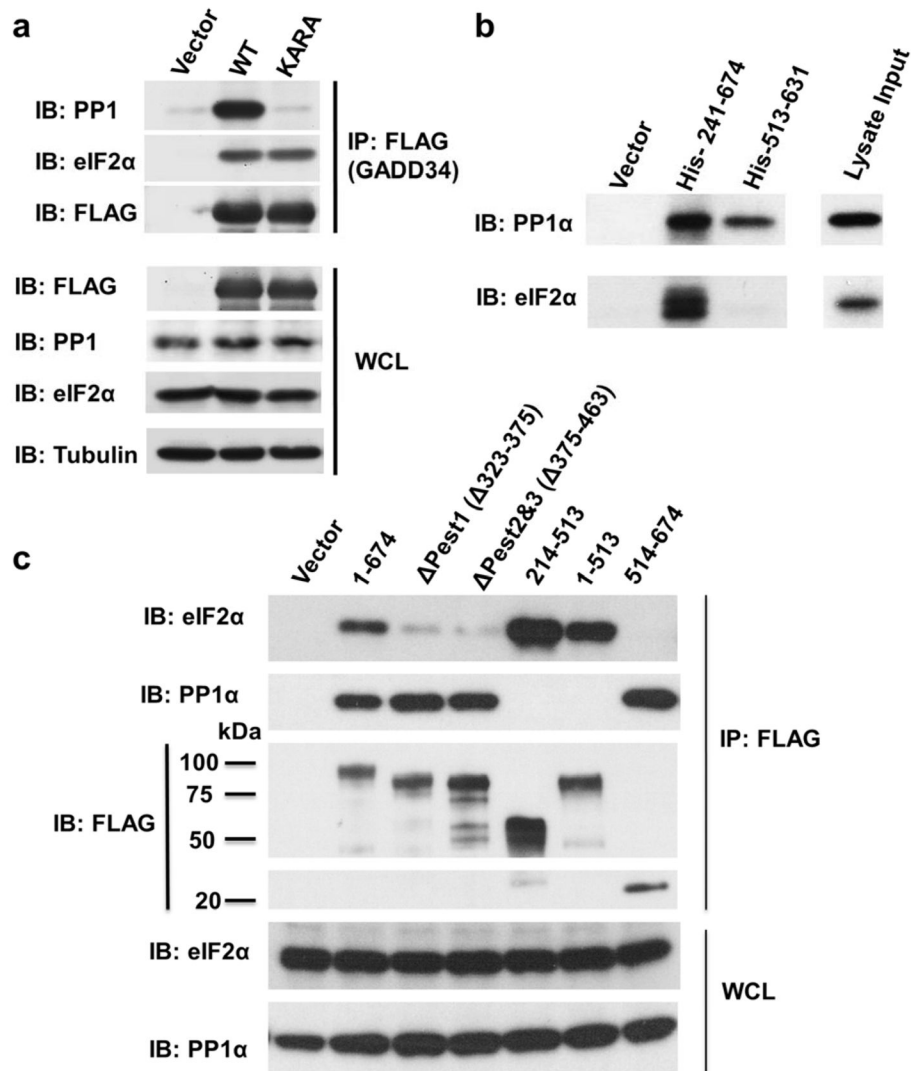


**Figure 1. The GADD34 PP1-binding domain is intrinsically disordered**  
 (a) GADD34 domain structure; ER localization domain, PEST domain (4 PEST repeats; labeled 1 – 4; each ~40 aa) and a PP1-binding domain (includes canonical PP1-binding motif RVxF [KVRF]). GADD34<sub>241-674</sub>, GADD34<sub>513-631</sub> and GADD34<sub>552-621</sub> are constructs used. (b) Annotated 2D [<sup>1</sup>H, <sup>15</sup>N] HSQC of GADD34<sub>552-621</sub>. The narrow <sup>1</sup>H<sup>N</sup> chemical shift dispersion is a hallmark of IDPs. (c) Secondary Structure Propensity (SSP) analysis of GADD34<sub>552-621</sub> reveals two preferred  $\alpha$ -helical secondary structures (helices  $\alpha$ 1 and  $\alpha$ 2). (d) Helices  $\alpha$ 1 and  $\alpha$ 2 have also reduced fast timescale motions.



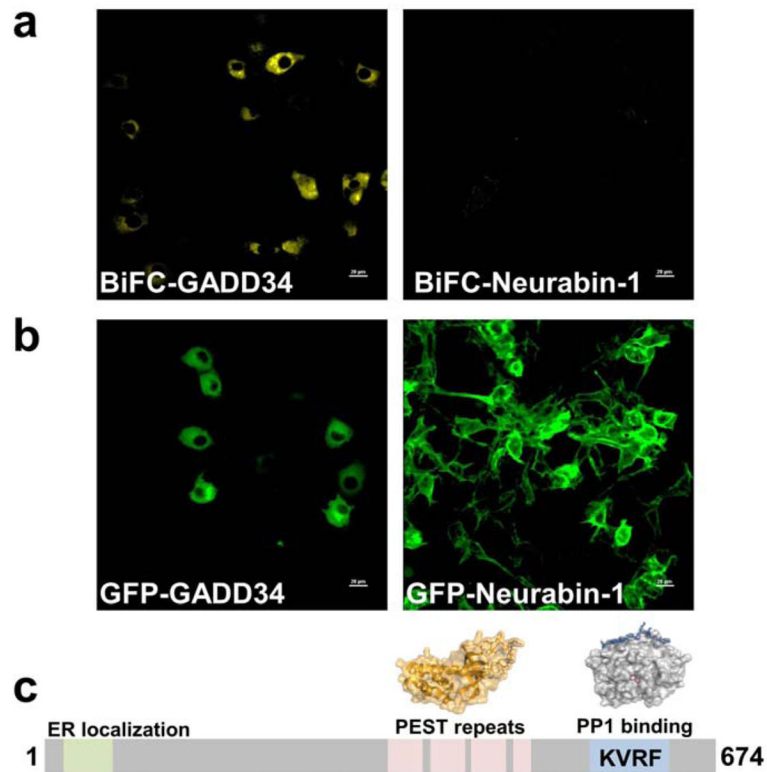
**Figure 2. The 3D structure of the GADD34:PP1 holoenzyme**  
 (a) GADD34 (residues 553-568, light blue, electron density  $2F_o - F_c$  contoured at  $1\sigma$ ) and PP1 $\alpha_{7-300}$  (gray, surface) form a complex. Two  $Mn^{2+}$  ions (pink spheres) are bound at the PP1 active site; bound phosphate is shown as sticks. No electron density was observed for GADD34 residues 552 or 569-591. (b) GADD34 PP1-binding domain with the two primary interaction sites (RVxF and  $\Phi\Phi$  residues are shown as sticks) highlighted. (c) SDS-PAGE of  $\sim 20$  GADD34<sub>552-591</sub>:PP1 $\alpha_{7-300}$  crystals; comparable migration of GADD34 from crystals and control (GADD34<sub>552-591</sub> alone) shows that no proteolytic degradation of GADD34<sub>552-591</sub> occurred during crystallization. (d) ITC of GADD34<sub>552-567</sub> and PP1 $\alpha_{7-330}$ .





**Figure 3. Recruitment of eIF2α is mediated by the GADD34 PEST domain**

(a) Immuno-precipitation (IP) experiment using WT- and KARA-GADD34. GADD34 recruits eIF2α from the HEK293T cell lysate independent of PP1 binding. (b) IP experiment using His<sub>6</sub>-GADD34<sub>241-674</sub> and His<sub>6</sub>-GADD34<sub>513-631</sub>. Only His<sub>6</sub>-GADD34<sub>241-674</sub> binds eIF2α (HEK293T cells lysate), while both His<sub>6</sub>-GADD34<sub>241-674</sub> and GADD34<sub>513-631</sub> bind PP1. (c) FLAG vector or FLAG-GADD34 proteins were expressed in HEK293 cells and IP using anti-FLAG-conjugated to agarose beads. The IP and whole cell lysates (WCL) were subjected to immunoblotting (IB) with indicated antibodies. Molecular weight markers (kD) are shown.



**Figure 4. Bimolecular Fluorescence Complementation (BiFC) assays highlight the role of GADD34 as a scaffolding protein**

(a) Flag-tagged GADD34 enhances the BiFC signal that results from the complementation of YFP from N-YFP-PP1 $\alpha$  and C-YFP-eIF2 $\alpha$ . Over-expression of neurabin-1, a neuronal PP1 regulatory protein (negative control) does not assemble the BiFC complex. (b) Localization of GFP-tagged GADD34 and neurabin-1 (COS-7 cell overexpression). (c) Model: GADD34 domain structure and the proposed PP1 $\alpha$  and eIF2 $\alpha$  binding sites (PP1 and eIF2 $\alpha$  structures are shown).