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Decoding the selectivity of eIF2 α holophosphatases and PPP1R15A inhibitors

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Abstract

The reversible phosphorylation of proteins controls most cellular functions. Protein kinases have been popular drug targets, unlike phosphatases, which remain a drug discovery challenge. Guanabenz and Sephin1 are selective inhibitors of the phosphatase regulatory subunit PPP1R15A (R15A) that prolong the benefit of eIF2 α phosphorylation, thereby protecting cells from proteostatic defects. In mice, Sephin1 prevents two neurodegenerative diseases, Charcot–Marie–Tooth 1B (CMT-1B) and SOD1-mediated amyotrophic lateral sclerosis (ALS). However, the molecular basis for R15A inhibition is unknown. Here we reconstituted human recombinant eIF2 α holophosphatases, R15A–PP1 and R15B–PP1, whose activity depends on both the catalytic subunit PP1 (protein phosphatase 1) and either R15A or R15B. This system enabled the functional characterization of these holophosphatases and revealed that Guanabenz and Sephin1 induced a selective conformational change in R15A, detected by resistance to limited proteolysis. This altered the recruitment of eIF2 α , preventing its dephosphorylation. This work demonstrates that regulatory subunits of phosphatases are valid drug targets and provides the molecular rationale to expand this concept to other phosphatases.

Introduction

The reversible phosphorylation of proteins controls virtually all aspects of cellular and organismal function, allowing them to adapt to sudden changes through the antagonistic action of protein kinases and phosphatases. Consequently, targeting phosphorylation offers a broad range of therapeutic opportunities, and kinases are prevalent drug targets in pharmaceutical research, with more than 3,000 approved and experimental drugs in this category¹. Although targeting phosphatases should in principle be as attractive as targeting kinases, phosphatases are largely untapped and only very few phosphatase inhibitors have been reported^{2, 3, 4, 5}.

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Contributions

M.C. designed, performed and analyzed all experiments, prepared the figures and helped with the manuscript. A.S. discovered compound C3 and performed cytotoxicity experiments. A.B. designed and guided the study and wrote the manuscript.

Competing financial interests

M.C. and A.B. are co-inventors on Great Britain patent application 1709927.6 on the activity assays and methods described in this manuscript.

The majority of protein phosphorylation events occur on serine and threonine residues, and PP1 is a major phosphatase catalyzing serine and threonine dephosphorylation^{6, 7, 8, 9, 10}. PP1 is a single-domain protein that assembles with one or two amongst more than 200 diverse regulatory subunits to form specific holophosphatase complexes^{6, 7, 8, 9, 10}. In cells, there is no free PP1, as this would be toxic, owing to the broad substrate specificity of free PP1 (ref. 8). Instead, regulatory subunits in cells form complexes with PP1 to restrict its specificity to cognate substrates^{7, 8, 10, 11}, thereby avoiding uncontrolled and promiscuous dephosphorylation events, which would be lethal. Because hundreds of holophosphatases share PP1 as a common catalytic subunit, catalytic inhibitors of PP1 inhibit hundreds of holophosphatases and are thereby toxic to cells^{3, 12}.

eIF2 α phosphorylation is an evolutionarily conserved and vital cellular defense system against many forms of stresses¹³. Phosphorylation of eIF2 α results in a decrease in protein synthesis, sparing the cellular resources to adapt to challenging conditions¹⁴. In mammals, the levels of eIF2 α phosphorylation are set by the cellular needs through the antagonistic action of four eIF2 α kinases and two eIF2 α holophosphatases¹⁴. The mammalian eIF2 α holophosphatases are composed of PP1 bound to either the stress-inducible regulatory subunit R15A (PPP1R15A, GADD34) or the functionally related and constitutively expressed regulatory subunit R15B (PPP1R15B, CreP)^{15, 16}. Guanabenz was discovered through a phenotypic assay: it protects cells from protein misfolding stress in the endoplasmic reticulum¹⁷. It does so by prolonging the benefit of eIF2 α phosphorylation by selectively binding and inhibiting R15A but not the related protein R15B. The selectivity of the inhibitor is important; whereas selective inhibition of R15A benefits cell fitness, inhibiting both eIF2 α holophosphatases is predicted to be lethal¹⁸, because this would result in a persistent inhibition of protein synthesis¹⁹. Indeed, Salubrinal, an inhibitor of eIF2 α dephosphorylation, is toxic to cells and *in vivo*^{3, 20}.

Improving proteostasis could in principle ameliorate a broad range of diseases associated with the misfolding of proteins²¹. Guanabenz could not be used to study R15A inhibition *in vivo*, because it has an undesirable activity: it is a potent α -2 adrenergic agonist²², which has side effects such as drowsiness, and even coma, at high doses in humans²³. In addition to the α -2 adrenergic activity, Guanabenz also reduces prion accumulation in yeast and in mammals²⁴. Thus, we developed a Guanabenz derivative, Sephin1, which selectively inhibits R15A but not R15B, while being devoid of both the α -2 adrenergic activity²⁵ and the anti-yeast-prion activity of Guanabenz²⁴. Thus, Sephin1 is a selective R15A inhibitor that lacks the known off-target activities of Guanabenz.

Sephin1 has suitable properties for *in vivo* studies and therefore was used to inhibit R15A in mice. Sephin1 is available in orally administered form, crosses the blood–brain barrier, and reaches concentrations in the brain known to inhibit R15A²⁵. When given to mice, it safely prevents the motor, morphological, and molecular defects associated with two otherwise unrelated protein-misfolding diseases: CMT-1B and a SOD1-related form of ALS²⁵. Importantly, genetic experiments from different groups validate the notion that R15A inhibition is a powerful therapeutic target in mouse models of CMT-1B (refs. 26,27) and SOD1-ALS²⁸. The prevention of neurodegenerative diseases in mice by Sephin1 was

achieved without detectable side effects²⁵, exemplifying the power and the benefit of selective inhibition of R15A.

There are more than 200 holophosphatases in mammals that could in principle be inhibited by the same paradigm of targeting their regulatory subunits. For this idea to become an attractive avenue for drug development research, the molecular basis for the selective inhibition of the R15A–PP1 holophosphatase needs to be elucidated. Phosphatases are challenging to study, and so far it has not been possible to reconstitute R15A inhibition *in vitro*^{29, 30}. Here we set out to overcome this challenge, aiming to elucidate the function of R15s and to reconstitute functional eIF2 α holophosphatases (R15A–PP1 and R15B–PP1) with recombinant proteins in order to reveal the molecular mechanisms of the selective R15A inhibitors.

A recombinant system recapitulates the selective activity of eIF2 α holophosphatases

A well-established property of PP1 regulatory subunits is to restrict the otherwise broad substrate selectivity of PP1 (ref. 8), defining regulatory subunits as inhibitors of PP1, because they block the ability of free PP1 to dephosphorylate noncognate substrates^{31, 32}. Recombinant R15A has been previously characterized using this paradigm and indeed inhibits PP1 from dephosphorylating an irrelevant substrate³³. *In vitro*, PP1 alone dephosphorylates eIF2 α , and addition of recombinant R15A has no measurable effect under these conditions³³. So far, reconstituting functional PP1 holophosphatases has been challenging, because regulatory subunits of phosphatases are natively unstructured³⁴. Here we expressed and purified a large fragment, known to bind Guanabenz and Sephin1 (refs. 17,25), of the regulatory subunit R15A^{325–636} (Supplementary Fig. 1). Similarly, we also expressed and purified the homologous fragment of R15B (R15B^{340–698}), as well as an unrelated regulatory subunit R3A (PPP1R3A, glycogen-targeting subunit of PP1) (Supplementary Fig. 1). PP1 was expressed and purified as previously described³⁵ (Supplementary Fig. 1). As previously reported³³, PP1 alone dephosphorylated eIF2 α , and the addition of R15A had no detectable effect under these conditions (Fig. 1a and Supplementary Fig. 2a). R15B also appeared inactive in this assay (Fig. 1a and Supplementary Fig. 2a). However, as anticipated^{32, 33}, R3A inhibited the dephosphorylation of eIF2 α by PP1 (Fig. 1a and Supplementary Fig. 2a). This confirms the well-established function of regulatory subunits as inhibitors of PP1 toward noncognate substrates^{31, 32}. In such an assay, neither of the R15A inhibitors Guanabenz or Sephin1 had any measurable effects (Fig. 1b), in contrast to the PP1 catalytic inhibitor calyculin A (Fig. 1c). Given that the assay used did not depend on the regulatory subunits, it is not surprising that it could not reveal the activity of the R15A inhibitors.

It therefore became crucial to first establish an assay to reveal and measure the positive function of R15s. The commonly used phosphatase assays measure protein dephosphorylation using stoichiometric amounts of PP1, but in cells, PP1 holoenzymes ought to be active at substoichiometric concentrations. Because total PP1 concentration in cells is estimated to be 0.2 μ M³⁶, and knowing that PP1 is a subunit of hundreds of

holoenzymes, the concentration of a given holoenzyme ought to be in a nanomolar range or below. Titrations of PP1 showed that the enzyme was capable of dephosphorylating eIF2 α at a stoichiometric concentration (1 μ M) relative to the substrate (also at 1 μ M), but its activity dropped with decreasing concentrations (Fig. 1d and Supplementary Fig. 2b,c). Whereas a substoichiometric concentration of PP1 (10 nM) relative to the substrate (1 μ M) had no effect on eIF2 α dephosphorylation, addition of R15A to substoichiometric concentrations of PP1 (10 nM) enabled the complete dephosphorylation of eIF2 α (Fig. 1e and Supplementary Fig. 2d). Likewise, addition of R15B converted PP1 (at 10 nM) into a proficient eIF2 α phosphatase (Fig. 1f and Supplementary Fig. 2e). Attesting to the selectivity of R15s, addition of R3A did not enable eIF2 α dephosphorylation by PP1 (at 10 nM) (Fig. 1g and Supplementary Fig. 2f). Conversely, the reconstituted R15 holoenzymes did not dephosphorylate an irrelevant substrate, phosphorylase a, further confirming the selectivity of the holoenzymes as well as the selectivity of the assay (Supplementary Fig. 3). The results obtained here reconcile the fundamental aspects of PP1 activity: in a nonphysiological paradigm, using a high concentration of isolated PP1, the enzyme is not selective, and at low concentrations, the activity of PP1 toward a given phosphosubstrate is only achieved upon addition of a cognate regulatory subunit. This demonstrates that R15s and PP1 are necessary and sufficient components of the eIF2 α holophosphatases. The recombinant system developed here has the unique property of reporting on the biological activity of holoenzymes. It reveals a positive function for R15s: converting PP1 into a holoenzyme capable of dephosphorylating eIF2 α .

Defining the domains of regulatory subunits required for holoenzyme activity

Having reconstituted functional R15 holoenzymes whose activity depends on the regulatory subunits, we next investigated the molecular basis for their activities. R15A and R15B contain a homologous PP1-binding region in their carboxy-terminal regions and have divergent amino-terminal regions of unclear function (Fig. 2a,b). Thus, we first characterized the recombinant and functional eIF2 α holophosphatases that we prepared. R15A bound PP1 with an affinity of 0.06 μ M, and this binding was largely encoded by the carboxy-terminal region of the protein, R15A^C, containing the PP1-binding site (Fig. 2c), as anticipated^{15, 33}. The binding affinity of the functional R15A to PP1 was similar to that measured in a previous study²⁹ with an R15A^{552–567} peptide containing the PP1-binding site. Likewise, R15B binds PP1, and this binding was also mediated by its carboxy-terminal region, R15B^C (Fig. 2d). Interestingly, the affinity of R15B for PP1 was lower than that of R15A (Fig. 2c,d). Because R15A is inducible¹⁵, it has to compete with existing holoenzymes to recruit PP1. The measured higher affinity of R15A for PP1, relative to that of R15B, could explain how the inducible R15A competes with the constitutive R15B to recruit PP1.

The PP1-binding regions of regulatory subunits have been well characterized⁸, but the functions of the other domains are unclear. Our recombinant system enabled us to study for the first time the contributions of the different domains of R15 to the activity of the reconstituted eIF2 α holophosphatases. Although the R15A^C fragment had a similar affinity

to PP1 to that of the functional R15A (Fig. 2c), it was unable to convert PP1 into an active holophosphatase (Fig. 2e). Likewise, the R15B^C fragment was fully competent for recruiting PP1 (Fig. 2d) but inactive in our phosphatase assay (Fig. 2f). In contrast to the carboxy-terminal fragments, functional R15s enabled dephosphorylation of eIF2 α (Fig. 2e,f). This shows that in addition to the carboxy-terminal regions of R15s, which are required to bind PP1, their amino-terminal regions are essential for R15–PP1 holoenzyme activity. This establishes a paradigm to study functional PP1 holoenzymes in which the activity of the holoenzyme depends on a functional regulatory subunit.

R15–PP1 has higher affinity for substrate than isolated PP1

To gain further mechanistic insights into the activity of the functional eIF2 α holophosphatases, we next aimed to measure the affinities of the different recombinant complexes to their substrates, using thermophoresis and a D95A mutant of PP1, PP1^{D95A}, with unaltered substrate binding but negligible activity³⁷. As anticipated^{37, 38}, PP1^{D95A} was catalytically inactive (Supplementary Fig. 4) but bound eIF2 α with ~0.65 μ M affinity (Fig. 3a). We next tested the binding of R15 to eIF2 α . Both R15A and R15B alone bound to eIF2 α (Fig. 3b,c). Because studies with opposite results have been reported^{29, 39}, we questioned which region of R15A binds to eIF2 α . No such studies had been performed with R15B. Thus, we tested which region of R15s bind to the substrate. We detected no binding of the isolated carboxy-terminal region of R15s to eIF2 α , whereas the amino-terminal regions of either R15 alone bound eIF2 α similarly to the functional R15s (Fig. 3b,c). Thus, binding of R15A and R15B to eIF2 α is encoded by their amino-terminal regions (Fig. 3b,c). The unrelated regulatory subunit R3A did not bind to eIF2 α (Fig. 3d). We next tested the binding of holophosphatase complexes to eIF2 α . The affinities of R15A–PP1^{D95A} and R15B–PP1^{D95A} for eIF2 α were, respectively, 5.4 and 3.0 times higher than the affinity of the isolated PP1^{D95A} for eIF2 α (Fig. 3e and Supplementary Table 1). The higher affinities of R15A–PP1 and R15B–PP1 for their cognate substrate eIF2 α relative to that of the PP1 catalytic subunit alone can explain why R15A and R15B convert free PP1 into a functional holoenzyme when using substoichiometric concentrations of PP1 (Fig. 1e,f). When R15 complexes were prepared with R15^C fragments, their affinities for eIF2 α were as low as that of the isolated PP1^{D95A} (Fig. 3e and Supplementary Table 1). This is in good agreement with the affinity measurements obtained with isolated fragments of regulatory subunits (Fig. 3b,c): given that the R15^C fragments do not bind to eIF2 α , these proteins cannot increase eIF2 α recruitment to PP1. This explains why the R15^C–PP1 complexes behaved like PP1 alone and did not dephosphorylate eIF2 α in our activity assay (Fig. 2e,f). The amino-terminal fragments of R15s did not alter the binding affinity of PP1^{D95A} to eIF2 α (Fig. 3e and Supplementary Table 1). This is not surprising, given that the R15^N fragments do not bind PP1 (Fig. 2c,d). In contrast to functional R15s, R3A decreased PP1^{D95A} affinity to eIF2 α (Fig. 3e and Supplementary Table 1). These results define the molecular basis for the dual functions of regulatory subunits: R15A and R15B increase the affinity of PP1 to their cognate substrate, thereby enabling its dephosphorylation, whereas R3A decreases PP1 affinity to a noncognate substrate and prevents its dephosphorylation. In addition, the data demonstrates that both the eIF2 α -binding amino-terminal region of R15s and their PP1-binding carboxy-terminal region are required for their functionality.

R15A sensitivity to limited proteolysis

Having recapitulated the function of regulatory subunits of holophosphatases in a recombinant system enabled us to study the selective R15A inhibitors Guanabenz and Sephin1. First, we confirmed that R15A directly binds Guanabenz and Sephin1, as previously reported^{17, 25} (Fig. 4a). Furthermore, we observed that the binding of R15A to the selective inhibitors Guanabenz and Sephin1 was not covalent because R15A, immobilized on biotinylated Guanabenz or biotinylated Sephin1, was eluted with excess of Guanabenz or Sephin1, respectively (Fig. 4a). Having confirmed that Guanabenz and Sephin1 directly bind R15A, we next investigated the consequences of this interaction and set out to examine whether Guanabenz and Sephin1 induced a conformational change in R15A. The experimental paradigm we employed was previously used to reveal a conformational change induced by a PTP1B inhibitor⁴⁰. To assess whether binding of R15A inhibitors induced a conformational change in their target, we measured the sensitivity of R15s to mild proteolysis in the presence or absence of inhibitors. As a control, we took advantage of a close chemical derivative of Guanabenz, compound C3 (Fig. 4b), which unlike Guanabenz, was inactive in cytoprotection from ER stress (Supplementary Fig. 5). As expected for natively unstructured proteins⁴¹, R15A and R15B were sensitive to a limited trypsin proteolysis (Fig. 4c). The addition of Guanabenz decreased the sensitivity of R15A to trypsin proteolysis (Fig. 4c). No such protective effects on the sensitivity to trypsin were seen when using Guanabenz and R15B (Fig. 4d), confirming the selectivity of Guanabenz for R15A. Similar results were obtained using Sephin1 (Fig. 4c,d). Importantly, the negative compound C3 did not alter the protease sensitivity of R15 to mild proteolytic treatment (Fig. 4c,d). Further attesting to the specificity of the findings, the compounds had no measurable effects on the protease sensitivity of MBP, showing that the compounds did not inhibit trypsin (Supplementary Fig. 6). This also suggests that the compounds do not induce aggregation of proteins. The observation that the interaction between R15A and its inhibitors was reversible (Fig. 4a) also indicated that the compounds did not cause aggregation of the proteins. To formerly address this possibility, we performed light scattering analysis. We found no aggregation of R15A at saturating concentration of Guanabenz, Sephin1 or C3 (1 mM), (Supplementary Fig. 7). However, Salubrinal at 50 μ M induced robust aggregation (Supplementary Fig. 7). Together these results confirm that the R15A inhibitors Guanabenz and Sephin1 directly and reversibly bind R15A. The binding of Guanabenz and Sephin1 to R15A selectivity alters the sensitivity to trypsin of R15A but not R15B, implying that they induce a conformational change in their target. These findings not only confirm the selectivity of Sephin1 and Guanabenz for R15A but also provide the beginning of a mechanistic insight into their mode of inhibition that we explored further.

R15A inhibitors selectively impair substrate recruitment

The selectivity of the inhibitors shown in previous studies^{17, 25} and confirmed here suggests that these inhibitors ought to target a divergent region of R15A and R15B, rather than their homologous carboxy-terminal regions. Knowing that the functional R15A specifically binds its inhibitors^{17, 25}, which results in altering protease sensitivity (Fig. 4c,d), and having established that functional R15 holoenzymes have an increased affinity for eIF2 α relative to the isolated PP1 (Fig. 3e), we tested whether the inhibitors altered substrate

recruitment. The inhibitors were unsuitable for thermophoresis experiments and thus, we conducted pulldown assays. We found that Guanabenz decreased the binding of R15A to eIF2 α (Fig. 4e, lanes 10, 11, and f). Unlike what we observed in cells^{17, 25}, we did not observe dissociation of the recombinant holophosphatases upon treatment with their respective inhibitors (Supplementary Fig. 8), suggesting that some cellular factors might be required for this dissociation. The decreased binding of R15A to eIF2 α in the presence of Guanabenz was selective, as no such effect was observed with Guanabenz and R15B (Fig. 4e, lanes 13, 14, and f). Likewise, Sephin1 selectively reduced the binding of eIF2 α to R15A (Fig. 4g, lanes 10, 11, and h) but not to R15B (Fig. 4g, lanes 13, 14, and h). This confirms the selectivity of the inhibitors and demonstrates that they compromise the ability of R15A to recruit the eIF2 α substrate. The conformational changes induced by Guanabenz and Sephin1 (Fig. 4c) provides a molecular rationale to explain the altered eIF2 α recruitment to R15A in the presence of the inhibitors.

A recombinant system recapitulates the selective inhibition of R15A inhibitors

The remaining question was whether we could reconstitute the selective inhibition of R15A–PP1 in an activity assay. Whereas recombinant R15A–PP1 and R15B–PP1 were both active eIF2 α holophosphatases, Guanabenz selectively inhibited R15A–PP1 but not R15B–PP1 (Fig. 5a, lanes 8, 9). Sephin1 also selectively inhibited R15A–PP1 in the recombinant system (Fig. 5b, lanes 8, 9). As observed before^{17, 25}, Guanabenz and Sephin1 did not inhibit PP1 (Supplementary Fig. 9). In contrast, we found that Salubrinal, a compound that induces eIF2 α phosphorylation in cells but whose target is unknown²⁰, inhibited PP1 activity (Supplementary Fig. 9d). This finding does not demonstrate that Salubrinal is a PP1 inhibitor. In fact, Salubrinal induced the precipitation of different proteins (R15A, eIF2 α and PP1) even at low concentrations (Supplementary Figs. 7 and 9e,f), unlike Guanabenz and Sephin1 (Supplementary Figs. 7 and 9g,h). Having found that the R15A inhibitors altered recruitment of eIF2 α , we suspected that Guanabenz and Sephin1 target the eIF2 α -binding amino-terminal region of R15A (R15A^N). The unstructured nature of R15A rendered mutagenesis studies inconclusive. Thus, we turned to a different approach and generated chimeras, swapping the R15^N regions of R15A and R15B to assess whether we could transfer the selective inhibition of Guanabenz and Sephin1 from R15A to R15B. Swapping the amino-terminal regions of the R15s generated the functional proteins R15A^N-R15B^C and R15B^N-R15A^C (Fig. 5c, lanes 6, 7). R15A^N-R15B^C–PP1 was inhibited by Guanabenz and Sephin1 (Fig. 5c, lanes 8, 10). In contrast, the R15B^N-R15A^C–PP1 chimera was largely insensitive to Guanabenz and Sephin1 (Fig. 5c, lanes 9, 11). Confirming the selectivity of the assays and of the R15A inhibitors, compound C3 was inactive and did not inhibit R15A–PP1 or R15B–PP1 (Fig. 5d). This establishes that a defined recombinant system containing only three components, R15, PP1 and the eIF2 α substrate, recapitulates the exquisitely selective inhibition of R15A by Guanabenz and Sephin1.

Discussion

Here we have developed a recombinant system composed of only three components, an R15 regulatory subunit, the PP1 catalytic subunit and the eIF2 α substrate, that faithfully recapitulates the physiological function of holophosphatases. This unique feature of the assay was key in order to enable functional studies of R15 holophosphatases and the discovery of the mechanism underlying the selective inhibition of R15A by Guanabenz and Sephin1.

Our recombinant holophosphatase activity assay, which solely depends on the regulatory and catalytic subunits of phosphatases, enabled for the first time the direct dissection of the role of regulatory subunits in the function and selectivity of holophosphatases. The mechanistic and functional study of recombinant R15 holophosphatases provides the molecular explanation for an old conundrum: how a seemingly promiscuous enzyme is in fact highly selective¹¹. *In vitro*, free PP1 alone dephosphorylates any phosphoserine or phosphothreonine protein, as well as artificial phosphosubstrates⁴¹. In cells, this does not occur, because PP1 is not free but bound to regulatory subunits. In our recombinant holophosphatase activity assay, we show that although PP1 alone can be an active phosphatase at high and nonphysiological concentrations, there is a strict dependence on R15 regulatory subunits in order for it to be capable of dephosphorylating eIF2 α when used at physiological concentrations. We demonstrate that this is due to an increased affinity of R15–PP1 for eIF2 α compared to PP1 alone. An unrelated regulatory subunit, R3A, had the opposite effect and decreased the affinity of PP1 to eIF2 α , demonstrating the selectivity of PP1 holoenzymes. We show here that the function of R15s is encoded by both their amino- and carboxy-terminal regions, which bind to the eIF2 α substrate and PP1 catalytic subunit, respectively. Consequently, both regions of R15s are essential for its activity in our holophosphatase assay. The reconstituted R15–PP1 holophosphatases were not only functional but also selective, as they were unable to dephosphorylate a noncognate substrate, phosphorylase a. These results provide the molecular explanation for the substrate-specifier function of regulatory subunits: they recruit the cognate substrate and prevent recruitment of noncognate substrates. Knowing that PP1 can only be detected as a complex with diverse regulatory subunits in cells⁸, where its concentration has been estimated to be $\sim 0.2 \mu\text{M}$ ³⁶, the finding that free PP1 is inactive at low concentrations may represent a safeguarding mechanism to ensure that it remains inactive during holoenzyme biogenesis, until bound to a regulatory subunit.

Phosphatases have long been thought to be undruggable. However, the notion that holophosphatases can be selectively inhibited by targeting their regulatory subunits is emerging^{17, 25}. This has recently been challenged by two studies^{29, 30}. The mechanistic insights into R15A holophosphatase function and inhibition provided here explain why, in one study, Guanabenz had no effect on a carboxy-terminal fragment of R15A (ref. 29): because it lacked the critical amino-terminal region. We defined that the amino-terminal region of R15A is not only required for its function but is responsible for inhibition by Guanabenz and Sephin1. In a second study, the authors did not observe inhibition of a recombinant R15A–PP1 complex and concluded that the effects of Guanabenz and Sephin1 were independent of R15A³⁰. Our R15–PP1 holoenzymes also appeared inactive when we

stopped the reaction after 20 min, as in the Crespillo-Casada *et al.*³⁰ study, but were active upon longer incubation (Supplementary Fig. 10). In addition, the recombinant proteins used here were different from those used in that study. Expression of functional PP1 in heterologous systems is notoriously difficult, and the properties of native and recombinant PP1 often differ with regard to selectivity as well as sensitivity to inhibitors and regulatory subunits³⁵. Thus, we followed an optimized protocol to produce recombinant PP1 with nearly native properties³⁵ by co-expressing it in *Escherichia coli* at 10 °C with the chaperones GroEL and GroES, in the presence of MnCl₂, which is known to stabilize the active site³⁵ (Online Methods). In the study by Crespillo-Casado *et al.*³⁰, PP1 was expressed at 18 °C without these chaperones. Moreover, their *in vitro* assays relied on the presence of actin, yet the physiological relevance of actin for R15 phosphatase function is unclear. Here, we clearly establish that actin is not required for the activity of R15 holoenzymes. It is important to highlight that controls consisting of repeating the standard and previously published assays^{17, 25} to assess the potency and selectivity of the inhibitors in cells were not performed in the study by Crespillo-Casado *et al.*³⁰.

Because little is known about the function of regulatory subunits other than their binding region to PP1, other labs have focused on studying this interaction, with the underlying assumption that the inhibitors ought to disrupt the R15A–PP1 interaction^{29, 30}. Although we observed that the R15A–PP1 complexes dissociate in cells treated with R15A inhibitors^{17, 25}, we expected this to be a consequence of an allosteric change in the holoenzyme rather than the result of a direct disruption of the R15A–PP1 interaction interface by the inhibitors. This is because the inhibitors are small and thereby unlikely to compete with the large R15A–PP1 interface²⁹. In addition, because this region is conserved in different regulatory subunits, small molecules disrupting the conserved PP1 binding region would probably not be selective.

Thus, understanding the mechanism of action of R15A inhibitors required an understanding of the function of the regulatory subunit. Here we show that the amino-terminal domains of R15 are essential for function, and this finding was a stepping-stone to elucidate the mechanism of action of the R15A inhibitors. We have recapitulated the selective inhibition of R15A–PP1 by Guanabenz and Sephin1 *in vitro* and revealed how inhibition is achieved: the inhibitors alter the protease sensitivity of R15A but not R15B, indicating that they induce a selective conformational change in R15A, compromising its substrate-binding function, ultimately resulting in a loss of activity in our holophosphatase assay. Our results not only explain the mechanism of action of R15A and its inhibitors but also provide mechanistic insights to rationalize our early observations, suggesting that the dissociation of R15A–PP1 complexes observed in cells^{17, 25} is a consequence, not a cause, of the inhibition. In cells, such a conformational change induced by the selective inhibitors is likely to render R15A prone to degradation. Remarkably, we found that selective inhibition by Guanabenz and Sephin1 can be transferred from R15A to R15B by swapping the amino-terminal region of the proteins, indicating that the amino-terminal region of R15A is responsible for inhibition by Guanabenz and Sephin1. Our findings suggest that these inhibitors are allosteric inhibitors of R15A that bind selectively to its amino-terminal region and induce a conformational change. As a result, the function of the regulatory subunit is compromised and dephosphorylation of the substrate is inhibited. A close derivative of Guanabenz,

compound C3, lacking the cytoprotective activity of Guanabenz in cells was inactive in the assay, confirming the relevance and selectivity of our holophosphatase activity assay. Thus, we provide here multiple lines of evidence in different and biochemically defined assays, demonstrating that Guanabenz and Sephin1 are selective inhibitors of R15A.

We have previously exemplified the power, the safety and the therapeutic benefit of selective inhibition of a regulatory subunit of a holophosphatase in neurodegenerative disease models associated with ER stress²⁵. Here we developed assays to reveal the function of R15 that lead us to uncover the molecular basis of the selective inhibition of R15A by Guanabenz and Sephin1. This work confirms and validates that both Guanabenz and Sephin1 (refs. 17,25) are selective inhibitors of R15A. Lastly, the suite of versatile assays described here are applicable to hundreds of holophosphatases, opening up a broad range of possibilities to study a large, mostly untapped class of enzymes.

Methods

Protein expression and purification

We cloned the cDNA encoding human PPP1R15A, PPP1R15B and PPP1R3A regulatory subunits into a pMAL-c5x-His vector, encoding for an amino-terminal MBP-tag and a carboxy-terminal His₆-tag. We cloned the following constructs: PPP1R15A amino acids 325–636 (R15A), 325–512 (R15A^N), and 513–636 (R15A^C); PPP1R15B amino acids 340–698 (R15B), 340–635 (R15B^N), and 636–698 (R15B^C); and PPP1R3A amino acids 1–240 (R3A). We obtained R15A^N and R15B^C chimeras were by swapping the amino-terminal regions (R15^N) of R15A and R15B proteins, described above, maintaining the same carboxy-terminal regions (R15^C), to produce R15B^N–R15A^C and R15A^N–R15B^C chimeras, respectively, using In-Fusion cloning (Takara). Empty pMAL-c5x-His vector, encoding for MBP-His₆ protein was used as a control where specified. All regulatory subunits were expressed in BL21 pLysS cells in Luria broth (LB) at 30 °C overnight. Proteins were purified by means of tandem affinity chromatography using HisTrap excel (GE Healthcare) followed by an MBPTrap HP column (GE Healthcare) using buffer A (50 mM Tris pH 7.4, 200 mM NaCl). Regulatory subunit proteins were dialyzed in buffer A and stored at –80 °C, freeze thawed only once and used within one month.

The cDNA encoding amino acids 7–330 of human PP1 α was cloned into a modified pGEX6p1 vector in which the vector's GST-tag was replaced by an amino-terminal Thio₆/His₆-tag (MGSDKIHSHHHH). The PP1^{D95A} mutant was obtained using site-directed mutagenesis³⁸. PP1 proteins were expressed and purified using a protocol adapted from ref. 35. PP1 proteins were expressed in BL21/pGro7 cells (Takara) in LB supplemented with 50 μ g/ml ampicillin, 35 μ g/ml chloramphenicol and 2 mM MnCl₂. Cells were grown at 35 °C until OD₆₀₀ 0.5. Expression of the pGro7 plasmid was then induced with 1 g/L l-arabinose, and the temperature was immediately lowered to 10 °C. At OD₆₀₀ 1.0, PP1 expression was induced with 0.1 mM IPTG. After 48 h expression at 10 °C, cells were harvested and resuspended in fresh LB supplemented with 2 mM MgCl₂ and 200 μ g/ml chloramphenicol to stop new protein synthesis, giving time for the recombinant PP1 to be folded by the *E. coli* folding machinery. Cells were incubated for a further 2 h at 10 °C and then harvested. Thio₆/His₆-PP1 was purified by affinity chromatography on a HisTrap excel column (GE

Healthcare) followed by size exclusion chromatography on a HiLoad 16/600 Superdex 200 pg column (GE Healthcare) using PP1 buffer (50 mM Tris pH 7.4, 1 M NaCl, 2 mM MnCl_2). Purified PP1 was stored at -80°C , in PP1 buffer, in stocks above 20 μM , and diluted in the appropriate buffer immediately before use. It is important to note that PP1 was not freeze thawed and was used within one month.

GST-tagged (amino-terminal) murine PERK kinase domain (amino acids 537–1114) (Addgene #21817, <https://www.addgene.org/21817/>) and His₆-tagged (carboxy-terminal) human eIF2 α (amino acids 1–185) solubility-enhanced mutant⁴² were expressed in BL21 pLysS cells in LB at 37°C for 6 h. GST-PERK was purified on GST Sepharose beads (GE Healthcare) followed by size exclusion chromatography on a HiLoad 16/600 Superdex 200 pg column (GE Healthcare) using kinase buffer (50 mM Tris pH 7.4, 100 mM NaCl, 10 mM MgCl_2 , 5 mM DTT). eIF2 α was purified by affinity chromatography on a HisTrap excel column (GE Healthcare), followed by size exclusion chromatography on a HiLoad 16/600 Superdex 200 pg column (GE Healthcare) using buffer A.

eIF2 α phosphorylation

eIF2 α was phosphorylated on residue Ser51 using purified PERK kinase, as described above. 1 mg of purified PERK, in a final volume of 1 ml, was incubated with 50 μl of GST Sepharose beads (GE Healthcare), pre-equilibrated with kinase buffer for 30 min at RT. Excess PERK was removed by washing the beads 3 times with 1 ml kinase buffer, by spinning the samples gently at 2,000 r.p.m. for 5 min at 4°C . 500 μg of purified eIF2 α , in a final volume of 1 ml, predialyzed in kinase buffer, was added to PERK-containing GST beads. 5 mM ATP (pH 7.4) was added to the reaction and phosphorylation was allowed to proceed for 1 h at 37°C with shaking at 350 r.p.m. The supernatant, containing phosphorylated eIF2 α , was collected. Phosphorylated eIF2 α was further purified by size exclusion on a HiLoad 16/600 Superdex 200 pg column (GE Healthcare) using dephosphorylation buffer (50 mM Tris (pH 7.4), 1.5 mM EGTA (pH 8.0), 2 mM MnCl_2). Elution fractions were run on 4–12% NuPAGE Bis-Tris gels (Life Technologies) and visualized by staining with InstantBlue Protein Stain (Expedeon). Samples containing pure phosphorylated eIF2 α (P-eIF2 α) were pooled and concentrated to 5 mg/ml, and stored at -80°C in small aliquots.

Phosphorylase b phosphorylation

Phosphorylase b was phosphorylated using radioactive ^{33}P isotope, following a protocol adapted from ref. 43. 10 mg of phosphorylase b (Sigma) was dissolved in 500 μl of reaction buffer (200 mM Tris (pH 7.4), 200 mM glycerol-1-phosphate, 200 μM CaCl_2 , 20 mM $\text{Mg}(\text{C}_2\text{H}_3\text{O}_2)_2$). 3.6 mg of phosphorylase kinase (Sigma) was dissolved in 1.8 ml of reaction buffer. 87.5 μl of phosphorylase b solution and 125 μl of phosphorylase kinase were made up to 245 μl with reaction buffer. After 10 min of gentle shaking at RT, the solution was centrifuged at 15,000 g for 5 min to remove protein precipitates. The supernatant was transferred to a new tube. The phosphorylation reaction was initiated by the addition of 1 μl of 10 mM ATP and 4 μl of $[\gamma\text{-}^{33}\text{P}]\text{ATP}$ 10 mCi/ml stock (NEG602H100UC, Perkin Elmer) and allowed to proceed for 2 h at 30°C with shaking at 350 r.p.m. To stop the reaction, the solution was added directly to a PD MiniTrap G-25 desalting column (GE Healthcare),

which was pre-equilibrated with dephosphorylation buffer (50 mM Tris (pH 7.4), 1.5 mM EGTA (pH 8.0), 2 mM MnCl_2), using the gravity protocol. Desalted protein samples were collected and a Bradford assay was used to measure the concentrations of protein. Phosphorylated phosphorylase b is known as phosphorylase a.

In vitro dephosphorylation of P-eIF2 α and 33P-phosphorylase a

PP1 and regulatory subunits were diluted to the appropriate concentration (as indicated in the figure legends) in dephosphorylation buffer immediately before use. Dephosphorylation reactions were performed in a final volume of 35 μl . Diluted PP1 was pre-incubated in the presence or absence of regulatory subunits (at a final concentration of 1 μM for experiments in Figs. 1 and 2 and Supplementary Figs. 3 and 4 and 50 nM for experiments in Fig. 5 and Supplementary Fig. 10), and/or compounds (all at 100 μM , except for calyculin A, which was used at 100 nM, and Salubrinal, which was used at 30 μM). All compounds were diluted in DMSO, and a DMSO vehicle was used in all control experiments. Pre-incubation was carried out for 15 min at room temperature.

The dephosphorylation reaction was then initiated by the addition of P-eIF2 α or ^{33}P -labeled phosphorylase a substrates at a final concentration of 1 μM . Samples were incubated at 30 °C for 16 h, with shaking at 350 r.p.m. Reactions were stopped by the addition of 4% SDS Laemmli sample buffer.

eIF2 α samples were analyzed by immunoblotting or Phos-tag gels. For immunoblotting, samples were diluted ten-fold, and 10 μl of the reactions were loaded on 4–12% NuPAGE Bis-Tris gels (Life Technologies). Immunoblotting was carried out using α -Phospho-eIF2 α (Ser51) (#9721, Cell Signaling) and total α -eIF2 α (ab26197, AbCam) antibodies. The epitope of total α -eIF2 α (ab26197, AbCam) (eIF2 α residues 50–150) overlaps with the phosphorylated residue Ser51. Therefore this antibody preferentially bound to nonphosphorylated eIF2 α compared to P-eIF2 α protein. To try and circumvent this bias, total α -eIF2 α (ab26197, AbCam) was left for a minimum of 24 h at 4 °C on the membrane. For Phos-tag gels, 15 μl of nondiluted samples were run on a 15% SuperSep Phos-tag acrylamide gel (Alpha Laboratories) and visualized by staining with InstantBlue Protein Stain (Expedeon).

^{33}P -labeled phosphorylase a samples were analyzed by phosphorimaging. 10 μl of samples were run on 4–12% NuPAGE Bis-Tris gels (Life Technologies) and visualized with InstantBlue Coomassie Protein Stain (Expedeon) to monitor total phosphorylase levels. To measure levels of phosphorylated ^{33}P -phosphorylase a, the gel was analyzed by phosphorimaging.

Protein binding to biotinylated compounds

Purified R15A protein was diluted to 1 μM in IP buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 0.1% Tween20, 10% glycerol). 1 μM R15A, in 100 μl volume, was precleared with 25 μl of Pierce Streptavidin Magnetic Beads (ThermoFisher) for 1 h at 4 °C with rotation at 20 r.p.m. The supernatants were collected and incubated with 0.5 mM biotinylated Guanabenz, Sephin1 or biotin control plus 25 μl of pre-equilibrated Pierce Streptavidin Magnetic Beads. Samples were incubated for 3 h at 4 °C with rotation at 20 r.p.m. The supernatant was

removed, and samples were thoroughly washed and transferred to a fresh Eppendorf tube. The beads were washed thoroughly with 5×1 ml interaction buffer, with 30 min incubation each time and then resuspended with 50 μ l of 4% SDS Laemmli sample buffer. Samples were run on 4–12% NuPAGE Bis-Tris gels (Life Technologies) and analyzed by immunoblotting using α -MBP HRP (E8038, NEB) to reveal R15A. Elution experiments were performed on beads containing R15A bound to biotinylated Guanabenz or biotinylated Sephin1 by adding 100 μ l of interaction buffer containing 2 mM Guanabenz or Sephin1, respectively, or DMSO vehicle control. Samples were incubated for 10 min at 4 °C with 20 r.p.m. rotation. 30 μ l of supernatant was added to 10 μ l of 16% SDS Laemmli sample buffer. 10 μ l sample was run on 4–12% NuPAGE Bis-Tris gel (Life Technologies) and analyzed by immunoblotting using α -MBP HRP (E8038, NEB) to reveal R15A.

Thermophoresis affinity measurements

Thermophoresis experiments were performed using a Monolith NT.115 instrument (NanoTemper Technologies). Purified PP1 and P-eIF2 α proteins were labeled using the Monolith NT Protein labeling Kit Red-NHS, following the manufacturer's instructions, and stored for no longer than a month at –80 °C. For thermophoresis experiments, all protein dilutions were carried out in thermophoresis buffer (50 mM HEPES (pH 7.4), 100 mM NaCl, 0.1% Tween20, 2 mM MnCl₂). Labeled proteins (50 nM) were mixed with equal volumes of 1:1 serial dilutions of the unlabeled binding partner. In holophosphatase-binding experiments, labeled P-eIF2 α was diluted to 50 nM in thermophoresis buffer already containing 10 μ M of the appropriate regulatory subunit, and then mixed as described above with 1:1 serial dilutions of unlabeled PP1. All experiments were carried out in enhanced-grade capillaries, using 100% LED power and 100% IR-laser (on for 25 s), at 20 °C. GraphPad Prism software was used to fit the data with a nonlinear regression (least-squares fit) curve and dissociation constants (K_D) were determined. Each measurement was repeated in three independent experiments, and mean K_D values ($\pm$ s.e.m.) are reported ($n = 3$; biological replicates).

Limited trypsin proteolysis

Purified R15A, R15B or MBP were diluted to 0.5 μ M in PBS (13.7 mM NaCl, 0.27 mM KCl, 0.8 mM NaHPO₄, 0.2 mM KH₂PO₄) pH 7.4, in a final volume of 200 μ l, and incubated for 15 min at room temperature with 100 μ M compound or DMSO vehicle. Reactions were initiated by the addition of 2.5 nM of trypsin from bovine pancreas (Sigma), made from the lyophilized powder in PBS. Reactions were allowed to proceed at 22 °C with shaking at 350 r.p.m. At time points 30 min, 1 h, 2 h or 3h, 30 μ l of sample was removed from the mix and digestion was stopped by the addition of 10 μ l of 16% SDS Laemmli sample buffer. Samples were run on 4–12% NuPAGE Bis-Tris gels (Life Technologies). Proteins were visualized by staining with InstantBlue Coomassie Protein Stain (Expedeon).

Pull down experiments

Purified MBP-tagged regulatory subunits (200 nM), P-eIF2 α (1 μ M) or PP1 (1 μ M), and 200 μ M compounds, or DMSO vehicle, were added as appropriate to 20 μ l amylose magnetic beads, pre-equilibrated with interaction buffer (50 mM Tris (pH 7.4), 200 mM NaCl, 0.05% Tween20) in 200 μ l volume. All protein dilutions were carried out in

interaction buffer. 5% of input sample was removed and added to 4% Laemmli SDS sample buffer for further analysis. The beads were then incubated for 10 min at 4 °C with shaking at 350 r.p.m. to allow R15s to bind to the beads, as well as for P-eIF2 α or PP1, and the compounds, where relevant, to bind to the R15s. The supernatant was removed, and samples were transferred to a fresh Eppendorf tube. The beads were washed thoroughly with 5 $\times$ 1 ml interaction buffer and then resuspended with 50 μ l of 4% SDS Laemmli sample buffer. Samples were run on 4–12% NuPAGE Bis-Tris gels (Life Technologies) and analyzed by immunoblotting using α -MBP HRP (E8038, NEB) to reveal R15s, α -eIF2 α (ab26197, AbCam) or α -PPP1A (ab137512, AbCam) antibodies. eIF2 α immunoblots were quantified and normalized, independently for R15A and R15B, against vehicle control samples. The means of three independent experiments were plotted $\pm$ s.e.m. ($n = 3$; biological replicates).

Visualization of protein precipitates

Purified P-eIF2 α (1 μ M) or PP1 (1 μ M) were diluted with dephosphorylation buffer and compounds were added, at the indicated concentration, in a final volume of 50 μ l. Samples were allowed to equilibrate for 5 min at room temperature before visualizing.

Light scattering

Light scattering experiments, to monitor the presence of protein aggregates, were performed with a Varian Cary Eclipse Fluorescence Spectrophotometer (Agilent), as used in ref. 44. R15A was diluted to 5 μ M, and 1 mM Guanabenz, 1 mM Sephin1, 1 mM C3, 50 μ M Salubrinal or DMSO vehicle control were added. Samples were incubated for 10 min at RT. In a range of 320–400 nm, soluble proteins do not absorb, whereas protein aggregates do. Therefore, light scattering was measured at 380 nm emission, 380 nm absorption to monitor the presence of aggregates. For each sample, 100 data points were collected over a period of 10 min, at 20 °C, with constant stirring.

Compound synthesis

Guanabenz, Sephin1 and the biotinylated derivatives of Guanabenz and Sephin1, were synthesized as previously described^{17, 25}. Salubrinal (Sal 003) was purchased from Sigma. Compound C3, (E)-2-((3-chloropyridin-2-yl)methylene)hydrazine-1-carboximidamide, was synthesized as follows.

3-chloropyridine-2-carbaldehyde (0.25 g, 0.001773 Mol) was dissolved in ethanol (10 mL) at room temp. 1-aminoguanidine hydrochloride (0.196 g, 0.001773 Mol) and sodium acetate trihydrate (0.241 g, 0.001773 Mol) were added, and the reaction was heated to reflux at 80 °C for 3 h. The reaction was added to a solution of saturated sodium bicarbonate. The solid was filtered off, and the solid residue was washed with demineralized water, hexane and ether. The solid was dried and triturated with diethyl ether. The product yield was 0.170 g (0.00086 Mol, 48%).

Cytoprotection experiments

We plated 40,000 cells/ml HeLa cells in a 96-well plate and treated them with different concentrations of compound, as indicated, or DMSO vehicle in the presence of 250 ng/ml tunicamycin for 72 h. To monitor cell death, we added a 1:2,000 dilution of CellTox green

dye (Promega) to the media. The growth of the cells was monitored over time and pictures were taken every 2 h with the IncuCyte ZOOM system and analyzed by the IncuCyte ZOOM software (Essen BioScience). To compare different compounds and their cytoprotective effect, a growth ratio for each time point was calculated:

$$\text{Growth ratio} = \frac{\text{Phase confluency (\%)} \text{ at } x \text{ hours}}{\text{Phase confluency (\%)} \text{ at } 0 \text{ hours}}$$

The end point of the assay (72 h) was chosen for generating the graphs.

Statistical analyses

Representative results of three independent experiments (biological replicates) are shown in all panels. GraphPad Prism software was used for all statistical analyses. Data are presented as means $\pm$ s.e.m. Data were analyzed using one-way or two-way ANOVA, as indicated in the figure legends. The level of significance was set at * P 0.05, ** P 0.01, *** P 0.001; n.s., not significant.

Data availability

Raw data used to obtain graphs in Figures 1d,2c,d,3,4f,h and 5 and Supplementary Figures 5 and 7 are available in Supplementary Table 2. Original images of all uncropped gels are available in Supplementary Data Set 1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Reconstitution of functional eIF2 α holophosphatases with recombinant proteins.

(a) Immunoblots showing P-eIF2 α and eIF2 α following a dephosphorylation reaction of 1 μ M P-eIF2 α by recombinant PP1 (1 μ M) in the presence or absence of 1 μ M of recombinant R15A, R15B or R3A. (b,c) Immunoblots of P-eIF2 α and eIF2 α following a dephosphorylation reaction of 1 μ M P-eIF2 α by PP1 (1 μ M) in the presence or absence of (b) R15A and its inhibitors Guanabenz or Sephin1 and (c) calyculin A. (d) A titration curve of P-eIF2 α (1 μ M) dephosphorylation by increasing PP1 concentrations. A representative immunoblot corresponding to this titration is shown in Supplementary Figure 2b. Data are means $\pm$ s.e.m. (n = 3 independent experiments). (e–g) Immunoblots of P-eIF2 α and eIF2 α following a dephosphorylation reaction of 1 μ M P-eIF2 α by PP1 (10 nM) in the presence or absence of 1 μ M (e) R15A, (f) R15B, or (g) R3A. All dephosphorylation reactions were carried out at 30 $^{\circ}$ C for 16 h. For all experiments, data shown is representative of one of three independent experiments. Uncropped images for blots shown are available in Supplementary Data Set 1.

Figure 2. Defining the R15 domains required for PP1 binding and eIF2 α holophosphatase activity.

(a,b) Schematics of the proteins studied here: (a) R15A and (b) R15B. Amino acid residues delimiting the amino-terminal and carboxy-terminal regions and the presence of an amino-terminal MBP-tag and carboxy-terminal His₆-tag are shown. The location of the PP1-binding region is indicated. (c,d) Thermophoresis binding curves of labeled PP1 binding to titrations of unlabeled (c) R15A (amino acids 325–636), R15A^N (amino acids 325–512), R15A^C (amino acids 513–636) or (d) R15B (amino acids 340–698), R15B^N (amino acids 340–635), R15B^C (amino acids 636–698). Dissociation constants (K_D) are means $\pm$ s.e.m. ($n = 3$ independent experiments). Thermophoresis raw data are available in Supplementary Table 2. (e,f) Immunoblots of P-eIF2 α and eIF2 α following a dephosphorylation reaction by PP1 (10 nM) in the presence or absence of (e) R15A, R15A^N, R15A^C or (f) R15B, R15B^N, R15B^C. Dephosphorylation reactions were carried out at 30 °C for 16 h. Data shown are representative immunoblots of three independent experiments; uncropped images for blots shown are in Supplementary Data Set 1.

Figure 3. Functional R15 holoenzymes have a higher affinity for their substrate than PP1.

(a) Thermophoresis binding curve of labeled P-eIF2 α binding to titrations of unlabeled PP1^{D95A}. K_D is the mean $\pm$ s.e.m. ($n = 3$; biological replicates). (b–d) Thermophoresis-binding curves of labeled P-eIF2 α binding to titrations of unlabeled (b) R15A, R15A^N, R15A^C (c) R15B, R15B^N, R15B^C, or (d) R3A. K_D are means $\pm$ s.e.m. ($n = 3$ independent experiments). Thermophoresis raw data are available in Supplementary Table 2. (e) K_D of labeled P-eIF2 α to titrations of unlabeled PP1^{D95A}, in the presence of saturating, and unlabeled, functional R15 (R15A or R15B), their nonfunctional carboxy-terminal fragments (R15A^C or R15B^C), their amino-terminal fragments (R15A^N or R15B^N), or R3A. The value of P-eIF2 α binding to PP1^{D95A} corresponds to that shown in a. K_D values are means $\pm$ s.e.m. ($n = 3$ independent experiments). Details in Supplementary Table 1. Statistical

significances, relative to PP1^{D95A} binding to P-eIF2 α , are shown. ** P 0.01, *** P 0.001, n.s., not significant (one-way ANOVA).

Figure 4. R15A inhibitors alter the protease sensitivity of R15A and selectively decrease substrate binding to R15A.

(a) Binding of R15A to biotinylated Guanabenz and biotinylated Sephin1 immobilized on streptavidin beads. Immunoblots of input and bound samples, probed with α -MBP (to reveal R15s) are shown. Bound samples (lane 3) were eluted with an excess of Guanabenz (top) or Sephin1 (bottom). Data shown are representative of three independent experiments. (b) The chemical structures of Guanabenz, Sephin1 and C3. (c,d) Coomassie-stained gels showing limited trypsin digestion of (c) R15A and (d) R15B in the presence or absence of

Guanabenz, Sephin1 or C3. Trypsin digestions were carried out using 2.5 nM of trypsin, and reactions were allowed to proceed for 0 h, 30 min, 1 h, 2 h and 3 h (left to right lanes of each gel, respectively) at 22 °C and terminated by the addition of 4% SDS Laemmli sample buffer. Data shown are representative of three independent experiments. **(e–h)** Binding of P-eIF2 α to MBP-tagged R15s immobilized on magnetic amylose beads in the presence or absence of **(e)** Guanabenz or **(g)** Sephin1. Immunoblots of input and bound samples, probed with α -MBP (to reveal R15s) or α -eIF2 α antibodies are shown. Data shown are representative of three independent experiments. **(f,h)** Levels of eIF2 α bound to R15s in the presence or absence of **(f)** Guanabenz or **(h)** Sephin1. eIF2 α immunoblots of three independent pull down experiments, as shown in **e** and **g** were quantified and normalized independently for R15A and R15B against DMSO control samples (lanes 10 and 13, for R15A and R15B, respectively, in **e** and **g**). Means $\pm$ s.e.m. ($n = 3$ independent experiments) are shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, n.s., not significant (two-way ANOVA). Uncropped images of blots shown are in Supplementary Data Set 1.

Figure 5. An activity assay with functional recombinant R15 holoenzymes recapitulates selective inhibition of R15A by Guanabenz and Sephin1.

(a,b) Immunoblots of P-eIF2α and eIF2α following a dephosphorylation reaction of 1 μM P-eIF2α by R15A-PP1 and R15B-PP1 holoenzymes in the presence or absence of (a) Guanabenz or (b) Sephin1. (c) Immunoblots of P-eIF2α and eIF2α following a dephosphorylation reaction of 1 μM P-eIF2α by R15A^N-R15B^C-PP1 and R15B^N-R15A^C-PP1 chimeric holoenzymes in the presence or absence of Guanabenz or Sephin1. (d) Immunoblots of P-eIF2α and eIF2α following a dephosphorylation reaction of 1 μM P-eIF2α by R15A-PP1 and R15B-PP1 holoenzymes in the presence or absence of C3. All dephosphorylation reactions were carried out using R15 at 50 nM and PP1 at 10 nM at 30 °C for 16 h. For all experiments, data shown are representative of three independent experiments. P-eIF2α levels were quantified and normalized against levels in lane 2 (P-eIF2α alone) and expressed as percentage relative to levels in lane 2. Uncropped images of blots shown are in Supplementary Data Set 1.