Post-translational Regulation of FNIP1 Creates a Rheostat for the Molecular Chaperone Hsp90

Highlights

- Casein-kinase-2-mediated sequential phosphorylation of the co-chaperone FNIP1
- FNIP1 relay phosphorylation leads to gradual activation of Hsp90 clients
- Serine/threonine protein phosphatase 5 (PP5) dephosphorylates FNIP1
- O-GlcNAcylation causes ubiquitination and proteasomal degradation of FNIP1

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In Brief

Sager et al. show that casein-kinase-2-mediated sequential phosphorylation of the co-chaperone FNIP1 leads to incremental inhibition of Hsp90 ATPase activity and gradual activation of both kinase and non-kinase clients. O-GlcNAcylation antagonizes phosphorylation of FNIP1, preventing its interaction with Hsp90, and consequently promotes FNIP1 ubiquitination and proteasomal degradation.
Post-translational Regulation of FNIP1 Creates a Rheostat for the Molecular Chaperone Hsp90

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INTRODUCTION

The molecular chaperone heat shock protein 90 (Hsp90) is essential in eukaryotes and is involved in looking after target proteins, also referred to as client proteins (Picard, 2002; Schopf et al., 2017; Verba and Agard, 2017). Hsp90 exists as a dimer, and each protomer consists of (1) an N-terminal domain, where the nucleotide-binding pocket resides; (2) a middle-domain; and (3) a C-terminal domain, which is constitutively dimerized with the other protomer. The N-M domains of Hsp90 are connected by a charged linker (Mayer, 2010; Prodromou, 2016; Röhl et al., 2013). Hsp90 chaperone function is linked to its ability to bind and hydrolyze ATP (Panaretou et al., 1998; Zierer et al., 2016), which allows for a series of conformational changes known as the chaperone cycle, wherein an Hsp90 dimer progresses from an N-terminally “open” to a “closed” conformation. Co-chaperones and post-translational modifications (PTMs) regulate the Hsp90 chaperone cycle and, consequently, the chaperoning and activity of the client proteins (Sager et al., 2018; Sahasrabudhe et al., 2017).

As the name suggests, folliculin-interacting protein 1 (FNIP1) was initially identified as a binding partner of the tumor suppressor folliculin (FLCN) (Baba et al., 2006). Our subsequent work revealed FNIP1 as a co-chaperone of Hsp90 that acts as a scaffold to load FLCN onto Hsp90 (Sager et al., 2018; Woodford et al., 2016). FNIP1 is also involved in chaperoning of both kinase and non-kinase clients. FNIP1 does not have any known functional domains; however, based on amino acid sequence alignments, conserved regions were identified and named as A–D. The C-terminal domain of FNIP1 (amino acids 929–1,166 or fragment D) preferentially interacts with the middle domain of Hsp90. This fragment and the full-length FNIP1 are potent inhibitors of Hsp90 ATPase activity (Woodford et al., 2016). Small-molecule inhibitors that target the nucleotide-binding pocket of the N-terminal domain of Hsp90 also inhibit its ATPase activity and lead to degradation of the client proteins (Neckers et al., 2018). However, FNIP1-mediated inhibition of Hsp90 ATPase activity appears to decelerate the chaperone cycle, not inhibit it completely, as overexpression of FNIP1 stabilizes and activates client proteins. This can also be reversed by the co-chaperone Aha1, which is the activator of the Hsp90 ATPase function and competes with FNIP1 for binding to Hsp90 (Woodford et al., 2016).

SUMMARY

The molecular chaperone Hsp90 stabilizes and activates client proteins. Co-chaperones and post-translational modifications tightly regulate Hsp90 function and consequently lead to activation of clients. However, it is unclear whether this process occurs abruptly or gradually in the cellular context. We show that casein kinase-2 phosphorylation of the co-chaperone folliculin-interacting protein 1 (FNIP1) on priming serine-938 and subsequent relay phosphorylation on serine-939, 941, 946, and 948 promotes its gradual interaction with Hsp90. This leads to incremental inhibition of Hsp90 ATPase activity and gradual activation of both kinase and non-kinase clients. We further demonstrate that serine/threonine protein phosphatase 5 (PP5) dephosphorylates FNIP1, allowing the addition of O-GlcNAc (O-linked N-acetylglucosamine) to the priming serine-938. This process antagonizes phosphorylation of FNIP1, preventing its interaction with Hsp90, and consequently promotes FNIP1 lysine-1119 ubiquitination and proteasomal degradation. These findings provide a mechanism for gradual activation of the client proteins through intricate crosstalk of post-translational modifications of the co-chaperone FNIP1.
Figure 1. Sequential Phosphorylation of the FNIP1 Co-chaperone by CK2 Kinase

(A) Endogenous FNIP1 protein was isolated from HEK293 cells, and the profile of interacting proteins was determined by MALDI-TOF.

(B) FNIP1 was immunoprecipitated (IP) from HEK293 cell lysates using anti-FNIP1 or immunoglobulin G (IgG) (control) and immunoblotted with indicated antibodies to confirm protein interactions.

(C) FNIP1-HA, FNIP1-D-HA, and empty vector (EV) were transiently expressed and isolated by IP from HEK293 cells. Indicated co-IP proteins were immunoblotted with indicated antibodies to confirm protein interactions.

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study, we used the FNIP1-D fragment (Hsp90-binding region of FNIP1) and identified a series of serine residues (938, 939, 941, 946, and 948) that were phosphorylated by casein-kinase-2 (CK2) both in vivo and in vitro. This led to stepwise binding of FNIP1 to Hsp90, incremental inhibition of Hsp90 ATPase activity, and gradual activation of both kinase and non-kinase clients. We further demonstrated that protein phosphatase 5 (PP5) dephosphorylates FNIP1, allowing for subsequent O-GlcNAcylation (addition of O-linked N-acetylglucosamine) of the priming serine-938. This antagonized phosphorylation of FNIP1 and disrupted its interaction with Hsp90 as well as promoted ubiquitination of FNIP1 lysine-1119 and eventual degradation of FNIP1 in the proteasome. These data provide a mechanism for gradual activation of the kinase and non-kinase client proteins through intricate crosstalk of PTMs of the co-chaperone FNIP1.

RESULTS

CK2 Sequentially Phosphorylates FNIP1 Co-chaperone

FNIP1 is a co-chaperone of Hsp90 (Woodford et al., 2016). To gain further insight into the binding partners and their involvement in regulation of FNIP1, we immunoprecipitated endogenous FNIP1 from HEK293 cells followed by mass spectrometry (MS) analysis. We identified Hsp90, CK2, O-linked N-acetylglycosamine (GlcNAc) transferase (OGT), and serine/threonine-protein phosphatase 5 (PP5) to interact with FNIP1 (Figure 1A). We confirmed these data by immunoprecipitation of FNIP1 from HEK293 cells followed by immunoblotting (Figure 1B). FLCN and FNIP2 proteins are known binding partners of FNIP1 (Woodford et al., 2016), and they were used as positive controls. FNIP1 does not have any known functional domains; however, our previous work has shown that its C-terminal region (amino acids 929–1,166 or fragment D) binds to Hsp90 and inhibits its ATPase activity (Woodford et al., 2016). Transient expression and immunoprecipitation of FNIP1-D-HA from HEK293 showed that the FNIP1-D fragment, like full-length FNIP1 hemagglutinin (HA), also binds to Hsp90, CK2, OGT, and PP5 (Figure 1C). We next showed that CK2 is capable of phosphorylating FNIP1 on the FNIP1-D fragment on serine, but not threonine, residues in vitro (Figures 1D and 1A).

We examined the sequence of the FNIP1-D fragment and found that S946 was the only serine to fit the canonical CK2 consensus sequence (Cesaro and Pinna, 2015). This serine, however, was present in a stretch of residues that included a number of other serine residues as well as multiple aspartic and glutamic acids. CK2 is well known to be capable of multisite phosphorylation, with non-canonical consensus sequences recognizing acidic residues or phosphorylated serine residues in close proximity to the serine of interest (Cesaro and Pinna, 2015). In fact, FNIP1-S938 was identified as a possible CK2 phosphorylation site in a systematic investigation for these non-canonical hierarchical consensus sequences (St-Denis et al., 2015). We therefore made non-phosphorylatable alanine mutants of this series of serine residues (S938, S939, S941, S946, and S948) and bacterially expressed and purified these mutants as well as the wild-type FNIP1-D fragment. We performed an in vitro kinase assay using CK2 and ATP followed by immunoblotting with pan-phosphoserine antibody, which showed a gradual reduction of serine phosphorylation from S948A to S938A (Figures 1E and S1B). Interestingly, this was not due to alteration of FNIP1-D binding to CK2. Our data here therefore suggest that CK2 phosphorylates these serine sites in the FNIP1-D fragment in a relay manner (Figure 1F).

PP5 Relay Dephosphorylation of FNIP1 Disrupts Its Interaction with Hsp90

PP5 is a serine/threonine-protein phosphatase and also a co-chaperone of Hsp90 (Schopf et al., 2017). Since PP5 interacts with FNIP1, we decided to check its ability to dephosphorylate FNIP1. Expression and purification of the FNIP1-D fragment as well as the non-phosphorylating alanine mutants (S938A, S939A, S941A, S946A, and S948A) from bacteria followed by in vitro phosphorylation with CK2 again confirmed serine phosphorylation of FNIP1-D in a relay manner (Figures 2A and S2A). Addition of PP5 to these reactions led to dephosphorylation of wild-type FNIP1-D, but not its non-phosphorylatable alanine mutants, even though PP5 interacted with all of the mutants (Figures 2A and S2B). We repeated this experiment in HEK293 cells by transiently expressing wild-type FNIP1-D-HA or its individual non-phosphorylatable alanine mutants (S938A, S939A, S941A, S946A, and S948A) followed by immunoprecipitation and incubation with recombinant and active PP5-glutathione S-transferase (GST). Immunoblotting of these samples produced similar results as our in vitro experiments, showing serine dephosphorylation of only wild-type FNIP1-D-HA (Figure 2B). We also saw that phosphorylation of FNIP1-D promotes its interaction with Hsp90; this interaction occurs gradually based on the phosphorylation status of the serine series, and the FNIP1-D-S938A mutation blocks both its phosphorylation and binding to Hsp90 (Figures 2B and S2C). Our findings suggest that PP5 dephosphorylates FNIP1-D in a relay manner by initially removing phosphate from the S948 residue. This is supported by the fact that PP5 completely dephosphorylates all the modified serine residues on wild-type FNIP1-D both in vitro and in vivo (i.e., no serine phosphorylation signal for wild-type FNIP1-D-HA), and the common feature among all of the phosphomutants is the lack of phosphorylation on S948. Therefore, initial dephosphorylation of S948 on FNIP1-D is essential for subsequent dephosphorylation of the other serine sites (Figure 2C).

Sequential Phosphorylation of FNIP1 Incrementally Inhibits Hsp90 ATPase Activity

The ability of Hsp90 to bind and hydrolyze ATP is coupled to its chaperone function (Panaretou et al., 1998; Zierer et al., 2016).

(D) Indicated FNIP1-His6 fragments were used as substrates of CK2α in an in vitro kinase assay. Phosphorylation of serine residues was assessed by immunoblotting using a pan-anti-phosphoserine antibody.
(E) FNIP1-D-His6 and the indicated non-phosphomutants were bacterially expressed and purified. These proteins were used in an in vitro kinase assay with CK2α kinase. Serine phosphorylation was detected by immunoblotting using a pan-anti-phosphoserine antibody.
(F) Schematic representation of the relay phosphorylation of serine residues in the FNIP1-D fragment.
Our data show that CK2 mediated relay phosphorylation of FNIP1 impacts its binding to Hsp90; however, the consequence of this interaction toward Hsp90 ATP binding and hydrolysis remains unknown. We have previously shown that overexpression of FNIP1 in HEK293 cells increases Hsp90 binding to ATP agarose (Woodford et al., 2016). Here, we showed that overexpression of FNIP1-D-HA in HEK293 cells is sufficient to enhance Hsp90 binding to ATP agarose (Figure 3A). Transient expression of non-phospho-FNIP1-D mutants (S938A, S939A, S941A, S946A, and S948A) impacted Hsp90 binding to ATP in a similar manner as their interaction with Hsp90 (Figure 3B). We have previously shown that FNIP1-D purified from HEK293 cells inhibits Hsp90 at a 0.4:1 molar ratio (Woodford et al., 2016). Since FNIP1-D from HEK293 cells is post-translationally modified, we sought to dissect the impact of the individual serine phosphorylation states on ATPase activity of Hsp90-FLAG (Figure S3A).

While we avoided use of phosphomimetic mutations for cell-based assays in this study, in order to capture the different intermediate phosphorylation states on bacterially expressed FNIP1-D for in vitro experiments, we constructed a series of sequential phosphomimetic mutants (Figures 3C and S3B). We next measured Hsp90 ATPase activity in the presence of bacterially expressed and purified wild-type FNIP1-D-His6

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Figure 2. PP5 Relay Dephosphorylation of FNIP1 and Its Disruption from Hsp90
(A) Recombinant FNIP1-D-His6 and indicated non-phosphomutants were phosphorylated in vitro by CK2α and then incubated with or without recombinant PP5-GST. Phosphorylation of serine residues was examined by immunoblotting using a pan-anti-phosphoserine antibody.
(B) HEK293 cells were transiently co-transfected with FNIP1-D-HA and the indicated non-phosphomutants, IP, and then incubated with recombinant PP5-GST. Samples were immunoblotted for serine phosphorylation and interaction with Hsp90. LE, long exposure; SE, short exposure.
(C) Schematic representation of PP5 mediated dephosphorylation of FNIP1-D serine residues.
(devoid of any PTMs) and showed that it can inhibit Hsp90 ATPase activity in a dose-dependent manner (Figures S3C and S3D). Since a 0.4:1 molar ratio of FNIP1-D-His6 to Hsp90 does not inhibit Hsp90 ATPase activity, we used the same amounts of the phosphomimetic serine FNIP1-D mutants and showed that FNIP1-D-His6-S938E/S939A (FNIP1-D EA) slightly inhibited (by 19%) the ATPase activity of Hsp90-FLAG (Figure 3 D).

Earlier, we showed that phosphorylation of S938 leads to relay phosphorylation of S939, S941, S946, and S948. Here, our data show that the FNIP1-D phosphomimetic S938E/S939E/S941E/S946E/S948E (ie phosphomimetic of all serine residues in the series) significantly inhibits the ATPase activity of Hsp90 by 79% (Figures 3 D and S3E). Our intermediate phosphomimetic serine FNIP1-D mutants led to gradual inhibition of Hsp90 ATPase activity similar to the pattern seen with Hsp90 binding and ATP binding. Taken together, our data presented here provide strong evidence that relay serine phosphorylation of FNIP1-D causes an incremental binding to Hsp90 and, consequently, gradually inhibits its ATPase activity.

Figure 3. FNIP1-D Serine Phosphorylation Gradually Inhibits Hsp90 ATPase Activity
(A) FNIP1-D-HA (wild-type [WT]) was overexpressed in HEK293 cells, and the cell lysates were challenged with ATP-agarose for 1 h at 4 °C, washed, and then incubated with or without 10 μM of the Hsp90 inhibitor ganetespib (GB) for an additional 1 h at 4 °C. ATP-agarose was washed, and ATP-bound Hsp90 was analyzed by immunoblotting. EV was used as control. 
(B) Non-phosphorylatable FNIP1-D-HA (WT) and its mutants were transiently expressed in HEK293 cells, and cell lysates were incubated with ATP-agarose. Hsp90 binding to ATP was examined by immunoblotting.
(C) Schematic representation of creating FNIP1-D phosphomimetic mutants for expression and purification from bacteria. EV was used as control.
(D) Inhibitory effect of the recombinant FNIP1-D-His6 phosphomimetic mutants on ATPase activity of Hsp90α-FLAG isolated from PC3 cells was measured in vitro. A Student’s t test was performed to assess statistical significance (n.s., not significant).
Figure 4. Relay Serine Phosphorylation of FNIP1 Gradually Activates Both Kinase and Non-kinase Clients

(A) RLM1-LacZ activity was measured in yeast expressing WT hHsp90α and WT FNIP1-FLAG and its non-phosphomutants. EV was used as a control. Cells were grown to mid-log phase and stressed with 8 mM caffeine for 4 h. Data are presented as mean ± SD derived from three independent experiments. A Student’s t test was performed to assess statistical significance (**p < 0.01). FNIP1-FLAG and Hsp90α were visualized by immunoblotting.

(B) Yeast with WT hHsp90α, expressing WT FNIP1-FLAG, and its non-phosphomutants were also co-expressing Ste11DN-cMyc under the GAL1 promoter. EV was used as a control. Cells were grown on glucose (-) or galactose (+) media, and Ste11DN-cMyc protein expression was examined by immunoblotting.

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Stepwise Phosphorylation of FNIP1 Gradually Activates Both Kinase and Non-kinase Clients

We previously reported that Lst4 is not an ortholog of FNIP1 in the budding yeast Saccharomyces cerevisiae (Woodford et al., 2016). Additionally, overexpression of FNIP1 under the GAL1 promoter caused lethality in yeast with Hsp90α as the sole copy of Hsp90 (Woodford et al., 2016). Here, we expressed FNIP1-FLAG under a significantly weaker and constitutive promoter, ADH1 (alcohol dehydrogenase 1), and showed that yeast cells were viable (Figures S4A and S4B). We next confirmed that FNIP1-FLAG under either the GAL1 or ADH1 promoter is capable of interacting with Hsp90α (Figure S4C). Like in HEK293 cells, FNIP1-FLAG is also subject to serine phosphorylation in yeast, and expression of the non-phosphomutants showed a gradual reduction in serine phosphorylation of FNIP1-S948A to S938A mutants (Figure S4 D). These mutants also bound in a similar manner to Hsp90α (Figure S4D). The bakers’ yeast S. cerevisiae has been proven to be a valuable tool to examine the effect of Hsp90 on chaperoning of its client proteins (Echtenkamp and Freeman, 2012). Here, we deciphered the impact of relay phosphorylation of FNIP1 on chaperoning of kinase and non-kinase clients in yeast. We used three well-established Hsp90 kinase clients: mitogen-activated protein (MAP) kinase Mpk1/Slt2, Ste11, and v-Src.

MAP kinase Mpk1/Slt2 is an endogenous kinase client of yeast Hsp90, which mediates a cell wall integrity pathway (Millson et al., 2005). We used the downstream reporter gene RLM1-LacZ to monitor Mpk1/Slt2 activity in yeast expressing empty vector (EV), wild-type FNIP1-FLAG, or its non-phosphomutants. Caffeine activates the RLM1-LacZ reporter (Truman et al., 2006), and we found that reporter activity was significantly increased in FNIP1-FLAG-expressing cells, and this effect was abrogated with the FNIP1-FLAG-S938A mutant (Figure 4A). Expression of individual non--phospho-FNIP1-FLAG mutants S939A, S941A, S946A, and S948A led to gradual activation of the reporter RLM1-LacZ and, hence, activation of the cell wall integrity pathway (Figure 4A). Another endogenous yeast kinase client is Ste11 (a Raf-1 ortholog) (Flom et al., 2008; Louvion et al., 1998), and a constitutively active variant, Ste11ΔN, expressed under the GAL1 promoter can readily be detected in yeast cells (Figure 4B). However, Ste11ΔN protein was moderately increased in cells expressing FNIP1-FLAG, whereas FNIP1-FLAG-S938A-expressing cells exhibited Ste11ΔN expression similar to that seen in the empty-vector-expressing cells. We observed a gradual increase in Ste11ΔN protein levels in cells expressing non-phospho-FNIP1-FLAG-S938A to S948A mutants correlating with the degree of FNIP1 serine phosphorylation (Figure 4B).

We next used another well-established Hsp90 kinase client, v-Src (Nathan and Lindquist, 1995). v-Src expression under the GAL7 promoter and v-Src-mediated tyrosine phosphorylation of proteins were detectable in yeast (Figure 4C). v-Src expression and activity were dramatically increased in yeast cells expressing FNIP1-FLAG (Figure 4C). Expression of FNIP1-FLAG-S938A reduced this effect to the same levels seen in yeast cells with EV (Figure 4C). Expression of the FNIP1-FLAG phosphomutants S939A, S941A, S946A, and S948A gradually increased v-Src protein levels and activity (Figure 4C).

We next explored the impact of FNIP1 and its phosphorylation in chaperoning of a set of well-established non-kinase clients: glucocorticoid receptor (GR) (Garabedian and Yamamoto, 1992; Picard et al., 1990; Pratt et al., 2004), heat shock transcription factor (HSF1), and cystic fibrosis transmembrane conductance regulator (CFTR) protein. We obtained data similar to the results obtained for kinase clients. More specifically, FNIP1-FLAG expression increased GR activity in yeast, whereas the S938A mutant had no effect compared to EV (Figure 4D). The expression of individual non-phospho-S939A, S941A, S946A, and S948A FNIP1-FLAG mutants led to a gradual increase of GR activity (Figure 4D). We observed a similar pattern with the heat shock element (HSE)-LacZ activity in yeast expressing wild-type FNIP1-FLAG and the phosphomutants (Figure 4E). Lastly, we assessed the impact of FNIP1 and its phosphomutants on the stability of CFTR protein, a client of Hsp90 (Loo et al., 1998; Youker et al., 2004). We expressed HA-tagged CFTR under a constitutive promoter, PGK1 (phosphoglycerate kinase), in wild-type yeast with Hsp90α expressing FNIP1-FLAG or its phosphomutants. The rate of CFTR protein degradation was determined by cycloheximide (CHX) chase analysis. Our data showed that overexpression of FNIP1-FLAG significantly improved CFTR stability compared to the EV-expressing wild-type yeast with Hsp90α (Figure 4F). The non-phosphomutant FNIP1-FLAG-S938A expressing cells had similar CFTR stability to the wild-type yeast with Hsp90α, and this was improved in mutants S939A to S948A (Figure 4F). Our data obtained for the clients were quantified and summarized in a heatmap (Figures 4G and S4E–S4H). FNIP1 expression in yeast appears to activate the kinase and non-kinase clients in a manner that is dependent on FNIP1 phosphorylation. This effect is completely abrogated with expression
of FNIP1-FLAG-S938A mutant and is gradually restored from S939A to S948A as phosphorylation increases. These data also mirror the pattern of FNIP1-FLAG phosphomutants binding to Hsp90 (Figure S4 D).

**O-GlcNAcylation of the Priming S938 Antagonizes FNIP1 Phosphorylation**

Our proteomic analysis as well as immunoprecipitation experiments identified OGT as a binding partner for FNIP1 and FNIP1-D (Figures 1A–1C). OGT modifies serine and threonine residues by the addition of GlcNAc (Zachara et al., 2015). To determine if OGT post-translationally modifies FNIP1-D, we bacterially expressed and purified FNIP1-D-His6 and its non-phosphomutants followed by the addition of active OGT and its substrate, UDP-GlcNAc. Our in vitro data showed that OGT-FLAG can modify FNIP1-D and forms a complex with the recombinant FNIP1-D-His6 and its non-phosphomutants; however, O-GlcNAcylation of FNIP1-D was completely abrogated in the S938A mutant (Figures 5A and S5A). This suggests that S938, which is the priming serine required for relay phosphorylation, can also be O-GlcNAcylated. We also obtained similar data in HEK293 cells and demonstrated that S938 is the only site in the FNIP1-D fragment that is subject to O-GlcNAcylation (Figure 5B). FNIP1-D phosphomutants are binding with the same affinity of wild-type FNIP1-D to OGT (Figure 5B). The sequential increase of FNIP1-D phosphorylation seems to go hand in hand with an equally increased affinity for Hsp90 (Figures 5B and S5B). Interestingly, overexpression of OGT-V5 in HEK293 cells completely abrogates serine phosphorylation of FNIP1-D, presumably by increasing O-GlcNAcylation of the S938 site and blocking the priming residue for the relay phosphorylation as well as by affecting CK2 activity (Tarrant et al., 2012) (Figures 5B and 5C).

**FNIP1 O-GlcNAcylation Is a Prerequisite for K1119 Ubiquitination and Proteasomal Degradation**

To gain further insight into regulation of FNIP1 by O-GlcNAcylation, we first examined the cellular distribution of FNIP1-FLAG and FNIP1-FLAG-S938A in HEK293 cells and showed that PTM of S938A does not affect its cellular localization (Figures 1A–1C). FNIP1-D-His6 and its non-phosphomutants binding was assessed by immunoblotting. (B) FNIP1-D-HA and its non-phosphomutants were co-expressed with either EV or OGT-V5 and immunoprecipitated with either HA or FNIP1-D antibodies. Serine phosphorylation and O-GlcNAcylation of FNIP1-D-HA and its mutants as well as their interaction with Hsp90 and OGT were assessed by IP and immunoblotting. (C) Schematic representation of O-GlcNAcylation of FNIP1.
inducible OGT knockout mouse embryonic fibroblasts (OGT(f/f) Cre-ERT2-GFP). In the presence of 4-hydroxytamoxifen (4-OHT), ERT2-Cre chimera dimerizes and translocates to the nucleus, where recombination of the loxP sites results in an Ogt null allele (Ogt is X linked). Treating these cells with 0.5 \( \mu \)M 4-OHT for 48 h led to loss of OGT (Figure S6 B). Cells were transfected with FNIP1-D-HA wild-type (WT) and S938A 24 h after 4-OHT induction and then extracted 24 h after transfection. Interestingly, OGT knockout caused an increase in the levels of WT FNIP1-D-HA, but not the S938A mutant, suggesting that O-GlcNAcylation may affect FNIP1 stability.

To verify this, we used 100 \( \mu \)M PUGNAC (an inhibitor of O-GlcNAcase [OGA]) to treat HEK293 cells overnight, which caused a shift in equilibrium from O-phosphorylation to O-GlcNAcylation (Figure 6B). Additionally, we co-expressed FNIP1-D-FLAG or S938A mutant with OGT-V5 to increase O-GlcNAcylation or CK2-HA to enhance serine phosphorylation of FNIP1-D in HEK293 cells (Figure 6B). We used these conditions to determine if O-GlcNAcylation or phosphorylation affects FNIP1-D stability. Treating these cells with 50 \( \mu \)M CHX was sufficient to conduct chase experiments. Our data showed that O-GlcNAcylation of FNIP1-D leads to its destabilization, whereas CK2-mediated phosphorylation protects FNIP1-D from degradation (Figure 6C). Consistent with these data, we further demonstrated that overexpression of OGT-V5 or inhibition of OGA with PUGNAC caused ubiquitination of FNIP1-D, whereas increased CK2-mediated phosphorylation or non-phosphorylatable S938A mutation completely protected FNIP1-D from ubiquitination (Figure 6D).

There are eight lysine residues (K982, K1055, K1071, K1074, K1094, K1117, K1119, and K1134) within the FNIP1-D fragment. We therefore mutated each lysine to an arginine residue in order to identify the ubiquitination site. We first showed that only the FNIP1-D-K1119R mutant is more stable than the other lysine mutants as well as the WT FNIP1-D fragment (Figure S6 C). Further experiments also revealed that FNIP1-D-HA-K1119R is unable to be ubiquitinated even with overexpression of OGT-V5 or increase O-GlcNAcylation or CK2-HA.
PUGNAc-mediated inhibition of OGA in HEK293 cells, therefore providing further evidence for ubiquitination of K1119 in the FNIP1-D fragment (Figure 6E). We repeated our chase experiments with FNIP1-D-HA and FNIP1-D-HA-K1119R in HEK293 cells either co-expressing OGT-V5 or treated with the OGA inhibitor PUGNAc. As expected, hyper-O-GlcNAcylation led to destabilization of WT FNIP1-D-HA (Figure 6F). However, this effect was abrogated with the FNIP1-D-HA-K1119R mutant (Figure 6F). Our data suggest that O-GlcNAcylation of FNIP1-D-S938 is a prerequisite for ubiquitination of K1119 and degradation of FNIP1-D (Figure 6G). Phosphorylation of FNIP1-D-S938 can block O-GlcNAcylation, ubiquitination, and degradation of FNIP1-D.

DISCUSSION

Research during the past few years has provided a clear picture on how co-chaperones and PTMs can be integrated in the functional cycle of Hsp90 and consequently the chaperoning and activity of the client proteins (Bachman et al., 2018; Sahasrabudhe et al., 2017; Verba et al., 2016). However, it is unclear whether optimal activity of the clients is achieved in a stepwise, controlled manner. Our data presented here provide a mechanism where CK2-mediated relay phosphorylation of the co-chaperone FNIP1 on S938, S939, S941, S946, and S948 leads to its interaction with Hsp90 and inhibition of Hsp90 ATPase activity incrementally, based on FNIP1 phosphorylation state. However, unlike small molecules that inhibit Hsp90 function and lead to client degradation (Neckers et al., 2018), FNIP1 appears to decelerate the ATPase activity and gradually activate the client proteins. The mechanism of FNIP1 deceleration of Hsp90 ATPase activity is likely very different from that of small-molecule inhibitors. Our data for the well-established non-kinase clients (HSF1, GR, and CFTR) are in agreement with our previous studies as well as other groups’ findings that slowing of the chaperone cycle through the actions of co-chaperones or PTMs benefits stability and activity of this class of Hsp90 clients (Mollapour et al., 2014; Sahasrabudhe et al., 2017; Wang et al., 2006). It is noteworthy that some of our in vivo experiments rely on overexpression of FNIP1 and its phosphomutants, which may derail the dynamics of the regulatory co-chaperone network. However, the use of appropriate controls and the constitutive ADH1 promoter in our functional yeast studies.
supports the idea that these effects are a result of phosphorylation of FNIP1.

What is puzzling to us, however, is how deceleration of the chaperone cycle leads to activation of kinases. This is not in agreement with previous studies with a hyperactive yeast Hsp90-T22I, which was shown to cause hyperactivation of the kinase clients Sit2/Mpk1 (Truman et al., 2006). Although FNIP1 binding decelerates Hsp90 ATPase activity in vitro, this appears to be a surrogate for the aggregate effect of the action of FNIP1 on Hsp90. The ATPase activity likely does not correlate directly to the activation of Hsp90 kinase clients, as there is a nuanced series of intermediate states that affect client protein maturation and cannot be captured by a single metric. While we can readily observe the effect of FNIP1 on Hsp90 function cumulatively through ATPase activity, FNIP1 seems to be affecting the chaperone cycle in other ways. FNIP1 prefers binding to the Hsp90 “closed” conformation, and its collective actions appear to be favorable for both kinase and non-kinase client activation.

We demonstrate here that degree of phosphorylation of FNIP1 allows titration of the activation of the client proteins. Taking advantage of phosphomutants, we were able to capture and functionally decipher the effect of the intermediate phosphorylation states of FNIP1 and consequent gradual activation of the clients. We currently lack the phospho-specific antibodies to phosphorylated FNIP1 that would be needed to determine the abundance of intermediate phosphorylation states of FNIP1 at steady state and dissect the role each site plays in client activation. Our findings presented here, however, provide a mechanistic understanding of how the chaperone machinery may respond to the needs of the cell. Control over the degree of FNIP1 phosphorylation can allow titration of client activation depending on cellular needs.

Despite the vast amount of knowledge gained from our data, there are, however, two broad and overarching questions that remain to be answered: how does FNIP1 decelerate Hsp90 ATPase activity at the atomic level, and how does the FNIP1 interaction with Hsp90 translate to the activation of client proteins? Our previous work provided some evidence that FNIP1 binds to the middle domain of Hsp90 and perhaps masks the catalytic residue within this domain. Additional work such as structural analysis would help identify contact sites between FNIP1 and Hsp90 and whether phosphorylated serine residues create a contact surface between FNIP1-D and Hsp90. Additionally, it is unclear how exactly FNIP1 exerts this regulation over client maturation and activation. For example, does FNIP1 directly play an important role on the Hsp90-client axis, or does it function through its effect on the dynamics of other co-chaperones?

The second part of our study focused on regulation and stabilization of FNIP1. Our interactome data identified both OGT and CK2 as binding proteins of FNIP1. We further demonstrated that PP5 dephosphorylates FNIP1 in a reverse pattern to its phosphorylation. In other words, PP5 dephosphorylates S948, which is essential for subsequent dephosphorylation of the S946, S941, S939, and S938. We further showed that OGT-mediated O-GlcNAcylation of FNIP1-S938 antagonizes its phosphorylation and consequently leads to ubiquitination of FNIP1-K1119 and ultimately its proteasomal degradation (Figure 7). These additional modifications provide further control over the stability and activity of FNIP1 and therefore its impact on the chaperoning of clients.

Although in this study, we are focusing on PTMs of FNIP1 and the impact on its regulation as well as chaperoning of the client proteins, it is worth mentioning that OGT is an Hsp90 client. Previous work has demonstrated O-GlcNAcylation of Hsp90 (Fierro-Monti et al., 2013; Frank et al., 2014; Zhang et al., 2012) as well as CK2, which leads to its decreased activity and degradation of this kinase (Tarrant et al., 2012). Here, we also confirmed OGT to be an Hsp90 client, and overexpression of FNIP1 caused a slight upregulation of OGT (Figures S7A and S7B). However, surprisingly, total O-GlcNAcylation of protein lysate was unaffected (Figure S7B). We speculate that the hyperactive FNIP1 co-chaperone may increase OGT client activity, which in turn leads to O-GlcNAcylation and degradation of FNIP1, serving as a negative feedback loop for FNIP1 regulation. Additionally, CK2 did not appear to be an Hsp90 client, since inhibition of Hsp90 did not lead to degradation of this kinase (Figure S7A). Ultimately, our study raises new questions with regards to relay phosphorylation of co-chaperones. How exactly is FNIP1 co-chaperone affecting the activation of Hsp90 client proteins? Does a similar relay mechanism exist in other co-chaperones of Hsp90 or indeed Hsp90 itself? How do they impact the chaperoning of client proteins? This concept warrants further investigation.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and two tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2019.01.018.
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AUTHOR CONTRIBUTIONS

R.A.S., M.R.W., S.J.B., A.J.B.-W., A.M.M., B.T.D., D.R.L., C.P., D.B., and M.M. performed experiments. R.A.S., M.R.W., T.A.H., N.E.Z., L.S.S., D.B., W.M.L., G.B., and M.M. designed experiments. R.A.S., M.R.W., G.B., and M.M. wrote the manuscript. M.M. conceived the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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### Key Resources Table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Rabbit anti-FLAG tag | Thermo Scientific | Cat# PA1-984B; RRID:AB_347227 |
| Mouse anti-FLAG tag  | Thermo Scientific | Cat# F3165 |
| Mouse anti-6x-His epitope tag (HIS.H8) | Thermo Scientific | Cat# MA1-21315; RRID:AB_557403 |
| Rat anti-Hsp90 (16F1) | Enzo Life Sciences | Cat# ADI-SPA-835; RRID:AB_11181205 |
| Mouse anti-GAPDH (1D4) | Enzo Life Sciences | Cat# ADI-CSA-335; RRID:AB_10617247 |
| Rabbit anti-FLCN (D14G9) | Cell Signaling Technology | Cat# 3697; RRID:AB_2231646 |
| Rabbit anti-PP5      | Cell Signaling Technology | Cat# 2289; RRID:AB_2168757 |
| Rabbit anti-OGT      | Cell Signaling Technology | Cat# 24083; RRID:AB_2716710 |
| Rabbit anti-CK2α     | Cell Signaling Technology | Cat# 2656; RRID:AB_2236816 |
| Rabbit anti-phospho-Akt S473 (D9E) | Cell Signaling Technology | Cat# 2289; RRID:AB_2315049 |
| Mouse anti-Akt (2H10) | Cell Signaling Technology | Cat# 2967; RRID:AB_331160 |
| Rabbit anti-GR (D6H2L) | Cell Signaling Technology | Cat# 12041; RRID:AB_2631286 |
| Mouse anti-O-GlcNAc (CTD110.6) | Cell Signaling Technology | Cat# 9875; RRID:AB_10950973 |
| Rabbit anti-HA tag (C29F4) | Cell Signaling Technology | Cat# 3724; RRID:AB_1549585 |
| Rabbit anti-myc tag (71D10) | Cell Signaling Technology | Cat# 2278; RRID:AB_490778 |
| Rabbit anti-V5 tag   | Cell Signaling Technology | Cat# 13202; RRID:AB_2687461 |
| Goat anti-GST        | GE Healthcare | Cat# 27-4577-01; RRID:AB_771432 |
| Mouse anti-phospho-serine (PSR-45) | Sigma-Aldrich | Cat# P5747; RRID:AB_477376 |
| Mouse anti-phospho-threonine (PTR-8) | Sigma-Aldrich | Cat# P6623; RRID:AB_477393 |
| Mouse anti-phospho-tyrosine (4G10) | Millipore | Cat# 05-321; RRID:AB_309678 |
| Mouse anti-v-src (clone 327) | Millipore | Cat# MABS193; RRID:AB_11205595 |
| Mouse anti-ubiquitin (P4D1) | Santa Cruz Biotech | Cat# sc-8017; RRID:AB_628423 |
| Rabbit anti-FNIP1 (181) | NCI (Baba et al., 2006) | N/A |
| Rabbit anti-FNIP2 (3G) | NCI (Hasumi et al., 2008) | N/A |
| Goat anti-FNIP1      | Antibodies-online.com | Cat# ABIN238670; RRID:AB_10775640 |
| Anti-mouse secondary | Cell Signaling Technology | Cat# 7076; RRID:AB_330924 |
| Anti-rabbit secondary | Cell Signaling Technology | Cat# 7074; RRID:AB_2099233 |
| Anti-rat secondary   | Cell Signaling Technology | Cat# 7077; RRID:AB_10694715 |
| Anti-goat secondary  | Santa Cruz Biotech | Cat# sc-2020; RRID:AB_631728 |
| Anti-mouse Alexa Fluor secondary | ThermoFisher Scientific | Cat# A-21203; RRID:AB_2535789 |
| **Bacterial and Virus Strains** | | |
| BL21(DE3)            | New England BioLabs | Cat# C2527I |

### Chemicals, Peptides, and Recombinant Proteins

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| SNX-2112            | Duke University; Dr. Timothy Haystead (Barrott and Haystead, 2013) | CAS# 908112-43-6 |
| CHX                 | Research Products International | Cat# C81040; CAS# 66-81-9 |
| Caffeine            | Sigma-Aldrich | Cat# C0750-500G |
| ATP                 | Sigma-Aldrich | Cat# FLAAS; CAS# 34369-07-8 |
| UDP-GlcNAc          | Sigma-Aldrich | Cat# U4375; CAS# 91183-98-1 |
| PUGNAc              | Sigma-Aldrich | Cat# A7229; CAS# 132489-69-1 |
| 4-Hydroxytamoxifen  | Sigma-Aldrich | Cat# SML1666; CAS# 68392-35-8 |

### Critical Commercial Assays

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Mirus TransIT-2020  | MirusBio | Cat# MIF5405 |
| PiPer Phosphate Assay | ThermoFisher Scientific | Cat# P22061 |

(Continued on next page)
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for reagents may be directed to and will be fulfilled by the Lead Contact Mehdi Mollapour (mollapom@upstate.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Plasmids
For mammalian expression pcDNA5-FNIP1-HA and pcDNA5-FNIP1-D-HA constructs were cloned previously (Woodford et al., 2016). FNIP1 and FNIP1-D were subcloned with a FLAG tag into pcDNA3 (see Table S2). pZW6-CK2x-HA was purchased from Addgene (Turowec et al., 2010) and cloned into pcDNA 3.1 pDestNV5 (ThermoFisher Scientific). OGT gene was subcloned into pcDNA3 plasmid with a FLAG tag (see Table S2). The EVs containing these tags were used and also referred to as controls. For bacterial expression, FNIP1-ABC and

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Anti-FLAG M2 affinity gel | Sigma-Aldrich | Cat# A2220; RRID:AB_10063035 |
| Anti-HA agarose | ThermoFisher Scientific | Cat# 26182; RRID:AB_2532162 |
| Protein G agarose | ThermoFisher Scientific | Cat# 15-920-010 |
| ATP agarose | Novus Biologicals | Cat# 510-0002 |
| Ni-NTA Agarose | ThermoFisher Scientific | Cat# 88221 |
| ProLong Gold antifade reagent with DAPI | ThermoFisher Scientific | Cat# P36935 |
| HEK293 (Female) | ATCC | Cat# CRL-1573; RRID:CVCL_0045 |
| PC3 (Male) | ATCC | Cat# CRL-1435; RRID:CVCL_0035 |
| MEFs OGT (f/y) Cre-ERT2 GFP (Male) | Dr. Natasha Zachara; CardioPEG CoreC4, Johns Hopkins University | Kazemi et al., 2010; O’Donnell et al., 2004 |

Experimental Models: Organisms/Strains

| PP30-Hsp90x | Piper et al., 2003 | n/a |
| Oligonucleotides | DNA primers Eurofins Genomics | See Table S2 |
| Recombinant DNA | pcDNAs-FNIP1-HA Baba et al., 2006 | n/a |
| pcDNAs-FNIP1-D-HA Baba et al., 2006 | n/a |
| pZW6-CK2x-HA Addgene (Turowec et al., 2010) | Cat# 27086 |
| pDONR221-OGT Harvard Plasmids | HsCD00042534 |
| pcDNA 3.1 pDestNV5 ThermoFisher Scientific | Cat# 12990010 |
| pYES2-FNIP1-FLAG Woodford et al., 2016 | n/a |
| p424-ADH ATCC | Cat# 87373 |
| pRSETA ThermoFisher Scientific | Cat# V53120 |
| pET-41a(+) Millipore | Cat# 70556 |
| pHCA/vGR Garabedian and Yamamoto, 1992 | n/a |
| PA526X Schena et al., 1989 | n/a |
| 2xRLM1-LacZ Truman et al., 2006 | n/a |
| 4XHSE-LacZ-pUp41a Truman et al., 2006 | n/a |
| pSM1152 (PProck-CFTR-HA) Youker et al., 2004 | n/a |
| YpRS426-GAL1-v-Src Murphy et al., 1993 | n/a |
| STE11-DN-cMYC-pYES2 This paper | n/a |

Deposited Data

| Mass spectrometry data | This study | PXD012229 |

Software and Algorithms

| GraphPad Prism 7.0 | GraphPad Software | https://www.graphpad.com/ |
FNIP1-D were subcloned into pRSETA containing an N-terminal His \(_6\) tag (ThermoFisher Scientific). Codon-optimized FNIP1-D was synthesized and subcloned into pET41 containing a C-terminal His \(_6\) tag. For yeast expression, FNIP1 was subcloned into p424ADH (ATCC\textsuperscript{®} 87373). pYES2-FNIP1-FLAG was cloned previously (Woodford et al., 2016). pHCA/GR (Garabedian and Yamamoto, 1992), constitutively expressing glucocorticoid receptor (GR) under control of the Alcohol dehydrogenase promoter (ADH1), the GR reporter vector pαS26X, a URA3 vector which expresses β-galactosidase (encoded by lacZ) as a reporter gene under control of a promoter bearing 3 × GR response elements (Schena et al., 1989), 2xRLM1-LacZ reporter plasmid (Truman et al., 2006), 4XHSE-LacZ-pUp41a (Truman et al., 2006), pSM1152 (P\(_{PGK}\)-CFTR-HA) plasmid (Yrouk et al., 2004), and YpRS426-GAL1-v-Src plasmid (Murphy et al., 1993) were all reported previously. STE11\_N-cMYC-pYES2 was created using primers in (Table S2). Point mutations were made using site-directed mutagenesis (see Table S2) and confirmed by DNA sequencing.

**Yeast Strains**
The yeast strain pp30 (MAT, trp1-289, leu2-3,112, his3-200, ura3-52, ade2-101, lys2-801, hsc82KANMX4, hsp82KANMX4) expressing Hsp90α-Ycplac111 as the sole Hsp90 was used in this study. These yeast strains were reported previously (Mollapour et al., 2011).

**Mammalian Cell Culture**
Cultured human embryonic kidney (HEK293) cells were grown in Dulbecco’s Modified Eagle Medium (DMEM, Sigma-Aldrich), PC3 cells were grown in RPMI-1640 medium (Sigma-Aldrich), and MEF-OGT cells were grown in low-glucose DMEM (Sigma-Aldrich). All media was supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich). HEK293 and PC3 cells were acquired from (American Type Culture Collection, ATCC). MEF-OGT cells were obtained from Dr. Natasha Zachara at the CardioPEG Core4 at Johns Hopkins University School of Medicine (Kazemi et al., 2010; O’Donnell et al., 2004). All cell lines were maintained in a CellQ incubator (Panasonic Healthcare) at 37°C in an atmosphere containing 5% CO\(_2\).

**METHOD DETAILS**

**Yeast Growth Media**
Yeast cells were grown on YPA (2% (w/v) Bacto peptone, 1% yeast extract, 2% glucose, 20 mg/liter adenine), YPGal (2% (w/v) Bacto peptone, 1% yeast extract, 2% galactose, 20 mg/liter adenine) and YPRaf (2% (w/v) Bacto peptone, 1% yeast extract, 2% raffinose, 20 mg/liter adenine). Selective growth was on dropout 2% glucose (DO) medium with appropriate amino acids (Adams et al., 1997). Medium pH was adjusted to 6.8 with NaOH before autoclaving.

**Bacterial Expression and Protein Purification**
FNIP1-ABC-His\(_6\), FNIP1-D-His\(_6\), and FNIP1-D phosphomutants were expressed and purified from E. coli strain BL21 (DE3). Transformed cells were grown at 37°C in LB with 50 mg/L ampicillin until OD\(_{600}\) = 0.6. The cultures were then cooled to 30°C, induced with 1mM IPTG, and grown to OD\(_{600}\) = 1.2. Cells were harvested by centrifugation and lysed by sonication in fresh lysis buffer without detergent (20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM MgCl\(_2\), protease inhibitor cocktail (Roche) and PhosSTOP (Roche)). After sonication Triton X-100 was added to a final concentration of 1% prior to pelleting insoluble debris by centrifugation. Supernatant was collected and FNIP1 fragment expression was assessed by immunoblotting. Isolation of FNIP1-His\(_6\) fragments was accomplished by two sequential Ni-NTA agarose (QIAGEN) pulldowns. Lysate was incubated with Ni-NTA agarose (QIAGEN) for 2hr at 4°C. Proteins bound to Ni-NTA agarose were washed three times with lysis buffer (as above) followed by two washes with 50mM imidazole in lysis buffer. They were then eluted in 500mM imidazole in lysis buffer and concentrated in 10K Amicon. Protein extraction from both yeast and mammalian cells was carried out using methods previously described (Mollapour et al., 2010). For immunoprecipitation, mammalian cell lysates were incubated with anti-FLAG or anti-HA antibody conjugated agarose beads (Sigma) for 2 hr at 4°C, or with anti-FNIP1 antibody for 1 hr followed by protein G agarose for 2 hr at 4°C. For ATP pulldown, lysates were incubated with ATP conjugated agarose for 1 hr at 4°C. Immunopellets were washed 4 times with fresh lysis buffer (20mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM MgCl\(_2\), 0.1% NP40, protease inhibitor cocktail (Roche), and PhosSTOP (Roche)) and eluted in 5x Laemmli buffer. Precipitated proteins were run on SDS-PAGE gel and Coomassie stained to confirm purity prior to use in assays.

**Protein Extraction, IP, ATP-pulldown and Immunoblotting**
Protein extraction from both yeast and mammalian cells was carried out using methods previously described (Mollapour et al., 2010). For immunoprecipitation, mammalian cell lysates were incubated with anti-FLAG or anti-HA antibody conjugated agarose beads (Sigma) for 2 hr at 4°C, or with anti-FNIP1 antibody for 1 hr followed by protein G agarose for 2 hr at 4°C. For ATP pulldown, lysates were incubated with ATP conjugated agarose for 1 hr at 4°C. Immunopellets were washed 4 times with fresh lysis buffer (20mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM MgCl\(_2\), 0.1% NP40, protease inhibitor cocktail (Roche), and PhosSTOP (Roche)) and eluted in 5x Laemmli buffer. Precipitated proteins were run on SDS-PAGE gel and transferred to nitrocellulose membranes. Co-immunoprecipitated proteins were detected by immunoblotting with indicated dilutions of antibodies recognizing 1:8000 FLAG, 1:10000 6x-His (ThermoFisher Scientific), 1:8000 Hsp90-835-16F1, 1:10,000 GAPDH (ENZO Life Sciences), 1:4000 FLCN, 1:1000 PP5, 1:2000 OGT, 1:2000 CK2α, 1:2000 p-Akt (S473), 1:4000 Akt, 1:2000 GR, 1:1000 O-GlcnAc, 1:2000 HA, 1:1000 myc, 1:2000 V5 (Cell Signaling), 1:10,000 GST (GE Healthcare), 1:1000 phospho-serine, 1:1000 phospho-threonine (Simga-Aldrich), 1:1000 phospho-tyrosine, 1:1000 v-src (Millipore), 1:1000 ubiquitin (Santa Cruz Biotechnology), 1:2000 FNIP1, 1:2000 FNIP2 (NCI), 1:1000 FNIP1 (antibodies-online.com). Secondary antibodies raised against mouse, rabbit, and rat (Cell Signaling) and goat (Santa Cruz Biotechnology) were used at 1:4000 dilution (See Key Resources Table).
Protein Purification from Mammalian Cells

Protein extraction was carried out using methods previously described (Mollapour et al., 2010). Purification was accomplished by two sequential immunoprecipitations. Hsp90β-FLAG for ATPase measurements was expressed in PC3 cells and OGT-FLAG for in vitro GlcNAcylation was expressed in HEK293 cells. Cell lysate was incubated with anti-FLAG antibody conjugated agarose beads (Sigma) for 2 hr at 4°C. Immunopellets were washed four times with fresh high salt lysis buffer (20 mM Tris-HCl (pH 7.4), 500 mM NaCl, 1 mM MgCl2, 1% NP40, protease inhibitor cocktail (Roche), and PhosSTOP (Roche)) and subsequently competed off with 3x FLAG-peptide (Sigma-Aldrich) in detergent-free fresh lysis buffer (20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM MgCl2, protease inhibitor cocktail (Roche), and PhosSTOP (Roche)) at 4°C for 1 hr two times. Protein was concentrated in 50K Amicon Ultra Centrifugal Filters (Millipore). A second round of immunoprecipitation, competition and concentration was then performed. Concentrations were determined using the Micro BCA Protein Assay Kit (Thermo Scientific) per manual protocol. Purified protein was run on an SDS-PAGE gel and Coomassie stained to confirm purity prior to use in assays.

Protein Extraction From Yeast

Yeast cells were collected from liquid culture by centrifugation and resuspended in 500μl of protein extraction buffer (20mM Tris-HCl (pH 7.4), 500 mM NaCl, 1 mM MgCl2, protease inhibitor cocktail (Roche), and PhosSTOP (Roche)) and two pellet volumes of acid washed glass beads. To lyse the cells, tubes were agitated using a bead beater (mini-Beadbeater 8, Biospec Products, USA) for 30 s at maximum speed and then 30 s on ice. This procedure was repeated 6X followed by (10,000Xg; 5 s) to pellet the beads and unbroken cells. The supernatant was transferred to a new microfuge tube and centrifuged (10,000Xg; 10 minutes) to pellet insoluble aggregates. Supernatant was then transferred to a fresh microfuge.

Assays for Hsp90 Client Activity in Yeast

Ste11αN and v-Src induction and activation were analyzed as described previously (Mollapour et al., 2010). Expressed Ste11αN-cMyc, v-Src protein were detected with cMyc mouse antibody (Cell signaling Technology), EC10 mouse antibody (Millipore) respectively. v-Src activity with 4G10 mouse anti-phosphotyrosine (Millipore). β-galactosidase assay for measuring GR-β-LacZ expression (Garabedian and Yamamoto, 1992), HSE-LacZ expression (Hjorth-Sørensen et al., 2001), and RLM1-LacZ (Truman et al., 2006) were described previously (Mollapour et al., 2014).

β-Galactosidase Assay

Yeast cells were grown overnight to exponential phase with a cell density of 2-3 x 10^6 cells per ml in 50ml of the same medium at 30°C. Then, dexamethasone (DEX) was added to a final concentration of 30 μM, followed by incubation at 30°C for 2.5 hr to activate the GR-β-LacZ. HSE-LacZ expressing yeast cells were heat shock at 39°C for 40 min. Cells were collected by centrifugation (2000 x g; 5 minutes), washed once with dH2O, and frozen at –80°C. β-Galactosidase activity was measured as previously described (Mollapour et al., 2011). Cell lysate (10 μl) was mixed with equal volume of 2XZ-buffer (60mM Na2HPO4, 60mM NaH2PO4, 5mM KCl, 0.5mM MgSO4, pH adjusted to 7.0). The mixture was added to 700 μl of 2mg/ml ONPG solution (O-Nitrophenyl-β-D-galactopyranoside dissolved in 1X Z buffer) prewarmed at 30°C. The reaction was stopped by adding 500 μL of 1M Na2CO3. The optical density at 420nm (OD420) of each reaction mixture was determined. The protein concentration of the lysate was determined by the BioRad assay. The β-galactosidase activity was calculated using the following formula: Enzyme Activity = 1000 x OD420/minute/[10 μL x protein concentration [μg/μl]]

Hsp90 ATPase Activity Measurement

Hsp90β-FLAG was isolated from PC3 cells and FNIP1-D-His6 WT and phosphomutants were isolated from bacteria as described above. ATPase activity of Hsp90β and its inhibition by FNIP1-D were measured using the PiPer Phosphate Assay Kit (Life Technologies) as described in the manufacturer’s protocol. Standard curve with linear fit line was created from 0-20μM P, final concentration reactions. 750ng of Hsp90β and aforementioned amounts of FNIP1-D were added to each reaction with 1mM ATP as substrate. Reactions were run in triplicate and incubated at 37°C for over 1 hour. 10μM GB (Synta) was used in drug reactions. ATP turnover was calculated as mmol P per mol Hsp90β per minute, and relative ATPase activity was calculated from those values, with the value of Hsp90β alone representing 100% activity.

In vitro FNIP1 Phosphorylation and Dephosphorylation

FNIP1-ABC, FNIP1-D fragment and its phospho mutants were isolated by incubating 2mg of protein extracts with 50 μl of Ni-NTA agarose (QIAGEN) for 2hr. The Ni-NTA agarose beads was washed with 30μl imidazole and then incubated with 50ng of baculovirus expressed and purified active CK2α-GST (SignalChem) for in vitro kinase assay. The assay was carried out in 50mM Tris-HCl (pH7.5), 10mM MgCl₂, and 0.2mM ATP, at 28°C for 15 min. The reaction was either quenched by addition of an equal volume of protein loading buffer, or washed 3X with 100mMNaCl, 50mMTris,pH7.5 followed by resuspension in a buffer containing 100mM NaCl, 50mM Tris pH7.5, 2 mM DTT, and 1mM MnCl₂. PP5 mediated dephosphorylation was performed as previously described (Dushukyan et al., 2017). The reaction was started by adding 0.25 μM PP5-GST and the samples were incubated at 30°C for 15 min. Samples were quenched by addition of an equal volume of protein loading buffer and followed by SDS/PAGE analysis.
**In vitro FNIP1 O-GlcNAcylation**

O-GlcNAcylation was performed *in vitro* using bacterially expressed and purified FNIP1-D-His\(_6\) and phosphomutants S938A, S939A, S941A, S946A and S948A. These proteins were bound to Ni-NTA agarose and then incubated with 10ng of OGT-FLAG purified from HEK293 cells and 0.2mM UDP-GlcNAc in 25μl reactions (50mM Tris-Cl, 12.5mM MgCl\(_2\), and 1mM DTT, pH7.5) for 15 min at 30 °C. The reaction was stopped by addition of protein loading buffer and SDS/PAGE analysis.

**Mass Spectrometry Analysis**

Visible bands were excised from the gel manually and cut into small pieces approximately 1 mm x 1 mm. These gel pieces were de-stained using 1:1 30 mM potassium ferricyanide: 100 mM sodium thiosulfate for 10 minutes. The destained gel pieces were then washed with 25 mM ammonium bicarbonate and acetonitrile alternatively for 5 minutes each wash. This cycle of 5 minute 25 mM ammonium bicarbonate wash followed by 5 minute acetonitrile wash was repeated 3 times. To further prepare the gel pieces for digestion, the gel pieces were then dehydrated in 100% acetonitrile. After removing all acetonitrile, 25 μl of porcine trypsin (Promega) dissolved in 25 mM ammonium bicarbonate at a concentration of 4 μg/ml was added to the gel pieces. The gel pieces were then kept at room temperature overnight (approximately 12-16 hours). Following digestion, the supernatant was transferred to a second tube, and acetonitrile was added to the gel pieces to complete the extraction of digested peptides. This extract was added to the first supernatant and this combined solution, containing the extracted peptides was frozen and lyophilized. The peptides were resuspended in 5 μl of 100:99:1 acetonitrile: water: trifluoroacetic acid immediately prior to spotting on the MALDI target. For MALDI analysis, the matrix solution consisted of alpha-cyano-4-hydroxycinnamic acid (Aldrich Chemical Co. Milwaukee, WI) saturating a solution of 1:1:0.01 acetonitrile: 25 mM ammonium citrate: trifluoroacetic acid. Approximately 0.15 μl of peptide solution was spotted on the MALDI target immediately followed by 0.15 μl of the matrix solution. This combined solution was allowed to dry at room temperature. MALDI MS and MS/MS data was then acquired using the ABSciEX TOF/TOF® 5800 Mass Spectrometer. Resultant peptide mass fingerprint and peptide sequence data was submitted to the UniProt database using the Mascot search engine to which relevance is calculated and scores are displayed. Data are presented in (Table S1) and (Figure 1A).

**Immunofluorescence Staining and Microscopy**

HEK293 cells were plated overnight on glass coverslips (#1) and then transiently transfected with pcDNA3-FNIP1-FLAG or pcDNA3-FNIP1-FLAG-S938A overnight. Cells were fixed in 4% paraformaldehyde for 20 minutes at room temperature, washed 3x with fresh PBS, and then permeabilized with 0.1% Triton X-100 at room temperature for 4 minutes followed 3 washes with fresh PBS. Cells were blocked in PB SAT (1% BSA, 0.5% Triton X-100 in PBS) for 30 minutes at room temperature. Cells were then incubated with primary mouse anti-FLAG (ThermoFisher Scientific) 1:250 in PB SAT for 2 hours at room temperature and then washed 3x with fresh PBSAT. Coverslips were then incubated with secondary anti-mouse Alexa Fluor® 594 (ThermoFisher Scientific) 1:900 in PB SAT at room temperature for 1 hour and then washed 3x with PBSAT followed by 2 washes with PBS. Coverslips were then mounted onto glass slides using ProLong® Gold antifade mounting media with DAPI (4′,6-diamidino-2-phenylindole) in fluorescence microscopy. (Thermo-Fisher Scientific). Images were obtained using a Zeiss LSM780 confocal microscope.

**Quantification and Statistical Analysis**

All statistics were performed using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, California, USA, https://www.graphpad.com). Statistical significance was ascertained between individual samples using a parametric unpaired t test. Trends were calculated through Pearson correlation coefficient testing, using the total number of phospho-sites present in each molecule as the X variable. Significance was denoted as asterisks in each figure: *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. Error bars represent the standard deviation (SD) for three independent experiments, unless it is indicated.

**Data and Software Availability**

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al., 2016) partner repository with the dataset identifier PRIDE: PXD012229.
Supplemental Information

Post-translational Regulation of FNIP1 Creates a Rheostat for the Molecular Chaperone Hsp90

Rebecca A. Sager, Mark R. Woodford, Sarah J. Backe, Alan M. Makedon, Alexander J. Baker-Williams, Bryanna T. DiGregorio, David R. Loiselle, Timothy A. Haystead, Natasha E. Zachara, Chrisostomos Prodromou, Dimitra Bourbouli, Laura S. Schmidt, W. Marston Linehan, Gennady Bratslavsky, and Mehdi Mollapour
| Protein                                      | MW       | Protein Score | C.I.% | Total Ion Score | C.I.% | Peptides                                                                 | Accession Number |
|----------------------------------------------|----------|---------------|-------|-----------------|-------|--------------------------------------------------------------------------|------------------|
| Folliculin-interacting protein 1             | 130471.9 | 658           | 100   | 437             | 100   | RTGLGAPGR MLSEYLR AFSEQGPLR NVLFDDSVK IVDALNEFR SQAVDQITR TCFPQQDQR LIVYQDCER IAIGVIFLSK RAFSEQGPLR WTVOASSQR NVLFDDSVKR RRNEDISVK GRNVLFDSSVK IAVGTEWDIPR TTICNLYTMR LLYFLTYFIR ELNEDRDSGIAR SQTTSLENGVFPR RSOTTSLENGVFPR LIEVASVQPNIANFGR GEIEESEYLVLTMRH IGEPWLTMMSTPEK SASFFAVHSNPMMPGR DTLISILPHGDKESSDKK VTFILGDSMPSDOSTELR SASLSSLITPFPSPNSSLTR DPDCGFSPWPLPEFDSQIR LIRASFFAHSPMMPGR ADNNTVINLGLNIGLSQFOSPR WSIEESFNLSDESCGPNPGIVR THPYNPLWAQLGDLYGAIGSPVR IGEPWLTMMSTPEKNHLCYR QVSSYYGGEQEDWAEEIIIFPSK |
| ATP-dependent RNA helicase A                 | 140869.1 | 92            | 99.988| 51              | 99.755| DFVNVLVR LAQFEPSQR GANLKDYYSR ELDADENDTPLGR AIEPPPLDAIEAEHTLR             | DHX9_HUMAN       |
| O-linked N-acetylglucosamine (GlcNAc) transferase | 116849.9 | 73            | 99.888| 28              | 97.516| TIVVTR AATGEEVPR SNGHIYDNR EQNIEEAVR VPNSVLWLLR GQLQEAIEHYR NRQEYEDIAVK EYQAADFEEAER DSQNPEAASYR LGTDLEYLKKVR IIFSPVAPKEEYVR EAIRISPFADAYSNMGTNLK FPAVGEPIQYAOQNMLPQNR IDPSTLQWMANILKRPNSVLWLLR |

**Table S1.** Cellular proteins associated with FNIP1. Related to Figure 1 and STAR Methods.
| Protein | MW | Protein Score | C.I.% | Total Ion Score | C.I.% | Peptides | Accession Number |
|---------|----|---------------|-------|----------------|-------|----------|-----------------|
| Heterogeneous nuclear ribonucleoprotein U-like protein | 95679.3 | 116 | 100 | | | GYFEHR QYNEEGR SGGGGYSQNR GLKAELAE QYNLEEGRK KRPYEENR GYFEHERDR NCAVEFNFQGQR VNELREELQR HLPSTEPDPHVVR LKVNELREELQR NYILDOTNYYQAQR SSGYPLIGFAYLWSGAR GPPGGNGGQFRNGGSSGGGNYR QNQFYTDQVIKQENESGYER APQQQQPPPQPPPQPPPQPPPQPPPQPPPQSYSPAR |
| Heat shock protein HSP 90-beta | 83212.1 | 218 | 100 | 130 | 100 | ALLFIPR KKMEESK TKPIWTR ADHEGPIGR SIYYITGESK IDIINPNQER APFDLFENKK RAPFDLFENK EQVANSAFVER HFSVEGQLEFR GVDSDEDPLNISR ELKIDIPNQER IVEKYIDOELNK EKEISDDDEEKEKGEK VILHLKEDQTEYLEER ALLFIPRRAPFDLFENKK YHTSOQSGEMTSLSVEYVR HNDDEQYAWESSAGSFTVR KRGFEVYVMTPEIDEYCVQLK |
| Heat shock protein HSP 90-alpha | 84606.7 | 154 | 100 | 97 | 100 | ALLFIPR TKPIWTR TKFENLC KFYEQFSK APFDLFENR DOVANSAFVER APFDLFENRK TDTGEPMGRGTK RAPFDLFENR HFSVEGQLEFR GVDSDEDPLNISR ELHINLIPQDR IVEKYIDOEELNK VILHLKEDQTEYLEER HNDDEQYAYESSAGSFTVR |
| Protein                                | MW     | Protein Score | C.I.% | Total Ion Score | C.I.% | Peptides                                                                 | Accession Number |
|----------------------------------------|--------|---------------|-------|-----------------|-------|--------------------------------------------------------------------------|------------------|
| Heat shock 70 kDa protein 1A/1B        | 70009  | 64            | 79.731| 19              | 0     | LLQDFFNGR, DAGVIAGLNVLR, TTPSYVAFTDTER, IINEPTAAIAJAYGLDR, GETKAFYPEEISSMVLT \ | HSP71_HUMAN      |
| Serine/threonine-protein phosphatase 5 | 56842.2| 27            | 0     | 27              | 0     | AFLEENNLDYIIR                                                            | PPP5_HUMAN       |
| Casein kinase II subunit alpha         | 45114.8| 83            | 99.988| 45              | 99.966| YDHQSR, FNDILGR, VYTDVNTHRPR, QLYQTLTDYDIR, TPALVFEHVNTDFK, GGPNIITLADIVKDPVSR, LIDWGLAEFYHPGQEQYVR, VLGTEDLYIDKYNIELDPR, EYWDYESHVVEWNGQDDYQLVR | CSK21_HUMAN      |
Figure S1

A) Indicated FNIP1-His<sub>6</sub> fragments were used as substrates of CK2α in an \textit{in vitro} kinase assay. Phosphorylation of serine residues was assessed by immunoblotting using pan-anti-phosphothreonine antibodies.

B) Purified FNIP1-D-His<sub>6</sub> and its non-phosphomutants were bacterially expressed and purified, run on an SDS-PAGE gel and coomassie stained.

Figure S1. CK2 mediated phosphorylation of FNIP1 \textit{in vitro}. (Related to Figure 1).
Figure S2. Recombinant FNIP1-D and PP5. (Related to Figure 2).

A) Purified FNIP1-D-His\textsubscript{6} and its non-phosphomutants were bacterially expressed and purified, run on an SDS-PAGE gel and coomassie stained.

B) Coomassie stained purified PP5-GST.

C) FNIP1-D-HA and indicated non-phosphomutants expressed in HEK293 cells, immunoprecipitated and then incubated with recombinant PP5-GST. Samples were immunoblotted for serine phosphorylation and Hsp90. Densitometry (optical density, OD) of the immunoblot in Fig. 2B are represented as mean ± S.D. Pearson correlation coefficients were calculated for both Hsp90 binding and phos-Ser. Students t-test was used to compare difference between WT +/- PP5-GST. Statistics were calculated using Graphpad, Prism 7. P>0.01 **, P>0.0001 ****
Figure S3

**Figure A**

Hsp90α-FLAG

**Figure B**

| FNIP1-D-His₆ | WT | S938A | EA | EEA | EEEA | EEEE |
|-------------|----|-------|----|-----|------|------|
| 250         | 130| 95    | 72 | 55  | 36   | 28   |

**Figure C**

% ATPase activity

(relative to Hsp90α only)

molar ratio FNIP1-D : Hsp90α

n.s.

*** p = 0.0001

**Figure D**

Hsp90 ATPase activity (mmol P/mmol Hsp90/min)

molar ratio FNIP1-D : Hsp90α

n.s.

n.s.

* p = 0.05

*** p = 0.0001

**** p = 0.00001

**Figure E**

Hsp90 ATPase activity (nmol P/mmol Hsp90/min)

Phos-Ser

*** p = 0.0001
Figure S3. Impact of phosphomimetic FNIP1-D mutants on Hsp90 ATPase activity. (Related to Figure 3).

A) Hsp90α-FLAG was expressed in PC3 cells and purified, and run on an SDS-PAGE gel followed by coomassie staining to assess purity.

B) FNIP1-D-His₆ and its phosphomimetics were expressed and purified from bacteria, run on an SDS-PAGE gel and coomassie stained.

C) Inhibitory effect of increasing amounts of recombinant wild-type FNIP1-D-His₆ on Hsp90α-FLAG ATPase activity (as % of Hsp90α alone) as measured in vitro. A Student’s t-test was performed to assess statistical significance (n.s. represents not significant; *P<0.05; ***P<0.001; ****P<0.0001).

D) Inhibitory effect of increasing amounts of recombinant wild-type FNIP1-D-His₆ on Hsp90α-FLAG ATPase activity (in mmol Pi/mol Hsp90/min) as measured in vitro. A Student’s t-test was performed to assess statistical significance (n.s. represents not significant; *P<0.05; ***P<0.001; ****P<0.0001).

E) Inhibitory effect of the recombinant FNIP1-D-His₆ phosphomimetic mutants on ATPase activity of Hsp90α-FLAG (in mmol P/mol Hsp90/min) isolated from PC3 cells was measured in vitro. A Student’s t-test was performed to assess statistical significance (n.s. represents not significant). p-value = 0.001 represents significance of Pearson correlation of the trend between number of phosphomimetic residues and effect on Hsp90 ATPase activity.
Figure S4

A

|          | EV | GAL1 | EV | ADH1 |
|----------|----|------|----|------|
| Glu      | Glu| Glu  | Glu| Glu  |
| Gal      | Gal| Gal  | Gal| Gal  |
| FNIP1-FLAG |    |      |    |      |
| Hsp90α   |    |      |    |      |

B

Glucose

GAL1-FNIP1-FLAG

EV

ADH1-FNIP1-FLAG

Galactose

EV

ADH1-FNIP1-FLAG

C

|       | FNIP1-FLAG | GAL1 | ADH1 |
|-------|------------|------|------|
| Glu   | Glu        | Glu  | Glu  |
| Gal   | Gal        | Gal  | Gal  |
| FNIP1-FLAG |   |      |      |
| Hsp90α |    |      |      |

D

|       | ADH1-FNIP1-FLAG | EV | WT | S938A | S939A | S941A | S946A | S948A |
|-------|-----------------|----|----|-------|-------|-------|-------|-------|
| Glu   | GAL1-FNIP1-FLAG |    |    |       |       |       |       |       |
| Glu   | Input           |    |    |       |       |       |       |       |
| Glu   | IP              |    |    |       |       |       |       |       |
| Glu   | Total           |    |    |       |       |       |       |       |
| Glu   | Hsp90α          |    |    |       |       |       |       |       |
| Glu   | Phos-Ser        |    |    |       |       |       |       |       |

E

Relative Ste11 band intensity (%)

F

Relative Phos-Tyr band intensity (%)

G

Relative v-Src band intensity (%)

H

Relative CFTR 60min band intensity (%)
Figure S4. Co-chaperone function of the phosphomimetic FNIP1-D mutants in yeast. (Related to Figure 4).
A) FNIP1-FLAG was expressed under either the GAL1 or ADH1 promoter in yeast expressing Hsp90α as the sole Hsp90. Cells were grown in glucose overnight and shifted to galactose for 12 hr. Expression of FNIP1-FLAG was examined by immunoblotting.
B) Yeast strains from (A) were spotted at 1:10 dilution series on YPED or YPGal media. Plates were incubated at 30°C for 2 days.
C) Hsp90α was immunoprecipitated from lysate from yeast strains in (A). Co-IP of FNIP1-FLAG was visualized by immunoblotting.
D) FNIP1-FLAG and its non-phosphomutants were expressed under the ADH1 promoter in yeast expressing Hsp90α as the sole Hsp90 and immunoprecipitated. Serine phosphorylation and interaction with Hsp90 were detected by immunoblotting. Short (SE) and long (LE) exposure of the radiographic film.
E) Densitometry (optical density, OD) of the Western blots in Figure 4B are represented as mean ± S.D.
F) Densitometry (optical density, OD) of the Phos-Tyr Western blots in Figure 4C are represented as mean ± S.D.
G) Densitometry (optical density, OD) of the v-Src Western blots in Figure 4C are represented as mean ± S.D.
H) Densitometry (optical density, OD) of the CFTR-HA bands at the 60 min time point in Figure 4F are represented as mean ± S.D.
Figure S5. Recombinant OGT. (Related to Figure 5).

A) OGT-FLAG was expressed in HEK293 cells and purified, run on an SDS-PAGE gel and coomassie stained.

B) Serine phosphorylation of FNIP-1-D-HA and its mutants were assessed by immunoblotting. Densitometry (optical density, OD) of the immunoblot in Fig. 5B are represented as mean ± S.D. Pearson correlation coefficients were calculated for Hsp90:FNIP-1D Binding (Blue) and Phos-Ser (Green). Statistics were calculated using Graphpad, Prism 7. P>0.01 **.
Figure S6. Post-translational modifications of FNIP1 and their impact on subcellular localization and degradation. (Related to Figure 6)

A) HEK293 cells were transiently transfected with FNIP1-FLAG wild-type or S938A mutant and then fixed and stained. DAPI was used for nuclear staining and cells were analyzed by immunofluorescence microscopy. Scale bar = 20µm.

B) Wild-type FNIP1-D-HA and S938A mutant were transiently transfected in induced and uninduced OGT knockout MEFS. Expression of FNIP1-D-HA was visualized by immunoblotting.

C) FNIP1-D-HA and its individual lysine to arginine mutants were transiently transfected in HEK293 cells. Their expression was analyzed by immunoblotting.
Figure S7. FINP1 co-chaperone does not affect O-GlcNAcylation. (Related to Figure 7).

A) HEK293 cells were treated with 2µM SNX-2112 (Hsp90 inhibitor) for the indicated amounts of time. OGT and CK2α stability were assessed by immunoblotting. Known Hsp90 clients phos-Akt (S473) and Akt were used as positive controls.

B) HEK293 cells were transiently transfected with FNIP1-HA. FNIP1 expression was probed with an endogenous FNIP1 antibody to demonstrate degree of overexpression. OGT and total O-GlcNAc levels in protein lysate were assessed by immunoblotting.