Protein Phosphatase 5 Is a Negative Modulator of Heat Shock Factor 1*

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Renaud Conde‡, Johnny Xavier§§, Christine McLoughlin‡§, Michael Chinkers¶, and Nick Osvencëê**

From the ‡Department of Anatomy and Cell Biology, College of Medicine, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5E5, Canada and the §Department of Pharmacology, University of South Alabama, Mobile, Alabama 36688

The major stress protein transcription factor, heat shock factor (HSF1), is tightly regulated through a multilayered activation-deactivation process involving oligomerization, post-translational modification, and interaction with the heat shock protein (Hsp90)-containing multichaperone complex. Conditions of proteotoxic stress, such as heat shock, trigger reversible assembly of latent HSF1 monomers into DNA-binding homotrimers that bind with high affinity to cognate heat shock elements. Transactivation is a second and independently regulated function of HSF1 that is accompanied by hyperphosphorylation and appears to involve a number of signaling events. Association of HSF1 with Hsp90 chaperone complexes provides additional regulatory complexity, however, not all the co-chaperones have been identified, and the specific molecular interactions throughout the activation/deactivation pathway remain to be determined. Here we demonstrate that protein phosphatase 5 (PP5), a tetratricopeptide domain-containing component of Hsp90-steroid receptor complexes, functions as a negative modulator of HSF1 activity. Physical interactions between PP5 and HSF1-Hsp90 complexes were observed in co-immunoprecipitation and gel mobility supershift experiments. Overexpression of PP5 or activation of endogenous phosphatase activity resulted in diminished HSF1 DNA binding and transcriptional activities, and accelerated recovery. Conversely, microinjection of PP5 antibodies, or inhibition of its phosphatase activity in vivo, significantly delayed trimer disassembly after heat shock. Inhibition of PP5 activity did not activate HSF1 in unstressed cells. These results indicate that PP5 is a negative modulator of HSF1 activity.

The cellular stress response protects against environmental or physiological perturbations that result in aberrant folding and aggregation of proteins. Stress induces rapid synthesis of a set of molecular chaperones known as heat shock proteins (Hsps)1 (1) that aid the folding, transport, regulation, and degradation of cellular proteins. The highly conserved transcription factor HSF1 is the key regulatory protein responsible for the up-regulation of Hsp expression in higher eukaryotes (1–3).

The mechanism of HSF1 regulation is highly complex, involving multifactorial control by phosphorylation, cellular compartmentalization, and protein-protein interaction with chaperone complexes. It is not yet known how these multiple layers of control are integrated to result in cellular regulation of HSF1 and the stress response. HSF1 exists under normal conditions as non-DNA-binding monomers, poised for rapid conversion to homotrimers with high affinity DNA binding activity to the heat shock elements in Hsp gene promoters (4, 5). This oligomeric switching involves rearrangement of hydrophobic interactions between 3 hydrophobic heptad repeats distributed along the HSF1 molecule (4, 6–8), and it is likely that there is dynamic recycling between monomeric and trimeric complexes during induction and recovery phases of the HSF1 activation/deactivation pathway.

Acquisition of transcriptional competence is a second independent step in the stress-activation process that is regulated through a central regulatory region (9) and multiple phosphorylation events (3, 10). Although hyperphosphorylation is generally associated with increased transcriptional activity, elucidating the effect of specific phosphorylation on HSF1 activity is proving to be a complicated task. Several kinases have been reported to phosphorylate HSF1 on specific residues and either enhance or repress its activities, including calcium/calmodulin-dependent protein kinase II (11), casein kinase 2 (12), protein kinase Ca (13), glycogen synthase kinase 3 (14, 15), and extra-cellular signal-regulated kinase 1 (16, 17).

In addition, there is evidence that the activities of HSF1 are influenced by Hsp90 multichaperone complexes (18-22). The role of Hsp90 chaperone complexes on transcription factor regulation has been most extensively characterized in the experimental model of steroid receptor maturation (23, 24). Hsp90 is the key molecular chaperone in mature receptor complexes together with p23 and one of the immunophilins (Cyp40, FKBP51, or FKBP52). Assembly of steroid receptor complexes is a highly dynamic process involving Hsp70 and accessory chaperones Hsp40, Hip, and Hop (24).

Using human HeLa cells extracts and rabbit reticulocyte lysates, Voellmy and colleagues (3, 19, 21) have shown Hsp90

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** To whom correspondence should be addressed: Dept. of Anatomy and Cell Biology, College of Medicine, University of Saskatchewan, 107 Wiggins Rd., Saskatoon, Saskatchewan S7N 5E5, Canada. Tel.: 306-966-4066; Fax: 306-966-4298; E-mail: osvenek@duke.usask.ca.

1 The abbreviations used are: Hsp, heat shock protein; Ab, antibody; HSF1, heat shock factor 1; PP5, protein phosphatase 5; EMSA, electrophoretic mobility shift assay; IP, immunoprecipitation; GR, glucocorticoid receptor; TPR, tetratricopeptide repeat; CMV, cytomegalovirus; HSE, heat shock element.
association with HSF1 monomers, as well as HSF1 trimer association with Hsp90-immunophilin-p23 complex, and have proposed a model for transcriptional repression of HSF1 activity by Hsp90. Our laboratory has examined HSF1 regulation by Hsp90 in the Xenopus oocyte model system, and reported HSF1 interaction with multiple components of the Hsp90 foldosome (18, 20). Hsp90, P23, and FKBP52 remain associated with the activated HSE binding HSF1 complex (20). Conversion to monomers after heat shock was accelerated by overexpression of Hsp90, Hsp70, Hip, and Hop and delayed by cyclophilins (Cyp-40, FKBP51, and FKBP52). HSF1 was activated in the absence of heat shock by microinjection of Abs against Hsp90 and p23, and microinjected Abs against several of the co-chaperones also affected the DNA-binding properties of HSF1. Further evidence supporting Hsp90-mediated control of HSF has been reported in yeast (25).

The precise molecular interactions and specific roles of individual components of the Hsp90 foldosome in regulating trimerization, DNA binding, and transcriptional activity, at each stage of the HSF1 activation/deactivation pathway, has not yet been determined. However, the initial studies with HeLa cells and Xenopus oocytes suggest that the multichaperone interactions that may regulate HSF1 share common features with the steroid receptor maturation pathway (22). PP5 (protein phosphatase 5) is a major component of steroid receptor-Hsp90 heterocomplexes, and is involved in the regulation of glucocorticoid receptor (GR) and estrogen receptor function (Refs. 26–30, for review, see Refs. 31 and 32). PP5 is a 58-kDa member of the PPP family of serine/threonine protein phosphatases that contains a tetratricopeptide repeat (TPR) domain not found in other members of this family. The N-terminal TPR domain of PP5 functions both to inhibit the C-terminal phosphatase domain and to mediate interactions with the MEEVD domain of Hsp90 (33). Hsp90 activates PP5 by disrupting TPR-phosphatase domain interactions, permitting substrate access to the active phosphatase domain (33, 34). Here we investigate the potential interaction of PP5 with HSF1-Hsp90 complexes, and subsequent regulation of HSF1 activities.

**EXPERIMENTAL PROCEDURES**

**Oocyte Manipulations—**Xenopus laevis oocytes were obtained, fractionated, and microinjected as described previously (14, 20). Chemical
Overexpression of PP5 Reduces HSF1 DNA Binding upon Heat Shock—To determine the potential role of PP5 in HSF1 regulation, oocytes were microinjected with pCMV6-PP5 (26), which codes for rat PP5 under control of the constitutively active CMV promoter. Western blot analysis (Fig. 1A) showed steadily increasing quantities of plasmid-derived PP5 over the time course of incubation, with a substantial increase relative to endogenous PP5 seen after 12 h of expression. Heat shock did not affect steady state levels of either endogenous or recombinant PP5, nor did overexpression of PP5 have any effect on the levels of HSF1, before or after heat shock.

The heat-inducible activation of HSF1 oligomerization/DNA binding was compared in un.injected controls and PP5-expressing (12 h) oocytes by EMSA (Fig. 1B). The amount of HSF1-HSE complex formation was significantly reduced in PP5-expressing oocytes relative to controls. DNA binding activity of other transcription factors such as CAAT box transcription factor (Fig. 1B, lower panel) or SP1 (data not shown) were not affected by expression of exogenous PP5, ruling out a general suppressive effect on DNA binding activities in the oocyte.

HSF1 activation was compared in oocytes expressing PP5 for 3, 6, or 12 h prior to heat shock (Fig. 1C). The reduction of HSF1 activation was proportional to the amount of exogenous PP5, suggesting a direct link between increased PP5 concentrations and HSF1 regulation. The relative reduction in HSF1 DNA binding activity was more prominent at the early phases of heat shock. After 60 min of heat shock, HSF1-HSE complex formation relative to controls was reduced by 20% in all samples, suggesting that lengthy heat shock diminished the impact of PP5 on activation.

We next tested the effect of PP5 overexpression on the induction of HSF1 by chemical stresses. HSF1 activation was reduced in PP5 expressing oocytes in response to salicylate, arsenite, cadmium, and ethanol treatments (Fig. 1D).

Subcellular Localization of PP5—We next compared the subcellular localization of endogenous and plasmid-derived PP5 in control and microinjected oocytes (Fig. 2). Endogenous PP5 was predominantly localized in the cytoplasm with low but detectable amounts in a single oocyte nucleus. Heat shock resulted in modest reproducible translocation of PP5 into the nucleus (lanes 5 and 6). Exogenous PP5 expressed from microinjected pCMV-FLAG-PP5 behaved similarly to endogenous PP5, both in relative distribution and nuclear translocation induced by heat shock. The total mass of the nucleus is ~5% of the whole cell, and longer film exposure revealed the presence of FLAG-tagged PP5 in the nucleus prior to heat shock (data not shown). Controls using the cytoplasmic marker IC3 and the nuclear marker proliferating cell nuclear antigen demonstrate the purity of subcellular fractions.

PP5 Interaction with HSF1—HSF1 is an exclusively nuclear protein in oocytes (35), and detection of nuclear PP5 made plausible a physical interaction between these proteins. Co-IP experiments were performed to test for interaction between endogenous PP5 and HSF1. Co-IP of PP5 using an Ab against HSF1 (Fig. 3A, lanes 1 and 2), and reciprocal pull-down of HSF1 using an Ab against PP5 (lanes 10 and 11) was observed, confirming a physical interaction between PP5 and HSF1 in oocytes. These interactions were detected in unshocked and heat-shocked samples with no consistent or reproducible difference observed in the co-IP signal after heat shock. This suggests that PP5 is a component of Hsp90 heterocomplexes with both inactive monomeric and activated trimeric HSF1. The protein-protein association here is presumably through interaction between the TPR domain of PP5 and the C terminus of Hsp90, although it remains possible that PP5 could...
interact directly with HSF1. Control IP reactions with unrelated Abs failed to bring down PP5 or HSF1 in both non-shocked or heat-shocked oocytes (Fig. 3A, lanes 4–9 and 12–18).

We examined whether exogenous plasmid-derived PP5 in microinjection experiments associates with HSF1 in a similar manner as endogenous PP5. Co-IP of FLAG-tagged PP5 with HSF1 was observed in both non-shocked and heat-shocked samples (Fig. 3B, lanes 3 and 4), showing a direct interaction between exogenous PP5 and HSF1 complexes. These data provide evidence of a biochemical link between PP5 overexpression and changes in HSF1 activity and functional properties observed in PP5 expressing oocytes (see Figs. 1 and 4–6).

To further confirm the presence of PP5 with activated HSF1 trimers, mobility supershift analyses were performed using Abs against PP5 and other components of HSF1-Hsp90 heterocomplexes. We have previously observed supershifts of HSF1-HSE complexes with anti-Hsp90, p23, and FKBP52 Abs (20), results consistent with findings of pull-down experiments of others using HeLa cell extracts (21). Distinct supershifted complexes with clearly reduced mobility were observed with Abs against PP5, HSF1, Hsp90, p23, Hip, and FKBP52 (Fig. 3C). This suggests that PP5 is a component of active trimeric HSF1 complexes along with Hsp90, p23, FKBP52, and Hip. HSF1-HSE complexes were not affected by Hop Abs (lanes 13 and 14), or control Abs against YY1 transcription factor or proliferating cell nuclear antigen (lanes 17–20). PP1 and PP2A Abs were also tested in these assays to test for possible interactions with the HSF1 heterocomplex, however, no supershift was observed (lanes 21–26).

Effect of PP5 Phosphatase Activity on the DNA Binding Activity of HSF1—Co-IP and mobility supershift experiments (Fig. 3) establish PP5 as a component of the HSF1-Hsp90 heterocomplex, and we observed significantly reduced activation of HSF1 DNA binding after expression of exogenous PP5 (Fig. 1). The next series of experiments were aimed at determining whether the inhibitory effect on HSF1 was because of the specific PP5 phosphatase activity. Oocytes did not survive treatments with arachidonic acid, a well established activator of PP5 (36, 37). Instead, in vivo activation of PP5 was accomplished by overexpression of the C-terminal portion of Hsp90 (C-90), which disrupts the autoinhibitory interaction between the TPR and catalytic domains of PP5 resulting in derepression of phosphatase activity (38). The magnitude of HSF1 activation was similarly reduced in both C-90 and PP5 expressing oocytes, with a similar temporal pattern of complex accumulation over the time course of heat shock (Fig. 4A, lanes 1–15). Okadac acid, a specific inhibitor of PP5, did not affect the pattern of HSF1 activation during heat shock, nor did it activate HSF1 in the absence of heat shock (Fig. 4A, lanes 21–25). Thus PP5 appears to negatively modulate HSF1 activity, but inhibition of PP5 alone is not sufficient to trigger trimerization/DNA binding.

Fostriecin, a specific inhibitor of PP2A at nanomolar concentrations (39), did not affect the magnitude or pattern of complex
either PP5 or C-90, or incubated with 4 µM fostriecin or 100 nM okadaic acid for 2 h prior to heat shock at 33 °C for the times indicated. Samples were subjected to EMSA with HSE, and quantification of DNA binding activity is shown below each panel. The data shown in each panel is representative of the effects observed in at least 3 repetitions of each experiment, and all oocytes used in each panel were obtained from a single ovary.

A, oocytes were microinjected into the nucleus with 7.5 milliunits of PP1 or PP2A enzyme, heat shocked at 33 °C for the indicated times, and subjected to EMSA with HSE. Uninjected oocytes are indicated (Control). The uninjected (−) and microinjected (+) samples were analyzed by Western blot to show the increase in PP1 or PP2A protein levels relative to endogenous protein (right panels). C, oocytes were microinjected with plasmid DNA coding for PP5 mutants K97A, R101A, or the TPR domain of PP5, exposed to the time course of heat shock at 33 °C, then subjected to EMSA with labeled HSE. Samples were analyzed by Western blot with anti-PP5 Ab to show the increase in PP5 and mutant proteins in the oocytes used in the EMSA and to assess the effect of TPR on endogenous PP5 levels (lower panel).

Non-shocked and heat-shocked samples are indicated (N and H).

HSF1 was also unaffected after elevation of PPP family members PP1 and PP2A (Fig. 4B). Therefore the diminishment of HSF1 activity observed after overexpression and C-90 activation was likely because of specific up-regulation of PP5 phosphatase activity.

It was possible that the effects observed in these experiments were because of destabilization or remodeling of endogenous Hsp90-chaperone complexes by overexpressed TPR-containing protein, or the C terminus of Hsp90. To test this, we used mutants of PP5, K97A, and R101A that contain amino acid substitutions in the TPR domain; these mutants display low affinity for Hsp90 in vitro, but retain full phosphatase activity (40). Expression of PP5-K97A and PP5-R101A diminished HSF1 activity in a similar manner as expression of wild-type PP5 or the PP5 activator C-90 (Fig. 4C, lanes 1-15). In addition, overexpression of the C-terminal PP5 TPR domain (26) had no significant effect (Fig. 4C, lanes 16-20). Therefore, inhibition of HSF1 observed in these experiments was likely because of PP5 phosphatase activity rather than competitive effects of microinjected proteins or remodeling effects on endogenous Hsp90 complexes.

**Effect of PP5 on Deactivation of HSF1 during Recovery**—The reduction of HSF1-HSE complex formation brought about by increased PP5 activity could be explained by a lowered rate of trimer formation during activation, or enhanced rate of trimer conversion to monomers during deactivation. We examined the profile of deactivation during the time course of recovery from heat shock to determine the effects of PP5 on disassembly of HSF1 trimers. The highest level of HSF1 activation was observed immediately after heat shock, and complexes disappeared by 30 min of recovery in controls (Fig. 5A, lanes 1-5). Reduced HSF1 activation was seen in PP5 expressing oocytes, and complexes disappeared more rapidly by 10 min (Fig. 5A, lanes 6-10, and E, lanes 7-12).

Activation of endogenous PP5 by expression of C-90 also resulted in an accelerated recovery profile (Fig. 5E, lane 13-18). This more rapid recovery could be because of a higher rate of deactivation brought about by increased PP5 activity, or to the fact that fewer complexes were present immediately after heat shock. No significant difference in the pattern of recovery was observed after elevation of PP1 or PP2A (Fig. 5B), or fostriecin-inhibition of PP2A (Fig. 5E, lanes 19-24), suggesting the effects on recovery are specific to PP5. An opposite effect was seen after inhibition of PP5 in vivo through microinjection of PP5 Abs, which resulted in a delayed deactivation profile (Fig. 5C, lanes 6-10) relative to untreated controls (lane 1-5) or controls microinjected with unrelated Abs (lanes 11-15). HSF1-HSE complexes were still detected at time points well after complexes had disappeared in controls. Microinjection of PP5 Abs decreased the interaction of PP5 with HSF1 (Fig. 5C, lanes 6-10) relative to untreated controls (lane 1-5), demonstrating that microinjected Abs had a direct effect on HSF1 complexes, and strengthening the link between perturbation of PP5 and effects on HSF1 activity. Okadaic acid inhibition of PP5 also prolonged deactivation (Fig. 5D). The converse effects on HSF1 recovery caused by PP5 activation or inhibition in these experiments suggest the phosphatase activity of PP5 contributes to the process of trimer deactivation.

**Effect of PP5 on the Transcriptional Activity of HSF1**—The DNA binding and transcriptional activities of HSF1 are separately regulated (20, 41, 42), so we examined the effect of PP5 on HSF1-mediated transcription. Transcriptional activity of the Hsp70 promoter in heat-shocked cells was completely abrogated by overexpression of PP5 (Fig. 6A, compare lanes 2 and 4). Transcription was not appreciably altered after inhibition of PP5, although slight increases were observed (Fig. 5E). Tran-
scription was not affected by microinjection of PP1 (Fig. 5B), however, it was reduced by microinjection of PP2A (Fig. 6C), and conversely, was enhanced by inhibition of PP2A phosphatase activity by fostriecin (Fig. 6D). No variation in transcription from the CMV reporter was observed. In all cases, the Hsp70 promoter was not significantly activated in non-shocked oocytes, and the level of heat-induced transcription was not affected by Me2SO (DMSO, panel F). Overall, these results suggest that PP5 has a negative regulatory role on HSF1-mediated transcription, although it is difficult to distinguish whether this effect was manifested through inhibition of transactivation potential per se, or if PP5 exerts its effects primarily at the level of trimer formation.

**DISCUSSION**

Our results demonstrate that PP5 is a component of the HSF1-Hsp90 complex, and suggests that it functions as a modulator of HSF1 activities. HSF1-PP5 interactions were demonstrated in co-IP assays as well as in EMSA supershift experiments (Fig. 3). HSF1 and PP5 co-IP was equal in both non-shock and heat-shocked cells, suggesting that PP5 interacts with both inactive HSF1 monomers and heat-activated trimers. Mobility supershift of HSF1 by anti-PP5 Abs further supports the interaction between PP5 and the trimeric form of HSF1. In these experiments, HSF1-HSE complexes formed in DNA binding assays were also recognized by Abs against Hsp90, p23, FKBP52, and Hip (Fig. 3). Therefore, PP5 is part of a large Hsp90-based chaperone complex that may function to assist in the conformational changes required for activation and deactivation (20, 21). We note that a PP5-HSF1 interaction had been observed in reconstitution assays with rabbit reticulocyte lysates (21), but the significance of this was not further explored. It should be emphasized that the HSF1-PP5 interactions reported here (Fig. 3) are between endogenous proteins, because co-IP was performed with non-microinjected oocytes. It is likely that interaction of PP5 with the HSF1-Hsp90 complex involves contact between the TPR domain of PP5 and the MEEVD motif of Hsp90 (29), however, direct contact between HSF1 and PP5 have not been ruled out. Exogenously expressed PP5 also associated with HSF1 in a similar fashion as the endogenous protein, therefore there is a direct biochemical link between the effects observed from perturbing PP5 activity and the activity of HSF1.

What is the function of PP5 in HSF1 complexes? Our results suggest that PP5 is a negative modulator of HSF1. The experimental approach to investigate the potential role of PP5 in this context was to examine the effects of overexpression, activa-
tion, or inhibition of PP5 on HSF1 activities in vivo in Xenopus oocytes. Exogenous PP5 diminished, but did not completely inhibit trimerization/DNA binding activity induced by heat shock (Figs. 1 and 4), and resulted in a more rapid time course of deactivation during recovery (Fig. 5). The same effects on activation and recovery were observed after stimulation of endogenous PP5 with the C-90 activator, suggesting that the phosphatase activity of PP5 plays a role in negative regulation of HSF1. Because similar reductions of HSF1 activity were also observed in experiments using a variety of chemical stressors (Fig. 1), we conclude that PP5 exerts a general regulatory effect on HSF1 regardless of its TPR domain, or that the point mutations are insufficient to disrupt Hsp90-PP5 interactions in vivo. This observation could prompt us to speculate that PP5 dephosphorylates targets outside the HSF1-Hsp90 complex, although overexpression of PP1 and PP2A had no effect. Further characterization of the specific substrate interactions of PP5 within the HSF1-Hsp90 complexes will be required to elucidate the precise mechanism by which it influences HSF1 activity.

The dose-dependent inhibition of HSF1 trimerization/DNA binding was most prominent at early time points of heat shock activation (Fig. 1C), however, we refrain from concluding that PP5 acts primarily as a repressor of HSF1 activation, but suggest that it functions to modulate the monomer-trimer equilibrium in favor of deactivation. We observed that HSF1 trimerization/DNA binding was not induced in the absence of heat shock by inhibition of PP5 with okadaic acid or microinjection of anti-PP5 Abs; induction was only observed in response to heat shock (Figs. 4 and 5). Inhibitors of PP5 phosphatase activity significantly delayed the deactivation of HSF1 trimers during recovery. Thus, the apparent inhibition of HSF1 at any point in the activation profile could be explained by an enhanced rate of trimer-monomer turnover brought about by elevated PP5 activity.

Reporter gene assays suggest that PP5 may also play an additional role in negative regulation of HSF1 transcriptional activity. Heat-inducible Hsp70 promoter activity was completely inhibited by overproduction of PP5 (Fig. 6). The results of transcription assays were consistent with reduced HSF1 DNA binding in cells expressing PP5. Because transcription is dependent on formation of HSE binding trimers, we cannot unambiguously determine whether PP5 exerts additional effects on the transcriptional activation domain apart from its effect on trimerization/DNA binding. In addition, the fact that we did not observe more dramatic increases in transcription after inhibiting PP5 with okadaic acid probably reflects the complexity of transcriptional regulation of HSF1 through multiple mechanisms.

Parallels could be drawn between the observations of PP5 in HSF1-Hsp90 complexes, reported here, and the existence of PP5 in Hsp90-GR and estrogen receptor heterocomplexes (26, 27, 30). The role of PP5 in steroid receptor signaling, and its substrates within Hsp90 heterocomplexes have not been identified (31). There are contradictory reports on the role of PP5 in GR regulation, because the use of dominant-negative mutants suggested PP5 is required for optimal GR signaling (26), whereas antisense-knockdown suggested it is antagonistic to GR signaling (28). PP5 has also been reported to negatively regulate ER-mediated transcription (30).

It is possible that there are conserved mechanisms by which PP5 influences the Hsp90 foldsome in the context of steroid receptor and HSF1 transcription factors. As with steroid receptor-Hsp90 heterocomplexes, there are several possible regulatory targets for PP5 in HSF1-Hsp90 complexes. The most obvious would be HSF1 itself, because it is phosphorylated by several kinases and is hyperphosphorylated upon heat shock. Also, it is possible that PP5 targets other cochaperones within the HSF1 complex. Finally, the heat shock-induced nuclear...
translocation of PP5 could reflect a requirement for PP5 in the stressed nucleus. Whether this translocation has an impact on HSF1 regulation (Fig. 2) is unknown. The precise molecular mechanism by which PP5 acts to negatively modulate HSF1 is currently under investigation.

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