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Report

Stress-Induced Phosphorylation of *S. pombe* Atf1 Abrogates Its Interaction with F Box Protein Fbh1

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Summary

The Atf1 transcription factor is critical for directing stress-induced gene expression in fission yeast [1]. Upon exposure to stress, Atf1 is hyperphosphorylated by the mitogen-activated protein kinase (MAPK) Sty1 [2, 3], which results in its stabilization [4]. The resulting increase in Atf1 is vital for a robust response to certain stresses [4]. Here we investigated the mechanism by which phosphorylation stabilizes Atf1. We show that Atf1 is a target for the ubiquitin-proteasome system and that its degradation is dependent upon an SCF E3 ligase containing the F box protein Fbh1. Turnover of Atf1 requires an intact F box, but not DNA helicase activity of Fbh1. Accordingly, disruption of Fbh1 F box function suppresses phenotypes associated with loss of Atf1 phosphorylation. Atf1 and Fbh1 interact under basal conditions, but this binding is lost upon stress. In contrast, a version of Atf1 lacking all intact MAPK sites still interacts with Fbh1 upon stress, indicating that the association between the F box protein and substrate is disrupted by stress-induced phosphorylation. Most F box protein-substrate interactions described to date are mediated positively by phosphorylation [5]. Thus, our findings represent a novel means of regulating the interaction between an F box protein and its substrate. Moreover, Atf1 is the first target described in any organism for the Fbh1 F box protein.

Results

Atf1 Is Ubiquitinated In Vivo

The levels of Atf1 are increased in a proteasome mutant, indicating that its stability might be controlled by ubiquitin-mediated proteolysis [4]. To test this, we purified ubiquitinated proteins from denatured extracts made from cells expressing histidine-tagged ubiquitin and immunoblotted with anti-Atf1 antiserum. We detected a smear of slowly migrating species characteristic of polyubiquitinated isoforms (Figure 1A). No smear was observed in immunoprecipitates from the control strain. Thus, we conclude that Atf1 is subjected to ubiquitin-mediated degradation by the 26S proteasome.

Atf1 Is Targeted for Proteolysis by the SCF^{Fbh1} Complex

Next we sought to identify the factors regulating Atf1 ubiquitination. Given its role in targeting many short-lived transcription factors and cell-cycle regulators for degradation [6, 7], we tested whether the SCF complex was involved. We examined the steady-state levels of Atf1 in a conditional mutant of the core SCF component Skp1. Upon incubation at the restrictive temperature, Atf1 protein accumulated in *skp1.A4* but not in wild-type, whereas the messenger RNA (mRNA) levels of *atf1* were unchanged (Figure 1B). Moreover, ubiquitination of Atf1 was reduced in the *skp1.A4* mutant (Figure 1C). These data are consistent with Atf1 being a substrate of an SCF complex. Next we tested whether Atf1 stability was regulated by other SCF components by analyzing strains containing mutations in 17 out of the 18 *S. pombe* genes encoding F box proteins [8–10]. To determine whether Atf1 stability was affected in these mutants, cycloheximide was added to shut off de novo protein synthesis and Atf1 levels were examined (Figure 2A; data not shown). Atf1 was clearly stabilized in *fbh1*Δ and to a lesser extent in *pof7*Δ, whereas in the other mutants, its levels decreased in a manner comparable to wild-type. We focused on Fbh1, given that we repeatedly observed stabilization of Atf1 in the *fbh1*Δ mutant. In contrast, we did not consistently observe an increase in Atf1 stability in *pof7*Δ (Figure 2A; see also Figure S1 available online). We do not know the reason for this, but we note that a nonreproducible accumulation of Cig2 has been observed in *pof7*Δ [10].

The levels of *atf1* mRNA were not affected in *fbh1*Δ, suggesting that Atf1 was regulated by Fbh1 in a posttranscriptional manner (Figure S2). Overexpression of *fbh1* in wild-type reduced Atf1 levels to approximately one-third of those transformed with the empty vector (Figures 2B and 2C; *fbh1* mRNA shown in Figures 2D and 2E). This further supports a link between Atf1 and Fbh1 in that overexpression of other F box proteins can result in reduced levels of their targets [11, 12]. In summary, our data suggest that Atf1 is targeted for proteasome-mediated degradation by an SCF^{Fbh1} E3 ubiquitin ligase.

The F Box Function, but Not DNA Helicase Activity, of Fbh1 Is Required for Targeting Atf1 for Degradation

Next we examined the relative contributions of the Fbh1 F box motif and DNA helicase activity to Atf1 degradation. The F box is situated at the N terminus of Fbh1, whereas the seven DNA helicase domains are spread across the rest of the protein (Figure 2F). We examined Atf1 stability in strains containing mutations within Fbh1 that are designed to disrupt either the interaction of the F box with Skp1 (L14A/P15A) or DNA helicase activity (D485N) [13]. Corresponding changes in other proteins are known to inactivate F box and helicase functions [14, 15]. Moreover, we verified that the *fbh1*$^{L14A/P15A}$ mutation abrogates binding between Fbh1 and Skp1 without affecting levels of Fbh1 protein (Figure S3). Following treatment with cycloheximide, Atf1 levels did not decrease in either *fbh1*Δ or *fbh1*$^{L14A/P15A}$ (Figures 2G and 2H). Accordingly, the half-life of Atf1 increased in the *fbh1*$^{L14A/P15A}$ mutant. In the wild-type background, the half-life of Atf1 was approximately 1.1 hr, consistent with previous results [4]. However, the stability of Atf1 was significantly increased in the *fbh1*$^{L14A/P15A}$ mutant: its levels did not decrease appreciably over 5 hr (Figures 2I

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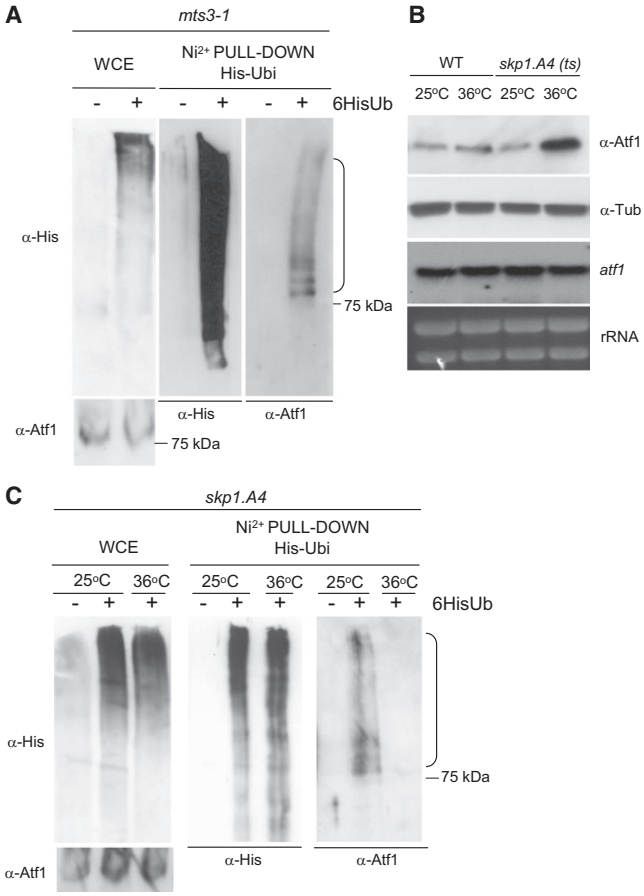

Figure 1. Atf1 Is Polyubiquitinated In Vivo in a Skp1-Dependent Manner

(A) Cultures of *mts3-1* transformed with either pREP41-6HisUbiquitin (+) or pREP41 alone (−) were grown to mid-log phase in Edinburgh minimal medium (EMM) at 25°C in the absence of thiamine and then shifted to 36°C for 3 hr. Histidine-tagged proteins were isolated by incubation with Ni-NTA resin. Ubiquitinated Atf1 was identified by immunoblotting of the purified proteins with anti-Atf1 antiserum. Immunoblotting was also performed with an anti-Histidine antibody (α-His). Molecular mass is indicated in kilodaltons. The following abbreviations are used: WCE, whole-cell extracts; IP, immunoprecipitation. The experiment was performed in a proteasome mutant strain (*mts3-1*) to prevent degradation of ubiquitinated conjugates [23]. The bracket corresponds to ubiquitinated forms of Atf1.

(B) Atf1 protein, but not mRNA, accumulates in a *skp1.A4* mutant. Cultures were grown to mid-log phase in yeast extract (YE) at 25°C and then shifted to 36°C for 3 hr. Protein samples were examined by immunoblotting with anti-Atf1 or anti-tubulin antiserum (α-Tub). Total RNA was extracted and examined by northern blotting with an *atf1* probe. Loading was assessed by examining ribosomal RNA (rRNA).

(C) The *skp1-A4* mutant was transformed with either pREP41-6His ubiquitin or pREP41 alone. Cultures were grown and ubiquitinated proteins were isolated as described in (A), except that proteasome inhibitor was added for 3 hr before harvesting. Purified ubiquitinated proteins were examined by immunoblotting as indicated. The bracket corresponds to ubiquitinated forms of Atf1.

and 2J). These data indicate that an intact F box motif is required for the function of Fbh1 in mediating Atf1 degradation. In contrast, when we treated the helicase mutant *fbh1*^{D485N} with cycloheximide, loss of Atf1 protein was comparable to wild-type (Figures 2G and 2H). Thus, we conclude that the DNA helicase activity of Fbh1 is not required for its role in mediating Atf1 degradation via SCF^{FBH1} activity.

Loss of Fbh1 F Box Function Suppresses the Defects Associated with Loss of Atf1 Phosphorylation

Next we asked whether the defects associated with reduced stability of Atf1 could be suppressed by loss of Fbh1 F box function. A mutant version of Atf1, lacking all eleven mitogen-activated protein kinase (MAPK) sites (Atf1-11M), is less stable than its wild-type counterpart and accumulates to a lesser extent upon stress. This reduction in Atf1 prevents *atf1-11M* cells from mounting a robust response to H_2O_2 [4].

Specifically, we hypothesized that abrogation of Atf1-11M targeting to the proteasome, through inactivation of Fbh1, might suppress the *atf1-11M* phenotypes. To test this, we combined the *atf1-11M* allele with either the *fbh1*$^{L14A/P15A}$ mutation or, as a control, with *pof9*Δ (which does not affect Atf1 stability; data not shown). Disruption of the Fbh1 F box function but not loss of *pof9* suppressed the H_2O_2 sensitivity of *atf1-11M* (Figure 3A). Consistent with this, loss of Fbh1 F box function resulted in an increase in Atf1-11M protein to levels comparable to wild-type; loss of *pof9* had no effect (Figure 3B). Finally, we observed a recovery of stress-dependent transcript levels in the Fbh1 F box mutant but not in *pof9*Δ, as illustrated by measuring *hsp9* and *gpx1* mRNA levels (Figures 3C and 3D). In summary, these data support the idea that degradation of Atf1 is mediated by SCF^{Fbh1} because the defects associated with loss of stress-induced stabilization of Atf1-11M can be suppressed by the F box mutant of Fbh1.

Stress-Induced Phosphorylation of Atf1 Inhibits Its Interaction with Fbh1

From our data, we made the following predictions: first, if Atf1 is a target of SCF^{Fbh1}, Atf1 and Fbh1 should bind to one another; second, this interaction would be weakened or abolished upon stress because Atf1 is stabilized under such conditions; and third, this interaction would be negatively regulated by stress-induced phosphorylation of Atf1. To address these predictions, we first sought to identify a physical interaction between Fbh1 and Atf1. To detect Fbh1 in yeast, we placed three copies of the Pk epitope at the 3′ end of the *fbh1* open reading frame (ORF), such that expression of the tagged protein was driven by the native *fbh1* promoter. Atf1 levels were not affected in this background (Figure S4). Moreover, *fbh1-3Pk* cells were wild-type with respect to cell length and exposure to Methyl methanesulfonate (MMS) (data not shown). Increased cell length and sensitivity to DNA-damaging agents are phenotypes displayed by *fbh1*Δ [13, 16]; thus, we conclude that the tags do not obviously disrupt Fbh1 function.

We performed immunoprecipitations in order to detect an interaction between Atf1 and Fbh1. In these experiments we observed two anti-Atf1 immunoreactive bands (Figure 4A, lane 5), the faster-migrating of which most likely represents a product of postlysis degradation (Figure S5). We did not observe Atf1 in immunoprecipitates of Fbh1 or vice versa (data not shown). In many cases, ubiquitination of a protein results in its prompt turnover by the proteasome; thus, if the fraction of Atf1 that is interacting with Fbh1 is being rapidly ubiquitinated and degraded, the interaction might not be observed. Thus, we performed immunoprecipitations upon proteasome inhibition. Atf1 was clearly observed in the immunoprecipitates from *fbh1-Pk mts3-1*, but not from the control untagged strain (Figure 4A, compare lanes 1 and 5). This interaction was lost upon osmotic stress (Figure 4A, compare lanes 5 and 6). The same results were observed with H_2O_2 (Figure S6), suggesting that loss of the interaction is a general response to stress rather than a stress-specific effect.

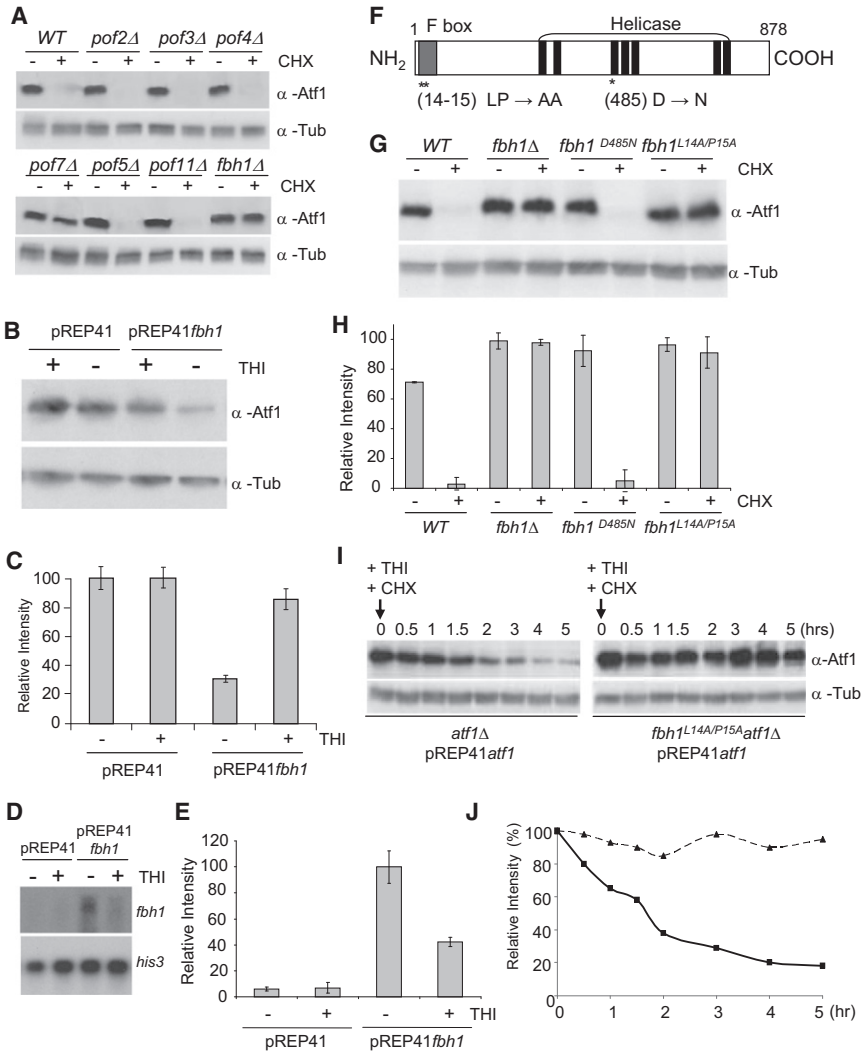

Figure 2. Loss of *fbh1* or Disruption to Its F Box Motif Results in an Increase in Atf1 Levels as a Result of Its Increased Stability

(A) Atf1 is stabilized in the *fbh1*Δ mutant. The indicated strains were grown at 30°C to mid-log phase in YE. Protein samples were prepared before (−) and after (+) overnight incubation with cycloheximide (CHX) at 100 μg/ml and were analyzed by immunoblotting.

(B) Overexpression of *fbh1* cDNA reduces the levels of Atf1. Wild-type cells were transformed with either pREP41 (the empty vector) or pREP41*fbh1* (resulting in expression of *fbh1* from the thiamine-repressible *nmt41* promoter). Exponentially growing cells were grown at 30°C in minimal medium (MM) in the absence (−) or presence (+) of 15 μM thiamine (THI). Addition of thiamine leads to repression of the *nmt* promoter; thus, the promoter is derepressed in its absence. Levels of Atf1 were examined by immunoblotting.

(C) The relative densities of the Atf1 bands were calculated by using the relevant loading control sample for each time point. The values represent the average of three experiments and are shown with standard deviation. The band with the highest intensity was designated as 100%, and the percentage intensities of the remaining bands were calculated relative to this.

(D) Northern blot of RNA extracted from samples used in (B) to examine the overexpression of *fbh1* cDNA. Loading was assessed with a probe recognizing the *his3* gene.

(E) Quantitation of *fbh1* mRNA from three independent experiments shown with standard deviation.

(F) Diagram of the F box protein Fbh1 indicating the locations of the F box and helicase domains and the amino acid changes corresponding to the *fbh1*$^{L14A/P15A}$ and *fbh1*^{D485N} alleles.

(G) Atf1 is not stabilized in the *fbh1* helicase mutant. Wild-type, *pof9*Δ, *fbh1*Δ, *fbh1*^{D485N}, and *fbh1*$^{L14A/P15A}$ cultures were grown to mid-log phase in YE at 30°C. Protein samples were prepared before and after overnight incubation with cycloheximide (100 μg/ml) and were analyzed by immunoblotting.

(H) Quantitation of the levels of Atf1 shown in (G) and in two further independent experiments shown with standard deviation.

(I) The *fbh1*$^{L14A/P15A}$ mutations result in stabilization of Atf1. *atf1*Δ and *fbh1*$^{L14A/P15A}$ *atf1*Δ cells containing the pREP41*atf1* plasmid with the *atf1* cDNA under the control of the *nmt41* promoter were grown in MM without thiamine to mid-log phase. Thiamine (15 μM) and cycloheximide (100 μg/ml) were added to repress the expression of the *atf1* gene and protein synthesis, respectively. Protein extracts were prepared from samples collected up to 5 hr and were analyzed by immunoblotting.

(J) The relative densities of the Atf1 signals were calculated for *atf1*Δ pREP41*atf1* (squares) and *fbh1*$^{L14A/P15A}$ *atf1*Δ pREP41*atf1* (triangles) as described in (C).

Similarly, Atf1-11M protein was also detected in Fbh1-3Pk immunoprecipitates, but the interaction was not lost upon stress (Figure 4B, lanes 15 and 16). The Fbh1 immunoprecipitates also contained Skp1 (Figure 4B, lanes 11–16), but unlike Fbh1-Atf1, Skp1 and Fbh1 still interacted upon stress. Further evidence that Atf1 phosphorylation by Sty1 is sufficient for inhibiting its binding to Fbh1 comes from ectopic activation of Sty1 in the absence of stress by use of either the constitutively active allele of the mitogen-activated protein kinase kinase (MAPKK) for Sty1 (*wis1-DD* [17]) or loss of the Pyp1 phosphatase [18]. In either case, levels of Atf1 are increased while the binding between Atf1 and Fbh1 is abrogated (Figure S7).

Atf1 has recently been shown to be phosphorylated by the Pmk1 MAPK in response to cell wall damage [19]. The effects that we observe here upon osmotic and oxidative stress appear to be mediated entirely through Sty1-mediated phosphorylation, given that Atf1 phosphorylation remains unchanged in *pmk1*Δ under such conditions, whereas Atf1 phosphorylation is lost in *sty1*Δ (Figure S8; [4]).

In summary, Atf1 and Fbh1 physically interact; this binding is negatively regulated upon stress through Sty1-mediated hyperphosphorylation of Atf1. This negative regulation ensures that Atf1 accumulates such that a robust response to stress can be mounted.

Discussion

To the best of our knowledge, this is the first in vivo evidence to demonstrate that substrate phosphorylation can negatively regulate its interaction with an F box protein. Most F box protein-substrate interactions described to date are mediated by phosphorylation. However, unlike Fbh1-Atf1, these interactions are mediated positively by phosphorylation [5]. We suggest that the mechanism described here might apply to

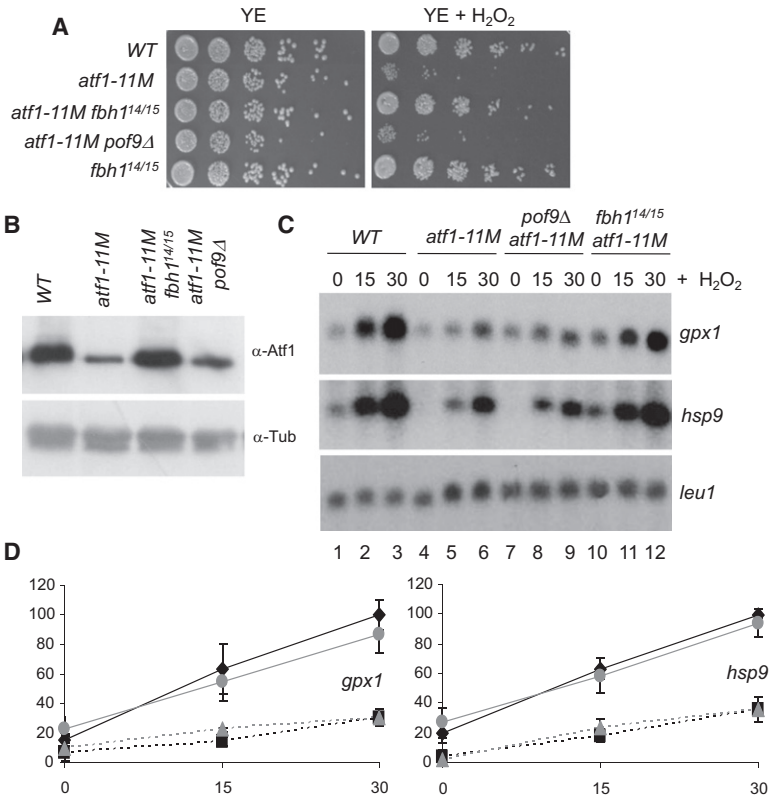

Figure 3. The Defects Associated with the *atf1-11M* Mutant Are Suppressed by the *fbh1*$^{L14A/P15A}$ Allele

(A) The H_2O_2 sensitivity of the *atf1-11M* mutant is suppressed by the *fbh1*$^{L14A/P15A}$ mutation (designated *fbh1*$^{14/15}$ in this figure). Cells taken from exponentially growing cultures of wild-type, *atf1-11M*, *atf1-11M fbh1*$^{L14A/P15A}$, *atf1-11M pof9*Δ, and *fbh1*$^{L14A/P15A}$ were spotted onto YE plates with and without 2 mM H_2O_2 and were incubated at 30°C for 4 days before being photographed.

(B) Levels of the Atf1-11M protein are restored by the *fbh1*$^{L14A/P15A}$ allele. Protein samples were prepared from wild-type, *atf1-11M*, *atf1-11M fbh1*$^{L14A/P15A}$, and *atf1-11M pof9*Δ cells grown to mid-log phase in YE at 30°C and were examined by immunoblotting.

(C) *atf1*-dependent gene transcription in *atf1-11M* and *atf1-11M fbh1*$^{L14A/P15A}$ cells. Wild-type, *atf1-11M*, *atf1-11M fbh1*$^{L14A/P15A}$, and *atf1-11M pof9*Δ cells were grown to mid-log phase in YE and stressed with 2 mM H_2O_2 for the times indicated (min). Total RNA was extracted and examined by northern blotting with DNA probes specific to the stress-induced genes *gpx1* and *hsp9* and the constitutively expressed *leu1* gene.

(D) Relative intensities of *gpx1* and *hsp9* were calculated by using the relevant loading control sample (*leu1*) at each time point for wild-type (black diamonds), *atf1-11M* (black squares), *atf1-11M fbh1*$^{L14A/P15A}$ (gray circles), and *atf1-11M pof9*Δ (gray triangles). The band with the highest intensity was designated as 100%, and the percentage intensities of the remaining bands were calculated relative to this. The *atf1-11M* allele is C-terminally epitope tagged with HA and 6His; thus, the "wild-type" strain shown here has the wild-type copy of *atf1* tagged in the same way. The results represent three independent experiments and are shown with standard deviation.

other F box proteins. Indeed, we note that budding yeast Sic1 shows reduced binding to the F box protein Cdc4 in a two-hybrid assay when threonine 173 is mutated to glutamate [20]. In addition to the in vivo interaction, we also detected an interaction between a region of recombinant Fbh1 and Atf1 in vitro (unpublished data).

In the absence of stress, Atf1 is basally phosphorylated by Sty1 [4]; Atf1 and Fbh1 interact, and Atf1 is constitutively turned over in an Fbh1-dependent manner. Upon stress, Atf1 is hyperphosphorylated at MAPK sites (Figure S9) by Sty1, which disrupts the interaction between Atf1 and Fbh1 (Figure 4C). This could be due to an accumulation of phosphorylated residues and thus potentially a graded response or alternatively dependent upon modification of specific sites. The Atf1-11M phosphorylation-site mutant is turned over more rapidly compared to wild-type Atf1 under nonstressed conditions [4], perhaps because of a conformational change resulting from a lack of basal phosphorylation. Alternatively, in the complete absence of phosphorylation, more than one degron may be available for targeting by the SCF^{Fbh1}, resulting in increased turnover (Figure 4C).

Atf1 is the first example of a substrate for any SCF^{Fbh1} complex. Interestingly, the mammalian homolog of Atf1, namely ATF-2, is also a target for ubiquitin-mediated degradation, which is prevented by phosphorylation at its N terminus [21]. Given our data, it is an intriguing possibility that human Fbh1 might target ATF-2 for degradation. In addition to Atf1, it seems likely that Fbh1, in common with other F box proteins, will direct multiple targets for ubiquitination via the SCF^{Fbh1}. Potential substrates are proteins involved in the homologous recombination pathway of DNA repair, because the F box mutant displays phenotypes associated with defective repair [13, 22]. It has been suggested that Fbh1 may promote degradation of such targets in order to suppress recombination [13]. It seems possible that, in this case, both the F box function and DNA helicase activity of Fbh1 may be required. In contrast, for Atf1, we found that only the F box motif plays a role in controlling Atf1 degradation, thus showing that, for this substrate at least, the two activities are separable.

In addition to the control of Atf1 by Fbh1, we also noticed that some reciprocal regulation might occur. Specifically, we noticed that the levels of Fbh1 varied according to the amount of Atf1 (Figure 4B). We find that this is mediated at the mRNA level (unpublished data) and thus could constitute a negative feedback mechanism that prevents an inappropriate accumulation of Atf1 in the absence of stress.

In summary, we have uncovered a novel mode of interaction between an F box protein and substrate; we propose that the mechanism of substrate interaction described here may serve to regulate other SCF-mediated events.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, one table, and nine figures and can be found online at http://www.cell.com/current-biology/supplemental/S0960-9822(09)01754-0.

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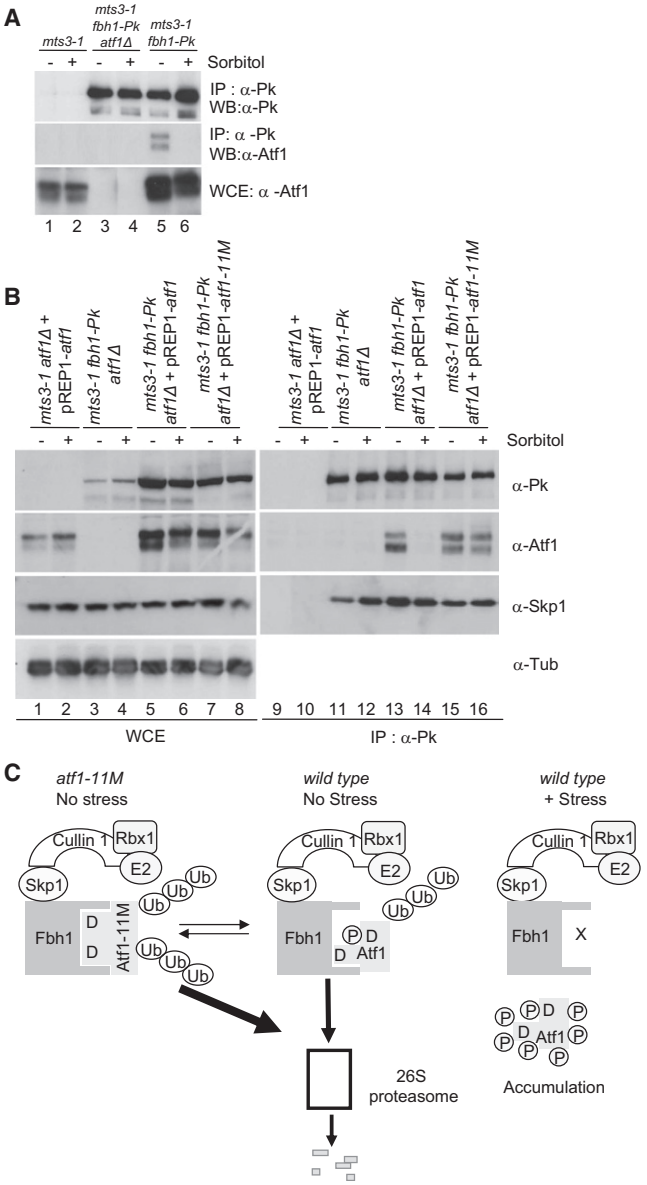

Figure 4. Binding between Atf1 and Fbh1 Is Negatively Regulated by Stress-Induced Phosphorylation

(A) Atf1 binds to Fbh1, but not upon exposure to stress. Immunoprecipitation was performed with anti-V5 agarose with protein extracts prepared from the indicated strains grown to mid-log phase in YE at 25°C and then shifted to 36°C for 3 hr. Cells were stressed by the addition of 1 M sorbitol for 20 min. Whole-cell extracts and immunoprecipitated proteins were analyzed by immunoblotting (WB).

(B) Stress-induced phosphorylation of Atf1 regulates binding to Fbh1. Immunoprecipitation was performed with anti-V5 agarose, with protein extracts prepared from the indicated strains grown as described in (A). Cells were stressed by the addition of 1 M sorbitol for 20 min. Whole-cell extracts and immunoprecipitated proteins were analyzed as described in (A).

(C) Model for the regulation of Atf1 by phosphorylation. See Discussion for details. Thicker arrow indicates decreased stability of Atf1-11 M compared to the wild-type Atf1 protein. D represents putative degron.

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