The SNF1 Kinase Ubiquitin-associated Domain Restrains Its Activation, Activity, and the Yeast Life Span**

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Background: The UBA domain in the AMP kinase family is poorly defined.
Results: This motif restrains kinase activity, resulting in decreased life span and oxidative stress resistance.
Conclusion: This inhibitory domain has defined influences with FOXOs on stress and aging.
Significance: The overactive kinase created by UBA mutations has positive stress resistance and aging influences that may translate to human metabolic benefits.

The enzyme family of heterotrimeric AMP-dependent protein kinases is activated upon low energy states, conferring a switch toward energy-conserving metabolic pathways through immediate kinase actions on enzyme targets and delayed alterations in gene expression through its nuclear relocalization. This family is evolutionarily conserved, including the presence of a ubiquitin-associated (UBA) motif in most catalytic subunits. The potential for the UBA domain to promote protein associations or direct subcellular location, as seen in other UBA-containing proteins, led us to query whether the UBA domain within the yeast AMP-dependent protein kinase ortholog, SNF1 kinase, was important in these aspects of its regulation. Here, we demonstrate that conserved UBA motif mutations significantly alter SNF1 kinase activation and biological activity, including enhanced allosteric subunit associations and increased oxidative stress resistance and life span. Significantly, the enhanced UBA-dependent longevity and oxidative stress response are at least partially dependent on the Fkh1 and Fkh2 stress response transcription factors, which in turn are shown to influence Snf1 gene expression.

The ability to shift between feasting and fasting is a central and essential metabolic shift that impacts biology at every level (1). Abundant nutrients can trigger reproductive readiness in multicellular organisms and promote cell growth and division in single-celled organisms by switching from catabolic to anabolic metabolic pathways through simple flipping of on and off switches (2). One switch that is active under times of energy depletion, such as during fasting, exercise, or oxygen depletion, is the nonhormonal central energy sensor and regulator known as the AMP-dependent protein kinase (AMPK)‡ (3–5) and its yeast ortholog SNF1 kinase (2, 6). All AMPK family members share distinct evolutionary homology-transcending primary sequences, tertiary protein folding, and quaternary protein associations of the heterotrimeric kinase complex (7–9). The yeast SNF1 kinase contains the catalytic Snf1 (α) subunit, the regulatory Snf4 (γ) subunit, and one of three β subunits Sip1, Sip2, or Gal83; the β subunits bridge the α and γ subunits in the complex and individually direct the subcellular location of the kinase (10, 11). The SNF1 kinase is important for the yeast stress response (12) and facilitates adaptation to glucose limitation, regulating the activity of metabolic enzymes and the transcription of metabolic genes. In addition to metabolic effects, this family of kinases has also been found to protect against oxidative stress and to impact the life span of model systems (13–16). As an enzyme complex that must efficiently respond to metabolic signals, its activity incorporates the rapid and reversible phosphorylation of its catalytic α subunit by well defined upstream kinases (Pak1, Elm1, and Tos3) (17) and phosphatases, with a major contributor being protein phosphatase I, Glc7-Reg1 (18–21). Further layers of regulatory finesse include the reversible physical allosteric association between the α and γ regulatory subunits and the nuclear translocation of the active trimeric complex to alter gene expression that aims to preserve the energy balance of the cell (10, 20, 22). Also conserved is acetylation and ubiquitination, recently reported for both mammalian AMPK and yeast SNF1 kinase complexes (23–26), that appear to have inhibitory roles in regulation of the kinase. Similarly, SUMOylation has been shown to be a negative regulator of Snf1 (27), clearly adding to the complexity of regulation of this central energy-sensing switch.

The current epidemic of lifestyle-related obesity, insulin resistance, and type 2 diabetes is overwhelming healthcare systems and budgets. The recommended lifestyle changes all stimulate AMPK; exercise, caloric restriction/weight loss, and muscle contraction all activate human AMPK with clinical metabolic benefits of improved glucose control and lowered cholesterol (28–30). In addition, a first line agent to treat type 2 diabetes, metformin, indirectly results in AMPK activation (31–33) as well as newer evidence of direct metformin influences on AMPK (33). Therefore, it is of great interest to understand how AMPK is regulated to gain insight into how this might be used to improve clinical health.
The catalytic subunits of the AMPK and AMPK-like family share very strong domain structure between all eukaryotes, including the yeast Snf1 (8, 34, 35). Relevant to this report is the presence within the majority of Snf1 subunits of a consensus motif for a ubiquitin associated domain, or UBA (20, 36), immediately adjacent to the catalytic domain. UBA domains are loose domains, including the yeast Snf1, that bind to ubiquitin (Ub) independently into stable surface structures (37). Their role continues to be clarified but includes the ability to bind to ubiquitin (Ub) in a linkage-specific (class 1, Lys-48-Ub4, and class 2, Lys-63-Ub4) or nonselective (class 4) manner via noncovalent associations between the hydrophobic UBA protein face and the globular head of the Ub moiety. Ub-binding UBA domains contribute to target proteolysis (Lys-48 linkages) or to cell trafficking (Lys-63), yet 25% of budding yeast proteins with UBA domains, including the yeast Snf1, have not been shown to bind ubiquitin of any linkage type, defined as class 3 (38). The importance of the UBA domain to Snf1 kinase activity and activation in yeast may clarify the importance of the role of ubiquitin in regulating yeast and higher eukaryotic AMPK. To date, complete deletion and point mutations within the yeast UBA domain resulted in a modest activating effect under both repressive and activating conditions (20). We report here the influence of mutations within conserved residues of the Snf1 UBA domain on the activity, activation, and allosteric associations between the Snf1 and the Snf4 subunits of the yeast Snf1 kinase, in addition to its influence on life span and oxidative stress resistance.

**Experimental Procedures**

**Creation of Snf1UBA Constructs**

Snf1UBA-GFP: Genomic Snf1 α Subunit with UBA Mutations and C-terminal GFP Tag—Two point mutations (G357A and G357V) in the Snf1UBA-HA presence were confirmed by sequencing. Snf1UBA-HA: 2-μm Plasmid Expressing Snf1 α Subunit with UBA Mutations and C-terminal HA Tag—YTH1510 was doubly transformed with both the linearized HA-tagged Snf1 plasmid (2 μm, TRP +, a kind gift from M. Schmidt (39)) with an internal SNF1 sequence deletion (BglII-NcoI digest) and the amplified linear PCR product containing full-length Snf1UBA coding sequence. Yeast plasmid and genomic DNA were retrieved (40) from TRP + colonies and transformed into XL-1 Escherichia coli to select for recircularized AMP + plasmids.

**Creation of FKH and SNF1 Deletion Strains**

All strains were based on S288c. Individual cassettes for snf1::kanMX6, fkh1::kanMX6, and fkh2::kanMX6 were amplified with 500 bp upstream and downstream. Integration was into YTH4269 with selection for Kanres and PCR confirmation. Combinations were created by crossing and tetrad dissection, scoring for markers, phenotypes, and confirmed by primer-specific amplification.

**Total Protein Extract and Western Blot Analysis**

Whole cell protein extracts from logarithmically growing cultures were prepared by standard bead beat protocol (42) in the presence of RIPA buffer, protease, and phosphatase inhibitors. Anti-phospho-AMPK (Cell Signaling), GFP (Covance), and HA (Roche Applied Science) antibodies were purchased,
and the chemiluminescent signal was captured on a VersaDoc molecular imager.

**Invertase Assay**

Yeast strains were grown to early log phase in 2%YPD (1% yeast extract, 2% peptone, and 2% glucose/dextrose). 1 \times 10^6 cells were moved to ice from the 2% glucose sample, based on absorbance (an A_{600} of 1 is approximately equivalent to 2 \times 10^7 cells). The remaining cells were washed and resuspended in YP 0.05% glucose for 2 h when 1 \times 10^6 cells were again removed as the low glucose, activated samples. Colorimetric measurement of invertase activity (glucose production from sucrose) was performed based on published protocols (18, 43, 44), with the following parameters. 50 \mu l of cell suspensions had 0.5 m sucrose (12.5 \mu l) added for 10 min at 37 °C, before stopping the reaction with K_2HPO_4 (75 \mu l). 500-\mu l Assay Mix (50 \mu l of 5000 units/ml glucose oxidase, 62.5 \mu l of 1 mg/ml peroxidase, and 375 \mu l of 10 mg/ml o-dianiside (suspended in 95% ethanol) into 25 ml 0.1 m potassium phosphate buffered to pH 7.0) was added to start the color reaction. After 20 min at 37 °C, the color was developed with 6N HCl (500 \mu l). A_{600} blanked to a no sucrose control gave values used to calculate invertase activity, reported as micromolars of glucose converted/min/10^6 cells. Activity was normalized (value of 1) to that of 2% glucose WT in each biological repeat. Statistical analysis was done using PRISM Version 6.0b software and two-way analysis of variance.

**Fluorescence Microscopy**

Fluorescence microscopy was used to determine the subcellular localization of Snf1-GFP and Snf1UBA-GFP. Logarithmically growing cultures were divided between nonactivating complete media (CM), with 2% glucose versus activating (CM 5% glycerol) conditions for 20 min. Live cells were moved to mounting medium containing DAPI (Sigma) for immediate fluorescence microscopy. Cells were viewed with an Olympus BX51 fluorescence microscope ×100 objective equipped with an Infinity 3–1 UM camera. Images were collected using Infinity Analyze software version 5.0. A minimum of 150 cells for each strain and condition was consecutively scored for colocalization of the GFP-tagged subunits and DAPI nuclear staining.

**Two-hybrid Analysis**

The yeast two-hybrid reporter strain (PJ69-4a, a gift from S. Fields) was doubly transformed with pairs of empty vectors (−ve control, pGAD-C2 and pGBDU-C2), the same backbones expressing unmodified Snf1 and Snf4 subunits (+ve control) or Snf1UBA and Snf4 or Reg1. 1 \times 10^6 cells of logarithmic cultures (in 3 \mu l volumes) of each of the three transformation sets were repeatedly spotted down the glucose gradient of the slant plates, grown at 30 °C until colonies were visible, and the image was scanned before and after color development. The glucose gradient slant plates involved sequential stacking and cooling of 20 ml of 2% glucose (bottom) and 0.05% glucose (top) media poured at ∼30° slant into Petri plates. Drop-out medium lacking leucine and uracil was used in both layers for dual plasmid maintenance. Plates were equilibrated overnight prior to use. Freshly prepared warm liquid X-Gal-agarose overlay medium (45) was layered to completely cover cells, solidified, and incubated at 30 °C, and images were scanned again after color development.

**Analysis of Life Span and Stress Resistance**

The snf1Δfkh1Δfkh2Δ strain was created by genetic crossing (snf1Δ (YTH1510) × fkh1Δ fkh2Δ (YTH2578)). Tetrad were picked and markers scored on G418 and YPSuc plates, with triple deletions confirmed by PCR. Oxidative stress was induced by exposure to hydrogen peroxide. The indicated strains were grown to late stationary phase in CM, with equal cell numbers left untreated or treated with 100 mm H_2O_2 for 60 min at 30 °C. All cells were washed and then plated on YPD for 2 days at 30 °C before scoring survival as a percentage compared with untreated. Replicative and chronological life span analyses were performed as described previously (46) using the indicated iso- genetic yeast strains.

**SNF1 and SNF4 Expression Analysis**

RNA was isolated (RNeasy, Qiagen) from logarithmically growing Snf1-GFP or fkh1Δ2Δ Snf1-GFP yeast strains that were transformed with empty vector (− , YEp24) or HA-pFkh1 or HA-pFkh2 (47) followed by reverse transcription. cDNA was used as template in amplification reactions, and equal reaction volumes were retrieved after the indicated number of cycles. Abundance was normalized to signal for ribosomal RNA at 26 cycles. VersaDoc quantitation was obtained from RedSafe nucleic acid stain signal (FroggaBio).

**Results**

**Generation of a Snf1 Construct with a Mutated UBA Domain—To gather a greater understanding of SNF1 kinase regulation and function in yeast, we focused on the role played by the UBA domain, a loose linear sequence present in yeast and mammalian AMPK-related kinase α subunits that fold into a conserved tri-helical structure characterized by a hydrophobic face (48). Such UBA surfaces can associate noncovalently with the globular head of ubiquitin or poly-Ub chains, yet this function has not yet been detected for the AMPK-related kinase class of human proteins (36) nor reported for the yeast α subunit, Snf1 (38). It has been recently published that mutations and deletions within the yeast Snf1 UBA sequence led to modest increases in phosphorylation of the SNF1 kinase itself and of an in vitro target, suggesting a natural inhibitory role of the UBA domain under normal conditions (20). The two amino acids selected for conservative replacement in our studies (Gly-357 and Leu-361) were based on their high degree of conservation within UBA motifs in general (38), as well as within the AMPK family specifically (36).

We replaced two highly conserved amino acid residues within the UBA domain of Snf1 (Snf1UBA) that spans amino acids 348–389 (Fig. 1A). The mutated Gly-357 (∗, G357A) and Leu-367 (∗, L367) (Fig. 1A) residues are adjacent on the hydrophobic face of the crystalized UBA motif protein (36) and are present in the majority of AMPK-like kinases (Fig. 1A). A striking exception is the absence of a convincing UBA domain consensus in human α subunits AMPKα1/α2 that lacks Gly-357 yet retains the Leu-367 residue. The Gly-357 residue was independently mutated in the human AMPK-like study (36), and different conserved mutations were previously introduced within yeast Snf1 (20).
Activation of the Snf1UBA Mutant Is Greater than Wild Type—Endogenous unmodified Snf1-GFP and the mutant Snf1UBA-GFP were expressed at similar levels and grew equivalently on sucrose, a nonfermentable carbon source that requires SNF1 kinase function for utilization (Fig. 1, B and C). This is a phenotype of the snf1/H9004 mutant that led to its yeast nomenclature (sucrose nonfermenting). An HA-tagged version of the same UBA mutation expressed from a high copy plasmid (Snf1UBA-HA) was similarly able to complement for growth on sucrose (data not shown). The relative activation of Snf1UBA was compared with wild type by assessing the following: (i) activating phosphorylation of Snf1 Thr-210(Thr(P)-210); (ii) nuclear import efficacy under activating conditions; and (iii) levels of allosteric subunit associations between the Snf1/H9251(Snf1) and Snf4/H9253 subunits. Activating conditions (0.05% low glucose) revealed a pervasive increase in the Thr(P)-210 phosphorylation of Snf1 UBA over Snf1 under both activating and repressive conditions for the endogenous (Fig. 2 A) and plasmid-expressed (Fig. 2B) UBA versions. This trend toward activation is consistent with the outcome from unrelated mutations within the yeast UBA consensus that included both a full UBA deletion (residues 347–398) as well as the combined mutation of Met-356/Tyr-358 or Leu-385 alone (20).

Activated SNF1 kinase relocates to the nucleus rapidly after stimulating conditions are introduced (11). The impact of UBA motif mutations on this mechanism was tested by making use of the Snf1-GFP and Snf1UBA-GFP fusion constructs and fluorescence microscopy. The Snf1-GFP subunit was efficiently shuttled to the nucleus within 20 min following stimulation (82%), as was the case with Snf1UBA-GFP (90%) (Fig. 2, C and D). The greater degree of phosphorylation observed for Snf1UBA-GFP under repressive conditions (2% glucose) does not result in a detectable increase in nuclear Snf1UBA localization, demonstrating that the phosphorylated Snf1UBA remains appropriately cytosolic under these conditions (Fig. 2D). It remains possible that Snf1UBA was shuttled at an increased rate over that of Snf1, which is beyond our capability to detect.

Activation of yeast and AMPK trimeric complexes also involves the allosteric juxtapositioning of the Snf1-Snf4 subunits, and the strength of their associations can be measured using the yeast two-hybrid system (41); β-galactosidase production correlates with the strength of the associations between the two proteins tested. Increased two-hybrid associations were observed between Snf4 and the Snf1UBA at all glucose concentrations, and they visibly exceeded the maximum associations detected between unmodified Snf1 and Snf4 (Fig. 2E). Together, these data provide evidence that the wild type UBA domain functions to inhibit SNF1 kinase activation at least in part by hindering α-γ associations.

Snf1UBA Mutant Activity Is Greater than Wild Type—Next, we analyzed the effect of the UBA mutation on SNF1 kinase enzymatic activity by indirectly measuring its ability to target and phosphorylate the transcriptional repressor Mig1 (49) as compared with unmodified Snf1. Upon activation, phosphorylated and the nucleus-shuttled Snf1 targets Mig1 for phosphorylation to release glucose-dependent repression of SUC2,
which encodes the invertase enzyme. Once active, invertase catalyzes the quantifiable biochemical conversion of sucrose to glucose and fructose (49). We show that Snf1UBA-GFP cells exhibit increased invertase activity most notably under activating conditions compared with unmodified Snf1-GFP (Fig. 3A).

This is correlated with a detectable increase in the overall intensity of the Mig1ph signal (Fig. 3B), whereas the associated Mig1ph “supershift” is only seen in low glucose.

We then measured the impact of the mutated UBA motif on the yeast replicative life span (RLS) to determine whether this homeostatic end point is also affected by Snf1 kinase activity. RLS measures the mitotic capacity of yeast cells by determining the number of daughter cells a single mother can produce (50). We previously demonstrated that Snf1 plays a role in extending yeast RLS via Mig1 inhibition (16), and others have shown that RLS is reduced when the Snf1 kinase Sip1αβ subunit is deleted (15, 51). Here, we show that under minor nutrient stress conditions (growth on 2% glucose dropout media), snf1Δ cells have an obvious decrease in RLS when compared with plasmid-borne Snf1-HA (16 versus 19 generations; Fig. 3C). However, when Snf1UBA-HA was expressed from the same high copy plasmid in snf1Δ cells, mean RLS was considerably increased beyond wild type levels to 27 generations.

We repeated the RLS experiment on CM, which induces a similar nutrient stress, using strains harboring endogenous SNF1UBA-GFP and SNF1-GFP alleles to avoid copy number bias. Our results demonstrate that the UBA mutation, under the control of the endogenous promoter, continues to confer extended mitotic longevity (Fig. 4A). Thus, increased SNF1 kinase activity due to the UBA mutation increases RLS regardless of its source of expression. Our results indicate that the increased α-γ subunit associations, and nuclear transcriptional
activity (invertase), play an important role in extending mitotic longevity as measured by the RLS assay.

Increased Life Span Conferred by the UBA Mutation Is Partially Dependent on the Yeast Forkhead Transcription Proteins Fkh1 and Fkh2—In mammalian cells it is known that activated AMPK phosphorylates FOXO3A and results in increased FOXO-dependent transcriptional activity without altering the cellular location of FOXO3A (52). The FOXO family of forkhead transcription factors promotes resistance to oxidative stress, suppresses tumor development, and enhances longevity/life span (13, 53). We have recently shown that the yeast FOXO orthologs, the F-box proteins Fkh1 and Fkh2, are redundant stress-responsive transcription factors that extend both RLS and yeast chronological life span (CLS) when their activity is increased (46). Although RLS measures how long a cell can remain mitotically active, the CLS assay measures how long a population of cells can remain metabolically active once they have exited mitotic growth and entered stationary phase (54). Furthermore, combined disruption of both FKH1 and FKH2 was required to decrease CLS, and the double mutants no longer responded to caloric restriction, indicating that Fkh1 and Fkh2 are critical responders to caloric restriction, a metabolic stress that also results in AMPK/SNF1 kinase activation. To date, however, Snf1 has only been shown to interact with the Hcm1 Forkhead protein (55) and not Fkh1 or Fkh2.

To test whether increased Snf1UBA-dependent RLS requires an evolutionarily conserved interaction with yeast Fkh1 and Fkh2, we genomically introduced the Snf1UBA-GFP allele, which confers a long lived phenotype, into a fkh1Δ fkh2Δ strain. Cells lacking both FKH1 and FKH2 cannot be used for RLS experiments because of severe flocculence (data not shown; see Ref. 46). The Snf1UBA-GFP allele, however, reduced flocculence in fkh1Δ fkh2Δ cells enough so that the triple mutants could be used in these RLS studies. The results show that introducing the FKH1 and FKH2 deletions into the Snf1UBA-GFP strain reduced RLS to below WT levels (Fig. 4A). This provides the first evidence that Snf1 requires functional Fkh1 and/or Fkh2 proteins to regulate RLS.

A reciprocal life span experiment was performed to test whether Snf1 and the Fkh1/2 proteins work together. Snf1 was deleted within the fkh1Δ fkh2Δ strain to generate the triple deletion mutant. Because we could not perform RLS due to flocculence, we performed CLS experiments. A previous global CLS study (56) found that snf1Δ strains, as well as strains deleted for any of the individual SNF1 kinase subunits (γ subunit, snf4Δ; β subunits, sip1Δ, sip2Δ, and gal83Δ), all had reduced CLS. We confirm here that snf1Δ cells are short lived when measured by CLS (Fig. 4B). snf1Δ CLS was found to be similar to fkh1Δ fkh2Δ CLS. Importantly, the snf1Δ fkh1Δ fkh2Δ CLS is similarly short lived. This result suggests that the SNF1 kinase works in the same pathway as Fkh1 and Fkh2 to maintain normal CLS. Taken together, Fig. 4, A and B, suggests the Forkhead proteins work downstream of Snf1.

Enhanced Stress Resistance Conferred by the UBA Mutation Is Dependent on the Yeast Forkhead Transcription Proteins Fkh1 and Fkh2—In addition to life span, the forkhead and Snf1 cellular functions also intersect for stress survival. An evolutionarily conserved stress response network utilizing AMPK
The expression of Snf1UBA-GFP in the fkh1/2Δ strain alleviates the stress defect of the fkh1/2Δ strain alone or augments the stress resistance over that of unmodified Snf1. This suggests that in order for the Snf1UBA strain to respond to oxidative stress, a functional Forkhead protein response system is paramount. Consistent with the findings for RLS, our data supports the
idea that Snf1 activates the Forkhead transcription factors in times of stress.

**Snf1 UBA Motif Impacts Life Span**

**Fkh1/2 Transcription Factors Are Required for SNF1 Expression**—Loss of Fkh1/2 resulted in an inability of the overactive Snf1UBA derivative to complement for oxidative stress resistance (Fig. 4C). The *Drosophila* Daf16/FOXO was recently reported to directly regulate the expression of an atypical AMPK-like subunit (60), and Fkh1 and Fkh2 affect the transcription of numerous yeast genes (61), leading us to ask whether the loss of Fkh1/2 activity resulted in decreased transcription of SNF1 kinase subunits. Fig. 5A shows that the total protein abundance of Snf1 UBA-GFP was decreased in the absence of Fkh1/2 and that overexpression of either forkhead protein from a plasmid partially restored Snf1 protein levels (Fig. 5B). This observation was mirrored at the transcriptional level where **SNF1** expression was decreased by the combined disruption of Fkh1/2 (Fig. 5, C and D), and subsequent Fkh1 or Fkh2 overexpression from a high copy plasmid partially restored **SNF1** expression toward baseline, with Fkh1 showing a greater induction of **SNF1** expression than Fkh2. These results were normalized to the rRNA signal to account for the differences within the isogenic strains tested (Fig. 5E). **SNF4** expression was negligibly impacted and was not influenced by **FKH1** or **FKH2** overexpression from a plasmid (Fig. 5, D and E).

**Mutations to the UBA Domain Do Not Impact Reg1 Interactions with Snf1**—Snf1 activation was increased overall and less repressed by high glucose levels upon introduction of conserved changes to the yeast UBA sequence (Fig. 2A and B), and the same sequence modifications enhanced Snf1-Snf4 allosteric associations even under repressive glucose levels (Fig. 2E). These defects are strikingly similar to those seen by disruption of the PP1 phosphatase activity that reverses the SNF1 kinase activating Thr-210 phosphorylation, specifically in **reg1Δ** strains (21). The PP1 regulatory subunit, Reg1, binds directly to Snf1 in a glucose-responsive manner, and **reg1Δ** strains are constitutively active for **SNF1** kinase, independent of glucose abundance (62, 63). To the best of our knowledge, the portion of the Snf1 subunit that Reg1 binds to has been delineated to the N-terminal catalytic domain of Snf1 and not pinpointed further (63). The Snf1 UBA motif is C-terminal to this, making a direct

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**FIGURE 5. ** **SNF1** expression requires Fkh1 and Fkh2. **A,** Western blot (WB) of whole cell lysates from logarithmically growing isogenic yeast strains in 2% glucose probed for GFP and tubulin protein abundance. **B,** isogenic strains with genomic Snf1 variants were transformed with high copy HA-tagged Fkh1 or HA-Fkh2 plasmids. Cells were grown in 2% glucose drop-out media. C, reverse transcription PCR analysis of **SNF1**, **SNF4**, **TUB1**, and rRNA transcript abundance of isogenic strains with, or disrupted for, **FKH1** and **FKH2** and with or without plasmid overexpression of HA-Fkh1 or Fkh2. −200-bp products for all. D, representative agarose gel of RT-PCR analysis of **SNF1**, **SNF4**, **TUB1**, and rRNA at 26 cycles from the strains indicated. E, VersaDoc quantitation of DNA signal at 26 PCR cycles (RedSafe; FroggaBio), normalized to rRNA expression for each condition. Mean ± S.D. of three biological repeats.
disruption to Reg1-Snf1 associations unlikely. However, indirect effects mediated through UBA sequence alterations affecting the binding of an intermediary (such as Sip5, which is reported to bind both Snf1 and Reg1 (64)) or by inducing structural changes within Snf1 may be detected as defects in Reg1-Snf1 associations. Two-hybrid analysis was used to compare Snf1-Reg1 and Snf1UBA-Reg1 interactions throughout a glucose gradient and demonstrated an indistinguishable intensity of color development (Fig. 6). The Snf1-Reg1 and Snf1UBA-Reg1 interactions exhibited the expected increase in association as the glucose concentrations dropped to 0.05% and are significantly lower than the positive control (Snf1-Snf4) in this assay. Because of the weak Reg1-Snf1 interactions observed even at the lowest glucose levels, subtle disruptions may not have been detected.

Taken together, the results presented here indicate that the natural UBA domain functions as a negative influence on SNF1 kinase activation and activity in part by influencing steady state phosphorylation and Snf1-Snf4 subunit interactions. The critical readout of the impact of the UBA mutation is seen in increased stress resistance and replicative life span. Our study shows that the independently described role for Snf1 and the Forkheads in life span and stress response may be due to actions within the same or overlapping pathways, where Forkheads act upstream of Snf1 to promote Snf1 gene expression, while completing a positive feedback loop by facilitating SNF1 kinase biological functions.

Discussion

Given the strong proclivity for regulatory mechanisms of SNF1 kinase/AMPK control to be conserved between yeast and higher eukaryotes, and the potential importance of the UBA domain in providing an additional layer of regulatory sophistication of the enzyme, we set out to further characterize the in vivo role of the UBA domain in the regulation of the SNF1 kinase. Here, evidence is presented confirming an endogenous regulatory role for the yeast UBA consensus in SNF1 kinase activation and activity, as well as a complex genetic and functional interaction with the Forkhead proteins Fkh1 and Fkh2.

The extensive presence of a loosely conserved UBA domain with the AMPK family catalytic α subunits opens the possibility of a regulatory role for this motif. Although UBA domains have the potential to recruit ubiquitinated proteins to their hydrophobic motif and thereby affect their activity and activation, this appears to be variable. Specifically, the yeast SNF1 kinase UBA domain, like other class 3 yeast UBA domains, has not been shown to bind to mono- or poly-Ub (38), an activity also not detected for the UBA domains in human AMPK-related kinases (36). Nonetheless, mutations to the α subunit UBA domains in yeast Snf1 and human AMPK-related proteins did affect activity, albeit reportedly in opposing directions. In Saccharomyces cerevisiae, it was found that complete deletions and selected mutations of the yeast Snf1 UBA motif did not impair phosphorylation or activation of the yeast SNF1 kinase; rather, there was a subtle increase in both (20). In contrast, the UBA domain was necessary for full activity when studied in AMPK-related protein kinases (36). This AMPK study mutated conserved consensus amino acids or completely deleted the UBA motif and found that this prevented phosphorylation and activation of the immunopurified α subunit fragments when analyzed in vitro, suggesting an activating function to this motif under normal conditions. It is possible, however, that the manipulation, truncation, and expression levels in this mammalian system were not representative of in vivo responses to activating signals.

UBA Domain Dampens SNF1 Kinase Activation through Modified Allosteric Associations—Replacement of two highly conserved amino acids within the Snf1 UBA sequence revealed a positive effect on the activity of the SNF1 kinase. Our investigations confirm and extend the observations that UBA sequence mutations positively effect the activation of the yeast kinase, shown specifically here to be through an enhancement of Snf1-Snf4 subunit associations, proportionately under both repressive and activating states (Fig. 2E) with reproducible enhancements of Thr(P)-210 and maintenance of efficient nuclear import (Fig. 2, A–D). Stimulatory conditions have been well documented to promote allosteric associations between these subunits (65–67), and this work indicates that the inherent function of the natural UBA domain may be to regulate SNF1 kinase activation by restraining α-γ associations. The mechanism underlying this modulation of association remains to be determined, yet a glucose-responsive association of a peripheral protein(s) with the hydrophobic face of the UBA domain may introduce steric hindrance between the α-γ subunits, thereby preventing full activation. A direct test of the physical associations between the PP1 regulatory subunit, Reg1, and Snf1 did not demonstrate a disruption by the conserved mutations within the UBA domain, as could have been predicted by the Snf1UBA phenotype (Fig. 6).

UBA Domain Dampens SNF1 Kinase Nuclear Activity—In addition to looking at the activation of SNF1 kinase, we also looked at UBA influences on activity. The activated SNF1 kinase targets nuclear transcriptional repressors to facilitate adaptation to non-glucose carbohydrate sources, such as the lifting of SUC2 gene repression and subsequent production of...
**SNF1 Kinase UBA Motif Influences Life Span**

invertase, an enzyme that cleaves sucrose into glucose and fructose (49). Invertase activity was increased over wild type by the presence of the Snf1UBA allele under activating conditions and less noticeably under repressive conditions, supporting an inhibitory role in controlling SNF1 kinase activity. This assay is a quantitative measure of SNF1 kinase enzymatic activity on its nuclear target, the transcriptional repressor Mig1. A qualitative increase in Mig1 phosphorylation signal intensity was observed in Snf1UBA cells under activating and repressive growth, yet the phosphorylation shift was limited to activating conditions only. Although there is a loss of glucose repression in the activation of SNF1 kinase, its nuclear activity remains well controlled, as seen by the maintenance of regulated nuclear import (Fig. 2, C and D). The observation that the Snf1UBA mutation did not effect normal cytosolic-nuclear partitioning is important, as it suggests that this regulatory step remains intact. This is emphasized by acknowledging that the strong allosteric association between subunits and the increased phosphorylation of Snf1UBA even under repressive conditions, do not translate to enhanced nuclear import in high glucose. Subsequently, measures of nuclear activity (invertase and Mig1ph) under glucose repression are therefore maintained, as observed (Fig. 3, A and B).

UBA Domain Influences Life Span in a Forkhead-dependent Mechanism—SNF1 kinase is known to be necessary for normal life span, as deletion of any subunit resulted in shortening of C. elegans (56). A striking effect of the UBA mutation is an enhanced RLS of S. cerevisiae beyond that of wild type. This effect was not diminished by lowering the copy number, as endogenous Snf1UBA-GFP expression levels (Fig. 4A) were as capable of promoting life span extension as high copy plasmid expression of Snf1UBA-HA (Fig. 3C). Performing the RLS assay on media with nutrient limitations revealed the need for Snf1 for RLS maintenance under stress conditions. This is in keeping with prior observations using yeast, worms, and mouse systems that nutrient limitations revealed the need for Snf1 for RLS maintenance. We demonstrate that although the introduction of the UBA mutations enhances both mitotic life span and oxidative stress resistance over unmodified Snf1, this cellular role is dependent on the presence of the Fkh1/2 proteins as Snf1UBA alone was not sufficient to overcome the fkh1Δ2Δ strain phenotypes. Taken together, a positive feedback loop can be envisioned. Initial stress recognition by the SNF1 kinase leads to the activation of the Forkheads that may be mediated by Hcm1. Once the Forkheads are active, they elicit a transcriptional stress response and longevity program, including the expression of SNF1, thus completing the loop.

Enhanced AMPK Activity Provides Health Benefits—There are several activating mutations within the AMPK/SNF1 kinase reported throughout the literature. A naturally occurring genetic mutation in pigs within the γ subunit yields lean muscular pigs prized by the food industry and is thought to be due to enhanced AMP binding (72). Dominant mutations within human AMPK α subunits have also demonstrated lowered % body fat (73) and lowered blood sugar and lipid levels (74). Furthermore, activation of AMPK by the oral antidiabetic drug metformin is currently in over 200 clinical trials of cancer therapy, indicating the world-wide interest in AMPK-dependent benefits to human health. Together, there is a clear clinical benefit to enhanced AMPK activity. Exercise and caloric restriction (with weight loss) alone are capable of transiently activating AMPK and yielding health gains, yet these treatment options are difficult to adhere to, as evidenced by the current epidemic of obesity and insulin resistance. Future identification of AMPK activators and repressors may yield new metabolic targets for many human conditions. UBA domain binding partners may play a key role in these approaches.

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**SNF1 Transcription Is Influenced by Yeast Forkhead Proteins**—A novel insight into one mechanism whereby Snf1 and Fkh1/2 mechanistically interact arose out of our observation that the Fkh1/2 combined deletion strain exhibited decreased abundance of the Snf1 protein. We have determined that this is due to loss of Fkh1/2-dependent SNF1 expression. SNF4 expression was not affected despite the analogous Daf16/Forkhead transcription factor in *Caenorhabditis elegans* contributing to the expression of the γ subunit of an atypical AMPK-related enzyme (60). In yeast, Fkh1 alone binds to the SNF1 promoter, consistent with its greater impact on returning SNF1 expression toward normal as compared with Fkh2 (Fig. 5E). Neither Fkh1 nor Fkh2 recognize the SNF4 promoter, and no impact on SNF4 expression was found when these proteins were overexpressed (Fig. 5E). However, the yeast forhead have complementary functions as combined deletion of Fkh1 and Fkh2 is required for aging and stress phenotypes (46); Fkh2 could therefore partially induce SNF1 expression by complementation. We demonstrate that although the introduction of the UBA mutations enhances both mitotic life span and oxidative stress resistance over unmodified Snf1, this cellular role is dependent on the presence of the Fkh1/2 proteins as Snf1UBA was not sufficient to overcome the fkh1Δ2Δ strain phenotypes. Taken together, a positive feedback loop can be envisioned. Initial stress recognition by the SNF1 kinase leads to the activation of the Forkheads that may be mediated by Hcm1. Once the Forkheads are active, they elicit a transcriptional stress response and longevity program, including the expression of SNF1, thus completing the loop.
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