Suppression of the HSF1-mediated proteotoxic stress response by the metabolic stress sensor AMPK

Siyuan Dai¹,‡, Zijian Tang¹,2,‡, Junyue Cao¹,‡, Wei Zhou¹,‡, Huawen Li¹, Stephen Sampson¹ & Chengkai Dai¹,*

Abstract
Numerous extrinsic and intrinsic insults trigger the HSF1-mediated proteotoxic stress response (PSR), an ancient transcriptional program that is essential to proteostasis and survival under such conditions. In contrast to its well-recognized mobilization by proteotoxic stress, little is known about how this powerful adaptive mechanism reacts to other stresses. Surprisingly, we discovered that metabolic stress suppresses the PSR. This suppression is largely mediated through the central metabolic sensor AMPK, which physically interacts with and phosphorylates HSF1 at Ser121. Through AMPK activation, metabolic stress represses HSF1, rendering cells vulnerable to proteotoxic stress. Conversely, proteotoxic stress inactivates AMPK and thereby interferes with the metabolic stress response. Importantly, metformin, a metabolic stressor and popular anti-diabetic drug, inactivates HSF1 and provokes proteotoxic stress within tumor cells, thereby impeding tumor growth. Thus, these findings uncover a novel interplay between the metabolic stress sensor AMPK and the proteotoxic stress sensor HSF1 that profoundly impacts stress resistance, proteostasis, and malignant growth.

Keywords AMPK; HSF1; metformin; proteostasis; tumorigenesis

Subject Categories Metabolism; Molecular Biology of Disease; Protein Biosynthesis & Quality Control

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Introduction
Organisms commonly encounter a wide variety of insults, both environmental and pathological. Many such insults, including heat shock, heavy-metal toxins, acidosis, ischemia/reperfusion, and oxidative damage, disturb cellular proteostasis and trigger the proteotoxic stress response (PSR), or heat shock response (Lindquist, 1986; Westerheide & Morimoto, 2005). This stress response is predominantly controlled by a transcription factor named heat shock factor 1 (HSF1) and is hallmarkmed by a drastic induction of heat shock proteins (HSPs) (Lindquist, 1986; Morimoto, 2008). HSPs are molecular chaperones, proteins that assist protein folding, trafficking, and degradation and thereby guard the proteome against the danger of misfolding and aggregation (Lindquist, 1986; Wolch, 1991). Thus, this stress response plays an essential role in preserving proteostasis and enhancing survival in the face of such stressors, antagonizes neurodegeneration, and prolongs normal lifespan (Hsu et al, 2003; Balch et al, 2008; Dai et al, 2012a; Kondo et al, 2013; Lin et al, 2013; Pierce et al, 2013). Contrasting sharply with these broadly acclaimed benefits, our and others’ recent studies have begun to reveal a surprising action of the HSF1-mediated stress response, whereby it is hijacked by malignant cells and facilitates oncogenesis (Dai et al, 2007, 2012a; Min et al, 2007; Meng et al, 2010; Jin et al, 2011; Scott et al, 2011).

Despite its evident importance in biology and human diseases, our understanding of the mechanisms through which this ancient adaptive stress response is regulated remains incomplete. In particular, little is known of how the PSR interacts with other cellular stress responses. One of such responses is the metabolic stress response that critically maintains cellular energy homeostasis. Metabolic stressors, through raising intracellular AMP/ATP ratios, instigate a low cellular energy state. By sensing increased AMP/ATP ratios, AMP-activated protein kinase (AMPK), a master metabolic regulator, becomes mobilized (Hardie, 2011). Mammalian AMPK is a protein complex comprising one catalytic α-subunit and two regulatory subunits: β and γ (Hardie, 2011). Upon activation, AMPK phosphorylates a wide array of downstream effectors to enhance ATP generation and reduce ATP expenditure (Hardie, 2011). This function of AMPK has proven crucial for cells to survive metabolic stress.

Herein, we report a novel interplay between the metabolic stress sensor AMPK and the proteotoxic stress sensor HSF1 and show that it has profound impacts on stress resistance, proteostasis, and tumor growth.

¹ The Jackson Laboratory, Bar Harbor, ME, USA
² Graduate Programs, Department of Molecular & Biomedical Sciences, The University of Maine, Orono, ME, USA
*Corresponding author: Tel: +1 207 288 6927; Fax: +1 207 288 6078; E-mail: Chengkai.Dai@jax.org
‡These authors contributed equally to this work

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Results

Metformin suppresses the HSF1-mediated PSR

In order to infer small molecules that potentially modulate the HSF1-mediated PSR, we queried the Connectivity Map (www.broadinstitute.org/cmap), a collection of gene expression profiles from human cells treated with bioactive molecules (Lamb et al., 2006), with a gene signature that was identified in human breast cancer cells following HSF1 knockdown (Santagata et al., 2013). As expected, perturbagens known to activate HSF1, including the proteasome inhibitor MG-262 and HSP90 inhibitors (alvespimycin, tanespimycin, and monorden), generate gene expression patterns opposite to the query HSF1-inactivation gene signature (Fig 1A). In contrast, two translation inhibitors, cephaline and emetine, generate expression patterns enriched for the query gene signature, congruent with their roles in suppressing HSF1 activation (Santagata et al., 2013). Intriguingly, metformin, a metabolic stressor and the most frequently prescribed medication for type II diabetes mellitus (T2D) worldwide (Dowling et al., 2011), also provokes gene expression changes concordant with HSF1 inactivation (Fig 1A).

Next, we experimentally verified the metformin-mediated suppression of the PSR by testing the ability of metformin to suppress heat shock-induced HSP expression. For HSP expression, we used an NIH3T3 reporter cell line that stably expresses a GFP-firefly luciferase fusion controlled by the human HSP70B (HSPA6) promoter (Dai et al., 2012a). As expected, heat shock-induced GFP expression in phosphate-buffered saline (PBS)-treated cells and metformin markedly blocked this induction (Fig 1B). Metformin also impaired induction of endogenous HSP72 and HSP25 proteins (Fig 1C), two classic stress-inducible HSPs. Congruent with transcriptional suppression by metformin, induction of Hsp72 (Hspa1a) and Hsp25 (Hspb1) mRNAs by heat shock was markedly impaired in metformin-treated cells compared to control cells (Fig 1D and E). While these notable effects were achieved using 10 mM metformin, a similar pattern of changes, albeit to a lesser extent (Supplementary Fig S1A and B), was also observed for 10 μM metformin, a concentration comparable to that found in the blood of T2D patients (Sum et al., 1992; Lalau et al., 2011; Dowling et al., 2012). Importantly, this defect in the PSR was not due to impaired cell viability following metformin treatment (Fig 1F).

To demonstrate impaired HSF1 activation, we directly examined HSF1–DNA binding. To accomplish this, we developed a new enzyme-linked immunosorbent assay (ELISA)-based technique to measure HSF1–DNA binding capability (Fig 1G, left panel). In this assay, nuclear HSF1 proteins competent for DNA binding are captured by ideal heat shock element (HSE) oligonucleotides that are pre-immobilized on a microtiter plate through biotin–avidin interactions. As expected, HSF1–DNA binding was markedly increased following heat shock (Supplementary Fig S1C); however, metformin evidently blocked this increase (Fig 1G, right panel).

While our new method is quantitative and high-throughput, it requires, similar to the prevailing chromatin IP (ChIP) technique, a large amount of DNA and provides only average values for a given cell population. To provide more detailed information on HSF1–DNA binding, we adapted the newly emerged proximity ligation assay (PLA) technique to visualize endogenous physical associations between HSF1 and genomic DNAs at the single-cell level. By means of two species-specific secondary antibodies conjugated with oligonucleotides, PLA converts protein–protein interactions in proximity into DNA signals (Clausson et al., 2011). We extended this technique to detect HSF1–DNA interactions in situ by utilizing a mouse anti-double-stranded DNA antibody in conjunction with a rabbit anti-HSF1 antibody (Fig 1H). The specificity of antibodies was validated by standard immunostaining. In HEK293T cells, the DNA antibody generated similar staining patterns with and without heat shock (Supplementary Fig S1D). This staining largely overlaid with that of the DNA dye DAPI, indicating a predominant DNA...
Figure 1.

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labeling irrespective of heat shock. Importantly, this staining was markedly reduced following DNase treatment (Supplementary Fig S1E), indicating specific DNA labeling. As a negative control, HEK293T cells stably expressing HSF1-targeting shRNAs did not display notable HSF1 staining (Supplementary Fig S1F). Congruently, no specific PLA signals were detected in these HSF1-deficient cells (Fig 1I, left panel). In cells expressing scramble shRNAs and without heat shock, only sporadic PLA signals, manifested as distinct nuclear foci, were observed (Fig 1I, right panel), indicating basal constitutive HSF1 activation. In contrast, heat shock greatly increased these signals, demonstrated by numerous bright nuclear foci (Fig 1I, right panel). Importantly, metformin potently blocked this increase (Fig 1I, right panel), demonstrating impaired in vivo HSF1–DNA binding by metformin.

HSF1 activation is a multi-step process, involving phosphorylation, nuclear translocation, and DNA binding. Next, we investigated whether metformin affected HSF1 nuclear translocation, a prerequisite for its DNA binding. As expected, heat shock caused most HSF1 proteins to translocate from the cytoplasm to the nucleus (Fig 1J). Importantly, metformin impeded this translocation (Fig 1J). Thus, metformin impairs HSF1–DNA binding at least in part through blockade of its nuclear translocation.

To determine whether metformin impairs the PSR at the organismal level, we utilized transgenic mice that express dual reporter genes, firefly luciferase and EGFP, both controlled by the mouse Hspa1l promoter (O’Connell-Rodwell et al., 2008). When analyzed with bioluminescence imaging, these mice enable noninvasive surveillance of the PSR in vivo. Under non-stress conditions, these reporter mice exhibited basal luciferase activities in mouth and limbs (Fig 1K; Supplementary Fig S1G). As expected, challenge with the proteasome inhibitor velcade, a potent proteotoxic stressor, markedly enhanced this luciferase activity (Fig 1K; Supplementary Fig S2F), indicating specific HSF1 induction by heat shock but also markedly blocked metformin-induced Hsp induction (Fig 1E and F), indicating the necessity of metformin for metformin-mediated HSF1 inactivation. The incomplete blockade of metformin effect was likely due to residual AMPK proteins (Fig 2D) and/or AMPK-independent mechanisms of metformin. To determine the sufficiency of AMPK activation for HSF1 suppression, we activated AMPK signaling through expression of a GST-tagged constitutively active mutant of the z1-subunit, AMPKzCA (Egan et al., 2011). AMPK activation was evidenced by elevated phosphorylation of RAPTOR, a known cellular substrate for AMPK (Gwinn et al., 2008), at Ser792 (Supplementary Fig S2E). Under both basal and heat shock conditions, this mutant impaired activation of the HSF1 reporter (Fig 2G), demonstrating that AMPK activation alone is sufficient to repress the transcriptional activity of HSF1. Together, these results indicate a negative regulation of the PSR by AMPK.

We next sought to elucidate the molecular mechanisms by which metformin inactivates HSF1. Through diminishment of mitochondrial ATP production, metformin activates AMPK, a key cellular sensor of metabolic stress (El-Mir et al., 2000). This activation is known to mediate numerous effects of metformin (Kahn et al., 2005). However, it has also been reported that metformin inhibits mTORC1 independently of AMPK (Kalender et al., 2010).

We first asked whether mTORC1 inhibition by metformin mediates HSF1 suppression. To address this, we examined induction of Hspa1l genes in cells treated with rapamycin, a specific mTOR inhibitor. Successful inhibition of mTORC1 by rapamycin was evidenced by a marked reduction of S6K1 phosphorylation (Supplementary Fig S2A). In stark contrast to metformin, in our experiments, rapamycin enhanced Hspa1l induction by heat shock (Supplementary Fig S2B and C), arguing against mTORC1 inhibition as a primary cause of HSF1 suppression by metformin. This result somewhat contrasts with a recent report, indicating that rapamycin suppressed HSF1 activation (Chou et al., 2012). Surprisingly, our results showed that whereas high doses of rapamycin suppressed HSF1, low doses activated HSF1 (unpublished observations), suggesting indirect regulation of HSF1 by mTORC1. As expected, metformin induced AMPK activation, evidenced by elevated Thr172 phosphorylation of AMPKz (Supplementary Fig S2A; Hawley et al., 1996). Moreover, two other mechanistically distinct AMPK activators, A-769662 and aminomidoazole carbamamide ribonucleotide (AICAR) (Corton et al., 1995; Göransson et al., 2007), also impaired the PSR (Fig 2A and B; Supplementary Fig S2D). A-769662 is a direct and specific AMPK activator, causing phosphorylation of the well-characterized AMPK substrate acetyl-CoA carboxylase (ACC) (Fig 2C; Göransson et al., 2007). These results suggest AMPK as a common mediator of HSF1 suppression.

To directly test this, we conditionally deleted the two Amppk isoforms, z1 and z2, in mouse embryonic fibroblasts (MEFs) through adenoviral Cre recombinase (Fig 2D). Compared to GFP transduction, Cre transduction in Amppkz1/z2 MEFs not only enhanced Hspa1l induction by heat shock but also markedly blocked metformin-induced Hspa1l suppression (Fig 2E and F), indicating the necessity of AMPK for metformin-mediated HSF1 inactivation. The incomplete blockade of metformin effect was likely due to residual AMPKz proteins (Fig 2D) and/or AMPK-independent mechanisms of metformin. To determine the sufficiency of AMPK activation for HSF1 suppression, we activated AMPK signaling through expression of a GST-tagged constitutively active mutant of the z1-subunit, AMPKzCA (Egan et al., 2011). AMPK activation was evidenced by elevated phosphorylation of RAPTOR, a known cellular substrate for AMPK (Gwinn et al., 2008), at Ser792 (Supplementary Fig S2E). Under both basal and heat shock conditions, this mutant impaired activation of the HSF1 reporter (Fig 2G), demonstrating that AMPK activation alone is sufficient to repress the transcriptional activity of HSF1. Together, these results indicate a negative regulation of the PSR by AMPK.

We reasoned that AMPK might directly phosphorylate HSF1. To test this, we first examined potential physical interactions between AMPK and HSF1 by co-immunoprecipitation (co-IP). As a positive control, while some endogenous ACC proteins were co-precipitated with endogenous AMPKz proteins in MEFs under basal conditions, metformin markedly enhanced this co-IP (Fig 2H). Importantly, in a similar pattern, endogenous HSF1 proteins were also co-precipitated with AMPKz (Fig 2H). In HEK293T cells, exogenous FLAG-HSF1 proteins were also co-precipitated with GST-AMPKzCA (Supplementary Fig S2F), indicating specific in vivo physical AMPK–HSF1 interactions. This interaction was then confirmed in situ by PLA. Using a rabbit antibody recognizing Thr172-phosphorylated AMPKz in conjunction with a mouse monoclonal anti-HSF1 antibody, we visualized endogenous AMPK–HSF1 interactions in Hsf1+/+ MEFs. Hsf1-/- MEFs served as negative controls. As expected, no specific PLA signals were observed in Hsf1-/- MEFs, irrespective of metformin treatment (Fig 2I). While only faint signals were detected in Hsf1+/+ cells treated with PBS, metformin treatment markedly augmented these signals (Fig 2I), indicating a specific and inducible interaction between endogenous HSF1 and AMPK proteins.

We next asked whether AMPK inactivates HSF1 through phosphorylation. The protein motif algorithm ScanSite (http://scansite.mit.edu/) predicted a potential phosphorylation site on HSF1 at serine 121 residue (Supplementary Fig S3A). To test this, we took advantage of a phosphorylation-specific antibody. The specificity of this antibody was validated using a phosphorylation-resistant mutant, HSF1S121A, in HSF1-deficient HEK293T cells. This antibody
Figure 2. AMPK suppresses HSF1 activation through physical interaction.

A–C Reporter cells were treated with and without 1 μM A-769662 for 3 h followed by heat shock at 43°C for 30 min and recovery at 37°C for 5 h. Hspa1a and Hspb1 mRNA levels were quantitated by qRT–PCR (mean ± SD, n = 3, Student’s t-test, ***P < 0.001). ACC phosphorylation was detected by immunoblotting (C).

D–F Primary MEFs were derived from Ampkα1fl/fl; Ampkα2fl/fl embryos and transduced with either adenoviral GFP or Cre to delete Ampkα1/2 in vitro. AMPKα levels were detected 4 days after transductions (D). Following viral transductions, Hsp mRNA levels were quantitated by qRT–PCR in MEFs with and without 10 mM metformin treatment overnight followed by 43°C heat shock for 30 min and recovery for 4 h (mean ± SD, n = 3, one-way ANOVA, n.s., not significant, *P < 0.05, **P < 0.01, ***P < 0.001).

G Transcriptional activities of HSF1 were measured by a dual reporter system consisting of two plasmids: the HSF1-dependent reporter pHSE-SEAP, in which ideal HSEs drive the expression of secreted alkaline phosphatase (SEAP), and the control reporter pCMV-Gaussia Luc, in which a CMV promoter drives the expression of secreted Gaussia luciferase. In HEK293T cells, either LacZ or GST-AMPKα1CA plasmid was co-transfected with the two reporter plasmids. After 24 h, the transfected cells were heat-shocked at 44°C for 45 min. The culture supernatants were collected to measure SEAP and luciferase activities 24 h after heat shock. SEAP signals were normalized to Gaussia luciferase signals (mean ± SD, n = 5, Student’s t-test, **P < 0.01, ***P < 0.001).

H, I Following treatment with 10 mM metformin overnight, endogenous AMPKα and HSF1 proteins were co-precipitated using anti-AMPKα agarose conjugates from lysates of immortalized Hsf1+/+ and Hsf1−/− MEFs (H). HC: heavy chain. Endogenous HSF1-AMPKα interactions in immortalized Hsf1+/+ and Hsf1−/− MEFs were visualized in situ by PLA (I). Experimental details are described in Materials and Methods. Scale bars 50 μm.

Source data are available online for this figure.
readily detected exogenously expressed HSF1 WT under basal conditions and metformin treatment enhanced this signal (Fig 3A). In contrast, the same antibody failed to detect exogenously expressed HSF1 S121A mutants under both basal and metformin treatment conditions (Fig 3A). Moreover, this antibody did not detect specific signals in Hsf1−/− MEFs (Supplementary Fig S3B). Importantly, both Ampkα1/2 deletion and Ampkα1/2 knockout largely blocked HSF1 Ser121 phosphorylation induced by metformin or A-769662 (Fig 3B; Supplementary Fig S3C), demonstrating the necessity of AMPK for this phosphorylation in vivo. Conversely, AMPKαCA was sufficient to induce Ser121 phosphorylation of HSF1 WT, but not HSF1 S121A (Fig 3C). Previously, it was reported that MAPK-activated protein kinase 2 (MK2) could phosphorylate the same site (Wang et al, 2006). However, MK2 knockdown failed to significantly block HSF1 Ser121 phosphorylation induced by metformin (Supplementary Fig S3D and E), indicating that MK2 is not important for this modification induced by metformin.

To address whether AMPK directly phosphorylates HSF1, we conducted in vitro kinase assays using immunoprecipitated AMPK complexes. Compared to IgG controls, precipitates of AMPKα antibodies weakly phosphorylated recombinant HSF1 proteins at Ser121 in the absence of AMP (Fig 3D). However, AMP markedly enhanced this phosphorylation and the AMPK inhibitor compound C blocked this event (Fig 3D; Zhou et al, 2001), indicating a specific action of AMPK for this phosphorylation. Western blotting also demonstrated the necessity of AMPK for this phosphorylation (Supplementary Fig S3E). Consistently, AMPKαCA expression did not reduce HSF1 WT levels (Supplementary Fig S3F), but also were refractory to AMPK-depletion of Ser121 phosphorylation induced by metformin (Supplementary Fig S3G). This reduction likely sufficed to induce Ser121 phosphorylation of HSF1 WT, but not readily detected exogenously expressed HSF1 WT under basal conditions and metformin treatment enhanced this signal (Fig 3A). In contrast, the same antibody failed to detect exogenously expressed HSF1 S121A mutants under both basal and metformin treatment conditions (Fig 3A).

In HEK293T cells that stably express an shRNA targeting the 3’ UTR of human HSF1, HSF1 WT or HSF1 S121A was expressed. Following treatment with 10 mM metformin overnight, the levels of HSF1 phosphorylation at Ser121 were detected by immunoblotting using a specific phospho-HSF1 Ser121 antibody (ABD01, Assay Biotechnology).

Primary Ampkα2/8, Ampkα2/6 MEFs were transduced with adenoviral GFP or Cre. Following treatment with 10 mM metformin overnight or treatment with 10 μM A-769662 for 3 h, HSF1 Ser121 phosphorylation was examined by immunoblotting.

HSF1 WT or HSF1 S121A was co-expressed with either Cre or GST-AMPKαCA in HSF1−/− HEK293T cells. HSF1 Ser121 phosphorylation was examined by immunoblotting.

AMPK complexes were immunoprecipitated from HEK293T cells treated with and without 10 mM metformin overnight. Aliquots of AMPK complexes were incubated with 400 ng purified recombinant His-tagged HSF1 proteins with and without 100 μM AMP or 20 μM compound C (CC). HSF1 Ser121 phosphorylation was detected by immunoblotting.

HSF1 activities were measured by the dual reporter system in HSF1−/− HEK293T cells. Either FLAG-HSF1 WT or FLAG-HSF1 S121A was co-expressed with LacZ or AMPKα1/2 (E). Following expression of HSF1 WT or HSF1 S121A, cells were treated with and without 10 mM metformin overnight (F) (mean ± SD, n = 5, one-way ANOVA, n.s., not significant, *p < 0.05, **p < 0.01, ***p < 0.001).

After transfection with FLAG-HSF1 WT or FLAG-HSF1 S121A plasmids for 3 days, HSF1−/− HEK293T cells were treated with and without 10 mM metformin overnight (H). Nuclear extracts were used to measure HSF1-DNA binding by the ELISA-based DNA binding assay using anti-FLAG antibodies (mean ± SD, n = 3, one-way ANOVA, n.s., not significant, *p < 0.05, **p < 0.01, ***p < 0.001).

Following transfections and metformin treatment as described in (G) and (H), cytoplasmic and nuclear proteins were extracted for immunoblotting using anti-FLAG antibodies. Source data are available online for this figure.

In response to metabolic stress, AMPK is mobilized to sustain cellular energy homeostasis and improve survival (Kahn et al, 2005). In light of the inactivation of HSF1 by AMPK that we discovered, we next asked whether metabolic stress impacts the cellular response to proteotoxic stress. To provoke metabolic stress, we applied either leucine or glucose deprivation to the NIH3T3 heat shock reporter cells described above. Similar to metformin treatment, deprivation of each nutrient suppressed GFP induction by heat shock (Fig 4A and B). This suppression was not due to impaired cell viability following nutrient deprivation (Supplementary Fig S4A and B). Mechanistically, nutrient deprivation impaired HSF1−DNA binding following heat shock (Fig 4C and D), thereby impairing HSP induction (Fig 4E and F). Congruent with diminished
Figure 3.
protein-chaperoning capacity following nutrient deprivations, protein Lys48 polyubiquitination, a key modification targeting proteins for proteasomal degradation and a surrogate indicator of protein misfolding (Pickart & Eddins, 2004), was aggravated under both basal and heat shock conditions (Fig 4E and F). In stark contrast, both nutrient deprivations induced expression of BIP/GRP78 (Fig 4E and F), a key endoplasmic reticulum (ER) chaperone and marker of the unfolded protein response (UPR), which responds to ER stress (Walter & Ron, 2011). Together, these results indicate that nutrient deprivations trigger the UPR but, intriguingly, suppress the PSR. As expected, both nutrient deprivations induced AMPKα Thr172 phosphorylation in NIH3T3 and HEK293 cells (Fig 4G and H; Supplementary Fig S4C and D). Congruent with HSF1 phosphorylation by AMPK, both nutrient deprivations also induced HSF1 Ser121 phosphorylation (Fig 4G and H; Supplementary Fig S4C and D). Of note, 4-h leucine starvation was required to induce evident AMPK phosphorylation (Fig 4G; Supplementary Fig S4C). These findings not only agree with HSF1 inactivation by AMPK (Fig 2), but also suggest AMPK activation as a common mechanism underlying the inhibitory effect of metabolic stress on the PSR.

Suppression of HSF1 activation by nutrient deprivations was further confirmed in HEK293 cells. Importantly, AMPKα1/2 knockdown not only elevated basal HSF1 activity but also reversed HSF1 suppression imposed by nutrient deprivations (Fig 4I and J). It has been known that AMPK activation inhibits mTORC1 (Gwinn et al., 2008). To determine whether mTORC1 inhibition following nutrient deprivations plays an important role in HSF1 inactivation, we measured HSF1 transcriptional activities under nutrient deprivations in the presence of high doses of rapamycin. While 500 nM rapamycin alone suppressed HSF1, combined nutrient deprivation further inactivated HSF1 (Supplementary Fig S4E), strongly suggesting that AMPK activation induced by nutrient deprivations is capable of suppressing HSF1 independently of mTORC1. Congruent with a direct regulatory mechanism, S121A mutation rendered HSF1 resistant to nutrient deprivation-mediated suppression (Fig 4K and L). Together, these results reveal that metabolic stress antagonizes the HSF1-mediated PSR, wherein AMPK activation plays an important role via phosphorylating HSF1 at Ser121.

**Proteotoxic stress suppresses AMPK**

In stark contrast to metabolic stress, our results revealed that heat shock markedly diminished AMPK Thr172 phosphorylation (Fig 5A and Supplementary Fig S5A). In parallel, HSF1 Ser121 phosphorylation was also reduced under heat shock (Fig 5A and Supplementary Fig S5A), suggesting AMPK inactivation. This was confirmed by in vitro kinase assays. Compared to AMPK complexes immunoprecipitated from cells without heat shock, complexes from heat-shocked cells exhibited markedly impaired capability to phosphorylate recombinant ACC1 proteins in vitro even in the presence of AMP (Fig 5B). Congruent with this defect, heat shock caused a specific reduction of γ2-subunit within immunoprecipitated AMPK complexes (Fig 5B). AMPKγ subunits play a critical role in binding AMP/ATP, and mutations in γ2-subunit are associated with cardiac hypertrophy (Blair et al., 2001; Hardie, 2011). To our surprise, 30-min heat shock sufficed to diminish total cellular γ2-subunits (Fig 5B). We reasoned that this reduction was likely due to protein instability. In support of this notion, an increase in γ2-subunit was detected in detergent-insoluble fractions of cellular protein extracts and a simultaneous decrease in this subunit was detected in detergent-soluble fractions (Fig 5C), suggesting oligomerization and/or aggregation of γ2-subunits under heat shock conditions. Thus, our results indicate that, at least through regulation of Thr172 phosphorylation of α-subunits and destabilization of γ-subunits, heat shock suppresses AMPK.

Given the pivotal role of AMPK in mediating the metabolic stress response, we were curious to know whether proteotoxic stress could interfere with cellular responses to metabolic stressors. Consistent with its inhibitory effect on AMPK, transient heat shock immediately proceeding nutrient deprivations significantly impaired induction of AMPK and ACC phosphorylation (Fig 5D and E), suggesting suppression of the metabolic stress response by heat shock.

We next asked whether attenuation of Ser121 phosphorylation during heat shock contributes to HSF1 activation. Compared to cells expressing HSF1WT, cells expressing HSF1S121A exhibited elevated HSP mRNAs, HSF1–DNA binding, and reporter activities under both basal and heat shock conditions (Fig 5F–H and Supplementary Fig S5B). Under basal conditions, expression levels of HSF1WT and HSF1S121A were comparable and, therefore, heightened activity of HSF1S121A was at least in part due to its enhanced nuclear translocation (Supplementary Fig S5C and D). Following heat shock, both HSF1WT and HSF1S121A proteins slightly increased (Supplementary Fig S5C). Interestingly, HSF1S121A mutants increased more compared to HSF1WT (Supplementary Fig S5C), congruent with a role of Ser121 phosphorylation in HSF1 stability. This elevated protein levels may also contribute to heightened HSF1 activities in cells expressing HSF1S121A under heat shock conditions, in addition...
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Figure 4.

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to enhanced nuclear translocation (Supplementary Fig S5D). Together, these results indicate that proteotoxic stress suppresses AMPK, which facilitates the PSR but impairs cellular responses to metabolic stressors. Intriguingly, metabolic and proteotoxic stressors exert opposite impacts on AMPK and HSF1 (Fig S5).

**Metabolic stressors disrupt proteostasis in tumor cells**

Our and others’ recent studies have pinpointed HSF1 as a potent facilitator of oncogenesis (Dai et al., 2007, 2012a; Min et al., 2007; Meng et al., 2010; Jin et al., 2011; Scott et al., 2011). In light of our new findings, we next asked whether HSF1 inactivation could contribute to the anti-neoplastic effects of metabolic stressors.

First, we examined the impact of 10 μM metformin, a clinically relevant concentration, on constitutive HSF1 activation in tumor cells. In WM115 and WM278 human melanoma cells, metformin impaired constitutive endogenous HSF1–DNA binding (Fig 6A) and diminished basal mRNA levels of HSP72/HSPA1A and HSP27/HSPB1 (Fig 6B). Conversely, AMPKα1/2 suppression not only elevated basal HSP expression but also antagonized metformin-mediated HSP suppression (Fig 6C and D). Compound C at 2 μM exerted similar effects, albeit to a lesser extent (Supplementary Fig S6A and B). To demonstrate HSP reduction at the protein level by metformin in tumor cells, prolonged treatment was applied to both block new synthesis of HSPs and deplete existing HSPs. Indeed, this prolonged treatment diminished HSP72 and HSP27 protein levels in a panel of diverse human tumor cell lines, ranging from melanoma (WM115 and A2058), to malignant peripheral nerve sheath tumor (MPNST) (S462), to colon carcinoma (RKO), and to breast adenocarcinoma (MCF-7 and MDA-MB-231) (Fig 6E; Supplementary Fig S6C–F).

Consistent with diminished HSP levels, metformin treatment elevated global protein Lys48 polyubiquitination in both detergent-soluble and detergent-insoluble fractions in diverse human tumor cell lines (Fig 6F and G; Supplementary Fig S6G–I). Importantly, cotreatment with compound C prevented HSP reduction, elevation of polyubiquitination, and increase in caspase 3 cleavage, which indicates apoptosis (Fig 6H). These results demonstrated a key role of AMPK in metformin-induced proteostasis disruption. Importantly, while HSF1 knockdown reduced HSP expression and elevated protein polyubiquitination, treatment of HSF1-deficient cells with metformin failed to further induce polyubiquitination (Fig 6I), indicating that metformin disrupts proteostasis mainly through HSF1 suppression. Increased ubiquitination of detergent-insoluble proteins suggested aggravated protein aggregation (Fig 6F–I). To investigate this effect of metformin in detail, we developed a new method to measure intracellular protein aggregation. Our method is based on the Coulter principle, which detects the alterations in electrical impedance produced by particles suspended in an electrolyte (Zwicker, 2010), and which we adapted to quantitate the sizes of protein aggregates extracted from live cells. To use this method to quantitate protein aggregation, we measured aggregation of the GFP-tagged polyglutamine expansion tract (polyQ79) protein (Sanchez et al., 2003). Proteins with polyQ expansions are prone to misfolding and aggregation and are casually implicated in human neurodegenerative disorders (Muchowski & Wacker, 2005). While LacZ-expressing HEK293T cells contained only small aggregates that were barely detectable, polyQ79 expression markedly enlarged aggregates (Fig 6J), validating our new approach for aggregate quantitation. Importantly, both metformin and AMPKα1/2 further enlarged these aggregates (Fig 6J), congruent with diminished HSP levels. Conversely, either HSF1 or a dominant negative AMPK mutant, AMPKαΔN (Mu et al., 2001), potently antagonized the enlargement of aggregates by metformin (Fig 6J), strongly suggesting a critical role of the AMPK–HSF1 pathway in metformin-induced protein aggregation. In further support of an aggravated protein aggregation, metformin caused a shift in distribution of polyQ proteins from detergent-soluble to detergent-insoluble fractions (Supplementary Fig S6J).

We previously demonstrated that HSF1 is essential to the growth and survival of tumor cells but dispensable for primary non-transformed cells (Dai et al., 2007). We reasoned that malignant cells might be more sensitive to metformin treatment than their non-transformed counterparts. To test this, we employed a pair of isogenic cell lines: immortalized MEFs and malignant derivatives of these cells that stably express oncogenic HRASG12V. In malignant MEFs, metformin impaired HSP72 expression, elevated protein polyubiquitination, and induced caspase 3 cleavage; in contrast, metformin had little effect on immortalized MEFs (Fig 6K). Interestingly, glucose deprivation exerted similar effects (Fig 6L) as well as aggravated polyQ aggregation (Fig 6M), all in a dose-dependent manner. Collectively, these results indicate that metabolic stressors suppress the HSF1-mediated PSR in malignant cells, thereby impairing their proteostasis and survival.

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**Figure 5.** Heat shock suppresses AMPK and interferes with cellular responses to metabolic stressors.

A Reporter cells were heat-shocked at 43°C for the indicated time. AMPKα Thr172 and HSF1 Ser121 phosphorylation was measured by immunoblotting.

B Immediately following heat shock at 43°C for 40 min, AMPK complexes were immunoprecipitated from HEK293 cells and in vitro kinase assays were performed as described in Figure 3D using recombinant human ACC1 proteins as substrates.

C Following heat shock at 43°C for 40 min, both detergent-soluble and detergent-insoluble fractions of cellular proteins were extracted from HEK293 cells as described in Materials and Methods for immunoblotting.

D, E Immediately following heat shock at 43°C for 40 min, HEK293 cells were subjected to nutrient deprivations for 4 h. AMPKα Thr172 and ACC Ser79 phosphorylation was measured by immunoblotting.

F–H HSF1WT or HSF1ΔN, A2780 Lys48 plasmids were transfected into HSF1-deficient HEK293T cells. Transfected cells were heat-shocked at 43°C for 40 min. HSP mRNAs were quantitated by qRT–PCR following overnight recovery. HSF1–DNA binding was measured immediately after heat shock (mean ± SD, n = 3, Student’s t-test, **P < 0.01, ***P < 0.001).

I Schematic depiction of the opposite impacts of metabolic and proteotoxic stress on AMPK and HSF1. While metabolic stress activates AMPK to suppress HSF1, proteotoxic stress suppresses AMPK to enhance HSF1 activation. Dashed arrow signifies HSF1 activation independent of AMPK.

Source data are available online for this figure.
Metformin inactivates HSF1 to retard tumor growth in vivo

Consistent with an HSF1 dependence of malignant cells, stable HSF1 knockdown markedly impaired proliferation of A2058 and S462 cells (Fig 7A; Supplementary Fig S7A). This impairment was correlated with aggravated protein polyubiquitination (Fig 7B). In line with a role in HSF1 inactivation, the proliferation of A2058 and S462 cells was also impaired by metformin (Fig 7C; Supplementary Fig S7B, C).
antagonized these metformin-induced changes but also suppressed metformin. Importantly, HSF1 overexpression not only potently suppressed protein expression and induced protein ubiquitination in HSF1WT-expressing cells, these effects were largely abolished in HSF1S121A-expressing cells (Fig 7H). In consequence, the proliferation of HSF1WT-expressing cells was less impaired by metformin compared to that of HSF1S121A-expressing cells under adherent conditions (Supplementary Fig S7H). Moreover, HSF1S121A-expressing cells were also more resistant to metformin treatment under anchorage-independent growth conditions. This was demonstrated in both suspension cultures and soft agar assays (Fig 7I and Supplementary Fig S7I). While in suspension cultures, cells expressing HSF1WT were sensitive to metformin at all glucose concentrations, they only responded to metformin at normal and low glucose concentrations in soft agar (Supplementary Fig S7I). Of note, glucose concentrations had a marked impact on cellular growth in soft agar. In low-glucose medium, the growth of both HSF1S121A and HSF1WT-expressing cells was severely impaired (Supplementary Fig S7I). Nonetheless, a slight metformin resistance was still observed in HSF1S121A-expressing cells (Supplementary Fig S7I). We reasoned that such stringent growth conditions might alleviate cellular dependence on HSF1, which is distinct from suspension growth conditions (Fig 7I). These results together suggest that HSF1 Ser121 phosphorylation plays an important role in metformin-mediated anti-neoplastic effects.

Evidence from human patients also supported the AMPK-HSF1 interaction. In a large-cohort study of patients developing clear cell renal cell carcinomas (ccRCC) (Cancer Genome Atlas Research Network, 2013), tumors showing elevated AMPKα Thr172 phosphorylation displayed reduced mRNA levels of HSPA1A, HSPA1B,
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Figure 6.

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HSPB1, and HSP90AA1, all of which are classic HSF1 transcriptional targets (Fig 7J), suggesting inhibition of HSF1. Moreover, ccRCC patients with high levels of AMPKα phosphorylation demonstrated better overall survival (Supplementary Fig S7J; Cancer Genome Atlas Research Network, 2013). Taken together, these results indicate that a clinically relevant concentration of metformin suppresses constitutive HSF1 activation and disrupts proteostasis within tumor cells, thereby retarding tumor growth (Fig 7K).

Discussion

Our findings unveil a novel interplay between AMPK and HSF1. AMPK inactivates HSF1 through phosphorylation of it at Ser121. This regulatory interaction is essential for maintaining homeostasis and coordination of metabolic and proteotoxic stress responses.

Metabolic stress activates AMPK, which subsequently inhibits activities of SREBP1 and mTORC1 (Gwinn et al, 2008; Li et al, 2011), key regulators of lipid and protein synthesis. Significantly, our findings indicate that AMPK likely plays an additional critical role in survival of metabolic stress. Similar to the case with lipid and protein synthesis, processes involved with chaperone-mediated protein folding and anti-aggregation also consume ATP (Welch, 1991). It becomes wasteful energetically to sustain excessive chaperoning capacity when overall protein synthesis is mitigated, as is the case during times of stress. Therefore, it is a logical action for AMPK to sense fluctuations of intracellular AMP and ATP levels, and to orchestrate AMPK and HSF1 transcriptional responses that form the core of the metabolic and proteotoxic stress responses.

Inactivation of HSF1 by amino acid starvation was reported previously (Hensen et al, 2012), although the underlying mechanisms remained elusive. Now, our findings reveal that the metabolic and proteotoxic stress responses, two fundamental cellular stress pathways, are interconnected through AMPK and HSF1. Whereas metabolic stressors activate AMPK but suppress HSF1, our results reveal that proteotoxic stressors, such as heat shock, disrupt the opposite effect at least in the cell types we examined (Fig 5I). Although the response to each individual stress is advantageous to survival of that stress, this antagonizing interaction between the two responses could have adverse impacts in the face of simultaneous exposure to the two stresses (Figs 4E and F, and 5D and E). While our results indicate diminished AMPK activities under heat shock in NIH3T3 and HEK293 cells (Fig 5A–C; Supplementary Fig S5A), it was previously reported that heat shock activated AMPK in rat hepatocytes (Corton et al, 1994), a highly metabolically active cell type. Thus, these results suggest that AMPK response to heat stress may be cell type-dependent.

Implications of AMPK–HSF1 interactions in human diseases and therapies

Evidence is emerging that implicates AMPK in neurodegeneration. Neuronal AMPK activation has been noted in Huntington’s disease and Alzheimer’s disease and contributes to neuronal death (Ju et al, 2011; Vingtedoux et al, 2011). Thus, it is tantalizing to speculate that AMPK activation may aggravate neurodegeneration in part through impairment of neuronal proteostasis. Exacerbation of polyQ aggregation inflicted by metformin, glucose deprivation, or activated AMPK (Fig 6J and M) supports this possibility. Further, our findings imply a potential adverse side effect of metformin on populations afflicted with neurodegenerative disorders, a notion that may deserve further investigation.

Figure 7. Metformin inactivates HSF1 to retard tumor growth.

A, B A2058 cells were transduced with lentiviral scramble or HSF1-targeting (hA6 and hA9) shRNAs. (A) Cell numbers were quantitated by Hoechst 33342 DNA staining (mean ± SD, n = 4, two-way ANOVA, ***P < 0.001). (B) Protein levels were measured by immunoblotting.

C A2058 cells stably expressing LacZ or HSF1 were grown in medium containing 4.5, 1.0, or 0.45 g/l glucose. Following treatment with 10 μM metformin, cell proliferation was measured by Hoechst 33342 DNA staining (mean ± SD, n = 5, two-way ANOVA, ***P < 0.001).

D, E 1 × 10^6 LacZ- or HSF1-expressing A2058 cells were transplanted into female NOD/SCID mice. One day after transplantation, metformin was administered via drinking water at 1 mg/ml. Tumor incidence (log-rank test; D) and volumes (E) were measured (mean ± SEM, two-way ANOVA n.s., not significant, *P < 0.05, **P < 0.01, ***P < 0.001). Tumor growth curves were fitted to exponential growth models to calculate tumor doubling time (DT).

F Individual proteins were measured by immunoblotting using lysates of three tumors from each treatment group.

G Protein levels were measured by immunoblotting using lysates of mouse liver tissues.

H A2058 cells stably expressing FLAG-HSF1WT or FLAG-HSF1Δ1224 were treated with 10 μM metformin for 7 days. Protein levels were measured by immunoblotting.

I A2058 cells stably expressing HSF1WT or HSF1Δ1224 were grown in high-glucose (50 mM) or low-glucose (5 mM) media for 10 days. Cell viability was measured by Guava flow cytometer using ViaCount® reagent (mean ± SEM, n = 8, Student’s t-test, n.s., not significant, **P < 0.01, ***P < 0.001).

J Tukey box plots showing the inverse correlation between AMPKα Thr172 phosphorylation and HSF1 mRNA expression in ccRCC. Patients were stratified on the median value of AMPKα Thr172 phosphorylation reverse-phase protein array (RPPA) scores (Student’s t-test).

K Schematic depiction of the suppression of HSF1 and disruption of proteostasis by AMPK in malignant transformation.

Source data are available online for this figure.
Figure 7.
Despite the importance of the HSF1-mediated PSR in oncogenesis, it remains poorly understood how malignant cells hijack this powerful adaptive mechanism. The negative regulation of HSF1 by AMPK, thus, may shed new light on this question. Previous studies have demonstrated many anti-neoplastic effects of the LKB1–AMPK pathway. LKB1 functions as a tumor suppressor and its germline mutations are causally linked to Peutz–Jeghers syndrome (Shackelford & Shaw, 2009). In addition, AMPK stimulates tumor suppressors including TSC2 and TP53 (Inoki et al., 2003; Jones et al., 2005), and its inactivation promotes oncogenesis (Faubert et al., 2013). Now our findings reveal an important role of the tumor-suppressive AMPK pathway in inactivating HSF1. Conceivably, inactivation of the LKB1–AMPK pathway could in a cell-autonomous fashion mobilize the PSR to assist malignant transformation. This resonates with the interaction between HSF1 and the tumor suppressor NF1 we discovered recently, in which NF1 deficiency sufficiently activates HSF1 to promote oncogenesis (Dai et al., 2012a). These negative regulations of HSF1 mediated by tumor-suppressive pathways highlight the notion that activation of HSF1 and its mediated PSR is deeply embedded within oncogenic processes.

Our findings also uncover a novel mechanism of action of metformin. The principal metabolic effects of metformin result from suppressed liver gluconeogenesis and enhanced muscle glucose uptake (Violett et al., 2012). In addition to these well-known anti-diabetic benefits, epidemiological studies have revealed reduced tumor incidence in T2D patients taking metformin (Evans et al., 2005; Garrett et al., 2012; Sadeghi et al., 2012). Preclinical investigations show that metformin impairs de novo oncogenesis in mice and impeded tumor growth in xenograft models (Anisimov et al., 2010; Memmott et al., 2010; Lliopoulos et al., 2011; Tomic et al., 2011). Despite this exciting promise, the proposed underlying mechanisms are diverse, ranging from reduced insulin levels, to suppressed mTORC1 activity, to activated TP53, and to impaired NF-κB signaling (Buzzai et al., 2007; Dowling et al., 2007; Hirsch et al., 2013).

Now our findings indicate that metformin suppresses the HSF1-mediated PSR. Importantly, due to the lack of proteotoxic stress, HSF1 is dispensable for the viability of primary cells and mice under non-stress conditions (Xiao et al., 1999; Dai et al., 2007). In stark contrast, malignant cells constantly endure proteotoxic stress from within and without and, in turn, develop an addiction to HSF1 to sustain their robustness (Dai et al., 2007, 2012b). Congruently, whereas in malignant cells a clinically relevant concentration of metformin and mild glucose deprivation suppressed constitutive HSF1 activation, aggravating proteomic imbalance and impairing survival, little impact was observed in non-transformed cells (Figs 6K and L, and 7F and G), indicating a promising therapeutic window. Moreover, our results showing partial resistance of tumors to metformin as a result of HSF1 expression (Fig 7D–F) highlight the importance of this specific mechanism to metformin’s overall anti-neoplastic effects and further imply that tumors with low levels of HSF1 expression may be more sensitive to metformin treatment. Our findings collectively suggest a novel unifying mechanism of action of metabolic stressors in malignancy—disruption of proteostasis via HSF1 inactivation.

Materials and Methods

An ELISA-based HSF1–DNA binding assay

Biotinylated ideal HSE (CTAGAACCTTCTAGAGCTTCTAG) oligonucleotides were self-anneled to form double-stranded DNA probes in annealing buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA). To immobilize these probes for HSF1 binding, 100 μl of 500 nM biotinylated HSE probes diluted in PBS was added to neutravidin-coated 96-well plates (Thermo Scientific) and incubated at 4°C overnight. After washing with PBS once, wells were incubated with 200 μl SuperBlock blocking buffer (Thermo Scientific) at RT for 1 h. After washing with 1× DNA binding buffer (10 mM Tris, 50 mM NaCl, 1 mM EDTA, 5% glycerol, pH 7.5) once, 100 μl nuclear proteins diluted in 1× DNA binding buffer was added to each well and incubated at RT for 40 min. The captured protein–DNA binding was stabilized by immediately incubating the wells with 1% formaldehyde diluted in 1× DNA binding buffer at RT for 5 min. Following washing 3 times with 1× DNA binding buffer, each well was incubated with 100 μl rabbit polyclonal HSF1 antibody (B7109, Assay Biotechnology) diluted 1:1,000 in SuperBlock blocking buffer at RT for 2 h. After TBS-T washing, each well was incubated with HRP-conjugated anti-rabbit IgG secondary antibodies diluted in the blocking buffer at RT for 1 h. Following extensive TBS-washing, colorimetric signals were developed using 1-Step Ultra TMB-ELISA substrate (Thermo Scientific).

To validate this method, nuclear extracts of heat-shocked Hsf1+/+ and Hsf1+− cells were used to serve as positive and negative controls, respectively. Assay specificity was further validated by pre-incubating nuclear extracts with non-biotinylated ideal HSE or a scramble oligonucleotide of the HSE (GGGACATTATTGGTGCAA-CATTAC).

Bioluminescence imaging

Transgenic Hsp70-luc-2A-eGFP reporter mice (FVB/NJ/C57BL/6J background) were obtained from The Jackson Laboratory. Mice of the same sex at 5–6 weeks of age were treated with metformin for 3 days via i.p. injection. Six hours before bioluminescence imaging, mice were challenged with a single dose of vecodace i.v. injection. Before imaging, XenoLight RediJect D-luciferin (150 mg/kg) was i.p. injected into mice. Mice were anesthetized with isoflurane, and luminescence signals were recorded using a Xenogen IVIS Lumina II system (Caliper Life Sciences). Images of both dorsal and ventral positions were captured. The total photon flux of each mouse was quantified using Living Image software.

Proximity ligation assay

Following treatments, cells were fixed with 4% formaldehyde in PBS for 15 min at RT. After blocking with 5% normal goat serum in PBS with 0.3% Triton X-100, a pair of rabbit and mouse primary antibodies were incubated simultaneously with fixed cells. To detect HSF1–DNA interactions, fixed cells were treated with 7 U/ml RNase at RT for 1 h before incubation with rabbit anti-HSF1 antibodies (H-311, Santa Cruz Biotechnology) and mouse monoclonal anti-ds DNA antibody (HYB331-01, Santa
Cruz Biotechnology) overnight at 4°C. To detect phospho-AMPKα–HSF1 interactions, fixed cells were incubated with rabbit monoclonal phospho-AMPKα Thr172 antibodies (40H9, Cell Signal Technology) and mouse monoclonal HSF1 antibodies (E-4, Santa Cruz Biotechnology) overnight at 4°C. All primary antibodies were used at 1:50 dilution in blocking buffer. Following incubation with Duolink PLA anti-rabbit Plus and anti-mouse Minus probes (OLINK Bioscience) at 37°C for 1 h, ligation, rolling circle amplification, and detection were performed using the Duolink In Situ Detection Reagents Red (OLINK Bioscience) as per manufacturer’s instructions. Nuclei were counterstained with Hoechst 33342. Finally, PLA signals were documented by a Leica TCS SP5 confocal microscope.

**Soluble and insoluble protein fractionation**

Equal numbers of cells were incubated with cell lysis buffer containing 1% Triton X-100 on ice for 20 min. The crude lysates were first centrifuged at 500 g for 2 min at 4°C. The supernatants were further centrifuged at 20,000 g for 20 min at 4°C. The final supernatants and pellets were collected as detergent-soluble and detergent-insoluble fractions, respectively. Insoluble fractions were further subjected to sonication for SDS–PAGE.

**Measurement of aggregate size**

Equal numbers of cells from different samples were lysed with cold cell lysis buffer. Following centrifugation at 20,000 g for 15 min at 4°C, detergent-insoluble pellets were further extracted with RIPA buffer 3 times. The final insoluble pellets were re-suspended in 10% SDS by pipetting and immediately subjected to aggregate sizing using a Multisizer™ 3 Coulter Counter equipped with a 20-μm aperture (Beckman Coulter).

**Animal studies**

Ampkα1<sup>fl/fl</sup> and Ampkα2<sup>fl/fl</sup> mutant mice on the C57BL/6J background were obtained from The Jackson Laboratory. Hsf1 mutant mice (129SvJ/BALB/c) were a generous gift from Dr. Ivor Benjamin (Xiao et al., 1999) and described previously (Dai et al., 2007, 2012b).

For tumor xenograft studies, 1 × 10<sup>6</sup> A2058 cells were s.c. injected into the left flanks of 9-week-old female NOD.CB17-Prkdc<sup>scid</sup>/J (NOD/SCID) mice (The Jackson Laboratory). One day after injection, mice were given 1 mg/ml metformin via water held in light-tight drinking bottles. This dose is calculated based on 1,000 mg/60 kg/day in humans. The equivalent mouse dose is 205 mg/kg/day, which corresponds to about 4 mg/day for a 20 g adult mouse consuming 4 ml water per day. Tumor sizes were measured by a caliper weekly, and tumor volumes were calculated following the formula 4/3πR<sup>3</sup>. All mouse experiments were performed under a protocol approved by The Jackson Laboratory Animal Care and Use Committee.

**Statistical methods**

All statistical analyses were performed using Prism 5.0 (GraphPad software). Statistical significance: *P < 0.05; **P < 0.01; ***P < 0.001.

**Supplementary information** for this article is available online: http://embj.embopress.org

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**Author contributions**

SD performed immunoblotting, bioluminescence imaging, flow cytometry experiments, body weight composition analysis, and in vivo shRNA delivery; ZT performed DNA binding, ChIP, and immunoblotting experiments; JC and W2 performed immunoprecipitation, PLA, and measurement of aggregate size experiments; HL performed cell growth and viability experiments; CD conceived the project and oversaw the studies; SS and CD wrote the manuscript.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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