



# SIRT1 associates with eIF2-alpha and regulates the cellular stress response

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**SIRT1 is a NAD<sup>+</sup> dependent protein deacetylase known to increase longevity in model organisms. SIRT1 regulates cellular response to oxidative and/or genotoxic stress by regulating proteins such as p53 and FOXO. The eukaryotic initiation factor-2, eIF2, plays a critical role in the integrated stress response pathway. Under cellular stress, phosphorylation of the alpha subunit of eIF2 is essential for immediate shut-off of translation and activation of stress response genes. Here we demonstrate that SIRT1 interacts with eIF2 $\alpha$ . Loss of SIRT1 results in increased phosphorylation of eIF2 $\alpha$ . However, the downstream stress induced signaling pathway is compromised in SIRT1-deficient cells, indicated by delayed expression of the downstream target genes CHOP and GADD34 and a slower post-stress translation recovery. Finally, SIRT1 co-immunoprecipitates with mediators of eIF2 $\alpha$  dephosphorylation, GADD34 and CreP, suggesting a role for SIRT1 in the negative feedback regulation of eIF2 $\alpha$  phosphorylation.**

**S**IRT1, the human ortholog of the yeast SIR2 protein, has been implicated in lifespan extension in model organisms, such as yeast, worms and flies<sup>1–5</sup>. SIRT1 plays a role in a wide range of cellular processes including metabolism, cell-cycle, cell growth and differentiation, apoptosis and cellular response to stress<sup>6</sup>, and have been shown to reduce age associated physiological changes in mice<sup>7, 8</sup>. By regulating various proteins, such as NF- $\kappa$ B, Ku70, p53, E2F1, p73 and the forkhead transcription factors (FOXOs)<sup>5, 9–12</sup> SIRT1 responds to cellular stress, thereby protecting cells from oxidative and genotoxic damage. Recently, SIRT1 was shown to regulate the heat shock factor 1 (HSF1), suggesting a role in protein homeostasis during cellular stress<sup>13</sup>. However, despite the involvement of SIRT1 in various types of cellular stress, it hasn't been implicated yet in the Integrated Stress Response (ISR) pathway, critical for halting protein synthesis and activating stress response genes during cellular stress.

The eukaryotic initiation factor 2-alpha (eIF2 $\alpha$ ) plays a critical role in regulating translation attenuation in response to stress signals. Translation is controlled by various extra and intra-cellular stimuli such as nutrients, growth factors, hormones and stress signals<sup>14</sup>. Initiation of translation is a critical checkpoint for protein synthesis, in which phosphorylation of eIF2 $\alpha$  plays a rate limiting role. Four different eIF2 $\alpha$  kinases, PERK, GCN2, PKR and HRI phosphorylate eIF2 $\alpha$  in response to distinct stress stimuli. In order to bind to the Met-tRNA<sup>Met</sup> and initiate translation, eIF2 must be associated with GTP, which is subsequently hydrolyzed to GDP in the initiation step. Thus, at the end of first round of translation initiation, and before eIF2 $\alpha$  can be recycled for a second round, eIF2 $\alpha$ -bound GDP must be exchanged for GTP in a reaction catalyzed by eIF2B. Phosphorylation of eIF2 $\alpha$  in response to cellular stress results in formation of a stable, inactive eIF2 $\alpha$ -GDP-eIF2B complex, thereby inhibiting the exchange to eIF2-GTP. The reduction in eIF2-GTP levels leads to a general reduction of global protein synthesis. However, translation of specific stress response genes, such as ATF4 and CHOP, is induced in response to stress, resulting in expression of genes important for metabolism, redox status, and apoptosis<sup>15, 16</sup>. Downstream of ATF4/CHOP is GADD34, whose expression correlates temporally with eIF2 $\alpha$  dephosphorylation later in the stress response signaling. GADD34 is a stress-inducible regulatory subunit of a holophosphatase complex that dephosphorylates eIF2 $\alpha$  and plays a role in translational recovery through feed back inhibition of eIF2 $\alpha$  phosphorylation. GADD34 associates with type 1 protein phosphatase (PP1) catalytic subunit to mediate dephosphorylation of eIF2 $\alpha$ . In addition to GADD34, a new member of the GADD34 family, CreP (CreP, the constitutive repressor of eIF2 $\alpha$  phosphorylation), has been shown to be responsible for maintaining the basal, steady state level of eIF2 $\alpha$  phosphorylation<sup>17</sup>.

Here we identified eIF2 $\alpha$  as a novel binding partner for SIRT1 in a yeast two-hybrid screen and examined the biological significance of the association between these two proteins. We demonstrate that depletion of SIRT1 results in increased and prolonged phosphorylation of eIF2 $\alpha$ . However, expression of the downstream target genes in the stress signaling pathway, CHOP and GADD34 is delayed and suppressed in absence of SIRT1 causing a relatively weaker post-stress translation recovery. The SIRT1-eIF2 $\alpha$  association, however, does not appear to be



regulated by stress conditions, indicating a constitutive role for SIRT1 in regulating eIF2 $\alpha$  phosphorylation. Consistent with this, we show that SIRT1 interacts with both the constitutive and induced dephosphorylators of eIF2 $\alpha$ , CreP and GADD34. Taken together, these results establish a novel binding partner for SIRT1 and suggest role for SIRT1 in modulating the eIF2 $\alpha$  mediated cellular stress response pathway.

## Results

**SIRT1 associates with eIF2 $\alpha$  *in vivo*.** To identify potential binding partners for SIRT1, we used a yeast two-hybrid screen. Full length SIRT1 fused to the Gal4-DNA binding domain (DBD) was used as the bait to screen approximately  $10.5 \times 10^6$  transformants of a human spleen cDNA library, fused to the GAL4 activation domain (AD) in the AH109 yeast strain as described<sup>18</sup>. A positive clone for eIF2 $\alpha$  was identified in the yeast two-hybrid screen. Interestingly, both SIRT1 and eIF2 $\alpha$  have been shown to regulate cellular stress response, largely by regulating transcription and translation, respectively.

The association between SIRT1 and eIF2 $\alpha$  was confirmed in mammalian cells by co-immunoprecipitation of endogenous SIRT1 and eIF2 $\alpha$  as well as transiently expressed epitope-tagged versions of the proteins. Immunoprecipitation of whole cell extracts from HeLa cells using anti-eIF2 $\alpha$  or anti-SIRT1 antibodies followed by Western blot analysis with anti-SIRT1 or anti-eIF2 $\alpha$  antibody respectively, demonstrated that the endogenous SIRT1 and eIF2 $\alpha$

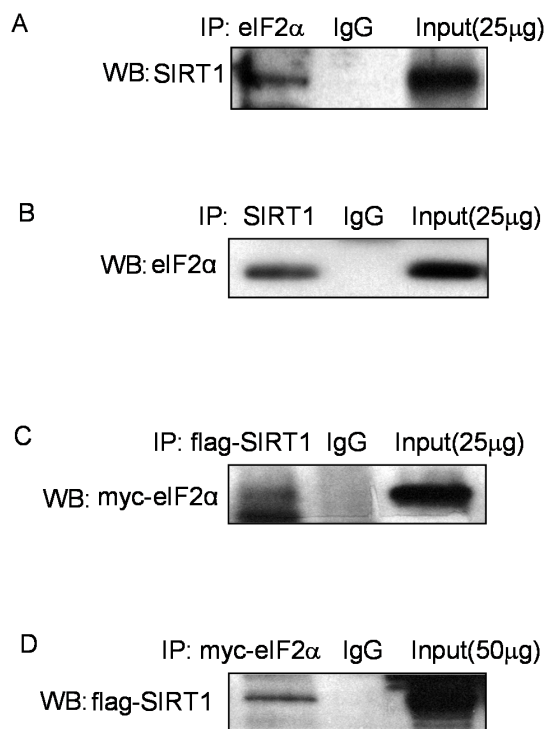
proteins interact under physiological conditions (Fig. 1A and B). Exogenously expressed Flag-tagged SIRT1 and myc-tagged eIF2 $\alpha$  also were co-immunoprecipitated from transfected HeLa cells (Fig. 1C and D).

**eIF2 $\alpha$  phosphorylation is enhanced in SIRT1 depleted cells.** After confirming the physiological association of SIRT1 and eIF2 $\alpha$ , we next investigated whether there was a regulatory overlap between these two proteins in mediating the cellular stress response. For this, HeLa cells depleted for SIRT1 by stable retrovirus-mediated expression of a shRNAi against SIRT1<sup>18</sup> were used. HeLa cells expressing non-targeted shRNAi were used as a negative control. SIRT1 depleted and matched-control cells were treated with stress conditions: amino-acid starvation, glucose starvation, serum deprivation, ER stress (tunicamycin) and oxidative stress (H<sub>2</sub>O<sub>2</sub>). As shown in Figure 2A, under all different conditions, the SIRT1 depleted cells showed higher levels of phosphorylated eIF2 $\alpha$  compared to matched control cells.

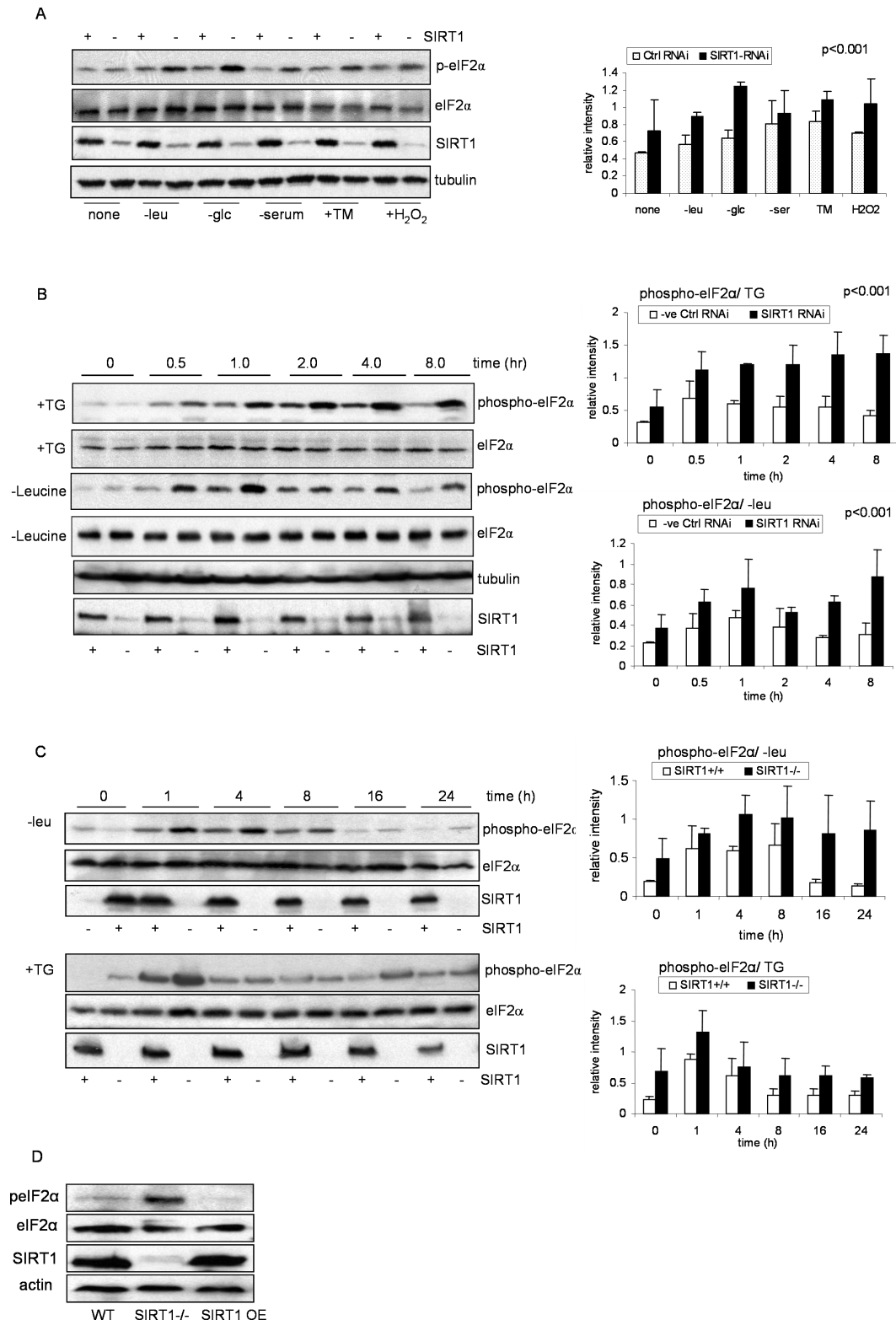
Since the increase and subsequent decline in phosphorylation of eIF2 $\alpha$  through a negative feedback loop are critical components of the cellular stress response, we examined the kinetics of eIF2 $\alpha$  phosphorylation in SIRT1 depleted and control HeLa cells. Cells were treated with two different stress conditions, ER stress (thapsigargin) and amino acid starvation (-leucine), for up to 8 hours and phosphorylation of eIF2 $\alpha$  analyzed by Western blotting. The SIRT1-depleted cells had higher phosphorylation of eIF2 $\alpha$  at all time points analyzed and, unlike the control cells, did not show the normal decline in phosphorylation at the later stages of the stress response (Fig 2B). This result was verified in murine cells using SIRT1-wild-type and SIRT1-null murine embryonic fibroblasts (MEFs). Consistent with the results in HeLa cells, the SIRT1 knock-out MEFs showed higher and prolonged phosphorylation of eIF2 $\alpha$  in response to leucine starvation and ER stress (Fig. 2C). Notably, SIRT1 deficient cells showed higher levels of phosphorylated eIF2 $\alpha$  even under no stress conditions, indicating a stress-independent role for SIRT1 on eIF2 $\alpha$  phosphorylation. Taken together, these results suggest a role for SIRT1 in eIF2 $\alpha$  dephosphorylation under prolonged stress conditions as well as in maintenance of lower basal level of eIF2 $\alpha$  phosphorylation in the steady state.

To confirm the role of SIRT1 in regulation of eIF2 $\alpha$  phosphorylation, we examined the ability to rescue the high level of eIF2 $\alpha$  phosphorylation in SIRT1 deficient cells in the steady-state by exogenously overexpressing SIRT1. For this, SIRT1 null MEFs stably overexpressing SIRT1 were generated by stable retroviral transduction. eIF2 $\alpha$  phosphorylation was assessed by measuring phospho-eIF2 $\alpha$  in WT, SIRT1 null and SIRT1-overexpressing-SIRT1null cells. As demonstrated in Fig. 2D, overexpression of SIRT1 in SIRT1-null MEFs rescued the basal lower level of eIF2 $\alpha$  phosphorylation, confirming a role for SIRT1 in regulating eIF2 $\alpha$  phosphorylation under steady-state conditions.

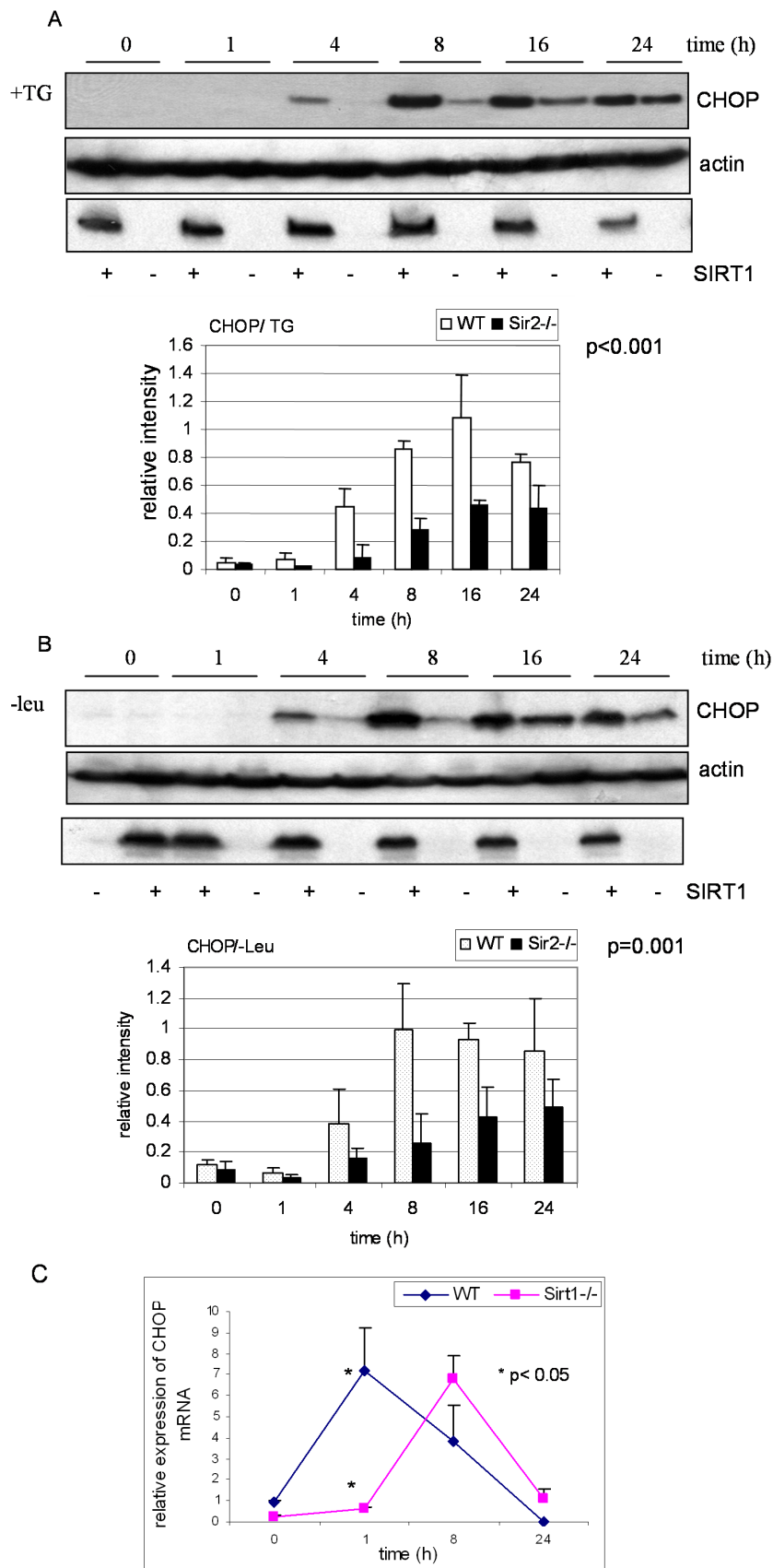
**Expression of the stress response protein CHOP/GADD153 is delayed in absence of SIRT1.** Since the SIRT1-deficient cells showed significantly higher levels of phosphorylated eIF2 $\alpha$ , we next examined the effect of SIRT1 depletion on the downstream effector proteins in the eIF2 $\alpha$ -stress signaling pathway. The SIRT1 knock-out and wild-type MEFs were subjected to ER stress and amino-acid starvation for various time periods and CHOP expression was measured by Western blot analysis. Surprisingly, the SIRT1 deficient cells, which exhibited higher levels of eIF2 $\alpha$  phosphorylation, showed reduced and delayed expression of the stress protein CHOP under both stress conditions, ER-stress (TG) (Fig 3A) and leucine starvation (Fig 3B). In contrast to the wild-type cells, which had significant induction of CHOP expression within 4 hr of stress treatment, 16–24 hr was required to achieve significant expression of CHOP in the SIRT1 deficient cells. We further confirmed the regulation of CHOP expression by SIRT1 by measuring CHOP mRNA



**Figure 1 |** (A) and (B) Co-immunoprecipitation of endogenous SIRT1-eIF2 $\alpha$ : Whole cell extracts from HeLa cells were immunoprecipitated for eIF2 $\alpha$  or SIRT1 using respective antibodies. Samples were then probed for SIRT1 and eIF2 $\alpha$  respectively, by Western blotting. (C) and (D) Co-immunoprecipitation of epitope tagged SIRT1-eIF2 $\alpha$ : HeLa cells were transfected with flag-tagged SIRT1 and myc-tagged eIF2 $\alpha$ . 24 hr post transfection, whole cell extracts were immunoprecipitated for SIRT1 or eIF2 $\alpha$  using flag or myc antibodies respectively, followed by Western blot analysis using anti-myc and anti-flag antibodies respectively. IgG antibody was used as a negative control for immunoprecipitation and 25–50  $\mu$ g of whole cell protein lysate was used as input. The data presented is a representative of multiple independent experiments.



**Figure 2 | (A)** eIF2 $\alpha$  phosphorylation in SIRT1 deficient HeLa cells in response to stress: SIRT1 depleted and matched-control HeLa cells were treated as indicated for 1 hr. The whole cell lysates were analyzed by Western blotting using phosphorylated and total protein antibodies for eIF2 $\alpha$ . Tubulin was used as a loading control. -leu: leucine starvation, -glc: glucose starvation, -serum: serum starvation, +TM: 2 $\mu$ g/ml tunicamycin, H<sub>2</sub>O<sub>2</sub>: 500 nM hydrogen peroxide, +: Negative control RNAi HeLa cells, -: SIRT1 RNAi HeLa cells. (B) and (C) Kinetics of eIF2 $\alpha$  phosphorylation in SIRT1 depleted HeLa cells (B) and SIRT1<sup>-/-</sup> MEFs (C): SIRT1-deficient and matched control cells were subjected to ER stress (thapsigargin) or leucine starvation for indicated time periods. The bar graphs represent the relative band intensities (mean  $\pm$  SD from two independent experiments). Combined two-tailed p value across all group sets was calculated using student's t-test D. Basal eIF2 $\alpha$  phosphorylation level in WT, SIRT1 null, and stably SIRT1 over-expressing null MEFs. Whole cell lysates were analyzed by Western blot analysis using phospho- or total protein antibody for eIF2 $\alpha$ . +: Negative control RNAi HeLa cells or SIRT1<sup>+/+</sup> MEFs, -: SIRT1 RNAi HeLa cells or SIRT1<sup>-/-</sup> MEFs.

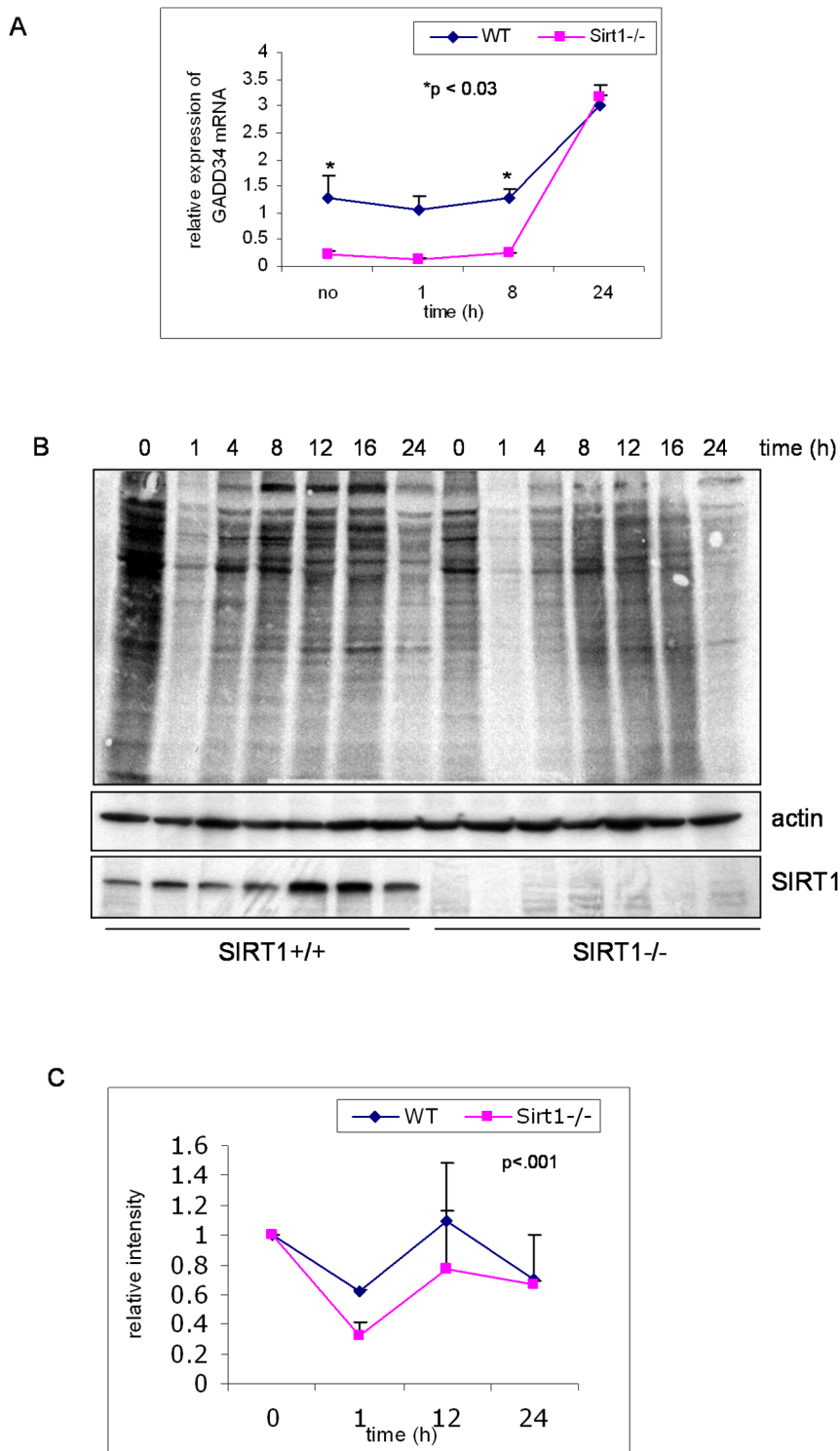


**Figure 3** | CHOP expression in SIRT1 deficient cells in response to ER stress (A) and amino acid (B) starvation: SIRT1 knock-out and WT MEFs were treated with thapsigargin (ER stress) or leucine starvation for indicated time period. Whole cell lysates were analyzed by western blot analysis. The bar graph represents the relative band intensities (mean  $\pm$  SD from two independent experiments). Combined two-tailed p value across all group sets was calculated using student's t-test. (C) mRNA expression of CHOP gene in SIRT1-deficient MEFs: WT and SIRT1 null MEFs treated with TG (2  $\mu$ M) for specified time-period and analyzed by qPCR. Data represent expression levels relative to untreated WT MEFs (assigned an arbitrary value of 1).



levels in WT and SIRT1-deficient cells by quantitative-PCR (Fig. 3C). These results suggest that the increased phosphorylation of eIF2 $\alpha$  in SIRT1-deficient cells could be a secondary effect downstream of impaired CHOP expression.

**SIRT1 deficient cells show slower translation recovery after stress treatment.** The stress signaling pathway is tightly regulated through a negative feedback loop to ensure that the transient shut-off of global protein synthesis in response to stress is reverted to retrieve



**Figure 4** | (A) Gene expression of GADD34 in SIRT1-deficient MEFs.: WT and SIRT1 null MEFs treated with or without TG (2  $\mu$ M) for 1 h, and cells were analyzed after specified time-period by qPCR. Data represent expression levels relative to untreated WT MEFs (assigned an arbitrary value of 1). The two-tailed p value for specified groups were calculated using student's t-test. (B) Post-stress translation recovery in SIRT1 deficient cells in response to ER stress: Wild-type and SIRT1 null mouse embryonic fibroblasts were treated with 2  $\mu$ M thapsigargin for 1 hr. Cells were then pulsed with 200  $\mu$ Ci/ml  $^{35}$ S labeled methionine for 30 min at each time point and new protein synthesis was assessed by incorporation of  $^{35}$ S in cellular protein. Actin was used as the loading control. +: SIRT1+/+ MEFs, -: SIRT1<sup>-/-</sup> MEFs. (C) The graph represents the relative band intensities of the blot in 'B' calculated using Image J software after normalization with the loading control actin. The combined two-tailed p value across all group sets was calculated using student's t-test.



normal functioning. The dephosphorylation of eIF2 $\alpha$  at later stages of the stress response is regulated by its downstream target GADD34 through a negative feedback mechanism. Thus, it is possible that the higher and sustained phosphorylation of eIF2 $\alpha$  in SIRT1-deficient cells is a result of the delayed expression of CHOP and consequent delay in the feedback dephosphorylation of eIF2 $\alpha$  at later stages of the stress response. Thus we examined the expression of GADD34 in response to stress in SIRT1-deficient and WT MEFs by quantitative PCR. As shown in Fig. 4A, the SIRT1-deficient cells show a significantly lower basal expression of GADD34, indicating a possible defect in eIF2 $\alpha$  dephosphorylation in SIRT1-deficient cells.

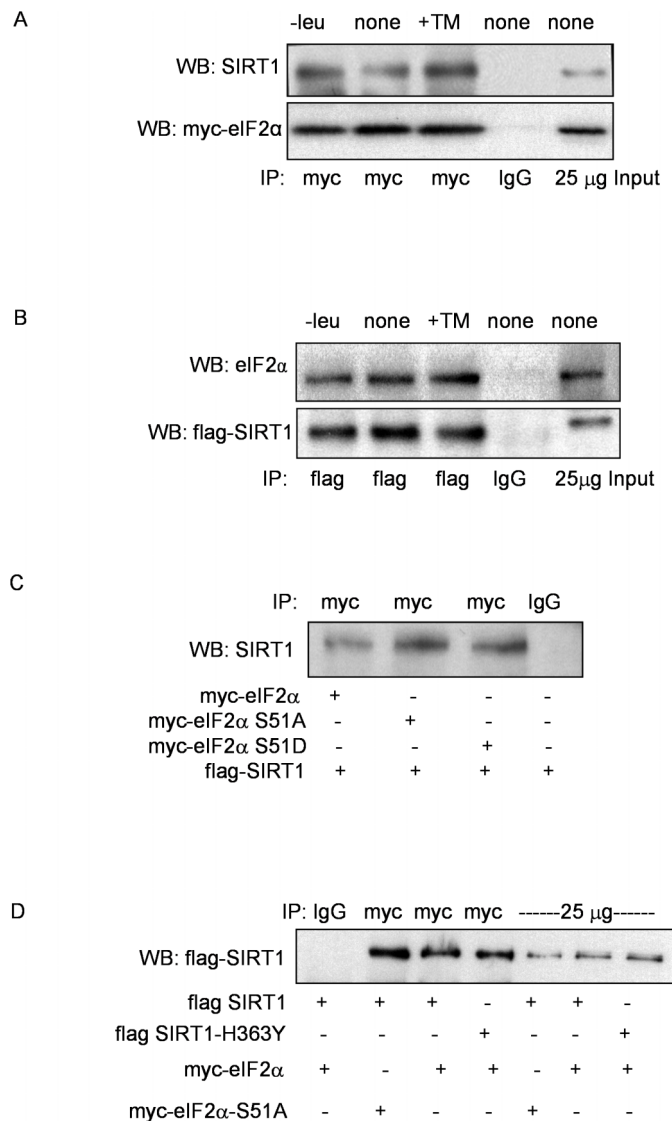
To determine if reduced GADD34 gene expression in SIRT1 deficient cells had any effect on post-stress translation recovery, we examined the levels of new protein synthesis in SIRT1-deficient versus wild-type cells in response to stress treatment. SIRT1 positive and negative MEFs were subjected to ER stress for 1 hr and new protein synthesis examined at specific time-points by measuring incorporation of radiolabeled <sup>35</sup>S-methionine. SIRT1-deficient cells showed a relatively slower translation recovery after stress treatment, as indicated by reduced protein synthesis (Figure 4B and C). Also, the inhibition of new protein synthesis immediately after stress was greater in the SIRT1<sup>-/-</sup> cells compared to wild-type cells, consistent with the observed higher eIF2 $\alpha$  phosphorylation. These results suggest a novel role for SIRT1 in directly modulating protein synthesis in response to cellular stress.

**SIRT1-eIF2 $\alpha$  association is independent of stress, SIRT1 deacetylase activity or eIF2 $\alpha$  phosphorylation.** Since SIRT1 regulated stress-induced eIF2 $\alpha$  phosphorylation and its downstream stress-signaling pathway, we next examined if SIRT1-eIF2 $\alpha$  association was regulated by stress. HeLa cells were transfected with flag-tagged SIRT1 and myc-tagged eIF2 $\alpha$  expression vectors and treated with two different stress conditions: amino acid starvation (-leucine) or ER stress (tunicamycin). Whole cell extracts were examined for SIRT1-eIF2 $\alpha$  association by co-immunoprecipitation using epitope antibodies. Surprisingly, we found that the interaction of SIRT1 with eIF2 $\alpha$  was not affected significantly by stress conditions (Fig 5A and B).

Cellular stress results in Ser51 phosphorylation of eIF2 $\alpha$ . Since SIRT1 depletion leads to higher phosphorylation of eIF2 $\alpha$ , we examined if the SIRT1-eIF2 $\alpha$  association was affected by the phosphorylation state of eIF2 $\alpha$ . HeLa cells were co-transfected with expression plasmids for SIRT1 and either a phosphorylation-mutant (eIF2 $\alpha$ S51A) or phosphorylation-mimetic (eIF2 $\alpha$ S51D) eIF2 $\alpha$  and their interaction analyzed by co-immunoprecipitation. As shown in Fig 5C, the SIRT1-eIF2 $\alpha$  interaction was not affected by the phosphorylation status of eIF2 $\alpha$ , further confirming that the association is not dependent on stress induction.

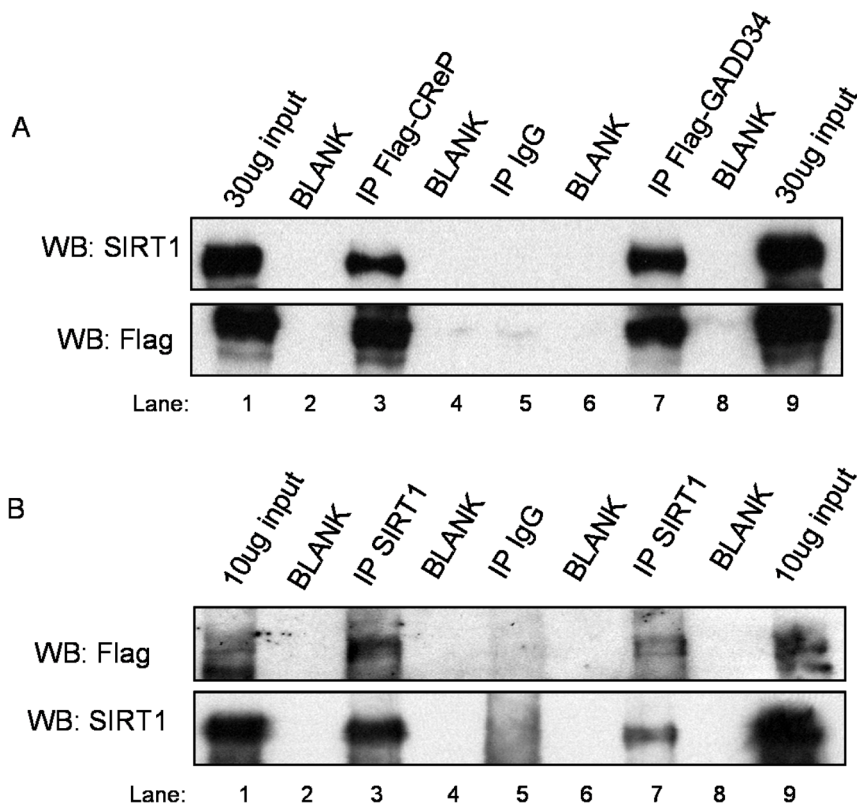
To determine if the catalytic activity of SIRT1 was important for SIRT1-eIF2 $\alpha$  association, we used the catalytic mutant SIRT1, SIRT1-H363Y, in which the catalytic domain of SIRT1 is mutated by substituting the histidine 363 residue with a tryptophan. Co-immunoprecipitation analysis using epitope-tagged SIRT1, SIRT1H363Y, eIF2 $\alpha$  or eIF2 $\alpha$ S51A showed no significant difference in SIRT1-eIF2 $\alpha$  association (Fig 5D).

**SIRT1 interacts with regulators of eIF2 $\alpha$  phosphorylation.** The SIRT1 depleted cells showed an increased phosphorylation of eIF2 $\alpha$  even without stress, indicating a potential defect in SIRT1 null cells in maintaining the basal low level of eIF2 $\alpha$  phosphorylation. CreP, the constitutive repressor of eIF2 $\alpha$  phosphorylation, is a family member of the GADD34 protein<sup>17</sup>. Unlike GADD34, the activity and expression levels of CreP is unaffected by stress stimuli, and knock-down of CreP results in a defect in the basal levels of eIF2 $\alpha$  de-phosphorylation in cultured cells<sup>17</sup>. Thus, it is possible that the association of SIRT1 with eIF2 $\alpha$ , as demonstrated to be a stress-independent process, acts at a constitutive level to maintain low basal level of eIF2 $\alpha$ . To investigate this, we examined



**Figure 5 | SIRT1-eIF2 $\alpha$  association under stress conditions: (A) and (B) HeLa cells were transfected with flag-tagged SIRT1 and myc-tagged eIF2 $\alpha$  expression plasmids. 24 h post-transfection, cells were either treated with vehicle alone or -leucine media or tunicamycin (2  $\mu$ g/ml) for 1 hr. Whole cell lysates were used for immunoprecipitation of eIF2 $\alpha$  or SIRT1 using anti-myc-tag or anti-flag-tag antibody respectively and Western blotted with flag for SIRT1 or eIF2 $\alpha$  antibody. IgG antibody was used as negative control of immunoprecipitation and 25  $\mu$ g whole cell lysate was used as input. The blots were also probed for the immunoprecipitated proteins. (C) Co-immunoprecipitation of SIRT1 with the phosphorylation mutant and mimetic eIF2 $\alpha$ . (D) Co-immunoprecipitation of the catalytic-mutant-SIRT1 with eIF2 $\alpha$ : HeLa cells were transfected with the indicated expression plasmids. 24 h post-transfection whole cell lysates were extracted and used for immunoprecipitation of eIF2 $\alpha$  using anti-myc-tag antibody and Western blotted with flag antibody for SIRT1. IgG antibody was used as negative control of immunoprecipitation and 25  $\mu$ g whole cell lysate was used as input. The data presented is a representative of multiple independent experiments.**

if SIRT1 interacted with the dephosphorylators of eIF2 $\alpha$ . As shown in Figure 6A and B, co-immunoprecipitation assay using flag-tagged-GADD34 and -CreP showed that SIRT1 interacts with both GADD34 and CreP. This result suggests a role for SIRT1 in eIF2 $\alpha$  dephosphorylation under both stress induction (potentially through GADD34) and steady-state conditions (potentially through CreP).



**Figure 6** | SIRT1 interacts with the proteins that mediate eIF2 $\alpha$  dephosphorylation: 293T cells were transfected either with flag-tagged GADD34 and myc-tagged SIRT1, or with flag-tagged CReP and myc-tagged SIRT1 expression plasmids. 48 h post-transfection whole cell lysates were used for immunoprecipitation of (A) GADD34 or CReP using anti-flag-tag antibody or (B) SIRT1 using anti-SIRT1 antibody and Western blotted with SIRT1 or flag respectively. IgG antibody was used as negative control of immunoprecipitation and 10–30  $\mu$ g whole cell lysate was used as input. Lanes 1& 3: cells transfected with flag-CReP and myc-SIRT1, Lanes 7&9: with flag-GADD34 and myc-SIRT1. The data presented is a representative of two independent experiments.

## Discussion

SIRT1 regulates a wide array of cellular processes ranging from metabolism, growth and differentiation to the cellular stress response. The wide range of SIRT1 targets is believed to underlie its potential as a regulator of longevity in mammals. Since most of the target substrates for SIRT1 are transcription factors, the predominant function of SIRT1 is thought to be regulation of transcription through the deacetylation of transcription factors and histones. Here we identify a novel binding partner for SIRT1, eIF2 $\alpha$ , which is a translational regulator important for mediating the integrated stress response. It is important to note that although, SIRT1 is a nuclear protein, we and others have demonstrated that SIRT1 also localizes in the cytoplasm of multiple cell types such as HeLa, 293, Jurkat and murine embryonic fibroblasts<sup>12, 19–21</sup>.

Phosphorylation of eIF2 $\alpha$  is one of the first steps in response to cellular stress. We examined the potential biological relevance of SIRT1-eIF2 $\alpha$  interaction in regulating the cellular stress response by investigating the effect of SIRT1 on eIF2 $\alpha$  phosphorylation in response to a variety of stress stimuli. We observed that the absence of SIRT1 results in an elevated and prolonged phosphorylation of eIF2 $\alpha$ , but reduced and delayed induction of the downstream stress response proteins CHOP and GADD34. It has been reported that under prolonged stressful conditions, cells lacking GADD34 have increased levels of phosphorylated eIF2 $\alpha$  and a sustained shutoff of protein synthesis, which interferes with expression of the downstream stress genes<sup>22, 23</sup>. A GADD34-holophosphatase complex tightly regulates the dephosphorylation of eIF2 $\alpha$  through a negative feedback loop in the later stages of the stress response in order to release the transient shut-off of protein synthesis caused by the initial stress response. Thus, it is possible that the elevated phosphorylation of eIF2 $\alpha$  in SIRT1-deficient cells is an indirect effect of impaired

dephosphorylation of eIF2 $\alpha$  at later stages of the stress signaling pathway. This model would be consistent with the observation that increased eIF2 $\alpha$  phosphorylation was associated with reduced, not increased, CHOP expression in SIRT1-deficient cells. We examined the effect of SIRT1 on GADD34 gene expression in response to stress and its potential impact on translation recovery. Our results demonstrated that GADD34 expression is also reduced in SIRT1-deficient cells, which could be responsible for a relatively weaker recovery from translation attenuation in SIRT1-deficient cells as compared to the SIRT1-positive cells.

The physical association of SIRT1 and eIF2 $\alpha$  indicate that in addition to transcriptional regulation of CHOP and GADD34, SIRT1 potentially regulates eIF2 $\alpha$  phosphorylation through direct interaction with proteins involved in eIF2 $\alpha$  phosphorylation or dephosphorylation. To understand the mechanistic importance of SIRT1-eIF2 $\alpha$  interaction, we examined if the deacetylase activity of SIRT1 or eIF2 $\alpha$  phosphorylation status affects the association between SIRT1 and eIF2 $\alpha$ . Our results demonstrate that the interaction of SIRT1-eIF2 $\alpha$  is independent of the catalytic activity of SIRT1 or stress regulated phosphorylation status of eIF2 $\alpha$ . The increased eIF2 $\alpha$  phosphorylation of SIRT1 depleted cells under normal condition suggested that the SIRT1 deficient cells potentially had a defect in maintaining the low steady-state level of phospho-eIF2 $\alpha$ . Additionally, the stress-independent association of SIRT1 with eIF2 $\alpha$  indicated that SIRT1 may have a constitutive role in the regulation of eIF2 $\alpha$  phosphorylation. This led us to examine the possible interaction of SIRT1 with mediators of eIF2 $\alpha$  dephosphorylation, GADD34 and CReP. Interestingly, we found that SIRT1 co-immunoprecipitates with both GADD34 and CReP. The interaction of SIRT1 with CReP, the constitutive de-phosphorylator of eIF2 $\alpha$ , further suggests a role for SIRT1 in maintaining the steady-state level of phospho-eIF2 $\alpha$ . In contrast, the



regulation of GADD34 by SIRT1 appears to be mediated both at the level of gene expression and through physical protein-protein interaction, further emphasizing the importance of SIRT1 in the stress-induced signaling pathway.

Our results suggest a model where SIRT1 regulates eIF2 $\alpha$  phosphorylation and the integrated stress response pathway, by affecting one or more proteins, and/or their recruitment to the eIF2 $\alpha$ -holophosphatase complex. The exact mechanism by which SIRT1 mediates the regulation of eIF2 $\alpha$  phosphorylation through GADD34 and/or CREP clearly warrants further investigation. Interestingly, eIF2 $\alpha$  phosphorylation was recently found to be required for stress-induced activation of PI3K-signaling<sup>24</sup>. Consistent with this, in addition to the demonstrated effect of SIRT1 depletion on eIF2 $\alpha$  phosphorylation, we also have observed increased mTOR activity under SIRT1 depletion<sup>21</sup>. Although, further investigation is needed to understand the precise mechanism through which the SIRT1-eIF2 $\alpha$  interaction regulates the stress signaling pathway, our results suggest that it involves both transcriptional and translational regulation of the integrated stress-response pathway.

## Methods

**Cell culture.** HeLa cells were purchased from American tissue culture consortium (ATCC) and cultured in DMEM media (Dulbecco's modified Eagle's media) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 100 U penicillin, 100 U streptomycin, 0.25 g/ml amphotericin B, HEPES buffer and 2 mM L-glutamine. The SIRT1 deficient HeLa cell line was generated using stable retroviral infection of shRNAi for SIRT1 as described in<sup>18</sup>. Cells were cultured under standard tissue culture conditions. The Sirt1-wild-type and Sirt1 null mouse embryonic fibroblasts (MEFs) were a gift from Dr. Michael McBurney, University of Ottawa, Canada. SIRT1-null MEFs stably expressing SIRT1 were generated by stable retroviral transfection of SIRT1-null MEFs with SIRT1-plasmid. The retroviral transduction and puromycin selection of SIRT1-expressing cells were performed as described in<sup>18</sup>.

**Stress treatments.** For various stress treatments, cells were seeded at about 70% confluency and cultured overnight as mentioned above. Cells were treated for indicated time periods in stress media such as: leucine negative media, glucose negative media, serum negative media, 2  $\mu$ g/ml tunicamycin (TM) and 2  $\mu$ M thapsigargin (TG) in normal growth media. Cells were then harvested using trypsin-EDTA for protein and mRNA isolation and analysis.

**Protein interaction assay, co-immunoprecipitation and western blotting.** The yeast two-hybrid assay was performed as described in<sup>18</sup>. The interaction between SIRT1 and eIF2 $\alpha$  was confirmed by co-immunoprecipitation assay. Whole cell lysates were made in NP-40 lysis buffer (20 mM TrisHCL, pH8.0, 137 mM NaCl, 10% glycerol, 1% nonidet P-40, 2 mM EDTA) with protease inhibitor cocktail (Sigma-aldrich). Protein concentration of the cell lysates was determined using a Bradford assay. For exogenous expression of epitope-tagged proteins, pCDNA-flag-SIRT1 and pCMV-myc-eIF2 $\alpha$  expression plasmids were transfected in HeLa cells using Lipofectamine<sup>TM</sup>2000 reagent as per manufacturer's protocol. 24 hr post-transfection, whole cell lysates were prepared as described above. For immunoprecipitation, 500  $\mu$ g of the cell lysate was incubated with specific protein antibody or epitope-tag antibody overnight at 4°C on a rotator. For SIRT1-GADD34/CreP CoIP, flag-tagged GADD34 and CreP (kindly provided by Dr. David Ron) were transfected in 293T cells. 48 h later, 2 mg of cell lysate was used for immunoprecipitation. As a negative control, normal IgG antibodies were used. Normal IgG for IP-SIRT1 and IP-Flag and -Myc is mouse-IgG, whereas for IP-eIF2 $\alpha$  the normal IgG is rabbit-IgG. Immunoprecipitated proteins were resolved by polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blot analysis. Antibodies used for immunoprecipitation are as followed: anti-SIRT1 (Upstate), anti-eIF2 $\alpha$  (Santa Cruz biotechnology), anti-flag-tag (Chemicon), anti-myc-tag 9E10 (Upstate), normal IgG (Santa Cruz biotechnology). Relative intensities were calculated after normalization with actin or total protein (where applicable) using image J software. Combined two-tailed p value across all group sets (or individual time point, where specified) from at least two independent experiments was calculated using student's t-test.

**Protein synthesis assay by <sup>35</sup>S-methionine incorporation.** SIRT knock-out and wild-type mouse embryonic fibroblasts were seeded in 6 well plates at a density of 4.5  $\times$  10<sup>5</sup> cells/well. Cells were stress treated for 1 hr with 2  $\mu$ M thapsigargin and incubated in normal growth media for the rest of the time. 30 min prior to the specific time points, cells were depleted for methionine and cysteine by replacing the media with methionine/cystein negative growth media (Gibco DMEM - methionine and -cystine). At specific time points, cells were pulsed with 200  $\mu$ Ci/ml <sup>35</sup>S-protein labeling mix (Easy-tag Express <sup>35</sup>S-protein labeling mix, Perkin-Elmer) and incubated at 37°C incubator for 30 min after adding the radioactivity. Protein extracts were resolved by SDS-PAGE and examined for radioactivity by exposure to Kodak BioMax MR-films.

**Quantitative PCR.** Total cell RNA was reverse-transcribed and assayed by quantitative real-time PCR on an instrument (MX3000P; Stratagene) using SYBR green incorporation. The expression of genes was normalized to that of  $\beta$ -actin and expressed relative to the indicated reference sample (average  $\pm$  SD of triplicate reactions). Relative expression was calculated using the  $\Delta\Delta C_T$  method. Primer sequence for CHOP: 5'GCAGCTGAGTCCCTGCCTTT3', 5'TGAAGTTTTTGATTCTTCTCTC3' and GADD34: 5'CAGAGGAAGAAGACAGCGATTGCG3', 5'TCAGGAAGGGACTTGTATGTGGG3'.

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## Author contributions

HSG performed all the experiments in and wrote the first draft of the manuscript. BR and PDR interpreted experiments, provided financial support and edited the manuscript.





## Additional information

**Competing financial interests:** The authors declare no competing financial interests.

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