

SIRT1 Protects against α -Synuclein Aggregation by Activating Molecular Chaperones

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α -Synuclein is a key molecule in the pathogenesis of synucleinopathy including dementia with Lewy bodies, Parkinson's disease, and multiple system atrophy. Sirtuins are NAD⁺-dependent protein deacetylases that are highly conserved and counter aging in lower organisms. We show that the life span of a mouse model with A53T α -synuclein mutation is increased by overexpressing SIRT1 and decreased by knocking out SIRT1 in brain. Furthermore, α -synuclein aggregates are reduced in the brains of mice with SIRT1 overexpression and increased by SIRT1 deletion. We show that SIRT1 deacetylates HSF1 (heat shock factor 1) and increases HSP70 RNA and protein levels, but only in the brains of mice with A53T and SIRT1 expression. Thus, SIRT1 responds to α -synuclein aggregation-induced stress by activating molecular chaperones to protect against disease.

Introduction

α -Synuclein is a 140 aa protein, mainly localized in presynaptic terminals in brain. Although the detailed physiological functions of α -synuclein are still not known, recent studies suggest that it plays a key role in synaptic functions (Dev et al., 2003; Eriksen et al., 2003). α -Synuclein is normally an unstructured and soluble protein. However, in the subgroup of neurodegenerative disorders termed "synucleinopathies," α -synuclein is known to polymerize into fibrils and to accumulate in pathologic hallmark inclusions, such as Lewy bodies (LB), Lewy neuritis (LN), and glial cytoplasmic inclusions (Takeda et al., 2006). α -Synuclein pathology is found in the dementia with Lewy bodies, Parkinson's disease (PD), and multiple system atrophy (Arima et al., 1998a,b). The LB and LN are characteristic of PD, and point mutations or gene multiplications of α -synuclein are responsible for familial PD. α -Synuclein pathology is also found in both sporadic and familial cases with Alzheimer's disease (Yokota et al., 2002). These findings suggest that abnormal α -synuclein metabolism plays a key role in neurodegenerative processes in synucleinopathies, but the precise underlying mechanisms remain unknown. The discovery of PD-related genes (α -synuclein, UCH-L1, parkin, LRRK2, PINK 1, DJ-1) led to the hypothesis

that misfolding of proteins and the dysfunction of the ubiquitin–proteasome pathway are pivotal to α -synuclein pathogenesis (Muchowski and Wacker, 2005; Morimoto, 2008; Broadley and Hartl, 2009). Mitochondrial dysfunction and oxidative stress in aging cause the accumulation of misfolded proteins, in addition to producing other deleterious events in dopaminergic neurons (Dauer and Przedborski, 2003).

Sirtuins are NAD-dependent deacetylases that counter aging in lower organisms and have a wide spectrum of metabolic and stress tolerance functions (Donmez and Guarente, 2010; Haigis and Sinclair, 2010). Recently, SIRT1 was shown to suppress β -amyloid production by activating the α -secretase gene ADAM10 (Donmez et al., 2010). Remarkably, SIRT1 also suppressed tau protein aggregation, responsible for tangles within affected neurons (Min et al., 2010). SIRT1 was shown to protect against neurodegeneration in p25 overexpression mice (Kim et al., 2007), as well as in Wallerian degeneration slow mice (Araki et al., 2004). Little is known about the effect of sirtuins in PD, but one study showed that the SIRT2 inhibitors rescue against α -synuclein-mediated toxicity in a cellular and a *Drosophila* model of PD (Outeiro et al., 2007). Albani et al. (2009) also showed that resveratrol, a putative SIRT1 activator, protects SK-N-BE cells from oxidative stress and α -synuclein-mediated toxicity. Therefore, it is very important to find out whether SIRT1 will be protective against α -synuclein aggregation as it is against Alzheimer's disease or will exacerbate the disease, as we consider sirtuin-based drug therapies.

Molecular chaperones, in particular heat shock proteins (Hsps) are crucial to control the aggregates in neurodegenerative diseases. Cells avoid accumulating potentially toxic aggregates by activating molecular chaperones that either refold or promote degradation of misfolded proteins by the proteasome. Of relevance to synucleinopathies, overexpression of Hsp70 is protective against α -synuclein aggregates (Auluck et al., 2002; Klucken et al., 2004; Luk

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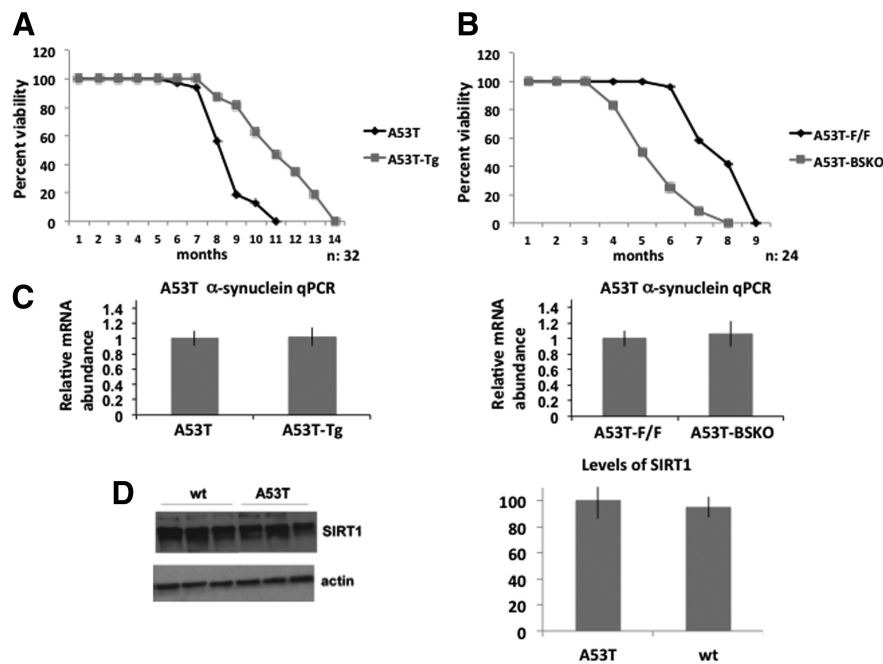


Figure 1. SIRT1 prolongs the life span of homozygous A53T mouse model—mice expressing α -synuclein gene with A53T mutation. **A**, Survival analysis of A53T and A53T mice overexpressing SIRT1 (A53T-Tg). $n = 32$ for each genotype. **B**, Survival analysis of A53T and A53T mice lacking SIRT1 in brain (A53T-BSKO). $n = 24$ for each genotype. Graphs show percentage of survival scored monthly. **C**, A53T α -synuclein RNA levels quantified from whole brains of A53T and A53T-Tg or A53T-F/F and A53T-BSKO mice by qPCR. $n = 4$ for each indicated genotype. **D**, Western blotting of SIRT1 in whole-brain extracts from A53T mice and wt littermates. $n = 6$ for each genotype. Quantification is shown on the right. A representative blot is shown. The statistical analysis in **C** and **D** was performed using Student's t test. Error bars in figures represent SEM.

et al., 2008). Furthermore, introducing an Hsp70 transgene into α -synuclein mutant transgenic mice led to significant reduction in detergent-insoluble α -synuclein aggregates and protected cells from toxicity (Klucken et al., 2004). Another study showed that Hsp70 reduced the amount of aggregated α -synuclein *in vivo* and *in vitro* (McLean et al., 2004). However, Shimshek et al. (2010) demonstrated that Hsp70 is not beneficial in a mouse model of α -synucleinopathy. Interestingly, SIRT1 has been shown to deacetylate heat shock factor 1 (HSF1), activate its binding to the Hsp70 promoter, and increase Hsp70 transcription under heat shock conditions in cultured cells (Westerheide et al., 2009). By bridging high-throughput genetic and transcriptional data, Yeager-Lotem et al. (2009) identified a relationship between α -synuclein toxicity and heat shock response, which is conserved from yeast through mammals. HSF1 appears to be downstream of the α -synuclein toxicity suppressor Gip2, since strains overexpressing Gip2 showed elevated concentrations of heat shock proteins (Yeager-Lotem et al., 2009).

Materials and Methods

Mouse strains. All mice used were males and in congenic C57BL/6. Transgenic A53T mice (Giasson et al., 2002) were purchased from The Jackson Laboratory and backcrossed for seven generations. A53T α -synuclein gene expression in this mouse model is driven by the prion promoter. For experiments, only homozygous A53T mice were used. SIRT1 transgenic mice have been described previously (Bordone et al., 2007). SIRT1 gene expression in this mouse model is driven by the actin promoter. SIRT1 brain-specific knock-out mice were generated by crossing a SIRT1 allele containing a floxed exon 4 (Cheng et al., 2003) with Cre-expressing mice driven by the brain-specific nestin promoter (Cohen et al., 2009; Donmez et al., 2010). All mice were housed at controlled temperature (25°C) and 12 h light/dark cycle.

Plasmids. The plasmid pBABE-mSIRT1 has been described previously (Li et al., 2007). The plasmids expressing mSIRT1 and Hsp70 were purchased from Addgene. The SIRT1-shRNA and the Hsp70-shRNA plasmids were purchased from Open Biosystems.

Immunohistochemistry. Mice were perfused with 4% paraformaldehyde, cryoprotected, sectioned 40 μ m thick, and collected at 150 μ m intervals. Twelve sections per brain were analyzed. Vectastain kit (Vector Laboratories) were used to perform α -synuclein and glial fibrillary acidic protein (GFAP) staining according to the manufacturer's directions by using α -synuclein (Abcam) and GFAP (Abcam) antibodies. α -Synuclein aggregates and GFAP staining were quantified using NIH ImageJ program. For proteinase K treatment, sections were covered with proteinase K working solution [8 mg of proteinase K (30 U/mg), 10 ml of TE-CaCl₂ buffer, pH 8.0, 10 ml of glycerol], incubated 10–20 min at 37°C in humidified chamber, cooled at room temperature for 10 min, rinsed in PBS Tween 20 twice for 2 min, blocked for 30 min, and then followed by the standard immunohistochemistry procedure with Vectastain kit.

Western blotting and immunoprecipitation. Mouse brains were homogenized in RIPA buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1% SDS, 150 mM NaCl, 1% NP-40, 0.1% sodium deoxycholate) including Complete protease inhibitor mixture (Roche) and centrifuged, and 100 μ g of the supernatant was loaded onto 4–15% gradient SDS-PAGE gels and immunoblotted with anti-SIRT1 (Millipore), α -synuclein, HSF1 (ab2923), Hsp70 (Abcam), and Ac-K (ImmuneChem) antibodies. For Western blotting using cells, cells were harvested and extracted in RIPA buffer as explained above. The extraction of detergent-soluble and -insoluble α -synuclein aggregates from brain was performed according to the reference (Kahle et al., 2001). The procedure is the following. After the mouse brain is homogenized in RIPA buffer, Triton X-100 was added to the final concentration of 1% to the total brain lysate before the sonication step. After sonication, the brain lysate was centrifuged at 1000 $\times g$. The supernatant from this step was centrifuged at 130,000 $\times g$. The pellet was then extracted in 5% SDS and once more centrifuged at 130,000 $\times g$. The supernatant from this step is the detergent-soluble fraction loaded onto the gel. The remaining pellet was extracted in 8 M urea and loaded onto the gel as the detergent-insoluble fraction. Western blotting experiments were performed with at least six mice from each genotype and age, and the representative blots are shown.

The immunoprecipitations were performed by using Pierce Direct IP kit (Thermo Fisher Scientific). Immobilizing the antibody covalently to agarose beads, this method results in purified antigen free from antibody contamination. To show the endogenous interaction between SIRT1 and HSF1, anti-SIRT1 antibody or normal rabbit serum (NRS) was coupled to beads, and then wild-type (wt) and SIRT1^{-/-} (SIRT1^{-/-}) mouse embryo fibroblasts (MEFs) were incubated with the beads. To determine HSF1 acetylation, anti-HSF1 antibody or NRS was coupled to beads, and then wt, SIRT1^{-/-} (SIRT1^{-/-}), and SIRT1-overexpressing (mSIRT1) MEFs were incubated with the beads. The eluate was blotted with anti-SIRT1, anti-HSF1, and anti-Ac-K antibodies. When performing IPs directly from mice brains, the brain extracts [wt, transgenic (Tg), wild-type (F/F), BSKO (F/F, Cre), A53T, A53T-Tg, A53T (A53T-F/F), A53T-BSKO (A53T-F/F, Cre)] were incubated with the beads. To determine HSF1 acetylation, anti-HSF1 antibody or NRS was coupled to beads, and then brains extracts (see above) were incubated with the beads. The eluate was blotted with anti-SIRT1, anti-HSF1, and anti-Ac-K antibodies.

RNA isolation and analysis. Total RNA from mice brains was isolated by using Trizol (QIAGEN). For real-time qPCR analysis, cDNA was

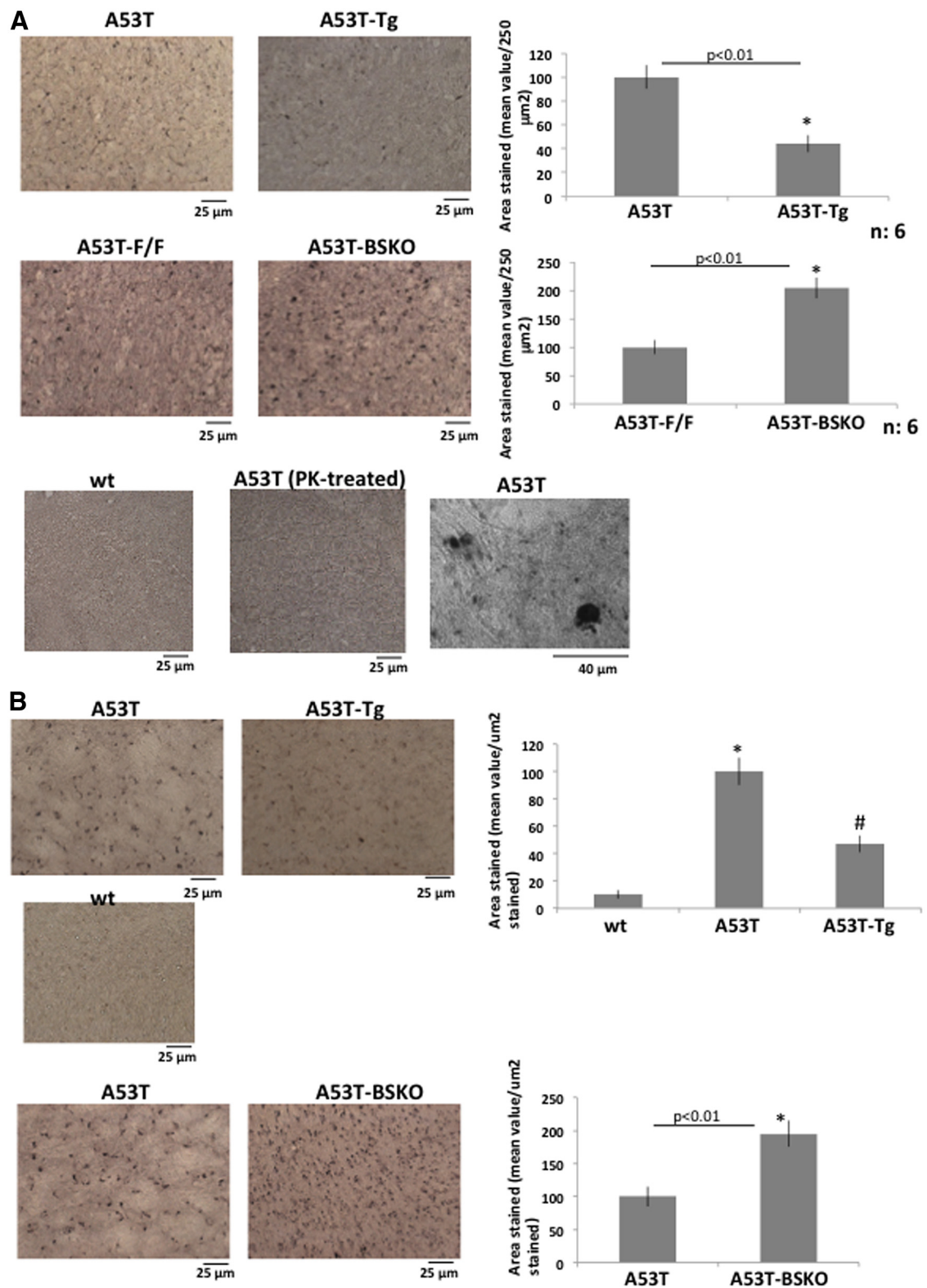


Figure 2. SIRT1 decreases α -synuclein aggregates in A53T mouse brain. **A**, Immunostaining of α -synuclein aggregates in cortical sections of 3.5-month-old A53T and A53T-Tg mice (top panel) and A53T-F/F and A53T-BSKO mice (middle panel). Quantification is shown on the right. $n = 6$ for each genotype. There were no α -synuclein aggregates observed in wt mice or proteinase K-treated cortical sections of A53T mice (bottom panel, left and middle, respectively). Higher magnification picture of α -synuclein aggregates is also demonstrated (bottom panel, right). The statistical analysis was performed using Student's *t* test, and significant differences are demonstrated by a single asterisk (*) indicating $p < 0.01$. Error bars in figures represent SEM. **B**, Immunostaining of gliosis in cortical sections of A53T, A53T-Tg, and wt mice (top and middle panels) and A53T-F/F and A53T-BSKO mice (bottom panel) is performed by using GFAP antibody. Quantification is shown on the right. $n = 6$ for each genotype. The statistical analysis was performed by two-way ANOVA in the top panel, and significant differences are demonstrated by single asterisk (*) or pound sign (#) indicating $p < 0.01$. In the bottom panel, Student's *t* test was performed for statistical analysis; significant differences are demonstrated by single asterisk (*) indicating $p < 0.01$. Error bars in figures represent SEM.

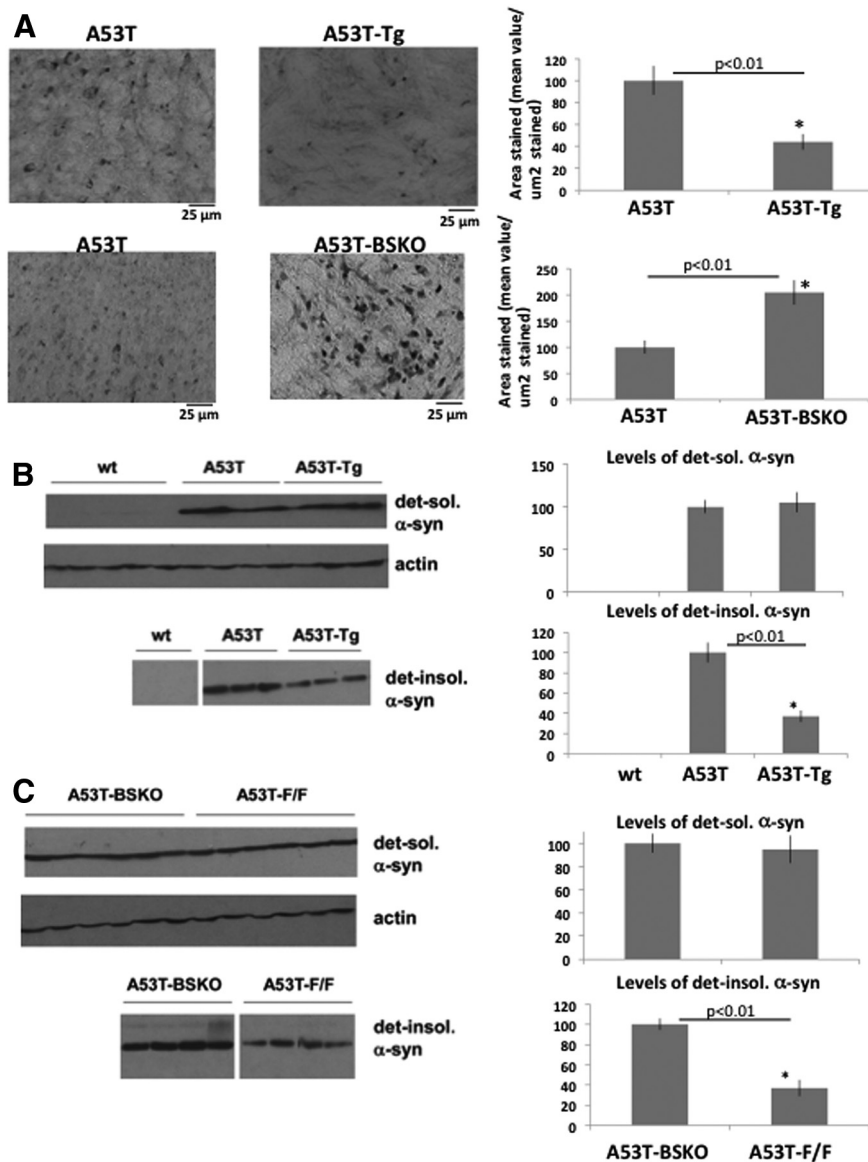


Figure 3. SIRT1 decreases α -synuclein aggregates in A53T mouse brain. **A**, Immunostaining of α -synuclein aggregates in the brainstem sections of A53T and A53T-Tg mice (top panel) and A53T-F/F and A53T-BSKO mice (bottom panel). Quantification is shown on the right. $n = 6$ for each genotype. The statistical analysis was performed using Student's t test, and significant differences are demonstrated by single asterisk (*) indicating $p < 0.01$. Error bars in figures represent SEM. **B**, Western blotting of detergent-soluble (top panel) and detergent-insoluble (bottom panel) α -synuclein fractions extracted from whole brain of indicated mice (1 mouse per lane). $n = 6$ for each genotype. Quantification is shown on the right. Representative blots are shown. **C**, Western blotting of detergent-soluble (top panel) and detergent-insoluble (bottom panel) α -synuclein fractions extracted from whole brain of indicated mice (1 mouse per lane). $n = 6$ for each genotype. Representative blots are shown. Quantification is shown on the right. The statistical analysis in **B** and **C** was performed using Student's t test, and significant differences are demonstrated by a single asterisk (*) indicating $p < 0.01$. Error bars in figures represent SEM.

synthesized from total RNA by SuperScript III reverse transcriptase (Invitrogen) with random primers. The cDNA was then subjected to PCR analysis with gene-specific primers in the presence of SYBR Green (Bio-Rad). Relative abundance of mRNA was obtained by normalization to 18S levels.

Cells and transfection. SIRT1^{+/+} and SIRT1^{-/-} MEFs have been described previously (Li et al., 2007). A53T α -synuclein cDNA was subcloned into the pTRE vector from Clontech. Stable cell lines were made by cotransfecting H4 cells with the pTRE-synuclein (hygromycin) and pTet-off (G418). Tet-off system from Clontech was used. The cells are cultured for 48 h after Dox removal to have the maximal α -synuclein expression. The plasmids expressing mSIRT1 and Hsp70 were purchased from Addgene. The SIRT1-shRNA and the Hsp70-shRNA plasmids were purchased from Open Biosystems. Transfections were performed by using Effectene transfection reagent (QIAGEN).

Toxicity assay. Toxicity was analyzed 24 h after transfection by using the ToxiLight assay from Lonza, according to the manufacturer's protocol.

Statistical analysis. The analysis was performed using Student's t test or two-way ANOVA. The type of statistical analysis used is indicated in each figure legend. Significant differences are demonstrated by single symbols (*, #, €, ¥) indicating $p < 0.01$. Error bars in figures represent SEM.

Results

SIRT1 prolongs the life span of homozygous A53T mouse

We first wanted to test whether SIRT1 could protect against α -synuclein pathology in a transgenic mouse bearing the human α -synuclein gene with the A53T mutation (Giasson et al., 2002), which causes familial early-onset PD. Although this mouse model is not a true model of PD, since these animals lack overt neurodegeneration of the nigrostriatal dopaminergic pathway and no loss of dopaminergic neurons is observed, it is regarded as a model of synucleinopathy and proteinopathy. For simplicity, we will refer to these mice as "A53T mice." This mouse model, when homozygous and backcrossed to C57BL/6 for seven generations, had a life span of 7–8.5 months. α -Synuclein aggregates appeared at ~2.5–3 months of age and they increased as mice aged. To test the effects of altering levels of SIRT1 in these A53T mice, they were crossed to SIRT1 transgenic mice (denoted as Tg) and SIRT1 brain-specific knock-out mice (denoted as BSKO). A53T mice are the littermate controls for the A53T-Tg mice and A53T-F/F mice are the littermate controls for A53T-BSKO (A53T-F/F, Cre) mice. The life span of A53T mice overexpressing SIRT1 (A53T-Tg) was significantly extended compared with A53T mice (Fig. 1A). In contrast, the life span of A53T mice lacking SIRT1 in brain (A53T-BSKO) was shortened compared with A53T (A53T-F/F) mice (Fig. 1B). SIRT1 Tg or SIRT1 BSKO mice have a roughly normal life span compared with wild-type mice (Jeong et al., 2011). Neither the SIRT1 transgene nor the knock-out allele affected expression of the A53T α -synuclein gene (Fig. 1C). A53T α -synuclein RNA levels in the brain were very similar when A53T mice were compared with A53T-Tg mice (Fig. 1C, left panel), and when A53T-F/F mice were compared with A53T-BSKO mice (Fig. 1C, right panel). Similarly, SIRT1 protein levels were similar when whole-brain extracts from A53T mice and wt littermates were compared (Fig. 1D).

SIRT1 decreases α -synuclein aggregates in A53T mouse brain

We then analyzed the status of α -synuclein aggregates by immunostaining and quantifying cortical α -synuclein aggregates in serial coronal cross sections of 3.5-month-old C57BL/6 littermates.

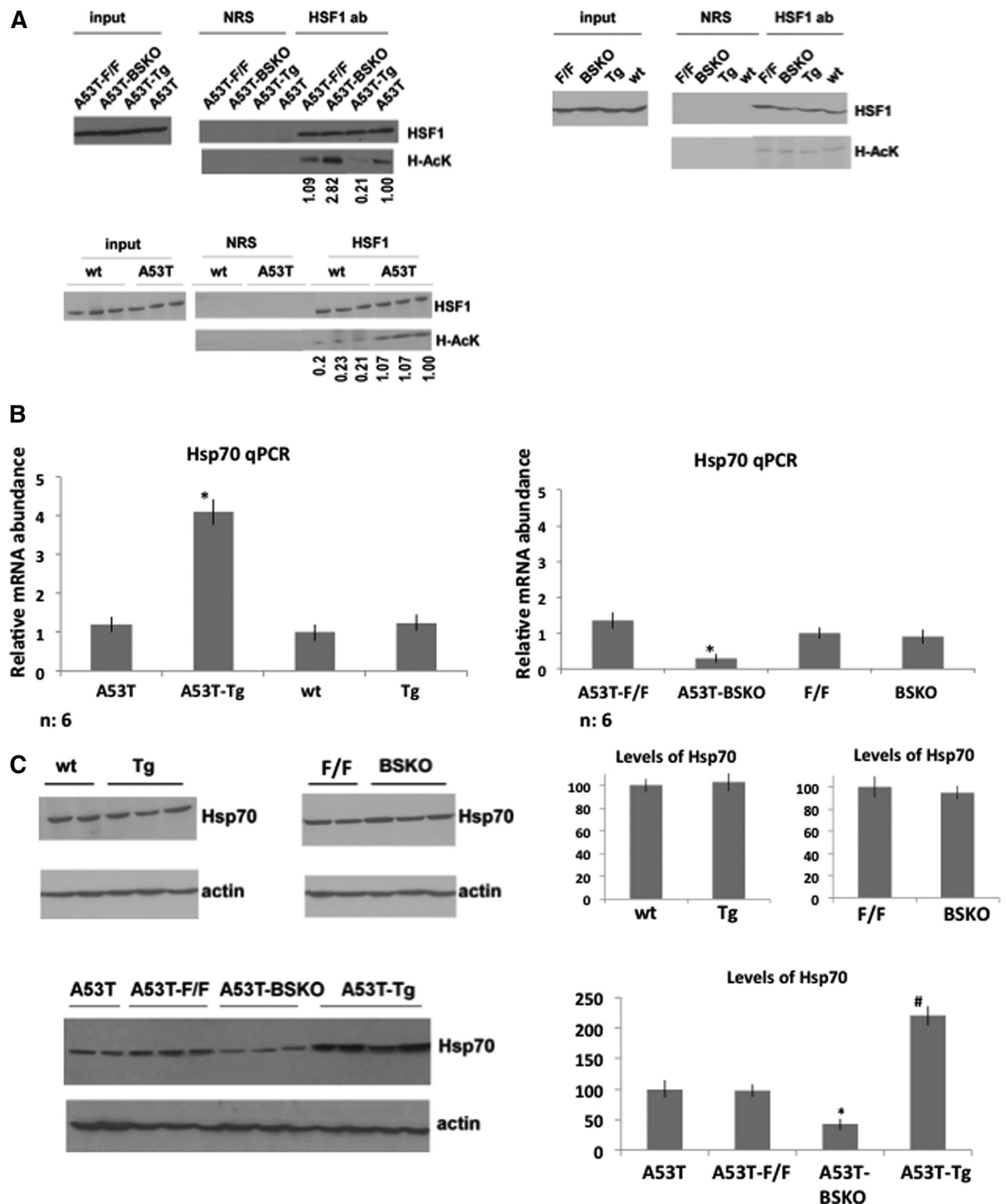


Figure 4. SIRT1 deacetylates HSF1 and increases Hsp70 levels in brains of A53T mice. **A**, Left, Whole-brain extracts from A53T mice: A53T-wt, A53T-Tg, A53T-F/F, and A53T-BSKO immunoprecipitated with NRS or anti-HSF1 antibody and blotted with anti-HSF1 or anti-acetyl lysine (H-AcK) antibodies. Right, Whole-brain extracts from mice without A53T α -synuclein gene: wt, Tg, F/F, and BSKO immunoprecipitated with NRS or anti-HSF1 antibody and blotted with anti-HSF1 and anti-acetyl lysine (H-AcK) antibodies. $n = 4$ for each genotype. Representative immunoblots are shown. Bottom panel, Whole-brain extracts from A53T mice and wt littermates were immunoprecipitated with NRS or anti-HSF1 antibody and blotted with anti-HSF1 and anti-acetyl lysine (H-AcK) antibodies. $n = 6$ for each genotype. Representative immunoblots are shown. Acetylated bands are quantified by using ImageJ program. **B**, Hsp70 RNA levels quantified from whole brains of mice by qPCR. $n = 6$ for each indicated genotype. The statistical analysis is performed by using two-way ANOVA; significant differences are demonstrated by a single asterisk (*) indicating $p < 0.01$. Error bars in figures represent SEM. **C**, Western blotting of Hsp70 protein extracted from whole brains of wt, Tg, F/F, and BSKO mice without (top panel) or with the A53T α -synuclein gene (bottom panel). $n = 4$ for each genotype. Representative immunoblots are shown. Quantification is shown on the right. For the top panel, statistical analysis was performed using (Figure legend continues.)

α -Synuclein aggregates were decreased in A53T-Tg mice compared with A53T mice (Fig. 2A, top panel), while α -synuclein aggregates were increased in A53T-BSKO mice compared with A53T-F/F mice (Fig. 2A, middle panel). We have not observed any α -synuclein aggregates in wt mice or proteinase K (PK)-treated A53T mice brain (Fig. 2A, bottom panel, left and middle, respectively). Higher magnification picture of α -synuclein aggregates is also demonstrated here (Fig. 2A, bottom panel, right). We have analyzed gliosis in cortical sections adjacent to those in Figure 2A by using an antibody against GFAP, which is an inflammation marker in brain. We observed that gliosis was decreased in A53T-Tg mice compared with A53T mice (Fig. 2B, top panel) and increased in A53T-BSKO mice compared with A53T-F/F mice (Fig. 2B, bottom panel). In A53T brain, α -synuclein aggregates are also seen in the brainstem. Similar to the cortex, α -synuclein aggregates were decreased in A53T-Tg mice compared with A53T mice (Fig. 3A, top panel), and increased in A53T-BSKO mice compared with A53T-F/F mice (Fig. 3A, bottom panel). We also used differential extraction methods (Kahle et al., 2001) (see Materials and Methods) to quantify detergent-soluble α -synuclein and detergent-insoluble aggregates. Interestingly, we did not observe any difference in the detergent-soluble α -synuclein species (Fig. 3B,C, top panels). In contrast, detergent-insoluble α -synuclein was decreased in A53T-Tg mice compared with A53T mice (Fig. 3B, bottom panel) and increased in A53T-BSKO mice compared with A53T-F/F mice (Fig. 3C, bottom panel).

SIRT1 deacetylates HSF1 and increases Hsp70 levels in brains of A53T mice

The differences in detergent-insoluble α -synuclein prompted us to test whether molecular chaperones may be mechanistically involved. Since SIRT1 deacetylates HSF1 under heat shock conditions in cultured cells (Westerheide et al., 2009), we checked whether SIRT1 deacetylates HSF1 in murine brains. We initially found that acetylation levels of HSF1 were very low and did not change in brains of SIRT1 Tg, wt, or BSKO (F/F, Cre) and wild-type (F/F) mice without the A53T α -synuclein gene (Fig. 4A, top right). Importantly, acetylation levels of HSF1 were decreased in A53T-Tg mice compared with A53T and increased in A53T-BSKO compared with A53T-F/F mice, indicating that SIRT1 deacetylates HSF1 in A53T mice brains (Fig. 4A, top left). This shows that SIRT1 affects the acetylation status of HSF1, but only in brains expressing A53T α -synuclein. Importantly, we also checked the acetylation level of HSF1 in A53T mice and compared with wt littermates and confirmed that A53T α -synuclein gene expression triggers HSF1 acetylation (Fig. 4A, bottom panel).

Since HSF1 binds to the Hsp70 promoter and activates its transcription, we also analyzed the Hsp70 RNA levels in brains of mice by quantitative RT-PCR (Fig. 4B). Hsp70 RNA levels were increased in A53T-Tg compared with A53T mice (Fig. 4B, left) and decreased in A53T-BSKO compared with A53T-F/F mice (Fig. 4B, right). The RNA levels of Hsp70 were not changed between wt and Tg brain or wt (F/F) and BSKO brain not expressing A53T α -synuclein (Fig. 4B). In addition, Hsp70 protein levels

were increased in A53T-Tg brains compared with A53T and decreased in A53T-BSKO compared with A53T-F/F (Fig. 4C, bottom panel), while the levels were not changed in wt, Tg, and SIRT1 BSKO brains not expressing A53T α -synuclein (Fig. 4C, top panel).

SIRT1 deacetylates HSF1, activates Hsp70, and protects against α -synuclein toxicity in cells

Our data show that overexpressed SIRT1 deacetylates HSF1 and activates Hsp70 only in A53T mice brains and not in mice brains without the A53T α -synuclein gene, where HSF1 acetylation is very low. This suggests that SIRT1 deacetylates HSF1 and activates chaperones under stress conditions induced by the α -synuclein gene to potentiate the heat shock response. To further test this idea, we subjected wt MEFs and SIRT1 KO MEFs to heat shock (1 h at 42°C) and then analyzed the interaction of endogenous SIRT1 with HSF1, as well as the activation of Hsp70. Only under heat shock conditions, did SIRT1 bind to HSF1 (Fig. 5A, compare left, right). Furthermore, SIRT1 deacetylated HSF1 after heat shock (Fig. 5B, compare left, right). Without heat shock, HSF1 acetylation was low regardless of SIRT1 levels (Fig. 5B, right). In addition, Hsp70 protein levels increased after heat shock in wild-type MEFs (Fig. 5C), but to a greater extent in SIRT1-overexpressing MEFs. However, Hsp70 levels were not induced in SIRT1^{-/-} cells after heat shock (Fig. 5C). These findings show that SIRT1 is required for induction of Hsp70 by heat shock in normal cells and that SIRT1 overexpression results in HSF1 deacetylation and hyperinduction of the heat shock response.

We next investigated whether SIRT1 would be protective against A53T α -synuclein toxicity by using H4 cells that express A53T α -synuclein controlled by the tet-off system. We note that A53T α -synuclein expression in H4 cells do not form observable oligomers/aggregates. Figure 5, E and F, shows the SIRT1 and Hsp70 protein levels, respectively, upon overexpression and knockdown. Readings were low and did not change when SIRT1 or Hsp70 was overexpressed in control cells not expressing A53T (Fig. 5D, 1, 2, 3). However, A53T triggered toxicity and SIRT1 overexpression decreased this toxicity (Fig. 5D, compare 4, 5), while silencing SIRT1 with shRNA increased toxicity (Fig. 5D, compare 4, 6). Moreover, overexpressing Hsp70 also decreased toxicity in this assay (Fig. 5D, compare 4, 7). Conversely, silencing Hsp70 increased toxicity compared with vector (Fig. 5D, compare 4, 8), and SIRT1 overexpression in these knockdown cells partially decreased toxicity (9), but not to the levels in cells with normal Hsp70 (4). Since these experiments did not clearly discern whether SIRT1 and Hsp70 worked via the same or different pathways, we next used a synthetic enhancement approach by silencing SIRT1 and Hsp70 at the same time. A53T toxicity levels in cells expressing both SIRT1 shRNA and Hsp70 shRNA (10) were not significantly higher compared with cells expressing shRNA for SIRT1 (6) or Hsp70 (8) alone. The latter experiment suggests that SIRT1 and HSF1 function in the same pathway to mitigate A53T toxicity. Scrambled-shRNAs did not have any effect on the toxicity levels (Fig. 5D, 11, 12).

Finally, silencing of SIRT1 increased acetylation of HSF1 and overexpression of SIRT1 decreased acetylation of HSF1, showing that SIRT1 deacetylates HSF1 in challenged H4 cells (Fig. 6, left panel). The degree of HSF1 deacetylation corresponded to the levels of Hsp70 RNA in these cells (Fig. 6, right panel). These data suggest that SIRT1 deacetylates HSF1 and can protect H4 cells from α -synuclein-dependent toxicity by activating Hsp70.

←

(Figure legend continued.) Student's *t* test, and significant differences are demonstrated by a single asterisk (*) indicating $p < 0.01$. For the bottom panel, statistical analysis was performed by using two-way ANOVA, and significant differences are demonstrated by single asterisk (*) and pound sign (#) indicating $p < 0.01$. Error bars in figures represent SEM.

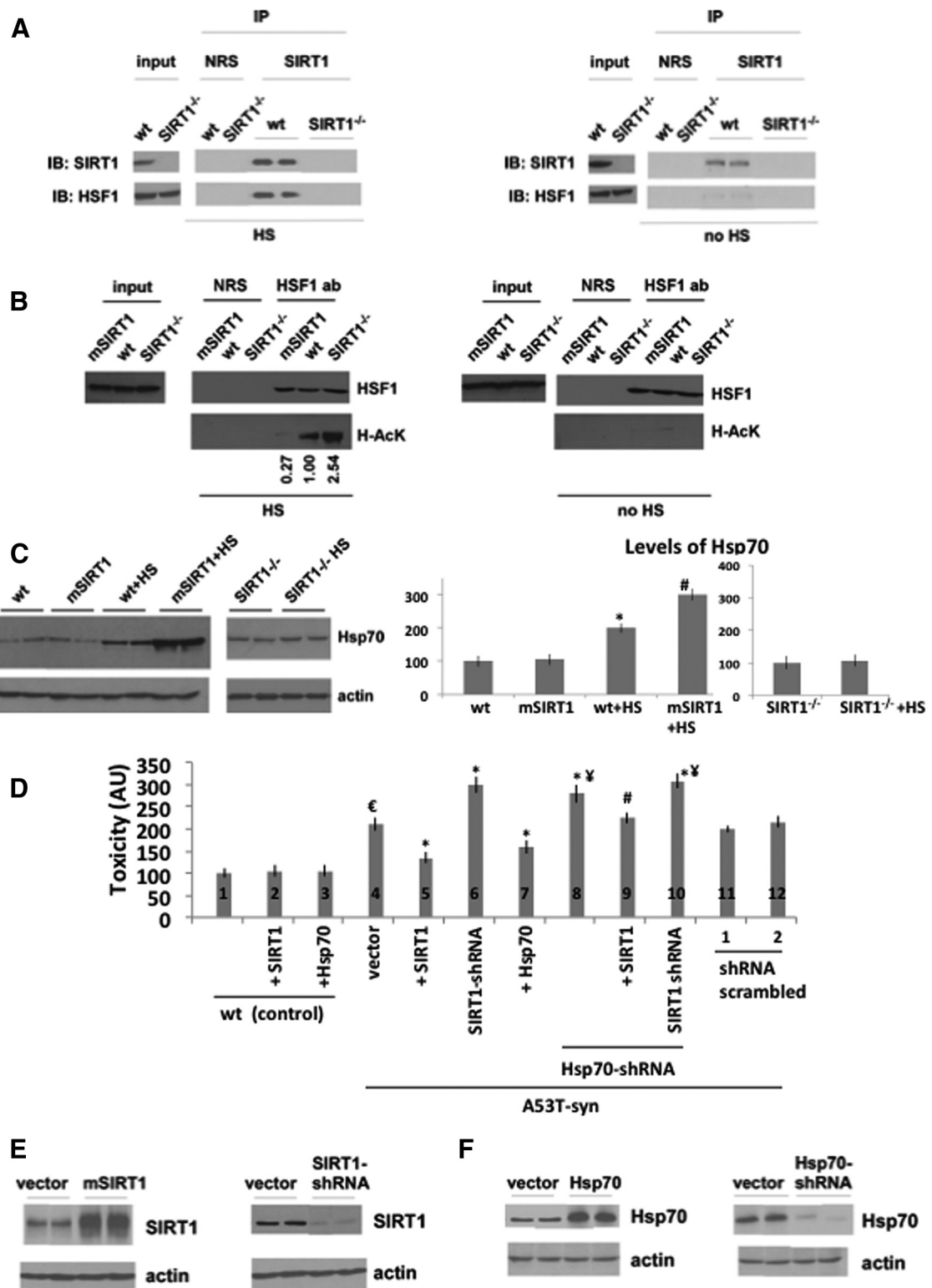


Figure 5. SIRT1 deacetylates HSF1 and activates Hsp70 in MEFs and protects against α -synuclein toxicity in cells. **A**, Cell lysates from wt and SIRT1^{-/-} MEFs were immunoprecipitated with NRS or anti-SIRT1 antibody after being subjected to heat shock (HS) and then blotted with anti-SIRT1 and anti-HSF1 antibodies (left panel). The control cells without heat shock (no HS) are shown on the right panel. The two proteins are shown to interact at endogenous levels only under heat shock conditions. **B**, SIRT1 deacetylates HSF1 only under heat shock conditions. Cell lysates from wt, SIRT1-overexpressing MEFs (mSIRT1), SIRT1^{-/-} MEFs were immunoprecipitated with anti-HSF1 antibody or NRS after being subjected to heat shock (HS) and analyzed by Western blotting with anti-HSF1 or anti-pan acetylated lysine (Ac-K) antibodies (left panel). The control cells without heat shock (no HS) are shown on the right panel. Acetylated bands are quantified by using ImageJ. **C**, Western blotting of Hsp70 protein from the lysates of wt, SIRT1-overexpressing (mSIRT1), and SIRT1^{-/-} MEFs with (HS) or without being subjected to heat shock. Actin serves as a loading control. The experiment was performed four times. Representative blots are shown. Quantification is shown below. Statistical analysis was performed using two-way ANOVA, and significant differences are demonstrated by a single asterisk (*) indicating $p < 0.01$. Error bars in figures represent SEM. **D**, Toxicity levels of H4 cells that express A53T α -synuclein by the inducible tet-off system (1 μ g/ml doxycycline). The toxicity levels were increased by removing doxycycline (samples 4–12). Overexpression of SIRT1 (2) or Hsp70 (3) had no effect on control cells (compare 1 with 2 or 3). However, overexpression of SIRT1 in A53T α -synuclein-expressing cells (compare 4, 5) or overexpression of Hsp70 (compare 4, 7) decreased toxicity. SIRT1 shRNA (6) or Hsp70 (Figure legend continues.)

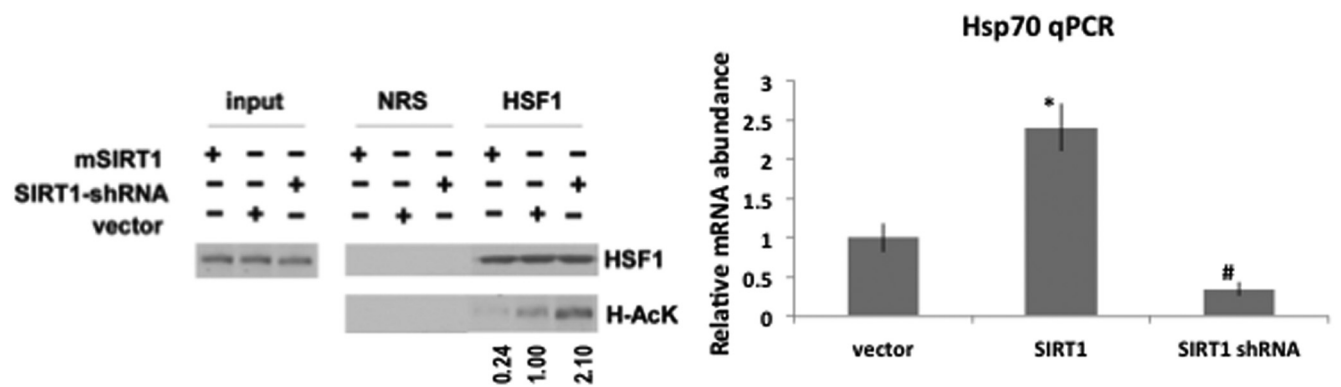


Figure 6. SIRT1 deacetylates HSF1 and activates Hsp70 in A53T α -synuclein-expressing H4 cells. Left, Lysates from A53T-overexpressing H4 cells transfected by SIRT1 (mSIRT1), SIRT1-shRNA, or vector immunoprecipitated with anti-HSF1 antibody or NRS and blotted with anti-HSF1 or anti-pan-acetylated lysine (Ac-K) antibodies. Right, Hsp70 RNA levels quantified from cell lysates of A53T-overexpressing H4 cells transfected by SIRT1 (mSIRT1), SIRT1-shRNA, or vector quantified by qPCR. The analysis was performed using two-way ANOVA, and significant differences are demonstrated by single asterisk (*) or pound sign (#) indicating $p < 0.01$. Error bars in figures represent SEM.

Discussion

We show in this study that SIRT1 protects against α -synuclein aggregation and A53T α -synuclein-induced toxicity. α -Synuclein aggregates in the A53T model result from misfolding of this protein. Decreases in these aggregates and mitigation of disease can occur by mechanisms that prevent the misfolding or clear the aggregates by degradation. Molecular chaperones like heat shock proteins perform these tasks in cells. Our findings show a close relationship between SIRT1 and the heat shock response. In MEFs, we show that SIRT1 is required for the thermal induction of the heat shock protein Hsp70, and overexpression of SIRT1 yields an increase in this induction. In mice, we observe a synthetic effect of A53T α -synuclein gene as a stressor and SIRT1 overexpression in the induction of Hsp70. In mice without the A53T α -synuclein gene, Hsp70 is not induced regardless of the levels of SIRT1, and the activator of its transcription, HSF1, is not acetylated. In A53T mice, an increase in HSF1 acetylation is triggered, which is likely to occur by a stress-induced conformational change that exposes the factor to HATs and deacetylases. However, the ability of this conformer to activate Hsp70 requires the additional step of deacetylation by SIRT1. When this occurs in SIRT1-overexpressing A53T mice, Hsp70 is induced, α -synuclein aggregation is suppressed, and survival is greatly extended. This finding is consistent with the earlier report that SIRT1 deacetylates and activates HSF1 (Westerheide et al., 2009). We did not observe high-molecular oligomeric species in our Western blotting assays. We observed a difference only in detergent-insoluble aggregates with SIRT1 overexpression or deletion indicating that SIRT1 decreases detergent-insoluble α -synuclein in these mice.

←

(Figure legend continued.) shRNA (8) increased toxicity (compare 4, 6; or 4, 8). When cells were transfected with Hsp70-shRNA during SIRT1 overexpression, the toxicity level was reduced but not to the level without the Hsp70 knockdown (compare 5, 9; not significant). When the cells were transfected with both SIRT1 shRNA and Hsp70 shRNAs (10), the toxicity level was increased (compare 4, 10) but was not higher than SIRT1 shRNA alone (6) or Hsp70 shRNA alone (8). The analysis was performed using two-way ANOVA. $^{\epsilon}p < 0.01$ indicates control (1) versus vector (4). $^{*}p < 0.01$ indicates vector (4) versus SIRT1 overexpression (5) or SIRT1 shRNA (6) or Hsp70 overexpression (7) or Hsp70 shRNA (8), or SIRT1 shRNA + Hsp70 shRNA (10). $^{*}p < 0.01$ indicates vector (4) versus Hsp70 shRNA (8) or SIRT1 overexpression plus Hsp70 shRNA (9) or SIRT1 shRNA plus Hsp70 shRNA (10). $^{*}p < 0.01$ indicates +SIRT1 (5) versus +SIRT1 plus Hsp70 shRNA (9). **E**, Western blotting of SIRT1 levels from the lysates of A53T-overexpressing H4 cells that are transfected by SIRT1 (left) or SIRT1-shRNA (right). Actin serves as a loading control. **F**, Western blotting of Hsp70 levels from the lysates of A53T-overexpressing H4 cells that are transfected by Hsp70 (left) or Hsp70-shRNA (right). Actin serves as a loading control.

In summary, our findings suggest that therapeutic strategies to activate SIRT1 in the brain may be useful in treating α -synuclein aggregation and related toxicity. The synthetic effect of α -synuclein-induced stress and SIRT1 activation in the induction of the heat shock response may also inform treatment. SIRT1-activating drugs may be effective after disease onset and function to mitigate progression of α -synuclein aggregation and related toxicity. Our findings further support the notion that genes activated by calorie restriction to mediate physiological adaptations, such as sirtuins, may be broadly beneficial in combating diseases of aging.

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