Sirt2-SUMOylation is essential for its tumor-suppressor function in neuroblastoma

Wenmei Lu
Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine
Qian Wang
Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine
Ci Xu
Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine
Haihua Yuan
Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine
Qiang Fan
Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine
Biying Chen
Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine
Renjie Cai
Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine
Danhong Wu
Shanghai Fifth People's Hospital, Fudan University
Ming Xu (mingxu@shsmu.edu.cn)
Shanghai Jiao Tong University School of Medicine https://orcid.org/0000-0002-3996-4304

Research

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Abstract

Background

SUMOylation is an important post-translational modification and participates in a variety of cellular physiological and pathological processes in eukaryotic cells. Sirt2, as a NAD^+-dependent deacetylase, usually exerts tumor-suppressor function. However, its SUMOylation roles in cancer cells have not been illustrated.

Methods

Ni^{2+}-NTA, GST-pull down and immunoprecipitation *in vitro* and *in vivo* were used for the Sirt2-SUMOylation or P38-Acetylation assay. Immunofluorescence and Nuclear/Cytosol Fractionation Kit were performed to identify the localization of Sirt2 with or without SUMOylation. The proliferation, soft-agar colony formation, migration and invasion were performed to detect the phenotypes of neuroblastoma cells *in vitro*, and the xenograft tumor model was conducted to verify Sirt2-SUMOylation role in tumorigenesis *in vivo*. R2 online database were used for the clinical analysis, including expression, survival curve and pathology stage.

Results

SUMOylation can occur in Sirt2 protein at both lysine 183 and lysine 340 sites. SUMOylation of Sirt2 does not affect its localization or stability, but involves in the P38-mTORC2-AKT cellular signal transduction *via* the direct deacetylation on P38. SUMOylation-deficient Sirt2 losses the capability of suppressing tumor processes and neuroblastoma cell shows a resistance to Sirt2-specific inhibitor AK-7.

Conclusion

We revealed an important function of SUMOylation on Sirt2, which is closely associated with the cellular signal transduction and is essential for suppressing the tumorigenesis in neuroblastoma.

Background

The human Sirt2 protein, a homolog of the budding yeast silent information regulator 2 (Sir2), is one member of the Sirtuin family of NAD^+-dependent deacetylases and ADP-ribosyltransferases [1, 2]. Accumulating evidence reveals that Sirt2 plays important functions in multiple cell events [3–5], which involves a variety of biological and pathological conditions, including development, neural activity, obesity, and carcinogenesis [2, 6, 7]. Accordingly, various Sirtuin inhibitors are also developed and

assessed the effects on the different diseases *via* targeting specific protein structure, biological activity, or signaling pathways [8, 9].

As an important deacetylation enzyme, Sirt2 requires the inevitable NAD-nicotinamide exchange reaction to deacetylate the specific lysine on its substrates, such as those found in histone H4, α-tubulin, p65 and p53 [10–13]. Traditionally, Sirt2 maintains genome integrity and suppresses tumorigenesis depending on its deacetylation activity [14]. Also, it acts as a substrate and can be affected by other enzymes or regulators after the post-translational modification (PTM). The histone acetyltransferase p300 is able to acetylate Sirt2 and attenuates its deacetylase activity to enhance the acetylation level of its specific substrates [15]. Sirt2 phosphorylation at Ser331 triggered by Cyclin-dependent kinases inhibits its catalytic activity and decreases the cell motilities [16, 17]. However, ERK1/2 interacts with Sirt2 and enhances the stability of Sirt2 protein as well as the enhanced deacetylase activity, which are probably dues to the Sirt2 phosphorylation occurrence at the other sites [18].

SUMOylation is a reversible post-translational modification involved in protein subcellular localization [19], stress response [20], transcriptional repression [21], DNA repair [22] and many other cellular processes [23, 24]. SUMO (small ubiquitin-like modifier) can be covalently attached to proteins as a monomer or a lysine-linked polymer *via* the sequential action of E1 complex SAE1/SAE2, E2 (UBE2I/UBC9) and E3 enzymes (PIAS, RANBP2 or CBX4), and can be removed by SUMO1/Sentrin-specific proteases SENPs. In Sirtuin family, Sirt1, Sirt3 and Sirt6 have been proved to be SUMOylated and show significant biological and physiological functions in cellular activity or tumor processes. The SUMOylation of Sirt1 increases its deacetylase activity and inactivates apoptotic proteins responding to genotoxic stress [25]. Similarly, Sirt6 SUMOylation also can increase its deacetylation activity and exerts a tumor suppressive function [26]. However, mitochondrial deacetylase Sirt3 activity can be retrained by SUMOylation, but SENP1 translocated into mitochondria upon metabolic stress will activate Sirt3 to reduce fat mass and antagonize high-fat diet induced obesity [27].

Here, we report that SUMOylation can also occur within the Sirt2 protein and ensures its deacetylase activity to suppress the tumor processes in neuroblastoma. In addition, *de novo* evidence reveals that Sirt2 is capable to deacetylate P38/MAPK directly and inhibits the P38-mTORC2-AKT signaling, which is also closely associated with its SUMOylation function.

Methods

SUMOylation assays. Three methods were used to determine SIRT2 SUMOylation. (1) *Ni-NTA pull-down assay*. Briefly, 293T cells were co-transfected with plasmid combo for 48 h and were lysed in imidazole contained Buffer 1 (6M Guanidine-HCl, 0.1M NaH_2PO_4, 0.01M Tris-HCl, 10 mM β-mercaptoethanol and 5 mM imidazole, pH8.0). The cell lysates were incubated with Ni-NTA beads at 4℃ overnight, and then the beads were successively washed by buffer 2 (8M Urea, 0.1M NaH_2PO_4, 0.01M Tris-HCl, 10 mM β-mercaptoethanol, pH8.0), buffer 3 (8M Urea, 0.1M NaH_2PO_4, 0.01M Tris-HCl, 10 mM β-mercaptoethanol, 0.2% TritonX-100, pH6.3) and buffer 4 (8M Urea, 0.1M NaH_2PO_4, 0.01M Tris-HCl, 10 mM β-

mercaptoethanol, 0.1% TritonX-100, pH6.3). Purified protein was eluted by elution buffer (200 mM imidazole, 0.15 M Tris-HCl, 30% Glycerol, 0.72 M β-mercaptoethanol and 5% SDS, pH6.7) as previously described [28] and determined by immunoblotting with anti-HA antibody. (2) *Immunoprecipitation assay*. 293T cells transfected with plasmid combo were harvested and lysed at 48 h in 1% SDS lysis buffer (containing 5 mM EDTA, 5 mM EGTA, 20 mM N-ethylmaleimide and a complete protease inhibitor cocktail). The cell lysate was diluted in pre-RIPA (20 mM NaH_2PO4/Na_2HPO_4, 150 mM NaCl, 1% Triton-100, 0.5% deoxycholic acid sodium salt) at a ratio of 1:10, then was incubated with 8μL of Protein A/G Beads and 2μL of anti-Flag antibody overnight at 4℃. After three times washing, the protein samples were subjected to western blotting analysis. (3) *GST-pull down assay*. *E. coli* BL21 (DE3) harboring plasmid pGEX-6p-1/Sirt2 with or without pE1E2S1 construct was stimulated by 0.2 mM IPTG for 10hrs at 16℃ and was lysed according to the instruction of B-PER Protein Extraction Reagent (ThermoFisher, USA). Lysate was then incubated with Glutathione Hicap Matrix (QIAGEN, Germany) overnight at 4℃. After three times washing with lysis buffer, western blot was performed to determine the Sirt2 SUMOylation *in vitro*.

Immunoprecipitation and acetylation assays. HEK293T cells were transfected with HA-tagged P38 with or without Flag-tagged Sirt2 for 48hrs and lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM EDTA and a complete protease inhibitor cocktail). For acetylation assay, HEK293T cells were cultured with 2 μM TSA for 16 hours before harvest. Cells lysate was incubated with Protein A/G Mix Magnetic Beads and proper antibodies at 4℃ overnight followed by immunoblotting to determine the interaction between P38 and Sirt2 protein, or the acetylation level of P38 *in vitro* and *in vivo*.

Cell proliferation assay. The CCK8 kit (Biotool, Shanghai, China) was used to measure the cell proliferation of the constructed stable SH-SY5Y cell lines according to the instruction. Briefly, 1000 cells/well were resuspended in 100μL medium and seeded into a 96-well plate. After cultured at 24 hours intervals for 3 days, the cells were treated with 10% CCK8 solution and incubated at 37℃ for one hour. The absorbance at 450 nm was measured with a microplate reader (ThermoFisher, USA). The experiments were performed independently in triplicate, and the data were presented as the mean ± S.E.M.

Migration and invasion assays. The migration assay was performed as previous description [29]. Briefly, SH-SY5Y stable cells harboring wild HA-tagged Sirt2 or its mutants were resuspended in 200 μl serum-free RPIM medium. Then the cells suspension was added into the upper uncoated (4×10^4 cells/well for migration) or Matrigel matrix-coated (8×10^4 cells/well for invasion) chambers (Corning, NY, USA). Necessarily, the complete medium containing 20% FBS was added into the lower chambers as a chemoattractant at 37℃ in humidified 5% CO_2. The cells that migrated or invaded to the lower side of the upper chambers were fixed and stained with 0.1% crystal violet solution for counting and analyzing. The experiment was performed independently in triplicate, and the data were presented as the mean ± S.E.M.

Soft agar colony formation assay. The designated cells were seeded into soft agar to determine the effect of anchorage-independent growth as described previously [29]. Briefly, 2 ml medium containing 0.6%

base agar gel (Ameresco, PA, USA) was solidified as the lower layer in six-well plates. 1000 cells were planted into 2 ml medium containing 0.35% base agar gel and 5% FBS and layered onto the base in 6-well palates. Cell colonies developed in soft agar were stained with 0.01% crystal violet after two weeks. The colonies were taken photos under inverted microscope and the number of colonies was counted using ImageJ software.

Xenograft tumor model. The SH-SY5Y cells xenograft tumor model was performed in the back of nude mice as described previously [29]. 5-week-old male BALB/c nude mice were subcutaneously injected on the back with 100 μl PBS containing 2×10^6 SH-SY5Y cells stably expressing Sirt2-shRNA, HA-Sirt2^{WT}, HA-Sirt2^{K183R}, HA-Sirt2^{K340R} or HA-Sirt2$^{K183/340R}$, respectively. The wide type SH-SY5Y cell was set as mock control. The operated mice were sacrificed at 2 weeks after xenograft and the tumor weight was measured. All the animal operations were conducted with the approval and guidance of Shanghai Jiao Tong University Medical Animal Ethics Committees.

Statistical analysis. All data were analyzed as means ± S.D. for western blotting, or means ± S.E.M for migration, invasion, soft agar and mouse xenograft experiments. Statistical calculations were performed by SPSS tool and the graphs were conducted by GraphPad Prism software. Differences between individual groups were analyzed using the t-test (two-tailed and unpaired). A p-value of < 0.05(*), < 0.01(**) or < 0.001(***) is considered of significance.

Results

Human Sirt2 can be SUMOylated in vitro and in vivo.

To determine whether Sirt2 can be SUMOylated, HEK293T cells were used to over-express HA-tagged Sirt2 with or without His-tagged SUMO1 and Flag-tagged UBC9, cell lysis after transfection were subjected to Ni-NTA pull-down assay and western-blot analysis. Two major shifted Sirt2 protein bands were observed and their levels could be enhanced or attenuated by E2 enzyme UBC9 or deSUMOylation enzyme SENP1, respectively (Fig. 1A). In consistent with that, the immunoprecipitation assay also showed a same outcome about Sirt2 SUMOylation events in HEK293T cells (Fig. 1B). To identify the endogenous SUMOylation on Sirt2 protein, SH-SY5Y cells stably expressing HA-Sirt2 were harvested and subjected to immunoprecipitation with HA antibody, which showed that Sirt2 SUMOylation indeed could happen *in vivo* according to the detection by SUMO1 antibody and HA antibody (Fig. 1C). In the prokaryotic expression system, *in vitro* GST-pull down assay also proved that GST-tagged SIRT2 could be SUMOylated when co-transfected with SUMO1, E1 and E2 enzymes in BL21 *E. Coli* host (Fig. 1D). The endogenous SUMOylation on Sirt2 was also identified in SH-SY5Y cells through immunoprecipitation assay with SUMO1 and Sirt2 antibodies (Fig. 1E). In addition to SUMO1 modification, SUMO2 and SUMO3 modifications were also positively identified in HEK293T cells (Fig S1A).

As an NAD^+-dependent deacetylase, we wonder whether Sirt2 SUMOylation could be stimulated by NAD^+ in cells. Surprisingly, 1 mM NAD^+ could obviously enhance the Sirt2 SUMOylation level but higher NAD^+

concentration would not help the further improvement (Fig. 1F). However, it had a significant and positive time effect on Sirt2 SUMOylation under 1 mM NAD^+ stimulation (Fig. 1G). On the contrary, EGF, H_2O_2, or even cisplatin (CPT) did not affect the SUMOylation level of Sirt2 (Fig S1B-D). Together, these evidences indicate that Sirt2 can be SUMOylated *in vitro* and *in vivo*, which can be specifically stimulated by NAD^+.

Sirt2-SUMOylation does not affect the localization and stability of Sirt2 in cells.

To determine which lysine was conjugated by SUMO1 molecule, SUMOplot™ Analysis Program (http://www.abgent.com.cn/tools/sumoplot) and GPS-SUMO 2.0 Online Service (http://sumosp.biocuckoo.org/online.php) were applied to predict the probability of SUMOylation sites within Sirt2 protein (Fig S2A). There were six potential SUMOylation sites predicted by both software with the different system approaches [30], including Lys183, Lys192, Lys216, Lys252, Lys301 and Lys340 (Fig. 2A and Fig S2A). Sequentially, substitution of these lysine (K) to arginine (R) respectively revealed that both K183R and K340R significantly attenuated the SUMOylation level of Sirt2 (Fig S2B and Fig. 2B). Intriguingly, the K183R mutation within the Sirt2 protein notably removed a shifted protein band but enhanced the level of another one, however, the K183R and K340R double mutations caused a significant decline of the total Sirt2 SUMOylation level according to both of the Ni-NTA pull-down and immunoprecipitation assays (Fig. 2B & C).

To assess whether SUMOylation on Sirt2 can affect its localization and stability in the cell, HA-tagged Sirt2 wide type (WT) or mutants were cloned into pGreenPuro-Dual vector (see description in Fig. 3G) and transferred into SH-SY5Y cells through lentiviral infection, respectively, and the even expression level was identified by western-blotting (Fig S2C). The nucleus/cytosol extraction assay revealed that SUMOylation did not alter the localization of Sirt2, whatever on K183, K340, or K183 + 340 (Fig. 2D & E). Moreover, the Immunofluorescence assay also showed a consistent outcome (Fig. 2F). Sirt2 protein stability was controlled by the proteasome system but not lysosomal pathway according to the protein level detection in cells after MG132 or Chloroquine treatment (Fig S2D), yet, the SUMOylation on Sirt2 did not significantly affect its stability by detecting the protein half-life after cell-treating with cycloheximide (CHX) (Fig. 2G). Taken together, these data indicate that both K183 and K340 are the main SUMOylation sites in Sirt2, but the SUMOylation on Sirt2 impacts neither of its localization or stability in neuroblastoma cells.

Sirt2-SUMOylation mainly inhibits P38-mTORC2-AKT signaling in neuroblastoma cells.

As an important deacetylation enzyme, Sirt2 participates in various cell activities such as aging, cell signal transduction and carcinogenesis [31], however, its signaling mechanisms in cells are still not well understood. Considering this, we silenced the expression of Sirt2 in SH-SY5Y cells through pGreenPuro-dual lentiviral system with the specific shRNA. The pP38, pAKT and pmTOR were significantly enhanced but pERK was short-term depressed when abrogating the Sirt2 expression after starvation following with complete culture medium stimuli (Fig. 3A left two lanes in 3B, Fig S3A). mTORC1 and mTORC2 are the two main sensors of mTOR signaling, while the activation of pS6K (pP70)/p4E-BP1 or pAKT reflects the

mTORC1 or mTORC2 signaling pathway, respectively. Here, Sirt2 silencing did not significantly enhance the activation of p4E-BP1 but did only slightly impact on pS6K (Fig. 3A, supplemental Fig S3A). These data indicated that Sirt2 mainly involved in the P38, AKT and mTORC2 signal transduction in neuroblastoma cells. To clarify the regulation role between P38 and mTORC2/AKT caused by Sirt2 abrogation, several inhibitors were applied for further analysis (Fig. 3B-E). Rapamycin as mTOR specific inhibitor can inhibit mTORC1 activation but has no effect on mTORC2, and it did not affect the level of pP38, pmTOR and pAKT under the Sirt2 silencing condition (Fig. 3B middle 4, 5 lanes & C). Also, AKT inhibitor (LY294002) did not impact the P38 activation, but significantly abolished the pmTOR and pS6K level (Fig. 3B right 3,4 lanes & C). Intriguingly, both pAKT and pmTOR levels were suppressed when P38 signal was blocked by SB202190 (Fig. 3B right 1, 2 lanes & C). In addition, pp242, an inhibitor of both mTORC1 and mTORC2, was applied to confirm that mTOR signal indeed did not affect the Sirt2-associated P38 activity (Fig. 3D & E). These data indicate that Sirt2 in neuroblastoma can mainly suppress P38-mTORC2-AKT signaling (Fig. 3A-E). The strategy of cell signal transduction mediated by the specific inhibitors showed that Sirt2 can only slightly or temporarily affect the mTORC1 or ERK signaling, but is mainly responsible for suppressing the P38-mTORC2-AKT signaling in neuroblastoma (Fig. 3F).

To rescue Sirt2 expression in SH-SY5Y cells harboring silenced endogenous Sirt2 for re-observing the signal transduction, we constructed a homemade lentivector based on pGreenPuro™ shRNA lentivector (System Biosciences, Switzerland), termed pGreenPuro-Dual, which contained two independent expression cassettes generating non-coding RNA transcripts (*i.e.* shRNA, miRNA or lncRNA) by H1 promoter, and producing mRNA for translation by CMV promoter, respectively (Fig. 3G). This all-in-one lentiviral vector can be used for recombinant lentivirus packaging to silence endogenous gene and express exogenous gene (*i.e.* wide type or mutants) with only once infection force on the target cell, which avoids the uncontrollable changes of cellular behavior caused by multiple viral infections and drug selections.

Then, the SH-SY5Y cells were subjected to infection by recombinant lentivirus harboring shRNA targeting to endogenous Sirt2-3'UTR and rescued exogenous HA-Sirt2-WT, -K183R, -K340R or -K183/340R, and cells infected with virus generating by pGreenPuro-Dual vector or pGreenPuro-Dual/shRNA were set as the system control. After puromycin selection, the endo- or exo-Sirt2 protein expression level in those cell lines was quantified by western-blotting (Fig S3B). Surprisingly, re-introduced Sirt2-WT suppressed the activation of P38, mTOR and AKT resulting from endo-Sirt2 ablation, while SUMOylation-deficient Sirt2 (K183R, K340R or K183/340R) can not resume the normal signal transduction in SH-SY5Y cells (Fig. 3H & I). Taken together, these evidences indicate that Sirt2-SUMOylation is critical in inhibiting the P38-mTORC2-AKT signaling in neuroblastoma.

Sirt2 is a promised deacetylase of MAPK/P38 and its enzyme activity relates to SUMOylation.

Acetylation of lysine residues within proteins is relevant to the physiological activities of targeted proteins. P38 was proved to be reversibly acetylated by PCAF/P300 and deacetylated by HDAC3, which

regulates its affinity for ATP binding and P38 kinase activity [32]. However, there have no evidence yet to illuminate the relationship between Sirt2 and P38. To investigate whether Sirt2 could directly regulate P38, the Immunoprecipitation assay revealed that Sirt2 could interact with P38 in cells (Fig. 4A). In consistent with that, the GST-pull down assay also proved that GST-Sirt2 recombinant protein could specifically bind to endogenous P38 in SH-SY5Y cells, but GST-Sirt1 protein could not (Fig. 4B). As one of the deacetylation enzymes, we wonder whether Sirt2 can regulate the acetylation level of P38. The immunoprecipitation assay showed that Sirt2 could obviously decrease both of the exogenous and endogenous P38 acetylation levels (Fig. 4C & D). Comparing with the known deacetylase HDAC3, Sirt2 showed a similar deacetylation ability to P38 protein (Fig. 4E). Moreover, the P38 acetylation level was obviously enhanced by treating with Sirt2-specific inhibitor AK-7 (Fig. 4F). Traditionally, acetylated proteins are more stable comparing to their unacetylated counterparts, because unacetylated lysines are usually targeted for ubiquitination-mediated proteasomal degradation [33]. Considering of this, we compared the half-life of P38 with or without Sirt2 expression, the result conversely showed that deacetylated P38 was more stable than the acetylated one (Fig. 4G & Fig S3C), which was consistent with a certain phenomenon that Sirt2 deficiency can stabilize NFATc2 to enhance its transcription activity [34]. To assess whether Sirt2-SUMOylation could affect its deacetylation activity, the SH-SY5Y stable cell lines harboring Sirt2-WT or -mutants were used for immunoprecipitation with acetyl-lysine antibody and the acetylated-P38 level indicated that SUMOylation significantly impaired the deacetylation ability of Sirt2 (Fig. 4H). Thus, these results together suggest that Sirt2 is a promised deacetylase of P38 and the enzyme activity is closely associated with its SUMOylation.

Sirt2-SUMOylation is critical in suppressing the phenotypes of neuroblastoma cells.

To assess the phenotypes induced by Sirt2-SUMOylation in neuroblastoma cell, we first detected the proliferation ability of SH-SY5Y cell lines with silenced Sirt2 or directly overexpressed Sirt2 variants (*i.e.* WT, K183R, K340R or K183/340R), which showed Sirt2 ablation or Sirt2-SUMOylation deficiency significantly facilitated the proliferation capability, but Sirt2-WT showed an opposite effect (Fig. 5A). Meanwhile, re-applied Sirt2-WT but not Sirt2-K183R, -K340R or -K183/340R resumed the proliferation in Sirt2-silenced cells (Fig. 5B). According to the soft-agar colony-forming assay, knock-down Sirt2 or overexpress Sirt2 mutants (K183R, K340R, or K183/340R) enhanced the clone formation, yet overexpressed Sirt2-WT significantly suppressed the clone formation (Fig. 5C upper panel and D). Similarly, re-applied Sirt2 variants in Sirt2-deficient cells showed almost the same outcomes (Fig. 5C lower panel and E). To inspect the cell mobility and invasiveness resulted from Sirt2-SUMOylation, the migration and invasion assays were conducted by using the cell chamber in 24-well plates. The results revealed that Sirt2 can significantly suppress the migration and invasion abilities of SH-SY5Y cells (Fig. 5F-K). But SUMOylation-deficient Sirt2, whatever overexpression (Fig. 5F, I upper panel & G, J) or rescued expression (Fig. 5F, I lower panel & H, K), intensified the cell capabilities of migration and invasion *in vitro*. In addition, we wonder whether Sirt2-SUMOylation affected the drug susceptibility, the IC50 of Cisplatin and AK-7 were detected in the mentioned cell lines above, respectively. The result showed that Cisplatin, as a frequently used chemical drug for neuroblastoma, did not have any different effect among SH-SY5Y cells with or without Sirt2 or its mutants comparing to control (Fig S3D). While

AK-7 proved to be able to inhibit Sirt2 activity and enhance the phosphorylation of P38 in SH-SY5Y according to our previous report [35], showed a significant different drug-effect between SH-SY5Y cells harboring Sirt2-WT and those harboring Sirt2-mutants (K340 mainly) (Fig. 5L). Together, these data reveal that Sirt2-SUMOylation plays an important role in inhibiting the malignant phenotypes in neuroblastoma cells and makes a contribution to the cell sensitivity to Sirt2-specific inhibitor AK-7 *in vitro*.

Sirt2-SUMOylation exerts tumor-suppressor property in mice and Sirt2 expression is closely related to the patient's prognosis.

In xenograft mouse model, the tumor size or weight was measured at 2 weeks after subcutaneous injection. Comparing with vector or mock group, both of weight and size of the tumors from the Sirt2-deficient or -mutants cell injection group were much greater. However, the overexpressed Sirt2-WT significantly suppressed the tumor growth (Fig. 6A & B). To trace expression changes of Sirt2 in the development and progression of neuroblastoma, a series of statistical analyses were performed using the R2 online database (https://hgserver1.amc.nl/cgi-bin/r2/main.cgi). Comparing to normal brain tissue, the Sirt2 expression is significantly suppressed in tumors from three different datasets (Fig. 6C). The survival curve also revealed that lower Sirt2 expression predicted a poor survival probability of either event-free or overall (Fig. 6D & E). Moreover, with the increase of tumor pathological stage, the expression of Sirt2 showed a downward trend according to the R2 dataset analysis, particularly in stage 4 (Fig. 6F). Moreover, neuroblastoma from stage 4 s which is similar to those from stage 1, 2 in molecular [36] showed the same Sirt2-expression pattern (Fig. 6F). Taken together, these all indicate that Sirt2 expression is closely associated with the development of neuroblastoma and the patient's prognosis. As well, SUMOylation on Sirt2 seems essential for suppressing the tumorigenesis of neuroblastoma in mice.

Discussion

Neuroblastoma, as an embryonal tumor of the autonomic nervous system, is the most common extracranial tumor in children and infants. Nearly half of all neuroblastomas occur in children younger than 2 years of age. Neuroblastoma accounts for approximately 6–10% of childhood tumors and 15% of childhood tumor mortality. Neuroblastoma is a neurosecretory tumor that may originate in any ridge of the sympathetic nervous system, typically in the adrenal medulla or paraspinal ganglia [37]. Recent advances in neuroblastoma research have focused on inhibition of cell signaling pathway, tyrosine kinase activity, differentiation or immunotherapy, and increasing evidence reveal that AKT presents a clinically relevant target in neuroblastoma [38, 39].

Human deacetylase Sirt2 which is closely related to yeast Hst2p prefers to localize to the cytoplasm in mammalian cells [10] and regulates various cellular processes through promoting deacetylation of multitudinous substrates in diverse cellular compartments. In cancer diseases, Sirt2 is frequently supposed to display tumor suppressor function [31, 40], however, its intrinsic mechanism is still not clearly illustrated. Post-translational modification (PTM) is an important process to ensure proteins to exert further cellular function in eukaryotes. To date, it has been reported that Sirt2 can be phosphorylated

or acetylated accompanying the deacetylation activity alteration, which directly or indirectly affects the biological function of its substrates [16, 18]. In this work, we identified a novel PTM-SUMOylation on Sirt2 at both K183 and K340 sites, which makes a great contribution to its tumor-suppressor function as well as the cell signal transduction. Because Sirt2 is an NAD^+-dependent deacetylase [41, 42], we also revealed that Sirt2-SUMOylation can be specifically and positively regulated by NAD^+ in the cell, which logically highlights the importance of SUMOylation to Sirt2 protein function (Fig. 1F & G). SUMOylation is called small ubiquitin-like modifiers originally because of the partial similarity to ubiquitination [43]. It often influences protein stability and subcellular localization [44]. However, we did not observe significant changes in either its localization or stability following a series of point-mutation assays (Fig. 2), which impelled us to speculate the other biological function of Sirt2-SUMOylation.

It has been reported that Sirt2 is closely associated with the activities of P38 and AKT in a variety of physiological conditions or diseases [45–47]. Hence, we investigated the regulatory relationship among Sirt2, P38, AKT, and even mTOR through using specific chemical inhibitors in neuroblastoma cells, which cleared that mTOR-AKT activation resulted from Sirt2-deficient mainly relied on P38 activation, because the mTOR or AKT signal was almost vanished after blocking P38 activity (Fig. 3B, right 1, 2 lanes). Similarly, we also demonstrated that Sirt2-mediated AKT activity is completely dependent on mTORC2 signaling in SH-SY5Y cells (Fig. 3B, right 3, 4 lanes & E). However, whether P38 can directly regulate mTOR are still in puzzle although there is some evidence about the crosstalk between P38/MAPK and mTOR signaling pathway in human glioblastoma cells [48]. More interestingly, SUMOylation-Sirt2 can significantly affect Sirt2-mediated P38-mTORC2-AKT signaling (Fig. 3H & I), which relighted an important significance of SUMOylation to Sirt2 function. Besides that, we have provided evidences that Sirt2 is a promising deacetylase of substrate P38 and directly impairs the phosphokinase activity of P38 *in vitro* and *in vivo*, which is in consistent with the earlier reports that acetylation of P38 enhances its kinase activity [32]. Likewise, the deacetylation activity of Sirt2 has also been proved to be related to its SUMOylation status (Fig. 4). With these considerations in mind, we propose that Sirt2 is involved in regulating the P38-mTORC2-AKT signaling axis through the direct deacetylation of P38, which is largely associated with its SUMOylation function.

Despite Sirt2 usually functions as a tumor suppressor in cancer diseases, most of the works focus on seeking its substrates to reflect the regulation mechanism in the process of tumor development, such as deacetylation of P73 [49] and HIF-1α [50]. However, little research reaches to the regulatory mechanism of its own intrinsic functions of Sirt2. According to our phenotype analysis *in vitro* and *in vivo*, we reveal that Sirt2-SUMOylation plays a critical role in promoting the processes of neuroblastoma as well as drug sensitivity to AK-7 in SH-SY5Y cells (Fig. 5, Fig. 6A & B). In the clinical, we showed that lower Sirt2 expression is closely associated with the tumor occurrence or progress and poor prognosis in patients (Fig. 6C-F), Nevertheless, there are still numbers of patients harboring high-Sirt2 expression present rapid progress and short survival time. Probably, it could be due to the Sirt2-SUMOylation deficiency which causes the activity loss of Sirt2 and further the aberrant activation of AKT. Unfortunately, we did not have

enough clinical samples and commercial Sirt2-SUMOylation specific antibodies to detect the association between Sirt2-SUMOylation status and patient survival outcomes.

Conclusions

In summary, our findings shed light on a novel biology insight that SUMOylation of Sirt2 is capable to inhibit the aberrant transduction of P38-mTORC2-AKT signal through the direct deacetylation effect on a new substrate MAPK/P38. Sirt2-SUMOylation seems to function as a ‘brake’ to control P38 acetylation level and avoid the excessive activation of P38-mTORC2-AKT and is essential for its tumor-suppressor function in neuroblastoma (Fig. 7), which may represent an innovative strategy for cancer prognosis or therapy.

Abbreviations

ADP
Adenosine Diphosphate
ATP
Adenosine Triphosphate
CDS
Coding Sequence
CHX
Cycloheximide
CPT
Cisplatin
CQ
chloroquine
EGF
Epidermal Growth Factor
FBS
Fetal Bovine Serum
GST
Glutathione S transferase
HDAC
Histone Deacetylase
IC50
50% Inhibiting Concentration
NAD
Nicotinamide Adenine Dinucleotide
NAM
Nicotinamide

PTM
Post-Translational Modification
SAE
SUMO1 Activating Enzyme
SENP
SUMO1/Sentrin Specific Peptidase
SUMO
Small Ubiquitin-like Modifier
TSA
Trichostatin A
UBC9
Ubiquitin Carrier Protein 9
UTR
Untranslated Region
WT
Wide Type

Declarations

Ethics approval and consent to participate

All animal studies were conducted with the approval and guidance of Shanghai Jiao Tong University Medical Animal Ethics Committees.

Consent for publication

Not applicable.

Availability of supporting data

All data presented to support the conclusions in this article are included in the main text or supplementary information files.

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

M.X. and D.W. designed and guided the whole work. W.L., Q.W., and C.X. performed most of the experiments; H.Y., B.C. Q.F. and R.C. helped with all experiments; Q.W. and C.X. collected and analyzed clinical data; M.X. and W.L. wrote the manuscript. All authors read and approved the final manuscript.

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Authors' information

Wenmei Lu and Qian Wang contributed equally to this work. Ming Xu and Danhong Wu are the corresponding authors.

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Figures

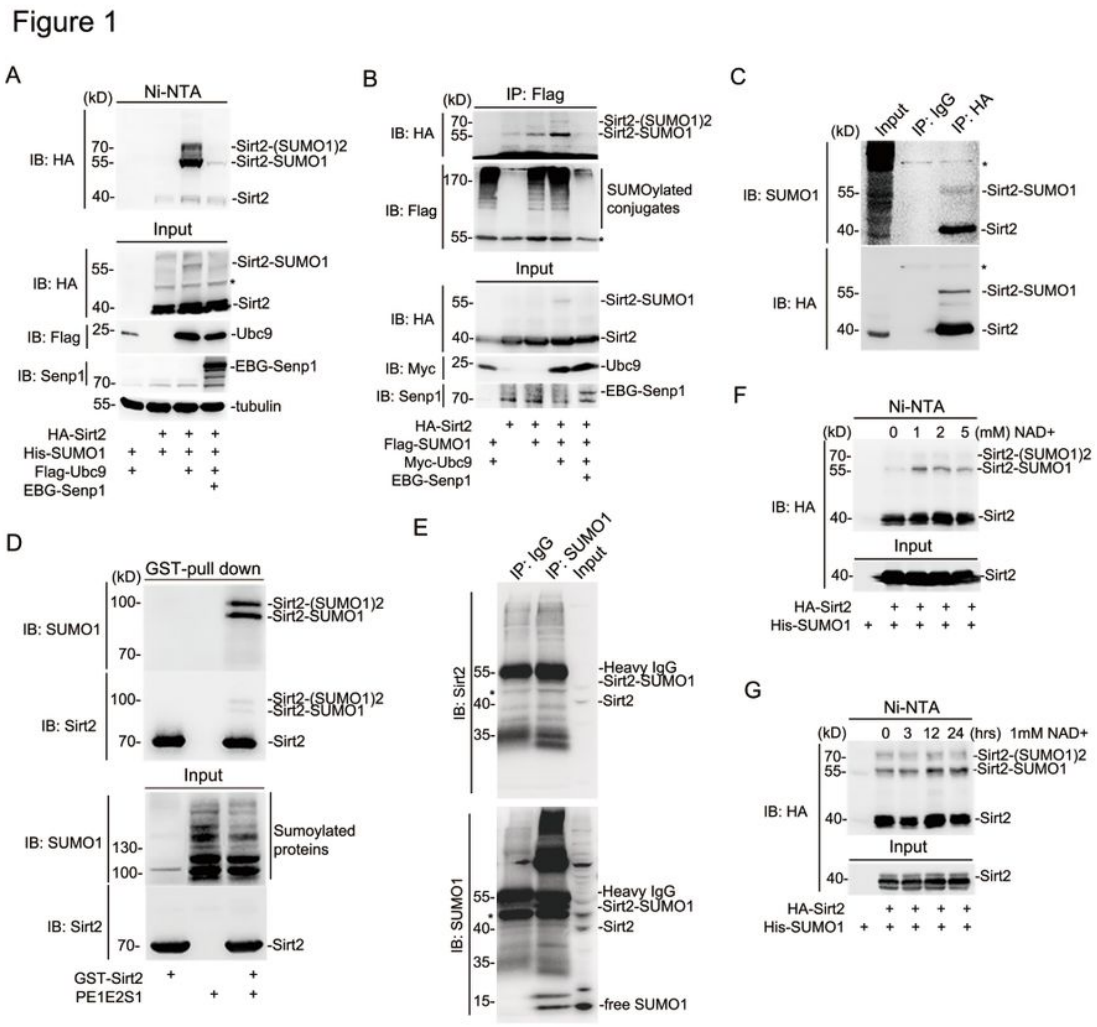

Figure 1

Human Sirt2 can be SUMOylated in vitro and in vivo. A. His-SUMO1 conjugates were isolated with Ni-NTA agrose under denaturing conditions and immunoblotted with antibodies against mouse-HA, mouse-Flag or rabbit-SENP1. B. Cell extracts were immumoprecipitated with mouse-Flag antibody and immonoblotted with antibodies against mouse-HA, mouse-Flag, mouse-Myc or rabbit-SENP1. C. SH-SY5Y cells overexpressing HA tagged Sirt2 were extracted and cell lysis was immumoprecipitated with mouse-HA or normal mouse IgG antibodies and immonoblotted with antibodies against rabbit-SUMO1 or mouse-HA. The light-chain-specific HRP-conjugated anti-mouse/rabbit IgG was used for the second antibody for immunoprecipitation assay. D. SUMOylation reaction was carried out in DE3 bacterial cells expressing GST tagged Sirt2. Bacterial extracts were immunobloted with antibodies against rabbit-SUMO1 or rabbit-Sirt2 after isolation with GST agrose. E. SH-SY5Y cell extracts were used for immunoprecipitated with antibodies against rabbit-SUMO1 and immunoblotted with rabbit-Sirt2 antibody. F&G. 293T cells expressing HA-Sirt2 and His-SUMO1 were treated with gradient concentration NAD+ (0, 1, 2 or 5mM) for 24 hours or treated with 1mM NAD+ for different time periods (0, 3, 12 or 24h). Cell extrats were immonoblotted with antibody against mouse-HA after the method of Ni-NTA pull down. The asterisks represent non-specific bands.

Figure 2

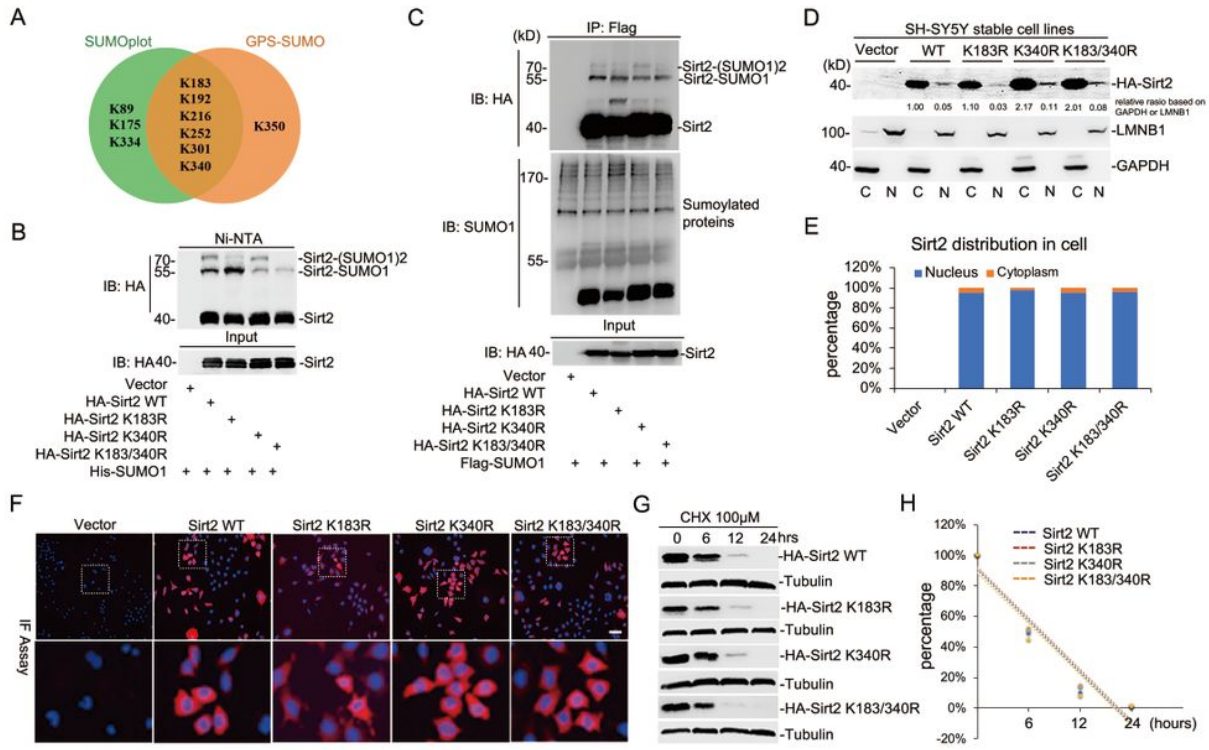

Figure 2

Sirt2-SUMOylation dose not affect the localization and stability of Sirt2 in cells. A. Putative SUMOylation sites of human Sirt2 predicted by SUMOplot and GPS-SUMO software was showed in pie chart. B&C. 293T cells were cotransfected with His-SUMO1/Flag-SUMO1 and HA-tagged Sirt2 or its mutants at K183 and K340. SUMOylated Sirt2 was isolated by Ni-NTA agarose or immumoprecipitated with mouse-Flag antibody and detected with antibodies against mouse-HA or -Flag using the method of western-blot. D&E. SH-SY5Y cell lines stably expressing HA-tagged Sirt2 or its mutants were extracted by Nuclear/Cytosol Fractionation kit. Cell extracts were subjected to immunoblot. Sirt2 distribution in cells were calculated by Image J software. F. The localization of HA-Sirt2K183R, HA-Sirt2K340R and HA-Sirt2K183/340R in SH-SY5Y cells were shown by immunofluorescence staining with anti-HA antibody. Nuclear DNA was stained with DAPI. G. SH-SY5Y cells expressing HA-Sirt2K183R, HA-Sirt2K340R and HA-Sirt2K183/340R were treated with 100 μM CHX for different time periods (0, 6,12 or 24h). Mixture extracts was immunoblotted by antibodies against HA and Tubulin. H. Its half-life period was showed as line graph according to the quantification of protein level by using Image J software.

Figure 3

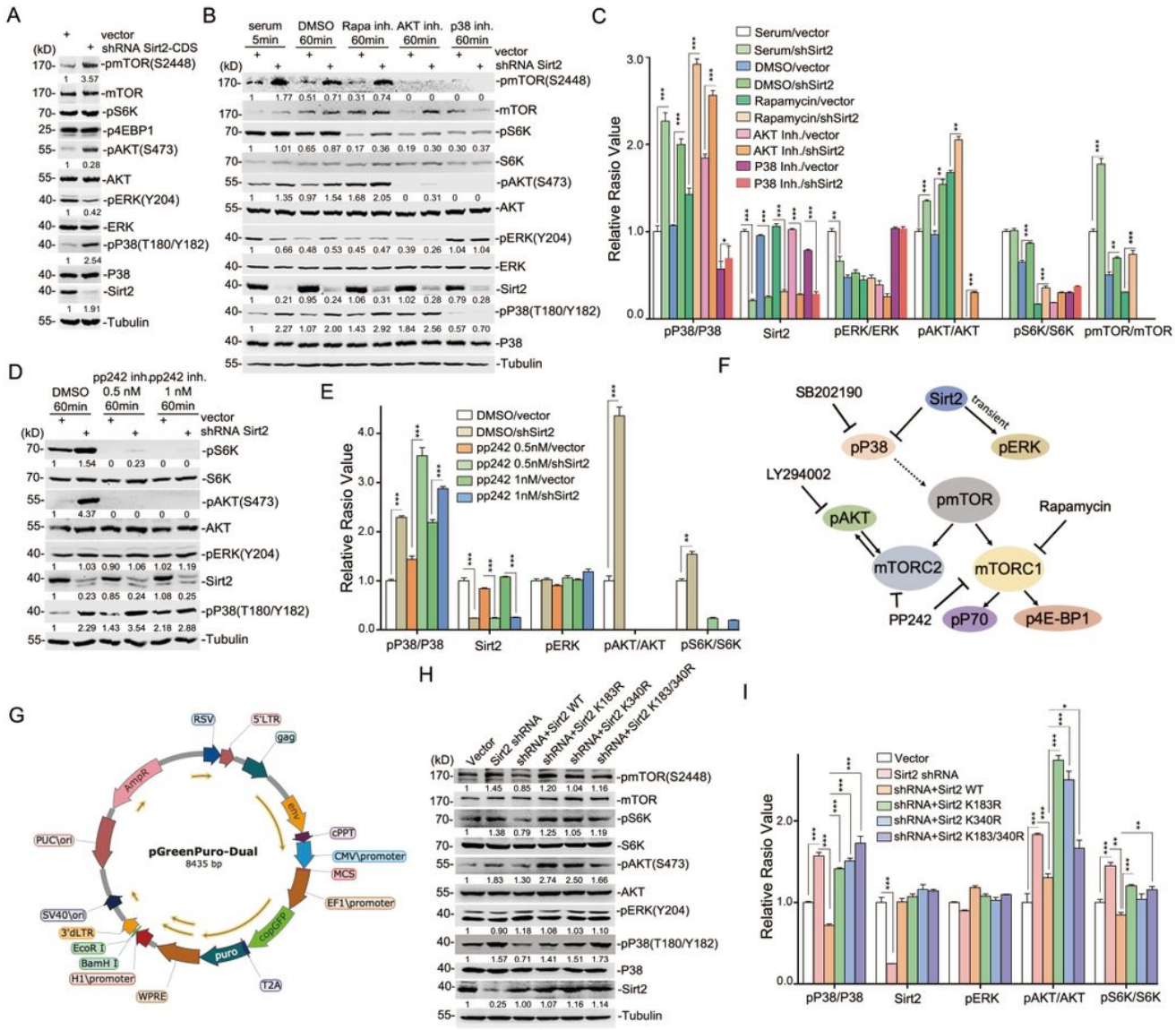

Figure 3

Sirt2-SUMOylation mainly suppresses P38-mTORC2-AKT signaling in neuroblastoma cell. A. SH-SY5Y cells infected with lentiviral expressing shRNA targeting to Sirt2 3'-UTR or control were starved for 2 hours and then stimulated by complete culture medium for 5 minutes. Cell lysis was collected and resolved by SDS-PAGE for western-blot analysis. B-E. SH-SY5Y cells infected with lentiviral expressing Sirt2 shRNA or control were treated with mTORC1 inhibitor Rapamysin (20ug/ml), AKT inhibitor LY294002 (50μM), P38 inhibitor SB202190 (20μM), pp242 (0.5μM or 1μM) or dimethylsulfoxide (DMSO) as a control for 60 minutes. Cell lysis was collected and resolved by SDS-PAGE for western-blot analysis (B&D). Protein levels were quantified by Image J software and showed as a graph (C&E). F. A snapshot of relationship of P38-mTOR-AKT signal mediated by Sirt2. G. The homemade lentivector contains CMV promoter for overexpressing foreign gene, H1 promoter for expressing shRNA and EF1a promoter for Puromycin and EGFP expression. The vector map was draw by using DNAMAN software. H&I. Cell lines of SH-SY5Y

harboring silenced endogenous Sirt2 with or without expressing exogenous wild type Sirt2 or its mutants were harvested for western-blot analysis (H). Protein levels were quantified by Image J software and showed as a graph (I). All the western-blot analysis was repeated at least three times. *p<0.05, **p<0.01, ***p<0.001.

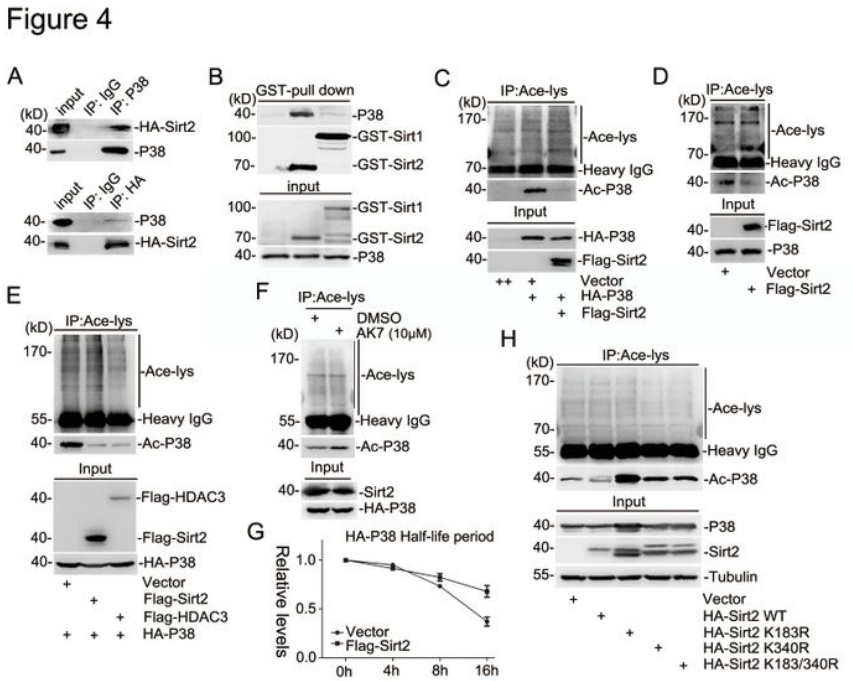

Figure 4

Sirt2 is a promised deacetylase of MAPK/P38 and its enzyme activity relates to SUMOylation. A. HEK293T cells were transfected with HA-Sirt2.Cell extracts were immumoprecipitated for western-blot analysis. B. GST-Sirt1 or -Sirt2 was expressed in DE3 bacterial cells. Purified recombinants by GST-pull down and was immunobloted with GST or P38 antibodies. C. Exogenous Flag-Sirt2 and HA-P38 were co-expressed in HEK293T cells. Cell lysis were subjected to immumoprecipitation with antibody against Ace-lysine and immunobloted with HA, Flag or Ace-lysine antibodies. D. HEK293T cell extracts with or without overexpressed Flag-Sirt2 were immumoprecipitated with Ace-lysine or normal IgG antibody and immunobloted with Flag, P38 or Ace-lysine antibodies. E. HEK293T cells co-transfected with HA-P38 and Flag-Sirt2/Flag-HDAC3 or its vector were harvested for immumoprecipitated with Ace-lysine antibody and immunobloted with antibodies against HA, Flag or Ace-lysine. F. HEK293T cells expressing HA tagged P38 were treated with AK7 (10μM) or DMSO for 24 hours. Cell extracts were immumoprecipitated with

Ace-lysine antibody against and immunobloted with antibodies against HA, Sirt2 or Ace-lysine. G. HEK293T cells expressing HA-P38 with or without exogenous Flag-Sirt2 were treated with 100μM CHX for different time periods, and then were harvested for western-blot analysis. The degradation rate of P38 was quantified as line diagram. H. HEK293T cells were transfected with or without wild type HA-Sirt2 or its mutants were harvested and extracted for immumoprecipitation with Ace-lysine antibody and immunoblotted with P38, HA, tubulin or Ace-lysine antibodies.

Figure 5

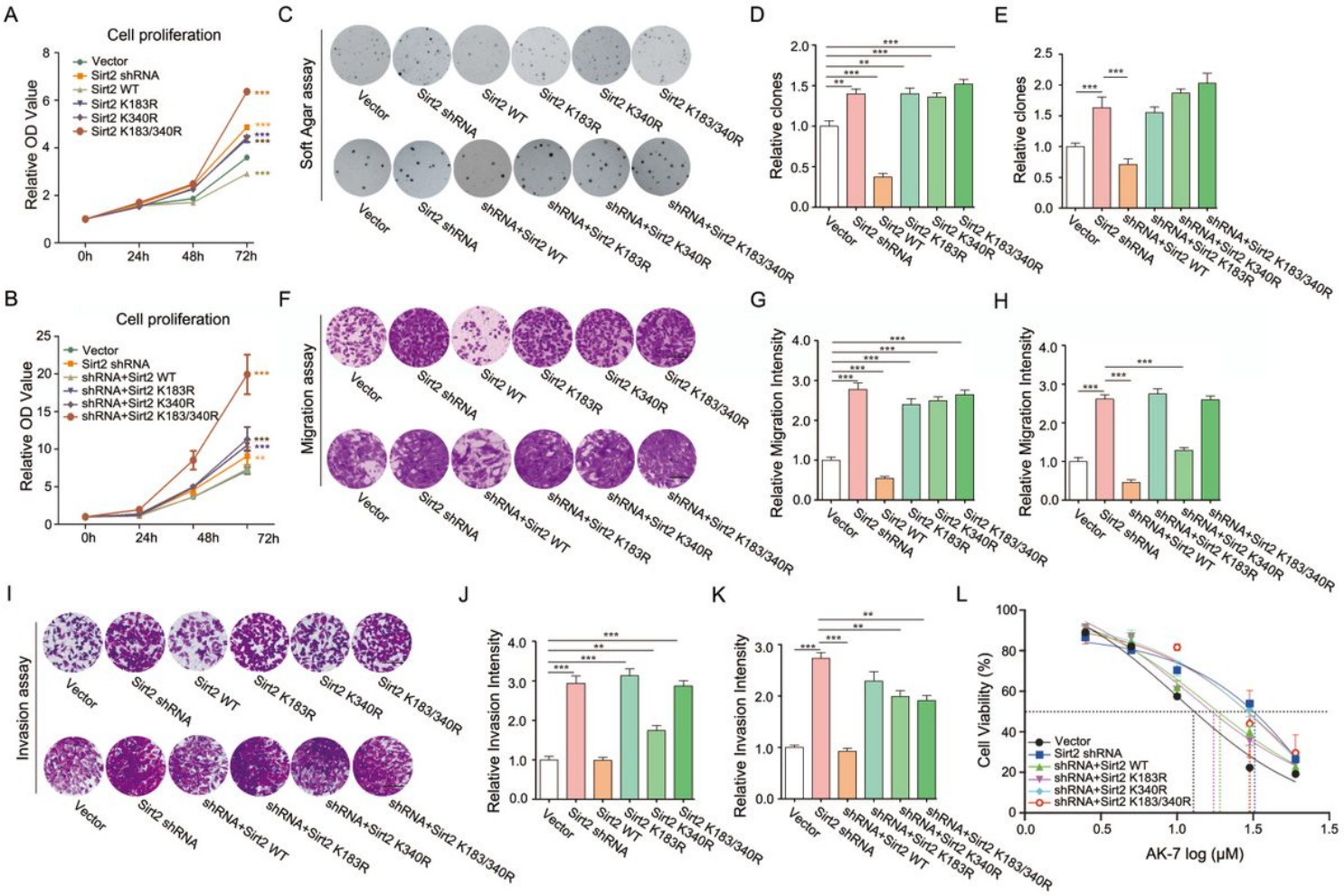

Figure 5

Sirt2-SUMOylation is critical in suppressing the phenotypes of neuroblastoma cell. A-H. SH-SY5Y cells lines stably over-expressing HA tagged Sirt2-WT, -mutants or shRNA targeting to Sirt2 3'-UTR were constructed with the pGreenPuro-Dual lentiviral system. The cell line harboring vector was set as negative control. SH-SY5Y cells lines stably expressing shRNA targeting to Sirt2 3'-UTR or co-expressing shRNA and Sirt2-WT or -mutants was also generated by pGreenPuro-Dual lentiviral system, respectively. The cell line harboring vector or shRNA was set as system control. Cells proliferation were detedcted with CCK-8 proliferation assay (A&B). 1000 SH-SY5Y cells were seeded in the top layer with 0.35% agar for clone formation assay. The photos were taken at 10 days after seeding. And the number of colonies were counted by Image J and analysed by SPSS software (C, D&E). 4×104 SH-SY5Y cells were seed in the

upper uncoated chamber. The photos were taken after 0.1% crystal violet staining at 24 hours post cell seeding. And the number of cells were scored by Image J and analysed by SPSS software (F, G&H). 8×104 SH-SY5Y cells were seed in the upper matrix-coated chamber. The photos were taken after 0.1% crystal violet staining at 48 hours post cell seeding. And the number of cells were scored by Image J and analysed by SPSS software (I, J&K). L. Cell viability was detected by treated with gradient concentration of AK7 for 48 hours. All the experiments were repeated at least three times. *p<0.05, **p<0.01, ***p<0.001.

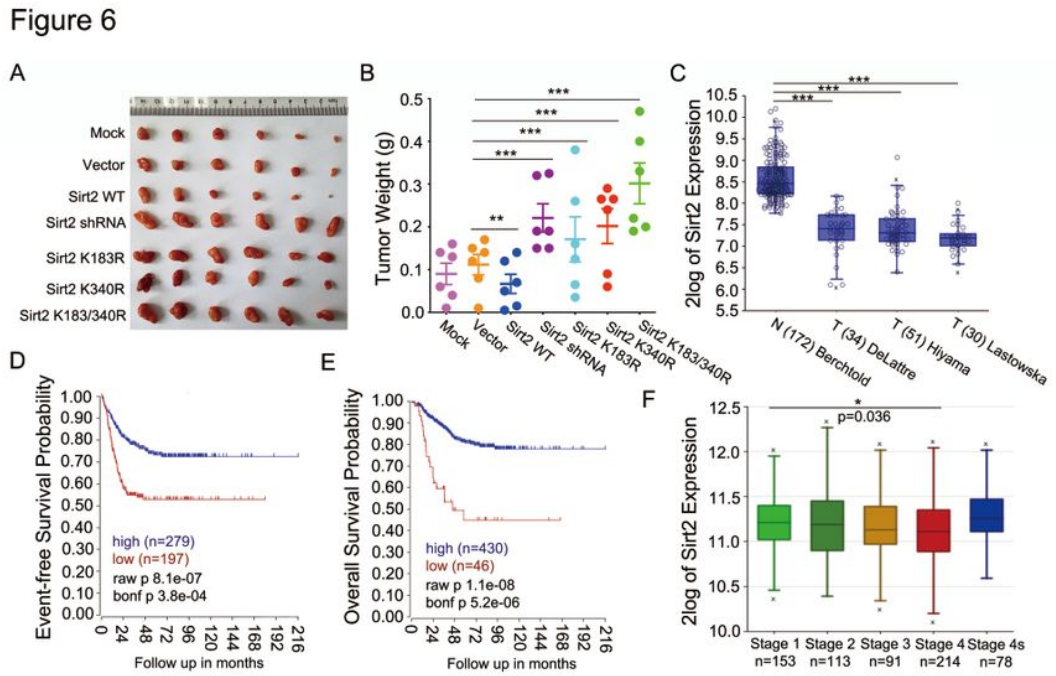

Figure 6

Sirt2-SUMOylation exerts tumor-suppressor property in mice and Sirt2 expression is close related to patient's prognosis. A-B. 5-week old nude mice were subcutaneously injected with 2×106 SH-SY5Y cells stably expressing Sirt2-shRNA, -WT or mutants. Mice were injected with naked or lentivector infected SH-SY5Y cell line was set as the mock or control group, respectively. All mice were sacrificed and tumors were dissected, photographed and weighted at 2 weeks (A). The weight of tumors were analysed by SPSS software (B). C. R2 online database was used for comparison among normal brain tissue (Berchtold 172 cases) and tumor tissues (DeLattre 34 cases; Hiyama 51 cases; Lastowska 30 cases). D-E. Kaplan curve about event-free survival probability (D) or overall survival probability (E) associated with Sirt2 gene expression was generated by R2 online software (Accession: GSE45547, 649 cases). 173 samples

lacking survival data was omitted from the analysis. F. Sirt2 expression levels were compared among patients with different pathological stages (Accession: GSE45547, 649 cases). One way analysis of variance (ANOVA) was used for the statistical analysis. *p<0.05, **p<0.01, ***p<0.001.

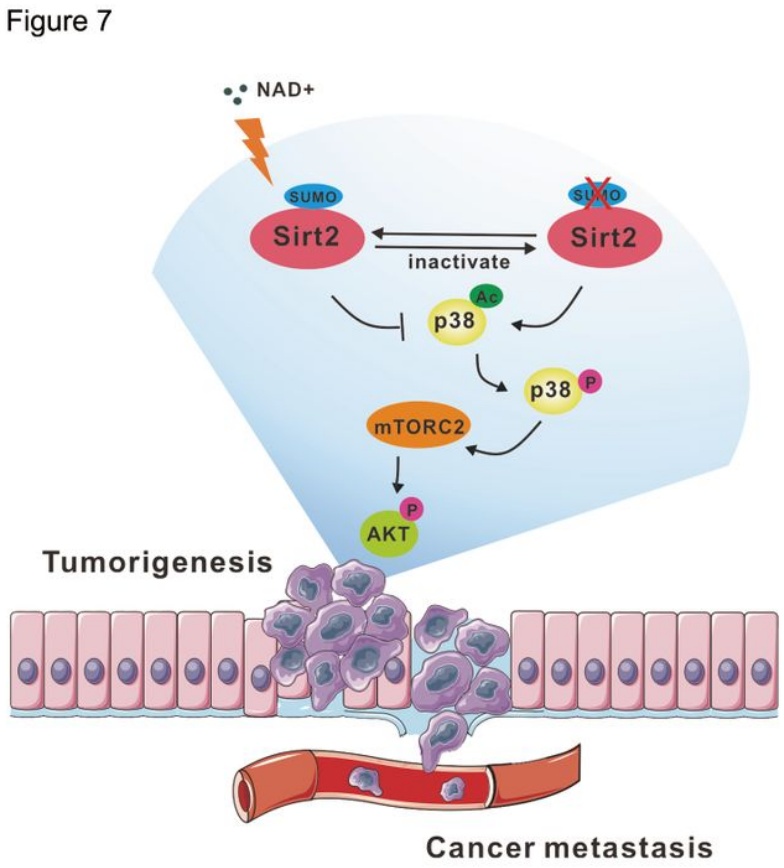

Figure 7

Graphical abstract. Sirt2 is an NAD+-dependent deacetylase and targets to both histone and non-histone protein for deacetylation. NAD+ induced SUMOylation is important for Sirt2 to activate its deacetylase activity. Meanwhile, non-histone protein MAPK/P38 is a new substrate of Sirt2, and its acetylation ensures the phosphokinase activity which can induce the AKT activation through mTORC2 complex. In neuroblastoma cell, Sirt2-SUMOylation seems to function as a 'brake' to control P38 acetylation level and avoid the excessive activation of P38-mTORC2-AKT. However, SUMOylation-deficient Sirt2 will decrease the capability of deacetylation on P38 and results to the aberrant activation of AKT, which ultimately promotes the tumorigenesis or cancer cell metastasis in the development of neuroblastoma.

Supplementary Files

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