

Lysine methylation of FOXO3 regulates oxidative stress-induced neuronal cell death

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FOXO transcription factors have a critical role in oxidative stress-induced neuronal cell death. A variety of post-translational modifications of FOXO family proteins have been reported, including phosphorylation, acetylation, ubiquitination and recently arginine methylation. Here, we demonstrate that the methyltransferase Set9 methylates FOXO3 at lysine 270. Methylation of FOXO3 leads to the inhibition of its DNA-binding activity and transactivation. Accordingly, lysine methylation reduces oxidative stress-induced and FOXO3-mediated Bim expression and neuronal apoptosis in neurons. Collectively, these findings define a novel modification of FOXO3 and show that lysine methylation negatively regulates FOXO3-mediated transcription and neuronal apoptosis.

Keywords: apoptosis; FOXO3; methylation; signal transduction

EMBO reports (2012) 13, 371–377. doi:10.1038/embor.2012.25

INTRODUCTION

Oxidative stress has long been implicated as an essential and causal factor in neuropathology of several neurodegenerative disorders. Upon oxidative stress, a series of genes involved in cell death and recovery are activated [1]. The FOXO transcription factors have a central role in oxidative stress-induced neuronal cell death [2–4]. FOXO promotes neuronal apoptosis in response to oxidative stress through inducing the expression of pro-apoptotic downstream genes including *Bim* and *FasL* [5–7].

Many post-translational modifications including phosphorylation, acetylation, ubiquitination and arginine methylation have

been shown to regulate FOXO's activity [8,9]. For example, it has been well documented that the phosphorylation of FOXOs by the serine–threonine kinase Akt increases their binding to 14-3-3 proteins and results in the translocation of FOXO proteins from the nucleus to the cytoplasm [10]. Previously, we demonstrated that serine/threonine kinase MST1 phosphorylation of FOXO proteins disrupts their interaction with 14-3-3 proteins and increases FOXO's nuclear accumulation [4].

Recently, methylation of lysines by different methyltransferases has been demonstrated to be important in regulation of both histone proteins and non-histone proteins. Set9, originally identified as a Histone H3 Lysine 4 (H3K4) methyltransferase [11], has now been shown to methylate many non-histone proteins such as p53, TAF10, DNMT1 and E2F1, and have an important regulatory role in diverse biological processes [12–16].

Here we identified the FOXO transcription factors as novel substrates of Set9. We found that Set9 methylates FOXO3 at lysine 270 and downregulates its transcriptional activity, which inhibits FOXO3-mediated neuronal cell death upon oxidative stress. Furthermore, we found that lysine methylation of FOXO3 reduces its DNA-binding activity independent of Akt-mediated phosphorylation. Taken together, we demonstrate a new signaling link between Set9 and FOXO3 in oxidative stress-induced neuronal cell death.

RESULTS AND DISCUSSION

Set9 interacts with and methylates FOXO3

Set9 is the first identified lysine methyltransferase that can methylate the non-histone substrates [12–16]. First, we characterized the interaction between Set9 and FOXO3. Upon expression in 293T cells, FOXO3 and Set9 formed a physical complex (Fig 1A). We also found that endogenous FOXO3 associated with Set9 in cerebellar granule neurons (CGNs), by using the anti-Set9 antibody for immunoprecipitation and FOXO3 antibody for immunoblotting (Fig 1B). As Set9 is a lysine methyltransferase, we next test whether Set9 might methylate FOXO3 protein. We found that Set9 methylated both FOXO3 and FOXO1 *in vitro* (Fig 1C). To delineate the methylation domains on FOXO3 by

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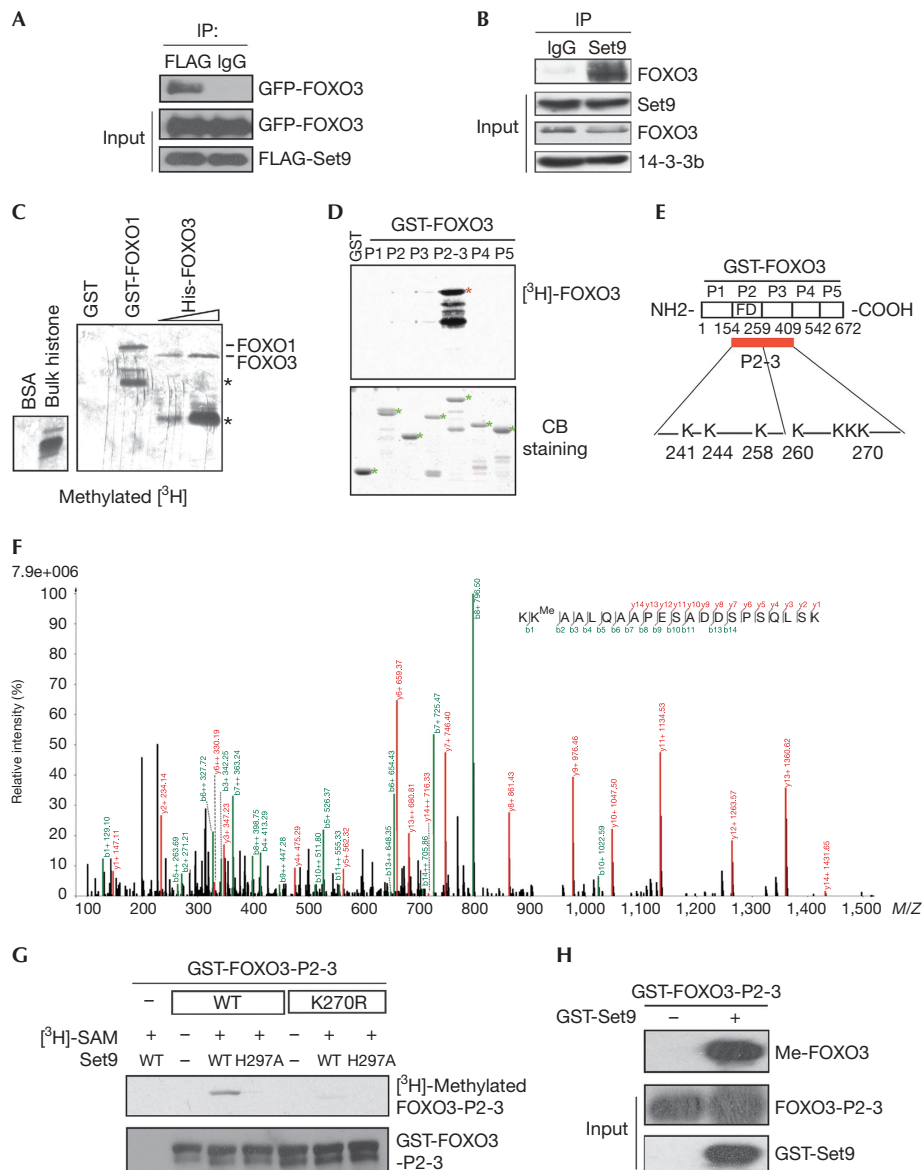


Fig 1 | Set9 interacts with and methylates FOXO3 at K270. (A) Lysates of 293T cells transfected with plasmids encoding GFP-FOXO3 and FLAG-Set9 were immunoprecipitated with anti-FLAG antibody or IgG. Western blot analysis was performed using anti-GFP or FLAG antibody. (B) Anti-Set9 or IgG immunoprecipitates from CGNs were immunoblotted with FOXO3 antibody. (C) Right panel: *In vitro* methylation assays were performed by incubating GST-FOXO3, His-FOXO1 or GST with recombinant Set9 in the presence of ^3H -SAM. The reaction products were subjected to SDS-PAGE, and the methylation of FOXO was detected by autoradiography. Left panel: BSA or bulk histone was used as the negative or positive substrate, respectively. Asterisks stand for the degraded species of FOXO1 or FOXO3 proteins. (D) Methylation assays were performed as in C, and the substrates are GST-fused proteins of the truncated fragments of FOXO3. Fig shows that the methylation mainly occurs in the fragment of GST-FOXO3 P2-3. Red asterisk indicates the methylated FOXO3 fragment; green asterisks stand for the GST-fused fragments of FOXO3. CB, Coomassie Blue. (E) The lysines in the fragment of GST-FOXO3 P2-3 (aa 154-409) are shown. (F) The fragmentation mass spectrometry analysis of the FOXO3 peptide KKMeAALQAAPESADDSPSLSK identified a mono-methylated residue at K270. (G) *In vitro* methylation assay was performed by incubating GST-FOXO3 P2-3 with recombinant WT-Set9 or H297A-Set9 in the presence of ^3H -SAM. (H) Immunoblotting assay shows that FOXO3 K270 mono-methylated antibody recognizes methylated FOXO3 P2-3 by Set9. aa, amino acid; BSA, bovine serum albumin; CGN, cerebellar granule neuron; GFP, green fluorescent protein; GST, glutathione *S*-transferase; IP, immunoprecipitation; Me-FOXO3, methylated FOXO3; SAM, *S*-adenosyl-methionine; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; WT, wild type.

Set9, different glutathione *S*-transferase (GST) fusion proteins encoding different FOXO3 regions (P1-P5 and P2-3, which is the fused P2 and P3) were used for the *in vitro* Set9 methylation assay

(Fig 1D, E). Interestingly, either P2 (forkhead domain) or P3 alone, or the other fragments, was not methylated by Set9, but the fused P2 and P3 (P2-3) were robustly methylated (Fig 1D), suggesting

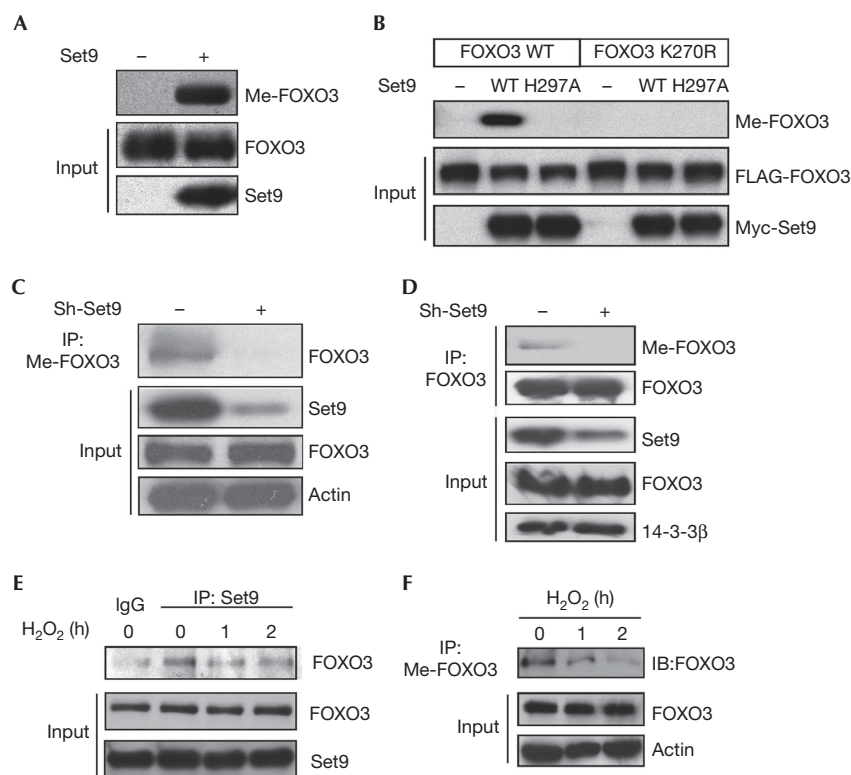


Fig 2 | Set9 methylates FOXO3 at K270 *in vivo*. (A) Lysates of 293T cells transfected with FLAG-FOXO3 together with Myc-Set9 or control vector were immunoblotted with anti-K270 mono-methylated antibody. (B) Lysates of 293T cells transfected with plasmids encoding FLAG-FOXO3 WT or K270R together with Myc-Set9 WT or H297A were immunoblotted with the methylation (K270 mono-methylated) antibody. (C) Anti-Me-K270 FOXO3 immunoprecipitates from 293T cells transfected with Set9 shRNA plasmids or control vector were blotted with anti-FOXO3 antibody. (D) Anti-FOXO3 immunoprecipitates from 293T cells transfected with Set9 shRNA plasmids or control vector were blotted with anti-Me-K270 FOXO3 or anti-FOXO3 antibody. The input was immunoblotted with indicated antibodies. (E) Anti-Set9 immunoprecipitates from primary CGNs treated with 200 μ M H₂O₂ from 0 to 2 h were immunoblotted with anti-FOXO3 antibody. (F) Anti-Me-K270 FOXO3 immunoprecipitates from CGNs and treated with 200 μ M H₂O₂ from 0 to 2 h were immunoblotted with anti-FOXO3 antibody. CGN, cerebellar granule neuron; IP, immunoprecipitation; WT, wild type.

that the structural integration of FOXO3 protein is required for Set9-mediated methylation.

To further map the methylation site(s), we performed mass spectrum analysis of the methylated FOXO3 P2–3, and Lys270 was found as a residue mono-methylated by Set9 (Fig 1F). Next, we mutated Lys270, which is a conserved site among all the FOXO family proteins, to arginine (K270R) for the *in vitro* methylation analysis. As expected, K270R mutation abolished Set9-mediated FOXO3 methylation. We also found that wild-type (WT) Set9, but not catalytically inactive Set9 (Set9 H297A), methylated FOXO3 *in vitro* (Fig 1G). Set9 has been mostly reported as a mono-methyltransferase on non-histone substrates [12–16]. In line with this, we found that FOXO3 was mono-methylated at K270 by Set9 *in vitro* by using the self-raised polyclonal antibodies that specifically recognized the mono-methylated FOXO3 at Lys270 (Fig 1H).

We next determined whether Set9 induces the K270 methylation *in vivo* by using the methylation-specific antibodies. Upon expression in cells, FOXO3 was found to be methylated by Set9 (Fig 2A). We also found that the methylation antibody recognized WT- but not K270R-FOXO3 in cells (Fig 2B). Furthermore,

Set9 H297A failed to induce FOXO3's methylation (Fig 2B). Consistently, knockdown of Set9 by using Set9 shRNA in cells abolished the methylation of FOXO3 (Fig 2C,D). Together, these experiments demonstrate that Set9 methylates FOXO3 at K270 *in vivo*. In response to oxidative stress, many post-translational modifications of FOXO proteins, such as phosphorylation, acetylation and arginine methylation, are dynamically altered [3,4,9,17]. Thus, we next examined whether oxidative stress alters the FOXO3 K270 lysine methylation. We found that the interaction between Set9 and FOXO3 reduced (Fig 2E), and the methylation levels of FOXO3 gradually decreased, in primary CGNs upon H₂O₂ treatment (Fig 2F). In addition, we observed that the H₂O₂ treatment reduced the methyltransferase activity of Set9 (supplementary Fig S2 online). Together, our data implicate that the methylation levels of FOXO3 are dynamically regulated and that oxidative stress reduces the methylation of FOXO3.

FOXO3 methylation suppresses its activity

It has been shown that lysine methylation is important in regulating the activity of transcription factors, such as p53 and

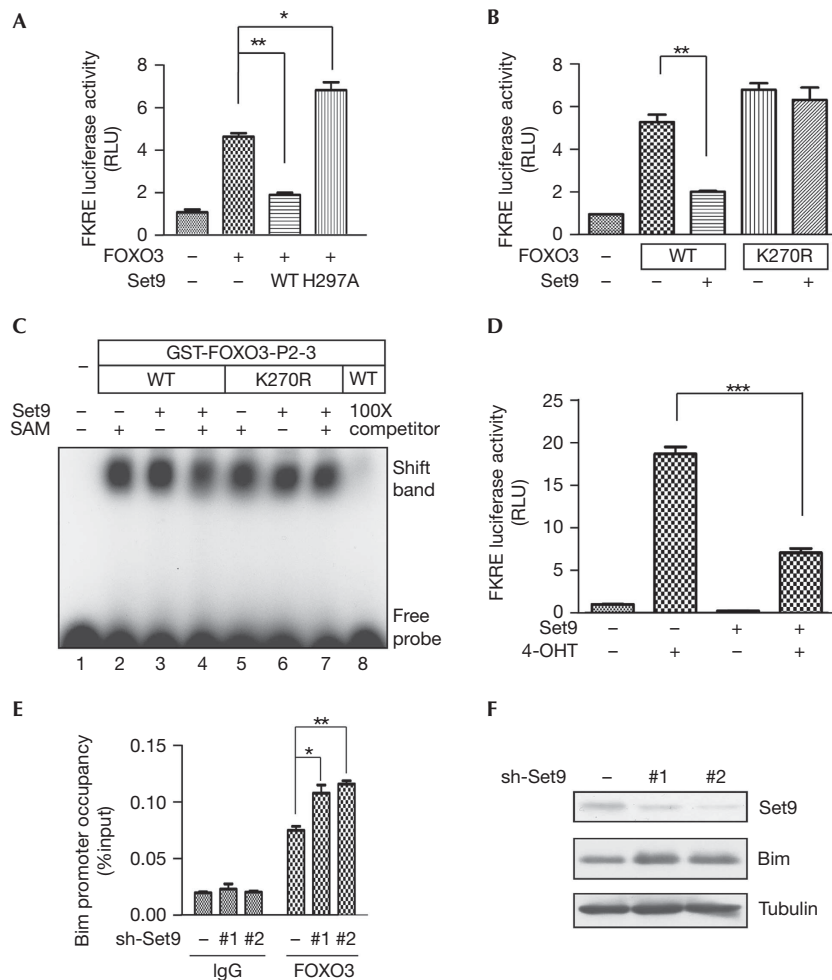


Fig 3 | Methylation of Lys 270 inhibits the DNA-binding activity of FOXO3 and FOXO3-dependent transcription. (A) 293T cells were transfected with FKRE luciferase reporter gene and tk-renilla reporter together with plasmids encoding Set9 WT or H297A mutant, as well as FOXO3 or the control vectors. Shown is mean \pm s.e.m. firefly/renilla luciferase relative to control vectors (*t*-test, $^{**}P < 0.01$, $^{*}P < 0.05$, $n = 3$). (B) 293T cells were transfected with FKRE-luciferase reporter gene and tk-renilla reporter together with plasmids encoding FOXO3 WT or FOXO K270R, as well as Set9 or the vector control. Shown is mean \pm s.e.m. firefly/renilla luciferase relative to control vectors (*t*-test, $^{**}P < 0.01$, $n = 3$). (C) The EMSA was performed using the indicated methylation products. 100X competitor indicates the amount of the cold probe. (D) MEF cells stably expressing inducible ER-FOXO3 TM (triple mutations on Akt phosphorylation sites) were transfected with Set9 together with FKRE-luciferase/tk-renilla reporter. At 24 h after transfection, cells were treated with or without 4-OHT for another 12 h before collecting. Set9 inhibits FOXO3 TM-dependent FKRE-luciferase reporter gene expression (*t*-test, $^{***}P < 0.001$, $n = 3$). RLU, relative light unit. (E) Crosslinked chromatin was prepared from Neuro2A cells transfected with Set9 shRNA plasmid (#1 or #2) or the control plasmid (sh-Luc) and immunoprecipitated with anti-FOXO3 antibody or IgG alone, followed by real-time PCR using primers specific to *Bim* promoter. (F) Lysates of Neuro2A cells transfected with Set9 shRNA plasmid (#1 or #2) or the control plasmid (sh-Luc) were immunoblotted with the indicated antibodies. EMSA, electrophoretic mobility shift assay; FKRE, forkhead response element; SAM, S-adenosyl-methionine; shRNA, small hairpin RNA; TM, triple mutation; WT, wild type.

E2F1 [12,14,18]. We next asked whether Set9-induced methylation of FOXO3 regulates its transcriptional activity. WT-Set9, but not the H297A mutant Set9, significantly suppressed FOXO3-mediated expression of FKRE (forkhead response element) promoter-driven luciferase in 293T cells (Fig 3A). Consistently, Set9 failed to suppress K270R-FOXO3-induced FKRE luciferase expression (Fig 3B). These results suggest that Set9 inhibits FOXO3's transcriptional ability. As K270 is a conserved site in the region next to the DNA-binding domain of FOXO3, we then examined whether the lysine methylation affects FOXO3's

DNA-binding activity. The recombinant WT or K270R FOXO3 P2-3 was methylated by Set9 *in vitro*, followed by the electrophoretic mobility shift assay (EMSA). We found that methylation inhibited the DNA-binding activity of WT-FOXO3 but not that of K270R-FOXO3 (Fig 3C). Thus, Set9-induced FOXO3 methylation might inhibit its transactivation through reducing its DNA-binding activity.

It is well known that Akt phosphorylates FOXO and increases the 14-3-3 binding, resulting in the increased cytoplasmic translocation and decreased transcriptional activity of FOXO [10].

We therefore examined whether Set9-mediated methylation of FOXO3 might affect subcellular localization. We found that oxidative stress induced FOXO3 nuclear accumulation (supplementary Fig S3A online), which is consistent with our previous findings [3,4], and expression of Set9 failed to affect the subcellular localization of FOXO3. Accordingly, Set9 knockdown failed to alter the nuclear translocation (supplementary Fig S3B online) and Akt-dependent phosphorylation at serine 253 (supplementary Fig S3C online). We also found that the methylation of FOXO3 did not affect its binding affinity with 14-3-3 proteins (supplementary Fig S3D online). To further confirm that the negative regulation of FOXO3 by Set9-induced methylation is independent of Akt-mediated phosphorylation, we performed FKRE promoter-driven luciferase reporter assay in MEF cells, which are stably expressed inducible ER-FOXO3-TM (triple mutations on Akt phosphorylation sites [19]). Interestingly, Set9 significantly repressed FOXO3-TM-induced FKRE luciferase expression (Fig 3D). The FOXO3 target gene *Bim* encodes a BH3-only protein, which activated neuronal cell death machinery upon oxidative stress treatment [3,4]. In ChIP (chromatin immunoprecipitation) assay, Set9 knockdown increased the direct binding of FOXO3 to the promoter of its target gene *Bim* (Fig 3E). In addition, Set9 knockdown increased the *Bim* protein expression in Neuro2A (Fig 3F). Taken together, Set9-induced methylation at K270 inhibits the DNA-binding activity and transactivation of FOXO3 protein independent of Akt-mediated phosphorylation.

Set9 inhibits FOXO3-mediated neuronal apoptosis

FOXO3 is important in oxidative stress-induced cell death [17,20]. We found that expression of Set9 repressed FKRE promoter-driven luciferase reporter activity (Fig 4A), whereas Set9 knockdown led to a considerable increase of the reporter activity in primary cultured CGNs (Fig 4B). We found that Set9 knockdown increased the hydrogen peroxide-induced expression of *Bim* in CGN cells (Fig 4C).

It has been shown that Set9 promotes DNA damage-induced apoptosis in tumour cells [12,14]. We next examined whether Set9 regulates neuronal cell death in response to oxidative stress. Set9 knockdown increased oxidative stress-induced cell death in CGNs (Fig 4D), suggesting that Set9's function in the regulation of cell death might depend on the cell types and/or specific cellular stresses. We also found that the knockdown of FOXO3 and Set9 together significantly suppressed neuronal apoptosis (Fig 4E), indicating that Set9 and FOXO3 shared a signalling cascade to regulate the neuronal cell death in response to oxidative stress. In other experiments, expression of exogenous Set9 reduced an RNA-mediated interference-resistant form of FOXO3 (FOXO3R WT)-induced cell death in the background of FOXO RNA-mediated interference (Fig 4F). In contrast to FOXO3R, Set9 failed to suppress FOXO3R K270R expression-induced neuronal apoptosis in response to oxidative stress (Fig 4F). Taken together, Set9-induced methylation of FOXO3 repressed FOXO3-mediated neuronal cell death under oxidative stress.

It has been shown that Set9-mediated methylation regulates the biological functions of its substrates through modulating the protein stability [13–15,21,22]. However, in this study, no protein level change of FOXO3 was observed. In contrast, Set9-mediated methylation inhibits the DNA-binding activity of FOXO3 both

in vitro and *in vivo* (Fig 3C,E), suggesting that lysine methylation of FOXO3 directly alters its DNA binding.

Recently, the acetylation, ubiquitination or arginine methylation of FOXO has been shown to interact with Akt-mediated phosphorylation [9,23,24]. Interestingly, we found that Set9 inhibited the transcriptional activity of Akt sites mutant FOXO3 (TM; Fig 3D). In addition, either expression or knockdown of Set9 in neurons had no effect on FOXO3's subcellular localization and Akt-dependent phosphorylation (supplementary Fig S3A–C online), as well as the 14-3-3 binding (supplementary Fig S3D online), indicating that Set9-induced methylation of FOXO3 might functionally inhibit its transcriptional activation independent of PI3K/Akt signalling. We also observed that Set9-mediated FOXO3 methylation does not alter the acetylation and protein stability of FOXO3 (supplementary Fig S4 online and unpublished observations). Furthermore, we found that the methylation site K270 is conserved among FOXO family proteins (supplementary Fig S5A online) and Set9 methylates FOXO1 at the corresponding site, K271, *in vitro* and in cells (supplementary Fig S5B–D online), suggesting the conservation of the lysine methylation in FOXO family members.

We further observed that FOXO3 levels at K270 decreased in response to oxidative stress (Fig 2E, F), which supports the conclusion that K270 methylation functions as a negative regulation of FOXO3's pro-apoptotic ability. The finding also raises a possibility of the demethylation of FOXO3 proteins. It has been reported that LSD1 (lysine-specific demethylase-1) demethylates Set9-mediated p53 and E2F1 upon DNA damage treatment [12,14]. However, we observed that LSD1 failed to demethylate FOXO3 protein *in vitro*, and LSD1 did not affect the transcriptional activity of FOXO3 in cells (unpublished observations). Thus, it will be interesting to identify the possible specific demethylase(s) for FOXO3 upon oxidative stress treatment in future studies.

In sum, our findings elucidated a new regulatory mechanism of FOXO3 in oxidative stress-induced neuronal cell death, in which Set9 reduces FOXO3 transcriptional activity through methylation at K270. Therefore, our present study provides an unappreciated post-translational modification of FOXO proteins, which might help explore a new regulatory machinery in the other biological hypotheses including cell metabolism and neurological diseases.

METHODS

Cell line culture, transfection and cell death assay. 293T and Neuro2A cells were maintained in DMEM medium supplemented with 10% fetal bovine serum at 5% CO₂ concentration. CGN cultures and transfections were performed as described [25]. At 24 h after H₂O₂ (60–100 μM) treatment, neuronal apoptosis assay was performed as described [26] by using the Zeiss Imager D1 microscope.

Immunoprecipitation and western blotting. Immunoprecipitation and western blot analysis were performed as described [27].

***In vitro* methylation assay.** A measure of 2 μg of the recombinant full-length GST-FOXO3 or fragmental proteins or bulk histone was incubated with 1.5 μg recombinant Set9 in the presence of 2 μCi ³H-S-adenosyl-methionine (SAM, Perkin-Elmer) or 0.1 μM SAM (Sigma) in the reaction buffer (50 mM Tris-Cl, pH 8.5, 5 mM MgCl₂, 0.8 mM DTT) at 30 °C for 1 h. The reaction products were loaded on SDS-polyacrylamide gel electrophoresis,

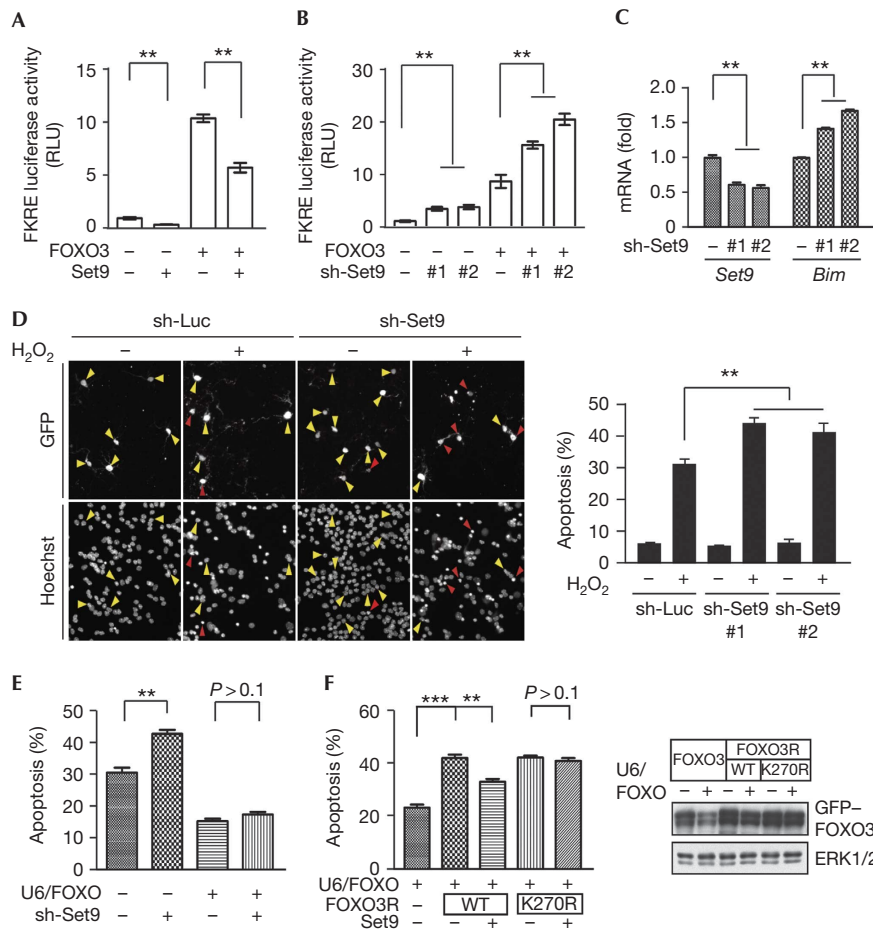


Fig 4 | Set9 inhibits oxidative stress-induced neuronal cell death via methylation of FOXO3. (A) CGNs were transfected with the FOXO3, Set9 WT, Set9 H297A or control vector together with FKRE-luciferase reporter gene and tk-renilla reporter. Set9 significantly inhibits FOXO3's transcriptional activity in CGNs (*t*-test, $P < 0.01$, $n = 3$). (B) CGNs were transfected with the FOXO3 or the control vector with or without Set9 shRNA plasmid (#1 or #2), together with FKRE-luciferase/tk-renilla reporter. Knockdown of Set9 considerably increases the transcriptional activity of FOXO3 (*t*-test, $P < 0.01$, $n = 3$). (C) Total RNA was isolated from cultured Neuro2A cells and cDNA was synthesized by reverse transcription, followed by real-time PCR. Knockdown of Set9 considerably increases the expression of *Bim* (*t*-test, $P < 0.01$, $n = 3$). (D) CGNs transfected with the Set9 RNAi plasmid (sh-Set9 #1 and #2) or control vector (sh-Luc) together with GFP vector were treated with H_2O_2 . Yellow arrowhead stands for the healthy cells and red arrowhead indicates apoptotic cells. Set9 knockdown increases cell apoptosis upon H_2O_2 treatment (ANOVA followed by Fisher's PLSD *post hoc*, $P < 0.01$, $n = 4$). (E) CGNs transfected with the Set9 RNAi plasmid (sh-Set9 #1) or control vector (sh-Luc), FOXO3 RNAi plasmid (U6/FOXO3) or its control vector U6 together with GFP vector were treated with H_2O_2 . Set9 knockdown increases FOXO-dependent cell apoptosis upon H_2O_2 treatment (ANOVA followed by Fisher's PLSD *post hoc*, $P < 0.01$, $n = 3$). (F) Left panel: CGNs transfected with the FOXO3 RNAi plasmid (U6/FOXO3) together with indicated plasmids were treated with H_2O_2 , and cell death was analysed as in E. Data are presented as mean \pm s.e.m. (ANOVA followed by Fisher's PLSD *post hoc*, $P < 0.01$, $n = 3$). Right panel: Lysates of 293T cells transfected with indicated plasmids were immunoblotted with anti-GFP or ERK1/2 antibody. ANOVA, analysis of variance; cDNA, complimentary DNA; CGN, cerebellar granule neuron; FKRE, forkhead response element; GFP, green fluorescent protein; mRNA, messenger RNA; RNAi, RNA-mediated interference; shRNA, small hairpin RNA.

followed by autoradiography or western blotting with the K270 me-FOXO3-specific antibody.

EMSA. EMSAs were performed as described previously [28]. The oligonucleotides sequences are as follows: 5'-CATAAACAAACA TAAACAACATAACAAA-3' and 5'-TTTGTTTATGTTTGTATG TTTGTTATG-3'. The FOXO3-DNA complexes were separated on 6% $1 \times$ TBE polyacrylamide gels and analysed by autoradiography.

Chromatin immunoprecipitation assay. Transfection of neuro2A cells (5×10^6 cells) with the indicated plasmids was performed

with the CHIP assay kit (Millipore no. 17-295) following the manufacturer's protocol. Briefly, FOXO3 antibody (Abcam no. ab-12162) or rabbit IgG was used for the immunoprecipitation of the DNA-protein immunocomplexes. Crosslinking was reversed by heating for 6 h at $65^\circ C$, followed by digestion with proteinase K. The purified DNA was subjected to quantitative PCR with BIM-ChIP primers as reported [29].

Statistical analysis. Statistical analysis of the data was performed with a two-tailed Student's *t*-test or one-way analysis of variance,

followed by Fisher's PLSD (protected least significant difference) *post hoc* test using the Origin software (Version 8). Data are presented as the mean \pm s.e.m. * P <0.05, ** P <0.01 or *** P <0.001 denotes statistical significance.

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

ACKNOWLEDGEMENTS

We thank members of the Yuan laboratory for discussion and critical reading of the manuscript. This work is supported by National Science Foundation of China (30870792, 81030025 and 81125010) and Ministry of Science and Technology of China (973-2009CB918704 and 2012CB910701).

Author contributions: QX, YH, LT, SP and CR performed the experiments. HY, M-qD, ZY and HC participated in study design and data analysis. QX and ZY wrote the paper.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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