Supporting information

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SI Materials and Methods

Materials and Chemicals. Human recombinant PDGF-BB, sodium selenite, hydrocortisone, apo-transferrin, β-estradiol, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), Actinomycin D, protamine sulfate, and chloroquine were purchased from Sigma-Aldrich. Cycloheximide was purchased from Calbiochem. The following antibodies were used: anti-FOXO1(Santa Cruz Biotechnology; CST), anti-FOXO3, anti-FOXO4 (Santa Cruz Biotechnology); anti-PDGFRA (Santa Cruz Biotechnology); anti-PDGFRB (Upstate); anti-total-AKT, anti-phospho AKT(Ser-473), anti-ERK1/2, anti-phospho FOXO3 (Ser-253) (CST); anti-β-Actin, anti-Flag (Sigma-Aldrich); anti-GFP(Abcam).

Plasmid Constructs. The human PDGFRA promoter was cloned into pGL3 basic vector using the following primers: forward 5′-GCG GAGCTC CCTCTTTCCCGGCAGAGCACCAAC-3′; reverse 5′-GGG CTCGAG CCGCAATGAATGTCCCACACATGG-3′. Mutant human PDGFRA luciferase reporter constructs were generated using KOD-plus and DpnI (TOYOBO).Primer sequences for the mutagenesis were: forward 5′-GCTTATTTCCTGACAGCTAGGGACTTAGAGCAAATG-3′; reverse 5′-CATTTGCTCTAAGTCCCTAGCTGTCAGGAAATAAGC-3′. For mouse PDGFRA luciferase reporter, the primer sequences were: forward 5′-GGA GGTACC CGTCAACACCTCCCCCTTC-3′; reverse 5′-GGG AGATCT CCGATACCCGGAGTG-3′. The primers for mPDGFRA mutant reporter were: forward 5′-GACTTCATTTCCTGACAGCTAGGGACTTTAAGCAAATG-3′; reverse 5′-CATTTGCTTAAAGTCCCTAGCTGTCAGGAAATGAAGTC-3′. The mutagenesis primer for constructing the resistant form were: PRA-Res-F: 5′-GTGACTACATG GATATGAAACAGGCAGACACTACACAGTATG-3′; PRA-Res-R: 5′-CATACTGTGTAGTGTCTGCCTGTTTCATATCCATGTAGTCAC-3′. The following primers were used to generate FOXO resistant form expression vector: FOXO1-Res-F: 5′-GCGTGCCGTATTTTAAAGATAAGGGTGACAGCAACAGCTC-3′; FOXO1-Res-R: 5′-GAGCTGTTGCTGTCACCCTTATCTTTAAAATACGGCACGC-3′. FOXO3-Res-F: 5′-GGAACTGGCAAGAGTTCATGGTGGATAATTAACCCTGATGG-3′; FOXO3-Res-R: 5′-CCATCAGGGTTAATTATCCACCATGAACTCTTGCCAGTTCC-3′.FOXO4-Res-F: 5′-CGTCCACGAAGCAGTTCTAACGCGAGTAGCGTCAGCACCC-3′; FOXO4-Res-R: 5′-GGGTGCTGACGCTACTCGCGTTAGAACTGCTTCGTGGACG-3′.

Cell Culture. Neuroblastoma cell lines SH-SY5Y and SK-*N*-SH were cultured in RPMI medium 1640 containing 10% FBS and penicillin (100 units/mL) and streptomycin (100 μg/mL). Cells were maintained at 37 °C in a saturated humidity atmosphere containing 95% air and 5% CO_2. Neuroblastoma cell differentiation was induced by treatment with 16 nM TPA plus 10 ng/mL PDGF-BB in SHTE medium (RPMI medium 1640 containing 30 nM sodium selenite, 10 nM hydrocortisone, 30 μg/mL apo-transferrin, and 10 nM β-estradiol).

QRT-PCR. Relative transcript expression levels of various genes were assessed by QRT-PCR. PDGFRA primers: forward 5′-GAGCATCTTTGACAACCTCTACAC-3′; reverse 5′-CCGGTACCCACTCTTGATCTTATTG-3′. PDGFRB primers: forward 5′-CAGCTCCGTCCTCTATACTGC-3′; Reverse 5′-GGCTGTCACAGGAGATGGTT-3′. Mouse PDGFRA primers: forward 5′-CCGGGTATCGGATTTTCTTT-3′; reverse 5′-GCCAGCCTCACTTCACTCTCC-3′. GAPDH primers: forward 5′-AGCCACATCGCTCAGACA-3′; reverse 5′-GCCCAATACGACCAAATCC-3′.FOXO1 primers: forward 5′-CTTCAAGGATAAGGGTGACAGCAAC-3′; reverse 5′-TGGATTGAGCATCCACCAAGAA-3′. FOXO4 primers: forward 5′-TGTACCCTACTTCAAGGACAAGG-3′; reverse 5′-CTCCCTCAGGGTTCAGCAT-3′. GAP43 primers: forward 5′-AGTGAGCAAGCGAGCAGAA-3′; reverse 5′-GTTGCGGCCTTATGAGCTT-3′; IGF-1R primers: forward 5′-ACTTACTCGGACGTCTGGTCCTTC-3′; reverse 5′-ATCTTGGGGTTATACTGCCAGCAC-3′; Insulin receptor primers: forward 5′-TCATCAACGGGCAGTTTGT-3′; reverse 5′-TTACAGATGGTCGGGCAAA-3′. For QRT-PCR analysis, each template was tested in triplicate. The abundance of each gene was determined relative to GAPDH.

Western Blotting. Cells were lysed on ice with 1% Triton X-100 lysis buffer containing 100 mM NaCl, 50 mM Tris, 2 mM EDTA, 40 mM glycerophosphate, 1 mM Na_4VO_3, 10 mM NaF, and protease inhibitors. Cell lysates were analyzed with indicated antibodies.

RNA Interference. Knockdown of individual FOXO family members and PDGFRA were achieved with hairpins targeting the following sequence: FOXO1 (1), FOXO3 (2), FOXO4- GCAGTTCAAATGCCAGCAGTG, PDGFRA- GGACATGAAGCAGGCTGATAC. The sequence for control shRNA was 5′-GCAAAGAAGGCCACTACTATA-3′. The shRNA was cloned into the pLV-H1-EF1α-Puromycin/Blasticidin lentiviral vector, and lentivirus were generated according to the manufacturer's protocol (Biosettia). The neuroblastoma cells were infected with lentivirus in the presence of 5 μg/mL protamine sulfate for 24 h.

Neurite Length Measurements. Neurites were analyzed using an Olympus IX51 microscope. Images were acquired with a CCD camera and Image-Pro Plus 6.0 analysis software (Olympus). Lengths were determined as the distance between the edge of the cell body and the tip of the growth cone. The length of each neurite was measured manually and the mean length of the neurites per cell was calculated. The analyses were carried out "blind" to avoid any subjective influences during the measurements. At least 150 cells from three randomly selected fields were counted from one experiment. Data represent mean ± SD of two independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Statistical Analysis. Statistical evaluation of the data was performed using Student *t* test. $P < 0.05$ was considered to indicate a significant difference. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

1. Potente M, et al. (2005) Involvement of Foxo transcription factors in angiogenesis and postnatal neovascularization. *J Clin Invest* 115:2382–2392.

2. Tsai WB, Chung YM, Takahashi Y, Xu Z, Hu MC (2008) Functional interaction between FOXO3a and ATM regulates DNA damage response. *Nat Cell Biol* 10:460–467.

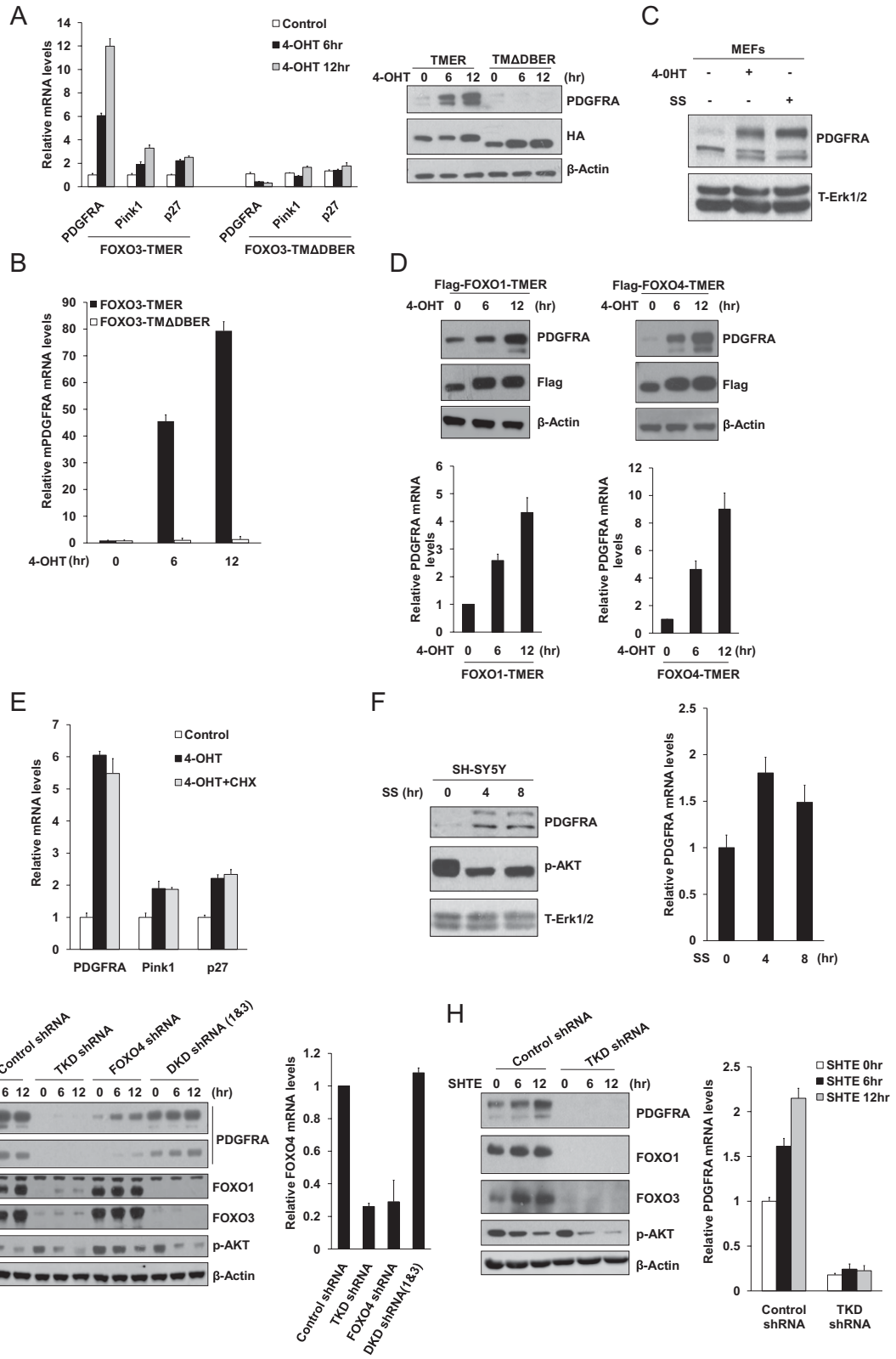

Fig. S1. FOXO-dependent induction of PDGFRA. (*A*) FOXO3-TM activates PDGFRA expression. SH-SY5Y cells stably expressing HA-FOXO3-TMER or TMΔDBER were exposed to 4-OHT (1 μM) for the indicated times. Total RNA and protein were then extracted. Levels of PDGFRA, Pink1, p27 mRNA were examined by QRT-PCR and normalized to GAPDH mRNA. The protein lysates were subjected to Western blotting with antibodies against PDGFRA, HA, and β-actin. Unless otherwise stated, all results shown are one trial representative of at least three independent experiments. (*B*) $p53^{-/-}$ FOXO3-TMER or TMΔDBER MEF cells treated with 4-OHT (1 μM) for 6 h or 12 h were assessed by QRT-PCR and normalized to GAPDH mRNA. QRT-PCR data are means ± SD from three independent experiments. (*C*) $p53^{-/-}$ FOXO3-TMER MEFs either treated with 4-OHT (1 μM) for 10 h or serum starvation for 24 h were subjected to Western blotting with

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indicated antibodies. T-Erk1/2, loading control. (*D*) Transactivation of PDGFRA by FOXO1/FOXO4. SH-SY5Y cells stably expressing Flag-FOXO1-TMER or Flag-FOXO4-TMER were treated with 4-OH (1 μM) for 6 h or 12 h. Total RNA and protein were extracted for QRT-PCR and Western blotting. GAPDH, normalization control. All data are means ± SD of three independent experiments. (*E*) FOXO-dependent induction of PDGFRA does not require de novo protein synthesis. SH-SY5Y FOXO3-TMER cells were treated with CHX for 30 min before the addition of 4-OHT to prevent de novo protein synthesis. After the addition of 4-OHT (1μM) for 6 h, relative levels of PDGFRA, Pink1, and p27 mRNA were determined by QRT-PCR. (*F*) Serum deprivation induces PDGFRA expression. SH-SY5Y cells were subjected to serum withdrawal for 4 h or 8 h. Lysates were prepared and analyzed by Western blotting with antibodies against the indicated proteins (*Left*). Levels of PDGFRA mRNA were assayed by QRT-PCR and normalized to control GAPDH mRNA (*Right*). (*G*) Simultaneously knocking down FOXO1/3/4 attenuates the induction of PDGFRA upon serum-free SHTE treatment. SH-SY5Y cells infected with control shRNA, FOXO4 shRNA, FOXO1/3 double knockdown shRNA (DKD), or triple knockdown shRNA (TKD) were assayed by Western blotting with indicated antibodies (*Left*). FOXO4 mRNA levels were detected by QRT-PCR (*Right*). (*H*) Depletion of FOXOs down-regulates PDGFRA in SK-*N*-SH cells. Cells infected with control shRNA or TKD shRNA were subjected to serum-free SHTE treatment for 6 h or 12 h. Total RNA and protein were then extracted. Cell lysates were subjected to Western blotting with antibodies against the indicated proteins (*Left*). Relative levels of PDGFRA mRNA were determined by QRT-PCR (*Right*).

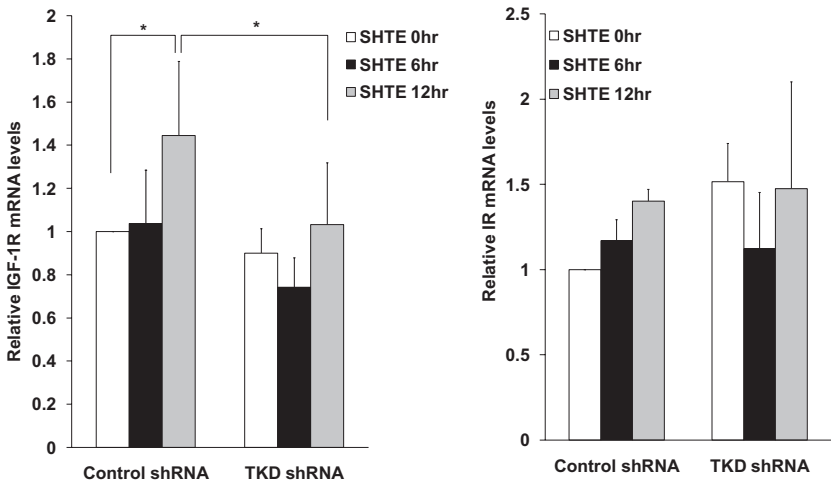

Fig. S2. IGF-1R and IR expression levels in SH-SY5Y cells expressing FOXO TKD shRNA. Cells expressing control shRNA or TKD shRNA were subjected to serum-free SHTE treatment. Total RNA were then assessed by QRT-PCR and normalized to GAPDH mRNA. QRT-PCR data are means ± SD from five independent experiments. **P* < 0.05.

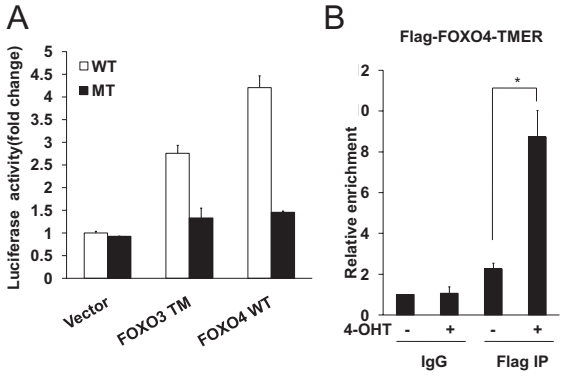

Fig. S3. (*A*) FBE mutation impaired transactivation of the mouse *pdgfra* promoter by FOXO proteins. 293T cells were cotransfected with either control vector or FOXO expression vector (FOXO3TM or FOXO4 WT) together with mouse *pdgfra* promoter luciferase reporters containing either intact (WT) or mutated (MT) FBE. Results were represented the mean and error of three independent experiments conducted in triplicates. (*B*) Ectopically expressed FOXO4 proteins physically bind to the *pdgfra* promoter. QRT-PCR was conducted after chromatin IP using samples from pBabe-Flag FOXO4-TMER SH-SY5Y cells that were either untreated (Con) or treated with 4-OHT. **P* < 0.05.

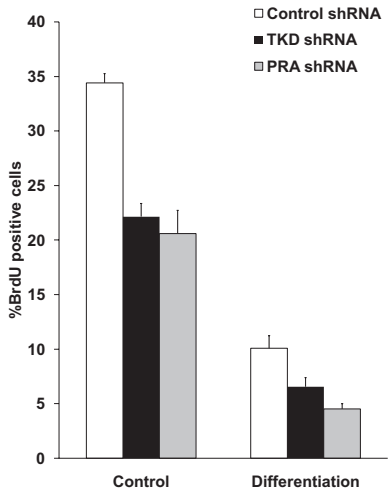

Fig. S4. SH-SY5Y cells expressing indicated shRNA were pulsed with BrdU (10 μg/mL) for 12 h, and then stained with anti-BrdU antibody followed by FACS analysis for BrdU-positive cells. Data represent mean ± SD of three independent experiments.

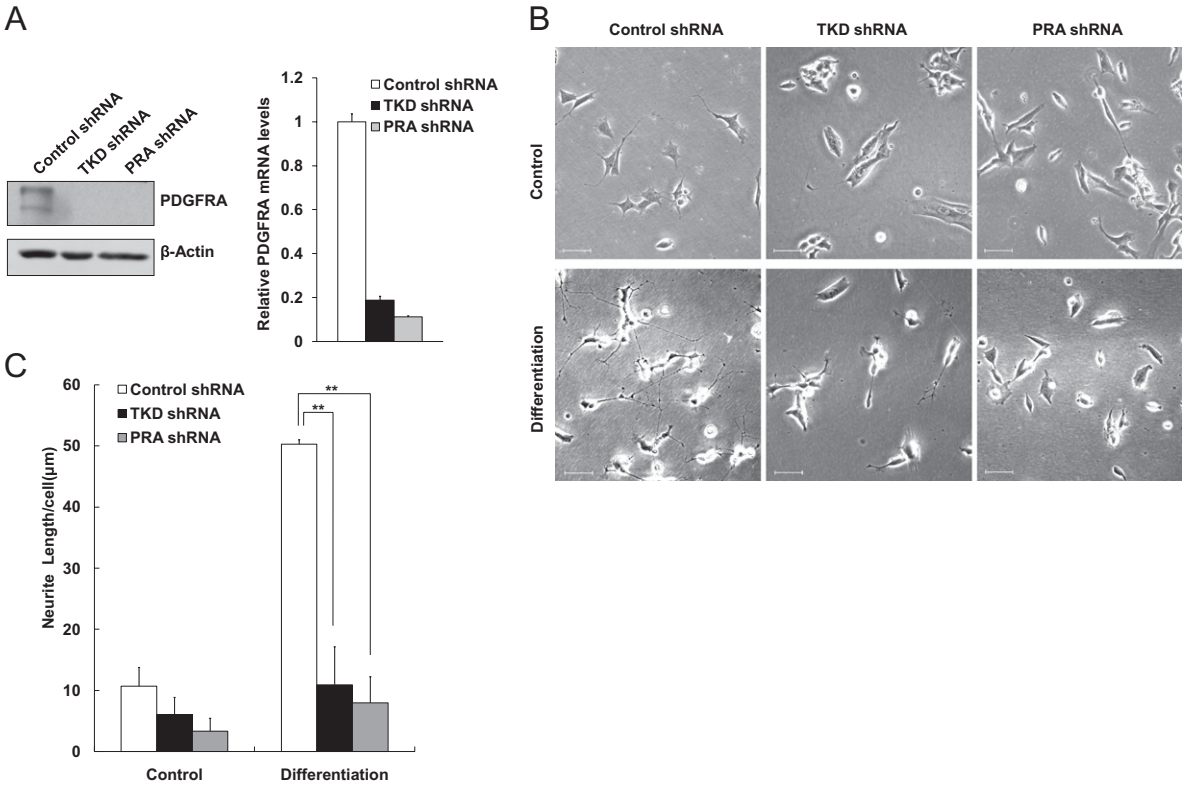

Fig. S5. Depletion of FOXOs or PDGFRA abolishes the differentiation ability of SK-*N*-SH cells. Cells expressing indicated shRNA (*A*) were cultured with STP medium for 72hr, cell morphology change was then analyzed by phase contrast microscopy (*B*), and by measuring average neurite length per cell (*C*). (Scale bars, 50 μm.)

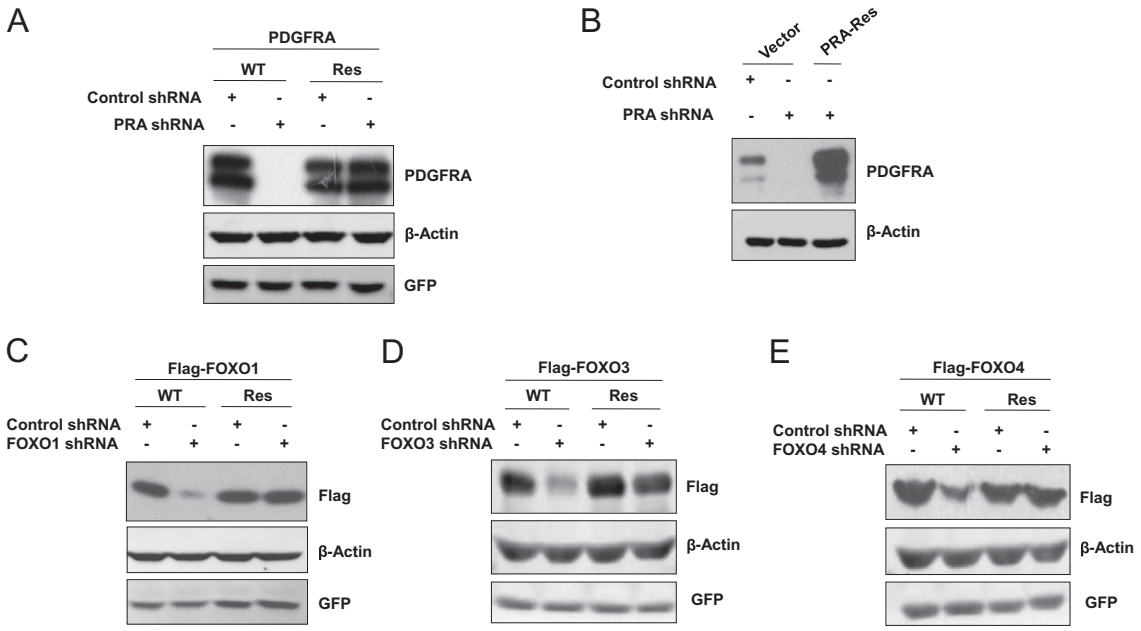

Fig. S6. Confirmation of shRNA resistant constructs. (*A*) 293T cells were cotransfected with indicated constructs. Total protein was extracted. The protein lysates were subjected to Western blotting with PDGFRA, β-Actin, GFP antibodies. Note that the endogenous level of PDGFRA in 293T is undetectable. GFP was used as the internal transfection efficiency control. (*B*) PRA-Res expression in PDGFRA knockdown cells. SH-SY5Y cells stably expressing indicated lentiviral vectors were collected and lysates were subjected to Western blotting with antibodies against PDGFRA and β-Actin. (*C*–*E*) Lysates of 293T cells transfected with indicated constructs were subjected to Western blotting with the indicated antibodies.

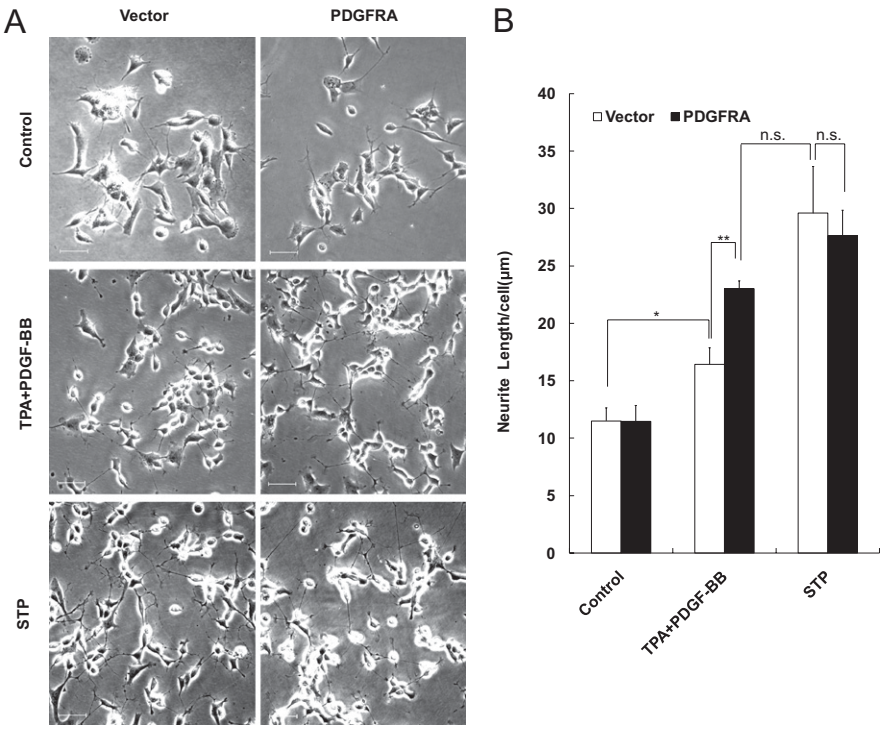

Fig. S7. Differentiation of SH-SY5Y cells stably expressing PDGFRA in the presence of TPA and PDGF-BB. Cells expressing either vector or PDGFRA were subjected to TPA/PDGF-BB or STP treatment. (*A*) Cell morphology change was then analyzed by phase contrast microscopy. (Scale bars, 50 μm). (*B*) Average neurite length per cell was analyzed. $*P < 0.05$; $**P < 0.01$; n.s., not significant.

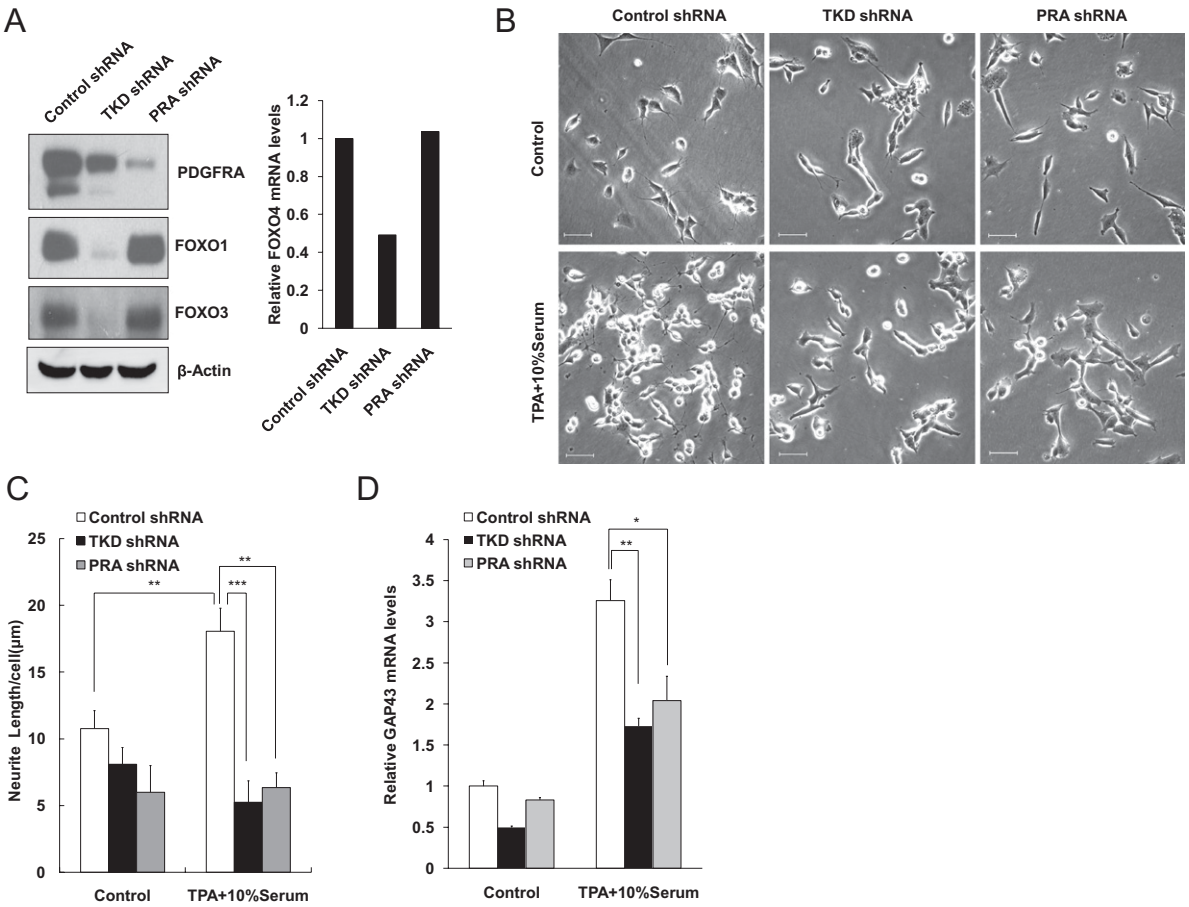

Fig. S8. Down-regulation of FOXO or PDGFRA attenuates SH-SY5Y differentiation upon TPA/serum treatment. (*A*) SH-SY5Y cells expressing indicated shRNA were subjected to Western blotting and QRT-PCR to determine the knockdown efficiency. (*B*) Cells in *A* cultured in growth medium (containing 10% FBS) were treated with 16 nM TPA to induce differentiation. Differentiation morphology change was analyzed by phase contrast microscopy. (Scale bars, 50 μm.) (*C*) Average neurite length per cell was quantified. (*D*) RNA was extracted, and GAP43 expression levels were analyzed by QRT-PCR.

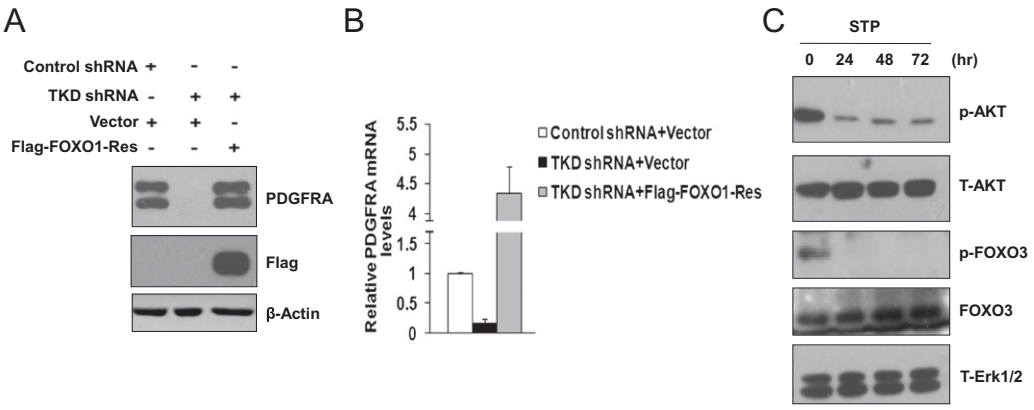

Fig. S9. (*A*) Expression of FOXO1-Res rescued the TKD shRNA-induced down-regulation of PDGFRA expression. SH-SY5Y cells stably expressing Flag-FOXO1-Res were infected with control shRNA or TKD shRNA. After differentiation for 48 h, cell lysates were analyzed by Western blotting. (*B*) Total RNA from cells in *A* were analyzed by QRT-PCR and normalized to GAPDH mRNA. (*C*) STP treatment inactivates Akt. SH-SY5Y cells treated with STP medium at different time points were subjected to Western blotting using indicated antibodies. T-Erk1/2, loading control.