Supplementary Data

Dyrk1A Phosphorylates p53 and Inhibits Proliferation of Embryonic Neuronal Cells

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I. Supplementary Methods

Cell Culture and DNA Transfection

H19-7 cells were maintained in DMEM containing 10% heat-inactivated FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 200 μg/μl G418. Cells were grown at 33°C in 5% CO_2. Human osteosarcoma U2OS cells, human breast carcinoma MCF-7 cells, and human embryonic kidney HEK293 cells were cultured at 37°C in DMEM containing 10% heat-inactivated FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. The p53-deficient human lung carcinoma H1299 cells were cultured at 37°C in RPMI medium 1640 containing 10% heat-inactivated FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Dyrk1A-overexpressing H19-7 cells (H19-7/Dyrk1A) were established and cultured as described previously (Park et al., 2007). Neural precursor hES-NP cells at stage IIa were derived from human embryonic stem cells (H-9, established at WiCell, Wisconsin, passages 35 - 38) and cultured as previously described (Park et al., 2005). All DNA transfections were performed using Lipofectamine and plus reagents (Invitrogen), according to the manufacturer's protocol.

Cortical neurons were isolated from E18.5 Sprague-Dawley rat embryos, and dissociated with 0.1% trypsin digestion. After 5 times of trituration with a fire-polished Pasteur pipette, the cells (2.0×10^6) were plated into poly-L-lysine- and laminin-coated 60-mm dishes and cultured in neurobasal medium supplemented with 2% B-27 and 0.5 mM L-glutamine in a humidified incubator with 5% CO_2 at 37°C. Half of the culture medium was changed every 3 days. For DNA transfection, the media was changed with transfection media (10 mM HEPES, 1 mM pyruvate, 0.6% glucose, and 2 mM glutamine in MEM), and a mixture of DNA (4 μg)

and Lipofectamine 2000 (5 μl) in Opti-MEM (Invitrogen) was added according to the manufacturer's protocol. After 4 h incubation, the media was changed with fresh neurobasal medium supplemented with 2% B-27 and 0.5 mM L-glutamine, and the neurons were cultured for 72 h.

DNA Constructs and RNA Interference

Mammalian constructs encoding wild-type and kinase-inactive rat Dyrk1A tagged with hemagglutinin (HA) (pSVL-HA-Dyrk1A WT and K188R) were a kind gift from W. Becker (Institut fur Pharmakologie und Toxikologie, Universitatsklinikum der RWTH, Germany). Plasmids encoding 6xHis-Xpress-tagged wild-type and K188R mutant Dyrk1A (pcDNA4/HisMax-Dyrk1A WT and K188R) were generated as described previously (Park et al., 2007). Plasmids encoding V5-6xHis-tagged rat Dyrk1A were made by amplifying the rat *Dyrk1A* gene by PCR with the forward primer 5'-GAATTCACGATGCATACAGGAGGAGAGA-3' and the reverse primer 5'-CTCGAGCGAGCTAGCTACAGGACT-3'. The PCR products were sub-cloned into a pcDNA3.1/V5-His vector (Invitrogen) containing *EcoR*I and *Xho*I sites. Prokaryotic construct expressing bacterial recombinant 6xHis-tagged Dyrk1A [pET-28a(+)-Dyrk1A] was generated through PCR amplification of rat *Dyrk1A* gene with the forward primer 5'-GAATTCACGATGCATACAGGAGGAGAGA-3' and the reverse primer 5'-CTCGAGCGAGCTAGCTACAGGACT-3' and sub-cloning into a pET-28a(+) vector (Novagen) containing *EcoR*I and *Xho*I sites.

To clone rat *p53* and generate a construct encoding V5-6xHis-tagged p53, we isolated mRNA from rat cerebrum tissue using Trizol reagents. This mRNA served as a template for the synthesis of a cDNA library with SuperScript III reverse transcriptase, which was used according to the manufacturer's protocol. Rat *p53* transcripts were specifically amplified from the cDNA library by PCR using the forward primer 5'-GAATTCATGGAGGATTCACAGTCGGA-3' and the reverse primer 5'-CTCGAGGTCTGAGTCAGGCCCCAC-3'. The PCR products were sub-cloned into a pcDNA3.1/V5-His vector containing EcoRI and XhoI sites. The construct encoding V5-6xHis-tagged wild-type human p53 was a kind gift from J. W. Cho (Yonsei University, Seoul, Republic of Korea). For purification of recombinant 6xHis-tagged human p53 proteins, a full-length fragment of human *p53* was amplified by PCR with the forward primer 5'-GAATTCATGGAGGAGCCGCAGTCAGATC-3' and the reverse primer 5'-

CTCGAGGTCTGAGTCAGGCCCTTCTGTC-3'. The PCR products were sub-cloned into a pET-28a(+) vector containing *EcoR*I and *Xho*I sites. Truncated forms of human *p53* were also sub-cloned into a pET-28a(+) vector in the same manner using the following primers: for $p53^{1-293}$, same forward primer used for wild-type and reverse primer 5'-CTCGAGCCCTTTCTTGCGGAGATTCTCTT-3'; for $p53^{1-167}$, wild-type forward primer and reverse primer 5'-CTCGAGCTGTGACTGCTTGTAGATGGCCA-3'; for $p53^{1-93}$, wild-type forward primer and reverse primer 5'-CTCGAGCAGGGGCCAGGAGGGGGCT-3'; for $p53^{94-293}$, forward primer 5'-GAATTCATGTCATCTTCTGTCCCTTCCCAGAAA-3' and the same reverse primer used for $p53^{1-293}$; for $p53^{94-393}$, same forward primer used for $p53^{94-293}$ and the wild-type reverse primer.

To construct p53-S15A mutant, we used pET-28a(+)-$p53^{1-167}$ plasmids as templates to perform PCR for site-directed mutagenesis, which was performed using a QuikChange XL site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. PCR was performed using the forward primer 5'-GCGTCGAGCCCCCTCTGGCTCAGGAAACATTTTCAG-3' (the site encoding Ala 15 is underlined) and the reverse primer 5'-CTGAAAATGTTTCCTGAGCCAGAGGGGGCTCGACGC-3' (the site encoding Ala 15 is underlined). p53-S33A and p53-S90A mutants were constructed using pcDNA3.1/V5-His-p53 plasmids as templates to perform PCR for site-directed mutagenesis in the same manner. PCR was performed using the following primers: for p53-S33A, the forward primer 5'-GAAAACAACGTTCTGGCCCCCTTGCCGTCCCAA-3' (the site encoding Ala 33 is underlined) and the reverse primer 5'-TTGGGACGGCAAGGGGGCCAGAACGTTGTTTTC-3' (the site encoding Ala 33 is underlined); for p53-S90A, the forward primer 5'-CCTGCACCAGCCCCCGCCTGGCCCCTGTCATCT-3' (the site encoding Ala 90 is underlined) and the reverse primer 5'-AGATGACAGGGGCCAGGCGGGGGCTGGTGCAGG-3' (the site encoding Ala 90 is underlined). All serine to alanine substitutions were confirmed by DNA sequencing.

Plasmids encoding Flag-tagged human p53 as well as the firefly luciferase reporter containing a synthetic p53 response element, the $p21^{CIP1}$ promoter, or the *Mdm2* promoter, were a kind gift from C. O. Joe (Korea Advanced Institute of Science and Technology, Taejon, Republic of Korea). The p53-specific siRNA and scrambled siRNA were designed and synthesized by Sigma-Proligo. The p53 siRNA duplex sequences were 5'-

rAUrArCrCrArCUrAUrCrCrArCUrArCrArATT-3' and 5'-UUrGUrArGUrGrGrAUrArGUrGrGUrA UTT-3'. Scrambled siRNA duplex sequences were 5'-rGrGrAUrArGUUrGrCrGrAUUrGrArGUUTT-3' and 5'-rArArCUrCrArAUrCrGrCrArArCUrAUrCrCTT-3'. These siRNA duplexes were transfected into cells using Lipofectamine and plus reagents, according to the manufacturer's protocol.

Lentivirus-mediated Transduction

For production of lentiviral particles containing Dyrk1A, either wild-type or a K188R *Dyrk1A* was amplified by PCR using the forward primer 5'-GAATTCTCATGCATACAGGAGGAGAGA-3' and the reverse primer 5'-CTCGAGGCCGAGCTAGCTACAGGACTC-3'. The resulting PCR products were then sub-cloned into a pENTR1A vector (Invitrogen) containing *Eco*RI and *Xho*I sites to generate an entry clone. An entry clone containing human p53 was also constructed in the same manner using the forward primer 5'-GAATTCACATGGAGGAGCCGCAGTCAGAT-3' and the reverse primer 5'-CTCGAGGAGTCTGAGTCAGGCCCTTCTGT-3'. *Dyrk1A* and *p53* were then transferred from pENTR1A entry clones into pLenti6/V5-DEST vectors using LR recombination reactions. Final constructs were subjected to DNA sequencing. Lentiviral particles containing either Dyrk1A or p53 were generated using 293FT cells and ViraPower lentiviral packaging mix, according to the manufacturer's protocol. In a few cases, transduction was performed using lentiviral particles generated by Macrogen (Seoul, Republic of Korea). H19-7, hES-NP, and U2OS cells were then transduced with 293FT supernatants containing lentiviral particles in the presence of polybrene (8 - 10 μg/ml) for 6 - 8 h. The cells were then transferred to fresh culture media and grown for 48 - 72 h.

Terminal dUTP Nick End Labeling (TUNEL) Assay

H19-7/Dyrk1A and H19-7/pTK cells (5.0×10^4 cells) were seeded onto poly-L-lysine-coated dishes and cultured overnight. Apoptotic cells were then estimated using the *in situ* cell death detection kit, TMR red (Roche Applied Science), according to the manufacturer's protocol.

Immunocytochemistry and Immunohistochemistry Analysis

H19-7 cells (5.0×10^4 cells) were seeded onto poly-L-lysine-coated coverglasses, and cells were mock-transfected or transfected with Dyrk1A-V5. Cells were then washed twice with PBS and immunostained as described previously (Park et al., 2007). Cells were stained with

mouse monoclonal anti-p53 and rabbit polyclonal anti-V5 antibodies. A TRITC-conjugated antibody against mouse IgG and a FITC-conjugated antibody against rabbit IgG were used to detect the primary antibodies. Where specified, the samples were stained with 4',6'-diamidino-2-phenylindole (DAPI), and visualized using a LSM 510 META confocal microscope (Carl Zeiss). Confocal data obtained at a wavelength of 405 nm for DAPI, 488 nm for FITC, and 543 nm for TRITC. Data were then processed using the Zeiss LSM Image Browser (Carl Zeiss).

For immunohistochemical analysis of Dyrk1A and p53, surgically isolated E14.5 mouse embryos were fixed on 4% paraformaldehyde/PBS and cryoprotected in 30% sucrose for 2 days at 4°C. After freezing in OCT compound (Sakura Finetek), coronal sections (10 μm) of frozen embryos were mounted on ProbeOn Plus slides (Fisher Scientific), and immunostained using the primary anti-Dyrk1A and anti-p53 antibodies (Cell Signaling Technology), and the secondary Alexa Fluor 488-conjugated anti-mouse IgG and Alexa Fluor 546-conjugated anti-rabbit IgG antibodies, respectively (Invitrogen).

Purification of Bacterial Recombinant Dyrk1A and p53 Proteins

BL21 bacteria were transformed with pET-28a(+)-Dyrk1A or pET-28a(+)-p53 constructs, and the protein expression was induced with isopropyl β-D-1-thiogalactopyranoside. Cells were sonicated in lysis buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 15 mM imidazole, and 2 mM phenylmethylsulfonyl fluoride), and centrifuged at 3,000 x *g* for 15 min at 4°C. The resulting supernatant was incubated with Ni^{2+}-NTA agarose beads overnight at 4°C. The beads were then pelleted by centrifugation at 1,000 x *g* for 3 min at 4°C and washed five times with washing buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 100 - 250 mM imidazole, and 2 mM phenylmethylsulfonyl fluoride). The recombinant proteins were eluted by adding elution buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 250 - 500 mM imidazole, and 2 mM phenylmethylsulfonyl fluoride).

In Vitro Binding Assay of p53 and Dyrk1A

To avoid endogenous p53 contamination, we transfected p53-null H1299 cells with V5-tagged wild-type Dyrk1A. Twenty-four hours later, cells were lysed in 1% Nonidet P40 lysis buffer. One milligram of cell lysate was incubated overnight at 4°C with anti-V5 antibody and recombinant 6xHis-tagged p53. The mixture was then incubated with 30 μl of a 1:1 Protein A-Sepharose bead suspension for 2 h at 4°C with gentle inverting.

Immunoprecipitation and immunoblot analyses were then performed.

Materials

Three separate anti-Dyrk1A antibodies were purchased from BD Biosciences, Abnova Corporation, and Cell Signaling Technology. Mouse monoclonal anti-p53, rabbit polyclonal anti-p53, six anti-phospho-p53 (Ser-6, -9, -15, -20, -37, and -46), anti-Cyclin D1, and anti-PTEN antibodies were purchased from Cell Signaling Technology. Anti-Flag, anti-phospho-serine, and anti-phospho-threonine antibodies were purchased from Sigma-Aldrich. Anti-$p21^{CIP1}$, anti-PCNA, anti-Bax, anti-Fas, and anti-Hsp90 antibodies were purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-V5 antibody was purchased from Abcam. Enhanced chemiluminescence reagent and [γ-^{32}p]ATP were purchased from PerkinElmer Life and Analytical Sciences. The following materials were purchased from Invitrogen: anti-Xpress, anti-V5 (mouse monoclonal), fluorescein isothiocyanate (FITC)-conjugated anti-rabbit or anti-mouse IgG, tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-IgG (anti-mouse or anti-rabbit IgG), horseradish peroxidase-conjugated IgG (anti-mouse or anti-rabbit IgG), Dulbecco's modified eagle's medium (DMEM), RPMI medium 1640, fetal bovine serum (FBS), neurobasal medium, B-27 supplement, Trizol reagents, SuperScript III reverse transcriptase, the 293FT cell line, a ViraPower lentiviral packaging mix, Gateway LR Clonase II enzyme mix, Ni^{2+}-NTA agarose beads, and BL21 cells. All other used chemicals used in this study were purchased from Sigma-Aldrich and USB Corporation.

II. Supplementary Figure Legends

Supplementary Figure S1 **Dyrk1A binds to the region of human p53 spanning amino acids 94 - 167 and phosphorylates the region of 1 - 93.** *A*. Identification of direct phosphorylation of human p53 by bacterial recombinant Dyrk1A through *in vitro* kinase assay. *B*. Schematic representation of p53 and its truncation mutants. p53 contains a transactivation domain (TA), N-terminal region of DNA-binding domain (DBD1), C-terminal region of DNA binding domain (DBD2), and oligomerization (OD) & regulatory domain (RD). The p53 mutants lack one or more of these domains. *C*. H1299 cells were mock-transfected or transfected with Dyrk1A-V5 for 24 h. One milligram of cell lysate was then incubated with one of the recombinant 6xHis-tagged human p53 truncation mutants overnight.

The samples were then immunoprecipitated with the anti-V5 antibody, and immunoprecipitates were probed with anti-V5 or anti-p53 antibodies. *D*. *In vitro* kinase assay showing phosphorylation of 6xHis-tagged human p53 truncation mutants by Xpress-tagged Dyrk1A. Note that Dyrk1A phosphorylates the N-terminal region of human p53 spanning amino acids 1-93. Asterisks indicate recombinant human p53 proteins and their phosphorylation signals.

Supplementary Figure S2 **Dyrk1A induces the expression of p53 target genes in H19-7 cells.** *A*. *In vitro* kinase assay showing p53 phosphorylation by anti-Dyrk1A immunocomplexes in H19-7 cells. After cells were transfected with plasmids encoding Xpress-tagged WT or kinase-inactive (K188R) Dyrk1A, anti-Xpress immunoprecipitates were incubated with recombinant 6xHis-tagged human p53 and [γ-^{32}p]ATP. The reaction products were separated by SDS-PAGE and analyzed by autoradiography. *B*. Luciferase activity of a *p21^{CIP1}* promoter-driven reporter in H19-7 cells transfected with or without plasmid encoding p53-V5. Luciferase activity was analyzed 24 h post transfection. Co-transfection of the pRL vector, which constitutively expresses *Renilla* luciferase, was used to normalize transfection efficiency. Data are expressed mean ± s.d. (n = 3; ***, $P < 0.001$, Student's *t*-test). *C* & *D*. Luciferase activity of *Mdm2* promoter-driven reporter in H19-7 cells (*C*) as well as in H19-7/pTK and H19-7/Dyrk1A cells (*D*). Cells were transfected with the luciferase reporter alone or with plasmids encoding Xpress-tagged WT or its K188R Dyrk1A. Luciferase activity was analyzed 24 h post transfection. Co-transfection of the pRL vector was used to normalize transfection efficiency. Data are expressed mean ± s.d. (n = 3; **, $P < 0.01$ and ***, $P < 0.001$, Student's *t*-test).

Supplementary Figure S3 **Attenuation of H19-7 cell proliferation and G1/G0-S phase transition is dependent on the kinase activity of Dyrk1A.** *A*. After H19-7 cells were transiently transfected with either wild-type or kinase-inactive (K188R) Dyrk1A, cell proliferation was measured using a terazolium-based assay. Data are expressed in arbitrary units (n = 16; ***, $P < 0.001$, Student's *t*-test). *B* & *C*. Cell cycle analysis of H19-7 cells transiently transfected with either wild-type or K188R Dyrk1A. Representative histogram images are shown at each time point after release from thymidine synchronization (2 mM for 24 h), and each cell phase is indicated by the lines (*B*). Data are expressed as the percentage of cell population in each phase (*C*) (n = 3; **, $P < 0.01$ and ***, $P < 0.001$, Student's *t*-test).

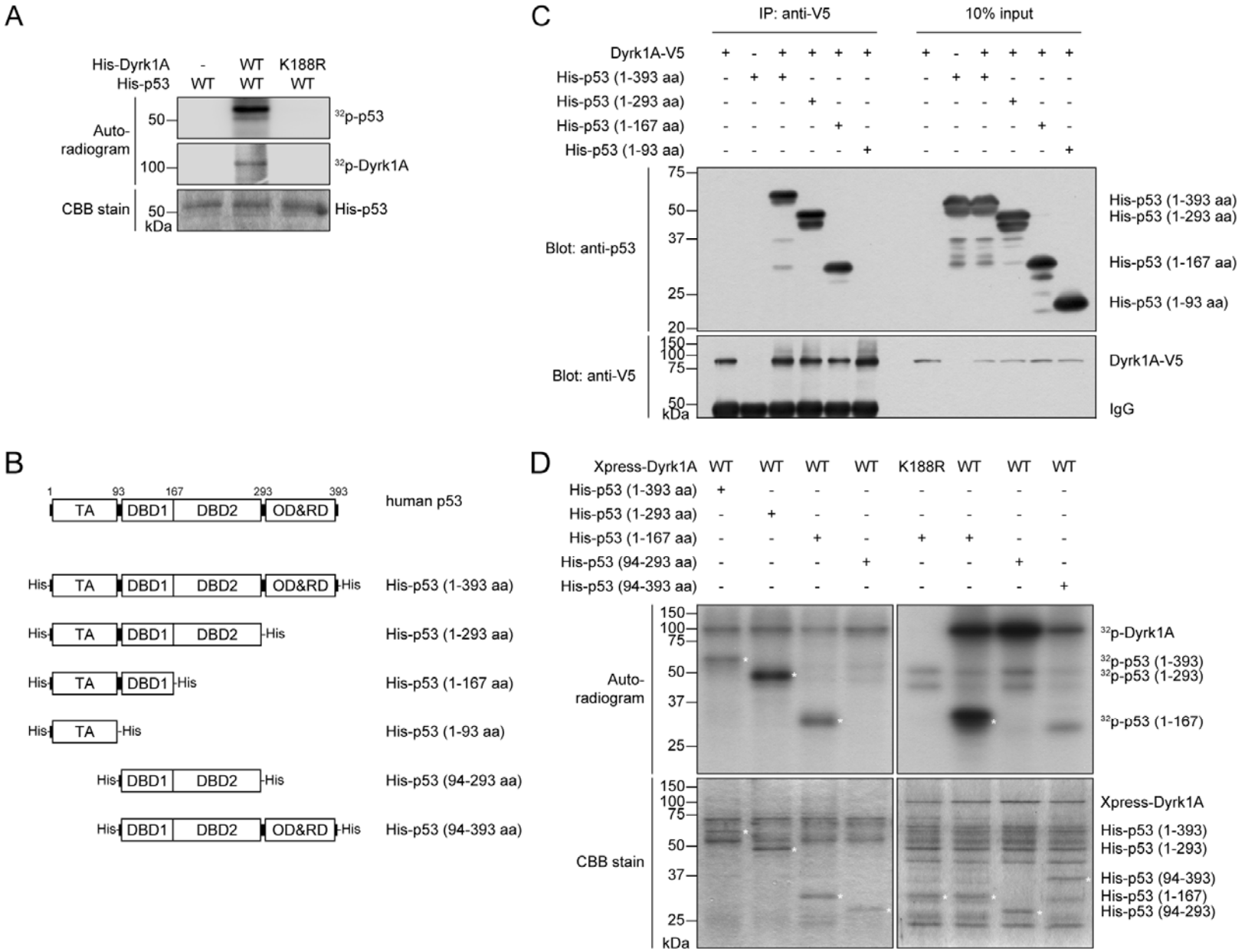
Supplementary Figure S4 **The protein levels of DYRK1A in the frontal cortices from DS patients and age-matched controls.** Band intensity was quantified and normalized to the intensity of HSP90 (Fig. 7*K*) (*, $P < 0.05$).

Supplementary Figure S5 **Dyrk1A interacts with and phosphorylates p53 at infant and adult stages.** *A* & *B*. Reciprocal co-immunoprecipitation/immunoblot analyses of Dyrk1A-p53 interactions in 7-week-old rat whole brain lysates. *C* & *D*. Immunoblot analysis of mouse p53 and p53-phosphoSer 18 levels in whole brain lysates from postnatal 3 day *DYRK1A* Tg mice and littermate controls (*C*), and in the hippocampus, frontal cortex, and cerebellum lysates from 2-month-old *DYRK1A* Tg mice and littermate controls (*D*).

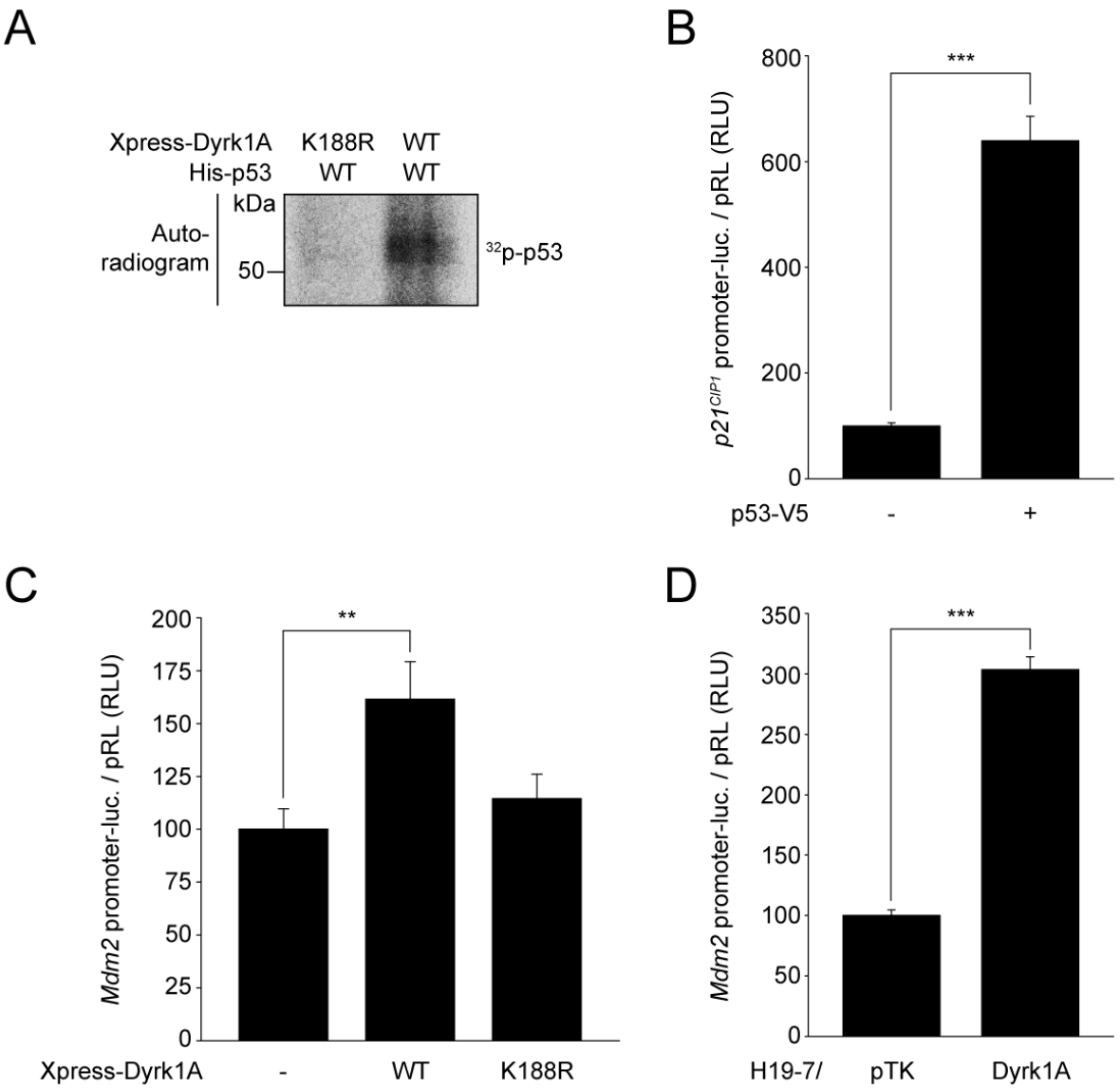
Supplementary Figure S6 **Changes in caspase-3 cleavage under the serum deprivation.** Immunoblot analysis of cleaved caspase-3 in H19-7/Dyrk1A and H19-7/pTK cells. The cells were incubated with N2 media and lysed for an immunoblot analysis.

Supplementary Figure S7 **Changes in cyclin D1 levels by Dyrk1A overexpression in H19-7 cells and E14.5 mouse brains.** *A*. Immunoblot analysis of cyclin D1 in H19-7/Dyrk1A cells and H19-7/pTK cells. Hsp90 served as a loading control. *B* & *C*. Immunoblot analysis of cyclin D1 in whole brain lysates from E14.5 *DYRK1A* Tg mice ($n = 3$) and littermate controls ($n = 3$). Band intensity was quantified and normalized to the intensity of Hsp90 (Fig. 7*I*). Data are expressed in arbitrary units ($P > 0.05$, Student's *t*-test).

Park et al., Supplementary Figure 1



Park et al., Supplementary Figure 2



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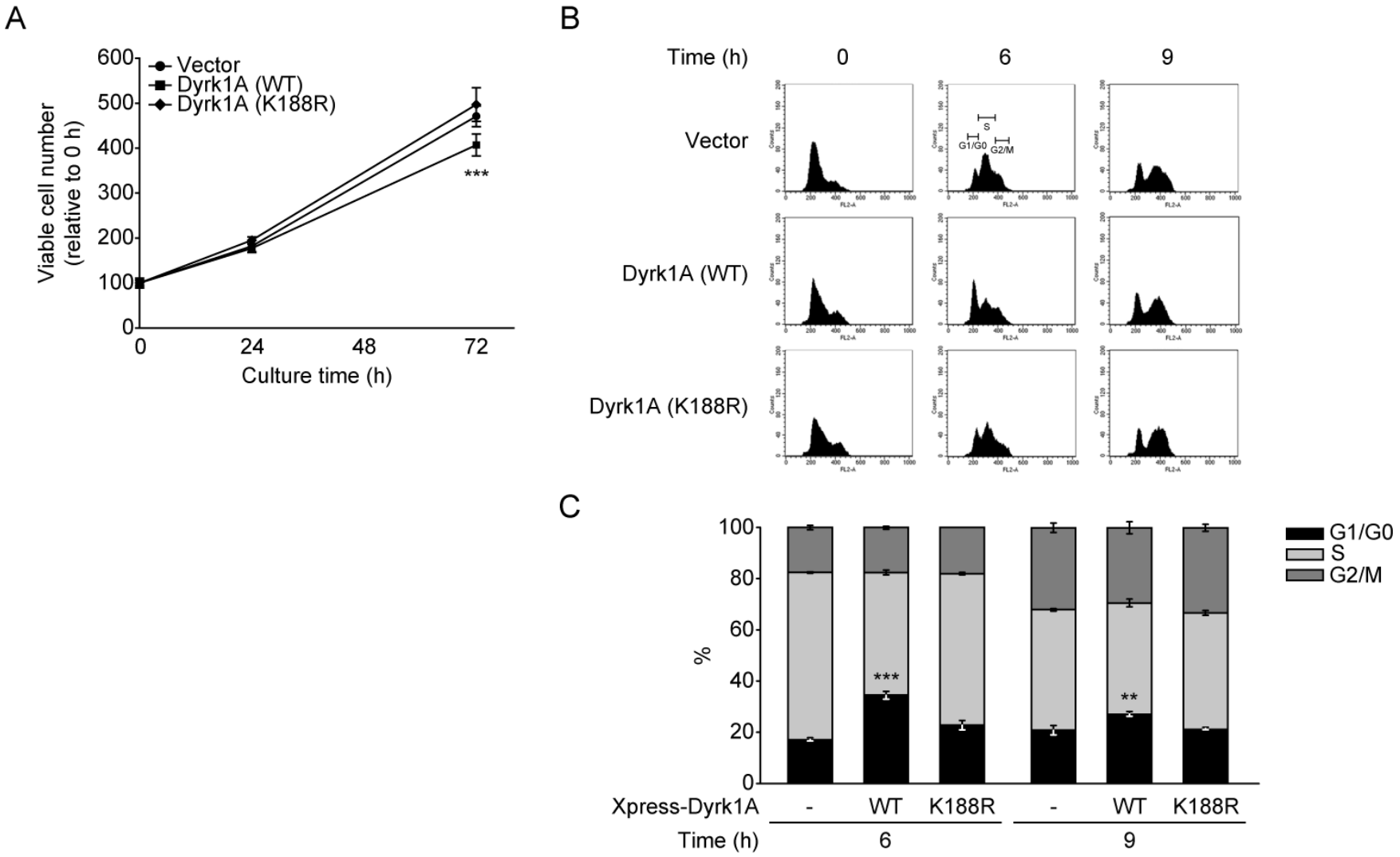

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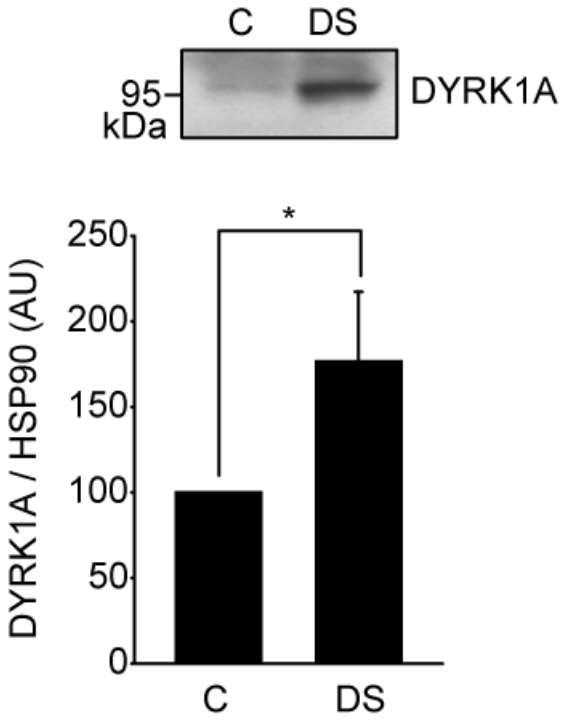

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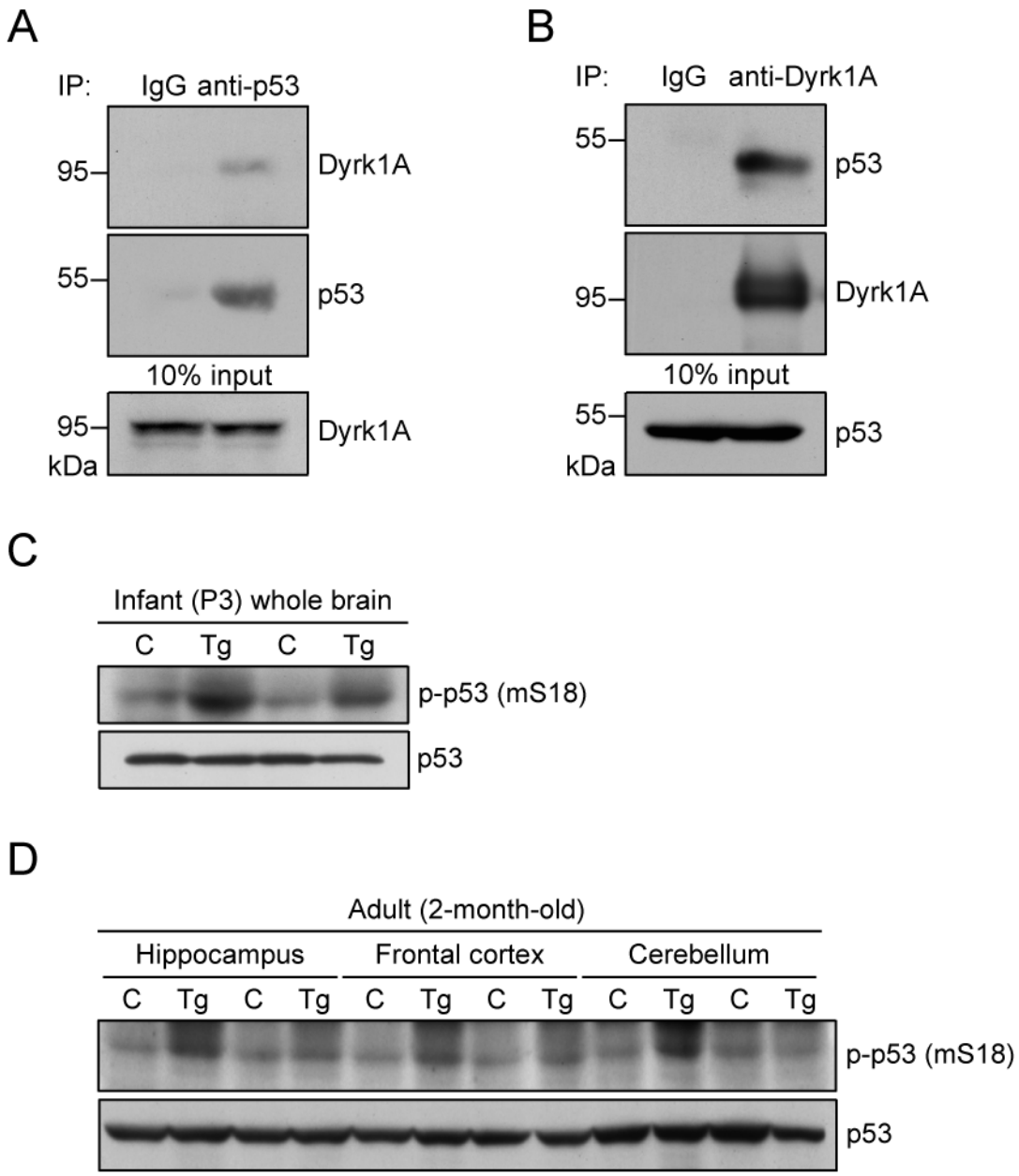
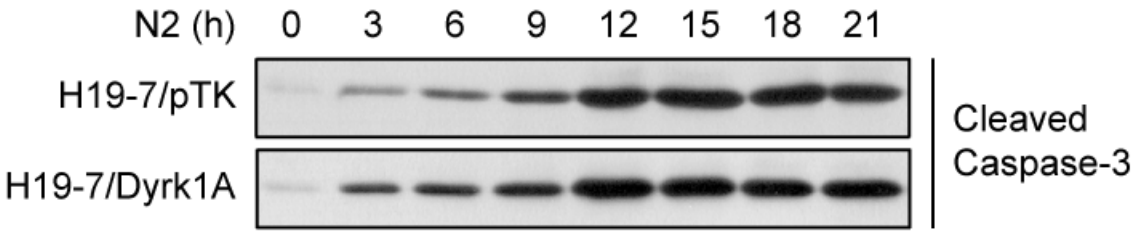


Park et al., Supplementary Figure 6



Park et al., Supplementary Figure 7