Supporting Information

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SI Materials and Methods

Plasmids and Reagents. The cDNAs for WT or deletion mutants of JFK were amplified by PCR and ligated into XbaI/EcoR I sites of a pcDNA3.1 vector that contains 1 or 3 copies of FLAG. The GST-JFK and EGFP-JFK expression plasmids were constructed by cloning full-length or mutants of JFK into pGEX-4T-3 and pEGFP-N vectors, respectively. All clones were confirmed by DNA sequencing. pCMV-(HA-Ub)4, pDNA3-Myc3-Fbw7, pDNA3-Myc3-Skp2, pDNA3-Myc3-COP1, pDNA3-Myc3-mβTrcp, pcDNA3-HA2-Roc1, and pcDNA3-hSkp1 were from Yue Xiong (Chapel Hill, NC). p53(R175H) was from Wei Gu (New York, NY). pG13-Luc and pWWP-Luc (p21-Luc) were from Bert Vogelstein (Baltimore, MD). The sources of antibodies against the following proteins were: hemagglutinin (HA; 12CA5), FLAG (M2), and β-actin (Sigma); cyclin E, cyclin D1, D2, D3, p27, and Keap1 (Santa Cruz Biotechnology); Skp1 and Roc1 (Zymed); Cul1 and Cul3 (Cell Signaling); and Myc (PL14), p53 (DO-1), and MDM2 (Ab-1; MBL). Polyclonal antibodies against JFK were raised against the C-terminal epitope of the JFK protein (CYPKTNALYFVRAKR) in rabbits. MG132 was from Sigma, λ protein phosphatase (λPPase) was from New England BioLabs, and curcumin was from Biomol.

Bioinformatics. The full length of JFK cDNA was cloned by RT-PCR from a mammary library (Clontech). The ORF, conserved domains, and chromosomal location of JFK were predicted with the databases in NCBI (www.ncbi.nlm.nih.gov). The theoretical molecular weight and isoelectric point of JFK were calculated with the database at the Expert Protein Analysis System server. The homologous alignment was analyzed by the ClustalW program (version 1.60) (1), and phylogenetic analysis was performed using the Jotun Hein method (2).

Cell Culture and Transfection. U2OS, H1299, and MCF-10A cells were from ATCC. $p53^{-/-}$/$Mdm2^{-/-}$ MEFs were from Guillermina Lozano (Houston, TX). Cells were maintained in DMEM (HyClone) supplemented with 10% FBS. All transfections were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. Luciferase assays were carried out according to the manufacturer's instructions (Promega). U2OS and H1299 cells were transfected with siRNA oligonucleotides twice at 24- to 48-h intervals and expanded as necessary to prevent contact inhibition. The sequences of siRNAs were: JFK siRNAs, 5′- GACUUGACCUAAACAGCAAtt-3′; MDM2 siRNA, 5′-AAUGGUUGCAUUGUCCAUGGCtt-3′; ARF-BP1 siRNA, 5′- UUGCUAUGUCUCUGGGACAtt-3′; COP1 siRNA, 5′-CUGACCAAGAUAACCUUGAtt-3′; and Pirh2 siRNA, 5′-UGUAACUUAUGCCUAGCUAtt-3′.

Immunoprecipitation and Western Blotting. Western blotting and immunoprecipitation were performed essentially the same as previously described (3–5). Briefly, H1299 or U2OS cells were transfected with JFK or p53 expression plasmid or specific siRNAs. Forty-eight hours after transfection, cellular lysates were prepared by incubating the cells in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, 2 mM EDTA) for 20 min at 4 °C. This was followed by centrifugation at 500 × *g* for 5 min at 4 °C. For immunoprecipitation, 500 μL of protein was incubated with specific antibodies (1–2 μg) for 12 h at 4 °C with constant rotation; 50 μL of 50% protein A or G agarose beads was then added and the incubation was continued for an additional 2 h. Beads were then washed 5 times using the lysis buffer. Between washes, the beads were collected by centrifugation at 500 × *g* for 5 min at 4 °C. The precipitated proteins were eluted from the beads by re-suspending the beads in 2× SDS/PAGE loading buffer and boiling for 5 min. The resultant materials from immunoprecipitation or cell lysates were resolved using 10% SDS/PAGE gels and transferred onto nitrocellulose membranes. For Western blot analysis, membranes were incubated with appropriate antibodies for 1 h at room temperature or overnight at 4 °C followed by incubation with a secondary antibody. Immunoreactive bands were visualized using Western blotting Luminol reagent (Santa Cruz Biotechnology) according to the manufacturer's recommendation.

GST Pull-Down Assay. GST pull-down experiments were performed as previously described (6–9). Briefly, equal amounts of GST fusion proteins (GST, GST-JFK, or JFK deletion mutants) were immobilized on 50 μL of 50% glutathione-Sepharose 4B slurry beads (Amersham Biosciences) in 0.5 mL of GST pull-down binding buffer (10 mM Hepes, pH 7.6, 3 mM $MgCl_2$, 100 mM KCl, 5 mM EDTA, 5% glycerol, 0.5% CA630). After incubation for 1 h at 4 °C with rotation, beads were washed 3 times with GST pull-down binding buffer and resuspended in 0.5 mL of GST pull-down binding buffer before adding 5 μL of in vitro transcribed/translated p53 for 2 h at 4 °C with rotation. The beads were then washed with 0.5 mL of ice-cold immunoprecipitation assay buffer and 1 mL of cold PBS solution. The bound proteins were eluted by boiling in 25 μL of loading buffer and resolved on SDS/PAGE.

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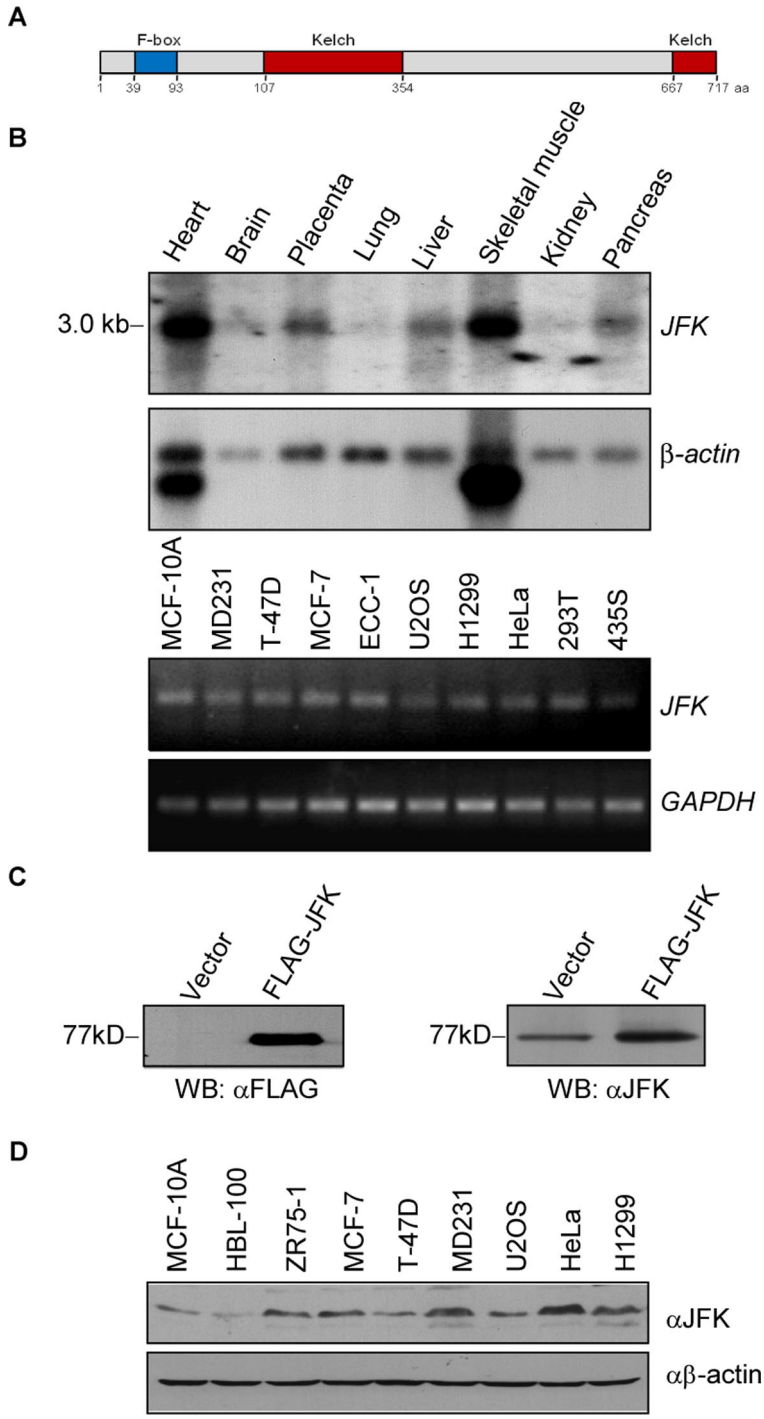

Fig. S1. Cloning and characterization of JFK. (*A*) Schematic representation of the structure of JFK protein. (*B*) The expression profiling of JFK. (*Upper*) Northern blotting analysis of *JFK* mRNA expression in different tissues. (*Lower*) RT-PCR analysis of *JFK* mRNA expression in different cell lines. The expression of *b-actin* and *GAPDH* was used as controls. (*C*) Western blotting analysis of JFK protein expression in U2OS cells that were transfected with vector or FLAG-JFK using anti-FLAG (*Left*) or anti-JFK (*Right*). (*D*) Western blotting analysis of JFK protein expression in a panel of normal (MCF-10A and HBL-100) and cancer cell lines. The expression of b-actin was used as the control.

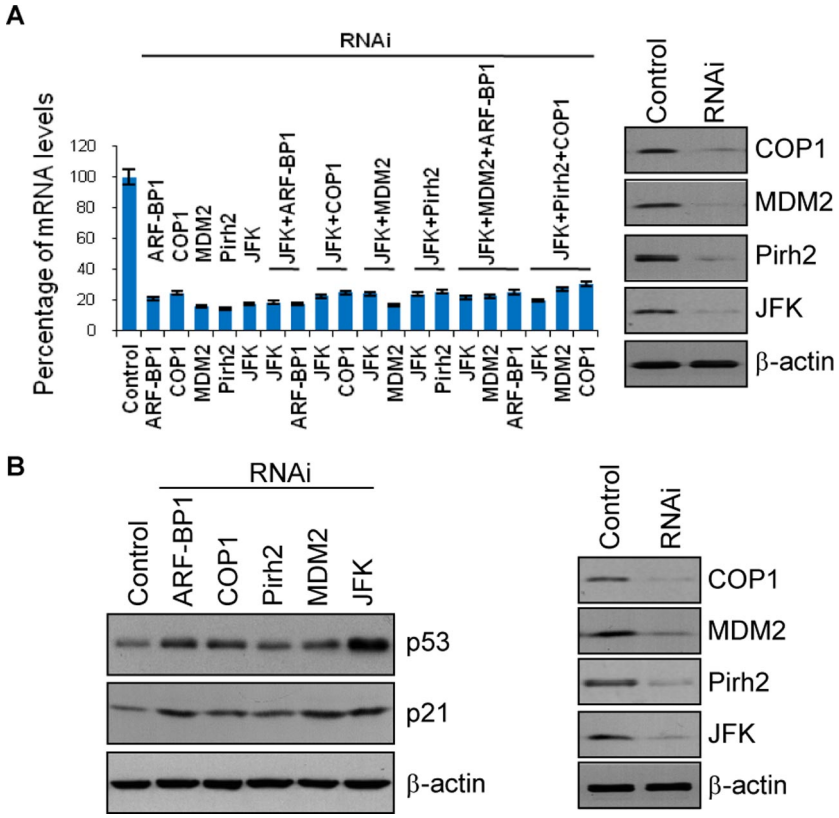

Fig. S2. JFK knockdown stabilizes p53 and activates the transcription of p21. (*A*) U2OS cells were transfected with siRNA oligonucleotides for JFK and/or for MDM2, Pirh2, COP1, or ARF-BP1 as indicated. Total RNAs were prepared for real-time RT-PCR analysis of mRNA expression of the indicated E3 ligases for knockdown efficiency, which is expressed as a function of the specific mRNA/GAPDH mRNA. The data were normalized to *GAPDH*. Each bar represents the mean ± SD for triplicate experiments. (*B*) Knockdown of JFK stabilizes p53 in normal MCF-10A cells. Cellular lysates from MCF-10A cells transfected with siRNA oligonucleotides for JFK, MDM2, Pirh2, COP1, or ARF-BP1 were immunoblotted with antibodies against the indicated proteins.

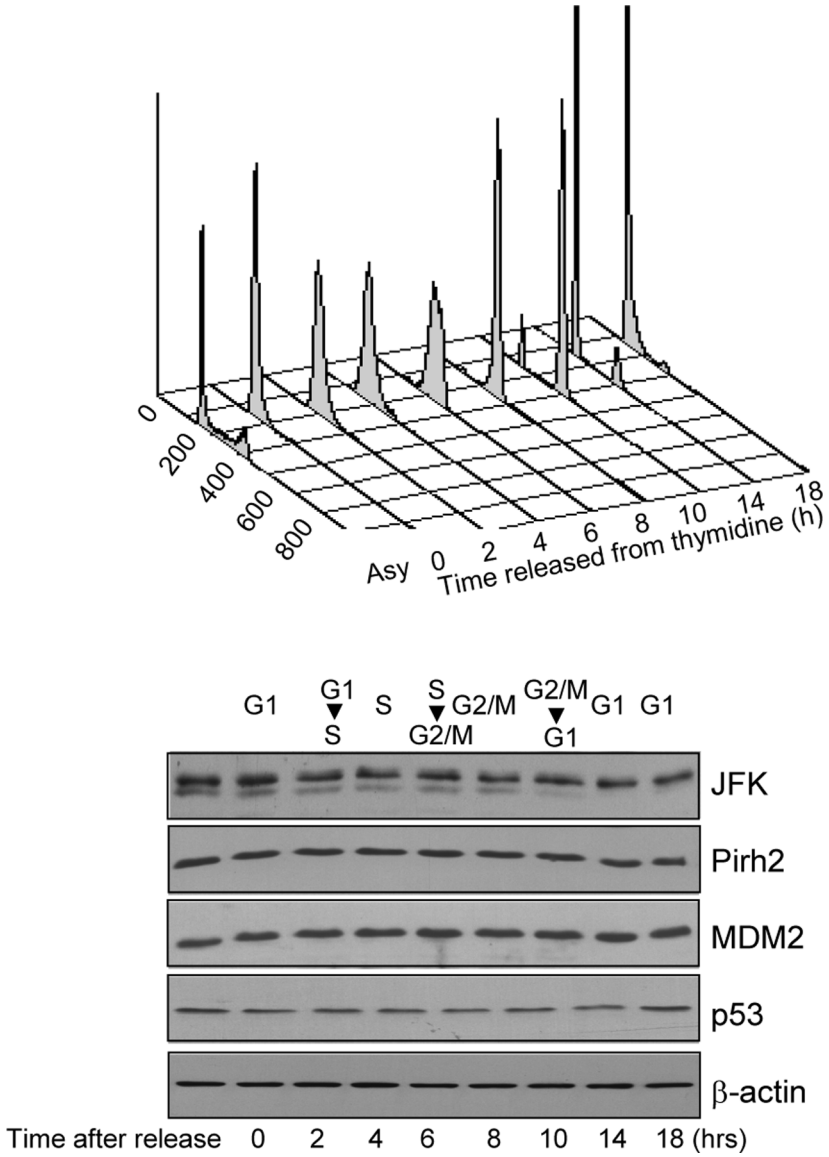

Fig. S3. Regulation of p53 by JFK is not cell cycle-dependent. (*Upper*) U20S cells were synchronized at G1/S border by double thymidine blocking, and were subsequently allowed to progress through the cell cycle for the indicated times. Flow cytometric analysis was used to monitor the progression of cells through the cell cycle. (*Lower*) Protein expression was analyzed by immunoblotting with antibodies against the indicated proteins. The first lane was non-synchronized cells. The expression of b-actin was used as the control.