Supplementary Information

Lin Yuan et al., Apak competes with p53 for direct binding to intron 1 of p53AIP1 to regulate apoptosis, Supplementary Information includes Supplementary Methods, References, three Supplementary Figures (including Figure Legends), and two Supplementary Tables.

Supplementary Methods

Expression, purification and refolding of recombinant Apak protein

*E.coli* BL21 cells containing His-tagged Apak expression plasmid pET28C-Apak were induced by 0.1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) at 30°C for 5–6 h to express Apak protein. The cells were suspended in 1× PBS containing 1 mM Phenylmethylsulphonyl- fluoride (PMSF) and then lysed by sonication. The insoluble fraction was collected by centrifugation and washed sequentially with 1× PBS containing 2 M Urea, 0.1% SDS, 1% NP-40 and 1% TritonX-100. The majority of the insoluble fraction after extensive washing contains Apak protein. The purified protein was solubilized in 1× PBS containing 8 M Urea, and was renatured by successive dialysis against refolding buffer (25 mM HEPES, pH 7.9, 50 mM NaCl, 0.1% NP-40, 1 mM PMSF, 0.16 mM dithiothreitol [DTT], 10 μM ZnSO₄, 10%glycerol) at 4 °C. The purification of His-tagged Apak protein was carried out using Ni-NTA superflow (Qiagen) according to the manufacturer's recommendation. The resulting protein was evaluated by coomassie blue staining and Western blot analysis with anti-His antibody following SDS-PAGE.
Cell culture and transfection

Human lung adenocarcinoma H1299 (p53-deficient) cells were grown in RPMI 1640 medium; human p53+/+ HCT116, and p53−/− HCT116 colon cancer cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Hyclone) and 1% penicillin-streptomycin (Mediatech) at 37°C with 5% CO2. Mammalian cells were transfected with Lipofectamine 2000 (Invitrogen) according to manufacturer's recommendations.

Antibodies

Apak antibody was raised in our lab and described previously [1]. Antibodies against p53AIP1 and 14-3-3σ were purchased from Abnova. p53 antibodies Ab-3 was from Oncogene, pAb421 from Abcam, and DO-1 from Santa Cruz. Other antibodies used were anti-Myc and anti-GFP (Clontech), anti-actin and GAPDH (Santa Cruz).

RNA interference

The Apak short hairpin RNA (shRNA) sequence (5’-GGGATTATTTGGAAGCCAA-3’) and the control sequence (5’-TGCGTTGCTAGTACCAAC-3’, non-targeting sequence) were cloned into RNAi-Ready pSIREN-DNR-DsRed-Express (Clontech). The short interfering RNAs (siRNAs) specifically targeting p53AIP1 (#1, 5’-GGGUUUCAGACUUCGUCAUGAG-UA-3’, #2, 5’-GAGCUUCAGAUCUGCUCAGAUA-3’) and non-targeting siRNA
(5’-UUCUCCGAACGUGUCACGU-3’) were synthesized by Shanghai GenePharm. All siRNA and shRNA transfections were performed with Lipofectamine 2000 (Invitrogen) and the RNA interfering efficiency was assessed by western blot analysis.

Western blotting analysis

Proteins were separated by SDS-PAGE and transferred to NC membrane. The membrane was blocked with 5% nonfat dry milk in TBST (50 mM Tris [pH 7.5], 200 mM NaCl, 0.05% Tween 20) at room temperature for 1 h. The membrane was then incubated with primary antibody with the same concentration of milk in TBST for 1 h at room temperature, washed three times with TBST for 15 min, then incubated with the HRP-conjugated secondary antibody at room temperature for 1 h. The membrane was again washed three times with TBST, treated with SuperSignal (Pierce Biotechnology) detection reagents and exposed to Kodak Light films.

DNA-protein blotting

Cells were transfected with wild-type and various deletion mutants of Apak respectively, and after 48 h, cell lysates were resolved on 15% SDS-PAGE and transferred to nitrocellulose membrane. The filters were blocked overnight at 4 °C in Apak refolding buffer (25 mM HEPES [pH 7.9], 50 mM NaCl, 0.1% NP-40, 1 mM PMSF, 0.16 mM dithiothreitol [DTT], 10 μM ZnSO₄, 10%glycerol) containing 5% dry milk. Then the filters hybridized with 10⁶ cpm of Biotin labeled oligonucleotides per ml for 8h at 4 °C in the binding buffer containing 10 μg of poly(dl-dC) per ml.
The filters were washed twice with binding buffer for 10 min, then incubated with the HRP-conjugated Streptavidin at room temperature for 15 minutes. The filters were washed three times with washing buffer.

**Apoptosis analysis**

The percentage of apoptotic cells was determined by analysis of Annexin V-positive cells as described [2].

**Reference**


Supplementary Table 1

The P53AIP1 intron 1-derived Apak recognition sequence, and mutation sequences

<table>
<thead>
<tr>
<th>Probe name</th>
<th>Sequence</th>
</tr>
</thead>
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<tr>
<td>Apak/p53-BS</td>
<td>CCTCCTCTCTGTGCCCCGGGCTTGTCGAGATG</td>
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<tr>
<td>Mut4</td>
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### Supplementary Table 2

**Primers for ChIP analysis**

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<th>Gene name</th>
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<td>GCGAGACTGTGGCCTTGTGT</td>
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</tr>
<tr>
<td>P53AIP1</td>
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<td>GADD45</td>
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<tr>
<td>P21</td>
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<td>GACAAAAATAGCCACCAGCCTC</td>
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</table>
**Legend to Supplementary Figure S1**

*E. coli* expressed His-tagged Apak protein was analysed by Coomassie blue staining (up) and Western blot analysis (bottom). Cell lysates from non-induced (lane 1), IPTG-induced (lane 2), soluble fraction (lane 3) of cell lysates and His-Apak protein after purification (lane 4), urea-solubilization and refolding (lane 5) were analyzed. Arrow indicates Apak protein.

**Legend to Supplementary Figure S2**

GFP-tagged Apak WT and the ZF truncate were transfected into HCT116 cells. Forty-eight hours later, the localization of Apak WT and ZF was visualized through a confocal microscope.

**Legend to Supplementary Figure S3**

HCT116 cells were transfected with GFP vector or GFP-tagged Apak. After treated with MMS (0.02%) for 3h, cells were harvested and analysed by immunoblotting with indicated antibodies.
Supplementary Figure S1

Supplementary Figure S2
Supplementary Figure S3

<table>
<thead>
<tr>
<th>Protein</th>
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<td></td>
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