

Supporting Online Material

Materials and Methods

Plasmids and reagents. Human DET1 cDNA was amplified by PCR from a HEK293 cell library and cloned into pEF6 myc-His (Invitrogen), a modified pFLAG CMV14 vector (Sigma) with a Glu-Glu tag and TEV cleavage site inserted 5' to the C-terminal 3xFLAG sequence, or a modified pFLAG CMV6 vector (Sigma) with a GST tag inserted 3' to the N-terminal FLAG sequence. Human COP1 and hCOP1 Δ 24 cDNA were amplified by PCR from MCF7 and HEK293 libraries, respectively, and cloned into pFLAG CMV6 (Sigma) or pEF6 myc-His (Invitrogen). The hCOP1 RING mutant was generated using a Quikchange Kit as directed by the manufacturer (Stratagene) to mutate C136 to A and C139 to A. The FLAG hCOP1 RING+cc deletion (amino acids 307-731) and the FLAG hCOP1 and hCOP1 Δ 24 C-terminal WD40 deletions (removing the C-terminal 206 amino acids) were generated by standard PCR techniques from the respective pFLAG CMV6 constructs. Human ATF2 cDNA was amplified by PCR from a Jurkat cell library and cloned into pcDNA3.1/His (Invitrogen). Ubiquitin-HA and c-jun 6xHis were kind gifts from Dirk Bohmann (University of Rochester, NY, USA). The c-jun cDNA was subcloned into pEF6 myc/His (Invitrogen) and the c-jun hCOP1 binding domain mutant was generated by mutating E 227 to A, E 228 to A, V 232 to A, and P 233 to A. Murine CUL4A was subcloned into a pcDNA3 vector with a myc epitope tag. HA-DDB1 and FLAG-DDB1 were kind gifts from Dr. Michel Strubin (University Medical Centre, Geneva, Switzerland) and Dr. Stephen Goff (Columbia University/HHMI, New York, USA), respectively. HA-tagged JunB and JunD were gifts

from Dr. Michael Karin (University of California, San Diego, USA), and the JunD cDNA was subcloned into pEF6 myc/His (Invitrogen). The c-fos construct was a gift from Dr. Tom Curran (St. Jude Children's Research Hospital, Chicago, USA). Polyclonal hCOP1 antibodies were generated in chickens (Gallus Immunotech) against hCOP1 amino acids 563-643 and monoclonal antibodies were raised in mice against hCOP1 71-270. Monoclonal hDET1 antibodies were generated in mice against hDET1 amino acids 64-189. Antibodies to the following epitopes and proteins were purchased from the indicated vendors: FLAG M2 monoclonal antibody and affinity gel (Sigma); c-jun (SC-45 and H-79), c-fos (K25), and CUL4 (C19) (Santa Cruz); c-jun (Upstate Biotech), c-myc (Roche); HA (Covance); DDB1 (Oncogene Research); ROC1 (Zymed); β -tubulin and actin, (ICN Biomedicals). CUL4A antibody was also a gift from Dr. Pradip Raychaudhuri (University of Illinois at Chicago, USA).

Cell culture and transfections. HEK293T cells were cultured in high glucose DMEM with 10% fetal bovine serum and 1x L-Glutamine, and U2OS cells were cultured in McCoy's 5A medium with 10% fetal bovine serum and 1x L-Glutamine. HEK293T cells were transfected with Geneporter2 Transfection Reagent (Gene Therapy Systems) and U2OS cells were transfected with Lipofectamine 2000 (Invitrogen) as recommended by the respective manufacturers.

hDET1 and hCOP1 message analysis and real-time RT-PCR. PCR analysis of hDET1 and hCOP1 tissue distribution was performed on human cDNA panels as directed

by the manufacturer (Clontech). For c-jun message analysis, RNA was prepared from cells transfected with highest dose of hDET or the hCOP variants (Fig. S8) or hDET and the highest doses of the hCOP variants (Fig. S9) or the indicated siRNA oligonucleotides (Fig. 4B, S11) and used for real-time RT-PCR analysis. Total RNA was isolated using Qiagen RNeasy mini kit (Qiagen) and treated with DNase (Qiagen) as recommended by the manufacturer. Probes were designed for c-jun and β -actin, and real-time RT-PCR analysis was performed using an ABI7700 sequence detector according to the manufacturer's recommendations using at least triplicate samples normalized to β -actin. Relative levels of c-jun and β -actin were calculated following the Standard Curve Method in separate tubes as outlined in the ABI Prism 7700 Sequence Detection System User Bulletin #2.

Immunoprecipitations and Western blotting. Unless otherwise noted, HEK293T cells were used for analysis of interactions between endogenous proteins and/or transiently expressed proteins. In some cases (Fig. 1C-E, S6, 2A, S7), cells were treated prior to collection with 25 μ M MG132 (Calbiochem). Cells were washed with PBS and subsequently lysed for 30 minutes at 4°C in a buffer containing 120 mM NaCl, 50 mM HEPES pH 7.2, 1 mM EDTA, 0.1% NP-40, and Complete protease inhibitor cocktail (Roche). Lysates were cleared by centrifugation for 30 minutes at 4°C at 21,000 x g, in some cases were pre-cleared for one hour by rotating at 4°C with Protein A+G beads (Pierce), and proteins were immunoprecipitated 2 hours to overnight with the indicated antibody or anti-FLAG affinity gel (Sigma) at 4°C. Immunocomplexes were captured by

rotating for 2-3 hours with Protein A, Protein G (Sigma) or Protein A+G affinity matrices pre-blocked in 1% BSA/PBS. Immunoprecipitates were washed three times with lysis buffer and in some cases two additional washes with lysis buffer containing 1M NaCl were included between the first and second washes. Samples were reduced and alkylated, proteins were separated by SDS-PAGE, and subsequently transferred onto PVDF membranes (Invitrogen) following standard procedures. Western blotting was performed as recommended by the respective antibody manufacturers.

FLAG elutions and analysis of co-eluting proteins. HEK293T cells were transfected with the indicated FLAG-tagged constructs as described above. In some cases (Fig 1A), cells were treated prior to collection with 25 μ M MG132 (Calbiochem). Cells were washed once with PBS, collected, and dounce homogenized in a hypotonic lysis buffer containing 10 mM NaCl, 1.5 mM MgCl₂, 10 mM Tris pH 7.5, 25 μ M MG132 (when necessary), and Complete protease inhibitor cocktail (Roche). Lysates were cleared by centrifugation and immunoprecipitated with anti-FLAG affinity gel (Sigma) 4 hours to overnight. Immunocomplexes were washed once with wash buffer #1 (20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol, Complete protease inhibitor cocktail) and three times with wash buffer #2 (20 mM Tris pH 7.4, 20% glycerol, 0.2 mM EDTA, 300 mM NaCl, 0.1% NP-40, Complete protease inhibitor cocktail) and rotated at least 2 hours in wash buffer #2. Samples were eluted with 300 μ g/mL 1x or 3xFLAG peptide (Sigma) according to the manufacturer's instructions. Eluted proteins were analysed by immunoblotting or were digested with trypsin and the

resulting peptides sequenced by capillary liquid chromatography-ion trap tandem mass spectrometry.

in vivo and in vitro ubiquitination assays. For *in vivo* ubiquitination assays, HEK293T cells were transfected as described above with the indicated constructs. Cells were treated with 25 μ M MG132 for 30 minutes prior to collection. Cells were collected, lysed, and lysates were cleared by centrifugation as described above but with 25 μ M MG132 and 10 mM N-ethylmaleimide added to the lysis buffer. To dissociate proteins 1% SDS was added to lysates, which were then heated at 90°C for 5 minutes. The samples were diluted 10-fold with a dissociation dilution buffer containing 1% NP-40, 0.5% deoxycholate, 120 mM NaCl, 50 mM HEPES, 1 mM EDTA, and Complete protease inhibitor cocktail (Roche). Myc-tagged c-jun was immunoprecipitated at 4°C by rotating 3 hours with anti-myc antibody followed by a 2-hour incubation with blocked Protein G beads. Samples were washed and prepared for Western blot analysis as described above. For *in vitro* ubiquitination assays, cells were transfected with the indicated constructs and FLAG-tagged proteins were eluted as described above. The elutions were combined as indicated using 1X TBS to normalize the reaction volumes and rotated at 4°C for 30 minutes to promote complex binding. A portion of each mixture was reserved for immunoblotting to ensure equal loading of the eluted components. The elution mixtures were included in the *in vitro* ubiquitination assays performed in duplicate 100 μ L reaction volumes containing the following components where indicated: 20 μ g N-terminal biotinylated ubiquitin (Boston Biochem), 0.4 μ g E1

(Calbiochem), 1.8 μ g UBCH5b (Boston Biochem), 30 μ L elution mixture, 1M sodium citrate, and 10 μ L 10X reaction buffer (300 mM HEPES pH 7.2, 20 mM ATP, 50 mM $MgCl_2$, 2 mM DTT). Reactions were incubated at 30°C for 1 hour with agitation at 750 rpm and were subsequently recombined for a final volume of 200 μ L. SDS (1% v/v) was added and reactions were heated at 90°C for 15 minutes. Samples were diluted to a final volume of 5 mL with the dissociation dilution buffer described above and 5 μ g of anti c-jun (Santa Cruz Biotech SC-45) and 6 μ L of anti-c-jun (Upstate Biotech) antibodies were added. Samples were rotated for 3 hours at 4°C, followed by an additional 3 hour incubation with 100 μ L Protein A/G Plus agarose (Santa Cruz Biotech). Samples were washed and prepared for Western blot analysis as described above. Proteins were separated by SDS-PAGE and immunoblotted with the indicated antibodies as described above.

RNAi and luciferase reporter assays. All siRNA oligos were synthesized with 3'dTdT overhangs by Dharmacon. The following sequences were used in HEK293T cells: hCOP1: AAU GGC CAC AGG UGG CAG AUA, AAC UGA CCA AGA UAA CCU UGA, and AAG ACU UGG AGC AGU GUU ACU (a mixture of all 3); hDET1: AAC GUU GAA AAG CCU CCU UGU and AAG ACU AUU CCC UCC AUA UCA (a mixture of both); DDB1: AAC UCC UUG GAG AGA CCU CUA; CUL4A: AAG CAU GAG UGC GGU GCA GCC; and ROC1: AAA UUG GAU GGA ACU GUG UUU. The following sequences were used in U2OS cells: hCOP1: AAC UGA CCA AGA UAA CCU UGA; hDET1: AAC GUU GAA AAG CCU CCU UGU and AAG ACU AUU CCC

UCC AUA UCA (a mixture of both); DDB1: AAC UCC UUG GAG AGA CCU CUA; CUL4A: AAG CAU GAG UGC GGU GCA GCC; and ROC1: AAA AGA CUU CUU CCA UCA AGC. Non-specific sequences included Scramble II Duplex (Dharmacon), AAA GUU CCA AUA GAA CCA GUC, AAU CAU UGU GAC GAG GUU CAG, or AAG GCU GCA CCG CAC UCA UGC. HEK293T cells and U2OS cells were transfected as described above three times at 24-hour intervals. Prior to the third transfection, the cells were split to 80% confluency. Cells used for immunoprecipitation and/or immunoblot analysis (Fig. 1E, 4B, S11) or for c-jun message analysis (Fig. 4B, S11) were transfected for the third time with oligos alone and collected 30 hours thereafter as described above. Cells used for assessment of hDET1+hCOP1-mediated c-jun degradation (Fig. 4E) were transfected for the third time with oligos alone, split again after 24 hours, co-transfected with the indicated hDET1 and hCOP1 constructs, and collected 30 hours thereafter. Cells used in AP-1 reporter assays (Fig. 4B, S11) were transfected for the third time with the appropriate oligos along with pAP1(PMA)-Luc (Clontech) and pRL-TK (Promega) at a 10:1 ratio. Basal AP-1 activity was measured 30 hours thereafter using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's recommendations. Cells that were used to determine whether AP-1 activity was detectable in response to PMA (Fig. 4A, S10) were transfected with pAP1(PMA)-Luc (Clontech) and pRL-TK (Promega) at a 10:1 ratio. Thirty hours thereafter, cells were treated with PMA or were left untreated, and cells were collected for analysis within 6 hours.

Pulse-chase analysis. HEK293T and U2OS cells were transfected with hDET1 or Scramble II Duplex siRNA as described above. After the second round of transfection, 2.5×10^6 cells were split into 10-cm dishes and transfected for a third round. The following day cells were washed 2x with PBS and incubated for 10 minutes in 6 mL Cys/Met-free medium containing 10% diafiltered FBS. Subsequently 175 μ Ci/mL Tran³⁵SLabel (ICN) was added to the medium and cells were labeled for 2 hours. Cells were washed 4x with PBS and incubated in regular cell-culture medium for the indicated times. The cells were then harvested in 0.5 mL PBS/TDS (1% Tween-20, 0.5% deoxycholate, 0.1% SDS) containing 30 mM sodium azide and 1 mM NaF with protease inhibitor cocktail tablets (Boehringer Mannheim), lysates were passed 15x through a 25-gauge needle, and were stored at -80°C until all timepoints were collected. The lysates were clarified by centrifugation and TCA precipitated counts were measured.

Immunoprecipitations were set up in 5 mL final volumes of PBS/TDS lysis buffer using equal numbers of counts. Lysates were incubated overnight with 5 μ g anti c-jun (SC-45, Santa Cruz Biotech) antibody overnight followed by a 2 hour incubation with 100 μ L Protein A/G Plus agarose (Santa Cruz Biotech) the following day. Samples were washed 4x with PBS/TDS lysis buffer and prepared for SDS-PAGE as described above. The gels were fixed, amplified (Amersham), dried, and exposed to film following standard protocols. Densitometry measurements were analyzed with NIH Image software.

FACS analysis and confocal microscopy. U2OS cells were transfected with three rounds of hDET1 or Scramble II Duplex siRNAs as described above. Following

transfection, cells were split and supplied with fresh medium as needed. Three days after the last round of transfection, cells were collected and processed for western blot analysis as described above. Cells were also fixed in 70% ethanol/PBS overnight at -20°C , stained with Propidium Iodide, and subsequently analyzed via FACS for sub-G1 content. For assessment of nuclear morphology, cells were grown on slides and fixed in freezing absolute methanol, washed in PBS, and coverslipped with DAPI-containing Vectashield mounting medium (Vector Labs). Cells were subsequently viewed using a Deltavision Deconvolution microscope.

Supporting Figure Legends

Figure S1. Sequence conservation among DET1 homologs. Sequence alignment was generated using the Clustal W algorithm. Identical residues are shaded. The protein sequences of mouse (Mm), rat (Rn), rice (Os), tomato (Le), Arabidopsis (At), potato (St), and fly (Dm) DET1 homologs are available under GenBank accession numbers NP_083861, XP_218833, BAB16336.1, CAA10993, CAB78141, AAM18188, and NP_524784, respectively. The human (Hs) protein sequence was translated from GenBank AK054603, with the starting ATG determined by homologies with mouse and rat DET1. The mosquito (Ag) and *Ciona intestinalis* (Ci) DET1 protein sequences were predicted from GenBank XM_321165 and AK112713, respectively. The *Dictyostelium discoideum* (Dd) sequence was derived from genomic (GenBank AC117075) and EST (GenBank BJ433632 and BJ371698) sequences with the rest of the DET1 sequence alignment as guidance.

Figure S2. Expression analysis of hDET1. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) control primers or hDET1-specific primers were used to amplify cDNA fragments prepared from the indicated sources.

Figure S3. Proposed architecture of the DCX^{hDET1-hCOP1} complex. The DCX^{hDET1-hCOP1} complex is compared to the SCF^{βTrCP} ubiquitin ligase. Boxed “F” and “X” indicate F- and X-boxes, respectively. β-cat: β-catenin substrate; S?: unknown substrate.

Figure S4. Sequence and domain conservation among COP1 orthologs. Sequence alignment was generated using the Clustal W algorithm followed by visual inspection and manual adjustment. Identical residues are shaded. (A) The C3HC4 RING finger domain

is underlined with asterisks indicating key cysteine and histidine residues. (B) The coiled-coil region is defined by the COILS prediction program and is underlined. (C) The six tandem WD-40 repeats revealed by Pfam analysis are underlined. Protein sequences of human (Hs), mouse (Mm), morning glory (In), pea (Ps), rice (Os), rose (Rc), tomato (Le), and Arabidopsis (At) COP1 are available under GenBank accession numbers NP_071902, AAD51094, AAG31173, CAA70768, BAA94422, AAK81856, AAC98912, AAB91983, respectively. Partial COP1 protein sequences of dog (Cf), cotton (Ga), and mosquito (Ag) were predicted from GenBank accession numbers BM537660, BF271046, XM_310428, respectively. The partial zebra fish (Dr) sequence was predicted from three EST clones (AL925436, AI959013 and BQ262083). The partial xenopus (Xl) sequence was predicted from three EST clones (BU915448, BE026293 and CA982158). The partial lettuce (Ls) sequence was predicted from two lettuce EST sequences (BQ851039 and BQ861976). The soybean (Gm) sequence was predicted from several EST clones (CA784346, AW472102, BF068639, BU080814, and BM143933). The chicken (Gg) sequence was predicted from multiple chicken EST sequences (BU422118, BU392251, BU435001, BU461738, and BU295711).

Figure S5. HCOP1 domains and relative location of splice sites. Upper schematic: Numbers indicate amino acid residues. Hatched areas indicate positions of splice sites. RING: RING finger domain. CC: coiled-coil domain. WD-40: WD40 domain. Lower amino acid sequence: Domains are shaded corresponding to the schematic. Spliced amino acids are boxed within the hCOP1 amino acid sequence.

Figure S6. FLAG-GST-hDET1 binds myc-hCOP1 but not myc-hCOP1 Δ 24. n/s: non-specific antibody control

Figure S7. Interaction of hCOP1 splice variants with DCX^{hDET1-hCOP1} subunits. FLAG-hCOP1, but not FLAG-hCOP1 Δ 24, binds the complete endogenous DCX^{hDET1-hCOP1} complex immunoprecipitated (IP) from U2OS cells.

Figure S8. FLAG-hCOP1, FLAG-hCOP1 Δ 24, the FLAG-hCOP1 RING mutant (MT), and myc-hDET1 do not affect c-jun turnover or message levels in HEK293T cells.

Figure S9. Myc-hDET1 transfected with FLAG-hCOP1, FLAG-hCOP1 Δ 24, or the FLAG-hCOP1 RING mutant (MT) do not affect c-jun message.

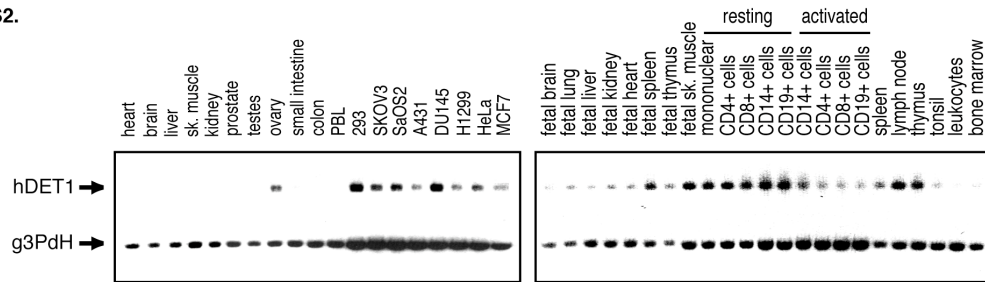
Figure S10. AP-1-driven luciferase reporter activity is detectable in U2OS cells in response to treatment with the positive stimulus phorbol 12-myristate 13-acetate (PMA).

Figure S11. siRNA-mediated reduction of DCX^{hDET1-hCOP1} subunit expression stabilizes endogenous c-jun protein (top panels) and increases basal AP-1 activity (lower graphs) in U2OS cells. n/s: non-specific control oligonucleotide.

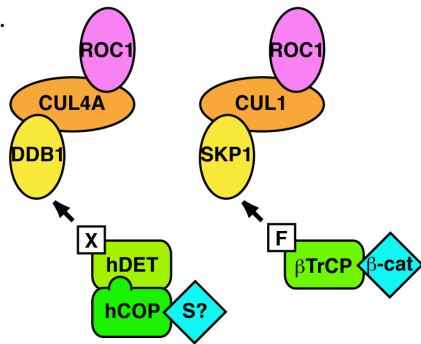
Figure S12. Pulse-chase analysis of endogenous c-jun in U2OS cells transfected with hDET1 siRNA or non-specific (n/s) control oligonucleotides. Reduction of hDET1 expression attenuates c-jun turnover.

Figures S2, S3
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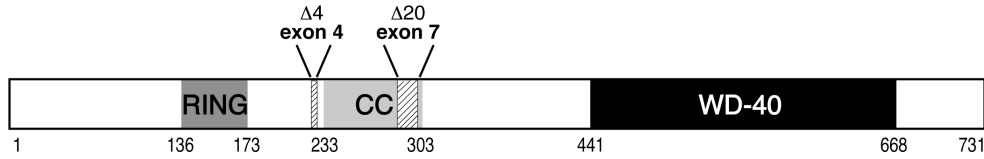
S2.



S3.

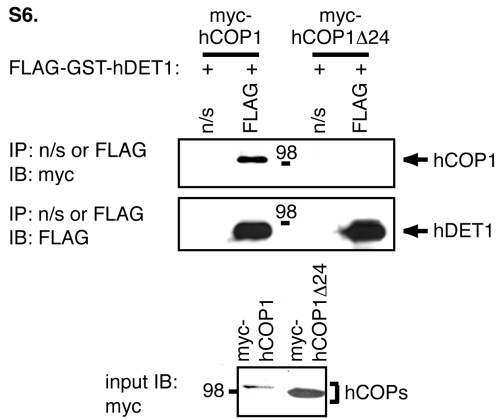


S5.

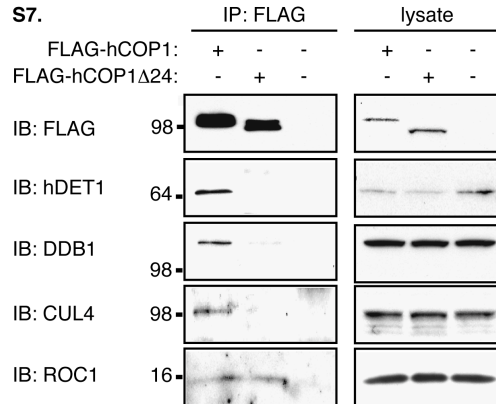


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 IEAKANVCCVKFSPSSRYHLAFGCADHCVHYYDLRNTKQPIIMVFKGHRKAVSYAKFVSGEEIVSASTDSQLKLWNV/GKP
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 GESNVLIAANSQGTIKVLELV

S6.



S7.



Figures S8-S12
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