

# Human De-Etiolated-1 Regulates c-Jun by Assembling a CUL4A Ubiquitin Ligase

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*Arabidopsis thaliana* De-etiolated-1 (AtDET1) is a highly conserved protein, with orthologs in vertebrate and invertebrate organisms. AtDET1 negatively regulates photomorphogenesis, but its biochemical mechanism and function in other species are unknown. We report that human DET1 (hDET1) promotes ubiquitination and degradation of the proto-oncogenic transcription factor c-Jun by assembling a multisubunit ubiquitin ligase containing DNA Damage Binding Protein-1 (DDB1), cullin 4A (CUL4A), Regulator of Cullins-1 (ROC1), and constitutively photomorphogenic-1. Ablation of any subunit by RNA interference stabilized c-Jun and increased c-Jun-activated transcription. These findings characterize a c-Jun ubiquitin ligase and define a specific function for hDET1 in mammalian cells.

Photomorphogenesis comprises the physiological changes that plants undergo in response to light. Negative regulators have been identified in *Arabidopsis* seedlings with mutated *DET* or constitutively photomorphogenic (*COP*) genes (1). A specific function for the protein affected in AtDET1 mutants (2) has not been determined, al-

though AtDET1 has been associated with an array of processes, including peroxisomal metabolism (3) and chromatin remodeling (4). The COP9 signalosome (CSN), a protein complex that activates cullin-containing multisubunit ubiquitin ligases (5, 6), is composed of other negative regulators of photomorphogenesis (CSN1 to CSN8). Ubiquitin ligases target substrates to the ubiquitin-proteasome system (UPS), the primary conduit for regulated degradation of cellular proteins (7). These negative regulators of photomorphogenesis are highly conserved in mammals, suggesting that they function in fundamental pathways that are common to divergent species.

To better understand these conserved processes, we investigated the function of hDET1 in

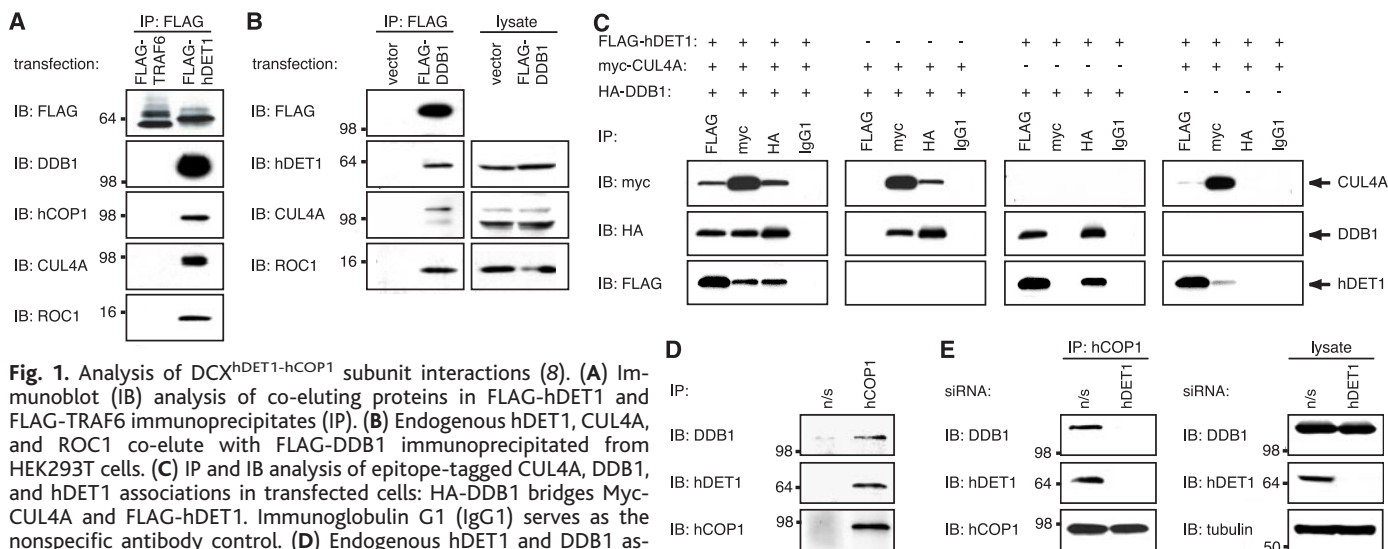
human cells. Analysis of *hDET1* cDNA revealed a highly conserved 550-amino acid protein (GenBank accession number AK054603) (fig. S1) (4, 8). *hDET1* expression was highest in the ovary, some lymphoid organs, resting leukocytes, and certain cell lines (fig. S2). Because DDB1 associates with CUL4A and ROC1 in mammalian ubiquitin ligase complexes (9–11), and AtDET1 and DDB1 interact to regulate photomorphogenesis (4), we speculated that hDET1 might also bind ubiquitin ligase components. To investigate this hypothesis, a proteomics approach was taken to identify hDET1-associated proteins. Epitope-tagged hDET1 (FLAG-hDET1) was immunoprecipitated from transfected human embryonic kidney (HEK293T) cells and eluted proteins were identified by mass spectrometry analysis. Peptides from DDB1, CUL4A, ROC1, and hCOP1 were detected in FLAG-hDET1 elutions but not in the control (FLAG-TRAF6), suggesting that these interactions were specific to hDET1 (12). Immunoblot analysis confirmed the presence of these proteins (Fig. 1A). FLAG-DDB1 elutions also contained endogenous hDET1, CUL4A, and ROC1 (Fig. 1B).

Collectively, these data suggested that DDB1 links hDET1 to CUL4A to form a ubiquitin ligase complex. To test this idea, epitope-tagged CUL4A, DDB1, and hDET1 were expressed in HEK293T cells and protein associations were analyzed by immunoprecipitation and immunoblot analysis. In the absence of hemagglutinin (HA)-DDB1 expression, the association between Myc-CUL4A and FLAG-hDET1 was attenuated (Fig. 1C).

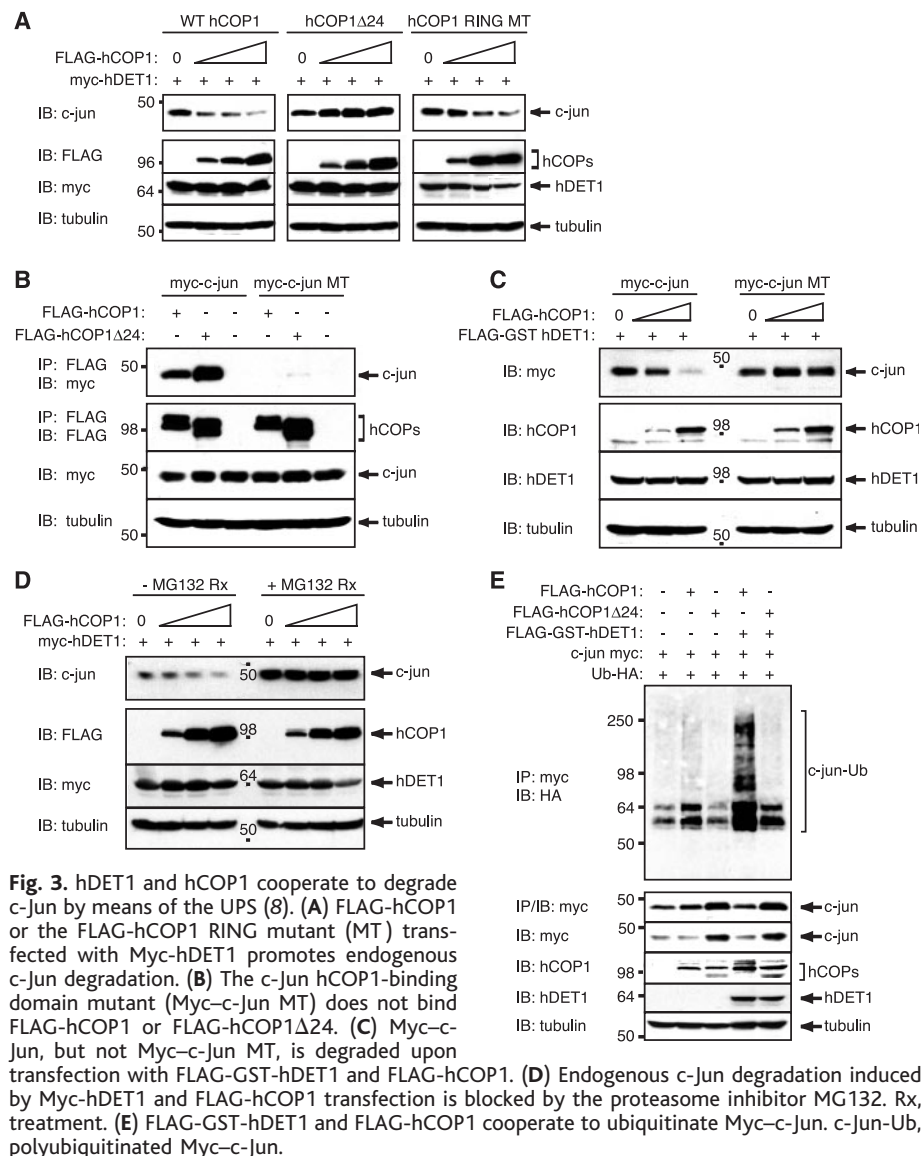
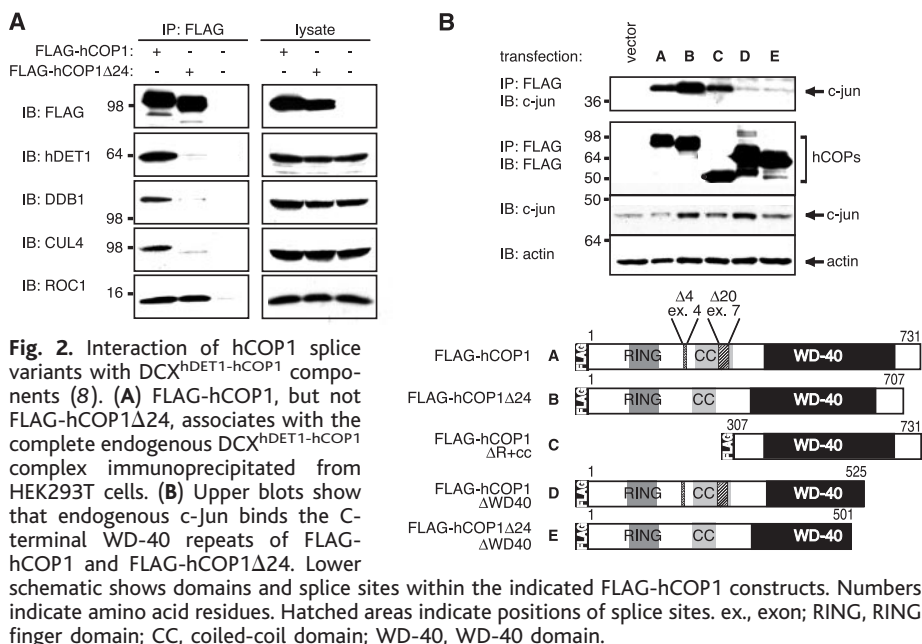
Although AtDET1 and AtCOP1 genetically interact to repress photomorphogenesis (1, 13), they have not been shown to physically associate. Decreasing hDET1 expression with small

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**Fig. 1.** Analysis of DCX<sup>hDET1-hCOP1</sup> subunit interactions (8). (A) Immunoblot (IB) analysis of co-eluting proteins in FLAG-hDET1 and FLAG-TRAF6 immunoprecipitates (IP). (B) Endogenous hDET1, CUL4A, and ROC1 co-elute with FLAG-DDB1 immunoprecipitated from HEK293T cells. (C) IP and IB analysis of epitope-tagged CUL4A, DDB1, and hDET1 associations in transfected cells: HA-DDB1 bridges Myc-CUL4A and FLAG-hDET1. Immunoglobulin G1 (IgG1) serves as the nonspecific antibody control. (D) Endogenous hDET1 and DDB1 associate with endogenous immunoprecipitated hCOP1. n/s, nonspecific antibody control. (E) siRNA-mediated ablation of hDET1 diminishes endogenous hCOP1 and DDB1 association. n/s, nonspecific oligonucleotide control.



interfering RNA (siRNA) oligonucleotides attenuated the association between endogenous hCOP1 and DDB1 (Fig. 1, D and E), suggesting that hDET1 links hCOP1 to DDB1.

These results indicate that hDET1, hCOP1, DDB1, CUL4A, and ROC1 form a multisubunit ubiquitin ligase (fig. S3). In keeping with the Skp1/CUL1/F-box (SCF) nomenclature (14), we propose the name DCX<sup>hDET1-hCOP1</sup> (for DDB1/CUL4A/X-box) for this complex, in which an undefined motif (the X-box) permits association between the heterodimeric hDET1-hCOP1 substrate adaptor and DDB1.

*hCOP1* was cloned to investigate its proposed role as a substrate-binding DCX<sup>hDET1-hCOP1</sup> component. Analysis of *hCOP1* cDNA revealed a highly conserved 731-amino acid protein (GenBank accession number NP\_071902) with predicted RING finger, coiled-coil, and WD-40 domains (fig. S4) (15, 16). hCOP1 splice variants were also cloned (12). The most prevalent variant, as determined by reverse transcription polymerase chain reaction (RT-PCR) and expressed sequence tag database analysis, lacked the four terminal amino acids of exon 4 and all 20 amino acids of exon 7 (hCOP1Δ24, GenBank accession number AY509921) (fig. S5). Because hCOP1Δ24 lacks part of the coiled-coil protein interaction domain (17) (fig. S5), interactions between hDET1 and the hCOP1 splice variants were investigated. FLAG-glutathione *S*-transferase (GST)-hDET1 bound Myc-hCOP1 but not Myc-hCOP1Δ24 (fig. S6). Furthermore, endogenous hDET1, DDB1, and CUL4A associated with FLAG-hCOP1 but not with FLAG-hCOP1Δ24 in HEK293T and in human osteosarcoma (U2OS) cells (Fig. 2A and fig. S7). These results suggest that hDET1 recruits hCOP1 to the DCX<sup>hDET1-hCOP1</sup> complex. Unexpectedly, hCOP1Δ24 also bound ROC1, but this was not mediated by CUL4A, suggesting that both hCOP1 splice variants might interact with other cullins. hCOP1 and hCOP1Δ24 bound endogenous CUL5, but not CUL1, CUL2, or CUL3 (11). However, it is also possible that RING finger heterodimerization mediates ROC1 binding to each hCOP1 splice variant (18, 19).

Because AtCOP1 promotes the degradation of basic region-leucine zipper (bZIP) transcription factors HY5 and HYH (13, 20) and hCOP1 binds Jun family bZIP transcription factors (16), we tested the ability of the potential substrate adaptors hDET1, hCOP1, and hCOP1Δ24 to bind human bZIP transcription factors. Both hCOP1 variants bound c-Jun, JunB, and JunD but not other bZIP transcription factors including c-Fos, ATF2, or hXBP-1 (12). No interaction between hDET1 and any bZIP transcription factor was detected (12). The interaction between endogenous c-Jun and the hCOP1 variants was dependent on the C-terminal WD-40 repeats common to both hCOP1 splice forms (Fig. 2B).

Given that hCOP1 and hCOP1Δ24 both bound c-Jun but differentially bound hDET1, we

investigated whether they differentially regulated c-Jun stability and whether the hCOP1 RING finger was required for this process. RING fingers mediate the function of certain ubiquitin ligases (21); although the AtCOP1 RING finger mediates in vitro polyubiquitination (22), it is dispensable for repressing photomorphogenesis (17). The expression of hCOP1, the hCOP1 RING mutant (Cys<sup>136</sup>→Ala<sup>136</sup>, Cys<sup>139</sup>→Ala<sup>139</sup>), or hDET1 had no effect on endogenous c-Jun protein or message levels (fig. S8), whereas hCOP1Δ24 expression caused a modest accumulation of endogenous c-Jun protein (fig. S8). In contrast, hDET1 expression with hCOP1 or the hCOP1 RING mutant, but not hCOP1Δ24, reduced endogenous c-Jun protein levels (Fig. 3A). No change in c-Jun mRNA was detected (fig. S9), indicating that these effects were post-transcriptional. These results imply that hDET1 binds hCOP1 and thereby recruits c-Jun to the DCX<sup>hDET1-hCOP1</sup> complex for ubiquitination and subsequent degradation. Although hCOP1Δ24 binds endogenous c-Jun (Fig. 2B), it cannot associate with the DCX<sup>hDET1-hCOP1</sup> complex (Fig. 2A and figs. S6 and S7) and sequesters c-Jun from degradation (Fig. 3A). Additionally, the hCOP1 RING finger is dispensable for c-Jun degradation (Fig. 3A), suggesting that the ROC1 RING finger is the functional ubiquitin ligase domain of the DCX<sup>hDET1-hCOP1</sup> complex.

To ascertain whether c-Jun binding to hCOP1 is required for DCX<sup>hDET1-hCOP1</sup>-mediated degradation, the hCOP1 interaction domain of c-Jun was mapped by deletional analysis to residues 146 to 296 (12). Alignment of this region with JunB, JunD, and *Arabidopsis* bZIP transcription factors HYH, STH, and STO revealed the consensus sequence Asp/Glu-Glu-x-x-Val-Pro, where x is any amino acid, similar to previous reports (16, 23). When mutated (MT) to Ala<sup>227</sup>-Ala-x-x-x-Ala-Ala<sup>233</sup>, Myc-c-Jun-MT binding to FLAG-hCOP1 and FLAG-hCOP1Δ24 was abrogated (Fig. 3B). This mutant was also resistant to hDET1-hCOP1-mediated degradation (Fig. 3C).

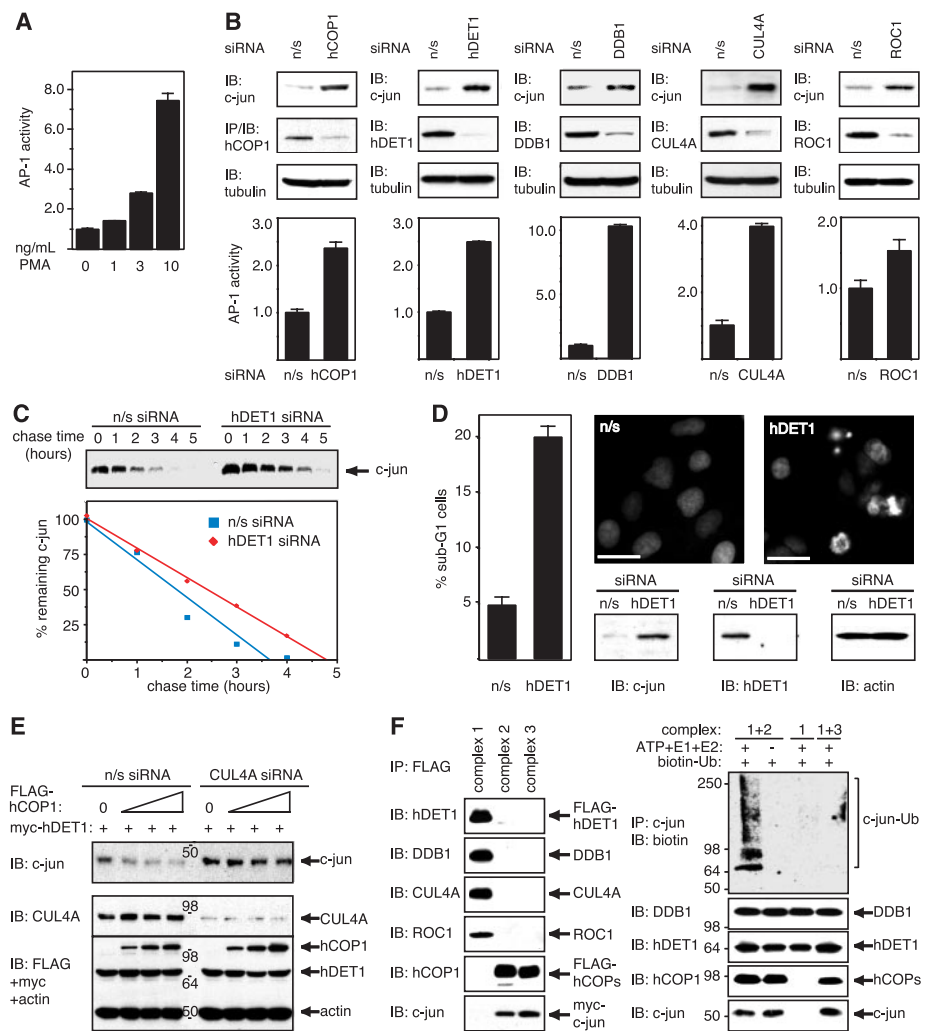
We next investigated whether the DCX<sup>hDET1-hCOP1</sup> complex promoted UPS-mediated c-Jun degradation. Treatment of cells with the proteasome inhibitor MG132 blocked hDET1-hCOP1-induced c-Jun degradation (Fig. 3D). Additionally, hDET1 expressed with hCOP1, but not hCOP1Δ24, promoted c-Jun ubiquitination in transfected cells (Fig. 3E).

To evaluate the physiological relevance of the DCX<sup>hDET1-hCOP1</sup> complex, we assessed whether ablation of each DCX<sup>hDET1-hCOP1</sup> subunit by siRNA would affect endogenous c-Jun stability and basal transcriptional activity. We first established that c-Jun transcriptional activity was detectable in cells transfected with an AP-1-driven reporter construct in response to the positive stimulus phorbol 12-myristate 13-acetate (PMA) (Fig. 4A and fig. S10). Reduced expression of each DCX<sup>hDET1-hCOP1</sup> component resulted in endogenous c-Jun protein accumula-

tion and also enhanced basal AP-1 activity in HEK293T and U2OS cells (Fig. 4B and fig. S11). No changes in c-Jun message were detected (12), suggesting that c-Jun accumulation was due to stabilization of c-Jun protein rather than enhanced c-Jun transcription. Pulse-chase analysis also indicated that the DCX<sup>hDET1-hCOP1</sup> complex promoted posttranslational c-Jun turnover, because reduced hDET1 expression stabilized c-Jun in both cell lines (Fig. 4C and fig. S12). Additionally, transfection with hDET1 siRNA,

but not control siRNA, induced apoptosis of U2OS cells (Fig. 4D). Indeed, constitutive c-Jun expression is proapoptotic (24), and severe AT-DET1 mutations are lethal (2). Collectively, these findings underscore the critical role of the DCX<sup>hDET1-hCOP1</sup> complex in maintaining proper cellular homeostasis.

To assess the interdependence of the DCX<sup>hDET1-hCOP1</sup> subunits in promoting c-Jun degradation, endogenous CUL4A protein was reduced with siRNA; this attenuated hDET1-hCOP1-in-



**Fig. 4.** Analysis of DCX<sup>hDET1-hCOP1</sup> function (7). (A) AP-1-driven luciferase reporter activity is detectable in HEK293T cells in response to the positive stimulus PMA. (B) siRNA-mediated reduction of DCX<sup>hDET1-hCOP1</sup> subunit expression stabilizes endogenous c-Jun protein (top panels) and increases basal AP-1 activity (lower graphs) in HEK293T cells. n/s, nonspecific control oligonucleotide. (C) Pulse-chase analysis of endogenous c-Jun in HEK293T cells transfected with hDET1 siRNA or nonspecific (n/s) control oligonucleotides. Reduction of hDET1 expression attenuates c-Jun turnover. (D) hDET1 siRNA induces apoptosis of U2OS cells cultured 3 additional days posttransfection. Left graph: percent of cells with sub-G<sub>1</sub> DNA content characteristic of apoptosis. Upper micrographs: condensed and fragmented chromatin in 4',6'-diamidino-2-phenylindole-stained nuclei of apoptotic cells. Scale bar, 30 μm. Lower immunoblots: analysis of endogenous proteins. (E) siRNA-mediated ablation of CUL4A expression (CUL4A siRNA) attenuates hDET1/hCOP1-induced c-Jun degradation. n/s siRNA, nonspecific siRNA control oligonucleotide. (F) In vitro ubiquitination of c-Jun by the DCX<sup>hDET1-hCOP1</sup> complex. Left panels: immunoblot analysis of elutions from FLAG immunoprecipitates. Complex 1 transfection: FLAG-hDET1. Complex 2 transfection: FLAG-hCOP1 and Myc-c-Jun. Complex 3 transfection: FLAG-hCOP1Δ24 and Myc-c-Jun. Right panels: elutions were combined for in vitro ubiquitination assays (top) and analyzed by immunoblotting.

duced c-Jun degradation (Fig. 4E). To investigate whether the DCX<sup>hDET1-hCOP1</sup> complex could ubiquitinate c-Jun in vitro, HEK293T cells were transfected to express FLAG-hDET1 (complex 1), FLAG-hCOP1 and Myc-c-Jun (complex 2), or FLAG-hCOP1Δ24 and Myc-c-Jun (complex 3), and proteins were eluted from FLAG immunoprecipitates (Fig. 4F). Similar to substrate adaptors of other multisubunit ubiquitin ligases (14, 21), the assembly of hCOP1 and hDET1 into the DCX<sup>hDET1-hCOP1</sup> complex promoted their degradation (12), necessitating the use of MG132 to stabilize the DCX<sup>hDET1-hCOP1</sup> complex (Figs. 1, A and C to E, and 2A; figs. S6 and S7). However, MG132 was omitted in this experiment to isolate the complexes with minimal associated endogenous hCOP1 and hDET1 (Fig. 4F). Elutions were mixed to promote hCOP1 and hDET1 binding and were subsequently added to in vitro ubiquitination assays. Ubiquitination of c-Jun was detected only when the complete DCX<sup>hDET1-hCOP1</sup> complex was assembled (Fig. 4F). hCOP1Δ24 did not promote effective ubiquitination of bound c-Jun (Fig. 4F), consistent with its inability to associate with the DCX<sup>hDET1-hCOP1</sup> ligase complex (Fig. 2A and figs. S6 and S7).

Although it is established that c-Jun stability is regulated by the UPS, the identity of any c-Jun ubiquitin ligase has been elusive (25, 26). Our results identify and characterize a c-Jun ubiquitin ligase. The DCX<sup>hDET1-hCOP1</sup> complex appears to be distinct from a previously reported unidentified c-Jun ubiquitin ligase (25, 26) because coexpression of JNK or deletion of the c-Jun delta domain did not protect c-Jun from hDET1-hCOP1-induced degradation (12). Because c-Jun regulates a myriad of cellular processes and can induce oncogenic transformation (24, 27), it is possible that several ubiquitin ligases exist for tight regulation of c-Jun activity, similar to p53 (7). It is also possible that DCX<sup>hDET1-hCOP1</sup> components have separate regulatory functions when not incorporated in the DCX<sup>hDET1-hCOP1</sup> complex, because recombinant AtCOP1 ubiquitinates LAF1 in vitro without other DCX<sup>hDET1-hCOP1</sup> subunits (22), and SKP1 and associated F-box proteins can function as components of SCF ubiquitin ligases and also as separate entities (21). The conservation of the proteins comprising the human DCX<sup>hDET1-hCOP1</sup> complex suggests that an orthologous ubiquitin ligase assembles in *Arabidopsis*. This could provide a mechanism for how AtDET1 and AtCOP1 regulate photomorphogenesis. Moreover, the presence of a cullin in the DCX<sup>hDET1-hCOP1</sup> complex could explain why CSN mutants are constitutively photomorphogenic (5).

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Materials and Methods  
Figs. S1 to S12

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# The Ubiquitin Ligase SCF<sup>Fbw7</sup> Antagonizes Apoptotic JNK Signaling

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Jun N-terminal kinases (JNKs) are essential for neuronal microtubule assembly and apoptosis. Phosphorylation of the activating protein 1 (AP1) transcription factor c-Jun, at multiple sites within its transactivation domain, is required for JNK-induced neurotoxicity. We report that in neurons the stability of c-Jun is regulated by the E3 ligase SCF<sup>Fbw7</sup>, which ubiquitinates phosphorylated c-Jun and facilitates c-Jun degradation. Fbw7 depletion resulted in accumulation of phosphorylated c-Jun, stimulation of AP1 activity, and neuronal apoptosis. SCF<sup>Fbw7</sup> therefore antagonizes the apoptotic c-Jun-dependent effector arm of JNK signaling, allowing neurons to tolerate potentially neurotoxic JNK activity.

The Jun N-terminal kinase (JNK) signaling pathway leading to c-Jun phosphorylation is implicated in neuronal apoptosis caused by a variety of conditions, including exposure to excitotoxins and some neurodegenerative disorders (1, 2). Genetic ablation of c-Jun phosphorylation results in protection from neuronal death, but how phosphorylated c-Jun regulates cell death is unclear (3, 4).

To determine whether JNK-mediated phosphorylation of c-Jun modifies the interaction of the c-Jun N terminus with cofactor molecules to modulate neurotoxic JNK signaling, we used a modified yeast-based, cytoplasmic two-hybrid screening to identify phosphorylation-dependent interactions (5) (Fig. 1A). The

Ras Recruitment System (RRS) is based on the complementation of a temperature-sensitive yeast *cde25* mutant, deficient in Ras activity, by human oncogenic Ras (RasV12). Ras function requires plasma membrane localization, which can be achieved through interaction between two hybrid proteins. A c-Jun leucine zipper mutant (JunΔLZ) was generated to avoid unwanted interaction with known partner proteins. It was fused to RasV12 (RasV12-JunΔLZ) and used as a bait (Fig. 1B). A constitutively active MKK7-JNK1 fusion protein was placed under the control of the methionine-regulated *MET3* promoter, to induce N-terminal phosphorylation of the RasV12-JunΔLZ bait fusion protein in a methionine-dependent manner (Fig. 1C) (6–8). After transfection of a brain cDNA library in which all encoded proteins were fused to a myristoylation signal and thus associated to the plasma membrane (8), yeast clones were screened for rescue of the *cde25* mutation at the restrictive temper-

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