array of substrates. In yeast, over 19 F-box proteins are known, in *A. thaliana* over 400, and in humans ~70 [2]. The family of SCF ligases in turn is the prototype for a superfamily of cullin-RING ligases that, like SCF, are modular enzymes comprising a cullin-RING subcomplex linked to a variable substrate receptor subunit (VHL box proteins for Cul2, BTB proteins for Cul3, and SOCS box proteins for Cul5). Altogether, the human genome may have the capacity to code for as many as 350 different CRLs.

Given the diversity of CRL substrate receptor proteins, two important questions emerge. First, how is the repertoire of CRLs dynamically controlled? Second, are distinct CRL complexes differentially regulated in a manner that depends on the identity of the substrate receptor? One partial answer to both of these questions is that F-box and other substrate receptors are often unstable proteins, and it is thought that they are targeted for degradation in part by 'autoubiquitination' within SCF-E2 complexes [2]. However, not all CRL substrate receptors are unstable, and thus there must be some means of differentially controlling their stability. There are multiple ways in which this might be accomplished. First, CRL ubiquitin ligase activity is negatively regulated by Cop9 Signalosome (CSN) *in vitro* [3-6]. CSN cleaves the ubiquitin-like protein Nedd8 from the cullin subunit of CRLs [3,7]. Attachment of Nedd8 to Cul1 strongly stimulates the ability of the Cul1-Hrt1/Roc1/Rbx1 catalytic core to promote ubiquitin chain synthesis by Cdc34 E2 enzyme [8-10]. Once Nedd8 is detached, CAND1 can bind Cul1 and displace Skp1, thereby preventing the recruitment of substrate to the catalytic core [11,12]. In addition to removing Nedd8, CSN also recruits a deubiquitinating enzyme to Cul1, Ubp12, that opposes ubiquitin polymerization [6,13]. Thus, CSN may play a key role in controlling the dynamics of individual CRL complexes and the overall repertoire of different CRL complexes in a cell.

CSN is a highly conserved protein complex found from yeast to humans. CSN is composed of eight subunits, termed Csn1-Csn8 [14] and each of these subunits contains high homology to components of the 26S proteasome lid subcomplex and eukaryotic Initiation Factor 3 (eIF3) [reviewed in [15]]. CSN has been found to play diverse roles in several different organisms [reviewed in [15]]. In *A. thaliana*, CSN components were identified in a screen for plants that displayed a constitutive photomorphogenic defect (plants develop in the dark as they would in the light). In *D. melanogaster*, mutations in Csn4 and Csn5 result in pleitropic effects, including activation of meiotic checkpoints [16,17] and failure of photoreceptor neurons to differentiate [18]. RNAi of Csn5 in *C. elegans* additionally results in pleitropic effects, including sterile worms and alterations in microtubules [19].

Although the molecular basis behind many of these phenotypes has yet to be elucidated, it is becoming evident that deneddylation of cullins catalyzed by the 'JAMM' metalloprotease active site motif in the Csn5 subunit is at least partially responsible. Transgenic *csn5* with mutations in the JAMM motif fails to correct the developmental defects of *csn5*-delete flies [20]. Moreover, failure to deneddylate Cul3 in *C. elegans* is implicated in accumulation of the microtubule severing protein Mei-1, which results in microtubule defects [19,21].

The effects of loss of deneddylation of cullin proteins are still not understood. Loss of CSN function causes a defect in cullin-based ligase activity *in vivo* suggesting that deneddylation promotes SCF activity [4,7,20-25]. However, *in vitro* data suggests a negative role for CSN in regulation of SCF [3-6]. What may account for this discrepancy? One hypothesis suggests that neddylation and deneddylation affect cycles of assembly of CRL complexes and the stability of substrate adaptor proteins *in vivo* [15,6].

In an effort to investigate how loss of deneddylation affects SCF activity, we conditionally silenced the catalytic subunit of CSN in mammalian cells. Suppression of Csn5 protein resulted in a significant decrease in the F-box proteins Skp2, cyclin F, Fbx7, Fbx4, and Fbw7. Moreover, mRNA transcripts for all but one of these F-box proteins were unaltered when *CSN5* was suppressed, suggesting the decreases in protein levels are post-translational. Consistent with this notion, treatment with proteasome inhibitors largely restored the levels of multiple F-box proteins and dominant-negative Cul1 prevented loss of cyclin F. Finally, we found a dramatic increase in the protein and activity levels of the SCF^{Fbw7} substrate cyclin E, suggesting that loss of F-box proteins in CSN-deficient cells results in substrate accumulation.

Results

Depletion of CSN5 in HEK293 cells is not lethal

To analyze the effect of loss of deneddylation in human cells, we utilized the doxycyline-inducible shRNA system developed by Clevers and colleagues [26] to conditionally down-regulate Csn5 protein levels. Eight days of doxycycline treatment of cells carrying the inducible Csn5-specific shRNA resulted in a drastic reduction in both Csn5 mRNA and protein levels when compared to induced cells expressing a scrambled shRNA (Figures 1A and 1B). By contrast, the levels of other CSN subunits showed little or no change (Figure 1C). Despite this significant drop in Csn5 protein levels, few morphological changes were observed, the cells continued to grow and divide, and little change in steady-state cell cycle distribution was detected by FACS (data not shown). This is consistent with prior observations on normal diploid BJ1 fibroblasts