Table 1: Plasmids used in this study

Plasmid	Reference	Description
pGC95	This study	Tetracycline inducible scrambled siRNA
pGC93	This study	Tetracycline inducible siRNA to human Csn5.
RJD 1419	This study	CMV Flag-Csn5
RJD 1500	This study	CMV Flag-Csn5 (ASA)
RJD 1501	This study	CMV Flag-Csn5 (D151N)
RJD 942	[34]	HA-Cul1
RJD 1192	[27]	HA-Cul1 (1–498)

was added. 500 uL of BBS (0.05 M BES, 0.28 M NaCl, 0.0015 M Na_2HPO_4, pH 7.0) was added and solution vortexed to mix. Cells were re-fed with fresh MEM supplemented with 2.5 uL/mL 25-hydroxycholesterol (Sigma). Precipitate was applied to cells and incubated for 4 hours prior to removal with PBS. Cells were re-fed with fresh MEM and incubated 24–48 hours. All plasmids used in this study are listed in Table 1.

Construction of stable cell lines

HEK293 cells were transfected with pGC95 (control scrambled shRNA, 5'-CGTGCAAGGTCAGTACATGTTCAAGAGACATGTACTGACCTTGCACG) or pGC93 (5'-TGCTCAGGCTGCTGCATATTTCAAGAGAATATGCAGCAGCCTGAGCA). 24 hours after transfection, cells were re-fed with MEM supplemented with 5 ug/mL puromycin and selected for 48 hours. Post-selection, cells were grown for 7–10 days and single colonies were selected. Two representative clonal expansions (1A and 2F) of pGC93 are shown.

mRNA analysis

RNA was purified from cells using Qiagen RNeasy kits. After purification, RNA was normalized by concentration used as a template for stratascript reverse transcriptase following manufacturers protocols (stratagene). Reverse transcribed RNA was then used for PCR towards Csn5, β-Actin, cyclin F, Fbx4, Fbx7, Fbw7, and Skp2.

Protein analysis

Native lysates were made by resuspending cell pellets in an equal volume of buffer A (25 mM TRIS (7.5), 150 mM NaCl, 0.3% Triton X-100, 1 mM EDTA and 1 mM DTT supplemented with 1 mM PMSF, 0.25 ug/mL pepstatin, and 5 ug/mL each of leupeptin, aprotinin, and chymotrypsin). Lysates were cleared by centrifugation and normalized using the Biorad Protein Assay (Biorad). Denatured lysates were made by resuspending cell pellets in an equal volume of boiling SDS buffer (2% SDS, 50 mM TRIS (7.5) and 5 mM DTT) and boiled for 5 minutes. Lysates were sonicated and cleared by centrifugation.

Antibodies

The following antibodies were used in this study: Fbw7 (Orbigen PAB-10563), cyclin F (Santa Cruz Biotechnology sc-952), cyclin E (Santa Cruz Biotechnology sc-198), Csn5 (Santa Cruz Biotechnology sc-9074), β-tubulin (Santa Cruz Biotechnology sc-9104) Fbx7 (Zymed 51–8000) and c-myc (Santa Cruz Biotechnology sc-42). Skp2 was generously provided by W. Krek and Fbx4 was generously provided by W. Boelens.

Histone H1 kinase assay

Native lysates were immunoprecipitated for 1 hour with antibodies toward cyclin E. Immunoprecipitates were washed 3 times in native lysis buffer (buffer A). Beads were resuspended in 10 uL of reaction mix (for 100 uL: 3 uL 5 mg/mL histone H1, 1.5 uL 4.5 mM ATP, 1.5 uL γ-^{32}P ATP, 94 uL of kinase assay buffer (10 mM TRIS (7.5), 10 mM $MgCl_2$, 50 mM NaCl, 2 mM EDTA, 1 mM DTT and 0.02% Triton X-100)). Reactions were incubated for 20 minutes at 21°C and analyzed by SDS-PAGE followed by autoradiography.

Abbreviations

CSN: COP9/signalosome

SCF: SKP1/Cullin/F-box protein complex

JAMM: Jab1/MPN domain metalloenzyme motif

BTB: "bric-a-brac/tramtrack/broad" protein complex

Authors' contributions

All experiments were performed by GC. Both GC and RJD conceived and designed the experiments. Both authors contributed to the writing of the manuscript and both authors read and approved the final manuscript.

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