Additional Methods

Cell culture and cell cycle synchronization. HeLa, U-2OS, RPE1-hTERT, HEK-293T, and H1299, HCT116 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS). For synchronization at G1/S, HeLa cells were cultured in the presence of 2 mM thymidine (Sigma) for 16 hours, washed twice with PBS, and cultured in fresh medium without thymidine for 8 hours. After another 16 hours in thymidine, cells were washed twice with PBS and cultured in fresh medium. siRNA oligos were transfected between the first and second thymidine block. To trap cells in prometaphase, nocodazole (100 ng/ml) was added five hours after the release from the thymidine block. In the experiments shown in Fig. 2c and Supplementary Fig. 1b, 10 μM MG132 was added for 3-6 hours prior to harvesting the cells.

Transient transfections. HEK-293T cells were transfected using the calcium phosphate method, as described[25]. U-2OS and HEK-293T were transfected using Exgene (Fermentas) according to the manufacturer's instruction. siRNA duplexes were transfected into subconfluent U-2OS or HeLa cells using HiPerfect reagent (QIAGEN) according to the manufacturer's instructions. Combined siRNA and DNA transfection was performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions

Purification and MudPIT Analysis. HEK-293T cells were transfected with constructs encoding either FLAG-HA-tagged Cyclin F wild type or FLAG-HA-tagged Cyclin F(1-270). Forty-eight hours after transfection, cells were collected and lysed in lysis buffer (LB: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 0.5% NP40, plus protease and phosphatase inhibitors). Cyclin F and associated proteins were immunopurified with anti-FLAG M2 agarose beads (Sigma). After washing, proteins were eluted twice by competition with FLAG peptide (Sigma). The eluate was then subjected to a second immunopurification with an anti-HA resin (12CA5 monoclonal antibody crosslinked to protein G Sepharose), prior to elution by competition with HA peptide (Roche). The final eluate was then precipitated with TCA.

TCA-precipitated proteins were urea-denatured, reduced, alkylated and digested with endoproteinase Lys-C (Roche), followed by modified trypsin (Roche), as described in[8,26]. Peptide mixtures were loaded onto 100 µm fused silica microcapillary columns packed with 5-µm C_{18} reverse phase (Aqua, Phenomenex), strong cation exchange particles (Partisphere SCX, Whatman), and reverse phase[27]. Loaded microcapillary columns were placed in-line with a Quaternary Agilent 1100 series HPLC pump and a LTQ linear ion trap mass spectrometer equipped with a nano-LC electrospray ionization source (ThermoFinnigan). Fully automated 10-step MudPIT runs were carried out on the electrosprayed peptides, as described in[8]. Tandem mass (MS/MS) spectra were interpreted using SEQUEST[28] against a database of 61430 sequences, consisting of 30552 human proteins (downloaded from NCBI on 2008-03-04), 177 usual contaminants (such as human keratins, IgGs,

and proteolytic enzymes), and, to estimate false discovery rates, 30712 randomized amino acid sequences derived from each non-redundant protein entry. Peptide/spectrum matches were sorted and selected using DTASelect[29] with the following criteria set: spectra/peptide matches were only retained if they had a DeltCn of at least 0.08 and a minimum XCorr of 1.8 for singly-, 2.0 for doubly-, and 3.0 for triply-charged spectra. In addition, peptides had to be fully-tryptic and at least 7 amino acids long. Combining all runs, proteins had to be detected by at least 2 such peptides, or 1 peptide with 2 independent spectra. Under these criteria the final FDRs at the protein and spectral levels were 1.6% and 0.13%±0.05, respectively. Peptide hits from multiple runs were compared using CONTRAST[29]. To estimate relative protein levels, Normalized Spectral Abundance Factors (NSAFs) were calculated for each detected protein, as described in[30-32].

Antibodies. The following rabbit polyclonal antibodies were used: Cyclin F (C-20; Santa Cruz Biotechnology), CP110 (A301-343A; Bethyl Laboratories), Skp1 (H-163; Santa Cruz Biotechnology), Centrin 2(N-17; Santa Cruz Biotechnology), phosphorylated Cdc2 (phospho-Tyr15; Santa Cruz Biotechnology), phosphorylated Histone H3 (phospho-Ser10; Millipore), p27 (Invitrogen), Cul1 (Invitrogen) and FLAG (Sigma). A second rabbit polyclonal antibody to CP110 and rabbit polyclonal antibodies against Cyclin A, Cyclin B, and the PSTAIRE peptide were previously described[10,33,34]. The following mouse monoclonal antibodies were used: Cyclin F (clone 2123D1a, ab50811; Abcam), α-tubulin (T5168; Sigma), and γ-tubulin (T5326; Sigma).

Gene silencing by small interfering RNA. The sequences of the oligonucleotides #1, 2, and 3, corresponding to the Cyclin F mRNA were CCAGUUGUGUGCUGCAUUA, UAGCCUACCUCUACAAUGA , and GCACCCGGUUUAUCAGUAA , respectively. The sequence of the CP100 siRNA has been previously reported[13]. A dsRNA oligo to LacZ mRNA (CGUACGCGGAAUACUUCGA) served as a negative control[25].

In vitro ubyquitylation assay. FLAG-tagged Cyclin F wild type or FLAG-tagged Cyclin F(LP/AA) were transfected into HEK-293T cells. Twenty-four hours after transfection, cells were incubated with MG132 for three hours, prior to lysis. Anti-Flag M2 agarose beads were used to immunoprecipitate the $SCF^{Cyclin\ F}$ complex. The beads were washed four times in lysis buffer and two times in ubiquitylation reaction buffer (URB: 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM MgCl2, and 1 mM DTT). Beads were then used for *in vitro* ubiquitylation assays, which were performed in a volume of 30 µl, containing 2 mM ATP, 5 µM E1 (Boston Biochem), 10 ng/µl Ubch3, 10 ng/µl Ubch5c, 1 µM ubiquitin aldehyde and 2.5 µg/µl ubiquitin (Sigma). The reactions were incubated at 30 °C for two hours and analyzed by immunoblotting with antibodies to CP110.

Statistical analyses. All data represented the average from at least three independent experiments, with at least 100 cells counted per experiment. Significance were calculated by ANOVA (Fig. 3a, 3b, 4b, Supplementary Fig. 7, and Supplementary Fig. 8) or double-tailed t-test (Fig. 4d, Supplementary Fig. 9) using a GraphPad Prism software. Differences were considered significant when p was inferior to 0.05.

Supplementary References

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