Supplemental Data

SCFβTrCP-Mediated Degradation of Claspin Regulates Recovery from the DNA Replication Checkpoint Response

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Supplemental Experimental Procedures

Plasmids
The N-terminal and C-terminal fragments of Claspin were separately amplified by RT-PCR using a cDNA library generated from HEK293T cells. The two RT-PCR products were sequentially inserted into pcDNA3.1-HA tag to obtain a plasmid containing full-length Claspin. Claspin mutants were generated using the QuickChange Site-directed Mutagenesis kit (Stratagene). For retrovirus production, both wild-type Claspin and Claspin mutants were subcloned into the retroviral vector LZRSpBMN by PCR. All cDNAs were sequenced. Plk1 constructs (wild type, K82R and T210D) were kindly provided by Prasad Jallepalli (MSKCC).

Antibodies
A mouse monoclonal antibody against Claspin was a made using a GST-Claspin fusion protein (amino acids 785-1056 of human Claspin) as the antigen. The phosphosite-specific antibody was generated by injecting rabbits with the
phosphopeptide DSPDS*GQGSYET, corresponding to amino acids 25-37 of human Claspin with a phosphoserine at position 30 (S*). The antibody was then purified from serum using protein A-Sepharose and subsequently by two rounds of affinity chromatography using both phospho- and nonphosphopeptide chromatography. Mouse monoclonal antibodies were from Zymed/Invitrogen (βTrCP1, Cul1, Plk1 and Skp1), Sigma (anti-FLAG), Santa Cruz Biotechnology (Chk1, p53 and cdc25A) and Covance (anti-HA). Rabbit polyclonal antibodies were from Zymed/Invitrogen (βTrCP1), Upstate (phospho-Ser10 Histone H3), Santa Cruz Biotechnology (Wee1, phospho-Tyr15 Cdk1) and Cell Signaling (phospho-Ser317 Chk1 and phospho-Ser15 p53). Rabbit polyclonal antibodies against Cdk1 (Carrano et al., 1999), cyclin A (Carrano and Pagano, 2001) and Wee1 (Watanabe et al., 2004) and the mouse monoclonal antibody to cyclin E (Faha et al., 1993) were previously described.

**Purification of βTrCP2 interactors**

HA-βTrCP2 was transfected into HEK293T cells using the calcium phosphate method (Bashir et al., 2004). Forty-eight hours later, cells were collected (after adding MG132 for the last 6 hours in culture) and lysed in lysis buffer (LB: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 0.5% Triton X-100, plus protease and phosphatase inhibitors). HA-βTrCP2 was immunoprecipitated with anti-HA affinity gel (Roche) and the immunocomplexes were washed 5 times with LB (15 minutes each). After washing, the HA-beads were incubated at 30°C for 2 hours with gentle agitation
in the presence of the following components: 50 mM Tris-HCl pH 7.5, 5 mM MgCl$_2$, 200 µM okadaic acid, 2 mM ATP, 0.6 mM DTT, 1 mM ubiquitin aldehyde, E1, Ubc3, Ubc5 and FLAG-Ubiquitin. HA-βTrCP2 and interacting proteins were eluted twice by competition with an HA peptide. FLAG-polyubiquitylated products generated in the ubiquitylation reaction were subsequently subjected to a second immunoprecipitation with anti-FLAG M2 affinity gel (Sigma) and then eluted by competition with a FLAG-peptide. The eluate was digested with trypsin and directly analyzed by liquid chromatography-tandem mass spectrometry.

**In vitro ubiquitylation assay**

Ubiquitylation and degradation assays were previously described (Bloom et al., 2003). Briefly, Claspin ubiquitylation was performed in a volume of 10 µl containing 50 mM Tris pH 7.6, 5 mM MgCl$_2$, 0.6 mM DTT, 2 mM ATP, 2 µl in vitro transcribed/translated unlabelled βTrCP1, 1.5 ng/µl E1 (Boston Biochem), 10 ng/µl Ubc3, 10 ng/µl Ubc5, 2.5 µg/µl ubiquitin (Sigma), 1 µM ubiquitin aldehyde, 160 nM Plk1 (Invitrogen), 1 µl $^{35}$S-methionine-labelled in vitro transcribed/translated Claspin NT as substrate. Virtually identical results were obtained using full-length Claspin with the exception that, due to the large size of this protein, the resolution of phosphorylated and ubiquitylated forms was less evident. Where indicated 2.5 µg/µl methylubiquitin (Boston Biochem), 2 µl in vitro transcribed/translated unlabelled Fbw5, 250 nM Cdk1-cyclin B (Invitrogen) or lower concentrations of Plk1 were used. The reactions were
incubated at 30 °C for the indicated times and analyzed by SDS-PAGE and autoradiography.

Supplemental References

Figure S1. High levels of Claspin in human fibroblasts immortalized with SV40 Large T antigen are still subjected to degradation in G2

Uninfected IMR-90 normal human fibroblasts (NHF) and IMR-90 cells retrovirally infected with SV40 Large T antigen (NHF + LT) were synchronized at G1/S using aphidicoline (APH) for 20 hours (indicated as time 0). Cells were subsequently washed and allowed to progress through the cell cycle for the indicated times. Protein extracts were analyzed by immunoblotting with antibodies to the indicated proteins.