CRL4-DDB1-VPRBP ubiquitin ligase mediates the stress triggered proteolysis of Mcm10

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Supplementary Materials and Methods

Cell culture, antibodies and western blotting. Human HeLa and 293T were grown at 37°C in DMEM supplemented with 10% fetal bovine serum. Caffeine and UCN-01 were purchased from Sigma and CGK733 was obtained from Calbiochem. UV irradiation was done using a UV crosslinker CL-1000 from UVP. Anti-human Mcm10 antibody was produced using recombinant His-tagged Mcm10 (cloned in pET28a vector), purified on nickel-NTA column (Qiagen). Rabbit were injected with recombinant protein along with complete Freund's adjuvant to obtain Mcm10 Ab. Anti-Mcm10 sera was affinity-purified using Mcm10 conjugated sepharose column. Mouse monoclonal anti-cyclin E, rabbit polyclonal anti-SKP2, rabbit polyclonal anti-cyclin B1 were purchased from Santa Cruz Biotechnology. Mouse monoclonal anti-PCNA was purchased from Cell Signaling Technology. Rabbit polyclonal anti-ROC1, rabbit polyclonal anti-DDB2, rabbit polyclonal anti-CDT2, rabbit polyclonal anti-CDT1, rabbit polyclonal anti-VprBP, rabbit monoclonal anti-cullin4a, mouse monoclonal anti-CDH1 and mouse monoclonal anti-Cdc20 were purchased from Abcam. Rabbit polyclonal anti-DDB1 was obtained from Gene Tex and Abcam. Mouse monoclonal anti-cullin1 was purchased from BD Pharmingen. Mouse monoclonal anti-HA and mouse monoclonal anti-myc were obtained from Sigma. Polyclonal goat anti-rabbit HRP and polyclonal rabbit anti-mouse HRP were obtained from Dako. Avidin D, Peroxidase labeled (Av-HRP), Concentrate was obtained from Vector Labs. For western blotting, cells were harvested in 1X SDS sample buffer. Equal amount of protein was separated on SDS-polyacrylamide gels and then transferred onto nitrocellulose membranes. Finally the results were assayed using the Enhanced Chemiluminescence method.

Cell cycle synchronization and BrdU labeling. Synchronization of specific RNAi depleted HeLa cells at mitotic phase was done using 9μM of RO-3306. Cells were blocked in RO-3306 for 18 h respectively and later the synchronized cells were obtained after a mitotic shake-off. After 3-4 washes with 1XPBS, the synchronized cells were released into drug-free medium followed by harvesting at different time-points. pMX-puro retroviral vector used to express NTD+ID and CTD domains of Mcm10 in U2OS cells has been described previously. 2 h after UV irradiation, the cells were harvested for evaluating the levels of the respective domains by anti-HA immunoblotting. 16-24 h post UV irradiation, HeLa cells were incubated with 100 μM BrdU followed by fixing with 4% formaldehyde, treatment with 2 M HCl and neutralization with 0.1 M sodium borate buffer (pH 8.5). The fixed cells were permeabilized with 0.2% Triton X-100, blocked with 3% BSA and incubated with a FITC-conjugated BrdU antibody.

RNA extraction and reverse-transcriptase PCR. For RNA extraction, the cell pellet obtained from the siRNA transfected cells was resuspended in TRIzol reagent (Invitrogen, Cat. No. 15596-018) followed by chloroform addition. The RNA from aqueous layer was precipitated using isopropanol and finally resuspended in RNase free water after 70% ethanol washes. For RT-PCR, RNA was quantified using NanoDrop spectrophotometer (NanoDrop Technologies, ND-1000). cDNA synthesis was carried out using 0. 25-1 μg RNA, 10 μM oligo dT_{20} primer, 1 mM dNTPs, 5X Mu-MLV reverse transcriptase buffer, RNase inhibitor (RNasin, Promega) and Mu-MLV reverse transcriptase enzyme (200 U/μl, Fermentas). The sequences of the primers used for PCR were as follows:
BETA-2 MICROGLOBULIN, forward primer: 5′GTTGACTTACTGAAGAATGGAGAGA3′,
reverse primer: 5′TCAATATTAAAAAGCAAGCAAGCAG3′,
beta-TRCP, forward primer: 5′ TGTGCCAGACTCTGCTTAAACCAA 3′,
reverse primer: 5′ TTGATCTGACTCTGACCACTGCT 3′,
CDT2, forward primer: 5′GAACCCATAGCATCCAAGTCTTTCC3′,

reverse primer: 5′GGAGAAGTCTTCAACCCAGTCATAT3′,
CUL1, forward primer: 5′GCTTGCATTGGTGACTTGGAGAGAC3′,
reverse primer: 5′GCCCCAATTCCACGTAAGACTGTAC 3′,
CDC20, forward primer: 5′ATCTCAGGCCATGGCTTTGC3′,
reverse primer: 5′GGTCCAACTCAAAACAGCGC3′,
CDH1, forward primer: 5′ AGTCAGAACCGGAAAGCCAA3′,
reverse primer: 5′ ACACATCGTTGCCGTCATCG3′,
CUL4A, forward primer: 5′ACAGCGATCGTAATCAATCCTGAGA3′,
reverse primer: 5′GGATAAACCTGAACAGGATCATGAT3′,
CUL4B, forward primer: 5′CTAAAGACATCATGATTCAGTTCAA3′,
reverse primer: 5′ACTATGTTTGCCTAGGTAAAATGTC3′.
DDB2, forward primer: 5′TCTGCTAGTAGCCGAATGGTGGTCA3′,
reverse primer: 5′TGTGCGGCAGCGAGTAGAGGAAG3′,
FBXW7, forward primer: 5′TGACGATTTTGATCAGTCTGA3′,
reverse primer: 5′ATGTAATTCGGCGTCGTTGTT3′,
MCM10, forward primer: 5′GAGAGAACAACTTGCCTATCTGGAA3′
reverse primer: 5′GACACGGCACTTCACTTCTCTGATG3′,
ROC1, 5′CTCTGGGCCTGGGATATTGTGGTTG3′,
reverse primer: 5′TTTTGAGCCAGCGAGAGATGCAGTG3′,

Construction of plasmids. Construction of pET28a-Mcm10 has been described previously. For construction of full-length Mcm10, Mcm10 was amplified from cDNA and then cloned in pCDNA3-HA vector. Full-length DDB1, Cul4a, Roc1, ubiquitin and VprBP were also amplified from cDNA and then cloned in pCDNA3-HA vector. Subsequently, respective genes were subcloned into other vectors. The primers used for cloning are as follows:

Cloning primers for **pCDNA3-HA-Mcm10:**

Full-length Mcm10 (1-875)
Forward primer:
5′GATCAGATATCGCATGGATGAGGAGGAAGACAATCTG 3′
Reverse primer:
5′ATAGTTTAGCGGCCGCTTATTTAAGGCTGTTCAGAAATTTAGC 3′

Fragment 1-240aa (NTD domain) with NLS
Forward primer:
5′GATCGATATCGCCCAAAAAAGAAGAGAAAGGTACAATTCATGGATGAGGAGGAAGACAA
TCTG 3′
Reverse primer:
5′ATAGTTTAGCGGCCGCTTATTCCCCAGAACTTCCTGGGGT 3′

Fragment 1-430aa (NTD +ID domain) with NLS
Forward primer:
5′GATCGATATCGCCCAAAAAAGAAGAGAAAGGTACAATTCATGGATGAGGAGGAAGACAA
TCTG 3′
Reverse primer:
5′ATAGTTTAGCGGCCGCTTACAGATCCGCACGCTTTGCGCT 3′

Fragment 240-430aa (ID domain) with NLS
Forward primer:

5′GATCGATATCGCCCAAAAAAGAAGAGAAAGGTACAATTCGAAACGACTCAACCCATCTGT
GTG 3′
Reverse primer:
5′ATAGTTTAGCGGCCGCTTACAGATCCGCACGCTTTGCGCT 3′

Fragment 607-875aa (CTD domain) with NLS
Forward primer:
5′GATCGATATCGCCCAAAAAAGAAGAGAAAGGTACAATTCCCTCCACGGACAGGATCCGAG
TTC 3′
Reverse primer:
5′ATAGTTTAGCGGCCGCTTATTTAAGGCTGTTCAGAAATTTAGC 3′

Cloning primers for **N6-myc pCDNA3 Mcm10:**
Forward primer:
5′GAATTCGATATCATGGATGAGGAGGAAGACAATCTG3′
Reverse primer:
5′ATAGTTTAGCGGCCGCTTATTTAAGGCTGTTCAGAAATTTAGC3′

Cloning primers for **pCDNA3-HA-DDB1:**
Forward primer:
5′GATCAGATATCGCATGTCGTACAACTACGTGGTAACGGC3′
Reverse primer:
5′ATAGTTTAGCGGCCGCCTAATGGATCCGAGTTAGCTC3′

Cloning primers for **N6-myc pCDNA3 DDB1:**
Forward primer:
5’GAATTCGATATCATGTCGTACAACTACGTGGTAACGGC 3’
Reverse primer:
5’ATAGTTTAGCGGCCGCCTAATGGATCCGAGTTAGCTC 3’

Cloning primers for **pCDNA3-HA-Cul4a:**
Forward primer:
5′GATCAGATATCGCATGGCGGACGAGGCCCCG3′
Reverse primer:
5′ATAGTTTAGCGGCCGCTCAGGCCACGTAGTGGTACTG3′

Cloning primers for **N6-myc pCDNA3 Cul4a:**
Forward primer:
5’GAATTCGATATCATGGCGGACGAGGCCCCG 3’
Reverse primer:
5’ATAGTTTAGCGGCCGCTCAGGCCACGTAGTGGTACTG 3’

Cloning primers for **pCDNA3-HA-Roc1:**
Forward primer:
5′GATCAGATATCGCATGGCGGCAGCGATGGATGTG3′
Reverse primer:
5′ATAGTTTAGCGGCCGCCTAGTGCCCATACTTTTGGAAT3′

Cloning primers for **N6-myc pCDNA3 Roc1:**
Forward primer:
5’GAATTCGATATCATGGCGGCAGCGATGGATGTG 3’

Reverse primer:
5'ATAGTTTAGCGGCCGCCTAGTGCCCATACTTTTGGAAT 3'

Cloning primers for **pCDNA3-HA-Ubiquitin:**
Forward primer:
5′GATCAGATATCGCATGCAGATCTTCGTGAAAACCCTT3′
Reverse primer:
5′ATAGTTTAGCGGCCGCCTAACCACCTCTCAGACGCAGGACCAG3′

Cloning primers for **pCDNA3-HA-VprBP (1093-1311):**
Forward primer:
5' GATCGATATCGCGAGAGTGGCTTCACCTGCTGT 3'
Reverse primer:
5' ATAGTTTAGCGGCCGCAGCTCCATACATCACTGT 3'

Cloning primers for **N6-myc pCDNA3 VprBP:**
Forward primer:
5′CCGGAATTCATGACTACAGTAGTGGTACAT3′
Reverse primer:
5′ATAGTTTAGCGGCCGCTCACTCATTCAGAGATAAGATGAT3′

Cloning primers for **C-Terminus VprBP Fragment (864 to 1507aa)**
Forward primer:
5′CCGGAATTCGCGGAGAAACAGCAACCGTGCT3′
Reverse primer:
5′ATAGTTTAGCGGCCGCTCACTCATTCAGAGATAAGATGAT3′

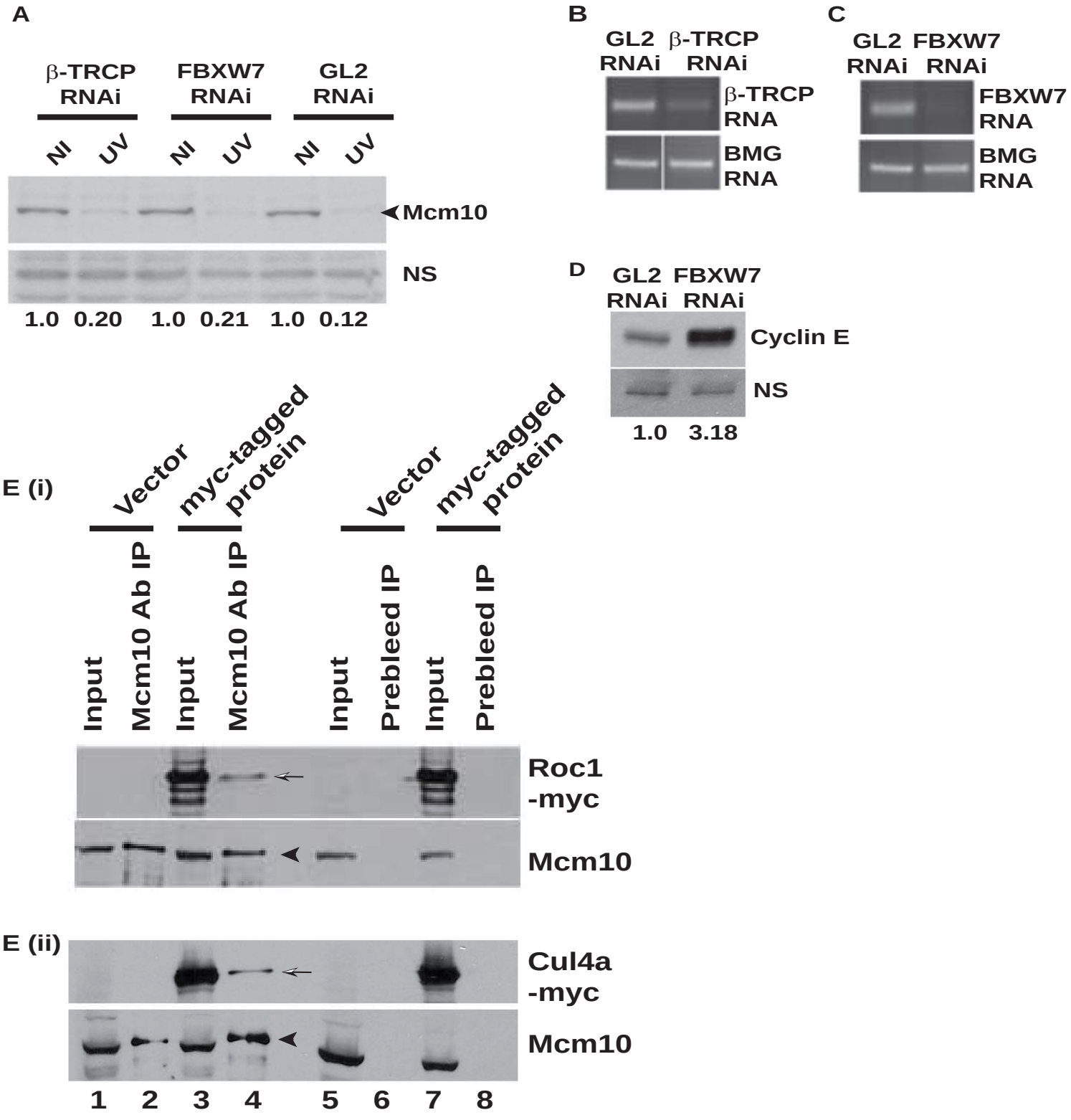
Figure Legends

Figure S1. (A-D) HeLa cells were transfected with *beta-TRCP*, *FBXW7* or control *GL2* siRNA and as described in Figure 2, the levels of Mcm10 protein were evaluated. The levels of mRNA (B-C) of the genes silenced by RNAi in part (A) were determined. Immunoblot in part (D) displays cyclin E stabilization after *FBXW7* RNAi. HeLa cells were transfected with *FBXW7* or control *GL2* siRNA and the levels of cyclin E protein were evaluated. The numbers indicate the levels of cyclin E protein following *FBXW7* siRNA depletion relative to control *GL2* transfected cells. **(E)** Mcm10 physically interacts with exogenously expressed Roc1 and Cul4a. 293T cells were transfected with pcDNA3 vector, pcDNA3-myc-Roc1 or pcDNA3-myc-Cul4a, lysed under mild conditions and immunoprecipitation was carried out with either anti-Mcm10 antibody or preimmune serum. Mcm10 band has been indicated by black arrowhead in the second and fourth panels (lane 4) while co-immunoprecipitation of Roc1-myc (part i) and Cul4a-myc (part ii) was evaluated by anti-myc antibody (indicated by shaded arrow in the first and third panels, lane 4).

Figure S2. Requirement of Roc1, Cul4, DDB1 and VprBP for UV-triggered Mcm10 degradation is confirmed by siRNA duplex targeting different regions of the target genes. **(A), (B), (D) and (F)** HeLa cells were transfected on three consecutive days with different *DDB1*, *ROC1*, *CUL4A+B* or *VPRBP* siRNA, targeting a different region than the siRNA duplex used in Figure 3 and 6. The new oligos used were *DDB1 (2)*, *ROC1 (2)*, *CUL4A (2)*, *CUL4B (2)* and *VPRB(2)* siRNA. 24 h after the last transfection, the cells were exposed to UV, harvested 4 h later and Mcm10 levels (black arrowhead) were analyzed in non-irradiated (NI) and UV-irradiated (UV) cells. The numbers in panel (A), (B), (D) and (F) indicate the levels of Mcm10 protein in UV-irradiated cells relative to non-irradiated cells after specific siRNA transfections. The decrease in the levels of DDB1 and VprBP proteins was confirmed by immunoblotting (A and F) while the decrease of *ROC1* (C), *CUL4A* and *CUL4B* (E) mRNA levels was confirmed by RT-PCR. **(G)** Cell cycle distribution was determined by flow cytometry of propidium iodide-stained DNA of VprBP depleted HeLa cells. The inset shows the percentage of total cells that are present in different phases. NS points to a non-specific band that displays equal protein loading in different lanes while β-2 microglobulin (BMG) serves as the internal RNA loading control.

Figure S3. (A) HeLa cells were transfected with either *GL2* or *DDB1* siRNA for three consecutive days and after the third transfection, they were incubated with DMSO or 9 μM RO-3306 for 18 h. Subsequently, the cells were exposed to UV, harvested 4 h later and Mcm10 levels were analyzed in non-irradiated (NI) and UV-irradiated (UV) cells. The numbers in panel (A) indicate the levels of Mcm10 protein in UV-irradiated cells relative to non-irradiated cells after the specific treatment. NS points to a non-specific band that displays equal protein loading. **(B)** Cell cycle distribution was determined by flow cytometry of propidium iodide-stained DNA of HeLa cells, as described in (A).

Figure S1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FBXW7 RNAi
GL2 RNAi
NI
UV
NI
UV
NI
UV
Mcm10
NS
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B
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β-TRCP RNAi
β-TRCP RNA
BMG RNA
C
GL2 RNAi
FBXW7 RNAi
FBXW7 RNA
BMG RNA
D
GL2 RNAi
FBXW7 RNAi
Cyclin E
NS
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E (i)
Vector
myc-tagged protein
Vector
myc-tagged protein
Input
Mcm10 Ab IP
Input
Mcm10 Ab IP
Input
Prebleed IP
Input
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Mcm10
E (ii)
Cul4a -myc
Mcm10
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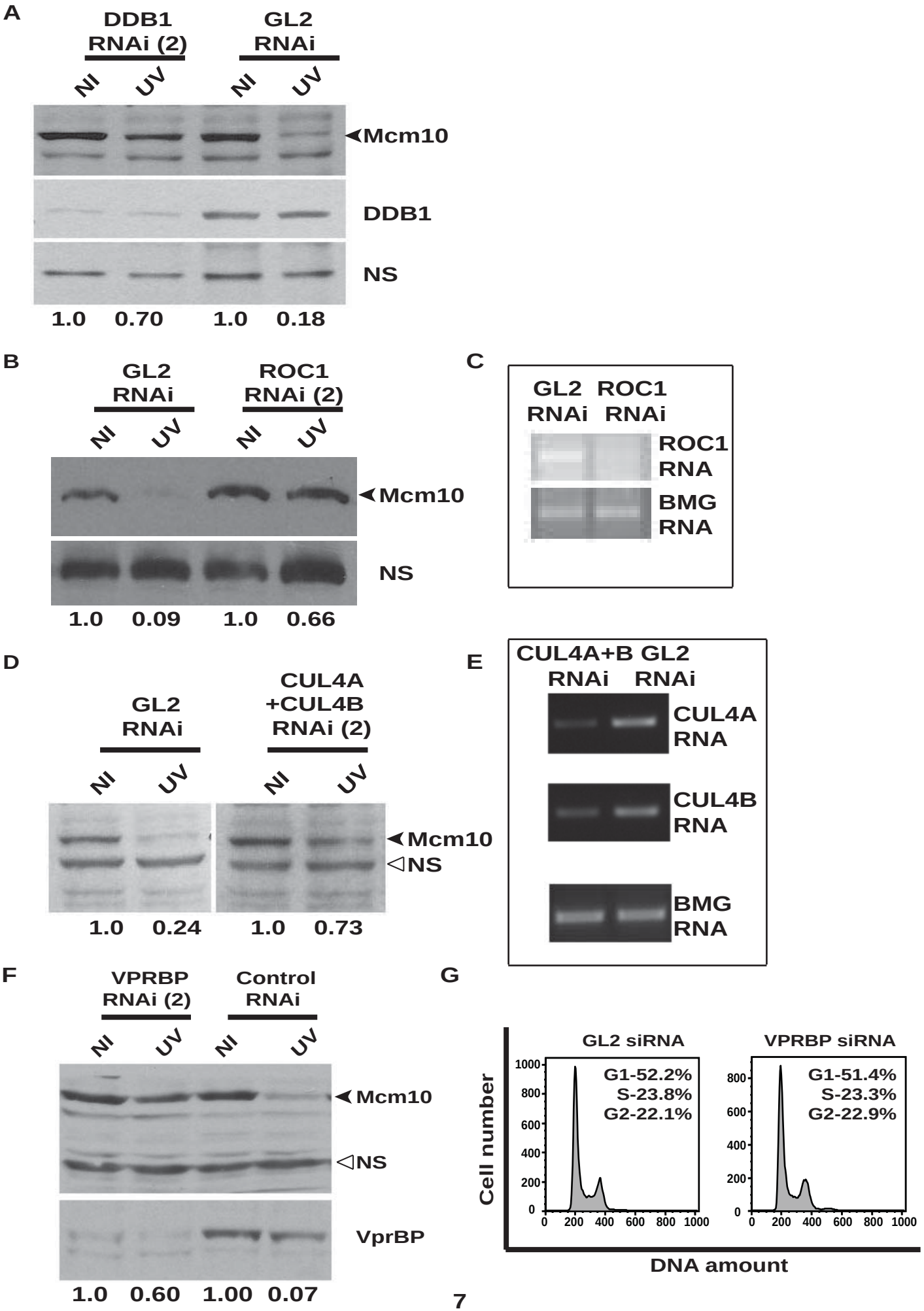
Figure S2


A
DDB1 RNAi (2)
GL2 RNAi
NI
UV
NI
UV
Mcm10
DDB1
NS
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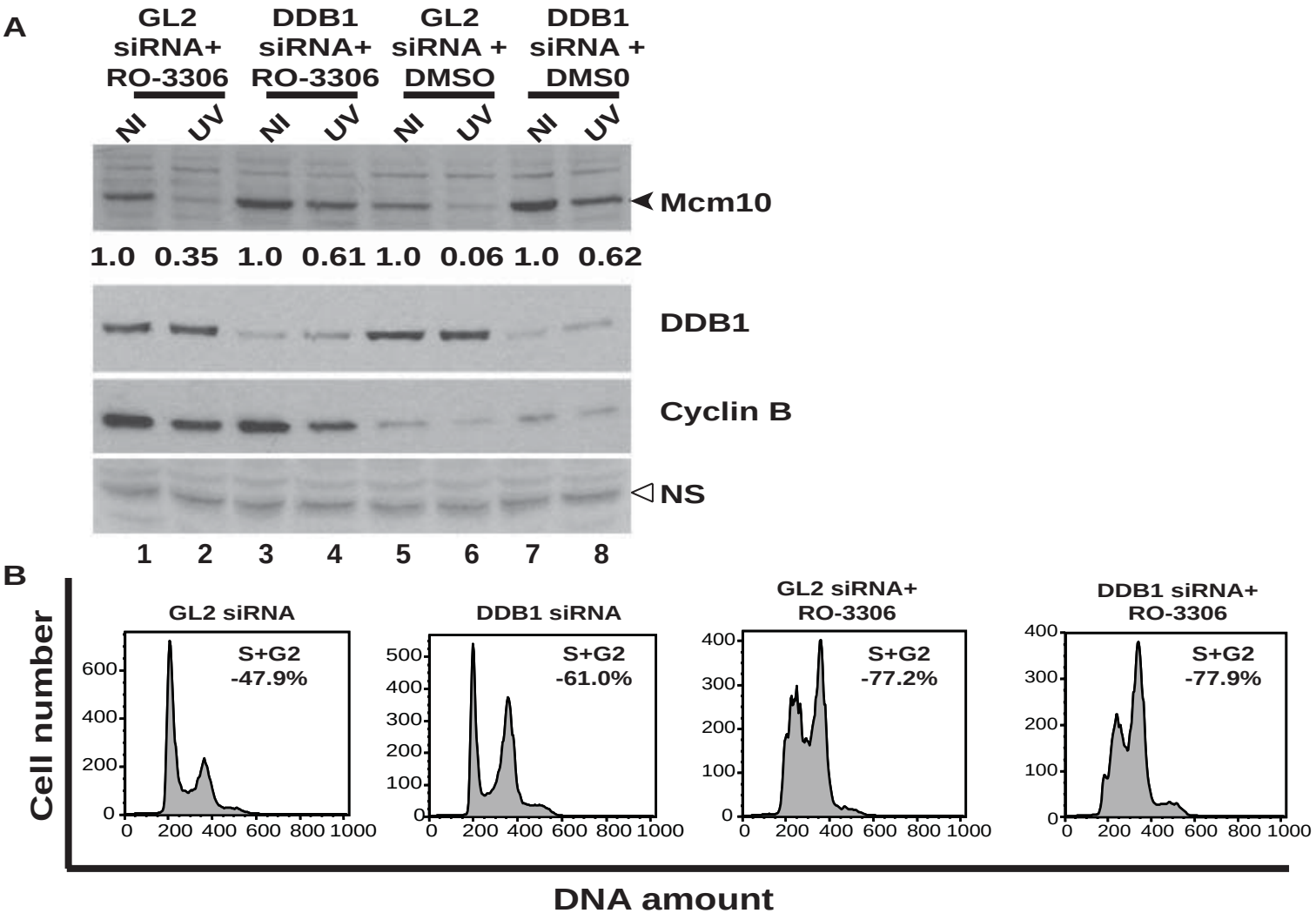
B
GL2 RNAi
ROC1 RNAi (2)
NI
UV
NI
UV
Mcm10
NS
1.0 0.09 1.0 0.66
C
GL2 RNAi
ROC1 RNAi
ROC1 RNA
BMG RNA
D
GL2 RNAi
CUL4A +CUL4B RNAi (2)
NI
UV
NI
UV
Mcm10
NS
1.0 0.24 1.0 0.73
E
CUL4A+B RNAi
GL2 RNAi
CUL4A RNA
CUL4B RNA
BMG RNA
F
VPRBP RNAi (2)
Control RNAi
NI
UV
NI
UV
Mcm10
NS
VprBP
1.0 0.60 1.00 0.07
G
GL2 siRNA
G1-52.2%
S-23.8%
G2-22.1%
VPRBP siRNA
G1-51.4%
S-23.3%
G2-22.9%
Cell number
DNA amount
0
200
400
600
800
1000

Figure S3


A
GL2 siRNA+ RO-3306
DDB1 siRNA+ RO-3306
GL2 siRNA + DMSO
DDB1 siRNA + DMS0
NI
UV
Mcm10
1.0 0.35 1.0 0.61 1.0 0.06 1.0 0.62
DDB1
Cyclin B
NS
1 2 3 4 5 6 7 8
B
Cell number
GL2 siRNA
S+G2 -47.9%
DDB1 siRNA
S+G2 -61.0%
GL2 siRNA+ RO-3306
S+G2 -77.2%
DDB1 siRNA+ RO-3306
S+G2 -77.9%
DNA amount