SUPPLEMENTAL INFORMATION

APC/C and SCF$^{Cyclin\ F}$ constitute a reciprocal feedback circuit controlling S-phase entry

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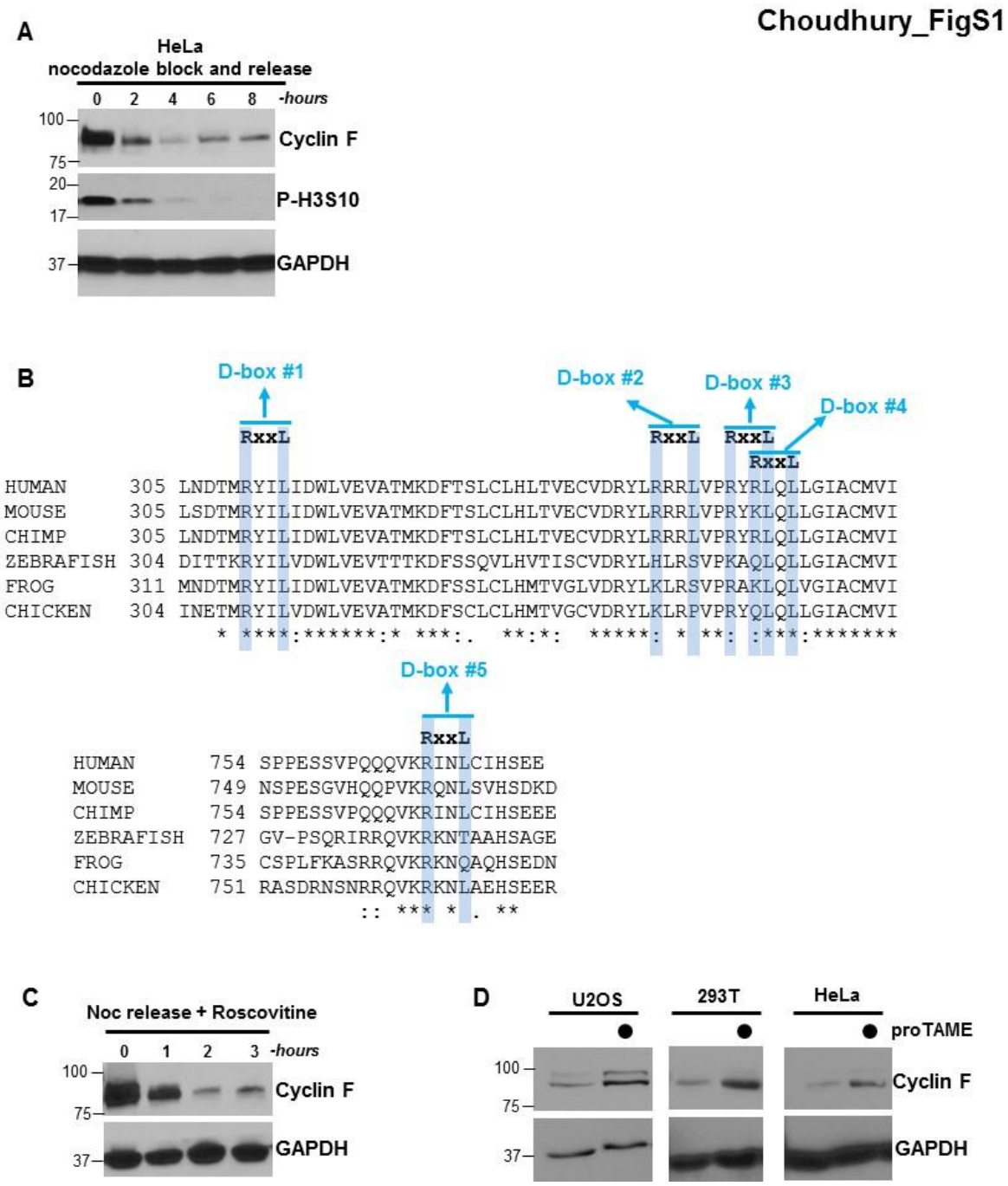
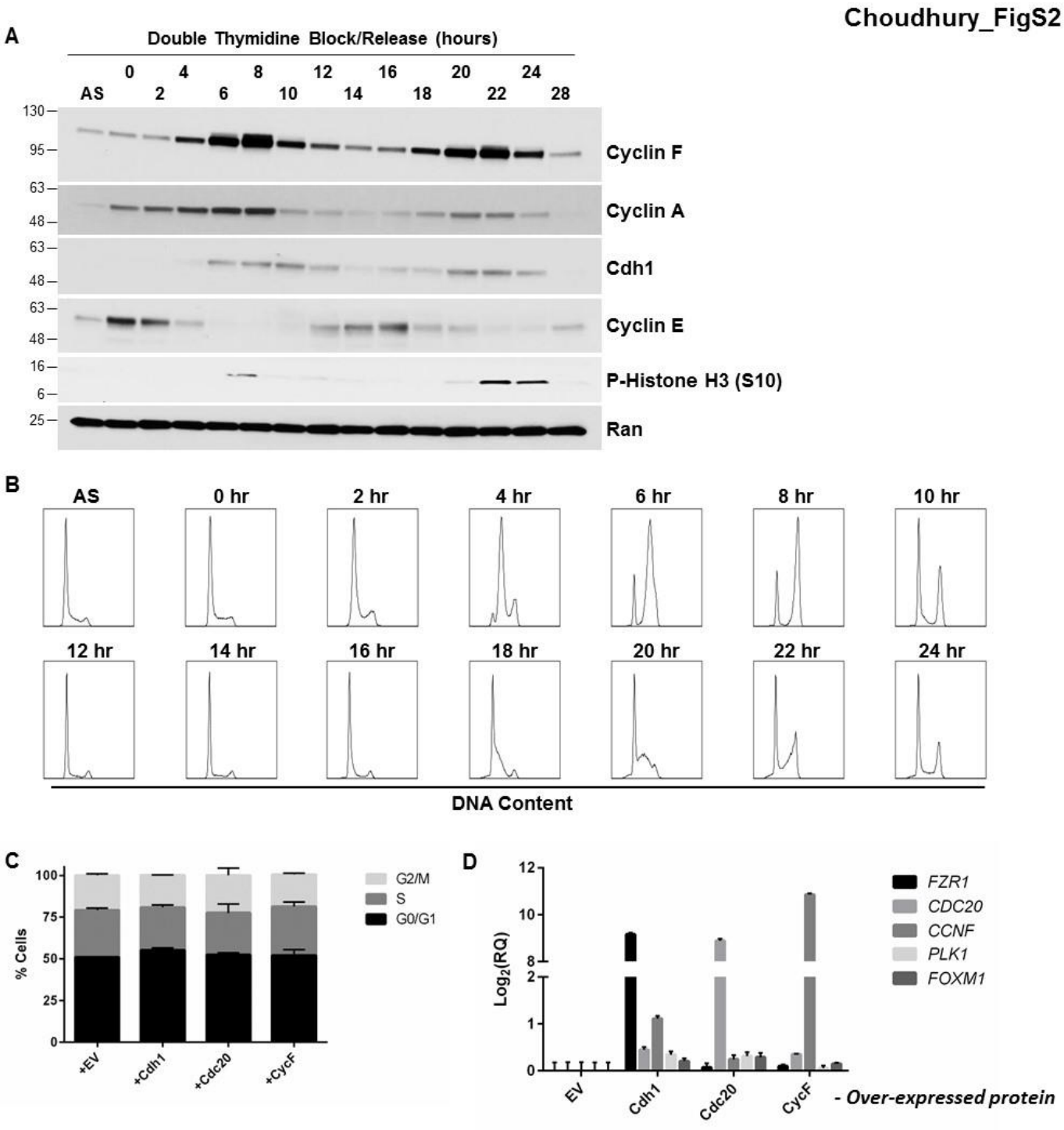
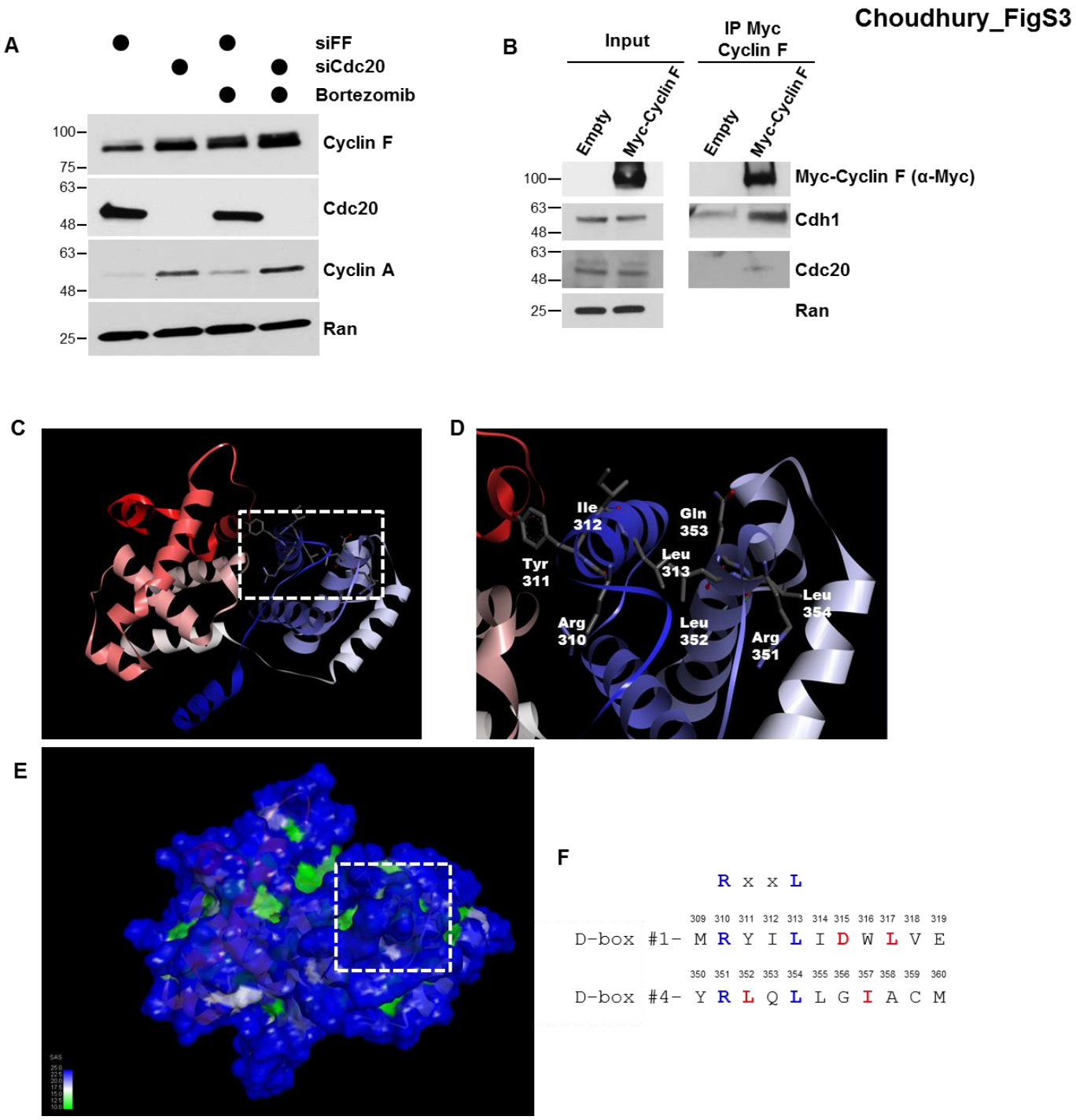
SUPPLEMENTAL FIGURES



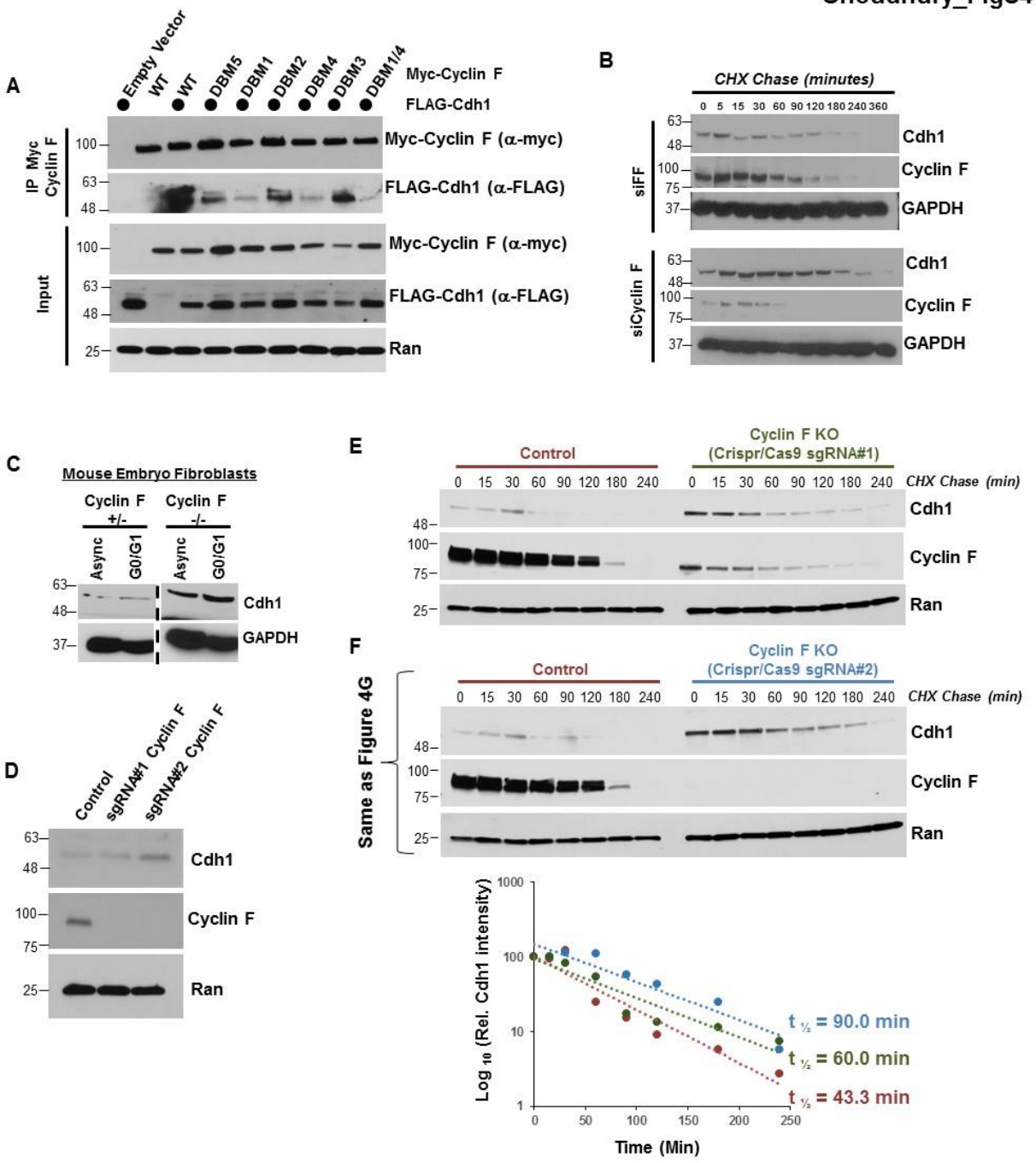
Supplemental Figure S1. Related to Figure 1. Cyclin F is degraded at mitotic exit, and in a cellular APC/C activation assay. (A) HeLa cells were synchronized in mitosis with nocodazole, released into the cell cycle and analyzed by immunoblot. (B) Alignment of Cyclin F sequences from human (*H.sapiens*), mouse (*M.musculus*), zebrafish (*D.rerio*), frog (*X.tropicalis*), chimp (*P.trogoldytes*) and chicken (*G.gallus*). Blue highlights indicate the positions of the putative D-box residues (RxxL). (C) Cyclin F abundance was evaluated in an APC/C activation assay. HeLa cells were arrested in nocodazole and treated with roscovitine to drive APC/C activation and mitotic exit. (D) U2OS, 293T and HeLa cells were treated with the APC/C inhibitor proTAME for 2.5 hours.



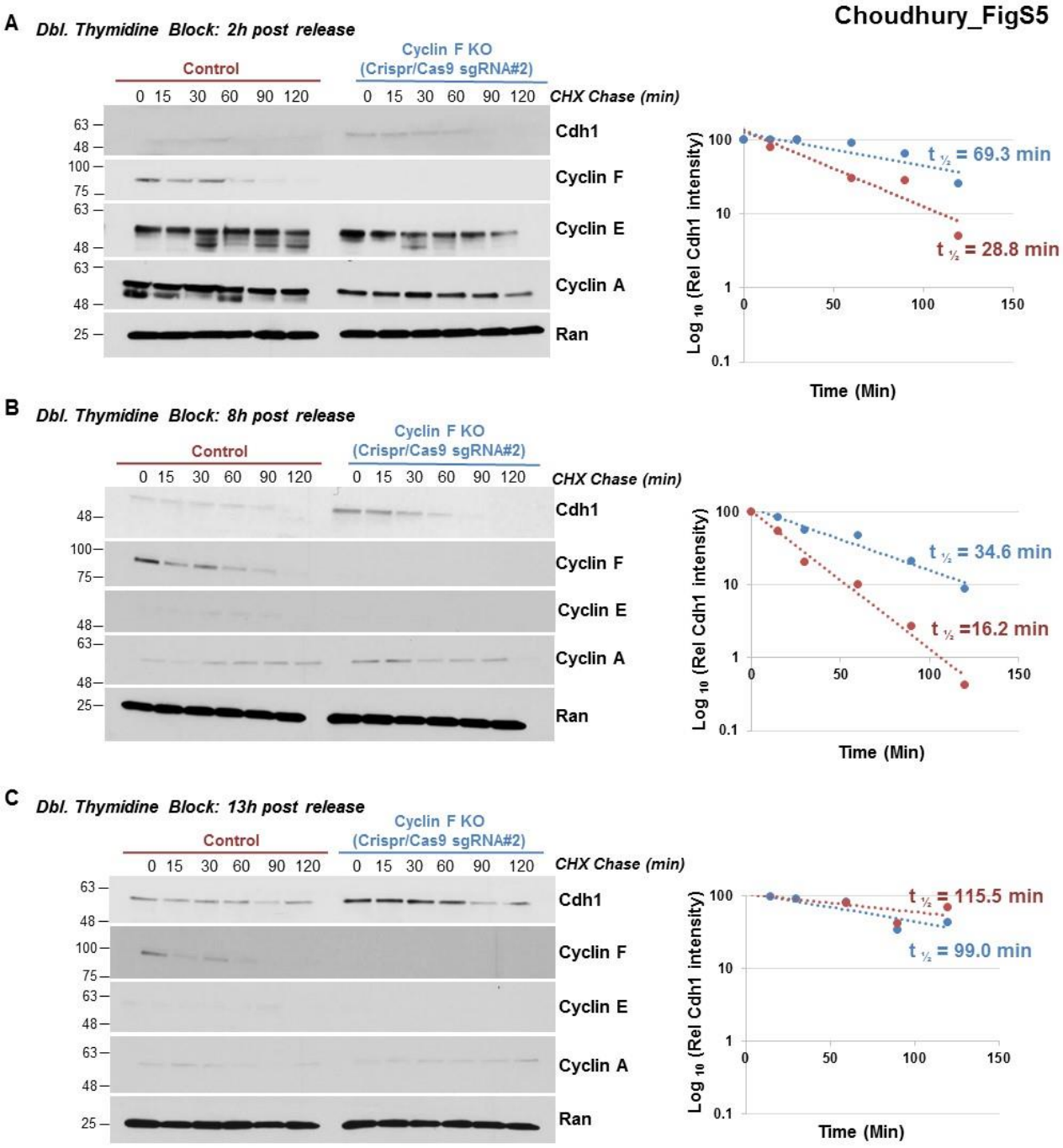
Supplemental Figure S2. Related to Figure 1. Cyclin F and Cdh1 regulation throughout the cell cycle. (A) HeLa cells were synchronized in early S-phase using double thymidine block and analyzed by immunoblot after release into the cell cycle. (B) Samples from the same experiment stained with propidium iodide and analyzed by flow cytometry. (C) Quantification of cell cycle profiles after Cdh1, Cyclin F and Cdc20 overexpression. Following transfection of 293T cells with genes shown on x-axis, cell cycle analysis was performed using propidium iodide staining and flow cytometry. Experiments was performed in triplicates. Error bars represent standard deviation. (D) Quantitative real-time PCR (q-RT-PCR) analysis of Fzr1/Cdh1, Cdc20, Cyclin F (CCNF), Plk1 and FoxM1 mRNA after overexpression of proteins shown on x-axis. q-RT-PCR was performed in triplicates. Error bars represent standard deviation.



Supplemental Figure S3. Related to Figure 3. Cyclin F protein levels are regulated by Cdc20 as well as Cdh1, and Cyclin F contains exposed D-box sequences on its surface. (A) U2OS cells were transfected with control (siFF) or Cdc20 siRNA for 24 hours and cells were either treated with DMSO or 200 nM bortezomib for another 4h prior to harvesting. (B) Myc-Cyclin F was expressed in 293T cells, and anti-Myc precipitates were analyzed by immunoblot. Both Cdh1 as well as Cdc20 co-purify in this assay. (C) Homology modeling of Cyclin F (aa 279-540) was obtained using human Cyclin B1 as a template (PDB ID:4Y72). The modelled protein is colored N-(Blue) to C-termini (Red) and the two D box regions is depicted by dotted box. (D) A zoomed in view of the D-boxes 1 and 4 with all the residues labelled. (E) Solvent accessibility surface (SAS) on Cyclin F protein indicate that the D-box region is exposed and can interact with solvent. D-box region is marked as in C. (F) Amino acid sequences of the two D-boxes and their corresponding positions are shown. Red indicates preferred amino acids.



Supplemental Figure S4. Related to Figure 4. Cdh1 stability throughout the cell cycle. **Cyclin F controls the stability and abundance of Cdh1.** (A) The conserved amino acids in the five putative D-boxes in Cyclin F were changed (RxxL→AxxA), alone and in combination. Binding to Cdh1 was measured by coIP following transient transfection of FLAG-Cdh1 and Myc-Cyclin F into 293T cells. Prior to lysis cells were treated with MG132. (B) Cyclin F and control (FF) siRNA were transfected into HeLa cells. After 48 hours cells were treated with cycloheximide, and time points were taken as indicated. Depletion of Cyclin F increased the half-life of Cdh1. (C) Cdh1 levels are increased in both asynchronous and synchronized (serum starved) Cyclin F null MEFs, compared to WT MEFs (note that blots separated by dotted line are taken from the same film). (D) Level of Cyclin F and Cdh1 in Crispr/Cas9 knockout cell lines. (E and F) Crispr/Cas9 technology was used to delete Cyclin F in HeLa cells. The half-life of Cdh1 is longest in the complete Cyclin F knock-out cell line (sgRNA#2, panel F), and shortest in control cells (panel E and panel F, left). In the cell line with residual Cyclin F (sgRNA#1, panel E), Cdh1 has an intermediate half-life. Graphs below immunoblots represent Cdh1 decay in control and Cyclin F knockout cells. Colors correspond to following cell lines: red (control), green (sgRNA#1), and blue (sgRNA#2).



Supplemental Figure S5. Related to Figure 4. Cdh1 stability throughout the cell cycle. Control and Cyclin F knockout HeLa cells were synchronized using double thymidine block and release for the indicated time. Cycloheximide (100 µg/ml) was added at 2h (A), 8h (B), and 13h (C) after release from thymidine and chase was performed for 2 hours. Cyclin F knock-out cell line (sgRNA#2) was used. Cyclin F knockout effects Cdh1 half-life at 2 and 8 hours after release, approximately in S and G2 phase, respectively. The half-life 13 hours after release, when cells are in the subsequent G1, is unaffected by Cyclin F knockout. Graphs at right represent Cdh1 half-lives in control and Cyclin F knockout cells. Red and blue coloring corresponds to control and Cyclin F knockout cells, respectively.

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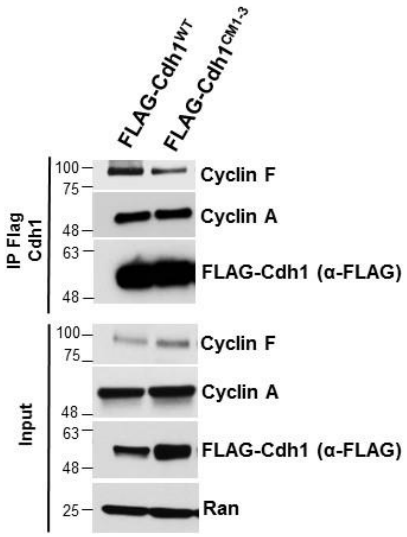
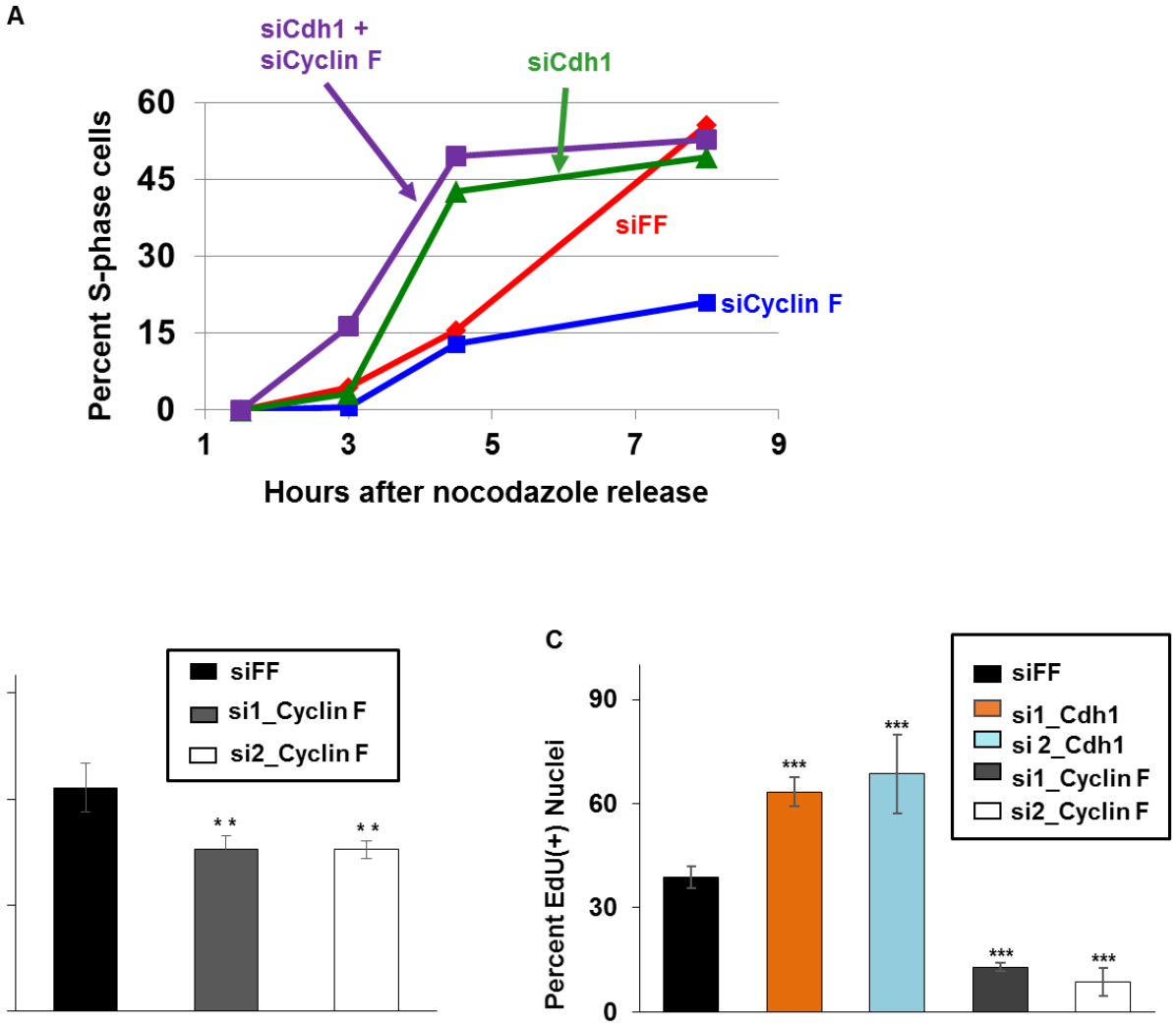


Supplemental Figure 6. Related to Figure 5. Cdh1 mutants that cannot bind Cyclin F are still competent to bind Cyclin A. Ectopically expressed Cdh1 (WT) and Cdh1 (Cy Mut1-3) were introduced into cells and purified on FLAG agarose. Cells were treated with MG132 prior to lysis and IP.



Supplemental Figure S7. Related to Figure 6. Cyclin F and Cdh1 have an antagonistic relationship in controlling S-phase entry. (A) U2OS cells were depleted using siRNA and synchronized in mitosis with nocodazole. Following release into the cell cycle S-phase entry was monitored by measuring EdU incorporation by flow cytometry. (B) U2OS cells were synchronized in mitosis with nocodazole following depletion with siRNA targeting firefly luciferase (FF) and Cyclin F, using two independent siRNAs. After 6h release from nocodazole, cells were pulsed with EdU for 30 minutes, fixed, and analyzed for EdU incorporation. The percent of nuclei that are EdU positive is shown (performed in triplicates, ** $p \leq 0.001$) (C) HeLa S3 cells were synchronized in mitosis with nocodazole after treatment with intended siRNAs and analyzed as above (performed in triplicates, *** $p \leq 0.0005$). p values were calculated using un paired t-test. Error bar indicate SD of mean.

SUPPLEMENTAL MATERIALS AND METHODS

Yeast strains and immunoblots

Yeast strains, listed below were derived from W303a using standard yeast genetics. Human Cyclin F (pCB112; a gift from Dr. Stephen J. Elledge) was transformed into strain CMY2052-2A (*MATa bar1::hisG, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1, loxP-9MYC-CDH1-KanMX4*) and transformants were selected on SC-ura plates. An isogenic negative control for Cdh1 immunoblotting was also included (*MATa bar1::hisG can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1, CDH1-KanMX4*). Lithium acetate-dependent transformation of yeast was performed as described previously. YPD and SC-ura media were prepared as described. Cells were grown in YPD to mid-log at 23°C. Immunoblots were probed with mouse monoclonal anti-Myc antibody 9E10 (University of Virginia Hybridoma Facility) and mouse monoclonal anti-TUBA4A antibody (Sigma B-5-1-2) for tubulin loading controls.

Plasmid construction

Sequences for PCR primers are detailed in supplemental table 1. PCR amplification of DNA was performed using High fidelity DNA polymerase. The Cyclin F full length ORF, obtained from the human ORFeome collection, was subcloned using gateway recombination cloning into a pDEST N-Myc vector. The human Fzr1/Cdh1 cDNA was PCR amplified and subcloned into pCDH5 vector (SBI, Biotechnologies Inc.) using *Xba*I and *Not*I restriction enzyme sites. Truncation mutants of Cdh1 were created by PCR amplification. FLAG tagged Cdh1 and Myc tagged Cyclin F vectors were mutated using standard site directed mutation protocols.

Crispr/Cas9 gene knockout

CRISPR lentiviral constructs for Cyclin F were generated by annealing together primers for sgCCNF1 (F: 5'-CACCGGCGAGCGCGGCGATGGGGAG-3'; R: 5'-AAACCTCCCCATCGCCGCGCTCGCC-3') and sgCCNF2 (F: 5'-CCACCGAGCGCGGCGATGGGGAGCGG-3"; R: 5'-AAACCCGCTCCCCATCGCCGCGCTC-3') and cloning the products into lentiCRISPR v2 as previously described (Sanjana et al., 2014). lentiCRISPR v2 was a gift from Feng Zheng (Addgene plasmid #52961). Transduced HeLa cells were single cell cloned by serial dilution, and Cyclin F-null populations were identified by immunoblot.

Gene Silencing by Small Interfering RNA

All siRNA transfection experiments were performed using lipofectamine 2000 (Life Technologies). Two siRNA oligonucleotides targeting Cdh1 (#1: 5´CCACAGGAUUAACGAGAAU3´ and #2: 5´GCACGGAGACCGCUUCAUC 3´) were used at 30 nM concentration for 48h. RNAi depletion of Cyclin F (#1: 5´ UAGCCUACCUCUACAAUGAUU3´ and #2: 5´GCACCCGGUUUAUCAGUAAUU 3´) was performed at similar concentrations for 48h. siRNA targeting firefly luciferase (siFF; 5´CGUACGCGGAAUACUUCGAUU 3´) was used as a control.

Antibodies used in this study

The following antibodies, and the indicated concentrations, were used in this study. Anti- FLAG was purchased from Sigma (using to detect FLAG-Cdh1; 1:1000, M2 anti Flag Mouse antibody F-1804). The following antibodies were purchased from Santa Cruz Biotechnology: c-Myc (used to detect Myc-Cyclin F; sc-40, 1:5000), Cyclin A (sc-751; 1:5000), Cyclin F (sc-952; 1:1000), GAPDH (sc-25778; 1:5000), Ran (sc-271376; 1:5000), Tubulin (sc-32293; 1:5000) and Cdc27 (sc-9972; 1:5000). The following antibodies were purchased from Cell Signaling Technologies: phospho-Histone H3 (Cat # 3377; 1:1000) and Cyclin E (Cat # 4129S; 1:2000). HA-tagged proteins were detected using anti mouse mAb (1:2000 from Covance cat #16B12). Cyclin-B (ab 32053) and Cdh1/Frz1 (ab 3242; 1:500) antibodies were from Abcam. All antibodies were diluted in 5% nonfat dried milk [prepared in phosphate buffered saline, 0.05% tween-20 (PBST)] and incubated overnight at 4°C and detected using HRP conjugated secondary antibodies (Jackson Immuno Research Laboratories Inc; 1:5000 dilutions) following standard procedures.

Primers used in this study

No.	Primer name	Sequence (5 ´to 3´)	Explanation

1	ME151	CACTCTAGAgccaccAtggattacaaggatgacgatgacaagATGGACCAGGACTATGAGCG	Forward primer for cloning of cdh1 into pcDH vector
2	ME152	CACGCGGCCGCTTACCGGATCCTGGTGAAGAGGTTG	Reverse primer for cloning of cdh1 into pcDH vector
3	ME068	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGCCACCATGGGGAGCGGCGGCGTGGTCCACTGTAG	Forward primer for cloning of cyclin F into gateway entry vector
4	ME112	GGGGACCACTTTGTACAAGAAAGCTGGGTAttacagcctcacaaggcccaggttcatg	Reverse primer for cloning of cyclin F into gateway entry vector
5	ME72	CCCAGCAACAGGTGAAGGCCATCAACGCATGCATACACAGTGAGG	CCNF aa 767-770 mutant forward primer
6	ME073	CCTCACTGTGTATGCATGCGTTGATGGCCTTCACCTGTTGCTGGG	CCNF aa 767-770 mutant reverse primer
7	ME90	CTCAATGACACAATGGCGTACATTGCGATCGACTGGCTGGT	CCNF aa 310-313 mutant forward primer
8	ME91	ACCAGCCAGTCGATCGCAATGTACGCCATTGTGTCATTGAG	CCNF aa 310-313 mutant reverse primer
9	ME76	GTGGACCGGTACCTGGCAAGAAGAGCGGTGCCGCGGTAC	CCNF aa 343-346 mutant forward primer
10	ME77	GTACCGCGGCACCGCTCTTCTTGCCAGGTACCGGTCCAC	CCNF aa 343-346 mutant reverse primer
11	ME78	AGGAGGCTGGTGCCGGCATATAGAGCCCAGCTGCTGGGC	CCNF aa 349-352 mutant forward primer
12	ME79	GCCCAGCAGCTGGGCTCTATATGCCGGCACCAGCCTCCT	CCNF aa 349-352 mutant reverse primer
13	ME80	TGGTGCCGCGGTACGCATTAGCAGCACTGGGCATCGCCT	CCNF aa 351-354 mutant forward primer
14	ME081	AGGCGATGCCCAGTGCTGCTAATGCGTACCGCGGCACCA	CCNF aa 351-354 mutant reverse primer
15	ME244	GGACCAGGACTATGAGGCGGCCGCGGCTGCCCAGGCCGTCATCCAGAATG	Forward primer for mutation of first overlapping Cyc binding box of Cdh1 (RRLLRQL) (aa 7-13)
16	ME245	CATTCTGGATGACGGCCTGGGCAGCCGCGGCCGCCTCATAGTCCTGGTCC	Reverse primer for mutation of first overlapping Cyc box of Cdh1 (RRLLRQL) (aa7-13)
17	ME246	CCCAGCAAGCACGGAGACGCCTTCGCCCCCTCCAGAGCCG	Forward primer for mutation of second Cyclin binding box of Cdh1 (RFI) (aa 47-49)
18	ME247	CGGCTCTGGAGGGGGCGAAGGCGTCTCCGTGCTTGCTGGG	Reverse primer for mutation of second Cyclin binding box of Cdh1 (RFI) (aa 47-49)

19	ME248	CTGACCGGGCACTCCTACGCC GTGGCGTACCTGGCAATGTCC C	Forward primer for mutation of C-terminal Cyclin binding box of Cdh1 (RV L) (aa 445-447)
20	ME249	GGGACATTGCCAGGTACGCCA CGGCGTAGGAGTGCCCGGTCA G	Reverse primer for mutation of C-terminal Cyclin binding box of Cdh1 (RVL) (aa 445-447)
29	ME349	GGGGACAAGTTTGTACAAAAA AGCAGGCTTCGAAGGAGATAG AGCCACCATGGACCAGGACTA TGAGCG	Forward primer for cloning Cdh1 full length in pDEST vector
30	ME350	GGGG AC CAC TTT GTA CAA GAA AGCTGGGTATTACCGGATCCT GGTGAAGAGGTTG	Reverse primer with stop codon for cloning Cdh1 full length in pDEST vector
31	ME351	GGGGACAAGTTTGTACAAAAA AGCAGGCTTCGAAGGAGATAG AGCCACCATGGGTGCCGGCAT CGAGAAGGTG	Forward primer for cloning Cdh1 (101-496 aa) in pDEST vector
32	ME352	GGGGACAAGTTTGTACAAAAA AGCAGGCTTCGAAGGAGATAG AGCCACCATGCTCAGCGTGGG GCTAGGCAC	Forward primer for cloning Cdh1 (201-496 aa) in pDEST vector
33	ME353	GGGGACAAGTTTGTACAAAAA AGCAGGCTTCGAAGGAGATAG AGCCACCATGCCGCCACTGCA GTCGGAGCG	Forward primer for cloning Cdh1 (301-496 aa) in pDEST vector
34	ME354	GGGGACAAGTTTGTACAAAAA AGCAGGCTTCGAAGGAGATAG AGCCACCATGCAAGTGTGCAA TCTGGCCTG	Forward primer for cloning Cdh1 (401-496 aa) in pDEST vector
35	ME218	GGATTAACGAGAATGAGAAG	Forward primer used for Cdh1 q-RT-PCR
36	ME219	ATTCTTGAGCAGGGCAGAG	Reverse primer used for Cdh1 q-RT-PCR
37	ME153	AGGACAAGCGCTATGGAGAA	Forward primer used for CCNF q-RT-PCR
38	ME154	TCTGTCTTCCTGGAGGCTGT	Reverse primer used for CCNF q-RT-PCR
39	ME412	GCTGAACTCAAAGGTCACAC	Forward primer used for Cdc20 q-RT-PCR
40	ME413	CTCAGGGTCTCATCTGCTG	Reverse primer used for Cdc20 q-RT-PCR