Supplemental Materials

Molecular Biology of the Cell

Wasserman et al.

1 **Supplementary Information**

2 **For**

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4 Cell cycle oscillators underlying orderly proteolysis of E2F8

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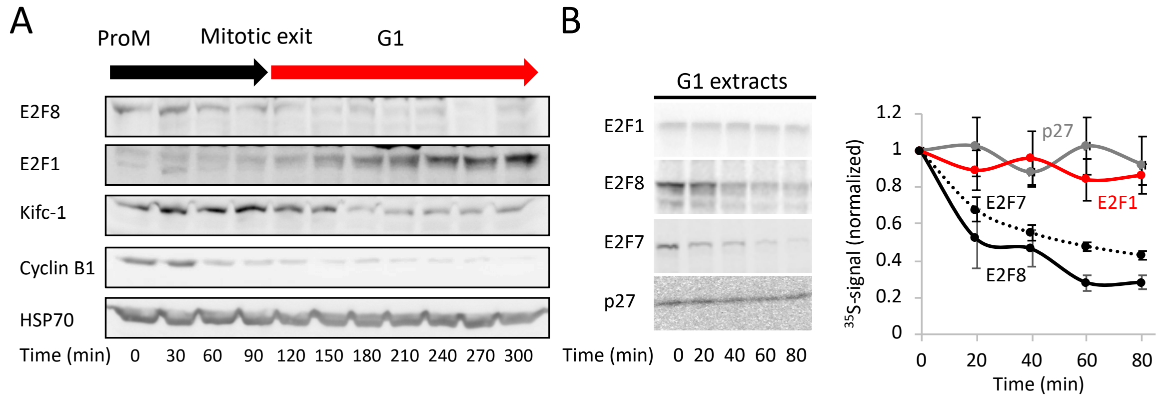


Figure S1: ***E2F1 is stable in APC/C^{Cdh1}-active G1 extracts.*** **(A)** Western blot analyses of E2F1, E2F8, Cyclin B1, Kifc-1 (APC/C^{Cdh1} targets) and HSP70 (loading control) in synchronous S3 cells released from a thymidine-nocodazole block into G1. Samples were harvested in 30 min intervals. **(B)** Time-dependent degradations of ^{35}S-labeled E2F1, E2F7, E2F8 and p27 (IVT products) in G1 extracts were assayed by SDS-PAGE and autoradiography. A set of source data and quantification of three experiments are shown (mean and SE values are plotted). In contrast to E2F8 and E2F7, E2F1 is stable in G1 extracts, like the SCF^{Skp2} target p27 (negative control).

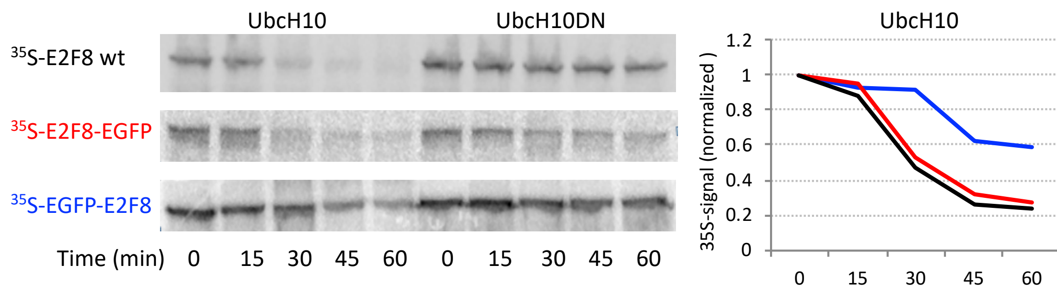


Figure S2: ***Proteolysis of EGFP-tagged E2F8 in G1 extracts.*** E2F8 was tagged with EGFP on either N- or C terminus. ^{35}S-labeled IVT product of tagged and untagged E2F8 were made, and their time-dependent degradations in G1 extracts were assayed by SDS-PAGE and autoradiography. Quantification of source data is plotted using matching colors.

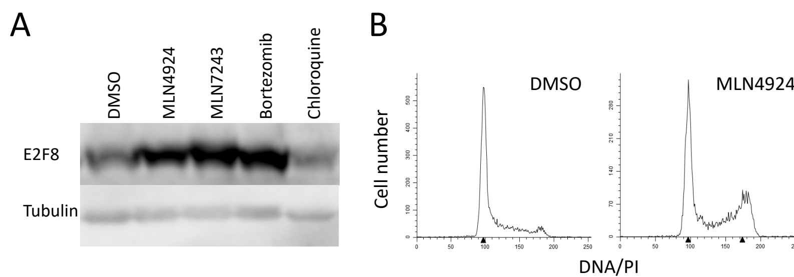
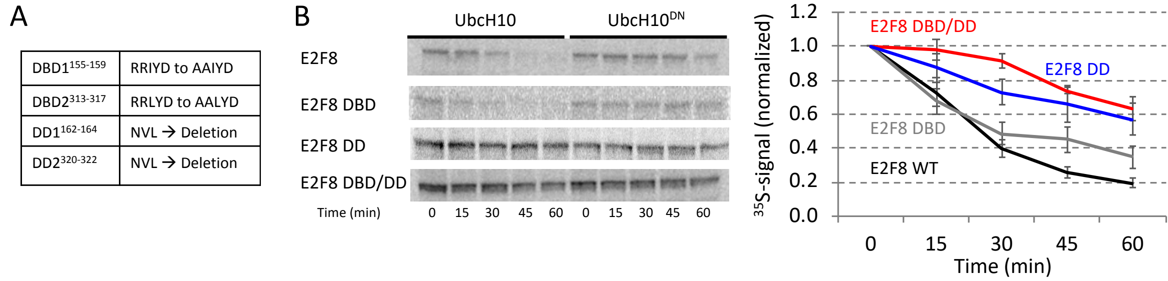


Figure S3: ***E2F8 dynamics following proteolysis perturbations.*** **(A)** HEK293 cells were treated with the depicted drugs for 5 h and harvested for Western blot analysis with anti-E2F8 and anti-Tubulin antibodies. Except for Chloroquine (100 μM), all drugs were used at a final concentration of 1 μM. **(B)** DNA distribution (PI staining) of HEK293 cells following 5 h treatment with DMSO and MLN4924.



DBD1$^{155\text{-}159}$	RRIYD to AAIYD
DBD2$^{313\text{-}317}$	RRLYD to AALYD
DD1$^{162\text{-}164}$	NVL → Deletion
DD2$^{320\text{-}322}$	NVL → Deletion

Figure S4. ***Degradation of E2F8 in G1 extracts is dependent on intact dimerization domains.*** **(A)** E2F8 variants with mutated DNA-binding domains (DBD1/DBD2) or dimerization domains (DD1/DD2) or both, were generated using point mutations or deletion. **(B)** Degradation of ^{35}S-labeled E2F8 variants (IVT products) was tested in G1 extracts supplemented with WT or dominant negative UbcH10 (UbcH10^{DN}). Time-dependent degradation was assayed by SDS-PAGE and autoradiography. Representative raw data and quantifications are shown. Mean E2F8 levels (^{35}S signals) normalized to max signal at t = 0 are shown (n = 3-4). Bars represent SE. An unexpected, albeit profound, dependency of E2F8 proteolysis on intact dimerization domains, but not the adjacent DNA-binding domains, is evident.

Degradation of E2F8 in G1 extracts is dependent on intact dimerization domains

E2F8-EGFP, but not EGFP-E2F8, is degraded in G1 extracts in a manner similar to untagged E2F8 **(Fig. S2)**. At some point during this study, we asked to complement our findings with *in vivo* quantification of fluorescently tagged E2F8 in live cells. Multiple attempts and strategies to generate stable cell lines with a constitutive (not inducible) expression of E2F8-EGFP failed. In addition, extensive efforts to knock in Venus (YFP) into an endogenous E2F8 locus also failed. Reasoning that fluorescently tagged E2F8, all the more so when overexpressed, might be cytotoxic, and focusing on E2F8 dynamics rather than function, we generated E2F8 lacking DNA binding domains (DBD1/2) with the assumption that dysfunctional E2F8-EGFP might be inert *in vivo*. Because E2F8 functions as a homo/hetero-dimer, we also deleted its two dimerization domains (DD1/2) to minimize potential dominant-negative effect of the modified E2F8 in cells **(Fig. 5A and Fig. S4A)**. Mutations were strategized based on the literature (Liu, Shats et al., 2013, Zalmas, Zhao et al., 2008). Conceptually, this experimental set up is valid only if the temporal proteolysis of the modified E2F8 variants is unchanged. To test that, we first assayed the proteolysis of the quadruple DBD/DD E2F8 mutant in G1 extracts. Surprisingly, this mutation nearly blocked E2F8 degradation **(Fig. S4B)**. It was the DD mutations, rather than the DBD mutations, that contributed most to this molecular phenotype. In view of these results, we decided to abandon this line of research. The data, however, have implications on the structure-to-function relationship of E2F8 with relevance to past and future research of atypical E2Fs.

Supplementary Tables

Table 1: List of DNA oligos used for cloning and mutagenesis

No.	Plasmid	F/R*	Oligo sequence	Description
1	pcDNA4/TO-Cyclin B1-DM	F	GACTGGATCCATGGCGCTCCGAGTCACCAG	For cloning a D-box mutant full-length human Cyclin B1 into a Tet-induced vector.
2		R	GACTCTCGAGTTACACCTTTGCCACAGCCTT	
3	pCS2-FA-E2F8-EGFP	F	GCATGGCCGGCCCACCATGGAGAACGAAAAGGAAA	For C-terminal tagging E2F8 with EGFP in pCS2-FA vector.
4		R	GCATACCGGTGTATGGACATCCTCTGTTGAG	
5	pCS2-His-E2F8-N80-EGFP	F	GCATGGCCGGCCCACCATGGAGAACGAAAAGGAAA	For cloning the ORF of E2F8 N-terminus (amino acids 1-80) in frame with His (N-terminal) and EGFP (C-terminal) tags in pCS2-FA vector.
6		R	GCATACCGGTGGATCCCATTTCTGATCTCTGTTGCGGATC	
7	pCS2-His-E2F8-C-EGFP	F	GCATGGCCGGCCCACCATGCTCGAGGACAGTGGTTC	For cloning the ORF of E2F8 C-terminus (amino acids 607-867) in frame with His (N-terminal) and EGFP (C-terminal) tags in pCS2-FA vector.
8		R	ATGCACCGGTGGATCCCAATGGACATCCTCTGTTGAGA	
9	pCS2-p27	F	ATATGGCCGGCCCACCATGTCAAACG	For cloning ORF of p27 into pCS2-FA vector.
10		R	GCATGGCGCGCCTTACGTTTGACGTCT	
11	pcDNA-E2F1	F	ATATGGTACCATGGCCTTGGCCGGGGCCCC	For cloning ORF of E2F1 into pcDNA3.1(+) vector.
12		R	GCGCGAATTCTCAGAAATCCAGGGGGGTGA	
13	pCS2-FA-His-E2F8-HA	F	GCATGGCCGGCCCACCATGGAGAACGAAAAGGAAA	For tagging E2F8 with C-terminal HA and cloning this PCR product into a pCS2-FA-His vector.
14		R	ATGCGGCGCGCCTTAAGCGTAATCTGGAACATCGTATGGGTACATATGGACATCCTCTGTTGAGAC	
15	pCS2-FA-E2F8 pCS2-His-E2F8-N80-EGFP	F	AGCCACATAAAAGGGGACTAATGAAAGCACCTCTGAAAGAA	E2F8 mutagenesis: for substituting Thr 20 with Ala. Phosphorylation site mutation.
16		R	TTCTTTCAGAGGTGCTTTCATTAGTCCCCTTTTATGTGGCT	
17	pCS2-FA-E2F8 pCS2-His-E2F8-N80-EGFP	F	CTTTGGCCCTTTAACCGCACCTACCAAGCCCAA	E2F8 mutagenesis: for substituting Thr 44 with Ala. Phosphorylation site mutation.
18		R	TTGGGCTTGGTAGGTGCGGTTAAAGGGCCAAAG	
19	pCS2-FA-E2F8 pCS2-His-E2F8-N80-EGFP	F	TGTGAGCCACATAAAAGGGGACTAATGAAAGATCCTCTGAAAGAATCCACC	E2F8 mutagenesis: for substituting Thr 20 with Asp. Phosphorylation site mutation.
20		R	GGTGGATTCTTTCAGAGGATCTTTCATTAGTCCCCTTTTATGTGGCTCACA	
21	pCS2-FA-E2F8 pCS2-His-E2F8-N80-EGFP	F	CTGACTTTGGCCCTTTAACCGATCCTACCAAGCCCAAGGAAGG	E2F8 mutagenesis: for substituting Thr 44 with Asp. Phosphorylation site mutation.
22		R	CCTTCCTTGGGCTTGGTAGGATCGGTTAAAGGGCCAAAGTCAG	
23	pCS2-FA-E2F8 pCS2-FA-His-E2F8-HA	F	GAAAAGGAAAATCTCTTTTGTGAGCCACATAAAGCGGGACTAATGAAAACAC	E2F8 mutagenesis: for substituting Arg 15 with Ala. RxL site mutation.
24		R	GTGTTTTCATTAGTCCCGCTTTATGTGGCTCACAAAAGAGATTTTCCTTTTC	
25	pCS2-FA-E2F8 pCS2-FA-His-E2F8-HA	F	GAGATCCGCAACAGAGATCAGAAAGCGGGTTTGTTTGACAA	E2F8 mutagenesis: for substituting Arg 81 with Ala. RxL site mutation.
26		R	TTGTCAAACAAACCCGCTTTCTGATCTCTGTTGCGGATCTC	
27	pCS2-FA-E2F8 pCS2-FA-His-E2F8-HA	F	TGGAAGATTTGGATAAAAGCAAGTTTAAAACAAAAATTGCGAGGTTGTATGATATAGCTA	E2F8 mutagenesis: for substituting Arg 313 with Ala. RxL site mutation.
28		R	TAGCTATATCATACAACCTCGCAATTTTTGTTTTAAACTTGCTTTTATCCAAATCTTCCA	
29	pCS2-FA-E2F8 pCS2-FA-His-E2F8-HA	F	AACTCTCTTTGTCCCACAGGCAAAACTGGAAGTCTCAACA	E2F8 mutagenesis: for substituting Arg 857 with Ala. RxL site mutation.
30		R	TGTTGAGACTTCCAGTTTTGCCTGTGGGACAAAGAGAGTT	

31	pCS2-FA-E2F8 pCS2-His-E2F8-N80-EGFP	F	GGCCCACCATGGAGAACGAAGCGGCAAATCTCTTTTGTGAGCCAC	E2F8 mutagenesis: for substituting Lys 5 and Glu 5 with Ala. KEN site mutation.
32		R	GTGGCTCACAAAAGAGATTTGCCGCTTCGTTCTCCATGGTGGGCC	
33	pCS2-FA-E2F8	F	GGAGGTGAGACGGTCTTCAGCAGCGAACTGTGCCAAAAACCTC	E2F8 mutagenesis: for substituting Lys 375 and Glu 376 with Ala. KEN site mutation.
34		R	GAGGTTTTTGGCACAGTTCGCTGCTGAAGACCGTCTCACCTCC	
35	pCS2-FA-E2F8 pCS2-His-E2F8-C-EGFP	F	GCAGAGTCCATTTTGTCTGGTGCAGCAAACTCAAGTGCT CTTTCCCC	E2F8 mutagenesis: for substituting Lys 657 and Glu 658 with Ala. KEN site mutation.
36		R	GGGGAAAGAGCACTTGAGTTTGCTGCACCAGACAAAATGGAC TCTGC	
37	pCS2-FA-E2F8	F	GGGTTTGTTTGACAACGGAAGTGGAGTACCTGAGGCCA AAGA	E2F8 mutagenesis: for substituting Arg 87 and Leu 90 with Gly and Val. RXXL site mutation.
38		R	TCTTTGGCCTCAGGTACTCCACTTCCGTTGTCAAACAAACCC	
39	pCS2-FA-E2F8	F	TACACTTGGCACGGGGGACACAATGTCAACAAAACCCT TG	E2F8 mutagenesis: for substituting Arg 183 and Leu 186 with Gly and Val. RXXL site mutation.
40		R	CAAGGGTTTTGTTGACATTGTGTCCCCCGTGCCAAGTGTA	
41	pCS2-FA-E2F8	F	GACGCATTTACGATATCGTGGAGAGTTTACATATGGTGAG	E2F8 mutagenesis: Deletion of NVL 162. Dimerization domain mutation.
42		R	CTCACCATATGTAAACTCTCCACGATATCGTAAATGCGTC	
43	pCS2-FA-E2F8	F	AACAAAAATTAGGAGGTTGTATGATATAGCTAGTAGCCT GGATCTTAT	E2F8 mutagenesis: Deletion of NVL 320. Dimerization domain mutation.
44		R	ATAAGATCCAGGCTACTAGCTATATCATACAACCTCCTAATTT TTGTT	
45	pCS2-FA-E2F8	F	CAGAGGAACTTAATGTTGAACGTGCAGCCATTTACGAT ATCGTGAACGTCC	E2F8 mutagenesis: for substituting Arg 155 and 156 with Ala. DNA-binding domain mutation.
46		R	GGACGTTCACGATATCGTAAATGGCTGCACGTTCAACATTAA GTTCCTCTG	
47	pCS2-FA-E2F8	F	CCATGTGGAAGATTTGGATAAAAGCAAGTTTAAAACAAAAATTGCGGCGTTGTATGA TATAGCTAATGTTC	E2F8 mutagenesis: for substituting Arg 313 and 314 with Ala. DNA-binding domain mutation.
48		R	GAACATTAGCTATATCATACAACGCCGCAATTTTTGTTTTAAA CTTGCTTTTATCCAAATCTTCC ACATGG	

* Primer orientation; forward (F) or Reverse complement (R)