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Brief Report

# Involvement of the SCF Complex in the Control of Cdh1 Degradation in S Phase

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## KEY WORDS

Cdh1, Cul1, SCF, S phase, degradation

## ABBREVIATIONS

| | |
|---|---|
| SCF | Skp1/Cul1/F-box protein, Roc1 |
| APC/C | anaphase promoting complex/cyclosome |
| CDK | cyclin dependent kinase |
| CHX | cyclohexamide |

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## ABSTRACT

The anaphase promoting complex/cyclosome (APC/C) is a multisubunit ubiquitin ligase that acts as a key regulator in the progression through mitosis (when mostly in complex with Cdc20) and as a stabilizer of the $G_1$ phase (when in complex with Cdh1). Cdh1 is an activator of APC/C, and it has previously been reported that it is capable of mediating its own degradation during $G_0$ and $G_1$. Herein, we show that the SCF complex (Skp1/Cul1/F-box protein/Roc1) intervenes in the surveillance of Cdh1 cellular abundance in S phase.

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## INTRODUCTION

The multisubunit ubiquitin-protein ligase anaphase promoting complex/cyclosome (APC/C), which is present throughout the cell cycle, presents peak activities in a cell specific manner from metaphase until late $G_1$ phase.[1,2] Ordered activation of APC/C by Cdc20 in metaphase and by Cdh1 in telophase is critical for the occurrence of anaphase and exit from mitosis, and ensures that the latter event does not take place before sister chromatid separation had been initiated. The binding of Cdh1 to the APC/C from late mitosis throughout $G_1$ promotes exit from mitosis by contributing to the degradation of mitotic cyclins[2,3] and maintains the $G_1$ state by preventing premature accumulation of positive regulators of the cell cycle.[1,4] Hence, the APC/C is essential for proper chromosome segregation, exit from mitosis, a stable $G_1$, and therefore an accurate DNA replication in S phase. Concerted crosstalk between signaling pathways orchestrating these events has to be maintained since their deregulation would cause genetic instability, which is a widely observed phenomenon in several human cancers.[4,5]

Cdh1 is active when present in its unphosphorylated form from late mitosis and throughout $G_1$, in part due to low cyclin dependent kinase (CDK) activity. When Cdh1 is unphosphorylated it binds the APC/C with high affinity and promotes its activation.[1-3] As decreased levels of Cdh1 induce premature entry into S phase,[6] it is crucial for a cell to keep Cdh1 levels under tight control in order to ensure flawless cell cycle progression. It has been shown by Listovsky et al[7] that Cdh1 activates the APC/C to mediate its own degradation in an RXXL-motif dependent manner. The RXXL motif, also referred to as the destruction box, in addition to the KEN box, is a consensus sequence that targets APC/C substrates for degradation.[1-3] The authors had addressed the question of Cdh1 degradation mainly in $G_0$ and in $G_1$, when $APC/C^{Cdh1}$ is active. It remains unclear whether auto-ubiquitination is the only means by which Cdh1 degradation is mediated, and whether this is cell phase specific. Here, we investigate the possibility that the SCF ubiquitin ligase complex plays a role in the degradation of Cdh1 during S phase.

## MATERIALS AND METHODS

**Cell lines, cell synchronizations and treatments.** 293T cells, T98G cells and Hela cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% calf serum. 293T cells were engineered to express a Cul1 deletion mutant (Cul1-N252) in a doxycyclin dependent manner.[8] Cul1-N252 cells were grown in DMEM supplemented with 10% calf serum, blastocydine (5 μg/ml) and hygromycine B (200 μg/ml). T98G cells and Hela cells were used for synchronization experiments, protein half-life studies and RNA interference transfections. Cells were synchronized in early S phase by treatment with aphidicolin (2 μg/ml), and in $G_0$ by serum starvation for 48 hours. They were released from serum starvation for 5 or 26 hours, and subsequently subjected to cyclohexamide treatment (100 μg/ml) in order to inhibit protein synthesis.

**Cell extraction and immunoblotting**. Protein extraction and immunoblotting were carried out as described previously.[9]

**RNA interference.** SiRNA duplexes were designed in accordance with recommendations (Dr. Ouathek Ouerfelli) and were synthesized by Memorial Sloan Kettering siRNA facility. Three duplexes were designed according to the sequence of human Cul1 as follows; Oligo 1: UUGUGCCUAC-CUCAAUAGATT; Oligo 2: GGUCGCUUCAUAAA-CAACATT; Oligo 3: AC-UGCUCAGGAUUGAU-ACATT. RNA interference was performed as described.[10] For experiments in which cells were synchronized with aphidicolin, a first cycle of siRNA transfection was performed for 24 hours, followed by a second cycle of 24 hours, during which aphidicolin was added 4 hours subsequent to the siRNA transfection.

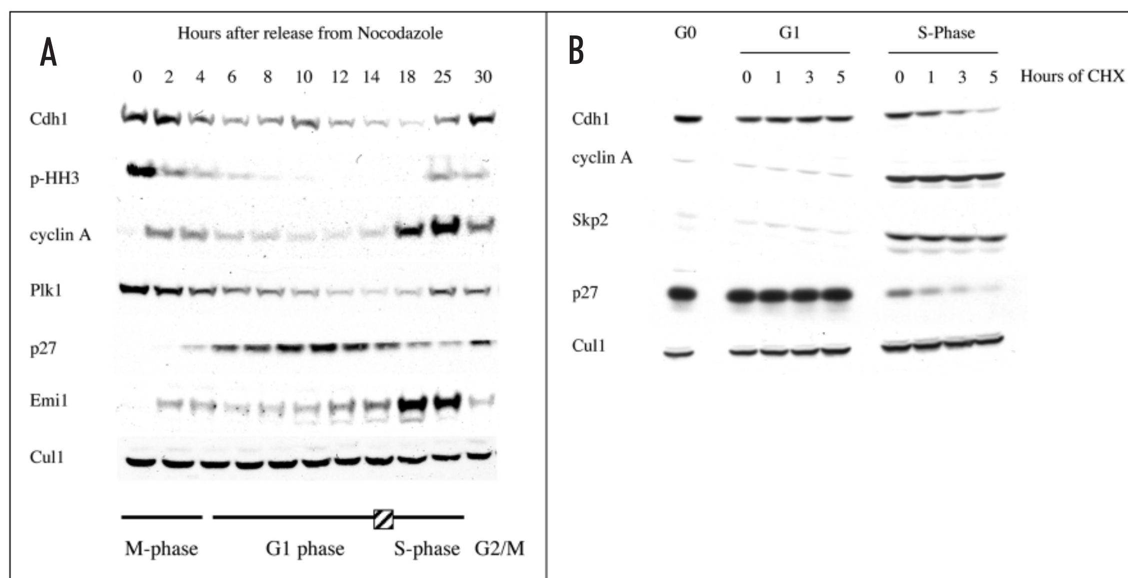


Figure 1. Levels of Cdh1 vary during the cell cycle. (A) Hela cells synchronized in prometaphase with nocodazole, followed by mitotic shake-off were released and harvested at the indicated time points. Cell extracts were immunoblotted with antibodies to Cdh1, phospho-Histone H3 (pHH3), cyclin A, Plk1, p27, Emi1 and Cul1. (B) T98G cells were synchronized in $G_0/G_1$ by serum starvation, then either harvested at $G_0$ without cyclohexamide (CHX) treatment (first lane), or released by supplementation with serum for 5 and 26 hours, into $G_1$ and early S phase, respectively. Cells in $G_1$ and S phase were subsequently treated with cyclohexamide and harvested at the indicated time points. Cell extracts were immunoblotted with the antibodies to the indicated proteins.

## RESULTS AND DISCUSSION

It is crucial for the cell that levels and activity of Cdh1 be kept under tight regulation to avoid untimely activation of the APC/C, which would be deleterious to the cell. Although expression levels of Cdh1 are high in early mitosis, its activity is low due to high CDK activity at this point of the cell cycle.[1-3] Cdh1 levels subsequently decline in late $G_1$ phase and in early S phase as illustrated in Figure 1A, in agreement with results published previously.[7,11] It has been shown that levels of Cdh1 are kept low in $G_0$ and $G_1$ due to Cdh1 mediating its own auto-ubiquitination.[7] Here we show that Cdh1 is also actively proteolyzed during S phase, as its half- life is significantly shorter in S phase as compared to the $G_1$ phase (Fig. 1B). In this experiment, Skp2 and cyclin A represent markers for synchronization of cells in S phase, and p27 is a positive control for the $G_1$ phase.

The question remained open as to whether autoubiquitination is the only factor responsible for Cdh1 destruction. SCF is known to target cell cycle regulatory proteins for proteolysis and hence regulating the $G_1$/S transition,[12-15] during which period the APC/C complex is indeed inactive due, in part, to Cdh1 degradation. We investigated whether an SCF ubiquitin ligase complex may be upstream of Cdh1 degradation in S phase. To explore this possibility we used cells that were engineered to express a dominant negative deletion mutant of Cul1 encoding 252 N-terminal residues (Cul1-N252), whereby the Tet-cassette is doxycylin-inducible.[8] The induction of Cul1-N252 leads to the sequestration of the adaptor protein Skp1 which links the F-box protein to Cul1, and subsequently

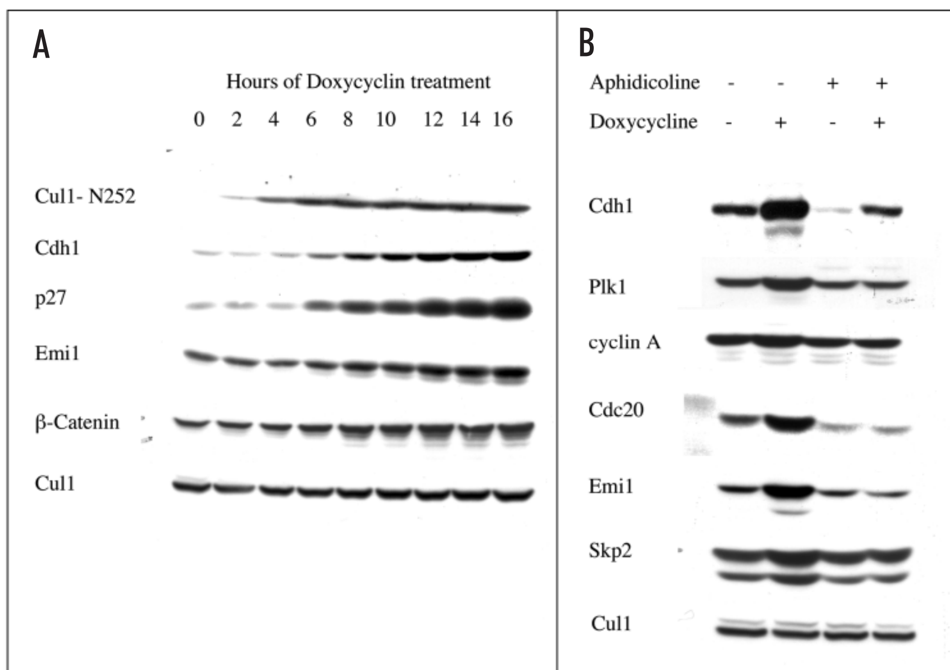


Figure 2. Inducible expression of a dominant negative Cul1 results in Cdh1 accumulation. (A) Induction of a dominant negative Cul1 deletion mutant (Cul1-N252) with doxycyclin for the indicated number of hours leads to accumulation of Cdh1in a time-dependent fashion. Cell lysates were also blotted for known Cul1-dependent substrates: p27, Emi1 and β-catenin. (B) Asynchronous cells or cells synchronized in early S phase with aphidicolin were treated or not with doxycyclin. Lysates were then immunoblotted for Cdh1 and some of its known substrates (cyclin A, Cdc20, Plk1 and Skp2), and for Emi1 as an SCF substrate.

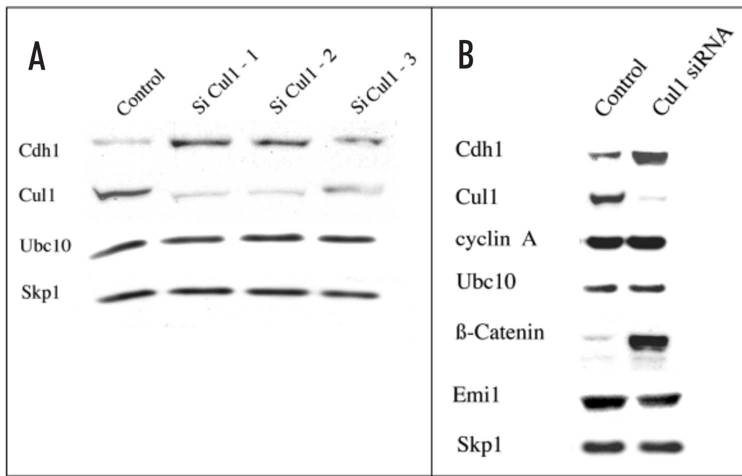


Figure 3. Transfection of Cul1 siRNA oligos results in increased Cdh1 levels. (A) T98G cells were either mock transfected (control) or transfected with one of three different Cul1 siRNA duplexes. Cells extracts were immunoblotted for Cul1 (to assess the efficiency of the siRNA), Cdh1, Ubc10, and Skp1 (as protein loading control). (B) Hela cells were transfected with a pool of two different Cul1 siRNA duplexes [1 and 2 used in the experiment shown in (A)], for two cycles. During the second cycle of siRNA, aphidicolin was added to the cells for 24 hours. Cells lysates were immunoblotted for Cul1, Cdh1, cyclin A, Ubc10, β-catenin and Emi1. Skp1 was used as protein loading control.

causes an accumulation of SCF substrates. Here we show that the induction of Cul1-N252 by doxycyclin leads to a significant accumulation of Cdh1 in asynchronous cells in a time-dependent manner (Fig. 2A), similarly to known SCF-complex substrates such as Emi1, β-Catenin, and p27.[14,15,16] These results suggest that an SCF complex may be involved (directly or indirectly) in the degradation of Cdh1. Next, to address the question of whether this equally applies in S phase, we synchronized cells with aphidicolin, and subsequently subjected them to doxycyclin treatment. In Figure 2B, we show that the scarcely detectable protein levels of Cdh1 in S phase were significantly enhanced when cells were treated with doxycycline. These results show that accumulation of Cdh1 in S phase correlates with the inhibition of the SCF complex by Cul1-N252, and imply that the SCF could be a contributing factor in keeping Cdh1 protein levels low in S phase. We also show that the induced increase in Cdh1 protein levels during S phase does not bring about the degradation of other known APC/$C^{Cdh1}$ substrates, such as Plk1, cyclin A, Cdc20 and Skp2, which is consistent with the fact that they are $G_1$ substrates of APC/$C^{Cdh1}$. These data indicate that despite the fact that Cdh1 expression is high, it is however maintained inactive partially due to CDKs' phosphorylation activity in S phase, and that the cell cycle is not hampered due to the unscheduled upregulation of Cdh1. Importantly, in contrast to the S phase situation, after induction of Cul1-N252, levels of Plk1, cyclin A, Cdc20 and Skp2 increase in asynchronous cells. This is likely to be the result of the accumulation of Emi1, an inhibitor of APC/C.[16] Accordingly Emi1 accumulates when SCF is inhibited in asynchronous cells, but not in cells synchronized in S phase (Fig. 2B). Thus, the S phase accumulation of Cdh1 is independent of Emi1 expression levels and cannot be attributed to an inhibition of APC/C by Emi1.

Results regarding Cdh1 upregulation due to disruption of the SCF complex were confirmed in a different set of experiments using small RNA interference aimed at silencing Cul1 expression. As illustrated in Figure 3A, there is a direct correlation between downregulation of Cul1 and upregulation of Cdh1 in asynchronous cells. Levels of Ubc10, another substrate of APC/$C^{Cdh1}$, are not altered following Cul1 silencing. Furthermore, when siRNA treatment was concomitant with aphidicolin treatment, Cdh1 accumulated significantly (Fig. 3B). These results consolidate the data obtained with the Cul1-N252 mutant (Fig. 2) implying that degradation of Cdh1 is downstream of an SCF complex. It ought to be emphasized that although Cdh1 is upregulated, it appears to be inactive, as one would expect it to be in S phase, as levels of cyclin A and Ubc10 do not decrease as a consequence of Cdh1 accumulation. Furthermore, the possibility that Cdh1 accumulation in S phase is due to an inhibition of its auto-ubiquitination/degradation may be ruled out considering the fact that Emi1 expression is not augmented following depletion of Cul1 in S phase (Fig. 3B).

The current paradigm indicates that Cdh1 degradation is mediated via auto-ubiquitination.[7] Our data suggest either that during S phase an SCF complex regulates the degradation of Cdh1 (mediated by APC/$C^{Cdh1}$ itself or via another ubiquitin ligase), or that an SCF complex directly targets (via an as yet unknown F-box protein) Cdh1 for degradation. It would be worthwhile to delve into the mode by which SCF is upstream of Cdh1 degradation. The phosphorylation of Cdh1 in S phase may indeed be involved in its recognition by SCF. In conclusion, crosstalk between APC/$C^{Cdh1}$ and the SCF complex may play a critical role in ensuring proper timing for the degradation of key cell cycle regulators, hence maintaining cell homeostasis.

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