Oncogene (2007) 26, 3406–3414

www.nature.com/onc

ORIGINAL ARTICLE

Cell cycle regulation of the human Six1 homeoprotein is mediated by APC^{Cdh1}

KL Christensen[1], JDG Brennan[2], CS Aldridge[2] and HL Ford[1,2,3]

[1]*Program in Molecular Biology, University of Colorado at Denver and Health Sciences Center, Fitzsimons Campus, Aurora, CO, USA;* [2]*Department of Obstetrics and Gynecology, University of Colorado at Denver and Health Sciences Center, Fitzsimons Campus, Aurora, CO, USA and* [3]*Department of Biochemistry and Molecular Genetics, University of Colorado at Denver and Health Sciences Center, Fitzsimons Campus, Aurora, CO, USA*

The Six1 homeoprotein is an important mediator of normal development, where it is critical for the proliferation of precursor cell populations that ultimately constitute the muscle, kidney and inner ear, among other organs. Interestingly, its overexpression has been observed in numerous cancers, where it contributes to the proliferative and metastatic ability of the cancer cells. Here we show that Six1 not only regulates the cell cycle, but is itself regulated throughout the cell cycle via ubiquitin-mediated proteolysis. The protein is present from the G_1/S boundary until mitosis, when it is degraded via the anaphase-promoting complex (APC) with its activating subunit Cdh1. However, unlike most identified APC^{Cdh1} targets, Six1 does not contain functional destruction or KEN box motifs that are necessary for its degradation. Instead, the Six1 protein contains multiple, as yet undefined, sequences within its N- and C-termini responsible for its degradation, including an N-terminal region that binds to Cdh1. Cell cycle regulation of Six1 occurs both transcriptionally and post-translationally via phosphorylation; therefore, this study demonstrates a third and novel mechanism of cell cycle-specific regulation of Six1, underscoring the importance of confining its activity to a defined cell cycle window from the G_1/S boundary to early mitosis.
Oncogene (2007) **26,** 3406–3414. doi:10.1038/sj.onc.1210122; published online 20 November 2006

Keywords: Six1; anaphase-promoting complex; Cdh1; proteasome; homeoprotein; cell cycle

Introduction

Six1 is an important developmental regulator that is required for the proliferation of progenitor cells during the development of numerous organs. (Li *et al*., 2003; Zheng *et al*., 2003; Ozaki *et al*., 2004). Gene amplification and overexpression of Six1 is observed in human cancers, where it confers developmental properties on adult cells leading to an increase in both proliferation and metastasis (Li *et al*., 2002; Coletta *et al*., 2004; Reichenberger *et al*., 2005; Yu *et al*., 2006). The pro-proliferative role of Six1 has been documented in both breast cancer and in rhabdomyosarcoma, although it has been most rigorously examined in breast cancer cells where it plays a role in entrance into and progression through S phase and in the G_2 cell cycle checkpoint (Ford *et al*., 1998; Coletta *et al*., 2004; Yu *et al*., 2006).

Similar to many other genes that influence cellular proliferation, Six1 is *itself* regulated throughout the cell cycle both at the transcriptional level (Ford *et al*., 1998) and post-translationally through phosphorylation (Ford *et al*., 2000). Here we show that a third level of cell cycle-specific regulation exists for Six1, which involves proteasome-mediated proteolysis.

Targeted degradation of critical regulatory molecules in the cell cycle is required to maintain the highly ordered series of events necessary for proper progression through each cell cycle stage. To achieve cell cycle-specific degradation, proteins are ubiquitinated, targeting them for proteolysis by the 26S proteasome. Ubiquitination is achieved by a cascade of three enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin ligase (E3), the last providing the substrate specificity for the pathway (Hershko and Ciechanover, 1998).

The E3 anaphase-promoting complex (APC) is active during mitosis and G_1 phase, regulating the expression of many proteins that are important in the control of anaphase entry and progression, mitotic exit, cytokinesis and DNA replication (Peters, 2002). The APC is activated by associating with either of two activating subunits, Cdc20 or Cdh1. These activators are thought to provide both timing and substrate specificity to the APC. Cdc20 becomes active first at the end of metaphase, allowing APC^{Cdc20}-dependent degradation of securin, sister chromatid separation and progression into anaphase. Cdh1 is activated in late mitosis and remains active in G_1. (Peters, 2002). Given their key roles in cell cycle regulation, it is perhaps not surprising

Correspondence: Dr H Ford, Department of Obstetrics and Gynecology, Mailstop 8309, 12800 E 19th Avenue, PO Box 6511, Aurora, CO 80045, USA.
E-mail: heide.ford@uchsc.edu
Received 28 July 2006; revised 6 October 2006; accepted 6 October 2006; published online 20 November 2006

that many APC substrates have been implicated as oncogenes (Park *et al*., 2005).

In this study, we demonstrate that protein levels of the Six1 oncogene fluctuate with cell cycle progression, increasing throughout S and G_2 phases, and abruptly disappearing in late mitosis. Degradation of Six1 is mediated through the ubiquitin–proteasome pathway dependent on APC^{Cdh1}. This identifies Six1 as the third transcription factor targeted by the APC, all of which play critical roles in development, are overexpressed in cancers, and may promote tumor progression. Unlike the other transcription factors, Six1 degradation by APC^{Cdh1} is independent of known destruction motifs, suggesting that it contains novel APC^{Cdh1} recognition motif(s). Although Six1's N-terminus binds to Cdh1, regions contained in both the N- and C-termini contribute to APC^{Cdh1}-mediated degradation, indicating that the Six1 degradation motif is complex, as is observed in other APC targets. This study demonstrates a third mechanism of cell cycle-specific regulation of Six1, underscoring the importance of confining Six1 activity to a defined window of the cell cycle encompassing the G_1/S boundary through early mitosis.

Results

Six1 expression fluctuates in the cell cycle

We have previously shown that Six1 promotes cell cycle progression (Ford *et al*., 1998; Coletta *et al*., 2004), and that Six1 transcript levels and phosphorylation state are regulated in the cell cycle (Ford *et al*., 1998, 2000). As the expression of many proteins with cell cycle regulatory functions is controlled by ubiquitin-mediated proteolysis, we asked whether Six1 protein levels were also regulated in a cell cycle-specific manner. To this end, we first examined Six1 protein levels throughout the cell cycle in 21PT human mammary carcinoma cells, which express high levels of endogenous Six1. 21PT cells, synchronized by serum starvation, were released into the cell cycle, and Six1 protein levels as well as cell cycle progression were monitored by Western blot analysis and flow cytometry. Figure 1 demonstrates that Six1 protein levels fluctuate dramatically during the cell cycle, increasing with progression through S phase and peaking in G_2/M. This pattern of expression is similar to that observed with cyclin B1 (Figure 1a), which is known to be degraded in a cell cycle-specific manner.

We previously observed that Six1 messenger RNA (mRNA) expression increases with progression through S phase following release from a mimosine-induced G_1 arrest (Ford *et al*., 1998). In addition, Six1 was recently identified as an E2F1 target (Young *et al*., 2003), further suggesting its transcriptional regulation at the G_1/S boundary. Thus, the changes in protein levels observed throughout the cell cycle could be due to cell cycle-specific transcriptional regulation of Six1, as opposed to regulation of protein stability. To determine if observed fluctuations in Six1 protein levels are in part due to regulation that is post-transcriptional, we transfected a cytomegalovirus (CMV) promoter-driven Six1 expression plasmid into MCF7 cells, which express low endogenous levels of Six1 protein, thus removing the normal transcriptional control conferred on Six1. After transfection, the cells were synchronized at the metaphase–anaphase transition using nocodazole, and then released into the cell cycle to examine Six1 levels. Figure 1b shows the loss of Six1 protein as the cells progress through mitosis, again with kinetics similar to endogenous cyclin B1. Thus, although it is still formally possible that Six1 mRNA stability may change with progression through the cell cycle, we have ruled out the possibility that transcriptional control alone is responsible for changes in Six1 expression through the cell cycle. This suggests that Six1 protein stability may be regulated in the cell cycle.

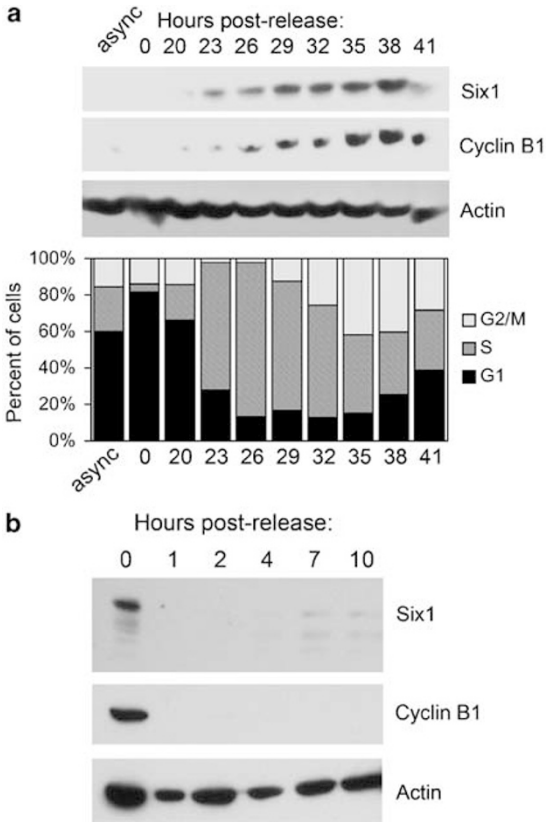

Figure 1 Six1 protein expression is regulated in the cell cycle. (**a**) Endogenous Six1 fluctuates with cell cycle progression. 21PT mammary carcinoma cells were synchronized by serum starvation, and nuclear extracts were collected at the indicated time points after release as well as from asynchronous cycling cells (async). Western blot analysis was performed with anti-Six1, anti-cyclin B1 and anti-β-actin antibodies. The lower panel depicts the percentage of cells in G_1, S and G_2/M phases as determined by flow cytometric analysis for each time point. (**b**) Cell cycle changes in Six1 protein levels are not dependent on transcriptional regulation. MCF7 cells were transfected with SIXFL to express Six1 constitutively in the cell cycle, and then synchronized at the metaphase–anaphase transition by nocodazole block. At the indicated time points after release, whole-cell lysates were collected and analysed by Western blot as in (**a**).

Six1 is degraded through the ubiquitin–proteasome pathway

As the kinetics of Six1 degradation are similar to that observed with cyclin B1, we investigated whether Six1, like cyclin B1, is subject to proteasome-mediated proteolysis. MCF7 cells transfected with Six1 were treated with 26S proteasome inhibitors, MG132 and LLNL, or with nonspecific protease inhibitors, LLM and E64. Six1 protein accumulated in the cells after treatment with MG132 and LLNL, but not with LLM and E64 (Figure 2a). Similar experiments were performed to look at endogenous Six1 and, as was observed with exogenous Six1, an accumulation of the protein was observed after treatment with LLNL and MG132, but not LLM and E64 (Figure 2a).

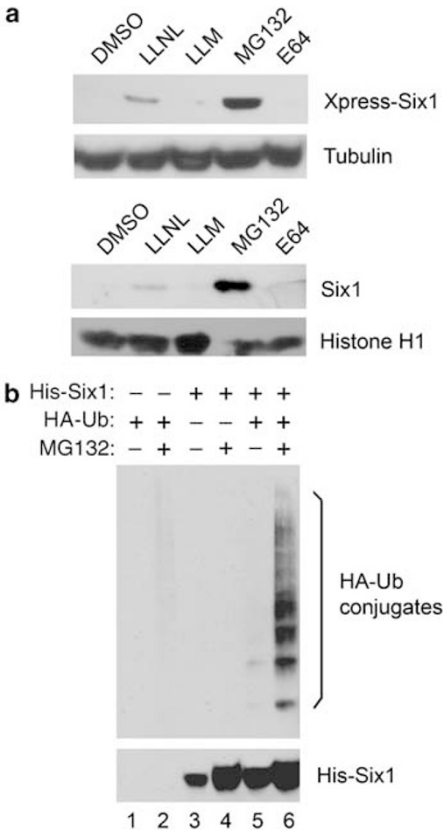

Figure 2 Six1 is degraded through the ubiquitin–proteasome system. (**a**) Six1 accumulates after inhibition of the proteasome. MCF7 cells were treated for 12 h with DMSO alone, proteasome inhibitors LLNL and MG132, calpain protease inhibitor LLM or cysteine protease inhibitor E64. In the upper panel, MCF7 cells were transfected with the Six1 expression plasmid SKMFL before treatment. Western blot analysis was performed on whole-cell lysates with an anti-Xpress antibody to detect exogenous Six1, and an anti-β-tubulin antibody as a loading control. In the lower panel, endogenous Six1 levels were examined in nuclear extracts collected after treatment. Western blotting was performed with an anti-Six1 antibody, and an anti-histone H1 antibody as a nuclear loading control. (**b**) Six1 is poly-ubiquitinated *in vivo*. MCF7 cells were co-transfected with His-Six1 and HA-ubiquitin (lanes 5 and 6) or empty vector control (lanes 3 and 4). A 12 h treatment with MG132 was used to inhibit the proteasome, allowing accumulation of polyubiquitinated substrates (lanes 2, 4, 6). Tagged-Six1 protein was purified from cell lysates with Ni-NTA beads and subjected to Western blot analysis. An anti-Xpress antibody was used to detect total Six1 bound to the beads (bottom panel), while an anti-HA antibody was used to detect ubiquitinated Six1 (top panel).

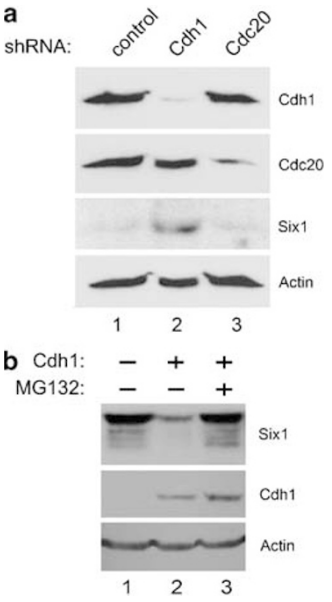

Figure 3 Six1 degradation is mediated by Cdh1. (**a**) RNAi knockdown of Cdh1 results in an accumulation of Six1. MCF7 cells were transfected with shRNA-expressing vectors, pSUPER (control, lane 1), pSUPER-Cdh1 (lane 2) or pSUPER-Cdc20 (lane 3). Nuclear extracts were collected 60 h post-transfection for Western blot analysis with anti-Cdh1, anti-Cdc20, anti-Six1 and anti-β-actin antibodies. (**b**) Six1 expression is reduced by Cdh1 overexpression, but restored by inhibiting the proteasome. Six1 was co-transfected with Cdh1 (lane 2 and 3) or empty vector (lane 1), followed by a 12 h treatment with MG132 (lane 3) to block proteasome-dependent degradation. Protein levels were assessed by Western blot analysis of whole-cell lysates with anti-Six1, anti-Cdh1 and anti-β-actin antibodies.

We next examined whether Six1 is tagged with polyubiquitin chains, as is observed on substrates targeted for degradation by the 26S proteasome (Hershko and Ciechanover, 1998). His-tagged Six1 was co-transfected with hemagglutinin (HA)-tagged ubiquitin into MCF7 cells, after which the cells were incubated in the absence or presence of MG132 to inhibit degradation and enrich for ubiquinated Six1. Six1 was then purified from cell lysates using nickel beads and examined for ubiquitination via Western blot analysis with an anti-HA antibody. Figure 2b demonstrates that Six1 is indeed ubiquitinated within the cell.

Six1 is degraded via the APC in conjunction with Cdh1

As Six1 expression in the cell cycle mirrors another APC substrate cyclin B1 (Figure 1), we investigated whether Six1, like cyclin B1, is a target of APC-dependent degradation. Short hairpin RNA (shRNA)-expressing vectors targeting APC activator subunits Cdc20 and Cdh1 were transfected into MCF7 cells to knockdown expression of these subunits and determine whether the absence of either activator would lead to an increase in Six1 stability. Six1 protein accumulated in cells when

Cdh1 was knocked-down, but not when Cdc20 was knocked-down, suggesting that Six1 is a target of APC^{Cdh1} (Figure 3a).

Cdh1 is present in limiting amounts in the cell, and its overexpression can increase APC activity (Fang *et al*., 1998). To confirm that Six1 is degraded downstream of APC^{Cdh1}, we performed *in vivo* degradation assays by transfecting Six1 along with Cdh1 into MCF7 cells. Co-transfection of Six1 with Cdh1 results in a reduction in Six1 protein levels (Figure 3b, lanes 1 and 2), which can be restored by the addition of the proteasome inhibitor, MG132 (Figure 3b, lane 3). This confirms that Six1 is degraded downstream of APC^{Cdh1}.

Six1 degradation is independent of D-box, KEN box, A-box and O-box motifs

Most APC^{Cdh1} substrates identified contain destruction box (D-box) or KEN box motifs that are necessary for APC-mediated degradation. When these sequences are mutated, APC-dependent degradation is blocked. Six1 contains two sequences resembling the KEN box in its C-terminus, and one potential D-box in its homeodomain (Figure 4a and b). Point mutations were generated in full-length Six1 that substitute the critical residues in these motifs with alanines (Figure 4b), and these mutants were tested in our Cdh1-overexpression assay to determine their role in mediating Six1 degradation. Surprisingly, none of these mutations, either singly or in combination, prevented degradation by Cdh1 overexpression (Figure 4c).

To date, two APC^{Cdh1} substrates have been described that do not contain functional D-box or KEN box sequences, but instead contain novel degradation motifs. In *Xenopus* Kid (Xkid) a novel sequence, G-X-E-N, functions as a APC^{Cdh1} degradation motif (Castro *et al*., 2003), but has not been found in other APC^{Cdh1} substrates. We found no sequences resembling the GxEN motif in Six1. Another novel degradation motif,

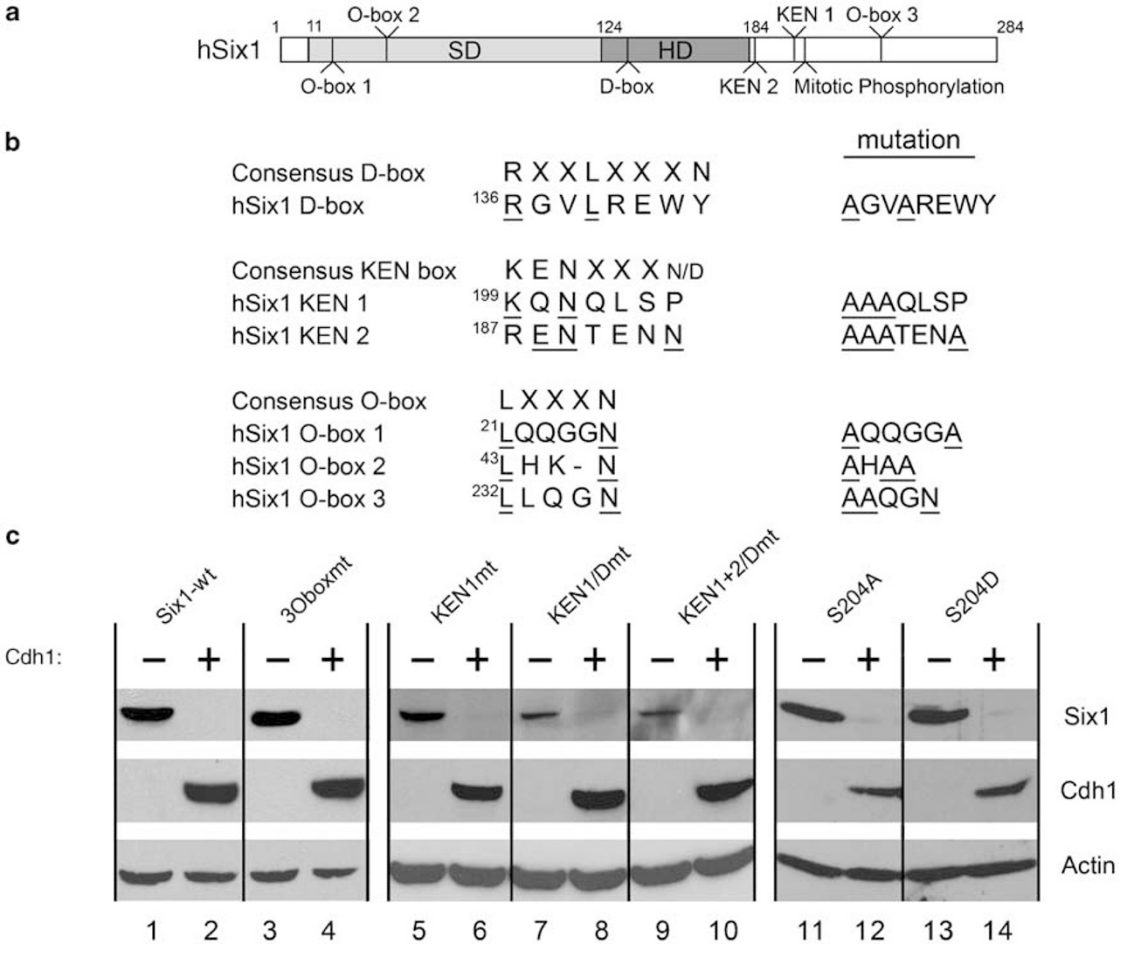

Figure 4 Known Cdh1 destruction motifs are not involved in Six1 degradation. (**a**, **b**) Six1 contains a weak homology D box, two weak homology KEN boxes, three O-box type motifs and a mitotic phosphorylation site at serine 204. (**b**) Mutations were made in full-length Six1 substituting critical residues in these motifs with alanines, as specified in the right hand column. (**c**) Cdh1-mediated degradation of Six1 is not dependent on the putative D-box, KEN box, O-box motifs or on serine 204 phosphorylation state. Six1 mutants were co-transfected into MCF7 cells with Cdh1 or empty vector control, protein levels were assessed by Western blot analysis of whole-cell lysates with anti-Xpress, anti-Cdh1 and anti-β-actin antibodies. Six1 constructs shown are mutated in all three O-boxes (3Oboxmt: lanes 3, 4); KEN box 1 (KEN1 mt: lanes 5, 6); KEN box 1 and D-box (KEN/Dmt: lanes 7, 8); KEN box 1 and 2, and D-box sequences (KEN1 + 2/Dmt: lanes 9, 10); serine 204 to alanine, non-phosphorylatable mutant, (S204A: lanes 11, 12); and serine 204 to aspartic acid, phospho-mimetic mutant, (S204D: lanes 13, 14).

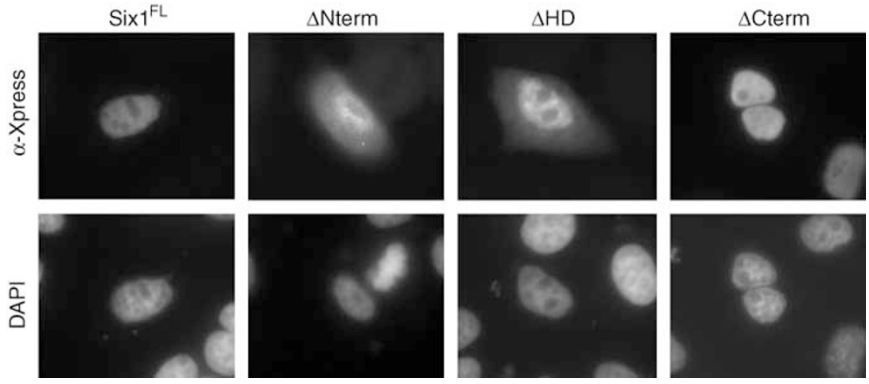

Figure 5 The N-terminus of Six1 is required for nuclear localization. Xpress-tagged Six1, and mutants lacking the N-terminus (ΔNterm), the homeodomain (ΔHD) or the C-terminus (ΔCterm), were transfected into MCF7 cells. Top panels show subcellular localization of Six1 by immunofluorescence using the anti-Xpress antibody, bottom panels show counterstaining of nuclei by DAPI. Representative images are shown.

the O-box, was identified in the *Drosophila* origin recognition complex large subunit (ORC1), and also found in *Drosophila* Asp and the *S. pombe* securin homolog Cut2 (Araki *et al*., 2005). Only two residues in the O-box sequence were found to be essential, L-X-X-X-N. We identified three sequences resembling the O-box in Six1 (Figure 4a and b), however, mutation of all three of these O-boxes did not stabilize Six1 when co-expressed with Cdh1 (Figure 4c).

Another degradation motif, the A-box (Q-R-V-L), is present in the Aurora kinases Aur-A and Aur-B, which also contain a functional KEN box (Crane *et al*., 2004; Nguyen *et al*., 2005). In Aur-A, a serine residue just downstream of the A-box is phosphorylated specifically in mitosis. Mutation of this serine (Ser51) to aspartate blocks APC-mediated degradation, indicating that dephosphorylation of this residue is necessary for degradation (Littlepage and Ruderman, 2002). It has been suggested that this phosphorylation helps to tightly control the timing of degradation during mitosis. Six1 does not contain any sequences resembling the QRVL of the A-box; however, as we have previously shown that it is hyperphosphorylated during mitosis (Ford *et al*., 2000), we investigated whether mitotic phosphorylation affects Cdh1-mediated degradation in a manner similar to that observed with the Aurora A kinase. Using mass spectrometry, serine 204 of Six1 was demonstrated to be specifically phosphorylated in mitosis (data not shown). To determine whether this mitotic phosphorylation regulates APC^{Cdh1}-dependent degradation of Six1, we generated both a non-phosphorylatable mutant of Six1 (Ser 204 to Ala substitution) and a phospho-mimetic mutant (Ser 204 to Asp substitution). When these mutants were tested for degradation in response to Cdh1 overexpression, neither stabilized Six1 (Figure 4c), suggesting that the mitotic-specific phosphorylation of Six1 at Ser 204 is not involved in Six1 degradation by APC^{Cdh1}. Overall, our results with Six1 mutants suggest that, like Xkid and ORC1, Six1 contains novel, degradation signals that are recognized by APC^{Cdh1}.

The Six1 N-terminal and C-terminal sequences both contribute to Six1 degradation

As Six1 contains no previously characterized degradation motifs, we created gross deletions of the amino terminus (ΔNterm), homeodomain (ΔHD), or carboxyl terminus (ΔCterm) to identify the region(s) necessary for APC^{Cdh1}-dependent degradation. Before assessing the contribution of each region to degradation, we examined the cellular localization of these proteins to ensure that differences in stability were not owing to differences in subcellular localization. Immunocytochemistry of MCF7 cells transfected with these Six1 deletion mutants demonstrated that similar to full-length Six1, ΔHD and ΔCterm were primarily nuclear, whereas ΔNterm was largely relocalized to the cytoplasm (Figure 5). This observation corroborates recent findings that the nuclear localization sequence (NLS) of Six2, whose N-terminus is 93% conserved to that of Six1 (Kawakami *et al*., 2000), resides in the N-terminus of the protein (Brodbeck *et al*., 2004). The ΔNterm construct was therefore redesigned with an NLS (NLSΔNterm) and nuclear expression in MCF7 cells confirmed by immunocytochemistry (data not shown). All mutants were then examined for stabilization when co-expressed with Cdh1 in MCF7 cells. ΔNterm and ΔCterm were both stabilized in the presence of Cdh1-overexpression, whereas ΔHD was degraded similar to full-length Six1 (Figure 6b). This suggests that both the N-terminus and C-terminus of Six1 contain sequences that contribute to degradation of Six1 via APC^{Cdh1}. Smaller deletions and numerous point mutations spanning the entire N- and C-termini were made. However, none of these more limited mutations blocked APC^{Cdh1}-mediated degradation (data not shown), suggesting that there are multiple redundant motifs within both the N- and C-termini.

The N-terminus of Six1 binds directly to Cdh1

Cdh1 binds to D-box and KEN box motifs in its substrates (Burton *et al*., 2005; Kraft *et al*., 2005). Although Six1 does not have functional D or KEN

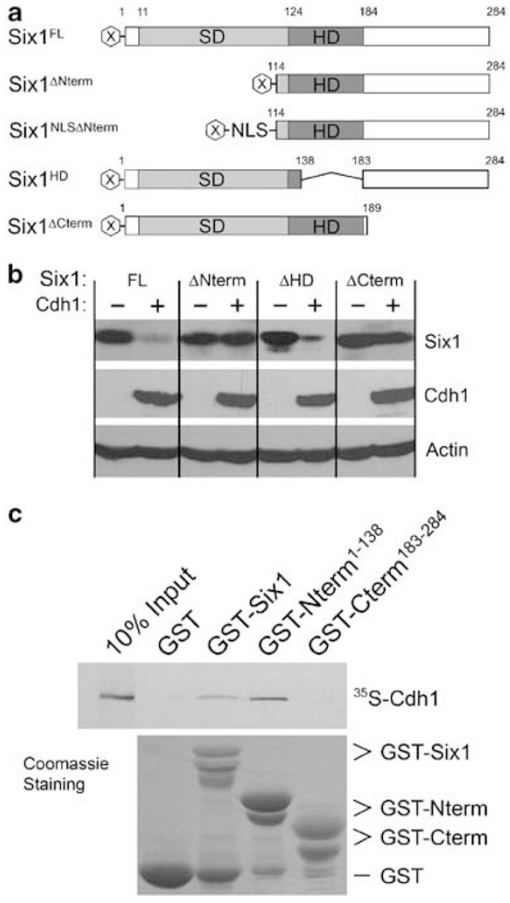

Figure 6 Six1 N- and C-termini are both required for efficient degradation, whereas only the N-terminus binds to Cdh1. (**a**) Diagram of Six1 deletions. The conserved Six domain (SD) is shown in light gray and homeodomain (HD) in darker gray. The Xpress epitope tag is indicated by a hexagon enclosed X. The SV40 NLS sequence (NLS) was added to the ΔNterm construct (NLSΔNterm) to force nuclear localization. (**b**) Deletion of either the N- or C-terminus stabilizes Six1 when co-expressed with Cdh1. Full-length Six1 or mutants lacking the Nterm (with an added NLS to force expression into the nucleus) (ΔNterm), the homeodomain (ΔHD) or the Cterm (ΔCterm) were co-transfected into MCF7 cells with Cdh1 or empty vector control. Whole-cell lysates were collected for Western blot analysis with anti-Xpress, anti-Cdh1 and anti-β-actin antibodies. (**c**) The N-terminus of Six1 binds to Cdh1. GST alone, or fused to full-length Six1, the Nterm of Six1 (amino acids 1-138) or the Cterm of Six1 (amino acids 183–284) were expressed in *E. coli*, then purified with Glutathione–sepharose beads. Fusion protein-bound beads were incubated with ^{35}S-labeled *in vitro* translated Cdh1, washed and then separated by 12% SDS–PAGE. Fusion proteins were visualized by Coomassie staining (lower panel), then dried, and bound-Cdh1 was visualized by autoradiography (upper panel).

boxes, we wished to determine whether the Six1 could bind to Cdh1, as observed for direct targets of APC^{Cdh1}. Glutathione *S*-transferase (GST) pull-down assays were performed with GST-Six1 fusion proteins and recombinant Cdh1. Both full-length GST-Six1 and GST-Nterm (1-138) bind to Cdh1, whereas the GST-Cterm (183-284) does not bind Cdh1 (Figure 6c). This suggests that Six1 is a direct target of Cdh1, and that the first 138 amino acids of the Six1 protein contain a novel degradation motif that recognizes and binds to the APC activator Cdh1. In contrast, the C-terminus does not bind directly to Cdh1 (Figure 6c), but is nevertheless also required for efficient Cdh1-mediated degradation (Figure 6b).

Discussion

Ubiquitin-mediated proteolysis is an important mechanism of confining expression of key cell cycle regulators to the appropriate cell cycle stages. The E3 ubiquitin ligase APC directs degradation of many proteins important in regulating progression through mitosis, exit from mitosis and events in G_1. Our studies show that the Six1 homeoprotein is regulated in a cell cycle-specific manner by ubiquitin-mediated proteolysis through APC^{Cdh1}. Targeted degradation of Six1 during late mitosis and G_1 indicate the importance of downregulating its activity during these phases of the cell cycle.

Six1 degradation is independent of known APC degradation motifs, suggesting that novel APC-targeting motifs exist. This is not surprising as known destruction motifs are poorly defined by only 2–3 critical residues. Recent evidence indicates that Cdh1 and Cdc20 interact specifically with the D-box and KEN box destruction motifs of their substrates (Burton *et al*., 2005; Kraft *et al*., 2005). Thus, it is has been suggested that substrates lacking canonical D-boxes may contain a three-dimensional interaction surface similar to a D-box but with differing primary amino acid sequence (Eytan *et al*., 2006). Our experiments suggest that the N-terminus of Six1 contains a novel motif mediating interaction with Cdh1 and directing APC^{Cdh1}-mediated degradation of Six1, as deletion of the N-terminus stabilizes Six1 to degradation mediated by APC^{Cdh1} and GST pull-down experiments indicate that the N-terminus alone is sufficient for binding to Cdh1.

In addition, Six1 contains a second domain in the C-terminus that is required for degradation. Our experiments demonstrate that deletion of either the N-terminus or the C-terminus stabilizes Six1 to degradation by APC^{Cdh1}. A double recognition motif such as this has been found in other APC^{Cdh1} substrates (Castro *et al*., 2005) and may be due to the fact that the core APC, as well as the activators Cdh1 and Cdc20, participate in substrate-binding interactions (Yamano *et al*., 2004; Passmore and Barford, 2005; Eytan *et al*., 2006). It is thus possible that the N-terminus of Six1 contains a novel motif important for Cdh1 binding, whereas the C-terminus contains an additional element that binds to the core APC.

This study demonstrates a third mechanism of cell cycle-specific regulation of Six1 expression; that of ubiquitin-mediated proteolysis. The concerted action of the three identified pathways (see Figure 7) confines Six1 activity to specific stages of the cell cycle. Six1 is regulated transcriptionally by E2F1 (Young *et al*., 2003), and its transcript levels increase as cells progress into S phase (Ford *et al*., 1998). This S-phase increase in Six1 transcription leads to an accumulation of Six1

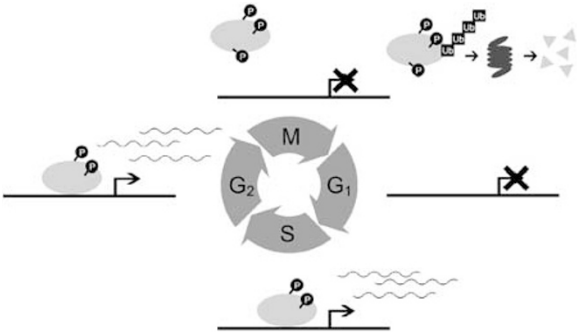

Figure 7 Proposed model of Six1 regulation by multiple mechanisms in the cell cycle. Six1 is transcriptionally activated at the G_1/S boundary by E2F1, and transcript levels increase with progression through S phase. Six1 protein represented by the light gray oval, can then be synthesized and bind to regulatory regions of its target genes during S and G_2 phases. Although Six1 is known to cooperate with cofactors (Kenyon *et al*., 2005), for simplicity only Six1 is shown in the diagram. Six1 exists as a phosphoprotein throughout the cell cycle, but is hyper-phosphorylated during mitosis, inhibiting its ability to bind DNA. Thus, it appears that Six1 activity is first downregulated by phosphorylation during mitosis, then during late mitosis Six1 is degraded via ubiquitin-mediated proteolysis directed by APC^{Cdh1}. Six1 levels stay low through G_1 phase until downregulation of APC^{Cdh1} activity, combined with transcriptional induction of Six1, allows Six1 levels to increase again at the G_1/S boundary.

protein, allowing activation and/or repression of its transcriptional targets. Six1 activity is then downregulated in mitosis by hyperphosphorylation, which inhibits its ability to bind DNA (Ford *et al*., 2000). Finally, later in mitosis, Six1 is ubiquitinated by APC^{Cdh1}, targeting it for degradation by the 26S proteasome. Continued APC^{Cdh1} activity presumably keeps Six1 levels low through G_1, after which levels increase again at the G_1/S boundary owing to increased transcription and increased protein stability. The multiple mechanisms of Six1 regulation throughout the cell cycle suggest that it is critical to confine the activity of this transcription factor to specific cell cycle stages, where it is expected to play an important role. Indeed, our previous studies in tissue culture cells have demonstrated that Six1 overexpression can alter progression through S phase and also through G_2/M (Ford *et al*., 1998; Coletta *et al*., 2004), times when Six1 activity would be expected to be high. Furthermore, a number of knockout studies in mice have demonstrated a critical role for Six1 in the proliferation of precursor cell populations, leaving no ambiguity about the importance of Six1 in proliferation *in vivo* (Zheng *et al*., 2003; Ozaki *et al*., 2004).

The APC is a central coordinator of cell cycle progression in mitosis and G_1, and the majority of APC targets are critical regulators of cell cycle related events (Peters, 2002). Degradation of Six1 by the APC is notable, as currently only two other transcription factors, HOXC10 (Gabellini *et al*., 2003) and Id2 (Lasorella *et al*., 2006), have been identified as APC targets. Like Six1, Id2 is a developmentally important transcription factor that affects cellular proliferation and tumor progression (Lasorella *et al*., 2001). Similarly, the homeoprotein HOXC10 has also been implicated in cell cycle regulation and is overexpressed in some cancers (Gabellini *et al*., 2003). It would be of interest to determine whether a number of transcription factors involved in regulating proliferation during development share this mechanism of regulation, and if disruption of APC-mediated degradation contributes to their aberrant expression in cancers.

Our work demonstrates that the Six1 homeoprotein and oncogene is a target of APC^{Cdh1}, and that its degradation is dependent on a novel motif for Cdh1 binding in the N-terminus of the protein. We suggest that appropriate degradation of Six1, mediated by the APC, is an important means of regulating its transcriptional activity appropriately both in the cell cycle and in development. To date, we have identified three independent mechanisms of Six1 regulation in the cell cycle, via transcription, phosphorylation and degradation. The existence of these overlapping pathways of regulation is indicative of the critical importance of confining Six1's activity from the G_1/S boundary through early mitosis.

Materials and methods

Mammalian cell culture and transfections

MCF7 and 21PT cells were grown as described (Ford *et al*., 1998, 2000). The proteasome and other cellular proteases were inhibited by culturing cells for 12 h in the presence of 25 μM MG132 (Sigma, St Louis, MO, USA), 25 μM LLM (Sigma), 25 μM LLNL (Sigma) or 25 μM E-64 (Calbiochem, San Diego, CA, USA) dissolved in dimethyl sulfoxide (DMSO). Transiently transfections were performed using Superfect (Qiagen, Valencia, CA, USA) according to the manufacturer's specifications with a plasmid:superfect ratio of 1:4.

Mammalian expression plasmids

The untagged, full-length Six1 cDNA expression plasmid (SIXFL) as well as the His and XPRESS-tagged full-length Six1 cDNA (SKMFL) were previously described (Ford *et al*., 1998, 2000). The previously described deletions ΔNterm, ΔHD and ΔCterm (Ford *et al*., 2000) were inserted into pcDNA3.1-HisC (Invitrogen, Carlsbad, CA, USA) to add His and Xpress tags. NLSΔNterm was generated using a 5′ PCR primer including the coding sequence for the Simian virus 40 (SV40) NLS (PKKKRKV) to amplify the ΔNterm sequence, and then inserted into pcDNA3.1HisC. Point mutations in full-length Six1 (as described in Figure 4) were generated by codon substitution using PCR mutagenesis and inserted into pcDNA3.1HisC.

Cell synchronizations

21PT cells were arrested in G_0/G_1 by culturing for 48 h in serum-free Dulbecco's modified Eagle's medium plus antibiotics, then released into the cell cycle by washing with phosphate-buffered saline and replacing with normal growth medium. For the MCF7 synchrony, cells were transfected with SIXFL, and 8 h later treated with 100 ng/ml nocodazole for 13 h. A mitotic shake-off was performed, and the cells were replated in complete medium to release from the arrest. For both synchronies, the cell cycle profile at each time point was examined using flow cytometry of propidium iodide stained cells.

Western blotting, antibodies
Nuclear extracts were prepared as described previously (Jamieson *et al*., 1991). Whole-cell lysates (Ford *et al*., 2000) and Western blotting was performed as described (Ford *et al*., 2000). Anti-cyclin B1 (GNS1), anti-cdc20 (H-175) and anti-histone H1 (AE-4) antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA; anti-cdc6 (DCS-180) and anti-cdh1 (DH01) antibodies from Neomarkers; anti-β-actin (AC-174) and anti-β-tubulin (2.1) from Sigma; anti-HA (AU1) from Covance, Berkeley, CA, USA; and anti-Xpress from Invitrogen. The anti-Six1 antibody was generated as described previously (Ford *et al*., 2000).

Identification of Mitotic Phosphorylation Site in Six1
Full-length Six1 cDNA was subcloned into the pMALC2X vector (New England Biolabs, Ipswich, MA, USA). pMAL and pMALSix1 were bacterially expressed and purified on amylose resin according to the manufacturer's protocol (New England Biolabs). Purified proteins (25–50 μg) (still attached to the resin) were incubated in interphase or mitotic extracts from *Xenopus laevis* oocytes (a gift from Todd Stukenberg) for 30 min at room temperature. After incubation, the proteins were electrophoresed on 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels, appropriate bands were cut from the gel, trypsinized, and subjected to ion trap mass spectrometry (Wu *et al*., 2000) by William S Lane at the Harvard Microchemistry and Proteomics Analysis Facility. Protein coverage of the Six1 protein incubated in the interphase extract was 53%, whereas coverage of the Six1 protein incubated in the mitotic extract was 36%. Only those peptides that were covered in both samples (31% of the Six1 protein) were used to assess mitotic-specific phosphorylation. The mitotic-specific phosphorylated peptide and residue identified by mass spectrometry was then confirmed via a targeted ion MS/MS analysis.

In vivo *ubiquitination assay*
MCF7 cells were co-transfected with HA-tagged ubiquitin (a generous gift from Dr Dirk Bohmann), pcDNA3.1 and/or His-tagged Six1. Cells were treated with either MG132 or DMSO, lysed in guanidine hydrochloride buffer (6 M GuHCl, 100 mM sodium phosphate buffer pH 8.0 and 10 mM imidazole), passed through a 25G needle to shear the DNA, and then incubated for 3 h at room temperature with Ni-NTA beads (Qiagen). Beads were washed three times each with guanidine hydrochloride buffer, guanidine hydrochloride buffer diluted 1:4 in 25 mM Tris pH 6.8/20 mM imidazole and 25 mM Tris pH 6.8/20 mM imidazole. Beads, with bound His-tagged Six1, were heated at 100°C in 2 × sample buffer supplemented with 200 mM imidazole for 10 min, then analysed by Western blot.

Cdh1 overexpression assay
The Xpress-tagged Six1, or the indicated mutant, was co-transfected into MCF7 cells with pCMV-HA-Cdh1 (a gift from Dr Kristin Helin) or empty vector. Whole-cell lysates were prepared 40–48 h post-transfection.

Immunocytochemistry
MCF7 cells were transfected with Xpress-tagged Six1, or appropriate mutants. Subsequently, cells were fixed in 0.7% formaldehyde then permeablized with 0.5% Triton X-100. Immunofluorescence was performed with an anti-Xpress antibody (1:000), biotinylated goat anti-mouse immunoglobulin G (1:15) (Calbiochem), and streptavidin-fluorescein (1:100) (Calbiochem); and then mounted in Vectashield (Vector Labs, Burlingame, CA, USA) containing 0.1 μg/ml 4′-6-diamidino-2-phenylindole (DAPI).

RNA interference
pSUPER, pSUPER-Cdc20 and pSUPER-Cdh1 plasmids (Brummelkamp *et al*., 2002) (gifts from Dr Reuven Agami) were transfected by electroporation as described previously (Agami and Bernards, 2000). The protocol was modified for 4 mm gap cuvettes to 300 V delivered in 15 pulses with 2 ms duration, 1.5 s interval. Nuclear extracts were prepared 60 h post-transfection.

GST pull-down experiments
Full-length Six1 cDNA, the N-terminus (encoding amino acids 1-138) and the C-terminus (encoding amino acids 183-284) were subcloned into pGEX2T (Pharmacia Biotech). GST-Six1, GST- Nterm, GST-Cterm, or GST alone were transformed into DH5α *Escherichia coli* cells (Invitrogen), and expression induced for 5 h with 100 μM isopropyl-thiogalactopyranoside. Cells were then resuspended in NETN buffer (Ford and Zain, 1995), lysed by sonication, and centrifuged. The supernatant was incubated with glutathione–sepharose Beads (Amersham, Piscataway, NJ, USA) in NETN plus 5% dry milk overnight at 4°C. The TNT® Coupled Transcription/Translation System (Promega, Madison, WI, USA) was used with ^{35}S-methionine (Amersham Pharmacia, Piscataway, NJ, USA) to *in vitro* translate Cdh1 from the pCS-Cdh1 vector (a gift from Dr Marc Kirschner). Radiolabeled Cdh1 was incubated with beads containing bound GST, GST-Six1, GST-Nterm or GST-Cterm, in binding buffer (50 mM Tris pH7.5, 120 mM NaCl, 2 mM ethylenediaminetetraacetic acid, 0.2% Igepal CA-630, 1 mg/ml bovine serum albumin (BSA)) for 1 h at 4°C. Beads were washed four times in binding buffer minus BSA, then heated to 100°C with 2 × sample buffer. Samples were electrophoresed on a 12% SDS–polyacrylamide gel electrophoresis (PAGE) gel, Coomassie stained, dried and visualized by autoradiography.

Acknowledgements

We thank William S Lane at the Harvard Microchemistry and Proteomics Analysis Facility for performing mass spectrometry analysis. We thank Carol Dacwag and Edith Kabingu for technical support. We are grateful to Paul Jedlicka, Andrew Bradford and members of the Ford lab for helpful discussions and critical reading of the manuscript. KLC was funded by a predoctoral fellowship from the National Science Foundation. This work was funded by a grant from the National Cancer Institute to HLF (RO1 CA095277).

References

Agami R, Bernards R. (2000). Distinct initiation and maintenance mechanisms cooperate to induce G1 cell cycle arrest in response to DNA damage. *Cell* **102**: 55–66.

Araki M, Yu H, Asano M. (2005). A novel motif governs APC-dependent degradation of Drosophila ORC1 *in vivo*. *Genes Dev* **19**: 2458–2465.

Brodbeck S, Besenbeck B, Englert C. (2004). The transcription factor Six2 activates expression of the Gdnf gene as well as its own promoter. *Mech Dev* **121**: 1211–1222.

Brummelkamp TR, Bernards R, Agami R. (2002). A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296**: 550–553.

Burton JL, Tsakraklides V, Solomon MJ. (2005). Assembly of an APC-Cdh1-substrate complex is stimulated by engagement of a destruction box. *Mol Cell* **18**: 533–542.

Castro A, Bernis C, Vigneron S, Labbe JC, Lorca T. (2005). The anaphase-promoting complex: a key factor in the regulation of cell cycle. *Oncogene* **24**: 314–325.

Castro A, Vigneron S, Bernis C, Labbe JC, Lorca T. (2003). Xkid is degraded in a D-box, KEN-box, and A-box-independent pathway. *Mol Cell Biol* **23**: 4126–4138.

Coletta RD, Christensen K, Reichenberger KJ, Lamb J, Micomonaco D, Huang L *et al*. (2004). The Six1 homeoprotein stimulates tumorigenesis by reactivation of cyclin A1. *Proc Natl Acad Sci USA* **101**: 6478–6483.

Crane R, Kloepfer A, Ruderman JV. (2004). Requirements for the destruction of human Aurora-A. *J Cell Sci* **117**: 5975–5983.

Eytan E, Moshe Y, Braunstein I, Hershko A. (2006). Roles of the anaphase-promoting complex/cyclosome and of its activator Cdc20 in functional substrate binding. *Proc Natl Acad Sci USA* **103**: 2081–2086.

Fang G, Yu H, Kirschner MW. (1998). Direct binding of CDC20 protein family members activates the anaphase-promoting complex in mitosis and G1. *Mol Cell* **2**: 163–171.

Ford HL, Kabingu EN, Bump EA, Mutter GL, Pardee AB. (1998). Abrogation of the G2 cell cycle checkpoint associated with overexpression of HSIX1: a possible mechanism of breast carcinogenesis. *Proc Natl Acad Sci USA* **95**: 12608–12613.

Ford HL, Landesman-Bollag E, Dacwag CS, Stukenberg PT, Pardee AB, Seldin DC. (2000). Cell cycle-regulated phosphorylation of the human SIX1 homeodomain protein. *J Biol Chem* **275**: 22245–22254.

Ford HL, Zain SB. (1995). Interaction of metastasis associated Mts1 protein with nonmuscle myosin. *Oncogene* **10**: 1597–1605.

Gabellini D, Colaluca IN, Vodermaier HC, Biamonti G, Giacca M, Falaschi A *et al*. (2003). Early mitotic degradation of the homeoprotein HOXC10 is potentially linked to cell cycle progression. *EMBO J* **22**: 3715–3724.

Hershko A, Ciechanover A. (1998). The ubiquitin system. *Annu Rev Biochem* **67**: 425–479.

Jamieson C, McCaffrey PG, Rao A, Sen R. (1991). Physiologic activation of T cells via the T cell receptor induces NF-kappa B. *J Immunol* **147**: 416–420.

Kawakami K, Sato S, Ozaki H, Ikeda K. (2000). Six family genes–structure and function as transcription factors and their roles in development. *Bioessays* **22**: 616–626.

Kenyon KL, Yang-Zhou D, Cai CQ, Tran S, Clouser C, Decene G *et al*. (2005). Partner specificity is essential for proper function of the SIX-type homeodomain proteins Sine oculis and Optix during fly eye development. *Dev Biol* **286**: 158–168.

Kraft C, Vodermaier HC, Maurer-Stroh S, Eisenhaber F, Peters JM. (2005). The WD40 propeller domain of Cdh1 functions as a destruction box receptor for APC/C substrates. *Mol Cell* **18**: 543–553.

Lasorella A, Stegmuller J, Guardavaccaro D, Liu G, Carro MS, Rothschild G *et al*. (2006). Degradation of Id2 by the anaphase-promoting complex couples cell cycle exit and axonal growth. *Nature* **442**: 471–474.

Lasorella A, Uo T, Iavarone A. (2001). Id proteins at the cross-road of development and cancer. *Oncogene* **20**: 8326–8333.

Li CM, Guo M, Borczuk A, Powell CA, Wei M, Thaker HM *et al*. (2002). Gene expression in Wilms' tumor mimics the earliest committed stage in the metanephric mesenchymal-epithelial transition. *Am J Pathol* **160**: 2181–2190.

Li X, Oghi KA, Zhang J, Krones A, Bush KT, Glass CK *et al*. (2003). Eya protein phosphatase activity regulates Six1-Dach-Eya transcriptional effects in mammalian organogenesis. *Nature* **426**: 247–254.

Littlepage LE, Ruderman JV. (2002). Identification of a new APC/C recognition domain, the A box, which is required for the Cdh1-dependent destruction of the kinase Aurora-A during mitotic exit. *Genes Dev* **16**: 2274–2285.

Nguyen HG, Chinnappan D, Urano T, Ravid K. (2005). Mechanism of Aurora-B degradation and its dependency on intact KEN and A-boxes: identification of an aneuploidy-promoting property. *Mol Cell Biol* **25**: 4977–4992.

Ozaki H, Nakamura K, Funahashi J, Ikeda K, Yamada G, Tokano H *et al*. (2004). Six1 controls patterning of the mouse otic vesicle. *Development* **131**: 551–562.

Park KH, Choi SE, Eom M, Kang Y. (2005). Downregulation of the anaphase-promoting complex (APC)7 in invasive ductal carcinomas of the breast and its clinicopathologic relationships. *Breast Cancer Res* **7**: R238–R247.

Passmore LA, Barford D. (2005). Coactivator functions in a stoichiometric complex with anaphase-promoting complex/cyclosome to mediate substrate recognition. *EMBO Rep* **6**: 873–878.

Peters JM. (2002). The anaphase-promoting complex: proteolysis in mitosis and beyond. *Mol Cell* **9**: 931–943.

Reichenberger KJ, Coletta RD, Schulte AP, Varella-Garcia M, Ford HL. (2005). Gene amplification is a mechanism of Six1 overexpression in breast cancer. *Cancer Res* **65**: 2668–2675.

Wu X, Ranganathan V, Weisman DS, Heine WF, Ciccone DN, O'Neill TB *et al*. (2000). ATM phosphorylation of Nijmegen breakage syndrome protein is required in a DNA damage response. *Nature* **405**: 477–482.

Yamano H, Gannon J, Mahbubani H, Hunt T. (2004). Cell cycle-regulated recognition of the destruction box of cyclin B by the APC/C in *Xenopus* egg extracts. *Mol Cell* **13**: 137–147.

Young AP, Nagarajan R, Longmore GD. (2003). Mechanisms of transcriptional regulation by Rb-E2F segregate by biological pathway. *Oncogene* **22**: 7209–7217.

Yu Y, Davicioni E, Triche TJ, Merlino G. (2006). The homeoprotein Six1 transcriptionally activates multiple protumorigenic genes but requires Ezrin to promote metastasis. *Cancer Res* **66**: 1982–1989.

Zheng W, Huang L, Wei ZB, Silvius D, Tang B, Xu PX. (2003). The role of Six1 in mammalian auditory system development. *Development* **130**: 3989–4000.