CUL-4A stimulates ubiquitylation and degradation of the HOXA9 homeodomain protein

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The HOXA9 homeodomain protein is a key regulator of hematopoiesis and embryonic development. HOXA9 is expressed in primitive hematopoietic cells, and its prompt downregulation is associated with myelocytic maturation. Although transcriptional inactivation of HOXA9 during hematopoietic differentiation has been established, little is known about the biochemical mechanisms underlying the subsequent removal of HOXA9 protein. Here we report that the CUL-4A ubiquitylation machinery controls the stability of HOXA9 by promoting its ubiquitylation and proteasome-dependent degradation. The homeodomain of HOXA9 is responsible for CUL-4A-mediated degradation. Interfering CUL-4A biosynthesis by ectopic expression or by RNA-mediated interference resulted in alterations of the steady-state levels of HOXA9, mirrored by impairment of the ability of 32D myeloid progenitor cells to undergo proper terminal differentiation into granulocytes. These results revealed a novel regulatory mechanism of hematopoiesis by ubiquitin-dependent proteolysis.

Keywords: CUL-4A/hematopoiesis/homeodomain/
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Introduction

The HOX homeodomain (HD) proteins are DNA-binding transcription factors that have long been recognized as key regulators of development and hematopoiesis (reviewed in McGinnis and Krumlauf, 1992; Magli *et al.*, 1997; Thorsteinsdottir *et al.*, 1997). In vertebrates, there are 39 HOX genes that are organized into four chromosomal clusters (HOXA, B, C and D), which can be classified into 13 paralogous groups based on their extensive sequence homology within the HD and their relative chromosomal locations within a cluster (reviewed in Gehring *et al.*, 1994). HOX genes are expressed at various stages during

hematopoietic development. Mounting evidence suggests that HOXA9, a member of the abdominal-B subclass of HOX genes, plays important roles in normal hematopoiesis as well as in leukemia development. Targeted disruption of HOXA9 in mice severely reduced the number of hematopoietic stem and progenitor cells, indicating that HOXA9 is required for maintaining the population of primitive hematopoietic cells (Lawrence *et al.*, 1997). Conversely, enforced expression of HOXA9 promotes proliferative expansion of primitive hematopoietic stem/progenitor cells and subsequently inhibits their differentiation (Thorsteinsdottir *et al.*, 1997; Fujino *et al.*, 2001). Aberrant expression of HOXA9 due to retroviral integration or chromosomal translocation induced acute myeloid leukemia (AML) in mice and humans (Borrow *et al.*, 1996; Nakamura *et al.*, 1996a, b; Kroon *et al.*, 1998). From a genetic analysis of 6817 genes, HOXA9 was identified as the most correlative of poor prognosis in leukemia (Golub *et al.*, 1999). These data highlight the importance of precise control of HOXA9 protein levels at various stages of hematopoiesis.

The abundance of a given cellular protein is regulated by the interplay between its biosynthesis and degradation. During normal hematopoietic development, HOXA9 is strongly expressed in the CD34⁺ populations enriched in early myeloid progenitors, and is turned off when cells exit the CD34⁺ compartment and undergo terminal differentiation (Sauvageau *et al.*, 1994; Lawrence *et al.*, 1997). A similar expression pattern of HOXA9 was documented in the murine 32D myeloid progenitor cells (Fujino *et al.*, 2001). Further, downregulation of HOXA9 mRNA in 32D cells is mirrored by the reduction of functional HOXA9 DNA-binding complexes (Fujino *et al.*, 2001). In conjunction with decreased biosynthesis, rapid turnover of HOXA9 would ensure low steady-state levels, which are necessary for proper execution of differentiation into myeloid lineages. The studies of the biochemical mechanisms controlling the activities of HOXA9 thus far have been focused primarily on transcriptional regulation and signal transduction. Little is known about how their cellular abundance is controlled at the post-translational level. Identification of proteins involved in the removal of HOXA9 will be necessary for understanding the elaborate regulatory circuitry governing hematopoiesis as well as embryonic development.

A major pathway for targeted elimination of cellular proteins is ubiquitin-dependent proteolysis, which is a cascade of enzymatic reactions involving the E1 ubiquitin-activating enzyme, the E2 ubiquitin-conjugating enzyme and the E3 ubiquitin-protein ligase. As a result, multiple ubiquitin moieties are conjugated to the substrate, which in turn allows its recognition and degradation by the 26S proteasome (reviewed in Hershko and Ciechanover, 1998). E1 and E2 function in the activation and transfer of

ubiquitin, while E3 confers substrate specificity and facilitates transfer of ubiquitin from E2 to substrates. Most E3s are multimeric protein complexes. The cullin family of proteins recently have been identified as an essential component of certain RING domain-type E3s and serve as docking platforms in the assembly of E3 complexes (reviewed in Deshaies, 1999). There are multiple cullins in mammals that organize distinct E3 complexes (Kipreos *et al.*, 1996), and participate in the regulation of diverse cellular processes such as cell cycle, signal transduction and transcriptional regulation (reviewed in Deshaies, 1999). Aberrant expression or amplification of cullin 4A (*CUL-4A*) was found in primary breast and hepatocellular carcinomas (Chen *et al.*, 1998; Yasui *et al.*, 2002). Mice bearing a homozygous disruption of *CUL-4A* died by 7.5 days post-coitum, indicating an essential role for *CUL-4A* in early embryonic development (Li *et al.*, 2002a). Recent studies identified the damaged DNA-binding protein 2 (DDB2) as a proteolytic target of *CUL-4A*, suggesting a role for *CUL-4A* in nucleotide excision repair (Chen *et al.*, 2001; Nag *et al.*, 2001). Other targets of *CUL-4A* remain to be identified.

In an effort to delineate the function of the *CUL-4A* ubiquitylation machinery, we carried out a yeast two-hybrid screen and identified HOXA9, which specifically interacted with *CUL-4A*. *CUL-4A* associated with HOXA9 and stimulated its ubiquitylation and degradation in mammalian cells. Enforced expression of *CUL-4A* in 32D myeloid progenitor cells abrogated the HOXA9-induced differentiation block and enhanced granulopoiesis in response to granulocyte colony-stimulating factor (G-CSF). Conversely, silencing of *CUL-4A* expression by RNA interference (RNAi) led to increased accumulation of HOXA9 and inhibition of granulocytic differentiation. These results provide compelling evidence that the stability of HOXA9 is specifically regulated at the post-translational level by the *CUL-4A* ubiquitylation machinery, and highlight the proteolytic targeting of HOXA9 as a novel means that cells employ to control hematopoiesis.

Results

Identification of HOXA9 as a *CUL-4A*-interacting protein

To gain insight into the function of *CUL-4A*, we performed a yeast two-hybrid screen of a human HeLa cell library (Gibco) using *CUL-4A* as a bait. From 5×10^6 transformants, we identified a *CUL-4A*-interacting clone that contains the C-terminal portion of HOXA9 (amino acid residues 161–271), designated HOXA9(C), which encompasses the HD and the tryptophan motif (ANWL) for binding to the PBX family of HOX cofactors (Figure 1A). To determine whether HOXA9 and *CUL-4A* can interact *in vivo*, HOXA9(C) was expressed in HeLa cells as a GST fusion protein along with the FLAG-tagged *CUL-4A* (F-*CUL-4A*). GST-HOXA9(C) was precipitated from cell extracts using glutathione-Sepharose 4B beads (Amersham) and bound proteins were analyzed by western blotting with antibodies against FLAG (M2) for F-*CUL-4A*, or GST for GST-HOXA9(C). F-*CUL-4A* was indeed detected in the GST-HOXA9(C) precipitates, but not in GST precipitates despite comparable F-*CUL-4A* levels in both (Figure 1B and C). This confirmed that the

Fig. 1. *CUL-4A* interacts with the HOXA9 homeodomain protein. (A) Schematic representation of the domain structure of HOXA9 and the HOXA9(C) fragment identified in a yeast two-hybrid assay as a *CUL-4A* interactor. MIM, MEIS1 interaction motif; HD, homeodomain; gray box, tryptophan consensus motif for PBX binding. (B) *CUL-4A* interacts with HOXA9 in mammalian cells. F-*CUL-4A* was co-transfected into HeLa cells with GST-HOXA9(C) or GST alone (in µg). GST or GST-HOXA9(C) was precipitated from 500 µg of cell extracts using glutathione-Sepharose 4B beads (Amersham), and immunoblotting was carried out to detect F-*CUL-4A* and GST-HOXA9(C) using the anti-FLAG (M2) or anti-GST monoclonal antibodies. (C) The expression of F-*CUL-4A* was detected from 200 µg of extracts by immunoblotting using the anti-FLAG (M2) antibody.

CUL-4A–HOXA9 complex also formed *in vivo*. In addition, F-*CUL-4A* and GST-HOXA9(C) co-fractionated in a glycerol gradient centrifugation, further suggesting the physical association between these two proteins (data not shown). These results also indicate that the C-terminal portion of HOXA9 is sufficient for interaction with *CUL-4A*.

Expression of *CUL-4A* in hematopoietic cells

To determine the expression and subcellular distribution of *CUL-4A* at different stages of hematopoietic differentiation, we performed an indirect immunofluorescence assay on primitive human CD34⁺ as well as differentiated primary hematopoietic cells using an affinity-purified anti-*CUL-4A* polyclonal antibody (Chen *et al.*, 2001). As shown in Figure 2A, *CUL-4A* is localized primarily in the cytoplasm of CD34⁺ cells, as well as in monocytes, macrophages, neutrophils (Figure 2A), and T and B lymphocytes (data not shown). Therefore, *CUL-4A* is expressed throughout hematopoietic development. By immunofluorescent confocal microscopy, ~5% of

Fig. 2. *CUL-4A* is expressed in primitive and differentiated hematopoietic cells. (A) An indirect immunofluorescence assay was carried out on the indicated cells using the anti-CUL-4A polyclonal antibody and secondary antibody conjugated to Cy3 (top). The same cells were also stained with DAPI and photographed under phase contrast lenses (bottom). (B) Subcellular localization of CUL-4A in primary CD34⁺ hematopoietic stem/progenitor cells by immunofluorescent confocal microscopy. CD34⁺ cells were also stained with the TO-PRO-3 dye for visualization of nuclei.

CUL-4A could also be detected in the nucleus of CD34⁺ cells (Figure 2B).

CUL-4A mediates ubiquitylation and proteasome-dependent degradation of HOXA9

Since CUL-4A is a member of the cullin family of ubiquitin–protein ligases and can bind to HOXA9, we first investigated whether CUL-4A could reduce the steady-state levels of the HOXA9 protein. In HeLa cells, co-expression of *CUL-4A* with *HOXA9* led to a considerable, CUL-4A dose-dependent downregulation of HOXA9 (Figure 3A). The mRNA levels of transfected *HOXA9* were not reduced in response to the ectopic expression of *CUL-4A* (Figure 3B), indicating that the CUL-4A-induced reduction of HOXA9 occurred at translational or post-translational levels. The decrease in HOXA9 was specific for CUL-4A, as overexpression of *CUL-1*, the cullin subunit of the SCF (SKP1, CUL-1 and F-box-containing substrate receptor) ubiquitin–protein ligase, did not affect the HOXA9 levels (Figure 3C). Further, *CUL-4A* expression was unable to downregulate HOXA9 in the presence of the synthetic vinyl sulfone proteasome inhibitor NLVS (Figure 3D), indicating that CUL-4A-dependent reduction of HOXA9 is mediated through the proteasome pathway.

The cullin homology domain of CUL-4A is responsible for binding the RING domain protein Rbx1/Roc1/Hrt1 and for connecting to the E2 ubiquitin-conjugating enzyme for

ubiquitin delivery. To assess whether the ubiquitin–protein ligase activity of CUL-4A was required for HOXA9 downregulation, we co-expressed *HOXA9* with a mutant form of CUL-4A that was deleted of the cullin homology domain [*CUL-4A*(Δ)] (Chen *et al.*, 2001). CUL-4A(Δ) was incapable of recruiting the E2 ubiquitin-conjugating enzyme due to the loss of Rbx1/Roc1/Hrt1 binding (Furukawa *et al.*, 2000), but still retained its ability to interact with HOXA9 (data not shown). In contrast to the downregulation of HOXA9 by the wild-type CUL-4A, ectopic expression of increasing amounts of *CUL-4A*(Δ) failed to degrade HOXA9, but rather resulted in a dose-dependent increase in HOXA9 accumulation (Figure 3E). Therefore, the cullin homology domain is required for CUL-4A-mediated reduction of HOXA9. Taken together, these studies provide strong evidence that the steady-state levels of HOXA9 are controlled by the CUL-4A ubiquitylation machinery.

To investigate whether CUL-4A-stimulated downregulation of HOXA9 was a result of accelerated HOXA9 degradation, we measured the turnover rate of HOXA9 by pulse–chase analysis. HOXA9 is a relatively stable protein with a half-life of 26 h in HeLa cells (Figure 3F). Ectopic expression of *CUL-4A* resulted in a dramatic shortening of the HOXA9 half-life to ~3.1 h, whereas *CUL-1* or *CUL-4A*(Δ) expression did not affect the turnover rate of HOXA9 (Figure 3F). Therefore, CUL-4A specifically

Fig. 3. CUL-4A stimulates HOXA9 degradation by the ubiquitin-proteasome pathway. (A) HeLa cells were transiently transfected with *HA-HOXA9* and increasing doses of *F-CUL-4A* (in μ g). The steady-state levels of HOXA9 and CUL-4A were determined from 100 μ g of cell extract by immunoblotting using antibodies against HA-HOXA9, F-CUL-4A or β -actin. A 2 μ g aliquot of pGREEN LANTERN-1 plasmid was routinely included in each transfection in this and the following experiments, and comparable 50–60% transfection efficiencies were observed. (B) mRNA levels of *HOXA9* were determined in the same transfected HeLa cells as in (A) by Northern blotting using a 32 P-labeled probe encompassing the 600 bp *HincII*–*BglIII* fragment of *HOXA9* cDNA. (C) *MYC-CUL-1* was transiently transfected into HeLa cells together with *HA-HOXA9* (in μ g) as in (A) to assess the effect of another cullin family member on HOXA9 stability. (D) Expression plasmids of *HA-HOXA9* and *F-CUL-4A* were transiently transfected into HeLa cells, and treated in either the absence or presence of 20 μ M proteasome inhibitor NLVS to examine the HOXA9 levels by immunoblotting. (E) Transfection of increasing amount of *CUL-4A*(Δ) (in μ g) in HeLa cells resulted in an increased accumulation of HOXA9, as assessed by immunoblotting as in (A). (F) CUL-4A accelerates the turnover rate of HOXA9. HeLa cells were transiently transfected with *MYC-HOXA9* alone, or together with *F-CUL-4A*, *F-CUL-4A*(Δ) or *MYC-CUL-1*. Pulse-chase analysis was conducted to determine the decay of HOXA9 over time, which was quantitatively measured using phosphorimager scanning. The percentage of HA-HOXA9 remaining was graphed on a logarithmic scale over time (in hours). (G) HeLa cells were transfected with the plasmids as indicated (in μ g). An *in vivo* ubiquitylation assay was conducted as described (Chen *et al.*, 2001). Ubiquitylated MYC-HOXA9 species are indicated on the right (*). The migration positions of molecular mass standards (in kDa) are indicated on the left. IgG, immunoglobulin heavy chain. Expression of *MYC-HOXA9* and *F-CUL-4A* was also detected from 100 μ g of each extract by immunoblotting using the anti-MYC (9E10) and anti-FLAG (M2) monoclonal antibodies.

Fig. 4. The homeodomain contains the degradation signal for CUL-4A-mediated HOXA9 proteolysis. **(A)** Schematic representation of HOXA9 (as in Figure 1A) and the HOXA9 HD truncation mutants. **(B)** HeLa cells were transfected with *MYC-HOXA9*(Δ 1), *MYC-HOXA9*(Δ 2) or *MYC-HOXA9*(Δ 3) along with increasing amounts of *MYC-CUL-4A* (in μ g). Cellular extracts were prepared and subjected to western blotting using antibodies against MYC tag (9E10) or β -actin. **(C)** Pulse-chase analysis on HeLa cells expressing *MYC-CUL-4A* together with *MYC-HOXA9*(Δ 1), *MYC-HOXA9*(Δ 2) or *MYC-HOXA9*(Δ 3). The half-lives of the individual HOXA9 truncations were determined as in Figure 3F. Band intensities were quantitatively measured by phosphorimager scanning, and the percentage of HOXA9 truncations remaining at each time point were graphed on a logarithmic scale over time (in hours).

reduces HOXA9 levels at post-translationally through promoting its degradation.

We next tested whether CUL-4A-mediated HOXA9 degradation is ubiquitin dependent. MYC-tagged HOXA9 (MYC-HOXA9) and hemagglutinin (HA)-tagged ubiquitin were transiently transfected into HeLa cells together with F-CUL-4A. Cellular proteins modified by HA-ubiquitin were immunoprecipitated by the anti-HA antibody and probed with the anti-MYC antibody (9E10) to detect ubiquitin-modified MYC-HOXA9. As shown in Figure 3G, a series of high molecular weight, ubiquitin-conjugated HOXA9 species were readily observed (Figure 3G, lane 2), and were significantly increased by ectopic *CUL-4A* expression (Figure 3G, lane 3). Treatment of these HeLa cells with the MG132 proteasome inhibitor resulted in a further increase in HOXA9 ubiquitylation (Figure 3G, lane 4), consistent with the stabilization of HOXA9 by the proteasome inhibitor (Figure 3D). Conversely, ectopic expression of *CUL-4A*(Δ) led to a dose-dependent inhibition of HOXA9 ubiquitylation (data not shown). Therefore, CUL-4A stimulates degradation of HOXA9 through promoting its ubiquitylation. Collectively, these studies provide strong evidence that HOXA9 is subjected to CUL-4A-mediated ubiquitylation and degradation.

The homeodomain is required for HOXA9 proteolysis

To determine the signal sequence required for CUL-4A-mediated HOXA9 degradation, we assessed the stability of

HOXA9 truncation mutants in response to ectopic expression of *CUL-4A*. HeLa cells were transiently transfected with MYC-tagged HOXA9(Δ 1), (Δ 2) and (Δ 3) truncation plasmids (Figure 4A), along with either the control vector pcDNA3 or increasing amounts of *MYC-CUL-4A*, and the steady-state levels of HOXA9 mutants were determined by immunoblotting. As shown in Figure 4B, ectopic expression of *MYC-CUL-4A* induced a dramatic decrease in both HOXA9(Δ 1) and HOXA9(Δ 2), which contained the HD of HOXA9. However, the steady-state levels of HOXA9(Δ 3), in which the HD was deleted but which retained the tryptophan motif (ANWL) located immediately N-terminal to the HOX HD for PBX binding, as well as the MEIS interaction motif (MIM) for binding to another HOX cofactor MEIS1, were not affected by *CUL-4A* expression (Figure 4B). Pulse-chase analysis in the presence of MYC-CUL-4A further showed that HOXA9(Δ 1) and HOXA9(Δ 2) were rapidly turned over with half-lives of 3 and 2.25 h, respectively. However, HOXA9(Δ 3) was stable, and no degradation was observed within the 5 h chase period (Figure 4C). Therefore, the C-terminal region encompassing the HD is required for CUL-4A-mediated HOXA9 degradation.

The HOX HD is highly conserved during evolution. The three-dimensional structure of the prototypic Antennapedia HD has been determined by nuclear magnetic resonance (NMR) spectroscopy, which revealed a tightly folded globular structure consisting of three helical domains separated by two flexible turns (Qian *et al.*, 1989). Helix III is primarily responsible for DNA binding,

while helix I is positioned away from DNA, and is thought to be involved in interaction with regulatory factors (Sharkey *et al.*, 1997). To define further the degradation signal within the HOXA9 HD required for CUL-4A-mediated degradation, we examined the stability of HOXA9(Δ 4), which contains the entire HOXA9(Δ 3) and the helix I region of HD (Figure 4A). In contrast to HOXA9(Δ 3), ectopic expression of *CUL-4A* reduced the steady-state levels of HOXA9(Δ 4) (Figure 5A). A vector-based small interfering RNA (siRNA) was generated for CUL-4A (nucleotides 760–778 from ATG) in the pSUPER vector (Brummelkamp *et al.*, 2002b), and demonstrated >90% reduction of CUL-4A in a transient co-transfection assay (Figure 5B). Co-expression of pSUPER-CUL-4A resulted in dramatic inhibition of the rapid turnover of HOXA9(Δ 4) (Figure 5C). Similar results were obtained with another HOXA9 deletion mutant that carries HOXA9(Δ 3) and all three α -helices of the HD (data not shown). Collectively, these results further defined the degradation signal within helix I of the HOXA9 HD.

***CUL-4A* overcomes the *HOXA9*-induced block of granulocyte differentiation in 32D (clone 3) myeloblast cells**

The interleukin-3 (IL-3)-dependent 32Dcl3 myeloid progenitor cells were originally derived from normal murine bone marrow (Greenberger *et al.*, 1983). In response to G-CSF, 32Dcl3 cells cease to proliferate and progressively differentiate into granulocytes. Fujino *et al.* (2001) recently demonstrated that *HOXA9* expression is down-regulated during G-CSF-induced granulocytic differentiation of 32Dcl3 cells. Enforced expression of *HOXA9* from the constitutive cytomegalovirus (CMV) promoter blocked G-CSF-dependent granulopoiesis and maturation, indicating that downregulation of *HOXA9* is critical for myeloid differentiation. Importantly, the *HOXA9* transgene is not subjected to transcriptional inhibition upon G-CSF treatment, unlike that with the endogenous *HOXA9*. Therefore, the 32Dcl3 cell line stably expressing *HOXA9* (designated 32D-HOXA9) provides a defined experimental model that highlights the function and post-transcriptional regulation of *HOXA9* in hematopoietic differentiation, and allowed us to investigate whether CUL-4A-mediated proteolysis of *HOXA9* affects granulocytic maturation. *MYC-CUL-4A* was first introduced into 32D-HOXA9 cells to generate stable 32Dcl3 cell lines expressing both *MYC-CUL-4A* and *HOXA9*. Two independent clonal 32D cell lines, designated 32D-HOXA9/*MYC-CUL-4A* (6-16) and (7-1), were analyzed further for the effect of CUL-4A on *HOXA9* degradation and on granulocytic differentiation in response to G-CSF. The parental 32Dcl3 cells expressed detectable levels of endogenous *HOXA9*, and the overall *HOXA9* levels increased ~5-fold in the stable 32D-HOXA9 cells (Figure 6A, lanes 1 and 2) (Fujino *et al.*, 2001). Stable expression of *MYC-CUL-4A* resulted in a dramatic reduction of intracellular *HOXA9* in 32D-HOXA9/*MYC-CUL-4A* cells to below its basal level seen in parental 32Dcl3 cells (Figure 6A, compare lanes 4 and 5 with lanes 1 and 2). This result indicates that CUL-4A efficiently degrades *HOXA9* in the 32Dcl3 myeloid progenitor cells, which is consistent with the biochemical studies of CUL-4A-induced *HOXA9* degradation in HeLa cells (Figure 3).

Fig. 5. The degradation signal of *HOXA9* is defined within the helix I of the *HOXA9* HD. (A) Ectopic expression of *CUL-4A* decreased the steady-state levels of *HOXA9*(Δ 4) in HeLa cells. (B) *CUL-4A* siRNA induced a dramatic decrease in *CUL-4A* levels. pSUPER-CUL-4A or pSUPER-p53 (control) were transiently co-transfected with MYC-CUL-4A in HeLa cells to assess the extent of *CUL-4A* knockdown by immunoblotting. (C) *CUL-4A* siRNA prolonged the half-life of *HOXA9*(Δ 4). Pulse-chase analysis was carried out in HeLa cells as in Figure 3F to determine the half-life of *HOXA9*(Δ 4) in the presence or absence of co-transfected pSUPER-CUL-4A. The percentage of *HOXA9*(Δ 4) remaining was graphed on a logarithmic scale over time (in hours).

We thus examined whether ectopic expression of *CUL-4A* could overcome the *HOXA9*-induced block of granulocytic differentiation (Fujino *et al.*, 2001). The course of G-CSF-induced 32D granulopoiesis can be classified into three distinct stages based on the characteristic nuclear morphology: (i) undifferentiated blasts (round or oval nucleus, lacy chromatin); (ii) intermediate cells including promyelocytes, myelocytes (round nucleus and condensed chromatin) and metamyelocytes (kidney-bean-shaped nucleus and more condensed chromatin); and (iii) terminally differentiated granulocytes (horseshoe-shaped nucleus or segmented nucleus with two or more lobes) (Tan-Pertel *et al.*, 2000). Exponentially growing 32D, 32D-HOXA9, 32D-HOXA9/pcDNA3 and 32D-HOXA9/*MYC-CUL-4A* (clones 6-16 and 7-1) were induced to differentiate for 8 days in the presence of 20 ng/ml of G-CSF. Figure 6B shows the morphology of 32D and stable 32D cell lines grown in IL-3-containing (left panels) or G-CSF-containing medium (right panels); the percentage of undifferentiated, intermediate and terminally differentiated cells was determined in day 8

Fig. 6. CUL-4A ablates the HOXA9-induced differentiation block of 32Dcl3 myeloid progenitor cells. (A) Western blotting to detect stable expression of *HOXA9* and *MYC-CUL-4A* in 100 μ g of 32Dcl3, 32D-HOXA9, 32D-HOXA9/pcDNA3 and 32D-HOXA9/MYC-CUL-4A (clones 6-16 and 7-1) extracts using antibodies against HOXA9, MYC tag or β -actin. (B) Wright-Giemsa staining of the indicated 32Dcl3 cell lines grown in the presence of 5 ng/ml of IL-3, or induced to differentiate with 20 ng/ml of G-CSF for 8 days. Hemocytograms of the resulting 32Dcl3 cell lines are indicated on the right. The percentages of myoblast, intermediate and granulocytic cells were obtained by counting a total of 500 cells in each experiment, and data are presented as mean $\pm$ SD from four independent experiments. (C) The indicated 32Dcl3 cell lines were either untreated or treated with 20 ng/ml of G-CSF for 8 days, and analyzed by flow cytometry for expression of MAC1 (mCD11b) as a marker for granulocytic differentiation. Histograms showed the quantitation of MAC1-FITC⁺ staining and M1 values in each column indicate the percentages of differentiated (MAC1-positive) 32Dcl3 cells.

G-CSF cultures. A significant portion of 32D cells underwent granulocytic maturation and showed the typical multinucleated granulocyte morphology upon G-CSF treatment (Figure 6B, top right panel). Enforced expression of *HOXA9* in the stable 32D-HOXA9 cells inhibited granulocytic differentiation, and the majority of the G-CSF-treated 32D-HOXA9 cells remained at the undifferentiated, mononucleated blast (65%) or intermediate stages (27%) (Figure 6B, upper middle panel) (Fujino *et al.*, 2001). Similar results were obtained with 32D-HOXA9/pcDNA3 cells (57% blast, 39% intermediates) (Figure 6B, lower middle panel). Only 4–8% of terminally differentiated cells were observed in these *HOXA9*-expressing 32D cells. In contrast, all the *CUL-4A*-expressing 32D-HOXA9/MYC-CUL-4A clones examined exhibited a degree of granulocytic differentiation comparable with that of the parental 32Dcl3 cells, with significantly higher levels of terminally differentiated (28%) and intermediate (47%) populations compared with the 32D-HOXA9 cells (Figure 6B, lower panel). Therefore, ectopic expression of *CUL-4A* restored the sensitivity in

32D-HOXA9 cells to G-CSF-induced granulocytic differentiation.

G-CSF-induced differentiation of 32D cells can also be quantitated by measuring the expression of the mature myeloid cell surface marker MAC1/mCD11b. To examine further the effect of *CUL-4A* on overcoming the *HOXA9*-mediated block of granulocytic differentiation, flow cytometry analyses were carried out to determine the percentage of MAC1-positive cells following 8 days of G-CSF treatment. As shown in Figure 6C, 13.27% of 32Dcl3 cells were MAC1 positive. Enforced expression of *HOXA9* in 32D-HOXA9 or 32D-HOXA9/pcDNA3 cells led to a marked reduction of MAC1-positive cells (0.75–5.37%) (Figure 6C). Expression of *MYC-CUL-4A* resulted in a 25-fold increase in MAC1-positive 32D-HOXA9/CUL-4A cells (19.10%) compared with the control 32D-HOXA9/pcDNA3 cells (0.75%). Noticeably, 32D-HOXA9/MYC-CUL-4A cells demonstrated greater increased sensitivity to G-CSF-induced differentiation (19.10%) than the parental 32Dcl3 cells (13.27%) (Figure 6C). This is consistent with a reduction of the overall levels of

Fig. 7. CUL-4A knockdown by RNAi resulted in increased HOXA9 levels and inhibition of granulocyte maturation. (A) 32Dcl3 cells were infected by pRS-CUL-4A or pRS recombinant retroviruses and selected in puromycin-containing medium for 3 days. The resulting 32D/pLS-CUL-4A or 32D/pLS cells were expanded and the steady-state levels of endogenous CUL-4A and HOXA9 determined by immunoblotting. (B) 32Dcl3, 32D/pRS-CUL-4A and 32D/pRS cells were treated with 20 ng/ml of G-CSF for 3 days, Wright–Giemsa stained, and the percentage of blast, intermediate and terminally differentiated populations determined from at least 200 cells as in Figure 6. The result shown is representative of three independent experiments.

HOXA9 protein by CUL-4A to below the basal levels in parental 32Dcl3 cells (Figure 6A). Furthermore, ectopic expression of *CUL-4A* could accelerate G-CSF-induced granulopoiesis of 32Dcl3 cells in the absence of *HOXA9* overexpression (Supplementary figure 1 available at *The EMBO Journal* Online). Collectively, these results indicate a role for CUL-4A in downregulating HOXA9 levels, thereby contributing to granulocytic maturation.

Silencing of endogenous CUL-4A expression by RNAi led to an increased HOXA9 accumulation and inhibition of granulocytic differentiation

To establish further the role of CUL-4A in HOXA9 degradation and granulopoiesis, we employed the siRNA approach to knock down CUL-4A in 32Dcl3 cells, in human HL-60 promyelocytic leukemia cells and in primary CD34⁺ hematopoietic stem/progenitor cells. Attempts to assess the hematopoietic role of CUL-4A in human HL-60 and CD34⁺ cells were not successful due to rapid cell death (within a week) upon transduction of the human CUL-4A siRNA, which knocked down >90% of the endogenous CUL-4A (data not shown). A vector-based siRNA for mouse CUL-4A, which targets nucleotides 1957–1979 (from ATG), was generated and expressed from the retroviral pRetroSUPER RNA interference vector (pRS) (Brummelkamp *et al.*, 2002a). 32D cells infected with the pRS-CUL-4A or control pRS retroviruses were first subjected to selection in puromycin-containing medium and then treated with G-CSF for granulocytic differentiation. Expression of the CUL-4A siRNA resulted in ~60% reduction of CUL-4A and a 2.1-fold increase in the accumulation of endogenous HOXA9 in 32D cells (Figure 7A). The residual 40% of CUL-4A is probably sufficient for maintaining cell survival, thus allowing us to examine whether CUL-4A knockdown affects G-CSF-induced granulocytic differentiation. As shown in Figure 7B, 32Dcl3 cells expressing CUL-4A

siRNA exhibited resistance to G-CSF-induced differentiation: 62% of 32D/pLS-CUL-4A cells exhibited blastic morphology, while 42% were seen with the control 32D/pRS cells. In marked contrast to the pRS-infected cells where 16% were terminally differentiated after G-CSF treatment, only 3% of 32D/pLS-CUL-4A cells exhibited multinucleated morphology (Figure 7B). Taken together, these loss-of-function studies indicate that CUL-4A plays an important role in permitting G-CSF-induced granulocytic differentiation of 32D cells by reducing the levels of HOXA9.

Discussion

Our understanding of the mechanisms controlling hematopoiesis and embryonic development is primarily at the level of transcriptional regulation. Although many transcription factors are uniquely expressed at specific stages of development, little is known about how cells remove these factors at the post-translational level, and what the consequences are if they fail to do so. We have demonstrated that the CUL-4A ubiquitylation machinery controls the stability of HOXA9 through ubiquitin-dependent proteolysis. It has been established that HOXA9 plays a critical role in stimulating the expansion of stem and progenitor cells, and are rapidly downregulated as cells commit to differentiation (Sauvageau *et al.*, 1994; Lawrence *et al.*, 1997). Enforced expression of *HOXA9* renders these cells resistant to differentiation signals and enhances their proliferative potential (Thorsteinsdottir *et al.*, 1997; Fujino *et al.*, 2001). The steady-state level of HOXA9 is a result of the equilibrium between its synthesis and degradation. Constitutive expression of *HOXA9* in 32D cells not only provides a well-defined model system for delineating a role for HOXA9 in granulocytic differentiation (Fujino *et al.*, 2001), but also allows the analysis of CUL-4A-mediated HOXA9

degradation without the complication of transcriptional downregulation of the endogenous *HOXA9* in response to G-CSF. Here we showed that stable ectopic expression of *CUL-4A* in 32D-HOXA9 cells reduced HOXA9 levels, efficiently removed the differentiation block imposed by HOXA9, and restored the sensitivity of these myeloid progenitor cells to G-CSF-induced granulopoiesis (Figure 6). Conversely, silencing of *CUL-4A* expression by RNAi induced an increased accumulation of HOXA9 and blocked granulopoiesis (Figure 7). These studies reveal striking correspondence between CUL-4A siRNA-mediated inhibition of granulocyte maturation (Figure 7) and the differentiation block imposed by enforced expression of *HOXA9* (Thorsteinsdottir *et al.*, 1997; Fujino *et al.*, 2001), and underscore the physiological significance of CUL-4A-dependent HOXA9 degradation in the regulation of hematopoietic development.

Removal of HOXA9 during hematopoietic differentiation is probably a combined outcome of many regulatory mechanisms. Besides transcriptional downregulation (Sauvageau *et al.*, 1994; Lawrence *et al.*, 1997; Fujino *et al.*, 2001), our data delineate a scenario where the CUL-4A-mediated ubiquitylation of HOXA9 contributes to rapid disappearance of the protein once its biosynthesis is decreased upon initiation of terminal differentiation. The activity of the CUL-4A machinery itself might also be regulated at different stages of hematopoiesis by the expression or the subcellular distribution of other as yet unidentified components of the CUL-4A complex, such as the putative substrate receptor(s). Another possibility is that specific post-translational modifications on HOXA9 signal its recognition by the CUL-4A machinery, and the absence of these modifications prevents inappropriate degradation of HOXA9 at different stages of hematopoiesis. In this regard, many substrates of the CUL-1 (SCF) ubiquitin–protein ligase must be phosphorylated first, in order to be recognized by specific F-box-containing substrate receptors for ubiquitylation and degradation (reviewed in Deshaies, 1999). Phosphorylation has been reported for HOX proteins, although the consequence is not fully understood (Jaffe *et al.*, 1997; Yaron *et al.*, 2001). Therefore, it would be of interest to assess the effect of HOXA9 phosphorylation on its association with CUL-4A and on HOXA9 stability.

Ectopic expression of *HOXA9* stimulates the expansion of myeloid progenitor cells and promotes AML in mice (Thorsteinsdottir *et al.*, 1997; Kroon *et al.*, 1998; Schnabel *et al.*, 2000). In a recent study, Li *et al.* (2002b) reported that enforced overexpression of *CUL-4A* inhibits the maturation of the myelo-monocytic leukemia cell line PLB-985 to granulocytes and monocytes in response to dimethylformamide (DMF) and phorbolmyristate acetate (PMA), respectively. The apparent discrepancy between this finding and our data may be due to the different cellular systems [PLB-985 myeloid leukemia cell line (Tucker *et al.*, 1987) versus normal bone marrow-derived diploid 32Dcl3 cell line (Greenberger *et al.*, 1983)]. In addition, different experimental conditions were employed for induction of granulocytic differentiation: PLB-985 cells were induced by treatment with the chemical inducer DMF, whereas 32Dcl3 maturation was triggered by exposure to the physiological regulator of granulopoiesis, G-CSF. Hence, the pathways involved might be different.

Furthermore, it is not known whether PLB-985 cells express significant levels of endogenous HOXA9 and what role HOXA9 plays in the DMF-driven differentiation of PLB-985 cells. In contrast, the role of HOXA9 in the maturation of 32Dcl3 in response to G-CSF has been clearly defined (Fujino *et al.*, 2001). In our system, enforced expression of *CUL-4A* in 32Dcl3 not only abolishes the inhibition of granulocytic differentiation in HOXA9-overexpressing cells (Figure 6), but also leads to an increase in differentiation of wild-type 32Dcl3 cells that mirrors the decline of the endogenous levels of HOXA9 (Supplementary figure 1).

Cullins generally function as scaffold proteins in the assembly of ubiquitin–protein ligase complexes, rather than directly recognizing the substrate (Deshaies, 1999; and references therein). Since purified CUL-4A and HOXA9 did not interact with each other *in vitro* (data not shown), it is conceivable that a substrate receptor mediates their association, and a yeast homolog of this putative receptor bridges such an interaction in the two-hybrid assay. By co-immunoprecipitation, we could detect the interaction of transfected CUL-4A and HOXA9 (Figure 1B), but have not been able to detect binding of the endogenous proteins. This is partly due to the poor efficiency of our HOXA9 and CUL-4A antibodies in immunoprecipitation. Moreover, the binding between ubiquitin–protein ligases and their substrates might be transient in nature, and robust proteasome-dependent degradation as a result of such interactions further complicates the detection of such protein–protein interactions. It is likely that only a fraction of the post-translationally modified HOXA9 could interact with a putative receptor of the CUL-4A machinery, and the resulting degradation of HOXA9 makes it difficult to detect the stable association of endogenous CUL-4A and HOXA9.

HD was originally defined as the DNA-binding domain (McGinnis and Krumlauf, 1992), and residues conserved among HOX HD proteins in helix III and the N-terminal arm preceding the homeodomain are specifically responsible for contacting DNA (Qian *et al.*, 1989). Our studies suggest that helix I of the HOXA9 HD also serves as a specific degradation signal recognized by the CUL-4A ubiquitylation machinery (Figure 5). Helix I of HOX HD, which is oriented away from DNA, is believed to participate in protein–protein interactions (Sharkey *et al.*, 1997). It is possible that the CUL-4A machinery directly targets not only ‘free’ HOXA9, but also the DNA-bound form of HOXA9 for degradation, and, if so, this would represent an irreversible means which cells utilize to attenuate HOXA9 transcriptional activity. Alternatively, DNA binding might affect the efficiency of HOXA9 degradation by CUL-4A. Moreover, HD is conserved among all the 39 members of vertebrate HOX proteins, but divergent from that of non-HOX proteins. Consistently, enforced CUL-4A expression had no effect on the stability of members of the TALE family (PBX1 and MEIS1) or the POU family (Oct-2) of HD proteins (data not shown). Future studies should determine whether the stability of other HOX paralogous members is also subjected to regulation by the CUL-4A ubiquitylation machinery.

Materials and methods

Cell culture, plasmids and antibodies

32Dcl3 and 32D-HOXA9 cells were generous gifts of Dr Takuro Nakamura and were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and 10% WEHI growth supplement. Zeocin (0.5 mg/ml; Invitrogen) was added to the medium for 32D-HOXA9 cells. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. To isolate human CD34⁺ hematopoietic stem cells, umbilical cord blood samples (obtained from the New York Blood Center) were subjected to centrifugation (400 g, 30 min) over Ficoll-Hypaque (Pharmacia) density gradients ($d = 1.077$). The mononuclear cells at the interphase were collected, and were used to purify CD34⁺ cells using the immunomagnetic MACS CD34 Isolation kit (Miltenyi Biotec Inc.) according to the manufacturer's instructions.

MYC-CUL-4A and MYC-CUL-1 expression plasmids (generous gifts of T.Ohta, St Marianna University, Kawasaki, Japan), F-CUL-4A, and F-CUL-4A(Δ), which carries an internal deletion of the cullin homology domain (residues 346–532), have been described previously (Chen *et al.*, 2001). cDNAs for HA- or MYC-HOXA9, or MYC-tagged HOXA9 truncation mutants were amplified by PCR from mouse HOXA9 (CMV-Hoxa-9, gift of C.Largman) respectively, and were cloned into the mammalian expression vector pcDNA3 (Invitrogen). CUL-4A siRNA oligos targeted to nucleotides 1957–1979 (from ATG) of mouse CUL-4A were synthesized and cloned into the retroviral pRETRO-SUPER (pRS) RNAi vector to generate recombinant retroviruses as described (Brummelkamp *et al.*, 2002a). SiRNA targeted to nucleotides 760–778 (from ATG) of human CUL-4A was also generated and cloned into the pSUPER RNAi vector (Brummelkamp *et al.*, 2002b). The identity of each cDNA was verified by sequencing. CUL-4A antibodies used in these studies were either raised against GST-CUL-4A and affinity purified (Chen *et al.*, 2001), or purchased from Santa Cruz. The HOXA9 polyclonal antibody was a generous gift of Dr Takuro Nakamura. Antibodies against FLAG (M2), HA, MYC and β -actin, as well as secondary antibodies used for immunoblotting or immunofluorescence assays were purchased from commercial sources and have been described (Chen *et al.*, 2001). The indirect immunofluorescence staining and confocal microscopy for the subcellular localization of CUL-4A were described in detail (Chen *et al.*, 2001).

Protein stability and ubiquitylation

Transient transfection in HeLa cells was carried out using plasmids as indicated in each figure (in μ g) by calcium phosphate precipitation (Promega) or by Eugene 6 reagent (Roche). The total amount of DNA was normalized in each transfection using the pcDNA3 expression vector. A 2 μ g aliquot of pGREEN LANTERN-1 plasmid was included in all the transient transfection experiments in order to measure the transfection efficiencies and to compare the steady-state levels of transfected proteins. Procedures for immunoblotting, immunoprecipitation, metabolic labeling by [³⁵S]methionine and cysteine for pulse-chase analysis of protein half-lives, and *in vivo* ubiquitylation assay have been comprehensively described (Chen *et al.*, 2001).

32Dcl3 hematopoietic differentiation assay

For establishing stable 32D cells, MYC-CUL-4A or the control pcDNA3 vector were transfected into 32D-HOXA9 or 32Dcl3 cells by electroporation, and selected in 1 mg/ml G418-containing medium for 2 weeks. Stable pcDNA3- or MYC-CUL-4A-expressing clones were isolated by serial dilution and expansion following the procedure described in detail (Freshney, 2000). For siRNA-mediated loss-of-function studies, 32Dcl3 cells were infected with the pRS-CUL-4A or the control pRS retroviruses for 12 h as described (Brummelkamp *et al.*, 2002a). Two more transductions were performed using the same procedure every 12 h. The infected 32Dcl3 cells were subjected to selection in 2.5 μ g/ml puromycin-containing medium for 2–3 days to eliminate the uninfected cells, and expanded in RPMI medium containing 5 ng/ml of IL-3 and 3 μ g/ml of puromycin.

For G-CSF-induced granulocytic differentiation, 1.5×10^6 32Dcl3 or 32D cells stably transfected with CUL-4A or infected with pRS-CUL-4A were washed and induced to differentiate in RPMI 1640 medium containing 0.5% FBS and 20 ng/ml of G-CSF (R&D systems) for a period of 8–9 days. A total of 2.5×10^5 of each cell population were used at the time of induction. At different time points after induction, viable cells were counted by trypan blue exclusion, and differentiating (G-CSF) as well as the control proliferating (IL-3) cells were centrifuged onto glass coverslips using a Cytospin-3 (Shandon), and stained with a Wright–

Giemsa staining kit (Fisher Scientific). The slides were examined under the microscope and myeloid differentiation was quantitatively analyzed by identifying cytoplasmic granules and nuclear fragmentation using the criteria previously described (Tan-Pertel *et al.*, 2000). Cells at different differentiation stages were counted and recorded for statistical analysis. A total of 2.5×10^5 cells were also incubated with fluorescein isothiocyanate (FITC)-labeled anti-MAC1 antibody (1:200 dilution, PharMingen) on ice for 30 min, and cell differentiation evidenced by positive MAC1 staining was assayed by fluorescence-activated cell sorting (FACS) analysis (Becton Dickinson FACS Calibre).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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