Ubiquitination-dependent cofactor exchange on LIM homedomain transcription factors

Heather P. Ostendorff*†, Reto I. Peirano*†, Marvin A. Peters*, Anne Schlüter*, Michael Bossenz*, Martin Scheffner* and Ingo Beth

* Zentrum für Molekulare Neurobiologie Hamburg (ZMNH), Universität Hamburg, Martinistrasse 85, 20251 Hamburg, Germany
† Institut für Biochemie I, Medizinische Fakultät, Universität zu Köln, Joseph-Schelmann-Strasse 52, 50931 Köln, Germany
†† These authors contributed equally to this work

The interactions of distinct cofactor complexes with transcription factors are decisive determinants for the regulation of gene expression. Depending on the bound cofactor, transcription factors can have either repressing or transactivating activities. To allow a switch between these different states, regulated cofactor exchange has been proposed; however, little is known about the molecular mechanisms that are involved in this process. LIM homedomain (LIM-HD) transcription factors associate with RING (RING finger LIM domain-binding protein) and with CLIMP (cofactor of LIM-HD proteins; also known as NLI, Ldb and Chip) cofactors. The co-repressor RING inhibits the function of LIM-HD transcription factors, whereas interaction with CLIMP proteins is important for the expression of the biological activity conferred by LIM-HD transcription factors.

We demonstrate a ubiquitination-dependent exchange of RING with DNA-bound LIM-HD transcription factors in vivo. Furthermore, we demonstrate a ubiquitination-dependent exchange of RING with DNA-bound LIM-HD transcription factors in vivo.

* M. Bossenz, M. A. Peters, A. Schlüter, M. Scheffner and I. Beth

Supplementary Information accompanies the paper on Nature's website (http://www.nature.com).

Acknowledgements
We thank K. Murphy and W. Sha for retroliter vectors; P. Andros, D. Bhattarcharya, Y. Refaeli and W. Sha for advice; M. Matloubian, T. Okada, D. Stainier and A. Weiss for the authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to J.G.C. (e-mail: cyster@uta.edu).
it efficiently mediated ubiquitination of LMO2 and LMO4 (Fig. 1c; see also Supplementary Information). The addition of either bacterially expressed full-length CLIM1 or CLIM2 inhibited the ubiquitination of LMO proteins. The presence of the LIM interaction domain in DN-CLIM, but not the dimerization-domain-containing CLIMΔC, was required for this inhibition, suggesting a competition with RLIM for LIM domains (Fig. 1d). CLIM1 and CLIM2 were also poly-ubiquitinated in the presence of RLIM (Fig. 1e, f), and this reaction was not affected by adding bacterially expressed LMO2 or Lhx3 proteins (data not shown). Furthermore, RLIM was able to poly-ubiquitinate 35S-labelled CLIM1 protein complexed with Lhx3, which was purified in glutathione S-transferase (GST) pull-down experiments using bacterially expressed GST–Lhx3 protein (Fig. 1e). Bacterially expressed C-RLIM lacking the LIM and CLIM interaction domains was not able to promote either CLIM or LMO2 ubiquitinations (data not shown), indicating that these reactions are dependent on the presence of the LIM and CLIM interaction domains. Furthermore, the RING finger protein ligase Mdm2 did not ubiquitinate CLIM cofactors and LMO2 protein in similar experiments (data not shown), demonstrating the specificity of the RLIM-mediated ubiquitination reactions (see Supplementary Information). These results demonstrate that RLIM is a ubiquitin protein ligase that is able to ubiquitinate LMO proteins and CLIM cofactors in vitro. Poly-ubiquitinated proteins are indicated by three asterisks.

Figure 1

RLIM is a ubiquitin protein ligase that is able to ubiquitinate LMO proteins and CLIM cofactors in vitro. Poly-ubiquitinated proteins are indicated by three asterisks.

a, 35S-labelled RLIM protein incubated with ubiquitin-conjugating (E2) proteins UbcH5 and UbcH7. b, Ubiquitin protein ligase activity is abolished in RING finger deletion mutants RLIMΔRING (residues 544–600 deleted) and RLIMΔ9 (residues 562–571 deleted). c, Bacterially expressed RLIM ubiquitinates 35S-labelled LMO2 proteins. Ubiquitinated proteins are indicated by an asterisk. 35S-labelled CLIM1 bound to GST–Lhx3 was purified in pull-down assays before the in vitro ubiquitination experiment. d, RLIM ubiquitinates CLIM cofactors in a dose-dependent manner.

Figure 2

RLIM targets CLIM2 and LMO2 proteins for degradation. Western blots on total protein extracts of CHO cells co-transfected with the constructs indicated, using specific Myc antiserum, are shown. a, Extracts of cells co-transfected with non-tagged, full-length RLIM and Myc-CLIM2 or Myc-LMO2. In vitro-translated and 35S-labelled Myc-tagged proteins are indicated. The messenger RNA levels in transfected cells were determined by northern blot using the 32P-labelled 6x Myc complementary DNA probe. b, Extracts of cells co-transfected with non-tagged full-length RLIM and Myc-Lhx3 or Myc-Isl1 expression vectors. c, Extracts of cells co-transfected with Myc-tagged RLIMΔ9 and Myc-CLIM2 expression vectors. d, Extracts of cells co-transfected with non-tagged, full-length RLIM and Myc-CLIM2 expression vector. Three hours before collection, cells were treated with 25 μM of the proteasome inhibitor LLNL (MG101).
protein ligase that is able to specifically ubiquitinate LMO and CLIM proteins in vitro.

In transfected cells, overexpression of RLIM induced complete degradation of Myc-tagged CLIM2 and LMO2 in a dose-dependent manner (Fig. 2a), whereas the protein levels of LIM-HD transcription factors Isl1, Lhx1 and Lhx3 were only weakly affected (Fig. 2b; see also Supplementary Information). This degradation was dependent on a functional RING finger, because co-transfection of the Myc-RLIMΔ9 construct abolished CLIM2 and LMO2 degradation (Fig. 2c and data not shown). Addition of the proteasome inhibitor LLNL to transfected cells partially impaired CLIM2 degradation, indicating proteolytic degradation through the 26S proteasome (Fig. 2d). To obtain evidence that endogenous RLIM is involved in endogenous CLIM degradation, we first compared cellular CLIM and RLIM protein levels. A reciprocal correlation between RLIM and CLIM protein levels was observed when comparing the gonadotrope pituitary cell line αT3 (ref. 19) to Chinese hamster ovary (CHO) cells and human embryonic kidney (HEK) 293 cells (Fig. 3a and data not shown), consistent with the idea that RLIM is involved in cellular CLIM degradation. Furthermore, when haemagglutinin (HA)-tagged full-length RLIM was transfected into the αT3 cell line, the endogenous CLIM levels (stained red) in the nuclei of transfected cells (stained green) dropped significantly (Fig. 3b). Conversely, in CHO, HEK 293 and ectodermal chick cells, endogenous CLIM levels increased on overexpression of the RLIMΔ9 construct, presumably owing to its dominant negative effect17 on endogenous RLIM (Fig. 3c; see also Supplementary Information). These results were confirmed by RNA interference (RNAi) experiments in which the inhibition of endogenous RLIM expression caused an augmentation of CLIM levels (Fig. 3d). Together, these experiments demonstrate that endogenous RLIM is involved in CLIM degradation, thus placing RLIM in a central position for the developmental control of CLIM proteins.

To investigate the mechanisms by which RLIM regulates LIM-HD transcription-factors, we performed transient transfections. RLIM acts as a transcriptional co-repressor of Lhx3-mediated transcriptional activation from the αGSU and prolactin promoters (Fig. 4a, b) through the recruitment of Sin3A (ref. 8), a component of the histone deacetylase co-repressor complex. RLIMΔ9 was able to inhibit CLIM-independent, but no longer CLIM-dependent, activation events mediated by Lhx3. Notably, RLIMΔ9 interacted as efficiently as RLIM with Lhx3 and Sin3A (see Supplementary Information). These results indicate that RLIM can exert its repressive functions on LIM-HD transcription factors by two distinct but not mutually exclusive mechanisms, that is, by recruitment of Sin3A and by degradation of CLIM. We next examined the protein regions of RLIM responsible for protein–protein interactions with nuclear LIM domains and with CLIM cofactors8. Use of truncated RLIM proteins in a stringent GST protein interaction assay restricted this

![Figure 3](image-url)  
**Figure 3** In vivo degradation of CLIM cofactors is mediated by RLIM. **a,** Western blots using CLIM and RLIM antisera showing reciprocal correlation of relative endogenous CLIM and RLIM protein levels in αT3 and CHO cell lines. **b,** Degradation of CLIM in transfected cells visualized by co-labelling with HA and CLIM antiserum. In αT3 cells, expression of HA-tagged, full-length RLIM (green) resulted in degradation of CLIM (red). **c,** Endogenous CLIM (red) levels augment on HA-RLIMΔ9 (green, which combines with red to show yellow) transfections in CHO and HEK 293 cells. **d,** Inhibition of RLIM expression by RNAi entails increased CLIM levels. HeLa cells were transfected with double-stranded RNA oligonucleotides (siRLIM) or with a single-strand sense control. Cells were visualized with RLIM (green) or CLIM (red) antiserum. An estimated 60–70% of the cells were transfected. In parallel experiments, protein levels were monitored by western blots. Independent experiments using a different siRLIM RNA led to similar results (not shown). Nuclei are visualized by blue staining.

![Figure 4](image-url)  
**Figure 4** Functional protein interactions of RLIM. **a,** CHO cells were co-transfected with the αGSU promoter, and Lhx3 and LIM cofactor expression plasmids. CLIM co-transfections resulted in a 2.7-fold increase of activation levels compared with Lhx3 alone8. Lhx3/CLIM-mediated activation events are inhibited by RLIM in a RING-finger-dependent fashion. **b,** RLIM-mediated inhibition of the prolactin promoter is not dependent on the RLIM finger. CLIM co-transfections do not influence activities of this promoter8. **c,** Mapping of the LIM and CLIM interaction domains. RLIM and CLIM interact with GST fusion proteins. NLS, nuclear localization domain; BD, basic domain; RING, RING H2 zinc finger. **d,** Mapping of the RLIM interaction domain on CLIM. DD, dimerization domain; NLS, nuclear localization domain; NID, LIM–interaction domain.
Cofactor exchange on LIM-HD proteins. D expressed cofactors CLIM1, RLIM and RLIM interact with the DNA-bound Lhx3–CLIM1 complex. Poly-ubiquitinated CLIM–Lhx3–DNA complexes are formed a combined EMSA/in vitro ubiquitination assay demonstrating RLIM- and ubiquitination-dependent displacement of CLIM from the DNA-bound Lhx3–CLIM complex (Fig. 5a). The fact that this effect required a functional RING finger and was dependent on Ubch5, indicates that RLIM is able to ubiquitinate CLIM cofactors associated with DNA-bound Lhx3 (see Supplementary Information). To obtain definitive evidence for the hypothesis that RLIM can associate with DNA-bound LIM-HD proteins by targeting CLIM for degradation, we tested the CLIM cofactor occupancy on the αGSU promoter using chromatin immunoprecipitation (ChIP). In agreement with the fact that αT3 cells synthesize αGSU, the LIM-HD transcription factor Lhx3 and CLIM cofactors appeared to be predominantly associated with this promoter, whereas the association of RLIM seemed significantly lower (Fig. 5b). Examination of the cofactor occupancy of this promoter in transfected αT3 cells showed that Myc-CLIM2 protein associated with the promoter (Fig. 5c). We detected enhanced association of RLIM with the αGSU promoter in cells expressing full-length Myc-RLIM. This association was dependent on a functional RING finger, as the RLIMΔ9 protein—which binds equally well to Lhx3 when compared with wild-type protein (see Supplementary Information)—was not able to bind efficiently to the endogenous αGSU promoter.

The finding that RLIM is able to ubiquitinate CLIM cofactors bound to LIM-HD proteins, that CLIM cofactors can compete with RLIM for binding to LIM domains, and that a ubiquitination/degradation-dependent exchange in cofactor occupancy on DNA occurs, provides an attractive mechanism for how cofactor exchange is mediated on LIM-HD proteins. Whether or not and in which order cofactor exchange occurs probably depends on LIM-HD, LMO, CLIM and RLIM nuclear concentrations, the ubiquitin-ligase activity of RLIM, and the availability of other LIM-domain interaction partners. This is demonstrated through the use of a model (Fig. 5d) showing the protein–protein interactions and ubiquitination events mediated by RLIM. As a growing number of transcription factor families have been shown to interact with several multiprotein complexes that confer multiple functions and activities, the degradation of selective protein members opens up intriguing possibilities of transcriptional regulation. Thus, regulated protein degradation together with competition for protein interaction domains probably represent general mechanisms used in combinatorial regulation of gene transcription.

Methods

Antibodies and in vitro ubiquitination assays

Polyclonal antisera against mouse RLIM and CLIM were generated in rabbits (see Supplementary Information) and used for cofactor detection in western blots, immunocytochemistry and ChIP assays. Myc polyclonal antisera and fluorescein isothiocyanate-labelled Myc and HA antisera (Santa Cruz) were used to detect Myc- and HA-tagged proteins, respectively. For RLIM-dependent ubiquitination, 2 μl of TNT rabbit reticulocyte lysate-translated proteins labelled with 35S (Promega) were incubated in the presence of 50 ng E1, 50 ng each of bacterially expressed Ubch5 and/or Ubch7 and 6 μg ubiquitin (Sigma), and various bacterially expressed proteins, as indicated, in a reaction volume of 50 μl. In addition, the reactions contained 25 mM Tris–HCl at pH 7.5, 50 mM NaCl, 2 mM dithiothreitol, 4 mM ATP and 6 mM MgCl2. After incubation for 2 h at 30 °C, the reactions were stopped, separated by SDS–polyacrylamide gel electrophoresis, and the 35S-labelled proteins detected by fluorography.
**Protein mutants and transient transfections**

We used the following protein mutants: RLIMΔ9 (residues 562–571 in the RING finger region), RLIMΔ1 (residues 1–543), CLIMΔC (1–271), LhxΔC (1–266), and proteins CLIM1, CLIM2 (ref. 21) and LMO2-RLIM, N-RLIM (1–207), M-RLIM (208–423), C-RLIM (403–600), DN-CLIM (225–341). Transient transfections and co-transfections were carried out using a Superfect transfection kit (Qiagen), as described previously27.

**Immunocytochemistry and RNAi**

RNA experiments were performed as reported24, transfecting HeLa cells with double-stranded RNA oligonucleotides (Pharmacia) containing human RLIM sequences25, or with a single-strand sense control (sequence information available on request) using the OligoFectamine kit (Invitrogen). Cells were visualized with RLIM or CLIM antisera and with a single-strand sense control (sequence information available on request) using the Oligofectamine kit (Invitrogen). Cells were visualized with RLIM or CLIM antisera and immunostaining of transfected cells were performed as reported25. The experiments were analysed and pictures taken on a confocal microscope (Leica DMIRBE).

**Protein–protein interactions and EMSA experiments**

The in vitro protein–protein interaction experiments were carried out with bacterially expressed GST fusion proteins and 35S-labelled proteins produced by in vitro transcription/translation using a TNT system28. For EMSA, protein levels of transfected cells were monitored by western blots using RLIM or CLIM antisera. Immunostaining of transfected cells were performed as reported25. The experiments were analysed and pictures taken on a confocal microscope (Leica DMIRBE).

**Chromatin immunoprecipitation assays**

ChIP assays were essentially done as described29, using anti-Lhx3/Plim21, anti-CLIM, anti-RLIM, and anti-Myc (BabCo) antisera, and oligonucleotides encompassing the region on the mouse EGU promoter that contains the LIM-HD binding site25,26 (see Supplementary Information).

Received 12 October 2001; accepted 17 January 2002.


**Structure of the HP1 chromodomain bound to histone H3 methylated at lysine 9**

Peter R. Nielsen*, Daniel Nietlispach*, Helen R. Mott†, Juliana Callaghan*, Andrew Bannister†, Tony Kouzaris†, Alexey G. Murzin†, Natalia V. Murzina* & Ernst D. Laue†

*Cambridge Centre for Molecular Recognition, Department of Cancer Research, University of Cambridge, 80 Tennis Court Road, Cambridge CB2 1GA, UK †Wellcome/Cancer Research UK, Institute of Cancer and Developmental Biology and Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QK, UK ‡MRC Centre for Protein Engineering, Hills Road, Cambridge CB2 2QH, UK

Specific modifications to histones are essential epigenetic markers—heritable changes in gene expression that do not affect the DNA sequence. Methylation of lysine 9 in histone H3 is recognized by heterochromatin protein 1 (HP1), which directs the binding of other proteins to control chromatin structure and gene expression1,2. Here we show that HP1 uses an induced-fit mechanism for recognition of this modification, as revealed by the structure of its chromodomain bound to a histone H3 peptide dimethylated at Nε of lysine 9. The binding pocket for the N-terminal groups is provided by three aromatic side chains, Tyr 21, Trp 42 and Phe 45, which reside in two regions that become ordered on binding of the peptide. The side chain of Lys 9 is almost fully extended and surrounded by residues that are conserved in other chromodomains. The QTAR peptide sequence preceding Lys 9 makes most of the additional interactions with the chromodomain, with HP1 residues Val 23, Leu 40, Trp 42, Leu 58 and Cys 60 appearing to be a major determinant of specificity by binding the key buried Ala 7. These findings predict which other chromodomains will bind methylated proteins and suggest a motif that they recognize.

Histone HP1 is present in the regulatory region of chromatin structure and thus gene expression4. Methylation at lysine 9 of histone H3 by Suv39 methyltransferase⁵ and its relatives strongly...