The H3K4me3 Histone Demethylase Fbxl10 Is a Regulator of Chemokine Expression, Cellular Morphology, and the Metabolome of Fibroblasts

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Background: Fbxl10 is a member of the JHDM family.

Results: We show that Fbxl10 functions as an H3K4me3 demethylase. The PHD domain recognizes H3K4me3 and H3K36me2 and shows E3 ligase activity. Using a microarray approach we identified target genes for Fbxl10.

Conclusion: Our data reveal a regulatory role of Fbxl10 in cell morphology, chemokine expression, and the metabolic control.

Significance: Fbxl10 plays a novel role in the regulation of target genes.

Fbxl10 (Jhdm1b/Kdm2b) is a conserved and ubiquitously expressed member of the JHDM (JmjC domain-containing histone demethylase) family. Fbxl10 was implicated in the demethylation of H3K4me3 or H3K36me2 thereby removing active chromatin marks and inhibiting gene transcription. Apart from the JmjC domain, Fbxl10 consists of a CxxC domain, a PHD domain, and an FBox domain. By purifying the JmjC and the PHD domain of Fbxl10 and using different approaches we were able to characterize the properties of these domains in vitro. Our results suggest that Fbxl10 is rather a H3K4me3 than a H3K36me2 histone demethylase. The PHD domain exerts a dual function in binding H3K4me3 and H3K36me2 and exhibiting E3 ubiquitin ligase activity. We generated mouse embryonic fibroblasts stably overexpressing Fbxl10. These cells reveal an increase in cell size but no changes in proliferation, mitosis, or apoptosis. Using a microarray approach we were able to identify potentially new target genes for Fbxl10 including chemokines, the noncoding RNA Xist, and proteins involved in metabolic processes. Additionally, we found that Fbxl10 is recruited to the promoters of Ccl7, Xist, Crabp2, and Ripk3. Promoter occupancy by Fbxl10 was accompanied by reduced levels of H3K4me3 but unchanged levels of H3K36me2. Furthermore, knockdown of Fbxl10 using small interfering RNA approaches showed inverse regulation of Fbxl10 target genes. In summary, our data reveal a regulatory role of Fbxl10 in cell morphology, chemokine expression, and the metabolic control of fibroblasts.
ized it as an H3K4me3 demethylase (12). In a recent work by Tzatsos and co-workers the authors postulate the option of Fbxl10 being a demethylase for both modifications (13). Whereas slight experimental differences in Fbxl10 expression and demethylase assay condition might explain different experimental results, it is still striking that similar approaches by overexpression of Fbxl10 in infected HeLa cells revealed in one study H3K36me2 and in the other study H3K4me3 as the Fbxl10 target (10, 12).

In addition, Fbxl10 is described as a nucleolar protein to repress rRNA transcription (12), to be involved in apoptosis (14) and inhibition of cellular senescence (10). Furthermore, Fbxl10 plays a role in hematopoietic stem cell self-renewal and proper generation of the neural tube in vivo (15, 16).

Therefore, we investigated in further detail the role of the JmjC domain and the yet uncharacterized PHD domain of Fbxl10. Our data clearly indicate that Fbxl10 functions as a H3K4me3 rather than a H3K36me1/2 demethylase. However, the PHD domain recognizes both H3K4me3 and H3K36me2. Notably, the PHD domain of Fbxl10 also shows E3 ligase activity in vitro, indicating a role for Fbxl10 in addition to histone demethylation. Overexpression of Fbxl10 in mouse embryonic fibroblasts (MEFs) resulted in an increase in both total cellular and nuclear size, but did not affect proliferation, mitosis, or apoptosis in these cells. Using a microarray approach we were able to identify 131 new putative target genes for Fbxl10 including a set of chemokines, noncoding RNA Xist and metabolic regulators. To verify selected target genes we showed direct binding of Fbxl10 to the promoters of Ccl7, Xist, Crabp2, and Ripk3 accompanied by a significant decrease in H3K4me3 whereas H3K36me2 remained unchanged. Knockdown of Fbxl10 inverted the regulation of Ccl2, Ccl7, Cxcl10, and Xist. Our data indicate a novel function of Fbxl10 in regulating metabolic processes and cell shape.

EXPERIMENTAL PROCEDURES

Protein Purification—Domains of Fbxl10 were cloned into the pGEX-4T1 expression vector (GE Healthcare) using the following primers: JmjC, 5'-gccgcatggttacacttcgg; RING, 5'-cggaattccacaccgccgtgtgccttgtgtgt and 5'-tgcggctagcctaacttagtttttgcaggagtttctcttccac. MEF cells were transformed in One Shot BL21 STAR (DE3)pLysS Escherichia coli (Invitrogen). Protein expression and purification were done using glutathione-Sepharose bead columns (GE Healthcare) according to the manufacturer’s instructions.

Western Blotting—Cells were harvested with radioimmunoprecipitation assay buffer, and samples were heated with Laemmli buffer at 95 °C for 5 min. Membranes were blocked in 5% milk or BSA and later washed with PBS-T or TBST-T. The following antibodies were used: β-actin (1:5000, A5441; Sigma-Aldrich), HA antibody (1:2500, 16B12; His Antibodies, Freiburg, Germany), PCNA (1:1000, ab29; Abcam, Cambridge, UK), histone H3 (1:5000, ab1791; Abcam), H3K4me2 (1:1000, pAb-035050; Diagenode, Liege, Belgium), H3K4me3 (1:1000, ab1012; Abcam), H3K9me2 (1:1000, 07-441; Upstate, Billerica), H3K27me3 (1:1000, pAb-069-050; Diagenode), H3K36me1 (1:1000, ab9048; Abcam), H3K36me2 (1:1000, 07-274; Upstate), H2Aub (1:500, 05-678; Upstate), GST (1:5000, 27-4577-01; Amersham Biosciences), ubiquitin antibody (1:2000, Z0458; Dako) and anti-mouse or anti-rabbit HRP antibodies (1:5000; Sigma-Aldrich).

PHD-Histone Binding Assay—Binding assays of 1 μg of GST-PHD and 10 μg of bulk histones (1:5000; Sigma-Aldrich) were performed as described by Taverna et al. (17). Samples were then analyzed by Western blotting with the indicated antibodies.

In Vitro Ubiquitination Assay—E3 ligase assay was performed in ubiquitination buffer (50 mM HEPES, pH 8.0, 4 mM ATP, 10 mM MgCl2, 0.2 mM ZnCl2, 50 mM NaCl, 11.4 μg of ubiquitin (Sigma-Aldrich), 200 mM (His)6UBE1 human recombinant protein (Boston Biochem, Boston, MA), 1 μM GST-UBC5a human recombinant protein (Boston Biochem) by adding purified PHD domain, equal amounts of GST or RING domain for 2 h at 30 °C. Samples were then analyzed by Western blotting.

Histone Demethylation Assay—Histone demethylation assay was performed in demethylation buffer (50 mM Tris-HCl, pH 7.9, 50 mM KCl, 10 mM MgCl2, 2 mM ascorbate, 1 mM α-ketoglutarate, 100 μM Fe2+). As substrate, 1 μg of bulk histone (1:5000; Sigma-Aldrich) or recombinant H3K4me3 or H3K36me2 (Active Motif, Rixensart, Belgium) was mixed with purified JmjC domain of Fbxl10. Samples were then incubated for 30 min at 37 °C and analyzed by Western blotting.

Generation of MEFs Stably Overexpressing Fbxl10—We used the Tet-Off system (Clontech) for inducible gene expression. The murine Fbxl10 cDNA was cloned into pUHD10-3 with an N-terminal HA tag and a Kozak sequence. Primers used for cloning pUHD10-3-HA-Fbxl10 were 5'-gctgatatacctcccgactggtctgagctgtctgtttgat and 5'-tcggattcaacccgctgctttgtg and 5'-cggatccggctgcttcggctgccttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgcttgctgg
H3K4me3 Histone Demethylase Fbxl10

Quantitative Real-time PCR (qRT-PCR)—RNA was extracted with PureLink RNA Micro scale kit (Invitrogen) according to the manufacturer’s protocol. cDNA syntheses were performed with 0.5 μg of RNA employing SuperScriptIII (Invitrogen), and qRT-PCRs were done as described by Lim et al. (18). Control values were set to 1, and hypoxanthine-guanine-phosphoribosyltransferase (Hprt) was chosen as a housekeeping gene. The following primers were used for qRT-PCR: Hprt, 5′-tctctcctcagacgcttt and 5′-cctggtctcatctgcttaa; Col2A1, 5′-cgctctcatgcgttcagg and 5′-ttatagctgtcggccatttc; Lama5, 5′-gccgaggaaacaccacta and 5′-gcaatctcttcattgctgcag; Xist, 5′-ctagctctctgctctactaca and 5′-agagagacacaacagctca; Sfrp2, 5′-gacaagacgctctgctca and 5′-tcacagacctctggagagct; Ccl2, 5′-ctacagcttggctca and 5′-gatactctggcttgtaagtg; Ccl5, 5′-tcaagagctctgagacagc and 5′-gagtgggtgtcagaccta; Ccl17, 5′-tcttaggctctggctata and 5′-tgcagatagcagcatgtggat; Cxcl10, 5′-gtcgcctgcttttcgcttggtgcttc and 5′-tcctgtttgctctcgactgc; Catgaccatccaccaacggcg.

Signaling) according to the manufacturer’s protocol. 10–15 μg of chromatin was immunoprecipitated with 3 μg of HA (ab9110; Abcam), H3K4me3 (ab1012; Abcam), or H3K36me2 (07-274, Upstate) antibody. Equal amounts of IgG antibodies (kch-819-015; Diagenode) were used as control antibody. ChIP after knockdown of Fbxl10 was performed as described previously (19). Recovered DNA was analyzed by TaqMan qRT-PCR using the following loci-specific primers: Xist, 5′-tctggtcctcattctgcagttgtgc and 5′-gattttgagagcttctctctgta; RipK3, 5′-gtcgctctggacagggtcagttc and 5′-tgatagctctgcattcaacag; Xlr, 5′-ttctggtgaggagaggcaagt and 5′-tccatcttcattcgtcctc; E2f5, 5′-gatgcctctgctagtcaggtcag and 5′-gatgatcatataaatccgctttgc; Tll1, 5′-aaagtgctctgcttgagat and 5′-aacttcacatcttgaagct.

Proliferation Assay—Proliferation was measured with the Click-iT EdU Alexa Fluor 488 Imaging Kit (Invitrogen) as recommended by the manufacturer. Percentage of proliferating cells was assessed by counting the number of positive cells compared with the total number of cells on 8 visual fields 3 and 24 h after EdU treatment.

Mitosis Assay—Cells were prepared for immunofluorescence as described above. Staining was done with the mitosis marker H3S10P antibody (1:400, ab5176; Abcam) and Alexa Fluor 594 goat anti-rabbit (1:400; Invitrogen). Ratio of mitotic to total cell number was measured by counting positive cells on 8 visual fields 24 and 48 h after plating.

Histone Preparation—Histone preparation was done using acidic extraction. Briefly, cells were incubated in Triton extraction buffer (PBS with 0.5% Triton X-200, 2 mM PMSF, and 0.02% Na3CO3, washed, and incubated overnight in 0.2 n HCl. Purified histones were analyzed by Western blotting as described above.

Microarray Analysis—Microarray experiments were performed with Affymetrix Mouse Gene St 1.0 arrays using 300 ng of total RNA. Analysis was performed according to Lim et al. (18).

Chromatin Immunoprecipitation (ChIP)—ChIP was performed with SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling) according to the manufacturer’s protocol. 10–15 μg

A

N

JmjC

CXXC

PHD

FBOX

C

B

α-H3

α-H3K4me3

α-H3K36me2

C

α-H3

α-H3K4me3

α-H3K36me2

FIGURE 1. Fbxl10 is a H3K4me3 demethylase consisting of four distinct domains. Fbxl10 exists in three isoforms. Two isoforms contain the four major domains: JmjC, CxxC, PHD, and Fbox. A and B, the third isoform lacks the JmjC domain (A). Incubation of JmjC domain of Fbxl10 together with bulk histone (B) resulted in demethylation of H3K36me2 but not in demethylation of H3K36me2 in vitro. C, specificity of H3K4me3 demethylation was verified by incubation with recombinant H3K4me3 and H3K36me2.

RESULTS

Fbxl10 Is a H3K4me3 Rather Than a H3K36me2 Histone Demethylase—Fbxl10 is a protein of the JmjC domain family which is expressed in three different isoforms (Fig. 1A). Isoform 1 represents the full-length Fbxl10 protein containing the four
main domains of the potential histone demethylase. Isoform 2 differs only slightly from isoform 1 in the 5'-UTR and coding region of the gene, still harboring all four main domains, whereas isoform 3 lacks the JmjC domain. The isoforms are known to be expressed in vivo as shown by Fukuda et al. (16). This is of particular interest because Fbxl10 research has focused mainly on its role as a histone demethylase requiring the presence of the JmjC domain. However, expression of isoform 3 suggests a potential function of the short isoform other than histone demethylation. With respect to the existing controversial data on the specificity of histone demethylation by Fbxl10, we first tested the capacity of a recombinant GST-tagged JmjC domain of Fbxl10 to demethylate H3K4me3 or H3K36me2 in vitro. Therefore, we purified the GST-tagged JmjC domain and incubated the recombinant protein and bulk histone as substrate. Changes in H3K4me3 and H3K36me2 were detected via Western blotting and compared with an input control. Incubation of the purified JmjC domain of Fbxl10 with bulk histones led to a strong reduction of the H3K4me3 signal compared with the input control. However, H3K36me2 methylation levels were not altered (Fig. 1B). To further validate the substrate specificity, we also tested a commercially available recombinant H3K4me3 or H3K36me2 as substrate. The strong decrease in H3K4me3 and the unchanged signals in H3K36me2 confirm that the JmjC domain of Fbxl10 functions as H3K4me3-specific demethylase (Fig. 1C).

The PHD Domain of Fbxl10 Binds to H3K4me3 and H3K36me2 and Shows E3 Ligase Activity—Recently, several reports described PHD domains beside bromo- and chromodomains as the main reader domains of histone modifications (7). To investigate the role and function of the PHD domain of Fbxl10, we purified the GST-tagged PHD domain of Fbxl10 and performed GST pulldown assays with bulk histones. We analyzed the binding of the PHD domain to histones by Western blotting using methylation state-specific antibodies.

Our results shown in Fig. 2A demonstrate binding of the Fbxl10 PHD domain to H3, H3K4me3, and H3K36me2. Other modifications like H3K4me2, H3K9me2, or H3K36me1 were not enriched by the pulldown experiments, indicating specific-
including proliferation, mitotic count, and apoptosis. Contradictory reports on Fbxl10 promoting or blocking proliferation (10, 12) prompted us to investigate proliferation of Fbxl10-overexpressing cells with Click-iT EdU Alexa Fluor 488 Imaging Kit which relies on the incorporation of EdU in the DNA of proliferating cells. The assay clearly demonstrated an equal amount of proliferating cells per time unit in D5 and control cells 3 and 24 h after treatment with EdU (Fig. 4B). These results were further validated by Western blotting of the proliferation marker PCNA (Fig. 4C) and identical mitotic rate of clone D5 and control cells determined by immunofluorescence against phosphorylated histone H3 24 and 48 h after plating (H3S10P; Fig. 4D).

To examine whether the stable overexpression of Fbxl10 has an effect on apoptosis we treated our cell lines with increasing concentrations of staurosporine, menadione, or thapsigargin known to induce apoptosis by different mechanisms. Cell viability was measured after 6 h of treatment using an MTT assay. At this time point the cell viability for both cell lines was decreased compared with untreated cell lines in a very similar manner. Results shown in Fig. 4E and supplemental Fig. 1 revealed an identical degree of apoptosis in both cell lines.

Fbxl10 was previously reported to confer specific changes in histone modification either directly via its JmjC domain or indirectly by its binding partners (10, 12, 23, 24). Whereas H3K4me3 and H3K36me2 were discussed as direct targets, H3K27me3 and H2Aub were reported as indirect targets because Fbxl10 was found in Polycomb complexes with H3K27 methylation activity and H2A ubiquitination capability (23, 24). H3K4me2 and H3K36me1 have not been described as Fbxl10 targets and were therefore used as negative controls. To investigate potential changes in global histone modification after Fbxl10 overexpression, we purified histones by acidic extraction and analyzed the histone modifications H3K4me2/3, H3K36me2, H3K36me, H3K27me3, and H2Aub (Fig. 4F). Indeed, global levels of histone modifications were not affected by overexpression of Fbxl10, indicating its function through gene locus-specific modifications.

Fbxl10 Regulates Genes Involved in the Cellular Metabolome and Anatomical Structures—To investigate gene expression patterns regulated by Fbxl10 we next analyzed transcriptome changes resulting from Fbx10 overexpression using a microarray approach. Comparing three independently reproduced transcriptomes of untransfected control cells and the D5 cell
clone, respectively, we identified 131 genes with a -fold change
>2 (supplemental Table 1) and an additional 133 genes with a
-fold change >1.72 (supplemental Table 2 and Supplemental
Fig. 2). The microarray data were released into the GEO data-
base for public access (accession number GSE34691).

To confirm these data, we validated selected sets of both up-
and down-regulated genes by qRT-PCR. Steady-state transcript
levels of Col2a1 and Lama5 were increased and expression of the
noncoding RNA Xist and Sfp2 were decreased confirming the
microarray analysis (Fig. 5A). Interestingly, among the 131
regulated genes we identified a group of chemokines (Ccl2,
Ccl5, Ccl7, and Cxcl10), strongly down-regulated in the D5 cell
down-regulated chemokine, Cx3cl1, was identified among the
133 genes with the slightly reduced threshold of >1.72-fold change.
Reduced expression levels of these chemokines were verified by qRT-PCR (Fig. 5B).

We then further verified the microarray data on protein level using a
Ccl2 ELISA, showing a significantly decrease of Ccl2 protein
in supernatant of D5 cells compared with control cells (supple-
mental Fig. 3). As fibroblasts are well known to produce chem-
okines (25, 26) our observation suggests an important role for
Fbxl10 in chemokine repression.

To assess whether the promoters of Xist and the chemokine
Ccl7 are direct or rather indirect targets of Fbxl10, we per-
formed ChIP assays using α-HA, α-H3K4me3, α-H3K36me2,
and IgG control antibodies followed by qRT-PCR. Results
shown in Fig. 5C clearly reveal direct binding of Fbxl10 to both
of these promoters. In addition, we observed significant reduc-
tion of H3K4me3 levels, emphasizing the function of Fbxl10 as
a gene locus-specific H3K4me3 demethylase at the promoters
of these target genes. H3K36me2 levels remained unchanged
at the Ccl7 and increased at the Xist promoter. IgG antibody was
used as a control and confirmed specificity of the HA,
H3K4me3, and H3K36me2 antibodies (Fig. 5C).

Functional classification of all differentially expressed genes
revealed that ~49% of all Fbxl10-regulated genes in fibroblasts
are involved in metabolic processes indicating a strong involve-
ment of Fbxl10 in metabolic regulation (Fig. 5D). From the 264
genesis significantly changed by a threshold of -fold change
>1.72, 101 genes are involved in metabolism (supplemental
Table 3 and supplemental Fig. 4A). Considering that the entire
genome list for metabolic processes contains only 1376 genes, we
calculate that overexpression of Fbxl10 in fibroblasts alters
expression of almost 8% of all genes involved in cell metabolism
(supplemental Fig. 4A).

As illustrated in supplemental Fig. 2 the metabolic regulators
Crabp2 and RipK3 were regulated by Fbxl10. We assessed
whether the promoters of Crabp2 and RipK3 are targets of his-
tone modification by Fbxl10. Indeed, we found that Fbxl10
binds to the promoter of these genes. We found significantly
reduced levels of H3K4me3 and a nearly unchanged
H3K36me2 level when Fbxl10 is overexpressed (Fig. 5E).

Biological pathway analysis of these metabolism genes indi-
cated that they are strongly involved in phosphor metabolism
and oxidation and reduction reactions (supplemental Fig. 4B).
Due to the enrichment of phosphor processes in this analysis
we asked whether main receptor tyrosine kinase pathways are
altered in D5 cells. However, using the PathScan RTK Signaling
Kit we could not detect different activation of these pathways
(supplemental Fig. 4C).

Gene ontology analysis revealed a further 26 genes belonging
to the cluster “anatomical structure morphogenesis.” In
accordance with Fig. 5A we could show here that Fbxl10 regu-
lates the expression of genes encoding cell adhesion molecules
and extracellular matrix proteins including Col2a1 and Lama5.

Further genes of this cluster like Efnb2 and Efna5 were previ-
ously described to alter cell morphology whereas Edn1 is
known to influence cell size and is involved in cellular hyper-
 trophy (27–29). These genes are of special interest regarding
the altered morphology of D5 compared with the control cell
line (supplemental Fig. 5).

To assess whether the knockdown of Fbxl10 leads to an
inverse regulation of previously identified target genes, we used
siRNA approaches against the demethylase in the control cell
line. Interestingly, reduction of Fbxl10 levels resulted in
increased Xist, Ccl2, Ccl5, Ccl7, and Cxcl10 RNA expression.

Other genes differentially expressed in D5 cells were not
affected by the knockdown of Fbxl10 (supplemental Fig. 6A).

ChIP analysis at the promoters of Xist, Ccl2, Ccl7, and Cxcl10
confirmed the qRT-PCR data and showed significantly
increased H3K4me3 levels and unchanged H3K36me2 levels at
both promoters (supplemental Fig. 6B).

**DISCUSSION**

**Fbxl10 Is a H3K4me3 Demethylase**—In this report, we show
that Fbxl10 functions as a H3K4me3 demethylase. Our data
obtained from purified JmjC domain in vitro are confirmed by
decreased gene locus-specific H3K4me3 levels upon binding of
Fbxl10 to its direct targets Xist, Ccl7, RipK3, and Crabp2. In
agreement with our data, Frescas et al. also observed H3K4me3
demethylation activity for Fbxl10 (12). This is further supported
by a very recent publication analyzing the demethylase function
of the *Drosophila* homolog Dkdm2 which was described as a
H3K4me3 demethylase in vivo (30). Nevertheless, it has to be
considered that histone demethylases act in multienzyme com-
plexes, and the specificity of histone modifiers may be depend-
ent on interaction with other proteins or modifications on his-
tone residues nearby (1, 5). Thus, we cannot completely exclude
a potential H3K36me2 demethylase function of Fbxl10 in vivo.
Distinct substrate specificity depending on the complex
involved has already been described for the histone demethyl-
ase LSD1 (31, 32). We also do not know whether the demethyl-
lation reaction of Fbxl10 is dependent on other modifications
on the histone tail. This has been described for H3K36me which
depends on H3P38 isomerization (33) or for the influence of
H3T6, which in consequence prevents removal of active methyl
marks from H3K4 during AR/LSD1-stimulated gene expres-
sion (34).

**The PHD Domain of Fbxl10 Binds to H3K4me3 and
H3K36me2**—In addition to bromo- and chromodomains, PHD
domains are well known for their function as histone readers,
recognizing and binding to methylated lysines on histones (7).
Interestingly, we identified H3K4me3 as a binding partner for
this PHD domain. Regarding the ability of Fbxl10 to demethyl-
late the same mark, this finding clearly offers a trans mode
action for Fbxl10. Although binding to H3K4me3 on one
nucleosome, Fbxl10 might demethylate the same mark on a neighboring histone or nucleosome. In addition to binding H3K4me3, we also showed for the first time the binding of a PHD domain to H3K36me2. Binding to this mark would offer a cis mode for Fbxl10 in that it binds to H3K36me2 and demethylates H3K4me3 on the same histone tail. As mentioned above we cannot completely exclude the previously proposed role of Fbxl10 as a H3K36me2 demethylase. Under these circumstances binding to H3K36me2 would also offer a trans mode of action for Fbxl10 (35).

Fbxl10 Exhibits E3 Ubiquitin Ligase Activity—Besides binding to histones some PHD domains were also reported recently to function as E3 ligase in ubiquitination (8) as well as sumoylation reactions (22). PHD domains are closely related to RING domains (20) which have already been identified as E3 ubiquitin ligases (21). Furthermore, the Fbox domain of Fbxl10 has been regarded as an anchor protein in E3 ubiquitin ligase complexes (9). These data prompted us to analyze whether the PHD domain of Fbxl10 also possesses E3 ubiquitin ligase potential. The PHD domain, however, is present in the short isoform 3 of Fbxl10 and might mediate a demethylase-independent protein function. As the ubiquitin pattern mimicked that of the RING domain of human XIAP and was abolished after the replacement of PHD, E1, E2, or ATP, these data unambiguously reveal a functional E3 ubiquitin cascade in which the PHD domain serves as an E3 ligase.

Previous reports postulated that Fbxl10 contributes to the BCOR complex and may be indirectly involved in H2A ubiquitination (23, 24). Offering bulk histones as substrate in the in vitro ubiquitination assay, however, did not result in H2A ubiquitination, indicating that Fbxl10 cannot ligate H2A with ubiquitin or might require an essential co-factor (data not shown).

Fbxl10 Regulates Chemokines, the Metabolome, and the Cell Anatomy in Fibroblasts—To get a deeper insight into Fbxl10 function, we generated a cell line stably overexpressing Fbxl10 and investigated Fbxl10-regulated target genes. Notably, we did not detect any changes in proliferation or response to different proapoptotic stimuli. Comparing our results with published data (10, 12) regarding effects of Fbxl10 on proliferation, it is important to point out that results from transient transfection of Fbxl10 are of limited value and may explain the different data sets. Moreover, we cannot exclude that distinct multienzyme complexes in different cell lines are favored by Fbxl10 leading to a complete different outcome. However, our stably transfected cell may better reflect the in vivo situation than the relative short time of transient overexpression or siRNA approaches (12).

Here, we provide the first insights into Fbxl10 transcriptome regulation by identifying a set of 264 genes differentially regulated by permanent Fbxl10 overexpression. Consistently, Cdkn2b, the most frequently described Fbxl10 target gene, was among our gene set regulated in fibroblasts. Fbxl10 was recently linked to senescence by regulating Cdkn2b expression (10, 36). In addition, we identified an entire set of chemokines and verified that Fbxl10 indeed binds directly to and demethylates histones of the Ccl7 promoter in a gene locus-specific manner. Remarkably, a knockdown of Fbxl10 led to inverse regulation of these chemokines.

Most interestingly, pathway analysis of all significantly regulated transcripts revealed that nearly 50% of all putative Fbxl10 target genes are linked to metabolic processes including the direct targets RipK3 and Crabp2 (37, 38). Further analysis showed significant increase of phosphor metabolism genes among the putative Fbxl10 targets. This finding strongly suggests an important role of Fbxl10 in cellular metabolism. Moreover, the analysis also emphasized our observation that Fbxl10 overexpression does not alter cell proliferation and mitotic index because genes association were not enriched in the microarray. Strikingly, the permanent overexpression of Fbxl10 resulted in an increase in total cellular and nuclear size. Consistently, regulation through Fbxl10 may provide a pathway in response to external signals by changes in cell morphology and metabolome.

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