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Research Article

The LIM-homeodomain transcription factor LMX1B regulates expression of NF-kappa B target genes

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ABSTRACT

LMX1B is a LIM-homeodomain transcription factor essential for development. Putative LMX1B target genes have been identified through mouse gene targeting studies, but their identity as direct LMX1B targets remains hypothetical. We describe here the first molecular characterization of LMX1B target gene regulation. Microarray analysis using a tetracycline-inducible LMX1B expression system in HeLa cells revealed that a subset of NF-κB target genes, including *IL-6* and *IL-8*, are upregulated in LMX1B-expressing cells. Inhibition of NF-κB activity by short interfering RNA-mediated knock-down of p65 impairs, while activation of NF-κB activity by TNF-α synergizes induction of NF-κB target genes by LMX1B. Chromatin immunoprecipitation demonstrated that LMX1B binds to the proximal promoter of *IL-6* and *IL-8* *in vivo*, in the vicinity of the characterized κB site, and that LMX1B recruitment correlates with increased NF-κB DNA association. *IL-6* promoter-reporter assays showed that the κB site and an adjacent putative LMX1B binding motif are both involved in LMX1B-mediated transcription. Expression of NF-κB target genes is affected in the kidney of *Lmx1b*^{-/-} knock-out mice, thus supporting the biological relevance of our findings. Together, these data demonstrate for the first time that LMX1B directly regulates transcription of a subset of NF-κB target genes in cooperation with nuclear p50/p65 NF-κB.

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Introduction

LIM-homeodomain (LIM-HD) proteins are transcription factors that belong to the family of LIM proteins, originally named for the LIM domain-containing transcription factors *Lin11*, *Isl1*, and *Mec3*. To date, at least 12 LIM-HD transcription factors have been characterized in mammals (LHX1–9, ISL1–2, LMX1A and LMX1B). They play key regulatory functions in cell-type specification during development, and many of them are associated with human diseases (reviewed in [1,2]). LIM-HD proteins feature two LIM

domains in their amino termini and a central homeodomain (HD). The HD is a highly conserved 60 amino acid domain that mediates the binding to specific DNA elements within target genes. The majority of the characterized HD recognizes AT-rich elements containing a 5'-TAAT-3' core motif (or ATTA on the other strand) (reviewed in [3,4]). The LIM domain is a conserved cysteine- and histidine-rich zinc-coordinating domain of approximately 50–60 amino acids, consisting of two tandemly repeated zinc fingers. The LIM domain is a multifunctional protein–protein interaction domain. It mediates interactions with other transcription factors

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bound in the vicinity, as well as with transcriptional cofactors that do not bind to DNA (reviewed in [1]), and is thus responsible for positively or negatively regulating the transcriptional activity of LIM-HD proteins. Several cofactors of LIM-HD have been described, including the CLIM coactivators (CLIM1/LDB2 and CLIM2/LDB1/NLI) and the corepressor R LIM. LDB1 is a cofactor of multiple LIM-containing proteins, and plays essential functions in development (reviewed in [5]). It regulates transcription through protein interactions via its C-terminal LIM interaction domain (LID) [6–9]. The RING H2 zinc finger protein R LIM is a negative transcriptional regulator of LIM domain-containing factors, through recruitment of histone deacetylase (HDAC) as well as through its ubiquitin ligase activity that targets both LDB1 and LIM-HD proteins for degradation [10,11].

The LIM-HD transcription factor LMX1B is expressed in multiple tissues, including kidney, brain, developing limb and eye, and cranial mesenchyme [12–21] (also reviewed in [2,22]). Gene targeting studies in mouse uncovered numerous functions of LMX1B. *Lmx1b* homozygous knock-out mice display limb defects, including dorsal-to-ventral conversion of the limbs and a lack of patella and nails [13]. These mice also display kidney defects, in particular abnormalities in the glomerular basement membrane, and impaired differentiation and maintenance of the podocytes, the cells specifically expressing LMX1B in the kidney [23], resulting in severe proteinuria and death [13,24–26]. In the brain, LMX1B is involved in the development of mesencephalic dopaminergic neurons [21] and of serotonergic neurons [17], and in the differentiation and migration of afferent sensory neurons [27]. It is also essential for the development of the tectum and the cerebellum [18]. Finally, LMX1B is required for the development of multiple tissues of the anterior segment of the murine eye [19].

Interestingly, the *Lmx1b* null mouse model mimics the human disease associated with LMX1B heterozygous mutations, nail-patella syndrome (NPS), also known as hereditary osteo-onychodysplasia (HOOD) and Fong's Disease. NPS is a rare autosomal dominant disorder characterized by various skeletal dysplasia (nail dysplasia, hypoplastic or missing patella, elbow dysplasia) in 70–90% of NPS patients, podocyte-associated nephropathy in 40% of NPS cases, open angle glaucoma in 10% of NPS patients, and often neuropathy (reviewed in [2,22,28]). Mutations in LMX1B concentrate within the exons encoding the LIM domains (LIM1 44%, LIM2 38%) and the HD (18%) [28], strongly supporting a role of LMX1B as a transcription factor and the importance of LIM domain-interacting cofactors.

LMX1B interacts through its LIM domains with the bHLH transcription factor E47 on the *insulin* gene minienhancer, resulting in synergistic gene activation [29,30]. LMX1B also interacts with the LDB1 cofactor *in vitro* [6–8,26,31]. Overexpression of LDB1 inhibits the synergistic activation by LMX1B and E47 in a reporter assay [7,32], very likely by competing for interaction to LMX1B LIM domains. We recently showed that LDB1 is essential for podocyte function *in vivo*, possibly as a cofactor of LMX1B transcriptional activity [26]. By contrast, E47 has no essential function in podocyte *in vivo*, and is thus an unlikely cofactor of LMX1B in the kidney [26]. Given LDB1 broad *in vivo* expression pattern and its role as a multifunctional adaptor protein, one would predict that specific cofactors of LMX1B exist *in vivo*, to regulate the tissue-specific expression of LMX1B target genes. However, tissue- and cell-specific LMX1B cofactors have not been identified thus far.

Immunohistochemical, *in situ* hybridization, and microarray analyses of *Lmx1b* null mice identified genes with deregulated expression (at RNA and/or protein level) in the various LMX1B-expressing tissues [13,18,19,24,25,33–35]. Thus, in the developing eye of *Lmx1b*^{-/-} mice, *Foxc1* and *Foxc2* are upregulated and *keratocan* is absent [19]. In the developing tectum and cerebellum of *Lmx1b* null mice, expression of *Wnt1*, *En1*, *Pax2* and *Gbx2* is reduced while expression of *Fgf8* is abolished [18]. In the developing limb, loss of dorsoventral asymmetry is associated with an altered expression pattern of the *sFrp2*, *Six1* and *Six2* genes [33] and with up- and downregulation of multiple genes [34]. In the kidney of newborn *Lmx1b*^{-/-} mice, expression of *Nphs2* (encoding podocin) is abolished, while expression of *Col4a3* and *Col4a4* (encoding the $\alpha(3)$ and $\alpha(4)$ chains of type IV collagen) and of *Cd2ap* is downregulated [13,24,25,35]. A/T-rich, so-called FLAT elements (FLAT-E: TAATTA, FLAT-F: TTAATA or TATTAA on the other strand) present in the promoter regions or introns of the genes encoding podocin, collagen IV α 3 and IV α 4, and CD2AP bind recombinant LMX1B *in vitro* [24,25,35], and drive LMX1B-mediated transcription of a reporter gene when present as multiple copies [24,35] but not from the native promoter [25]. In addition, expression of the genes encoding podocin, collagen IV α 3 and IV α 4, and CD2AP is not affected in the kidney of NPS patients [36]. Therefore, in the absence of *in vivo* molecular characterization of their regulation by LMX1B, it remains unclear whether deregulation of these genes in *Lmx1b* null mice is the consequence of an impaired or blocked differentiation program, or whether they represent *bona fide* LMX1B direct target genes.

We describe here the first molecular characterization of LMX1B target gene regulation *in vivo*. To identify putative LMX1B target genes, human LMX1B was expressed in HeLa cells using a tet-off inducible system. Genome-wide and quantitative RT-qPCR expression studies revealed that several NF- κ B target genes (*IL-6*, *IL-8*, *IL-1 β* , *IFN- β* , ...) and multiple interferon-stimulated genes (ISGs) were upregulated upon LMX1B expression. We found that while ISGs were induced in response to IFN- β , NF- κ B target genes were directly activated by LMX1B. Chromatin immunoprecipitation assays demonstrated that LMX1B protein is recruited to the promoter of the NF- κ B target genes *IL-6* and *IL-8*. Using NF- κ B p65-specific siRNAs, we showed that induction of NF- κ B target genes by LMX1B requires a functional p50/p65 NF- κ B transcription factor. Interestingly, gene expression analysis in *Lmx1b* null mice revealed that the NF- κ B targets induced by LMX1B in HeLa cells were upregulated in the kidney of *Lmx1b*^{-/-} mice, suggesting LMX1B-mediated transcriptional repression of these genes in murine kidney, whereas they remained unaffected in a kidney tubuli-derived cell line (LLC-PK₁) upon LMX1B expression. Our data thus demonstrate for the first time that LMX1B regulates transcription of a subset of NF- κ B target genes in cooperation with p50/p65 NF- κ B, probably through recruitment of cell-specific LIM-interacting cofactors.

Materials and methods

Plasmids

The human LMX1B pcDNA3-derived (Invitrogen) expression vector (p3M/myc-LMX1B) used for co-transfection in luciferase reporter assays has been described [26]. The pUHD10-3/myc-

LMX1B expression vector was generated by subcloning the myc tagged-LMX1B cDNA isolated from p3M/myc-LMX1B into the XbaI site of pUHD10-3 (a kind gift from H. Bujard, ZMDH, Heidelberg, Germany; [37]). The pUHD10-3/myc-LMX1B C95F construct was generated by introducing a single nucleotide change (TGC to TTC) within LMX1B cDNA, using the QuickChange site-directed mutagenesis kit (Stratagene), resulting in a single amino acid substitution (cysteine to phenylalanine) at position 95 of the corresponding LIM2 domain. The human wild-type and NF- κ B mutated motif-containing *IL-6* promoter (–1168/+11) fused to the *Firefly* luciferase reporter gene (designated IL-6P.wt-Luc and IL-6P.NF- κ B-Luc respectively; [38]) were obtained from the BCCM/LMBP plasmid collection (accession number LMBP 4495 and LMBP 4496 respectively). Site-directed mutagenesis of the FLAT element at position –146 relative to the transcription start site (TTAATA to TCGAGA) was performed on both IL-6P.wt-Luc and IL-6P.NF- κ B-Luc plasmids using the QuickChange site-directed mutagenesis kit (Stratagene), according to manufacturer's instructions, generating plasmids IL-6P.FLAT-Luc and IL-6P.FLAT.NF- κ B-Luc respectively. The promoter-less Δ IL-6P-Luc *Firefly* luciferase reporter control was obtained by excising the entire human *IL-6* promoter region (–1168/+11) from plasmid IL-6P.wt-Luc using HindIII. The (NF- κ B)₄-Luc *Firefly* luciferase reporter (pNF κ B-Luc, Clontech) contains 4 copies of the canonical κ B site upstream of a TATA-like minimal *HSV-TK* promoter. Vector backbones of (NF- κ B)₄-Luc and IL-6P-Luc *Firefly* luciferase reporters are identical, as revealed by sequence alignment using the Vector NTI software (Invitrogen). The internal control pRL-null *Renilla* luciferase reporter was from Promega.

Cells

To establish the tet-off human HeLa (HtTA) cell lines expressing the human wild-type LMX1B protein (HtTA-LMX1B wt) and its mutated counterpart LMX1B C95F (HtTA-LMX1B C95F), the parental HtTA-1 cell line (a kind gift from H. Bujard, ZMDH, Heidelberg, Germany; [37]) was transfected with plasmids pUHD10-3/myc-LMX1B wt and pUHD10-3/myc-LMX1B C95F respectively. Stable transfection was performed using Poly-L-Ornithin [39], in the presence of a plasmid conferring resistance to either hygromycin (pWE4; HtTA-LMX1B wt) or puromycin (pWE3; HtTA-LMX1B C95F) [40]. Antibiotic-resistant clones were isolated and tested for proper LMX1B expression in doxycycline-free medium. HtTA-LMX1B wt clone #34 and HtTA-LMX1B C95F clone #351 were chosen for this study. The tet-off porcine kidney tubuli-derived LLC-PK₁ (LtTA) cell line expressing human wild-type LMX1B protein (LtTA-LMX1B wt, clone #16) was established by stably transfecting plasmid pUHD10-3/myc-LMX1B wt into the parental LtTA-2,22 [41], as described above for the HtTA-LMX1B wt cell line. HtTA- and LtTA-derived cells were grown in Dulbecco's modified Eagle's medium (DMEM high glucose with L-Glutamine; PAA) containing 10% FBS (Gibco/Invitrogen), 200 μ g/ml Geneticin (PAA), 30 ng/ml doxycycline (SIGMA), and either 300 μ g/ml Hygromycin B (PAA) (HtTA- and LtTA-LMX1B wt cells) or 500 ng/ml Puromycin (PAA) (HtTA-LMX1B C95F cells). Medium was changed every 2 days. For induction of LMX1B expression, about 2×10^5 cells were seeded in a 6-well Petri dish and incubated in doxycycline-free medium at 37 °C, 5% CO₂ for the indicated times (up to 4 days). For single time-point experiments, cells were harvested 4 days after doxycycline removal. Stimulation with

recombinant human TNF- α (20 ng/ml; ImmunoTools) was performed for either 1 h (RT-qPCR and ChIP analyses) or 8 h (luciferase assay). For the IFN- β neutralizing antibody experiment, cells were incubated for 4 days in doxycycline-free medium in the presence of increasing amounts (0.2–1.8 μ g/ml) of anti-human IFN- β neutralizing antibody (AF814, R&D Systems). Antibody-containing medium was replaced after 2 days. Transient transfections were performed using Lipofectamine 2000 (Invitrogen) in the absence of serum and antibiotics. These were added to the medium 4–5 h post-transfection, as recommended by the manufacturer.

DNA microarray analyses

Two biological replicates of non-expressing HtTA-LMX1B cells (grown in doxycycline-containing medium) and of LMX1B-expressing HtTA-LMX1B cells (grown for 4 days in doxycycline-free medium), and three kidney samples from wild-type and *Lmx1b*^{-/-} knock-out mice were processed for gene expression array analyses, using an Affymetrix (human HtTA-LMX1B cells) or an Agilent (mouse kidneys) platform. Total RNAs were isolated as described below. Quality control of the isolated RNA samples and sample processing for microarray analysis were performed at a regional German Affymetrix Service Provider and Microarray Core Facility, "KFB – Center of Excellence for Fluorescent Bioanalytics" (www.kfb-regensburg.de). For hybridization on Affymetrix Human Gene 1.0 ST Arrays, sample preparation was carried out in accordance with the Affymetrix GeneChip™ Whole Transcript (WT) Sense Target Labeling Assay Manual (Rev. 2). Three-hundred nanograms of DNase-treated total RNA were used to generate Biotin-labeled sense strand (ss) DNA. Following fragmentation and terminal labeling, ssDNA products (5.5 μ g) were hybridized to the array for 16 h at 45 °C at 60 rpm in a rotating chamber. Hybridized arrays were washed and stained in an Affymetrix Fluidics Station FS450, and the fluorescent signals were measured with an Affymetrix GeneChip™ Scanner 3000-7G. Signal intensity calculation was performed using the RMA algorithm as implemented in the Affymetrix Expression Console 1.1 software. For mouse expression arrays, DNase-treated total RNA (1 μ g) was synthesized into Cyanine 3 labeled cRNA (One-Color Microarray-Based Gene Expression Analysis Protocol, version 1.0.1, Agilent Technologies). Labeled cRNA (1.5 μ g) from each sample was hybridized onto Agilent Whole Mouse Genome 4 \times 44 K Expression Arrays, washed and scanned using an Agilent DNA Microarray Scanner according to manufacturer's instructions. Microarray data was extracted using Agilent Feature Extraction Software. For both human and mouse analyses, a significance analysis was performed with ArrayAssist Software (Agilent). Transcripts showing a fold change above 2-fold and a *p*-value below 0.05 were considered as significantly regulated. The microarray data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO Series accession number GSE12008.

mRNA analysis

Total RNAs were isolated using the Nucleospin RNA II kit (Macherey-Nagel), including an on-column DNase I treatment to eliminate genomic DNA contamination, following the manufacturer's recommendations. For RT-qPCR analyses, 1 μ g RNA was used

for cDNA synthesis with the iScript cDNA synthesis kit (BioRad) following the manufacturer's protocol. Quantitative real-time PCR was performed on a RotorGene 3000 (Corbett Research) with SYBR Green I and HotStarTaq (Qiagen). Data were normalized to either human Lamin A/C mRNA (HtTA-derived cell lines) or S9 ribosomal mRNA (mouse kidney and LtTA-LMX1B wt cell line), and expressed as relative mRNA levels, as previously described [42–44]. Human-specific forward and reverse real-time PCR primers used in this study are as follows. Lamin A/C: TCGGTACGGCTCTCATCACT and CTCGTCGTCCTCAACCACAGT; LMX1B: GTGTGAACGGCAGCTACGC and TCATCTCGCTCTTACCGG; ISG15: GCTGGTGGTGACAAATGC and CGGCCCTGTATTCTCTCAC; G1P3: GCAGAAGGCGGTATCGCTT and TCTTACTGCTCCACCCC; IL-6: CTGCGCAGCTTTAAGGAGTTC and CTGAGGTGCCCATGCTACATT; IL-8: CTGCAGCTCTGTGGAAGGTG and TCTGTGTTGGCGCAGTGTG; IL-1 β : CCTGAGCTCCG-CAGTGA AAA and CAGAGGT-CCAGGTCCCTGGAA; IFN- β : TCAGAAGCTCTGTGGCAATT and GTTCATCTGCTCTTGAGGCA; NF- κ B p50: GGCTACACCGAAGCAATTGAA and GAGTGGGCTGAGAGGTGG; NF- κ B p65: GATCTGCCGAGTGAACCGA and GATCTCATCCCCACCGAGG; IFNAR1: ACACCATTTGCAAAGCTCAG and TTAACCATCCAAAGCCACA; I κ B- α : CTGTGATCCTGAGTCCGAGA and GGGTGGTG CAGGACTGAGTC; STAT1: CCGCACCTT-CAGTCTTTCC and ATGCAG- GGCTGTCTTTCCAC; PKD2: GGAAG-CAGCACTAACCGAGA and GCCGTAGTCAAGATGCACA. Murine-specific forward and reverse real-time PCR primers used in this study are as follows. Podocin: GCGAGCGACCAGAGGAAG and GAGCGAGGACAAGAAGCC; ISG15: TTGCCAGAAAGATTGGTGTG and GGTAAGACCGTCTGGAGCA; IL-6: TTGGGACTGATGCTGGTGAC and TCAGAATTGCCATTGCACAAC. The porcine-specific forward and reverse real-time PCR primers for IL-6 were GAACTCCCTCTCCA-CAAGCG and GGCAGTAGCCATCACCAGAAG respectively. Primers specific for S9 and mouse LMX1B have been already described [26,43]. Real-time PCR primers specific for additional investigated genes presented in Table 1 are available upon request.

Protein analysis

For whole cell extract analysis, cells were lysed in Brij lysis buffer as already described [42–44]. For nuclear and cytosolic lysates, cells were lysed in buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM MgCl₂, 1 mM dithiothreitol, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, and 0.5 mM phenylmethylsulfonyl fluoride) containing 0.625% NP-40, centrifuged at 17,000 \times g for 20 s and the supernatant harvested (cytosolic fraction). The nuclei were washed once in buffer A and nuclear proteins were extracted for 1 h under vigorous shaking in buffer C (20 mM HEPES [pH 7.9], 400 mM KCl, 5 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, and 0.5 mM phenylmethylsulfonyl fluoride). Nuclear membranes were eliminated by centrifugation (15 min at 17,000 \times g) and the supernatant harvested (nuclear fraction). Cell lysates were analyzed by Western blot as previously described [26,42–44]. LMX1B protein detection was performed using the formerly described mouse hybridoma supernatant 193-67 (diluted 1:10) [26]. NF- κ B p65 antibody was from Santa Cruz Biotechnology (sc-372; diluted 1:1000). Quality of the nuclear and cytosolic extraction was verified with an antibody directed against the nuclear protein HDAC1 (Upstate Biotechnology, 05-614; diluted 1:1000). Equal protein loading of whole cell extracts was verified with an anti-actin antibody (SIGMA A-2066; diluted 1:5000).

Short interfering RNA (siRNA)-mediated knock-down

NF- κ B p65 and non-specific scramble I (ScI) siRNA duplexes were synthesized using the Silencer siRNA Construction Kit (Ambion #1620), as formerly described [42,44]. Absence of non-specific stimulation of the interferon response upon siRNA transfection [45] was verified by RT-qPCR (not shown). siRNA sequences for NF- κ B p65 (REL1096) and ScI have been described [44,46]. siRNAs were transfected using Lipofectamine 2000 (Invitrogen). Briefly, 2×10^5 HtTA-LMX1B wt cells were seeded on a 6-well Petri dish in the absence or presence of doxycycline. The next day, cells were transfected with 5 nM siRNA and 2 μ l Lipofectamine 2000 following the manufacturer's recommendations. Transfection in the absence of siRNA was performed in parallel. TNF- α stimulation (20 ng/ml; ImmunoTools) was performed 1 h before harvest as indicated. Cells were harvested 72 h post-transfection (and thus 4 days post-doxycycline removal) for protein and RNA analysis. No siRNA-transfected and ScI-transfected control cells behaved identically (not shown) and thus only the ScI data are shown.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed as previously described [42–44] on 3.3×10^6 HtTA- or LtTA-LMX1B wt cells grown for 4 days in the absence or presence of doxycycline, using the formerly described LMX1B-specific rabbit polyclonal antiserum BMO8 (20 μ l; [26]), the NF- κ B p65 antibody from Santa Cruz Biotechnology (30 μ l or 6 μ g; sc-372), or no antibody (protein A-sepharose; GE Healthcare) as a control. Immunoprecipitated genomic DNA was analysed by real-time PCR as previously described [42–44] and data were expressed as a percentage of input DNA. For LMX1B ChIPs, the no antibody control yielded the same background level as the +Dox control performed in the presence of antibody, and thus in some figures only the +Dox control is shown. Forward and reverse real-time PCR primers used in this study to amplify human genomic DNA are as follows. IL-6 (-1237): TGGTTTCAGCCTGAAGCCAT and ACTAG-GTTGTCCTCCAGTCTCC; IL-6 (-921): CCTCCTTGACATCCCCAAC and GTCTCCTGGAGCCTGAAAT; IL-6 (-536): TCACAGGGAGAGC-CAGAACA and CCAAGCCTGGGATTATGAA; IL-6 (-151): GCCATGC-TAAAGGACGTCACA and GGGCTGATTGAAACCTTATTAAGAT; IL-6 (+1046): TTTCGCCAAACCAGCCTTGT and GGAGTCCAGAGGTGG-TAGGGA; IL-8 (-2572): TTTTCGCCTAAGCCATT and AAGATAAC-CATTTGAGTCCCTGCATA; IL-8 (-114): CAAATAGGAAGTG-TGATGACTCAGG and TTTGCAACTGATGGCCCAT. Forward and reverse real-time PCR primers specific for porcine genomic DNA are as follows. IL-6 (-25): CCCCACCCTCCAACAAAGA and TCCT-GGAGGGCAGATGGA; LamA/C (+108): ATCACCCGGCTACAGGA-GAAG and GGTCGATGTAAACCGCCAAG. Numbers in brackets indicate the mean position of the generated amplicon (relative to the transcription start site or in the case of porcine LamA/C relative to the translation start site).

Luciferase reporter assays

HtTA-1 cells were transfected using Lipofectamine 2000 (Invitrogen). Briefly, 1×10^5 cells were seeded on a 24-well Petri dish in doxycycline-containing medium. The next day, cells were transfected with 0.4 μ l Lipofectamine 2000 and the following plasmids: *Firefly* luciferase reporter (0.2 μ g), *Renilla* luciferase reporter (15 ng), and either the LMX1B-expressing vector (0.1 μ g) or the

Table 1 – Selected ISGs and NF-κB target genes upregulated in HtTA-LMX1B cells^a (A) and in the kidney of *Lmx1b* knock-out mice^b (B)

Gene description	Gene symbol	Accession	Reference	A. HtTA-LMX1B		B. Mouse kidney	
				Fold induction ^c Array	RT-qPCR	Fold induction ^c Array	RT-qPCR
LIM homeobox transcription factor 1, beta	<i>LMX1B</i>	NM_002316	–	31.06	273	n.a.	n.a.
Interferon-stimulated genes (ISGs)							
Dickkopf homolog 1 (<i>Xenopus laevis</i>)	<i>DKK1</i>	NM_012242	[72]	18.63	31.0	3.11	n.d.
Interferon, alpha-inducible protein 27	<i>IFI27</i>	NM_005532	[65]	15.41	n.d.	4.10	n.d.
2'-5'-oligoadenylate synthetase 2, 69/71 kDa	<i>OAS2 (OAS3)</i>	NM_002535	[72]	13.17	n.d.	4.19	n.d.
myxovirus (influenza virus) resistance 2 (mouse)	<i>MX2</i>	NM_002463	[65]	12.07	n.d.	3.65	n.d.
2',5'-oligoadenylate synthetase 1, 40/46 kDa	<i>OAS1</i>	NM_016816	[72]	11.06	n.d.	3.16	n.d.
Interferon, alpha-inducible protein 6	<i>IFI6 (G1P3)</i>	NM_022872	[65]	8.30	17.3	–	n.d.
Interferon-induced protein with tetratricopeptide repeats 1	<i>IFIT1 (IFI56)</i>	NM_001548	[64]	5.58	9.66	6.33	n.d.
Interferon induced with helicase C domain 1	<i>IFIH1 (MDA5)</i>	NM_022168	[70]	5.36	n.d.	2.86	n.d.
Interferon-induced protein 44-like	<i>IFI44L</i>	NM_006820	[72]	4.53	n.d.	–	n.d.
Complement component 4 binding protein, alpha	<i>C4BPA (C4BPB)</i>	NM_000715	[65]	4.18	n.d.	–	n.d.
Signal transducer and activator of transcription 1, 91 kDa	<i>STAT1</i>	NM_007315	[85]	4.18	7.38	2.28	n.d.
Interferon-induced protein with tetratricopeptide repeats 2	<i>IFIT2</i>	NM_001547	[65]	4.16	n.d.	n.s.	n.d.
5'-nucleotidase, ecto	<i>NT5E (CD73)</i>	NM_002526	[71]	4.13	2.75	n.s.	1.75
Interferon-stimulated transcription factor 3, gamma 48 kDa	<i>ISGF3G (IRF9)</i>	NM_006084	[65]	4.02	4.30	2.28	1.98
Dual specificity phosphatase 6	<i>DUSP6</i>	NM_001946	[72]	3.96	n.d.	n.s.	n.d.
Interferon-induced protein with tetratricopeptide repeats 3	<i>IFIT3</i>	NM_001031683	[65]	3.95	n.d.	3.01	n.d.
CD274 molecule	<i>CD274 (B7-H1)</i>	NM_014143	[80]	3.89	n.d.	n.s.	n.d.
ISG15 ubiquitin-like modifier	<i>ISG15</i>	NM_005101	[65]	3.86	7.45	6.12	3.19
Interferon-induced protein with tetratricopeptide repeats 5	<i>IFIT5 (RI58)</i>	NM_012420	[77]	3.84	n.d.	–	n.d.
Interferon induced transmembrane protein 1 (9–27)	<i>IFITM1</i>	NM_003641	[65]	3.49	n.d.	n.s.	n.d.
Signal transducer and activator of transcription 2, 113 kDa	<i>STAT2</i>	NM_005419	[85]	3.31	6.00	2.34	n.d.
Interferon-induced protein 35	<i>IFI35</i>	NM_005533	[63]	3.21	n.d.	n.s.	n.d.
Interferon, gamma-inducible protein 16	<i>IFI16 (m: Ifi204)</i>	NM_005531	[65]	3.06	1.79	2.46	n.d.
SP100 nuclear antigen	<i>SP100</i>	NM_001080391	[67]	2.97	n.d.	n.s.	n.d.
Complement component 1, s subcomponent	<i>C1S</i>	NM_201442	[66]	2.77	n.d.	n.s.	n.d.
2'-5'-oligoadenylate synthetase-like	<i>OASL</i>	NM_003733	[72]	2.66	n.d.	6.84	n.d.
Dual specificity phosphatase 5	<i>DUSP5</i>	NM_004419	[72]	2.55	n.d.	–	n.d.
Toll-like receptor 3	<i>TLR3</i>	NM_003265	[75]	2.52	2.55	n.s.	n.d.
Interferon-induced protein 44	<i>IFI44</i>	NM_006417	[72]	2.33	n.d.	n.s.	n.d.
Complement factor H	<i>CFH</i>	NM_000186	[72]	2.21	n.d.	n.s.	n.d.
Toll-like receptor 4	<i>TLR4</i>	NM_138554	[75]	2.16	2.77	n.s.	n.d.
Promyelocytic leukemia	<i>PML</i>	NM_033240	[67]	2.02	n.d.	n.s.	n.d.
Complement component 1, r subcomponent	<i>C1R</i>	NM_001733	[65]	2.01	n.d.	n.s.	1.35
Interferon regulatory factor 7	<i>IRF7</i>	NM_004031	[65]	1.68	7.20	4.93	2.34
Interferon stimulated exonuclease gene 20 kDa	<i>ISG20</i>	NM_002201	[65]	1.65	n.d.	n.s.	n.d.
NF-κB target genes							
Chemokine (C-X-C motif) ligand 10	<i>CXCL10 (IP-10)</i>	NM_001565	[78]	9.94	n.d.	2.50	n.d.
Interleukin 6 (interferon, beta 2)	<i>IL6</i>	NM_000600	[74]	8.20	12	n.s.	2.84
Chemokine (C-C motif) ligand 5	<i>CCL5 (RANTES)</i>	NM_002985	[76]	5.13	n.d.	n.s.	n.d.
Interleukin 8	<i>IL8</i>	NM_000584	[73]	5.28	51	–	n.d.
B-cell CLL/lymphoma 6 (zinc finger protein 51)	<i>BCL6</i>	NM_001706	[79]	1.88	n.d.	n.s.	n.d.
Chemokine (C-X-C motif) ligand 11	<i>CXCL11</i>	NM_005409	[82]	1.82	n.d.	n.s.	n.d.
Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	<i>NFKBIA</i>	NM_020529	[81]	1.71	1.02	n.s.	n.d.
Chemokine (C-X-C motif) ligand 2	<i>CXCL2 (MIP-2)</i>	NM_002089	[84]	1.68	n.d.	n.s.	n.d.
Chemokine (C-X-C motif) receptor 4	<i>CXCR4</i>	NM_001008540	[68]	1.66	n.d.	n.s.	n.d.
Interleukin 1, beta	<i>IL1B</i>	NM_000576	[69]	1.54	21	3.91	3.90
Interferon, beta 1	<i>IFNB1</i>	NM_002176	[51]	1.21	24	n.s.	no signal
Chemokine (C-C motif) ligand 2	<i>CCL2 (MCP-1)</i>	NM_002982	[83]	1.05	0.65	5.76	n.d.

^a Fold induction in HtTA-LMX1B cells grown for 4 days in the absence of doxycycline relative to doxycycline-treated cells.

^b Fold induction in *Lmx1b*^{-/-} relative to *Lmx1b*^{+/+} mice.

^c Fold induction ≥ 2 are in bold; – gene is absent from mouse array; n.s. not significant (array); n.d. not determined (RT-qPCR); n.a. not applicable.

corresponding empty pcDNA3 vector (0.1 µg) to adjust to equal DNA amount. Twenty-four hours post-transfection, cells were treated with TNF-α (20 ng/ml) for 8 h, as indicated, washed with PBS and

lysed in PLB buffer (Promega). Protein concentration was determined by a Bradford-based quantification method (Roti-Quant, ROTH) and luciferase activity determined using the Dual-luciferase reporter

assay system (Promega), following the manufacturer's protocol, and the Centro XS³ LB 960 microplate luminometer (2 s delay and 10 s measurement time; Berthold Technologies). Transient transfections and luciferase assays were performed each time in duplicate. Data were captured with the Berthold MikroWin 2000 software. *Firefly* luciferase activity was normalized to *Renilla* luciferase activity, and corrected for the promoter-less Δ IL-6P-Luc reporter-mediated basal activity. Data were expressed relative to the promoter activity in unstimulated pcDNA3-transfected cells (arbitrarily set to 1). Identical results were obtained in the HtTA-LMX1B wt cell line maintained in the presence of doxycycline (not shown).

Mice

Conventional *Lmx1b* knock-out mice have been described [25]. *Lmx1b*^{-/-} homozygous mice die soon after birth. Therefore, on the day of birth, offspring was killed, kidneys were removed and snap-frozen in liquid nitrogen, and genomic DNA was isolated from the head for genotyping. Total RNA was isolated from a half kidney of three wild-type homozygous and three *Lmx1b*^{-/-} homozygous mice using the Nucleospin RNA II kit (Macherey-Nagel), for RT-qPCR and microarray analyses (kidney), as described above.

Results

NF-κB target genes and interferon-stimulated genes are upregulated in LMX1B-expressing HeLa cells

To identify putative LMX1B target genes, LMX1B was expressed in HeLa cells using a tetracycline-dependent inducible (tet-off) system (HtTA-LMX1B wt cells; [37]). Upon withdrawal of the tetracycline antibiotic doxycycline, LMX1B expression in HtTA-LMX1B wt cells was detected after ~1–2 days at the RNA level (Fig. 1A, -Dox and data not shown) and after ~3 days at the protein level (Fig. 1B, middle panel). LMX1B protein levels in HtTA-LMX1B wt cells reached a plateau after 4 days in doxycycline-free medium (Fig. 1B).

To identify LMX1B-regulated genes, a gene expression profiling study was performed on LMX1B-expressing cells grown for 4 days in the absence of doxycycline. The same cells grown in the presence of doxycycline were used as a control. Total RNAs were isolated and used to hybridize a whole human genome array. Upon LMX1B expression, two main classes of genes were induced: (i) interferon-stimulated genes (ISGs) such as *ISG15*, *G1P3*, *STAT1*, *STAT2*, *IRF7*, *IRF9*, *TLR3* or *TLR4*, and (ii) NF-κB target genes including *IL-6*, *IL-8* and *IL-1β* (Table 1, section A). Induction of these genes was validated by real-time RT-PCR (Fig. 1A and Table 1A).

Induction of NF-κB targets and interferon-stimulated genes by LMX1B requires a functional LIM2 domain

Induction of ISGs and NF-κB target genes in LMX1B-expressing HeLa cells is dependent on LMX1B protein and is not the consequence of a non-specific effect of doxycycline removal, since expression of these genes remained unaffected in the parental HtTA-1 cells grown for 4 days in the absence of doxycycline (Figs. 1B and C). Similarly, no upregulation of ISGs and NF-κB targets was detected in an HtTA cell line expressing the unrelated protein H-ferritin [47] (data not shown), demonstrating that this gene expression pattern is not an

artefact of protein overexpression. Moreover, induction of these genes by LMX1B is dependent on a functional LIM2 domain, since ISGs and NF-κB targets were no longer induced upon doxycycline withdrawal in a HeLa tet-off cell line expressing comparable levels of the LMX1B C95F mutant protein (Figs. 1B and C, HtTA-LMX1B C95F). This single cysteine to phenylalanine mutation within the LIM2 domain of LMX1B was identified in a patient with nail-patella syndrome [28,48] and is expected to disrupt its zinc finger structure [49] and thus its functionality. These data hence demonstrate that LMX1B specifically upregulates expression of ISGs and NF-κB target genes in HeLa cells, and that this activating effect requires a functional LIM protein interaction domain.

IFN-β expression is induced by LMX1B and is responsible for induction of interferon-stimulated genes

Interestingly, several well-known NF-κB target genes such as *NFKBIA* (encoding the NF-κB inhibitor IκB-α), *CCL2* (MCP-1), or the NF-κB subunits *p50* and *p65*, were not induced following LMX1B expression (Fig. 1A, Table 1A, and data not shown), suggesting that LMX1B regulates expression of a subset of NF-κB target genes. By contrast, multiple ISGs were induced, suggesting a more global effect. This prompted us to investigate whether one of the interferon genes was upregulated in LMX1B-expressing HeLa cells. We found that expression of *IFN-β* (Fig. 1A and Table 1A) but not of *IFN-α* or *IFN-γ* (not shown) was induced upon LMX1B expression. This observation hence suggests that induction of *IFN-β*, possibly as a NF-κB target gene [50,51], is responsible for the downstream induction of ISGs. In support to this idea, the *IFN-β* receptor chains *IFNAR1* and *IFNAR2* [52] were expressed at the RNA level in HtTA-LMX1B wt cells (Fig. 1A and data not shown).

To experimentally explore whether *IFN-β* induction is accountable for ISG upregulation, expression of ISGs and NF-κB target genes was monitored in HtTA-LMX1B wt cells grown for 4 days in doxycycline-free medium in the absence or presence of increasing amounts (0.2–1.8 μg/ml) of *IFN-β* neutralizing antibodies. The specific blockade of the *IFN-β* signalling pathway prevented induction of the ISGs by LMX1B (Fig. 2A, *G1P3*, *STAT1*) without affecting LMX1B-dependent induction of the NF-κB target genes (Fig. 2A, *IL-6*). Interestingly, induction of the *IFN-β* gene itself was partially inhibited by the presence of *IFN-β* neutralizing antibodies in the medium (Fig. 2A), in agreement with the observation that *IFN-β* regulates its own expression [52], and confirming that it is also regulated independently of the *IFN-β* pathway, likely as a NF-κB target. These results therefore demonstrate that (i) the LMX1B-dependent induction of NF-κB target genes (*IL-6*, *IL-8*, *IL-1β*, *IFN-β*) occurs upstream of the ISG induction, (ii) the NF-κB target *IFN-β* is responsible for the induction of ISGs as a secondary response, and (iii) *IFN-β* itself regulates its own expression, generating an auto-amplification loop (Fig. 2B).

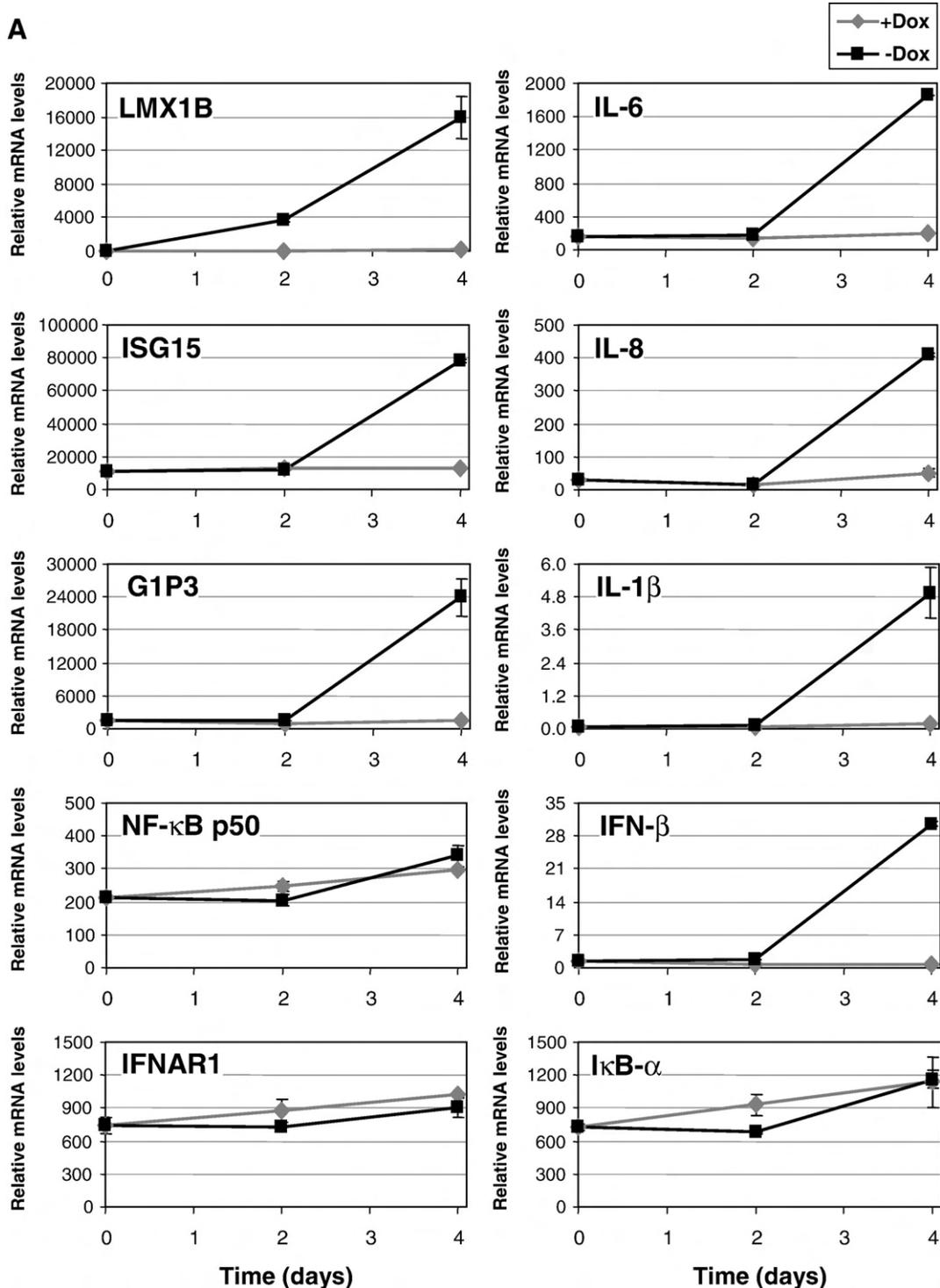
LMX1B directly regulates NF-κB target gene expression, in cooperation with p50/p65 NF-κB

Our finding that induction of NF-κB target genes occurs upstream of the *IFN* pathway in LMX1B-expressing cells raises the possibility of a direct transcriptional regulation by LMX1B. NF-κB target genes are normally induced in response to numerous extracellular stimuli by the NF-κB family of transcription factors. This family consists of 5 members: *p50*, *p52*, *p65* (RelA), RelB and c-Rel, that regulate

transcription as homo- and heterodimers. Their activity is controlled by a family of inhibitory I κ B proteins which control their nuclear translocation and DNA binding activity to specific κ B sites [5'-GG(A/G)N(A/T)(C/T)(C/T)CC-3'] (reviewed in [53]). Two major signalling pathways have been characterized, the classical (or canonical) NF- κ B signalling pathway, and the alternative (or non-canonical) NF- κ B pathway. The classical pathway is induced mainly in response to pathogens and pro-inflammatory cytokines such as TNF- α or IL-1 β . It results in the activation and nuclear translocation of p50/p65 NF- κ B dimers, and in the downstream transcription of genes encoding chemokines and pro-inflammatory cytokines (IL-6,

IL-8, IL-1 β , ...) (reviewed in [54]). By contrast, the alternative pathway is induced by a distinct set of cytokines (LT β R, CD40L and BAFF) and results in the activation of p52/RelB NF- κ B heterodimers, which regulate transcription of genes involved in lymphoid organ maintenance and in B cell maturation (reviewed in [54]). The specific induction of various pro-inflammatory cytokines and chemokines by LMX1B (Table 1 and Fig. 1) strongly suggests that these genes are induced through the p50/p65-dependent classical NF- κ B pathway [46,55,56].

We therefore explored the possible implication of p50/p65 NF- κ B in the activation of NF- κ B target genes by LMX1B. We first



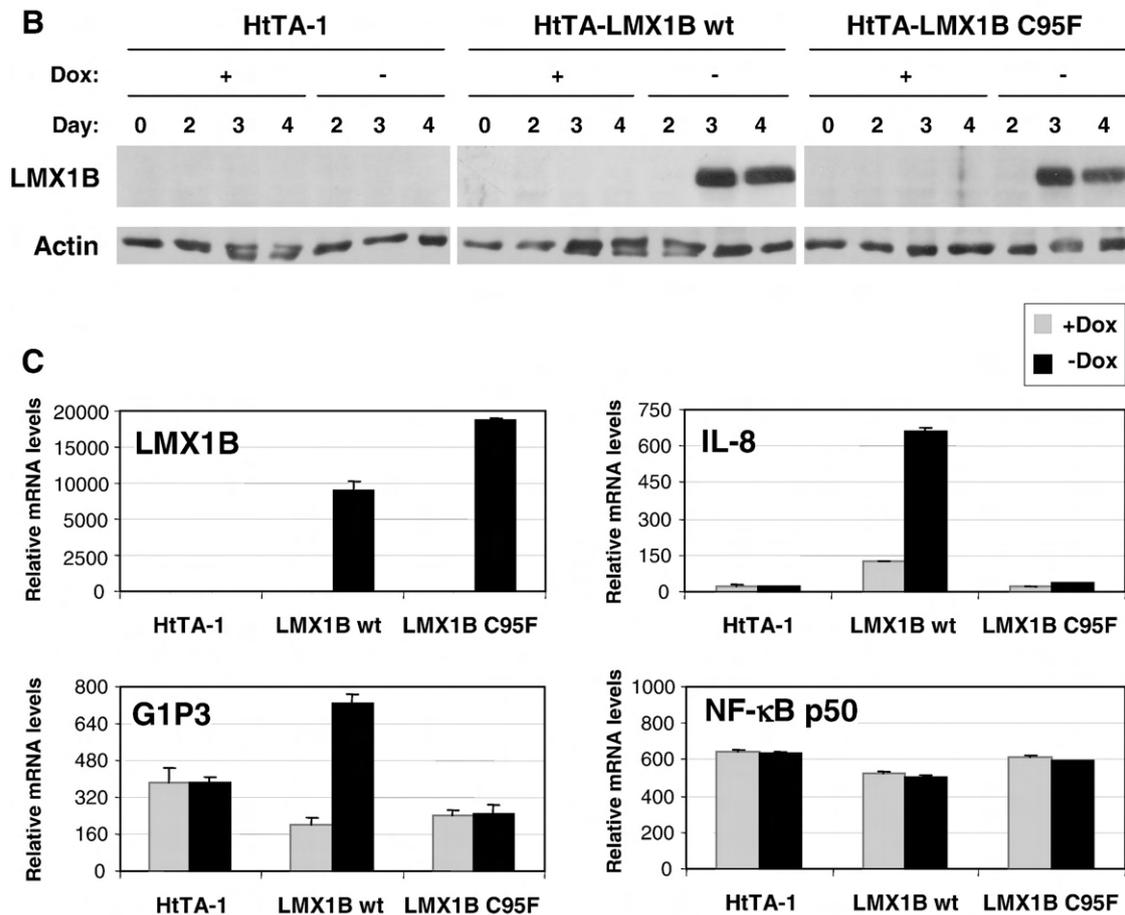


Fig. 1 – NF- κ B target genes and interferon-stimulated genes (ISGs) are upregulated in LMX1B-expressing HeLa cells. (A) RNAs were isolated from HtTA-LMX1B wt cells grown for the indicated times in the presence (+Dox) or absence (–Dox) of doxycycline, and analyzed by RT-qPCR using primers specific for NF- κ B target genes (IL-6, IL-8, IL-1 β , IFN- β , NF- κ B p50 and I κ B- α), interferon-stimulated genes (ISG15 and G1P3), and the IFN- β receptor gene (IFNAR1) as a control. (B) Equal amounts of whole cell protein lysates from parental HtTA-1, HtTA-LMX1B wt and HtTA-LMX1B C95F cell lines grown in the presence or absence of doxycycline for the indicated times, were subjected to Western blot analysis using an antibody directed against either LMX1B or actin as a loading control. (C) Induction of NF- κ B targets and ISGs is dependent on a functional LMX1B protein. HtTA-1, HtTA-LMX1B wt and HtTA-LMX1B C95F cells were grown for 4 days in the presence (+Dox) or absence (–Dox) of doxycycline and mRNA levels were analyzed by RT-qPCR, as described above.

determined whether LMX1B is acting directly at the nuclear level, or indirectly for instance by upregulating a component of the cytosolic activation pathway, leading to p50/p65 nuclear translocation. Nuclear and cytosolic fractions from non-induced and 4 day-induced HtTA-LMX1B wt cells were analyzed by Western blot using NF- κ B p65-specific antibodies. While nuclear translocation of NF- κ B p65 could be readily detected upon stimulation of non-induced cells by TNF- α , a classical activator of the NF- κ B pathway [57] (Fig. 3, compare lane 4 to lane 3), nuclear p65 protein levels remained unchanged upon LMX1B expression (Fig. 3, compare lane 8 to lane 7). Similar results were obtained at earlier time points of LMX1B induction (1 to 3 days of doxycycline removal; not shown). This observation argues against an indirect activation of the upstream NF- κ B signalling pathway by LMX1B, and rather suggests a regulation at the nuclear level.

We next addressed whether regulation of NF- κ B target gene expression by LMX1B is dependent on NF- κ B itself. Expression of the p65 subunit of NF- κ B was knocked down using RNA

interference. Seventy-two hours after transfection of p65-specific or non-specific (Sc1) siRNAs into HtTA-LMX1B wt cells, a maximum reduction in p65 protein levels was achieved (Fig. 4A), corresponding to a ~70% reduction at the RNA level (Fig. 4B). In both LMX1B-expressing cells and TNF- α -stimulated cells as a control, NF- κ B p65 knock-down resulted in a ~50% decrease in NF- κ B target gene induction (Fig. 4B, IL-6, IL-8 and IFN- β). This effect was specific for NF- κ B target genes, since mRNA levels of an unrelated gene remained unaffected upon NF- κ B p65 siRNA transfection (Fig. 4B, PKD2). These results therefore demonstrate that induction of NF- κ B target genes by LMX1B requires a functional p50/p65 NF- κ B transcription factor. It also suggests a possible functional interaction between LMX1B and NF- κ B.

To address whether LMX1B functionally cooperates with NF- κ B, the stimulating effect of TNF- α on expression of NF- κ B target genes was evaluated in HtTA-LMX1B wt cells expressing LMX1B. TNF- α stimulation of LMX1B-expressing cells resulted in a synergistic increase in mRNA levels of the NF- κ B target genes regulated by

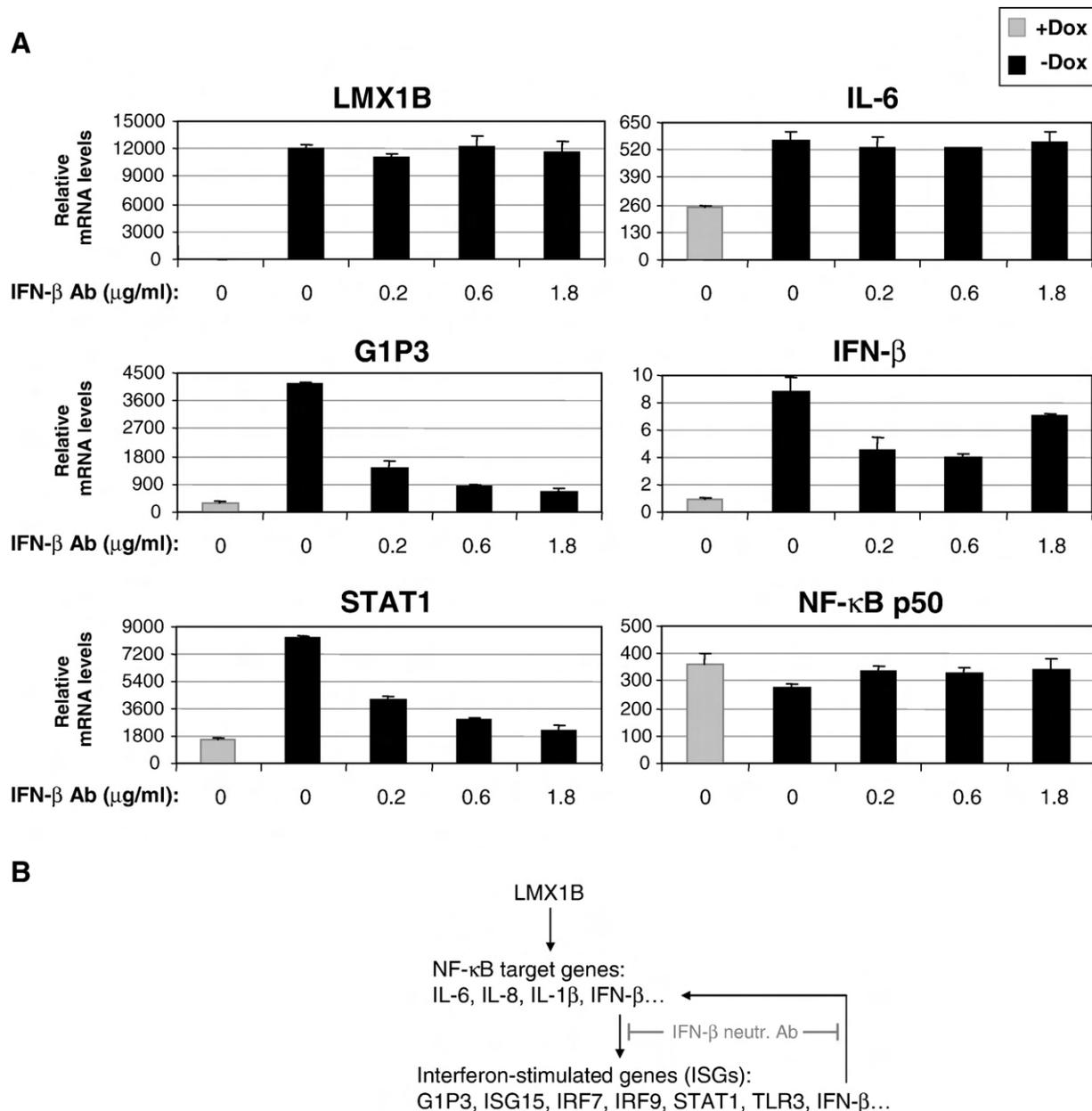


Fig. 2 – Interferon-stimulated genes are induced in response to IFN-β. (A) Doxycycline-treated (+Dox) and untreated (-Dox) HtTA-LMX1B wt cells were grown for 4 days in the presence or absence of increasing amounts of IFN-β neutralizing antibodies (IFN-β Ab), and mRNA levels were analyzed by RT-qPCR, as described in legend to Fig. 1. **(B)** Model of regulation of NF-κB target genes and interferon-stimulated genes (ISGs) by LMX1B in HtTA-LMX1B wt cells. LMX1B induces expression of several NF-κB targets, including IFN-β which in turn induces expression of ISGs, as well as its own expression. IFN-β neutralizing antibodies prevent both LMX1B-mediated ISG induction and IFN-β-mediated auto-amplification loop.

LMX1B (Fig. 4C, *IL-6*, *IL-8*, *IL-1β* and *INF-β*, compare +Dox to -Dox). Importantly, this cooperative effect was not observed upon TNF-α stimulation of a NF-κB target not regulated by LMX1B (Fig. 4C, *NF-κB p50*; see also Fig. 1 and 2), and is thus LMX1B-dependent. Altogether, these data demonstrate that LMX1B functionally cooperates with NF-κB to activate expression of a subset of NF-κB target genes. No physical interaction between LMX1B and NF-κB proteins could be however evidenced in co-immunoprecipitation assays (data not shown), suggesting that functional cooperation does not take place at the protein level.

LMX1B binds to the promoter of the NF-κB target genes *IL-6* and *IL-8* in vivo

To investigate whether LMX1B/NF-κB functional interaction takes place at the chromatin level, we examined the ability of LMX1B and NF-κB to bind to the NF-κB target genes *in vivo* using chromatin immunoprecipitation (ChIP). Promoter sequence analysis of the investigated NF-κB target genes revealed the presence of one or more consensus FLAT (5'-TAAT-3' core-containing) motif(s) in the vicinity of the characterized κB sites within the proximal promoters of the

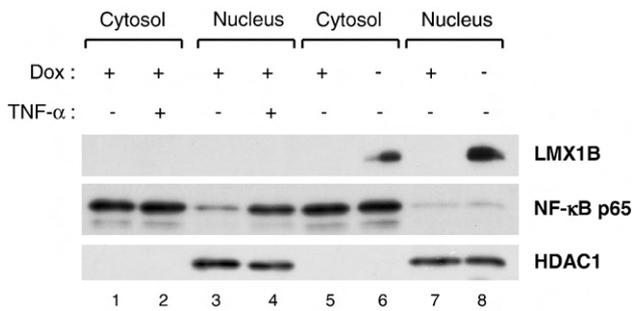


Fig. 3 – LMX1B does not affect NF- κ B nuclear translocation. HtTA-LMX1B wt cells were grown for 4 days in the presence or absence of doxycycline (Dox), or stimulated with TNF- α for 1 h to activate the NF- κ B signalling pathway as a control. Nuclear and cytosolic lysates were subjected to Western blot analysis using antibodies specific for LMX1B, NF- κ B p65 and the nuclear protein HDAC1 to check for nuclear and cytosolic extraction integrity. Comparable results were obtained after 1, 2 or 3 days of doxycycline removal (not shown).

human *IL-6* and *IL-8* genes (Fig. 5A). In addition, the FLAT element within the *IL-6* promoter was conserved in sequence and position between several species (Fig. 5A), suggesting functional conservation. ChIP assays were first performed using LMX1B-specific antibodies on HtTA-LMX1B wt cells grown for 4 days in the absence or presence of doxycycline. Immunoprecipitated genomic DNA was analyzed by real-time PCR using primers amplifying the proximal promoter region of the human *IL-6* and *IL-8* genes that contains the conserved FLAT element(s) along with the κ B site (Figs. 5B and C, positions -151 and -114 relative to the transcription start site respectively), as well as upstream and downstream control primers (Figs. 5B and C). LMX1B was strongly and exclusively associated with the proximal region of the *IL-6* (Fig. 5B, position -151) and *IL-8* (Fig. 5C, position -114) promoters. This association was specific since no significant signal was detected in doxycycline-treated cells (Figs. 5B and C, +Dox) and in ChIP assays performed in the absence of LMX1B antibodies (Fig. 5C, No Ab). In addition, LMX1B overexpression did not lead to ubiquitous FLAT element binding *in vivo*, since no signal was detected within the second intron of the *IL-6* gene that contains two adjacent consensus FLAT elements (Fig. 5B, position +1046). These data thus demonstrate that LMX1B is specifically recruited to the *IL-6* and *IL-8* proximal promoters, possibly through binding to the FLAT element flanking the κ B site.

We next monitored NF- κ B association with the *IL-6* and *IL-8* promoters by ChIP using antibodies directed against the p65 subunit of NF- κ B (Fig. 5C). A control ChIP experiment was performed on TNF- α -stimulated HtTA-LMX1B wt cells in the absence of LMX1B (+Dox), and revealed the expected recruitment of NF- κ B to its binding site (Fig. 5D, upper graph, position -114). NF- κ B p65 binding was specific since it was neither detected over the upstream control region (Fig. 5D, upper graph, position -2572), nor in the no-antibody control ChIP (Fig. 5D, upper graph, No Ab). We then asked whether LMX1B recruitment in HtTA-LMX1B wt cells grown in the absence of doxycycline is accompanied by an increase in NF- κ B binding, possibly explaining the requirement for NF- κ B p65 for LMX1B-mediated transactivation and the cooperative effect of both transcription factors on target gene expression described above. Upon doxycycline withdrawal, a slight increase in NF- κ B

binding was specifically detected on its recognition site (Fig. 5D, lower panel, -Dox, compare position -114 to -2572). Such increase was not detected in the no-antibody control ChIP (Fig. 5D, lower panel, No Ab). This enhanced NF- κ B p65 DNA binding was however moderate, being approximately 10-fold below the binding detected upon TNF- α stimulation (Fig. 5D, upper graph). Although a weaker signal could be due to a reduced antibody accessibility in the ChIP assay in LMX1B-expressing cells compared to TNF- α -stimulated cells, it most likely reflects the low level of nuclear p65 proteins in LMX1B-expressing cells (Fig. 3, lane 8) [58]. Yet, NF- κ B recruitment tightly correlated with LMX1B association to the same promoter region (Fig. 5C), hence suggesting a LMX1B-dependent DNA association. Similar results were obtained on the human *IL-6* promoter (data not shown). Together, these ChIP data demonstrate that LMX1B binds to the proximal promoter region of human *IL-6* and *IL-8* *in vivo*, and that LMX1B recruitment results in a slight increase in NF- κ B p65 binding to its nearby recognition site.

The conserved FLAT and κ B elements within the *IL-6* proximal promoter are both involved in LMX1B-mediated transcription

Functional assays were then carried out to determine whether LMX1B and NF- κ B DNA binding to the *IL-6* promoter *in vivo* is required for transcriptional gene activation. Luciferase reporter assays were performed using reporter constructs bearing the human *IL-6* promoter (-1168/+11; [38]), either wild-type or mutated within the proximal FLAT element (-146) and/or κ B site (-74) (Fig. 6A). Transcriptional effect of LMX1B and NF- κ B was evaluated in HtTA-1 cells upon co-transfection of a LMX1B expression plasmid and TNF- α stimulation respectively.

In the absence of co-transfected LMX1B, TNF- α stimulation of HtTA-1 cells resulted in a 4-fold activation of the wild-type *IL-6* promoter (Fig. 6B, IL-6P.wt, -LMX1B, black bar), in accordance with published data [38]. Mutation of the FLAT element did not affect NF- κ B-mediated transcriptional activation of the *IL-6* promoter (Fig. 6B, IL-6P.FLAT, -LMX1B, black bar). By contrast and as expected [38], mutation of the κ B site abolished NF- κ B-mediated transcription (Fig. 6B, IL-6P.NF- κ B and IL-6P.NF- κ B.FLAT, -LMX1B, black bars).

LMX1B co-transfection resulted in a 3-fold activation of the wild-type *IL-6* promoter (Fig. 6B, IL-6P.wt, +LMX1B, grey bar), demonstrating the ability of LMX1B to activate transcription from the *IL-6* promoter. By contrast, the control (NF- κ B)₄-luciferase reporter containing four κ B sites in tandem fused to a minimal promoter, but no FLAT motif, was not stimulated by LMX1B (Fig. 6B, (NF- κ B)₄, +LMX1B, grey bar). Mutation of the FLAT element strongly inhibited activation of the *IL-6* promoter by LMX1B (Fig. 6B, IL-6P.FLAT, +LMX1B, grey bar), indicating that transactivation of the *IL-6* promoter by LMX1B is mainly mediated by the proximal FLAT element. Mutation of the κ B site alone did not influence activation by LMX1B (Fig. 6B, IL-6P.NF- κ B, +LMX1B, grey bar). However, mutation of both FLAT and κ B elements completely abolished LMX1B-mediated transactivation (Fig. 6B, IL-6P.FLAT.NF- κ B, +LMX1B, grey bar), suggesting that the κ B site might play some minor role in LMX1B-mediated transactivation.

TNF- α stimulation in the presence of LMX1B resulted in a slightly enhanced transactivation of the wild-type *IL-6* promoter (Fig. 6B, IL-6P.wt, +LMX1B, black bar). In contrast to the cooperative effect observed *in vivo* in the context of chromatin (Fig. 4) however, this positive effect was only additive, hence revealing that regulatory

components are missing in the reporter assay to achieve cooperative transactivation. As expected, this additive effect was dependent on LMX1B binding to its FLAT element since it was lost upon mutation of the FLAT motif (Fig. 6B, IL-6P.FLAT, +LMX1B, black bar).

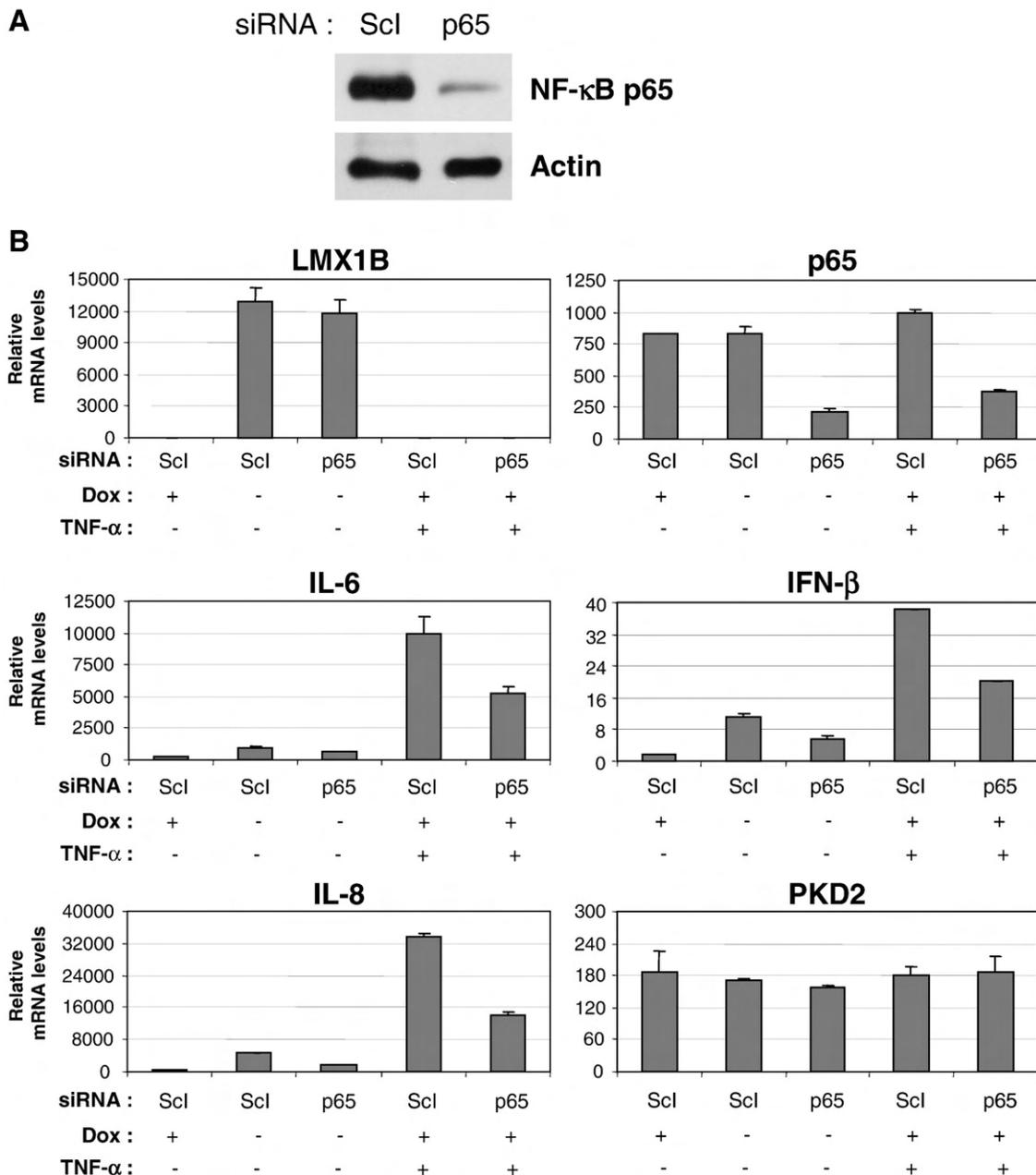
Intriguingly, mutation of the κ B motif not only inhibited NF- κ B-mediated transcription, it also impaired LMX1B-mediated transactivation of the *IL-6* promoter (Fig. 6B, IL-6P.NF- κ B, +LMX1B, black bar). This observation suggests that transactivation by LMX1B involves a cofactor also required for NF- κ B-mediated transcription. This common cofactor would become limiting following the massive nuclear translocation of NF- κ B upon TNF- α stimulation, while hindered from binding to DNA (mutated κ B site). If correct, this proposition would predict that reciprocally LMX1B overexpression would impair NF- κ B-mediated transactivation of a reporter non-responsive to LMX1B. Indeed, NF- κ B-mediated transactivation of the (NF- κ B)₄-luciferase reporter was significantly inhibited upon LMX1B overexpression (Fig. 6B, (NF- κ B)₄,

+LMX1B, black bar), hence supporting the idea that both LMX1B and NF- κ B recruit a common coactivator required for their activity.

Altogether, these luciferase reporter assays demonstrate that LMX1B can activate transcription from the *IL-6* promoter mainly through the proximal FLAT element flanking the κ B site. These assays also indicate the possible recruitment of a common coactivator by LMX1B and NF- κ B, and suggest that chromatin components are likely involved in the functional cooperation observed between both transcription factors *in vivo*.

Expression of NF- κ B target genes and interferon-stimulated genes is upregulated in the kidney of *Lmx1b*^{-/-} knock-out mice

We showed that in HeLa cells LMX1B directly activates expression of a subset of NF- κ B target genes, leading to the indirect upregulation of multiple interferon-stimulated genes (ISGs). We also showed that



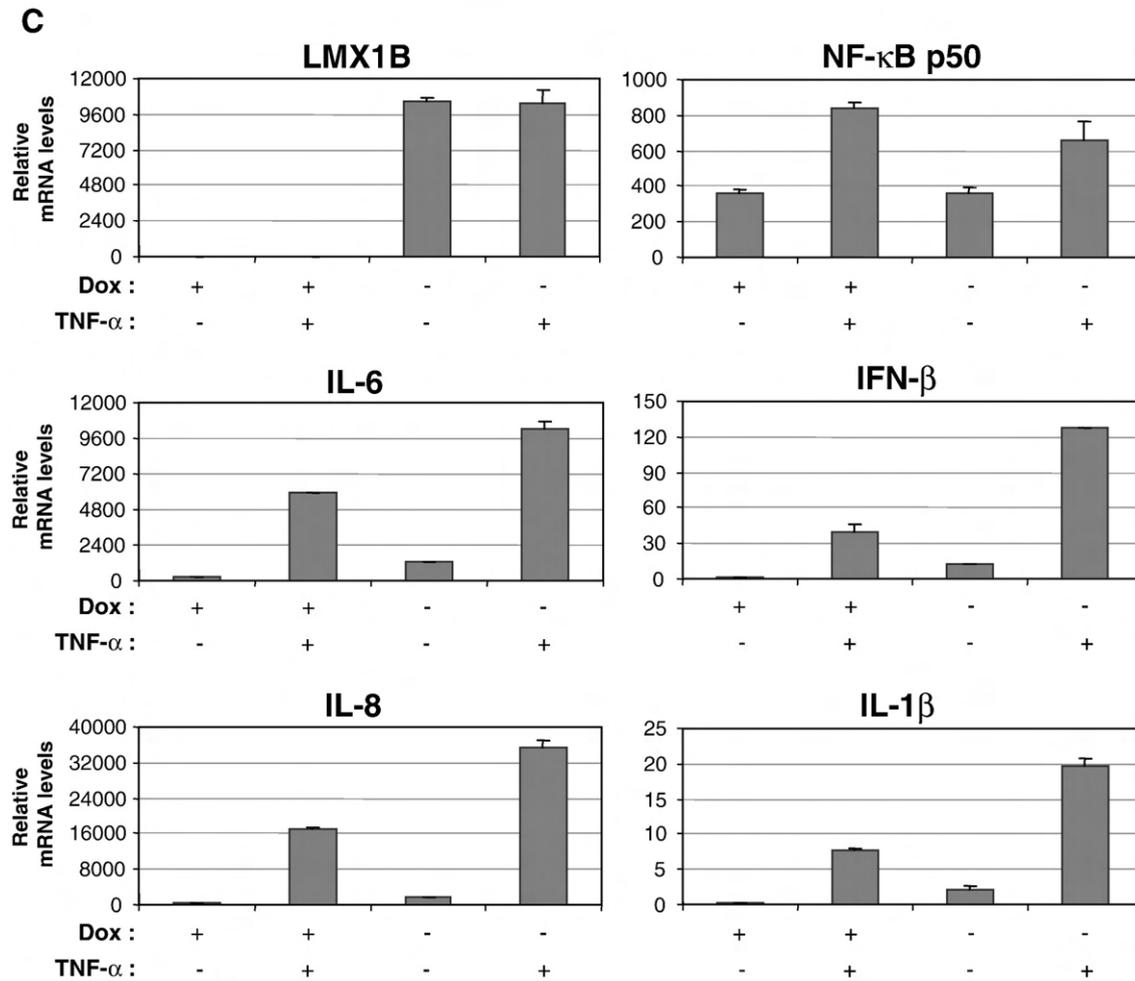


Fig. 4 – NF- κ B is required and cooperates with LMX1B to regulate expression of NF- κ B target genes. (A, B) LMX1B-mediated induction of NF- κ B targets is dependent on NF- κ B. H τ TA-LMX1B wt cells grown in the presence or absence of doxycycline (Dox) were transfected at day 1 with either a non-specific (Sc1), or a NF- κ B p65-specific siRNA. Seventy-two hours later, cells were stimulated with TNF- α for 1 h or left unstimulated as indicated, and harvested for Western blot (A) or RT-qPCR (B) analysis. Non-transfected cells were processed in parallel and provided identical results as the Sc1-transfected cells (not shown). (A) Whole cell lysates from doxycycline-treated transfected cells were immunoblotted against NF- κ B p65, as well as actin to control for equal protein loading. (B) RT-qPCR was performed using primers specific for NF- κ B target genes (IL-6, IL-8, IFN- β) and an unrelated control gene (PKD2). NF- κ B p65 mRNA levels were reduced by ~70% upon siRNA transfection (p65) while LMX1B mRNA levels remained unaffected (LMX1B). (C) LMX1B and NF- κ B functionally cooperate to induce NF- κ B target genes. H τ TA-LMX1B wt cells were grown for 4 days in the presence or absence of doxycycline (Dox), and stimulated with TNF- α for 1 h to activate NF- κ B transcriptional activity or left unstimulated. RNAs were isolated and analyzed by RT-qPCR as described for Fig. 1, using primers specific for LMX1B as a control, and for LMX1B-dependent (IL-6, IL-8, IL-1 β and IFN- β) and -independent (NF- κ B p50) NF- κ B target genes.

activation of the NF- κ B target *IL-6* by LMX1B can be reproduced in a reporter assay in these cells. Since HeLa cells do not normally express LMX1B though, it raises the question of the biological significance of these observations. To address that question, *in vivo* expression studies were undertaken using conventional *Lmx1b* knock-out mice [25]. These mice display podocyte-associated nephropathy, leading to severe proteinuria and death soon after birth [13,24,25]. Total RNAs were isolated from kidneys of three newborn wild-type and *Lmx1b*^{-/-} knock-out mice and analysed by RT-qPCR and gene expression profiling array. In the kidney of *Lmx1b*^{-/-} knock-out mice, *Lmx1b* mRNAs were undetectable and expression of the putative LMX1B target gene *podocin* was abolished (Fig. 7A), as previously described [24,25]. Interestingly, gene expression profiling

revealed that a significant number of the ISGs and NF- κ B targets identified in HeLa cells were differentially expressed in the kidney of *Lmx1b*^{-/-} knock-out mice (Table 1, section B), supporting the biological relevance of our observations. Unexpectedly however, their expression was upregulated in the knock-out mice (Table 1B). Several of them, including *Isg15*, *Irf-7*, *Irf-9*, *IL-6* and *IL-1 β* were validated by real-time RT-PCR (Fig. 7A and Table 1B). These data suggest that the physiological function of LMX1B in the kidney is to repress expression of NF- κ B target genes and ISGs. A similar pattern was observed in other mouse tissues expressing LMX1B such as eye and limb (data not shown). This is in apparent contradiction with the data obtained in H τ TA-LMX1B cells, in which expression of the same genes was induced by LMX1B (Fig. 1 and Table 1A). This is also in

apparent contradiction with our luciferase reporter assays demonstrating that LMX1B can activate transcription from the *IL-6* promoter (Fig. 6). Alternatively, these observations raise the

possibility that cell- and tissue-specific cofactors are implicated in transcriptional regulation by LMX1B, dictating either activation or inhibition of the target gene.

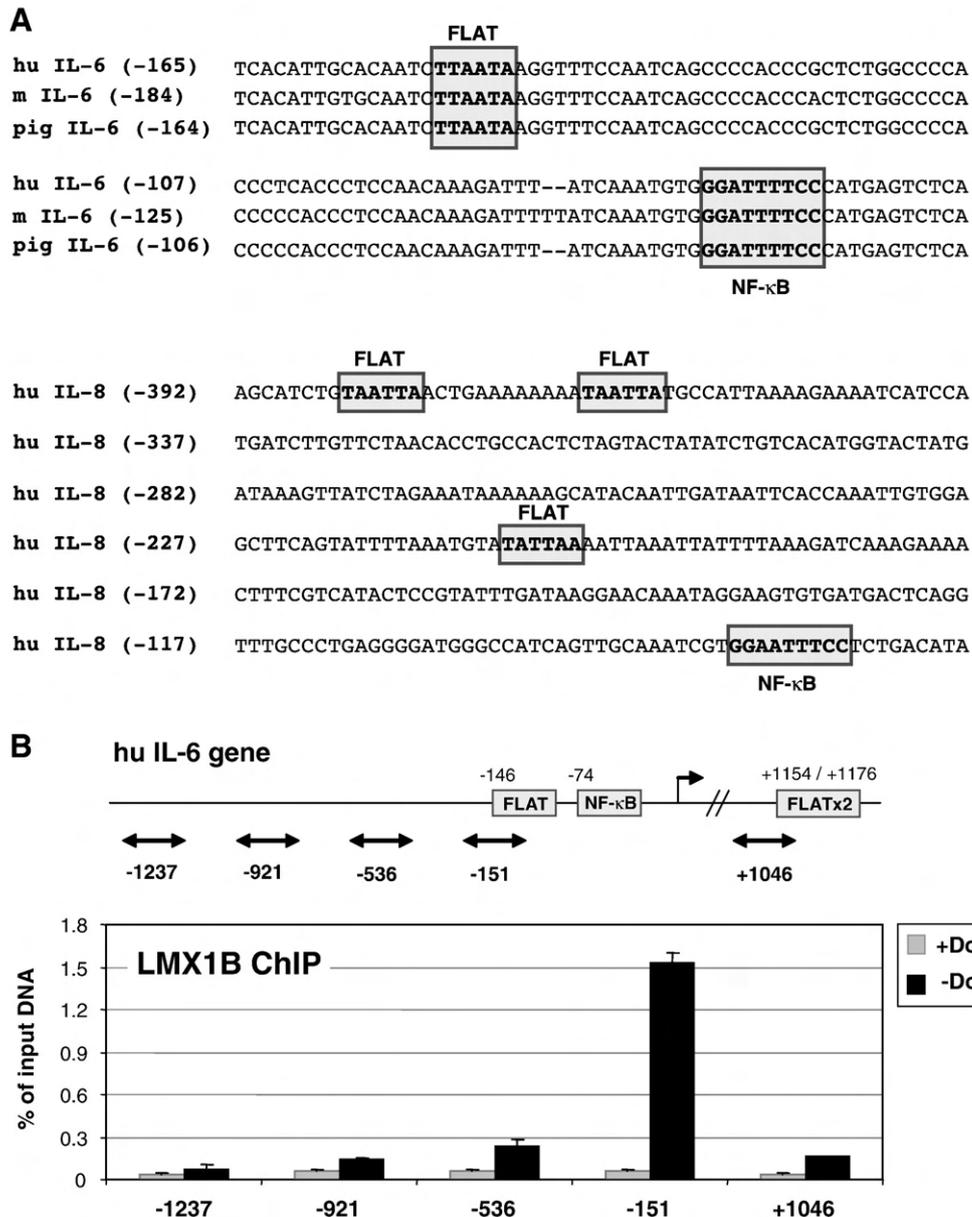
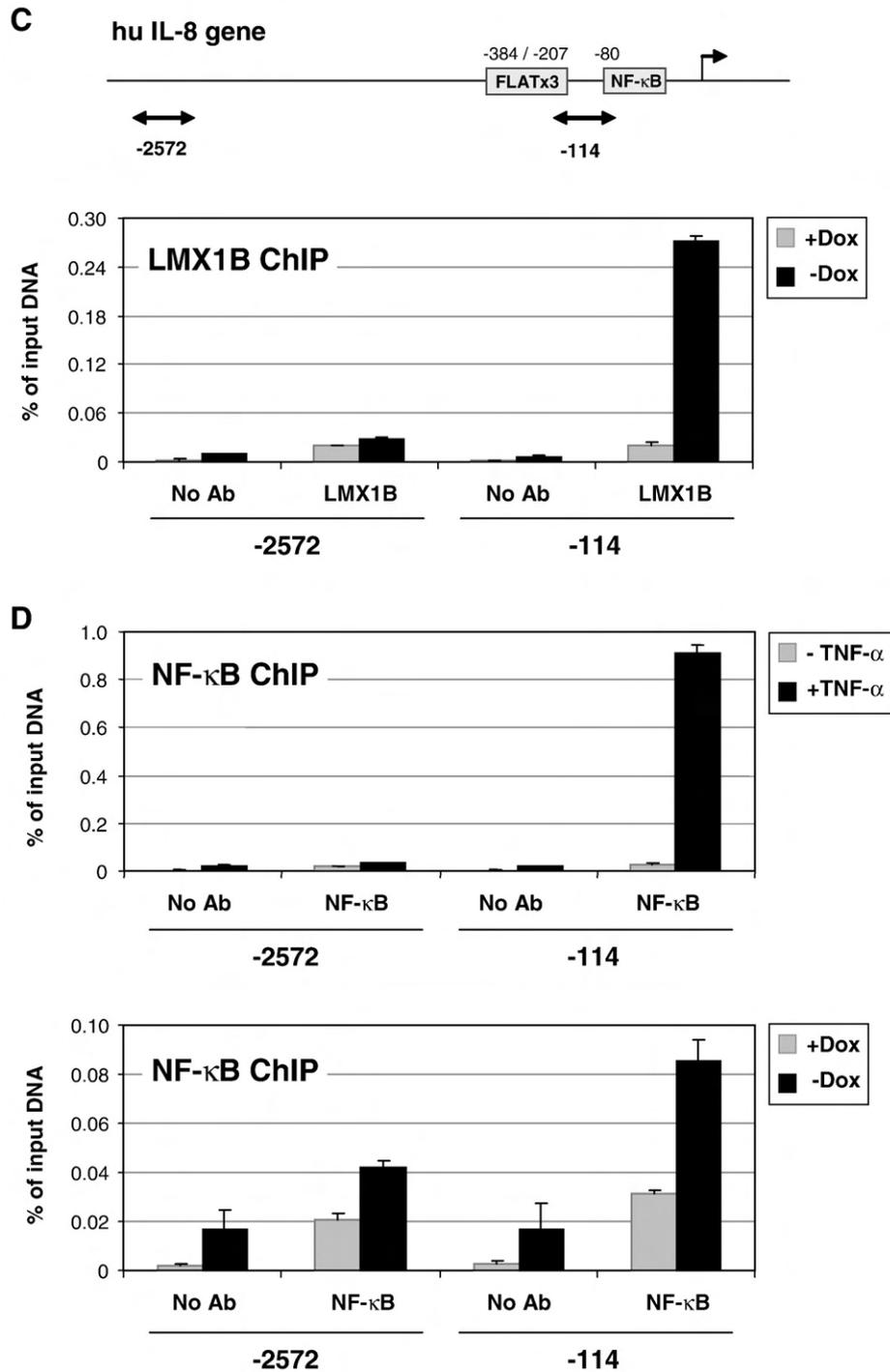


Fig. 5 – LMX1B is recruited to the human *IL-6* and *IL-8* promoters *in vivo*. (A) Nucleotide sequences of the human *IL-6* and *IL-8* promoters showing the conserved FLAT-F motif (TTAATA) within the *IL-6* promoter of several species (position -146 relative to the transcription start site of the human promoter), and the presence of 3 FLAT elements (TAATTA and TATTAA, between positions -384 and -207 relative to the transcription start site) within the human *IL-8* promoter. In both genes, the FLAT motifs are located in the vicinity of the identified κB site (positions -74 and -80 respectively relative to the transcription start site). (B–C) LMX1B chromatin immunoprecipitation (ChIP) was performed from doxycycline-treated (+Dox) and untreated (-Dox) HtTA-LMX1B wt cells as described in Materials and methods. Immunoprecipitated genomic DNA was analyzed by real-time PCR using primers specific for the human *IL-6* (B) and *IL-8* (C) genes. No-antibody control ChIPs yielded similar background signals as LMX1B ChIPs from doxycycline-treated cells, and are only shown in panel C for the sake of clarity. Upper panel shows a schematic representation of the gene, together with the FLAT and κB motifs (grey boxes) and the position (relative to the transcription start site) of the investigated amplicons. (D) Association of nuclear NF-κB with DNA is increased in LMX1B-expressing cells. NF-κB p65 ChIP was performed from unstimulated and TNF-α-stimulated HtTA-LMX1B wt cells grown in the presence of doxycycline as a control experiment (upper graph), and in doxycycline-treated (+Dox) and untreated (-Dox) HtTA-LMX1B wt cells (lower graph). Genomic DNA was analyzed using primers specific for the human *IL-8* promoter. The LMX1B and NF-κB ChIPs shown in panels (C) and (D) were performed from the same cross-linked cells for direct comparison.



LMX1B* recruitment to the *IL-6* promoter in LLC-PK₁ cells is not sufficient to mediate transcriptional activation *in vivo

To explore the cell-specific regulation of LMX1B activity, expression of NF-κB target genes was investigated in a distinct cellular context. LMX1B was expressed in the porcine kidney tubuli-derived cell line LLC-PK₁ using the same tet-off inducible system as for HeLa cells (HtTA-LMX1B), generating the so-called LtTA-LMX1B cell line (Fig. 7B). Similarly to HeLa cells, LLC-PK₁ cells do not express endogenous LMX1B. Remarkably, expression of *IL-6* and of none of the investigated NF-κB target genes and ISGs was regulated by LMX1B

in LLC-PK₁ cells (Fig. 7B and data not shown). Furthermore, a microarray analysis comparable to the one undertaken in HeLa cells (Table 1) revealed that few genes were differentially regulated by LMX1B in LLC-PK₁ cells (not shown). To exclude the possibility that absence of gene regulation was the consequence of a defect in LMX1B recruitment to its target genes in these cells, chromatin immunoprecipitation was performed and revealed that LMX1B was efficiently recruited to the *IL-6* proximal promoter in LLC-PK₁ cells (Fig. 7C). These data therefore indicate that LMX1B binding to DNA is not sufficient to regulate expression of downstream target genes *in vivo*. They also imply that other factors are necessary, thus supporting our

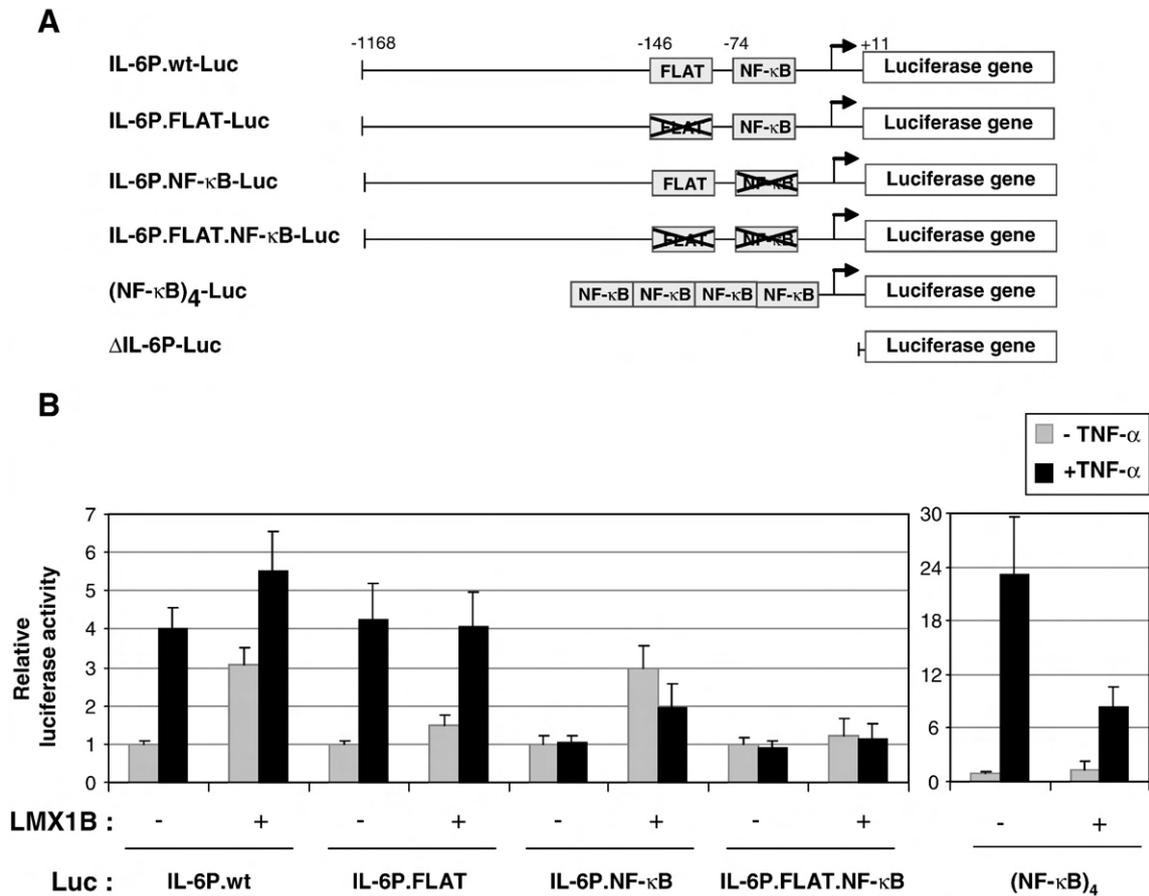


Fig. 6 – The FLAT and NF- κ B recognition motifs are involved in transcriptional regulation of the human *IL-6* promoter by LMX1B and NF- κ B. (A) Schematic representation of the *Firefly* luciferase reporter constructs used in this study. The proximal FLAT and κ B motifs within the human *IL-6* promoter (grey boxes) were mutated (crossed grey box) individually or together (IL-6P-Luc derivatives), and transfected in parallel to control *Firefly* luciferase reporters containing either four κ B sites fused to a minimal promoter [(NF- κ B)₄-Luc] or a total deletion of the human *IL-6* promoter (Δ IL-6P-Luc). (B) HTA-1 cells were co-transfected with the indicated *Firefly* luciferase reporter plasmid (Luc) and a LMX1B-expression vector (or an equal amount of the corresponding empty control vector), as described in Materials and methods. Twenty-four hours later, cells were stimulated with TNF- α for 8 h (black bars) or left unstimulated (grey bars). Luciferase activity was monitored as described in Materials and methods and expressed relative to basal promoter activity (=1). Histograms represent an average of at least 3 independent experiments, each performed in duplicate.

proposition that differentially expressed cofactors are required for positively or negatively regulating LMX1B transcriptional activity in distinct cellular contexts (Fig. 8).

Discussion

Using various *in vitro* and *in vivo* approaches, we demonstrate for the first time that LMX1B directly regulates expression of several NF- κ B target genes and, by inducing IFN- β , indirectly regulates expression of interferon-stimulated genes (ISGs).

LMX1B, transcriptional activator and repressor

Transcriptional activation by LMX1B was previously shown using reporter constructs containing multiple copies of a FLAT element fused to a minimal promoter [24,29,35], but to the best of our knowledge was never shown from a native promoter. We show here for the first time that in HeLa cells LMX1B activates

transcription of a luciferase reporter driven by the native human *IL-6* promoter (-1168/+11). Transcriptional activation of the endogenous *IL-6* gene in HeLa cells was confirmed by expressing LMX1B using a tet-off system and gene expression profiling, which identified a number of NF- κ B target genes upregulated as well. We were therefore surprised to identify the same genes upregulated in the kidney of *Lmx1b* knock-out mice, supporting a repressive – rather than activating – effect of LMX1B. This apparent discrepancy might suggest that our tet-off expression system yields non-physiological observations. However, several pieces of evidences argue against such possibility. First, as discussed above, our data are in accordance with the previous description of transcriptional activation of luciferase reporters by LMX1B [24,29,35], and with reports of both positive and negative functions attributed to LIM-HD transcription factors [1,2]. Second, we demonstrated that the activating function of LMX1B in our tet-off expression system was neither the result of doxycycline treatment nor of aberrant protein overexpression. In that regard, LMX1B overexpression in another tet-off cell line (LL-CPK₁) was not sufficient to trigger induction of

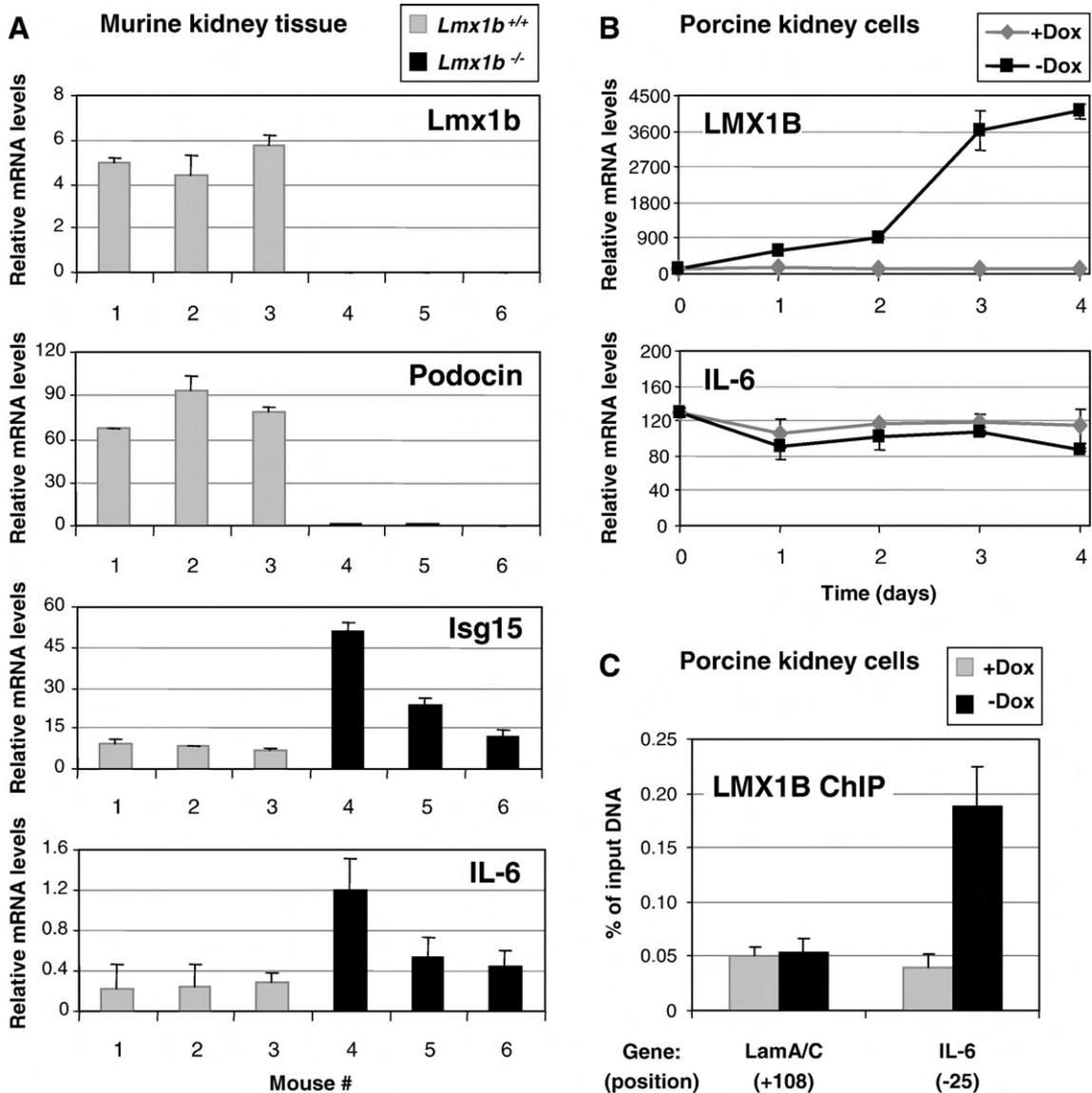


Fig. 7 – NF- κ B target genes and ISGs are upregulated in the kidney of *Lmx1b*^{-/-} knock-out mice but not in LLC-CPK₁ cells expressing LMX1B. (A) RNAs were isolated from the kidney of wild-type (*Lmx1b*^{+/+}) and homozygous knock-out (*Lmx1b*^{-/-}) mice, and analyzed by RT-qPCR using primers specific for mouse *Lmx1b* and the putative LMX1B target gene podocin as controls, as well as for the mouse NF- κ B target IL-6 and interferon-stimulated gene *Isg15*, as described above. (B) NF- κ B target genes and ISGs are not regulated by human LMX1B in the porcine LLC-CPK₁ cell line. Tet-off inducible L_tT_A-LMX1B cells were grown in the absence (-Dox) or presence (+Dox) of doxycycline for the indicated times and RNAs were analyzed by RT-qPCR using human (LMX1B) and porcine (IL-6) specific primers. LMX1B expression was also verified at the protein level by Western blot analysis (not shown). (C) LMX1B is recruited to the IL-6 promoter in LLC-CPK₁ cells. LMX1B ChIP was performed from doxycycline-treated (+Dox) and untreated (-Dox) L_tT_A-LMX1B cells as described above for H_tT_A-LMX1B cells. Immunoprecipitated genomic DNA was analyzed by real-time PCR using primers specific for the porcine IL-6 proximal promoter (mean position -25 relative to the transcription start site). Due to limited porcine genomic sequence availability, control primers were designed within the Lamin A/C (LamA/C) coding sequence (mean position +108 relative to the translation start site).

NF- κ B target genes. Third, a point mutation within the LIM2 domain of LMX1B abrogated its activating function in HeLa cells, demonstrating the specificity of this effect and the importance of a functional LIM2 domain, in accordance with the literature [1]. We therefore strongly believe that the data obtained in the HeLa tet-off cell line are biologically relevant, and that they actually reveal a unique characteristic of LMX1B, as either a positive or negative transcriptional regulator depending on the cellular context.

LMX1B activity is regulated by cell-specific cofactors

We found that while LMX1B positively regulates expression of NF- κ B target genes in HeLa cells, it represses the same genes in the mouse kidney – likely in the podocytes exclusively expressing LMX1B [23] – but is not sufficient to regulate expression of these genes in the kidney tubuli-derived cell line LLC-CPK₁. A similar downregulation of NF- κ B target genes by LMX1B in mouse kidney

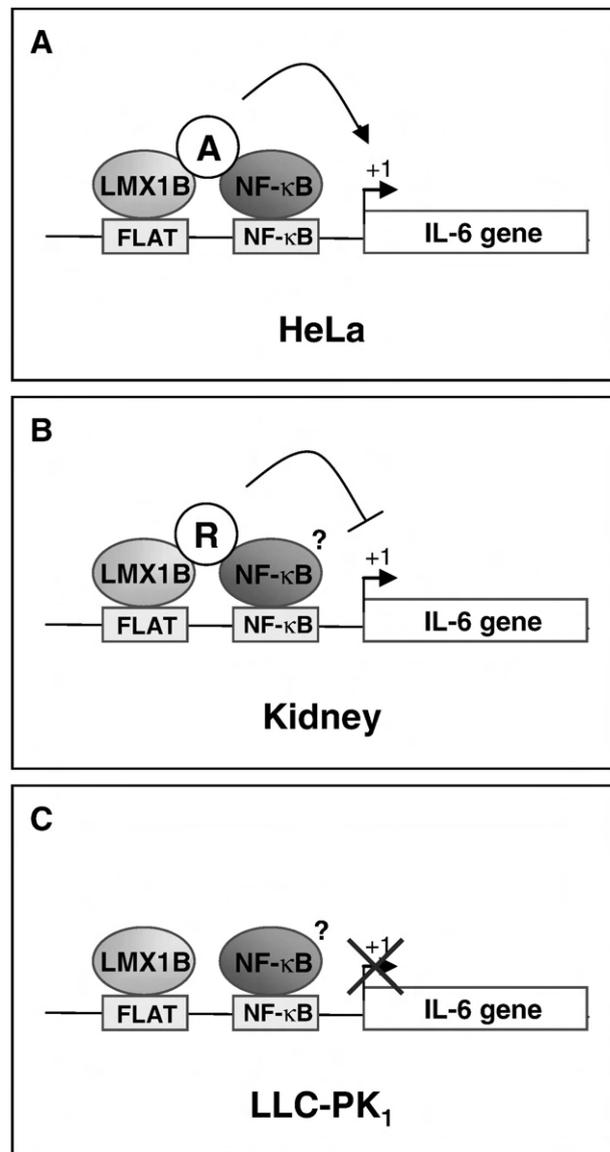


Fig. 8 – Model of cell-specific regulation of NF-κB target genes by LMX1B. (A) In HeLa cells, LMX1B binds to the FLAT element within the proximal promoter of NF-κB target genes such as *IL-6* and *IL-8*. LMX1B recruitment leads to an increase in nuclear p50/p65 NF-κB binding to its nearby recognition site, possibly through protein–DNA complex stabilization via recruitment of a common coactivator (“A”). NF-κB activation by TNF-α in LMX1B-expressing cells results in enhanced transcription of the target gene. By contrast, prohibiting either NF-κB or LMX1B association to DNA in TNF-α-stimulated cells (by mutating or in the absence of their recognition motif respectively) partially inhibits LMX1B- or NF-κB-mediated transcription respectively, probably through sequestration of coactivator “A” by nuclear NF-κB or LMX1B in excess. (B) In mouse kidney, absence of coactivator “A” and likely presence of corepressor “R” might result in inhibition of NF-κB target gene expression. Whether NF-κB is also involved in this repressive effect remains to be shown. (C) In the porcine kidney cell line LLC-PK₁, Although LMX1B binds to its recognition site in the context of chromatin, NF-κB target genes are not regulated by LMX1B, suggesting an absence of both coactivator “A” and corepressor “R”. Altogether, our data support a model of tissue-specific regulation of LMX1B target genes based on differential expression of specific cofactors. These tissue- and cell-specific cofactors remain to be identified.

was observed using podocyte-specific inducible *Lmx1b* knock-out adult mice (A.R., unpublished data). Although upregulation of NF-κB target genes was detected in other LMX1B-expressing tissues in the conventional *Lmx1b* knock-out mice, we cannot rule out that part of this expression pattern is the result of proteinuria-induced inflammation in the kidney. We are currently addressing that point through gene expression analysis of glomerulus preparations

isolated from podocyte-specific inducible *Lmx1b* knock-out mice, at early time points upon *Lmx1b* knock-out.

Collectively, our data support a model involving cell-specific cofactors positively or negatively regulating LMX1B transcriptional activity (Fig. 8). First, ChIP assays showed that while LMX1B is able to bind to the endogenous *IL-6* promoter in HeLa and LL-CPK₁ cells, it only supports transcriptional activation in HeLa cells. Absence of

regulation in LLC-PK₁ cells cannot be attributed to expression of human LMX1B in porcine cells since human and porcine LMX1B share 100% identity. Second, a functional LIM2 protein interaction domain is essential for LMX1B-mediated gene regulation [1,2]. Third, mutation of the NF- κ B recognition motif within the *IL-6* promoter–luciferase reporter partially impaired LMX1B-mediated transcription in the presence of TNF- α , and reciprocally LMX1B expression inhibited NF- κ B-mediated transcription of a reporter lacking a FLAT element. These observations suggest that both LMX1B and NF- κ B transcription factors require a common cofactor to activate transcription in HeLa cells. Although these assays suggest a competition for binding to this common coactivator, this factor might be involved in the LMX1B/NF- κ B synergy observed *in vivo* on expression of endogenous NF- κ B target genes. An alternative explanation for the observed reciprocal inhibition would be that LMX1B and NF- κ B directly interact with each other. However, we were unable to detect such interaction in co-immunoprecipitation experiments. Since intact DNA binding sites are necessary for LMX1B-mediated transcription, our data rather evoke the recruitment of a common coactivator or adaptor protein to pre-bound LMX1B and NF- κ B transcription factors. Such coactivator might stabilize the LMX1B/NF- κ B/chromatin complex, possibly explaining the increase in NF- κ B binding detected by ChIP. Since NF- κ B promoter occupancy directly correlates with transcriptional activity [58], an increase in NF- κ B binding would ultimately result in enhanced transcription of the target gene.

So far, the multifunctional and ubiquitously expressed protein LDB1 is the only characterized cofactor of LMX1B [6–8,26,31]. Cell-specific cofactors of LMX1B hence remain to be identified. One possible candidate is iASPP (also called Nkip1 or RAI), a nuclear protein directly interacting with and inhibiting the activity of NF- κ B p65 [59,60]. iASPP was identified in a yeast two-hybrid screen by our group as a possible interacting partner of LMX1B (R.W., unpublished data), raising the possibility that it might act as an adaptor protein between LMX1B and NF- κ B p65. Although both iASPP/LMX1B and iASPP/NF- κ B interactions could be confirmed by co-immunoprecipitation from eukaryotic cells, no complex containing all three proteins could be detected (not shown), suggesting that the interaction of iASPP with LMX1B and NF- κ B is mutually exclusive. In addition, iASPP did not significantly affect LMX1B-mediated expression of NF- κ B target genes in transient transfection assays, making iASPP an unlikely cofactor of LMX1B-mediated transcription. Our tet-off HeLa and LLC-PK₁ cell lines, as well as murine kidney tissues, represent valuable experimental systems to search for LMX1B-specific cofactors using biochemical approaches.

LMX1B/NF- κ B cooperation is regulated by chromatin

Inhibition of NF- κ B activity by RNA interference impeded while stimulation of NF- κ B activity by TNF- α enhanced LMX1B-mediated induction of NF- κ B target genes. This demonstrates that LMX1B and NF- κ B transcription factors functionally cooperate to regulate expression of these genes in HeLa cells. Other examples of functional cooperation involving either LMX1B or NF- κ B and other homeobox-containing proteins have been reported. The bHLH transcription factor E47 interacts with LMX1B bound in its vicinity on the *insulin* gene minienhancer, and synergistically activates transcription together with LMX1B [29,30]. On the other hand, the homeobox-containing protein HOXB7 functionally

cooperates with p50/p65 NF- κ B to activate transcription from an artificial reporter gene *in vitro* [61]. Although we showed that p50/p65 NF- κ B is involved in activation of NF- κ B target genes by LMX1B in HeLa cells, it remains to be determined whether it is also involved in LMX1B-mediated repression of these genes in the mouse kidney. Similarly, it remains to be shown whether the NF- κ B signalling pathway in LLC-PK₁ cells is functional. Impairment of p50/p65 NF- κ B activity might also explain, at least in part, the absence of induction of NF- κ B target genes noted in these cells.

A direct protein–protein interaction between LMX1B and NF- κ B could not be demonstrated by co-immunoprecipitation from whole cell extracts. Although a weak interaction might not be detectable in such conditions, our results suggest that LMX1B/NF- κ B functional cooperation in HeLa cells rather takes place at the chromatin level. In support to this, the possible common coactivator suggested by our reporter assays, while required, was not sufficient to support LMX1B/NF- κ B synergy in that assay. Furthermore, LMX1B recruitment to the *IL-6* and *IL-8* promoters *in vivo* correlated with an increased NF- κ B DNA association in cells with very low nuclear NF- κ B protein levels. Since NF- κ B DNA binding *in vivo* is a dynamic process in which NF- κ B only transiently binds to κ B sites [58], an increase in NF- κ B promoter occupancy therefore suggests a stabilization of the NF- κ B/chromatin complex or/and a greater chromatin accessibility. Correlation of the transcriptional state and chromatin accessibility (e.g. by Chromatin Accessibility by Real-Time PCR or CHART-PCR [43,44]) using our tet-off HeLa- and LLC-PK₁-derived cell lines would bring valuable information about differences in chromatin structure at these promoters and about the possibility of a LMX1B-induced chromatin reorganization. Indeed, although LMX1B was efficiently recruited to the porcine *IL-6* promoter in LLC-PK₁ cells, we cannot exclude that chromatin organization at this promoter impedes transcriptional initiation.

Regulation of NF- κ B target genes by LMX1B is promoter-specific

We found that in HeLa cells LMX1B binds to the promoter region of human *IL-6* and *IL-8* genes, in close proximity to the well-defined κ B site. DNA association is likely to occur via the identified FLAT element(s) adjacent to the NF- κ B motif, as demonstrated by our luciferase reporter assays. It should be mentioned that, by contrast to *IL-6* and *IL-8*, the promoter region of the NF- κ B target genes *IL-1 β* and *IFN- β* only contained degenerated FLAT elements in the vicinity of the κ B motif, and that only negligible LMX1B binding was detected by ChIP (not shown). Weaker binding to non-consensus FLAT elements combined with hindered antibody accessibility due to promoter occupancy for instance by enhanceosomes, as is the case for *IFN- β* [62], might explain this result. It remains therefore unclear at this point whether activation of the NF- κ B target genes *IL-1 β* and *IFN- β* by LMX1B follows the same molecular mechanism as for *IL-6* and *IL-8*. Our data however clearly showed that LMX1B does not regulate expression of all NF- κ B target genes, suggesting a promoter-specific mechanism of regulation.

Disease implications

Altogether, the data presented in this study support a model of cell-specific regulation of NF- κ B target genes by LMX1B, involving the binding of both LMX1B and NF- κ B p50/p65 transcription

factors to their respective recognition sites, and their stabilization through recruitment of specific cofactors, directing either activation or repression of transcription (Fig. 8). Besides spatial regulation of LMX1B activity, our observations imply that specific LMX1B cofactors are likely to play an essential role in the temporal regulation of LMX1B target genes during development.

The contribution of deregulation of NF- κ B target gene (and ISG) expression in the pathology of nail–patella syndrome (NPS) has not been evaluated thus far. There is to date no report of inflammatory- or immune-related disorder in NPS patients, as would be predicted from the present study. On the other hand, since our data suggest a tissue-specific regulation of NF- κ B target gene expression, no global exacerbation or attenuation of the inflammatory response would be expected. A more comprehensive study of a possible alteration of the immune-response in NPS patients would be necessary to address that point, and might open the door to novel therapeutic intervention in NPS with the use of pro- and anti-inflammatory drugs.

Acknowledgments

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