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The podocyte-specific inactivation of *Lmx1b*, *Ldb1* and *E2a* yields new insight into a transcriptional network in podocytes

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Abstract

Patients with nail-patella syndrome, which among other symptoms also includes podocyte-associated renal failure, suffer from mutations in the *LMX1B* gene. The disease severity among patients is quite variable and has given rise to speculations on the presence of modifier genes. Promising candidates for modifier proteins are the proteins interacting with LMX1B, such as LDB1 and E47. Since human kidney samples from patients are difficult to obtain, conventional *Lmx1b* knock-out mice have been extremely valuable to study the role of Lmx1b in podocyte differentiation. In contrast to findings in these mice, however, in which a downregulation of the *Col4a3*, *Col4a4* and *Nphs2* genes has been described, no such changes have been detected in kidney biopsies from patients. We now report on our results on the characterization of constitutive podocyte-specific *Lmx1b*, *Ldb1* and *E2a* knock-out mice. Constitutive podocyte-specific *Lmx1b* knock-out mice survive for approximately 2 weeks after birth and do not present with a downregulation of the *Col4a3*, *Col4a4* and *Nphs2* genes, therefore they mimic the human disease more closely. The podocyte-specific *Ldb1* knock-out mice survive longer, but then also succumb to renal failure, whereas the *E2a* knock-out mice show no renal symptoms for at least 6 months after birth. We conclude that *LDB1*, but not *E2A* is a promising candidate as a modifier gene in patients with nail-patella syndrome.
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Keywords: LMX1B; LDB1; E2A; Podocyte; Slit diaphragm; Foot processes; Nail-patella syndrome

Introduction

Latest since the positional cloning of *NPHS1*, the gene mutated in patients with congenital nephrotic syndrome of the Finnish type (Kestilä et al., 1998), it has become clear that podocytes are essential components of the glomerular filtration barrier. *NPHS1* codes for nephrin, an integral membrane protein with immunoglobulin-like domains in the extracellular NH_2-terminus and a short cytoplasmic tail, which contributes to the formation of the slit diaphragm (Holthöfer et al., 1999; Holzman et al., 1999; Ruotsalainen et al., 1999), a proteinaceous structure connecting the interdigitating foot processes of podocytes. Following the identification of nephrin, a number of other proteins have been associated with the slit diaphragm such as podocin, CD2AP, Neph1 and TRPC6. Podocin is the product of the *NPHS2* gene, which is mutated in patients suffering from steroid-resistant nephrotic syndrome (Boute et al., 2000). The importance of CD2AP for podocyte function was first appreciated from the finding that podocytes of *Cd2ap* knock-out mice lose their foot processes (Shih et al., 1999), later on it

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was also demonstrated that patients with a loss of one *CD2AP* allele are more prone to develop glomerulosclerosis (Kim et al., 2003). Both podocin and CD2AP have been demonstrated to form a complex with nephrin (Schwarz et al., 2001; Shih et al., 2001), which has important implications for the regulation of the slit diaphragm. Neph1 is a paralogue of nephrin, which has also been localized to the slit diaphragm (Barletta et al., 2003), where it obviously serves an essential function because *Neph1* knock-out mice develop proteinuria and die soon after birth (Donoviel et al., 2001). So far, however, no human disease has been associated with mutations in *NEPH1*. The positional cloning of the *TRPC6* gene, which is mutated in patients with focal-segmental glomerulosclerosis (Reiser et al., 2005; Winn et al., 2005), came as a big surprise because it encodes a cation channel and therefore represents a new paradigm in the podocyte field. It has been hypothesized that TRPC6 transduces extracellular signals, possibly mechanical stimuli, to podocytes, but so far the appropriate stimulus for TRPC6 in podocytes is unknown. It is an interesting hypothesis, however, because mutations in the gene encoding α-actinin-4 also cause focal-segmental glomerulosclerosis (Kaplan et al., 2000) and α-actinin-4 may therefore represent a downstream target of TRPC6.

While a plethora of structural proteins have been identified in the podocyte (for an extensive review, see Pavenstädt et al., 2003), it is a complete mystery how the expression of the respective genes in the podocyte is regulated. In order to address this question we have turned to another hereditary disease, nail-patella syndrome, which was named after the characteristic deformities observed in patients. A portion of the patients, possibly up to 40%, also exhibit kidney abnormalities (Sweeney et al., 2003), typically a thickened glomerular basement membrane with fibrillar inclusions and electron-lucent areas. In addition, the disappearance of podocyte foot processes has been observed (Ben-Bassat et al., 1971; del Pozo and Lapp, 1970). Although nail-patella syndrome was one of the first hereditary diseases for which genetic linkage was established (Renwick and Lawler, 1955), it took more than 40 years before mutations in *LMX1B* were identified in patients (Dreyer et al., 1998; McIntosh et al., 1998; Vollrath et al., 1998). *LMX1B* codes for a transcription factor with two zinc-binding LIM domains at its NH_2-terminus, a DNA-binding homeodomain in the middle and a putative transcriptional activation domain at its COOH-terminus. The identification of *LMX1B* was facilitated by the parallel generation of the *Lmx1b* knock-out mouse, which suffers from multiple defects and also shows features typical of nail-patella syndrome (Chen et al., 1998). Most important in this context is the fact that the podocytes do not develop foot processes and are not connected with each other by slit diaphragms (Miner et al., 2002; Rohr et al., 2002). Remarkably, evidence has been presented that LMX1B regulates the expression of other genes with essential functions in the podocyte because podocytes of *Lmx1b* knock-out mice no

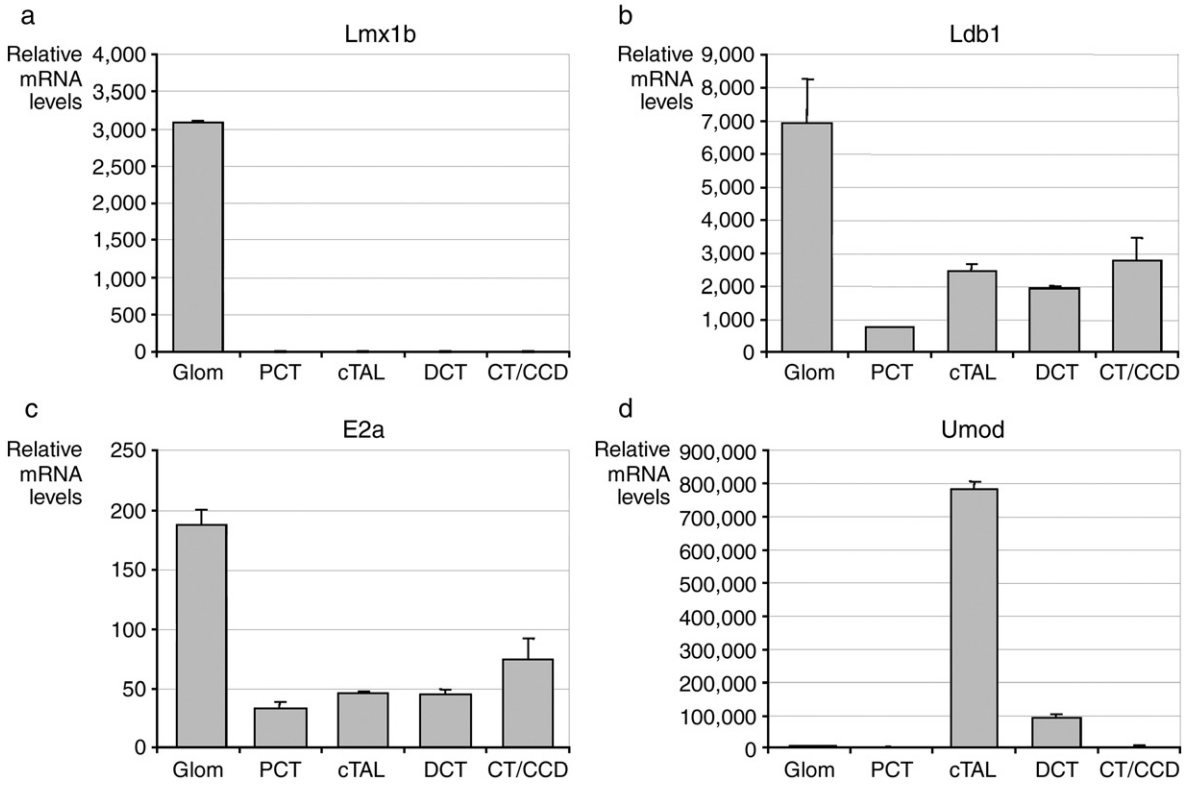


Fig. 1. Expression pattern of the *Lmx1b*, *Ldb1* and *E2a* genes along the nephron. Total RNA was extracted from glomeruli (Glom), proximal convoluted tubules (PCT), cortical thick ascending limbs (cTAL), distal convoluted tubules (DCT) and connecting tubules/cortical collecting ducts (CT/CCD). Analysis by quantitative PCR showed that whereas Lmx1b mRNA was specifically detected in glomeruli (a), Ldb1 and E2a mRNAs were present in all nephron segments isolated (b, c). The purity of the preparation can be appreciated from the fact that uromodulin mRNA was confined almost exclusively to the cortical thick ascending limb as described in the literature (Kumar and Muchmore, 1990) (d).

longer produce podocin (Miner et al., 2002; Rohr et al., 2002) and the α3 and α4 chains of collagen IV (Morello et al., 2001). This phenomenon has been explained by the binding of LMX1B to AT-rich regions in the first intron of the *COL4A4* gene (Morello et al., 2001) and in the promoter region of the *NPHS2* gene (Miner et al., 2002; Rohr et al., 2002). However, the analysis of kidney biopsies from patients has been contradictory to the findings in the mouse because podocin and the α3 and α4 chains of collagen IV were still detected in the biopsies (Heidet et al., 2003). One further discrepancy between the patients and the *Lmx1b* knock-out mice concerns the fact that nail-patella syndrome is inherited in an autosomal-dominant fashion, but *Lmx1b* (+/−) mice do not develop a podocyte phenotype (Rohr et al., 2002). It therefore appears urgent to generate a more suitable animal model in order to better understand the pathogenesis of nail-patella syndrome in general and the role of LMX1B in the podocyte in particular.

Materials and methods

Microdissection of nephron segments and collecting ducts, quantitative PCR

Nephron segments for quantitative RT-PCR were obtained by a modified collagenase digestion protocol (Schafer et al., 1997) from male C57Bl/6 mice with a weight of 20–24 g. Collection of the cortical nephron segments was carried out over a time period of 3–4 h after onset of digestion. After the various tubular segments were separately transferred to a culture dish containing MEM/0.25% BSA, their total length was measured. At least

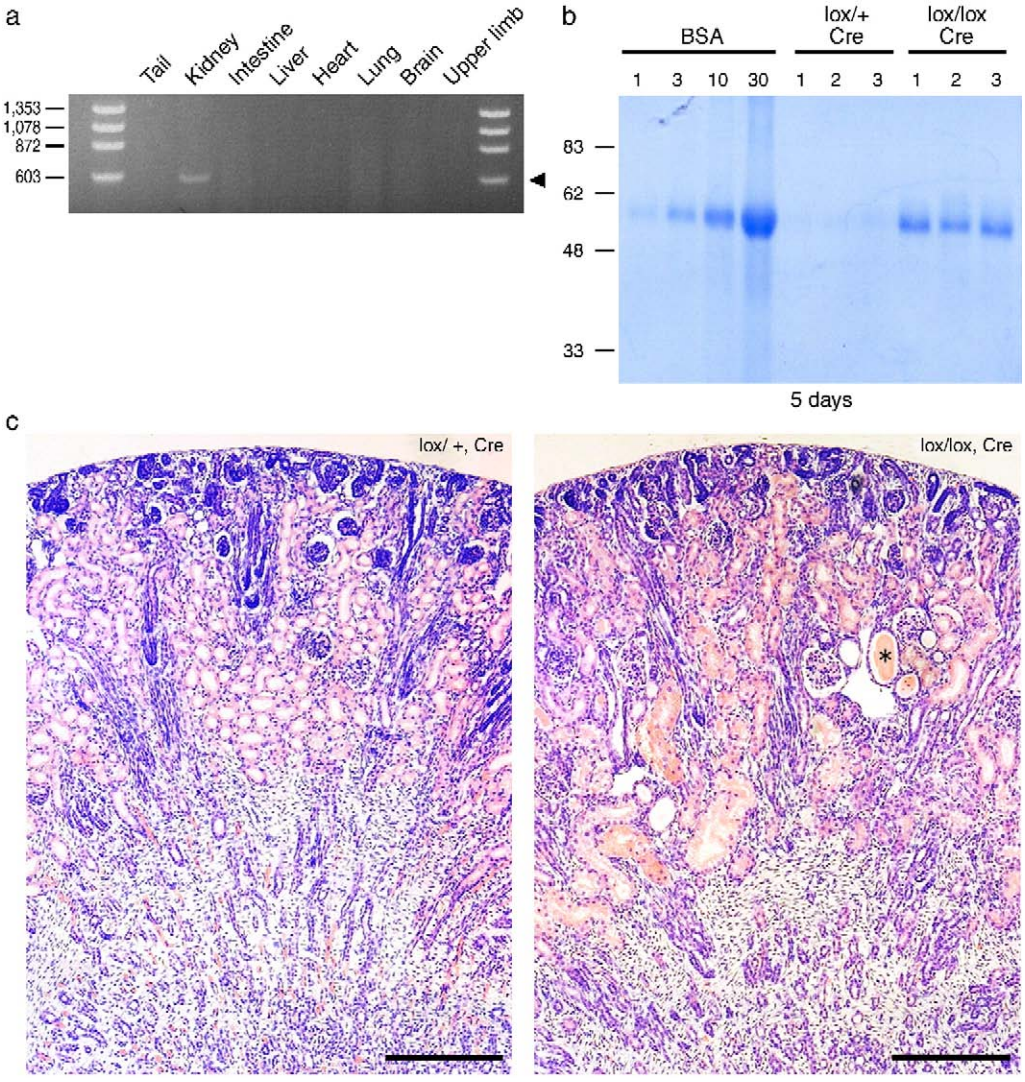
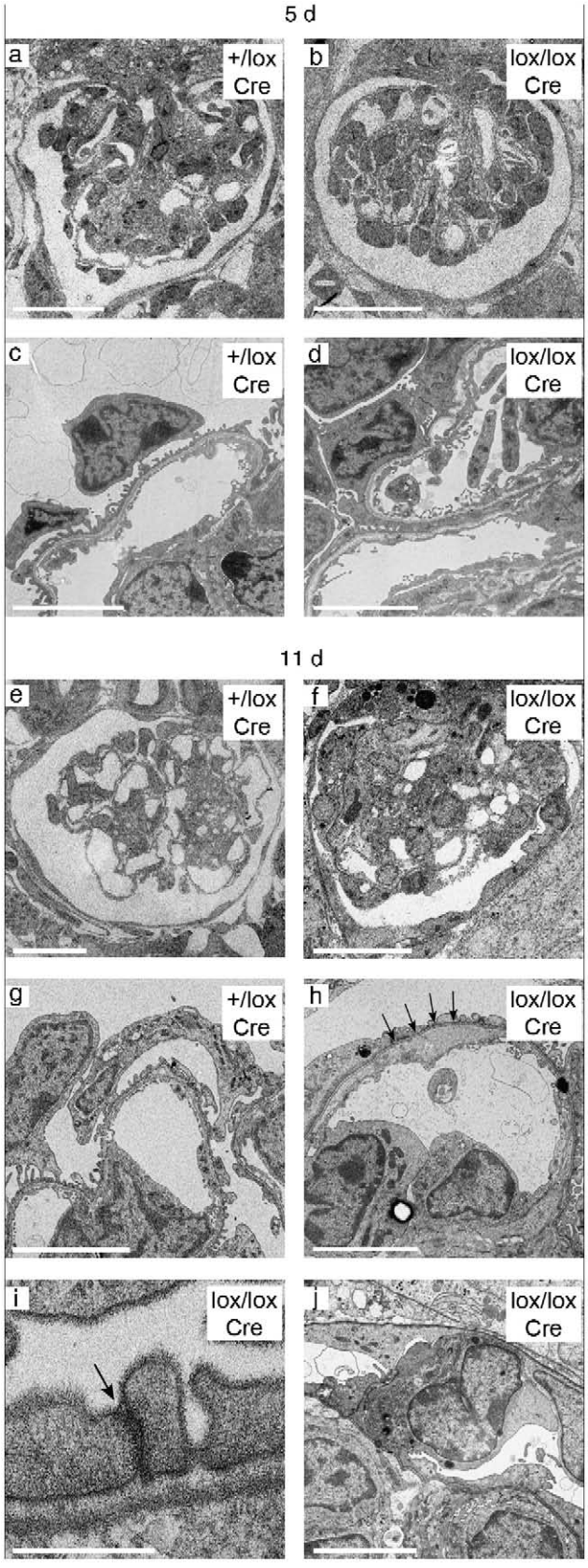


Fig. 2. Podocyte-specific inactivation of *Lmx1b* leads to proteinuria. (a) Genomic DNA was isolated from the indicated tissues and organs. Two hundred nanograms of genomic DNA was used in a PCR reaction with specific primers which yielded an expected ~600-bp product (arrow head) only in the kidney. (b) Three 5-day-old mice of the indicated genotypes were sacrificed to collect urine from their bladder. After centrifugation, 2 μl of urine was analyzed on a denaturing polyacrylamide gel together with 1 μg, 3 μg, 10 μg and 30 μg of bovine serum albumin (BSA). Following staining with Coomassie Brilliant Blue it could be easily appreciated that the three mice with two floxed *Lmx1b* alleles had developed albuminuria. (c) Histological sections of 5-day-old mice were stained with H&E. No gross abnormalities were observed in mice with two floxed *Lmx1b* alleles except for occasional dilated tubular profiles filled with an eosinophilic, probably proteinaceous material (asterisk). Scale bars, 100 μm.

11 mm of each segment was pooled, in addition at least 22 glomeruli were collected. Samples were transferred to 50 µl GTC solution (4 M guanidine thiocyanate, 0.5% *N*-lauryl-sarcosinate, 25 mM sodium citrate, 700 mM β-mercaptoethanol) and stored at −80 °C until RNA was extracted. Nephron samples from 3 mice were pooled and RNA was extracted using the Nucleospin RNA II kit (Macherey-Nagel) following the manufacturer's recommendations. After elution of the RNA with 45 µl of H_2O, 15 µl was used for cDNA synthesis with the iScript cDNA synthesis kit (BioRad) again following the manufacturer's recommendations. Quantitative PCR was performed on a Rotor-Gene 3000 (Corbett Research) with 1.5 µl of the cDNA reaction using HotStarTaq (Qiagen) and the oligonucleotides listed below. Data were normalized to S9 ribosomal mRNA and expressed as relative mRNA levels.



Lmx1b Fwd	5′-CAGCAGCGAAGAGCTTTCAA-3′
Lmx1b Rev	5′-GTCTCTCGGACCTTCCGACA-3′
Ldb1 Fwd	5′-GTGGAAGGCCGGTTGTACC-3′
Ldb1 Rev	5′-GCCACGTCTTTATCCGCATC-3′
E47 Fwd	5′-CTACTCCCCGGATCACTCCA-3′
E47 Rev	5′-CTCCATCTTGCTCAGGCCA-3′
Umod Fwd	5′-TGCACCGATCCTAGTTCCGT-3′
Umod Rev	5′-CATCTACCCTGCATTCTTCGC-3′
S9 Fwd	5′-GCAAGATGAAGCTGGATTAC-3′
S9 Rev	5′-GGGATGTTCACCACCTG-3′

Generation of podocyte-specific knock-out mice

All the mouse lines mentioned here were maintained on a C57Bl/6 background. The P2.5-Cre transgenic mice contain a Cre expression cassette under control of the *NPHS2* promoter and show Cre activity exclusively in podocytes (Moeller et al., 2003). The floxed *Lmx1b* allele was generated by introducing loxP fragments upstream and downstream of exons 4 and 6, respectively (this part of the gene encodes the homeodomain). Mice with the floxed *Lmx1b* allele can live for 2 years without showing a phenotype. The floxed *Ldb1* allele was generated by introducing loxP sites upstream and downstream of exons 1 and 9, respectively, mice with the floxed *Ldb1* allele again show no phenotype. The floxed *E2a* allele was generated by introducing a loxP site upstream of the exons encoding the basic/helix–loop–helix region and a loxP site downstream of the last exon (Pan et al., 2002). The respective floxed mice were crossed twice with the P2.5-Cre mice to inactivate *Lmx1b*, *Ldb1* and *E2a* specifically in podocytes.

Genotype determination and Cre-mediated recombination analysis

Genomic DNA was isolated from tail biopsies (Laird et al., 1991) and used subsequently for genotype analysis. The floxed *Lmx1b* allele was detected by PCR using the primers Lmx1b (5′-lox) and Lmx1b (3′-lox). To detect the floxed *Ldb1* allele, the primers Ldb1G and Ldb1sense were used, and to detect the floxed *E2a* allele, the primers YZ 104 and YZ 150 primers were used. The Cre transgene was either detected by Southern blot or by PCR using the primers CreF1 and CreR1 primers.

To determine Cre-mediated recombination, genomic DNA from 11-day-old *Lmx1b*$^{lox/lox}$; *NPHS2*:Cre, 19-day-old *Ldb1*$^{lox/lox}$; *NPHS2*:Cre and adult *E2a*$^{lox/lox}$; *NPHS2*:Cre mice was extracted using the NucleoSpin Tissue kit (Macherey-Nagel) according to the manufacturer's instructions. PCR amplification was performed using the primer pairs LmxCreF1 and LmxCreR1 for *Lmx1b*, Ldb1CreF1 and LdbCreR1 for *Ldb1*. In the case of *E2a*, PCR was performed with three oligonucleotides (PGKneo For m, E2Aflox For m, and YZ 198). The primer pair PGKneo For m/YZ 198 is specific for the non-recombined allele (PCR product of 1.2 kbp), whereas the

Fig. 3. Ultrastructural changes after podocyte-specific inactivation of *Lmx1b*. (a–d) In 5-day-old animals, no obvious structural changes are seen at a low magnification (a, b). At a higher magnification, foot processes can be seen in the *Lmx1b*$^{+/lox}$; *Nphs2*:Cre, but not in the *Lmx1b*$^{lox/lox}$; *Nphs2*:Cre mice (c, d). (e–j) At 11 days of age, glomeruli of *Lmx1b*$^{lox/lox}$; *Nphs2*:Cre mice show distinct sclerotic changes (e, f). At a higher magnification, a thickened glomerular basement membrane (arrows in h), the loss of slit diaphragms (arrow in i) and adhesions to Bowman's capsule (i) can be seen. Scale bars, 20 µm (a, b, e, f), 5 µm (c, d, g, h, j), 0.5 µm (i).

primer pair E2Aflox For m/YZ 198 is specific for the recombined allele (PCR product of 0.5 kbp).

Lmx1b (5′-lox)	5′-AGGCTCCATCCATTCTTCTC-3′
Lmx1b (3′-lox)	5′-CCACAATAAGCAAGAGGCAC-3′
Ldb1G	5′-CTTATGTGACCACAGCCATGCATGCATGTG-3′
Ldb1sense	5′-CAGCAAACGGAGGAAACGGAAGATGTCAG-3′
YZ 104	5′-ATGTGTGGTGGCCCACACTTGT-3′
YZ 150	5′-ACATGGCTGAATATCGACGGT-3′
CreF1	5′-CCTGGAAAATGCTTCTGTCCG-3′
CreR1	5′-CAGGGTGTTATAAGCAATCCC-3′
LmxCreF1	5′-GTACCTCCTGTGAGGATGCC-3′
LmxCreR1	5′-ACAGGGCAGAGGGAAAGTG-3′
Ldb1CreF1	5′-CTCCAAGCATCCCACTTAGCTAGC-3′
Ldb1CreR1	5′-ATGTGTCTGCCACACAGAATCTGC-3′
PGKneo For m	5′-CTGCCCATTCGACCACCAAGCG-3′
E2Aflox For m	5′-GTGTCTGCACTCCAGATTGTGCCTGTT-3′
YZ 198	5′-CGGATCCATCCTCGTCTTCATTGGTACTG-3′

Perfusion fixation, light and electron microscopy

Mice at postnatal days 5, 11 and 19 were perfused through the distal abdominal aorta for 3 min using 4% paraformaldehyde (PFA)/1× PBS. Then kidneys were removed and fixed overnight in 4% PFA/1× PBS for paraffin embedding and in 2% glutaraldehyde/1× PBS for electron microscopy. After incubation with cacodylate-buffered 1% OsO_4 for 2–3 h, kidneys were dehydrated in ethanol and acetone before being embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and then visualized using a transmission electron microscope (Philips CM12) equipped with a slow-scan CCD digital camera.

Determination of proteinuria

Two microliters of urine was combined with 50 µl of sample buffer (125 mM Tris pH 6.7, 2.5% SDS, 10% glycerol, 2.5% β-mercaptoethanol, 0.01% bromophenol blue), boiled for 5 min and then loaded onto a 10% SDS–polyacrylamide gel. After electrophoresis the gel was stained in Coomassie Brilliant Blue R250.

Immunohistochemistry

Paraffin-embedded sections were used for all antibodies except in the case of the anti-collagen IV α4 antibody. After deparaffinization, sections were subjected to microwave treatment (5×5 min in 10 mM sodium citrate pH 6.0) and blocked overnight in 2% BSA/1× PBS before the primary antibodies were applied. The following antibodies were used: a polyclonal rabbit anti-WT1 antibody (diluted 1:500; Santa Cruz Biotechnology), a polyclonal rabbit anti-nephrin antibody (diluted 1:1000) (Holzman et al., 1999), a polyclonal rabbit anti- α-actinin-4 antibody (diluted 1:1000) (kind gift from M. Pollak), the polyclonal rabbit anti-podocin antibody P35 (diluted 1:1000) (kind gift from C. Antignac) (Roselli et al., 2002) and a polyclonal rabbit anti-CD2AP antibody (diluted 1:4000) (kind gift from A. Shaw) (Dustin et al., 1998). After three washes, the sections were stained with a Cy3-conjugated goat anti-rabbit IgG antibody (diluted 1:300; Dianova) for 1 h at room temperature.

For staining with a polyclonal rabbit anti-collagen IV α4 antibody (diluted 1:800) (kind gift from J. Miner) (Miner and Sanes, 1994), 7 µm thick cryosections of snap-frozen kidneys were fixed in ethanol for 10 min at −20 °C and then air-dried. After incubation for 1 h with 6 M urea/0.1 M glycine pH 3.5 at room temperature, the sections were blocked overnight with 5% non-fat dry milk solution. The next day, sections were washed with 1× PBS and stained overnight with the anti-collagen IV α4 primary antibody.

Generation of polyclonal and monoclonal anti-LMX1B antibodies

Histidine-tagged fragments of the human LMX1B protein comprising amino acids 31–395 and amino acids 322–395 were produced in *Escherichia coli*.

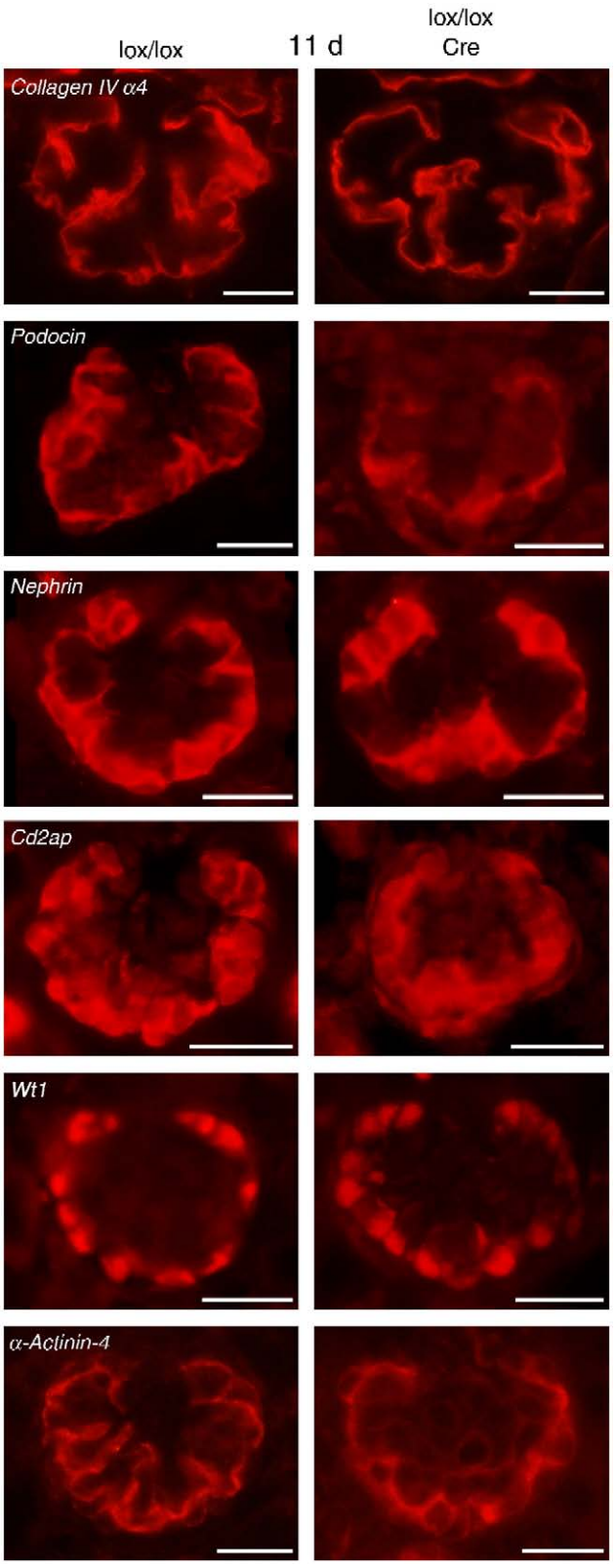


Fig. 4. Synthesis of disease-associated proteins after podocyte-specific inactivation of *Lmx1b*. All the indicated proteins were still present at comparable levels in the glomeruli of $Lmx1b^{lox/lox}$; *Nphs2*:Cre mice as demonstrated by immunofluorescence. Glomeruli of $Lmx1b^{lox/lox}$ mice are shown as controls. Scale bars, 20 µm.

After their purification on Ni^{2+} columns, the recombinant peptides were used to produce rabbit polyclonal antiserum and mouse monoclonal antibodies, respectively, according to standard protocols (Harlow and Lane, 1988). Rabbit antiserum BMO8 and hybridoma 193-67 yielded the best results in preliminary tests and were chosen for subsequent experiments.

Expression plasmids and transient expression in COS-7 cells

The human LMX1B and the murine Ldb1 cDNAs were subcloned into a derivative of the expression plasmid pcDNA3 which permits the synthesis of myc-tagged proteins in mammalian cells. Two days after transient transfection of COS-7 cells according to established protocols (Ausubel et al., 1996), the cells were lysed in Brij lysis buffer (10 mM Tris–HCl pH 7.5, 150 mM NaCl, 2 mM EDTA pH 8.0, 0.875% Brij 97, 0.125% NP40) for 15 min on ice. Insoluble material was removed by centrifugation and 500 μg of the supernatant used for co-immunoprecipitation.

Preparation of rat glomerular extracts

Glomeruli were isolated from adults rats according to a sieving technique. After the kidney homogenate was purified over sieves with pore sizes of 106 μm, 180 μm and 75 μm, the resulting glomerular fractions were lysed in Brij lysis buffer (see above) for 30 min with constant agitation. Two milligrams of total protein was used for the subsequent co-immunoprecipitation assay.

Co-immunoprecipitation

Protein lysates were precleared for 1 h at 4 °C against protein G sepharose 4 Fast Flow (GE Healthcare) equilibrated in Brij lysis buffer (see above). One milliliter of the anti-LMX1B hybridoma 193-67 or regular culture medium (RPMI 1640, 10% FCS) was pre-incubated overnight at 4 °C with 25 μl of protein G sepharose beads in the presence of Brij lysis buffer. After washing with Brij lysis buffer, the antibody-loaded beads were incubated for 5 h with the precleared protein lysate. Immune complexes were washed at least 4 times in Brij buffer, before the beads were resuspended in 40 μl of 2× sample buffer. After boiling, 20 μl was separated on a denaturing polyacrylamide gel and subsequently transferred onto a PVDF membrane (Millipore), where Lmx1b was detected with the rabbit antiserum BMO8 (diluted 1:2000) and Ldb1 with the affinity-purified rabbit polyclonal anti-Ldb1 antiserum 4508 (a kind gift of Drs. Gordon Gill and Samuel Pfaff, San Diego (Jurata et al., 1996); diluted 1:4000). Primary antibodies were visualized with an HRP-coupled anti-rabbit antibody (diluted 1:20,000; Sigma) and a chemiluminescence reagent (Perkin-Elmer).

Results

Mice with a constitutive podocyte-specific inactivation of Lmx1b are more similar to patients with nail-patella syndrome than conventional Lmx1b knock-out mice

The conventional *Lmx1b* knock-out mice have been a very valuable tool towards a better understanding of podocyte differentiation (Chen et al., 1998; Miner et al., 2002; Morello et al., 2001; Rohr et al., 2002). A big disadvantage of these mice, however, is the fact that they die on the day of birth. Furthermore, they do not precisely reflect nail-patella syndrome because (i) *Lmx1b* (+/−) mice do not develop a podocyte phenotype, although the human disease is inherited in an autosomal-dominant fashion, and (ii) conventional *Lmx1b* knock-out mice show a downregulation of the *Col4a3*, *Col4a4* and *Nphs2* genes (Miner et al., 2002; Morello et al., 2001; Rohr et al., 2002), whereas such a change has not been observed in the kidneys of patients (Heidet et al., 2003). Our goal therefore was to establish mice with a podocyte-specific constitutive inactivation of the *Lmx1b* gene in order to confirm the vital importance of Lmx1b in mice and to establish a better model for nail-patella syndrome.

Although it has been unequivocally demonstrated that the inactivation of *Lmx1b* leads to a podocyte phenotype, it has not

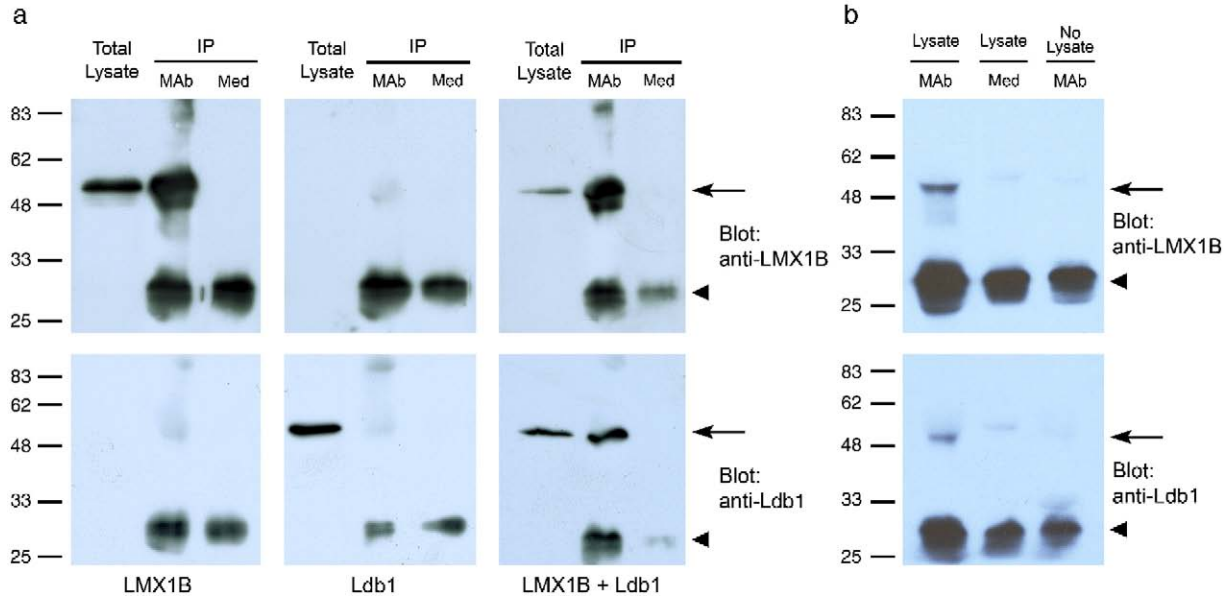


Fig. 5. Co-immunoprecipitation of LMX1B and LDB1. (a) COS-7 cells were transiently transfected with expression plasmids encoding human myc-tagged LMX1B (left), murine myc-tagged Ldb1 (middle) and both myc-tagged proteins (right). The recombinant proteins were easily detected in 10 μg of total lysate (all three panels), furthermore Ldb1 co-precipitated with LMX1B (right). (b) Complexes of Lmx1b and Ldb1 were also detected in lysates of rat glomeruli. Bands specific for LMX1B/Lmx1b and Ldb1 are indicated by arrows, a non-specific signal due to immunoglobulin light chains and/or medium is indicated by an arrow head. MAb, monoclonal anti-LMX1B antibody 193-67; Med, RPMI 1640/10% FCS.

been ruled out that LMX1B is also important for the function of other nephron segments and of the collecting duct. We therefore isolated RNA from various nephron segments and from collecting ducts and subjected the corresponding cDNAs to quantitative PCR analysis with primers specific for Lmx1b. This clearly demonstrated that *Lmx1b* is exclusively expressed in the glomerulus and not in other portions of the kidney (Fig. 1a), and therefore strongly suggested that the podocyte damage in the conventional *Lmx1b* knock-out mice was indeed the only change in the kidney. In order to confirm the latter assumption, we established mice with a floxed *Lmx1b* gene and podocyte-specific Cre activity (Moeller et al., 2003). These mice typically did not survive longer than 14 days after birth. Analysis of genomic DNA isolated from various tissues confirmed that the *Lmx1b* gene underwent recombination exclusively in the kidney (Fig. 2a) and indicated that the animals died from renal failure. This assumption was further supported by the finding that *Lmx1b*$^{lox/lox}$; *Nphs2*:Cre mice developed massive albuminuria already 5 days after birth (Fig. 2b) and by the presence of tubular protein casts detected by light microscopy (Fig. 2c).

Since all these data pointed to structural alterations in the glomerular filtration barrier, we next carried out an electron microscopical investigation of the kidneys. Five days after birth, no major changes of glomeruli were observed at a low magnification (Figs. 3a, b). At a high magnification, however, we already observed a loss of foot processes over distinct areas (Figs. 3c, d). Six days later the structural alterations were more prominent and the kidneys had developed focal-segmental glomerulosclerosis (Figs. 3e, f). Foot process effacement was abundant and was accompanied by thickening of the glomerular basement membrane (Figs. 3g, h) and by loss of slit diaphragms (Fig. 3i). Furthermore, adhesions between the glomerular tuft and Bowman's capsule were found (Fig. 3j). We reasoned that in analogy to the conventional *Lmx1b* knock-out mice these

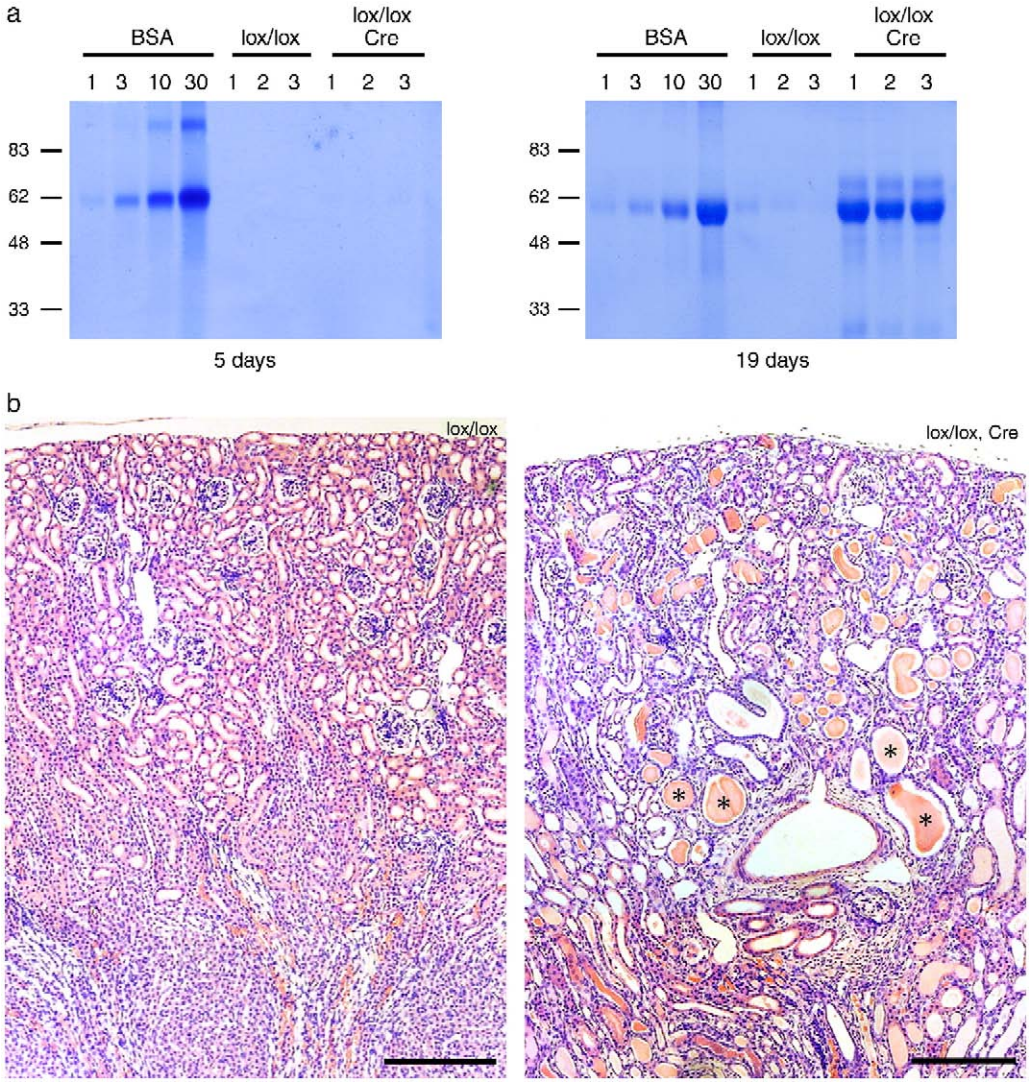
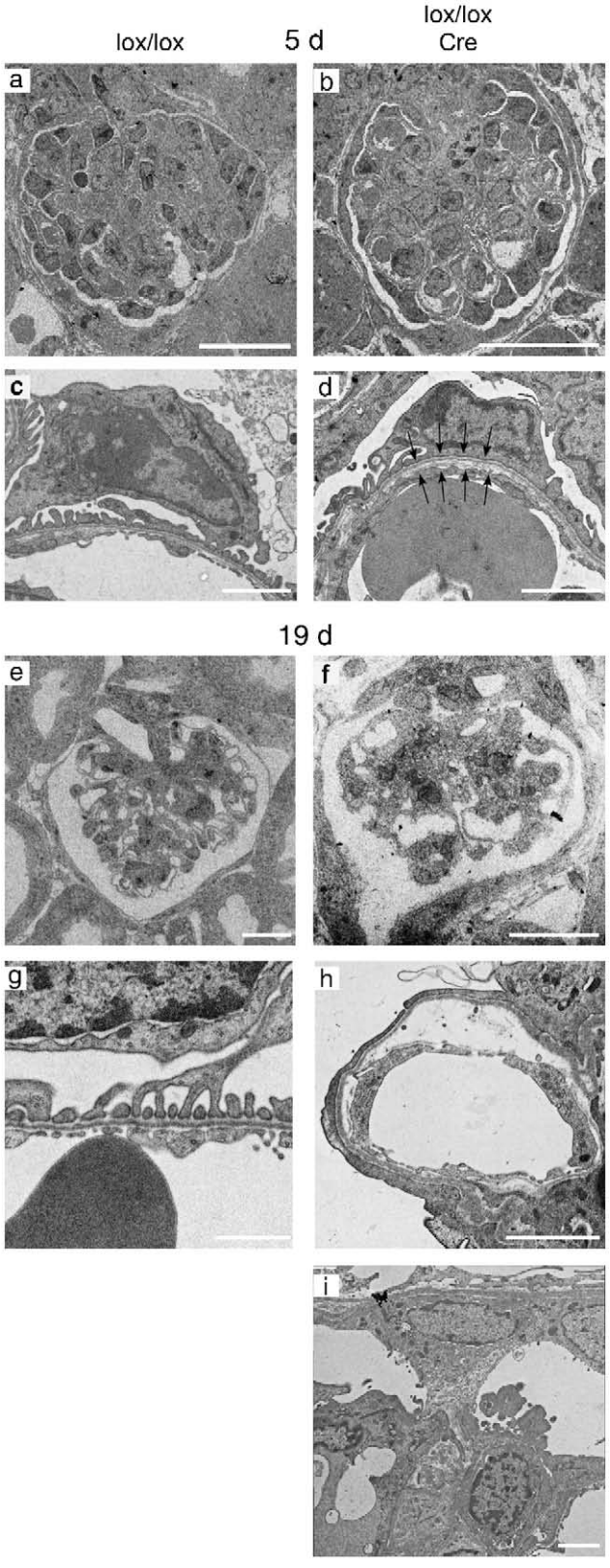


Fig. 6. Podocyte-specific inactivation of *Ldb1* leads to proteinuria. (a) Three 5-day- and 19-day-old mice of the indicated genotypes were sacrificed to collect urine from their bladder. After centrifugation, 2 μl of urine was analyzed on a denaturing polyacrylamide gel together with 1 μg, 3 μg, 10 μg and 30 μg of bovine serum albumin (BSA). Upon staining with Coomassie Brilliant Blue, albuminuria could be seen in the three mice with two floxed *Lmx1b* alleles at 19 days, but not at 5 days of age. (b) Histological sections of 19-day-old mice were stained with H&E. Frequent dilated tubular profiles filled with an eosinophilic material can be seen in the *Ldb1*$^{lox/lox}$; *Nphs2*:Cre mice (asterisks). Scale bars, 100 μm.

structural changes could be explained by a downregulation of the *Col4a3*, *Col4a4* and *Nphs2* genes. To our surprise, however, we were still able to detect the α3 and α4 chains of collagen IV as well as podocin by immunohistochemistry (Fig. 4), and the same was true for all the other proteins we examined, such as nephrin, Cd2ap, Wt1 and α-actinin-4 (Fig. 4). By real-time PCR with total kidney RNA from 11-day-old animals, the mRNA levels for podocin, nephrin and Wt1 had dropped to 25%, 35% and 50%, respectively (2 *Lmx1b*$^{lox/lox}$ and 3 *Lmx1b*$^{lox/lox}$; *Nphs2*:Cre mice).

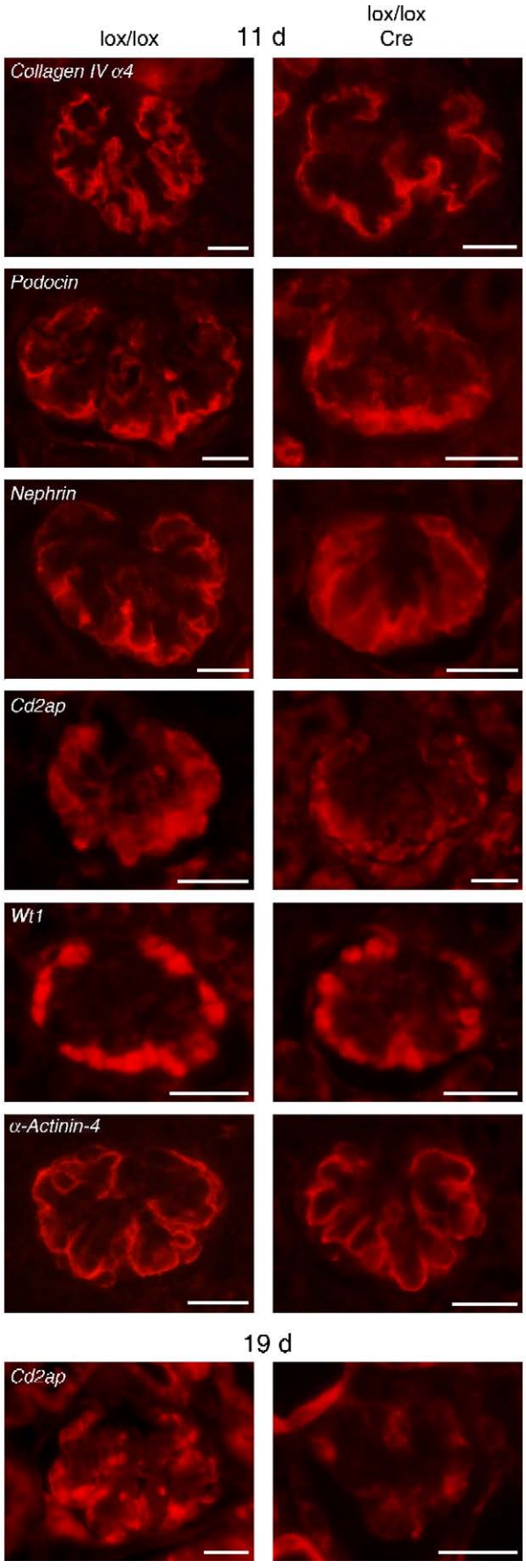


The podocyte-specific inactivation of Ldb1, but not of E2a, leads to a podocyte phenotype

It is obvious that LMX1B cannot act by itself but rather needs additional co-factors which may also help to determine the tissue-specific activities of LMX1B during kidney, limb, eye and brain development. These co-factors may further turn out to be important modifier genes for the penetrance of nail-patella syndrome whose clinical presentation is quite variable (Sweeney et al., 2003). So far two proteins have been shown to interact with LMX1B, they are E47 (Johnson et al., 1997), together with E12 one of the products of the *E2A* gene, and LDB1 (also known as NLI and CLIM2) (Marini et al., 2003). When we performed a yeast two-hybrid screen with the two LIM domains of LMX1B as bait and a human kidney cDNA library as prey, we among other proteins also identified LDB1 (data not shown), thus confirming a previous report (Marini et al., 2003). It is not clear, however, whether *LDB1* is expressed in the podocyte, whether the interaction between LMX1B and LDB1 also takes place in podocytes and whether this interaction is essential for podocyte function. Conventional *Ldb1* knock-out mice die in utero before kidney development has even started (Mukhopadhyay et al., 2003) and therefore are of little use to answer these questions.

In order to gain more insight into the functional significance of the interaction between LMX1B and LDB1, we first investigated the expression pattern of the murine *Ldb1* gene in various portions of the kidney. In contrast to *Lmx1b*, *Ldb1* was widely expressed in various nephron segments, among them the glomerulus, and in the collecting duct (Fig. 1b). While this provided further indirect evidence that LMX1B and LDB1 also interacted with each other in podocytes, we next sought to establish firm biochemical data for the association of the two proteins. Using newly generated mouse monoclonal and rabbit polyclonal anti-LMX1B antibodies, we were able to specifically detect LMX1B in transiently transfected COS-7 cells, and the same was true for Ldb1 with a previously described polyclonal antibody (Jurata et al., 1996) (Fig. 5a). Immunoprecipitation with the anti-LMX1B antibody also precipitated Ldb1 and therefore

Fig. 7. Ultrastructural changes after podocyte-specific inactivation of *Ldb1*. (a–d) In 5-day-old animals, no obvious structural changes are seen at a low magnification (a, b). At a higher magnification, occasional foot process effacement and a split glomerular basement membrane (arrows in d) can be seen in the *Ldb1*$^{lox/lox}$; *Nphs2*:Cre mice (c, d). (e–i) At 19 days of age, glomeruli of *Ldb1*$^{lox/lox}$; *Nphs2*:Cre mice look atrophied (e, f). At a higher magnification, foot process effacement is widespread, endothelial fenestrations are largely missing and endothelial cells have lifted off the glomerular basement membrane (g, h). Adhesions to Bowman's capsule (i) were also noticed. Scale bars, 20 μm (a, b, e, f), 2 μm (c, d, h, i), 1 μm (g).



served as a good basis to investigate the interaction of the endogenous proteins. Glomerular protein extracts were prepared from adult rats and incubated with the monoclonal anti-LMX1B antibody. Subsequent Western blot analysis not only demonstrated the presence of Lmx1b in glomeruli, but we could also show that the endogenous Ldb1 co-immunoprecipitated with Lmx1b (Fig. 5b).

These finding encouraged us to take a closer look at mice with a podocyte-specific inactivation of the *Ldb1* gene. Most of those mice died at around 4 weeks of age, although very few survived up to 3 months, thereby suggesting that LDB1 also is an essential protein in podocytes but that the podocyte-specific inactivation of *Ldb1* results in a milder phenotype than the inactivation of *Lmx1b*. This interpretation was supported by the urine analysis of the *Ldb1* knock-out mice at 5 and 19 days of age. At 5 days of age we saw no albuminuria in contrast to 5-day-old *Lmx1b* knock-out mice, yet a pronounced proteinuria had developed at 19 days (compare Fig. 6a with Fig. 2b). Similar to the situation in the podocyte-specific *Lmx1b* knock-out mice, dilated tubules with protein casts were seen in kidney sections (Fig. 6b). We therefore were not surprised to detect a gradual loss of foot processes in the podocyte-specific *Ldb1* knock-out mice (Figs. 7d, h) as well as a split glomerular basement membrane by electron microscopy (Fig. 7d). In contrast to what we observed after the podocyte-specific inactivation of *Lmx1b*, we repeatedly saw that the glomerular endothelial cells had lifted off the basement membrane (Fig. 7h). In our analysis of the expression pattern of various genes with importance to the podocyte, we again found no differences between the kidneys from knock-out and wild-type mice, with one exception: Cd2ap protein levels were markedly reduced in many glomeruli (Fig. 8). By real-time PCR with total kidney RNA from 11-day-old animals, the mRNA levels for podocin had dropped to 61%, whereas those for nephrin and Wt1 had even risen slightly to 104% and 103%, respectively (4 $Ldb1b^{lox/lox}$ and 3 $Ldb1b^{lox/lox}$; *Nphs2*:Cre mice).

Conventional *E2a* knock-out mice are viable and develop an immunological defect (Zhuang et al., 1994), but so far no careful analysis of the kidney phenotype has been performed. As a matter of fact it is not even clear whether *E2a* is expressed in podocytes. By quantitative RT-PCR we were able to detect E2a mRNA in many portions of the kidney including the glomerulus (Fig. 1c), which suggested that an interaction between LMX1B and E47 could also take place in podocytes. Since we wanted to rule out any indirect effects which might for example result from an interaction between glomerular endothelial cells and podocytes, we again resorted to the podocyte-specific inactivation of the *E2a* gene. In stark contrast to our findings with the podocyte-specific *Lmx1b* and *Ldb1* knock-out mice, animals

Fig. 8. Synthesis of disease-associated proteins after podocyte-specific inactivation of *Ldb1*. All the indicated proteins were still present at comparable levels in the glomeruli of $Lmx1b^{lox/lox}$; *Nphs2*:Cre mice at 11 days of age as determined by immunofluorescence. At 19 days of age, however, Cd2ap levels had markedly dropped in many glomeruli of $Ldb1^{lox/lox}$; *Nphs2*:Cre mice. Glomeruli of $Ldb1^{lox/lox}$ mice are shown as controls. Scale bars, 20 μm.

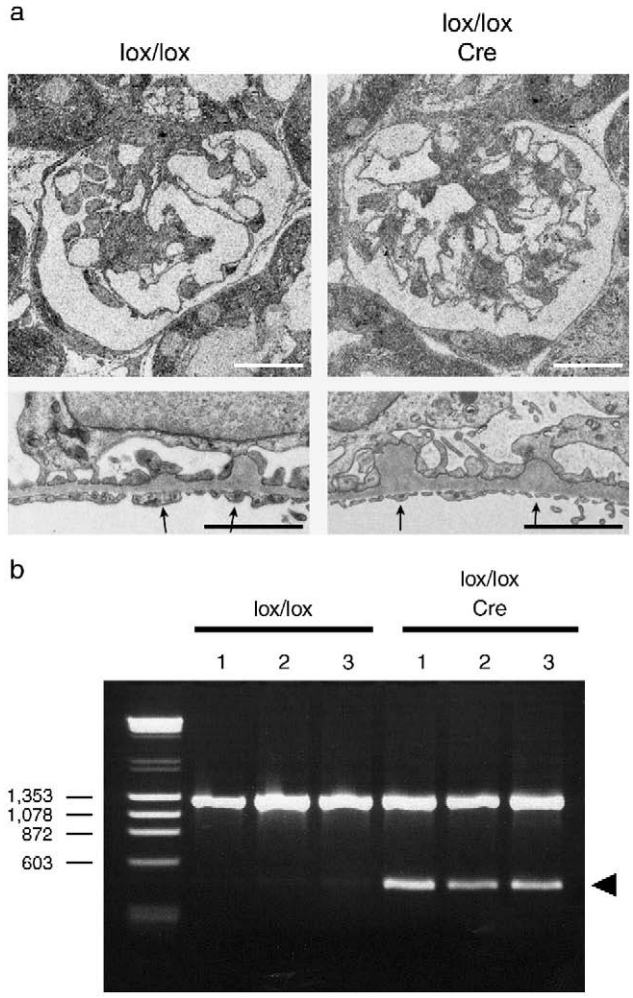


Fig. 9. Podocyte-specific inactivation of *E2a*. (a) On an ultrastructural level, only age-specific changes, i.e. humps extending towards Bowman's space (Duan and Nagata, 1993), were noticed in both $E2a^{lox/lox}$ and $E2a^{lox/lox}$; *Nphs2*:Cre mice (arrows). (b) Cre-mediated recombination of the *E2a* gene was easily detectable in $E2a^{lox/lox}$; *Nphs2*:Cre mice (the ~0.5 kbp band obtained after recombination is indicated by an arrow head). Scale bars, 20 μm (upper panels), 2 μm (lower panels).

with podocyte-specific inactivation of *E2a* survived for at least 6 months without any signs of renal failure. Neither were we able to detect albuminuria (data not shown) nor were ultrastructural alterations seen in the podocytes and the glomerular basement membrane in comparison to age-matched control mice (Fig. 9a). Cre-mediated recombination was detected in genomic DNA isolated from kidney (Fig. 9b), and an absence of Cre activity was therefore ruled out as a trivial explanation for the lack of a phenotype.

Discussion

Although conventional knock-out mice have been tremendously useful to elucidate the function of many proteins, they suffer from severe drawbacks. Since both alleles of the gene of interest are inactivated from the one-cell stage onwards, efficient compensatory mechanisms may develop in order to substitute for the inactivated gene and therefore no obvious phenotype can be observed. On the other hand, embryonic or perinatal lethal phenotypes obviously attest to the importance of a gene, but they prevent further experiments or mask phenotypes developing later in life. The tissue-specific inactivation of genes using the Cre-lox technology offers great promise to circumvent these problems as demonstrated in our study on the podocyte-specific inactivation of *Lmx1b*, *Ldb1* and *E2a*.

Conventional *Lmx1b* knock-out mice do not survive for longer than 24 h after birth (Chen et al., 1998), the cause of death has not been determined. Our data clearly show that the podocyte-specific inactivation of *Lmx1b* using Cre driven by a *NPHS2* promoter fragment leads to a phenotype different from that of the conventional *Lmx1b* knock-out mice. The podocyte-specific *Lmx1b* knock-out mice survive for up to 2 weeks after birth, and the podocytes develop foot processes initially and lose them subsequently. It therefore appears that Lmx1b not only is important for the initial differentiation of podocytes but also for maintenance of the podocyte phenotype; further experiments in adult mice are necessary to support this interpretation. We were surprised to see that podocin and the α3 and α4 chains of collagen IV were still produced after podocyte-specific inactivation of *Lmx1b* although these proteins are clearly absent in podocytes of conventional *Lmx1b* knock-out mice (Miner et al., 2002; Rohr et al., 2002). The presence of podocin in particular is remarkable because both podocin mRNA and protein are no longer produced in the conventional *Lmx1b* knock-out mice (Miner et al., 2002; Rohr et al., 2002). It has to be kept in mind that Cre is under control of a human *NPHS2* promoter fragment, therefore Cre will be synthesized at the same time as podocin and only then Cre-mediated recombination of both *Lmx1b* alleles will take place, upon which no more Lmx1b is produced. Once the Lmx1b protein levels have dropped under a critical threshold, the transcription of Lmx1b target genes and finally the podocyte will be affected. We can think of at least two scenarios why podocin is still present in the podocyte-specific *Lmx1b* knock-out mice. For one, Lmx1b may be necessary for the initial activation of *Nphs2* (as in the conventional *Lmx1b* knock-out mice where foot processes and slit diaphragms never develop) but not for maintaining its expression in the differentiated podocyte (as in the podocyte-specific *Lmx1b* knock-out mice where podocytes first differentiate normally and lose their foot processes and slit diaphragms subsequently). Alternatively, a scenario can be envisioned in which the loss of Lmx1b affects more fundamental cellular processes which are not only necessary for podocyte development but also during limb and eye development (e.g. cytoskeletal rearrangements). If these fundamental cellular processes are not carried out, the podocyte will not even reach a developmental stage in which more cell-specific programs will be switched on such as the transcription of genes responsible for the formation of foot processes and slit diaphragms. In the latter model, *Nphs2* is not a direct target gene of Lmx1b. Clearly, a crucial next step towards a better understanding of Lmx1b will be the unequivocal identification of its target genes.

Mutations in *LMX1B* have not been detected in all patients suffering from nail-patella syndrome. This may be due to the

fact that some patients carry mutations in the regulatory regions or in the introns of *LMX1B*, which are more difficult to detect, alternatively additional genes might be mutated in these patients. *LDB1* has been considered such a candidate gene but was not found to be mutated in patients (Marini et al., 2003). In hindsight that may not be a big surprise because of the widespread expression of *LDB1* and the early embryonic lethal phenotype of the *Ldb1* knock-out mice (Mukhopadhyay et al., 2003), therefore mutations in *LDB1* may not be compatible with life. The conventional *Ldb1* knock-out mice die before kidney development has even begun and therefore yielded no information on the importance of LDB1 for the podocyte (Mukhopadhyay et al., 2003). Our results now show that LDB1 is essential for podocytes and that the phenotype of mice with a podocyte-specific inactivation of *Ldb1* is similar but not identical to that of mice with a podocyte-specific inactivation of *Lmx1b*. A possible explanation for the less severe phenotype of podocyte-specific *Ldb1* knock-out mice could be that the loss of Ldb1 will not completely disrupt the transcriptional complex involving Lmx1b, and Lmx1b therefore will still function to a certain degree. LDB1 was originally described as a transcriptional activator of LIM domain proteins (Bach et al., 1997), and it also associates with LMX1A, a closely related paralogue of LMX1B (Iannotti et al., 1997). Somewhat surprisingly, LDB1 did not increase the activation of an insulin minienhancer reporter construct by LMX1A (Jurata and Gill, 1997), but it rather inhibited the transcriptional synergism between LMX1A and E47, which binds to the insulin minienhancer as well (Jurata and Gill, 1997). In the absence of E47, LDB1 had no effect on the transcriptional activity of LMX1A (Jurata and Gill, 1997). This contrasts with subsequent results obtained with LMX1B: despite using the same insulin minienhancer and transiently transfecting the same cells, LDB1 was able to inhibit LMX1B even in the absence of E47 (Dreyer et al., 2000). Therefore at present we cannot predict the effect of the lack of LDB1 on the target genes of LMX1B, this crucial issue will have to await the identification of LMX1B target genes in podocytes.

Patients with nail-patella syndrome exhibit quite variable disease severity, and proteins interacting with LMX1B are natural candidates as modifier genes. Our data point towards *LDB1* as a promising candidate, whereas the *E2A* gene is rather unlikely to influence disease severity in nail-patella syndrome. The *E2A* gene gives rise to two alternatively spliced transcripts, E12 and E47, of which the latter is translated into a protein enhancing the transcriptional activity of LMX1B (Dreyer et al., 2000; Johnson et al., 1997) and of LMX1A (German et al., 1992; Jurata and Gill, 1997). However, no obvious kidney phenotype has been described in the conventional *E2a* knock-out mice but this could be due to compensatory mechanisms. We therefore inactivated *E2a* specifically in podocytes to prevent compensatory mechanisms as much as possible, and again did not observe a phenotype. The easiest explanation would be that *E2A* is not expressed in podocytes, although we were able to detect the E2a mRNA in glomeruli and in a conditionally immortalized murine podocyte cell line (data not shown). Even if E47 is present in podocytes, it may not act in concert with LMX1B because due to the DNA-binding properties of E47 a target gene will have to contain binding sites for both LMX1B and E47, probably in close neighborhood. Whatever the explanation, at this point we have to conclude that with our tools available and at the level of analysis possible E2A does not play an essential role in podocytes. This study shows how important it is to confirm data from cell culture experiments in vivo. If LMX1B indeed is a transcriptional activator protein, then other co-factors will have to mediate its activity.

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