Integrin-linked Kinase Controls Renal Branching Morphogenesis via Dual Specificity Phosphatase 8

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ABSTRACT

Integrin-linked kinase (ILK) is an intracellular scaffold protein with critical cell-specific functions in the embryonic and mature mammalian kidney. Previously, we demonstrated a requirement for Ilk during ureteric branching and cell cycle regulation in collecting duct cells in vivo. Although in vitro data indicate that ILK controls p38 mitogen-activated protein kinase (p38MAPK) activity, the contribution of ILK-p38MAPK signaling to branching morphogenesis in vivo is not defined. Here, we identified genes that are regulated by Ilk in ureteric cells using a whole-genome expression analysis of whole-kidney mRNA in mice with Ilk deficiency in the ureteric cell lineage. Six genes with expression in ureteric tip cells, including Wnt11, were downregulated, whereas the expression of dual-specificity phosphatase 8 (DUSP8) was upregulated. Phosphorylation of p38MAPK was decreased in kidney tissue with Ilk deficiency, but no significant decrease in the phosphorylation of other intracellular effectors previously shown to control renal morphogenesis was observed. Pharmacologic inhibition of p38MAPK activity in murine inner medullary collecting duct 3 (mIMCD3) cells decreased expression of Wnt11, Krt23, and Slo4c1. DUSP8 overexpression in mIMCD3 cells significantly inhibited p38MAPK activation and the expression of Wnt11 and Slo4c1. Adenovirus-mediated overexpression of DUSP8 in cultured embryonic murine kidneys decreased ureteric branching and p38MAPK activation. Together, these data demonstrate that Ilk controls branching morphogenesis by regulating the expression of DUSP8, which inhibits p38MAPK activity and decreases branching morphogenesis.


Renal branching morphogenesis, defined as growth and branching of the ureteric bud (UB) and its derivatives, is essential to mammalian kidney development. The UB grows, branches, and differentiates to form the collecting ducts and pelvis of the mature kidney. Further, UB tips induce adjacent metanephric mesenchyme cells to undergo the process of nephrogenesis. Defects in renal branching morphogenesis cause congenital renal hypoplasia, characterized by abnormal collecting-system morphology and function and low nephron number.

The UB arises as a direct evagination of the intermediate mesoderm-derived Wolfian duct. Extracellular ligands and cell-surface receptors in multiple distinct molecular signaling pathways function during the induction and early patterning of the UB. In contrast, a limited number and variety of intracellular molecules that function within these signaling pathways to control ureteric branching have been identified. In vitro treatment of embryonic urogenital explants with pharmacologic inhibitors revealed distinct roles for extracellular signal-regulated kinase...
(ERK), protein kinase B (AKT), and p38 mitogen-activated protein kinase (p38MAPK) during renal development. Furthermore, ERK and AKT can be activated downstream of the tyrosine kinase receptor, RET, in the UB tip domain, suggesting that intracellular kinase signaling is functionally important during ureteric branching.

Integrin-linked kinase (ILK) is a scaffold protein that was initially identified through its ability to interact with the cytoplasmic domain of β-integrins. Investigation of ILK function has revealed a requirement for ILK upstream of intracellular kinases AKT, glycogen synthase kinase 3β, and p38MAPK. While each of AKT, glycogen synthase kinase 3β, and p38MAPK have been shown to play a role in controlling renal branching morphogenesis, their role downstream of ILK in the embryonic kidney has not been defined. Nor is it predicted by their functions in nonrenal tissues because their regulation by ILK is context-dependent. Previously, we demonstrated that Ilk is required for renal branching morphogenesis in vivo and controls UB branching via p38MAPK in vitro. However, the role of p38MAPK in vivo and the genes that act downstream of Ilk during the early stages of branching morphogenesis have not been previously defined.

In this study we characterize the intracellular signaling pathways and UB transcriptome in the early embryonic Ilk-deficient kidney to determine the primary role of Ilk in the regulation of renal branching. Our results demonstrate that Ilk regulates the expression of a distinct subset of UB branching genes through both p38MAPK-independent and -dependent pathways. Our data also demonstrate upregulation of a phosphatase, dual-specificity phosphatase 8 (DUSP8), not previously implicated in kidney development. We demonstrate a functional role for DUSP8 in attenuating activation of p38MAPK and p38MAPK-dependent genes and inhibiting branching morphogenesis. Together, these results describe a novel DUSP8-mediated mechanism by which ILK controls renal branching morphogenesis.

RESULTS

Mice with conditional deletion of Ilk in the developing UB (Ilk−/−UB) have decreased UB branching at E12.5. However, the underlying molecular mechanisms are unknown. We investigated these mechanisms using whole-genome–based analysis of gene expression in intact kidney tissue isolated at E12.5 (n=6 kidneys per sample, three biologic replicates per genotype). At the time of dissection, the presence of a branching phenotype in Ilk-deficient kidneys was documented using green fluorescent protein (GFP) expression driven by the Hoxb7 promoter (Figure 1). Gene expression changes were investigated through microarray analysis using Affymetrix GeneChip Mouse 430 2.0 arrays. Expression levels were normalized to wild-type (WT) and then analyzed for differential expression between Ilk−/−UB and WT samples using the Bioconductor limma package in R statistical program.

To determine changes in expression levels for individual genes, the top table of genes based on the t-statistic ranking was generated. Using a statistical cutoff of P<0.003 to identify changes in the expression of individual genes, we identified 131 downregulated probe sets and 96 upregulated (Figure 1C, Supplemental Tables 2 and 3). The magnitude of changes in gene expression, particularly for downregulated genes, was twofold or lower. This may be due to the fact that UB gene expression was assayed in whole tissue in which UB cells constitute a minority of cells. While a gene expression analysis in isolated UB cells may have generated gene expression changes of higher magnitude, the quality of RNA isolated from flow-sorted UB cells derived from mutant mice precluded a whole-genome gene expression analysis. Notwithstanding these considerations, consistent with genetic deletion of Ilk, the most statistically significant gene expression change was a downregulation of Ilk in Ilk−/−UB kidneys.
Gene Ontology Analysis Suggests Abnormal Regulation of Tube Morphogenesis Genes

To define the global state of gene expression in an Ilk-deficient state, we performed pathway analysis to identify gene ontology (GO) biologic processes that are enriched within the downregulated gene set. Sixty-one biologic process GO terms were statistically enriched in the downregulated gene list (Supplemental Table 4). Within the downregulated gene set, GO terms involved in epithelial tube development as well as the regulation of organ morphogenesis were enriched (Table 1). These terms are highly consistent with the mutant phenotype and a putative role for Ilk in controlling the expression of genes involved in branching morphogenesis and tube morphogenesis.

A Subset of Ilk-Dependent Genes have a UB-Specific Expression Pattern

Because knockdown of Ilk was specific to the UB lineage, next we investigated genes expressed in the ureteric lineage and which function in ureteric branching. Accordingly, the whole-kidney gene data set was filtered for genes that are known to be expressed in the UB by crossreferencing the downregulated genes with the microarray and in situ hybridization expression data available in GUDMAP (www.gudmap.org).14 This procedure resulted in reduction of the 131 downregulated genes to 14 genes with confirmed UB-specific expression, including Wnt11, a UB-tip-specific gene essential for UB development15 (Table 2). While a subset of these genes expressed, including Hes1, Gja1, Fgf9, Fgf1, Npnt, Cxcr4, Arg2, Bmp2, and LAMA1 involved in branching morphogenesis and tube morphogenesis.

Ilk Deficiency Causes a Specific Decrease in the Activation of p38MAPK

Because previous studies in nonrenal tissues have implicated ILK in the control of kinase activity,7,8 we investigated the state of kinase signaling in E13.5 Ilk−/− UB embryonic kidney tissue protein lysates. Consistent with our prior results in vitro,12 phosphorylation of p38MAPK, an indicator of p38MAPK activation, was significantly decreased by 61% in Ilk−/− UB embryonic kidney tissue (Figure 3, A and B). In contrast, we observed no statistically significant difference in the expression of the phosphorylated forms of AKT and ERK in Ilk−/− UB embryonic kidney tissue as compared with WT (Figure 3, A and B), nor was there a significant difference in the expression of the functionally active form of β-catenin protein, in which residues Ser33, Ser37, and Thr41 are not phosphorylated (Figure 3, A and B). Consistent with the analysis of protein expression, expression of the TCF/Lef-LacZ reporter allele, a surrogate measure of canonical Wnt target gene activation, was comparable in Ilk−/− UB and WT kidney tissue (Figure 3C).

p38MAPK Activation is Required for the Expression of a Subset of Ilk-Dependent Genes

We investigated the functional role of p38MAPK in regulating expression of Ilk-dependent genes in the UB by inhibiting p38MAPK function. Inhibition of p38MAPK was achieved through treatment of explanted kidneys with commercially available p38MAPK inhibitors SB203580 and SB202190. Following two days of treatment with SB203580 or SB202190, cultured E12.5 embryonic kidneys displayed abnormal UB branch morphology (Figure 4, A–C) and 40% or 66% fewer branches, respectively, compared with vehicle-treated littermate kidneys (Figure 4D). Next, we determined the effect

Table 1. Selected GO terms enriched in genes decreased in Ilk−/− UB kidneys

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<th>P Value</th>
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<td>GO:0051094</td>
<td>Positive regulation of developmental process</td>
<td>1.66E-07</td>
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<td>1.39E-06</td>
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<td>GO:0035295</td>
<td>Tube development</td>
<td>2.25E-06</td>
<td>HES11SOX8GXJAJ1IFGF9IFGF1NPNTICXR4IARG2IBMP2LAMAX1AJ1HIF1AWNT11ETV4ETV5ISRPRY1ISPRY21LKG</td>
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of p38MAPK inhibition on the expression of the six Ilk-dependent UB genes using qRT-PCT. While levels of Sox8, CXCR4, and Myb were not significantly affected by inhibition of p38MAPK in kidney explant culture (Figure 4E), expression of Wnt11, Krt23, and Slco4c1 were each significantly decreased (Figure 4F). These data indicate that Ilk acts via p38MAPK-dependent and -independent mechanisms to regulate genes expressed in the developing UB.

**Overexpression of DUSP8 Decreases Both p38MAPK Activation and Expression of UB Tip–Specific Genes**

GO analysis of the complete transcriptome identified altered regulation of MAPK phosphatases in Ilk−/− UB kidneys (Table 3). At the individual gene level, the p38MAPK phosphatase, Dusp8, was among the most significantly upregulated genes in Ilk−/− UB kidneys (Table 4). We confirmed upregulation of DUSP8 in Ilk−/− embryonic kidneys by qRT-PCR analysis (n=6/genotype, two technical replicates; Figure 5A).

While our published work demonstrated that Ilk activates p38MAPK, the mechanism underlying their interaction is not understood. Accordingly, we investigated the functional contribution of DUSP8 to p38MAPK activity in the UB lineage using inner medullary collecting duct cells (mIMCD3) in which we varied DUSP8 expression and stimulated p38MAPK signaling using EGF12 (Figure 5B). mIMCD3 cells respond to EGF within 15 minutes with a 52.2% increase in p38MAPK phosphorylation (Figure 5, C and D). In contrast, no increase in p38MAPK phosphorylation was observed in DUSP8-overexpressing mIMCD3 cells. While a further increase in p38MAPK was observed by 30 minutes following administration of EGF in mIMCD3 cells, no change in p38MAPK phosphorylation was observed in DUSP8-overexpressing mIMCD3 cells. Our data demonstrate that overexpression of DUSP8 results in a significant decrease in the expression of Wnt11 and Slco4c1 mRNA (Figure 5E). Krt23 was also decreased, but the change in expression was not statistically significant. mRNA levels of Myb and Sox8 were unchanged. Because CXCR4 mRNA was undetected in both mIMCD3- and DUSP8-overexpressing mIMCD3 cells, we could not evaluate its regulation by DUSP8. Together, these data show that Wnt11 and Slco4c1, both of which are regulated by p38MAPK, are also regulated by DUSP8.

**Adenovirus-Mediated DUSP8 Overexpression in Embryonic Kidney Explants Decreases UB Branching**

We investigated the functional contribution of DUSP8 in renal branching morphogenesis in intact kidney tissue. E12.5 WT kidney explants were treated with either GFP-expressing recombinant adenovirus (Ad-Gfp) or adenovirus carrying Gfp and Dusp8 (Ad-Gfp-Dusp8) for 5 days at 106 plaque-forming units/ml. Transduction efficiency of Dusp8 transcript was analyzed in three independent experiments by qRT-PCR analysis (n=9/group, three technical replicates; Figure 6A). A minus-reverse transcription (RT) negative-control sample was included in qRT-PCR experiments to establish the effective removal of adenoviral DNA in the RNA samples. The mRNA of Dusp8 was 68-fold more abundant in the Ad-Gfp-Dusp8–treated explants compared with control explants. Levels of DUSP8 protein were remarkably higher in Ad-Gfp-Dusp8–treated explants compared with control explants (Figure 6B). Next, we measured the effect of DUSP8 overexpression on ureteric branching. Following 5 days of treatment with Ad-Gfp-Dusp8, ureteric branches were analyzed by whole-mount immunofluorescence analysis of cytokeratin expression (Figure 6, C and D). Quantification of ureteric tips demonstrated a significant branching phenotype in the DUSP8-overexpressing kidneys, with an average of 45 UB branch tips compared with the 74 branch tips present in the control explants (n=14/group) (Figure 6E).

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**Table 2. Selected UB-expressed mRNA transcripts downregulated in Ilk−/− UB kidneys (P<0.003)**

<table>
<thead>
<tr>
<th>Probe ID</th>
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<th>Fold Change</th>
<th>P Value</th>
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<td>Chemokine (C-X-C motif) receptor 4</td>
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<td>1435303_at</td>
<td>TAF4B RNA polymerase II, TATA box binding protein-associated factor</td>
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<td>1422165_at</td>
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In parallel experiments we investigated the effect of DUSP8 overexpression on p38MAPK activity in embryonic kidney explants. Experiments were designed in a manner parallel to those using mIMCD3 cells, using EGF to stimulate p38MAPK activity (Figure 5). Three independent experiments were performed, each consisting of three explants isolated from different mice and treated individually and then pooled to generate sufficient protein for Western blot analysis. The levels of phosphorylated p38MAPK, controlled for total p38MAPK, in each treatment group were compared with Ad-GFP plus EGF (no DUSP8). Treatment of explants with Ad-GFP-DUSP8 or Ad-GFP did not significantly change p38MAPK

Figure 2. Validation of Ilk-dependent genes confirmed differential expression in the UB. (A) Results of qRT-PCR analysis of UB-expressed genes downregulated in Ilk<sup>−/−</sup> UB kidneys. Significantly decreased genes Ilk, Sox8, Wnt11, CXCR4, Slco4c1, Myb, and Krt23 are shown (P<0.05). (B) In situ hybridization analysis of gene expression in tissue sections demonstrated normal expression of (B and C) Ret and (R and S) Sox9 in UB tips. (D–Q) Decreased UB-specific expression of Ilk-target genes was confirmed. Arrows indicate UBs.
activation compared with Ad-GFP plus EGF. In contrast, treatment with Ad-GFP-DUSP8 plus EGF decreased p38MAPK activation by 62% \((P < 0.02)\) compared with the Ad-GFP plus EGF control (Figure 6F). Together, these data show that DUSP8 decreases branching morphogenesis and p38MAPK activation in intact murine kidney tissue.

**DISCUSSION**

A requirement for *Ilk* expression in ureteric cells during branching morphogenesis has been demonstrated in the murine kidney.\(^1\)\(^1\) Yet, the molecular mechanisms that underlie its actions have not been previously defined in vivo. Our previous analysis of intracellular signaling downstream of ILK in cultured collecting duct cells with gain or loss of ILK expression suggested that ILK controls tubule morphogenesis via p38MAPK.\(^9\) In this study, we demonstrate that *Ilk* controls p38MAPK-dependent expression of genes involved in renal branching morphogenesis by regulating the expression of DUSP8, which inhibits p38MAPK activity. We first identified genes that are regulated by *Ilk* in ureteric cells using a whole-genome mRNA expression analysis of whole-kidney mRNA in mice with *Ilk* deficiency targeted to the UB. The expression of six genes with expression in ureteric tip cells – *Wnt11*, *Krt23*, *Slo4c1*, *Myb*, *Sox8*, and *Cxcr4* – was downregulated in *Ilk*-deficient tissue. Next, we demonstrated that kidney tissue with *Ilk* deficiency is characterized by decreased phosphorylated p38MAPK but no significant decrease in the phosphorylation of other intracellular effectors (ERK, AKT, or \(\beta\)-catenin) previously shown to control renal morphogenesis. We then showed that inhibition of p38MAPK activity controls the expression of *Wnt11*, *Krt23*, and *Slo4c1*. Upregulation of DUSP8, a dual-specificity phosphatase, in *Ilk*-deficient kidney tissue suggested a mechanism by which ILK controls p38MAPK and ureteric tip cell gene expression. Our data show that DUSP8 overexpression in murine-collecting duct cells inhibited p38MAPK activation and expression of *Wnt11* and *Slo4c1* and decreased ureteric branching and p38MAPK activation in intact embryonic kidney tissue. Our data support a model of ILK signaling during UB branching (Figure 7). Our model suggests that ILK acts upstream of p38MAPK to regulate *Wnt11*, *Krt23*, and *Slo4c1* expression in the UB tip to stimulate UB branching. ILK also regulates expression of *Sox8*, *CXCR4*, and *Myb* in the UB. Our model further proposes that ILK negatively regulates the expression of DUSP8, a protein phosphatase, which can act to modulate p38MAPK activation and downstream target gene expression.
Ilk-Dependent Genes and Control of Renal Branching Morphogenesis

The expression of genes (Wnt11, Cxcr4, and Sox9) expressed in the ureteric tip and with demonstrated functions in ureteric branching are decreased in Ilk−/− UB mice. Wnt11-deficient mice are characterized by renal hypoplasia and decreased ureteric branching.15 This phenotype is similar to that observed in Ilk−/− UB mice and suggests that Wnt11 deficiency in the absence of Ilk is critical to the genesis of renal hypoplasia. We also observed decreased expression of Cxcr4, a chemokine encoding gene that is highly
expressed in the UB tip. CXCR4 \textsuperscript{-/-} mice are characterized by a mild degree of renal hypoplasia. Pharmacologic inhibition of CXCR4 in embryonic kidney explants inhibits branching morphogenesis. Moreover, the overall state of chemokine signaling is misregulated in Ilk \textsuperscript{-/-} UB kidneys (Supplemental Table 5, Table 3). Together, these observations suggest that loss of Sox9 and alone to control branching morphogenesis. However, the effects of Sox9 deficiency on branching morphogenesis are far less severe than those observed in mice with deficiency of both Sox8 and Sox9, suggesting that loss of Sox8 may act in combination with the loss of Wnt11 and Cxcr4 to disrupt branching morphogenesis. We identified loss of expression of other genes in ureteric tip cells, but without known functions during branching morphogenesis, in Ilk \textsuperscript{-/-} UB mice. Keratin 23 is an intermediate filament protein, which could play a structural role specific to the ureteric tip. The function of Sox9-2, a unique marker of the ureteric tip domain and a uremic toxin transporter protein, remains to be defined.

Growth factor signaling via the RET receptor is crucial for renal branching morphogenesis. Ret expression and phosphorylation is not decreased in Ilk \textsuperscript{-/-} UB kidney tissue. Interestingly, UB isolated from Ilk knock-out (K220A or K220M) mutant mice is unresponsive to the RET ligand, and GDNF and GDNF-responsive genes are downregulated in Ilk \textsuperscript{-/-} UB kidneys. Phosphorylation of specific tyrosines in the cytoplasmic domain of RET causes phosphorylation of multiple distinct pathways downstream of RET. Because loss of Ilk results in alterations in a subset of GDNF-dependent genes, including Myb and CXCR4, it is possible that Ilk may be acting downstream of a distinct phosphorylation site of RET to regulate a subset of signals downstream of RET.

Table 3. Selected GO terms differentially regulated in Ilk \textsuperscript{-/-} UB kidneys

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<td>GO:0019838</td>
<td>Growth factor binding</td>
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Table 4. Selected mRNA transcripts upregulated in Ilk \textsuperscript{-/-} UB kidneys (P<0.003)

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DUSP8 is a Novel Modulator of p38MAPK Activity in the Murine Embryonic Kidney

We identified DUSP8 as a novel ILK target that modulates p38MAPK activity. To our knowledge, this is the first reported functional role of DUSP8 in regulating branching morphogenesis. In fact, the body of evidence related to the role of phosphatases during kidney development has previously been limited to proteins that interact with RET. The tyrosine phosphatase SHP2 promotes signaling downstream of RET; loss of Shp2 in the developing UB decreases both ERK

Figure 5. DUSP8 overexpression results in altered p38MAPK phosphorylation and decreased UB gene expression. (A) Dusp8 overexpression in Ilk−/−UB kidneys was confirmed by qRT-PCR (P<0.05). (B) Following Dusp8 cDNA transfection and stable colony selection, qRT-PCR analysis demonstrated successful overexpression of Dusp8 in mIMCD3 cells (DUSP8 o/e) (P<0.05). (C and D) After 15 minutes of EGF treatment in culture, levels of phosphorylated (phospho) p38MAPK are comparable between WT and DUSP8-overexpressing cells. After 30 minutes of EGF treatment, phospho-p38MAPK levels in DUSP8-overexpressing mIMCD cells are significantly decreased compared with WT cells (P<0.001). (E) In an EGF-stimulated manner, DUSP8-overexpressing mIMCD cells have significantly decreased the expression of Wnt11 and Slco4c1 (*Wnt11, P<0.05; **Slco4c1, P<0.001). Inhibition of Krt23 and Myb in DUSP8-overexpressing cells was observed, but not statistically significant.
activation in UB tips as well as ureteric branching.26 The phosphatase PTEN antagonizes PI3K activity downstream of the RET receptor affecting branching morphogenesis through the regulation of the localization and generation of phosphoinositol 3–5 triphosphates.27

The DUSP family of proteins are specific to MAPKs. DUSPs dephosphorylate the serine-threonine and tyrosine residues in the kinase domain, thereby inactivating the kinases. In Ilk-deficient kidney tissue, the effect of ILK deficiency was specific to p38MAPK because phosphorylation of ERK and AKT was unchanged in contrast to a significant decrease in phosphorylation of p38MAPK. Three members of the DUSP family – DUSP8, DUSP10, and DUSP16 – have been identified in nonrenal tissues and shown to modulate p38MAPK activity.28–30 These observations are consistent with our data, which demonstrate that DUSP8 decreases p38MAPK phosphorylation and expression of genes that are modulated in their expression by p38MAPK in the embryonic kidney. Interestingly, attenuation of p38MAPK activation by DUSPs can lead to positive regulation of ERK phosphorylation and activity.31 Our finding that phosphorylation of ERK is unchanged suggests that abnormal ERK activity does not contribute to the phenotype in Ilk\(^{−/−}\)UB kidneys.

The regulation of DUSP8 expression is incompletely understood. Given our previous demonstration of a BMP7-ILK-p38MAPK signaling axis and our observation that DUSP8 modulates p38MAPK activation, an autoregulatory feedback loop to regulate its expression is likely. If so, our model (Figure 7) suggests that p38MAPK activation leads to decreased phosphorylated p38MAPK activity. Loss of negative inhibition in Ilk\(^{−/−}\)UB kidneys would cause excessive DUSP8 expression leading to decreased branching morphogenesis.

In summary, we demonstrated that Ilk is crucial for the sustained activation of p38MAPK and downstream gene expression in the developing ureteric cell in lineage. Decreased branching morphogenesis in Ilk\(^{−/−}\)UB mice is due, in part, to increased expression of DUSP8, which attenuates p38MAPK signaling. Ilk is required in the UB for the expression of specific markers of the tip cell domain, as well as expression of renal branching genes crucial for morphogenesis.

**CONCISE METHODS**

**Mice**

Mice with conditional ILK deficiency targeted to the ureteric lineage were generated using Hoxb7-Cre-EGFP\(^{32}\) and Ilk\(^{loxP}\) mice\(^{33}\) to generate Hoxb7-Cre-EGFP;Ilk\(^{loxP}\) (Ilk\(^{−/−}\)UB) progeny with a specific
Global Gene Expression Analysis in Mouse Kidney Tissue

E12.5 embryos were dissected and kidneys removed. GFP fluorescence and branching pattern were used to identify mutant and WT kidneys prior to RNA stabilization in RNAlater (Invitrogen). Due to the absence of GFP expression in both heterozygote and WT littersmates, the genotypes were confirmed by PCR prior to RNA isolation. Six kidneys were pooled in each biologic replicate prior to RNA isolation. Whole kidneys were cross hybridized with the TCF/LEF-β-galactosidase reporter strain.34 All mice were housed at the Toronto Centre for Phenogenomics according to the guidelines of the Canadian Council for Animal Care.

Microarray Data Set Analysis

The six samples were analyzed and compared using the Bioconductor limma package in the R statistical program. Briefly, the limma package was used to perform background correction, robust multiarray (GC-RMA) normalization, expression calculation, and comparative analysis using a contrast matrix between WT and Ilk−/− UB samples to determine log-fold change and differential expression for each probe. The limma package calculates the expression values for each probe and ranks the probe list by statistical significance.35 To investigate the contributions to the mutant phenotype of genes with common attributes, the relative enrichment of GO terms in the downregulated gene list was investigated using the Toppgene server (false discovery rate, P<0.05).36 The complete data set was analyzed by the program Ermine37 to identify globally altered molecular pathways.

qRT-PCR

RNA was isolated using Qiagen RNeasy Micro Kit and cDNA was generated using First Strand cDNA Synthesis (Invitrogen) from total RNA. Real-time PCR was performed using the Applied Biosystems 7900 HT Fast RT-PCR system with a PCR reaction mix containing a cDNA sample, SYBR green, and gene-specific primers (Supplemental Table 1). Relative mRNA expression levels were determined using the standard curve method and individual expression values were normalized by comparison to β2-microglobulin. In embryonic kidney explants treated with adenovirus, relative mRNA expression levels were determined using the comparative CT method and individual expression values were normalized by comparison to glyceraldehyde 3-phosphate dehydrogenase.

In Situ mRNA Hybridization, Western Analysis, and Immunocytochemistry

Whole embryos were fixed in 4% paraformaldehyde in PBS for 16 hours at 4°C. In situ hybridization was performed as described,38 on paraffin-embedded sections (4 μm) using dioxxygen-labeled cDNA probes for Ret, Wnt11, Sox8, Sox9, Slco4c1, Krtn23, Myb, and CXCR4. For Western blot analysis, kidneys or cultured cells were lysed in radioimmunoprecipitation assay buffer and subjected to immunoblotting as described.39 The following primary antibodies were used at a dilution of 1:1000: p38MAPK, 9212; P-p38MAPK, 9211; ERK, 4695; P-ERK, 9101; AKT, 9272; P-AKT, 9275; nonphospho (Active) β-catenn, 8480 (Cell Signaling Technology), DUSP8, NBPI-58302 (from Novus Biologicals), P-RET (Tyr-1062)-R, sc-20252-R (Santa Cruz Biotechnology), RET (C-19), sc-167 (Santa Cruz Biotechnology), and Actin, A5441 (Sigma-Aldrich). E12.5 urogenital ridges were stained for β-galactosidase expression as previously described.39

Cell Culture

Both mIMCD-3 and DUSP8-overexpressing mIMCD3 cells were grown in monolayer in DMEM-Ham’s F12 medium (DMEM-F12), supplemented with 10% FBS (Gibco), 1% penicillin-streptomycin in an incubator with 5% CO2 at 37°C. The medium for DUSP8-overexpressing mIMCD3 cells was supplemented with 8 μg/ml G418. DUSP8 (NM_008748) was overexpressed in mIMCD3 cells using a TrueClone full-length cDNA clone from Origene (MC203407). Cells

![Figure 7. Model of the role of ILK in UB branching. ILK acts upstream of p38MAPK to regulate Wnt11, Krtn23, and Slco4c1 expression in the UB tip to stimulate UB branching. Ilk−/−UB samples were of sufficient RNA quality (RNA integrity number >9) and 400 ng of each sample was amplified for array analysis using the Ambion IVT kit. Each sample was amplified, hybridized, and scanned according to the standard protocol by The Centre for Applied Genomics, The Hospital for Sick Children, Toronto, Canada.](image-url)
were transfected using Turbofect reagent (R0531; Thermo Fisher Scientific), according to the manufacturer’s instructions, and stable clones were generated following G418 selection over one week. EGF stimulation experiments were performed in triplicate after 18 hours of serum starvation in culture using 10 ng/ml EGF (E4127; Sigma-Aldrich).

Cultured Embryonic Kidney Explants

Kidneys were dissected from E12.5 Hoxb7-Cre-EGFP mice and grown in culture on filter membrane inserts for 5 days. For imaging of ureteric branches, explants were fixed for 1 hour in methanol at −20°C, permeabilized with 0.1% Triton X-100 for 10 minutes, blocked with Protein Block Serum Free (Dako) for 1 hour and incubated overnight with cytokeratin mAb (1:200; Sigma-Aldrich). After washes in PBS, staining was visualized by incubation with Alexa Fluor 488 goat anti-mouse antibody (1:2000 dilution; Sigma-Aldrich). For adenosine virus treatment, embryonic kidneys dissected at E12.5 were grown in culture on filter membrane inserts for 5 days. Ad-Gfp and Ad-Gfp-Dusp8 were purchased from Vector Biolabs and added daily to culture medium at 107 plaque-forming units/ml for 5 days. For imaging of ureteric branches, explants were fixed using the cell permeabilization filter membrane inserts for 2 days in the presence of DMSO vehicle control or chemical inhibitors SB203580 (20 μM, V1161; Promega) and SB202190 (10 μM, S7067; Sigma-Aldrich) (n=3 kidneys/treatment). For adenosine virus treatment, embryonic kidneys dissected at E12.5 were grown in culture on filter membrane inserts for 5 days. Ad-Gfp and Ad-Gfp-Dusp8 were purchased from Vector Biolabs and added daily to culture medium at 107 plaque-forming units/ml for 5 days. For imaging of ureteric branches, explants were fixed for 1 hour in methanol at −20°C, permeabilized with 0.1% Triton X-100 for 10 minutes, blocked with Protein Block Serum Free (Dako) for 1 hour and incubated overnight with cytokeratin mAb (1:200; Sigma-Aldrich). After washes in PBS, staining was visualized by incubation with Alexa Fluor 488 goat anti-mouse antibody (1:2000 dilution; Invitrogen). The number of UB tips was quantified using the cell counter plugin in ImageJ. For assays of p38MAPK activation, 12.5 embryonic kidney explants were treated for 2 days and then analyzed.

Statistical Analyses

Results were analyzed for statistical differences between two groups using the t test, on GraphPad Prism software (version 6.0). P<0.05 was considered statistically significant. Values are given as mean ± SEM.

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DISCLOSURES

None.

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