The Wnt/JNK signaling target gene alcam is required for embryonic kidney development

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ABSTRACT
Proper development of nephrons is essential for kidney function. β-Catenin-independent Wnt signaling through Fzd8, Inversin, Daam1, RhoA and Myosin is required for nephric tubule morphogenesis. Here, we provide a novel mechanism through which non-canonical Wnt signaling contributes to tubular development. Using Xenopus laevis as a model system, we found that the cell-adhesion molecule Alcam is required for proper nephrogenesis and functions downstream of Fzd3 during embryonic kidney development. We found alcam expression to be independent of Fzd8 or Inversin, but to be transcriptionally regulated by the β-Catenin-independent Wnt/JNK pathway involving ATF2 and Pax2 in a direct manner. These novel findings indicate that several branches of Wnt signaling are independently required for proximal tubule development. Moreover, our data indicate that regulation of morphogenesis by non-canonical Wnt ligands also involves direct transcriptional responses in addition to the effects on a post-translational level.

KEY WORDS: DM-GRASP, Xenopus laevis, alcam, Kidney, Pronephros

INTRODUCTION
The Xenopus embryonic kidney has become an attractive model system with which to examine nephron formation and to model human kidney diseases. The pronephros has a simple structure and contains one single functional nephron at each body side. The pronephros is formed by inductive signals from adjacent tissues, mesenchymal-epithelial transition (MET), epithelial tubulogenesis, morphogenetic movements and vasculogenesis. Genes that are necessary for Xenopus pronephros development are also required for the development of the more-complex mammalian meso- and metanephros (Carroll and Vize, 1999; Dressler, 2006; Ryffel, 2003). Based on molecular studies, the Xenopus pronephros can be subdivided into different segments that are homologous to the segments of the metanephric nephrons of mammals (Raciti et al., 2008). In Xenopus, the pronephric tubule branches at its most proximal end to generate three ciliated structures called nephrostomes that receive fluid derived from the kidney filter: the glomerulus, which in Xenopus is called glomus.

Wnt proteins are extracellular glycoproteins that bind to Frizzled (Fzd) receptors, thereby activating β-Catenin-dependent (canonical) and β-Catenin-independent (non-canonical) signaling pathways (Kestler and Kuhl, 2008), including the Wnt/ROCK, the Wnt/JNK and the Wnt/Ca2+ branches (Schulte, 2010). Wnt signaling is crucial for kidney development (Merkel et al., 2007) and misregulation of Wnt signaling contributes to congenital kidney diseases. Mutations in the Wnt signaling mediator Inversin, for example, are the cause of nephropathesis type II (Otto et al., 2003), and polycystic kidney disease has been linked to gain and loss of canonical, as well as changes in non-canonical, Wnt signaling (Lancaster and Gleeson, 2010; Luyten et al., 2010).

Wnts regulate different aspects of nephrogenesis, including differentiation, proliferation, MET, tubulogenesis and morphogenesis. Fzd8 and Inversin have been shown to be required for morphogenetic movements of the proximal and intermediate tubule (Lienkamp et al., 2010; Satow et al., 2004). This process also involves Daam1, WGEF and RhoA, and regulation of the cytoskeleton (Miller et al., 2011). Recent data indicated that morphogenesis of the tubule requires convergent extension movements that are reached by mediolaterally oriented cell intercalations. This process involves Myosin, a protein regulated by the RhoA-activated kinase ROCK (Lienkamp et al., 2012). Wnt4 is expressed in the metanephric mesenchyme that develops into the mature nephron through several intermediates (Diez-Roux et al., 2011; Stark et al., 1994). Cultures of metanephric mesenchyme treated with Wnt4 generate nephrons in the absence of the ureteric bud (Kispert et al., 1998). In Xenopus, Wnt4 has been shown to be required for proximal tubule development (Naylor and Jones, 2009; Saulnier et al., 2002). Recent evidence indicated that Wnt4 induces nephrogenesis by β-Catenin-independent signaling (Burn et al., 2011; Tanigawa et al., 2011). It remained unclear whether regulation of β-Catenin-independent Wnt signaling in this context also involves transcriptional responses in addition to its effects at the post-translational level.

Alcam is a member of the neuronal immunoglobulin-like domain superfamily of cell-adhesion molecules, and promotes cell adhesion and signaling (Corbel et al., 1996; DeBernardo and Chang, 1996). We previously reported alcam expression during Xenopus embryogenesis (Gessert et al., 2008), including its expression in the developing pronephros. With respect to kidney development, Alcam expression was also reported in the metanephric mesenchyme and the developing tubules in chicken (Tsukamoto et al., 2006). Publicly available microarray data indicate that Alcam is also expressed in the developing metanephros of mouse E15.5 embryos (http://www.gudmap.org/). Taken together, these data indicate a conserved expression of alcam during nephrogenesis. However, analyses regarding a potential function of Alcam during pronephros development are not yet available. In addition, earlier experiments suggested that alcam is regulated by non-canonical Wnt signaling (Gessert et al., 2008; Lapointe et al., 2012; Prieve and Moon, 2003). It remained elusive how alcam is regulated by β-Catenin-independent Wnt signaling and whether alcam is involved in Wnt mediated aspects of nephrogenesis.

By using the Xenopus laevis pronephros as a model system, we here show for the first time that Alcam is essential for proper
tubulogenesis, Alcam functions downstream of Fzd3 and is regulated by Wnt/JNK signaling involving ATF2 and Pax2 in a direct manner. These data suggest a new mechanism by which Wnt signaling coordinates pronephric tubulogenesis.

RESULTS
Alcam is required for nephron development in the Xenopus embryonic kidney

Alcam expression in Xenopus pronephric tissue starts around stage 22/23 during epithelial tubule formation. Thereafter, alcam transcripts were detected throughout the whole pronephric tubule (Fig. 1A; supplementary material Fig. S1B).

To study the potential function of Alcam during Xenopus pronephros development, we performed knock down experiments using a well-characterized Alcam antisense morpholino oligonucleotide (MO). This MO is able to block the translation of an alcam-GFP fusion construct (Gessert et al., 2008) and reduced the amount of endogenous Alcam protein in vivo (supplementary material Fig. S1A). In all following experiments, gfp RNA was co-injected as a lineage tracer (Tecza et al., 2011) and manipulation was performed on only one side of the embryo, leaving the other side unaffected as an internal control. Control MO was injected in all experiments as an injection control.

Alcam-depleted embryos revealed a pronephric phenotype affecting the proximal part of the pronephros, as shown by whole mount in situ hybridization against the γ-subunit of the Na+/K+-ATPase, fxyd2, at stage 36 (Fig. 1B) (Raciti et al., 2008). About 51% of embryos revealed a significant reduction of the fxyd2 expression domain in the anterior part of the tubule. This phenotype was restored by co-injection of an alcam RNA that is not targeted by the Alcam MO (Gessert et al., 2008), indicating the specificity of the Alcam MO-induced effect. A more-detailed quantification of the total tubular surface indicated that the proximal and intermediate tubule convolute was significantly reduced upon Alcam depletion (Fig. 1C). This reduction was similar in extent to that recently observed upon knock down of Inversin (Lienkamp et al., 2010). Again, this phenotype was rescued by alcam RNA co-injection. In addition, the three nephrostomes did not properly form and were not separated upon Alcam downregulation, as revealed by lhx1 staining (Fig. 1D). Furthermore, expression of foxc1, a marker gene for the proximal pronephros, was reduced in about 63% of Alcam morphant embryos.

Fig. 1. Alcam is essential for embryonic kidney development in Xenopus. (A) Alcam is expressed during Xenopus laevis pronephros development in particular in the anterior proximal part (arrows). Lateral views with anterior towards the right are shown. Dotted lines indicate the level of the transverse sections. (B) In situ hybridization at stage 36 indicates that Alcam is required for normal expression of fxyd2 in the proximal tubule (arrow). Loss of fxyd2 upon Alcam depletion is rescued by alcam RNA co-injection. (C) Quantification of the tubule convolute area demonstrates a significant reduction in size upon loss of Alcam function compared with Control MO-injected embryos. This phenotype is significantly restored by alcam co-injection. Measurements of individual embryos are indicated. Median values are also shown. Embryos were analyzed from three independent experiments. (D) At stage 36, injection of Alcam MO leads to a fusion of the three nephrostomes (arrow), which is rescued by alcam RNA co-injection. (E) Embryos injected with Alcam MO show a severe reduction in foxc1 expression (arrow), which is rescued by co-injection of alcam RNA. Quantitative representations are shown. Lateral views with anterior towards the left (injected side) or towards the right (uninjected side) are shown. ap, anterior-posterior; dv, dorsal-ventral; n, number of independent batches of embryos; N, number of analyzed embryos in total. *P≤0.05, **P≤0.01, ***P≤0.001 and ****P≤0.0001.
embryos (Fig. 1E). Both phenotypes were restored upon co-injection of alcam RNA (Fig. 1D,E). Taken together, these data indicate that a loss of Alcam results in disturbed morphogenesis of the proximal and intermediate tubules. The rescue experiments revealed that the phenotype observed upon Alcam MO injections is specific.

We next analyzed the Alcam MO-induced phenotype in more detail. At an earlier time point of development, expression of pronephros-specific marker genes such as lhx1, dll1, wt1, wtnt4 and fzd3 was not affected in Alcam-depleted embryos (supplementary material Fig. S2). These data indicate that Alcam is not required for general specification and early differentiation of the pronephros. Next, we investigated the segmentation of the pronephros. Based on a large scale whole-mount in situ hybridization screen, the Xenopus pronephros can be subdivided into different segments that are homologous to the different segments of the mammalian metanephric nephron (Raciti et al., 2008) (Fig. 2A). To this end, we used slc7a8 to label PT1, slc26a11 to label PT2, slc5a1 to label PT2 and PT3, slc12a1 to stain IT1, IT2 and DT1, and clenk to label IT1, IT2, DT1, DT2 and CT at stage 36 (Fig. 2B-F). Alcam downregulation led to reduced domains of gene expression, particularly in proximal (PT1-3), intermediate (IT1 and IT2) and distal parts (DT1) of the pronephros, indicating that these segments are established but are smaller in size. The expression of clenk in the connecting tubule was not affected. As expression of all genes was not completely abolished, we conclude that the gross segmentation of the pronephros into proximal, intermediate, distal and connecting tubules is not disturbed in Alcam-deficient embryos.

**Loss of Fzd3 phenocopies the loss of Alcam**

Alcam expression was recently shown to be affected by Wnt signaling (Gessert et al., 2008; Prieve and Moon, 2003). Therefore, we next searched for Wnt ligands and Fzd receptors that are expressed in the pronephros similar to alcam. For further experiments, we focused on wtnt4, fzd3 and fzd8 as they are prominently expressed in the anterior part of the Xenopus pronephros (Maurus et al., 2005; Satow et al., 2004; Saulnier et al., 2002; Shi et al., 1998) (supplementary material Fig. S1B). Wnt4 has been shown to be required for proximal tubule development in Xenopus (Naylor and Jones, 2009; Saulnier et al., 2002). Furthermore, studies identified Fzd3 as a likely receptor for fzd3 (supplementary material version of JNK1) (Lei et al., 2002). Fzd3 regulates expression through JNK1

To investigate whether regulation of alcam by Fzd3 occurs through a β-Catenin-dependent or -independent Wnt pathway, we made use of two well-described Dishevelled (dsh) deletion constructs designated dshΔDIX and dshΔDEP. DshΔDEP activates β-Catenin-dependent Wnt signaling, whereas dshΔDIX acts through β-Catenin-independent Wnt signaling branches (Boutros et al., 1998; Itoh et al., 2000; Kishida et al., 1999; Li et al., 1999).

We injected Fzd3 MO, together with either dshΔDIX or dshΔDEP RNA, and monitored expression of alcam. Interestingly, alcam downregulation by Fzd3 depletion could only be restored by co-injecting dshΔDIX but not dshΔDEP RNA (Fig. 4A), indicating a regulation of alcam through β-Catenin-independent Wnt signaling. As dshΔDIX activates the non-canonical Wnt signaling mediator jun N-terminal kinase JNK (Boutros et al., 1998), we aimed to rescue the Fzd3 MO phenotype through introducing a constitutively active version of JNK (caJNK1) (Lei et al., 2002). caJNK1 RNA injection led to a modest but significant rescue of alcam expression (Fig. 4B). Injection of higher caJNK1 amounts is not possible due to gastrulation defects. These data indicate that alcam regulation by Fzd3 occurs through β-Catenin-independent Wnt/JNK signaling.

**The Alcam promoter contains a Fzd3 response element**

We next analyzed the 5' upstream regulatory region of alcam. We determined the transcriptional start site by 5'RACE and isolated a genomic region upstream of the transcription initiation site (supplementary material Fig. S6). We started our analysis with a 3.1 kb fragment including the 5'UTR and the regulatory region 2.7 kb upstream of the transcription start site that we cloned in front of the luciferase reporter gene (~2.7 kb-luc) (Fig. 5A). This construct revealed transcriptional activity in untreated animal cap cells (ACs) of Xenopus embryos (Fig. 5B). Upon inducing a pronephric fate in ACs by treatment with activin A and RA (retinoic expression was otherwise normal (Fig. 3E; supplementary material Fig. S4) similar to the phenotype observed upon Alcam depletion (compare with Fig. 1D). These data suggest that alcam expression in the pronephros depends on Wnt4 and Fzd3 function. As the phenotype observed upon loss of Fzd3 function phenocopied the loss of Alcam, whereas downregulation of Wnt4 resulted in a broader phenotype, we focused in the rest of our study on characterizing the correlation between Fzd3 and Alcam.

To further validate our observation that alcam expression is downstream of Fzd3, we analyzed the Fzd3 knock down phenotype in more detail. Loss of Fzd3 led to defects in proximal tubules at stage 36, as indicated by fxyd2 expression (Fig. 3C). This phenotype was restored by co-injection of alcam RNA. Measurements of the tubular surface area revealed that the proximal and intermediate tubule convolute was significantly reduced in size (Fig. 3D). Again, this phenotype was rescued by alcam RNA co-injection. Moreover, Fzd3 depletion led to fused nephrostomes (Fig. 3E) and a downregulation of foxc1 expression (Fig. 3F), similar to the situation observed upon Alcam MO injections (compare with Fig. 1D,E). Both phenotypes were restored by alcam RNA co-injection. Analyses of segmentation markers indicated that Fzd3 depletion most strongly affected proximal, intermediate and distal tubule development (supplementary material Fig. S5), similar to the Alcam depletion phenotype (compare with Fig. 2).

Taken together, these loss of function and rescue data indicate that alcam is downstream of Fzd3 and that this regulation is of functional significance. These observations raised the question of which Wnt signaling pathway is alcam regulated through?
acid), we observed a strong upregulation of promoter activity in comparison with untreated ACs (Fig. 5B).

We next tested whether the alcam regulatory region responds to a loss of Fzd3 and co-injected the ALGAM luciferase construct together with a Fzd3 MO to measure luciferase activity in pronephric ACs. Inhibiting Fzd3 function led to a significant reduction of luciferase activity (Fig. 5C). These results indicate that the isolated −2.7 kb upstream fragment contains regulatory elements required for alcam regulation by Fzd3. We next deleted parts of the fragment generating −2.2 kb and −0.8 kb fragments fused to the reporter gene (−2.2 kb-luc and −0.8 kb-luc; Fig. 5A). Both fragments revealed transcriptional activity in pronephric ACs, although at lower levels (Fig. 5B). Whereas the −2.2 kb-luc fragment responded to Fzd3 downregulation (Fig. 5D), the −0.8 kb-luc fragment
construct did not (Fig. 5E). These data indicate that the Fzd3 response element is likely located between −2152 and −781 of the alcam promoter.

We then tested whether the −2.7 kb-luc construct responds to canonical or non-canonical Wnt signaling. As in *Xenopus* ACs, the −2.7 kb-luc construct showed an activity in MDCK (Madin Darby canine kidney) cells (Fig. 6A). Treatment of MDCK cells with Wnt11 (Fig. 6C), a non-canonical Wnt ligand, but not Wnt3a (Fig. 6B), a canonical Wnt ligand, resulted in a significant upregulation of reporter activity. Also in HEK293 cells, treatment with Wnt3a or the GSK3β inhibitor BIO did not result in any activation of the alcam reporter, although both treatments activate the Wnt/β-Catenin reporter TOPFlash (supplementary material Fig. S7A,B). Transfecting MDCK cells with caJNK1, however, activated −2.7 kb-luc (Fig. 6C). Similarly, caJNK1 activated the reporter in HEK293 cells (supplementary material Fig. S7C). Other non-canonical Wnt signaling mediators such as constitutively active versions of CamKII (Kühl et al., 2000) or NF-AT (Borchers et al., 2006) had no effect on −2.7 kb-luc (supplementary material Fig. S7C). To confirm these observations in *Xenopus*, we used the pronephric AC system and investigated the influence of modulating JNK activity on the alcam promoter. Indeed, inhibition of JNK by SP600125 treatment led to a significant downregulation (Fig. 6D), whereas overexpression of caJNK1 resulted in an upregulation of promoter activity (Fig. 6E). Taken together, these results obtained in different cell lines...
supported our findings in whole embryos that the alcam reporter responds to non-canonical Wnt/JNK signaling.

**Pax2 regulates the Fzd3 response element**

Our experiments identified a Fzd3 responsive element (Fzd3RE) located between −2152 and −781 of the alcam promoter (Fig. 5). A close inspection of this region revealed the presence of seven AP1/ATF2 and two Pax2 sites clustered in a small region of 265 nucleotides (supplementary material Fig. S6). Deleting this region within −2.7 kb-luc to generate −2.7 kb-Δ-luc resulted in a dramatically reduced promoter activity (Fig. 5F). Moreover −2.7 kbΔ-luc no longer responded to Fzd3 depletion (Fig. 5F).

Previous work by others has linked ATF2 to β-Catenin-independent Wnt signaling (Schambony and Wedlich, 2007). Chromatin immunoprecipitation indicated that ATF2 binds in the 265 bp region that depends on Fzd3 function, supporting a role for ATF2 in alcam regulation (Fig. 7A). Pax2 is another transcription factor that is activated by β-Catenin-independent Wnt signaling at the post-translational level by phosphorylation through JNK. These earlier studies additionally indicated that phosphorylation of Pax2 by JNK correlates with an increased transactivation of a direct target gene of non-canonical Wnt signaling.

In combination with the functional analyses, our data suggests a novel mechanism how non-canonical Wnt signaling regulates alcam expression and thereby tubular development in the embryonic kidney.

**DISCUSSION**

Our data make the following novel contributions: (1) we show for the first time, that the cell-adhesion molecule Alcam is required for nephrogenesis; (2) we demonstrate that alcam expression in the pronephros depends on Fzd3 function and the activation of a β-Catenin-independent Wnt signaling pathway; and (3) we identify for the first time a response element in the regulatory region of a direct target gene of non-canonical Wnt signaling.

**Requirement of Alcam for nephrogenesis**

Tubule formation during nephrogenesis requires the transition of mesenchymal cells into epithelial cells (mesenchymal-epithelial transition, called MET). Previous findings suggested Alcam to be involved in adherens junction formation (Choe et al., 2013; Jannie et al., 2012) and in the establishment of epithelial apical-basal polarity, two processes occurring during MET. Later during tubulogenesis, the proximal part enlarges the surface area that is required for proper and efficient reabsorption of water, ions and small compounds. This is achieved by proliferation of tubular cells and morphogenetic movements (Fischer et al., 2006; Karner et al., 2009; Lienkamp et al., 2012). These convergent extension movements require the action of several Wnt components, such as Fzd8 or Inversin (Lienkamp et al., 2010). The similarity between our Alcam and Fzd8/Inversin MO phenotypes suggests that Alcam might also be required for these movements. A detailed analysis of...
Cell migration in Alcam morphant embryos will shed light onto this issue. It is also tempting to speculate that a loss of epithelial polarity upon loss of Alcam causes disturbed morphogenetic movements of the tubule, finally resulting in the observed phenotype. A detailed analysis of epithelial cell polarity will be required in the future to fully understand the Alcam MO phenotype at the cellular level.

Wnt signaling during nephrogenesis

β-Catenin-dependent and -independent Wnt pathways are important for kidney development. β-Catenin-dependent Wnt signaling was previously shown to be required for specification, differentiation and proliferation of pronephric cells, as well as ureteric bud formation (Iglesias et al., 2007; Lyons et al., 2009; Maretto et al., 2011).

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**Fig. 5. Identification of a Fzd3-responsive element in the alcam promoter.** (A) alcam reporter constructs generated in this study. Regulatory regions are linked to the luciferase gene as reporter. (B) The −2.7 kb fragment is active in untreated and in activin A/retinoic acid (RA)-treated Xenopus animal cap explants. The −2.7 kb fragment is active in pronephric animal cap cells, whereas the −2.2 kb, −0.8 kb and −2.7 kbΔ fragments show considerable lower activities. (C) The activity of the −2.7 kb fragment is reduced upon Fzd3 MO injection. (D) The −2.2 kb fragment reacts to loss of Fzd3. (E) The −0.8 kb fragment does not react to Fzd3 depletion. (F) Deleting a small part of the −2.7 kb fragment is sufficient for loss of promoter activity. luc, luciferase reporter gene; n, number of independent experiments; RLU, relative light units; n.s., not significant. *P≤0.05, **P≤0.01, ****P≤0.0001.

**Fig. 6. The alcam promoter is regulated through β-Catenin-independent Wnt in MDCK and AC cells.** (A) The −2.7 kb fragment is active in MDCK cells. (B) The promoter does not respond to treatment of MDCK cells with Wnt3A. (C) The promoter responds to transfection of MDCK cells with Wnt11. Activity of the −2.7 kb-luc reporter is increased upon co-transfection with caJNK1. (D,E) The promoter activity of the −2.7 kb fragment is reduced by JNK inhibition (SP600125 treatment) (D), whereas the JNK overexpression leads to a significant upregulation of promoter activity (E) in activin A/retinoic acid-treated pronephric animal cap cells (ACs). n, number of independent experiments; luc, luciferase reporter gene; RLU, relative light units. *P≤0.05, **P≤0.01.
β-Catenin-independent Wnt signaling is involved in murine metanephric and convergent extension movements (Karner et al., 2009). These findings (Gessert et al., 2008; Lapointe et al., 2012; Prieve and Moon, 2010; Miller et al., 2011; Satow et al., 2004). These data suggest that β-Catenin-independent Wnt signaling regulates the cytoskeleton to ensure proper cell behavior in the proximal tubule (Fig. 7D). A more recent study indicated that this process is achieved by Myosin-dependent mediolateral cell intercalation (Lienkamp et al., 2012). Of relevance, neither loss of Fzd8 nor loss of Inversin affected alcamin expression in our study, suggesting that the Wnt/ROCK signaling branch is not involved in alcamin regulation. By contrast, we provide a novel mechanism showing that a Wnt/JNK/Alcam branch is required for tubulogenesis. At the molecular level, we found alcamin to be regulated by Fzd3 signaling. Our data strongly support earlier findings (Gessert et al., 2008; Lapointe et al., 2012; Prieve and Moon, 2003; McCoy et al., 2011; Park et al., 2012). β-Catenin-independent Wnt7b and Wnt9 are important for tubule morphogenesis because of their ability to regulate asymmetric cell divisions (Yu et al., 2009) and convergent extension movements (Karner et al., 2009). β-Catenin-independent Wnt4 is involved in murine metanephric tubulogenesis (Burn et al., 2011; Tanigawa et al., 2011). In line with these findings, the expression of alcamin is not detectable during specification but is present during tubulogenesis. Work by others showed that Wnt/RhoA/ROCK signaling regulates morphogenetic movements. This pathway involves Fzd8, Inversin, Daam1, the Rho-GEF WGEF and RhoA, resulting in a reduced tubular convolute based on defects in cell migration (Lienkamp et al., 2010, 2011; Miller et al., 2011; Satow et al., 2004). These data suggest that β-Catenin-independent Wnt signaling regulates the cytoskeleton to ensure proper cell behavior in the proximal tubule (Fig. 7D). A more recent study indicated that this process is achieved by Myosin-dependent mediolateral cell intercalation (Lienkamp et al., 2012). Of relevance, neither loss of Fzd8 nor loss of Inversin affected alcamin expression in our study, suggesting that the Wnt/ROCK signaling branch is not involved in alcamin regulation. By contrast, we provide a novel mechanism showing that a Wnt/JNK/Alcam branch is required for tubulogenesis. At the molecular level, we found alcamin to be regulated by Fzd3 signaling. Our data strongly support earlier findings (Gessert et al., 2008; Lapointe et al., 2012; Prieve and Moon, 2003), demonstrating that alcamin is a direct target gene of β-Catenin-independent Wnt signaling at the transcriptional level. Therefore, we can add alcamin to the currently very short list of two direct target genes of β-Catenin-independent Wnt signaling in vertebrates: e4f2 (Maurus et al., 2005) and pcdh8 (Schambony and Wedlich, 2007). Our novel findings and earlier reports by others suggest that two independent non-canonical Wnt branches are required for proper nephrogenesis: (1) a Fzd8/Inversin branch required for cytoskeletal rearrangements (Lienkamp et al., 2010, 2012; Miller et al., 2011); and (2) a Fzd3/JNK branch regulating expression of the cell-adhesion molecule alcamin (this study). This situation is remarkably similar to the situation during Xenopus gastrulation. During this process, it was shown that Wnt5a, but not Wnt11, regulates the expression of the cell-adhesion molecule pcdh8 (PAPC) at the transcriptional level, whereas Wnt11 likely regulates the actin cytoskeleton (Schambony and Wedlich, 2007). Earlier studies also revealed an antagonism of β-Catenin-dependent and -independent Wnt signaling with respect to regulation of cell proliferation in the pronephros (McCoy et al., 2011). The exact mediators of non-canonical Wnt signaling in this context have not been determined yet but might include CamKII or NF-AT, which can both antagonize Wnt/β-Catenin signaling (Kühl et al., 2000; Saneyoshi et al., 2002). Taken together, a complex picture concerning the role of Wnt signaling during nephrogenesis is emerging. It will be of interest to determine whether and how these signaling branches are interconnected during this process.

Our study provides the first promoter analysis of a direct target gene of β-Catenin-independent Wnt signaling. It will be of interest to identify other target genes of Fzd3 in pronephric tissue and to analyze whether the regulation of these genes also involves a similar Fzd3-responsive element. Further work will be required to analyze the regulation of these genes in the context of β-Catenin-independent Wnt signaling. This will help to uncover the molecular mechanisms underlying the transition from an endodermal to an epithelial phenotype in the developing kidney.
the regulation of *alcam* by ATF2. As ATF2 does not function as a homodimer but requires the formation of heterodimers with other bZIP (basic zipper) transcription factors (Bhoumik and Ronai, 2008), the identification of ATF2 interaction partners in the pronephros will be highly relevant.

Interestingly, we also found Pax2 and ATF2 sites in the *alcam* promoter of mouse and human origin approximately 1.9 kb upstream of the transcription start point (data not shown). This suggests that the regulation of Alcam through non-canonical Wnt signaling might be conserved cross-species. Our findings should foster work into this direction. Of note, a detailed expression analysis revealed the presence of *Fzd3* during murine tubulogenesis (Diez-Roux et al., 2011), suggesting a conserved regulation of *Alcam* by *Fzd3*. However, *Fzd3*−/− mice do not have a kidney phenotype (Luyten et al., 2010; Wang et al., 2002), which might be because *Fzd3* has been shown to act redundantly with *Fzd6* in several processes, including neural tube closure, auditory hair cell orientation and eyelid closure (Wang et al., 2006; Wang and Nanshan, 2007). In line with this, *Fzd6* expression has been described in the proximal tubule of the murine metanephros (Diez-Roux et al., 2011) and in the human fetal kidney (Takahara et al., 1998).

Taken together, our novel findings support the notion that *alcam* is a direct target gene of Wnt/JNK signaling.

**MATERIALS AND METHODS**

More detailed experimental procedures are provided in the supplementary material.

**Animals**

*Xenopus* embryos were staged as described previously (Nieuwkoop and Faber, 1994). All experiments using *Xenopus* were performed in agreement with the German law.

**Morpholino oligonucleotide (MO), RNA and plasmid injections**

Antisense morpholino oligonucleotides (MOs) were obtained from Gene Tools (OR, USA). *Alcam*, Wnt4 and *Fzd3* MOs were used as previously described (Cha et al., 2007; Deardorff et al., 2001; Gessert et al., 2008; Saulnier et al., 2002). To target pronephric tissue, the V2 blastomere in eight-cell stage embryos was injected (Huang et al., 1998; Moody and Kline, 1990) with 20 ng of Alcam MO, 10 ng Wnt4 MO or 15 ng of Fzd3 MO. As a control, the standard Control MO from Gene Tools was used.

**QPCR**

Total RNA was isolated from ACs and reversed transcribed using random primers and Superscript II Reverse Transcriptase (Invitrogen). Semi-quantitative RT-PCR was performed using the Phire Hot Start II PCR Master Mix (Finnzymes).

**In situ hybridizations**

Whole-mount *in situ* hybridization and embryo sections were carried out as described previously (Gessert et al., 2007; Hemmati-Brivanlou et al., 1990). Semi-quantitative RT-PCR

Total RNA was isolated from ACs and reversed transcribed using random primers and Superscript II Reverse Transcriptase (Invitrogen). Semi-quantitative RT-PCR was performed using the Phire Hot Start II PCR Master Mix (Finnzymes).

**5 RACE, *alcam* promoter isolation and cloning of deletion constructs**

The complete 5′UTR of *Xenopus laevis*, *alcam* was isolated by 5′RACE using the GeneRacer Kit (Invitrogen). To identify the upstream regulatory region, a genomic *Xenopus* Lambda Fix II phage library (Stratagene) and an *alcam*-specific DNA probe were used. To analyze the 5′UTR upstream regulatory region of *alcam*, a 3.1 kb fragment (5′UTR and the regulatory region 2.7 kb upstream of the transcription start site) was cloned into pGL3 basic (Promega), generating −2.7 kb-luc. Deletion constructs were cloned by inverse PCR using the −2.7 kb-luc plasmid as a template. Constructs were amplified using Pfu Ultra II DNA Polymerase (Stratagene) or Phusion DNA Polymerase (Finnzymes) and re-ligated with Ligate-IT Rapid Ligation Kit (USB). All cloned plasmids were verified by sequencing.

**Luciferase assay**

HEK293 or MDCK cells were transfected with promoter-luc together with the Renilla luciferase vector phRL-TK (Promega) and 0.1 µg/well of caJNK1/pCS2+, caNFAT/pCS2+, caCAMKI/pCS2+, Wnt11/pCMV2 or pax2, then lysed after 24 h or 30 h (pax2, Wnt11). For Wnt3a treatment, cells were incubated for 10 h with 200 ng/ml Wnt3a (R&D). BIO (Calbiochem) treatment carried out for 24 h with 5 µM BIO or DMSO as control. For control experiments, 0.2 µg/well Super8XTOPFlash vector was co-transfected with phRL-TK (0.05 µg/well, Promega) and treated with Wnt3a or BIO. Luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega).

**Chromatin immunoprecipitation**

ACs were fixed with formaldehyde and nuclei were collected by centrifugation. After lysis, sonication was performed using a Branson Sonifier 250. Immunoprecipitation was performed with ATF2 (Cell Signaling) or Pax2 antibodies (Abcam). DNA was purified using the PCR purification kit (Qiagen). PCR was performed using Phire Hot Start II PCR Master Mix (Finnzymes).

**Statistical**

The nonparametric Mann-Whitney rank sum test was used to determine statistical differences (Prism, Version 5.0d, Irvine, USA). A *P*-value ≤0.05 was considered to be significant. In all figures, statistical significances are indicated as: *P*≤0.05, **P**≤0.01, ***P***≤0.001 and ****P***≤0.0001.

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**Competing interests**

The authors declare no competing financial interests.

**Author contributions**


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**Supplementary material**

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.107938/-/DC1

**References**


Cloning
cajNK1 was obtained from addgene (pcDNA3FlagMKK7B2Jnk1a1). Based on this plasmid, the insert was amplified using the proof reading Pfu Ultra II HS DNA Polymerase (Stratagene) and the following primers: JNK_l: 5’-TCT AGA AAG CTT CGG CAG CCA ACA T-3’; JNK_r: 5’-TCT AGA AAG CTT GAG CTC GAG TCA CT-3’. 5’ ends of amplification primers were armed with XbaI binding sites. The PCR product was ligated into pSC-B (Stratagene) and subsequently transferred into pCS2+ with XbaI (NEB). All cloned plasmid were verified by sequencing.

Semi-quantitative RT-PCR
Total RNA was isolated from animal cap explants at indicated stages using the peqGOLD RNAPure Kit (PEQLAB) following the manufacturer's protocol. For cDNA synthesis, Superscript II RNase H reverse transcriptase (Invitrogen) and random primers were used. The semi-quantitative RT-PCR has been performed using the Phire Hot Start II PCR Master Mix (Finnzymes) according to the manufacture’s protocol. Primer sequences are: GAPDH_for: 5’-GCC GTG TAT GTG GAA TCT-3’; GAPDH_rev: 5’-AA GTT GTC GTT GAT GAC CTT TGC-3’; foxc1_for: 5’-TCA GAA TTT GCT GCA GTC ATA GAC-3’; foxc1_rev: 5’-GTC AGG GCT CCA TCT ACA GTC-3’; alcam_for: 5’-TGC CTA CCA CAT AAC CGA CA-3’; alcam_rev: 5’-TAC CGG AGC AGG ACA CT-3’.

5’RACE, alcam promoter isolation, and cloning of deletion constructs
To identify the complete 5’UTR of Xenopus laevis alcam gene, we performed a 5’RACE (rapid amplification of cDNA ends) using Xenopus embryos at stages 25 and 35 and the GeneRacer Kit (Invitrogen) according to the manufacture’s protocol. The sequence of the reverse gene specific primer (reverse GSP) was based on the published Xenopus laevis alcam gene (Acc. No. BC073670): 5’-CCC AAC CAA GCG TGG GCC ACC-3’. To identify the upstream regulatory region of Xenopus alcam gene, we made use of a genomic Xenopus Lambda Fix II phage library (Stratagene) and an alcam specific DNA probe with a length of 566bp (nucleotide 1-566 based on Acc. No. BC073670). Transcription factor binding sites were identified using TFSearch (Parallel Application TRC Laboratory, RWCP, Japan (Heinemeyer et al., 1998)) and PROMO (ALGGEN (Farre et al., 2003; Messeguer et al., 2002)). To analyze the transcriptional activity of the 5’UTR upstream regulatory region of the alcam gene, we cloned a 3.1kb fragment (5’UTR and the regulatory region 2.7kb upstream of the transcription start site) into the luciferase reporter vector pGL3 basic (Promega) called -2.7kb-luc. Deletion constructs were cloned by an inverse PCR using the -
2.7kb-luc plasmid as template and the following primers: pGL3_rev: PO₄-5’-AAG CTT ACT TAG ATC GCA GAT CTC-3’; -2.2kb_for: PO₄-5’-ACC CGG GAA GGC CCC CTA TGT-3’; 0.8kb_for: PO₄-5’-GTA GTT GTG TTT TCT ACC TAT-3’; -2,7kb_for: PO₄-5’-CAT TCA AGC ATA GAA ATA AAT TGT CA-3’; -2,7kb_for: PO₄-5’-GTA GTT GTG TTT TCT ACC TAT-3’; -2,7kb_for: PO₄-5’-GTA GTT GTG TTT TCT ACC TAT-3’. The constructs have been amplified using the Pfu II Ultra Polymerase (Stratagene) or the Phusion Polymerase (Finnzymes) and re-ligated with Ligate-IT™ Rapid Ligation Kit (USB). All cloned plasmids were verified by sequencing.

**Chromatin immunoprecipitation in Xenopus**

*Xenopus laevis* animal cap explants were collected in 1ml dissociation buffer (1mM HEPES, 8.8mM NaCl, 0.1mM KCl, 0.03mM Ca(NO₃)₂x4H₂O, 0.04mM CaCl₂x2H₂O, 0.08mM MgSO₄x7H₂O) and subsequently treated with 40µl of 37% formaldehyde for 10 min at 37°C to crosslink protein-DNA interactions. After treatment with 125µl 1 M glycine for 5 min at RT, ACs have been collected and washed twice with 1x PBS. 500µl of Nuclear Isolation Buffer (5mM PIPES, 85mM KCl, 0.5% NP40, pH8) + PhosStop/CompleteMini (Roche) has been added to the ACs and incubated on ice for 10 min. The nuclei were collected by centrifugation for 10 min at 6000 rpm and 4°C. 350µl lysis buffer (50mM HEPES, 150mM NaCl, 1% TritonX-100, 1.5mM EDTA) + PhosStop/CompleteMini was added and incubated for 10 min on ice. Sonication was performed using a Branson Sonifier 250 with the following setting: 20 times 20 cycles at 50% duty cycle, output control 3. The lysate was diluted 1:10 in ChIP dilution buffer (0.01% SDS, 1.1% TritonX-100, 1.2mM EDTA, 16.7mM Tris/HCL, pH 8.1, 167mM NaCl) + PhosStop/CompleteMini and after preclearing with 240µl Protein-A Sepharose (Sigma) for 1 h at 4°C, 10 µl of the ATF2 (Cell Signaling) or Pax2 antibody (abcam) was added and incubated over night slowly rotating at 4°C. 100µl Protein-A Sepharose were added and incubated for 4h at 4°C. After washing with 1ml high salt buffer (500mM NaCl, 1.1% TritonX-100, 0.01% SDS), 1ml low salt buffer (150mM NaCl, 1.1% Triton-X100, 0.01% SDS) and 1xTE (50mM Tris/HCl, pH 7.5, 1mM EDTA) twice, lysates were incubated with elution buffer (100mM NaHCO3, 1% SDS, 200mM NaCl, 100mM EDTA, 50mM Tris/HCl, pH 7.5, 0.2µg/µl Proteinase K) at 68°C over night. DNA was purified using the PCR purification kit (Qiagen) according to manufacturer’s protocol. PCR was performed using Phire Hot Start II PCR Master Mix (Finnzymes) according to the protocol. Primer sequences used are: Fzd3RE_for: 5'-ATG CCT AAC CGT GTT TTT CC-3'; Fzd3RE_rev: 5'-CCG TGT CTC CAA GTC CAA GT-3'; control_for: 5'-TGC CTA CCA CAT AAC CGA CA-3'; control_rev: 5'-TAC CGG AGC AGG ACA CTT CT-3'

**References for Supplementary Material**


Suppl. Figure 1. A. Western blot analysis reveals reduced proteins level upon Alcam MO in comparison to Control MO injections. B. At stage 23 alcam, fzd3, and wnt4 are expressed in the pronephros anlage (arrows). At stages 28, 32, and 34 fzd3 and wnt4 transcripts are localized in the proximal part of the pronephros (arrows) whereas alcam and fz8 are expressed in the proximal, intermediate, distal (arrows), and connecting (arrowheads) part of the pronephros. Lateral views of embryos are shown. Anterior is to the left.
Suppl. Figure 2. Alcam depletion has no effect on the expression of pronephric marker genes at early stages of *Xenopus* development. Alcam MO injected embryos do not show any reduction in expression of *lhx1* at stages 23 (A) and 28 (B) as well as *dll1* (a marker for the anterior proximal pronephros) (C), *wt1* (a marker for the glomus) (D), *wnt4* (E), and *fzd3* (F) at stage 28. A-F, Lateral views of the embryos with anterior to the left (injected side) or to the right (uninjected side) are shown.
Suppl. Figure 3. Loss of Fzd8 or Inversin does not affect alcam expression. At stage 36, Fzd8 or Inversin depletion affects the expression of fxyd2 in the proximal tubule, whereas knockdown of neither Fzd8 nor Inversin has an effect on the expression of lhx1 at stage 36, or alcam or foxc1 at stage 28. Lateral views with anterior to the left (injected side) or to the right (uninjected side) are given. Quantitative representations are given. n, number of independent experiments; N, number of analyzed embryos in total. n.s., not significant.
Suppl. Figure 4: Loss of Wnt4 leads to a severe downregulation of *lhx1* in the entire pronephros (arrow and arrowhead) whereas loss of Fzd3 results in fused nephrostomes in the proximal pronephros (white arrowheads). Lateral views with anterior to the left (injected side) or to the right (uninjected side) are given.
Suppl. Figure 5: A. Schematic illustration of a *Xenopus* pronephros according to Raciti et al., 2008. nt, nephrostome; PT, proximal tubule; IT, intermediate tubule; DT, distal tubule; CT, collecting tubule. At stage 36, the embryos injected with Fzd3 MO reveal changes in the expression of B. the amino acid transporter *slc7a8* (marker for PT1 of the proximal tubule); C. the anion exchanger *slc26a11* (marker for PT2 of the proximal tubule); D. the sodium/glucose cotransporter *slc5a1* (marker for PT2 und PT3 of the proximal tubule); E. the sodium/potassium/chloride cotransporter *slc12a1* (marker for IT1 und IT2 of the intermediate and DT1 of the distal tubule); F. the voltage sensitive chloride channel *clcnk* (marker for IT1 and IT2 of the intermediate, DT1 and DT2 of the distal, and the connecting tubule) on the injected side. Uninjected or Control MO injected side of the embryos display no pronephros phenotype. Quantitative representations are given. Lateral views of embryos with anterior to the left (injected side) or to the right (uninjected side) are shown. n, number of independent batches of embryos; N, number of analyzed embryos in total; uninj, uninjected. * p≤0.05, ** p≤0.01.
Suppl. Figure 6. Sequence of the identified regulatory region of *alcam*. AP1/ATF2 binding sites are indicated by grey boxes, Pax2 binding sites by a green box. The region deleted in the -2.7kb∆-luc reporter is also indicated. The different constructs used are highlighted.

Suppl. Figure 7. JNK1 activates the *alcam* promoter.
A. Promoter activity of the TOPFlash reporter indicates a strong increase after treatment of HEK293 cells with Wnt3 or BIO, respectively in comparison to the untreated vector. B. The *alcam* promoter does not respond to treatment of HEK293 cells with Wnt3a or BIO, respectively. C. Activity of the -2.7kb-luc reporter is increased upon co-transfection with caJNK1 but not upon co-transfection of HEK293 cells with caCAMKII or caNF-AT, respectively. RLU, relative light units.