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The Role of Non-canonical Planar Cell Polarity Wnt Signals on Cell Proliferation in Developing Amphibian Pronephroi

by

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Abstract

Wnt family signals are responsible for the control and regulation of numerous developmental processes throughout the amphibian *Xenopus laevis*. While many pathways exist, little evidence has been put forth describing a role for any of these within the developing kidney, or pronephros. In this investigation, wnt family signals were down-regulated unilaterally in *Xenopus* embryos and the effects upon kidney development assayed. The two methods utilized included injections of artificial extra-cellular wnt antagonists and injections of intra-cellular pathway specific antagonists. As a result, non-canonical planar cell polarity, or PCP, wnt signals were shown to have an inhibitory effect upon cell proliferation in the pronephros in wild-type *Xenopus* embryos.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
L	Litre
mL	Millilitre
µL	Microlitre
nL	Nanolitre
g	Gram
Mg	Milligram
Mg	Microgram
Ng	Nanogram
M	Molar (mol/litre)
mM	Millimolar
U	Unit (enzymatic)
B	Beta
A	Alpha, anti-
Hh	Hedgehog
BMP	Bone morphogenetic protein
Wnt	Wingless/Int
RTK	Tyrosine receptor kinase
C. elegans	*Caenorhabditis elegans*
TGF-β	Transforming growth factor beta
GDNF	Glial cell-derived neurotropic factor
D. melanogaster	*Drosophila melanogaster*

X. laevis	*Xenopus laevis*
FGF	Fibroblast growth factor
CRD	Cysteine-rich domain
SFRP	Secreted frizzled-related protein
Frz	Frizzled
Dvl	Dishevelled
GSK3	Glycogen synthase kinase 3
APC	Adenomatous polyposis coli
TCF/LEF	T-cell factor/lymphoid enhancer factor
PCP	Planar cell polarity
A/T/G/C/U	Adenosine/thymine/guanine/cytosine/uridine
NTP	Nucleotide triphosphate
ATP/TTP/GTP/CTP/UTP	A/T/G/C/U triphosphate
JNK	c-Jun terminal kinase
NO	Nitrouse oxide
NF	Nieuwkoop and Faber
WT	Wilm’s tumour
RNA	Ribonucleic acid
mRNA	Messenger RNA
DNA	Deoxyribonucleic acid
cDNA	Complementary DNA
lhx1	Lim homeobox 1
nFrz	n-terminal frizzled

Dvl-PDZ	Dishevelled, PDZ domain deletion
DshD2	Dishevelled deletion 2
DshD4	Dishevelled deletion 4
EGFP	Enhanced green fluorescent protein
memGFP	Membrane-bound EGFP
PH3	Phosphorylated histone 3
DαM	Donkey anti-mouse
IgG	Immunoglobin G
DNP	Dinitrophenol
DIG	Digoxigenenin
AP	Alkaline phosphatase
HRP	Horse radish peroxidase
MMR	Marc's modified ringer's
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
EDTA	ethylenediaminetetraacetic acid
MOPS	3-(N-morpholino)propanesulfonic acid
HCG	Human chorionic gonadotropin
MBS	Modified Barth's saline
MEMFA	MOPS, EDTA, magnesium, formaldehyde
TAE	Tris-acetate buffer with EDTA
TBE	Tris-borate buffer with EDTA
TBST	Tris-buffered-saline with Tween20
BSA	Bovine serum albumin

SSC	Sodium citrate buffer
MAB	Maleic acid buffer
DMF-TEA	Dimethylformamide-triethylamine
NHS	N-hydroxysuccinimide
FITC	Fluorescein isothiocyanate
ECM	Extra-cellular matrix
DiI	1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate

Epigraph

Here one must leave behind all hesitation: here every cowardice must meet its death.

-Dante Alighieri; The Inferno, Canto III

Chapter One: Introduction

1.1 Importance

The kidneys are absolutely essential to the survival of any vertebrate organism. In terrestrial animals such as humans, the kidneys serve to filter metabolic wastes and excess solutes from the blood and eliminate them while conserving the maximum amount of water, as well as producing a number of important hormones and serving to monitor blood pH and pressure (Bard, 2003). Homer Smith, in 1953, stated “our kidneys constitute the major foundation of our philosophical freedom. Only because they work the way they do has it become possible for us to have bones, muscles, glands, and brains”. While this statement is guilty of some degree of hyperbole, it remains true that when kidney failure occurs, defined as less than 10% of normal kidney function, death is certain unless measures such as kidney transplant or dialysis are carried out (Menon *et al*., 2008). However, these remedial methods are not completely effective, and in essence merely delay the inevitable. Many causes of renal failure exist, including congenital disease, drug and/or toxin exposure, infection and chronic disease such as diabetes. Despite the critical nature of the organ system, many facets of kidney development are uncharacterized. Much of this is due to the complicated structure of the mammalian kidney, which contains many thousands of individual functional units known as nephrons (Fig. 1.1). Furthermore, organogenesis in general is a complicated process, requiring many different inductive signals to properly specify and pattern the organ in question, and the kidney is no different (Bard, 2003). While much is known about the role of inductive signalling in a wide variety of tissues, much remains to be uncovered regarding the role of inductive signalling in kidney organogenesis.

Fig 1.1 Embryonic Kidneys

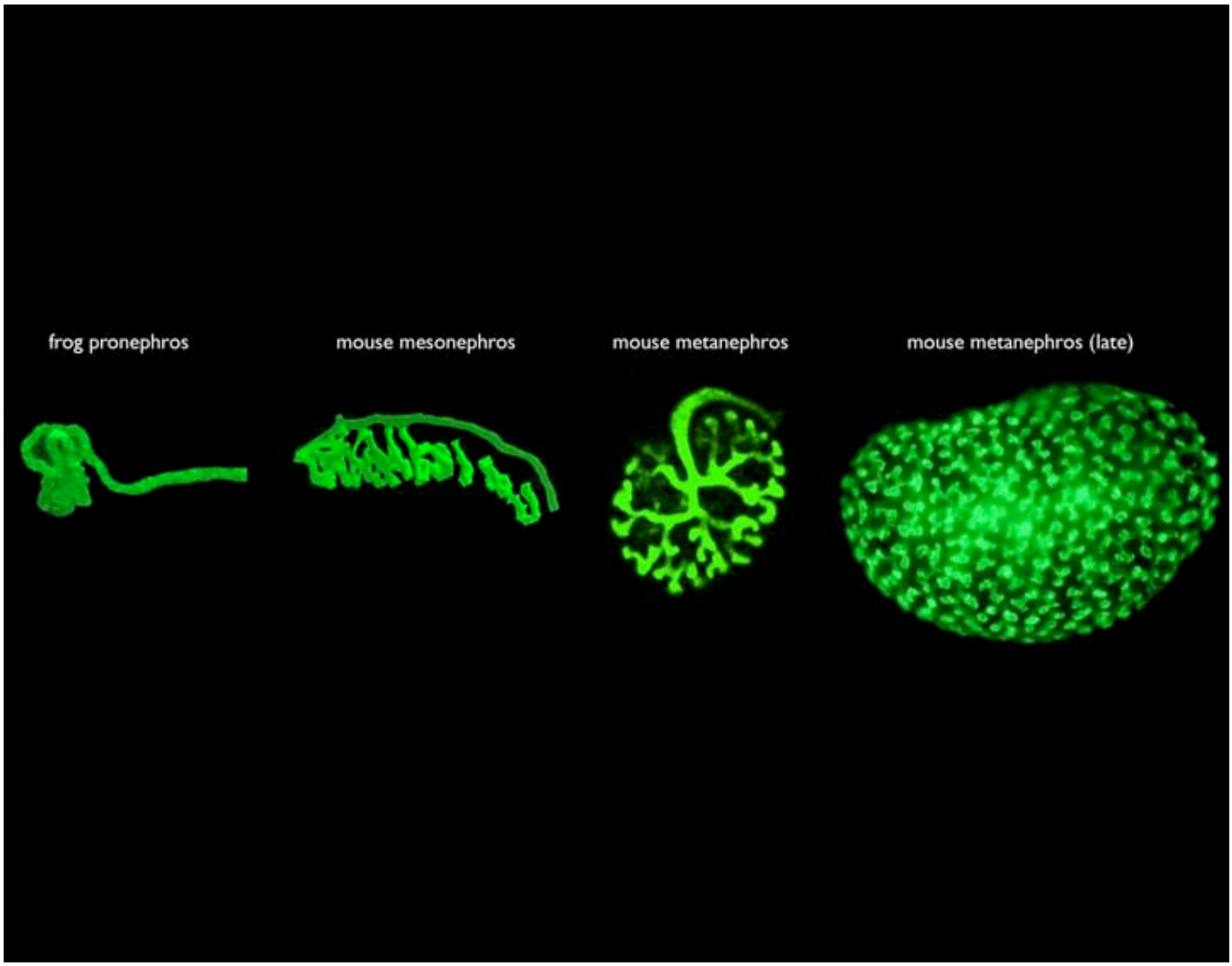

Fig 1.1 Embryonic Kidneys. Mammals undergo three stages of kidney development, the pronephros, mesonephros and adult metanephros. The adult metanephros is made up of thousands of individual nephrons. Each individual nephron in the metanephros is functionally similar to the frog pronephros. Images are not at shown at the same scale. Courtesy of P. Vize.

1.2 The kidney as a model of inductive interactions

One of the most prevalent and overarching concepts in the field of developmental biology is that of inductive interactions and their function via molecular signalling pathways. From the earliest forays into the way in which one tissue affects the developments of another in an embryo, researchers have struggled to discover the manner in which these interactions occur. As a result of these experiments, six main families of signalling molecules have been discovered to date. These include hedgehog (hh), bone morphogenetic proteins (BMPs), wingless/int1 (wnt), steroid hormone receptor, tyrosine receptor kinase (RTK), and notch (Martinez, Arias, and Stewart, 2002). These six families of molecules are now considered to be the primary effectors of what are known as inductive interactions; interactions between tissues of differing developmental histories in which one tissue alters the fate of another (Grobstein, 1956). Inductive interactions have time and again been shown to be one of the most important processes responsible for specification and patterning during a multitude of developmental steps, ranging from the establishment of the major embryonic axes to the determination of subsets of cells within an organ (Grobstein, 1956). An example of the importance of inductive interactions in whole-embryo patterning is that of dorso-ventral axis specification in the frog, *Xenopus laevis*. In order to properly delineate cells with a dorsal fate from ventral, wnt family signals must be active in the dorsal side of the embryo, resulting in the formation of Spemann's organizer and the subsequent formation of dorsal structures (Smith and Harland, 1991, Sokol *et al*., 1991, Tao *et al*., 2005). On a smaller

scale, inductive interactions are often responsible for specification of cells in organogenesis, for example the role of the notch family in determining the rectal fate of cells in the AB lineage in *C. elegans* (Edgar *et al*., 2001). While many types of induction occur during development, inductive interactions which occur through the association of two major components, a soluble ligand and membrane-bound receptor molecule, are responsible for directing many important processes within the developing embryo (Saxén *et al*., 1976). One of the hallmarks of the paradigm of soluble inductive signals is the remarkable specificity observed in this system. This specificity is due to the large number of members within each broad signalling family, combined with the large number of receptor molecules able to interact with those members. Each individual ligand/receptor combination can enact a different and potentially unique developmental effect, with the many permutations allowing for a huge number of possibilities (Okada, 2004). A characteristic example of the possible signal/receptor combinations is the BMP signal family, which are members of the transforming growth factor-β (TGF-β) superfamily of signalling proteins. This family of protein signals includes at least ten distinct members, with two type I receptors and a type II receptor combining to bind the various signals (Heisenberg and Solnica-Krezel, 2008). Not only can different developmental processes be dictated by which of the ten members are being expressed by the inducting tissue, but also by which combination of receptor molecules is being expressed on the competent responding cells.

In the developing kidney, the inductive interaction most studied has been that between the ureteric bud and the metanephric mesenchyme during adult kidney formation (Bard, 2003). In this particular example, a system of reciprocal induction patterns both

the ureteric bud, from which the collecting ducts and ureter are derived, and the metanephric mesenchyme, which gives rise to the nephrons and Bowman's capsules (Saxén and Sariola, 1987)(Fig 1.2). Initially, the metanephric mesenchyme secretes the inductive factor glial cell-line derived neurotropic factor (GDNF), which associates with the GDNF receptor ret, expressed on the cell membranes of the ureteric bud (Xu *et al.* 1999, Schuchardt *et al.* 1996). This association induces the ureteric bud to invade the metanephric mesenchyme, initiating the formation of the kidney. Reciprocally, the migrating bud, due to reception of the mesenchymal GDNF signal, produces wnt6 and 9b. These signals diffuse into the mesenchyme and induce it to aggregate and epithelialize via production of cadherin and laminar proteins (Ekblom *et al.* 1994, Carroll *et al*. 2005). Production of wnt4, induced from wnt6 and 9b reaching the mesenchymal mesenchyme, acts as an autocrine signal to complete epitheliazation of the mesenchyme into nephrons (Stark *et al*. 1994). Branching of the ureteric bud is completed by wnt11 and wnt7b signals in the tips of the ureteric bud in response to a return of GDNF from the epithelialized nephrons (Majumdar *et al*. 2003, Yu *et al*. 2009).

While copious evidence exists to suggest that inductive interactions are responsible for patterning of the adult kidney, until recently little credence was given to the idea that inductive interactions occurred between the individual functional regions of the nephron. However, studies have intimated that induction may indeed play a role in differentiating between the functional regions of the embryonic kidney (Zhou and Vize, 2004, Brandli, 1999, Hensey *et al.* 2002)(Fig. 1.3). Despite these findings, the nature and identity of the signals responsible for many processes in kidney organogenesis remain poorly characterized and represent the bulk of current research into kidney development.

Fig 1.2 Mammalian nephron

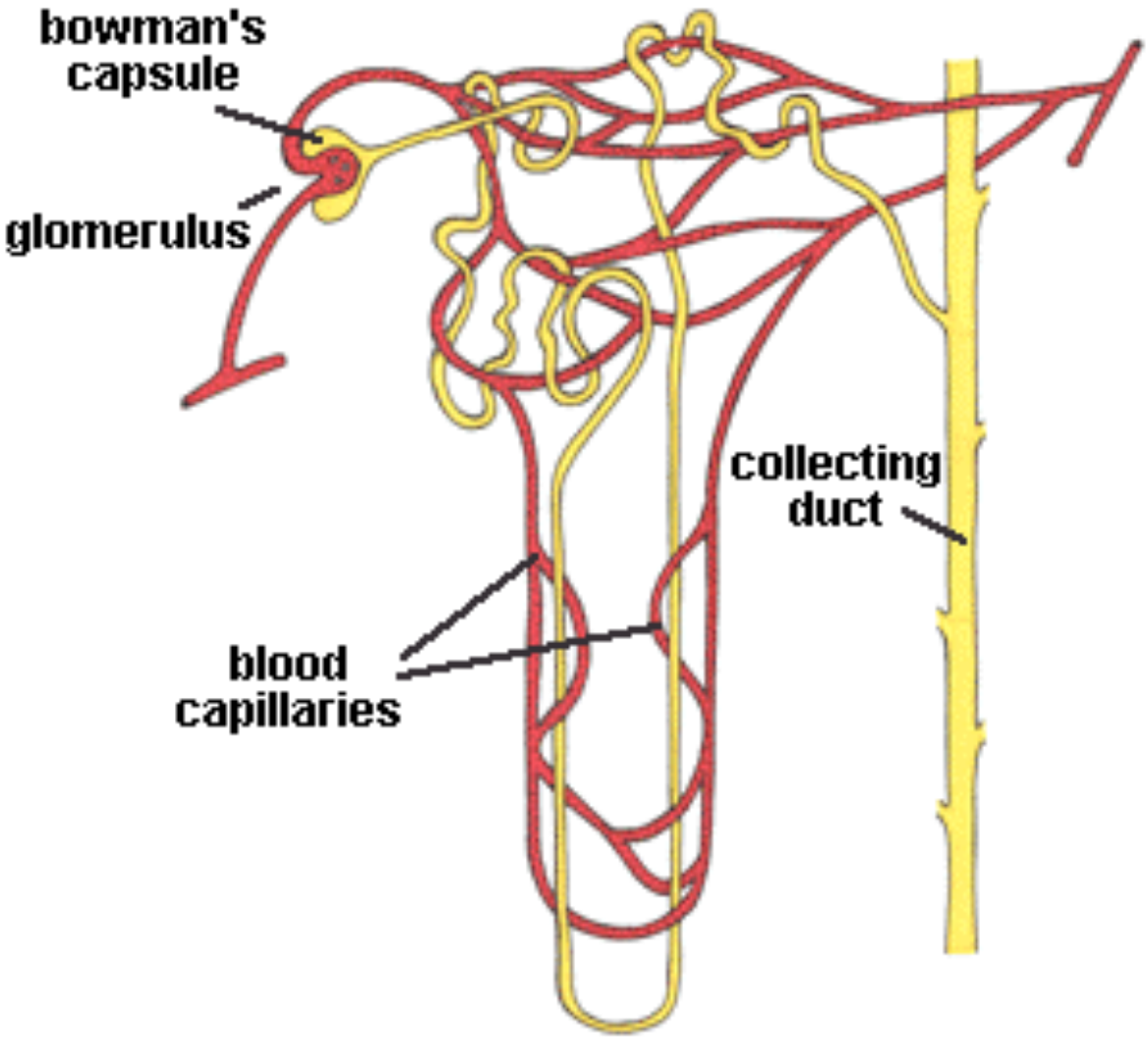

Fig 1.2 Mammalian nephron. Blood filters through the glomerulus and the filtrate is directly collected by Bowman’s capsule. Blood vessel systems surround both kidneys for reuptake of important nutrients. Courtesy of P. Vize.

Fig 1.3 Functional segments of the amphibian pronephros

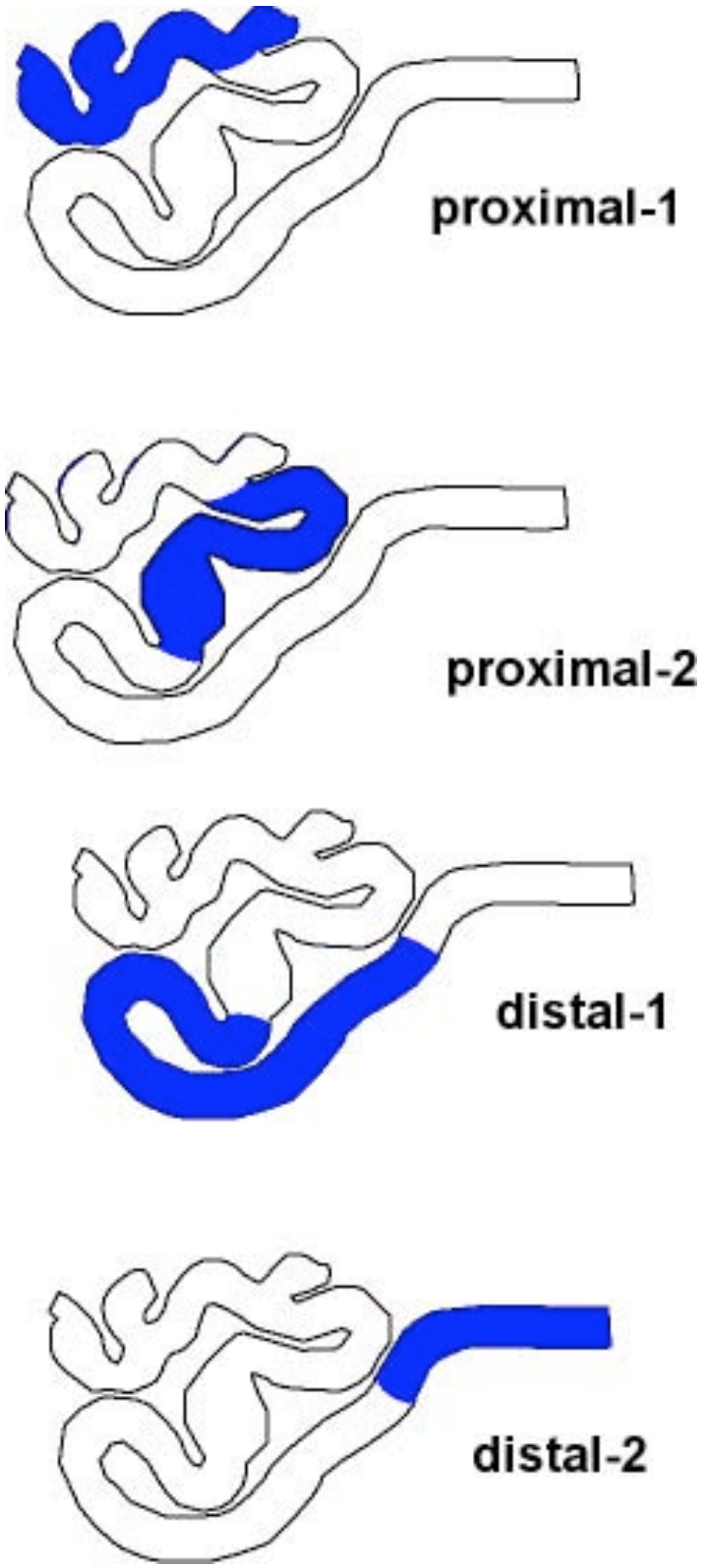

Fig 1.3 Functional segments of the amphibian pronephros. In pronephroi the filtrate is not directly collected by a Bowman’s capsule, but rather drains into a coelom and is directed into the proximal tubules by ciliated nephrostomes. Each pronephric segment is defined by differential expression of genes relating to various resorption and secretory mechanisms. Courtesy of P. Vize.

1.3 The kidney as a model of organogenesis

With any process of sufficient complexity, teasing information about its inner workings is difficult at best and often requires several lines of evidence from related sources to verify accuracy. A poignant example of this in the context of developmental biology is that of organogenesis. Organogenesis is the process of specification and organization of a number of tissues to produce a functional organ in the embryo and is a widely studied topic (Wingert and Davidson, 2008, Rubin, 2007). One of the most perplexing aspects of organogenesis is the amount of coordination required at the cellular level to successfully build an organ. As any organ is made up of a multitude of different tissues and cell types, and as all of these tissues come together in a very stereotypical and closely regulated fashion from widely disparate regions of the embryo, some manner of conserved coordination between the varied cell types is required. The molecular basis of this coordination is the subject of much research. Many cellular mechanisms have been identified in the development of organs, with a great deal of variation depending on the organ specified. In general, however, some of the main processes that must occur to build any organ include cell proliferation, specification, motility via various mechanisms, recruitment of endothelium and myriad others. Organogenesis has been studied across many species and tissues, with many model organisms, such as *C. elegans* and *D. melanogaster* having very well documented organogenic processes.

One of the most thoroughly studied examples of organogenesis, and the complexity inherent, is the vertebrate heart. Initial specification of pre-cardiac mesoderm occurs, as with many specification events, due to a gradient of soluble signals and a

corresponding gradient of inhibitory molecules. In this particular case, signals from the anterior endoderm consisting of fibroblast growth factor (FGF) and BMP family molecules work to actively specify both cardiac and blood precursor cells from competent mesoderm on both sides of the embryo (Schultheiss *et al*., 1995). Wnt molecules, specifically wnt3a and wnt8, induce this competent mesoderm to form blood precursors rather than cardiac cells. However, a gradient of the inhibitory molecules Cerberus, Dickkopf, and Crescent, produced by the anterior endoderm, act to antagonize these wnts and allow cardiac cells to be specified (Marvin *et al*., 2001, Schneider and Mercola, 2001, Tzahor and Lassar, 2001). Specified cells then migrate from each side of the embryo towards the ventral midline, along a gradient of the extracellular matrix protein fibronectin (Linask and Lash, 1988). After fusing and undergoing epitheliazation along the midline, looping of the then tubular heart into its final form begins. This process is largely based upon breaking of symmetry between the left and right sides of the heart and is driven by the asymmetric localization of proteins Lefty and Nodal (Srivastava *et al*., 1995). While several processes are required for the proper looping of the heart, one of the most important is differential cell division, likely directed by the expression of wnt11 exclusively in the right side of the heart (Flaherty and Dawn, 2008).

As indicated by this much simplified example, organogenesis of any organ requires many concurrent signals coordinated both spatially and temporally to produce a functioning organ. However, these findings tend to encourage more questions and as such many of the specifics of organogenesis remain uncharacterized in complex organisms. One of the most important emerging principles of organogenesis, and development in general, is that cellular mechanisms controlling organogenesis tend to be conserved

among various organs, and largely, across species. Gene products that are responsible for a certain mechanism in one tissue, be that specification, motility signals, proliferation, or others, are often reused in the same manner in other tissues at other times. For example, members of the FGF family of signalling molecules promote proliferation in a multitude of tissues in the developing embryo, such as eye and bone, rather than promoting a unique mechanism in each subset of cells (Hayashi *et al*., 2008, Deng *et al*., 2008). As such, revelations regarding the function of one of these tools in a particular tissue or organ are often widely applicable to other tissues within the organism and often across species as well. In the context of the kidney, then, mechanisms responsible for specification and organization of the kidney are likely also responsible for similar functions across the organism. Indeed, the embryonic kidney is regarded as a classic model of organogenesis among developmental biologists, mainly due to the easily apparent pathologies associated with disruptions to its formation (Bard, 2003).

1.4 Wnt signalling and kidney development

1.4.1 Canonical signals

Of the wnt pathways, the first to be elucidated is known as the canonical wnt pathway, whereas the other subsequently discovered pathways are labelled as non-canonical. The canonical wnt pathway acts through the binding of the wnt glycoprotein to frizzled (frz), the transmembrane receptor of wnt signals, triggering activation of the intracellular transducer dishevelled (dvl). In the absence of dvl activation, cytoplasmic β-catenin is constantly phosphorylated by glycogen synthase kinase 3 (GSK3) and subsequently degraded by a complex consisting of adenomatous polyposis coli protein

(APC) and axin (Huang and He, 2008, Sokol and Wharton, 2007, Widelitz 2005, Willert and Jones, 2006). Activation of dvl leads to its dimerization and association with axin/GSK3 and ultimately resulting in the stabilization of cytoplasmic β-catenin and its transduction into the nucleus to enact transcription along with the TCF/LEF family of transcription factors (Zeng *et al.,* 2005)(Fig. 1.4). Canonical wnt signals have been shown to enact many types of responses in many systems, including upregulation of cell proliferation (Logan and Nusse, 2004). For example, limb growth fails in *wnt5a* null mice due to a lack of mitosis in limb buds (Yamaguchi *et al*. 1999). Expansion of the central nervous system is also reliant on cell division, and is faulty in *wnt1* mutant mice (Megason and McMahon, 2002).

In the kidney, mouse studies have described a role for β-catenin in the branching of the ureteric bud, while ureteric bud-derived wnt-9b has been shown to have a role as an inducer of nephrogenesis (Bridgewater *et al*., 2008, Kispert *et al*., 1996, Carroll *et al*., 2005). Furthermore, the necessity of nuclear β-catenin in terminal differentiation of renal progenitors has also been demonstrated in mice (Schmidt-Ott *et al*., 2007, Marose *et al.*, 2008). In other experimental systems, wnt family signals have been implicated in the patterning of segmentation of the zebrafish hindbrain (Nasevicius and Ekker, 2000), a process involving many of the same transcription factors and signals as kidney segmentation, further solidifying a role for wnts in the developing kidney. In amphibians, *in situ* hybridization showing the existence of frz8, a receptor for wnt signals, in the distal regions of embryonic *Xenopus laevis* pronephroi provides preliminary evidence of the existence of wnt signalling in the kidney (Satow *et al*., 2004). Morpholino-based knockdowns of wnt4 in amphibian kidney have produced deletions of the proximal

Fig. 1.4 Overview of two main wnt signaling pathways

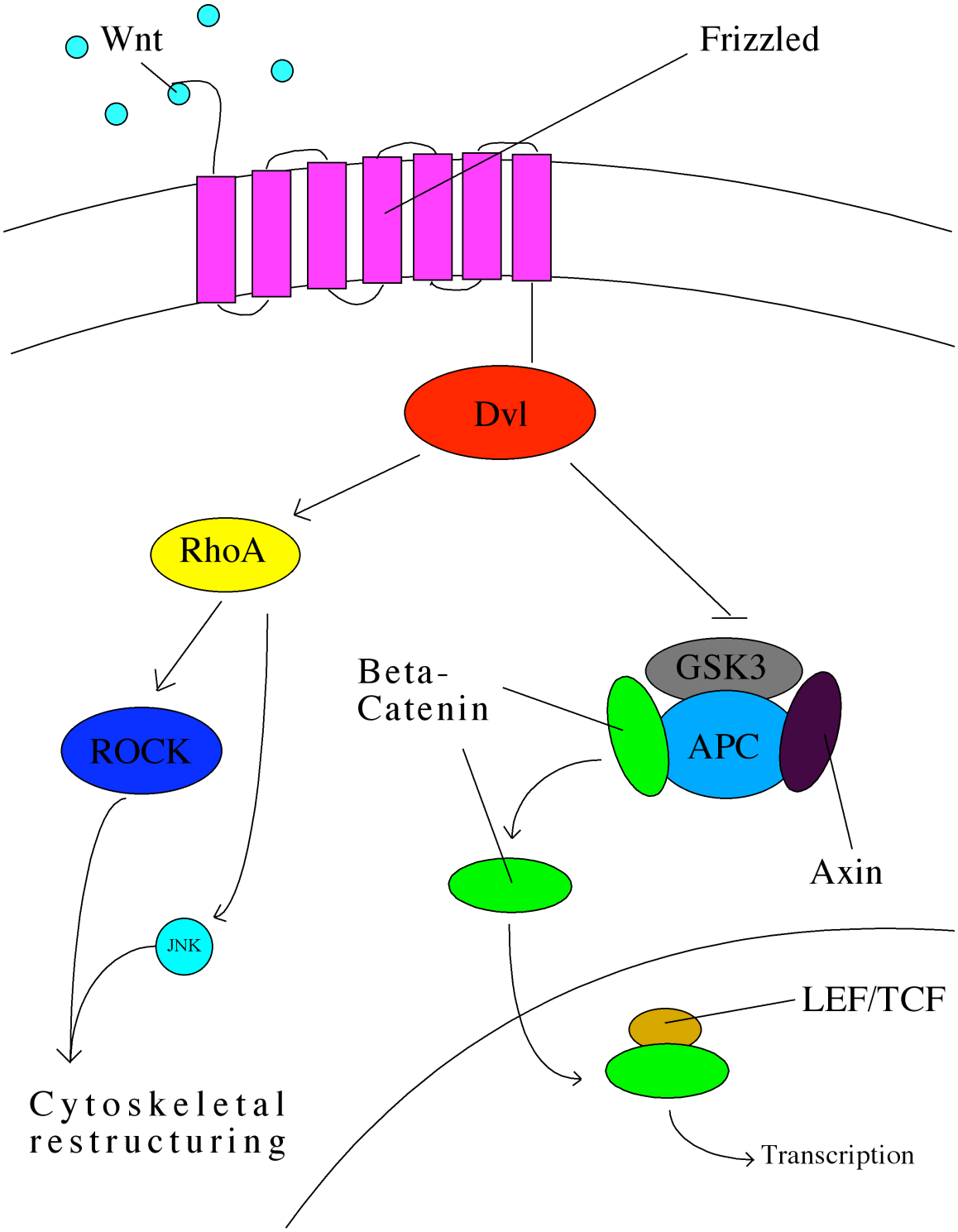

Fig. 1.4 Overview of two main wnt signaling pathways. Illustrated are canonical β-catenin and planar cell polarity pathways. Both pathways share a common element in that Dvl (red) acts as an intracellular transducer for all.

tubules while leaving the distal tubules unaffected, suggesting a role for frz8 specific to that segment (Saulnier *et al*., 2002). Interestingly, frz8 has only been detected in the regions unaffected by wnt4 knockdown, implying more receptors and signals that are as yet uncharacterized in the kidney. However, later studies have shown a role for wnt4 in early patterning of both *Xenopus* and zebrafish kidneys, with down-regulation leading to a loss of all kidney segments, making a consensus on the role of canonical signalling in *Xenopus* elusive (Lyons *et al*., 2009).

1.4.2 Non-canonical signals

One non-canonical pathway in particular is relevant in regards to the kidney, the planar cell polarity, or PCP, pathway. This pathway, as with the canonical pathway, requires a wnt signal, with wnt11 being the most commonly associated family member. Binding of wnt to frz subsequently activates the intracellular signal transducer dvl (Carreira-Barbosa *et al.,* 2003, Sokol, 1996). However, unlike canonical wnt signalling, the PCP pathway does not rely upon cytoplasmic β-catenin but rather acts through a phosphorylation cascade involving Rho GTPases and c-Jun n-terminal kinases (JNK) that ultimately restructures the actin cytoskeleton (Fig. 1.5)(Wallingford and Habas, 2005). Despite the depth of understanding of canonical wnt signals, the non-canonical pathways are not as well understood, with the PCP pathway falling directly in this category (Sharpe *et al*., 2001). What has been shown is that this particular pathway affects both proximal-distal patterning and convergent extension in a number of organisms, ranging from fruit flies to mice (Strutt and Strutt, 2005, Wallingford *et al.,* 2002). Furthermore, PCP signals have been demonstrated to affect the polarity of cell divisions both within mouse kidneys,

possibly in conjunction with convergent extension to enact lengthening of the kidney during morphogenesis, and in vertebrate heart looping (Fischer *et al*., 2006, Flaherty and Dawn, 2008). Unlike canonical signals, however, no studies have yet definitively described a role for wnt signals, acting through the PCP pathway, in the control of proliferation. On a similar note, despite the abundance of evidence pointing towards a role for non-canonical wnt-signalling in the amphibian kidney, no investigations have, to date, been carried out on how this signalling pathway affects kidney development in *Xenopus*.

1.4.3 Wnts and antagonism

The wnt signalling family plays many roles throughout development. One of the distinguishing characteristics of this family of molecules is the multiple pathways that wnt signals can act through to induce an effect. Currently, there are three main pathways that have been elucidated (Martinez, Arias, and Stewart, 2002), with a fourth pathway recently discovered (Schambony and Wedlich, 2007). Wnt signals often operate on a system of activation/antagonism by soluble antagonists possessing a cysteine-rich domain (CRD) similar to that found on the extracellular region of the receptor frizzled (Lin *et al*., 1997)(Fig. 1.5). These CRD antagonists are found nearly ubiquitously in systems where wnt signals are activated and serve to limit the effective area in which the soluble and diffuse wnt signal can act (Soltanoff *et al*. 2009). Naturally occurring CRD wnt antagonists, including Crescent, Frzb, Cerberus, Dickkopf, SFRP2 and 4, and others, are specific in which wnt signals are bound and antagonized, and by differential expression of both wnt signal and antagonist, enact processes of remarkable variety from the same

Fig 1.5 CRD wnt inhibition

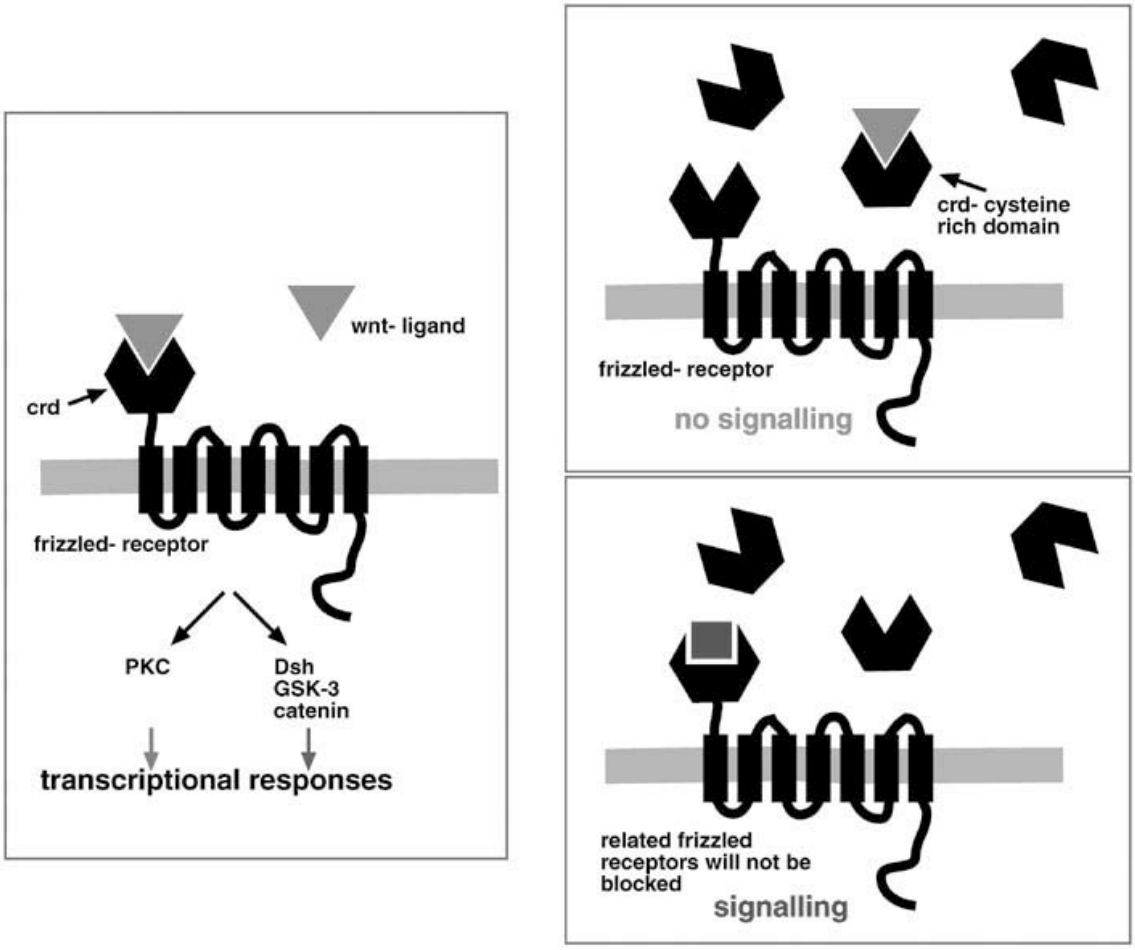

Fig. 1.5 CRD wnt inhibition. Inhibitors possessing a CRD domain act to interfere with the ability of wnt glycoproteins ability to interact with the frizzled receptor. Antagonists are specific as to which wnt molecule is antagonized. Courtesy of P. Vize.

small number of family members. Naturally occurring CRD inhibitors are particularly important in the development of head and brain structures, particularly Cerberus, frzb, and Dickkopf (Bouwmeester *et al*., 1996, Leyns *et al*., 1997, Glinka *et al*., 1998).

This concept of inhibition by competition for binding sites of wnt glycoproteins has been co-opted by researchers to down-regulate wnt signals broadly. As the essential means of down-regulation is the mimicry of the wnt-binding domain of frizzled, clones coding for solely the n-terminal, extra-cellular portion of frizzled have been developed that recapitulate the antagonistic effect of natural CRD inhibitors (Deardorff *et al*., 1998). As frizzled proteins often bind to more than one wnt signal, this provides an avenue to down-regulate several wnts at once (Logan and Nusse, 2004). Similarly, if the identity of the wnt signal in question is unknown, or the presence of a wnt signal only suspected, expression of these artificial wnt antagonists can serve to reliably down-regulate wnt signalling non-specifically (Papadopoulou and Edlund, 2005).

1.4.4 Intracellular wnt inhibition

While inhibition by competition is an effective tool for down-regulation of wnt signals broadly, in certain cases a more specific manner of wnt antagonism is required. In these cases, researchers have developed a technique for interfering with wnt signalling in a pathway-dependent rather than signal specific manner. This is accomplished by producing specific deletions of the intracellular transducer of wnt family signals, dishevelled. In these deletions, domains of the protein involved with associations with down-stream effectors are partially or completely deleted, interfering with the ability of

dvl to effect certain pathways of wnt signals (Rothbacher *et al*., 2000). These proteins contain deletions of the DIX, PDZ, or DEP domains (listed by location from N to C terminus) which are involved with various functions. The DIX (named after Dishevelled and axin) domain interacts with the c-terminal region of axin and is commonly associated with canonical signals (Zeng *et al*., 1999, Smalley *et al*., 1999). The PDZ domain (after PSD95, DigA, and zo-1) interacts primarily with other PDZ domains involved with sub-membraneous localization and is related to PCP signalling (Kennedy, 1995, Ponting *et al*., 1997). Lastly, the DEP domain (Dishevelled, Egl-10, Pleckstrin) is involved with associations with G-proteins and is believed to play a role with calcium-dependent pathways (Ponting and Bork, 1996). While these domains likely have further roles that are yet uncharacterized, experiments have been carried out with these deletion constructs demonstrating their efficiency at knocking-down the known functions carried out by each domain (Rothbacher *et al*., 2000). These molecules, then, can be over-expressed to out-compete endogenous dvl molecules for binding of activated frz, effectively stopping signal transduction down a specific pathway.

1.4.5 Pathway interactions

As exemplified by the descriptions of heart development and mammalian adult kidney morphogenesis above, both canonical and non-canonical wnt signals often act in organogenesis to direct varying processes. In the heart, canonical wnt signals (wnt 3a and 8) and their inhibition act to specify cardiac mesoderm, while non-canonical PCP signals (wnt11) help drive looping of the heart (Marvin *et al*., 2001, Schneider and Mercola, 2001, Tzahor and Lassar, 2001, Flaherty and Dawn, 2008). In the metanephros, canonical

signals (wnt6, wnt4 and wnt9b) induce aggregation and epitheliazation of metanephric mesenchyme, while PCP signals (again acting via wnt11) induce branching and invasion of the mesenchyme by the ureteric bud (Ekblom *et al*., 1994, Carroll *et al*., 2005, Majumdar *et al*., 2003, Yu *et al*., 2009). To date, many studies have indicated that, in general, canonical signals act to pattern or specify, whereas PCP signals enact morphogenetic processes (Logan and Nusse, 2004). However, no evidence exists that both signals are directly interacting in cardiogenesis and nephrogenesis. Conversely, wnt signal pathways have repeatedly been shown to interact in the patterning of tissues and organs within *Xenopus*, often in an antagonistic manner (Weidinger and Moon, 2003). Canonical signals have been implicated in up-regulating the rate of cell division in some tissues (Logan and Nusse, 2004). Conversely, nitric oxide (NO) signals, possibly working through the PCP pathway, are known down-regulators of cell proliferation globally in pre-gastrulation *Xenopus* embryos (Peunova *et al*., 2007). As noted, non-canonical wnt signals have been shown to play a role in the proper orientation of cell division within the mouse kidney (Fischer *et al*., 2006). Recently, however, a complimentary role in establishing the axis of division in kidney cells has been attributed to canonical signals, lending credence to the hypothesis that different wnt signalling pathways interact to pattern tissues (Yu *et al*., 2009). As the effect of developmental signals is often conserved between tissues, there is no reason to assume that this operation of different wnt signals acting at cross purposes to pattern tissues is not a global phenomenon. As such, wnt pathway antagonism may occur in a similar manner in other tissues and organisms, such as the *Xenopus* pronephros. Indeed, as evidence of wnts in proliferation exists in *Xenopus,* and evidence of wnts in kidney development exists in mouse models, it is not

unlikely that wnt signals influence, in the *Xenopus* pronephros, the rate of cell proliferation.

1.5 Pronephric morphogenesis in vertebrates

The mammalian kidney is made up of thousands of functional units known as nephrons, all which drain into a collecting duct on route to the bladder. In mammals, this adult kidney is known as the metanephros. During embryogenesis several intermediate stages of kidney arise, known, in chronological order of appearance, as the pronephros and mesonephros (Bard, 2003) (Fig. 1.1). In frogs and related amphibians, the adult kidney is itself a mesonephros, with embryonic filtration occurring through a pronephros (Nieuwkoop and Faber, 1994). Organogenesis of this embryonic kidney reflects that of the adult kidney organogenesis described above, although much less well-characterized. Pronephric progenitor cells are specified from the intermediate mesoderm post gastrulation, at Nieuwkoop and Faber stage 12.5 (NF 12.5) in *X. laevis*, and are identified by expression of the paired box transcription factor pax8 (Carroll and Vize, 1999). The original kidney anlage is subsequently subdivided into tubule and glomus progenitors by NF stage 22 in *Xenopus*, distinguishable by expression of the WT-1 transcription factor specifically in the glomus (Carroll and Vize, 1996). Approximately 10 hours post specification, at NF stage 20, kidney precursors segregate from surrounding mesenchyme to form a discrete clump of pronephric cells and begin to migrate caudally (Nieuwkoop and Faber, 1994). This process is completed approximately 15 hours later, at NF stage 34, when the migrating pronephric duct fuses with the rectal diverticulum as it extends rostrally (Gillespie and Armstrong, 1985, Nieuwkoop and Faber, 1994). A wave of

epitheliazation then occurs, propagating from the rostral end of the pronephros to the caudal, with expression of the solute transporter atp1a1 acting as a marker of kidney epithelium (Zhou and Vize, 2004). Following epitheliazation, the kidney is functional in amphibians, and begins producing urine by NF stage 35. This system is ideally suited to experimentation as the pronephros is essentially a single giant nephron, possessing all of the functional domains present in adult mammalian nephrons (Fig. 1.3). This embryonic nephron is large enough to be seen with the naked eye in amphibians, and, with the application of various antibody stains or *in situ* hybridization protocols, individual functional domains can be visualized (Zhou and Vize, 2004).

1.6 *Xenopus* as an experimental system

Aside from the easily visualized kidney, *Xenopus* has several other features that make it amenable to research. A second advantage to *Xenopus* as a model organism is the short development time of the embryo (Nieuwkoop and Faber, 1994). As an organism that undergoes metamorphosis, the time from embryo to fully formed tadpole is measured in a matter of days, and can be easily manipulated by changes in temperature. This allows for experiments to be scored and repeated in very short spans of time, in comparison to a more complicated model system. A third advantage is the large number of eggs and embryos produced by the frog. These embryos are large enough for easy manipulation, including microinjection of various mRNAs and other reagents (Soreq, 1985). This makes it possible to alter the gene expression of the embryo without time-consuming transgenics. Furthermore, the early frog embryo undergoes low levels of cell mixing, so it is possible to target injections to a specific blastomere or group of tissues without

affecting surrounding cells, increasing the experimental power of the system (Urban *et al*., 2006). Lastly, *Xenopus* is amenable to a number of gene manipulation techniques, including morpholino knockdowns (Mooulton and Tan, 2008) and, as mentioned, mRNA injections to express virtually any gene that can be cloned, a venture made much more accessible by the recent sequencing of *X. tropicalis*, a closely related species (Pollet and Mazabaud, 2006).

1.7 Experimental rationale and hypothesis

Suppression of cell proliferation may be very important to proper kidney morphogenesis, not only to ensure proper numbers of cells but also to maintain the stability of the actin cytoskeleton during the complicated cell movements required during the extension of the kidney primordium. As significant evidence exists to suggest that PCP and canonical wnt signals act as repressors and enhancers of proliferation respectively, and considering the redundant nature of inductive signals in differing systems, it is reasonable to assume that they act in the same fashion within the *Xenopus* pronephros. The hypothesis behind this investigation is that wnt family signals are affecting the proximal-distal specification of the *Xenopus* pronephros. Previously, exploration of the effects of wnt signals have focused on mammals during the formation of the mesonephros, while any effects earlier in development have not been addressed. In this investigation, the role of the wnt pathway within the early pronephros will be investigated, by (1) down-regulating wnt signals broadly by non-specific inhibition of soluble ligand, (2) down-regulating PCP signals specifically via dominant-negative inhibition of the intra-cellular transducer dvl, and (3) assaying rates of cell proliferation

within the pronephros both with and without inhibition of the PCP pathway. The *Xenopus* model organism provides a suitable environment in which to explore mechanisms of organogenesis at this very early stage of kidney development.

Chapter Two: Materials

2.1 Plasmids

Plasmids containing cDNA coding for various markers of kidney cells as well as injectable mRNAs are utilized in this study (Fig. 2.1, Tables 2.1 and 2.2). Plasmid containing lhx1 (Genbank accession #CO554633, Open Biosystems Clone #7296469 in pBluescript SK-) is linearized with *Xho*1 and transcribed from a T7 promoter. Pax8 (Genbank accession #CF521632, Open Biosystem clone #7012125 in pCMV-SPORT6) is linearized with *Bam*HI and transcribed with T7. Slc12a1 (Genbank accession #AI732841, Open Biosystems Clone #1323002 in pCMV-SPORT-6) is digested with *Sma*I and transcribed with T7. Atp1a1 (Genbank accession #BG161352, Open Biosystems clone #3400277 in pCMV-SPORT6) is digested with *Sma*I and transcribed with T7. cDNA encoding mRNA transcripts for nFrz8 (generously donated by Dr. Peter Klein, obtained in pCS2+) is linearized with *Not*I and transcribed from an SP6 promoter to generate mRNA. cDNA for both Dvl-PDZ proteins, DshD2 and DshD4 (also generously donated by Dr. John Wallingford, based on Xenopus *Dvl2*, Genbank accession #U431552, in CS2) are linearized with *Not*I and transcribed from an SP6 promoter to generate mRNA. Injections in embryos are traced with an EGFP fused to a ras farnesylation sequence, which labels cell membranes. cDNA for this mRNA (memGFP, donated by Dr. John Wallingford, in CS2+) is linearized with *Not*I, and mRNA was transcribed from an SP6 promoter.

Fig 2.1 Examples of markers utilized

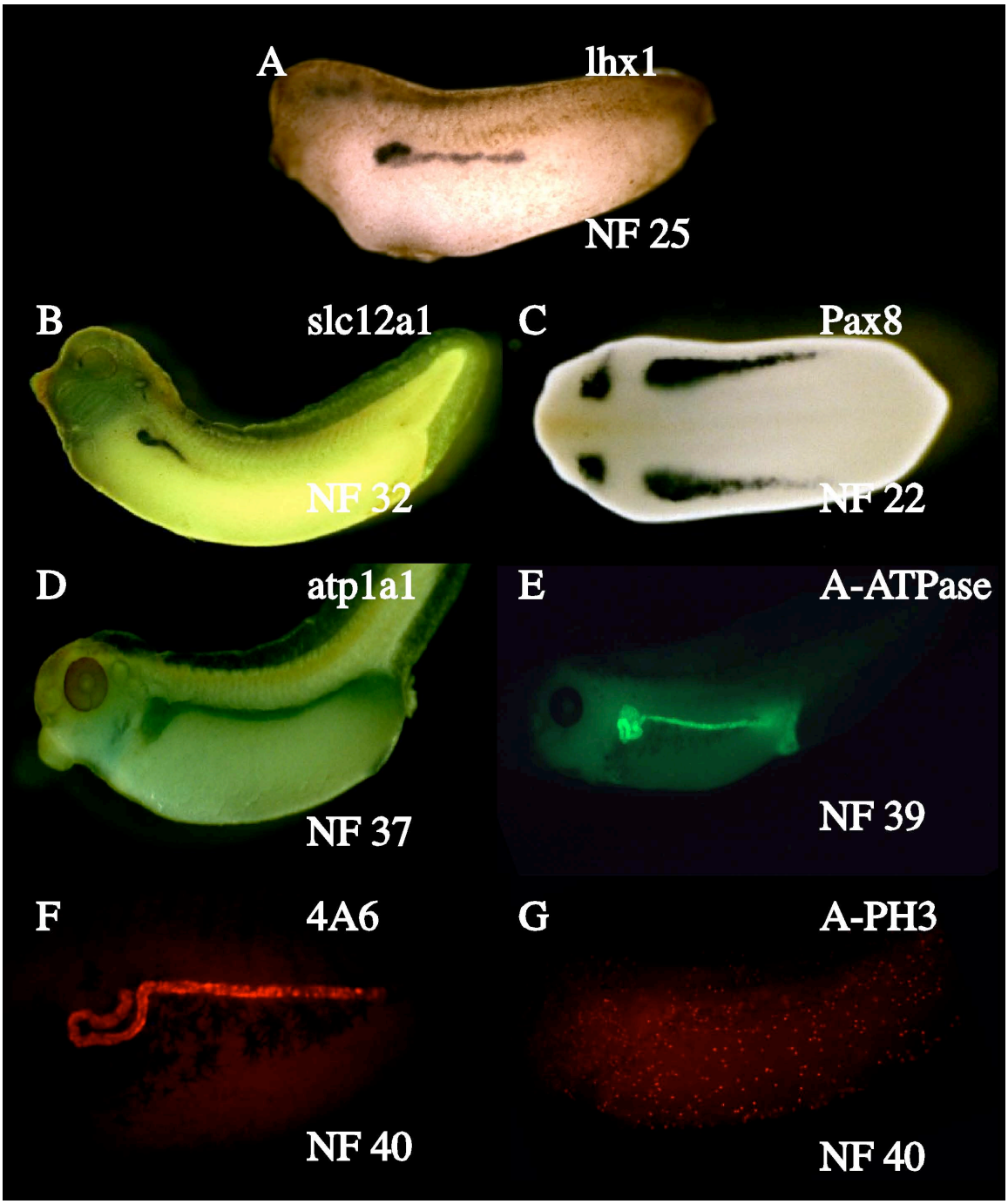

Fig. 2.1 Examples of markers utilized. Shown are kidney markers lhx1 (A), slc12a1 (B), Pax8 (C), atp1a1 (D), anti-Na^+/K^+ ATPase (A-ATPase)(E), 4A6 (F), and anti-phospho-histone 3 (A-PH3)(G). Pax8 and lhx1 are early markers of pronephric mesoderm, with expression detectable at stage 12.5 and peaking at approximately stage 25 (Carroll and Vize, 1999). Slc12a1 is a marker of mid-development pronephroi expressed solely in the distal tubules. Slc12a1 is detected first at stage 30 and remains until adulthood (Zhou and Vize, 2004). Atp1a1 is a marker of late pronephric epithelium detectable at stage 33 and remains highly expressed throughout development (Zhou and Vize, 2004). Anti-Na^+/K^+ ATPase is a monoclonal antibody (Millipore) targeting the protein coded for by atp1a1, and as such is detected slightly later, at stage 35. 4A6 is a monoclonal antibody targeting proteins found on the cell membranes of distal tubules and ducts of late-stage embryos, detectable at stage 40 and upwards (Vize *et al*., 1995). Anti-phospho-histone 3 is a monoclonal antibody marking cells in G2-M phase and is detectable at all stages (Saka and Smith, 2001). Images are of both colorimetric and fluorescent detection methods according to most commonly used protocols, although it is important to note that any of the included markers can be detected via both methods.

Table 2.1

Insert	Plasmid	Restriction Enzyme	Polymerase	Genbank Accession #
lhx1	pBluescript SK-	Xho1	T7	CO554633
pax8	pCMV-SPORT6	BamHI	T7	CF521632
slc12a1	pCMV-SPORT6	SmaI	T7	AI732841
atp1a1	pCMV-SPORT6	SmaI	T7	BG161352
nFrz8	pCS2+	NotI	SP6	-
DshD2	CS2	NotI	SP6	-
DshD4	CS2	NotI	SP6	-
memGFP	CS2+	NotI	SP6	-

Table 2.2

Marker	Cellular localization	Tissue localization	Temporal localization	Comments
lhx1	mRNA	Pronephros, neural ectoderm	Pronephros NF stg. 15 to adult	Expression lost in distal tubules at NF stg. ~32
pax8	mRNA	Pronephros, neural ectoderm	Pronephros NF stg. 12.5 to adult	
slc12a1	mRNA	Pronephros, distal tubules	NF stg.30 to adult	
atp1a12	mRNA	Pronephros, ear	Pronephros NF stg. 33 to adult	Expression highest in duct and distal tubules
α-Na/K ATPase	cell membrane	Pronephros, ear	Pronephros NF stg. 35 to adult	Monoclonal antibody
4A6	cell membrane	Pronephros, distal tubules and duct	NF stg. 39.5 to adult	Monoclonal antibody
α -PH3	nuclear	Whole organism (G2-M phase cells)	All stages	Rabbit polyclonal antibody

2.2 Enzymes

Enzymes utilized in this study include the restriction enzymes *Not*I, *Xho*I, *Bam*HI, and *Sma*I. All restriction enzymes are purchased from New England Biolabs. Transcription enzymes utilized include T7 (AM2082) and SP6 RNA polymerases, purchased from Ambion or as part of their respective kits as detailed. RNAse A is purchased from Sigma (R 5000). DNAse enzyme is purchased from Ambion (2238G2). RNAse inhibitor is used in some reactions and is purchased from Ambion (AM2682).

2.3 Antibodies

Antibodies against kidney proteins were used also. Antibodies used in this manner include the monoclonal duct marker 4A6 (Vize *et al*., 1995), as well as monoclonal antibodies targeting the kidney epithelial marker Na/K ATPase (Millipore 16-243). When GFP is used as a lineage tracer, signal was boosted using a rabbit anti-GFP antibody directly conjugated to the Alexa 647 fluor (Invitrogen A31852). Antibodies are visualized using either fluorescently labeled secondaries (donkey anti-mouse (DαM) Alexa 488 Molecular Probes cat. #A21202, DαM Alexa 546 Molecular Probes Cat. #A10036) or a tyramide amplification procedure (Vize *et al*., 2009). Cell proliferation in the kidney is investigated using rabbit IgG antibodies against phosphorlyated histone H3 (Upstate Biotech, Cat. #06-570) and detected with peroxidase-conjugated anti-rabbit IgG secondaries (Jackson Immunoresearch 111-035-144) as described in chapter 3, section 3.5. Antibodies against DNP and DIG are used in *in situ* hybridization. Both peroxidase and alkaline phosphatase linked antibodies are used in this technique. Anti-DNP-HRP antibodies are purchased from Perkin-Elmer (NEL747B001KT) and Anti-DNP-AP

antibodies are purchased from Mirus (MIR 6011). Anti-DIG-HRP and anti-DIG-AP antibodies are purchased from Roche (11 207 733 910, 11 093 274 910, respectively).

2.4 Kits

Kits are utilized for generation of DIG-labeled RNA probes, transcription of mRNA, purification of RNA, and isolation of plasmid DNA from microbial cultures. RNA purification (AM1908) and mRNA transcription kits (AM1340) are purchased from Ambion. Probe labeling kits are purchased from Roche (11 175 025 910). Plasmid isolation kits are purchased from Promega (A7640). Cytological nuclear counterstain kit (C-7590), including propidium iodide and SYTOX stains, are purchased from Molecular Probes.

2.5 Solutions and Buffers

All chemical components were purchased from Sigma, BDH, or Omni unless otherwise noted.

2.5.1 Embryo culture/microbial culture:

1X Marc's Modified Ringers (MMR)

0.1 M NaCl

2.0 mM KCl

1mM $MgSO_4$

2 mM $CaCl_2$

5 mM HEPES (pH 7.8)

0.1 mM EDTA

Human Chorionic Gonadotropin (HCG) Sigma, 9002-61-3

1 U/µL HCG in aqueous solution

1X Modified Barth's Saline (MBS)

88 mM NaCl

1 mM KCl

0.7 mM $CaCl_2$

1mM $MgSO_4$

5 mM HEPES (pH 7.8)

2.5 mM $NaHCO_3$

LB-Agar

10 mg/ml Tryptone

5 mg/ml yeast extract

10 mg/mL NaCl

15 mg/mL Agar

L-Broth

10 mg/ml Tryptone

5 mg/ml Yeast extract

0.5 mg/ml NaCl

2.5.2 Embryo fixation:

MEMFA

0.1 M MOPS (pH 7.4)

2 mM EDTA

1 mM $MgSO_4$

3.7% formaldehyde (w/v)

Bouin's Fix

10 mg/ml picric acid

9.25% formaldehyde (w/v)

5% glacial acetic acid (v/v)

2.5.3 Gel Electrophoresis and nucleotide handling:

Tris buffer with EDTA (TE buffer)

10 mM Tris (pH 7.5)

1 mM EDTA

1X TAE electrophoresis buffer

40 mM Tris-acetate (pH 8.3)

1 mM EDTA

1X TBE electrophoresis buffer

90 mM Tris-borate (pH 8.3)

2 mM EDTA

2.5.4 In situ hybridization and immunohistochemistry:

Tris-buffered saline with Tween20 (TBST)

12 mM Tris (pH 7.65)

152 mM NaCl

0.1 % Tween20 (v/v)

Triethanolamine

0.1 M Triethanolamine (pH 7.0-8.0) (Sigma T 1502)

Hybridization Buffer

50% formamide (v/v)

1 mg/ml Torula RNA (type IX, Sigma R 3629)

1x Denhart's solution

0.02% BSA (w/v)

0.02% polyvinylpyrrolidone

0.02% Ficoll 400

0.1% Tween 20 (v/v)

10 mM EDTA

5x SSC

20X sodium citrate buffer (SSC)

175.3 mg/ml NaCl

88.2 mg/ml sodium citrate (pH 7.0)

Maleic acid buffer (1X MAB)

100 mM maleic acid

150 mM NaCl

BM Block

2% BM block (Boehringer Mannheim 1096 176)

1X MAB

Goat Block

20% goat serum (v/v)

2 mg/ml BSA

TBST

Alkaline phosphatase buffer (AP buffer)

100 mM tris pH 9.5

50 mM $MgCl_2$

100 mM NaCl

0.1% Tween20 (v/v)

2 mM levamisol

0.2% TBTI

TBST

0.01 M Imidazole

0.2% BSA (w/v)

1% TBTI

TBST

0.01 M Imidazole

1% BSA (w/v)

Tyramide substrates

Tyramide substrates are prepared by combining 10 mg/ml tyramide in DMF-TEA (Dimethylformamide-triethylamine) with 10 mg/ml of N-hydroxysuccinimide ester (NHS ester) in dimethylformamide in the following ratios:

FITC: 4 ml NHS ester to 1.4 ml tyramide

Cy3: 1 ml NHS ester to 0.33 ml tyramide

The tyramide solutions are stored by adding an equal volume of 100% ethanol at -20°C

DNP Labelled ribonucleotides

100 mM C/G/A/U (Roche 11 277 057 001)

10 mM DNP-11-UTP (PerkinElmer 02118)

Transcription Buffer

10X commercial stock (Ambion 8151G)

2.5.5 Miscellaneous

Ficoll 400

2% (w/w) (Sigma 26873-85-8) in 0.2x MMR

Antibiotic/antimycotic

1% (v/v) (Sigma A 5955) in 0.2x MMR

Methyl salicylate

100% (Sigma M6752)

Chapter Three: Methods

3.1 Generating embryos

All experiments are carried out in *Xenopus laevis* embryos unless otherwise noted.

1. Female *Xenopus laevis* are injected subcutaneously with 600 µl of HCG and left at 18°C for ~15 hours.

2. Male *Xenopus* testes are harvested and placed in a solution of MBS containing 5% (v/v) goat serum and 700 µM $CaCl_2$ and stored at 4°C.

3. Eggs are harvested from females and fertilized with ¼-⅓ of a testicle, crushed in filtered water.

4. Fertilization is allowed to occur for 4 minutes, after which eggs are flooded with filtered water and allowed to divide (approximately 1h30)

5. Eggs are dejellied with a solution of 2% cysteine in 0.1x MMR, pH 7.9-8.0, until jelly coat is no longer visible and eggs are not sticky

3.2 Microinjections

For the purposes of the experiments described below, two major types of injection protocol are utilized. The first type, known as epidermal targeting, involves injection with a very fine needle of various reagents into a presumptive epidermal blastomere of a 4-8 cell embryo (Fig. 3.1). This technique allows secreted proteins to be expressed in the skin overlying the presumptive kidney, allowing for distribution onto the pronephros (Urban *et al*., 2006). The second major type is known as pronephric targeting. This type of injection is made in the same manner, however the injections are targeted to the dorsal

blastomere fated to become pronephric tissue (Fig. 3.1)(Wallingford *et al*., 1998). Both injection types are carried out unilaterally in order to utilize the uninjected side as a control.

All injections are carried out concurrently with either the fluorescent tracer FITC or mRNA encoding mem-GFP to mark injected cells. Concentration of mRNA in injection solutions is ~40ng/µl, with injection volumes of approximately 5-10 nl per embryo. With tracer taken into account (1:1 ratio of tracer to mRNA) total amount of mRNA per injection ranges from 0.1-0.3 ng. These experiments are typically performed on batches of 200 embryos, with approximately 50 from each batch being reserved and grown to late stage to confirm the functionality of the injections.

1. Dejellied embryos are transferred into a solution of 2% Ficoll 400 in 0.2x MMR for microinjections.

2. Needles are drawn from RNAse-free 3.5” Drummond capillary tubes using a needle puller (Narishige PC-10) and ground to sharpness with a needle grinder (Narishige EG-44).

3. Embryos are injected according to reagent (see above). Injections are carried out with a Nikon PLI-188 injection machine.

4. Injected embryos are rested at 13°C for 2 hours, then transferred into 0.2x MMR with 1% broad spectrum antibiotic/antimycotic

5. Embryos are incubated at 13°C until desired stage is reached. For stage 21/22, ~48 hours, stage 40/41, 1 week-10 days depending on conditions.

Fig 3.1 Microinjection sites

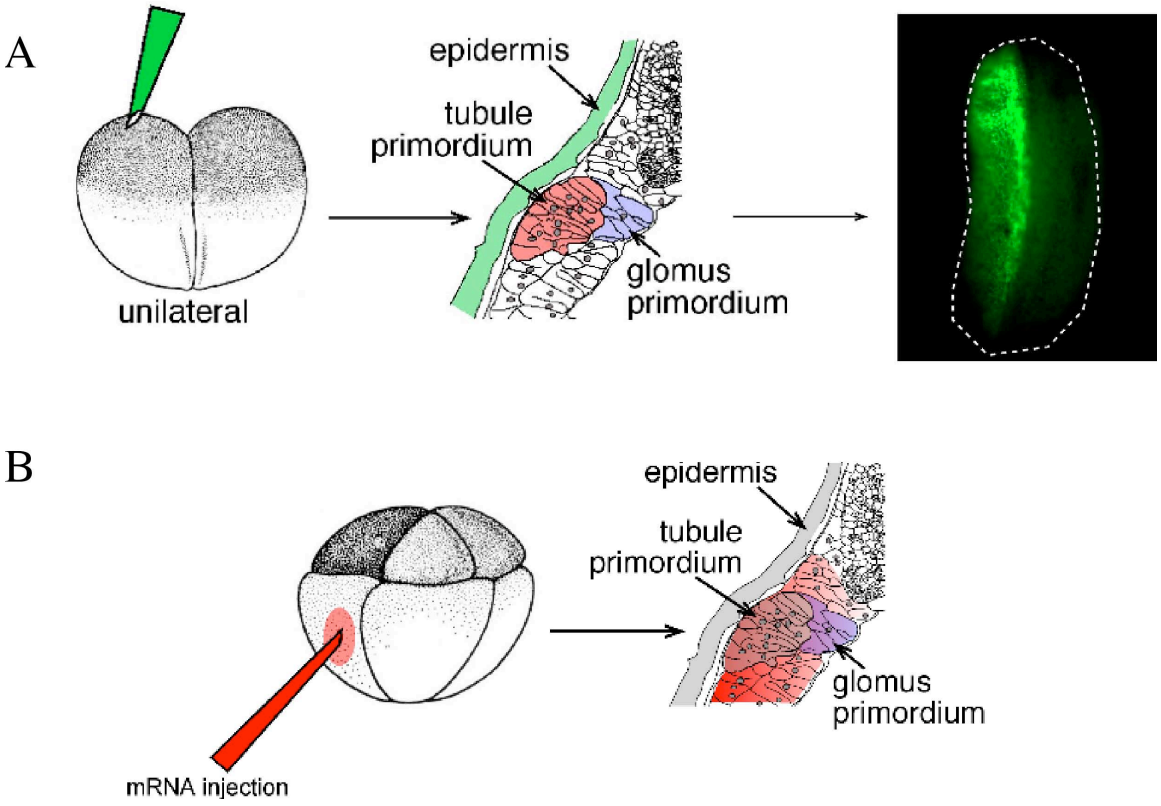

Fig. 3.1. Microinjection sites. Microinjections targeted to the epidermis (A) result in mRNAs being expressed in the skin overlying the kidney region, allowing soluble proteins to filter down onto the pronephros. Injections targeted to the pronephros itself (B) allow for expression within pronephric cells. Courtesy of P. Vize

6. Embryos are fixed at desired stage with MEMFA for 2 hours, then dehydrated in methanol and stored at -20°C.

3.3 Generation of RNA probes

Probes for kidney markers lhx1, pax8, slc12a1 and atp1a1 are utilized. Probes are labeled with uridines conjugated with either dinitrophenol (DNP) or digoxigenin (DIG).

3.3.1 Bacterial transformation and plasmid purification:

1. 50 µl DH5α compentent cells, 150 µl cold $CaCl_2$ and ~1 ng of circular plasmid DNA are combined in a 1ml eppendorf tube. Cells are incubated on ice for 30 minutes.

2. Tube is placed at 42° C for 2 minutes

3. Tube is replaced on ice for a further 5 minutes.

4. Cells are plated onto L-agar with 100 µg/ml ampicillin.

5. Plates are incubated at 37°C overnight.

6. Single colony from plate is selected and used to inoculate 100-125 ml of L-broth containing 100 µg/ml ampicillin. Broth is incubated overnight at 37°C.

7. Plasmids are purified from bacterial culture with Wizard plus Midipreps DNA purification system.

7.a. Cells are pelleted by centrifugation at 10,000 x g for 10 minutes. Supernatant is poured off and centrifuge tubes are blotted to remove excess liquid.

7.b. Cell pellet is resuspended in 3 ml of cell resuspension solution.

7.c. 3 ml of cell lysis solution is added and mixed by inversion.

7.d. 3 ml of neutralization solution is added and mixed by inversion.

7.e. Tubes are centrifuged at 14,000 x g for 15 minutes.

7.f. Supernatant is decanted to a new centrifuge tube.

7.g. 10 ml of DNA purification resin is added to the supernatant and mixed by swirling.

7.h. Resin/supernatant mixture is applied to a Midicolumn and a vacuum is applied to draw solution through the column.

7.i. 15 ml of column wash solution is added to the column and drawn through as per 7.h.

7.j. Step 7.i. is repeated

7.k. Resin is dried by applying vacuum for a further 30 seconds. Midicolumn filter is removed with a razor or suitable sharp object and transferred to a 1.5 ml eppendorf tube.

7.l. The filter is centrifuged for 2 minutes at 10,000 x g to remove residuum.

7.m. 300 µl of preheated TE buffer is added to midicolumn filter and incubated for 1 minute.

7.n. TE buffer is eluted by centrifugation at 10,000 x g for 20 seconds. Midicolumn filter is discarded.

8. DNA is stored at 4°C

3.3.2 Vector digestion

1. 2 µg of DNA (20 µl of 0.1 µg/µl), 5 µl enzyme buffer (New England Biolabs), 24 µl H_2O and 1 µl (10 U) digestion enzyme are combined in a 1.5 ml eppendorf tube.

2. Tube is incubate at recommended temperature for 2 hours (37°C or room temperature, depending on enzyme used)

3. 1 µl of tube mixture is applied to TAE-agarose electrophoresis gel to determine extent of digestion

4. Linearized plasmid is purified and concentrated through phenol-chloroform/ethanol precipitation

4.a. Volume of solution is adjusted to 200 µl with TE buffer (151 µl added)

4.b. 100 µl of phenol is added to solution and thoroughly mixed

4.c. 100 µl of chloroform is added to the solution and thoroughly mixed

4.d. Tube is centrifuged for 1 minute at 10,000 x g

4.e. Top phase of solution is transferred to new 1.5 ml eppendorf tube

4.f. 400 µl of 100% ethanol is added to solution and mixed

4.g. 60 µl of 3 M potassium acetate is added to solution and mixed. Incubate tube on ice for 5 minutes.

4.h. Tube is centrifuged at 10,000 x g for 5 minutes.

4.i. Supernatant is removed and replaced with 500 µl of 70% ethanol (v/v) and immediately centrifuged at 10,000 x g for 5 minutes.

4.j. Supernatant is removed and pellet is dried by vacuum centrifuge for 10 minutes.

4.k. Pellet is resuspended in 20 µl of RNAse free H_2O.

5. Purified template is stored at -20°C

3.3.3 Probe transcription and purification

1. 5 µl of 0.5 µg/µl DNA template, 2 µl 10x transcription buffer , 2 µl labeled nucleotide mixture, 1 µl RNAse inhibitor, 8 µl H_2O, and 2 µl (20 U) T7 RNA polymerase are combined in a 1.5 ml eppendorf tube and mixed thoroughly.

2. Reaction mixture is incubated at 37°C for 3 hours

3. 1 µl of reaction mixture is applied to a TBE-agarose electrophoresis gel to determine extent of transcription

4. 1 µl of DNAse is added to reaction mixture and incubated at 37°C for 15 minutes

5. Probe is purified using Ambion RNA purification kit.

5.a. Reaction mixture is brought to 100 µl with elution solution (80 µl added)

5.b. 350 µl of binding solution concentrate is added to the sample and mixed by pipetting.

5.c. 250 µl of 100% ethanol is added to the sample and mixed by pipetting.

5.d. Sample is applied to filter catridge, catridge is placed into a collection and elution Tube.

5.e. Filter/tube assembly is centrifuged for 1 minute at 10,000 x g. Eluate is discarded.

5.f. 500 µl wash solution is added to the filter. Filter/tube assembly is centrifuged for 1 minute at 10,000 x g.

5.g. Step 5.f. is repeated. Eluate is discarded.

5.h. Filter catridge is transferred to a fresh collection and elution Tube. 50 µl of elution solution is applied to the centre of the filter membrane. Filter/tube assembly is incubated at 70°C for 5 minutes.

5.i. RNA is eluted by centrifugation for 1 minute at 10,000 x g.

5.j. Steps 5.h. and 5.i. are repeated with the same elution tube.

6. Probe is stored at -80°C.

3.4 mRNA generation

1. Vector containing cDNA of interest are linearized and purified as described in section 3.3.2 above.

2. mRNA transcripts are generated using an Ambion capped RNA transcription kit.

2.a. 5µl of RNAse-free water, 10 µl of NTP/CAP solution, 2 µl of transcription buffer, 1 µg of linearized, purified DNA template and 2 µl of SP6 enzyme are combined in a 1.5 ml eppendorf tube and incubated at 37°C for 3 hours.

2.b. mRNA yield is approximated by applying 1µl of reaction mixture to a TBE-agarose electrophoresis gel.

2.c. DNA template is removed by adding 2-4 U of DNAse to the reaction mixture and incubating at 37°C for 15 minutes.

3. mRNA is purified by G50 sephadex column, using 10 aliquots of 200 µl TE buffer to elute mRNA.

4. Column fractions are assayed for the presence of mRNA by electrophoresis on a TBE-agarose gel.

5. Fractions not containing mRNA are discarded and mRNA containing fractions are concentrated via ethanol precipitation as outlined in section 3.3.2.

6. Concentrated mRNA is quantified by spectrophotometry (Nanodrop)

7. mRNA is stored at -20°C

3.5 *In situ* hybridization

In situ protocols are widely utilized to analyze both experimental and control samples. Fluorescent development is carried out through either use of a peroxidase

conjugated secondary antibody and a multi-channel tyramide amplification procedure or a chromogenic BM purple reaction (Vize, McCoy, and Zhou, 2009).

3.5.1 Probe Hybridization

1. Suitable embryos are selected and placed into RNAse free 4 ml glass vials.

2. Embryos are rehydrated in methanol series; washed in 75% methanol for 5 minutes with horizontal rocking.

3. Repeat step 2 with 50% methanol.

4. Repeat step 2 with 25% methanol.

5. Embryos are washed twice for 5 minutes in TBST with horizontal rocking.

6. Solution is replaced with 0.1 M triethanolamine and washed for 5 minutes with horizontal rocking.

7. Repeat step 6.

8. 12.5 µl acetic anhydride is added and embryos are incubated for 5 minutes.

9. Repeat step 8.

10. Embryos are washed for 5 minutes in TBST

11. All but 1 ml of TBST is removed from the vial and 250 µl of hybridization buffer is gently added. The buffer layer is allowed to filter down over the embryos.

12. TBST and buffer are removed from the vial and replaced with another 250 µl aliquot of hybridization buffer. Tubes are incubated vertically at 60°C with gentle rocking for 10 minutes.

13. Hybridization buffer is removed and replaced with fresh buffer. Tubes are incubated vertically at 60°C with gentle rocking for 4-6 hours.

14. Hybridization buffer is removed and replaced with fresh buffer containing ~0.5 µg/ml of each desired antisense RNA probe. Up to two probes can be hybridized, one DNP labeled, one DIG labeled. Probes are hybridized overnight at 60°C with gentle rocking.

15. Solution containing probe is removed and embryos are incubated vertically for 10 minutes in 250 µl of fresh hybridization buffer at 60°C with gentle rocking.

16. Embryos are washed three times in 2x SSC for 20 minutes at 60°C with horizontal rocking.

17. Wash solution is replaced with 200 µl of 2x SSC containing 20 µg.ml RNAse A and incubated vertically for 30 minutes at 37°C.

18. Excess RNAse is removed with a ten minute wash with 2x SSC at room temperature with horizontal rocking.

19. Embryos are washed twice with 0.2x SSC at 60°C with horizontal rocking.

20. Embryos are washed twice for a total of 10 minutes with MAB at room temperature with horizontal rocking.

3.5.2 Antibody Incubation

1. Embryos are blocked for 1 hour with 1 ml of MAB containing 2% BM blocking agent .

2. Blocking solution is replaced with a solution of MAB with 2% BM block containing either a 1/100 (for HRP-conjugated antibodies) or 1/1000 (for AP-conjugated antibodies) dilution of α-DNP or α-DIG antibodies, depending on nature of probe. Antibodies are incubated overnight at 4°C.

3. Excess antibody is removed by multiple washes with MAB followed by at least one wash with TBST for a combined total of 3 hours at room temperature.

4. Transcripts are detected by development with various substrates

4.a. Chromogenic Reaction

4.a.1. Embryos are washed twice with alkaline phosphatase buffer for a total of 10 minutes.

4.a.2. AP buffer is replaced with 1 ml BM purple (Boehringer Mannheim 1442 074) and incubated at room temperature until staining is apparent.

4.a.3. Embryos are rinsed with TBST and then fixed for 2 hours with Bouin's Fix.

4.a.4. Bouin's fix is washed away with multiple washes in 75% methanol (until wash is no longer yellow)

4.a.5. Samples are photobleached with a solution of 30% H_2O_2 and 70% methanol overnight on a bleaching lamp at room temperature.

4.b. Fluorescent Reaction

4.b.1. Embryos treated with HRP antibodies are washed in a solution of 0.2 % TBTI for 30 minutes at room temperature.

4.b.2. Replace 0.2% TBTI with a solution of 1% TBTI and a 1/100 dilution of tyramide substrate (FITC or Cy3 conjugated) and incubate for 30 minutes at room temperature

4.b.3. The reaction is brought to 0.001% H_2O_2 by serial dilution of a 30% stock solution and incubated for a further 30 minutes at room temperature.

4.b.4. Fluorescent reaction is stopped by washing samples in TBST twice for 10 minutes at room temperature, then overnight at 4°C.

4.b.5. For second channel fluorescence, the first HRP antibody is deactivated to prevent cross-reaction by a one hour incubation with 3% H_2O_2, then washed 3 times for 15 minutes at room temperature in TBST.

4.b.6. Second antibody is applied using the same protocol as outlined in the antibody incubation section as above using an antibody against the complementary probe. Conversely, immunohistochemistry can be utilized as outlined below rather than a second RNA probe. In either case, a second tyramide procedure is followed as in section 4.b.

5. Embryos are transferred into a solutions of TBST with 0.01 M EDTA and stored at 4°C.

3.6 Immunohistochemistry

Immunohistochemistry utilizing fluorescent substrates is widely utilized in this investigation, and is functional in conjunction with *in situ* stains of the kidney (Fig. 3.2). A procedure has been developed that will result in embryos fluorescently labeled both for kidney cells and mitotic cells, and can be easily analyzed through confocal microscopy (Fig. 3.3). To date, this technique is unpublished in *Xenopus* and represents a novel technology for visualization of tissue-specific sub-cellular markers.

1. Suitable embryos are selected and placed into 4 ml glass vials.

2. Embryos are rehydrated in methanol series; washed in 75% methanol for 5 minutes with horizontal rocking.

3. Repeat step 2 with 50% methanol.

4. Repeat step 2 with 25% methanol.

5. Embryos are washed twice for 5 minutes in TBST with horizontal rocking.

6. Embryos are blocked for 1 hour in goat block.

Fig. 3.2 Tissue-specific proliferation assay

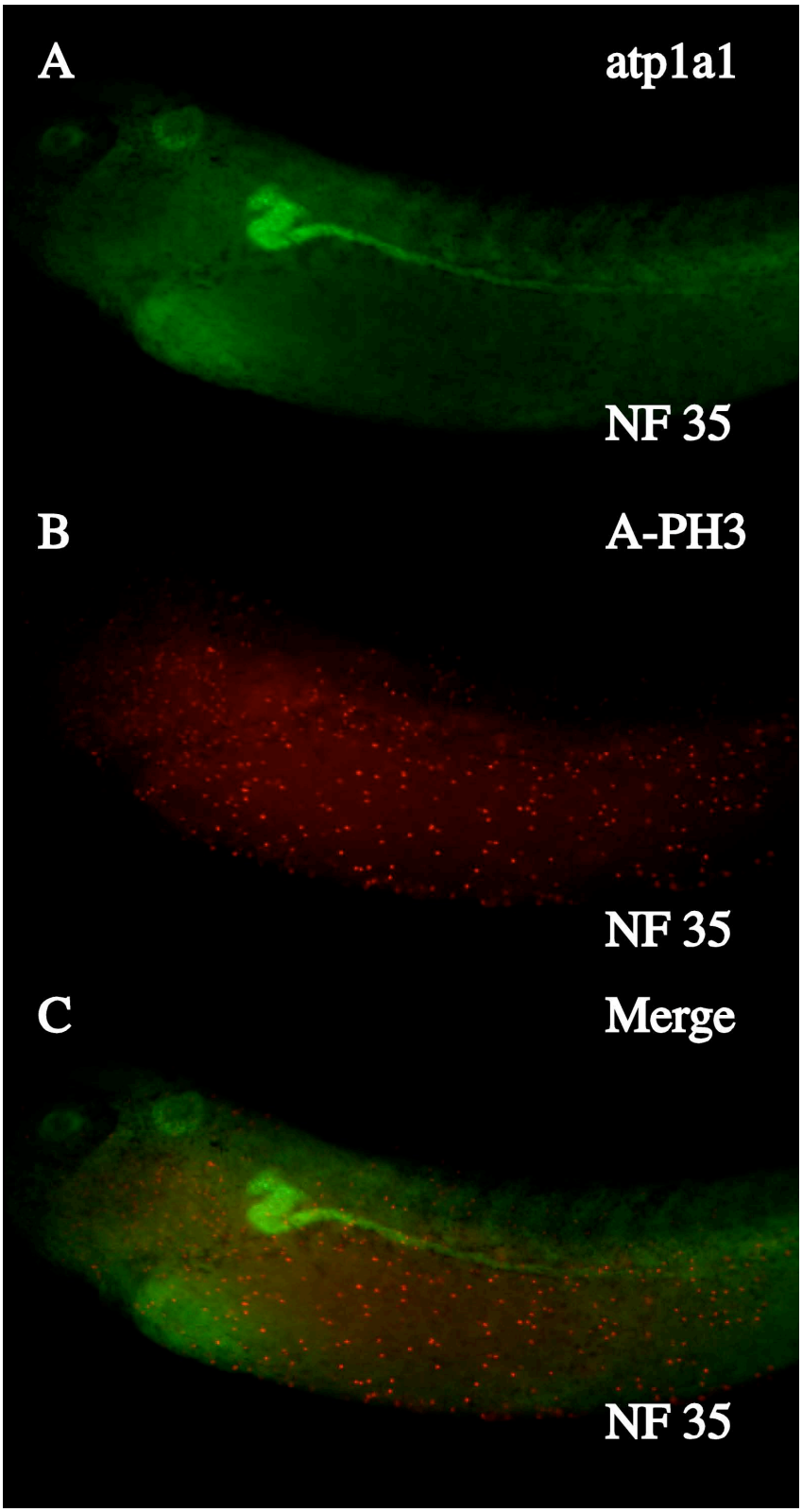

Fig. 3.2 Tissue-specific proliferation assay. An example of fluorescent *in situ* hybridization (atp1a1) (A) combined with a novel form of fluorescent immunohistochemistry (A-PH3) (B). When both channels are merged (C), this technique allows for accurate counts of proliferative cells to be determined within a specific tissue or organ. This technique can be carried out with virtually any tissue marker, *in situ* or otherwise.

Fig. 3.3 Tissue specific proliferation assay viewed via confocal microscopy

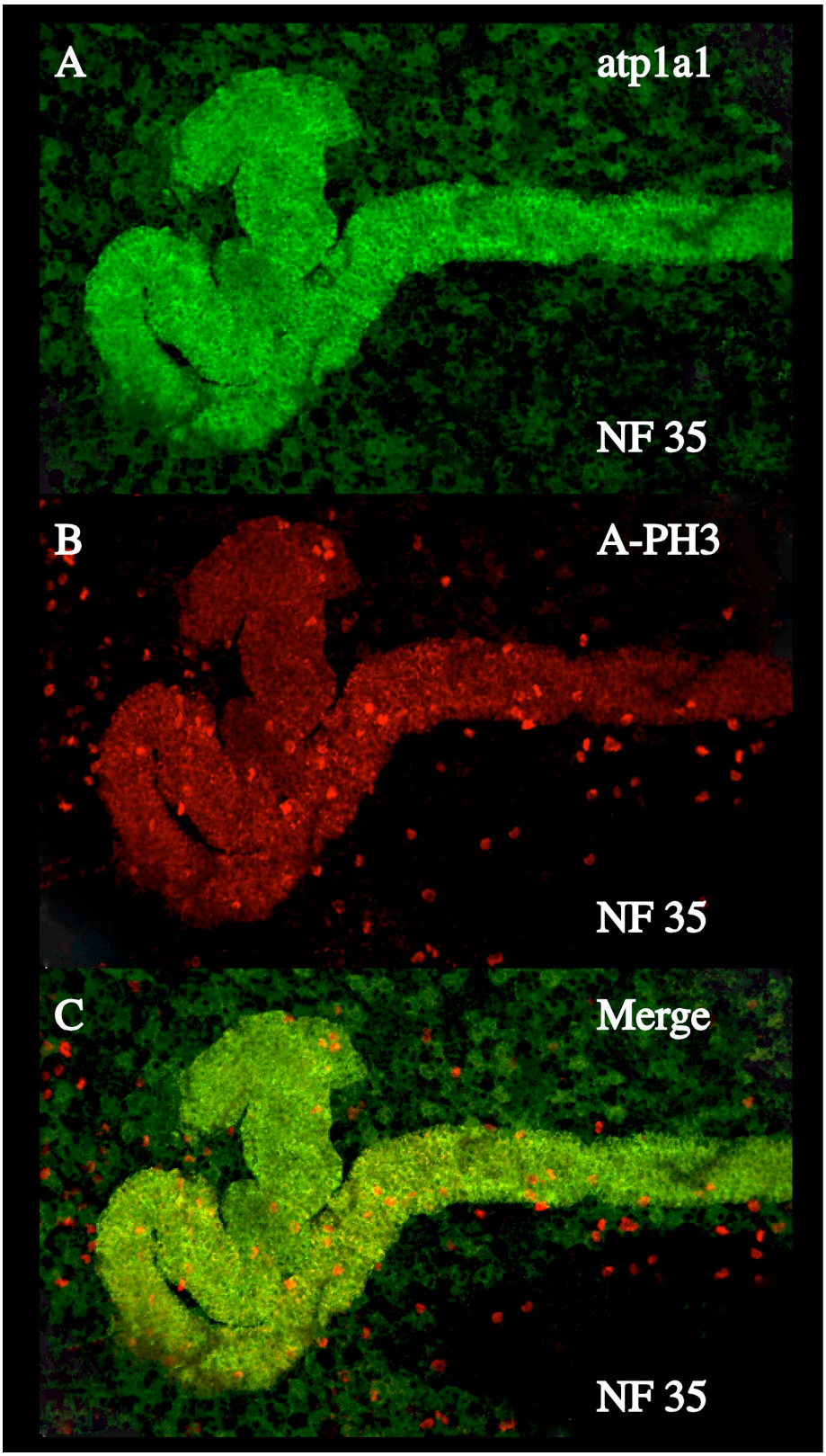

Fig. 3.3 Tissue specific proliferation assay viewed via confocal microscopy. *In situ* stain for atp1a1 in green (A), immunohistochemistry for anti-phospo-H3 in red (B). Confocal imaging of samples labelled for both tissue specific markers and proliferative cell markers allows for fine distinction between proliferative cells that are in the plane of the tissue in question or displaced in the z-axis (C).

7. Goat block is replaced with goat block containing primary antibody (4A6: ¼ dilution, α-Na/K ATPase 1/200 dilution, α-PH3 1/500 dilution, α-GFP 1/100 dilution) and incubated overnight at 4°C.

8. Excess antibody is removed by multiple washes in TBST with a combined duration of 3 hours. As α-GFP tracer antibody is directly conjugated to the Alexa 647 fluor, no further steps are required. For all other antibodies, the protocol is continued.

9. Step 6 is repeated.

10. Goat block is replaced with goat block containing donkey anti-mouse IgG coupled to HRP (1/200 dilution), or in some cases, rabbit anti-mouse IgG coupled to Alexa fluors 488 or 546 (4A6 only, 1/100 dilution) and incubated overnight at 4°C.

11. Step 8 is repeated.

12. In the case of HRP conjugated secondary antibodies, a tyramide development procedure is then carried out identical to that described in step 4.b of section 3.5.2. In the case of direct fluorescence (Alexa fluor secondaries), no development step is required.

3.7 Confocal microscopy

Samples prepared for confocal microscopy, except where noted, are dehydrated in methanol series and cleared with methyl salicylate prior to observation. Those samples not being labeled with a proliferative marker were counterstained with either SYTOX green or propidium iodide at 1% concentration to delineate nuclei. The confocal system used is a Leica model DMIRE2 scope with a Leica TCS SL scanner and lasers.

Chapter Four: Results

4.1 Manipulating wnt signals results in altered kidney morphology

To investigate the possibility of either canonical or non-canonical wnt signals being important in kidney formation, a broad-spectrum approach was taken to down-regulate wnt signals in the kidney through the use of an injected mRNA. This mRNA codes for the n-terminal region of the pronephric wnt receptor frz8 (nFrz8), and contains a deletion of the trans-membrane and c-terminus domains. When translated, this produces a soluble, non-membrane bound frz8 that is exported from the cell and proceeds to bind to and sequester wnt molecules in the ECM, preventing activation of endogenous frz molecules (Deardorff *et al*., 1998). Frz8 is known to bind to at least three different molecules, wnt1, wnt5 and wnt11, and the soluble version likely adheres to several more (Papadopoulou and Edlund, 2005). This makes nFrz8 an excellent molecule for down-regulating wnt signalling globally, and, as it is microinjected as described above, allows it to be targeted to the epidermis adjacent to the developing kidney in order to be diffused onto the kidney primordia itself.

The resultant embryos displayed two related phenotypes. The first was evident at approximately at NF stage 25, approximately 27 hours post fertilization. At this stage, kidney cells have been specified and kidney morphogenesis is just beginning to occur (Nieuwkoop and Faber, 1994, Vize *et al*., 1997). It is important to remember that injected mRNA has only a short half-life of approximately 24 hours in living *Xenopus* (Vize *et al.*, 1991), and as such the effect of any injections is likely occurring early in development. Embryos injected with this reagent display an enlarged kidney with a

peculiar pattern of punctuate cells along the length of the pronephros. The phenotype suggests a lack of cell adhesion or motility, or an increase in proliferation, and is unique among phenotypes resulting from injections described to date (Fig. 4.1). This phenotype was observed in approximately 10% of injected embryos (N >100)(Table 4.1). Later stage embryos, at NF stage 38-40, when kidneys are functional, showed similarly enlarged tubules, with a small proportion of the samples displaying a striking wavy or sinuous kidney, possibly resulting from the disruption of kidney morphogenesis earlier in development (Fig. 4.2). Again, this phenotype is occurring well after the effects of the injected mRNA have ceased and as such, is a result of an earlier perturbation. This later phenotype was present in a reduced fraction of injected samples, being ~4% penetrant (N >100) (Table 4.1). Despite the low percentage of affected embryos, absolutely no control embryos displayed a related phenotype and furthermore this late stage phenotype is unique among samples injected with mRNAs and bore further investigation.

4.2 Injections of Dvl-PDZ recapitulate changes in morphology

As NFrz8 down-regulates many wnt signals concurrently, an effort was made to determine which pathway was responsible for the phenotype observed. One method of determining this is by using a number of clones of mutant dvl2 which act as dominant-negatives for each of the known pathways (Rothbacher *et al*., 2000). These mRNAs code for an intracellular protein, and are therefore targeted into the kidney region itself, rather than the overlying ectoderm. These clones act by possessing specific deletions of domains regulating each wnt pathway, and upon injection, the number of inactive dvl

Fig. 4.1 Early stage phenotype resulting from injections of nFrz8

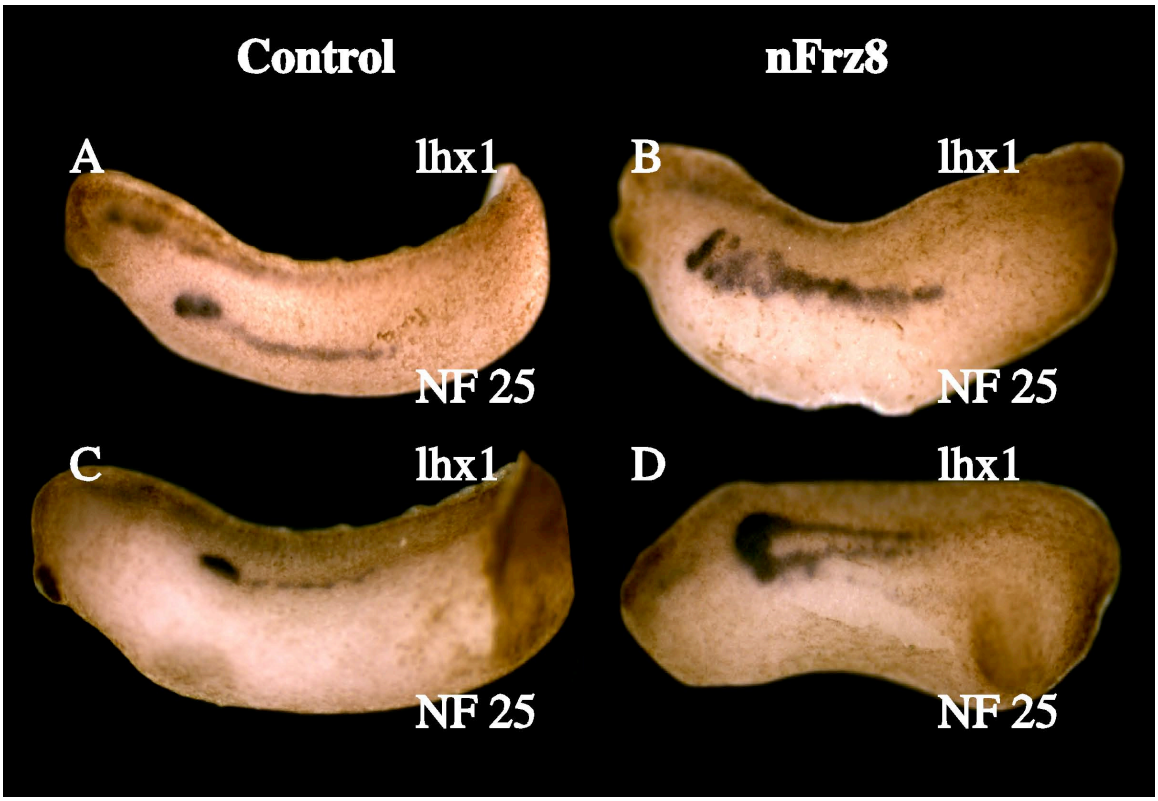

Fig. 4.1 Early stage phenotype resulting from injections of nFrz8. Control samples in A and C, injected samples in B and D. All sample are assayed for pronephric tissue with the marker lhx1 at stage 25. Samples injected with nFrz8 displayed an enlarged and discontinuous kidney in comparison to controls.

Fig. 4.2 Late stage phenotype resulting from nFrz8 injections

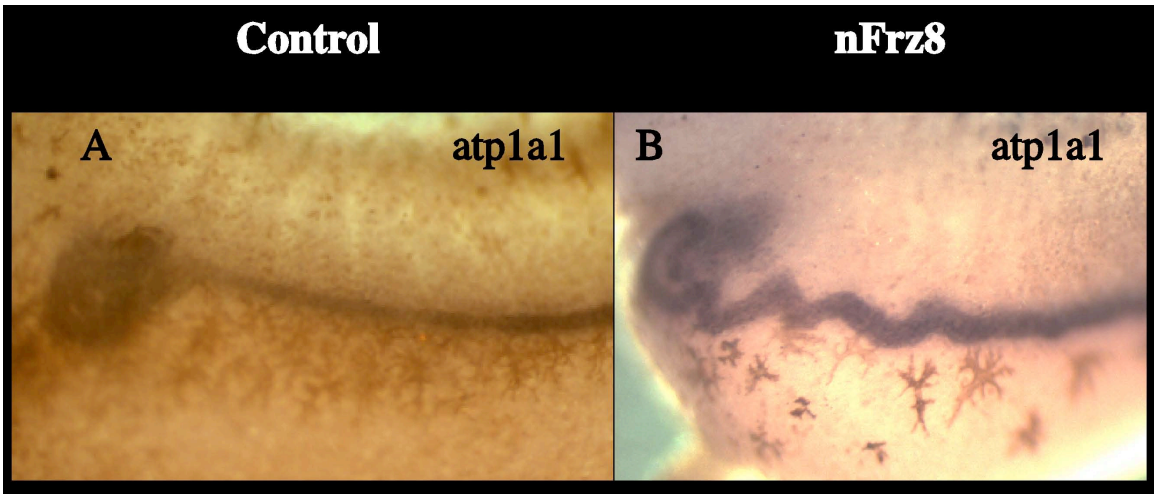

Fig. 4.2 Late stage phenotype resulting from nFrz8 injections. Embryos are stained via *in situ* for the kidney marker atp1a1. Samples injected with nFrz8 (B) displayed enlarged and sinuate kidneys. Control pronephroi (A) are characteristically smooth and straight.

Table 4.1

Stage examined	# embryos examined	# phenotypes	% penetrance
Control NF 25	50	0	0
nFrz8 NF 25	112	12	10.7%
Control NF 28	25	0	0
Dvl-PDZ NF 28	207	41	19.8%
Control NF 40	50	0	0
nFrz8 NF 40	103	4	3.9%
Dvl-PDZ NF 40	201	20	9.9%

molecules act to out-compete the endogenous functional dvl, again down-regulating wnt activity at the intracellular level. Those mutants that mimic the nFrz8 phenotype can be assumed to knockdown the pathway responsible for the original phenotype. This experiment was carried out with two specific clones of dvl2, DshD2 and DshD4 (Fig 4.3). These mRNAs code for a dvl molecule lacking all or part of the PDZ domain of the protein (Dvl-PDZ), interfering with the ability of the protein to bind with factors specific to the PCP pathway.

Both DshD2 and DshD4 created a phenotype similar, but not identical, to that seen in the nFrz8 experiments. While both clones did produce phenotypes, DshD2 produced affected samples at a higher rate and as such was utilized primarily in further experiments. Again, at earlier stages, an enlarged kidney was seen in a large fraction of injected samples, with nearly 20% of samples affected (N >200)(Table 4.1). However, unlike the nFrz8 injections, the lack of cell cohesion observed was not present when injected with Dvl-PDZ (Fig. 4.4). Despite these incongruities, later stage embryos displayed a concertina phenotype indistinguishable from that produced from nFrz8 injections in multiple independent rounds of microinjection. More specifically, approximately 10% of injected embryos assayed at stage 41 displayed kidneys ducts noticeably larger than controls, with wavy ducts when assayed through immunohistochemistry (N >200)(Fig. 4.5, Table 4.1). These samples were further examined through confocal microscopy and affected ducts were determined to be, on average, over 150% the diameter of uninjected ducts, with wild-type ducts averaging 50 microns (8 samples) versus injected, affected ducts averaging 78 microns (5 samples, multiple areas measured) (Fig. 4.6, Table 4.2). Furthermore, affected ducts were counted

to contain twice as many cells in cross-section as uninjected ducts (Fig. 4.7, 4.8 and Table 4.2)

Fig. 4.3 Deletion constructs of Dvl2

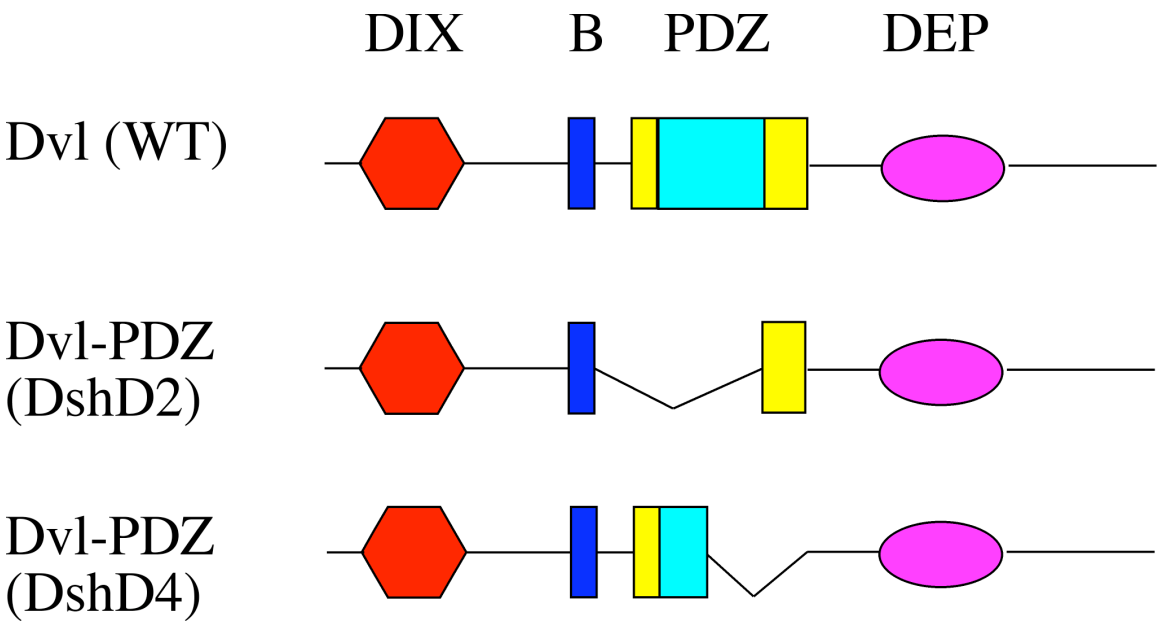

Fig. 4.3 Deletion constructs of Dvl2. Deletions of the PDZ domain of Dvl2 limit the ability of Dvl to associate with PCP-specific signalling components. The other functional domains of the protein (DIX, B, DEP) are left unchanged. DshD2 and DshD4 constructs differ in the extent and location of the PDZ deletion, as shown, with DshD2 missing residues 707 through 990, and DshD4 missing residues 894 through 1134 (Rothebacher *et al.,* 2000).

Fig. 4.4 Early phenotype resulting from injections of Dvl-PDZ

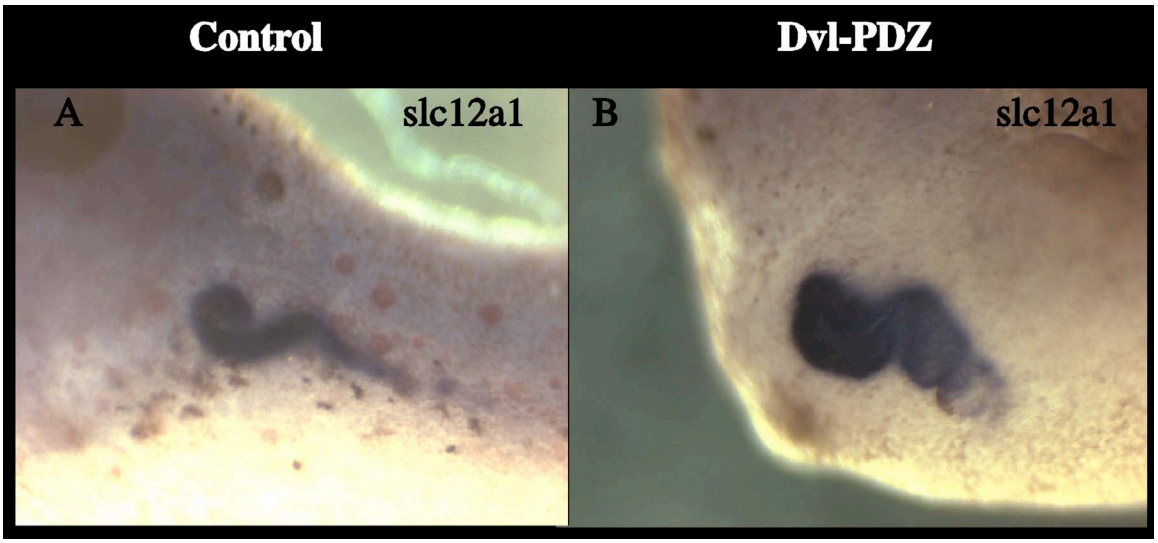

Fig. 4.4 Early phenotype resulting from injections of Dvl-PDZ. Embryos are stained via *in situ* for the distal tubule marker slc12a1. Embryos injected with Dvl-PDZ (B) display enlarged kidney tubules compared with controls (A), however without the apparent lack of cell adhesion seen in nFrz8 injected embryos. Enlargement is most prevalent in the most caudal section of the distal tubule. Samples are NF stage 32. Image in B is a contralateral view of the same sample as A.

Fig. 4.5 Confocal images of late stage phenotypes resulting from Dvl-PDZ injection

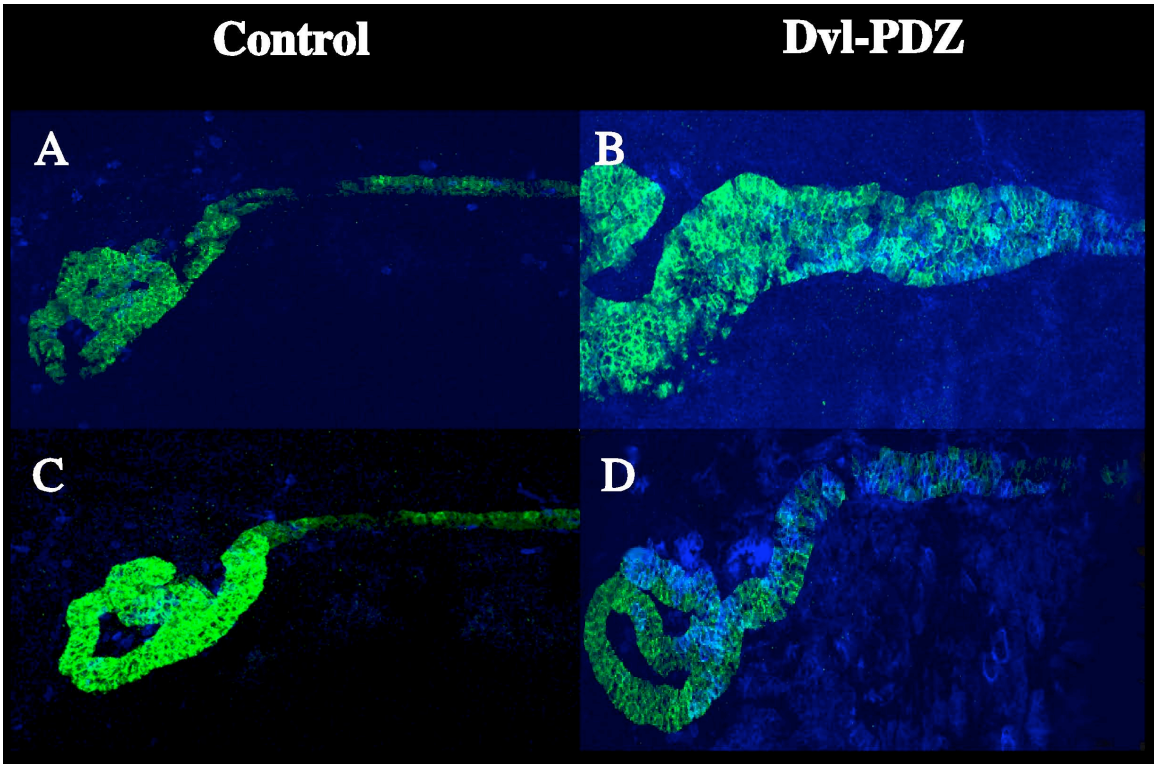

Fig. 4.5 Confocal images of late stage phenotypes resulting from Dvl-PDZ injection. All photos are maximal projections of confocal Z-stacks. Kidney cells are marked in green by the monoclonal antibody 4A6. Dvl-PDZ injections are traced with memGFP and boosted with anti-GFP polyclonal antibodies, in blue. Injected samples (B, D) show enlarged distal tubules and ducts in comparison with controls (A, C). B and D illustrate the range of phenotypes exhibited, with B exemplifying the most severe, D the least. Samples are NF stage 41. Images B and D are contralateral views of the same samples as A and C, respectively.

Fig. 4.6 Confocal Micrographs of Dvl-PDZ injected ducts

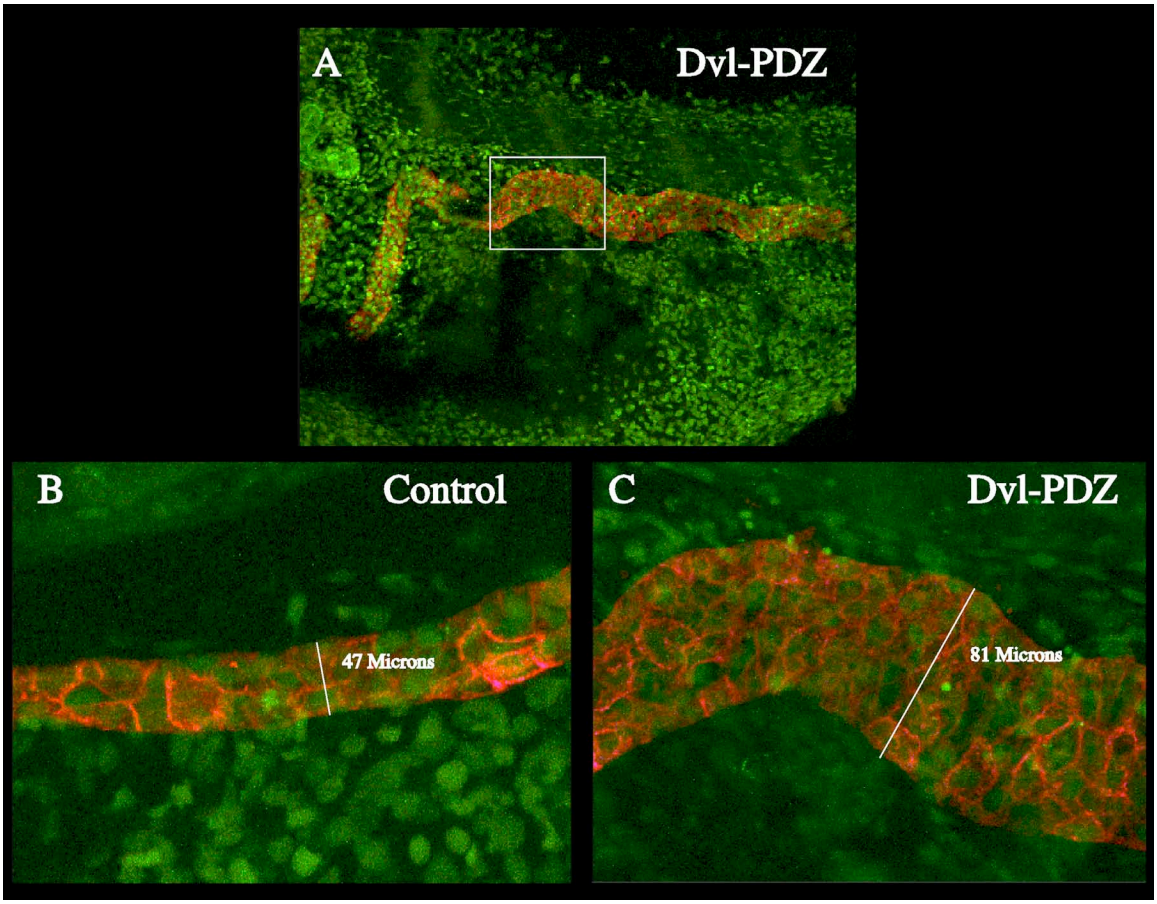

Fig. 4.6 . Confocal Micrographs of Dvl-PDZ injected ducts. Ducts are stained with 4A6 antibody (red) and counterstained with the nuclear marker sytox green. All photos are maximal projections of confocal Z-stacks. A and C illustrate ducts injected with Dvl-PDZ, B is an uninjected control. Image in C is a close up of the indicated region in A. Injected ducts displaying altered morphology are, on average, ~50% larger than control ducts. Samples are NF stage 40.

Fig. 4.7 Optical transverse section of Dvl-PDZ injected kidney ducts

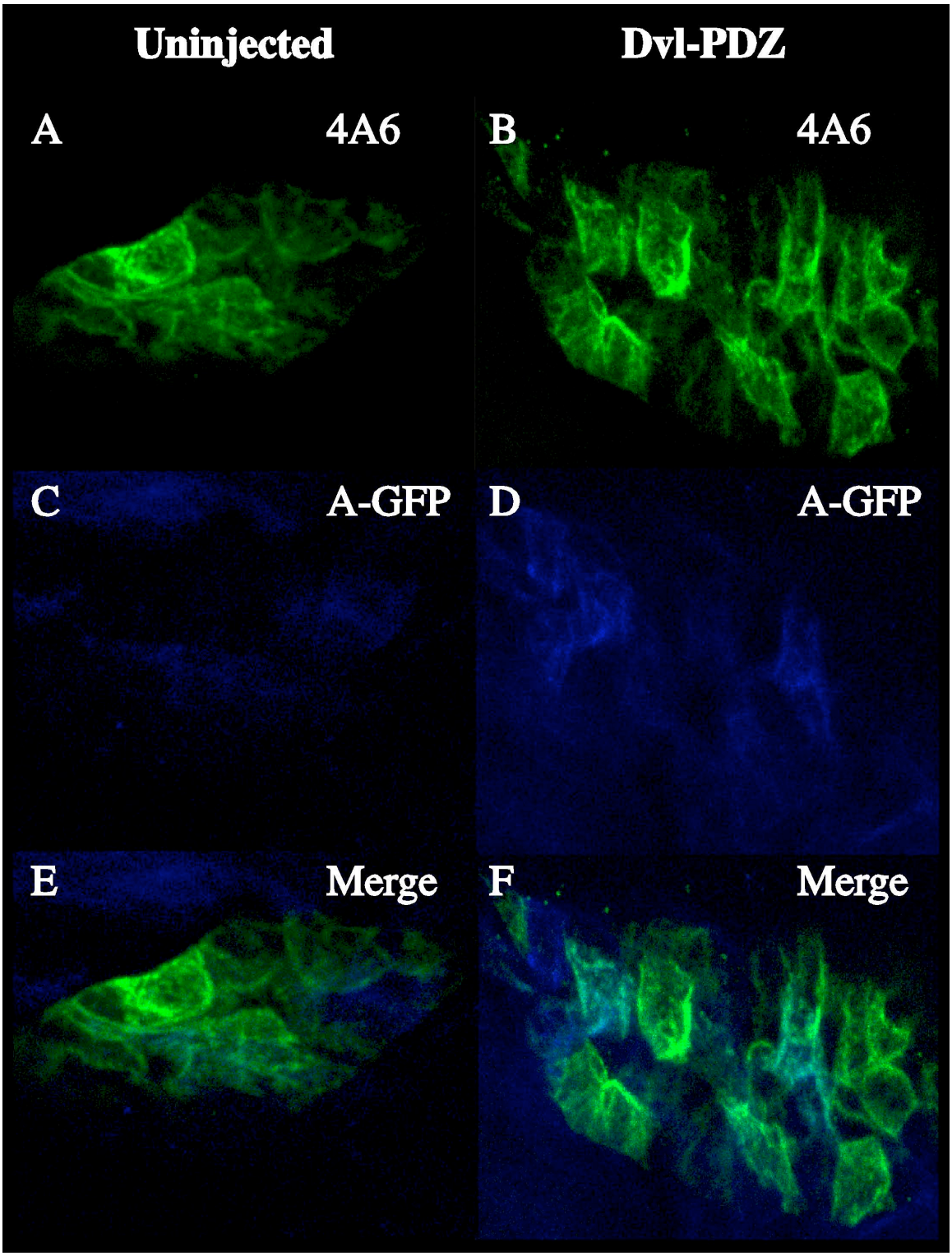

Fig. 4.7 Optical transverse section of Dvl-PDZ injected kidney ducts. Injected ducts (B, D, F) are determined by the presence of memGFP detected via polyclonal A-GFP antibodies. Ducts injected with Dvl-PDZ possess more cells than uninjected ducts (A, C, E) and are lacking a well-defined lumen. Sample is NF stage 40. Both columns display contralateral views of the same sample.

Fig. 4.8 Further optical transverse sections of Dvl-PDZ injected kidney ducts

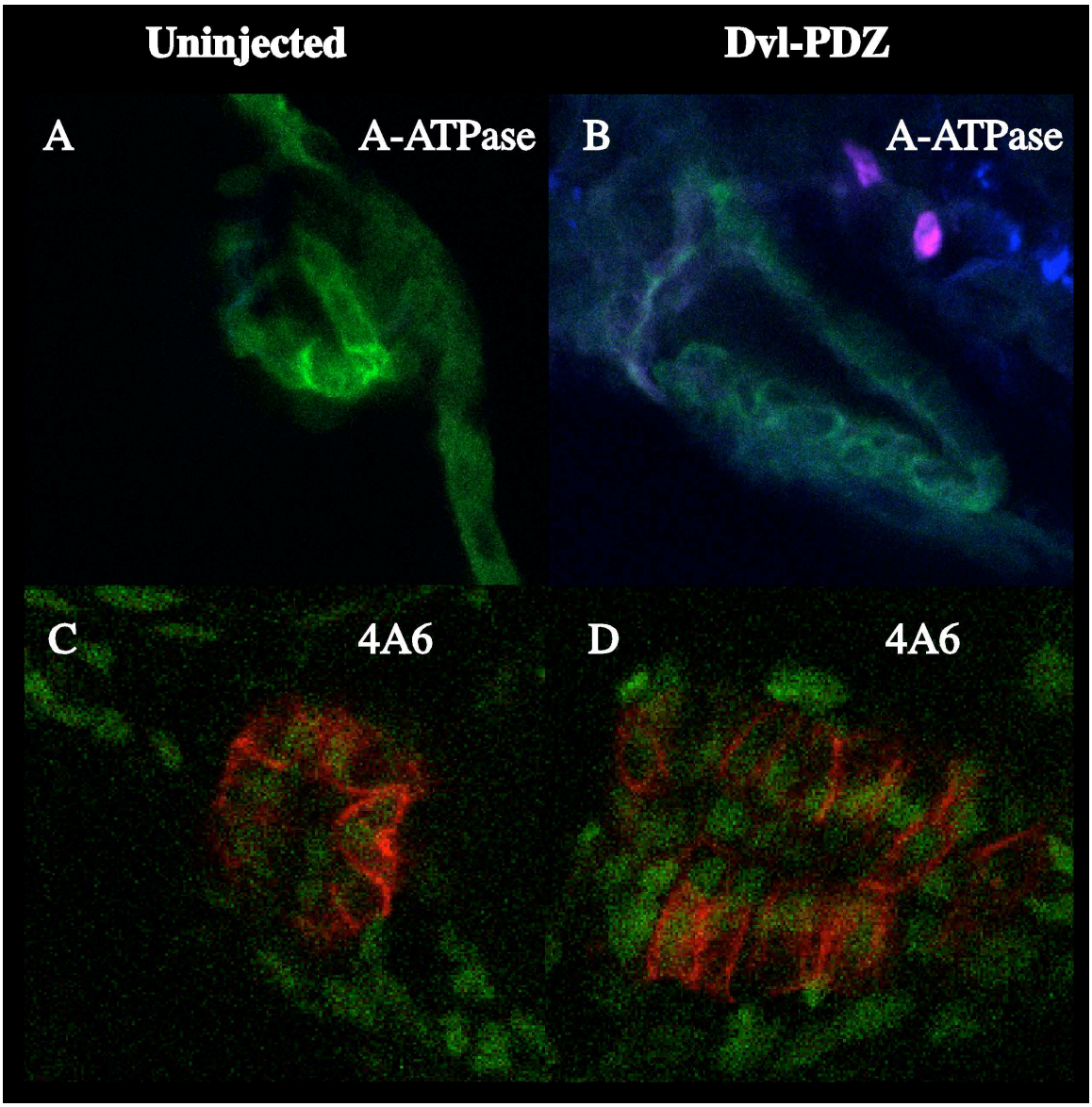

Fig 4.8 Further optical transverse sections of Dvl-PDZ injected kidney ducts. Top row (A, B) exhibits a single sample marked with anti-ATPase antibodies in green as well as injection tracer in blue. Bottom row (C, D) exhibits a sample marked with 4A6 antibodies in red, and the nuclear marker sytox green. Note the marked increase in duct diameter and cell number in both injected samples. All samples are NF stage 40. Images in panels B and D are contralateral views of the same samples as A and C, respectively.

Table 4.2

Sample #	Diameter (μm) (Control)	Diameter (μm) (Dvl-PDZ)	# cells in cross section (control)	# cells in cross section (Dvl-PDZ)
1	47	81	9	15
2	52	77	9	18
3	51	75	9	23
4	50	79		
5	48	78		
6	50			
7	49			
8	53			
Mean	50	78	9	18.7

4.3 Dvl-PDZ acts to repress cell proliferation

As both injected dominant negative dvl mRNAs have been shown to down-regulate the PCP pathway (Rothbacher *et al*., 2000, Wallingford and Habas, 2005), it is likely that inhibition of planar cell polarity signalling leads to the enlarged kidney. As it is clear that abrogation of the PCP pathway results in larger cell numbers in the kidney, it is reasonable to hypothesize that planar cell polarity signalling acts, in wildtype embryos, to inhibit cell division within the kidney. To examine this possibility, embryos injected with Dvl-PDZ were assayed for cell division by staining with antibodies targeting phosphorylated histone 3 (α-PH3)(Saka and Smith, 2001). These samples, when counterstained for kidney tissues via *in situ* hybridization and examined through confocal microscopy, allow for accurate counts of phospho-H3 positive cells specifically within the kidney primordium.

This technique was utilized exclusively on stage ~21/22 embryos, as these allow for the closest temporal correlation between the activity of the mRNA and the ability to detect kidney progenitor cells via lhx1and pax8 probes. Embryos were scored as injected or uninjected based upon the presence of a GFP tracer co-injected with the Dvl-PDZ (Fig. 4.9). Subsets of injected samples were sequestered and grown to late stage to be assayed for the concertina kidney phenotype as a positive control. Dvl-PDZ was found to significantly increase the number of proliferative cells by nearly 60% ($p<0.05$) in injected kidneys (Table 4.3)(Fig 4.10)(Fig 4.11), with late stage positive controls showing rates of enlarged kidneys similar to that previously determined when assayed with *in situ* hybridization (~10%, N>50).

Fig. 4.9 Confocal multi-channel fluorescent tissue-specific proliferation assay on Dvl-PDZ injected samples

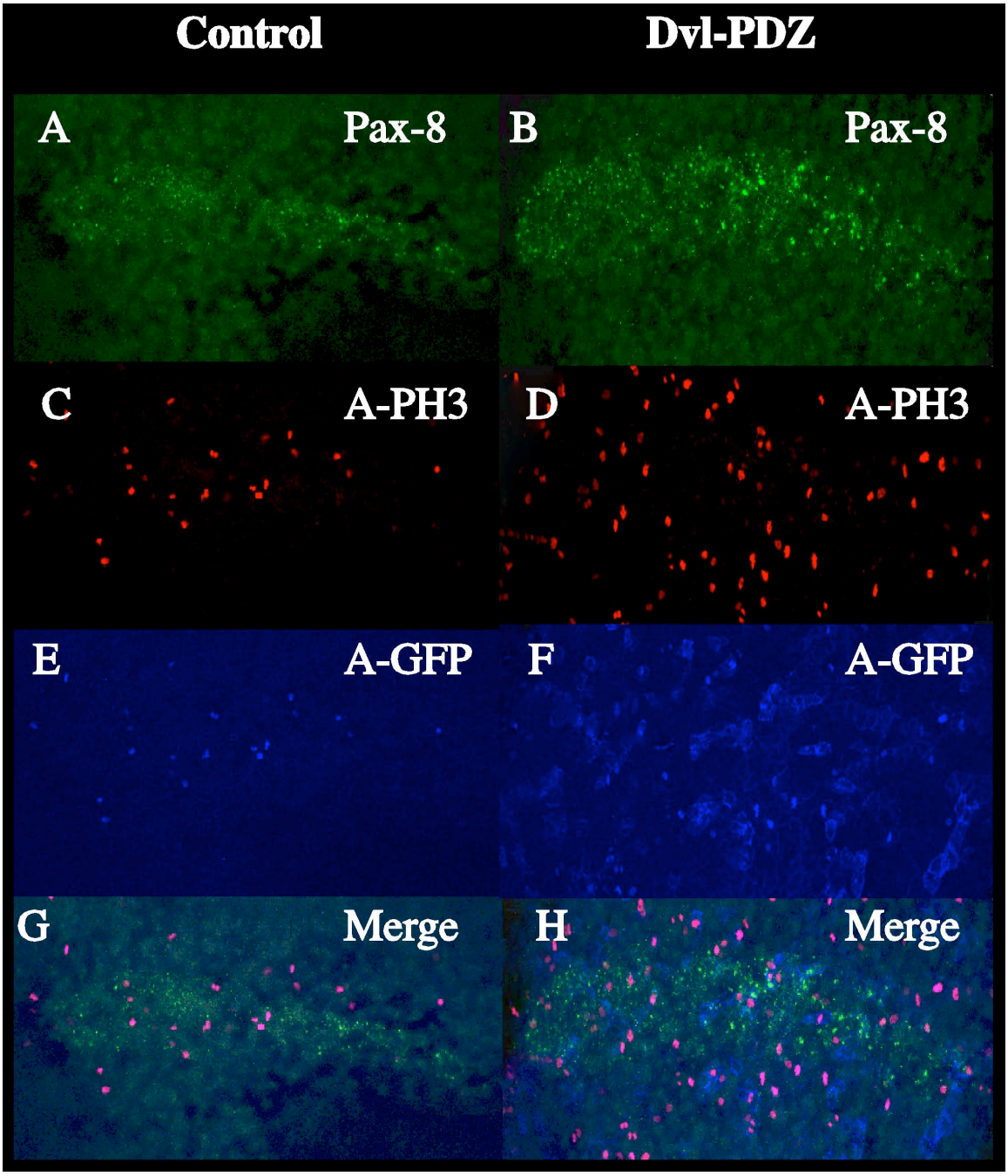

Fig. 4.9 Confocal multi-channel fluorescent tissue-specific proliferation assay on Dvl-PDZ injected samples. Kidney cells are marked via *in situ* against the pax8 marker in green. Proliferative nuclei are marked with A-PH3 in red. Dvl-PDZ injected side (B, D, F, H) is labelled with memGFP boosted by anti-GFP antibodies in blue. Images are maximal projections of z-stacks, and as such, A-PH3 positive cells which exist outside of the plane of the kidney have been removed for accurate kidney-specific counts of cells. Sample is at NF stage 21/22. Both columns display contralateral views of the same sample.

Table 4.3

DshD2 injected samples	# A-PH3 + nuclei (Injected side)	# A-PH3 + nuclei (Uninjected side)	Control side 1	Control side 2
1	0	3	9	10
2	23	19	12	9
3	18	20	4	8
4	35	28	14	15
5	6	1	15	17
6	32	12	12	14
7	14	11		
8	37	29		
9	23	8		
10	14	2		
11	21	14		
12	23	11		
13	14	6		
14	15	9		
Mean	19.64	12.36	11	12.167
Standard deviation	10.44	8.880	4	3.656
Standard error	0.74578	0.6343	0.6667	0.6093
p-value, paired	0.000347		0.1361	

Fig. 4.10 Cell proliferation in stage 21/22 Dvl-PDZ injected pronephroi

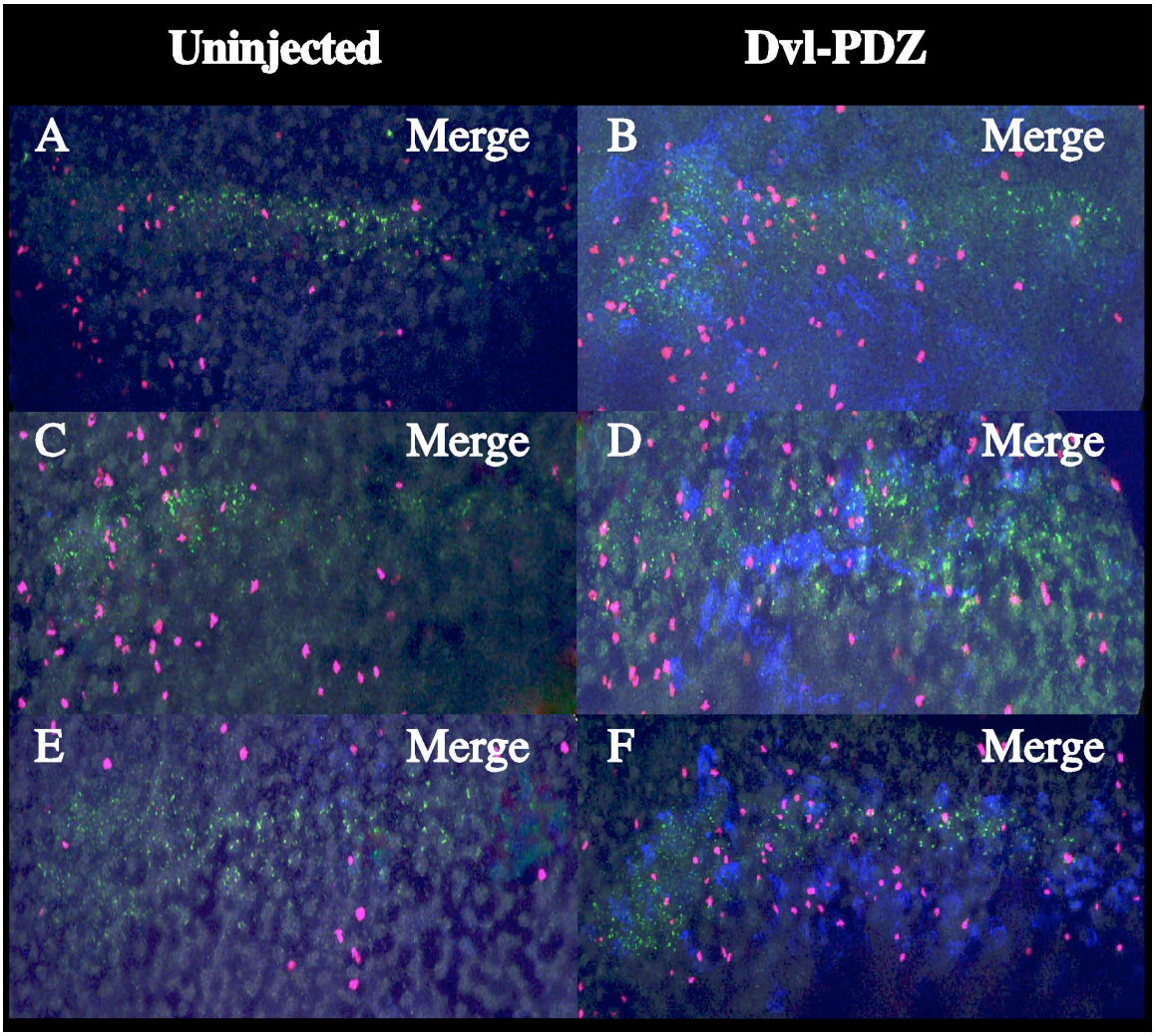

Fig 4.10 Cell proliferation in stage 21/22 Dvl-PDZ injected pronephroi. Embryos are assayed for pax8 in green, A-PH3 in red and imaged by confocal microscopy. Included in the left column (A, C, E) are the uninjected sides of samples, Dvl-PDZ on the right (B, D, F). Injected sides are determined by the presence of memGFP as boosted by anti-GFP antibodies, in blue. Injected sides (B, D, and F) show increased numbers of A-PH3 positive cells within the kidney. Images are maximal projections of z-stacks, and as such, A-PH3 positive cells which exist outside of the plane of the kidney have been removed for accurate kidney-specific counts of cells. Both columns display contralateral views of the same samples.

Fig 4.11 Anti-phospho-H3 positive cell counts in DshD2 injected versus control embryos

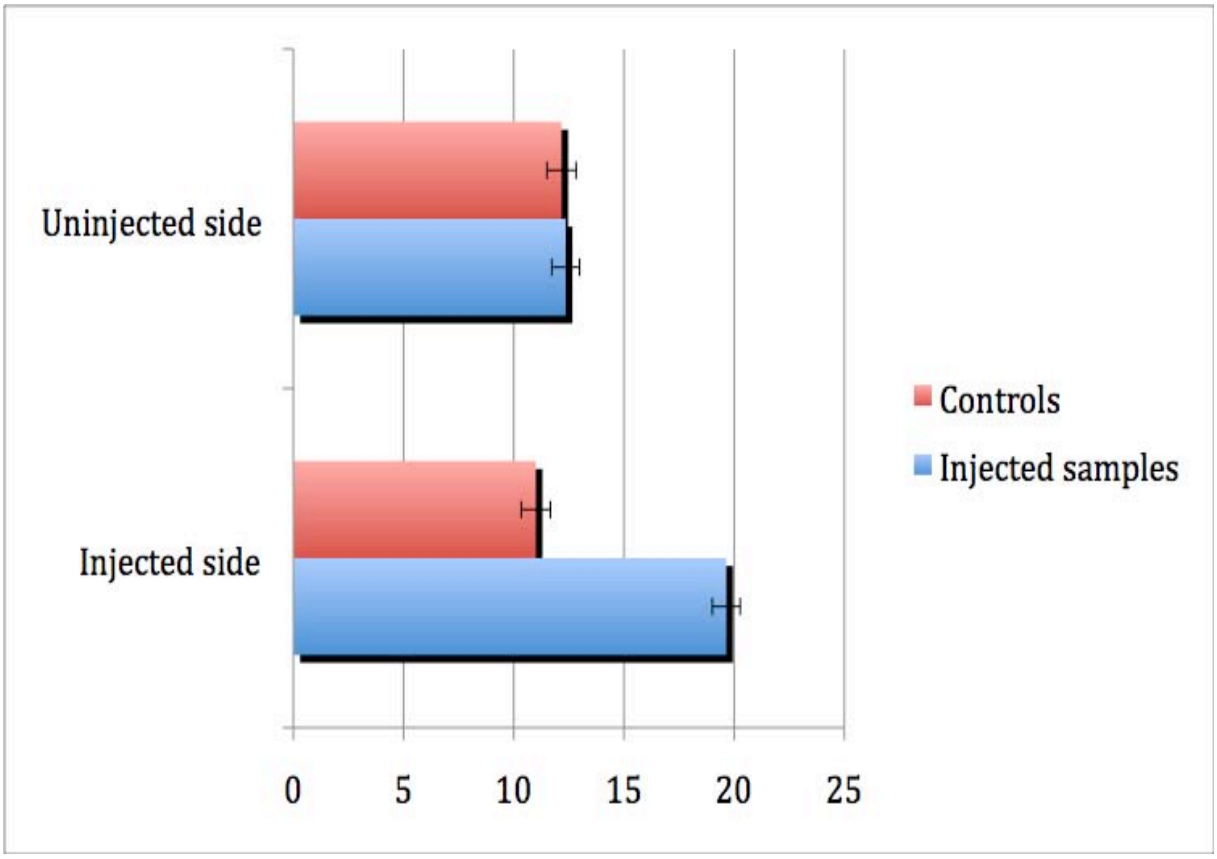

Fig 4.11 Anti-phospho-H3 positive cell counts in DshD2 injected versus control embryos. No significant difference exists between right and left sides of control embryos injected solely with GFP tracer (red). Embryos unilaterally injected with DshD2 (blue) show a statistically significant increase in A-PH3 positive cell nuclei on the injected side, as marked by co-injected GFP tracer. Bars indicate standard deviation from the mean.

Chapter Five: Discussion

5.1 Summary and limitations

In this investigation, the role of non-canonical wnt signals in the kidney was examined. It was demonstrated that injections affecting non-canonical signals, most likely acting through the PCP signal transduction pathway, affected cell proliferation in the kidney. Initially, injections of a broad-spectrum wnt inhibitor (nFrz8) resulted in a uniquely enlarged kidney duct. This effect was recapitulated by injections of a intracellular inhibitor of wnt signals (Dvl-PDZ). Upon further inspection, it was noted that the enlarged kidney possessed an increased number of pronephric cells at late stages. Embryos injected with Dvl-PDZ were seen to possess larger numbers of cells positive for a proliferative marker at early stages of development, while simultaneously displaying no obvious early morphogenetic defects. As previously noted, PCP signals are believed to act primarily via cytoskeletal restructuring, a process that is of utmost importance to both mitosis and cell migration. As kidney morphogenesis involves migration of kidney cells, it may be advantageous to limit the extent of proliferation in order to maintain a solid actin cytoskeleton, conceivably through the influence of PCP wnt signals.

Previously, the most prevalent wnt molecule known to act through a non-canonical pathway, wnt11, was not thought to be present in the early amphibian pronephros (Matthews *et al.,* 2008). This raises the question of whether or not another wnt ligand is acting to activate the PCP pathway, or if indeed a wnt signal is needed at all. Some evidence exists to suggest intracellular dvl can act independently of wnt signals entirely, positing a novel manner to control kidney development (Axelrod *et al*., 1996). However, if this is indeed the case, one would assume that the extracellular and intracellular

methods of down-regulation used in the study would produce different phenotypes. Furthermore, evidence has shown a role for wnt11 and 11b in early specification of the pronephros, with the signal originating in the inducing tissues of the anterior somites from stage 12.5 (Tételin and Jones, 2009). This points to the fact that the receptor for wnt11 is likely present within the pronephros at the stages of interest, and makes it unlikely that dvl is being activated independent of a wnt signal. Another possibility for the identity of the wnt signal responsible comes from very recent evidence in mouse models. A very similar phenotype to that produced by Dvl-PDZ can be recapitulated by abrogation of wnt 9b signalling in the metanephros, and has been traced to activity within the PCP pathway (Karner et al., 2009). While not necessarily applicable to frog models, wnt9b remains an intriguing candidate for the identity of the wnt signal responsible for the observed phenotypes.

One interesting factor in this thesis was the differing early stage phenotypes created by nFrz8 and the Dvl-PDZ mRNA injections. N-terminal truncated versions of Frz proteins in general are believed to be capable of binding to a more varied array of wnt signals than their membrane bound cousins and therefore are likely down regulating several unknown wnt signals. The punctuate and disorganized cells of the nFrz8 phenotype may be the result of the promiscuous nature of the soluble frz8 affecting more than one signalling pathway and therefore causing adhesion or morphogenetic defects in addition to the enlarged kidney. Indeed, the proliferative assay utilized on Dvl-PDZ injected embryos was not carried out on nFrz8 injected samples. As this is the case, it remains that the effects of nFrz8 on cell proliferation have not yet been directly observed and as such could be creating the concertina phenotype through a different mechanism

than that of Dvl-PDZ entirely. However, as this particular phenotype has never been reported previously in response to any injected mRNA, despite the abundance of varying mRNA injections carried out by this lab, this possibility is very remote.

One of the primary limitations in this study is the nature of the influence on wnt signals. Whether through extra-cellular (nFrz8) or intra-cellular (Dvl-PDZ) means, non-canonical wnt signals were only down-regulated. Ideally, to firmly establish a model of regulation of proliferation in the kidney, PCP signals should also be up-regulated. Unfortunately, to date, no effective method of specifically up-regulating PCP signals has been developed. One possibility would be to upregulate wnts, such as wnt11, that signal through the PCP pathway. This issue is complicated by the fact that the identity of the specific wnt signal or signals down-regulated by nFrz8 is not known, and could conceivably be due to more than one wnt, or none at all. Furthermore, according to the results of this study, up-regulation of PCP may result in less proliferation and therefore a smaller pronephros. This result is difficult to distinguish from a generally toxic effect of a gene manipulation, and any results would most likely become confounded with artifacts. In addition, wnt11 is involved in many organs and developmental processes, and any phenotype obtained by simple wnt11 mRNA injections would affect multiple facets of development as the wnt11 molecule diffuses across the embryo. If some manner of restricting wnt11 to the pronephros was developed, a very possible and relevant improvement to this study would be up-regulation of PCP family signals.

Another limitation of note in this study is the nature of the Dvl-PDZ clones that were utilized. While published literature shows that the two proteins utilized do in fact down-regulate PCP signalling, the effect of the specific deletions on other signalling

pathways has not been conclusively determined (Rothbacher *et al*., 2000). Some researchers have suggested that the DshD2 molecule used in this study may in fact have a excitatory effect on the canonical pathway (unpublished data). While this may be the case, DshD4 has not been shown to have any regulatory effect on any pathway other than PCP. Furthermore, reagents which upregulate canonical wnt signalling will, when injected into ventral blastomeres of a 4-cell embryo, result in the production of a second Spemann organizer and subsequently a mirroring of the dorsal-ventral axis (Smith and Harland, 1991). As neither of these constructs, when injected in a similar manner, produced axis duplications (data not shown), it is unlikely that any up-regulatory effect on the canonical wnt pathway is occurring. As both proteins were indistinguishable in the phenotypes they caused, it can be assumed that abrogation of PCP signalling specifically is the most likely explanation for the observed effects. Regardless, this uncertainty limits the ability to draw conclusions from studies involving Dvl-PDZ clones, as any effects resulting from their expression may conceivably be due to an unknown regulatory effect on an entirely different signalling pathway.

5.2 Canonical signalling

Canonical signals may also play an important role in determining the extent of cell proliferation in the developing pronephros. As noted previously, several lines of evidence point towards a role for β-catenin based transcriptional regulation in control of proliferation in a multitude of systems. Furthermore, preliminary evidence of a proliferation regulation mechanism based upon canonical wnt signalling exists in *Xenopus* kidneys (unpublished data). Lithium salts act to stabilize β-catenin in the cell,

allowing it to act as a transcription factor in much the same way as stabilization due to wnt signals (Logan *et al*. 1998). Embryos treated with 0.3 M lithium for one hour at NF stage 21/22 showed increased numbers of proliferative cells in a number of injected kidneys in confocal stacks, albeit in statistically inconclusive numbers. Furthermore, embryos injected with a construct consisting of an Engrailed repressor domain fused to the LEF transcription factor activated in a pulse for one hour at stage 21/22, creating an artificial repressor of canonical wnt targeted genes, showed a difference in numbers of proliferative cells between injected and control samples (Fig 5.1). While preliminary, this evidence does point towards a continuation of the general trend of antagonization of differing wnt signals in development, and remains an important avenue for future research.

5.3 Other possibilities

Cell apoptosis may account for the differences in cell number. This possibility is very unlikely, however, as apoptosis has not been shown to occur in the pronephros to any appreciable degree (Urban *et al*., 2006, Lenkowski *et al*., 2008). In addition, studies carried out on PCP signalling in mouse metanephroi have not reported any effect on apoptosis (Karner *et al*., 2009). Cell migration has not been substantially investigated in this investigation, and could possibly contribute to the phenotypes observed. As PCP signals are well-known to affect morphogenetic processes, it is expected that some type of morphogenetic defect is likely occurring (Strutt and Strutt, 2005, Wallingford *et al.*, 2002). However, as the mRNA injected is almost fully degraded by the point in time in which cell migration is occurring in the pronephros, any contribution to the late-stage

phenotype observed is likely slight (Vize *et al.,* 1997). A relatively simple manner in which to examine this would be to label the migrating pronephros at stage 32-34 in Dvl-PDZ injected and control samples and observe the degree of migration in injected versus uninjected sides. Again, some efforts have been made to examine this effect, however at the time of writing insufficient data had been collected to substantiate any hypothesis. Other investigations to illuminate the possibility of cell movement being disrupted by Dvl-PDZ include cells being marked via injection of fluorescent cell lineage tracers, such as mRNA coding for membrane-bound GFP, into the kidney. These embryos can be dissected and recombined with unlabelled embryos and, as the kidney duct migrates backwards towards the posterior of the embryo and into the unlabeled tissue, monitor cell movements through time-lapse confocal microscopy (Wang *et al*., 2006). Alternatively, kidney cells could be stained with DiI locally to mark them and examined in a similar fashion. While this method removes the necessity for dissection, it may stain more cells than those intended as DiI is applied topically rather than microinjected and is less conducive to exact localization. A defect in epitheliazation could be contributing to the phenotypes observed, most importantly to that of the nFrz8 injections. However, as certain markers of late stage kidney used, namely atp1a1 and the antibody 4A6, are themselves markers of epithelialized cells, it is apparent that some degree of epitheliazation is occurring in both nFrz8 and Dvl-PDZ injected kidneys. Despite this, some manner of over epithelialization or improper formation of an epithelial sheet cannot be ruled out with the experiments detailed above. Lastly, in order to determine if cell adhesion is being affected, possible avenues include injections of Dvl-PDZ into frogs sensitized to cell adhesion defects, either by physiological means such as lowering

calcium concentration in their growth media, or genetic means such as morpholino knockdowns of cadherins. Furthermore, the presence of important adhesion molecules such as cadherins can be assayed through fluorescent *in situ* analysis to determine if the levels of these proteins are affected. On a similar note, the late stage phenotype obtained from Dvl-PDZ injections may have possibly resulted from a defect in chemotactic path-finding behaviour of the kidney duct as it migrates caudally. However, if this were the case one would expect to see signs of a concertina duct from the beginning of morphogenesis, NF stage 25, onwards. As the sinuate kidney phenotype was never observed prior to stage 41, this possibility can safely be discounted. As the data obtained conclusively demonstrates a role for PCP in proliferation, any contribution made to the observed phenotypes by these alternate processes must act in concert with the changes in proliferation.

Fig 5.1 The effect of disruption of the canonical wnt pathway on cell division within the pronephros

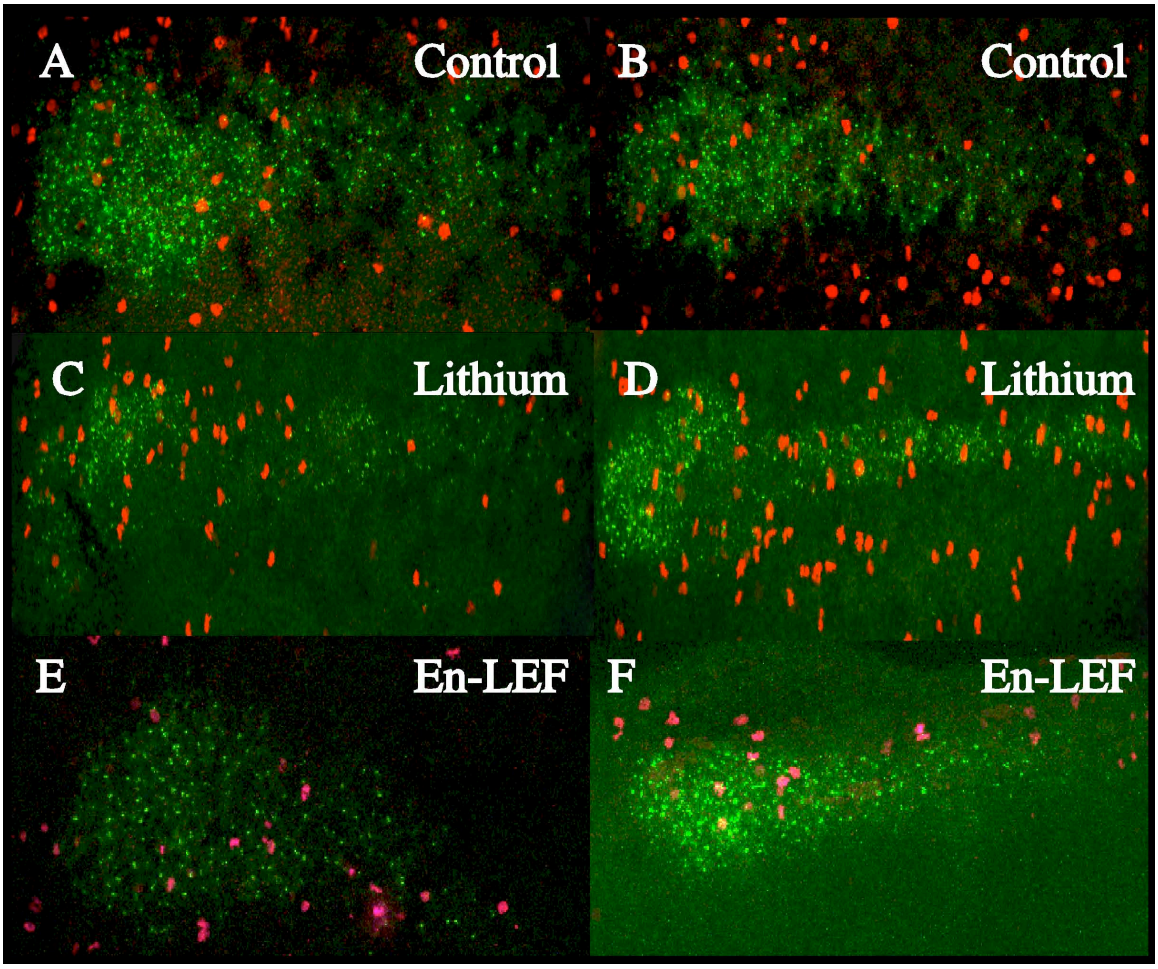

Fig 5.1 The effect of disruption of the canonical wnt pathway on cell division within the pronephros. Embryos are marked with Pax8 in green, A-PH3 in red. Embryos treated with lithium (C, D) appear to have increased numbers of A-PH3 positive cells within the kidney compared to controls (A, B). Conversely, embryos injected with En-LEF (E, F) appear to have fewer A-PH3 positive cells compared with controls. All samples are stage NF 21/22. Both columns display contralateral views of the same samples.

5.4 Conclusion

The sum total of experiments detailed in this investigation suggest a role for PCP pathway signalling in the control of proliferation within the amphibian pronephros. Furthermore, some evidence exists to suggest that canonical signalling may play a complementary role in the control of cell division, following a precedent observed in several other systems. These results can be synthesized into a model of wnt signal control where PCP and canonical pathways interact to balance the amount of cell proliferation in the migrating pronephros (Fig 5.2). Reagents that interfere with the action of one of the wnt signalling pathways tend to push this balance towards proliferation or its absence. As noted, coordinated control of proliferation is crucial during complex morphogenetic movements such as those involved in kidney pronephros extension. However, much work remains to be done to fully understand how proliferation is controlled within the developing pronephros.

Fig 5.2 Model of wnt involvement within control of cell division in the pronephros

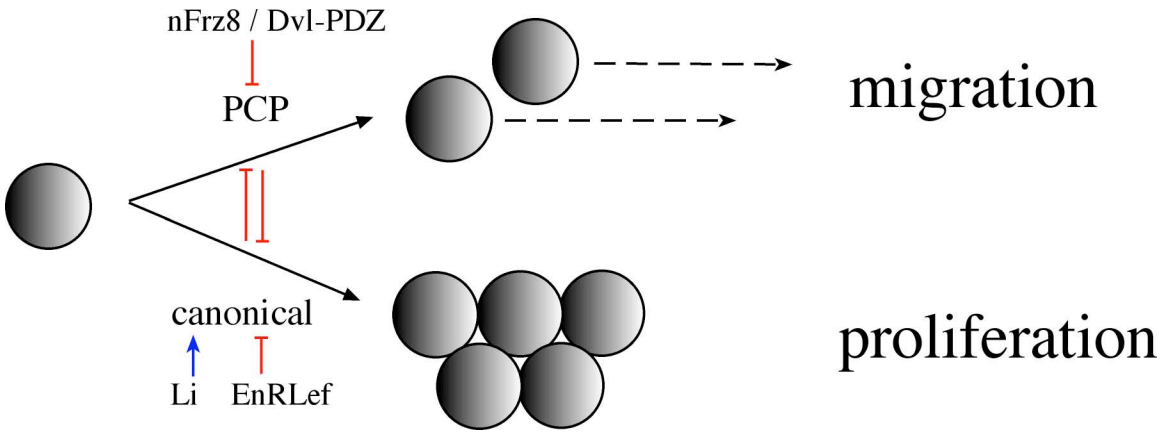

Fig 5.2 Model of wnt involvement within control of cell division in the pronephros. Canonical signals upregulate cell proliferation and repress migration, PCP signals repress proliferation and promote migration. Canonical signals are increased by the presence of lithium (Li), which stabilizes β-catenin within the cell. The engrailed-LEF construct (EnRLef) acts as a down-regulater of canonical signalling by repressing target genes of TCF/LEF. PCP signals are repressed by the presence of Dvl-PDZ, which interferes with intra-cellular signal transduction. nFrz8 downregulates PCP signals, and possibly canonical signal or others, by sequestering wnt molecules away from endogenous frz in the ECM.

References

Axelrod, J. D., Matsuno, K., Artavanis-Tsakonas, S., Perrimon, N. (1996). Interaction between Wingless and Notch signalling pathways mediated by disheveled. Science 271, 1826-1832

Bard, J. (2003). The metanephros. In “The Kidney; from normal development to congenital disease” (P.D. Vize, Woolf, A., Bard, J. B., Ed.). Academic Press, Amsterdam

Bouwmeester, T., Kim, S., Sasai, Y., Lu, B., De Robertis, E. M. (1996). Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann’s organizer. *Nature* 382(6592), 595-601

Brandli, A. W. (1999). Towards a molecular anatomy of the Xenopus pronephric kidney. *Int. J. Dev. Biol*. 43, 381-395

Bridgewater, D., Cox, B., Cain, J., Lau, A., Athaide, V., Gill, P.S., Kuure, S., Sainio, K., Rosenblum, N. D., (2008). Canonical WNT/beta-catenin signaling is required for ureteric branching. Dev. Biol. (Epub ahead of print)

Cadigan, K.M. and Nusse, R. (1997). Wnt signaling: a common theme in animal development. *Genes Dev*. 11, 3286-3305

Carreira-Barbosa, F., Concha, M. L., Takeuchi, M., Ueno, N., Wilson, S. W., and Tada, M. (2003). Prickle 1 regulates cell movements during gastrulation and neuronal migration in zebrafish. *Development* 130, 4037-4046

Carroll, T. J., Park, J. S., Hayashi, S., Majumdar, A., McMahon, A. P. (2005). Wnt9b plays a central role in the regulation of mesenchymal to epithelial transitions underlying organogenesis of the mammalian urogenital system. *Dev. Cell*. 9(2), 283-292

Carroll, T. J., Vize, P. D. (1996). Wilm's tumor suppressor gene is involved in the development of disparate kidney forms: evidence from expression in the Xenopus pronephros. *Dev. Dyn*. 206(2), 131-138

Carroll, T. J., Vize, P. D. (1999). Synergism between Pax-8 and lim-1 in embryonic kidney development. *Dev. Biol*. 214(1), 46-59

Deardorff, M. A., Tan, C., Conrad, L. J., Klein, P.S. (1998). Frizzled-8 is expressed in the Spemann organizer and plays a role in early morphogenesis. *Development* 125, 2687-2700

Deng, Z. L., Sharff, K. A., Tang, N., Song W. X., Luo, J., Chen, J., Bennett, E., Reid, R., Manning, D., Xue, A., Montag, A. G., Luu, H. H., Haydon, R. C., He, T. C. (2008). Regulation of osteogenic differentiation during skeletal development. Front Biosci 1;13, 2001-2021

Denver, R., J., Hu, F., Scanlan, T. S., Furlow, J. D. (2009). Thyroid hormone receptor subtype specificity for hormone-dependent neurogenesis in *Xenopus laevis*. *Dev. Biol.,* 326, 155-16

Edgar, L.G., Carr, S., Wang, H., and Wood, W.B. (2001). Zygotic expression of the caudal homolog pal-1 is required for posterior patterning in Caenorhabditis elegans embryogenesis. *Dev. Biol*. 229, 71–88.

Ekblom, P., Ekblom, M., Fecker, L., Klein, G., Zhang, H. Y., Kadoya, Y., Chu, M. L., Mayer, U., Timpl, R. (1994). Role of mesenchymal nidogen for epithelial morphogenesis in vitro. *Development* 120(7), 2003-2014

Fenton, R. A., Knepper, M. A. (2007). Mouse models and the urinary concentrationg mechanism in the new millennium. *Physiol. Rev*. 4, 1083-1112

Fischer, E., Legue, E., Doyen, A., Nato, F., Nicolas, J. F., Torres, V., Pontoglio, M. (2006). Defective planar cell polarity in polycystic kidney disease. *Nat. Genet*. 38, 21-23

Gillespie, L. L., Armstrong, J. B., Steinberg, M. S. (1985). Experimental evidence for a proteinaceous presegmental wave required for morphogenesis of axolotl mesoderm. *Dev. Biol*. 107(1), 220-226

Glinka, A., Wu, W., Delius, H., Monaghan, A. P., Blumenstock, C., Niehrs, C. (1998). Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. *Nature* 391(6665) 357-362

Grobstein, C. (1956). Inductive tissue interaction in development. *Adv. Cancer. Res.* 4, 187-236

Hayashi, T., Mizuno, N., Kondoh, H. (2008). Determinative roles of FGF and wnt signals in iris-derived lens regeneration in newt eye. *Dev. Growth Differ*. 50(4), 279-287

Heisenberg C. P., Solnica-Krezel, L. (2008). Back and forth between cell fate specification and movement during vertebrate gastrulation. *Curr. Opin. Genet. Dev.* 18(4), 311-316

Hensey, C., Dolan, V., Brady, H. R. (2002). The Xenopus pronephros as a model system for the study of kidney development and pathophysiology. *Nephrol. Dial. Transplant* 17, 73-74

Huang, H., He, X. (2008). Wnt/beta-catenin signaling: new (and old) players and new insights. *Curr. Opin. Cell Biol*. 20, 119-125

Karner, C. M., Chirumamilla, R., Aoki, S., Igarashi, P., Wallingford, J. B., Carroll, T. J. (2009). Wnt9b signalling regulates planar cell polarity and kidney tubule morphogenesis. *Nature Genetics* 41, 793-799

Kennedy, M. B. (1995). Origin of PDZ (DHR, GLGF) domains. *Trends Biochem. Sci*. 20, 4414-4422

Kispert, A., Vainio, S., Shen, L., Rowitch D. H., McMahon, A. P. (1996). Proteoglycans are required for maintenance of wnt-11 expression in the ureter tips. *Development* 122, 3627-3637

Lenkowski, J. R., Reed, J. M., Deininger, L., McLaughlin, K. A. (2008). Perturbation of organogenesis by the herbicide atrazine in the amphibian Xenopus laevis. *Environ. Health Perspect.* 116(2), 223-230

Leyns, L, Bouwmeester, T., Kim, S. H., Piccolo, S., De Robertis, E. M. (1997). Frzb-1 is a secreted antagonist of Wnt signaling expressed in the Spemann organizer. *Cell* 88(6), 747-756

Lin, K., Wang, S., Julius, M. A., Kitajewski, J., Moos, M. Jr., Luyten, F. P. (1997). The cysteine-rich frizzled domain of Frzb-1 is required and sufficient for modulation of Wnt signaling. *Proc. Natl. Acad. Sci. USA* 94(21), 11196-11200

Linask, K. K., Lash, J. W. (1988). A role for fibronectin in the migration of avian precardiac cells. I. Dose-dependent effects of fibronectin antibody. *Dev. Biol*. 129(2), 315-323

Logan, C. Y. and Nusse, R. (2004). The Wnt signaling pathway in development and disease. *Annu. Rev. Cell Dev. Biol*. 20, 781-810

Logan, C. Y., Miller, J. R., Ferkowicz, M. J., McClay, D. R. (1998). Nuclear beta-catenin is required to specify vegetal cell fates in the sea urchin embryo. *Development* 126(2), 345-357

Lyons, J. P., Miller, R. K., Zhou, X., Weidinger, G., Deroo, T., Denayer, T., Park, J. I., Ji, H., Hong, J. Y., Li, A., Moon, R. T., Jones, E. A., Vleminckx, K., Vize, P. D., McCrea, P. D., 2009. Requirement of Wnt/beta-catenin signaling in pronephri kidney development. *Mech. Dev*. 126(3-4), 142-159

Majumdar, A., Vainio, S., Kispert, A., McMahon, J., McMahon, A. P. (2003). Wnt11 and Ret/GDNF pathways cooperate in regulating ureteric branching during metanephric kidney development. *Development* 130(14) 3175-3185

Marose, T. D., Merkel, C. E., McMahon, A. P., Carroll, T. J. (2008). β-Catenin is necessary to keep cells of ureteric bud/Wolffian duct epithelium in a precursor state. *Dev. Biol.* 314, 112-126

Martinez Arias, A., Stewart, A. (2002). *Molecular Principles of Animal Development.* New York: Oxford University Press

Marvin, M. J., Di Rocco, G., Gardiner, A., Bush, S. M., Lassar, A. B. (2001). Inhibition of Wnt activity induces heart formation from posterior mesoderm. *Genes Dev*. 15(3), 316-327

Matthews, H. K., Broders-Bondon, F., Thiery, J. P., Mayor, R. (2008). Wnt11r is required for cranial neural crest migration. Dev Dyn. 237(11), 3404-3409

Megason S. G., McMahon, A. P. 2002. A mitogen gradient of dorsal midline wnts organizes growth in the CNS. *Development* 129, 2087-2098

Menon, V., Wang, X., Sarnak, M. J., Hunsicker, L. H., Madero, M., Beck, G. J., Collins, A. J., Kuseck, J. W., Levey, A. S., Greene, T. (2008). Long-term outcomes in nondiabetic chronic kidney disease. *Kidney Int.* (Epub ahead of print)

Moulton, J. D., Yan, Y. L. (2008). Using Morpholinos to control gene expression. *Curr. Protoc. Mol. Biol*. 26, 26-28

Nasevicius A., Ekker S. C. (2000). Effective targeted gene knockdown in zebrafish. *Nat Genet* 26, 216-220

Nieuwkoop P.D., and Faber, J. (1994). *Normal table of* Xenopus laevis *(Duadin).* Garland, New York.

Okada, T. S. 2004. From embryonic induction to cell lineages: revisiting old problems for modern study. *Int. J. Dev. Biol*. 48(8-9), 739-442

Papadopopoulou, S., Edlund, H. (2005). Attenuated Wnt signaling perturbs pancreatic growth but not pancreatic function. *Diabetes,* 54 (10), 2844-2851

Peunova, N., Scheinker, V., Ravi, K., Enikolopov, G. (2007). Nitric Oxide Coordinates Cell Proliferation and Cell Movements During Early Development of *Xenopus*. *Cell Cycle* 6(24), 3132-3144

Pollet, N., Mazabraud, A. (2006). Insights from Xenopus genomes. *Genome Dyn*. 2, 138-153

Ponting, C. P., Phillips, C., Davies, K. E., Blake, D. J. (1997). PDZ domains: targeting signalling molecules to sub-membraneous sites. *BioEssays* 19, 469-479

Ponting, C. P., Bork, P. (1996). Pleckstrin's repeat performance: a novel domain in G-protein signalling? *Trends Biochem. Sci*. 21, 245-246

Ribeiro, I., Kawakami, Y., Buscher, D, Raya, A., Rodriguez-Leon, J., Morita, M., Esteban, C. R., and Belmonte, J. C. I. (2007). Tbx2 and Tbx3 regulate the dynamics of Cell Proliferation during Heart Remodeling. *PLoS ONE* 2(4)

Rothbacher, U., Laurent, M. N., Deardorff, M. A., Klein, P.S., Cho, K. W., Fraser, S. E. (2000). Dishevelled phosphorylation, subcellular localization and multimerization regulate its role in early embryogenesis. *EMBO J* 19, 1010-1022

Rubin, D. C. (2007). Intestinal morphogenesis. *Curr. Opin. Gastroenterol*. 23(2), 111-114

Saka, Y., and Smith, J. C. (2001). Spatial and Temporal Patterns of Cell Division during Early *Xenopus* Embryogenesis. *Dev. Biol*. 229, 307-318

Satow, R., Chan, T., Asashima, M. (2004). The role of *Xenopus* frizzled-8 in pronephric development. *Biochem. Biophys. Res. Comm.* 321 487-494

Saulnier, D. M., Ghanbari, H., Brandli A. W. (2002). Essential function of Wnt-4 for tubulogenesis in the Xenopus pronephric kidney. *Dev. Biol*. 248, 13-28

Saxén, L., Lehtonen, E., Karkinen-Jaaskelainen, M., Nordling, S., Wartiovaara, J. (1976). Are morphogenetic tissue interactions mediated by transmissible signal substances or through cell contacts? *Nature* 259(5545), 662-663

Saxen, L., Sariola, H. (1987). Early organogenesis of the kidney. *Pediatr. Nephrol*. 1(3), 385-392

Schambony, A., Wedlich, D. (2007). Wnt-5A/Ror2 Regulate Expression of XPAPC through and Alternative Noncanonical Signaling Pathway. *Dev. Cell*. 12, 779-792

Schmidt-Ott K. M., Masckauchen T. N., Chen X., Hirsch B. J., Sarkar A., Yang, J., Paragas, N., Wallace V. A., Dufort, D., Pavlidid, P., Jagla, B., Kitajewski, J., Barasch, J. (2007).

Beta-catenin/TCF/LEF controls a differentiation-associated transcriptional program in renal epithelial progenitors. *Development* 134, 3177-3190

Schneider V. A., Mercola, M. (2001). Wnt antagonism initiates cardiogenesis in Xenopus laevis. *Genes Dev*. 15(3) 304-315

Schuchardt, A., D'Agati, V., Pachnis, V., Costantini, F. (1996). Renal agenesis and hypodysplasia in ret-k- mutant mice results from defects in ureteric bud development. *Development* 122(6), 1919-1929

Schultheiss, T. M., Xydas, S., Lassar, A. B. (1995). Induction of avian cardiac myogenesis by anterior endoderm. *Development* 121(12), 4203-4214

Sharpe C., Lawrence N., Martinez Arias A. (2001). Wnt signaling; a theme with nuclear variations. *Bioessays* 23, 311-318

Smalley, M. J., Sara, E., Paterson, H., Naylor, S., Cook, D., Jayatilaka, H., Fryer, L. G., Hutchinson, L., Fry, M. J., Dale, T. C. (1999). Interaction of axin and Dvl-2 proteins regulates Dvl-2-stimulated TCF-dependent transcription. *EMBO J* 18(10), 2823-2835

Smith, W. C., Harland, R. M. (1991). Injected *Xwnt-8* RNA acts early in Xenopus embryos to promote formation of a vegetal dorsalizing center. *Cell* 67, 753-765

Sokol, S. Y., Wharton jr., K. A. (2007). WNTers in La Jolla. Development 134, 3393-3399

Sokol, S. Y.(1996). Analysis of Dishevelled signalling pathways during *Xenopus* development. Curr. Biol. 6(11), 1456-1467

Sokol, S., Christian, J. L., Moon, R. T., Melton. D. A. (1991). Injected Wnt RNA induces a complete body axis in Xenopus embryos. *Cell* 67, 741-752

Soltanoff, C. S., Yang, S., Chen, W., Li, Y. P. (2009). Signaling networks that control the lineage commitment and differentiation of bone cells. *Crit Rev Eukaryot Gene Expr.* 19(1), 1-46

Soreq, H. (1985). The biosynthesis of biologically active proteins in mRNA-microinjected Xenopus oocytes. *CRC Crit. Rev. Biochem*. 18(3), 199-238

Srivastava, D., Cserjesi, P., Olson, E. N. (1995). A subclass of bHLH proteins required for cardiac morphogenesis. *Science* 270(5244), 1995-1999

Stark, K., Vainio, S., Vassileva, G., McMahon, A. P. (1994). Epthelial transformation of metanephric mesenchyme in the developing kidney regulated by Wnt-4. *Nature* 372(6507). 679-683

Strutt, H., and Strutt, D. (2005). Long-range coordination of planar polarity in *Drosophila. Bioessays* 27, 1218-1227

Tao, Q., Yokota, C., Puck, H., Kofron, M., Birsoy, B., Yan, D., Asashima, M., Wylie, C. C., Lin, X., Heasman, J. (2005). Maternal wnt11 activates the canonical wnt signalling pathway required for axis formation in Xenopus embryos. *Cell* 120(6), 857-871

Tételin, S., Jones, E. A. (2009). Xenopus wnt11b is identified as a potential pronephric inducer. *Dev. Dyn*. Epub ahead of print; doi :10.1002/dvdy.22012

Tzahor, E., Lassar, A. B. (2001). Wnt signals from the neural tube block ectopic cardiogenesis. *Genes Dev*. 15(3), 255-260

Urban, A. E., Zhou, X., Ungos, J. M., Raible, D. W., Altmann, C. R., Vize, P. D. (2006). FGF is essential for both condensation and mesenchymal-epithelial transition stages of pronephric kidney tubule development. *Dev. Biol*. 297(1), 103-117

Vize, P. D., Seufert, D. W., Carroll, T. J., Wallingford, J. B. (1997). Model systems for the study of kidney development: use of the pronephros in the analysis of organ induction and patterning. *Dev. Biol*. 188(2), 189-204

Vize, P.D., McCoy, K.E., Zhou, X. (2009). Multichannel wholemount fluorescent and fluorescent/chromogenic *in situ* hybridization in *Xenopus* embryos. *Nature Protocols* ePub ahead of print; doi:10.1038/nprot.2009.69

Wallingford, J. B., Carroll, T. J., Vize, P. D. (1998). Precocious expression of the Wilm's tumor gene xWT1 inhibits embryonic kidney development in Xenopus laevis. *Dev. Biol*. 202(1), 103-112

Wallingford, J. B., Habas, R. (2005). The developmental biology of dishevelled: an enigmatic protein governing cell fate and cell polarity. *Development* 132, 4421-4436

Wallingford, J.B., Fraser, S.E., and Harland, R.M. (2002). Convergent extension: the molecular control of polarized cell movement during embryonic development. *Dev. Cell* 2, 695-706

Wang, J, Hamblet, N.S., Mark, S., Dickinson M. E., Brinkman, B. C., Segil, N., Fraser, S. E., Chen, P., Wallingford, J. B., Wynshaw-Boris, A. (2006). Dishevelled genes mediate a conserved mammalian PCP pathway to regulate convergent extension during neurulation. *Development* 133, 1767-1768

Weidinger, G., Moon, R. T. (2003). When Wnts antagonize Wnts. *Jour. Cell. Biol.* 162 (5), 753-755

Widelitz, R. (2005). Wnt signaling through canonical and non-canonical pathways: recent progress. Growth Factors 23, 111-116.

Willert, K., Jones, K. A. (2006). Wnt signaling: is the party in the nucleus? Genes Dev. 20, 1394-1404.

Wingert, R. A., Davidson, A. J. (2008). The zebrafish pronephros: a model to study nephron segmentation. *Kidney Int*. 73(10), 1120-1127

Xu, P. X., Adams, J., Peters, H., Brown, M. C., Heaney, S., Maas, R. (1999). Eya1-deficient mice lack ears and kidneys and show abnormal apoptosis of organ primordial. *Nat. Genet*. 23 113-117

Yamaguchi, T. P., Bradley, A., McMahon. A. P., Jones, S. 1999. A wnt5a pathway underlies outgrowth of multiple structures in the vertebrate embryo. *Development* 126, 1211-1223

Yu, J., Carroll, T. J., Rajagopal, J., Kobayashi, A., Ren, Q., McMahon, A. P. (2009). A Wnt7b-dependent pathway regulates the orientation of epithelial cell division and

establishes the cortico-medullary axis of the mammalian kidney. *Development* 136, 161-171

Zeng, L., Fagotto, F., Zhang, T., Hsu, W., Vasicek, T. J., Perry W. L., Lee, J. J., Tilghman, S. M., Gumbiner, B. M., Costantini, F. (1997). The mouse Fused locus encodes Axin, an inhibitor of the Wnt signalling pathway that regulates embryonic axis formation. *Cell* 90(1), 181-192

Zeng, X., Tamai, K., Doble, B., Li, S., Huang, H., Habas, R., Okamura, H., Woodgett, J., He, X. (2005). A dual-kinase mechanism for Wnt co-receptor phosphorylation and activation. *Nature* 438, 873-877

Zhou, X., and Vize, P.D., (2004). Proximo-distal specialization of epithelial transport processes within the *Xenopus* pronephric kidney tubules. *Dev. Biol.* 271, 322-338