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PRIMER

# Wilms' tumour 1 (WT1) in development, homeostasis and disease

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## ABSTRACT

The study of genes mutated in human disease often leads to new insights into biology as well as disease mechanisms. One such gene is Wilms' tumour 1 (*WT1*), which plays multiple roles in development, tissue homeostasis and disease. In this Primer, I summarise how this multifaceted gene functions in various mammalian tissues and organs, including the kidney, gonads, heart and nervous system. This is followed by a discussion of our current understanding of the molecular mechanisms by which WT1 and its two major isoforms regulate these processes at the transcriptional and post-transcriptional levels.

KEY WORDS: WT1, Developmental disorders, Homeostasis, Molecular mechanisms of disease

## Introduction

The Wilms' tumour 1 (*WT1*) gene was first identified in 1990 as a strong candidate predisposition gene for Wilms' tumour (Call et al., 1990; Gessler et al., 1990), which is a paediatric kidney cancer that affects 1 in 10,000 children (Charlton and Pritchard-Jones, 2016). Since then, numerous studies have confirmed that *WT1*, which maps to chromosome 11p13, is mutated in the germline or somatically in ∼15% of Wilms' tumour cases (Charlton and Pritchard-Jones, 2016). Wilms' tumour has fascinated pathologists for over a century as it is one of the classic examples of how cancer arises through development gone awry (Hastie, 1994). *WT1* behaves as an archetypal tumour suppressor gene, as both alleles need to be deleted or inactivated for tumours to develop (Knudson and Strong, 1972). However, it is not as simple as this because the majority of tumours with *WT1* mutation also have gain-of-function mutations in the β-catenin (*CTNNB1*) gene and a proportion have a double dose of IGF2 through chromosome 11 loss of heterozygosity (Huang et al., 2016). Moreover, WT1 is expressed in a wide range of adult tumour types, including those derived from epithelial, mesenchymal, haematopoietic and neuronal tissue, even though it is not expressed in the corresponding healthy tissue, and this has led to the proposition that WT1 functions as an oncogene in these tumours. At present, there is scant evidence to support this concept. However, this widespread tumour expression has led to WT1 being the number one target for cancer immune therapy (Nishida and Sugiyama, 2016).

Although our understanding of the molecular and cellular mechanisms by which tumours arise through *WT1* mutation is incomplete, studies of the *WT1* gene over the past 25 years have led to broad insights into a number of phenomena. These include: (1) the mechanisms underpinning the development, homeostasis and disease of tissues arising from the intermediate and lateral plate mesoderm; (2) cellular switching events during development,

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particularly those involving mesenchyme-to-epithelial transitions (MET) and the reverse process, epithelial-to-mesenchyme transition (EMT); (3) the cellular origins of mesenchymal progenitors for a variety of tissue types, pinpointing the key role of the mesothelium; and (4) fundamental aspects of transcription and epigenetic regulation. WT1 is also an example of how protein isoforms differing by just a few amino acids may have profoundly different functions. Here, I provide an overview of the *WT1* gene, highlighting how it functions in the development and homeostasis of various organs and tissues, and how its mutation can lead to disease.

## *WT1* structure, evolution and isoforms

The mammalian *WT1* gene is ∼50 kb in length, encoding proteins from as many as ten exons. There are at least 36 potential mammalian WT1 isoforms, the diversity created through a combination of alternative transcription start sites, translation start sites, splicing and RNA editing (Fig. 1A). All isoforms include four C2H2 Kruppel-like zinc fingers similar to those found in the SP1 family of transcription factors. All non-mammalian vertebrates express only two isoforms, which differ by just three amino acids (lysine, threonine and serine; KTS) inserted by an alternative splice between zinc fingers 3 and 4 (Fig. 1B). Outside the vertebrates, there appears to be a WT1 orthologue in amphioxus but it is not clear whether invertebrates encode WT1 orthologues other than proteins with zinc fingers similar to those in the SP1 family.

Although the functional relevance of the many WT1 isoforms is unclear, the importance of the +KTS and −KTS isoforms has been highlighted by the identification of splice site mutations in patients with Frasier syndrome (Barbaux et al., 1997). These individuals have male-to-female sex reversal and suffer from focal segmental glomerulosclerosis (FSGS; i.e. scarring of the glomeruli) of the kidney. The dominant *WT1* mutations create one allele that only produces the −KTS isoforms, leading to a reduced +KTS/−KTS isoform ratio. This suggests that both isoforms are essential and have different functions. Further support for this notion came from a study in which mice expressing only the +KTS or −KTS isoforms were created through gene targeting (Hammes et al., 2001). Mice lacking either of these isoforms die neonatally through incomplete kidney development, although the lack of −KTS isoforms leads to a more severe developmental phenotype than loss of +KTS isoforms (Hammes et al., 2001). By contrast, mice that specifically lack a mammalian-specific 17 amino acid insertion encoded by an alternative exon 5 exhibit no observable phenotype (Natoli et al., 2002). This is surprising, as the 17 amino acid domain acts as a transcriptional activator through interaction with the prostate apoptosis response factor PAR4 (PAWR) (Richard et al., 2001), and a mutation in this domain has been identified in a Wilms' tumour (Schumacher et al., 1997), attesting to its importance in humans at least. Similarly, no phenotype is observed in mice lacking the extended isoforms of WT1, i.e. the isoforms produced via the use of a mammalian-specific alternative translation start site (Miles et al., 2003).

DEVELOPMENT
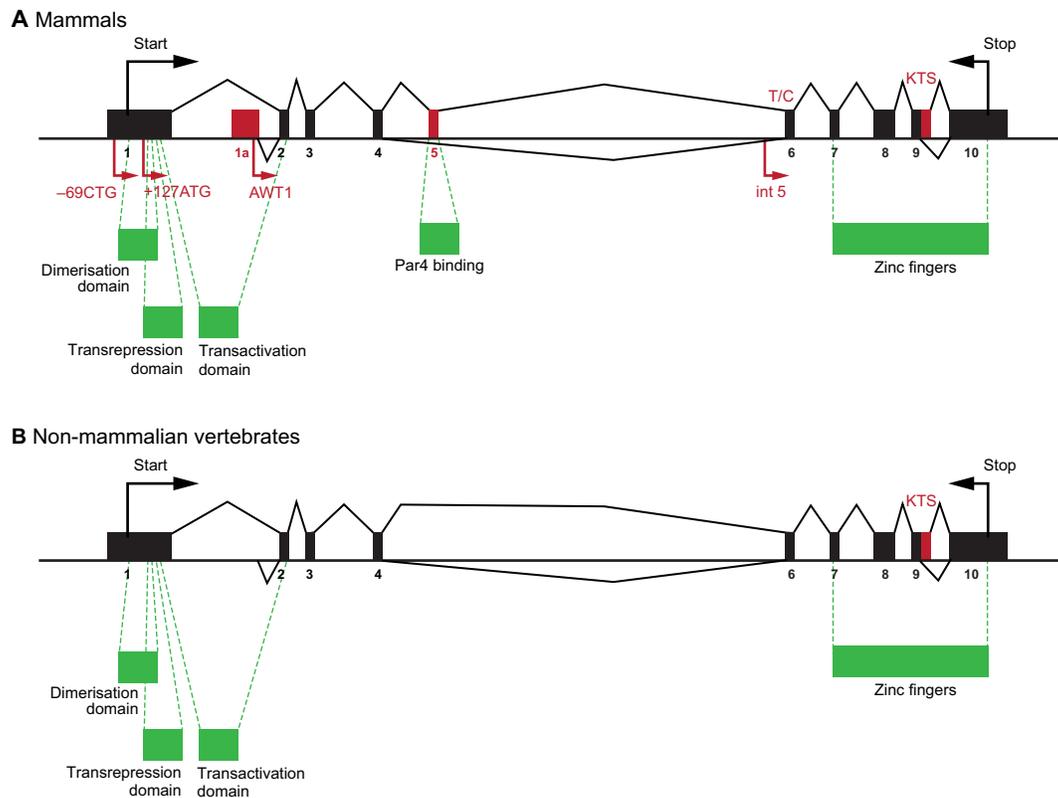
**Fig. 1. Structure of the *WT1* gene and the multiple isoforms it encodes.** (A) Variant transcriptional and translational start sites, alternative splices and RNA editing sites within the mammalian *WT1* gene are depicted in red; these can give rise to at least 36 potential mammalian WT1 isoforms. int 5, intron 5. (B) In non-mammalian vertebrates only two isoforms exist: one that includes the lysine, threonine and serine (KTS) insert and one that does not.

## Diseases arising through germline *WT1* mutations

Germline *WT1* mutations may lead not only to the eponymous tumour but also to glomerulosclerosis of the kidney, gonadal dysgenesis and, in rare cases, congenital diaphragmatic hernia (CDH) and heart disease. There are several human syndromes resulting from *WT1* hemizygosity or mutation and these have been very revealing about *WT1* gene function. For example, deletions associated with WAGR syndrome (Wilms' tumour, aniridia, genitourinary anomalies and retardation), which result in *WT1* haploinsufficiency, lead to Wilms' tumour in 70% of cases and a range of gonadal anomalies, most frequently undescended testes in males and streak gonads and bicornate uterus in females (Riccardi et al., 1978). On the other hand, heterozygous *WT1* point mutations, predominantly in the zinc finger domain, lead to the more extreme phenotypes found in Denys-Drash syndrome (DDS) (Pelletier et al., 1991). Children with DDS often develop Wilms' tumour and always suffer from mesangial sclerosis of the glomerulus, resulting in end-stage renal disease. Males with DDS have incompletely formed, ambiguous or female external genitalia; the testes may be normal, malformed, undescended or ambiguous. There is evidence to support the notion that the DDS mutations act in a dominant-negative mode, with the mutant WT1 protein dimerising with the wild-type protein, thus explaining the more severe phenotype compared with haploinsufficiency (Little et al., 1995). Patients with Frasier syndrome, which arises through an imbalance of the −KTS/+KTS WT1 isoforms (see above), have FSGS, and XY males with this condition exhibit female external genitalia and streak gonads (Barbaux et al., 1997). A fourth, less clear syndrome, and one that overlaps with DDS, is Meacham syndrome, which is characterised by CDH, ambiguous genitalia and complex congenital heart defects but no renal abnormalities. Surprisingly, two patients with Meacham syndrome have exactly the same *WT1* missense mutations as seen in two cases of DDS with a different spectrum of anomalies (Suri et al., 2007).

The spectrum of phenotypic anomalies that result from *WT1* mutations maps well onto the developmental expression domains of WT1. In mice, expression of WT1 is first detected in the proliferating coelomic epithelium and intermediate mesoderm at E9. Expression continues in the derivatives of the coelomic epithelium, the diaphragm, gonads and mesothelial lining of organs and in the kidney, which derives from the intermediate mesoderm (Armstrong et al., 1993). The phenotypes reported for the mouse *Wt1* knockout are dramatic and concord well with the disease spectrum. Indeed, *Wt1* null mice have no kidneys or gonads, exhibit CDH, and die at ~E13.5, presumably due to cardiac problems (Kreidberg et al., 1993). The mutant mice also suffer from hypoplastic lungs and have been shown to lack a spleen (Herzer et al., 1999) and adrenal glands (Moore et al., 1999; Bandiera et al., 2013).

## WT1 function in kidney development, homeostasis and disease

The kidney diseases arising through *WT1* mutation reflect the function of this gene at multiple stages of kidney development and tissue homeostasis (Fig. 2). Hence Wilms' tumours involving *WT1* mutations are likely to arise from the undifferentiated metanephric mesenchyme – a tissue that gives rise to the kidney – whereas *WT1* mutations that give rise to glomerulosclerosis reflect a role for WT1 in the differentiation and maintenance of a fascinating specialised kidney cell type, the podocyte.
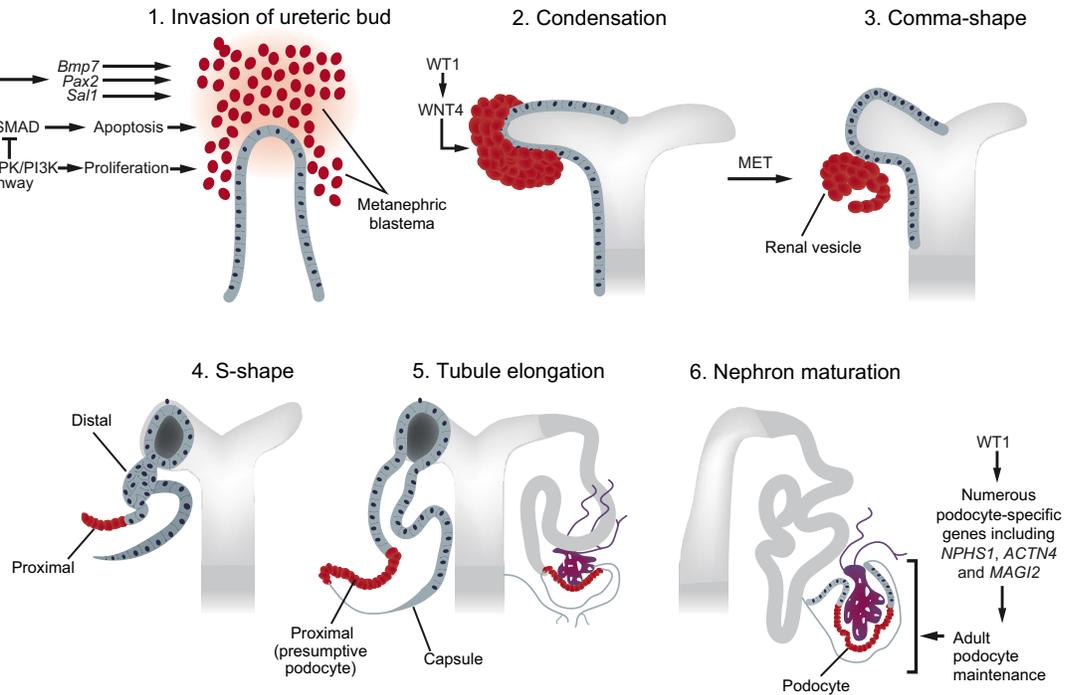
**Fig. 2. The role of WT1 at different stages of kidney development.** The various stages of kidney development are shown. WT1 expression is depicted in red. In the first stage, the ureteric bud invades the metanephric mesenchyme. WT1 is expressed at low levels and is required for mesenchyme maintenance. Key transcriptional target genes of WT1 at this stage are indicated. In the second stage, the mesenchyme condenses around the bud and the levels of WT1 and its target WNT4 increase. The next key stage involves MET, which is driven by WNT4 and requires WT1. The nephron then forms through several intermediate structures, including the comma-shaped and S-shaped bodies. WT1 expression becomes restricted to the proximal region of these structures, which will form the glomerulus. The highest level of expression is in the presumptive, differentiating and mature podocytes. In line with this, WT1 is required for podocyte differentiation, structure, function and maintenance through the activation of numerous podocyte-specific genes. The proximal portions of the developing nephron develop into tubular structures that do not express WT1.

The kidneys develop from two interacting and cross-signalling components, namely the metanephric mesenchyme and the ureteric buds, both of which are derived from the intermediate mesoderm. These two components develop into nephrons (the functional units of the kidney) and branching ureters (the ductal system of the kidney), respectively. A crucial factor here is the activation in the mesenchyme of the master regulator WNT4, which is necessary and sufficient to induce the MET that precedes the formation of nephrons from so-called cap mesenchyme (Stark et al., 1994). Formation of the segmented nephron then involves several intermediate stages including the comma-shaped and S-shaped bodies (Fig. 2). The final nephron is composed of the glomerulus, the proximal and distal tubules and the loop of Henle.

WT1 levels vary throughout this process of kidney development. WT1 is first expressed at low but detectable levels in the undifferentiated mesenchyme, and levels increase dramatically as the mesenchyme condenses around the bud prior to MET. Levels then stay high throughout nephrogenesis, becoming restricted to the proximal half of the S-shaped body and eventually the podocyte cells derived from this structure (Fig. 2). These expression levels correlate with a functional requirement for WT1 during kidney development; in *Wt1* knockout mice the bud fails to invade the mesenchyme, which degenerates through apoptosis (Kreidberg et al., 1993). To explore the mechanism by which WT1 ensures mesenchyme maintenance, chromatin immunoprecipitation (ChIP) coupled to mouse promoter microarrays (ChIP-chip) was used to identify WT1 target genes using embryonic kidney extracts (Hartwig et al., 2010). Among the 1600 potential WT1 target genes were several known to be essential for kidney development

within the mesenchyme (Fig. 2). These included *Pax2*, *Sal1* and *Bmp7*, the expression of which was shown to depend on WT1 in nephrogenic organ culture (Hartwig et al., 2010). In an elegant study, it was further shown that WT1 is essential for mesenchyme survival and proliferation through direct transcriptional regulation of two cross-talking signalling pathways (Motamedi et al., 2014). WT1 transcriptionally activates the expression of several FGFs that are essential for mesenchyme proliferation, while repressing BMP/SMAD signalling, which induces an apoptotic response in the mesenchyme (Fig. 2). In line with this, it was shown that recombinant FGFs can rescue the survival of *Wt1* null mesenchyme and suppress an apoptotic response induced by BMPs (Motamedi et al., 2014).

Key roles for WT1 during subsequent stages of kidney development can explain its function with respect to Wilms' tumour and glomerulosclerosis. There are several subtypes of Wilms' tumour, classified according to their pathology. The most common form is known as triphasic, comprising blastemal, epithelial and stromal elements. These tumours exhibit an architecture remarkably similar to that of the developing kidney. Wilms' tumours arising through *WT1* mutation, on the other hand, are mainly stromal and may often contain elements of heterotypic tissue, most commonly muscle but, more rarely, cartilage, bone and fat (Schumacher et al., 2003). WT1 is essential for the MET that underlies nephron differentiation, partly through transcriptional activation of *Wnt4* (Sim et al., 2002; Davies et al., 2004; Essafi et al., 2011). Furthermore, if *Wt1* is deleted just prior to MET, the aberrant kidneys formed express a transcriptome similar to that observed in human Wilms' tumours arising through *WT1* mutation (Berry et al.,

2015). It has therefore been hypothesised that *WT1* mutant tumours arise through defective MET. However, this does not seem to equate with the stromal-predominant nature of the tumours. To address this, the origin of Wilms' tumours was investigated by developing a series of mouse Wilms' tumour models (Huang et al., 2016) using different Cre lines to delete *Wt1* while simultaneously activating oncogenic *Ctnnb1* or increasing the dose of IGF2, either in the nephron progenitors or the stroma. This approach revealed that tumours only develop when these genetic manipulations occur in the mesenchymal nephron progenitors, not in the stroma. Although tumour histology in these mice does not necessarily recapitulate that seen in humans with the same genetic alterations, tumours arising through *Wt1* loss and increased IGF2 dose do express high levels of undifferentiated mesenchyme markers. Furthermore, deletion of *Wt1* in the mesenchyme leads to activation of myogenic markers, providing a molecular explanation for the formation of ectopic muscle in Wilms' tumours with *WT1* mutation (Berry et al., 2015).

By contrast, glomerulosclerosis, for example that seen in children with DDS and Frasier syndrome, is due to abnormalities of kidney podocytes – specialised cells that form a filtration barrier with endothelial cells. It is now clear from several studies that WT1 is essential both for podocyte differentiation and podocyte maintenance throughout adult life (Moore et al., 1999; Hammes et al., 2001; Chau et al., 2011; Berry et al., 2015; Gebeshuber et al., 2013). Recent ChIP-seq studies have shown that WT1 binds to the promoters and enhancers of around half the 200 podocyte-specific genes identified (Kann et al., 2015a; Lefebvre et al., 2015; Dong et al., 2015). A subset of these target genes was shown to be downregulated upon *Wt1* deletion, 11 of these specifically in mice lacking +KTS isoforms. Remarkably, WT1 bound to 18 of the 31 genes mutated in human podocyte disease, including *Nphs1*, *Nphs2* and *Actn4*. Through bioinformatics analysis, it was proposed that WT1 is part of a podocyte transcription network that includes WT1, FOX-class transcription factors, LMX1B and TCF21 (Kann et al., 2015a; Lefebvre et al., 2015; Dong et al., 2015). Targets of this network include the Hippo signalling system, implicating this pathway in podocyte development or maintenance.

### WT1 and the adrenal-gonad axis
As is the case for the kidney, WT1 plays roles at different stages during the development and homeostasis of the gonads and adrenal glands (Bandiera et al., 2015). These two steroidogenic organs develop from a common adrenogonadal primordium (AGP) arising from the coelomic epithelium. As discussed above, humans with *WT1* mutations have a range of gonadal abnormalities but no adrenal anomalies to this author's knowledge. *Wt1* null mice completely lack gonads and adrenal glands, pointing to a crucial role in the formation/survival of the AGP. A vital factor for AGP survival is the steroidogenic factor SF1 (NR5A1), which is a WT1 transcriptional target (Wilhelm and Englert, 2002). Hence, the reduction of SF1 levels in *Wt1* knockout mice is likely to be a major contributory factor to adrenal/gonad agenesis. It will be interesting to see if a WT1-regulated FGF/SMAD loop also functions to protect AGP survival, as in the kidney (Motamedi et al., 2014).

The gonadal anomalies in male patients with *WT1* mutation can be attributed to the role of WT1 in the differentiation and maintenance of Sertoli cells – the somatic cells that support germ cells during their development. The key factor in determining maleness and Sertoli cell development is the *SRY* gene on the Y chromosome. Expression of SRY is restricted to pre-Sertoli cells over a brief time window, and it has been shown that WT1 cooperates with GATA 4 to transcriptionally activate *SRY* (Miyamoto et al., 2008). In particular, the +KTS isoforms appear

to be more efficient in activating the *SRY* promoter in cooperation with GATA4 (Miyamoto et al., 2008), perhaps providing an explanation for the sex reversal observed in Frasier syndrome and in mice deficient for +KTS isoforms (Barbaux et al., 1997; Hammes et al., 2001). There are other striking similarities between the gonad and kidney that might involve WT1. Embryonic Sertoli cells must go through MET and polarisation prior to cord formation. Although WT1 has not yet been shown to be directly required for this process, it is one of the five factors, along with SF1, DMRT1, SOX9 and GATA4, that can cooperate to induce fibroblasts to a mature Sertoli cell fate via MET (Buganim et al., 2012). In addition, WT1 not only promotes Sertoli cell differentiation but suppresses the formation of Leydig cells – another specialised cell type in the gonad that produces testosterone in the presence of luteinizing hormone. Indeed, WT1 ablation in the testis leads to the transdifferentiation of Sertoli cells into foetal-like Leydig cells (Zhang et al., 2015).

The adrenal gland comprises two major components: the outer cortex, which arises from the AGP, and the inner medulla, which arises from neural crest ectoderm. The cortex houses steroidogenic cells that produce corticosteroids and aldosterone. At E9.75, AGP cells arising in the genital ridge are still bipotential, expressing WT1, GATA4 and SF1. As the gonadal and adrenal primordium split away from each other, WT1 and GATA 4 are downregulated in the precursors of the cortical steroidogenic cells, which still express and require SF1. Downregulation of WT1 was shown to be essential for development of the steroidogenic cells, as ectopic expression of a −KTS, but not a +KTS, WT1 isoform in the SF1-expressing progenitors blocks the differentiation of steroidogenic cells, maintaining the cells in a progenitor state (Bandiera et al., 2013). Hence, in both the developing gonad and adrenal gland, WT1 represses the differentiation of steroidogenic cells.

### WT1 function in EMT: implications for heart and diaphragm development
Whereas WT1 plays key roles in MET during kidney and gonad development (as discussed above), in the developing heart and diaphragm it is required for EMT.

In the developing heart, WT1 expression is mainly restricted to its mesothelial lining, the epicardium. *Wt1* knockout mice have smaller ventricles, a thinner epicardium and have a much reduced coronary vascular system (Kreidberg et al., 1993). Cell fate analysis has shown that the epicardium is the source of coronary vascular progenitors, particularly for the smooth muscle component of the vasculature but also for 20% of the embryonic endothelial cells (Cano et al., 2013). These progenitors, or epicardium-derived cells (EPDCs), arise from the epicardium via EMT, or perhaps more accurately mesothelial-mesenchyme transition (MMT). When *Wt1* is inactivated specifically in the epicardium, embryos die at E16.5 and the coronary vasculature is severely depleted; although the epicardium remains mostly intact, there is a dramatic reduction in EMT and in the resultant production of EPDCs (Martínez-Estrada et al., 2010). Mechanistically, it was shown that WT1 is required for EMT/ MMT in part through transcriptional activation of *Snai1*, an EMT inducer, and repression of the key epithelial component E-cadherin (*Cdh1*). In another study using *Wt1* null mice, it was shown WT1 also regulates EMT through its action, direct or indirect, on the WNT and retinoic acid (RA) signalling pathways (von Gise et al., 2011). There is much evidence of cross-talk between the developing epicardium and myocardium, the former producing factors required for myocardial growth. One of these factors is RA, the synthesis of which is reduced in *Wt1*-deleted epicardium (Guadix et al., 2011). This can be explained by the finding that WT1 is required for full transcriptional activation of the gene encoding

RALDH2 (ALDH1A2), a key component of the RA synthetic pathway (Guadix et al., 2011). WT1 also represses, directly and indirectly through IRF7, the inhibitory chemokines CXCL10 and CCL5 that inhibit epicardial cell migration and myocardial proliferation, respectively (Velecela et al., 2013). Another key upstream factor in coronary vascular development is the neurotrophin receptor TRKB (NTRK2), which appears to be a direct WT1 transcriptional target in the epicardium (Wagner et al., 2005a).

As mentioned above, *WT1* mutations can also lead to CDH, which is perhaps the most common serious birth defect, affecting 1 in 3000 births. CDH is characterised by incomplete formation or muscularisation of the diaphragm, which leads to herniation of the stomach, spleen, liver or intestines into the pulmonary cavities. The most prevalent form, observed in 90% of cases, is known as Bochdalek-type CDH and is characterised by a defect in the posterolateral area of the diaphragm, which is mostly lateralised to the left side. New insight into the mechanisms underpinning CDH has come from a recent study in which *Wt1* was deleted in the mesenchyme of the septum transversum (the tissue that gives rise to the diaphragm) of mice (Carmona et al., 2017). These mutant mice develop CDH with characteristics of the Bochdalek form. Using lineage tracing, it was shown that the post-hepatic plate coelomic epithelium normally gives rise to mesenchyme that populates the pleuroperitoneal folds, thus isolating the pleural cavities prior to the migration of the somatic myoblasts. However, when *Wt1* is deleted from this region this process fails, seemingly owing to defective EMT. It has been known for some time that normal diaphragm development requires RA and that a deficit of RA can lead to CDH (Sugimoto et al., 2008). The recent Carmona et al. (2017) study showed that, as with the epicardium, the levels of RALDH2 are reduced specifically in the post-hepatic mesenchymal plate of the mutant mice. Importantly, supplementation of the maternal diet with RA can partially rescue the phenotype.

### WT1-expressing mesothelium: a source of mesenchymal progenitors

A significant proportion of the vertebrate body comprises polarised epithelial cells, while another major component is non-polarised, more motile mesenchymal cells. These two cell types can switch between types via the processes of EMT and MET, and waves of EMT and MET are indeed vital for development. However, although often underappreciated, there are also stable cell populations that have an intermediate epithelial/mesenchyme state (Chau and Hastie, 2012). These cells are polarised, have adherens junctions but express high levels of mesenchymal markers including vimentin. Pre-eminent amongst these, as the name suggests, is the mesothelium that lines the body cavity and the organs therein. The mesothelium protects tissues from adhesion and plays key roles in fluid transport and inflammation (Kawanishi, 2016). However, over the past decade it has become clear that mesothelia are also a source of mesenchymal progenitors for diverse cell types within tissues, including fibroblasts and more specialised cells. In addition, it has been shown that WT1 is expressed at high levels in the proliferating coelomic epithelium and its mesothelial derivatives during development. Using lineage tracing, via a *Wt1* locus-driven Cre recombinase to activate a reporter in mesothelial cells and their progeny (Zhou et al., 2008), the identity and fate of such mesothelial-derived cells has been investigated.

As discussed above, a role for the mesothelium as a source of mesenchymal progenitors was first shown in the heart, where the epicardium produces progenitors for vascular smooth muscle and some endothelium. Soon after, it was shown that the serosal mesothelium is a major source of progenitors for the smooth muscle component of the gut and lung vasculature (Wilm et al., 2005; Que et al., 2008). Furthermore, the lung mesothelium is a source of progenitors for endothelial cells, bronchial musculature and tracheal and bronchial cartilage (Cano et al., 2013), while in the liver, the mesothelium is the source of a subset of hepatic stellate cells that play a key role in tissue fibrosis (Asahina et al., 2011) (Fig. 3). WT1-expressing mesothelium also produces progenitors for the interstitial cells of Cajal, the intestinal pace makers (Carmona et al., 2013) (Fig. 3). Perhaps most surprising was the finding that visceral fat depots have a WT1-expressing mesothelial lining that provides progenitors for a significant proportion of visceral white adipose tissue (WAT) (Chau et al., 2014) (Fig. 3). There are two major classes of WAT: visceral and subcutaneous. Excess visceral fat predisposes to major diseases including heart disease, type 2 diabetes and cancer, whereas subcutaneous fat is thought to be protective. The nature and origin of progenitors for subcutaneous WAT and visceral fat depots have been the subject of much speculation (Billon and Dani, 2012), but it has now been shown that a significant proportion of all visceral WAT, but not subcutaneous WAT, arises from WT1-expressing progenitors and that these arise from the mesothelium (Chau et al., 2014) (Fig. 3).

In some tissues, WT1 expression is downregulated towards the end of gestation or postnatally; the timing of this downregulation varies depending on the organ, e.g. it is fast in the lungs (Cano et al., 2016), occurs at an intermediate rate in the heart (Smart et al., 2011) and is slow in the liver (Ijpenberg et al., 2007). Potentially interesting from a regenerative medicine perspective is that WT1 expression is reactivated in the adult epicardium following cardiac ischaemia, and this is associated with epicardial cell proliferation and the production of new EPDCs, which have the potential to generate new coronary vasculature and, controversially, cardiomyocytes (Smart et al., 2011).

It is also of interest that the other two major differentiated cell types that express WT1 at high levels – the kidney podocytes and gonadal Sertoli cells – are also epithelial with mesenchymal properties. It is unclear whether mutation of *WT1* or other stresses leads to an epithelial-mesenchymal imbalance in these cells.

### WT1 in the nervous system: key roles in sensory neuron differentiation

WT1 expression is not restricted to the mesoderm and its derivatives during embryonic development. It is also expressed in a small number of symmetrically placed neurons in the ventral spinal cord, the roof of the fourth ventricle of the brain and the developing sensory nervous system (Armstrong et al., 1993). Although the functional significance of WT1 expression in the ventricle and spinal cord is yet to be revealed, it has been shown that WT1 is essential for the development of retinal, olfactory and taste bud neurons, with the +KTS and −KTS isoforms appearing to play different roles.

In the developing retina, for instance, WT1 expression becomes restricted to the presumptive retinal ganglion layer and is absent from adult retinas (Wagner et al., 2002a). *Wt1*$^{-/-}$ mice exhibit much thinner retinas than control animals, and the number of proliferating cells in E12 mutant embryos is reduced by 90% relative to wild-type controls (Wagner et al., 2002a). Furthermore, a significant proportion of retinal ganglion cells is lost by apoptosis in mutant embryos, and the growth of optic nerve fibres is disturbed. This phenotype is reminiscent of that observed in knockouts for the *Pou4f2* transcription factor (Gan et al., 1996). Accordingly, *Pou4f2* transcript levels are reduced dramatically in *Wt1* mutant retinas and
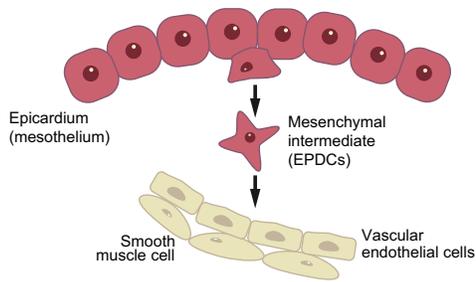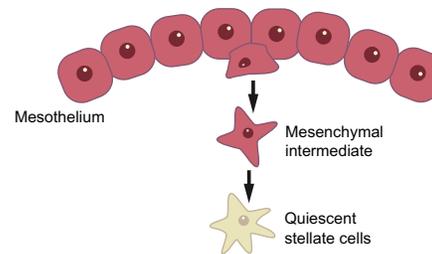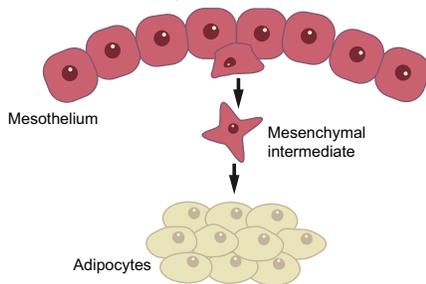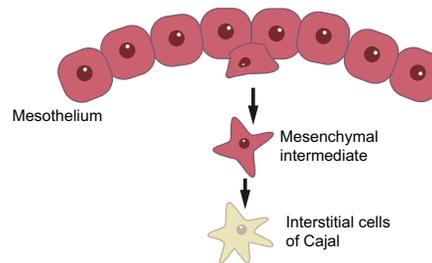
**Fig. 3. Specialised cell types arising from the mesothelium.** WT1 expression (red) is observed in the mesothelium and the mesenchymal progenitors it produces, but not in the differentiated progeny of these progenitors. For instance, WT1 is expressed in: (A) the mesothelial lining of the heart (the epicardium) and epicardium-derived cells (EPDCs); (B) the liver mesothelium and its mesenchymal progenitors that give rise to a subset of hepatic stellate cells; (C) the mesothelial lining of visceral white adipose tissue and the mesenchymal progenitors that give rise to adipocytes; and (D) the intestinal mesothelium and its progenitors that form interstitial cells of Cajal.

WT1 was shown to transactivate the *Pou4f2* promoter (Wagner et al., 2002a). Subsequently, it was shown that the retinal defects are more severe in mice lacking the −KTS than the +KTS isoforms (Wagner et al., 2005b).

WT1 is also expressed in the developing olfactory epithelium from E9.5 to E18.5, and both *Wt1* null mice and those specifically lacking +KTS isoforms exhibit a thinner olfactory epithelium than wild-type animals (Wagner et al., 2002a). By contrast, animals lacking −KTS isoforms appear to have normal olfactory epithelia. Mechanistically, it was shown that the expression of two key transcription factors with crucial roles in olfactory neuron development, MASH1 (ASCL1) and neurogenin 1, is reduced in *WT1* +KTS mutants. Moreover, the +KTS but not −KTS isoforms can induce the expression of these two neurogenic genes.

More recently, it was shown that the role of WT1 in sensory system development also extends to taste buds (Gao et al., 2014). In mammals, taste buds are located in specialised areas called papillae, of which there are three types: fungiform, foliate and circumvallate (CV). WT1 is expressed in the developing CV papillae located on the back of the tongue, and this expression continues into adult life, principally in taste buds. In mice lacking WT1, the CV papillae fail to form and the levels of three signalling molecules implicated in gustatory development (BMP4, PTCH1 and LEF1) are reduced. In line with this, WT1 was shown to bind to the promoter regions of the endogenous *Ptch1* and *Lef1* genes and their expression was shown to be dependent on WT1.

## WT1 in adult tissue homeostasis and disease

Although early studies established that WT1 plays crucial roles during development, it was not clear whether the gene continues to function in adult life and whether it is implicated in adult disease. WT1 continues to be expressed in just a few locations in the adult mouse: kidney podocytes, supporting gonadal cells, the mesothelial lining of organs and 1% of bone marrow cells. To address the continuing requirement for WT1 into adult life, a ubiquitous deletion of the gene was induced in 6-week-old mice (Chau et al.,

2011). The results were dramatic and unanticipated: the mice died ~10 days after the initiation of *Wt1* deletion. These animals suffered from severe glomerulosclerosis with loss of podocyte foot processes, atrophy of the spleen and exocrine pancreas, and widespread fat and bone loss. Although the mechanisms underlying these dramatic phenotypes remain unclear, it was hypothesised that they reflect a combination of systemic, local paracrine and cell-autonomous factors operating downstream of WT1 (Chau and Hastie, 2012). Proteomic analysis revealed a 95% reduction in circulating IGF1 levels in these mice (Chau et al., 2011). As IGF1 regulates both bone and fat growth, it is reasonable to conclude that reduction in this key signalling molecule contributes to the widespread bone and fat reduction. However, the fat loss might also reflect a cell-autonomous role for WT1 as it continues to be expressed in visceral WAT progenitors in adult life. Furthermore, preliminary evidence suggests that WT1 deletion leads to a reduction in these progenitors (Chau et al., 2014).

A rare population of WT1-expressing cells also persists in the cortex of the adult adrenal gland, and lineage tracing has shown that these cells are able to differentiate into steroidogenic cells throughout life (Bandiera et al., 2013). These progenitors also express GATA4, GLI1 and TCF21. Importantly, gonadectomy activated this cell population, leading to their differentiation into gonadal steroidogenic tissue (Bandiera et al., 2013). This defines a novel paradigm whereby a response to organ loss is the recreation of hormone-producing cells at an ectopic site.

WT1 is also expressed at high levels in a variety of adult epithelial tumours and in some leukaemias, and this has led to much endeavour in the pursuit of immune therapies targeting WT1 epitopes (Nishida and Sugiyama, 2016). In the majority of cases, it is still unclear whether WT1 overexpression is helping to drive the carcinogenic state or is merely a consequence. However, in an shRNA screen for genes cooperating with KRAS, WT1 was identified as a key regulator of oncogenesis and senescence downstream of KRAS (Vicent et al., 2010). Moreover, *Wt1* loss reduces tumour burden in a mouse model of KRAS-driven lung

cancer (Vicent et al., 2010). Consistent with this, *Wt1* loss was shown to lead to decreased proliferation and to senescence in a human lung cancer cell line dependent on oncogenic KRAS (Vicent et al., 2010). Much evidence has been published to suggest that WT1 can increase the survival of various cancer cells through anti-apoptotic functions. For example, a mechanism by which WT1 anti-apoptotic function can be modulated in response to cytotoxic drugs in cell lines and cell-free systems has been reported (Hartkamp et al., 2010). WT1 is cleaved into unstable fragments by the protease HTRA2, the expression of which is induced by anti-cancer drugs. This leads to downregulation of WT1 and a resulting increase in cell survival through upregulation of C-MYC and JUNB, which are normally repressed by WT1. It has also been reported (Wagner et al., 2014) that WT1 is often expressed in the vasculature and stroma of a variety of adult cancers, rather than in the epithelial components themselves. Using lung cancer and melanoma xenograft models, it was shown that the host vasculature and stroma invading the tumour express WT1 but that the nearby vasculature and stroma do not express detectable levels. In addition, when Tie2-Cre was used to delete *Wt1* in the host endothelial, haematopoeitic and myeloid suppressor cells, tumour growth and metastases are impaired, and pre-existing tumours regress (Wagner et al., 2014). Mechanistically, WT1 was shown to transcriptionally activate both *Pecam1* and *c-Kit* explaining, at least in part, its proangiogenic functions.

Surprisingly little is known about the pathways and factors that act upstream to regulate WT1 expression. However, it has been shown that WT1 expression is induced in coronary vasculature following ischaemia (Wagner et al., 2002b). It was then reported that WT1 is induced by hypoxia, with compelling evidence showing that the hypoxia-inducible factor HIF1 transactivates WT1 directly (Wagner et al., 2003). The possibility that WT1 plays a physiological role in response to ischaemia remains to be explored.

### Molecular mechanisms of WT1 action

Although there is much evidence that WT1 functions as a transcription factor, several studies support post-transcriptional functions, via RNA interactions, for WT1. Furthermore, as summarised below, the data suggest that the −KTS isoforms may function mostly as transcriptional regulators, whereas the +KTS isoforms act predominantly post-transcriptionally, although this is not black and white as the +KTS isoforms can bind DNA and regulate transcription and the −KTS isoforms can bind RNA and shuttle to the cytoplasm. Genetic studies have shown that the two isoforms function differently at the later stages of genitourinary development and in sensory organ differentiation (Hammes et al., 2001; Wagner et al., 2005b). However, mice lacking the −KTS or +KTS isoforms do not, unlike null mice, exhibit defects in the early stages of genitourinary development or cardiovascular development (Hammes et al., 2001). This suggests that the two isoforms perform identical or compensatory functions during these processes.

### Effects on transcription and chromatin

WT1 is a transcription factor that binds to DNA targets through its four zinc fingers. Early cell-free studies showed that WT1, the −KTS isoforms in particular, binds to a consensus site, 5′-GCGGGGGCG-3′ (Rauscher et al., 1990). This consensus is identical to that found for EGR1, which has only three zinc fingers, very similar to WT1 zinc fingers 2-4. Crystallographic and NMR analyses of WT1 zinc finger interaction with DNA confirmed that only zinc fingers 2-4 insert deeply into the major groove, where they make base-specific contacts (Stoll et al., 2007). The first zinc finger

does not contribute to binding specificity but helps anchor WT1 to the DNA. Cell-free studies also showed that the +KTS isoform binds to a slightly different sequence (Bickmore et al., 1992). A molecular explanation for this came through NMR analysis, which showed that the KTS insertion increases the flexibility of the linker between zinc fingers 3 and 4, thus abrogating binding of finger 4 to its cognate site in the major groove (Laity et al., 2000).

Recent studies identifying physiological target genes (summarised in Table 1) using ChIP-ChIP and ChIP-seq have brought much clarity to this area. Several slightly different genomic binding sites have been identified, one identical to that shown to interact with the +KTS isoforms *in vitro* (Motamedi et al., 2014; Kann et al., 2015a; Lefebvre et al., 2015; Dong et al., 2015). So far, most of the validated transcriptional targets appear to be −KTS specific, with few genuine +KTS targets identified. Through ChIP-ChIP and ChIP-seq, several thousand potential WT1 transcriptional targets during kidney development have been identified. However, so far, only a small subset of these have been validated as genuine targets, the expression of which changes as a result of WT1 mutation in developing tissue.

WT1 can act as either a transcriptional activator or repressor (Fig. 4A) depending on its binding partners (for a review see Toska and Roberts, 2014). Accordingly, there are repressor and activator regulatory domains at residues 71-101 and 180-250, respectively (Fig. 1). The repression domain was used to isolate a novel co-repressor, BASP1, that clearly plays a role in the downregulation of some WT1 targets *in vivo* (Carpenter et al., 2004; Essafi et al., 2011). Several other co-activators, co-repressors and transcription factors, including p53 (TRP53), have been shown to interact with WT1, modulating its target sites and activities, at least in cell lines (Toska and Roberts, 2014). Furthermore, several post-translational modifications, including sumoylation and phosphorylation, have been shown to regulate WT1 subcellular localisation and function (reviewed by Toska and Roberts, 2014).

Recent studies have revealed that WT1 can also regulate gene expression by modulating the epigenetic landscape, and this is relevant for disease progression (Rampal et al., 2014; Wang et al., 2015). Loss-of-function *WT1* mutations are observed in a subset of acute myeloid leukaemia (AML), as are mutations in the genes encoding TET family proteins, including TET2 and IDH1/2 (Rampal et al., 2014; Wang et al., 2015). These enzymes are involved in converting 5-methylcytosine (5mC) in the genome to 5-hydroxymethylcytosine (5hmC), often leading to increased gene expression. Importantly, it was noted that *WT1* and TET gene mutations are mutually exclusive and this led to the hypothesis that WT1 might work in the same pathway as TETs. Indeed, it was shown that WT1 interacts with TET2, recruiting it to target genes to activate their expression (Rampal et al., 2014; Wang et al., 2015). Accordingly, loss of function of WT1, TET2 or TET3 leads to a reduction in 5hmC and a similar impaired haematopoietic differentiation phenotype.

WT1 also seems to modulate the state of chromatin domains far beyond its binding sites. For example, WT1 is a transcriptional activator of *Wnt4* in developing kidney mesenchyme undergoing MET, but a repressor of *Wnt4* in epicardial cells poised for EMT, and these roles appear to involve global changes in chromatin access (Essafi et al., 2011). Transactivation and repression require the co-activator CBP/p300 (CREBBP/EP300) and co-repressor BASP1, respectively (Essafi et al., 2011). Deletion of *Wt1* in kidney mesenchyme leads to loss of *Wnt4* expression and the switching of the whole 130 kb *Wnt4* locus, which lies between two CTCF binding sites, from an active chromatin configuration to a repressed

**Table 1. Validated WT1 transcriptional targets that are relevant for developmental processes in different tissues**

| Gene | Cell/tissue type | Activated (A) or repressed (R) | Biochemical activity of protein | References |
|---|---|---|---|---|
| Fgf16/20 | Kidney mesenchyme/nephron progenitors | A | Signalling upstream of MAP kinase/ PI3 kinase | Motamedi et al., 2014 |
| Bmper | " | A | Inhibits BMP signalling | Motamedi et al., 2014 |
| Bmp7 | " | A | Usually signals via SMADS | Hartwig et al., 2010 |
| Sal1 | " | A | Transcription factor | Hartwig et al., 2010 |
| Pax2 | " | A | Transcription factor | Hartwig et al., 2010 |
| Heyl | " | A | Transcription factor/Notch signalling | Hartwig et al., 2010 |
| Cxxc5 | " | A | Transcription factor | Hartwig et al., 2010 |
| Lsp1 | " | A | F-actin binding | Hartwig et al., 2010 |
| Pbx2 | " | A | Transcription factor | Hartwig et al., 2010 |
| Plxdc2 | " | A | Plexin domain-containing protein | Hartwig et al., 2010 |
| Rps6ka3 | " | A | Protein kinase | Hartwig et al., 2010 |
| Scx | " | A | Transcription factor | Hartwig et al., 2010 |
| Sox11 | " | A | Transcription factor | Hartwig et al., 2010 |
| Gas1 | " | A | FGF signalling | Kann et al., 2015b |
| Wnt4 | " | A | Signalling via NFAT | Sim et al., 2002; Essafi et al., 2011 |
| Podxl | Podocytes | A | Anti-adhesive membrane protein | Palmer et al., 2001 |
| nephrin (Nphs1) | " | A | Renal filtration barrier | Wagner et al., 2004; Guo et al., 2004 |
| Magi2 | " | A | Assembly of slit diaphragm | Dong et al., 2015; Lefebvre et al., 2015; Kann et al., 2015a |
| Nphs2 | " | A | Component of slit diaphragm | Dong et al., 2015; Lefebvre et al., 2015; Kann et al., 2015a |
| Mafb | " | A | Transcription factor | Dong et al., 2015 |
| Scel | " | A | In skin cornified envelope | Ratelade et al., 2010 |
| Sulf1 | " | A | Sulfatase | Ratelade et al., 2010; Schumacher et al., 2011 |
| Snai1 | Epicardium/embryoid bodies | A | Activates EMT | Martínez-Estrada et al., 2010 |
| E-cadherin (Cdh1) | Epicardium | R | Epithelial cell adhesion | Martínez-Estrada et al., 2010 |
| Wnt4 | " | R | Signalling | Essafi et al., 2011 |
| Raldh2 (Aldh1a2) | " | R | Retinoic acid synthesis | Guadix et al., 2011 |
| Cxcl10 | " | R | Chemokine | Velecela et al., 2013 |
| Ccl5 | " | R | Chemokine | Velecela et al., 2013 |
| Sf1 | Adrenal-gonadal primordium | A | Transcription factor | Wilhelm and Englert, 2002 |
| Gli1 | Developing adrenal gland | | Transcription factor in SHH pathway | Bandiera et al., 2013 |
| Tcf21 | " | | Transcription factor | Bandiera et al., 2013 |
| Sry | Developing male gonad | A | Transcription factor | Miyamoto et al., 2008 |
| Lef1 | Posterior taste field | A | Transcription factor | Gao et al., 2014 |
| Ptch1 | " | A | Receptor for SHH | Gao et al., 2014 |
| Bmp4 | " | A | Signalling via SMADS | Gao et al., 2014 |

In all cases, WT1 has been shown to interact with these genes in the appropriate tissue by ChIP, whether in the primary paper listed or in a subsequent publication. In all cases, Wt1 mutation or overexpression leads to a change in the expression of the target gene in the appropriate tissue.

state. Conversely, deletion of *Wt1* in the epicardium switches the chromatin between these two CTCF sites from a repressed to an active conformation and this is associated with activation of *Wnt4* expression. This switching of states is referred to as 'chromatin flip-flop' (Essafi et al., 2011). It has been proposed that WT1 activates the *Wnt4* domain in the kidney mesenchyme to allow access of other transcriptional activators of *Wnt4*, including β-catenin and SIX2, whereas in the epicardium WT1 keeps the locus in a silent state, preventing access of *Wnt4* activators.

**Effects through RNA binding and post-transcriptional regulation**
The first indication that WT1 might also function post-transcriptionally came with the observation that the +KTS isoform specifically localises and interacts with splice factors in kidney cells (Larsson et al., 1995). It was subsequently revealed that WT1 can be incorporated into functional spliceosomes in cell-free systems (Davies et al., 1998). These observations were soon followed up by experiments showing that both major WT1 isoforms, but not EGR1, can bind RNA derived from exon 2 of *Igf2*, the +KTS isoform showing higher affinity (Caricasole et al., 1996). The RNA interaction occurred through the zinc fingers

and required the atypical first zinc finger. Subsequently, it was shown that both zinc finger 1 and the KTS insertion are important for WT1-RNA interactions in *Xenopus* oocytes (Ladomery et al., 2003). Using systematic evolution of ligands by exponential enrichment (SELEX), three high-affinity RNA-binding motifs have been identified for the WT1-KTS isoform (Bardeesy and Pelletier, 1998), and structural studies have shown that both the sequence and secondary structure of RNA determine binding specificity and affinity for WT1 (Zhai et al., 2001). It has also been demonstrated that, although the majority of WT1 is in the nucleus, some is present in the cytoplasm, located on actively translating polysomes, with all isoforms shuttling between the nucleus and cytoplasm (Niksic et al., 2004). Furthermore, WT1, specifically the +KTS isoform, was shown to recruit a viral RNA segment to polysomes (Bor et al., 2006). Additional circumstantial evidence for post-transcriptional functions comes from reports that WT1 interacts with RNA-binding proteins, often in an isoform-dependent manner. These include the splice factors U2AF65 (U2AF2) (Davies et al., 1998) and RBM4 (Markus et al., 2006), HNRPU (HNRNPU) (Spraggon et al., 2007) and the Wilms' tumour associated protein WTAP (Little et al., 2000). The latter protein is very topical as it is an
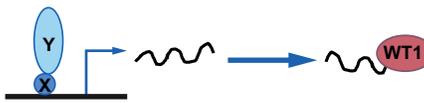
**A** Transcriptional regulation

**Activation**
Direct DNA binding
Interaction with other transcription factors
Formation of co-activator complexes
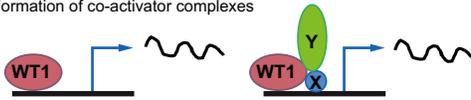
**Repression**
Direct DNA binding
Interaction with other transcription factors
Formation of co-repressor complexes
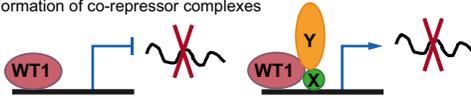
**B** Post-transcriptional regulation

**RNA stability**
Direct RNA binding
Interaction with 3′UTR of mRNAs

**Potential roles in splicing and translation**

**Fig. 4. The role of WT1 in transcriptional and post-transcriptional gene regulation.** (A) WT1 can function at the transcriptional level, acting to either activate or repress transcription. (B) WT1 can also function post-transcriptionally by regulating RNA stability and possibly RNA degradation, splicing and translation.

essential component of the complex that carries out m6A RNA methylation (Ping et al., 2014), linking m6A methylation to splicing.

In spite of all this circumstantial evidence, experiments demonstrating a post-transcriptional role for WT1 through RNA interaction have been lacking. The field has been hampered by a lack of identification of physiological RNA targets. This has now been addressed to some degree by a recent study that used UV crosslinking and sequencing to identify endogenous RNAs interacting with WT1 in M15 kidney cells and partially differentiated embryonic stem cells (Bharathavikru et al., 2017). This analysis showed that WT1 interacts with and regulates sets of mRNAs involved in appropriate developmental processes, including cell adhesion, cell migration, kidney and cardiovascular development. The interaction is mainly at the 3′UTR and often with secondary structures, supporting the NMR analysis. Transcriptomic and functional experiments support a model in which WT1 controls the stability of these mRNAs, most of which do not appear to be transcriptional targets. However, some genes, for example podocalyxin, are regulated by WT1 at the transcriptional and post-transcriptional levels, raising the possibility that WT1 might chaperone single genes through multiple stages of the gene expression cascade (Fig. 4B). Given these new findings, it will be essential to measure transcription rates, rather than just steady-state levels of mRNA, to prove that targets are regulated at the transcriptional level when WT1 levels are altered.

An intriguing non-canonical function for WT1 has also been identified (Shandilya et al., 2014), revealing that it regulates the mitotic checkpoint in cultured cell lines through direct interaction with the spindle assembly checkpoint protein MAD2. Clearly, this novel observation could have implications for cancers arising through *WT1* mutation or misregulation, but the relevance of these findings in an organism context still need to be ascertained.

## Conclusions

WT1 has been a valuable tool for understanding complex aspects of tissue development and homeostasis while also revealing some of the mechanisms that underpin human disease. Recent studies showing that WT1 regulates mRNA turnover through 3′ UTR interactions dovetail well with new findings highlighting the importance of microRNA processing pathway genes in the aetiology of Wilms' tumour (Astuti et al., 2012; Wegert et al., 2015). Further studies will be required to elucidate the physiological significance and the spectrum of post-transcriptional mechanisms by which WT1 mediates its myriad effects.

At present, we are very much underestimating the potential complexity of WT1 itself, never mind the complex networks in which it functions, warranting a more systems-based approach. For

example, how do the two major isoforms overlap or differ in their functions, and what is the role of the other mammalian-specific isoforms? In this regard, we must take into consideration the fact that all the WT1 isoforms have the potential to dimerize, so the homodimers and heterodimers might have very different functions. Moreover, we have little knowledge of WT1 post-translational modifications and whether or how these might affect WT1 activity. We also know very little about the factors that operate upstream to regulate WT1 expression.

Most experiments so far have shown that WT1 is essential in cellular or developmental processes but have not addressed its instructive roles. Much more work will be needed to identify the cell-autonomous and non-cell-autonomous mechanisms by which WT1 regulates homeostasis. It will be fascinating to study how the different WT1-expressing mesenchymal progenitors respond to tissue damage as well as cellular and environmental cues. Finally, it will be important to determine with rigour whether WT1 plays a significant role in adult cancer and whether this information can be translated into clinical benefit and therapeutic applications.

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