Life, sex, and WT1 isoforms--three amino acids can make all the difference

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Life, Sex, and WT1 Isoforms—Three Amino Acids Can Make All the Difference

Minireview

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Perhaps the biggest surprise to come from the human genome sequence is the low gene number estimate relative to expectation (International Human Genome Sequencing Consortium, 2001; Venter et al., 2001). This has now turned the spotlight on posttranscriptional events as a means for generating increased proteome complexity. Many genes encode multiple protein isoforms, usually through alternative splicing but also through the use of alternative promoters, alternative translational start sites, or RNA editing. We are now faced with the huge task of identifying the functions of all these protein isoforms that may number several hundred thousand in total.

The situation is epitomized by the Wilms' tumor suppressor gene, *WT1* (for a recent review see Little et al., 1999). Mutations in this gene in humans may lead to the eponymous childhood kidney cancer, to severe kidney disease (glomerular nephropathy) or gonadal dysgenesis, often in the form of male-female sex reversal. Studies in mouse have shown that WT1 is essential for the development of the kidneys, gonads, and several other mesodermally derived tissues (Herzer et al., 1999; Little et al., 1999; Moore et al., 1999).

In all mammals investigated, the *WT1* gene may encode up to 24 protein isoforms through a combination of alternative splicing, alternative translational start sites and RNA editing. All these proteins share four C-terminal C_2H_2 zinc fingers and an N-terminal proline/glutamine-rich regulatory region. The situation in fish—and probably other nonmammalian vertebrates—is much simpler. They appear to express only two WT1 isoforms, those differing by just three amino acids, KTS, inserted between zinc fingers 3 and 4 by exon extension through an alternative donor splice site (Figure 1).

The conservation of these two isoforms over 450 million years of evolution suggests that they may perform some distinct functions. As described in a recent *Cell* paper, Hammes et al. have tested this hypothesis by examining mice that can produce only WT1 (−KTS) or WT1 (+KTS) variants, respectively (Hammes et al., 2001). These studies provide conclusive evidence that the two classes of isoform do perform distinct functions in genitourinary development, but also that they can substitute for each other in a number of developmental processes. Perhaps most interesting is the finding that the +KTS variants are essential for the male sex determination program. Last but not least, the authors have generated an excellent animal model for studying the mechanisms underlying the nephropathy associated with human Frasier syndrome and Denys Drash syndrome (DDS).

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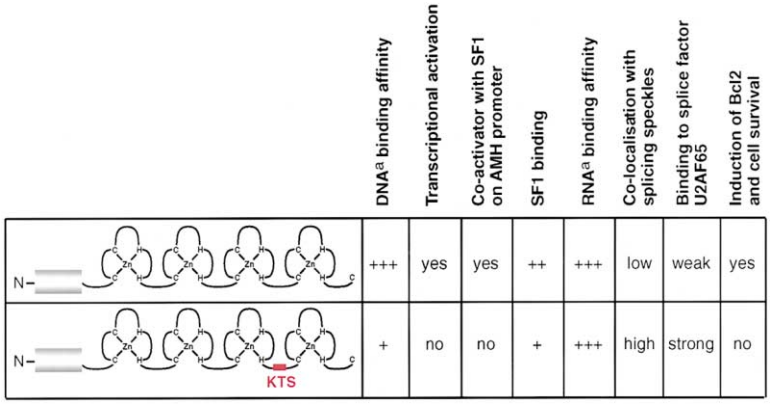
The Role of WT1 in Genitourinary Development: The Story So Far

Previous studies in human and mouse have demonstrated that WT1 is essential for the development of kidneys and gonads. The metanephric or permanent kidney develops through the interaction of two tissues, the mesonephric duct and metanephric mesenchyme, both derivatives of the intermediate mesoderm. Induction of the metanephros in the mouse starts at around E10.5 when a signal from the mesenchyme induces ureteric buds to grow out of the mesonephric duct and invade the mesenchyme. Signals from the ureteric buds then induce the metanephric mesenchyme to differentiate into epithelial cells that are the precursors of the nephron. Following a series of patterning and morphogenetic events, these epithelia organize into the components of the nephron, the proximal and distal tubules, the loop of Henle and the glomerulus. Signals from the mesenchyme on the other hand induce the buds to bifurcate. Eventually, the nephron will fuse with the buds which will become the collecting ducts. WT1 is expressed at low levels in the undifferentiated mesenchyme and levels increase dramatically during induction. By the time the nephron intermediate or S-shaped body has formed, WT1 expression becomes restricted to the region that is destined to become the visceral epithelium (podocytes) of the glomerulus. These podocytes extend foot processes which are linked by the slit membranes that play the key role in ultrafiltration. Expression persists in mature podocytes throughout life. *Wt1* knockout mice have no kidneys at all and the mesenchymal cells die through apoptosis.

The gonads also derive from the intermediate mesoderm though much less is known about induction events. WT1 is expressed in the undetermined genital ridge and then at high levels in the Sertoli cells of the testis and granulosa cells of the ovary. No gonads are detected in *Wt1* null mice. As the null mice completely lack kidneys and gonads, it has not been possible to define the roles played by WT1 in the later stages of nephrogenesis and gonad formation, including sex determination. Humans with *WT1* mutations leading to DDS and Frasier syndrome do have podocyte abnormalities and gonadal dysgenesis often including sex reversal, arguing for a role in these processes. However, the exact roles played by the WT1 isoforms and molecular mechanisms involved cannot be elucidated in human studies. The new mouse mutants are now starting to clarify the picture (Hammes et al., 2001).

Wt1 null mice die at midgestation and this appears to be due to a cardiac abnormality arising through incomplete formation of the epicardium. Recent studies have shown that the null mice also lack adrenal glands and spleen (Moore et al., 1999; Herzer et al., 1999). The +KTS and −KTS variants appear to be expressed at constant



	DNA[a] binding affinity	Transcriptional activation	Co-activator with SF1 on AMH promoter	SF1 binding	RNA[a] binding affinity	Co-localisation with splicing speckles	Binding to splice factor U2AF65	Induction of Bcl2 and cell survival
	+++	yes	yes	++	+++	low	weak	yes
	+	no	no	+	+++	high	strong	no

Figure 1. Different Properties of −KTS and +KTS WT1 Isoforms

[a]Relative RNA and DNA binding affinities depend on the particular sequence being investigated though this reflects the overall trend. This is a selective list chosen to reflect the main thrust of the review and is not meant to be comprehensive.

ratios throughout all these tissues and at various stages of development.

The Different Properties of WT1 Proteins Containing or Lacking the KTS Insert

To appreciate the new findings, we must consider them in the light of previous genetic, biochemical and cellular evidence supporting distinct roles for these two classes of WT1. Prior to this new study, the most compelling evidence came from human genetics. Children with Frasier syndrome develop a relatively late onset glomerular nephropathy and XY individuals have dysgenetic gonads, often showing female external features. Most children with this condition have heterozygous *WT1* mutations that disrupt the splice site normally leading to the KTS insertion (Little et al., 1999). Thus, only the −KTS isoforms are produced from the mutant allele, resulting in a predictable reduction in the ratio of WT1 (+KTS) to WT1 (−KTS) variants. The inescapable conclusion from the human studies is that the two isoform classes are likely to have some nonoverlapping functions.

Initially, it was assumed that WT1 functions only as a transcription factor. However, a body of evidence has accumulated to support an additional role in RNA processing (Little et al., 1999; Davies et al., 1998). Figure 1 summarizes some different properties of +KTS and −KTS variant proteins. Taken together the evidence supports a role for −KTS proteins in transcription and +KTS proteins in RNA processing.

While much effort has been expended on pursuing a transcriptional repressor function for WT1, it now seems that transcriptional activation is much more likely to be physiologically relevant (English and Licht, 1999). −KTS isoforms have been shown to activate a number of endogenous genes in cell culture as well as to transactivate the promoters of these genes. These include the *Bcl2* gene that plays a pivotal role in inhibiting apoptosis; WT1 (−KTS) simultaneously induces Bcl2 expression and cell survival in the human rhabdoid cell line G401 (Mayo et al., 1999). Consistent with its role as a tumor suppressor, WT1 (−KTS) also appears to directly activate the *p21* gene, leading to G1 cell cycle arrest. WT1 (−KTS) can also induce the expression of the *amphiregulin* gene which encodes an EGF-like protein coexpressed with WT1 during kidney development (Lee et al., 1999). A fourth likely target is the *DAX-1* gene which, when overexpressed in mice or humans, induces male-to-female sex reversal (Kim et al., 1999). A particularly compelling candidate WT1 (−KTS) target in light of the new studies is Sry, the testis determining factor (Hossain and Saunders, 2001). WT1 (+KTS) variants on the other hand were unable to induce the expression of any of these genes or to transactivate their promoters. Furthermore, induction of +KTS variants in human SAOS cells had no detectable influence on the levels of any mRNAs examined using a microarray containing 8,000 genes (Lee et al., 1999). Undoubtedly, the inability of WT1 (+KTS) variants to function as transcriptional activators is due mainly to their weaker interaction with DNA.

The New Findings: +KTS and −KTS WT1 Have Overlapping and Distinct Functions in Genitourinary Development

Hammes et al. have used gene targeting to create mutant mice that can only express WT1 (+KTS) or WT1 (−KTS) forms, respectively. The latter mice (Frasier mice) were created by mimicking a mutation found in humans with Frasier syndrome. The first surprise, given the differences discussed above, is that both sets of mice were much less severely affected than homozygous null animals. Hence in both cases the homozygotes survived through to birth so we can conclude that the heart develops normally—or at least to a degree that is compatible with perinatal survival. Second, unlike the null animals, nephrogenic induction seemed to initiate normally and almost complete nephrons were produced. Furthermore, the adrenal glands and spleen were normal. So in all these respects, heart development, early nephrogenesis, spleen, and adrenal development, WT1 (+KTS) and WT1 (−KTS) seem to perform identical functions. It is only at the later stages of genitourinary development that the differences between the two sets of mice become obvious.

Together the knockouts now provide concrete evidence that WT1 is required for the formation and structural integrity of the podocyte cells of the glomerulus. In each case severe impairment of kidney function led to death soon after birth. In the Frasier homozygotes the podocytes formed but their architecture was disrupted and foot process formation was impaired. Kidney development was much more severely affected in KTS homozygotes. The kidneys were hypodysplastic and the number of glomeruli was reduced. The glomeruli that did form were extremely abnormal; the expression of several podocyte markers was barely detectable, suggesting that WT1 (−KTS) is essential for the differentiation of the podocyte.

In the case of the gonad, a similar situation was observed. Remarkably all the homozygous XY Frasier mice developed as females. Hence WT1 (+KTS) is essential for the male determination program but not for formation of a female gonad. Homozygous KTS gonads on the other hand were much smaller, consisting mainly of undifferentiated mesenchyme and hardly any differentiated tissue. One possible explanation was the increase in apoptosis found in the anterior region of the gonad. It is difficult to assess whether −KTS variants are also required for male determination as there was very little differentiated tissue in XY KTS animals. However, male-specific markers were expressed in small clusters of cells, leading Hammes et al. to conclude that WT1 (−KTS) is unlikely to be required for male determination.

The assumption in all these experiments is that the phenotypes observed in the two knockouts are due to loss of − or +KTS variants. One caveat here is that there is a concomitant increase in the remaining variant and this overexpression could be causing the phenotype. This is particularly germane in Frasier homozygotes where the levels of WT1 (−KTS) have increased 2-fold. Although this scenario is formally possible, I feel it is unlikely for several reasons. First, in humans and mice a similar range of kidney and gonad phenotypes, including sex reversal, are observed in situations where overall WT1 levels are known to be reduced. Second, no studies have been described in which overexpression leads to any phenotype. Finally, in humans with Frasier syndrome, glomerular disease and sex reversal are observed when there is only likely to be a 50% increase in WT1 (−KTS) levels. This issue could be addressed by examining the phenotype of compound heterozygotes having the Frasier mutation opposite the *Wt1* null mutation. These mice would lack WT1 (+KTS) variants but express only the wild-type dose of WT1 (−KTS) variants.

WT1 in Development: Reconciling the Old and the New

The most surprising finding is that the + and −KTS isoforms appear to function identically through the early stages of nephrogenesis and in heart, adrenal, and spleen development. If the main function is at the transcriptional level, it is difficult to equate this with any of the known candidate target genes. As yet, +KTS proteins have not been shown to activate any genes transcriptionally, including those regulated by the −KTS forms. Perhaps this means that the relevant physiological targets may have very different binding sites to those identified so far that could accommodate both WT1 (+KTS) and WT1 (−KTS) variants. Alternatively, could each be working at the posttranscriptional level? Both + and −KTS isoforms can bind RNA, and, unlike the situation for DNA, may show similar affinities for the same sequence (Zhai et al., 2001). However, we would still have to explain the fact that the proteins appear to have different nuclear localization patterns. This idea has now received strong in vivo support from the new studies. In kidney nuclei from mice expressing only WT1 (+KTS), the protein gave a striking speckled pattern assumed to represent colocalization with splicing factors. On the other hand, in Frasier homozygotes the protein gave a much more diffuse pattern. One significant caveat here, and with respect to all the biochemical studies, is that WT1 molecules have been shown to dimerize in vitro and in the two-hybrid system. If this happens in vivo and there is heterodimerization between the + and −KTS isoforms, they could be influencing the location (and activities) of each other, something that is supported by the new findings and discussed by Hammes et al.

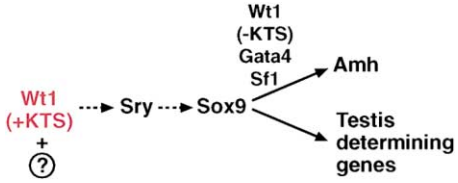


Figure 2. Molecular Pathways Controlling Sex Determination in XY Mammals

The figure is modified from Figure 1 of Koopman (2001). The dotted line from WT1 (+KTS) to Sry indicates that we know neither whether the interaction is direct nor whether it is at the transcriptional or posttranscriptional level.

One aspect that is consistent with previous biochemical and cellular findings is the increase in apoptosis in gonads and (presumably) kidneys of KTS mice that no doubt accounts in part for the hypodysplasia of both tissues. Thus, −KTS isoforms may well induce cell survival in vivo by activating Bcl2 as has been documented in cell culture studies (Mayo et al., 1999). This idea is reinforced by the phenotype of *Bcl2* null mice which also have hypodysplastic kidneys and reduced numbers of glomeruli (Veis et al., 1993).

Much interest has been aroused recently by new studies identifying some of the crucial components of the podocyte foot processes, in particular nephrin which constitutes the slit membrane through which ultrafiltration takes place. Mutations in nephrin and other components including α-actinin4, CD2AP, and podocin all lead to nephrotic syndromes, that in some cases share features with Frasier syndrome and DDS (Khoshnoodi and Tryggvason, 2001). It will be interesting to determine whether any of these genes are targets of WT1, particularly the +KTS variants. Heterozygous Frasier mice develop variable nephropathy including focal segmental glomerular sclerosis as seen in the human syndrome but also more severe diffuse mesangial sclerosis characteristic of DDS. These mouse models will be invaluable for dissecting the cellular and molecular mechanisms underlying these syndromes.

A Role for WT1 in the Male Determination Pathway

As reviewed recently in *Cell*, *Sry,* the mammalian testis determining gene, sits at the top of a gene cascade required for driving the undetermined genital ridge along the male pathway (Koopman, 2001). One of the crucial factors downstream of *Sry* is Sox9, which like Sry contains an HMG-like DNA binding domain (Figure 2). It is still unclear whether Sry is a direct activator of Sox9 expression. Sry exerts a number of cellular effects on the undetermined gonad including an increase in cellular proliferation and stimulation of Sertoli cell differentiation. In XX animals, DAX-1, an orphan nuclear receptor, acts as a repressor of male gene expression by binding to the related receptor Sf1.

One of the important gaps in understanding concerns the nature of factors operating upstream of Sry. Hammes

et al. now show that XY Frasier homozygous mice lacking WT1 (+KTS) isoforms develop along the female pathway. Furthermore, there is a dramatic reduction in Sry expression in these animals. So it appears that WT1 (+KTS) may be one of the crucial factors operating upstream of Sry (Figure 2).

That WT1 might act upstream of Sry was suggested previously by Hossain and Saunders (2001) who showed that WT1 (−KTS) but not WT1 (+KTS) could activate the endogenous *Sry* gene and transactivate its promoter. This seems to be at odds with the finding that mice lacking WT1 (+KTS) isoforms showed sex reversal and downregulation of *Sry*. However, it still remains to be seen whether WT1 (−KTS) activates Sry in vivo and one could envisage a scenario in which the two WT1 isoform types work together perhaps to increase Sry transcripts both at the transcriptional and posttranscriptional level.

Kim et al. (1999) proposed a compelling alternative mechanism to explain the sex reversal seen in Frasier syndrome, drawing on their finding that WT1 (−KTS) can activate Dax-1 expression. They suggested that the presumed increase in WT1 (−KTS) levels might induce Dax-1, leading to repression of the male phenotype. Support for the idea that Dax-1 is a physiological target has now come from analysis of KTS mice which show reduced levels of Dax-1 in the developing gonad. However, preliminary analysis by Hammes et al. (2001) suggests that Dax-1 levels are not increased in developing homozygous Frasier gonads, arguing against this hypothesis. As discussed above, more work will have to be done to determine whether overexpression of WT1 (−KTS) leads to any phenotype and in particular to sex reversal as posited by Kim et al. (1999).

Some recent findings raise the prospect of some shared molecular mechanisms operating in the sex determination pathways between flies and mammals, possibly involving alternative splicing. Last year a WT1 associating protein, WTAP, was identified (Little et al., 2000). WTAP and WT1 partially colocalize in splicing speckles and the two proteins interact in vitro and in vivo. Recently it has become apparent that WTAP is the mammalian homolog of *Drosophila* female-lethal-2-D (fl(2)d) which is required for the female-specific alternative splicing of sex-lethal (Sxl) pre-mRNA (Penalva et al., 2000). Sxl controls the processes of sex determination, sexual behavior, and dosage compensation and itself encodes a splice factor. Although there is no direct evidence showing that fl(2)d is a splice factor, the *fl(2)d* gene was picked up in a screen for loci that affect the splicing of *Ultrabithorax* mRNAs (Burnette et al., 1999); also the partial colocalization of WTAP with nuclear speckles would support such a role. Taken together, these findings support a role for WT1 in regulating sex determination through alternative splicing.

Perspectives

These new studies provide conclusive evidence that two alternative splice products differing by only three amino acids can have profoundly different biological functions. However, although the new findings are illuminating, the list of unanswered questions may now be longer than before; for example what are the molecular mechanisms and targets underlying both the shared and distinct functions of the +KTS and −KTS variants? Are they operating as transcription factors, in RNA processing or both, perhaps linking the two processes? Availability of the new mutant mice should help address these issues. It is not clear whether there are any other zinc finger proteins in which an insertion resulting from an alternative splice might shift the properties from those of a transcription factor toward a role in RNA metabolism. However, there are many examples where alternative splicing alters the DNA binding specificity and transcriptional targets of proteins. One that immediately comes to mind involves WT1's near neighbor PAX6 which is required for eye, nose, brain, and pancreas development. A conserved alternative splice which inserts an additional 14 amino acids into the PAX6 paired domain alters the DNA binding properties and likely target genes (Epstein et al., 1994). As with WT1, splice-site mutations that change the ratio of the two isoforms cause a distinct human syndrome. If these two examples are representative, we can confidently expect that functional proteome complexity will be much greater than gene number.

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