

# Frasier syndrome is caused by defective alternative splicing of *WT1* leading to an altered ratio of *WT1* +/-KTS splice isoforms

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Received November 28, 1997; Revised and Accepted January 9, 1998

The Wilms' tumor gene *WT1* plays a key role in genitourinary development and subsequent normal function. Homozygous mutations of *WT1* can be found in ~15% of Wilms' tumors. Furthermore, somatic heterozygous loss of *WT1* is known to lead to cryptorchidism and hypospadias in males. A much more severe phenotype is seen in patients with Denys–Drash syndrome which results from heterozygous dominant-negative mutations of the gene. Characteristic features are mesangial sclerosis with early kidney failure, varying degrees of gonadal dysgenesis and high risk of Wilms' tumors. Here we show that a related disease, Frasier syndrome, characterized by focal glomerular sclerosis, delayed kidney failure and complete gonadal dysgenesis, is probably caused by specific intronic point mutations of *WT1* that preferentially affect a CpG dinucleotide. Disruption of alternative splicing at the exon 9 splice donor site prevents synthesis of the usually more abundant *WT1* +KTS isoform from the mutant allele. In contrast to Denys–Drash syndrome, no mutant protein is produced. The splice mutation leads to an imbalance of *WT1* isoforms *in vivo*, as detected by RT-PCR on streak gonadal tissue. Thus, *WT1* isoforms must have quite different functions, and the pathology of Frasier syndrome suggests that especially gonadal development may be particularly sensitive to imbalance or relative underrepresentation of the *WT1* +KTS isoform.

## INTRODUCTION

The *WT1* gene originally was isolated as a Wilms' tumor suppressor gene (1,2). Since it is mutated in no more than 15% of Wilms' tumors, other genes probably play a more important role

in this tumor type (3,4). The gene encodes a nuclear zinc finger protein that can bind to DNA, and it is thought to function as a transcriptional regulator. Numerous genes have been proposed as targets for *WT1*, but the physiological relevance of these observations remains unclear (5).

The *WT1* gene is alternatively spliced yielding four isoforms (6,7). This affects exon 5 ( $\pm 17$  amino acids) and an alternative splice donor site at the end of exon 9, leading to the presence or absence of the tripeptide sequence KTS between zinc fingers 3 and 4. The +KTS and -KTS forms of *WT1* show different, but partly overlapping DNA-binding specificities (8). In transient transfection assays with reporters containing putative target gene promoters, these differences could be verified to a varying extent. However, *WT1* may function not only as a transcriptional regulator since specific RNA binding and co-localization with nuclear splicing factor have been demonstrated (9–11).

The importance of normal *WT1* function in development and growth control has been well documented. Knockout mice fail to develop kidneys due to massive apoptosis of metanephrogenic mesenchyme (12). They also show additional defects of mesothelia, heart and lungs, absence of gonadal development and they die around day 14 of gestation. Negative growth regulatory potential is evident from the observation that *WT1* expression can significantly reduce tumor formation of G401 Wilms' tumor cells in nude mice (13). In several cell types, introduction of *WT1* leads to increased apoptosis (14,15).

Gene dosage of *WT1* is critical especially for male development. A heterozygous loss of *WT1* as seen in WAGR syndrome patients leads to hypospadias and cryptorchidism (16). No genitourinary anomaly has been described for female individuals with *WT1* hemizyosity.

Dominant-negative mutations of *WT1* as seen in Denys–Drash syndrome show a much more dramatic phenotype (17). Effective *WT1* levels in cells are probably reduced below 50% since *WT1* protein can dimerize, resulting in abundant non-functional homo- and heterodimers of mutant *WT1* protein (18). Kidney function is severely impaired through diffuse mesangial sclerosis, leading to nephrotic syndrome and kidney failure within the first 2 or 3

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**Table 1.** Clinical characteristics of Frasier syndrome patients

|        | karyotype | onset of proteinuria | renal failure / dialysis | age at last follow-up | histological diagnosis                                    | gonadal development                        | syndromal tumor            | WT1 intron 9 |
|--------|-----------|----------------------|--------------------------|-----------------------|---|--|----------------------------|--------------|
| Che    | 46 XY     | 7 m                  | 12 yr                    | 17 yr                 | n.i.  | complete gonadal dysgenesis, streak gonads | gonadoblastoma             | +4 C > T     |
| Del    | 46 XY     | < 5 yr               | 13 yr                    | 17 yr                 | n.i.  | complete gonadal dysgenesis, streak gonads | gonadectomy                | +4 C > T     |
| Mar    | 46 XY     | n.i.                 | n.i.                     | n.i.                  | n.i.  | complete gonadal dysgenesis, streak gonads | n.i.                       | +4 C > T     |
| ws131  | 46 XX     | < 10 yr              | 11 yr                    | 37 yr                 | membranoproliferative glomerulonephritis                  | little, if any impairment                  | none                       | +5 G > A     |
| ws131B | 46 XY     | < 17 yr              | 19 yr                    |                       | focal glomerular sclerosis                                | complete gonadal dysgenesis, streak gonads | no gonadal or kidney tumor | +5 G > A     |
| ws130  | 46 XY     | 4 yr                 | 16 yr                    | 21 yr                 | focal sclerosing glomerulopathy with segmental hyalinosis | complete gonadal dysgenesis, streak gonads | gonadectomy                | +4 C > T     |
| DG     | 46 XY     | < 5 yr               | 10 yr                    | 16 yr                 | focal segmental glomerular sclerosis                      | complete gonadal dysgenesis, streak gonads | granulosa cell tumor       | +5 G > A     |
| JA *   | 46 XY     | 2 yr                 | 8 yr                     | 13 yr                 | no diagnosis reached                                      | complete gonadal dysgenesis, streak gonads | gonadectomy                | +5 G > A     |
| VM #   | 46 XY     | 2 yr 8 m             | 16 yr                    | n.i.                  | focal segmental glomerular sclerosis                      | complete gonadal dysgenesis, streak gonads | gonadectomy                | +5 G > A     |
| CS §   | 46 XY     | n.i.                 | yes                      | n.i.                  | glomerular sclerosis                                      | complete gonadal dysgenesis, streak gonads | gonadectomy                | +5 G > A     |

\*Patient JA has been reported before (25).

#Data for patient VM are taken from Bardeesy *et al.* (26).

§Data for patient CS are taken from Bruening *et al.* (22).

n.i.: no information obtained.

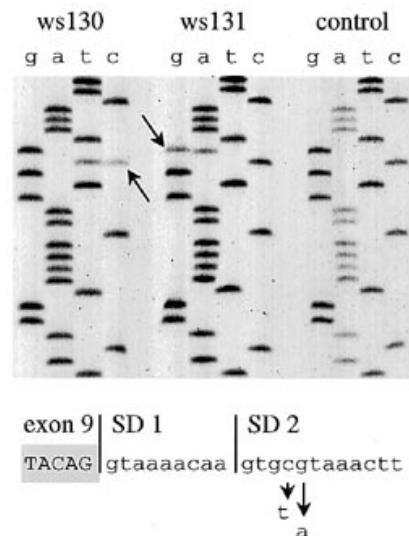
years of life in most cases (19). Gonadal development is disturbed to varying degrees. The most frequent diagnoses are 46 XY pseudohermaphrodites.

Frasier syndrome has been distinguished from Denys–Drash syndrome by Moorthy *et al.* (20) who established that there are clear differences in gonadal dysgenesis, progression of kidney disease and tumor risk. Initial attempts to find *WT1* mutations in Frasier patients failed since only exons were scanned at that time (21). Re-analysis of published patients and extension of this work has now provided clear evidence that Frasier syndrome is caused by mutations of the alternative splice donor site of exon 9.

## RESULTS

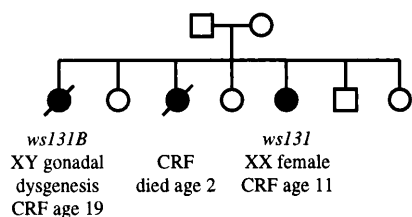
Genomic DNA from patients Che, Mar, Del, ws130, ws131 and DG could be isolated from blood cells. For ws130B, only paraffin embedded DNA from a colon tumor biopsy and DNA from a fibroblast cell line were available. Exon 9 of the *WT1* gene could be amplified reliably, and PCR products were sequenced directly without intermediate cloning steps. Possible cross-contamination of samples was ruled out by genotyping samples with the highly polymorphic marker *D11S533* (11q13).

Direct sequencing revealed a double band at the start of intron 9 at position +4 in patients Del, Mar, Che and ws130 (Fig. 1, Table 1). Instead of C alone, two nucleotides, T and C, could be read with equal intensity. Thus, the DNA is heterozygous for a C→T point mutation in intron 9. This mutation has not been described before. Scanning of the *WT1* sequence for possible splice donor sites with the program HSPL (<http://www-hgc.lbl.gov/projects/splice.html>) identified both splice donor sites at the end of exon 9. In the +4 mutant sequence, the second splice donor site had a markedly reduced score of 0.55 instead of 0.76 for the normal sequence. It is likely that this severely impairs the use of the second alternative



**Figure 1.** Frasier patients ws130 and ws131 show heterozygous point mutations in the second splice donor site of *WT1* exon 9. Direct sequencing of PCR-amplified DNA from the boundary of exon 9–intron 9 was performed with primer DDS1. Arrows point to the position of double bands identifying the respective mutations in ws130 and ws131, while unambiguous sequence is seen in control DNA. The schematic drawing below the autoradiograms depicts the exon–intron boundary with both alternative splice donor sites (SD1, SD2). The mutations affect nucleotides +4 and +5 as shown.

splice donor site, like the +5 G→A mutation described previously (22). Exactly the same +5 G→A mutation was detected—again in heterozygous form—in patient DG who presented with typical Frasier syndrome (Table 1).



**Figure 2.** Pedigree of the Frasier syndrome family. There are three affected siblings who suffered complete renal failure (CRF). For patient II-3 who died at age 2, no karyotype is available.

### WT1 intron 9 mutation in familial Frasier syndrome

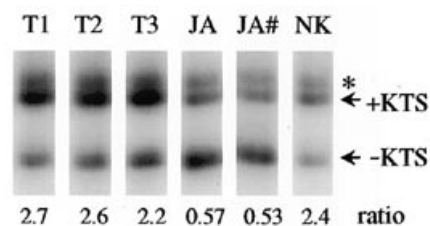
A very interesting case of possible familial Frasier syndrome had been published by Kinberg *et al.* (23). Three of the female children had kidney failure at ages 2, 11 and 16 years (Fig. 2). Typical focal glomerular sclerosis and XY gonadal dysgenesis had been diagnosed in ws131B. The membranoproliferative glomerulonephritis diagnosed in ws131 can be part of the clinical spectrum in longitudinal studies of focal glomerular sclerosis. Notably, the karyotype of ws131 is 46 XX, and only one additional possible case of Frasier syndrome has been reported with a 46 XX karyotype (24).

When DNA from blood cells of patient ws131 was analyzed, the +5 G→A mutation in intron 9 of *WT1* was identified again (Fig. 1). The sister with typical Frasier syndrome (ws131B) has died from colon cancer, but we were able to obtain a tissue biopsy from the colon tumor as well as DNA from a fibroblast cell line. Upon PCR amplification and direct sequencing, the same +5 G→A mutation could be detected in a heterozygous state in both specimens.

### The *WT1* intron 9 mutation affects alternative splicing *in vivo*

The most likely effect of these heterozygous mutations of *WT1* would be a reversal of the ratio of +/–KTS isoforms. The +/–KTS ratio usually is ~2:1 in all tissues tested. Biallelic expression of a heterozygous mutation would produce ratios of 2:1 and 0:3 from the respective chromosomes, yielding a combined ratio of 1:2. Archival gonadal tissue and an ovarian granulosa cell tumor could be retrieved from two of the patients. This allowed analysis of *in vivo* expression of *WT1* isoforms. RT-PCR was performed with exon-specific primers to quantitate isoform ratios in these cases in comparison with independent control samples. An invariable preponderance of the +KTS isoform (+/–KTS ratio of 1.5–2.8) was found in cDNA from 20 Wilms' tumors, as well as in two normal kidney samples and three different fetal kidney samples (examples in Fig. 3). On the contrary, there was a disturbed balance of isoforms in sections from streak gonadal tissue of patient JA, with a +/–KTS ratio between 0.48 and 0.57 in repeat experiments. In sections from the granulosa cell tumor of patient DG, *WT1* isoform ratios appeared to be similarly altered (not shown), although RT-PCR was less reliable, perhaps due to low expression of *WT1* or partial RNA degradation in the embedded tissue.

To rule out possible contamination of RNA samples with cloned DNA, expression of the *WT1* (CA)<sub>n</sub> repeat was tested.



**Figure 3.** The *WT1* splice mutations alter the +/–KTS isoform ratio of *WT1*. RT-PCR analysis of *WT1* expression was performed on RNA from Wilms' tumors (T1–T3), gonadal tissue from Frasier syndrome patient JA and human normal kidney (NK). The JA# sample is derived from an independent analysis of the same gonadal tissue block to demonstrate reproducibility. The hemi-nested primer pairs used (Fras-1/2 and DDS1/Fras-1) span two exons and one exon, respectively. Arrows identify the resulting PCR products of both isoforms. The weaker band (\*) above the +KTS isoform is probably derived from DNA heterodimers produced during PCR since it is missing in PCR of cloned *WT1* isoforms, but present when a mixture of cloned isoforms is used.

Both, genomic DNA and cDNA from DNase I-treated RNA samples were amplified with primers LK-GT1 and LK-GT2. Patients JA and DG were heterozygous in their genomic DNA and both showed biallelic expression of *WT1*.

### DISCUSSION

Eight patients with Frasier syndrome have been analyzed in the present study. In all cases, heterozygous mutations of the second alternative splice donor site of exon 9 were found. In addition to the previously described +5 G→A mutation, a +4 C→T mutation was detected. Available data suggest that the phenotypic consequences of both mutations are indistinguishable. The fact that *WT1* intron 9 mutations were detected in all cases studied provides strong evidence that these mutations represent the molecular cause of Frasier syndrome.

The +5 G→A mutation in patient JA has been described before, but the patient was classified as having Denys–Drash syndrome at that time (25). The similarity of clinical and molecular features of all Frasier patients listed in Table 1 suggests, however, that a re-classification would be appropriate. Similarly, two other cases (CS and VM) with *WT1* exon 9 splice mutations may in fact have Frasier syndrome (22,26). Neither patient developed Wilms' tumor and they had glomerular sclerosis and complete gonadal dysgenesis with streak gonads. Renal failure in VM only occurred at 16 years of age, unusually late for Denys–Drash syndrome. All clinical data are thus compatible with Frasier syndrome (see Tables 1 and 2).

While this manuscript was under review, two other groups reported on *WT1* mutations in eight additional patients with Frasier syndrome (27,28). Two new mutation sites at positions +2 and +6 of intron 9 could be found, and all results are in agreement with the present study. Even after merging all data, there is still a striking bias in the distribution of mutations: 15 of the 18 cases analyzed to date show the +4 C→T and +5 G→A mutations. This mutation hotspot probably results from the potential to deaminate 5-methylcytosine at the +4/+5 CpG dinucleotide.

The presence of alternative splice site mutations in Frasier syndrome highlights the importance of a precisely balanced expression of *WT1* isoforms for its correct function. The +5 G→A mutation has been shown previously to abolish alternative splicing

**Table 2.** Comparison of Denys–Drash and Frasier syndrome

|                     | Denys-Drash syndrome  | Frasier syndrome  |
|---------------------|---|---|
| Kidney pathology    | Diffuse mesangial sclerosis<br>Early kidney failure at age 0-3 yrs  | Focal segmental glomerular sclerosis<br>Delayed kidney failure at age 10-20 yrs   |
| Gonadal development | Variable impairment of development, broad spectrum of intersex phenotypes<br>Often partially developed gonads   | Complete sex reversal in 46 XY individuals, little or no impairment in 46 XX females<br>Streak gonads in 46 XY                            |
| Tumor risk          | High risk of Wilms' tumors since allele loss can lead to complete inactivation of WT1<br>Gonadoblastomas are rare   | No Wilms' tumors reported. Allele loss would only lead to loss of +KTS isoform expression<br>High risk of gonadoblastoma in streak gonads |
| WT1 gene            | Mostly missense mutations within the zinc finger domain and premature stop codons<br>Likely < 50% WT1 protein function due to dominant negative effect of mutant form | Splice donor site mutations in intron 9<br>No mutant WT1 protein, but altered ratio of zinc finger isoforms                               |

in transient transfections using minigene constructs (22). Although such tests have not been done for the +4 C→T mutation, it can be anticipated that this mutation will also affect alternative splicing as predicted by computer programs. Importantly, we have been able to demonstrate altered WT1 isoform ratios *in vivo* through an RT-PCR analysis of tissues expressing WT1. The ratio of +/-KTS isoforms in kidney or Wilms' tumors was in the range 1.5–2.8, which is identical to values obtained by other laboratories (6,29). In contrast, two samples of gonadal or tumor tissue from Frasier patients showed altered ratios of WT1 isoforms, with a ratio of ~0.5. Very similar results have been obtained by semi-quantitative RT-PCR with lymphocyte RNA from two patients with +4 C→T and +6 T→A mutations (27). Since the WT1 protein can dimerize (18), inversion of the +/-KTS ratio from ~2:1 to 1:2 will have an even stronger effect on the levels of WT1 +KTS and -KTS homodimers. In particular, the concentration of WT1 +KTS homodimers will decrease dramatically.

In this context, it is important to keep in mind that *WT1* can be expressed from only one allele—at least in placenta and brain of some individuals (30). If such a silencing of one *WT1* allele occurred in some of the terminally differentiated cells, for example in non-dividing podocytes of the kidney, it is conceivable that cells with active mutant *WT1* cannot produce any WT1 +KTS protein at all. This is of importance for the disease mechanism, and it would be interesting in future to assess allele-specific expression in affected cell types at the single cell level.

Since Frasier and Denys–Drash syndromes show partial clinical overlap it is worthwhile delineating specific characteristics for each disorder. Table 2 builds upon a previous classification by Moorthy *et al.* (20) and includes new data from genetic analyses that may explain differences in clinical presentation and disease course. The invariable presence of complete gonadal dysgenesis in all 46 XY Frasier patients suggests that the correct ratio of WT1 isoforms is absolutely critical for male gonadal development. On the other hand, the dominant-negative mutations seen in Denys–Drash syndrome may be variably permissive for male development, resulting in a broad spectrum of intersex and hermaphrodite phenotypes in karyotypic males. It should be noted that in individuals with a 46 XX karyotype gonadal development is generally less impaired or even normal (31).

The opposite situation is seen for kidney pathology. While Denys–Drash syndrome cases develop early nephrotic syndrome and renal failure, this only happens much later and over a longer

period of time in Frasier syndrome. Histological findings are also different—diffuse mesangial sclerosis with expansion of mesangial matrix and subcapsular atrophy and immaturity (Denys–Drash) instead of focal glomerular sclerosis affecting only a fraction of glomeruli or segments of glomerular tufts (Frasier). It is also interesting that WAGR deletion patients with only one functional *WT1* allele (50% WT1 function) have not been reported to have any impairment of kidney function, although hypospadias and cryptorchidism are clearly present in males.

The type of *WT1* mutation also has significant impact on the tumor risk in Frasier and Denys–Drash syndrome. In the latter, the dominant-negative mutant allele is defective and loss of the second allele—according to the two-hit model—may be the most important step in tumor formation already. In contrast, Frasier patients have one normal copy of *WT1* and one which can only produce the shorter isoform. Allele loss would thus lead to cells that cannot produce the +KTS isoform of WT1, but still have large amounts of the -KTS isoform. In this respect, it is interesting to note that tumorigenicity of the G401 Wilms' tumor cell line in nude mice can be suppressed by +KTS and -KTS isoforms to the same extent (13). This may explain why Frasier syndrome mutations apparently do not increase the risk of Wilms' tumor.

On the other hand, there is a high frequency of gonadoblastomas in Frasier patients (20). This may be due to the obligatory presence of streak gonads which carry a high risk of malignancy. The rather low incidence of gonadoblastoma in Table 1 may be due in part to prophylactic gonadectomy. In Denys–Drash syndrome, there are fewer cases of streak gonads with concomitantly lower incidence of gonadoblastoma. There may, however, still be differences in the state at which gonadal development is halted, resulting in further modulation of tumor risk.

Transmission to multiple offspring may not be uncommon in Frasier syndrome. The sisters ws131 and ws131B have the same mutation and a similar course of kidney disease. Transmission to a third female child in the same family appears likely, although the onset of kidney disease at 6 months with failure around age 2 is not typical (23). Interestingly, there are only two reports of possible 46 XX Frasier patients, one of them being patient ws131 who appears to have little if any problems of gonadal development. A second case has been described by Bailey *et al.* (24), but DNA was not available for study. The authors describe primary hypogonadism and sexual infantilism in their patient. This is completely different from our case and may suggest that the



patient described by Bailey *et al.* may not have Frasier syndrome. The paucity of 46 XX karyotypes reported may be due to underdiagnosis as these patients suffer primarily from nephrotic syndrome and kidney failure. Since females may in general have much reduced or missing gonadal problems, the correct diagnosis could be missed. The highly specific mutations detected in all cases presented may provide a facile way to identify these patients in dialysis and transplant units.

## MATERIALS AND METHODS

### Isolation of DNA and direct sequencing

Genomic DNA from white blood cells was purified by phenol/chloroform extraction and ethanol precipitation as described previously (3). From paraffin-embedded material, ten 5 µm sections were used for DNA extraction according to standard procedures (32). Briefly, sections were directly incubated in 200 µl of lysis buffer (50 mM Tris, pH 8, 1 mM EDTA, 0.5% Tween-20 and 200 µg/ml proteinase K) at 65°C overnight, followed by boiling for 10 min with 100 µl of Chelex 100 slurry. After centrifugation, the aqueous phase was stored at -20°C.

Amplification of *WT1* exon 9 was carried out with intronic primers WT9S (cattgttagggccgaggcta) and WT9A (ctttccaatcctctcatca) (3) and 50 ng of blood DNA or 1 µl of DNA purified from section material using standard conditions (30–40 cycles, 60°C annealing and 30 s each for denaturation, annealing and extension steps). In cases of low yield from section material, a reamplification for 15 cycles with hemi-nested primers DDS1 (gcgaaagtctcccggctcc) and WT9A was performed. PCR products were run on agarose gels and purified with Nucleotrap matrix (Macherey & Nagel, Düren, Germany). Direct sequence analysis was carried out with primers WT9A (antisense) and DDS1 (sense) using the ThermoSequenase [<sup>33</sup>P]dideoxy-terminator kit (Amersham).

For microsatellite analysis, primer sequences and PCR conditions were taken from GDB. Products were visualized by [<sup>32</sup>P]dCTP incorporation and autoradiography after separation on sequencing gels for the *WT1* (CA)<sub>n</sub> repeat (7). The *DIIS533* repeat could be analyzed directly by agarose gel electrophoresis.

### RT-PCR analysis

For paraffin-embedded archival specimens, a nucleic acid extraction protocol described by O'Driscoll *et al.* (33) was used. Ten 5 µm sections were deparaffinized with xylene and ethanol, air dried and suspended in 200 µl of digestion buffer (10 mM Tris, pH 8, 100 mM NaCl, 25 mM EDTA, 0.5% SDS and 100 µg/ml proteinase K). After digestion for 16 h at 50°C, total nucleic acids were extracted by phenol/chloroform extractions and subsequent ethanol precipitation. The nucleic acid pellet was resuspended in 100 µl of water. RT-PCR was performed on duplicate samples using the Boehringer Mannheim Titan one tube RT-PCR kit according to the instructions supplied (single tube reaction with 30 min cDNA synthesis, followed by 35 cycles of PCR). Primers were Fras-1 (ccagctcaaaagacaccaaag, exon 8) and Fras-2 (tttctgcaacttgccacc, exon 10). To quantitate *WT1* splice isoforms, a second round of hemi-nested PCR with primers DDS1 and Fras-2 and 0.1 µl of the first PCR reaction was performed with 15 cycles and addition of [<sup>32</sup>P]dCTP. Products were separated on a non-denaturing polyacrylamide gel, dried and exposed to X-ray

film. Quantitation was performed on a Molecular Dynamics Phospho-Imager.

## ACKNOWLEDGEMENTS

The authors would like to thank the patients involved in this study. We are grateful to Professors R. Dumas, C. Marty-Double, C. Angle, Drs M. Malone, J. Miller, M. Nolte, L. Rees and L. Roffman, and Ms D. Kuntzelman for providing specimens or clinical information. A.K. is funded by the Twistington Higgins Research Fellowship from the National Kidney Research Fund. This work was funded by grants from the Deutsche Forschungsgemeinschaft (Ge539-3/8).

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