Nuclear Transport of Wilms’ Tumour Protein Wt1 Involves Importins α and β

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Wilms’ tumour protein • Transcription factor • Nuclear transport • Importin • Nuclear localisation signal

Abstract
Background/Aims: Wilms’ tumour protein, Wt1, is a zinc finger molecule, which is required for normal embryonic development. Mutations of the WT1 gene can give rise to childhood cancer of the kidneys. Different Wt1 isoforms exist, which function either as transcription factors or have a presumed role in mRNA processing. Previous studies suggested that Wt1 undergoes nucleocytoplasmic shuttling, and cytoplasmic Wt1 was higher in malignant than in normal cells. The aim of this study was to analyse the molecular pathways along which Wt1 shuttles between the cytoplasm and nucleus. Methods: Interaction of Wt1 protein with various importin α subtypes and importin β was assessed in pull-down assays and co-immunoprecipitation experiments. Nuclear localisation signals (NLS) were identified by combining site-directed mutagenesis with subcellular immunodetection of the transfected Wt1 variants. Results: Wt1(+/-KTS) proteins were found to interact with importin α1 and importin β in vitro and in living cells in vivo. A NLS that was necessary and sufficient for nuclear import could be mapped to the third Wt1 zinc finger. Mutation of this NLS strongly weakened binding of Wt1 to importins. Conclusion: Nuclear translocation of Wilms’ tumour protein involves importins α and β, and a NLS in the third zinc finger.

Introduction
Loss-of-function mutations of the Wilms’ tumour gene-1 (WT1) are responsible for approximately 15% of renal childhood cancers known as nephroblastoma [1-3]. Nephroblastoma is one of the most frequent paediatric tumours and emerges, when the pluripotent progenitor cells in the immature kidney continually proliferate instead of differentiating to glomeruli and tubules [4]. Surprisingly, high Wt1 mRNA and protein levels were detected in various malignancies including carcinomas of the lung [5], colon [6], breast [7], and acute leukaemias [8-10]. These observations suggested that wild-type Wt1 does not only prevent Wilms’ tumour formation but may even promote malignant cell proliferation under certain conditions [11]. Besides its role as a tumour suppressor and putative oncogene Wt1 is necessary for normal embryonic
development. Thus, mice with homozygous \textit{Wt1} defect (\(\textit{Wt1}^{-/-}\)) are embryonic lethal, and exhibit agenesis of the kidneys and gonads in addition to abnormalities of the mesothelium, heart, spleen, retina and several other tissues [12-16]. A common paradigm of \textit{Wt1} expressing cells is their capability to switch between a mesenchymal and epithelial state. A failure of epithelial-mesenchymal transition (EMT) in the absence of \textit{Wt1} may lead to programmed cell death thereby impeding normal development [17].

The human \textit{Wt1} gene on chromosome 11p13 encodes an approximately 55 kDa protein whose domain structure comprises a proline/glutamine-rich N-terminal sequence implicated in transcriptional regulation and nuclear speckling (Fig. 1) [18-20]. Other functional motifs include a self-association domain and a putative RNA recognition sequence (Fig. 1) [21]. Four contiguous Cys2-His, zinc fingers at the C-terminus are necessary for sequence-specific nucleic acid binding of \textit{Wt1} (Fig. 1) [18, 19]. At least two dozens \textit{Wt1} isoforms are generated by alternative mRNA splicing, protein translation from variable start points, RNA editing, and post-translational modifications [22]. Among the two alternative splicing events the usage of an alternative splice donor site at the end of exon 9 results in the insertion of three additional amino acids, lysine, threonine, serine (KTS), between zinc fingers 3 and 4 (Fig. 1) [23, 24]. \textit{Wt1} proteins with the KTS tripeptide inclusion have a reduced DNA binding affinity compared to the -KTS forms [25] and are presumably involved in mRNA processing [26-29], whereas the \textit{Wt1} (-KTS) proteins function as transcription factors [30-33].

It has previously been reported that both \textit{Wt1} isoforms (+/-KTS) experience nucleocytoplasmic shuttling [28, 34]. Depending on the specific cell type up to 50% of total cellular \textit{Wt1} protein was detected in the cytoplasm [28], and the proportion of \textit{cytoplasmic} \textit{Wt1} was significantly higher in malignant than in normal cells [5, 7]. This raises the interesting possibility that a disparate subcellular allocation (nucleus vs. cytoplasm) contributes to the dual functions (tumour suppressor vs. oncogene) of \textit{Wt1} in normal and tumour cells. These observations also direct attention to the largely unknown molecular mechanisms that mediate nucleocytoplasmic trafficking of \textit{Wt1} protein.

The transport of larger proteins (above 40-60 kDa) through the nuclear pore complex is usually mediated by a specific nuclear transport receptor in the cytoplasm. The transport receptors recognise their substrates via specific signals of the cargo proteins [35]. In a wide variety of proteins the targeting information is encoded by “classical” nuclear localisation signals (NLS), which are composed of either one or two clusters of basic amino acids (monopartite or bipartite NLS). NLSs are contained by at least one copy in many proteins destined to undergo nuclear import [36, 37], which are bound by various importin subtypes. “Classical” NLS usually interact with importin \(\alpha\), which in turn are bound by importin \(\beta\) [38, 39]. On the other hand, importin \(\beta\)-like importins can directly act as nuclear translocators for nuclear cargo proteins [35, 40]. Nuclear export is mediated by exportins using a leucine-rich nuclear export signal (NES) which is recognised by CRM1 [41, 42]. In the present study we addressed the role of the “classical” nuclear import receptors importins \(\alpha/\beta\) in the distribution of \textit{Wt1} protein between the cell nucleus and the cytoplasm.

### Materials and Methods

**Tissue culture and cell transfections**

Human embryonic kidney (HEK) 293 cells (catalogue no. ACC 305) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The cells were kept at 37°C in a humidified tissue culture incubator (21% \(O_2\), 5% \(CO_2\)) and routinely split at a 1:10 ratio twice per week. The culture medium was DMEM nutrient (PAA Laboratories, Pasching, Austria) supplemented with 10% FCS (Bioclone KG, Berlin, Germany), 100 IU/ml penicillin (Invitrogen GmbH, Karlsruhe, Germany), 100 \(\mu\)g/ml streptomycin (Invitrogen, Karlsruhe, Germany), and 290 \(\mu\)g/ml L-glutamine (PAA Laboratories). Cell transfections were performed at approximately 50% confluence in 24-well tissue culture plates using 0.3 \(\mu\)g plasmid DNA and 1 \(\mu\)l Fugene6® reagent per well according to the manufacturer’s instructions (Roche Diagnostics, Mannheim, Germany).

**Plasmids**

A synopsis of the various expression constructs is provided in Table 1. All plasmids were generated by PCR amplification using full-length cDNA of the murine \textit{Wt1} gene as template. Plasmid pEEG-C1 has been derived from pEGFP-C1 (BD Biosciences, Clontech, Heidelberg, Germany) by insertion of a \textit{BglII/XhoI} fragment N-terminal of the multi cloning site (MCS) and a second EGFP at the N-terminus as \textit{NheI} fragment [43]. Due to its molecular mass of approximately 80 kDa the EGFP-EGFP-GST fusion protein expressed from pEEG-C1 is retained in the cell cytoplasm. Plasmid pEEG-C1 was therefore used to test different portions of the \textit{Wt1} cDNA ligated into the \textit{SalI/BamHI} restriction sites for the presence of NLS.

**Immunocytochemistry**

Immunostainings of acetone:methanol (3:2 ratio) -fixed cells were performed as described in detail elsewhere [44, 45]. The following antibodies were used at 1:100 dilutions in ready-
to-use antibody diluent (Zymed Laboratories Inc., Berlin, Germany): rabbit polyclonal anti-Wt1 antibody (WT180, Lot. no. E057, catalogue no. sc-846, Santa Cruz Biotechnology, Heidelberg, Germany), anti-Flag M2 monoclonal antibody from mouse (product code F 3165, Sigma-Aldrich, Hamburg, Germany). The reaction products were visualised by incubation (1.5 hours at room temperature) with Cy3- and Cy2-conjugated secondary antibodies at 1:200 dilutions, respectively. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) as indicated. Labelled cells were viewed under an epifluorescence microscope (Axiovert S100, Zeiss, Jena, Germany), which was connected to a digital camera (Spot RT Slider, Diagnostic Instruments, Sterling Heights, USA) equipped with the Metamorph V6.1 software (Molecular Devices Inc. Downington, USA). Likewise, the EGFP fluorescence signal was monitored with the digital camera system.

Protein expression and purification

Recombinant human importins α1, α3, α4, α5, α7 and β were expressed as GST-fusion proteins and purified as described [46], importin β was also expressed as His-tagged protein. Briefly, transformed E. coli BL21/pRep4 were grown with vigorous shaking at 37°C in LB medium. At mid-log phase of growth, protein overexpression was induced by 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 4 h at 25°C. The cells were collected and disintegrated in a French Press. The lysate was clarified (1 h, 22,000 rpm, SS 34 rotor) and incubated for 2-3 h at 4°C with glutathione-Sepharose (GE Healthcare Europe GmbH, München, Germany). The sepharose was washed 3 times and the protein was eluted by incubation with elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM reduced glutathione). Protein samples were dialysed against dialysis buffer (50 mM HEPES-KOH, pH 7.5, 200 mM NaCl, 5% glycerol). If required the protein was concentrated in centrifugal filter devices (Millipore, Schwalbach, Germany). His-tagged importin β was separated from the protein extract by metal affinity chromatography using BD TALON metal affinity resin (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France) according to the manufacturer’s instructions. The protein concentrations were measured using the RC DC protein assay (Bio-Rad, München, Germany).

**GST-importin pull-down assays**

GST or GST-importins were allowed to bind to glutathione-Sepharose 4B. GST-importin pull-down assays were carried out with purified GST as a negative control as described elsewhere [47]. In a typical experiment 100 µl beads were pre-equilibrated in IP-buffer (20 mM Hepes pH 7.5, 100 mM KOAc, 0.5 mM EGTA, 5 mM MgOAc, 250mM sucrose, 4°C), mixed with 15 µg GST protein or 15 µg GST-fusion proteins with or without His-tagged importin β and incubated at 4°C for 1 h. The Wt1 coding sequences - either full-length or truncated/mutated - were transcribed and translated in vitro in the presence of [35S]-methionine (TNT Coupled Reticulocyte Lysate System, Promega, USA) according to the manufacturer’s protocol. After incubation, 10 µl of the reaction batch were allowed to bind to the immobilised fusion proteins. [35S]-labelled methionine was obtained from Hartmann Analytic (Braunschweig, Germany). After washing three times with IP-buffer, the sepharose beads were dissolved in 60 µl Laemmli

| PLASMID NAME | WT1 AMINO ACIDS | VECTOR BACKBONE |
|--------------|-----------------|-----------------|
| p477         | 1 to 449 (full-length Wt1+KTS) | pCMV - 3Tag     |
| p483         | 1 to 449 (full-length Wt1+KTS) | pcDNA3          |
| p546         | 1 to 341        | pcDNA3          |
| p563         | 1 to 325        | pcDNA3          |
| p583         | 323 to 449      | pEEG - C1       |
| p583 - mutC/D| 323 to 449 with mutation of predicted NLS C and D | pEEG - C1       |
| p583 - mutE  | 323 to 449 with mutation of predicted NLS E  | pEEG - C1       |
| p583 - mutD  | 323 to 449 with mutation of predicted NLS D  | pEEG - C1       |
| p592         | 323 to 395      | pEEG - C1       |
| p593         | 323 to 385      | pEEG - C1       |
| p594         | 323 to 430      | pEEG - C1       |
| p721         | 1 to 449 (full-length Wt1+KTS) with mutation of predicted NLS E | pcDNA3          |
| p755         | 323 to 449      | pCMV - 3Tag     |
| p755-E       | 323 to 449 with mutation of predicted NLS E  | pCMV - 3Tag     |

**Table 1. Overview of the various Wt1 expression constructs used.**

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buffer [48]. Proteins were separated by SDS-PAGE (10%) and visualised by Coomassie Brilliant Blue staining. To detect the [35S]-labelled proteins, the dried gels were autoradiographed (16-24 h). Binding was visualised by analysing the data with the PCBAS 2.09 g software (Raytest Isotopenmessgeräte GmbH, Germany).

**Co-immunoprecipitation experiments**

The cells were solved in immunoprecipitation buffer (50 mM Tris pH 7.4, 1mM EGTA, 400 mM NaCl, 0.1% IGEPAL, 1.0 mM NaF) and lysed by sonification. The cell lysate was incubated with antibodies (Importin α1: BD Transduction Lab; Importin β: C-19, catalogue no. sc-1863, Santa Cruz Biotechnology). Antibodies and bound proteins were precipitated using ProteinG Sepharose (4Fast Flow, GE Healthcare Bio-Sciences). Wt1 protein was detected with an anti-Wt1 antibody (catalogue no. sc-192, Santa Cruz Biotechnology).

**Results**

Wt1 proteins interact with importins α1 and β in *vitro* and *in vivo*

Pull-down assays were performed to explore whether Wt1 proteins interact with various importin α/β molecules in *vitro*. For this purpose GST-tagged importins were immobilised to glutathione-Sepharose 4B and incubated with [35S]-methionine labelled full-length Wt1 proteins (+/-KTS isoforms). Bound proteins were separated by SDS-PAGE and subsequently visualised by autoradiography. Wt1(+KTS) and Wt1(-KTS) proteins were found to interact with importins β and α1 and, much less avidly, with importin α4 (Fig. 2). In contrast, Wt1 proteins did not bind to importins α3, α5, and α7 or the GST moiety (Fig. 2). The previously recognised interaction of importin α3 with hypoxia-inducible factor (HIF)-1α [46] was proven as a quality control for the pull-down assays (Fig. 2, left panel).

Co-immunoprecipitation experiments were carried out to test for binding of Wt1 proteins with importins α1, α3, α4 and β in native cells *in vivo*. We used murine mesonephros-derived M15 cells, because - to our knowledge - M15 cells have the highest endogenous Wt1 protein content among permanent cell lines [26]. Immunoprecipitations were performed with whole-cell lysates from M15 cells using polyclonal goat antibodies against importins α1, α3, α4 and β, respectively. The precipitated material was bound to protein G sepharose beads and loaded on SDS-PAGE. Separated proteins were immunoblotted using a polyclonal anti-Wt1 antibody (WT180, Lot. no. E057, Santa Cruz Biotechnology). Obviously, the ≈55 kDa Wt1 protein was co-immunoprecipitated with antibodies against importins α1 and β, but not with importin α3 and α4 antibodies (Fig. 3A).

**Interaction of Wt1 with importins α1/β and nuclear protein localisation require an intact zinc finger domain**

Importins α and β bind to nuclear localisation signals (NLS) that usually consist of positively charged amino acid residues [49]. Since bioinformatic analysis (PredictNLS) predicted putative NLSs mainly in the Wt1 zinc finger domain, we examined whether binding to importins α1 and β was abolished upon truncation of the Wt1 protein at its C-terminus. Indeed, a Wt1 fragment (p546, Table 1), which lacked the C-terminal zinc finger...
region, failed to interact with importins α1 and β, whereas the full-length Wt1(+KTS) protein was pulled-down by both importins (Fig. 3B).

Since the Wt1 zinc finger domain was apparently necessary for interaction with importins α1 and β, we explored whether the C-terminal portion was also crucial for nuclear localisation of the Wt1 protein. For this purpose HEK293 cells were transfected with expression constructs encoding either the four zinc fingers extending from amino acids 323 to 449 (p546), and GST-importins (α1, β) as described in the methods section. 1: importin β, 2: importin α1, 3: GST. The autoradiography below the gel demonstrates that interaction of Wt1 protein with both importins was dependent on an intact zinc finger domain.

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A NLS in the third zinc finger mediates nuclear import of Wt1 protein

Next we examined whether the Wt1 zinc finger domain conferred nuclear localisation to a heterologous protein, giving an indication that the region is a sufficient NLS. To this end we made use of a construct (pEEG-C1) carrying a duplicated EGFP expression cassette that was fused to an N-terminal GST fragment [43]. Upon transfection the EGFP-EGFP-GST protein was restricted
to the cell cytoplasm unless it contained an additional NLS which enabled nuclear import [43]. Notably, inclusion of the Wt1 zinc finger domain resulted in a shift of the fluorescence signal from the cytoplasm to the cell nuclei (Fig. 5). The critical amino acid sequence could be mapped to the third Wt1 zinc finger by transfecting HEK293 cells with various C-terminally truncated EGFP-EGFP-GST fusion constructs (Fig. 5). This becomes evident from a comparison of the nuclear and cytoplasmic distribution of proteins expressed from p592 and p593, respectively (Fig. 5).

Sequence analysis revealed a putative NLS (…RKFSR…) in zinc finger 3 of the Wt1 protein, which also fulfilled the criteria for possible interaction with importins α1 and β (Fig. 6A). To determine the function of this element the amino acids arginine (R) and lysine (K) were substituted by alanine (A). The Wt1 zinc finger domain with the mutated sequence (…AAAFSA…) was fused to the EGFP-EGFP-GST in plasmid p583-mutE. Unlike the wild-type protein (p583), which was readily visible in the nuclei, the mutated fusion protein (p583-mutE) was retained in the cytoplasm of transfected HEK293 cells (Fig. 6B). Mutation of two other putative NLS (C and D in Fig. 6A) in zinc fingers 1 and 2, either alone or in combination, did not change the nuclear fluorescence (Fig. 6B).

We then investigated whether the identified NLS was also critical for translocation of Wt1 protein to the nucleus. For this purpose the amino acid mutation (…AAAFSA…) was introduced in the Wt1 zinc finger domain, which was expressed as a Flag-tagged protein. While the wild-type zinc finger region (p755), like the full-length Wt1 protein (p477), was clearly restricted to the nuclei, the mutated protein (p755-mutE) could additionally be detected in the cell cytoplasm (Fig. 6C).

The predicted NLS in the Wt1 zinc finger domain interacts with importins β and α1

Finally, we examined whether the identified NLS in the third Wt1 zinc finger did also interact with importins α and β. To this end in vitro pull-down assays were performed with different GST-importins and [35S]-methionine labelled full-length Wt1(+KTS) protein of either wild-type or with mutation of the NLS (p721). Interaction of
Fig. 6. Identification of a nuclear localisation signal (NLS) in zinc finger 3 of the Wt1 protein. Panel A: Amino acid sequence of the C-terminal zinc finger domain of Wt1 protein. Sequences of the four zinc fingers are underlined and predicted NLSs (C, D, E) are boxed. The amino acids printed in bold were substituted by alanin (A) in the mutated constructs. Panel B: Detection of GFP in transfected HEK293 cells. Cell nuclei were sometimes stained blue with DAPI. Plasmid p583 contained the wild-type Wt1 zinc finger domain (amino acids 323-449) in the pEEG-C1 vector [43]. Mutations in plasmids p583-mutD, p583-mutC/D, and p583-mutE refer to the schematic drawing above. Strikingly, mutation of a predicted NLS in zinc finger 3 abrogated nuclear import of the EGFP-EGFP-GST fusion protein (p583-mutE). Panel C: Immunocytochemistry of HEK293 cells that had been transfected with constructs expressing either the full-length Wt1(+KTS) protein (p477) or the C-terminal zinc finger domain with (p755-mutE) and without (p755) mutation of the predicted NLS (E) in zinc finger 3. Expressed sequences were contained in the pCMV-3Tag vector, and visualised with an anti-Flag antibody. Note the cytoplasmic staining of p755-mutE in comparison to the exclusively nuclear localisation of p755. Blue staining of the cell nuclei was performed with DAPI.

Fig. 7. Interaction of Wt1 protein with importin β is substantially weakened upon mutation of the NLS in zinc finger 3. GST-coupled importin β was used in pull-down assays with in vitro transcribed full-length Wt1(+KTS) protein and a Wt1 molecule with mutated NLS (E) in zinc finger 3 (p721). Interaction of the mutated variant with GST-importin β was clearly diminished in comparison to the wild-type Wt1 protein. 1: importin β, 2: GST. Lane “Wt1(p721) input” was loaded with the input material from the in vitro transcription/translation using plasmid p721 as a template. The two shorter bands in the lower part of the “Input Wt1(p721)” lane presumably reflect truncated Wt1 proteins due to incomplete in vitro transcription/translation from the plasmid DNA.

the mutated Wt1 molecule with importin β was obviously reduced, albeit not fully abolished, in comparison to the wild-type protein (Fig. 7). Likewise, mutation of the predicted NLS weakened interaction with importin α1/α4 (data not shown). These results indicate that the pre-
Predicted NLS in zinc finger 3 of the Wt1 protein is indeed the main interacting domain with importin β.

Discussion

Wt1 proteins comprise a family of zinc finger molecules with important roles in development and disease [50]. Their functional complexity is accounted for - at least in part - by the existence of multiple isoforms, which are expressed in a characteristic spatio-temporal pattern. Besides other mechanisms, i.e. post-translational modifications [51, 52], the shuttling of Wt1 protein between the nuclear and cytoplasmic cell compartments may represent a relevant switch for regulating its biological activity [28, 34], which adds one distinct level of gene expression control [53]. Indeed, previous studies have shown that a significant portion of Wt1(+/−KTS) isoforms reside in the cell cytoplasm and associate with actively translating polyosomes [28, 29, 54]. These observations suggested a role for Wt1 in the control of protein translation in addition to its previously recognised functions as a transcription factor and putative mRNA processing molecule [26, 27, 30, 31].

Our results showing direct interaction of Wt1(+/−KTS) proteins with importins α1 and β suggest that nuclear Wt1 import involves the importin α/β pathway (Figs. 2, 3). This notion is further supported by the observation that mutation of a predicted NLS in the zinc finger domain of Wt1 reduced the binding affinity for importins β and α1 and abrogated nuclear import capability of an expressed protein (Figs. 6, 7). Since polypeptides with molecular masses below 40-50 kDa can passively diffuse into the nucleus [55], truncated Wt1 variants were fused to a duplicated EGFP-GST fusion protein to narrow-down the signals required for nuclear targeting (Figs. 5, 6) additional sequences outside of zinc finger 3 may also be involved in nuclear import of Wt1. This possibility is supported by the observation that deletion of zinc fingers 3 and 4 in the full-length Wt1 protein generated mutants that were still capable of nuclear targeting [62, 63].

The fact that importin β directly binds to a cargo protein is a rare event [64] and that importin α can bind to the same amino acid sequence is novel to our knowledge and awaits further clarification aimed at analysing the mutual interaction of these molecules at the identified NLS. This finding also suggests some functional redundancy in the nuclear import machinery for Wt1, which is not surprising given the outstanding importance of the Wt1 protein in development and disease [22, 50]. The NLS, which we pinpointed in the murine Wt1 protein, is fully conserved in the human polypeptide. Strikingly, in a study of 156 diverse tumours the third Wt1 zinc finger was identified as a hotspot for mutations in Wilms’ tumours, but in no other urogenital neoplasms [65]. It is a frequent observation that malignant cells contain substantial amounts of Wt1 in the cytoplasm, while their normal, i.e. non-transformed counterparts usually exhibit nuclear protein localisation [5, 7]. This raises the interesting possibility that cytosolic retention of Wt1 due to mutational disruption of nuclear import signals may contribute to the malignant phenotype in some tumours. Thus, one would predict that Wt1 exerts different functions in the cell cytosol and the nucleus, which may impose an oncogenic risk, e.g. due to proteins undergoing nucleocytoplasmic shuttling has been identified so far. Performing in silico analysis of Wt1 we determined regions showing some analogy to the NLS of SV40 “PKKKRKV” and nucleoplasmin “AVKRPAATKKAGQAKKKKLD” [60, 61]. The results of an earlier study indicated that fusion of either zinc finger 1 or zinc fingers 2 and 3 of Wt1 protein to β-galactosidase generated polypeptides that were capable of nuclear targeting [62]. In contrast, a β-galactosidase fusion protein including Wt1 zinc fingers 3 and 4 was retained in the cell cytoplasm [62]. These findings suggested the presence of at least two separable nuclear targeting signals, one in zinc finger 1 and another one in zinc fingers 2/3, of the Wt1 protein [62]. However, the exact localisation of the predicted NLS within the DNA binding domain and the core sequence motifs necessary for protein targeting to the nucleus have remained unknown. While our current results verify the importance of a NLS in the third zinc finger (Figs. 5, 6) additional sequences outside of zinc finger 3 may also be involved in nuclear import of Wt1. This possibility is supported by the observation that deletion of zinc fingers 3 and 4 in the second zinc finger (Figs. 5, 6) additional sequences outside of zinc finger 3 may also be involved in nuclear import of Wt1. This possibility is supported by the observation that deletion of zinc fingers 3 and 4 in the third zinc finger (Figs. 5, 6) additional sequences outside of zinc finger 3 may also be involved in nuclear import of Wt1. This possibility is supported by the observation that deletion of zinc fingers 3 and 4 in the full-length Wt1 protein generated mutants that were still capable of nuclear targeting [62, 63].

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diverse target gene selectivity and/or behaviour in a dominant-negative fashion.

In summary, by the identification of a functional NLS in the third Wt1 zinc finger this study extends the current knowledge of mechanisms underlying nuclear translocation of Wt1 protein. Wt1 can interact with the importins α1 and β, which are likely to regulate the cargo specificity and transport rate into the nucleus.

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