The Wilms' tumour suppressor protein, WT1, undergoes CRM1-independent nucleocytoplasmic shuttling

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Abstract The Wilms' tumour suppressor gene (WT1) encodes a zinc finger-containing nuclear protein essential for kidney and urogenital development. Initially considered a transcription factor, there is mounting evidence that WT1 has a role in post-transcriptional processing. Using the interspecies heterokaryon assay, we have demonstrated that WT1 can undergo nucleocytoplasmic shuttling. We have also mapped the region responsible for nuclear export to residues 182–324. Our data add further complexity to the role of WT1 in trancriptional and post-transcriptional regulation.

Key words: WT1; Nucleocytoplasmic shuttling

1. Introduction

The Wilms' tumour suppressor gene (WT1) encodes a nuclear protein essential for kidney and urogenital development [1]. WT1 contains four tandem C-terminal C_2H_2 zinc fingers (ZFs) [2,3] and structural modeling suggests that the N-terminus contains an RNA recognition motif (RRM) [4]. There are four major isoforms of WT1, ranging in size from 52 to 54 kDa, generated by alternate splicing of exon 5 and exon 9. This results in variable insertion of a 17 amino acid sequence near the middle of the protein and 3 amino acids (KTS) between ZFs 3 and 4. Isoform A lacks both splices while isoform D contains both. Isoforms B and C contain either the 17 amino acid sequence or the KTS respectively [5]. Two nuclear localisation sequences (NLSs) have been identified in the ZF domain of WT1 [6], one in ZF 1 and another in ZF 2. This was based on the ability of ZF 1 alone or ZFs 2 and 3, but not ZFs 3 and 4, to direct β-galactosidase to the nucleus. However, the precise residues that are required for nuclear localisation have not been identified.

The high similarity of the WT1 ZFs to those of early growth response gene 1 (EGR1) led to the assumption that WT1 is a transcription factor [7]. Since then a number of putative WT1 target genes have been identified for which WT1 can act as a transcriptional activator or repressor [8]. However, these target genes have not been verified in vivo and recent data suggests that few of the original target genes are genuine [9]. There is also mounting evidence to suggest that WT1 has a post-transcriptional role. This includes co-localisation of endogenous WT1 with splicing proteins in nuclear speckles [10], binding of WT1 (particularly the C and D isoforms) to the splicing protein U2AF65 [11], enrichment of WT1 by oligo dT chromatography [12], and in vitro binding of all isoforms of WT1 to RNA [13].

Since a number of multifunctional regulatory proteins with both transcriptional and post-transcriptional activity shuttle between the nucleus and cytoplasm [14,15], we investigated whether WT1 could be exported from the nucleus. We have demonstrated that WT1 can shuttle between the nucleus and cytoplasm and have identified the region of WT1 that contains a nuclear export sequence (NES).

2. Materials and methods

2.1. Plasmid construction

Cloning of mouse WT1 A and D cDNAs has been described previously [16]. These cDNAs were subcloned into pcDNA3.1 (Invitrogen). To create the RRM mutant, the WT1 D cDNA was digested with PpuMI and re-ligated, resulting in the deletion of the sequence encoding amino acids 8–180. WT1Δ(182–324) was created by amplification and subsequent ligation of residues 1–181 and residues 325–449. Other mutants were created by standard polymerase chain reaction (PCR) techniques. To create Gal4 fusion constructs, PCR fragments were subcloned into a vector derived from pSG424 that contains the N-terminal 147 amino acids of Gal4 [17]. To generate Rev1.4-green fluorescent protein (GFP) fusion constructs, WT1 constructs were subcloned into pRev(1.4)-GFP [18]. Rev-GFP [18] was used as a control for leptomycin B (LMB)-sensitive export.

2.2. Cell culture and transfections

HEK 293T, NIH 3T3 and COS-7 cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% foetal calf serum (Life Technologies), 1% L-Gln (Life Technologies) and 1% penicillin–streptomycin (Life Technologies) in a 37°C, 5% CO_2 incubator.

2.3. Interspecies heterokaryon assay

Nucleocytoplasmic shuttling was assayed using a heterokaryon assay [19]. Human HEK 293T cells were transfected with expression vectors, grown overnight and seeded with an equal number of murine NIH 3T3 cells (2×10^4 cells/ml) in fibronectin-coated eight-well chamber slides (Labtek). To determine whether endogenous WT1 could shuttle, murine M15 cells were seeded with non-transfected human HEK 293T cells that do not express WT1. After co-culturing for 3.5 h, cycloheximide (Calbiochem) was added to a final concentration of 100 μg/ml to inhibit protein synthesis. After a further 0.5 h, heterokaryons were formed by incubating the cells for 1 min with polyethylene glycol (45% polyethylene glycol, 10% DMSO in HEPES buff-

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er, pH 7.4). Individual wells were washed with fresh medium, incubated with medium containing 100 μg/ml of cycloheximide and cultured for a further 4 h. When LMB was used, it was added to the cells 1 h before fusion and for the remainder of the experiment at a concentration of 10 ng/ml. Formation of heterokaryons was assessed using phase contrast analysis to determine intact nuclear membranes, and Hoechst staining to verify the presence of mouse and human nuclei. For quantification, the first twenty heterokaryons seen on a slide were counted as long as the nuclei were intact based on phase contrast images. The fluorescence in the murine nucleus of a heterokaryon containing the GFP-Rev mutant was used as the basis for assessing whether active shuttling or diffusion was taking place. Only heterokaryons in which active shuttling was taking place were counted as shuttling. The standard error from two separate experiments was determined.

2.4. Immunofluorescence analysis

In most cases WT1 was detected using the mouse anti-WT1 6F-H2 monoclonal Ab (Dako), which binds to residues 1–181 of WT1, followed by Alexa 594-conjugated anti-mouse IgG (Molecular Probes). The RRM mutant was detected using the rabbit anti-WT1 C-19 polyclonal antibody (Santa Cruz), which binds to residues 431–450 of WT1, followed by Cy3-conjugated anti-rabbit IgG. Hoechst 33258 (Sigma) was used to stain the nuclei.

3. Results

3.1. Both WT1 A and D can be exported out of the nucleus

To determine whether WT1 could be exported out of the nucleus, we used the interspecies heterokaryon assay [19], which is commonly used to assay protein export. Human HEK 293T cells were transfected with an expression vector for WT1 D (Fig. 1A) and then fused with murine NIH 3T3 cells to generate interspecies heterokaryons. Neither of these cell lines express endogenous WT1. To identify heterokaryons, cells were stained with Hoechst 33258. While human nuclei appear diffusely stained, mouse nuclei appear spotted (Fig. 1B). WT1 D was detected in the murine nucleus of a heterokaryon (Fig. 1B), indicating that WT1 D can be exported out of the human nucleus and imported into the mouse nucleus. Labelling of cellular proteins with ^{35}S-Met and Cys demonstrated efficient blockage of protein synthesis by cycloheximide (Fig. 1C).

To confirm that shuttling of WT1 was not due to diffusion, cells were co-transfected with plasmids that express either WT1 D or GFP fused to a Rev mutant (Rev1.4), which lacks an NES [18]. In the same heterokaryon, shuttling of WT1 D (52 kDa) but not the GFP-Rev1.4 mutant (40.5 kDa), was observed (Fig. 1D). These data clearly demonstrate that WT1 is actively exported from the nucleus.

A heterokaryon assay with WT1 A shows that it also shuttles from the human to the mouse nucleus (Fig. 1E), indicating that neither of the alternate splice sites contains an NES. In addition, endogenous WT1 synthesised by the murine mesonephric M15 cell line was also shown to undergo nucleocytoplasmic shuttling (Fig. 1F), indicating that our data is physiologically relevant.

3.2. Export of WT1 is not CRM1 dependent

CRM1 is an export receptor for several proteins with diverse cellular functions that are characterised by the presence of a leucine-rich consensus sequence; $LX_{(2-3)}hX_{(2-3)}LXL$, where X is any amino acid and h can be F, I, L, V or M [20,21]. A similar leucine-rich sequence was identified between residues 442 and 449 of WT1 (Fig. 2A). To determine whether this sequence was a CRM1-binding NES, leucine residues 445, 447 and 449 were mutated to alanine residues. Heterokaryon assays using this 'NES mutant' showed that the mutant protein was exported (Fig. 2B), indicating that the C-terminal leucine-rich sequence was not responsible for CRM1-mediated export. To confirm that export is not CRM1-mediated, the heterokaryon assay was carried out in the presence of LMB, which inhibits export via CRM1 [19]. The results in Fig. 2C show that LMB did not inhibit export of WT1 D. However, LMB inhibited export of GFP fused to Rev (Fig. 2D), which is exported by CRM1 [20]. We concluded that the export of WT1 is not CRM1 dependent.

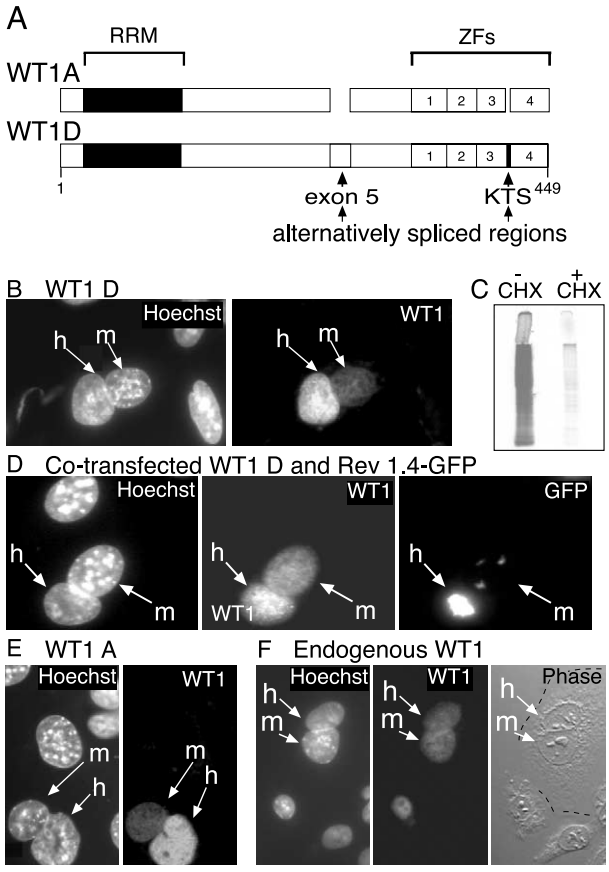

Fig. 1. Nucleocytoplasmic shuttling of WT1. A: Schematic representation of the WT1 A and D isoforms. B: Heterokaryon of an NIH 3T3 cell and a HEK 293T cell transfected with pcDNA3.1 WT1 D, and visualised with Hoechst or a WT1 antibody as described in Section 2. Human (h) and mouse (m) nuclei of heterokaryons are indicated with arrows. C: Autoradiograph of an sodium dodecyl sulfate gel containing total cell lysate from cells that were incubated with ^{35}S-labelled Met and Cys in the presence or absence of cycloheximide (CHX). D: Heterokaryon with an NIH 3T3 cell and a HEK 293T cell co-expressing WT1 D and Rev1.4-GFP. E: Heterokaryon of an NIH 3T3 cell and HEK 293T cell transfected with pcDNA3.1 WT1 A. F: Heterokaryon of an M15 cell and a HEK 293T cell.

3.3. The NES of WT1 is present between residues 182 and 324

To identify the location of the NES of WT1, we generated a series of WT1 deletion mutants (Fig. 3A). These included a series of C-terminal truncations (WT1 del ZF 4, WT1 del ZF(3–4) and WT1 del ZF(2–4)) and a mutant in which the N-terminal residues 8–180 were deleted. This mutant is referred to as the RRM mutant since it lacks the putative RRM (amino acids 20–111) [4]. Western blot analyses of WT1 D and the mutants generated are shown in Fig. 3B.

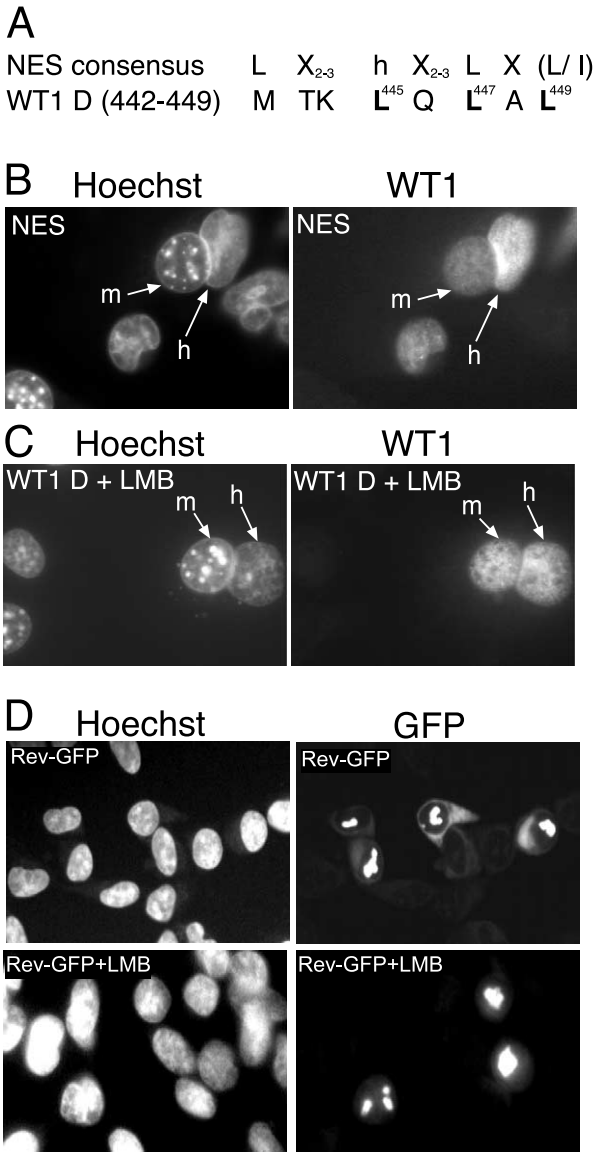

Fig. 2. Determination of CRM1-dependent nucleocytoplasmic shuttling of WT1. A: Alignment of the C-terminal residues 442–449 of WT1 D with the leucine-rich NES consensus sequence, h = hydrophobic amino acid. Leucine residues 445, 447 and 449 mutated to alanine residues in the NES mutant are indicated in bold. B: Heterokaryons from an assay with the NES mutant. C: Heterokaryons from an assay with WT1 D in the presence of LMB. Heterokaryons were visualised as described in the legend to Fig. 1. D: Cells transfected with pRev-GFP after incubation with or without LMB.

All four mutants were used in heterokaryon shuttling assays (Fig. 3C). Deletion of N-terminal residues 8–180 had no effect on nucleocytoplasmic shuttling. Removal of ZFs 3–4 also did not affect shuttling. However, when ZF 2, was also deleted, the protein was partially cytoplasmic. This demonstrates that ZF 2 is required for efficient nuclear localisation of WT1, but precluded the use of the heterokaryon assay to study nuclear export of this mutant.

To generate further C-terminal truncated proteins that were nuclear localised, we generated a series of truncated WT1 proteins fused to the N-terminal 147 amino acids of Gal4 (Fig. 4A). This region of Gal4 contains an NLS that targets Gal4 to the nucleus [22]. COS cells were transfected with these constructs and the subcellular localisation of WT1 was examined (Fig. 4B). Gal4-WT1(1–181) was nuclear, however, Gal4-WT1 del ZF(1–4) was partially cytoplasmic. This subcellular distribution indicates the presence of an NES between resi-

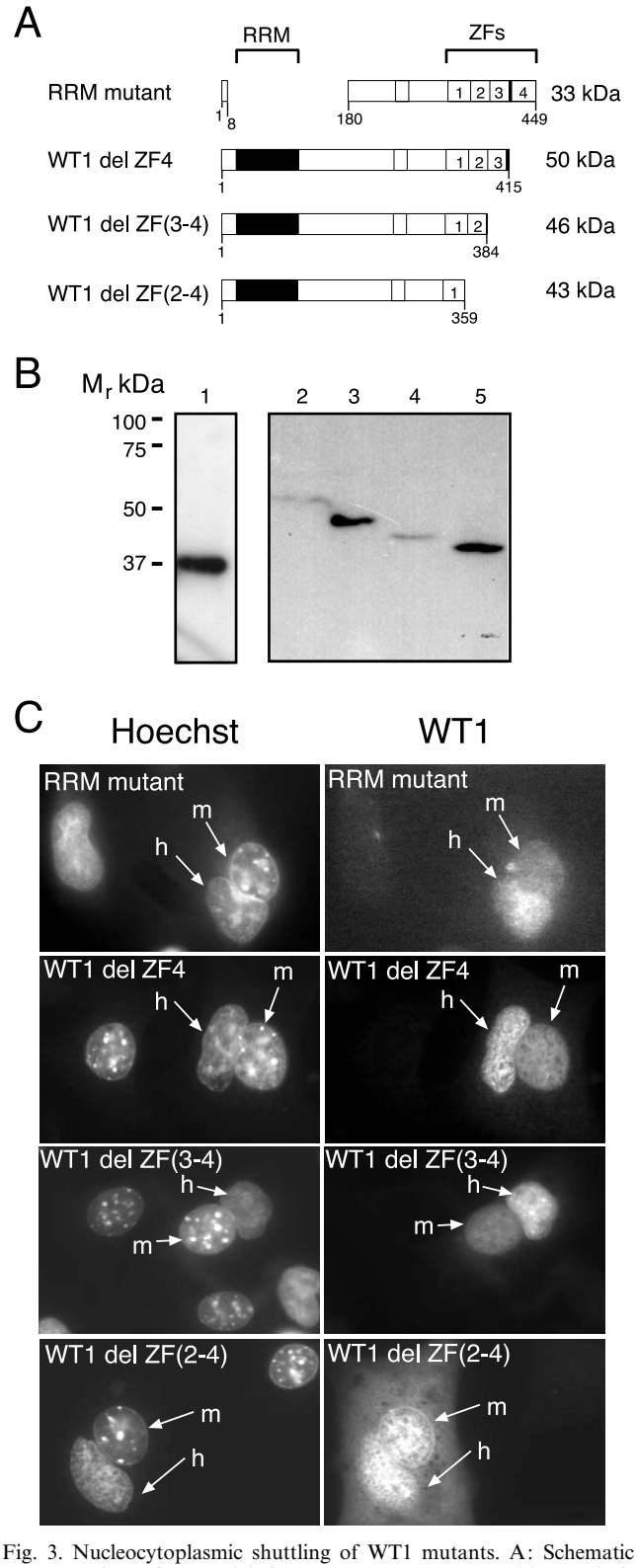

Fig. 3. Nucleocytoplasmic shuttling of WT1 mutants. A: Schematic representation of WT1 deletion mutants. B: Western blot analysis of WT1 and WT1 deletion mutants; 1, RRM mutant; 2, WT1; 3, WT1 del ZF 4; 4, WT1 del ZF(3–4); 5, WT1 del ZF(2–4). C: Heterokaryons from assays with WT1 deletion mutants. Heterokaryons were visualised as described in the legend to Fig. 1.

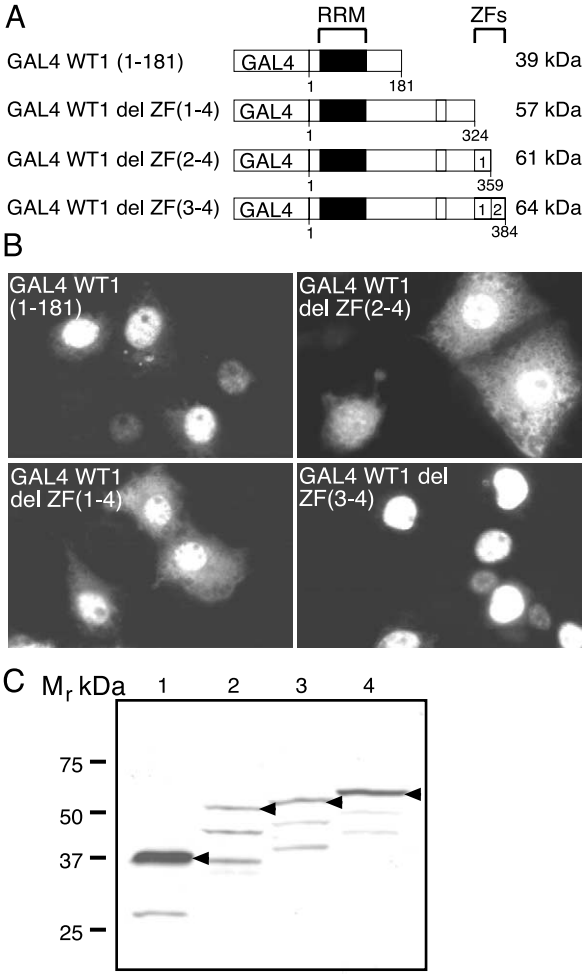

Fig. 4. Subcellular localisation of Gal4-WT1 fusion proteins in COS-7 cells. A: Schematic representation of WT1 mutants fused to Gal4. B: COS-7 cells expressing Gal4-WT1 fusion proteins were immunostained with a WT1 antibody as described in Section 2. C: Western blot analysis of Gal4-WT1 fusion proteins; 1, Gal4-WT1(1–181); 2, Gal4-WT1 del ZF(1–4); 3, Gal4-WT1 del ZF(2–4); 4, Gal4-WT1 del ZF(3–4).

dues 182 and 324, which can partially override the NLS of Gal4. Surprisingly, Gal4-WT1 del ZF(2–4), which contains the Gal4 NLS and one NLS of WT1, was also partially cytoplasmic. However, Gal4-WT1 del ZF(3–4) was nuclear. This demonstrates the requirement for ZF 2 for efficient nuclear localisation in the presence of the presumptive NES. Western blot analysis of the Gal4 fusion proteins (Fig. 4C) showed that a higher fraction of Gal4-WT1 del ZF(1–4) and Gal4-WT1 del ZF(2–4) was degraded compared with the other two proteins. We surmise that this is due to partial cytoplasmic localisation of these two proteins.

3.4. WT1 can mediate the export of Rev1.4-GFP

To unequivocally demonstrate that WT1 contains an NES, and to confirm the location of the NES, we tested the ability of WT1 and WT1 mutants to export GFP-Rev1.4, which lacks an NES. We generated a series of Rev1.4-GFP fusion proteins that contained either full length WT1 D, or the region containing the NES (WT1(182–324)) or WT1 without the NES (WT1Δ(182–324)). These proteins are shown schematically in Fig. 5A. The proteins were used in interspecies heterokaron assays and representative heterokaryons from each assay are shown in Fig. 5B. The percentage of heterokaryons that could export, based on GFP fluorescence, was determined for each protein (Fig. 5C). While shuttling could be detected in a few heterokaryons containing Rev1.4-GFP, upon fusion to WT1, there was an increase in the number of heterokaryons in which shuttling was detected. This was increased further upon fusion of Rev1.4-GFP with WT1(182–324). Again, this is consistent with the data in Fig. 4B and reiterates the role of ZF 2 in efficient nuclear localisation in the presence of an NES. As expected, upon deletion of residues

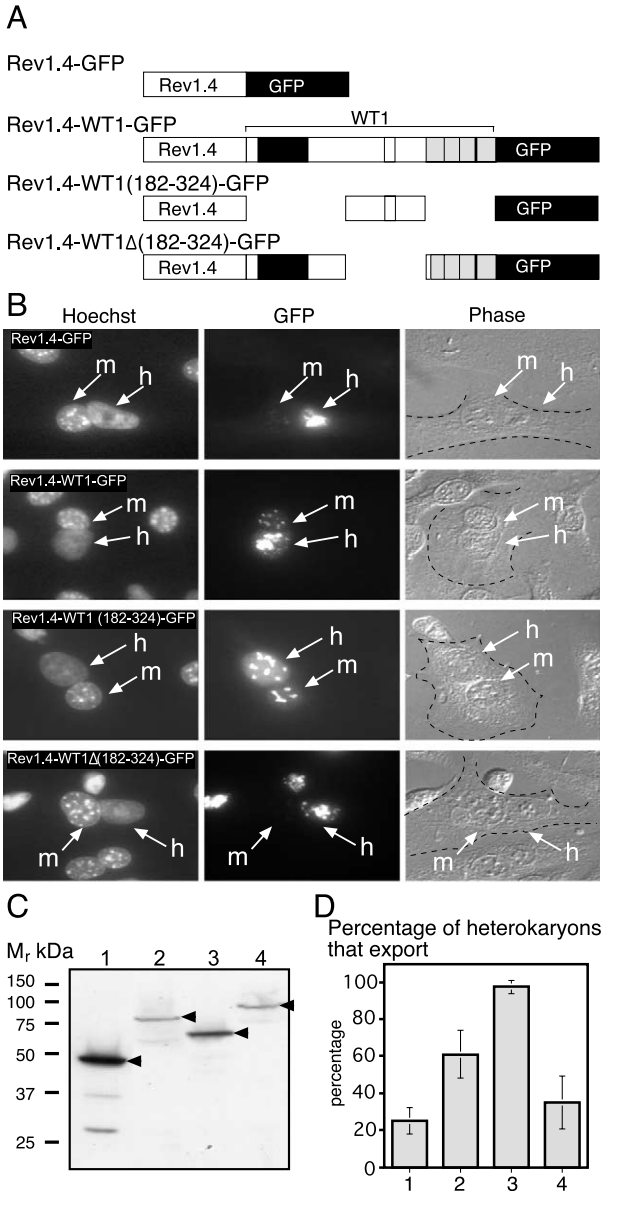

Fig. 5. Nucleocytoplasmic shuttling of Rev1.4-GFP-WT1 fusion proteins. A: Schematic representation of WT1 and WT1 mutants fused to Rev1.4-GFP. B: Heterokaryons from assays with Rev1.4-GFP and WT1 fusion proteins. Heterokaryons were visualised as described in the legend to Fig. 1. C: Western blot analysis of GFP fusion proteins; 1, Rev1.4-GFP; 2, Rev1.4-WT1Δ(182–324)-GFP; 3, Rev1.4-WT1(182–324)-GFP; 4, Rev1.4-WT1-GFP. D: Graphical representation of the percentage of heterokaryons in which protein shuttling was detected; 1, Rev1.4-GFP; 2, Rev1.4-WT1-GFP; 3, Rev1.4-WT1(182–324)-GFP; 4, Rev1.4-WT1Δ(182–324)-GFP.

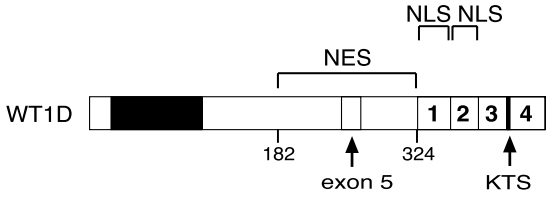

Fig. 6. Schematic representation of WT1 D with the NLSs and NES indicated.

182–324, the number of heterokaryons in which shuttling was detected was decreased compared with that for the full length WT1 fusion protein. We infer that the nuclear export activity of WT1 is transferable to a protein that cannot export and is localised to residues 182–324.

4. Discussion

Using the interspecies heterokaryon assay, we have shown that both endogenous and recombinant WT1 can be exported from a human nucleus and imported into a mouse nucleus. We have demonstrated that in the same heterokaryon, WT1 shuttles while a known non-shuttling Rev1.4-GFP mutant does not. We have also shown that the nuclear export activity is transferable to a protein that lacks an NES. And finally, we have mapped the NES to residues 182–324 (Fig. 6). Mapping of the NES was primarily based on the observation that Gal4-WT1(1–181) (39 kDa) was nuclear while Gal4-WT1 del ZF(1–4) and Gal4-WT1 del ZF(2–4) (57 and 61 kDa respectively) were partially cytoplasmic. This is supported by data showing that fusion of residues 182–324 to Rev1.4-GFP, which lacks an NES, conferred nucleocytoplasmic shuttling.

The region of WT1 that the NES was localised to contains a transcriptional activation domain as well as the alternately spliced sequence encoded by exon 5. However, the latter is not responsible for export since WT1 A, which lacks this sequence, is also exported. An analysis of the WT1 sequence between 182 and 324 failed to identify any of the recently identified non-classical export motifs [23–27]. This suggests that WT1 contains a distinct, as yet undefined, NES. In addition, export is not via CRM1, adding WT1 to the growing list of proteins that are exported via unique export receptors [23,25–27].

Although WT1 is exported, it does not accumulate in the cytoplasm. Therefore, the protein does not appear to be sequestered in the cytoplasm to regulate its nuclear activity unless this occurs under specific physiological conditions. Based on the heterokaryon assays, the protein is rapidly re-imported. WT1 may play a role in the export of cargo from the nucleus. The protein may shuttle in and out of the cell together with hnRNPs, as observed for a number of hnRNP-associated proteins [15,17]. This is consistent with data suggesting that WT1 has a post-transcriptional role [10–13]. WT1 may also play a role in the export of nuclear co-activators such as FHL2 and CBP with which it binds [28,29].

Nucleocytoplasmic shuttling of WT1 is directed by distinct NES and NLS regions rather than a single signal. Our data is in agreement with previous work showing that NLSs are present in ZFs 1 and 2 [6] and we demonstrated the role of ZF 2 in efficient nuclear retention. Hence ZF 2 may be critical for binding with a nuclear component, thus preventing nuclear export of WT1. This is consistent with gel filtration and sedimentation experiments, which have shown that nuclear WT1 is normally a part of a complex [12]. It is unlikely that WT1 is retained in the nucleus by a direct interaction with DNA or RNA since deletion of ZF 4, which strongly reduced the binding of WT1 to DNA and RNA [13], had no effect on the nuclear localisation of WT1. Rather the protein is likely to be retained in the nucleus by binding to other proteins. The ZFs of WT1 have been shown to mediate binding to U2AF65, CBP, and par-4 [11,29,30], with ZFs 1 and 2 being critical for the interaction with CBP. The C-terminus of WT1, which contains the ZFs, is also required for binding to p53, WTAP and Ciao 1 [31–33]. Export may thus be dependent on dissociation from WT1 binding factors and may explain why some heterokaryons expressing Rev1.4-GFP fused to full length WT1 did not shuttle.

There was no difference in the shuttling of WT1 A and D, although when expressed alone, WT1 A co-localises predominantly with transcription factors while WT1 D co-localises more with nuclear speckles [10]. This is not surprising since the isoforms interact via a self-association domain and can form oligomers in vivo [34,35]. As all the isoforms can bind RNA and both WT A and D are exported, this lends support to the argument that the isoforms do not have independent functions but rather interact together to effect the role of WT1 in transcriptional and post-transcriptional control. Since splicing takes place co-transcriptionally, co-ordination of these two roles is quite likely.

In conclusion, we have shown that WT1 can undergo nucleocytoplasmic shuttling and that an NES is present between residues 182 and 324. Further research will be required to establish the nature of the WT1 export complex and the nature of the opposing ZF 2-mediated retention signal.

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