

The Wilms' tumour protein (WT1) shuttles between nucleus and cytoplasm and is present in functional polysomes

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Mutations of the Wilms' tumour-1 (*WT1*) gene in humans can lead to childhood kidney cancer, life-threatening glomerular nephropathy and gonadal dysgenesis. The WT1 protein is normally expressed in the developing genitourinary tract, heart, spleen and adrenal glands and is crucial for their development, however its function at the molecular level is yet to be fully understood. The protein is predominantly nuclear and there is evidence that the two different isoforms of WT1 (–KTS and +KTS) are involved in two different steps of gene expression control: transcription and RNA processing. In this study we report a novel property of WT1, namely that it shuttles between the nucleus and cytoplasm. Moreover, western blot analysis showed that between 10 and 50% of total cellular WT1 can be detected in the cytoplasm depending on the cell type. A significant proportion of cytoplasmic WT1 is in association with ribonucleoprotein particles (RNPs), which strengthens the idea of its involvement in RNA metabolism. Furthermore, we report that WT1 is associated with actively translating polysomes, extending even further the potential roles of WT1 and opening the possibility that it is involved in the regulation of translation. Interestingly, despite the functional differences between two of the WT1 isoforms (+/–KTS) within the nucleus, both isoforms share the shuttling property and are found in translating polysomes.

INTRODUCTION

The tumour suppressor gene *WT1* encodes a protein which plays a critical role in normal development of the kidney, gonads, spleen and heart (1–3). Mutations in this gene can lead to Wilms' tumour of the kidney, glomerular sclerosis and gonadal dysgenesis. These features are found variably in three different syndromes arising through *Wt1* mutations, namely WGAR, Denys–Drash and Frasier syndromes (4,5).

The WT1 protein is predominantly nuclear and contains four Kruppel-type C2-H2 zinc fingers at the C-terminus, suggesting it functions as a transcriptional regulator. Over the past decade a great deal of evidence has accumulated to show that WT1 can indeed function as a transcriptional regulator: as an activator, a repressor or a co-activator (4). However, it has also become clear that the function of WT1 is more complex and that it is likely to be involved in at least two cellular processes: transcription control and RNA metabolism, depending on isoform differences (6,7). There are two major WT1 isoforms, which differ by the insertion of three amino acids, KTS, between zinc fingers 3 and 4 as the result of alternative splicing. The –KTS WT1 isoform binds to DNA with higher

affinity and appears to be more active in transcriptional regulation. A GC-rich DNA sequence, GCGGGGGCG, is recognized by WT1 (8), and the promoters of genes containing this sequence such as *EGR1* (9), *c-myc* (10), *WT1* (11) and *PAX2* (12) have been shown to be regulated by WT1 *in vitro*. Recently, a new binding site for the WT1–KTS isoform, GCGTGGGAGT, has been identified in *Sf1*, *amphiregulin* and *Bcl2* genes, all shown to be physiological targets of WT1 (13–15).

The first connection between the WT1 protein and RNA metabolism was established by Larsson *et al.* (16), who showed that the +KTS isoform co-localized preferentially with splicing factors within nuclear speckles. Soon after that Caricasole *et al.* (17) presented data showing that RNase but not DNase treatment can impair its localization within the nucleus. A putative RNA recognition motif in amino acids 11–72 of WT1 has been identified (18). The suggestion that WT1 is indeed involved in RNA processing and very likely in splicing was further supported by data showing that WT1 (+KTS more than –KTS) directly associates with the constitutive splicing factor U2AF65, which is part of the splicing machinery that recognizes the 3' splice site. However, U2AF65 binding is

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not needed for the speckled distribution of WT1, as shown by WT1 mutants unable to bind to U2AF65 that still co-localize with splicing factors in nuclear speckles (19). Another WT1 binding protein, WTAP, is also involved in splicing. WTAP is a homologue of the *Drosophila* protein FL(2)D that is required for female-specific splicing of Slx and Tra pre-mRNAs mediated by 3' splice site choice (20,21). *In vitro*, WTAP can replace FL(2)D in the regulation of female splicing of Tra pre-mRNA and is also found to be present in functional spliceosomes (22,23). Moreover, the WT1-RNA metabolism connection was further strengthened by the observations that WT1 co-purifies with nuclear poly(A)⁺ ribonucleoproteins. Interestingly, despite the suggestion of functional differences between the two isoforms (+/-KTS), both can co-sediment with RNPs on density gradients from fetal kidney cell line M15 nuclear extracts (24).

Despite the accumulating evidence of WT1 involvement in RNA processing, it is still not clear what role WT1 plays in this cellular process nor have *in vivo* target RNAs been identified. Caricasole *et al.* (17) showed that both WT1-KTS and WT1+KTS can bind to exon 2 sequences of the murine IGF-II transcript and that this binding was mediated by zinc finger 1. The importance of zinc finger 1 in RNA binding was also confirmed *in vivo* (25).

Over the past few years it has become apparent that a significant number of transcription and/or splicing factors shuttle to the cytoplasm where they may acquire a new function. The cytoplasmic function attributed to shuttling proteins is mostly nucleo-cytoplasmic transport of mRNA or RNA stability. Interestingly, some of these proteins are found to be present in functional ribosomes, indicating their involvement in translation (reviewed in 26).

Given these considerations and the fact that WT1 is also associated with RNPs we decided to test whether WT1 shuttles from the nucleus to the cytoplasm. In this paper, we report that WT1 is a shuttling protein and that a significant fraction of endogenous WT1 protein is present in the cytoplasm. We show that both WT1 isoforms, -KTS and +KTS, shuttle between the nucleus and the cytoplasm. Furthermore, cytoplasmic WT1 is present within mRNP complexes, and subsequently is associated with ribosomes and actively translating polysomes.

These new data strengthen the idea that WT1 plays a role in post-transcriptional processes and extend its potential range of functions in the cell.

RESULTS

WT1 protein shuttles between nucleus and cytoplasm

Several RNA processing factors have been shown to shuttle continuously between the nucleus and the cytoplasm, which is suggestive of their involvement in cytoplasmic functions (reviewed in 26). WT1's previous characterization showed it to be a nuclear protein that acts as a transcriptional regulator and also is implicated in mRNA metabolism. To address the question of whether WT1 shuttles between nucleus and cytoplasm we performed a heterokaryon analysis. This assay has been previously used to study the nucleocytoplasmic shuttling activities of several other proteins involved in RNA

processing, such as hnRNP and SR proteins (27-29). WT1-expressing M15 cells, derived from mouse mesonephros were fused to HeLa cells (which do not express WT1 protein) to form heterokaryons. Prior to fusion, the cells were treated with cycloheximide, so that no further protein synthesis takes place in the heterokaryons. The inhibitory effect of cycloheximide on protein synthesis under the conditions used for shuttling was confirmed by *in vivo* labelling the newly synthesized M15 cellular proteins with ³⁵S-Met, from the cycloheximide treated and untreated cells (data not shown). At 1 h post-fusion, the cells were fixed and stained to examine the distribution of the WT1 protein by immunofluorescence microscopy with an anti-WT1 polyclonal antibody (C19). To distinguish the mouse and human nuclei, the cells were stained with the dye DAPI, which gives a characteristic staining of intranuclear bodies in the mouse nuclei. Detection of WT1—only expressed in mouse M15 cells—within the human HeLa nuclei in the heterokaryons is indicative of shuttling. This was indeed the case as shown by the presence of WT1 in both M15 and HeLa nuclei in all of the tested interspecies heterokaryons. This showed that endogenously expressed WT1 was exported from the M15 nuclei into the cytoplasm of the heterokaryons and subsequently into the human HeLa nuclei (Fig. 1A).

Subcellular localization of WT1

This novel shuttling property of WT1 prompted us to closely investigate the intracellular distribution of this protein in WT1 expressing cell lines. Western blot analysis of nuclear and cytoplasmic fractions prepared from two WT1 expressing cell lines, M15 and mouse mesothelioma AC29, revealed that WT1 is predominantly nuclear, as detected by the C19 antibody. However, both cytoplasmic fractions were positive for WT1 as well. A rough comparison with a series of nuclear dilutions revealed that the proportion of cytoplasmic WT1 varied between tested cell lines, being ~1/10 of the total cellular WT1 protein in M15 cells and ~1/2 in the AC29 cell line (Fig. 1B, compare lanes 1 and 5). The same blots were re-probed with an antibody against the nuclear WT1-associating protein, WTAP. The absence of WTAP in the cytoplasm ruled out the existence of cross-contamination between nuclear and cytoplasmic extracts. WT1 was also observed in the cytoplasm of differentiated ES cells (Fig. 1B), strengthening the view that this reflects the normal *in vivo* situation.

Both the -KTS and +KTS WT1 isoforms are shuttling proteins

Two of the WT1 isoforms, +KTS and -KTS, show different nuclear sub-localization patterns and are suggested to have different, or at least partially different, molecular functions. Interspecies heterokaryon assays were therefore performed to test whether the shuttling activity of WT1 is affected by the presence of these three amino acids between zinc fingers three and four. HeLa cells were transfected with plasmids encoding full-length T7-tagged WT1 protein with or without the KTS residues. Twenty-four hours post-transfection cells were fused with mouse NIH3T3 cells and heterokaryon formation between transfected HeLa cells and non-expressing NIH3T3 cell was performed as described above. Upon fixation, immunofluorescence

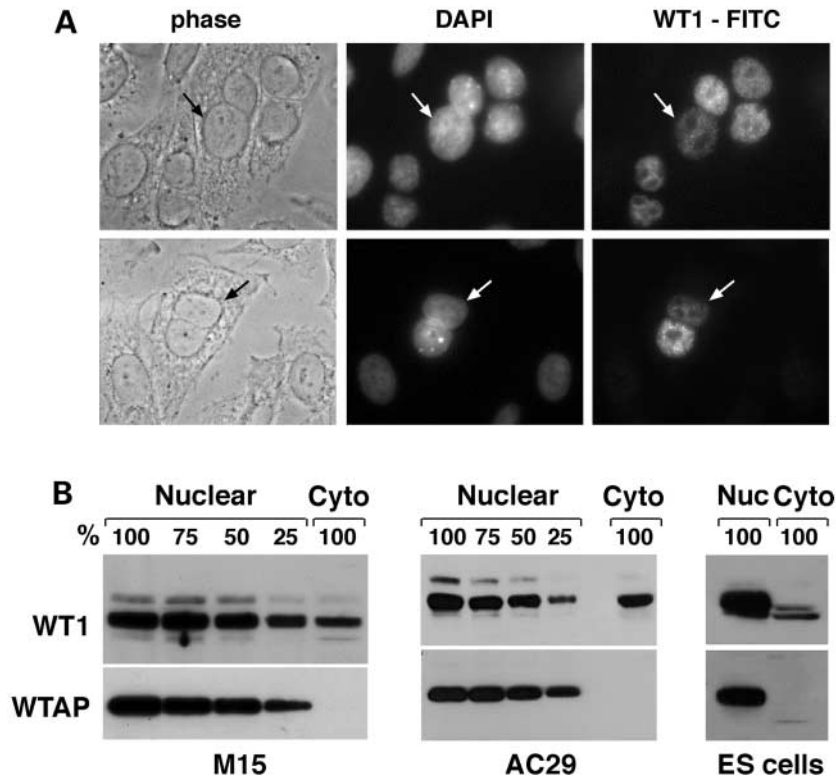


Figure 1. WT1 protein shuttles and is present in the cytoplasm of M15 cells. (A) WT1-expressing M15 cells were treated with cycloheximide to prevent further protein synthesis and subsequently incubated with nonexpressing HeLa cells. The cells were fused in the presence of polyethylene glycol to form heterokaryons. The cells were incubated for 1 h in the presence of cycloheximide, followed by fixation. WT1 localization is detected by immunofluorescence, using anti-WT1 (C19) rabbit polyclonal antibody and fluorescein isothiocyanate (FITC) conjugated secondary antibody (right panel). Phase images reveal heterokaryon formation (left panel). DAPI was used for differential staining of mouse and human nuclei within heterokaryons (middle panel). Arrows indicate HeLa nuclei in which positive staining is the result of WT1 shuttling. (B) Subcellular distribution of WT1 protein in M15, AC29 and ES cells. Nuclear and cytoplasmic extracts were prepared from mouse mesonephros, M15, mouse mesothelioma, AC29, and differentiated ES cells. The nuclear fraction was separated from cytoplasm by centrifugation and volumes were adjusted to be equal. For M15 and AC29 a graded series of nuclear extract concentrations was loaded in lanes 1 (40 μ g) to 4, and a volume of cytoplasmic extract equivalent to lane 1 was loaded in lane 5. Western blot was performed by using anti-WT1 (C19) rabbit polyclonal antibody. Anti-WTAP rabbit polyclonal antibody was used as a negative control for cross-contamination between nuclear and cytoplasmic extract.

was performed using a mouse monoclonal anti-T7 antibody. In all tested heterokaryons where HeLa were positive for epitope-tagged WT1 protein, the protein was also detected in NIH3T3 nuclei. This was irrespective of the presence of the KTS residues (Fig. 2). To rule out the possibility that the observed effect is the result of incomplete inhibition of protein synthesis or an effect influenced by the expression vector, a non-shuttling T7 epitope-tagged SC35, was used as a negative control (Fig. 2) (27). By comparison with WT1 +/-KTS isoforms where 100% of tested heterokaryons were positive for shuttling, in the control experiment 96% of heterokaryons showed no detection of SC35 in NIH3T3 nuclei. These results showed that, despite having different nuclear properties, both WT1-KTS and WT1+KTS isoforms are shuttling proteins.

Cytoplasmic WT1 is in association with poly(A) RNP

The cytoplasmic function of many shuttling proteins remains unclear. The best-described group of shuttling proteins is those which share the following features: (a) in the nucleus they are involved in transcription and/or post-transcriptional processes; (b) they are exported out of the nucleus in a complex with

mRNA; and (c) once in the cytoplasm (in a complex with RNA), they continue to regulate the last steps of gene expression.

As nuclear WT1 is present in RNA complexes (24), we decided to test whether cytoplasmic WT1 could also be detected in RNP complexes. For this purpose M15 cytoplasmic extracts were incubated with oligo(dT) cellulose. After extensive washing the RNA was eluted from the beads with sterile water. A small but significant proportion of WT1 was detected in the eluate by western blot analysis with anti-WT1 antibody, confirming an association of WT1 with cytoplasmic mRNPs (Fig. 3). Interestingly, a significant amount of the protein remains unbound (compare lanes marked A⁻ and A⁺ in Fig. 3). We can speculate that maybe the binding of cytoplasmic WT1 to the RNA is not favoured by the conditions used in this experiment. However, we cannot exclude the possibility that not all cytoplasmic WT1 is in association with RNP. A negative control, Hsp90 β was not bound to the oligo(dT) cellulose under these conditions. To rule out the possibility that the WT1 protein itself has high affinity for oligo(dT) cellulose we have previously shown that *in vitro* synthesised WT1 in RNase treated reticulocyte lysates does not bind to the cellulose (24).

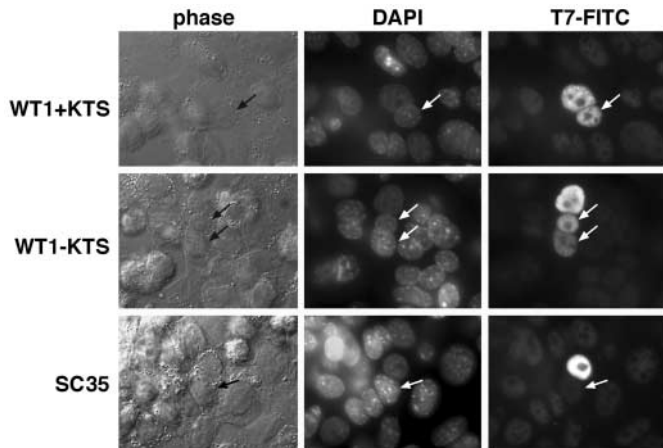


Figure 2. The shuttling property is common for WT1 +/-KTS isoforms. HeLa cells were transfected with expression plasmids encoding either T7 epitope-tagged WT1+KTS, WT1-KTS isoform or non-shuttling SC35. At 24 h post-transfection cells were treated with cycloheximide and fused with mouse NIH3T3 to form heterokaryons. The cells were incubated further for 1 h in the presence of cycloheximide, followed by fixation. The localisation of the expressed proteins was detected by anti-T7 mouse monoclonal antibody, followed by FITC secondary antibody (right panels). Phase images reveal heterokaryon formation (left panels). DAPI was used for differential staining of mouse and human nuclei within heterokaryons (middle panels). Arrows indicate NIH3T3 nuclei, which are positive for WT1 staining as a result of WT1 nucleocytoplasmic shuttling. Absence of shuttling for SC35 confirms reliability of the experiment.

Moreover, we here show a further control experiment in which the beads were saturated with oligonucleotides containing stretches of 10–18 adenines prior to incubation with the extract. WT1 was not detected in these eluates (lane marked A⁺/comp in Fig. 3). Altogether, these results show that WT1 binding to the beads is mediated by poly(A) RNA.

Cytoplasmic WT1 is associated with actively translating polyribosomes

To better define the sublocalization of cytoplasmic WT1, M15 cytoplasmic lysates were centrifuged through 10–50% sucrose gradients, allowing the separation of various components of the RNA machinery. Gradients were fractionated and the presence of WT1 in each fraction was studied by western blot analysis using mouse monoclonal anti-WT1 antibody (H2). An antibody against ribosomal protein S6 (rpS6), an integral component of the small ribosomal subunit, was used as marker for the ribosome profile on the gradient. Each blot was further re-probed with anti-Hsp90 β . This protein does not associate with the ribosomes and is present at the top of the gradient. The pattern of WT1 in the fractions followed closely the pattern of S6 protein: it was detected throughout the sucrose gradient, and co-fractionated with ribosomal subunits and poly-ribosomes. In addition, the sedimentation profile of WT1 was affected in EDTA-treated extracts, where intact ribosomes and polysomes have been destabilised. Under these conditions WT1 was distributed more narrowly across the gradient, sedimenting primarily with the 60S and 40S subunits. By comparison, the

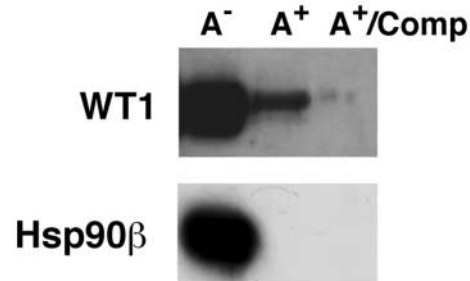


Figure 3. Oligo(dT) chromatography of M15 cytoplasmic extract. Cytoplasmic extract from M15 cells was incubated with oligo(dT) cellulose. After extensive washing with binding buffer, the bound material was eluted in sterile water. A⁻, flow-through; A⁺, elution from oligo(dT) cellulose; A⁺/comp, eluted material from the oligo(dT) cellulose previously treated with poly(A) oligonucleotide competitor. Western blot was performed by using anti-WT1 (C19) rabbit polyclonal antibody, followed by HRP secondary antibody. Anti-Hsp90 β rabbit polyclonal antibody was used as a negative control.

distribution of Hsp90 β was not affected by EDTA treatment (Fig. 4A).

Furthermore, we performed another experiment in which polysomes from M15 cells were pelleted by longer centrifugation through a 10–50% sucrose gradient. Western blot analysis of the pellet confirmed the presence of WT1 in poly-ribosomes which are marked by the presence of rpS6 and absence of Hsp90 β . Extracts treated with EDTA failed to pellet WT1 or rpS6 (Fig. 4B). Altogether these results suggest that the WT1 protein is associated with the translation machinery, and in particular a subset of cytoplasmic WT1 is associated with actively translating ribosomes.

WT1 protein consists of numerous isoforms in the range of 52–62 kDa (Fig. 4C). With the exception of the +KTS/-KTS isoforms, it is still not clear how the isoform differences affect the properties of WT1 at the molecular level. When the samples from the non-EDTA treated gradient were re-loaded on the 8% SDS-PAGE, it was possible to distinguish between some of these isoforms. As shown in Figure 4D, the isoforms containing two different start sites (ATG and CTG) and those containing or missing exon 5 are present in cytoplasm and all can be detected in heavy polysomal fractions as well as the ribosomal subunits and RNPs. These results indicate that the cytoplasmic localization and, in particular, association with the translating ribosomes, might be common for these four isoforms.

Both +KTS and -KTS WT1 isoforms co-fractionate with actively translating polysomes

As the +KTS and -KTS WT1 isoforms in the nucleus are known to have partially different localization and functions, we wanted to test if these differences extend to its localization in the cytoplasm and distribution on the sucrose gradient. As these isoforms differ in only three amino acids and no KTS specific antibody exists, it is impossible to distinguish their distribution on sucrose gradients containing M15 extracts. For this reason the cytoplasmic extracts from COS-7 cells transfected with constructs expressing either T7 tagged +KTS or -KTS WT1 isoforms were separated across 10–50% sucrose gradients and the distribution of WT1 isoforms was analysed by western blotting. We found that both WT1 isoforms were

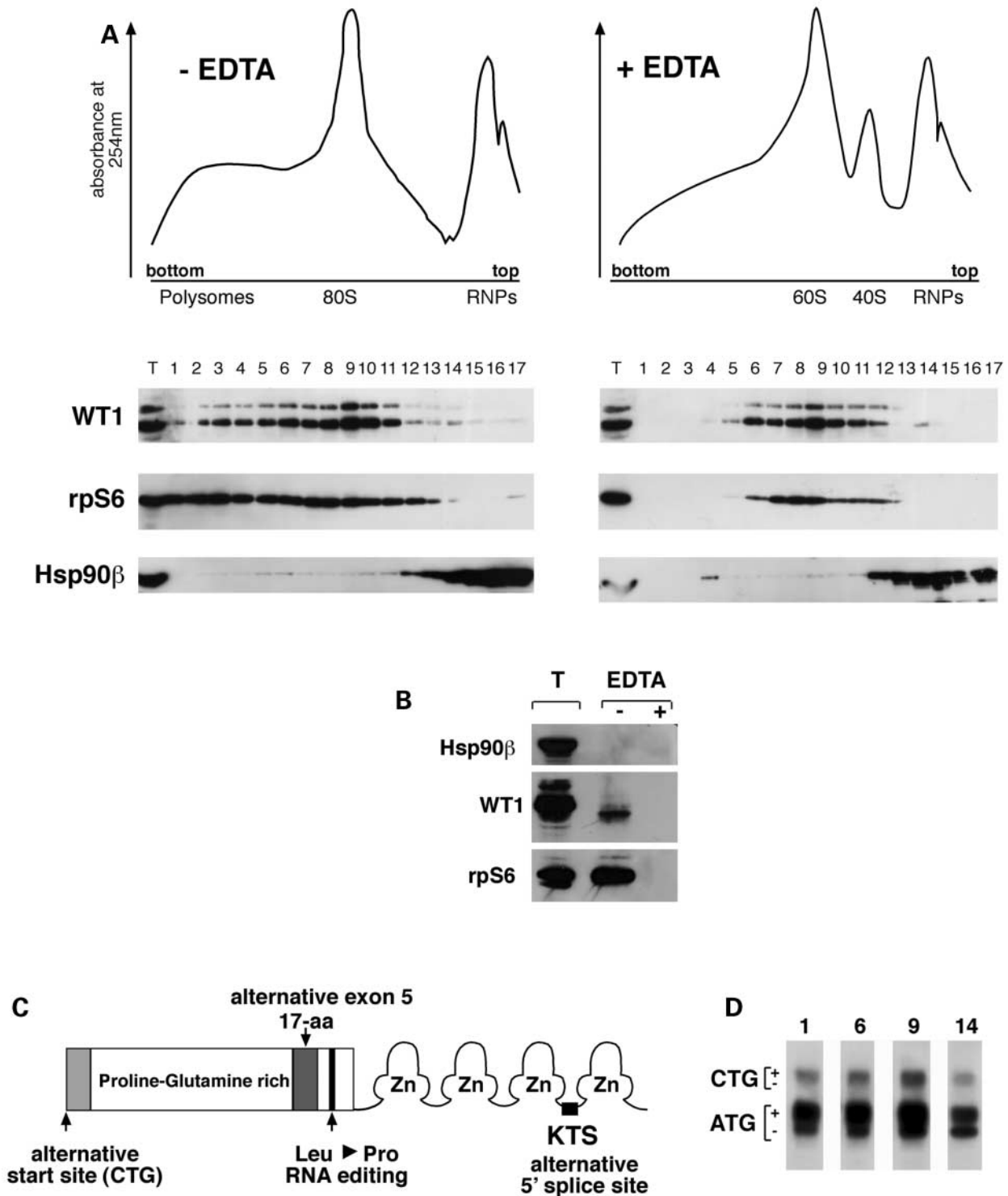


Figure 4. Association of endogenous WT1 with translating polysomes. **(A)** The comparison of the gradients from untreated (left panel) and EDTA treated (right panel) M15 extracts. The M15 cytoplasmic extracts were loaded onto 10–50% sucrose gradients and centrifuged for 2 h 20 min. Fractions were collected and the UV absorbance profiles of the cytoplasmic RNA complexes at 254 nm were determined (upper panels). Polysomes, ribosomal subunits 80S, 60S, 40S and RNP are indicated. Protein fractions were precipitated and loaded on the 12% SDS–PAGE. T, total cytoplasmic extract, 1–17, sucrose gradient fractions starting with the bottom fraction. **(B)** Polysomes from M15 cells were pelleted by centrifugation for 4 h 40 min. Pelleted polysomes were resuspended in loading buffer and loaded on the 12% SDS–PAGE. EDTA-treated extracts were incubated with 30 mM EDTA for 15 min prior the centrifugation. Blots were probed with (a) H2, anti-WT1 mouse monoclonal antibody, (b) anti-S6 rabbit polyclonal antibody, and (c) anti-Hsp90β rabbit polyclonal antibody. **(C)** Schematic presentation of mammalian WT1 protein isoforms. **(D)** Fractions 1, 6, 9 and 14 from M15 sucrose gradient used in **(A)** were loaded on 8% SDS–PAGE gel for better separation and probed with H2 antibody. Fraction 1 represents the isoform pattern detected in the fraction with heavy polysomes; 6, light polysomes; 9, monosomes; 14 RNPs. +, WT1 including exon 5; –, WT1 isoform missing exon 5.

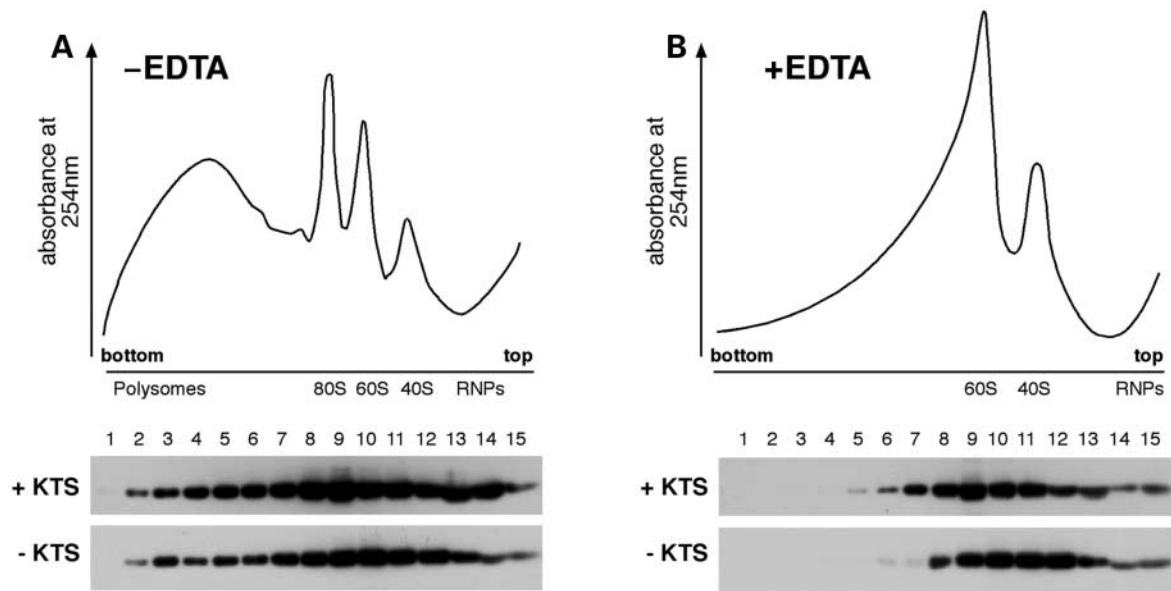


Figure 5. Association of WT1+KTS and WT1-KTS isoforms with polysomes. COS-7 cells were electroporated with constructs carrying T7 tagged either WT1+KTS or WT1-KTS cDNA isoform. Cytoplasmic extracts were collected 48 h later, loaded on 10–50% sucrose gradient and centrifuged for 2 h 20 min. Fractions were collected and the absorbance profiles of the gradients at 254 nm were determined (upper panels). Polysomes, ribosomal subunits (80S, 60S, 40S) and RNP are indicated. Protein fractions were precipitated and loaded on the 12% SDS-PAGE. EDTA-treated extracts were incubated with 30 mM EDTA for 15 min prior the centrifugation. Blot was probed with anti-T7 mouse monoclonal antibody. 1–15, sucrose gradient fractions starting with the bottom fraction.

broadly distributed between the mRNP complexes, ribosomal subunits and polysomes. Furthermore, the treatment with EDTA and subsequently the dissociation of ribosomal subunits caused the shift of both isoforms towards the individual subunits indicating that association of WT1 with the translational machinery is common for both WT1 isoforms (Fig. 5).

Taken together, these results demonstrate that WT1, a protein involved in transcriptional regulation and with a proposed role in RNA metabolism, shuttles continuously between the nucleus and cytoplasm, suggesting a putative involvement in cytoplasmic functions. The reported association of WT1 with polysomes suggests a role for WT1 in translational regulation.

DISCUSSION

So far extensive evidence has accumulated to suggest that WT1 acts as a complex multifunctional nuclear protein involved in at least two levels of gene expression control: transcription and RNA processing. Our finding that WT1 is not restricted to the nucleus but shuttles continuously between the nucleus and the cytoplasm adds a new level of complexity to the potential roles of WT1 at the cellular level. We showed that WT1 can be detected in the cytoplasm of various cell lines. The nucleo-cytoplasmic ratios among tested cell lines appear to be different, most likely indicating that the net result of shuttling kinetics between these cell lines is different. It would be interesting to see if these differences can be observed *in vivo* and if they are tissue-specific.

However, the main question raised by these findings is what, if any, is the functional significance of nucleo-cytoplasmic

shuttling of WT1? Firstly, the data showing that a significant proportion of cytoplasmic WT1 is in a complex with RNPs provides additional support to the idea that WT1 is involved in RNA metabolism, not only in the nucleus but also in the cytoplasm. Furthermore, our observation that the WT1 protein is present in functional polysomal complexes strongly suggests that, in addition to its known nuclear functions, WT1 might be involved in translation. These findings raise the possibility that WT1 might be a multifunctional component of protein complexes that regulate three steps of gene expression: transcriptional control, RNA processing and translational regulation.

This finding can be seen in the context of the recent discovery that transcription and post-transcriptional processes in nucleus and cytoplasm are intimately coupled (30). There is a growing list of RNA binding proteins involved in gene expression control within the nucleus that have shuttling properties and are involved in cytoplasmic regulation. Some members of the hnRNP family (such as hnRNP A1, hnRNP L and hnRNP I/PTB), which were originally thought just to accompany mRNA into the cytoplasm, have been found to control at least three cytoplasmic events: mRNA localization, mRNA turnover and mRNA translation (reviewed in 26,31). Furthermore, some DNA binding proteins involved in transcriptional control, are also thought to have cytoplasmic functions. For example, the tumour suppressor p53 has been suggested to be in a complex with polysomes through its association with mRNA (32). The orphan receptor DAX1, which acts as a transcriptional repressor, was also found to have the same property (33).

The WT1 protein is an interesting example of a multifunctional protein whose nuclear function seems to be divided between two different isoforms, +KTS and -KTS. Despite

their spatial and functional differences in the nucleus, our results from transfection experiments show that both isoforms can shuttle into the cytoplasm and both are found to be associated with functional polysomes. This result seems contradictory to the proposed different functions for the two isoforms. However, this is not unexpected when taking into consideration that these different isoforms show some redundancy at the biochemical and genetical level (7,34). For example, genetic studies in mice have shown clearly that the two isoforms can substitute for each other in the early stages of the genitourinary development and in the heart (35). Given this overlap of the WT1 isoforms properties we can speculate that the two isoforms might associate with dynamic protein complexes at different stages of gene expression within the nucleus, and remain a stable component all the way to translation. Because WT1 can self associate (36), it is also possible that the isoforms can recruit each other at the different steps of gene expression process. Our data from sucrose gradients of transfected +KTS and -KTS extracts show that *in vitro* each isoform co-sediments with polysomes independently of the presence of the other one. It remains to be seen if this reflects the *in vivo* situation.

Although the most likely possibility is that WT1, a component of nuclear mRNPs, is exported to the cytoplasm bound to mRNA, we cannot exclude at this stage the possibility that WT1 may be exported from the nucleus independently of mRNA export, as reported for hnRNP I/PTB (37). It is possible that, instead, direct modifications to the WT1 protein itself trigger its dissociation from the RNA and export from the nucleus. For example, there is evidence that at least two serine residues within zinc fingers 2 and 3 can be phosphorylated by forskalin treatment (38). Furthermore, it has been shown that phosphorylation of EWS/WT1 by PKA causes its accumulation in the cytoplasm, suggesting that such a mechanism could modulate the transport of that protein throughout the nuclear membrane (39). Therefore, it may be possible that the phosphorylation state of WT1 determines its subcellular localization. Once in the cytoplasm the WT1 could reassociate with RNPs as they are becoming engaged in translation or associate directly with translation machinery itself. This could explain why not all cytoplasmic WT1 is in the complex with RNP, as well as the observations that in the sucrose gradients the WT1 signal in RNP fractions are weak and become stronger as RNP become part of translation machinery. If this hypothesis that WT1 dissociates from RNP in the nucleus and reassociates with cytoplasmic RNP, turns out to be true, it would be interesting to see if WT1 binds to the same RNA targets within nucleus and cytoplasm.

It has been previously shown that WT1 accumulates in the cytoplasm in tumours of different tissue origins, such as rhabdomyosarcomas (40), some breast cancers (41) and colorectal adenocarcinomas (42). It is believed that in these tumour types WT1 plays an oncogene-like role rather than functioning as a tumour suppressor gene. Our data raise the possibility that the potential oncogenic role of WT1 could be in regulating translation rather than nuclear processes. It remains to be seen whether cytoplasmic WT1 in tumour cells is associated with polysomes. Furthermore, a mislocalization *per se*, rather than overexpression, might have an oncogenic potential.

The association of WT1 with polysomes extends even further the potential roles of this protein. Our future experiments will address the functional significance of nucleocytoplasmic shuttling and whether WT1 regulates translation of specific mRNAs.

MATERIALS AND METHODS

Cell lines and transfections

Cell lines used in these experiments were HeLa, COS7, NIH 3T3, mouse mesonephros-derived, M15 and mouse mesothelioma, AC29. All cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum except AC29 which were cultured in RPMI 1640 with 10% fetal calf serum. Transfection of COS7 cells for sucrose gradient analysis was done by electroporation, with 10 µg of vector per 75 cm flask of confluent COS7 cells, in a Biorad electroporator under the following conditions: 1 kV, 25 µF. The two constructs used were full-length mouse WT1 cDNA, + and -KTS subcloned into the pCGT7 vector as described before (24).

Western blot analysis and antibodies

Protein samples isolated from M15 nuclear or cytoplasmic extracts and sucrose gradient fractions were resolved by 12% SDS-PAGE if not stated otherwise. Proteins were then transferred to Hybond-P membranes (Amersham Pharmacia Biotech). Non-specific binding sites were blocked by incubation of the membrane with 5% non-fat dry milk in TBST (20 mM Tris, pH 7.5, 137 mM NaCl and 0.1% Tween 20). Proteins were detected using the following primary antibodies diluted in 5% non-fat dry milk in TBST: mouse monoclonal anti-T7 (Novagen) 1:10 000; rabbit polyclonal anti-WT1 (C19, Santa Cruz), 1:2000; rabbit polyclonal anti-rpS6 (Cell Signalling) 1:1000; mouse monoclonal anti WT1 (H2, DAKO) 1:250. Following washing in TBST, blots were incubated with the appropriate secondary antibodies conjugated to horseradish peroxidase (Amersham) and detected with ELC plus western blot detection system (Amersham).

Heterokaryon formation assay

Nucleocytoplasmic shuttling of endogenous WT1 protein was assayed by the heterokaryon formation assay using WT1-expressing mouse M15 cells and non-expressing HeLa cells. M15 cells were seeded on the coverslips at a density of 50%, followed by co-incubation with an excess of HeLa cells for 3 h in the presence of 50 µg/ml of cycloheximide. Thirty minutes before fusion cells were incubated in DMEM containing cycloheximide at a concentration of 100 µg/ml. For the fusion, each coverslip was inverted onto a drop of 50% PEG for 2 min, following by washing in PBS and incubation in fresh DMEM containing 100 µg/ml of cycloheximide for 1 h. Cells were then washed fixed and immunostained as described. Heterokaryon assay of the different WT1 isoforms was performed by electroporating HeLa cells with a plasmid containing cDNA encoding the protein of interest and fusing with NIH 3T3 as

described in Cazalla *et al.* (43). At least 50 heterokaryons were analysed for each experiment.

Cellular extract preparation

Cells were washed twice in ice cold PBS and scraped following the centrifugation at 4000 rpm at 4°C for 3 min. Cells were then resuspended in hypotonic swelling buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂ and protease cocktail). After 10 min incubation on ice, 10% NP40 was added and samples were centrifuged for 1 min at 14 000 rpm. The supernatant was removed and stored as a cytoplasmic fraction. The pellet was further resuspended in lysis buffer (20 mM Hepes pH 7.9, 600 mM KCl, 0.2 mM EDTA, 1 mM DTT, protease inhibitor). Upon incubation on ice for 30 min the solution was centrifuged for 10 min at maximum speed in a microfuge. The supernatant was removed, TCA precipitated and used as a nuclear fraction.

Oligo(dT) chromatography

M15 cells were trypsinized, washed in PBS and resuspended in lysis buffer (10 mM Tris pH 7.5, 5 mM NaCl, 2 mM MgCl₂, 8% glycerol, 1 mM DTT, 100 µ/ml RNase inhibitor). After 10 min incubation on ice, the lysate was passed through a 25G needle and centrifuged for 10 min. The supernatant was collected and the concentration of NaCl was adjusted to 250 mM NaCl. Pre-swollen oligo(dT) cellulose (Amersham) was added to the cytoplasmic extract and incubated by rotation for 2 h at 4°C. The cellulose was then washed three times with washing buffer (10 mM Tris, 250 mM NaCl, 2 mM MgCl₂). PolyA RNA together with PolyA+ bound protein was eluted from the cellulose with warm sterile water. Eluted fractions were TCA precipitated and the pellet was resuspended in 50 µl of 2 × SDS loading buffer, half of which was loaded on the 12% SDS-PAGE gel. When oligo(dT) cellulose was saturated with polyA oligonucleotides (25 µg of oligonucleotides/10 mg of cellulose) the oligonucleotides were incubated with pre-swollen oligo dT cellulose for 2 h at 4°C followed by incubation with the extract described above.

Polysome profiling by sucrose gradient fractionation

Cells were washed twice with ice-cold PBS containing 150 µg/ml of cycloheximide and scraped from the plate. Cells were pelleted at 1000 rpm in a clinical centrifuge, washed three times with PBS containing cycloheximide (150 µg/ml) and PMSF (1 mM) and finally resuspended in lysis buffer (20 mM Tris pH 7.5, 100 mM KCl, 5 mM MgCl₂, 0.3% NP40, RNase inhibitor, protease inhibitor). Lysed cells were pelleted by centrifugation 10 000 rpm in a cold microfuge. The resulting supernatant was then layered onto 10 ml linear 10–50% sucrose gradients containing 20 mM Tris, pH 7.5, 5 mM MgCl₂, 100 mM KCl and samples were centrifuged in a Beckman TH641 rotor at 37 000 rpm for 2 h and 20 min at 4°C. Following centrifugation, the gradients were fractionated using a Pharmacia Superfrac fraction collector and the absorbance of cytosolic RNA at 254 nm was recorded by an inline UV monitor (Pharmacia). Protein was precipitated by addition of trichloroacetic acid (TCA) and centrifuged at the maximal speed for

10 min. The pellet was washed twice with the ice-cold acetone resuspended in the loading buffer. A desired amount of the protein from each fraction was loaded on the 12% SDS-PAGE and analysed by western blot. For polysome pelleting, the extracts were prepared and centrifuged through sucrose gradients as described above except, the centrifugation time was 4 h and 40 min. The pellet was rinsed with the sucrose buffer and resuspended in a loading buffer.

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