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INSTITUTO NACIONAL DE CANCEROLOGIA

INFORME DE ACTIVIDADES
COMISION DE ESTUDIOS EN EL EXTERIOR

PRESENTADO A: DOCTOR CARLOS CASTRO

PRESENTADO POR: AMPARO SERNA ALARCON

Enero 11 de 2000

Durante mi comisión de estudios en el exterior realizada en la Fundación Tetamantti en la ciudad de monza Italia, tuve la oportunidad de colaborar con el Dr. Corrado Caslini en la realización de un proyecto que consistía en caracterizar funcionalmente los productos del gen ZNF162 identificado en dicho laboratorio. De esta investigación se genero un articulo el cual se presento en el XXVII Simposio de la Sociedad Italiana de Cancerología donde fue seleccionado para su presentación oral, actualmente este articulo se encuentra en revisión por parte del comité de Journal of Biological Chemistry para su publicación.

Con la elaboración de este trabajo he logrado ampliar mis conocimientos teóricos y prácticos en diferentes estrategias de biología celular y molecular los cuales se podrán aplicar en diferentes proyectos que estén en curso o que se puedan desarrollar en el futuro.

SOCIETÀ ITALIANA DI CANCEROLOGIA

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EUROPEAN ASSOCIATION FOR CANCER RESEARCH

XXVII SIMPOSIO DELLA SOCIETÀ ITALIANA DI CANCEROLOGIA

XXVII SYMPOSIUM OF THE ITALIAN CANCER SOCIETY

Istituto Nazionale per lo Studio e la Cura dei Tumori - Centro Didattico

Si certifica che il Dr./Prof.

Amparo SERNA

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whether the effect of c-erbB-2-transfection depends on the p53 status of the transfected cells. To this aim, the ovarian carcinoma cell line IGROV1 carrying a wild-type p53 and the cisplatin-resistant subline carrying a mutated-p53 were transfected with the c-erbB-2 gene. While the wild type-p53 IGROV1 was found to be growth-inhibited by c-erbB-2 transfection, the mutated-p53 variant was not.. IGROV1 transfectants also showed an increased expression of p53. Also removal of c-erbB-2 overexpression by cytoplasmic single chain antibodies in SKOV3, a null p53 cell line, and in its variant transfected with wild-type p53 suggests that c-erbB-2 is associated with proliferation only in cells lacking a functional p53.

Partially supported by A.I.R.C.

THE PTEN/MMAC1 GENE IS DOWN-REGULATED IN A SUBSET OF THYROID CARCINOMAS; PTEN RE-EXPRESSION INTO THYROID TUMOR CELLS INHIBITS PROLIFERATION

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The cytoplasmic dual-specificity phosphatase pTEN/MMAC1/TEP1 has recently been identified as the tumor suppressor gene most frequently mutated and/or deleted in human tumors. Germline mutations of pTEN give rise to several related neoplastic disorders including Cowden disease (CS), an autosomal dominantly-inherited cancer syndrome. Patients affected by CS show increased risk of breast and thyroid tumors. However, pTEN mutations in sporadic thyroid carcinomas have been rarely detected. In this study, we confirm that pTEN mutations in sporadic thyroid cancer are infrequent as we found only 1/26 thyroid tumors that carries a mutation and 1/8 tumor cell lines harboring a heterozygous deletion of pTEN gene. In addition, we have also investigated the expression of pTEN gene by Northern blot, RT-PCR and Western blot in sporadic thyroid tumors and cell lines. We report that pTEN expression -both at the mRNA and at the protein level- was drastically reduced in 7/8 tumor-derived cell lines and in 24/61 primary tumors. Finally, we demonstrate that pTEN may act as a suppressor of thyroid cancerogenesis as the constitutive re-expression of pTEN into 3 different thyroid tumor cell lines that have lost pTEN expression (TPC-1, ARO and FB-1, respectively) markedly inhibited cell growth. Such an arrest correlated with a significant increase in the expression of cyclin-dependent kinase inhibitor $p27^{kip1}$ and a concomitant decrease in in the activity of G1 cyclin-dependent kinase CDK2. In conclusion, our findings demonstrate that the inactivation of pTEN function may play a role in the development of thyroid tumors and that one key target of pTEN suppressor activity is represented by $p27^{kip1}$.

THE STAR FAMILY MEMBER ZNF162 (ZFM1/SF1) PROTEIN SHUTTLES CONTINUOUSLY BETWEEN THE NUCLEUS AND THE CYTOPLASM

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ZNF162, also known as ZFM1 or SF1, is a STAR family member of KH-domain containing RNA-binding proteins with potential dual function in signal transduction and activation of RNA (STAR). STAR family members include GRP33, Sam68, GLD-1, Who/How, Xqua, and Qk1, in which two conserved QUA1 and QUA2 domains of homology are contiguous with the KH domain, resulting in a larger STAR domain of 170 amino acids. Recently, ZNF162 (SF1) was identified as the mammalian ortholog of the yeast pre-mRNA branchpoint sequence binding protein (BBP) and a novel E complex component in mammalian splicing pathway. Furthermore, ZNF162 (ZFM1) was shown to repress transcription of both stage-specific activator protein (SSAP) of sea urchin and EWS human oncoprotein of Ewing's sarcoma tumors. At least seven alternatively spliced ZNF162 variants have been identified, all containing an N-terminal nuclear localization signal (NLS), a STAR domain, and a Zn-knuckle RNA-binding domain and diverging thereafter in their C-terminus. We cloned two of these isoforms, named $ZNF162_{B3}$ and $ZNF162_{B4}$. The first exhibits a putative ATP-binding motif (P-loop) and tyrosine-rich C-terminus, while the latter shows a proline-rich C-terminus, including a PPLP ligand for the WW-domain of formin-binding protein 11 (FBP11) and putative ligands for the SH3-domain of Abl. Here we report that both $ZNF162_{B3}$ and $ZNF162_{B4}$ are nucleo-cytoplasmic shuttling proteins continuously transported between the nucleus and the cytoplasm. At steady state, we show identical nuclear distribution pattern for both ectopically expressed proteins with distinct cytoplasmic speckled accumulation of $ZNF162_{B4}$, which is conferred by its proline-rich C-terminus. The N-terminal 141 amino acids portion of both ZNF162 proteins is required for shuttling, which is partially sensitive to the CRM1-mediated nuclear export inhibitor leptomycin B (LMB). Deletion of the only NLS domain delocalizes both proteins mostly in the cytoplasm of HeLa cells without preventing their shuttling ability. That indicates the existence of another nuclear shuttling domain inside the N-terminus. In addition, deletion of Zn-knuckle, STAR or QUA2 domain does not impair the nucleo-cytoplasmic shuttling ability of both proteins, therefore excluding their possible involvement in mRNA transport. Additional mutants are now been tested to positively identify the region responsible for shuttling.

The STAR Family Protein ZNF162 (SF1) Shuttles between the Nucleus and the Cytoplasm through the Amino Terminus

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Summary

The ZNF162 protein, also known as SF1, belongs to the growing STAR family of RNA-binding proteins with potential dual function in signal transduction and activation of RNA. ZNF162 shares with all STAR members a hnRNP K homologue (KH) domain contiguous with a QUA2 domain in a larger conserved domain named STAR. Here, using an interspecies heterokaryon assay, we found that $ZNF162_{B3}$ and $ZNF162_{B4}$ isoforms are nucleo-cytoplasmic shuttling proteins rapidly transported between nucleus and cytoplasm. These proteins show peculiar and transcription independent subcellular localization, while N-terminal NLS deletion mutants or $ZNF162_{HDF}$ and $ZNF162_{HB4}$ isoforms lacking the N-terminus show a similar broad intracellular distribution in cytoplasm and nucleus. By deletion mutant analysis we found that the N-terminal 120 amino acids of $ZNF162_{B3}$ and $ZNF162_{B4}$, and not the STAR domain and their alternatively spliced C-termini, is required for nucleo-cytoplasmic shuttling. Therefore, we reveal an additional function conferred to $ZNF162_{B3}$ and $ZNF162_{B4}$ by sequences N-terminal to

the KH domain. In addition, $ZNF162_{B3}$ and $ZNF162_{B4}$ nuclear export is a temperature dependent process partially sensitive to CRM1-inhibitor LMB. Thus, $ZNF162_{B3}$ and $ZNF162_{B4}$ share with QKI-5 STAR protein the ability to shuttle between nucleus and cytoplasm.

Introduction

The human zinc finger gene *ZNF162*, also known as *zinc finger gene* in the *MEN1* locus (*ZFM1*) (1) or *splicing-factor 1* (*SF1*) (2), [1] encodes for an RNA-binding protein which belongs to the evolutionarily conserved family of proteins called STAR, with a potential dual function in signal transduction and activation of RNA (3). STAR family members include: *Artemia salina* GRP33 (4); mouse and human SAM68 (5, 6); *Caenorhabditis elegans* GLD-1 (7); mouse and human T-STAR/ETOILE (8); *Drosophila melanoganster* who (9), Zebrafish Zqua (10), *Xenopus laevis* Xqua, mouse and human QKI (11, 12). All these proteins show a QUA1 and QUA2 regions of homology to QKI (11) contiguous with the hnRNP K homologue domain in a larger conserved STAR domain of 170 amino acids.

The *ZNF162/ZFM1* gene was first identified as incorrect candidate for the MEN1 syndrome, at locus 11q13 (1, 13). At least seven alternatively spliced *ZNF162* isoforms have been identified, all coding for a putative N-terminal NLS, and two central RNA-binding domains: a divergent STAR domain of 130 amino acids with a conserved KH domain flanking a QUA2 domain, and a Zn-finger domain of the retroviral Zn-knuckle family (1, 2, 14, 15). Two out of four inducibly expressed *ZNF162* isoforms, named $ZNF162_{B3}$ and $ZNF162_{B4}$ were cloned upon GM-CSF-stimulation of a human myeloid leukemia cell line (15). The former exhibits tyrosine-rich C-terminus with a putative ATP/GTP-binding motif (P-loop), while the latter shows proline-rich C-terminus including a PPLP motif of binding for the WW domain of FBP11 (16).

An alternative 5' noncoding region that shifts the beginning of translation at the internal Met_{116} has predicted other ZNF162 isoforms, referred as $ZNF162_{HD}$s. $ZNF162_{HD}$ isoforms therefore lack the N-terminal monopartite NLS (1).

The ZNF162 protein was also purified as splicing factor SF1, an RNA-binding protein required for the assembly of mammals and yeast pre-splicing complex (2, 17). SF1 binds specifically to the pre-mRNA branchpoint consensus sequence (BPS) at the 3' side of the intron through its KH RNA-binding domain (18-20). This BPS recognition is facilitated by interaction between SF1 and U2AF65, another essential component of the pre-splicing complex, which binds to the polypyrimidine tract adjacent to BPS (20). Thus, during the early steps of spliceosome assembly interactions between SF1/U2AF65 at the intronic 3' splice site and U1 small nuclear ribonucleoprotein (U1snRNP) at the 5' splice site generate the pre-mRNA commitment complex that bridges the ends of the intron favoring its recognition and subsequent removal.

Many nuclear proteins involved in pre-mRNA splicing were shown to shuttle continuously between nucleus and cytoplasm (21-23). Furthermore, several other cellular or viral RNA-binding proteins have been suggested to shuttle from the nucleus to the cytoplasm (24-28). Among them, the mouse QKI-5 isoform of STAR family member QKI has been shown to shuttle rapidly between nucleus and cytoplasm and a novel nuclear localization sequence, termed STAR-NLS, has been identified at the C-terminus (29).

Here we report that both the ZNF162$_{B3}$ and ZNF162$_{B4}$ isoforms are nucleo-cytoplasmic shuttling proteins rapidly transported between nucleus and cytoplasm in a transcription independent manner. We show that the N-terminal 120 amino acids domain of both ZNF162$_{B3}$ and ZNF162$_{B4}$ are sufficient for shuttling, which is partially sensitive to the exportin 1/CRM1-mediated nuclear export inhibitor LMB. The monopartite NLS inside the N-terminus confers steady state nuclear localization of both ZNF162$_{B3}$ and ZNF162$_{B4}$ isoforms. Finally, deletion of the STAR domain does not affect the nucleo-cytoplasmic shuttling ability of the two proteins.

Experimental procedures

DNA constructions - Full-length ZNF162$_{B3}$ and ZNF162$_{B4}$ cDNA sequences were obtained by RT-PCR and subcloned in pcDNA3 (Clontech) as described previously (15). FLAG-tagged ZNF162$_{B3}$ and ZNF162$_{B4}$ refer to the proteins encoded by cDNA modified to include an N-terminal FLAG epitope (DYKDDDDK). The FLAG-tagged pcDNA3/ZNF162$_{B3}$ and pcDNA$_3$/ZNF162$_{B4}$ plasmids encoding two epitope-tagged constructs were made by subcloning a double strand HindIII/NcoI overhang oligonucleotide into the HindIII and NcoI sites of pcDNA3/ZNF162$_{B3}$ and pcDNA3/ZNF162$_{B4}$ parent vectors. The overhang oligonucleotide contained an ATG codon (underlined below) in a Kozak consensus sequence (30) followed by a sequence encoding the FLAG epitope, which was obtained by synthesis and annealing of two 48-bp oligonucleotides, FLAG-sense 5'-AGCTTCTAGAGCGGCCGCCATGGACTACAAGGAC GACGATGACAAGGC-3' and FLAG-antisense 5'-CATGGCCTTGTCATCGTCGTCCTTGTA

GTCCATGGCGGCCGCTCTAGA-3'. The FLAG-tagged $ZNF162_{B3}$ and $ZNF162_{B4}$ cDNAs were subcloned into the XbaI cloning site of pUHD-10S expression vector, under control of a tetracycline (*tet*)-responsive promoter, pTet (31).

The constructs containing the $ZNF162_{HB4}$ and $ZNF162_{HDF}$ variants lacking the first 115 N-terminal amino acids were generated as follows. Total RNA was extracted from the GM-CSF dependent human myeloid leukemia cell line GF-D8 as previously described (15). After random-primer reverse transcriptase synthesis of complementary DNA the $ZNF162_{HB4}$ and $ZNF162_{HDF}$ variants were amplified with the following sense and antisense oligonucleotides, 5'-CCACACGATCTTGAGTAGCACAC-3' and 5'CGGATCCTGGAGAGAAGGGAAAGGAA-3'. To generate the FLAG-tagged $ZNF162_{HB4}$ and $ZNF162_{HDF}$ variants the amplified DNA fragments were digested at 5' end with *BspEI*, filled in with DNA polymerase I (Klenow fragment) and then digested at 3' end with *BamHI*. The $ZNF162_{HB4}$ and $ZNF162_{HDF}$ DNA fragments were subcloned into the filled in *NcoI* and the *BamHI* site of pFLAG-CMV-2 mammalian expression vector (Sigma).

ΔNLS_{14-19} mutated versions of $ZNF162_{B3}$ and $ZNF162_{B4}$ were constructed by megaprimer PCR with FLAG-tagged pcDNA3/$ZNF162_{B3}$ and pcDNA3/$ZNF162_{B4}$ as DNA template. The sequence of the ΔNLS-antisense oligonucleotide used in combination with FLAG-sense primer is 5'-GTGTCTTGGTTCCAGCGGCTTGGGAAGTCCAACGGCCGTG-3'. The amplified DNA fragments were used as forward megaprimer and 5'-GGTTGTGCTGCATGGGATGTC-3'

as reverse primer in a second PCR reaction. The amplified DNA fragments were digested with *HindIII / AflII* and subcloned in the *HindIII* and *AflII* sites of both FLAG-tagged pcDNA3/ZNF162$_{B3}$ and pcDNA3/ZNF162$_{B4}$ vectors. ΔSTAR$_{142-261}$ mutants of ZNF162$_{B3}$ and ZNF162$_{B4}$ were constructed by digesting the FLAG-tagged pcDNA3/ZNF162$_{B3}$ and pcDNA3/ZNF162$_{B4}$ with *BspHI* and *AflII*, filling in the ends with DNA polymerase I (Klenow fragment), and recircularizing the vectors by blunt end ligation. ΔNLS/ΔSTAR$_{B3}$ and ΔNLS/ΔSTAR$_{B4}$ double mutants were generated by swapping the ΔSTAR$_{B3/B4}$ N-terminal region for the NLS-deleted one of ΔNLS mutants.

The deletion construct encoding ZNF162$_{1-262}$ was generated by digesting FLAG-tagged pcDNA3/ZNF162$_{B4}$ with *AflII* and *EcoRI*, filling in the ends with DNA polymerase I (Klenow fragment), and recircularizing by blunt end ligation. The ZNF162$_{1-371}$ truncated protein was obtained by *EcoRI* digestion of two EcoRI sites of FLAG-tagged pcDNA3/ZNF162$_{B4}$ vector and recircularization. The ΔNLS/ZNF162$_{1-371}$ double mutant was generated by swapping the ZNF162$_{1-371}$ N-terminus with that of ΔNLS mutants. To generate ZNF162$_{1-120}$/GFP fusion protein the coding sequence of enhanced GFP was obtained by digesting the pEGFP-N1 vector (Clontech) with *HindIII* and *KpnI*. The *HindIII* end was filled in with DNA polymerase I (Klenow fragment) and the EGFP fragment was then inserted into the filled in *BspEI* site and the *KpnI* site of FLAG-tagged pcDNA3/ZNF162$_{B4}$. That generated an in frame fusion between the first 120 amino acids of ZNF162 with the entire EGFP protein.

Cell colture and transfections - The human epithelial carcinoma cell line HtTA-1 (kindly provided by Drs. Manfred Gossen and Hermann Bujard, Ref. 31), an HeLa subclone that constitutively expresses the *tet*-responsive transactivator TetR-VP16, tTA, was routinely maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (DMEM-F), 0.5 mg/ml G418 and 1 μg/ml tetracycline. The mouse embryonic fibroblasts NIH-3T3 and the monkey kidney cell line COS7 were maintained in DMEM-F.

HtTA-1 cells were stably co-transfected by calcium phosphate/DNA coprecipitation of either pUHD/ZNF162$_{B3}$ or pUHD/ZNF162$_{B4}$ with the hygromycin B-resistance plasmid pGEM-HYG at a 10:1 molar ratio, and selected by 3-4 weeks growth in DMEM-F 0.5 mg/ml G418, 0.5 mg/ml hygromycin B, and 1 μg/ml tetracycline. Single hygromycin B-resistant colonies were expanded, and routinely maintained at subconfluence in DMEM-F, 0.4 mg/ml G418, 0.4 mg/ml hygromycin B, 1 μg/ml tetracycline. Clones expressing FLAG-tagged ZNF162$_{B3}$ (HtFB3 clones) or ZNF162$_{B4}$ (HtFB4 clones) were repeatedly washed with PBS containing 2% FCS to remove tetracycline, and detected by western analysis with a αFLAG mAb. For transient transfections HtTA-1 cells were plated on coverslips into 35 mm colture dish, grown in DMEM-F and transfected by calcium phosphate/DNA precipitation of 10 μg of plasmid DNA per 35-mm dish of subconfluent cells in the absence of tetracycline.

The myeloid leukemia cell line U937T (kindly provided by Dr. Gerard Grosveld, St Jude Children's Research Hospital, Memphis, TN) was obtained by stable transfection of U937 cells with the pUHD-pTet/tTApuro vector (kindly provided by Drs. David Schatz and Dario Vignali, St Jude Children's Research Hospital, Memphis, TN). The vector encodes the tTA *trans*-activator, whose expression is autoregulated by *tet* operator sequences in the promoter. The U937T cell line was routinely cultured in RPMI 1640 medium supplemented with 10% FCS (RPMI-F), 0.5 μg/ml puromicin and 1 μg/ml tetracycline. Electroporations were used to stably co-transfect exponentially growing U937T cells with a 10-fold molar excess of pUHD/ZNF162$_{B3}$ or pUHD/ZNF162$_{B4}$, and pGEM-HYG. Briefly, the cells were electroporated by Gene Pulser apparatus (BioRad) at 200 V, 960 μF. Diluted cell suspensions were grown in 24-well cell culture plates (Costar) for 48 h at 37°C, 5% CO_2. Selection for drug resistance was based on growth for 3 to 5 weeks in RPMI-F containing 1 μg/ml tetracycline, 0.5 μg/ml puromicin and 0.5 mg/ml hygromycin B. After selection the cell populations were tested by western analysis for the expression of FLAG-tagged ZNF162$_{B3}$ (UtFB3 clones) and ZNF162$_{B4}$ (UtFB4 clones) using αFLAG mAb. Positive clones were routinely maintained in RPMI-F, containing 0.5 μg/ml puromicin, 0.4 mg/ml hygromycin B and 1 μg/ml tetracycline.

Antibodies and western analysis - The α-ZNF162$_{B4}$ rabbit polyclonal antibody (αB4 pAb) directed against ZNF162$_{B4}$ amino acids 448-468 was purchased from Research Genetics, Inc.

(Huntsville, AL). The ^{448}DQYLGSTPVGSGVYRLHQGKG^{468} epitope was chemically synthesized and coupled to a GST protein. The obtained GST-B4$_{448-468}$ fusion protein was employed as antigen to raise the αB4 pAb. The antiserum was collected 4 weeks after the booster injection and 10-15 days after further injections. Immunoglobulin G fraction of αB4 pAb was affinity-purified by Sepharose CL4B-protein A chromatography (Pharmacia Biotech), resuspended in PBS, 0.2% BSA, 50% glycerol and stored at –20°C. Additional immunochemical reagents included murine αFLAG mAb M2 (Sigma), horseradish peroxidase-conjugated sheep α-mouse Ig (Amersham), and horseradish peroxidase -conjugated donkey α-rabbit IgG (Amersham).

HtTA-1 and U937T subclones (~2 x 10^6) or HtTA-1 cells expressing ZNF162 isoforms or mutants were harvested 48 h after tetracycline removal or transient transfection. The cells were washed twice with ice cold PBS and lysated in 100 μl of 1X sample buffer. The cell lysates were boiled 5 min and runt on 8-10% SDS-PAGE and subsequently blotted on nitrocellulose membrane (BioRad). Membranes were blocked for 16 h with 5% skim milk powder in PBS (5X BLOTTO) at 4°C, incubated 1 h with 1:2000 dilution of affinity purified αB4 pAb or 10 μg/ml αFLAG mAb in 5X BLOTTO at room temperature and washed 3 times in 1X PBS, 0.05% Tween 20 (TPBS), 10 min each. The filters were then incubated for 1 h with 1:4000 dilution of horseradish peroxidase-conjugated donkey α-rabbit IgG or 1:2000 dilution of horseradish peroxidase-conjugated sheep α-mouse Ig and washed 4 times in TPBS at room temperature. The

ZNF162 proteins were then detected by chemiluminescence following SuperSignal ULTRA protocol (Pierce).

Actinomycin D and DRB treatments - After tetracycline withdrawal HtFB3, HtFB4, UtFB3 and UtFB4 clones expressing epitope-tagged $ZNF162_{B3}$ or $ZNF162_{B4}$ proteins were incubated in media containing 5 µg/ml actinomycin D (Sigma) or 100 µM DRB (Sigma) for 3 h. In both cases, cycloheximide was added at 100 µg/ml 1 h before and during the treatment.

Indirect immunofluorescence - Transfected HtTA-1 cells, HtFB3 or HtFB4 clones (~9×10^4) were plated on coverslips into 35 mm colture dish and grown for 24÷48 h with DMEM-F without tetracycline at 37°C, 5% CO_2. UtFB3 and UtFB4 cells were diluted at cell density of 1×10^5 cells/ml and coltured in RPMI-F without *tet*, for 24÷48 h. U937 cells were cytocentrifuged at 500 rpm on microscope slides and air dried for 20 min. Cells were fixed in 3.7% paraformaldehyde, washed 3 times with PBS and permeabilized in 0.2% Triton-X100 PBS at room temperature. After 3 additional washes with PBS, the slides or coverslips were pre-incubated with 5% skim milk powder, 1% normal horse serum in PBS for 30 min at room temperature and then incubated with αFLAG mAb M2 (10 µg/ml) or αB4 pAb (1/800), for 1h at room temperature in a humifyed chamber. αB4 pAb pre-immune serum was used as negative control at the same dilution. Cells were washed 4 times with PBS and then incubated with FITC-conjugated goat α-mouse IgG (1:200, Sigma) or Texas red-conjugated goat α-rabbit IgG (1:100,

Sigma), for 30 min at room temperature. DAPI (Sigma) was added to 0.5 μg/ml with the last washing step with PBS. The samples were examined in a Zeiss Axiophot microscope equipped with epifluorescence optics for DAPI/rhodamine/Texas red and FITC. Images were acquired with a Photometrics CCD camera and analyzed using the Iplab Spectrum software.

Heterokaryon formation and LMB treatment - HeLa/NIH-3T3 heterokaryons were prepared by a modification of the procedure described (32, 33). Transiently transfected HtTA-1 cells, HtFB3 or HtFB4 clones were extensively washed to remove tetracycline. After trypsinization ~9×10^4 cells were plated on glass coverslips into 35 mm colture dish in DMEM-F with or without 1 μg/ml *tet* and incubated at 37°C, 5% CO_2. Forty-eight hours later ~2×10^5 mouse NIH-3T3 fibroblasts were seeded onto the same coverslip and incubated 3 h with DMEM-F containing or not 2 nM LMB. One hour before cell fusion a final concentration of 100 μg/ml cycloheximide with or without 100 nM LMB (kindly provided by Dr. Barbara Wolff, Novartis) was added to the dish. Then, the coverslips were washed with PBS and placed cell-side down onto a drop of prewarmed 50% polyethylenglycol 3000-3700 (Sigma). After 2 min the coverslips were removed and extensively washed with PBS. The heterokaryons were then returned to prewarmed DMEM-F, 100 μg/ml cycloheximide, containing or not 100 nM LMB for additional 1 to 2 h of incubation before fixation. Immunofluorescence with αFLAG mAb or αB4 pAb was performed as

described above, except that Hoechst 33258 (Sigma) was added to 5 μg/ml at the time of incubation with the secondary antibodies.

Low temperature incubations - HtTA-1 cells transiently transfected with either $ZNF162_{1-120}$/GFP fusion protein or GFP alone were shifted 48 h post-transfections to DMEM-F containing 100 μg/ml cycloheximide for 1 h to inhibit protein synthesis. The coverslips were incubated with DMEM-F, 100 μg/ml cycloheximide for 3 h at 37°C or 4°C and then fixed and analyzed by fluorescence microscopy. Fixation and permeabilization were done as described above except that for cells incubated at 4°C all procedures were performed at 4°C.

Results

Subcellular localization of ZNF162$_{B3}$ and ZNF162$_{B4}$ isoforms - The cDNAs encoding for FLAG epitope-tagged constructs of both ZNF162$_{B3}$ and ZNF162$_{B4}$ proteins (Fig. 1A), were generated and subsequently subcloned into pUHD-10S expression vector under control of a tetracycline (*tet*)-responsive promoter. Both constructs were tested by transient transfection into the HeLa subclone HtTA-1 (31). The ectopically expressed proteins were western analyzed using a commercially available α-FLAG M2 monoclonal antibody (αFLAG mAb) or a specific α-ZNF162$_{B4}$ antiserum (αB4 pAb) raised against a C-terminal epitope of ZNF162$_{B4}$. As shown in Figure 1B, both FLAG-tagged proteins are recognized by the αFLAG mAb, while the αB4 pAb is able to recognize specifically both ectopic and endogenous ZNF162$_{B4}$ but it does not detect the ZNF162$_{B3}$ variant. αB4 pAb is therefore a good tool to discriminate between ZNF162$_{B3}$ and ZNF162$_{B4}$ in both western and subcellular localization analyses.

We used the *tet* repressor-based conditional expression system (31), as modified by Shockett and collaborators (34), to establish stable cell clones expressing epitope-tagged ZNF162$_{B3}$ or ZNF162$_{B4}$ isoforms. The pUHD-10S vector lacking an insert, pUHD/ZNF162$_{B3}$ or pUHD/ZNF162$_{B4}$, were stably transfected into the parental recipient cell line HtTA-1 and the U937 subclone U937T. We used the U937 cell line because it is representative of the myeloid cell setting in which we identified and cloned both the ZNF162$_{B3}$ and ZNF162$_{B4}$ isoforms (15).

Western blotting analysis employing the αFLAG mAb was performed 48 h after removing tetracycline from the medium to identify stable HeLa (HtFB3, HtFB4) or U937 (UtFB3, UtFB4) subclones with *tet*-regulated ZNF162$_{B3}$ or ZNF162$_{B4}$ protein expression (Fig. 2A), and three of each type were chosen for further studies.

First, we performed immunofluorescence study to determine the subcellular distribution of conditionally expressed ZNF162$_{B3}$ and ZNF162$_{B4}$ proteins at steady state (Fig. 2B and 2C). In a representative UtFB3 clone (Fig. 2B), the subcellular distribution of conditionally expressed ZNF162$_{B3}$ protein is entirely nuclear, with punctated nucleoplasmic distribution and exclusion of nucleoli (panel a). Conversely, in UtFB4 clones the ectopic ZNF162$_{B4}$ protein shows punctated nuclear localization, with exclusion of nucleoli, and weak cytoplasmic distribution, as clearly indicated by αFLAG mAb and αB4 pAb overlapping signals (panels b, c). It is remarkable that in UtFB4 cells lacking the αFLAG mAb staining (green) and therefore not expressing the FLAG-tagged ZNF162$_{B4}$, staining by αB4 pAb clearly shows both nuclear and cytoplasmic localization of endogenous ZNF162$_{B4}$ (panel c, arrowhead). In HeLa subclones (Fig. 2C), both conditionally expressed ZNF162$_{B3}$ and ZNF162$_{B4}$ proteins localize predominantly in the nuclei with punctated nucleoplasmic distribution and exclusion of the nucleoli (panels a-c). The cytoplasmic localization of ZNF162$_{B4}$ isoform was not restricted to U937 cells, since further experiments of transient transfection in COS cells showed restricted nuclear localization of ZNF162$_{B3}$, and nuclear and cytoplasmic distribution of ZNF162$_{B4}$ (data not shown).

The ZNF162$_{B3}$ and ZNF162$_{B4}$ subcellular localization is transcription independent - Many RNA-binding proteins involved in pre-mRNA splicing accumulate in the cytoplasm of cells incubated with inhibitors of RNA polymerase II (22, 23, 35). These proteins are seen to shuttle continuously between nucleus and cytoplasm, and the inhibition of transcription interferes with their nuclear reimport for reasons that are not fully understood, whereas export remain unaffected (21). We tested whether the ZNF162$_{B3}$ and ZNF162$_{B4}$ subcellular localization in both U937 and HeLa cell setting was somehow affected by block of ongoing transcription in a transcription inhibition assay.

In figure 2B, immunofluorescence analysis of a representative UtFB3 clone (panel d) shows no effects on the nuclear distribution of conditionally expressed ZNF162$_{B3}$ protein following 3 h exposure to RNA polymerase II inhibitor actinomycin D in the presence of the protein synthesis inhibitor cycloheximide. Cycloheximide alone has no effect, and was included together with the transcription inhibitor to prevent further protein synthesis from pre-existing mRNA. In UtFB4 actinomycin D-treated cells (panels e, f) the only visible effect consists in rounding up of ZNF162$_{B4}$ cytoplasmic speckles, as clearly evidenced by overlapping staining with both αFLAG mAb and αB4 pAb antibodies.

In HeLa subclones, after treatment with actinomycin D the conditionally expressed ZNF162$_{B3}$ or ZNF162$_{B4}$ proteins remain mainly in the nucleus (Fig. 2C panels d-f). In these cells,

the effect of transcription inhibition on the rRNA accumulation results in a reduction or complete disappearance of nucleoli and consequent distribution of FLAG-tagged proteins all over the nucleus, as revealed by αFLAG mAb or αB4 pAb immunostaining. Furthermore, exposure of the same $ZNF162_{B3}$ or $ZNF162_{B4}$ expressing clones to DRB, another RNA polymerase II inhibitor, gave the same results (data not shown).

Thus, $ZNF162_{B3}$ and $ZNF162_{B4}$ distribution in ether HeLa or U937 cell lines appear to be unaffected by inhibition of transcription, therefore suggesting a possible transcription independent nuclear transport of ZNF162 proteins.

The N-terminal NLS confers steady state nuclear localization of $ZNF162_{B3}$ and $ZNF162_{B4}$ isoforms - Functional domains of $ZNF162_{B3}$ and $ZNF162_{B4}$ proteins include a putative N-terminal NLS. The N-terminal NLS (Fig 1A), comprising amino acids 13-19 (^{13}PSKKRKR^{19}), contains positively charged residues and its sequence matches the consensus sequence for classical monopartite NLS (36). Interestingly, the predicted isoforms referred as $ZNF162_{HD}$s (1) begin translation at the internal Met_{116} and therefore lack the N-terminal 115 amino acids that include the NLS (Fig. 3A).

In order to determine whether the NLS could be solely responsible for the nuclear import of ZNF162 proteins, we constructed pcDNA3 expressing vectors encoding for FLAG-tagged deletion mutants of $ZNF162_{B3}$ and $ZNF162_{B4}$ isoforms, designated ΔNLS_{B3} and ΔNLS_{B4}, in

which amino acids 14 to 19 and therefore the entire monopartite NLS are removed from the proteins (Fig. 3A). Furthermore, two FLAG-tagged $ZNF162_{HD}$ variants, designated $ZNF162_{HDF}$ and $ZNF162_{HB4}$, were amplified from the myeloid leukemia cell line GF-D8 (37) and subcloned into pFLAG-CMV2 expression vector. The wild type $ZNF162_{B3}$, $ZNF162_{B4}$, $ZNF162_{HDF}$ and $ZNF162_{HB4}$ isoforms and the ΔNLS_{B3} and ΔNLS_{B4} mutants were transiently expressed in HtTA-1 cells in the absence of tetracycline.

In proliferating HeLa cells, both $ZNF162_{HB4}$ and $ZNF162_{HDF}$ variants show an equal nuclear and cytoplasmic distribution pattern (Fig. 3B) like ΔNLS_{B3} and ΔNLS_{B4} mutants (Fig. 3C). These results demonstrate that in HeLa transfected cells the lack of amino terminal NLS does not prevent the entry of ZNF162 variants into the nucleus. However, this monopartite NLS is responsible for the steady state nuclear localization of both $ZNF162_{B3}$ and $ZNF162_{B4}$ isoforms. Furthermore, these data show that the isoforms $ZNF162_{HB4}$ and $ZNF162_{HDF}$, lacking the amino terminus and therefore the NLS, similarly localize both in the nucleus and in the cytoplasm.

$ZNF162_{B3}$ and $ZNF162_{B4}$ isoforms shuttle rapidly between the nucleus and the cytoplasm - Due to the ability of the pre-mRNA binding protein hnRNP K to shuttle in and out of the nucleus (38) we wanted to test whether this was the case for $ZNF162_{B3}$ and $ZNF162_{B4}$ isoforms, both sharing with hnRNP K the KH RNA-binding domain. First, we constructed interspecies heterokaryons between the HtFB4 clones with *tet*-regulated expression of $ZNF162_{B4}$ and mouse

NIH-3T3 fibroblasts (Fig. 4A). HtFB4 clones were grown 48 h in the absence of tetracycline to induce the expression of $ZNF162_{B4}$ and then fused to NIH-3T3 cells. Before fusion and throughout the experiment the cells were treated with cycloheximide to prevent de novo synthesis of $ZNF162_{B4}$ protein in the heterokaryons. One to 2 h after fusion the cells were fixed and double stained with αFLAG mAb and αB4 pAb to analyze the FLAG-tagged protein distribution by immunofluorescence microscopy (Fig. 4 a-d). The cells were also stained simultaneously with the Hoechst 33258 dye, which allows to easily identifying human/mouse interspecies heterokaryons due to its characteristic staining of satellite DNA in the mouse nuclei.

The heterokaryon assay shows the migration of $ZNF162_{B4}$ from HeLa donor nuclei to NIH-3T3 acceptor nuclei (arrowheads). This indicates that the FLAG-tagged protein was exported from the HeLa nuclei to the shared cytoplasm, as proved by phase contrast analysis, and subsequently imported into the mouse nuclei. Nonetheless, by immunofluorescence analysis using αFLAG mAb the $ZNF162_{B3}$ protein showed identical shuttling ability when conditionally expressed in HtFB3 clones and subsequently fused with NIH-3T3 mouse cells to form interspecies heterokaryons (Fig. 4B, panels a-c). Detection of FLAG-tagged $ZNF162_{B3}$ protein into the mouse acceptor nuclei was achieved 1 to 2 h from cell fusion.

Therefore, both $ZNF162_{B3}$ and $ZNF162_{B4}$ isoforms shuttle rapidly between nucleus and cytoplasm in a way that is independent from transcription.

ZNF162$_{B3}$ and ZNF162$_{B4}$ nucleo-cytoplasmic shuttling is partially inhibited by LMB - After demonstrating that ZNF162$_{B3}$ and ZNF162$_{B4}$ are nucleo-cytoplasmic shuttling proteins we investigated the potential involvement of exportin 1/CRM1 in their nuclear export, (39, 40), which is sensitive to LMB (41).

Here we show that LMB treatment of HeLa subclones conditionally expressing ZNF162$_{B4}$ (Fig.4A, panels e-h) or ZNF162$_{B3}$ (Fig.4B, panels d-f) partially inhibits the shuttling of both proteins into NIH-3T3 nuclei in heterokaryon assay. We found that, under our experimental conditions, only 20-30% of heterokaryons is sensitive to LMB treatment, showing a decreased or complete absence of either ZNF162$_{B3}$ or ZNF162$_{B4}$ nuclear protein export. This LMB partial effect is clearly shown in figure 4B (panels d-f), in which two HeLa/NIH-3T3 cell fusions are depicted where one exhibits a complete block of ZNF162$_{B3}$ shuttling (arrows) while the other shows clear nucleo-cytoplasmic transport (arrowheads).

This result argues against a primary role played by CRM-1 in nuclear export of ZNF162 proteins, supporting the hypothesis of two or more nuclear export mechanisms.

ZNF162$_{B3}$ and ZNF162$_{B4}$ nucleo-cytoplasmic shuttling does not require the KH RNA binding domain - The ZNF162 proteins contain a divergent STAR domain divided into a KH and QUA2 homologous regions.

We constructed $\Delta STAR_{B3}$ and $\Delta STAR_{B4}$ mutants by deleting residues 142 to 261 from the corresponding wild-type proteins (Fig. 5A). The two FLAG-tagged mutants were transiently expressed in HtTA-1 cells and their subcellular localization analyzed by indirect immunostaining with either αFLAG mAb or αB4 pAb antibodies. At steady state, like the corresponding wild-type isoforms, both chimeric proteins localize in the nuclei with punctated nucleoplasmic distribution and exclusion of nucleoli (Fig. 5B, panels a, b). To determine whether the KH and QUA2 domains were involved in the partial nuclear localization of ΔNLS_{B3} and ΔNLS_{B4} mutants we generated $\Delta NLS/\Delta STAR_{B3}$ and $\Delta NLS/\Delta STAR_{B4}$. Both double mutants displayed identical nuclear and cytoplasmic distribution similar to that of ΔNLS mutants and $ZNF162_{HD}$ variants lacking the NLS (Fig. 5B, panels c, d). Hence, deletions of the entire STAR domain along with the six amino acids of NLS do not hamper nuclear localization of the recombinant proteins, suggesting the presence of another nuclear import activity outside the STAR domain.

In order to determine whether the KH and QUA2 domains are somehow involved in nucleo-cytoplasmic shuttling the ΔSTAR mutants were tested by heterokaryon assay. As shown in Figure 6, deletion of the STAR domain does not prevent the rapid nucleo-cytoplasmic shuttling of both $\Delta STAR_{B4}$ (panels a-c) and $\Delta STAR_{B3}$ (panels d-f) chimeric proteins. Thus, the KH and QUA2 domains are not required for shuttling.

We conclude that ZNF162 nucleo-cytoplasmic transport does not require the RNA-binding ability conferred by the KH domain.

The ZNF162 N-terminus is sufficient for nucleo-cytoplasmic shuttling - To investigate the sequences required for nucleo-cytoplasmic shuttling of $ZNF162_{B3}$ and $ZNF162_{B4}$ proteins we generated FLAG-tagged C-terminal deletion mutants (Fig. 5A) and determined their subcellular distribution and shuttling ability after transient transfection of HeLa cells and subsequent fusion with NIH-3T3 fibroblasts. At steady state, $ZNF162_{1-371}$ and $ZNF162_{1-262}$ mutants show predominantly nuclear localization (Fig. 5C, panels a, b). On the other hand, the ΔNLS/$ZNF162_{1-371}$ double mutant shows a broad cytoplasmic and nuclear distribution like for ΔNLS and ΔNLS/ΔSTAR mutants (data not shown). Once again that suggests the presence of another nuclear import activity outside the STAR and the C-terminal sequences.

In the heterokaryon assay, both $ZNF162_{1-371}$ and $ZNF162_{1-262}$ mutants demonstrate a rapid shuttling ability conferred by signals inside the $ZNF162_{B3}$ and $ZNF162_{B4}$ N-terminus (Fig. 6, panels g-l). We therefore tested directly whether the N-terminus, which should be spliced out from the putative $ZNF162_{HD}$ variants, is able to promote full nuclear import and nucleo-cytoplasmic transport of $ZNF162_{B3}$ and $ZNF162_{B4}$ proteins. We generated the $ZNF162_{1-120}$/GFP mutant, in which the first 120 amino acids of the ZNF162 protein were fused to GFP. We transfected HeLa cells, fixed and then analyzed them by direct fluorescence microscopy. As expected, $ZNF162_{1-120}$/GFP localizes predominantly in the nucleus of transfected cells, while the

GFP alone is evenly distributed throughout the cell (Fig. 5D, panel a, b). Thus, the ZNF162 N-terminal NLS activity can be transferred to heterologous proteins.

When analyzed in interspecies heterokaryon $ZNF162_{1-120}$/GFP nuclear export occurs and consequently the protein shuttles between the heterogeneous nuclei of the heterokaryon within 1 h of the fusion (Fig. 6 panels m-o). Hence, the ZNF162 N-terminus is sufficient for nucleo-cytoplasmic transport of $ZNF162_{B3}$ and $ZNF162_{B4}$ proteins, and the rapid kinetics of their shuttling suggests that it is an active process.

According to previous studies, nuclear import of classical NLS-bearing proteins is blocked at low temperature (21, 42). Conversely, temperature-dependent nuclear export does not occur at low temperature and clearly differs from the passive diffusion of NLS-bearing proteins under the same conditions (43). To determine the effects of low temperature on the nuclear export of ZNF162 proteins, we transfected $ZNF162_{1-120}$/GFP fusion protein into HeLa cells and then incubated them for 3 h at 4°C before fixation and fluorescence microscopy analysis. At low temperature, if export is an active process, the protein will remain entirely nuclear without passive diffusion in the cytoplasm (43). We found that at low temperature incubation the $ZNF162_{1-120}$/GFP remains almost entirely nuclear, while the GFP alone is invariably distributed throughout the cell (Fig. 5D panels c, d).

These results indicate that ZNF162 nuclear export is a temperature dependent process and that the N-terminal 120 amino acids of the protein can impart this property onto GFP.

Discussion

The ZNF162/SF1 STAR family protein encodes for several isoforms most of which, like $ZNF162_{B3}$ and $ZNF162_{B4}$, are identical for the first 447 residues and differ thereafter at their C-terminus (1, 2, 14, 15). Here, using an interspecies heterokaryon assay, we show that $ZNF162_{B3}$ and $ZNF162_{B4}$ are shuttling isoforms rapidly transported between the nucleus and the cytoplasm. This shuttling ability relies on their shared N-terminal 120 amino acids domain, as demonstrated by deletion mutant analysis.

A new function of the ZNF162 N-terminus - Thus, our data add an additional function to the N-terminus of the STAR family member ZNF162.

Previous studies identified the N-terminal 137 amino acids of ZNF162/ZFM1 as transcriptional repressor domain required for modulation of both Stage-specific activator protein (SSAP) of sea urchin and EWS oncoprotein of Ewing's sarcoma tumors (14, 44). The same sequences N-terminal to the KH domain were seen to mediate the interaction between ZNF162/SF1 and U2AF65, another essential component of the mammals and yeast pre-splicing complex (20). This SF1/U2AF65 cooperative interaction facilitates the SF1 recognition of the pre-mRNA branchpoint sequence at the intronic 3' acceptor site, which is inhibited by PKG-I phosphorylation of SF1 on Ser_{20}, leading to a block of pre-spliceosome assembly (45-47).

Interestingly, Wang et al. (47) noticed that the unique PKG-I phosphorylation site (^{17}RKRS^{20}) overlaps to the monopartite NLS (^{13}PSKKRKR^{19}) of ZNF162/SF1 (Fig. 1A). We

show here that the NLS is responsible for the steady state nuclear localization of both ectopic $ZNF162_{B3}$ and $ZNF162_{B4}$ proteins in HeLa cells. In fact, NLS deletion mutants or $ZNF162_{HDF}$ and $ZNF162_{HB4}$ isoforms (1), beginning translation at the internal Met_{116} and therefore lacking the N-terminal shuttling domain, show a similar broad nuclear and cytoplasmic distribution.

An intriguing possibility could be that phosphorylation by cytoplasmic PKG-I of the PKG-I site may also regulate the NLS-mediated ZNF162 nuclear import by the importin-α/β complex. Several examples of phosphorylation-dependent nuclear import signals have been now identified in both cellular and viral proteins (48-50). For instance, phosphorylation by cAMP-dependent protein kinase A (PKA) of Ser_{312} positioned 22 amino acids N-terminal to the NLS of a family of transcription factors was seen to facilitate the NLS/importin interaction (48). In the same manner, PKG-I phosphorylation of $ZNF162_{B3}$ and $ZNF162_{B4}$ at Ser_{20} could positively or negatively regulate their NLS-dependent nuclear import and consequently their transcriptional repressor and splicing activity inside the nucleus.

Although we demonstrated that NLS plays a main role on the nuclear import of $ZNF162_{B3}$ and $ZNF162_{B4}$ proteins, other sequences may be relevant for their subcellular distribution. Indeed, at steady state the ectopically expressed $ZNF162_{B3}$ is merely observed in the nucleoplasm while $ZNF162_{B4}$ is also localized in the cytoplasm, even though the cell type may influence the relative cytoplasmic distribution of the protein. A possible explanation of this peculiar subcellular distribution relies on the $ZNF162_{B3}$ and $ZNF162_{B4}$ alternatively spliced C-

termini. For instance, the presence in the $ZNF162_{B4}$ C-terminus of proline-rich sequences, including a PPLP binding motif for the WW domain of FBP1 (16), may account for protein-protein interactions responsible for the peculiar cytoplasmic retention of the protein in certain cell types.

Furthermore, ΔNLS_{B3} and ΔNLS_{B4} deletion mutants or $ZNF162_{HDF}$ and $ZNF162_{HB4}$ isoforms still retain the ability to enter the nucleus. We demonstrated that both the STAR domain and the C-terminal sequences of $ZNF162_{B3}$ and $ZNF162_{B4}$ are not relevant for this NLS-independent nuclear localization. Thus, additional nuclear import activity could still rely on sequences spanning amino acids 263 to 371, including the Zn-knuckle.

We found that $ZNF162_{B3}$ and $ZNF162_{B4}$ nuclear export is an active process, partially sensitive to the CRM1-mediated nuclear export inhibitor LMB (41). Exportin 1/CRM1, a member of the importin-β superfamily, binds specifically to leucine-rich NESs (39, 51). Two leucine-rich regions potentially organized as classical NES are present respectively at the N-terminus and QUA2 domain of ZNF162 proteins. By STAR deletion mutants analysis we demonstrated that the QUA2 domain is not required for shuttling. Thus, the N-terminal leucine-rich region alone, or each potential NES apart, could be responsible for the LMB-sensitive nucleo-cytoplasmic shuttling observed for both $ZNF162_{B3}$ and $ZNF162_{B4}$. However, nuclear export mechanisms other than the CRM-1-mediated pathway should be considered to explain the shuttling ability of these proteins. Currently, we are trying to characterize the role of the potential

NESs, together with the identification of other sequence elements inside the N-terminus involved in the ZNF162 nuclear export.

Nucleo-cytoplasmic shuttling of STAR family proteins - Similar to many RNA binding proteins the mouse QKI-5 isoform of STAR family member QKI was shown to shuttle rapidly between the nucleus and the cytoplasm in a way that is dependent on RNA polymerase II-mediated transcription (29). QKI-5 shares with two other STAR family proteins, SAM68 and T-STAR/ETOILE, a novel nuclear localization sequence at their C-terminus, called STAR-NLS. This 7-amino acids STAR-NLS domain is necessary for nuclear localization of all three proteins. Furthermore, in association with other unidentified sequence elements, STAR-NLS may be responsible for QKI-5 nucleo-cytoplasmic shuttling, although the lack of other shuttling sequences may account for the inability to shuttle of T-STAR/ETOILE (29). The role of QKI-5 shuttling protein in RNAs nuclear export and/or signal transduction from the cytoplasm to the nucleus has not been elucidated.

SAM68 was shown to interact with HIV-1 Rev response elements partially substituting as well acting in synergy with Rev in RRE-mediated gene expression (52). As expected, deletion of KH domain was seen to completely abolish this RRE-transactivation activity. Moreover, SAM68 RRE nuclear export was insensitive to LMB, suggesting an alternative CRM-1-independent nuclear export pathway, likely conferred by C-terminal sequence elements (29, 53). Thus, even if not formally demonstrated, these data support a potential nucleo-cytoplasmic

shuttling of SAM68. In addition, SAM68 was demonstrated to be phosphorylated by Src or Src family tyrosine kinases and target of Grb-2, phospholipase $C\gamma$-1, and Ras-GAP signaling molecules in mitotic cells (54-56). Therefore, SAM68 may function as a cell cycle-associated signal transduction molecule involved in post-transcriptional regulation of gene expression.

Our findings show that deletion of the STAR domain does not impair the nucleo-cytoplasmic shuttling of both $ZNF162_{B3}$ and $ZNF162_{B4}$, indicating that RNA-binding conferred by the KH domain may not be required for the nucleo-cytoplasmic shuttling. This data agrees with the transcription independent nuclear transport observed for both proteins in either HeLa or U937 cells. Therefore, although we cannot exclude a possible involvement of $ZNF162_{B3}$ and $ZNF162_{B4}$ in RNAs transport, we suggest the ZNF162 shuttling be most likely involved in signal transduction. In fact, the $ZNF162_{B3}$ and $ZNF162_{B4}$ isoforms were cloned as GM-CSF up-regulated transcripts following stimulation with GM-CSF of human myeloid leukemia cells (15). Furthermore, enhanced expression of alternatively spliced isoforms of the *ZNF162/ZFM1* mouse homolog was detected in spleen macrophages (57). We propose that, at least in the case of this hematopoietic lineage, ZNF162 isoforms may be involved in the transduction of extracellular signals (e.g. GM-CSF) inside the nucleus where in turn they regulates gene expression by transcriptional and/or post-transcriptional mechanisms.

These results indicate the nucleo-cytoplasmic shuttling as a common feature shared by few if not most of the STAR proteins. That support the idea of multiple and perhaps related functions exerted by these STAR proteins in both nuclear and cytoplasmic compartments.

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767

Footnotes

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[1] The abbreviation used are: hnRNP K, heterogeneous nuclear ribonucleoprotein K; KH, hnRNP K homologue domain; QKI, quacking; QUA1 and QUA2, QKI homologue domains; MEN1, multiple endocrine neoplasia type 1 syndrome; NLS, nuclear localization sequence; GM-CSF, granulocyte-macrophage colony stimulating factor; FBP11, formin-binding protein 11; DRB, 5,6-dichlorobenzimidazole riboside; LMB, leptomycin B; GFP, green fluorescence protein; PKG-I, cGMP-dependent protein kinase-I phosphorylation site; RT-PCR, reverse-transcriptase polymerase chain reaction; DAPI, 4,6-diamidino-2-phenylindole; NES, nuclear export signal; HIV-1, human immunodeficiency type 1 virus; RRE, Rev response element.

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