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Identification of Tino: a new evolutionarily conserved *bcl-2* AU-rich element RNA binding protein.

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Modulation of mRNA stability by regulatory cis-acting AU-rich elements (ARE) and ARE binding proteins (AUBPs) is an important posttranscriptional mechanism of gene expression control. We previously demonstrated that the 3'-untranslated region of *bcl-2* mRNA contains an ARE that accounts for rapid *bcl-2* downregulation in response to apoptotic stimuli. We also demonstrated that the *bcl-2* ARE core interacts with a number of AUBPs, one of which is AUF1/hnRNP D, known for its interaction with mRNA elements of others genes. In an attempt to search for other *bcl-2* mRNA-binding proteins, we used the yeast RNA Three-Hybrid System assay (RNA THS) and identified a novel human protein that interacts with *bcl-2* ARE. We refer to it as *Tino*. The predicted protein sequence of *Tino* reveals two N-terminal hnRNP K homology motifs (KH) for nucleic acid binding and a C-terminal RING domain, endowed with a putative E3 ubiquitin-protein ligase activity. In addition the novel protein is evolutionarily conserved; the two following orthologous proteins have been identified with *protein-protein* BLAST: PEM-3 of *Ciona savignyi* and MEX-3 of *Caenorhabditis elegans*. Upon binding, *Tino* destabilizes a chimeric reporter construct containing the *bcl-2* ARE sequence, revealing a negative regulatory action on *bcl-2* gene expression at the posttranscriptional level.

INTRODUCTION

The fate of mRNAs has recently emerged as an important point of regulation of gene expression. mRNA localization, stability and protein translation are closely controlled. In general, these mechanisms are based on the cooperation between cis- and trans-acting elements. Interactions among regulatory factors constitute the mRNP, an integrated dynamic platform for RNA-protein and protein-protein interactions acting on the cellular fate of each bound mRNA (1). It is now clear that this fate is determined within the nucleus, where pre-mRNAs are "coated" by hnRNP proteins (heterogeneous nuclear RiboNuclear Proteins). Some of them are able to shuttle between the nucleus and the cytoplasm (nucleocytoplasmic shuttling): in this manner nuclear and cytoplasmic events of mRNA metabolism are interconnected (2). mRNP particle assembly is so integral to gene expression control that it is functionally conceptualized as a posttranscriptional "operon" (3, 4).

One of the more investigated class of cis-acting factors are the AU-rich elements (ARE). AREs are involved in mRNP assembly and they are responsible for mRNA half-life control (5). AREs were initially found in the 3'UTR of the mRNAs of early response genes such as *c-fos*, *c-myc*, *c-jun,* which code for powerful transcriptional activators, and GM-CSF, IL-2 , IL-3, IL-6, which code for growth factors and cytokines. These mRNAs are finely regulated in response to external stimuli and are subject to rapid turnover (6, 7). AREs are well recognizable as one or more AUUUA motifs in 3'UTRs of mRNAs, often linked in a typical nonameric unit UUAUUUA(U/A)(U/A). A more recent ARE classification is reported in the ARED database (8), where more than 800 ARE harbouring mRNAs are classified on the basis of the number of AUUUA motifs they possess.

AREs bind to a growing number of AU-rich element binding proteins (AUBPs). Among these proteins are AUF1/hnRNP D, ELAV-like (Embryonic Lethal Abnormal Vision), hnRNP A1, A2 and C proteins, belonging to the wide family of hnRNPs; these proteins contain the RRM motif (RNA Recognition Motif) as their RNA interacting domain (9, 10, 11, 12). Other classes of RNA binding proteins participating in

ARE decay have been recently identified. TTP (Tristetraprolin) is the prototype of ARE binding proteins that possess a characteristic CCCH zinc finger domain (13). Another example is the KH-type splicing regulatory protein (KSRP), a KH-type protein first identified as a splicing factor (14). Recently, this protein was also implicated together with AUF1/hnRNP D and TTP proteins in the exosome recruitment on ARE mRNAs (15).

Considering the complexity of proteins that can bind a particular cis-acting element, we have chosen the yeast RNA THS technique to identify other *bcl-2* ARE binding proteins by a library screening assay. The RNA THS allowed us to clone two cDNAs: $p40^{AUF1}$ isoform cDNA and a novel human gene on which we have focused in this work. Here we report its isolation and characterization. We refer to this protein as pTino. The novel protein, prevalently localized in the nuclear-perinuclear compartments of the cell, is a novel regulator of *bcl-2* gene expression acting at the posttranscriptional level.

EXPERIMENTAL PROCEDURES

RNA Three-Hybrid library screening assay - The yeast strain L40-coat (*Mata, ura 3-52, leu2-3, 112, his3-200, trp1-1, ade2, LYS2::(LexA-op)-HIS3, LexA-MS2coat (TRP1))* and plasmids pIIIA/MS2-1, pIIIA/MS2-2, pIIIA/IRE-MS2 were gifts from Dr. M. Wickens (University of Wisconsin). The RNA expression vector that we chose for hybrid RNAs transcription was pIIIA/MS2-1. The human *bcl-2* ARE cDNA, cloned into the *SmaI* site of the pIIIA/MS2-1, spans nucleotides 944 to 1050 of the human *bcl-2* mRNA (GenBank™ accession number M14745). The resulting plasmid, pIIIA/MS2-B2ARE, was constructed as previously described (16). A derivative of yeast L40-coat containing pIIIA/MS2-B2ARE, was transformed with a human placenta MATCHMAKER cDNA library (# HL4025AH, CLONTECH). Double transformants were plated on synthetic media lacking leucine and histidine. 3 mM 3-aminotriazole (Sigma Chemical Co.) was used to select for relatively high levels of *HIS3* reporter gene activation. About 1.2×10^7 yeast transformants were screened. After a week, 1040 white colonies were picked up and assayed according to the described procedures for RNA THS (17). The RNA-dependent false-positives clones were identified at the end of the screening by mating assay, using the yeast R40-coat derivative strain (*Matα, ura 3-52, leu2-3, 112, his3-200, trp1-1, ade2, LYS2::(LexA-op)-HIS3, LexA-MS2coat (TRP1))* containing the unrelated RNA, 5'IRE-MS23', and the deletion mutant version of *bcl-2* ARE, 5'MS2-B2ΔARE3', whose nonameric motif was deleted and as a positive control the 5'MS2-B2ARE3'. The B2ΔARE cDNA sequence was deleted of the UUAUUUAU motif, ranging from nucleotides 1005 to 1012 of the human *bcl-2* mRNA, and cloned in the *SmaI* site of the pIIIA/MS2-1, giving rise to pIIIA/MS2-B2ΔARE vector.

RNA Three-Hybrid System mapping assays - 14 different and partially overlapping segments of *bcl-2* ARE were cloned into the *XmaI* and *SphI* sites of the pIIIA/MS2-2. The first 11 segments (from B2ARE1 to B2ARE11) were PCR-amplified using Pfu DNA Polymerase (Stratagene) and pIIIA/MS2-B2ARE plasmid as template or, only for the segment named B2ΔARE, the pIIIA/MS2-B2ΔARE. The primers used

were: fFW1: 5'-TCCcccgggTCAGCTATTTACTGCCA-3'; fFW2: 5'-TCCcccgggGCCAAAGGGAAATATCA-3'; fFW3: 5'-TCCcccgggATTTGTTACATTATTAAG-3'; fFW4: 5'- TCCcccgggATTTATTTATTTAAGACAG-3'; fFW5: 5'-TCCcccgggATTTAAGACAGTCCCATC-3'; fRV1: 5'- ACCTgcatgcTAAATGATATTTCCCTT-3'; fRV2: 5'-ACCTgcatgcTAATAATGTAACAAATAA-3'; fRV3: 5'- ACCTgcatgcTCTTAAATAAATAAATCT; fRV4: 5'-ACCTgcatgcCAAAGACAGGAGTTTTGA-3'; fRV5: 5'-ACCTgcatgcGATTTCCAAAGACAGGAG-3'. The B2ARE1 segment was PCR amplified with fFW1 and fRV2, B2ARE2 with fFW2 and fRV3, B2ARE3 with fFW3 and fRV4, B2ARE4 with fFW4 and fRV5. The B2ARE5 segment was PCR amplified with fFW1 and fRV1 primers, B2ARE6 with fFW2 and fRV2, B2ARE7 with fFW3 and fRV3, B2ARE8 with fFW4 and fRV4 and B2ARE9 with fFW5 and fRV5. B2ARE and B2ΔARE were amplified with fFW1 and fRV5. All 11 B2AREs were checked for their transcription in yeast baits using Northern analysis (data not shown). B2ARE10, B2ARE11 and B2ARE12 segments were synthesized as complementary oligonucleotides, with protruding ends forming *XmaI* and *SphI* sites. The L40-coat derivative clones, containing different combinations of hybrid RNAs and activation domain-fused proteins, were assayed for HIS3 and LacZ gene activation.

Plasmids - The 107-bp segment of human *bcl-2* mRNA located in the 3'UTR from nucleotide 944-1050 (GenBank™ accession number M14745; 16), was PCR-amplified from pBS-SK-H-Bcl-2 and inserted into the unique *BglII* restriction site downstream of the rabbit β-globin cDNA of the pTRE2pur vector (CLONTECH). The resulting plasmid is pTRE2pur-B2ARE. pTRE2pur vector harbouring the predicted ORF of *Tino*, named pTRE2pur/Tino, was produced as follows. A 1485 nucleotide long fragment was PCR-amplified from the pACT2 vector containing the human *Tino* cDNA with 5'-GGATCCGCCACCATGACCGAGTGCGTC-3' forward and 5'-GATATCTCAATACTGTCGTTGAAGGGC-3' reverse primers, and cloned into the *BamHI* and *EcoRV* sites of the pTRE2pur. pQE-TriSystem vector (Qiagen) harbouring the predicted ORF of *Tino*, named pQE-TriSystem/Tino, was produced as follows. A 1485

nucleotide long fragment was PCR-amplified from the pACT2 vector containing the human *Tino* cDNA with 5'-CATGCCATGGCAATGACCGAGTGCGTC-3' forward and 5'-CGCCTCGAGATACTGTCGTTGAAGGGC-3' reverse primers, and cloned into the *NcoI* and *XhoI* sites of the pQE-TriSystem.

Cell culture and transfection - RPE and HeLa cell lines were purchased from American Type Culture Collection (ATCC; www.atcc.org), HeLa Tet-Off cell line was purchased from CLONTECH. RPE cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium (Euroclone) and Ham's F12 medium (Euroclone) supplemented with 10% fetal bovine serum, 2 mM L-Glutamine and 0.1 µg/ml penicillin/streptomycin. HeLa cells were maintained in Dulbecco's modified Eagle's medium (Euroclone) supplemented with 10% fetal bovine serum, 2 mM L-Glutamine and 0.1 µg/ml penicillin/streptomycin. HeLa Tet-Off cells were maintained in Dulbecco's modified Eagle's medium (Euroclone) supplemented with 10% tetracycline-free fetal calf serum (CLONTECH), 2 mM glutamine, 50 IU/ml penicillin and 50 µg/ml streptomycin, and 100 µg/ml G418 (Sigma Chemical Co.). Recombinant *Tino* protein was analyzed by western blot of the protein lysates extracted from HeLa Tet-Off cells transfected with 4 µg of pTRE2pur/Tino (and pTRE2pur as control) or 4 µg of pQE-TriSystem/Tino (and pQE-TriSystem a control) performed in 6-well plates. For RPA experiments, HeLa Tet-Off cells were transiently transfected in 6-well plates with 50 ng of pQE-TriSystem/Tino or pQE-TriSystem plasmid, and 20 ng pTRE2pur-B2ARE or pTRE2pur as control, in the presence of 10 µg of plasmid DNA carrier. SuperFect Transfection Reagent (Qiagen) was used according to the manufacturer's instructions.

RNA extraction and RT-PCR - Mid-log cells were detached from culture cell dishes and about 1 x 10^6 cells were recovered for RNA extractions using RNeasy mini kit (Qiagen). Total RNA was extracted from RPE, SH-SY5Y, K562, NB4, A431, HUVEC, HEK 293, HeLa and Jurkat T cells. 1 µg of total RNA was reverse-transcribed using 0.5 µg of random examers (New England Biolabs) and 1 µl of Improm-II™

Reverse Transcriptase (Promega) following the manufacturer's recommendation. The reverse transcribed product was amplified in a 50 µl volume containing 25 pmol of gene specific primers, 50 mM Tris-HCl pH 9.0, 1.5 mM $MgCl_2$, 15 mM $(NH_4)SO_4$, 0.1% Triton X-100, 2.5% DMSO and 0.7 - 1 units of DyNAzyme^{TM} EXT DNA Polymerase (Finnzymes). PCR was performed for 34 cycles, with annealing at 59 °C, using 5'- GTCAACATGACCGAGTGCG-3' forward and 5'- GGATGATGGAGAAGTGTTCGG-3' reverse primers flanking the major intron site within *Tino* cDNA.

Northern blotting analysis - For tissue distribution analysis, a premade Northern blot containing approximately 2 µg of poly(A) RNA per lane was used (Human MTN^{TM} Blot, CLONTECH). Hybridization conditions were performed according to the manufacturer's instructions. The cDNA probe was derived after *ApaI* restriction enzyme cutting of the novel gene (nucleotides 521 – 1118 of *Tino* cDNA). The cDNA fragment was gel purified and then subjected to the random priming reaction for radioactive labelling, using 24 ng of DNA and [α-^{32}P]-dATP (3,000Ci/mmol; Amersham Biosciences) with the DECA Prime II kit (Ambion).

5'- and 3'-Rapid Amplification of cDNA Ends (5'- and 3'-RACE) - The Human Placenta FirstChoice^{TM} RACE Ready cDNA Kit (Ambion) was used for 5'- and 3'-RACE analysis of *Tino* transcripts. It provides a mix of two different types of cDNA population for each type of analysis. The cDNAs serves as templates in nested PCRs using adapter sequence-specific primers (provided with the FirstChoice^{TM} RACE Ready cDNA kit) and gene-specific primers. The sequences of the gene-specific primers used are as follows: 5'GSP1, 5'-ATGTGCGCCTCGATCTCCTC-3' (nucleotides 485-466 of *Tino* cDNA) and 5'GSP2, 5'-CTGCTGGATGCGCTTGATGG-3' (nucleotides 375-356 of *Tino* cDNA) for 5'-RACE; 3'GSP1, 5'-ATTCGCGTGGAGACGGAGAC-3' (nucleotides 1411-1430 of *Tino* cDNA) and 3'GSP2, 5'-GAAGCCGTTTTCTGATTTGACTTTTCTCGCCG-3' (nucleotides 1627-1658 of *Tino* cDNA) for 3'-

RACE. The DyNAzyme EXT^{TM} Polymerase (Finnzymes) was used in all nested RLM-RACE PCRs, according to the manufacturer's instructions.

GENSCAN analysis - The GENSCAN software is available at http://genes.mit.edu/GENSCAN.html. The predicted additional 5'-exon of *Tino* gene was amplified using the following forward primers: G1, 5'-ATGCCCAGCTCGCTCGGCCA-3'; G2, 5'-TGGCGCTGGACCAGCTGTCG-3' and G3, 5'-TGGCGACACGGACGAGGA-3'. The reverse primers were: R1, 5'-GCGTGGCGCGGATGATGGAG-3' (nucleotides 232-213 of *Tino* cDNA) and R2, 5'-TCCCAGTGACCGCGAACAC-3' (nucleotides 439-421 of *Tino* cDNA). The PCR profile time and temperatures were as follows: initial denaturation 94°C for 3 min followed by 35 cycles (94 °C for 30 s, 62 °C for 1 min 30 s, 72 °C for 1 min 30 s) with a final elongation step at 72 °C for 5 min.

Antibodies and Western blotting analysis - Rabbit polyclonal anti-pTino antibody was raised against three synthetic peptides from pTino. The peptides were the following: pep1, NH2-CKIKALRAKTNTYIK-COOH (aa 24-38); pep2, NH2-**C**KRIQQRTHTYIVTPGRDKE-COOH (aa 121-139); pep3, NH2-**C**KTPNQGRRPPTATA-COOH (aa 199-212). The peptides were synthesized at the PRIMM s.r.l. (Milan, Italy) synthesis facility using the solid-phase peptide synthesis method with Fmoc-chemistry and purified by reverse-phase HPLC. The amino-terminal amino acid for each peptide was coupled to the keyhole limpet hemocyanin (KLH) protein, then the three KLH coupled-peptides were injected into two rabbits and serum was collected after the second injection. The antisera were purified by affinity column chromatography using CNBr-Sepharose resin (Pharmacia) for each purification, aliquoted and kept at -20 °C until use. For total protein preparation and Western analysis, cells were collected, washed with ice-cold phosphate-buffered saline, and lysed with RIPA lysis buffer (100 µl/well; 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 5 µg/ml Aprotinin, 5 µg/ml Leupeptin, 1% Triton X-100, 1% Sodium deoxycholate, 0.1% SDS). For Western blotting, 25 µg of total proteins were separated on 12.5%

SDS-PAGE in 1X Tris-glycine-SDS running buffer (ICN Biomedical Inc.), transferred with the Trans-blot SD/Semi-Dry Transfer cell (Bio-Rad) onto nitrocellulose membranes (Schleicher & Schuell) in 25 mM Tris, 192 mM glycine, 20% methanol, and 0.1% SDS at 0.330 A for 30 min in cold room. Membranes were blocked with 5% skim milk in PBS-T at RT for 1 hr prior to addition of rabbit polyclonal anti-pTino antibody (1:1,000), diluted in PBS-T overnight at 4 °C, or mouse monoclonal anti-His(C-term) antibody (Invitrogen, #R930-25) (1:5,000), diluted in PBS-T with 5% skim milk overnight at 4 °C. After washing, membranes were incubated with peroxidase-conjugated secondary antibodies for 1 hr at RT (1:10,000, rabbit, Amersham Biosciences; 1:5,000, goat, Sigma Chemical Co.) and washed again prior to detection with ECL Plus reagents on ECL Hyperfilm (Amersham Biosciences).

***In Vitro* Translation -** The ORF fragment of *Tino* gene was PCR-amplified from plasmid pQE-TriSystem/Tino using the following primers: the forward primer 5'-GTTAATACGACTCACTATAGGGAAATAATAGTCAACATGACCGAGTGCGT-3', containing the T7 promoter sequence (*u*reverse primer 5'-CACTTAGTGATGGTGATGGTGATGGTGGTGCTCGA-3', containing the nucleotide sequence for the 8 histidine stretch present in the DNA template. The PCR product was incubated with the TNT-coupled transcription-translation reticulocyte lysate (Promega) in the presence of cold methionine, according to the manufacturer's instructions.

Gel Electrophoresis Mobility Shift Assay (GEMSA) - Mobility-shift assays were performed using a radiolabeled 107-nucleotide long RNA corresponding to the human *bcl-2* mRNA located in the 3'UTR from nucleotide 944-1050 (GenBank^{TM} accession number M14745) as described in Lapucci et al. (16). Increasing volumes of reticulocyte lysate product were incubated for 20 min at RT with 2 fmol (2 x 10^4 cpm/fmol) of ^{32}P-labeled *bcl-2* ARE riboprobe in the presence of 10 mM Tris (pH 7.5), 100 mM KOAc, 5 mM $Mg(OAc)_2$ acetate, 2 mM dithiothreitol, 100 mM spermine, 10% glycerol, 5 units of RNasin

(Promega), 50 µg of heparin and 0.2 µg/µl of yeast tRNA (total volume, 20 µl). Samples were electrophoresed in a 6% acrylamide gel (polyacrylamide:bisacrylamide/60:1), dried and analyzed using the Cyclone^{TM} Storage Phosphor System (Packard Biosciences UK).

Intracellular localisation - HeLa cells were seeded at very low density (about 10,000 cells/cm^2) and grown on glass cover slips (15x15 mm) in 12-well plates. Harvested cells were washed 2 times with 1 ml of cold PBS and fixed for 20 min in 3.7% paraformaldehyde in PBS. Following 3 washes for 2 min each with PBS, the cells were permeabilized with 1 ml of 0.25% Triton X-100 in PBS for 5 min at RT and washed 3 times for 2 min each in PBS. The following incubations were performed in the dark. Nuclei were stained with Hoechst 33258 (Sigma Chemical Co., #B2883, *blue fluorescence*) diluted 1:1,000 in PBS for 30 min at 37 ^{o}C. The cells were washed 3 times for 5 min at RT and incubated with 1 ml of blocking buffer (3% BSA, 0.1% Triton X-100 in PBS) for 1 hour at RT. The cells were incubated with primary rabbit polyclonal anti-Tino antibody diluted 1:500 in blocking buffer overnight at 4 ^{o}C and the next day washed 3 times for 15 min each in washing buffer (0.1% Triton X-100 in PBS). They were then incubated with secondary FITC-conjugated anti rabbit antibody (Chemicon, # AP156F, *green fluorescence*) diluted 1:800 for 60 min at RT. Following a final 3 washes with 1 ml of washing buffer for 5 min each at RT, the samples were dried, mounted onto glass slides and examined with a Nikon fluorescence microscope using B-2A filter for FITC, G-2A filter for Cy-3 and UV-2A filter for Hoechst 33258.

RNase Protection Assay (RPA) - HeLa Tet-Off cells were seeded in 6-well plates at a density of 1.5x10^5 cells/well 1 day prior to transient transfections. Two days post transfection cells were treated with 2 µg/ml doxycycline (CLONTECH), and total RNA was collected at various time points and harvested in Lysis Solution containing guanidine thiocyanate (Ambion). Lysates were passed through shredder columns (Qiagen) and stored frozen at -20 °C. The plasmids pGAPM, containing a portion of the human

glycealdeyde-3-phosphate dehydrogenase (GAPDH) coding region (18), and pBSΔβ (19), containing the rabbit β-globin cDNA, were linearized with *DdeI* and *NcoI* enzymes respectively, and used for *in vitro* run-off transcriptions to produce specific antisense-riboprobes. 10 fmol of ^{32}P-labeled riboprobes were generated using T7 (pGAPM) and T3 (pBSΔβ) RNA polymerases in the presence of [α-^{32}P]-UTP (800 Ci/mmol; Amersham Biosciences) according to the manufacturer's instructions for the MaxiScript Kit (Ambion). Riboprobes were synthesized to yield transcripts with specific activities of 1–2 x 10^4 cpm/fmol, and they were added (10 fmol each) to the total RNA isolated according to the Direct Protect^{TM} Lysate RPA Kit (Ambion). Protected RNA fragments were fractionated by denaturing gel electrophoresis, dried and analysed using a Cyclone^{TM} Storage Phosphor System (Packard). The GAPDH bands intensities were used to normalize β-globin bands to correct for loading errors. RNA half-lives and first-order decay constants were calculate from plots of percent of remaining RNA versus time using Prism 3.03 (GraphPad Software).

RESULTS

Cloning of *Tino* - In order to identify trans-acting factors that interact with the *bcl-2* ARE, a human placenta cDNA library was screened by the RNA THS. The bait RNA sequence used in the yeast RNA Three-Hybrid library screening was the 107 nucleotide long *bcl-2* ARE (107mer) located in the 3'UTR of *bcl-2* mRNA. For the synthesis of the hybrid RNA, the 107mer cDNA was cloned downstream of the MS2 sequence in the pIIIA/MS2-1 RNA expression plasmid. The chimeric RNA is 5'MS2-B2ARE3'. Of the initial 1040 *white* yeast double transformants recovered from SC-LEU-HIS+3AT 3 mM plates, 11 were selected for sequencing reactions at the end of the screen. Indeed only these clones demonstrated binding specificity for the *bcl-2* ARE, as verified by yeast mating assays using the bait negative controls 5'IRE-MS23', the unrelated RNA, and 5'MS2-B2ΔARE3', the 107mer sequence deleted of the UUAUUUAU segment. After sequencing, three different ISTs (Interacting Sequence Tags) were selected for analysis. The first cDNA sequence identified (two ISTs), shared by six independent yeast clones, was the 40 KDa isoform of AUF1 protein, $p40^{AUF1}$, an ARE binding protein that already we demonstrated to interact *in vitro* with the *bcl-2* ARE (16). The third IST, shared by five independent yeast clones, encoded a novel protein as determined using *nucleotide-nucleotide* BLAST. Consequently, the presence of a previously uncharacterised human gene was revealed which we named *Tino* (**Fig. 1**).

A second independent RNA Three-Hybrid library screening assay with a modified and improved version of the RNA THS was applied (unpublished results). Also with this modified RNA THS, $p40^{AUF1}$ and *Tino* were recovered; in particular the same IST of the novel gene was cloned. The LacZ reporter gene assay indicated that *Tino* protein (pTino) is the IST most specifically interacting with the *bcl-2* ARE. Indeed a strong decrease in the transactivation signal was observed when this fusion protein was tested with 5'IRE-MS23' or 5'MS2-B2ΔARE3' RNAs for RNA-protein interactions. Thus we focused on the

novel gene *Tino* (GenBank™ accession number AF458084). Recently, a related cDNA sequence, KIAA2031 (GenBank™ accession number AB107353), was obtained from sequencing projects of large human transcripts with function unknown (20).

***Tino* protein interacts *in vitro* with the 107mer ARE -** In order to confirm the interaction of the novel protein with the 107mer sequence, a gel electrophoresis mobility shift assay (GEMSA) was performed. The pTino was produced using the reticulocyte lysate system because of the difficulties producing recombinant protein either in bacteria or insect cells, probably due to its toxicity. Increasing amounts of *in vitro* synthesized *Tino* protein mix were incubated with the radioactively labelled 107mer ARE; efficient complex formation was observed with pTino, whose intensity parallels the volume of the protein synthesis mix (**Fig. 2**).

***Tino* and AUF1 proteins recognize different binding sites of the 107mer ARE -** Using the RNA THS we identified the site of the *bcl-2* ARE bound by pTino. In agreement with the results of the binding specificity test performed with the mating assay, pTino recognizes the motifs possessing the nonamer region (B2ARE2, B2ARE3 and B2ARE7); importantly, the RNA-protein interaction is lost (B2ARE4) without the adenine stretch just upstream of this element (**Fig 3A, 3B**). To determine the minimal motif bound by pTino, we tested B2ARE11 and B2ARE12 segments. We concluded that the 26 nt long sequence B2ARE11, containing both the adenine stretch and the nonamer, is the binding site of pTino (**Fig 3A, 3B**).

In order to compare the binding profiles of pTino and AUF1 on the *bcl-2* ARE, we have mapped the binding sites of $p40^{AUF1}$ (aa 94 - 297), the other relevant protein found in the library screening, and $p37^{AUF1}$ (aa 1 - 257), that recently we disclosed to be able to interact with the *bcl-2* ARE in RNA THS (16). $p40^{AUF1}$ is able to bind the segments within the first 75 nts of the *bcl-2* ARE (B2ARE1, B2ARE2, B2ARE3, B2ARE5, B2ARE6 and B2ARE7), which identify a larger RNA recognition sequence (**Fig 3A,**

3B). We determined that p40^{AUF1} interacts independently with B2ARE10, B2ARE11 and the B2ARE12 motifs (**Fig 3A, 3B**). p37^{AUF1} recognizes the motifs B2ARE1, B2ARE2, B2ARE3 and B2ARE7, showing that its binding site is immediately upstream of the nonamer region. Accordingly, we found that p37^{AUF1} is able to interact with B2ARE10 (**Fig 3A, 3B**). We conclude that pTino and AUF1 isoforms display different, and partially overlapping, interaction profiles with the *bcl-2* ARE.

Genomic structure and analysis of the human *Tino* gene - The cloned *Tino* cDNA contains 2,132 bp (**Fig. 4A**) that fully overlap human chromosome 19 within the 19p13.3 band, forming three exons on the corresponding genomic DNA region (**Fig. 4B**). The predicted first exon is short, containing only 67 bp, and it is separated from the second exon by a 10,541 bp intron with canonical GT-AG splice sites; the second (1,347 bp) and the third (718 bp) exon are only 187 bp apart. These 187 bp constitute a short intron possessing the AT-AG dinucleotides as splicing consensus boundaries for the donor and the acceptor site, respectively. This splicing junction AT-AG is a non-canonical splice site belonging to the AT-AC group controlled by the novel type of U12-based spliceosome (21), whose consensus sequences, /ATATCCTTT for the donor site and YAG/ for the acceptor site, are present in the 187 bp boundaries. The *Tino* cDNA sequence completely overlaps with another described human mRNA sequence of unknown function, recently cloned (KIAA2031; GenBank^{TM} accession number AB107353) (20). Thus *Tino* and KIAA2031 appear related. The short intron between exon 2 and 3, arising from the genomic distribution of *Tino* sequence, is retained in the KIAA2031 cDNA and consequently may represent an *alternative* intron. *Tino* and KIAA2031 mRNAs can be considered as alternatively spliced transcripts. However, this is not the only difference between the two sequences: the 5' end of the KIAA2031 cDNA is longer than that of our cloned DNA; indeed an additional 121 bp are present. These additional 121 bp represent the upstream adjacent region on the genomic sequence of the first exon of 67 bp.

Tino cDNA gives rise to a predicted partial protein of 490 amino acids (**Fig. 4C**). pTino is an RNA binding protein possessing two KH domains within its N-terminal region, with the KH1 domain spanning from amino acid 1 to 69 and the KH2 domain from amino acid 96 to 163. In addition, a C-terminal RING domain spanning from amino acid 424 to 463 is predicted, whose general function is an E3 ubiquitin-protein ligase activity. The sequence of the protein pTino completely overlaps with the KIAA2031 protein (515 aa) except for the C-terminus, where alternative 19 amino acids are present. The two predicted proteins differ starting from amino acid 471 of pTino - 511 of KIAA2031 - at which 19 amino acids (*RVETETPQPGGASALQRQY*) for pTino or 4 amino acids (*HIFS*) for KIAA2031 are present. Curiously, the last 4 amino acids and the stop codon of KIAA2031 are encoded by the 187 bp *alternative* intron present in the transcript (**Fig. 4C**). Moreover, an additional 40 amino acids from the KIAA2031 cDNA can be predicted at the N-terminus compared to pTino. KIAA2031 protein is probably a partial product since its initial amino acid is a proline and not a methionine.

A searching for homologous proteins recovered the putative ORF of the KIAA2009 sequence (GenBank™ accession number AB095929), whose locus lies on human chromosome 15q25.2 (**Fig. 5**). pTino may be a prototype of a protein family whose members are the KIAA2009 product and another putative member located on human chromosome 1q22. The latter is predicted because of the high identity score of local alignment obtained with our cDNA sequence and human EST sequences overlapping with that DNA region (GenBank™ accession numbers BE315075; BG746475; BQ947909). No functionally characterized homologous sequences were obtained among other mammalian species, but *Tino* homologies are predicted by automated computational analysis from the available GenBank™ EST sequences in *Mus Musculus* (GenBank™ accession number XP_137153) and in *Rattus Norvegicus* (GenBank™ accession number XP_218846) species. More interestingly, the sequence comparison with known sequences from other organisms revealed a high degree of conservation of *Tino* protein. Two

orthologous proteins exist, PEM-3 (Posterior End Mark-3) from *Ciona Savignyi* (22) and MEX-3 (Muscle EXcess-3) from *C. Elegans* (23). These proteins possess the highest sequence identities concentrated at the level of the two KH domains for the nucleic acid binding, with PEM-3 sharing 74% identity and MEX-3 69% identity (**Fig. 5**).

Expression and size determination of the *Tino* mRNA and protein - We examined the expression profile of endogenous *Tino* gene using human Northern blots containing poly(A) RNA from different healthy tissues (**Fig. 6A**). A predominant ≈ 3.1 - 3.2 kb transcript was detected, which was present at different levels in all the human tissues tested including heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas. A putative splicing variant was also detected in heart and skeletal muscle tissues, with an apparent molecular size of ≈ 6.5 kb. These data indicate that most probably, *Tino* and KIAA2031 cDNA are related to partial transcripts.

Also, the expression data from different human immortalized – RPE (retinal pigment epithelia) - and neoplastic cell lines - SH-SY5Y (neuroblastoma), K562 (chronic myelogenous leukemia), NB4 (acute promyelocytic leukemia), A431 (epidermoid carcinoma), HUVEC (primary human endothelial cells), HEK 293 (human embryonal kidney), HeLa (adenocarcinoma) and Jurkat T (acute T cell leukemia) - were obtained. Total RNA was extracted, reverse transcribed and amplified with gene specific primers. The specific band was detected in all the tested cell lines, with the exception of K562 cells where a very faint band was detectable (**Fig. 6B**).

Ectopical expression of the *Tino* gene was examined by western blotting analysis. Total protein extracts from transiently transfected HeLa Tet-Off cells were collected and analyzed with the anti-pTino or the anti-His(C-term) antibody (**Fig. 6C**). Following transfection of the *Tino* ORF with the pTRE2pur based vector, the recombinant protein was immunodetected (**Fig. 6C, lane 4**). By contrast, no signal was detected in the total protein extract prepared from HeLa Tet-Off cells transfected with the empty vector (MOCK) (**Fig. 6C, lane 3**). Recombinant pTino runs in SDS-PAGE with an apparent molecular mass of

about 60 kDa, although the calculated mass from the cDNA sequence is about 50 kDa. The peptide antibody specifically recognised the recombinant protein. As a further proof of the specificity, a band of identical size is detectable in HeLa Tet-Off protein extracts probed with the anti-pTino or the anti-His(C-term) antibody, following transient transfection of the His-tagged *Tino* ORF (**Fig. 6C, lanes 2, 5**). By contrast, neither the His-tagged nor the untagged protein was detectable with the relative antibodies in the protein extract of the corresponding controls (MOCK) (**Fig. 6C, lanes 1, 6).** The slight difference in molecular mass between the recombinant proteins expressed from the tetracycline-sensitive promoter or the constitutive promoter is due to the histidine stretch present in the recombinant protein produced by the second kind of vector. The probing of protein extracts with the anti-pTino antibody detects three others strong bands, two of which possess an apparent molecular weight of about 70-75 kDa and one of about 39 kDa. However, adsorption experiments with specific peptides allowed identification of an unspecific band (**Fig. 6D**).

Analysis of 5' and 3' UTR regions of *Tino* mRNA by Rapid Amplification of cDNA Ends (RACE) analysis - From Northern Blotting analysis we concluded that the cloned *Tino* cDNA does not correspond to the full-length mRNA; we then used RNA T4 ligation mediated RACE (RLM-RACE) for 5' and 3' transcript end cloning. With specific primers for 5' and 3' ends, nested-PCR reactions from human placenta cDNA were performed, and the amplicons were cloned in the pCRII vector (Invitrogen) through TA cloning and then sequenced. By means of 3'-RACE we cloned the 3' end of the mRNA and verified that it is perfectly overlapping with the *Tino* and KIAA2031 cDNAs (data not shown); this fact suggested that the missing mRNA region of *Tino* is in the 5' end. Despite many attempts, we were unable to clone the true 5' end of the transcript by RLM-RACE; the cloned products showed deletions of the known extreme 5' end at the ligation point.

Analysis of 5' end of *Tino* mRNA by GENSCAN - In order to clone the missing 5' end of *Tino* cDNA we applied a predictive method that defined the entire gene. The GENSCAN program (24) enabled us to

analyse the genomic region that we assumed contained our gene, a 21,942 bp region, from bp 1,493,021 to bp 1,514,963 of chromosome 19 (GenBank™ accession number NT_011255.13). This DNA region flanks the neighbouring MBD3 gene (Methyl-CpG Binding Domain protein 3). The predicted peptide completely overlaps the KIAA2031 protein and, except for the four last amino acids, with pTino and gives rise to a putative 651 amino acid protein. In addition, the major intron and the predicted poly(A) signal overlap with the structure of our gene (data not shown). However, the N-terminal region of the predicted protein is 136 amino acids longer than KIAA2031; these additional amino acids correspond to a 408 bp exon, reaching a length of about 3,100 bp when added to the 2,441 bases of the KIAA2031 sequence and to the ≈ 200 bp of the poly(A) tail. Thus, to validate this prediction, we designed three forward primers within this region, and we used them for PCR amplification reactions starting from total RNA extracted from RPE and HeLa cells and reverse transcribed with random primers (**Fig. 7A**). In both cell lines, the G2 forward primer alone, paired with the R1 reverse primer encompassing the first splice site junction of the gene, gave rise to PCR products. PCR amplicons can be obtained also with the G2-R2 primers (data not shown). On the other hand, no amplification products were obtained when G1-R1 and G3-R1 primer pairs were used. We focused on the G2-R1 major products, identified as two bands, that we gel purified and sequenced. The sequencing data demonstrate that the *Tino* cDNA was detected (**Fig. 7B**). For each sequenced product, the relative sequence in the genomic context and the putative N-terminal region of *Tino*/KIAA2031 protein are shown. The major PCR product adds 77 bp to the 5' end of the KIAA2031 transcript. Also the human EST fs06d08.y1 (GenBank™ accession number CD674038) contains a 5' end similar to the *Tino*/KIAA2031 gene; indeed it is almost completely overlapping with this product, and the two sequences share 98% identity over the additional 77 bp.

Localization of pTino - We determined the intracellular localization of endogenous pTino. Immunofluorescence revealed that pTino was localized predominantly in the nucleus, where local accumulation in clusters is visible, and also in the perinuclear region. No expression was observed in the

nucleoli. In addition, a spotted distribution of the protein is visible in the cytoplasm (**Fig. 8A, 8B**). Nuclear staining faded away when anti-pTino antibody was preincubated with its peptide antigens. However the faint cytoplasmic staining remained (data not shown).

pTino influences the levels of a β-globin/ARE chimeric transcript - We proceeded to study the function of *Tino* by its transient transfection into HeLa Tet-Off cells. We linked a β-globin reporter gene that is under the transcriptional control of a doxycycline-sensitive promoter (25) to the 107mer ARE sequence in the pTRE2pur/B2ARE construct. Upon transient transfection, β-globin/ARE **mRNA of uniform length** is synthesized. Addition of doxycycline blocks transcription of the β-globin/ARE gene. The functional contribution of *Tino* to ARE-directed mRNA decay was examined by cotransfection of repressible reporter plasmids encoding either the β-globin mRNA linked to the 107mer ARE sequence or the β-globin mRNA alone. HeLa Tet-Off cells were cotransfected with the pQE-TriSystem alone (MOCK) or with pQE-TriSystem/Tino (**Fig. 9**). Upon transient transfection into HeLa Tet-Off cells, reporter mRNA synthesis was allowed for 48 hours until addition of doxycycline. RNA levels of reporter constructs were determined by RNase Protection Assay. Control β-globin mRNA lacking the ARE was intrinsically stable and *Tino* did not induce its decay, as observed during the 6 hour interval (**Fig. 9, panel A, quantification in panel C**). β-globin/ARE mRNA decays faster with an half-life of 6.3 hr. When *Tino* was cotransfected, the half-life of the chimeric RNA was reduced to 2.9 hr, indicating an enhanced decay rate (**Fig. 9, panel B, quantification in panel C**). We conclude that the *bcl-2* ARE destabilizes the β-globin/ARE mRNA and ectopic expression of *Tino* acts through the *bcl-2* ARE to enhance this effect.

DISCUSSION

Here we describe pTino, a novel human evolutionarily conserved protein that interacts with the AU-rich element of *bcl-2* mRNA. *Tino* gene was identified following two serial library screenings of two different versions of the yeast RNA THS. The IST of *Tino* resulted from analysis of its binding specificity with the *bcl-2* ARE sequence at the end of RNA-protein interaction screenings. The RNA THSs were reconstituted by yeast mating assays in order to validate the binding specificity interaction of the novel cloned protein with the *bcl-2* ARE. *Tino* protein did not transactivate the LacZ reporter gene in the presence of the unrelated 5'IRE-MS23' RNA bait, and did not significantly interact with the hybrid RNA 5'MS2-B2ΔARE3' where the *bcl-2* ARE is deleted of the nonamer (**Fig. 1**). In contrast, RNA-protein complex formation both *in vivo* and *in vitro* occurs with the wild-type *bcl-2* ARE sequence (**Fig. 1, 2**). This indicates that the nonamer is needed for RNA-protein complex formation. However, the nonamer is not the only motif of *bcl-2* ARE required for the interaction of pTino. We mapped the binding site of pTino and disclosed that it must also contain the adenine stretch immediately upstream of the nonamer (**Fig. 3A, 3B**). Despite this relatively long sequence (5' - AGAAAAAAAGAUUUAUUUAUUUAAGA - 3') is not shared with other ARE harbouring human mRNAs, we can not exclude the possibility that pTino could interact with other motifs.

The cloned cDNA is a 2,132 bp segment whose protein product has two K homology (KH) domains (**Fig. 4A, 4C**). The conserved KH motif, first identified in human heterogeneous nuclear ribonucleoprotein K (hnRNP K), is shared by a wide variety of nucleic-acid binding proteins, many of which take part in the complex network of protein-protein and RNA-protein interactions regulating gene expression in eukaryotes (26). KH domains consist of an α-β fold with a topology similar to that found in ribosomal proteins (27). Like other RNA binding motifs, KH domains are found in either one or multiple copies; for example hnRNP K and human OncoNeural Ventral Antigen-1 (NOVA-1) possess three copies, Fragile X Mental Retardation protein 1 (FMR-1) two copies, and even 14 copies are present in chicken Vigilin. Two

KH domains are predicted at the N-terminus of pTino. At the C-terminus a RING module is predicted. This RING-finger is a specialized type of Zn^{2+}-finger domain of 40 amino acids that bind two atoms of zinc, probably involved in mediating protein-protein interactions. Based on the cysteine/histidine pattern, the variant of RING module present in pTino is the C3HC4-type. The RING domain has been implicated in a range of biological processes. E3 ubiquitin protein ligase activity is ascribed to the RING domain of c-*Cbl* protein, which is able to recruit an E2 ubiquitin-conjugating enzyme and its own substrates (28). It is believed that E3-like activity is a general function of the RING domain.

Evidence of the high degree of inter-species conservation of pTino is revealed from database searches using *protein-protein* BLAST (**Fig. 5**). pTino is probably the prototype of a small protein family of KH-containing RNA binding proteins. Indeed, transcripts (KIAA2009) and EST sequences of unknown genes have been cloned and predicted to codify polypeptides similar to pTino, especially with respect to the KH domains. Two putative other members of the protein family can be found on human chromosomes 15q25.2 and 1q22. Among the ancient bilaterian animals, such as ascidians and nematodes, two orthologous proteins, PEM-3 (*Ciona Savignyi*) and MEX-3 (*Caenorhabitis Elegans*) respectively, have high homology with pTino (**Fig. 5**). PEM-3 and MEX-3 proteins are RNA binding proteins whose biological relevance appears during embryonic development (22, 23). Many crucial decisions, such as the timing of cell division and cell-fate determination, are made in the early phases of embryonic development, when little or no transcription occurs and gene expression often relies on posttranscriptional controls (29). The MEX-3 (Muscle EXcess-3 protein) protein is implicated in anterior-posterior differentiation of *C. Elegans* embryos, in particular *mex-3* expression is required for proper muscle development of the worm. We know that MEX-3 is implicated in gene expression control of the *pal-1* gene, and that this control is dependent on the 3'UTR region of the *pal-1* mRNA. In particular, MEX-3 is a negative regulator of *pal-1* gene expression, possibly repressing PAL-1 translation by a direct interaction with the corresponding mRNA region (30). Indeed, recombinant MEX-3 protein was

demonstrated to bind to the *pal-1* 3'UTR *in vitro* (31). The evolutionarily conserved pTino interacts with the cis-acting element of the antiapoptotic *bcl-2* gene. Apparently, there is no relation between the two genes, *pal-1* and *bcl-2*, but the final output of their expression control in muscle progenitor cells could be the same. For example, BCL-2 is expressed in human skeletal muscle cells at an early stage of myogenic differentiation, promoting clonal expansion (32). BCL-2 protein levels decrease along the myogenic pathway from muscle stem cell to myofiber. All these observations suggest that *Tino* may be a negative regulator of the *bcl-2* gene during embryonic development.

MEX-3 acts in the cytoplasm during the early cleavage stages of the embryonic cell and is localized in P-granules (23), which are cytoplasmic structures containing RNAs and RNA binding proteins playing a role in mRNA processing or packaging (33). By contrast, we observed that endogenous protein is mostly distributed in the nuclear-perinuclear cell compartment (**Fig. 8**).

Northern blot analysis of the transcript of *Tino* indicates an apparent size of about 3.1 Kb present in all tested poly(A) RNAs, extracted from human healthy tissues (**Fig. 6A**). Placenta shows the stronger hybridization signal, while in the heart and skeletal tissue additional putative ≈ 6.5 Kb splicing variant is present. However, due to the low level of expression of this product, it can also be either a related transcript or a processing intermediate. The recently cloned KIAA2031 (20) cDNA is located in the same genetic locus as *Tino* on chromosome 19, suggesting that *Tino* and KIAA2031 are the same gene. Both sequences completely overlap except for two differences: the KIAA2031 transcript possesses a 187 nucleotide short intron containing the stop codon, which is not present in the *Tino* transcript. This raises the possibility that the two mRNAs are the result of the alternative splicing known as *intron retention* (34).

Northern analysis suggested that *Tino* and KIAA2031 sequences do not correspond to the full-length mRNA, indicated by the size discrepancies between the expected and detected transcripts. In order to identify the 5' and 3' ends of the mRNA of *Tino*, we applied two different approaches: PCR-based 5'- and 3'-RACE and the gene prediction program GENSCAN. RACE analysis showed that the missing part

of the transcript is the 5' end. The 3'-RACE product was completely overlapping with the terminal 3'UTR of *Tino* and KIAA2031. Through the GENSCAN analysis, we detected two RT-PCR products in HeLa and RPE cells (**Fig. 7A**), extending by 198 and 159 nucleotides the 5' ends of *Tino* cDNAs. We believe these represent alternative splicing products. Their sequences were aligned within the corresponding genomic region and translated into protein. At the genomic level they form two short introns (**Fig. 7B**). The splicing consensus boundaries are GC/CG for the longer and GA/CG for the shorter form. These acceptor and donor site pairs are neither canonical nor non-canonical splice sites, nevertheless we can not exclude the possibility that the transcripts derive from alternative splicing because of the presence of a branch site and pentamer (CTGCC) functioning as intronic splicing enhancer (35). This kind of pentamer accurately identifies human short introns. It represents the major contribution to intron recognition when computational analyses are performed. At the protein level, the difference seems to be functionally sound. Indeed, the two products codify proteins differing only at a particular phosphorylation site, whose consensus sequence is RXRXXS/T, which is the substrate of Protein Kinase B/Akt. This site, in the form *RGRPGT*, is present in the longer product (**Fig. 7B**).

The antibody obtained with a peptide based method allows to detect three major bands in western blot, possibly corresponding to the endogenous pTino: a doublet of about 70 - 75 kDa and one single band of about 39 kDa (**Fig. 6C, lanes 3 and 6**). The lower band in the upper doublet appears to be unspecific because is clearly detected even when the antibody is blocked by the specific peptides (**Fig. 6D**). The size of the specific upper band suggests the presence of an extra sequence with respect to the recombinant pTino. *Bona fide* the extra sequence corresponds to the unknown N-terminus of the novel protein, while the lower band derives probably from proteolytic processing yielding the band of about 39 kDa.

What is the functional significance of the physical association between pTino and the *bcl-2* ARE? To address this question, we used an inducible β-globin reporter gene with the *bcl-2* ARE linked to the

3'UTR. Transient transfection of *Tino* significantly accelerated (2 fold) mRNA reporter decay when the 107mer ARE motif was present in the mRNA 3'UTR (**Fig. 9**). Since the destabilizing protein AUF1 is another *bcl-2* mRNA AUBP (16), we suppose that pTino is part of the mechanism of posttranscriptional control of *bcl-2* expression and that probably acts "cooperatively" with AUF1 (**Fig. 3A, 3B**). In particular, the pooling of the binding sites of Tino and AUF1 over 48 nucleotides of the *bcl-2* ARE - where pTino partially shares its binding site with $p40^{AUF1}$ and is contiguous to the binding site of $p37^{AUF1}$, while the latter completely overlaps the binding site of $p40^{AUF1}$ - suggests a strictly coordinated mode of action that could explain different outputs of *bcl-2* expression in basal condition and during apoptosis. The ARE-based decay pathway is a cytoplasmic event, while the subcellular localization of the endogenous pTino we observed is mostly nuclear and perinuclear (**Fig. 8**). Accordingly, we suspect that the *bcl-2* mRNA destabilizing properties can be enhanced by pTino redistribution to the cytoplasm upon apoptosis. One example of regulated ARE binding protein is furnished by CUGBP2 (CUG-Binding Protein 2), a multifunctional protein containing three nonidentical RRMs motifs that is capable of apolipoprotein B and COX-2 AU-rich sequence interaction. Interestingly, CUGBP2 is an highly conserved AU-rich element binding protein able to relocate from the nucleus to the cytoplasm following apoptotic stimuli, and thus able to repress COX-2 mRNA translation (36, 37). Our future research on *Tino* will comprise to examine its functional participation in cellular life and death, both in physiological and pathological conditions, and to verify its cellular redistribution and transport pathway.

FOOTNOTES

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The nucleotide sequence relative to the novel gene Tino reported in this paper has the GenBank™ accession number AF458084.This sequence was submitted on December 12, 2001.

The abbreviations used are: RNA THS, RNA Three-Hybrid System; ARE, AU-rich element; mRNP, messenger ribonucleoprotein; hnRNP K, heterogeneous nuclear ribonucleoprotein K; TTP, Tristetraprolin; KSRP, KH-type splicing regulatory protein; AUF1, AU-rich factor 1; IST, interacting sequence tag; PEM-3, posterior end mark-3; MEX-3, muscle excess protein-3; CUGBP2, GUG-binding protein 2; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction; GEMSA, gel electrophoresis mobility shift assay; RPA, RNase Protection Assay

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FIGURE LEGENDS

Figure 1. *In vivo* interaction of *bcl-2* ARE and *Tino* by reconstituted RNA THS: verifying binding specificities by mating assay. Two positives cloned by the two library screening assays - $p40^{AUF1}$ and pTino - were used to confirm their specificity of interaction with the ARE motif. The hybrid RNAs used as negative controls were 5'MS2-B2ΔARE3' (the mutant RNA, deleted of nonamer motif) and 5'IRE-MS23' (the unrelated RNA). The RNA expression vectors were transformed in isogenic R40 coat yeast strain, and the selected transformants were mated with 9 different yeast harbouring ISTs L40-coat cells. The diploid cells containing the RNA binding proteins p40 LINE-1, hnRNP A/B, Fibrillarin, the transcriptional transactivator TWIST and the unrelated protein Haemoglobin are the negative controls. pTino and $p40^{AUF1}$ derive from two independent screenings of the library. The diploids (three independent clones for each type of RNA/protein combination containing yeast) were selected on SC-URA-LEU plates and replicated on filter for LacZ reporter gene transactivation assay.

Figure 2. Gel shift assay. pTino was expressed using an *in vitro* system for coupled transcription and translation synthesis, using PCR products as DNA templates. Increasing amounts of *in vitro* synthesized *Tino* protein mix were incubated with the 107mer ARE motif radioactively labelled and caused efficient

RNA-protein complex formation (lanes from 1 to 6). By contrast, no RNA-protein complex formation is obtained when *Tino* protein mix was incubated in the presence of an unrelated RNA radioactive labelled probe (β-globin RNA, 340 nt, lanes 8, 9), or the *in vitro* synthesized Luciferase protein is incubated with the 107mer ARE probe (lane 7).

Figure 3. Mapping assays. The RNA THS was used to determine the binding sites of pTino, $p37^{AUF1}$ and $p40^{AUF1}$ on the *bcl-2* ARE. For this goal, 42 L40-coat derivative yeast clones, each containing a different combination of hybrid RNA and RNA binding protein, were constructed. The upper panel (**A**) shows the sequences of the ARE segments cloned into the pIIIA/MS2-2 (*on the right*) and the results of HIS3 and LacZ reporter gene activation for each L40-coat derivative clone (*on the left*). HIS3 and LacZ genes were tested at the same time. Indeed, a single yeast colony for each yeast clone, was grown as a circular spot of about 5 mm of diameter for two days on synthetic media lacking uracile, leucine, histidine, and containing 10 mM 3-aminotriazole, selecting for HIS3 activating cells. Afterwards, the yeast cells were covered with a thin layer of soft-agar containing 0.1% SDS, for cellular permeabilization, and 0.04% X-GAL as a substrate of the β-galactosidase. The lower panel (**B**) summarizes the results of RNA THS mapping assays. The binding sites of the RNA binding proteins pTino (*gray*), $p37^{AUF1}$ (*white and fine black outline*) and $p40^{AUF1}$ (*white and thick black outline*) are indicated by the coloured lines subtended along the minimal motifs of *bcl-2* ARE recognized by each protein. The pentamers AUUUA and the nonamer motifs AUUUAUUUAUUUA are underlined in each panel; the base substitution T981->G of the *bcl-2* ARE (16) is in red (**A**).

Figure 4. A) Nucleotide and amino acid sequence of *Tino* cDNA. Numbers *on the left* indicate nucleotides,

and numbers in parentheses *on the right* indicate amino acids. The stop codon is indicated by an asterisk. The predicted ORF reveals 490 amino acids. The two KH domains (hnRNP K homology domain) for RNA binding are underlined; the RING domain is in bold characters and underlined. The polyadenilation signal in the 3'UTR is *in white on a black background*.

B) Genomic structure of the *Tino* gene. The genomic organization of *Tino* and KIAA2031 are shown. Introns are shown as lines, and exons as boxes. Coding regions are depicted in *gray*, non-coding regions in *white*. Numbers above indicate nucleotide positions within the two cDNAs. Splice-pair dinucleotides GT-AG (canonical splice site) and AT-AG (non-canonical splice site) are indicated below.

C) Modular structures of *Tino* and KIAA2031 proteins. Schematic representation of the protein motifs within *Tino* and KIAA2031 proteins. Numbers above indicate the amino acids of the predicted structural motifs, KH1, KH2 and RING domains. The representation also shows the sequence of the alternative C-terminal ends of the two proteins.

Figure 5. Protein sequence alignment. The protein sequence comparison among pTino (*Homo sapiens*), KIAA2009 (*Homo sapiens*), PEM-3 (*Ciona savignyi*, GenBank™ accession number AB001769) and MEX-3, isoform a, (*Caenorhabditis elegans*, GenBank™ accession number U67864) protein sequences is shown (CLUSTALW 1.8). PEM-3 and MEX-3 orthologous proteins possess the highest identity within the two KH domains (the amino acids in bold type). The RING domain is underlined. Identical amino acid residues are shared in *gray* (in *light gray* if they are shared with pTino sequence or in *dark gray* if they are not) and marked with an asterisk. Similar amino acids are marked with two vertical points. Secondary structural features (*beta-sheets* as β; *alpha-helices* as **H**) are shown above and they correspond boxed amino acids.

Figure 6. A) Expression profile of *Tino* by Northern analysis. In order to determine the tissue distribution of *Tino* mRNA, a premade Northern membrane blotted with 5 µg of poly(A) RNAs from normal human tissues (CLONTECH) was hybridized with a specific probe. The highest hybridization signal is with the poly(A) RNA extracted from human placenta; two bands are visible in lanes 1 and 6, with the heart and skeletal muscle RNAs. From this analysis the estimated *Tino* mRNA size is about 3,100 – 3,200 nt. A putative ≈ 6.5 Kb splicing variant present in heart and skeletal muscle is marked by *arrowheads*.

B) Expression profile of *Tino* mRNA by qualitative RT-PCR in human cell lines. Total RNA extracted from the human RPE cell line (retinal pigment epithelia) and human transformed cell lines (SH-SY5Y, K562, NB4, A431, HUVEC, HEK 293, HELA and Jurkat T) was reverse-transcribed with random primers and the initial 223 bp of *Tino* cDNA was amplified with specific primers.

C) Analysis of recombinant pTino in HeLa Tet-Off cells. Western blot analysis of recombinant pTino was carried out with the total protein extracts of transiently transfected HeLa Tet-Off cells, using the mouse monoclonal anti-His(C-term) antibody (α-HIS tag) or the rabbit polyclonal anti-pTino antibody (α-pTino). HeLa Tet-Off cells were transfected with either the pQE-TriSystem/Tino vector containing the *Tino* ORF linked to the histidine tag (lanes 2 and 5) or doxycycline-sensitive pTRE2pur/Tino containing the *Tino* ORF alone (lane 4). The corresponding controls (MOCK) are in lanes 1 and 6, for the first, and lane 3, for the second type of vector. The polyclonal antibody recognizes the recombinant pTino in the range of about 60 KDa. *M* is the molecular weight marker.

D) Adsorption experiments. To identify the band(s) corresponding to the endogenous product(s) of *Tino*, a Western Blotting analysis with the rabbit polyclonal anti-pTino antibody (α-pTino) adsorbed with the specific peptides was performed. In particular, the peptides were incubated 1 hr at RT with α-pTino at an 1:20 (antibody/peptides) molar ratio. Thus, total protein extracts of HeLa Tet-Off cells were immunoblotted with the antibody/peptides mix (*pep*) or with the primary antiboy alone (-). *unsp* indicates the unspecific protein product probed by the anti-pTino antibody; it corresponds to the lower band of the

doublet spanning from about 70 to 75 kDa.

Figure 7. A) GENSCAN analysis. Schematic strategy (*on the left*) and RT-PCR products (*on the right*) of analysis of 5' end of *Tino* with R1 and R2 reverse primers derived from *Tino* cDNA and G1, G2, and G3 forward primers designed with GENSCAN software.
B) GENSCAN RT-PCRs. Distributions of the *upper* and *lower* RT-PCR products (*capital letters and underlined*) on the chromosome 19. Consensus splice-sites are in *gray*, pentameric intronic enhancers in *dark gray*, and branch sites in *white on a black background*. The first nucleotide (G) of the *Tino* sequence cloned with the RNA THS is in *gray on a black background*. The relative protein sequences of the *upper* and *lower* products with underlined novel predicted amino acids are shown under each genomic context.

Figure 8. Immunolocalization of endogenous *Tino* protein in HeLa cells. Immunocytochemically stained protein pTino demonstrating a prevalent nuclear and perinuclear localization (**B**) with FITC-conjugated anti-rabbit secondary antibody (*green fluorescence*) (**A**) Nuclear DNA was counterstained with Hoechst 33258 (*blue fluorescence*).

Figure 9. mRNA stability analysis with pTino. HeLa Tet-Off cells were transiently cotransfected with vector only (MOCK) or a vector encoding pTino (TINO) and a vectors producing reporter RNA (**A**) β-globin or (**B**) β-globin+ARE of the *bcl-2* 3'UTR. The synthesis of the reporter RNAs was inhibited by doxycycline 48 h posttransfection, and total RNA was isolated at different time points. Reporter β-globin RNAs were quantified by RNase Protection Assay. Endogenous GAPDH mRNA was used for normalization. Half-lifes of reporter RNAs with or without *Tino* were calculated from first order decay constant (*k*) (See Materials and Methods).

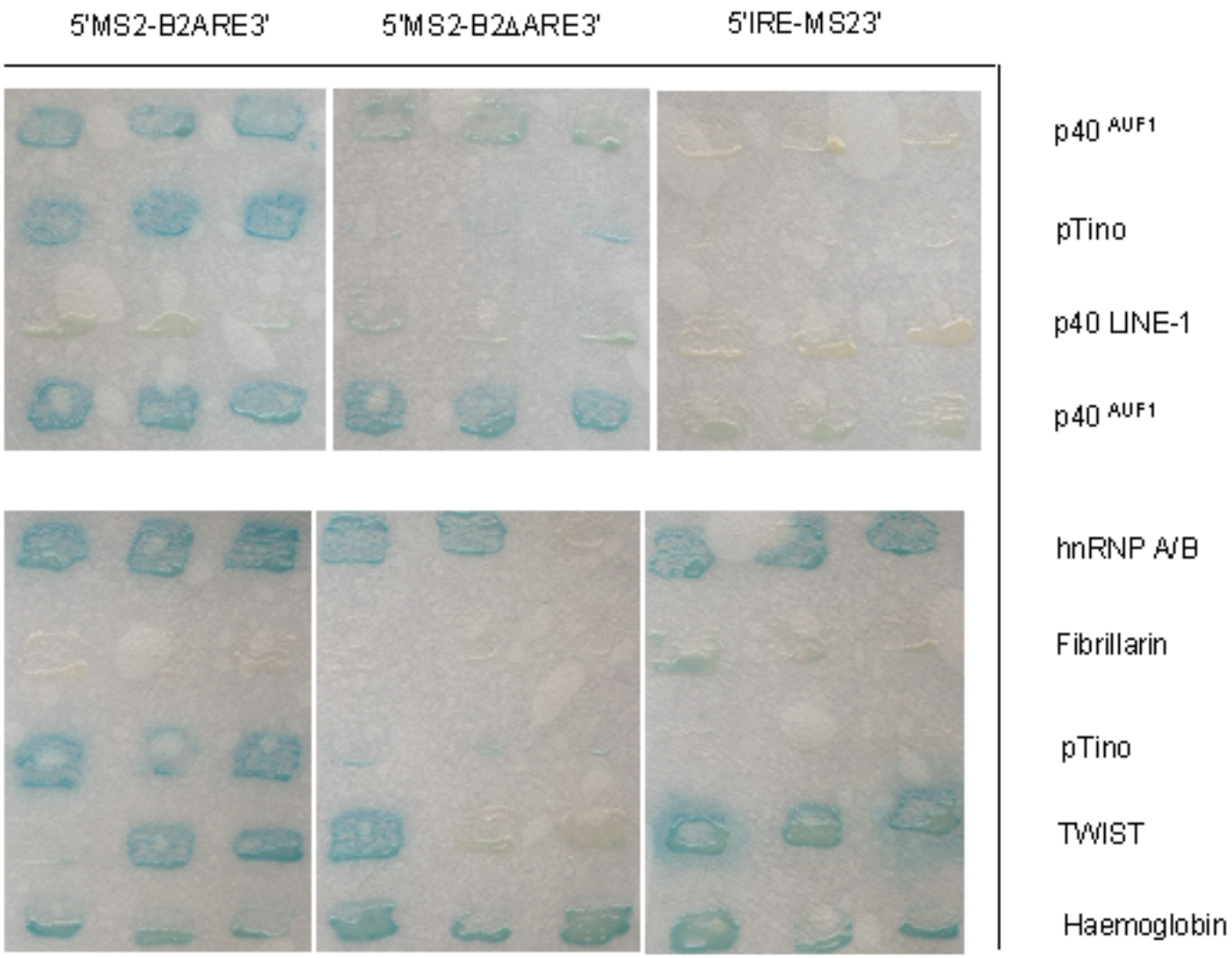


Figure 1

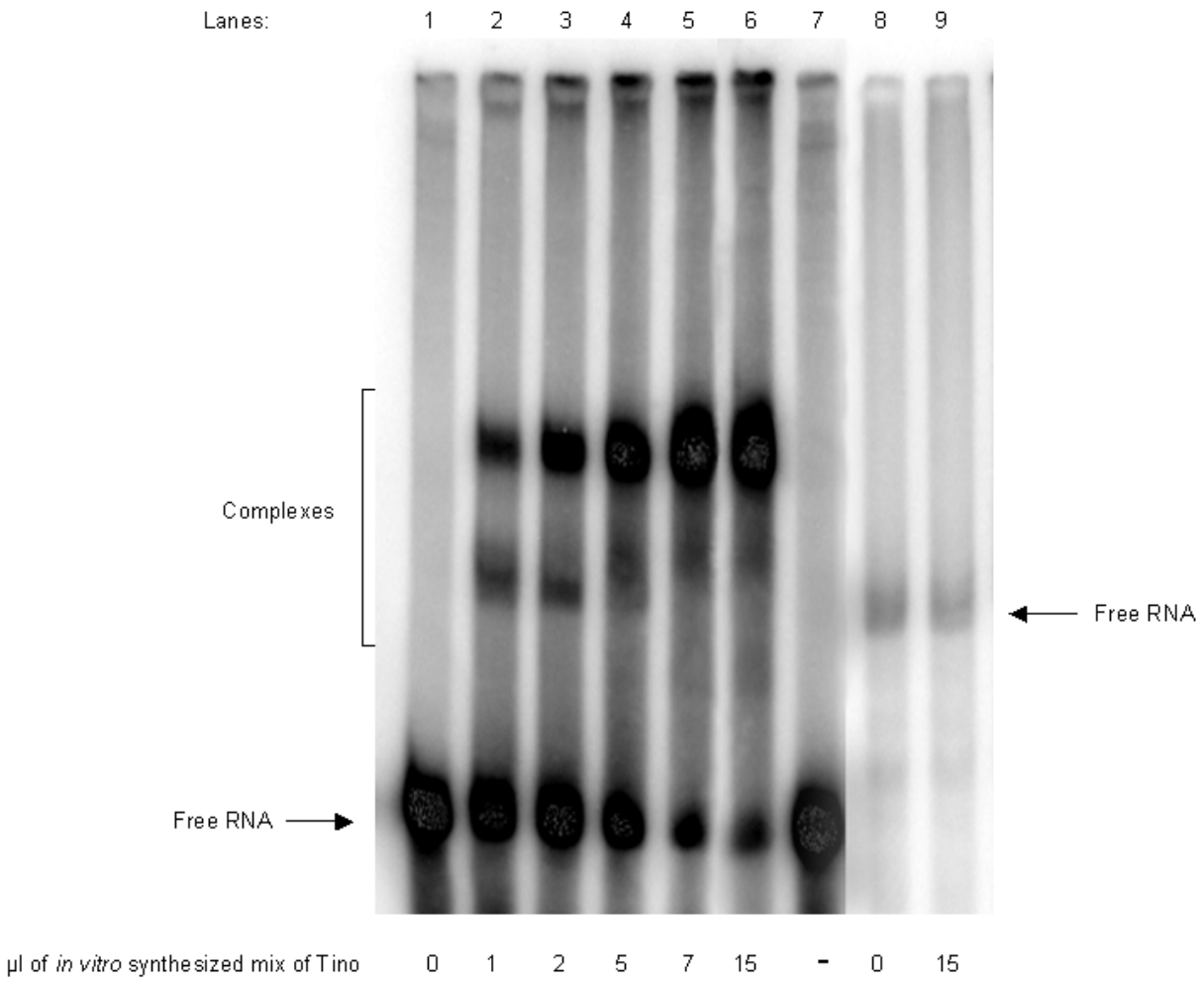


Figure 2

Figure 3

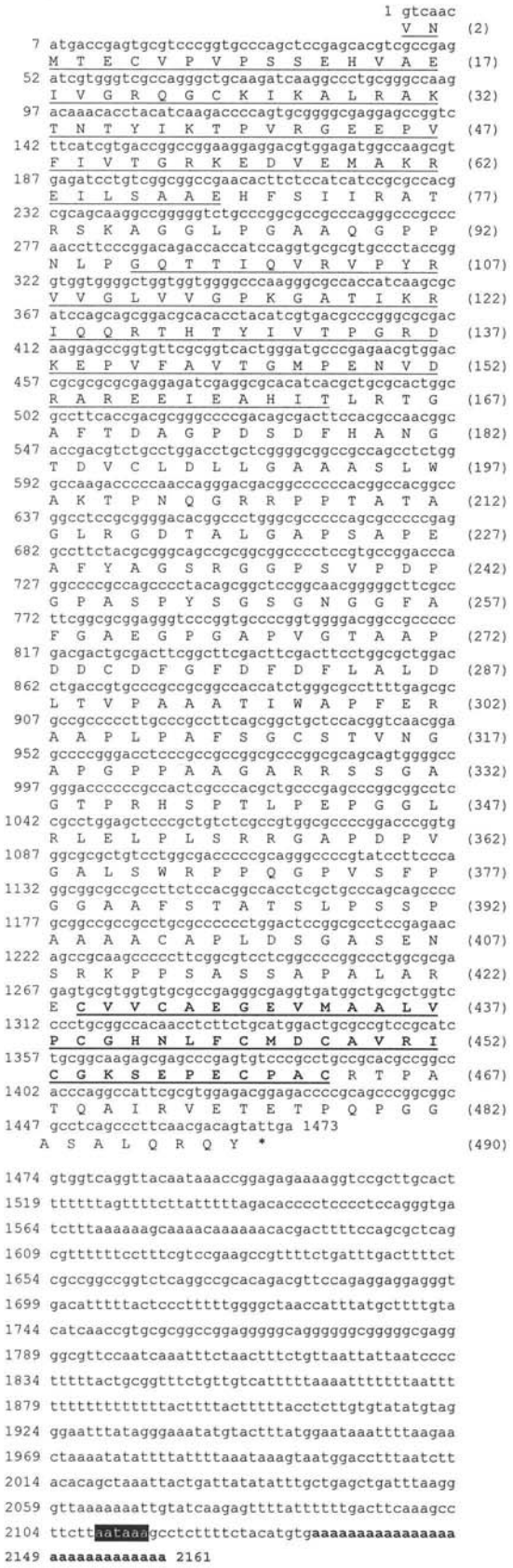
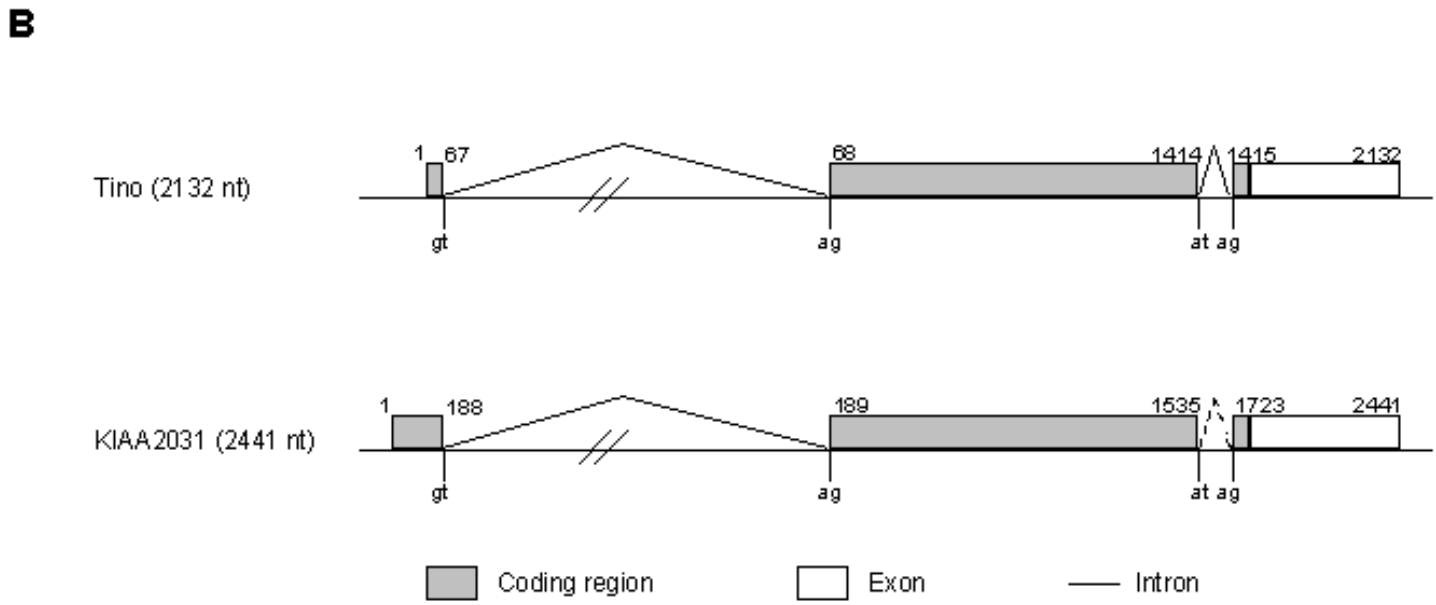


Figure 4 A



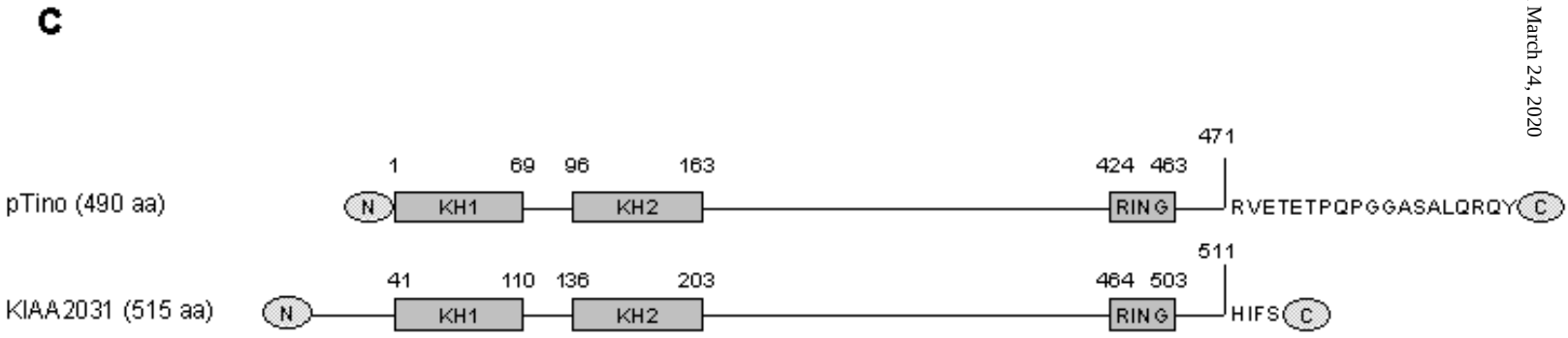
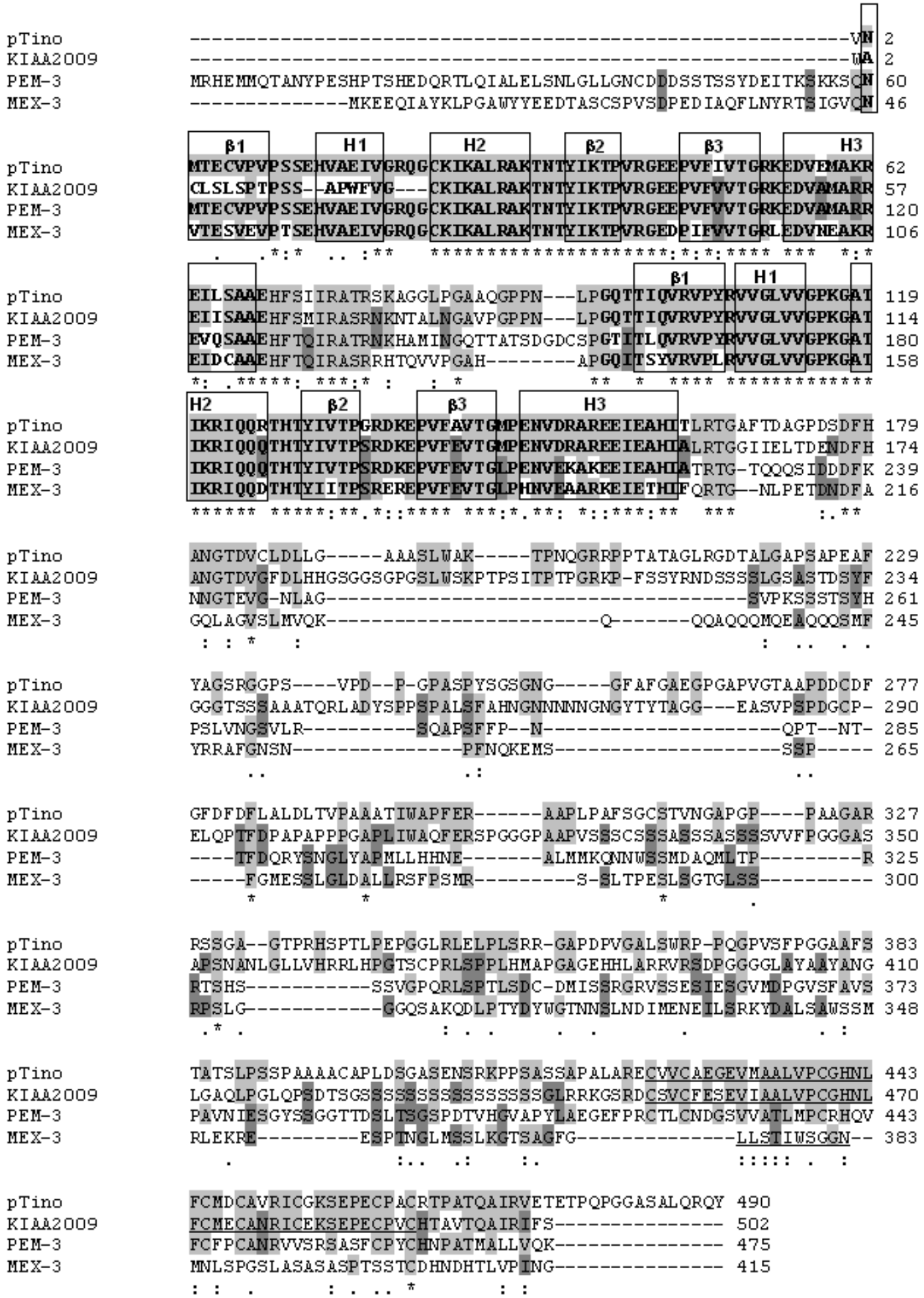


Figure 4



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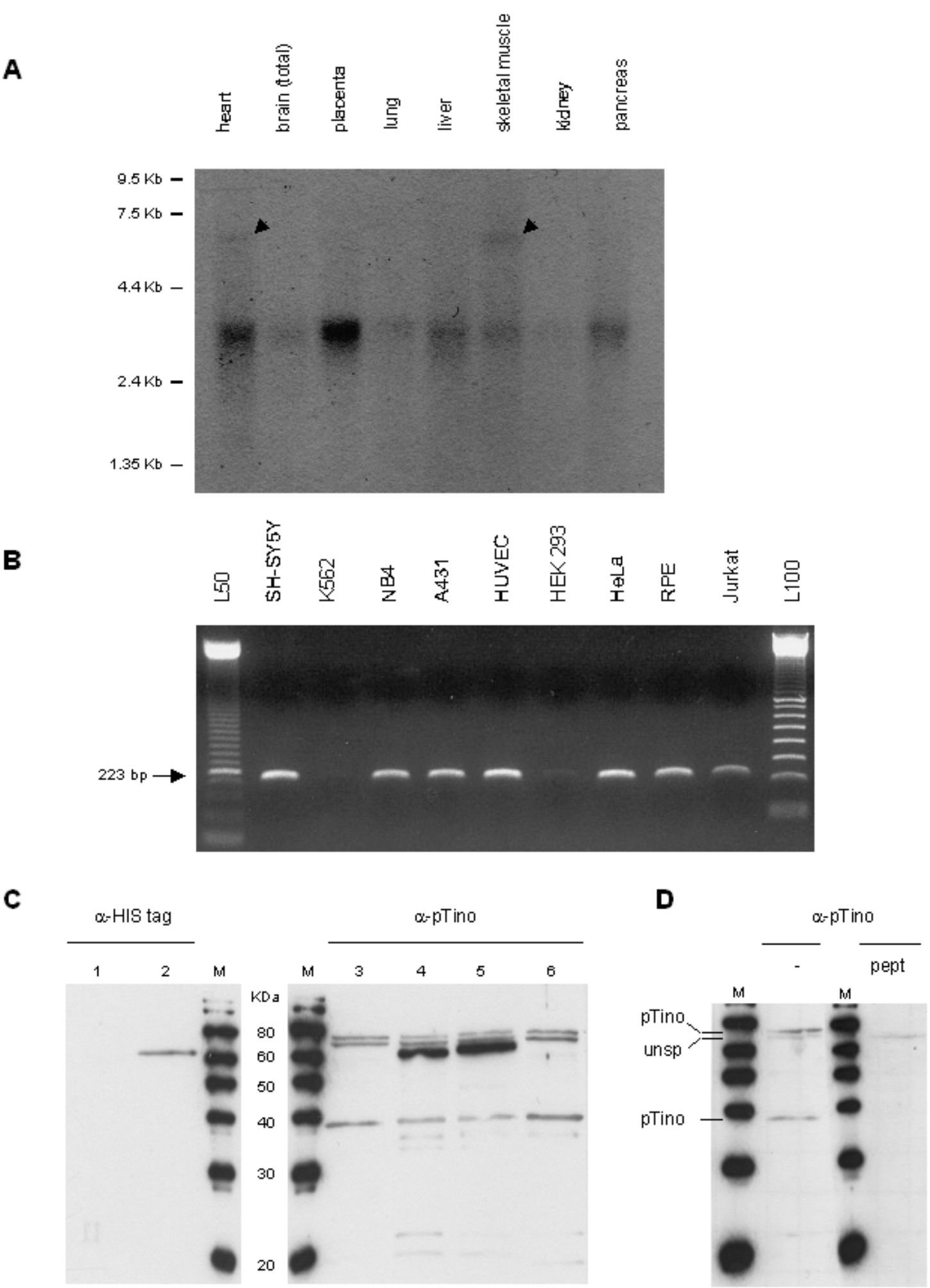
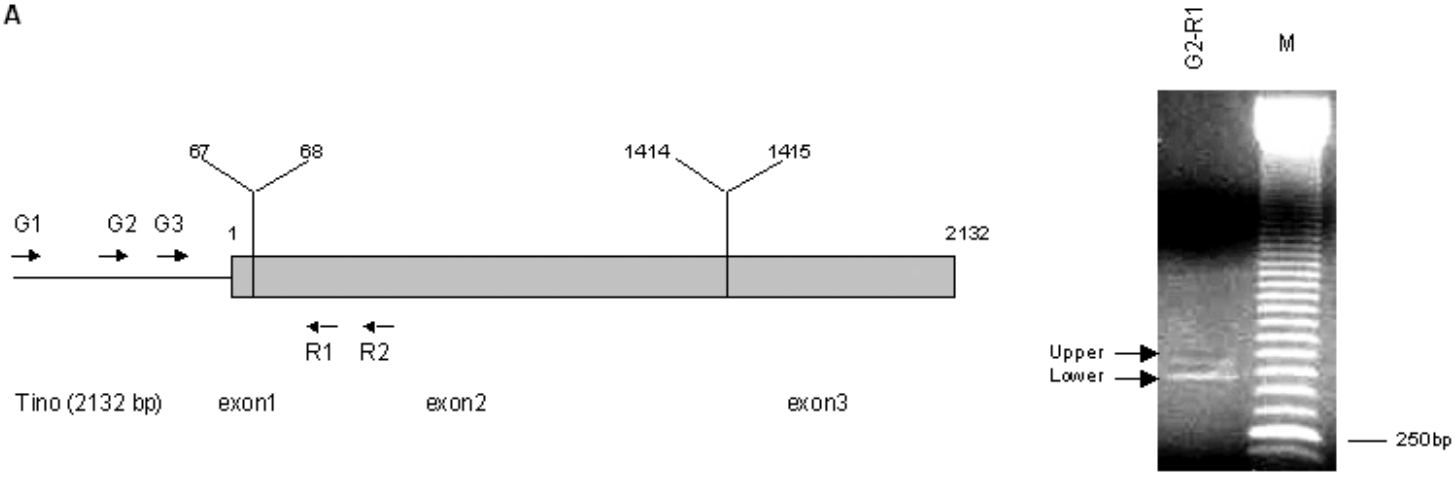


Figure 6

A



B

Genomic context on chr 19 (reverse strand), *upper* band

```
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cgcgccccgg ccgccgcccg aacccgacga cgcggccgcc gcgctccgcc  1507877
TGGCGCTGGA CCAGCTGTCG GCGCTCGGGc TGGGGGGCGC TGGCGACACG  1507827
GACGAGGAGG GGGCGGCCGG GGACGgcgca gcggcggcgg ggggcgcgga  1507777
cggcgggggcg gctccggagc ctgtgccccc cgacggacct gaggccggcg  1507727
cgcccccgac cctggccccc gccgtggccc ccgggtcgct gccgctgctg  1507677
gaccccaacg cgagtccccc gccgCCGCCG CCGCCCCGGC CGTCGCCCCC  1507627
CGACGTGTTC GCGGGCTTCG CGCCCCACCC CGCGGCCCTG GGGCCCCCGA  1507577
CGCTGCTGGC CGACCAGATG AGCGTGATCG GCAGCCGCAA GAAAAGCcTC  1507527
AACATGACCG AGTGCGTCCC GGTGCCCAGC TCCGAGCACG TCGCCGAGAT  1507477
CGTGGGTCGC CAGGgtgagt ggccctcgtc gggggttgct gtcctggggg  1507427
```

Predicted protein sequence

```
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MSVIGSRKKSVNMTECVPVPSSEHVAEIVGRQ
```

Genomic context on chr 19 (reverse strand), *lower* band

```
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cgcgccccgg ccgccgcccg aacccgacga cgcggccgcc gcgctccgcc  1507877
TGGCGCTGGA CCAGCTGTCG GCGCTCGGGC TGGGGGGCGC TGGCGACACG  1507827
gacgaggagg gggcggccgg ggacggcgca gcggcggcgg ggggcgcgga  1507777
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CGACGTGTTC GCGGGCTTCG CGCCCCACCC CGCGGCCCTG GGGCCCCCGA  1507577
CGCTGCTGGC CGACCAGATG AGCGTGATCG GCAGCCGCAA GAAAAGCcTC  1507527
AACATGACCG AGTGCGTCCC GGTGCCCAGC TCCGAGCACG TCGCCGAGAT  1507477
CGTGGGTCGC CAGGgtgagt ggccctcgtc gggggttgct gtcctggggg  1507427
```

Predicted protein sequence

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TECVPVPSSEHVAEIVGRQ
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Figure 7

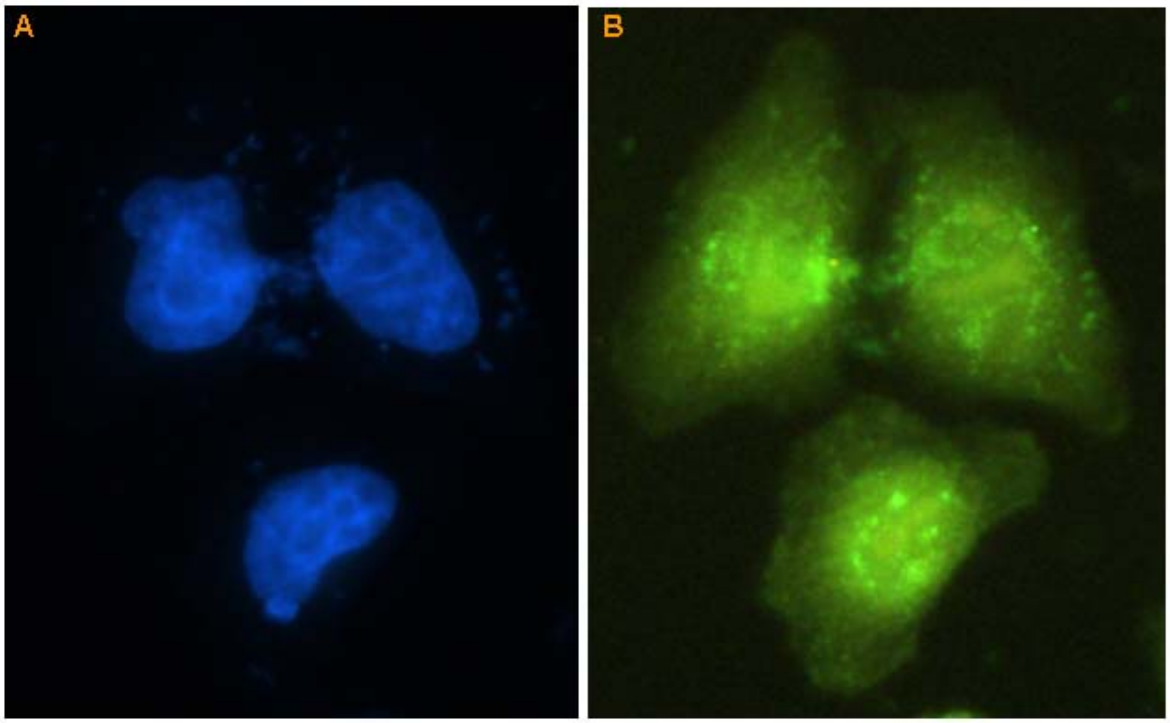
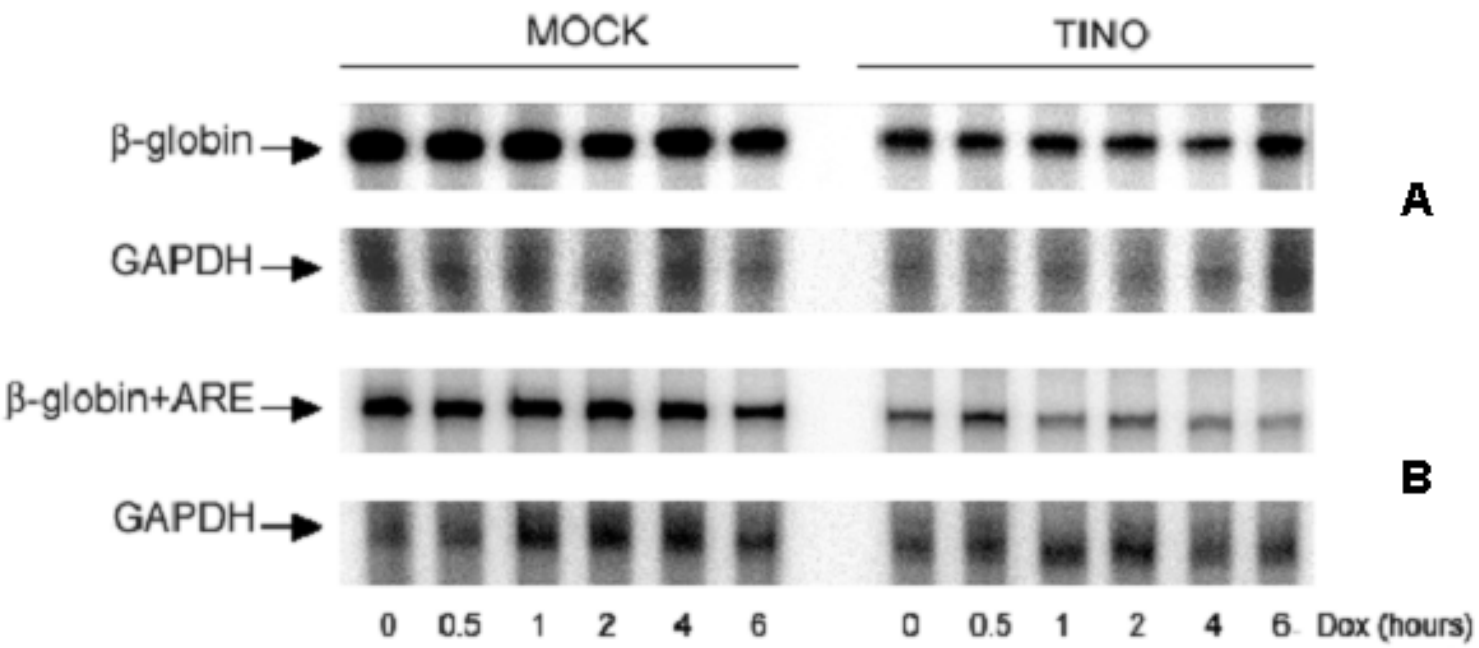
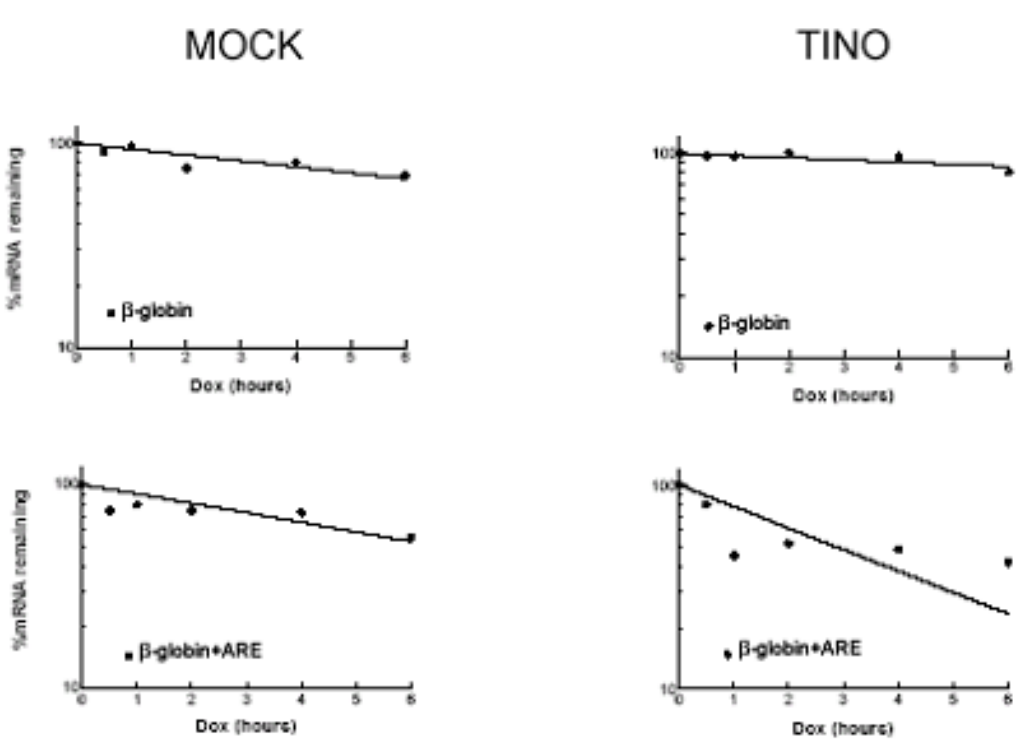


Figure 8





RNA	k (± S.E.) (h^{-1})	$t_{1/2}$ (h)
β-globin (mock)	0.07 ± 0.01	10
β-globin (Tino)	0.025 ± 0.006	28
β-globin+ARE (mock)	0.11 ± 0.02	6.3
β-globin+ARE (Tino)	0.24 ± 0.07	2.9

Figure 9

Identification of Tino: A new evolutionarily conserved bcl-2 AU-rich element RNA binding protein

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