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Ki-1/57 and CGI-55 ectopic expression impact cellular pathways involved in proliferation and stress response regulation

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ABSTRACT

Ki-1/57 (HABP4) and CGI-55 (SERBP1) are regulatory proteins and paralogs with 40.7% amino acid sequence identity and 67.4% similarity. Functionally, they have been implicated in the regulation of gene expression on both the transcriptional and mRNA metabolism levels. A link with tumorigenesis is suggested, since both paralogs show altered expression levels in tumor cells and the Ki-1/57 gene is found in a region of chromosome 9q that represents a haplotype for familiar colon cancer. However, the target genes regulated by Ki-1/57 and CGI-55 are unknown. Here, we analyzed the alterations of the global transcriptome profile after Ki-1/57 or CGI-55 overexpression in HEK293T cells by DNA microchip technology. We were able to identify 363 or 190 down-regulated and 50 or 27 up-regulated genes for Ki-1/57 and CGI-55, respectively, of which 20 were shared between both proteins. Expression levels of selected genes were confirmed by qRT-PCR both after protein overexpression and siRNA knockdown. The majority of the genes with altered expression were associated to proliferation, apoptosis and cell cycle control processes, prompting us to further explore these contexts experimentally. We observed that overexpression of Ki-1/57 or CGI-55 results in reduced cell proliferation, mainly due to a G1 phase arrest, whereas siRNA knockdown of CGI-55 caused an increase in proliferation. In the case of Ki-1/57 overexpression, we found protection from apoptosis after treatment with the ER-stress inducer thapsigargin. Together, our data give important new insights that may help to explain these proteins putative involvement in tumorigenic events.

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1. Introduction

Ki-1/57 (also named HABP4) has been discovered as an intracellular cross-reactant of the monoclonal antibody Ki-1, the first to specifically detect malignant cells in Hodgkin's lymphoma [1]. The protein CGI-55, also named SERBP1 (or PAIRBP1), has been first described as an RNA binding protein involved in the regulation of PAI-1 mRNA stability, by binding to its mRNA 3′UTR [2,3]. Ki-1/57 and CGI-55 share 40.7% identity and 67.4% similarity, which suggest that they might be paralogs, possibly with similar or redundant functions in human cells [4]. Yeast two-hybrid system protein–protein interaction data were obtained [4–12] and suggested that both are involved in multiple steps in gene expression regulation, although their exact roles and possible gene targets in human cells remain to be identified.

Although the relationship of Ki-1/57 with Hodgkin's disease is still unknown, further indications for the involvement of these proteins in tumorigenic processes have been reported in the literature. Familial colon cancer risk-associated SNP haplotypes have been found in strong

Abbreviations: HABP4, hyaluronan binding protein 4; PAIRBP1, plasminogen activator inhibitor RNA-binding protein 1; HEK293, human embryonic kidney 293 cells; TF, transcriptional factor; ER, endoplasmic reticulum; SG, stress granule; PB, processing bodies; GO, gene ontology; SUMO, small ubiquitin-like modifier; RBP, RNA-binding proteins; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PI, propidium iodide; SERBP1, serpine mRNA binding protein 1

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linkage disequilibrium with the haplotype block containing the *Ki-1/57* gene (referred as *HABP4*) [13]. Overexpression of CGI-55 has been detected in ovarian cancer and was correlated with advanced tumor stage [14]. Also, high expression levels of CGI-55 have been found in human lung cancer tissues and in a human giant cell lung carcinoma cell line with high metastatic potential [15]. Furthermore, analysis of different metastatic sites in individuals with prostate cancer reported altered gene expression of CGI-55 in lymph node, liver and bone metastasis [16].

The protein partners identified for Ki-1/57 by yeast two-hybrid assays are involved in different gene expression regulatory processes. Many of them are directly or indirectly involved in transcriptional regulation, such as the chromatin remodeling protein CHD3 [4] and the transcription factors MEF2C and p53 [5,6]. Others are involved in the regulation of pre-mRNA splicing (hnRNPQ and SFRS9 [7]) and translation (RACK1 [17], CIRP [18], FMRP and the ribosomal protein RPL38 [19]).

The participation of Ki-1/57 in RNA metabolism has been further confirmed through its in vitro binding to U-rich RNA probes and its ability to mediate pre-mRNA splicing and translation activities of reporter genes in transfected cells [7]. Furthermore, Ki-1/57 could be found in the large molecular weight complexes of 43–48S translation pre-initiation in sucrose gradient co-sedimentation experiments [8]. Together, the interaction of Ki-1/57 with proteins involved in splicing, chromatin remodeling, transcription and translation suggests that Ki-1/57 might be involved in regulating integrated gene expression mechanisms [20].

The structural features of Ki-1/57 are also in agreement with this apparent high promiscuity of protein–protein interaction partners. Several biophysical methodologies have been approached to confirm that Ki-1/57 belongs to the growing list of eukaryotic intrinsically disordered proteins [21]. The function of this kind of proteins includes regulation of transcription and translation, cellular signal transduction and other cellular processes that require high structural flexibility to allow specific binding to multiple partners [22]. Additionally, the lack of structure of these proteins provides accessibility to post-translational modification at several sites [23], consistent with the fact that Ki-1/57 can be phosphorylated on serine/threonine residues by the protein kinase C [9] and methylated on arginines by the arginine methyltransferase PRMT1 [10].

The subcellular localization of Ki-1/57 reflects its involvement with gene expression regulation processes as well. It is located in the cytoplasm, the nuclear pores and the nucleus, where it can be found in association with nucleoli and other smaller nuclear bodies [24]. This subcellular distribution can depend on different stimuli, as observed during cell treatment with the methylation inhibitor Adox and the PKC pathway inducer PMA, which leads to a relocation of Ki-1/57 to the cytoplasm [9]. In untreated cells, Ki-1/57 can partially localize to nuclear speckles [7], a place for post-transcriptional splicing and storage of splicing factors [20]. On the other hand, upon Adox treatment, Ki-1/57 can relocalize to other dot-like substructures in the nucleus, such as nucleoli, where ribosomal biogenesis and maturation take place, and also GEMS and Cajal bodies, which are important sites for spliceosomal and non-spliceosomal snRNP biogenesis, maturation and recycling [7,20].

CGI-55 alike localizes to cytoplasm and nucleus, where it can also be observed in a punctuated fashion in the perinuclear region, the nucleoli and p80-coilin-positive coiled bodies [11]. Additionally, CGI-55 has been found in cytoplasmic stress granules (SGs) and processing bodies (p-bodies) under stress conditions [25,26]. These substructures are cytoplasmic storage sites for non-translating mRNA, and their assembly is dynamically related with the inhibition of the translation initiation and the disassembly of polysomes [27,28].

Here, we set out to obtain additional functional information on these proteins from global gene expression data. Cultured cells transiently overexpressing Ki-1/57 or CGI-55 had their total mRNAs profiled by Affymetrix microarray gene expression technology. This revealed that the affected genes are mostly involved in cellular processes such as proliferation, apoptosis and cell cycle control. In further assays, we observed that Ki-1/57 and CGI-55 overexpressing cells showed reduced cell growth, mainly due to G1 phase arrest. Consistent with the alteration in apoptosis related gene expression, we found that Ki-1/57 overexpression can protect cells from apoptosis under treatment with the ER-stress inducer thapsigargin. Together, these data add new clues for the functional role of Ki-1/57 and CGI-55 in human cells yielding important information that contribute to explain their so far putative involvement in tumorigenic events.

2. Materials and methods

2.1. Plasmid constructs and siRNAs

Cloning of the complete cDNA encoding Ki-1/57 in fusion with the N-terminal Flag tag into pcDNA6 (pcDNA6-flag-Ki-1/57) has been previously described [29]. The N-terminally FLAG-tagged CGI-55 construct cloned into pcDNA6 Myc/His (Invitrogen) was kindly provided by John L. Goodier (University of Pennsylvania School of Medicine). Knockdown of Ki-1/57 and CGI-55 was performed using siRNA and scrambled siRNA from Ambion® In Vivo. HABP4 siRNA #87, #88, and #89 were tested for Ki-1/57 and SERBP1 siRNA #1, #2 and #3 for CGI-55. Under the tested conditions HABP4 siRNA #89 (siRNA Ki-1/57) and SERBP1 siRNA #3 (siRNA CGI-55) generated the lowest expression level of Ki-1/57 and CGI-55, respectively, compared to control cells (scrambled siRNA).

2.2. Cell culture, transient transfection and siRNA transfection

HEK293T cells were cultivated in high-glucose Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 1% penicillin G-streptomycin (Invitrogen) and 10% heat-inactivated fetal bovine serum (FBS, Invitrogen, South America) at 37 °C and 5% CO_2. For microarray experiments cells were transfected by using the calcium phosphate method [7]. For MTS assay of Ki-1/57 and CGI-55 overexpressing cells, Lipofectamine Reagent (Invitrogen) was used according to manufacturer's instructions. Transfection of cells for qRT-PCR, EdU incorporation assay, cell cycle analysis and apoptosis assay were performed with linear 25 kDa polyethylenimine (PEI, Polysciences). Briefly, cells growing in a 6-well plate were transfected with 3 μg DNA and 10 μL PEI (1 mg/mL) per well. Or cells plated in 24-well plates were transfected with 1 μg DNA and 3.3 μL PEI per well. For knockdown experiments, siRNA transfections were performed with cells growing in 6-well plates using linear 25 kDa polyethylenimine (PEI, Polysciences) with a final siRNA concentration of 50 nM and 16 μL PEI (1 mg/mL). The transfection reagent was removed after 16 h.

2.3. Total RNA isolation and microarray analysis

Total RNA was extracted from cells using TRIzol® (Invitrogen) according to the manufacturer's instructions and quantified by $OD_{260\ nm}$ measurements using Nanodrop® (Thermo Scientific). Isolation of mRNA was performed with Oligotex mRNA mini Kit (Qiagen) following the manufacturer's protocol. mRNA (1 μg) of control and overexpressing Ki-1/57 or CGI-55 cells was used for microarray analysis on human genome U133 Plus 2.0 Gene Arrays (Affymetrix, Santa Clara, CA). Labeling and washing were performed according to the standard Affymetrix protocol. The arrays were scanned using a GeneChip Scanner 3000 (Affymetrix). Data analysis and quality control were done using GCOS (Gene Chip Operating Software version 1.4). Quantity normalization and subsequent data processing were performed using ArrayAssist x.5 software package (Stratagene).

2.4. Quantitative real-time RT-PCR (qRT-PCR)

Total RNA was extracted from cells using TRIzol® (Invitrogen) and treated with DNAse I RNAse Free (Thermo Scientific). 5 μg RNA was reverse transcribed using SuperScript® III Reverse Transcriptase (Invitrogen) with oligo $(dT)_{20}$ primers. qRT-PCR was performed with a 7500 real-time PCR system (Applied Biosystems) with specific primers (Supplementary Table 1). Each real-time RT-PCR reaction (in 20 μL) contained 2 × SYBR Green PCR Master Mix (Applied Biosystems), primers and template cDNA (1/100 of the cDNA reaction). The cycling conditions consisted of an initial, single step of 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. PCR amplifications were performed in triplicates for each sample. Gene expression levels were quantified relative to the expression of β-actin endogenous control, using an optimized comparative Ct (ΔΔCt) value method.

2.5. Western blot analysis

HEK293T cells were collected at indicated times after transfection and lysed in lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 1% NP-40, 0.1% Triton X-100 and complete protease inhibitor set, Roche). Subsequently, the lysate was treated with DNAse I (Promega) and RNAse A and cleared at 14.000 ×g at 4 °C for 20 min. Protein concentrations were determined using the BCA Protein Assay Reagent Kit (Thermo Scientific). Proteins from total cell lysates were separated by SDS-PAGE electrophoresis and transferred onto a PVDF membrane. After blocking with 5% skim milk for 1 h, membranes were hybridized with one of the following: monoclonal anti-FLAG M2 antibody (Sigma) or monoclonal anti-β-tubulin antibody (Abcam). After washing with TBST, membranes were incubated with a horseradish peroxidase-conjugate secondary antibody and detection was performed using the Western Blotting Luminol Reagent kit (Santa Cruz Biotechnology), according to the manufacturer's recommendations.

2.6. In silico PPI analysis

The retrieved Ki-1/57 and CGI-55 interacting partners from yeast two-hybrid screens, together with the up- and down-regulated genes identified from overexpression of Ki-1/57 and CGI-55 microarrays were integrated in interaction networks using the Integrated Interactome System (IIS) platform, developed at the Brazilian Biosciences National Laboratory, Brazil (http://www.lge.ibi.unicamp.br/lnbio/IIS/) [30]. The enriched biological processes from the Gene Ontology (GO, http://www.geneontology.org/) database were calculated in each network using the hypergeometric distribution [30]. The enriched transcription factors for the up- and down-regulated genes were retrieved using the Enrichr software [31] and mapped in each network. The interaction networks were visualized using Cytoscape 2.8.3 software [32].

2.7. Phylogenetic analysis

A phylogenetic hypothesis was inferred by Bayesian inference (BI) using MrBayes v3.2.2 [33] to provide an evolutionary discussion about the function of proteins encoded by the CGI-55 (*SERBP1*) and Ki-1/57 (*HABP4*) genes. First, sequence data from these proteins were downloaded from the UniProt database (http://www.uniprot.org/). To root the phylogenetic tree, the VIG protein encoded by vasa intronic gene (vig) from *Drosophila melanogaster* was selected as an outgroup. Sequences were aligned using MAFFT [34] and the alignment was manually inspected, being subsequently trimmed using G-blocks [35]. The JTT + G model [36] was calculated by ProtTest 3.3 [37] as the best-fit amino acid substitution model, according to AIC and BIC criteria. The BI phylogenetic trees were calculated using the Bayesian Markov Chain Monte Carlo (MCMC) method with 1×10^7 generations and a sample frequency of 10^3, considering the JTT + G substitution model. The parameter convergence was analyzed in TRACER v1.5.0 (http://beast.bio.ed.ac.uk/tracer), and the chain reached a stationary distribution after 1×10^5 generations. Then, 1% of the generated trees was burned to produce the consensus tree.

2.8. MTS cell proliferation assay

HEK293T cells were seeded in 96-well plates, cultured to 60–70% confluence and transfected with pcDNA6-flag-Ki-1/57, pcDNA6-flag-CGI-55 or pcDNA6 (control). For knockdown experiments cells were transfected with siRNA against Ki-1/57 (siRNA #89), siRNA against CGI-55 (siRNA #3) or scrambled siRNA. 16 h following transfection the medium was replaced with Dulbecco's modified Eagle's medium for normal medium (10% FBS) or serum starvation (1% FBS). At the indicated time, cell proliferation was tested using the MTS assay (CellTiter 96® AQueous One Solution; Promega) according to manufacturer's protocol.

2.9. EdU incorporation assay

Cells were seeded in 6-well plates, transfected with pcDNA6-flag-Ki-1/57, pcDNA6-flag-CGI-55 or empty pcDNA6 and maintained under standard conditions (10% serum) or under serum starvation (1% serum). 24 or 48 h after transfection, cells were pulsed with EdU, incubated for further 4 h and harvested by trypsinization (0.25% trypsin, 0.53 mM EDTA). Cells were seeded on poly-L-lysine (Sigma)-treated cover slips, fixed with 4% paraformaldehyde and permeabilized using 0.5% Triton X-100 for 20 min at room temperature. Incorporation of EdU was observed by incubating fixed cells with 2% BSA in PBS for 30 min and Alexafluor 488 for a further 30 min under Cu(I)-catalyzed click reaction conditions, as described by the manufacturer (Click-iT® EdU Imaging Kits, Invitrogen). Cells were washed with PBS, and mounted on slides using Vectashield with DAPI (Vector Laboratories) for fluorescent microscopy. Images were taken on Confocal Laser Scanning Microscope Leica TCS SP8 (magnification ×100). Column graph shows the percentage of EdU-positive cells compared to total cells (± SE). Experiments were performed in duplicate biological experiments. A minimum of 400 cells was counted for each replicate.

2.10. Cell cycle analysis

Cells were seeded in 6-well plates, transfected with pcDNA6-flag-Ki-1/57, pcDNA6-flag-CGI-55 or pcDNA6 and maintained under normal conditions (10% serum) or under serum starvation (no serum) before analysis. Following 48 h after transfection, floating cells were collected and then added to the attached cells that have been harvested by trypsinization. Cells were washed twice with cold PBS, fixed with 2 mL of ice-cold 70% ethanol in PBS and incubated at 4 °C overnight. The pellets were collected by centrifugation and resuspended in PBS solution containing 60 μg/mL of propidium iodide, 0.5% Triton X-100 and 250 μg/mL of RNAse A. After incubation for 30 min in the dark at 37 °C followed by 30 min incubation at 4 °C, cells were analyzed for DNA content using a FACS Canto II (BD Pharmingen) and FACS DIVA software. The cell cycle distribution is shown as the percentage of cells containing 2n (G1 phase), 4n (G2 and M phases) and S phase judged by propidium iodide staining. The experiment was performed in triplicate biological experiments.

2.11. Apoptosis assays

HEK293T cells were cultured in 6-well plates to 60–70% confluency, and then transfected with pcDNA6-Flag-Ki-1/57, pcDNA6-Flag-CGI-55 or pcDNA6. 8 h after transfection, cells were treated with 30 μM cisplatin, 3 μg/mL actinomycin D or 10 μM thapsigargin for 16 h. Cells were then detached from plates with trypsin (0.25% trypsin, 0.53 mM EDTA), combined with floating cells and stained with Annexin V-FITC and propidium iodide (BD Pharmingen, San Diego, CA, USA) according

to the manufacturer's protocol. Data were collected on a FACS Canto II (BD Pharmingen) and analyzed by FACS DIVA software. Results shown in bar graphs represent annexin V positive cells and include early and late apoptosis.

2.12. Statistical analysis

Data are displayed as mean ± standard deviation (SD). Comparisons were analyzed by Student's *t*-test and a p-value < 0.05 was considered to indicate statistical significance. A statistical association between the frequency of transcription factors classes of genes modified by Ki-1/57, CGI-55 and the whole human genome was tested, using two-sided Fisher's exact test.

3. Results

3.1. Global gene expression analysis

The association of Ki-1/57 with proteins involved in regulatory mechanisms of transcription, RNA metabolism and translation regulation suggests a possible function for this protein in the regulation of gene expression. Looking for additional functional information, we performed microarray global gene expression analysis of HEK293T cells transiently overexpressing Ki-1/57 or CGI-55 (Fig. 1) by using Affymetrix microarray technology (Fig. 2). With a cutoff of 2-fold compared to cells transfected with an empty pcDNA6, the expression of 413 genes was altered by Ki-1/57 overexpression, with 88% of them down-regulated (363 down and 50 up-regulated genes) (Fig. 2A, top panel).

Similarly, the RNA expression profile of CGI-55 overexpression was also investigated. We found that 217 genes had differential expression and 90% of them were also down-regulated (190 down and 27 up-regulated genes) (Fig. 2A, bottom panel). These results suggest that the functional role of these proteins in gene regulation might be predominantly repressive. Interestingly, we found that Ki-1/57 and CGI-55 can alter at least 20 genes in common, with most of them being repressed by both proteins (Fig. 2B). This observation along with the amino acid sequence similarity and our previous yeast two-hybrid protein–protein interaction data reinforces the hypothesis of paralogy between these proteins [7].

3.2. Ki-1/57 and CGI-55 are paralogs that arose by a genome duplication in the baseline leading to the chordates

In the Pfam database, CGI-55 (SERBP1) and Ki-1/57 (HABP4) proteins are classified in the HABP4 family of hyaluronan-binding proteins (accession PF04774). This family includes proteins that have been observed to bind *in vitro* to hyaluronan (a glucosaminoglycan) or that are involved in the regulation of mRNA stability [38]. However, proteins classified in this family present a diverse architecture of domains and motifs, and it is not known whether these proteins share a common function. The initial reports of a hyaluronan binding activity for Ki-157 (HABP4) could not be shown to be of *in vivo* relevance.

Among proteins of the HABP4 family, the VIG protein of *D. melanogaster* shares significant similarity with CGI-55 and Ki-1/57. This protein is a component of the RISC complex, the RNA-induced silencing complex [39], and was selected as an outgroup in the phylogenetic analysis. Here, the molecular evolution of CGI-55 and Ki-1/57 proteins was assessed by Bayesian inference. The phylogenetic tree shows that these proteins are indeed paralogous as previously hypothesized and possibly originated from a major genome duplication event (Fig. 3) at the baseline leading to the chordates. The so-called HABP4 domain has as a characteristic to be enriched in several positively charged amino acids. This may be the explanation for the initial described binding of the negative charged hyaluronan but also may act as an RNA or nucleic acid binding module.

In addition, the analysis of the architecture of domains and motifs shows that CGI-55 and Ki-1/57 proteins have a distinct composition of glycine/arginine rich motifs (RGG / RXR) flanking the HABP4 domain (Fig. 3). These RGG/RXR motifs can be found mainly in proteins involved in RNA processing and transcriptional regulation [7]. Further

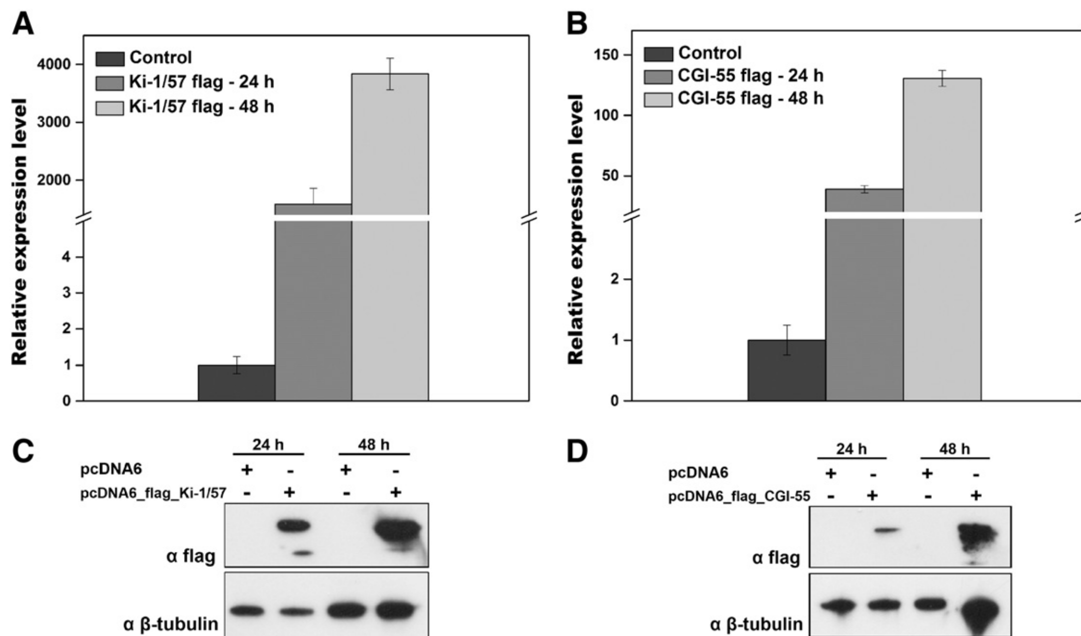


Fig. 1. Ki-1/57 and CGI-55 overexpression in HEK293T cells. HEK293T cells were seeded in 6-well plates, transfected 48 h later with pcDNA6-flag-Ki-1/57 (A, C) and pcDNA6-flag-CGI-55 (B, D) and compared with control (pcDNA6). At indicated time points (24 h and 48 h after transfection), cells were collected and resuspended in Trizol® for RNA extraction or in RIPA buffer for total protein extraction. Relative expression level of Ki-1/57 (A) and CGI-55 (B) was determined by quantitative RT-PCR experiments. The relative amount of each mRNA was normalized to β-actin. Data in histograms represent means (of triplicate experiments) ± SD. Western blot of cells transfected with pcDNA6-flag-Ki-1/57 (C) and pcDNA6-flag-CGI-55 (D) using anti-flag antibody and comparing with cells transfected with pcDNA6 vector. The antibody anti-β-tubulin was used as a loading control.

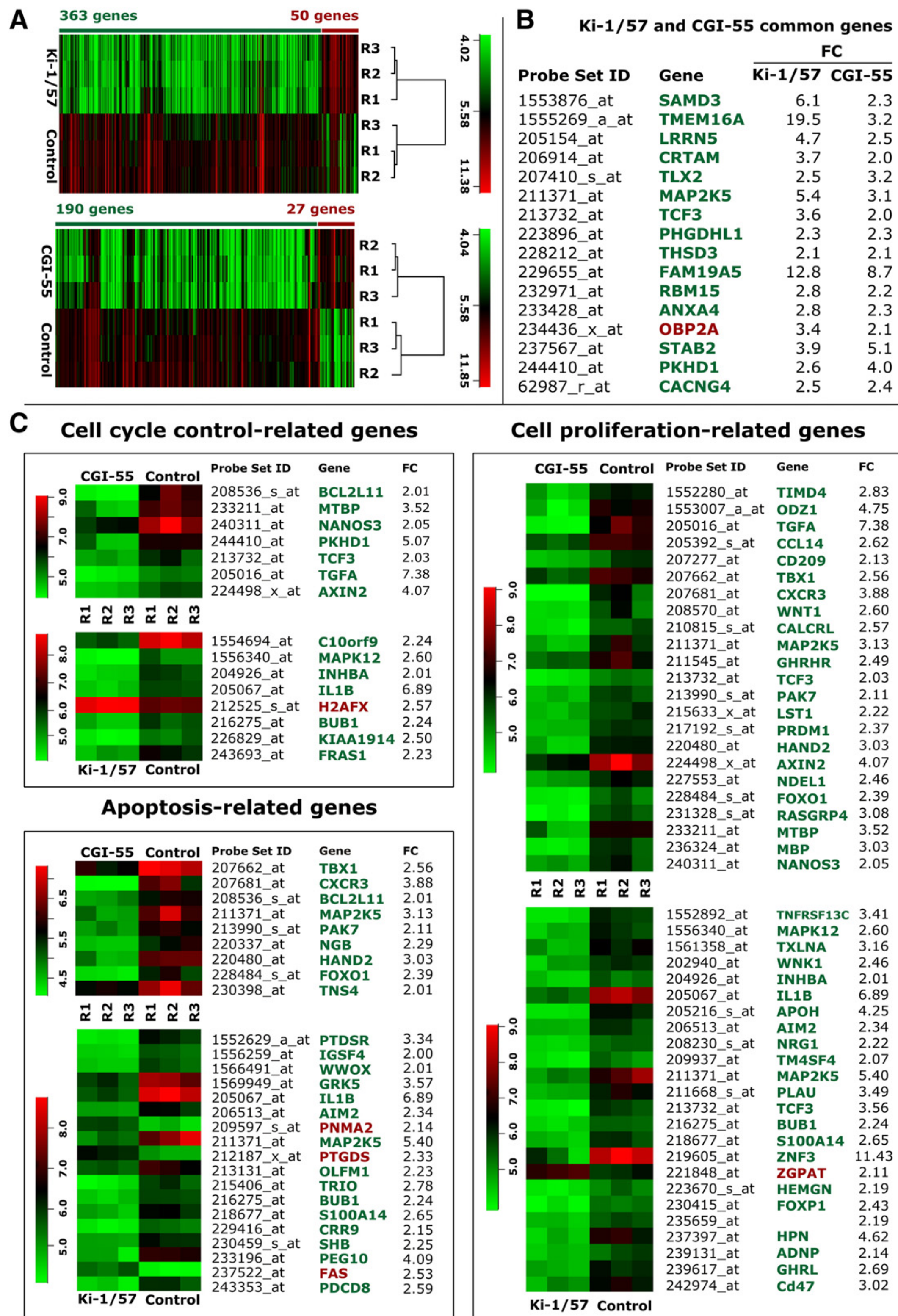


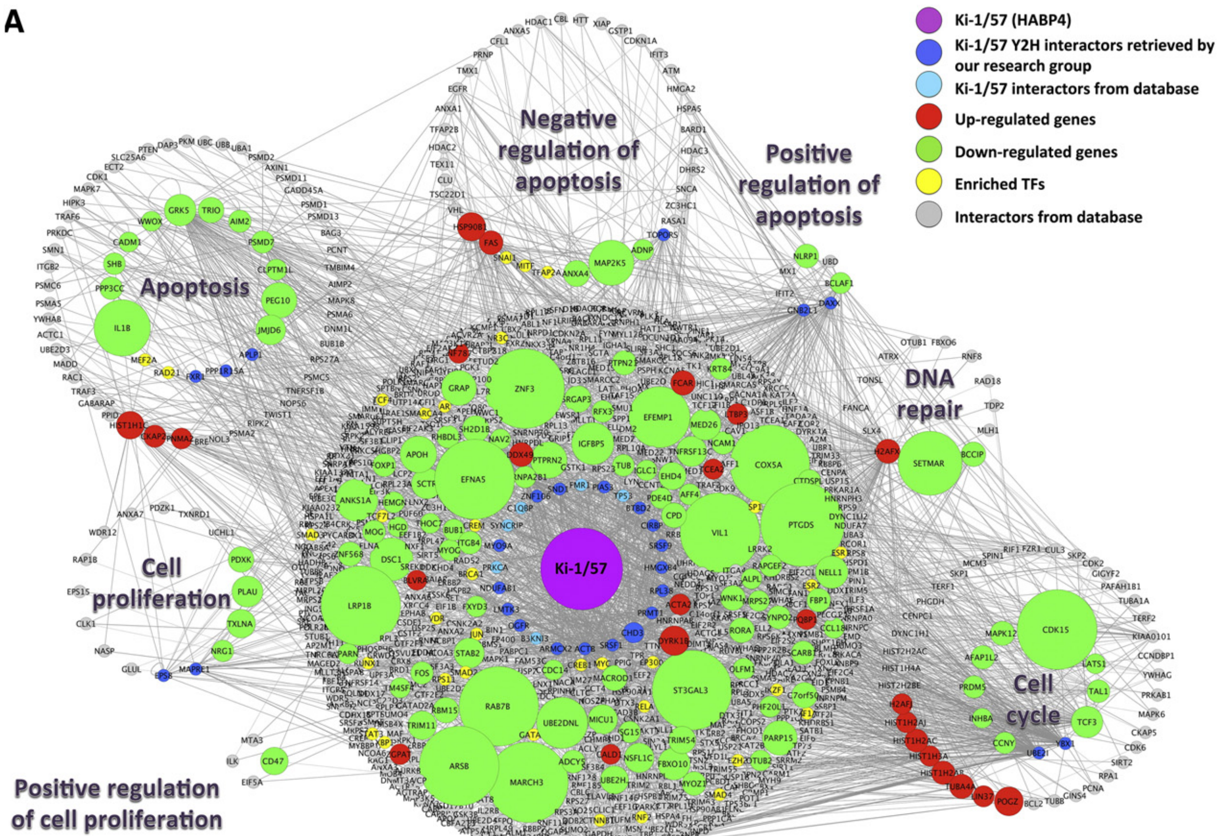
Fig. 2. Ki-1/57 and CGI-55 overexpression changes global mRNA profiles of HEK293T cells. (A) Heat maps show flag-CGI-55 and flag-Ki-1/57 transfected cells mRNA profile 48 h after transfection. Each line represents a gene in one of the analyzed replicates with fold change > 2 and $p < 0.05$. Both down-regulated (green) and up-regulated (red) RNAs were identified. (B) Common genes affected by Ki-1/57 and CGI-55. Gene names in green color represent down-regulated genes and, in red color, up-regulated genes. (C) Gene Ontology (GO) term analysis was performed to identify biological processes annotated among Ki-1/57 and CGI-55 regulated genes. The depicted genes were selected based on the following GO term annotation: apoptosis, cell cycle control and cell proliferation.

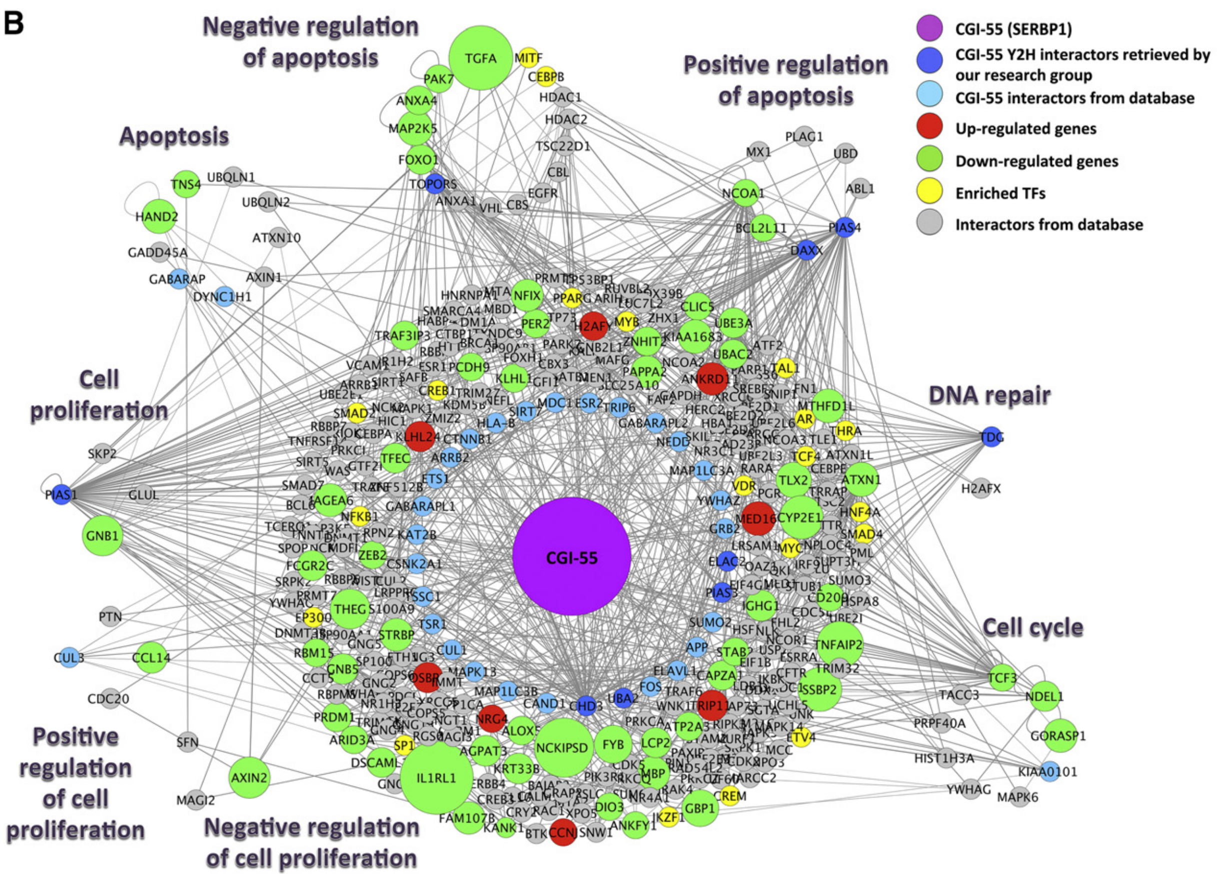
analysis of the sequence alignment shows that the CGI-55 protein presents three insertions rich in polar residues in HABP4 domain that differ from Ki-1/57 protein. Together, these findings can explain both the common origin and the functional differences between CGI-55 (SERBP1) and Ki-1/57 (HABP4) proteins, observed in gene expression analysis and in biological assays.

3.3. Functional network analyses

Biological process analyses were performed using Gene Ontology (GO) annotation to specify and identify pathways among target genes after Ki-1/57 and CGI-55 overexpression. The analyzed genes were involved in several biological processes (transcription regulation, RNA splicing, translation), but most evidently they were related to life/death cellular decision pathways such as apoptosis (9 genes for CGI-55 and 18 for Ki-1/57), cell proliferation (23 for CGI-55 and 24 for Ki-1/57) and cell cycle (7 for CGI-55 and 8 for Ki-1/57) (Fig. 2C). In order to find out functional interconnections between the microarray gene expression and the previously obtained protein–protein interaction data [4–12] we built up networks for Ki-1/57 and CGI-55. The top enriched GO biological processes among the yeast two-hybrid partners (dark blue), the up-regulated genes (red), the down-regulated genes (green) and the background intermediary proteins (gray) are detailed by clustering the proteins in the networks (Fig. 4A and B). In yellow, the enriched transcription factors described in different databases [31] related to the up and down-regulated genes identified by Ki-1/57 and CGI-55 microarrays (called here "enriched transcription factors", Fig. 4) are represented. The enriched transcription factors were classified according to Wingender et al. [40] and the frequency of each TF class was determined. We observed that the frequency of TF classes assigned to the genes targeted by Ki-1/57 and CGI-55 was significantly different from the frequency found in the human genome, but not from each other (Ki-1/57 vs. CGI-55) (Fig. S2). These TFs (yellow nodes in the networks) are particularly related to the apoptotic process (Fig. 4A and B). Furthermore, transcriptional factors were targeted by Ki-1/57 (e.g. BCLAF1, Fos, ADNP) and CGI-55 (e.g. PRDM1, TCF3, TBX1) overexpression on the observed global gene expression panel.

Fig. 3. Evolutionary analysis of CGI-55 and Ki-1/57 amino acid sequences. Top: the majority-rule consensus tree was obtained by Bayesian MCMC coalescent analysis of 26 HABP4 family protein sequences obtained from the UniProt database (http://www.uniprot.org/). Beside each node, the posterior probability values (PP) (expressed in percentage) calculated by using the best trees found by MrBayes are shown. Below: details of sequence alignments of the so-called HABP4 domain (hyaluronan binding protein 4).


A
Ki-1/57 (HABP4)
Ki-1/57 Y2H interactors retrieved by our research group
Ki-1/57 interactors from database
Up-regulated genes
Down-regulated genes
Enriched TFs
Interactors from database
Negative regulation of apoptosis
Positive regulation of apoptosis
Apoptosis
DNA repair
Cell proliferation
Cell cycle
Positive regulation of cell proliferation
Ki-1/57


B
CGI-55 (SERBP1)
CGI-55 Y2H interactors retrieved by our research group
CGI-55 interactors from database
Up-regulated genes
Down-regulated genes
Enriched TFs
Interactors from database
Negative regulation of apoptosis
Positive regulation of apoptosis
Apoptosis
Cell proliferation
DNA repair
Cell cycle
Positive regulation of cell proliferation
Negative regulation of cell proliferation
CGI-55

As observed in the networks, one of the most representative biological processes altered by Ki-1/57 and CGI-55 overexpression is cell proliferation. Representative modified genes by Ki-1/57 overexpression includes the repression of α-taxilin (TXLNA), a protein correlated with proliferative activity of hepatocellular carcinoma and the metastatic and invasive potential of renal cell carcinoma [41,42], as well as PLAU, PDXK, NRG1, also involved in promoting cell proliferation. CGI-55 overexpression decreased expression of proliferative genes as well (GNB1 and CCL14). Considering previous yeast two-hybrid assay results, in which we also found proteins related to proliferation control, like MAPRE1 for Ki-1/57 and CUL3 for CGI-55, these data collectively pinpoint that both Ki-1/57 and CGI-55 are involved directly or indirectly in repressive mechanisms of cellular proliferation control.

Ki-1/57 and CGI-55 overexpression also modified the expression of genes involved in apoptosis. Ki-1/57 overexpression, for example, repressed the expression of the apoptosis related genes GRK5, IL1B, PEG10 and PPP3CC (Fig. 4A). More specifically, Ki-1/57 expression leads to the repression of negative apoptosis regulators such as MAP2K5, ADNP, ANXA4, as well as the positive regulator BCLAF1. Moreover, Ki-1/57 expression increased the mRNA levels of HSP90B1, an endoplasmic reticulum chaperone that protects against ER stress-induced apoptosis [43]. These observations might suggest an involvement of Ki-1/57 in molecular decision mechanisms of death or protection against death during stress situations, such as ER stress, genotoxic or chemotherapeutic agents.

In the same way, CGI-55 overexpression also affected apoptosis related genes, such as HAND2, TNS4, specifically, the negative regulators MAP2K5, PAK7 and FOXO1, and the positive regulators NCOA1 and BCL2L11. These alterations of the expression of apoptosis related genes are in agreement with previous protein–protein interaction data, as both Ki-1/57 and CGI-55 interact with DAXX [6], a signaling protein that binds specifically to the Fas death domain [44] and TOPORS, a protein that mediates the p53 cellular response in DNA damage [45].

Ki-1/57 overexpression down-regulated Cyclin-dependent kinase 15 (CDK15), TCF3, MAPK12, INHBA, Cyclin-Y (CCNY), and up-regulated Tubulin alpha-4A (TUBA4A) and LIN37, which are proteins associated with cell cycle or cell division control [46–49]. CGI-55 overexpression also led to the down-regulation of cell cycle control genes, such as nuclear distribution protein NDEL1 and GORASP1 [50,51]. Protein–protein interaction data also support the involvement in cell division mechanisms, such as the case of UBE2I and YBX1 (Ki-1/57 interacting protein) and KIAA0101 (CGI-55 interacting protein), all of them involved in cell cycle control.

Interestingly, Ki-1/57 overexpression increased the expression of 50 genes, with 10 of them assigned as DNA histones. We speculate they could be involved in chromatin compaction during the gene repression process caused by Ki-1/57 overexpression. Besides, they may be associated to other biological processes affected by Ki-1/57, like cell cycle and apoptosis [52,53]. This could be the case of H2AX (H2AFX), which is responsible for recruiting multiple proteins to chromatin during DNA damage/repair response [54]. In addition, Ki-1/57 overexpression seems to affect histone function through down-regulating the histone-lysine N-methyltransferase (SETMAR), which is responsible for methylation of lysine residues in histone proteins [55].

All these observations together prompted us to validate specific targets enriched in relevant biological processes and further investigate whether the overexpression of Ki-1/57 and CGI-55 influences cell cycle, proliferation and apoptosis under stress conditions.

3.4. Validation of the microarray data

To further validate the microarray data findings, we analyzed by quantitative real-time PCR (qPCR) representative mRNAs involved in highly enriched biological processes targeted by Ki-1/57 and CGI-55 overexpression. For comparison, the average fold change in gene expression levels, determined by microarray and qPCR analysis, was log2 transformed and is shown in Fig. 5A and B. We were able to confirm genes involved in apoptosis (A), cell proliferation (P) and cell cycle (CC) targeted by Ki-1/57 and CGI-55 overexpression. For MAP2K5, a protein kinase that mediates a signal cascade involved in growth factor-stimulated cell proliferation, microarray data had shown a 5.4-fold and 3.13-fold reduction in expression, targeted by Ki-1/57 and CGI-55, respectively. qPCR results show 3.6-fold reduction for CGI-55. Comparable results were observed for other tested genes. Although there was a variation in the fold change values determined by these two methods, the expression trends were consistent.

Next, we further validated our microarray data by performing knockdown experiments of Ki-1/57 and CGI-55 (Fig. 5C and D). Optimization experiments have shown that a good down-regulation of Ki-1/57 expression occurred at all time points after transfection (Fig. 5C), whereas CGI-55 worked best only after 24 and 48 h, showing significant recovery after 72 h (Fig. 5D). Although we performed expression analysis at all time points after knockdown, most test points did not show significant differences. The most significant results were obtained for 5 genes after 96 h knockdown of Ki-1/57 (Fig. 5E) and after 48 h knockdown of CGI-55 (Fig. 5F). These results largely confirmed those of the overexpression, meaning that genes that were up or down-regulated upon overexpression, had an inverted characteristic upon knockdown. For example, CADM1 was repressed by overexpression of Ki-1/57 (Fig. 5A) and, upon knockdown (Fig. 5E, 96 h), it was found to be more expressed. On the other hand, the histone HIST1H2AB, for example, was up-regulated when Ki-1/57 was overexpressed (Fig. 5A), but was found to be repressed when Ki-1/57 was knocked down (Fig. 5E).

3.5. Cell proliferation, cell cycle and apoptosis analysis

To evaluate cell viability, MTS assays were performed with cells overexpressing flag-tagged Ki-1/57 or CGI-55. The results suggested that the overexpression of both proteins may lead to a significant reduction of cell growth either under normal or serum-deprived conditions (Fig. 6A, B). We also analyzed the effect of knockdown of Ki-1/57 and CGI-55 on HEK293T cell viability (Fig. 6C). No apparent differences were observed for Ki-1/57 (data not shown), but indeed, we found an increased viable cell number at 72 h of transfection with CGI-55 siRNA, under 10% serum condition (Fig. 6C). Although knock down experiments may not necessarily yield opposite results of overexpression assays, at least for CGI-55 we were able to observe such an effect (Fig. 6A, C).

The percentage of proliferating cells was also evaluated by measuring the incorporation of EdU in dividing cells. In agreement with cell viability experiments, the overexpression of Ki-1/57 and CGI-55 led to a reduced percentage of EdU incorporation. This result

Fig. 4. Interaction networks of Ki-1/57 and CGI-55 from previous yeast two-hybrid screening data and altered gene expression revealed by microarrays. The selected most relevant enriched GO biological processes among the yeast two-hybrid partners (dark blue), the up-regulated genes (red), the down-regulated genes (green), the enriched transcription factors (yellow) and the background intermediary proteins (gray) are depicted in the (A) Ki-1/57 and (B) CGI-55 networks by clustering the proteins involved in each of the biological processes with a circle layout. Clusters were assigned only to selected most relevant enriched biological processes containing at least one protein from the yeast two-hybrid screening or microarray data; proteins belonging to more than one biological process were assigned to clusters with the best enrichment p-values. More specific biological processes are shown only for proteins with more specific annotation in GO database. The histone HIST1H1C was assigned to "Apoptosis", and histones H2AFJ, HIST1H2AB, HIST1H2AC, HIST1H2AJ, HIST1H4A, HIST2H2AC and HIST2H2BE from the Ki-1/57 network were assigned to "Cell cycle", according to the Reactome database. The nodes sizes of up- and down-regulated proteins/mRNAs are depicted proportional to their fold change ($FC \geq 2.0$, $p \leq 0.05$). The protein-protein interaction networks were built using the IIS platform [30] and visualized using the Cytoscape software.

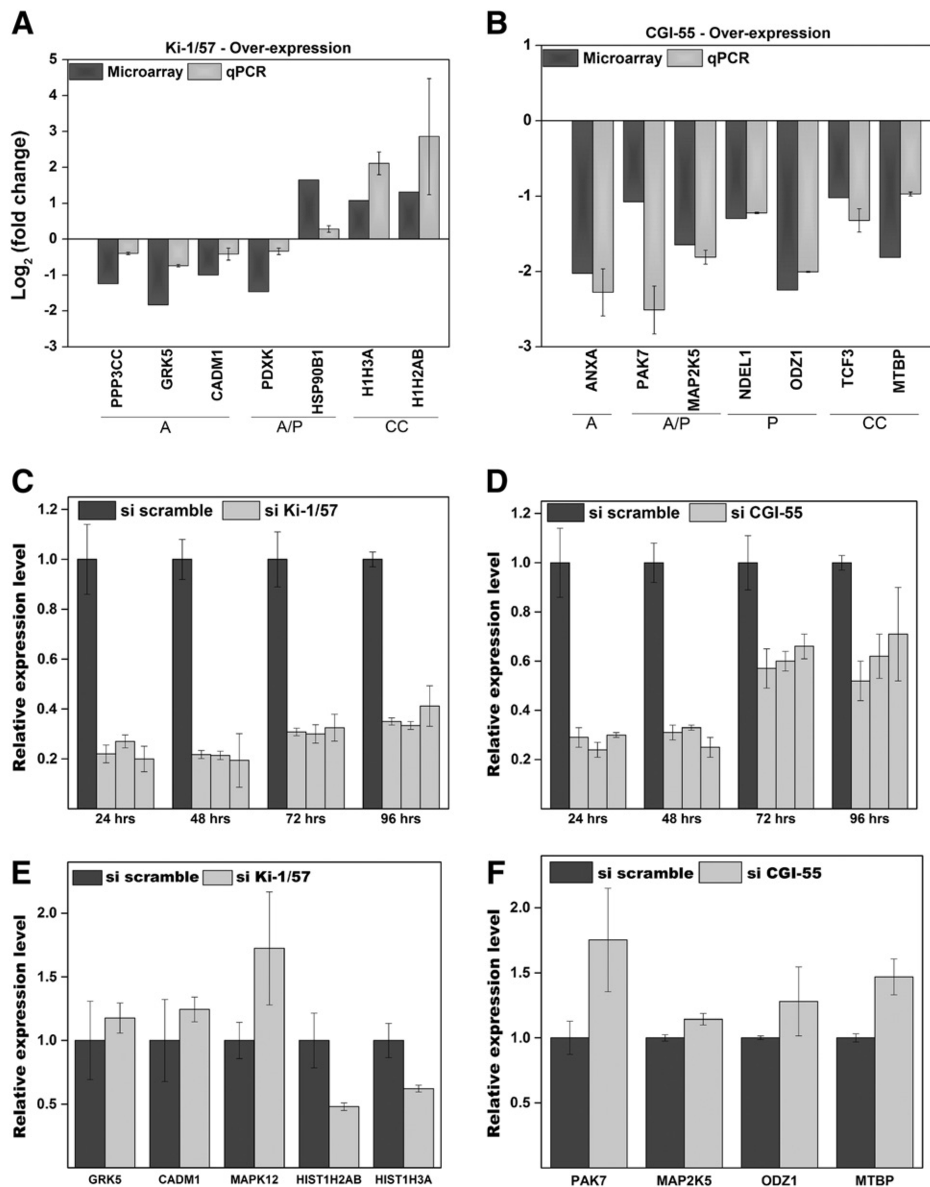


Fig. 5. Validation of microarray data by quantitative RT-PCR after Ki-1/57 and CGI-55 overexpression or siRNA knockdown. Fold change of mRNA of genes targeted by overexpression (48 h after transfection) of Ki-1/57 (A) and CGI-55 (B) in microarrays and confirmative quantitative RT-PCR experiments were Log_2 transformed and blotted together for comparison. The relative amount of each mRNA tested was normalized with β-actin. The specific primers are listed in Supplementary Table 1. Data in histograms are means (of triplicate experiments) ± SD. A stands for genes involved in apoptosis, P for cell proliferation and CC for cell cycle. (C, D) HEK293T cells were seeded in 6-well plates and transfected 24 h later with siRNA against Ki-1/57 (C) and CGI-55 (D). A scrambled siRNA was used as negative control upon the same conditions. At indicated time points (24, 48, 72 and 96 h after transfection), cells were collected and resuspended in Trizol® for RNA extraction. The expression levels of Ki-1/57 and CGI-55 genes were determined by quantitative RT-PCR experiments. (E, F) Genes found to have different expression levels in microarray experiments after Ki-1/57 and CGI-55 overexpression were analyzed in knockdown experiments (E) Ki-1/57 knockdown reverses the effects observed upon overexpression. Genes down-regulated by Ki-1/57 overexpression were up-regulated upon knockdown, 96 h after transfection with siRNA. Histones (HIST1H2AB and HIST1H3A), that were up-regulated after overexpression were down-regulated upon Ki-1/57 knockdown. (F) Genes found to be down-regulated by CGI-55 overexpression were up-regulated 48 h after siRNA CGI-55 transfection. The relative amount of each mRNA tested was normalized by β-actin. Data in histograms are means of triplicate experiments ± SD.

was most evident under serum starvation conditions, possibly due to the lack of interfering mitogenic factors present in the serum (Fig. 6D).

We further determined the effects of Ki-1/57 and CGI-55 on cell cycle progression (Fig. 6E). Under normal growth conditions (10% serum), no significant differences were observed. However, under serum starvation, a G1 phase arrest was observed in cells overexpressing either Ki-1/57 or CGI-55.

Given that cell proliferation, cell cycle progression and apoptosis are tightly related processes and that both Ki-1/57 and CGI-55 affected the expression of apoptosis related genes, we then also examined the apoptosis response of Ki-1/57 and CGI-55 overexpressing cells to stress. Under thapsigargin (an ER-stress inducer) treatment, Ki-1/57 overexpressing cells exhibited diminished apoptosis when compared to pcDNA-transfected control cells (Fig. 7A). CGI-55 overexpressing cells had an increased apoptosis rate, however no significant differences were observed under the tested stress conditions (Fig. 7B).

4. Discussion

By using microarray global gene expression analysis, we identified approximately 400 and 200 genes affected by Ki-1/57 and CGI-55 overexpression, respectively. These data along with protein–protein interaction data previously obtained in our group were used to build up enriched functional networks considering relevant top enriched GO biological processes regulated by Ki-1/57 and CGI-55. Many of the target

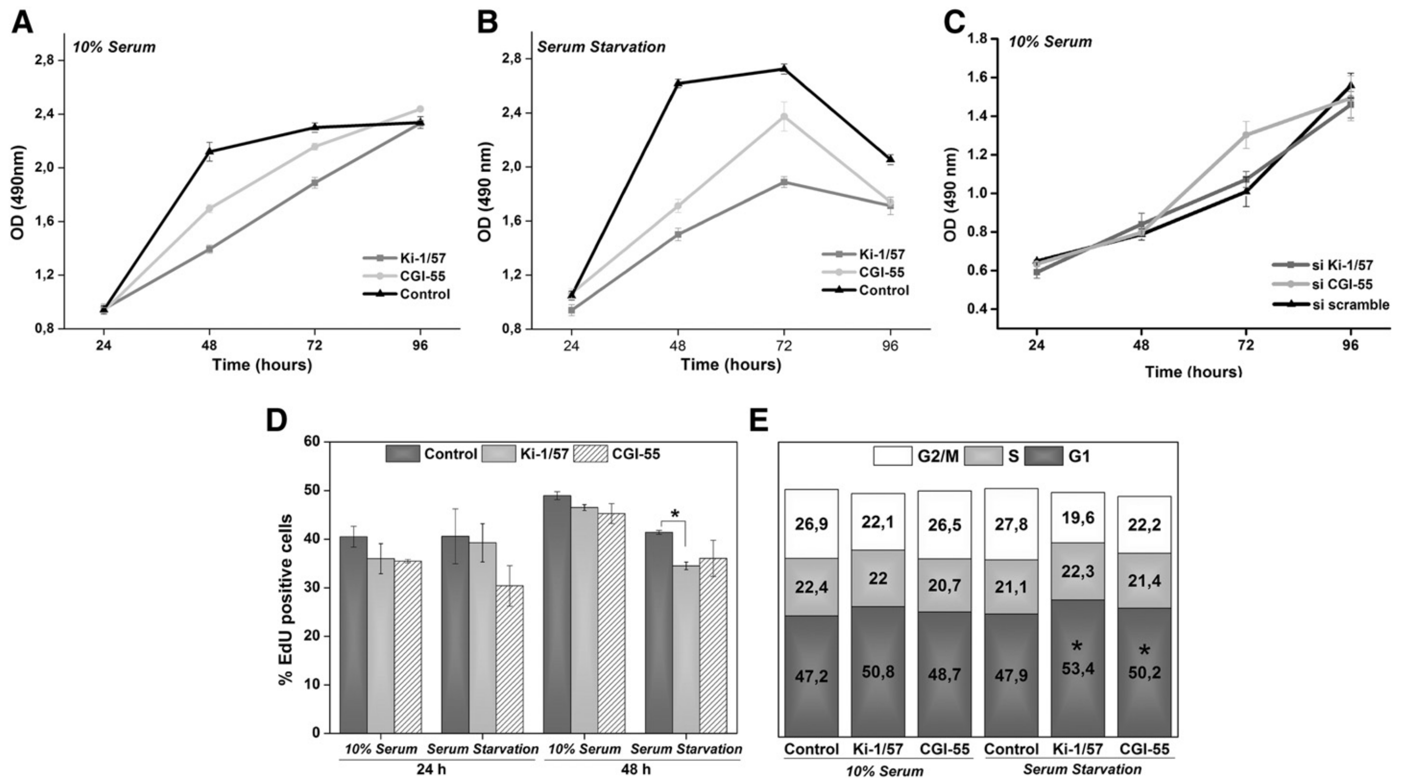


Fig. 6. Analysis of cell viability, proliferation and cell cycle. (A-C) MTS assay was performed to determine the effect of Ki-1/57 and CGI-55 overexpression or knock down on cellular viability. (A, B) HEK293T were seeded in 96-well plates and transfected 48 h later with pcDNA6-flag-Ki-1/57, pcDNA6-flag-CGI-55 or empty pcDNA6 vector. After 16 h the medium was substituted with medium containing 10% (10% Serum, A) or 1% fetal bovine serum (Serum Starvation, B). At indicated time points (24, 48, 72 and 96 h after changing media), MTS assay was performed and results are represented as mean OD (±SD) of 4 repeats. (C) MTS assay of CGI-55 and Ki-1/57 knockdown cells. Cells were seeded in 96-well plates, transfected 24 h later with, siRNA for CGI-55, Ki-1/57 or the control scrambled siRNA. After 16 h the medium was substituted with medium containing 10% fetal bovine serum. At indicated time points (24, 48, 72 and 96 h after changing media), MTS assay was performed and results are represented as mean OD 490 nm (±SD) of 3 repeats. (D) EdU incorporation assay. HEK293T were seeded in 6-well plates and processed as described above. The percentage of EdU-positive cells at 24 or 48 h after transfection was obtained for two conditions, 10% or 1% serum (Serum Starvation). More than 400 cells were counted for each condition. Each bar represents mean ± SD of 2 independent experiments. *p < 0.01 (E) DNA flow cytometry analysis of cell cycle profiles in HEK293 pcDNA6 (control), flag-Ki-1/57 or flag-CGI-55 transfected cells. Cells were seeded in 6-well plates in triplicate, transfected and analyzed 48 h later. After transfection, medium was changed 24 h before analysis for medium containing 10% FBS or medium with no serum (Serum Starvation). Data are presented as percentage of cells distributed in G1, S and G2/M cell cycle phase (mean). *p < 0.05 vs. pcDNA6 transfected cells.

genes were involved in regulation of transcription or other aspects of mRNA metabolism, a result consistent with the previously obtained protein–protein interaction data (Fig. S1) [4–12]. Although the exact mechanisms could not be pinpointed here, it is worth noting that most of the genes targeted by Ki-1/57 and CGI-55 overexpression were down-regulated (~90%), indicating that these proteins could act predominantly as repressors of gene expression.

Of particular functional interest, a representative biological process affected by Ki-1/57 and CGI-55 overexpression was involved in life/death decisions. Genes involved in apoptosis, proliferation and cell cycle control showed alteration in their expression levels in both microarray data and qPCR analysis. Expression of mRNA encoding GRK5, a kinase that interacts and phosphorylates p53 during the regulation of apoptosis and G2/M-phase transition [56,57] was

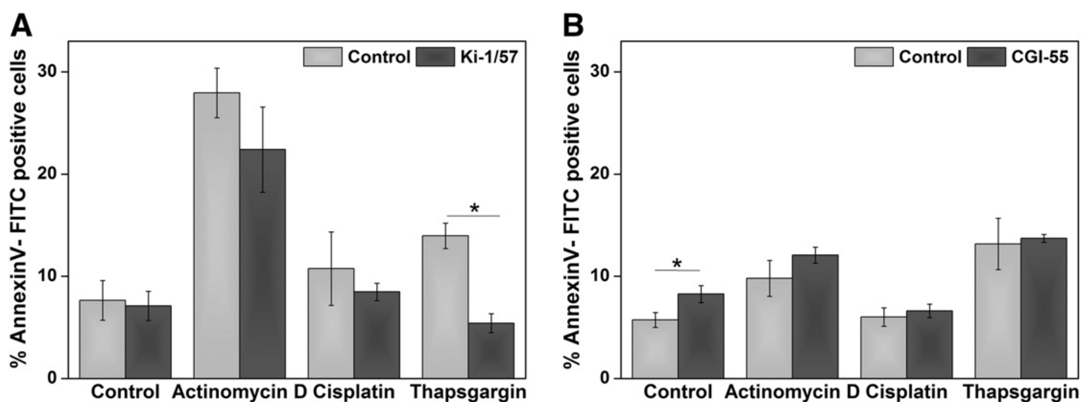


Fig. 7. Apoptosis analysis of cells overexpressing Ki-1/57 and CGI-55 under stress conditions. HEK293T cells were seeded in 6-well plates, transfected 48 h later with pcDNA6-flag-Ki-1/57 (A) and CGI-55 (B) and compared with control (pcDNA6). 8 h after the transfection cells were treated with 3 μg/mL Actinomycin D, 30 μM Cisplatin or 10 μM thapsigargin for 16 h, completing 24 h after transfection. Cell apoptosis was analyzed with annexin V-FITC and PI staining by flow cytometry. Experiments were performed in triplicate and repeated two times; each bar represents mean ±. S.D. *p < 0.01 (A) and *p < 0.05 (B) vs. pcDNA transfected cells.

down-regulated by Ki-1/57. The mRNA encoding HSP90B1 (GRP94), a calcium binding protein that inhibits apoptotic signals by maintaining calcium homeostasis in the ER [58] showed increased expression. The expression of TCF3, an important transcription factor that regulates cell proliferation and cell cycle regulatory genes [46], was a target for CGI-55 overexpression. MTBP, a protein that interacts with MDM2 (a negative regulator of p53) and has a crucial role in mitotic progression and proliferation in a p53-independent manner [59–61], is also affected by CGI-55.

Considering these information, we sought to further confirm whether Ki-1/57 and CGI-55 overexpression would affect cell life/death decisions. We observed reduced proliferation for both proteins in MTS and EdU incorporation experiments, and in both cases, the reduction was intensified in cells maintained with 1% fetal bovine serum. The reduced proliferation could be due to the arrest of cell cycle progression, since under serum starvation, Ki-1/57 and CGI-55 overexpression induced G1 cell cycle arrest. On the other hand, the overexpression of Ki-1/57 did not yield any alterations in the number of apoptotic cells. CGI-55 overexpression, in contrast, increased apoptosis under normal conditions. Despite the sequence similarity between these proteins, it seems they have acquired distinct functional properties during evolution. This seems to be confirmed by these proteins differences in the protein–protein interaction profile, the apoptosis data set and their distinct sets of target genes. While the apoptosis genes down-regulated by CGI-55 were mostly negative apoptotic regulators, Ki-1/57 targeted positive and negative regulators, increasing or reducing their expression. This is in agreement with the finding that CGI-55 overexpression promotes a higher apoptosis rate because it causes a repression of negative regulators of apoptosis. In the case of Ki-1/57 overexpression, an inhibitory effect on apoptosis could only be seen after thapsigargin treatment, indicating that positive and negative influences on apoptosis may be in balance under absence of thapsigargin.

Ki-1/57 and CGI-55 have cytoplasmic and nuclear localizations, where they can be frequently observed in dot-like structures or nuclear bodies such as Cajal bodies, nuclear speckles and PML-nuclear bodies (PML-NBs) [4,7,8,10,11,26,62]. These granules are nuclear substructures that respond to basic physiological processes as well as various forms of stress. PML-NBs for instance localize at the proximity of active transcription sites directly regulating the function of several transcription factors and responding to viral attack and genome instability, by abducting, modifying or degrading different protein partners [63,64]. Considering the localization in such granules and the panel of genes involved in apoptosis that were up or down-regulated by Ki-1/57 and CGI-55 overexpression, we then speculated that these two proteins would play some role in protecting cells against stress conditions. The overexpression of Ki-1/57 seemed to reduce the amount of apoptotic cells during treatment with different stress reagents, most significantly thapsigargin. The same was not observed in cells overexpressing CGI-55, which did not lead to an increase of the cells sensibility to the stressing agents.

We would like to point out that post-translational modifications are an additional important factor that adds further regulatory complexity to apoptosis signaling. For example in rat ovarian granulosa cells, CGI-55 and PGRMC1 form a receptor complex required for the anti-apoptotic effects of progesterone and the response of PGRMC1 to progesterone functions depends on PGRMC1 sumoylation status [65]. Moreover, small ubiquitin-like modifier (SUMO) can post-translationally modify PML and other PML-NB components. PML interacts with itself and other proteins through sumoylation and SUMO-interacting motif SIMs [63] and sumoylation of PML is required for the recruitment of components of PML-NBs [66]. CGI-55 interacts with the SUMO activating enzyme subunit 2, UBA2, and with the SUMO E3 ligases PIAS1 and −3 [11]. Moreover, a systematic study on SUMO modifications in response to heat shock, identified CGI-55 as a SUMO substrate [67]. In addition, CGI-55 and Ki-1/57 possess different predicted sumoylation sites (data not shown). Therefore, the association of Ki-1/57 and CGI-55 to PML-NBs would involve their SUMO modification and might influence the participation of these two proteins in apoptosis response, but likely in a differential manner.

The influence of Ki-1/57 overexpression on histones should also be pointed out. Whereas histone overregulation might in part explain the overall repressive effect of Ki-1/57 overexpression, some gene loci are found to be preferentially associated with PML-NBs, as histone gene clusters [68,69]. Moreover, not only transcription factors are found in PML-NBs, but also histone-modifying enzymes and other chromatin regulators, suggesting a role for PML-NBs in chromatin regulation [64]. Therefore, we can speculate that the up-regulation of histone genes by Ki-1/57 overexpression might be via PML-NBs. In this respect, DAXX, a Ki-1/57 and CGI-55 interacting partner and component of PML-NB, may regulate chromatin dynamics by binding to histone deacetylases and chromatin remodeling proteins [70]. Moreover, Ki-1/57 overexpression down-regulated the histone-lysine N-methyltransferase (SETMAR), responsible for methylation of lysine residues in histones, a pivotal process for heterochromatin formation [55]. It is important to highlight that some of the histones triggered by Ki-1/57 overexpression are related to stress response, such as H2AX, which is responsible for recruiting multiple proteins to chromatin during DNA damage/repair response [54]. This reinforces the possible role of Ki-1/57 in regulating gene expression in response to stress.

The enriched transcription factors from the functional networks of targeted genes by Ki-1/57 and CGI-55 overexpression were classified and the frequency of each TF class determined. We observed that the frequency of TF classes of the genes targeted by Ki-1/57 and CGI-55 was significantly different from the frequency found in the human genome, but not from each other (Ki-1/57 vs. CGI-55) (Fig. S2). This might implicate in Ki-1/57 and CGI-55 roles as corepressors of genes regulated by specific classes of TFs. It is important to note the increased frequency of the immunoglobulin fold class, which includes the p53 family TF, a well-established interacting partner of Ki-1/57 [10]. Together with the amino acid sequence similarity, the expression regulation of common genes identified by the microarray study and our previous yeast two-hybrid protein-protein interaction data, the TF classes analysis also strengthens the hypothesis of paralogy between these proteins [7].

Ki-1/57 and CGI-55 are RBPs that under stress conditions localize on SGs and PBs [25–27] and almost 90% of genes regulated by them were down-regulated, demonstrating a repressor activity. Many of the genes targeted by Ki-1/57 and CGI-55 are associated with the regulation of transcription and mRNA metabolism, and with cellular fate pathways, as apoptosis, cell cycle and proliferation. We speculate that these two proteins could act directly or indirectly via global repression of genes involved in response to stress conditions, possibly involving cytoplasmic and/or nuclear bodies, and may act on several different levels of gene expression regulation: from transcriptional level, through transcriptional factors or chromatin remodeling, via mRNA stability and splicing to translation initiation.

The high structural flexibility of intrinsically disordered proteins allows the binding to multiple partners and access of post-translational modifications at several sites. Post-translation modification of RNA-binding proteins is an energetically economical way to adapt to stress conditions, without new protein synthesis, and allows rapid and reversible protein regulation. Ki-1/57 and CGI-55 are both methylated, phosphorylated and we speculate that they are also sumoylated. This could determine their cellular localization and mode of action in response to different stress conditions.

5. Conclusions

Ki-1/57 and CGI-55 are paralogous regulatory proteins and have been functionally implicated in the regulation of gene expression on both the transcriptional and mRNA metabolism levels. The preliminary characterization of their protein interaction profiles, their complex subcellular localization and post-transcriptional modifications together with the finding of altered expression of these proteins in diverse cancer

cells had suggested that they may be involved in the process of tumorigenesis. Furthermore, the gene for Ki-1/57 is found in a region of a chromosome which contains only four genes and represents a haplotype for familiar colon cancer. Here, we characterized the alterations of the global transcriptome profile after Ki-1/57 or CGI-55 ectopic overexpression in HEK293 cells by DNA microchip technology and identified 363 or 190 down-regulated and 50 or 27 up-regulated genes for Ki-1/57 and CGI-55, respectively, of which 20 were shared between both proteins. This latter finding together with an evolutionary analysis, strongly suggests that both proteins are paralogs, which originated through an event of a whole genome duplication in the baseline of chordate evolution. Furthermore, the great majority of the genes with altered expression are associated to proliferation, apoptosis and cell cycle control processes. In further experiments, we could observe that overexpression of Ki-1/57 and CGI-55 results indeed in reduced cell proliferation, mainly due to a G1 phase arrest. In the case of Ki-1/57 overexpression, we found protection from apoptosis after treatment with the ER-stress inducer thapsigargin. Our transcriptome data together with a correlation of the published protein interactome data suggest that Ki-1/57 and CGI-55 have many overlapping functions and furthermore that they act mainly as repressors that affect the expression of genes involved in proliferation and cell cycle as well as apoptosis regulation. Taken together, our data provide important new insights that may help to explain these protein putative involvements in tumorigenic events.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2014.08.016.

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