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# The effects of telomere shortening on cancer cells: A network model of proteomic and microRNA analysis

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ABSTRACT

Previously, we have shown that shortening of telomeres by telomerase inhibition sensitized cancer cells to cisplatinum, slowed their migration, increased DNA damage and impaired DNA repair. The mechanism behind these effects is not fully characterized. Its clarification could facilitate novel therapeutics development and may obviate the time consuming process of telomere shortening achieved by telomerase inhibition. Here we aimed to decipher the microRNA and proteomic profiling of cancer cells with shortened telomeres and identify the key mediators in telomere shortening-induced damage to those cells.

Of 870 identified proteins, 98 were differentially expressed in shortened-telomere cells. 47 microRNAs were differentially expressed in these cells; some are implicated in growth arrest or act as oncogene repressors. The obtained data was used for a network construction, which provided us with nodal candidates that may mediate the shortened-telomere dependent features. These proteins' expression was experimentally validated, supporting their potential central role in this system.



## 1. Introduction

Telomeres, located at both ends of chromosomes, are composed of tandem TTAGGG repeats. Together with their shelterin protein complex they provide stability to the whole genome by preventing chromosome ends from being recognized as double strand breaks [1]. Telomeres incrementally erode in most somatic cells upon each round of DNA replication until they reach critical short length which eventually initiates a cessation of cell growth termed cellular replicative senescence [2].

Telomere shortening triggers genetic changes eventually leading to cell senescence. In addition to changes in the expression of genes located close to telomeres, termed Telomere Position Effect (TPE) [3], telomere shortening may affect additional genes, located elsewhere in the genome.

The molecular events associated with telomere shortening are not fully clarified yet. However, many experimental data derived from both lower eukaryotes and mammalian cells suggest several mechanisms in this regard. Telomere shortening can induce DNA damage response that eventually leads to cell cycle arrest [4,5]. In addition, the heterochromatin near telomeres may change, altering the regulation of genes and causing senescence [6]. It is of note, however, that the effect of telomere shortening on global expression has not been studied extensively in cancer cells.

Telomere shortening can be prevented by the action of the enzyme telomerase, a unique reverse transcriptase that synthesizes TTAGGG repeats at telomeric ends. Telomerase is not expressed in most somatic cells but retains activity in proliferative stem cells and in male germ line cells. However, in >90% of malignancies telomerase is highly active and plays a crucial role in malignant transformation and perpetuation of the malignant clone [7]. Its uniqueness and essentiality to cancer cells define the telomere–telomerase system as an attractive anticancer target [8].

Numerous studies have shown that telomerase inhibition causes apoptosis of cancer cells and shrinkage of tumors and enhances the sensitivity of tumor cells to antineoplastic treatments [9–12]. Although telomerase inhibition *per se* may exert antitumor effects by targeting the enzyme's "extracurricular" functions, most of the data, including ours, point to telomere shortening as the crucial end point which mediates the damage to cancer cells [9]. Previously we demonstrated that the extent of damage to cancer cells is related to the degree of telomere shortening. Continuous shortening of telomeres resulted in death of cancer cells *in vitro* and

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growth inhibition of tumors *in vivo*. Telomere shortening also differentially sensitized cancer cells to cisplatin, and not doxorubicin or vincristine [9]. In addition, cells with shortened telomeres migrate slower comparing to their intact original counterparts. These cells possess a higher DNA damage index and fail to repair DNA damage imposed by cisplatinum [9]. Although proven to be fruitful, the rationale behind targeting telomerase as an anticancer strategy still bears some disadvantages: Telomere shortening is achieved mainly by inhibiting telomerase activity [13]. This is a time consuming process, which is an obvious disadvantage in clinical settings. In addition, it may harm other telomerase expressing cells (e. g. stem cells) during a critical window of therapeutic treatment. Finally, cells with inhibited telomerase may overcome the inhibition by elongating telomeres by ALT (alternative lengthening of telomerase) mechanisms [14]. Clearly, a "biological short-cut" to telomere shortening would be advantageous.

Relatively little is known about the molecular mechanisms mediating the cancer cell damage induced by telomere shortening. Characterization of these pathways may be beneficial in developing a targeted therapy aimed at directly producing a "shortened telomere phenotype" in cancer cells bypassing telomerase inhibition. Therefore we set to identify the most important "key players" which mediate the phenotypic changes of cancer cells with shortened telomeres.

In order to better understand the molecular changes contributing to the shortened telomere phenotype, we employ a systematic approach, comparing the expression of miRNAs and proteins in cancer cells with intact or with shortened telomeres. The identification of proteins that may be central in mediating the effects of telomere shortening may allow targeting them in order to achieve directly the desired cellular phenotype.

In order to prioritize the biologically relevant connections from the large experimental data, a bioinformatic approach was utilized. This network approach also enables the identification of new proteins/miRNAs that may be relevant but did not show up in our results. We considered several candidate network models, each reflecting a different mode of telomere length control: telomere/telomerase machinery, apoptosis, DNA repair and cell migration. The most plausible option was chosen by measuring the correspondence of the inferred network with cisplatinum sensitivity, a unique characteristic of the cells with shortened telomeres. Subsequently, we experimentally validated the network's results for several candidate proteins by measuring the dynamics in their expression following telomere shortening. Indeed, the proteins that appeared to be more central in the network exhibited a higher degree of change in cells with shortened telomeres compared to intact cells.

## 2. Materials and methods

### 2.1. Experimental system

SK-N-MC (neuro-epithelial neuroblastoma/Ewing sarcoma) cells were exposed twice a week to telomerase inhibitor, GRN163 (5 μM) for about 20 months (kindly donated by Dr. S. Gryaznov, Geron Corp. Menlo Park, CA, USA). The telomerase inhibitor was then withdrawn from the growth media and the cells were further grown for 3 additional population doublings to allow for elongation of the shortest telomeres and complete reconstitution of telomerase activity. The control intact cells were maintained in the culture medium without the inhibitor. Telomere length was determined until shortening of more than 50% of the original length. Another set of control cells were created by withdrawing the inhibitor of telomerase from the growth medium until the telomeres reached their original size.

### 2.2. Telomere length determination

TRF lengths were measured by a non-radioactive Southern blot technique. DNA samples were extracted from the cells by the Genomic DNA purification kit (Gentra, Minneapolis, MN, USA) according to the manufacturer's instructions, digested for 16 h by *Hinf*III and *Rsa*I and separated on 0.8% agarose gels. After transfer to positively charged nylon membranes, samples were hybridized with a digoxigenin-labeled probe $(TTAGGG)_4$ (Roche Applied Science, Mannheim, Germany) for 16–18 h. The membranes were then exposed to chemiluminescence-sensitive films and the average TRF lengths were calculated by the Versa Doc Imaging System, using Quantity One software (Bio-Rad Laboratories).

### 2.3. miRNA profiling

#### 2.3.1. RNA isolation

RNA was isolated by using the EZ-RNA II commercial kit (Biological Industries, Beit Haemek, Israel) according to the manufacturer's instructions provided. Cells subjected to miRNA profiling were intact wild type cells, cells with shortened telomeres and cells that their telomeres were firstly shortened and then returned to their original size after the withdrawal of the telomerase inhibitor from their growth media.

#### 2.3.2. miRNA microarray

Custom miRNA microarrays have been described previously [21]. Briefly, ~900 DNA oligonucleotide probes representing miRNAs were spotted in triplicates on coated microarray slides (Nexterion® Slide E. Schott, Mainz, Germany). Each RNA sample was labeled by ligation of an RNA-linker, p-rCru-Cy/Dye (Dharmacon Lafayette, CO: Cy3 or Cy5) to the 3′ end and was incubated with the labeled RNA for 12–16 h at 42 °C and then washed twice. Arrays were scanned at a resolution of 10 μm and images were analyzed using SpotReader software (Niles Scientific Portola Valley, CA). Controls included: (i) synthetic small RNAs spiked into each RNA sample before labeling to verify labeling efficiency and (ii) probes for abundant small RNAs to validate RNA quality. Microarray spots were combined and signals normalized as described previously [15].

#### 2.3.3. Data analysis

The data included two samples: SK-N-MC cells with intact telomeres and SK-N-MC cells with shortened telomeres. A total of 170 miRNA probes had a signal that passed the minimal threshold of 300 in at least one of the samples. For each of those miRNA molecules the signal fold change was calculated. The chosen cutoff was of at least two fold.

### 2.4. Proteomic analyses

Cells were grown in the presence of SILAC-containing medium according to the protocol described by Mann and Ong [16]. Basically, the control intact cells were grown in the presence of isotope labeled L-arginine and L-lysine amino acids and the cells with shortened telomeres were maintained in a standard RPMI medium, containing "light" arginine and lysine. Both cell cultures were grown for 5 population doublings and lysed and proteins were extracted and separated on a 10% polyacrylamide gel. The mixed protein lane was divided into 10 pieces and subjected to quantitative mass spectrometry as described in [17]. This analysis was performed in the Smoler Proteomics Center at the Technion Haifa, Israel. Altogether we found 17 over-expressed and 81 under-expressed proteins. The chosen cutoff was again two fold, after correcting the data to a 1:1 ratio.

### 2.5. Significance of overlaps

We measure the significance of an overlap between two sets using a standard hypergeometric score:

$$\sum_{k=|S\cap A|}^{\min\{|S|,|A|\}} \frac{\binom{|S|}{k}\binom{N-|S|}{|S\cap A|-k}}{\binom{N}{|S\cap A|}}.$$

S and A are the compared sets (*e.g.*, S is the set of proteins that are miRNA targets and A is the set of proteins associated with doxorubicin sensitivity). The background N is the overall number of protein coding genes in the human genome [N ~ 21,700; [18]].

### 2.6. Network construction

Network analysis was conducted using the ANAT software for inference of functional protein networks [19]. The inference in ANAT is based on a database of human protein–protein interactions (~40 K interactions connecting ~10 K proteins obtained from the Human Protein Reference Database [HPRD]; [20]) and from large-scale screens including yeast two hybrid [21,22] and mass-spec [23] analysis. The interactions in the database are assigned with a confidence value based on the amount of supporting experimental evidence. We added a collection of miRNA–target interactions to the protein–protein interactions in ANAT's database. These interactions were obtained from the MAMI database (http://mami.med.harvard.edu/) using the medium–high level of confidence (sensitivity: 0.68; specificity: 0.5). We assigned the miRNA–target interactions with a confidence value of 0.5 (equal to their estimated specificity).

We used the anchored-network algorithm in ANAT for inferring high confidence networks that connect the experimentally determined proteins and miRNAs. The networks inferred by this algorithm are built around a specific set of nodes termed "anchor set". For each input node (in this case the experimentally determined proteins) the resulting network includes at least one path to at least one member of the anchor set [18,24].

The different anchor sets that we analyzed represent different mechanisms of action through which the inferred proteins and miRNAs are affected by changes in telomere length. We considered four groups of proteins: (i) telomere-binding proteins and the telomerase machinery, (ii) DNA repair, (iii) apoptosis and (iv) cell migration. The lists were assembled manually based on available literature data [25–28] (Supplemented Table S1). We constructed a network around each possible combination ($2^4 - 1 = 15$ altogether) of groups. In each run the anchor set given as input to ANAT consisted of the union of the members in the respective sets plus all the experimentally identified miRNAs.

In addition to the input nodes (*i.e.*, the anchor set and the experimentally determined proteins) the inferred networks include other proteins that act as mediators. For each of these added nodes we computed a centrality score, defined as the number of experimentally determined proteins whose paths to the anchor visit this node (see Table S3).

We evaluated the plausibility of the networks by measuring the overlap between these "added" proteins and proteins that are associated with the sensitivity to cisplatinum (Supplemental Table S2A). To derive the list of proteins associated with the sensitivity to cisplatinum we manually combined several published data sources [29–31]. We added to the list genes whose yeast ortholog was shown to confer resistance or sensitivity to cisplatinum upon knockout [32].

All information regarding protein functions is derived from the website http://www.genecards.org [63].

### 2.7. Validation of predicted changes in protein expression

Protein expression was measured in cells with shortened telomeres and in the parental control cells by Western blotting. The expression of

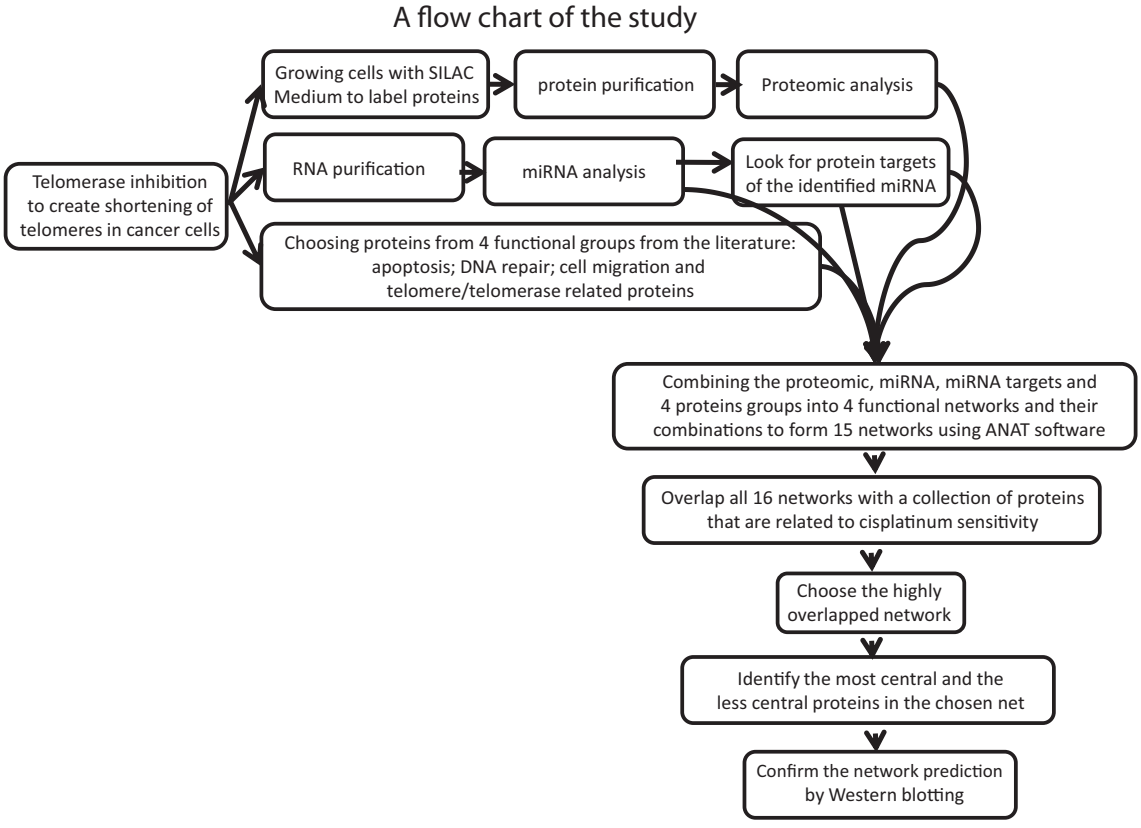


**Fig. 1.** A flow chart of the study (see text for details).

**Table 1**
miRs that were differentially expressed in the cells with shortened telomeres.

| | WT/shortened telomere signal |
|---|---|
| *A. Highly expressed miRs in the shortened telomere cells* | |
| hsa-miR-199a-5p | 0.0077 |
| hsa-miR-22 | 0.010 |
| hsa-miR-130a | 0.011 |
| hsa-miR-221 | 0.0209 |
| hsa-miR-10b | 0.028 |
| hsa-miR-129-3p | 0.0417 |
| hsa-let-7b | 0.0427 |
| hsa-miR-199a-3p | 0.0684 |
| hsa-miR-455-3p | 0.0689 |
| hsa-miR-181b | 0.0709 |
| hsa-miR-138 | 0.0793 |
| hsa-miR-193a-3p | 0.0848 |
| hsa-miR-361-5p | 0.104 |
| hsa-miR-148a | 0.1156 |
| hsa-miR-199b-5p | 0.1224 |
| hsa-miR-181a | 0.1362 |
| MID-00575 | 0.1368 |
| hsa-miR-505 | 0.137 |
| hsa-miR-663 | 0.144 |
| hsa-let-7d | 0.1491 |
| hsa-let-7c | 0.1509 |
| hsa-let-7a | 0.152 |
| hsa-miR-143 | 0.157 |
| hsa-miR-128 | 0.1571 |
| hsa-miR-27a | 0.1661 |
| hsa-miR-23a | 0.1718 |
| hsa-miR-421 | 0.1842 |
| MID-00135 | 0.1897 |
| hsa-miR-26a | 0.2069 |
| *B. Highly expressed miRs in the WT cells with intact telomeres* | |
| hsa-miR-451 | 217.0668 |
| hsa-miR-126 | 86.4861 |
| hsa-miR-886-5p | 76.2617 |
| hsa-miR-486-5p | 47.8322 |
| hsa-miR-223 | 46.4312 |
| hsa-miR-142-5p | 44.3716 |
| hsa-miR-886-3p | 24.5214 |
| hsa-miR-574-3p | 20.7693 |
| hsa-miR-21 | 11.5308 |
| hsa-miR-486-3p | 10.4617 |
| hsa-miR-146b-5p | 9.3262 |
| hsa-miR-18a* | 8.9537 |
| hsa-miR-339-5p | 7.0669 |
| hsa-miR-19a | 7.0316 |
| hsa-miR-17* | 5.9754 |
| hsa-miR-22 | 5.8548 |
| hsa-miR-550* | 4.418 |
| hsa-miR-130b | 4.364 |
| hsa-miR-125a-5p | 3.8808 |
| hsa-miR-574-5p | 3.6005 |
| hsa-miR-140-3p | 3.5167 |
| MID-00536 | 3.2574 |
| hsa-miR-483-5p | 3.0799 |
| hsa-miR-192 | 3.0604 |
| hsa-miR-92a | 3.054 |
| hsa-miR-185 | 2.9958 |
| hsa-miR-801 | 2.981 |
| MID-00405 | 2.8886 |

each protein was normalized to the expression of a house keeping gene. Protein concentration was determined by the Bradford assay (Bio-Rad Laboratories). Identical protein amounts were subjected to PAGE. The expression of all proteins was detected by the following antibodies (dilution of each antibody is denoted in parenthesis): anti-RPS6KA1 (1:500), anti-JNK1 (1:500), and anti-BCLXL (1:500) were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Anti-phosphorylated and total PRKDC (1:1000, 1:3000 respectively) and anti-phosphorylated and total Ku70 (1:1000, 1:3000 respectively) were purchased from Abcam, Cambridge, UK. Anti-c-Myc (1:800) was purchased from Millipore MA, USA. Anti-Pinx1 (1:1000) was purchased from Abnova, Taipei, Taiwan. Anti-tubulin (1:1000) was purchased from Cell Signaling, MA, USA.

Hybridization to all antibodies was followed by fluorescence-labeled secondary antibodies (LI-COR Biosciences, Lincoln, NE, USA). Visualization and quantification of the protein levels were performed by using the Odyssey Infrared Imaging System (LI-COR Biosciences NE, USA).

### 2.8. Study design

The flow chart that delineates the design of our study is described in Fig. 1. Telomeres were shortened by exposing SK-N-MC cells to GRN163, specific telomerase inhibitors. These cells underwent two major analyses: proteomic analyses and microRNA expression analyses. The obtained data was combined with the protein group which includes all the putative targets of the microRNA and with four different groups of proteins that may have a biological involvement in the phenotype characterizing cancer cells with short telomeres. All data was used for creating networks based on protein–protein interaction databases and compared to the group of cisplatinum sensitivity related proteins. The network with the highest overlap was chosen for further studies. After finding the most central proteins (nodes) in the network, the prediction of the network as to changes in the expression of several proteins was examined by Western blotting.

## 3. Results

### 3.1. Telomere shortening alters the miRNA profile of cells

To induce shortening of telomeres, SK-N-MC cells were exposed to GRN163, a template antagonist of telomerase. Administration of GRN163 resulted in 70–90% inhibition of telomerase activity (not shown). This inhibition persisted up to 72 h and repeated measurements throughout the 20 months of the experiment verified continuous inhibition of telomerase at this range. The telomeres were shortened in a range of 20–30% and over 50% after 3 and 20 months, respectively (see [9]).

We next analyzed the profile of the microRNAs of cells with shortened *versus* intact telomere length by screening over 900 miRNAs. The miRNA profile of the control intact cells with intact telomeres was similar to that of the cells whose telomeres were initially shortened and then re-elongated to their original length after withdrawal of the telomerase inhibitor from the growth medium (not shown).

Comparison of the miRNA profiles of the cells with shortened telomeres to those with intact telomeres revealed significant differences in 57 miRNAs. 29 miRNAs were upregulated and 28 miRNAs were downregulated in the cells with shortened telomeres (Table 1). Validation of 19 out of these 57 miRNAs verified the changes predicted by the miRNA analysis (Supplemented Fig. S6). Some of these miRNAs have biological functions that may be related to the "shortened telomere" phenotype (Table 2). For example, miR-199 and the Let7 family of miRNAs are implicated in growth arrest and apoptosis. Others such as miR-181, miR-148, and miR-143 target oncogenes and are considered tumor suppressors. Among the miRNAs that were down-regulated in the cells with shortened telomeres, some are involved in proliferation-, growth- and cancer-related processes (miRs-121, 192, 19, 17 and 18 which are part of the 17–92 cluster). Part of these miRNAs were also reported in our previous paper [9].

Interestingly, out of 3565 putative proteins that comprise the above miRNAs targets, 107 are related to cisplatinum resistance (out of about 500 proteins that are listed in Table S4) that are known to confer resistance to cisplatinum (Table S2A and [29–32]). This overlap is statistically significant ($P = 0.006$; see Materials

**Table 2**
Biological relevance of miRs that were differentially expressed in cells with shorter telomeres.

| miR symbol | Putative relevance to the phenotype of cells with shorter telomeres |
|---|---|
| *Upregulated in cells with shortened telomeres* | |
| hsa-let-7a | Possess anti-growth effects in cancer cells *in vitro* (lung cancer, melanoma) and *in vivo* (mouse models of lung cancer) [46] |
| hsa-let-7b | |
| hsa-let-7c | |
| hsa-let-7d | |
| hsa-miR-128 | May be involved in Alzheimer pathology, or in aging process in general [47] |
| hsa-miR-138 | Targets telomerase, is downregulated in squamous cell carcinoma of the tongue, anaplastic thyroid carcinoma and papillary thyroid carcinoma [48] |
| hsa-miR-143 | A tumor suppressor miR, targets ERK5 and may potentially target KRas [49] |
| hsa-miR-148a | A tumor suppressor gene, targets human DNMT3b protein, resulting in growth suppression [50] |
| hsa-miR-181a | Targets oncogenes such as: the oncogenes 70-kDa zeta-associated protein and Tcl1 in CLL [51] |
| hsa-miR-181b | |
| hsa-miR-193a-3p | A pro apoptotic miR, activates the caspase cascade V [52] |
| hsa-miR-199a-3p | A pro-apoptotic agent, targets MET and ERK2 proto-oncogenes [53] |
| hsa-miR-199a-5p | |
| *Upregulated in cells with intact telomeres* | |
| hsa-miR-125a-5p | Is implicated in cancer [54] |
| hsa-miR-140-3p | Is involved in cell growth in lung carcinoma cells, targets histone deacetylase 4 which binds 53bp1 and involved in DNA repair [55] |
| hsa-miR-146b-5p | Is associated with inflammatory processes, is induced by NF-kappaB [56] |
| hsa-miR-17 | miRs 17, 18a and 19a belong to the 17–92 cluster which is associated with processes related to proliferation and aggressiveness of malignancies and |
| hsa-miR-18a | defined as pro-tumorigenic [57–59]. |
| hsa-miR-19a | |
| hsa-miR-192 | Its expression activates the Survivin promoter in lung A549 cells, regulates cancer cell growth [60] |
| | Targets SIP1 |
| hsa-miR-21 | Targets sprouty2 and promotes cellular outgrowths and carcinogenesis. It targets tumor suppressor genes in invasion and metastasis [61]. |

References are denoted by parenthesis.

and Methods). The same comparison to the groups of proteins related to the sensitivity to doxorubicin [33–37] or vincristine [38–41,62] did not reveal a significant overlap (the proteins that are related to doxorubicin sensitivity are listed in Table S4). These results are in line with our previous data showing that sensitivity to cisplatin (and not other drugs such as vincristine or doxorubicin) is a typical characteristic of a telomere shortened phenotype of cancer cells [9].

### 3.2. Telomere shortening affects protein expression of cells

Cells with shortened or intact telomeres were subjected to proteomic analysis performed by comparative mass spectrometry on SILAC labeled proteins. This analysis identified 870 proteins out of which 81 were upregulated and 18 downregulated (although to a lower extent) in the shortened telomere cells (a ratio of 2 of protein expression was defined as a cutoff of significance). The list of differentially expressed proteins is given in Table 3A, B. Five proteins were selected for validation of the proteomic results and all presented the same tendency of expression: three were upregulated and two were downregulated as predicted by the proteome analyses (Supplemental Fig. 5).

Functional analysis using the Panther software [http://www.pantherdb.org] showed that most of these proteins (32% of all differentially expressed proteins) are associated with nucleic acid binding (Figs. 2A, B). For instance, the DNA-damage binding protein DDB1 exhibited elevated levels under telomere shortening. Other examples include HNRPC and HNRPD, which are both heterogeneous nuclear ribonucleoprotein RNA binding proteins which form complexes with heterogeneous nuclear RNA. These proteins are associated with pre-mRNAs in the nucleus and appear to influence pre-mRNA processing and other aspects of mRNA metabolism and transport.

### 3.3. Network data

To gain more understanding of the functional context in which the identified proteins and miRNAs operate, we inferred a network of physical interactions (protein–protein and miRNA–target) that connects them. Our inference strategy [19] requires a network anchor: a small set of nodes that serve as mediators for the effects of telomere shortening on protein levels.

In the anchor set, we considered the differentially expressed miRNA supplemented with a functionally relevant group of proteins. We considered four groups, representing four systems of putative biological relevance: apoptosis; DNA repair; cell migration; and telomere/telomerase (see Materials and Methods).

A separate network was inferred for each combination of systems (15 altogether). To select for the most plausible network, we evaluate their association with selective sensitization to cisplatinum, one of the defining features of the shortened telomere phenotype. Next, we evaluated the overlap between the nodes in each of the resulting networks and a set of 523 proteins known to be associated with sensitivity or resistance of cancer cells to cisplatinum (Materials and Methods, Supplemented Tables S2A and S2B). The highest scoring network, on which we concentrated, is the one built around the apoptosis and telomere–telomerase machinery systems. This network includes 39 out of the 523 cisplatinum-related proteins ($P < 1 \times 10^{-15}$, a hypergeometric P-value which was computed according to the hypergeometric distribution). In contrast, the overlap to proteins related to doxorubicin resistance or sensitivity with proteins from the whole scale of the networks was very low as detected by similar types of calculations (Supplemented Table S2A).

The chosen network includes most of the microRNAs listed in Table 2, which may have a biological relevance to the phenotype of cancer cells with shortened telomeres (Fig. 3). The interactive network is shown in the following link: www.cs.tau.ac.il/~roded/Uzieletal-network.cys. To view the interactive network please download the cytoscape software from the website: www.cytoscape.org. The miRNAs included in the network are: miR-181b, Let7a–d, miR-17, miR-138, miR-199a, miR-143, and miR-18a. Seven of them (181b, 138, Let7 a–d, 199a, and 18a) are connected to IGF2BP2 which was upregulated in our proteomic analysis.

### 3.4. Network usage for selection of candidates

We scored the proteins in the network based on a criterion of network centrality. This identified a small group of 13 central proteins,

**Table 3**
Proteomic results.

| Protein | Accession number | (Short tel/WT) | |
|---|---|---|---|
| | | Average | SDV |
| *A. Proteins that were highly expressed in cells with shortened telomeres compared to the intact wild type cells* | | | |
| Interleukin enhancer-binding factor 3 — *Homo sapiens* | Q12906 | 10 | 0.08 |
| Brain acid soluble protein 1 — *Homo sapiens* | P80723 | 7.14 | 0.08 |
| ATP synthase subunit O, mitochondrial precursor — *Homo sapiens* | P48047 | 6.67 | 0.04 |
| Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial precursor | P10515 | 6.67 | 0.16 |
| Golgi apparatus protein 1 precursor — *Homo sapiens* | Q92896 | 6.25 | 0.06 |
| Mitochondrial 28S ribosomal protein S29 | P51398 | 5.56 | 0.08 |
| Dolichyl-diphosphooligosaccharide—protein glycosyltransferase subunit 2 precursor | P04844 | 5.56 | 0.05 |
| Protein FAM84B | Q96KN1 | 5.26 | 0.16 |
| Pyruvate carboxylase, mitochondrial precursor | P11498 | 5 | 0.17 |
| Caprin-1 | Q14444 | 4.76 | 0.04 |
| Ras GTPase-activating protein-binding protein 1 | Q13283 | 4.76 | 0.03 |
| Insulin-like growth factor 2 mRNA-binding protein 1 | Q9NZI8 | 4.76 | 0.06 |
| Sodium/potassium-transporting ATPase subunit alpha-1 precursor | P05023 | 4.55 | 0.10 |
| Bifunctional 3′-phosphoadenosine 5′-phosphosulfate synthetase 1 | O43252 | 4.55 | 0.02 |
| Heterogeneous nuclear ribonucleoproteins A2/B1 | P22626 | 4.35 | 0.09 |
| F-box only protein 22 | Q8NEZ5 | 4.35 | 0.18 |
| Heterogeneous nuclear ribonucleoproteins C1/C2 | P07910 | 4.17 | |
| Insulin-like growth factor 2 mRNA-binding protein 2 | Q9Y6M1 | 4.17 | 0.08 |
| ATP synthase subunit b, mitochondrial precursor | P24539 | 4 | 0.16 |
| Heterogeneous nuclear ribonucleoprotein D0 | Q14103 | 4 | 0.18 |
| Insulin-like growth factor 2 mRNA-binding protein 3 | O00425 | 4 | 0.04 |
| ADP/ATP translocase | P05141 | 4 | |
| Myristoylated alanine-rich C-kinase substrate | P29966 | 4 | 0.16 |
| Membrane-associated progesterone receptor component 1 | O00264 | 3.85 | 0.10 |
| Dolichyl-diphosphooligosaccharide—protein glycosyltransferase subunit 1 precursor | P04843 | 3.85 | 0.08 |
| Eukaryotic translation initiation factor 5B | O60841 | 3.7 | 0.17 |
| ATP synthase subunit alpha, mitochondrial precursor | P25705 | 3.7 | |
| 60S ribosomal protein L26 | P61254 | 3.7 | 0.06 |
| 60S ribosomal protein L27 | P61353 | 3.7 | 0.17 |
| Staphylococcal nuclease domain-containing protein 1 | Q7KZF4 | 3.57 | 0.00 |
| 60S acidic ribosomal protein P1 | P05386 | 3.57 | 0.03 |
| ATP-binding cassette sub-family F member 1 | Q8NE71 | 3.57 | 0.07 |
| ATP-dependent RNA helicase DDX3X | O00571 | 3.57 | 0.09 |
| Nucleophosmin | P06748 | 3.57 | 0.16 |
| Phenylalanyl-tRNA synthetase alpha chain | Q9Y285 | 3.57 | 0.02 |
| 60S ribosomal protein L27a | P46776 | 3.45 | 0.05 |
| 40S ribosomal protein S20 | P60866 | 3.45 | 0.21 |
| P16615\|AT2A2_HUMAN Sarcoplasmic/endoplasmic reticulum calcium ATPase 2 — *Homo sapiens* (human) | P16615 | 3.45 | 0.39 |
| Keratin, type I cytoskeletal 10 | P13645 | 3.45 | 0.21 |
| Non-POU domain-containing octamer-binding protein | Q15233 | 3.33 | 0.25 |
| 4F2 cell-surface antigen heavy chain | P08195 | 3.33 | 0.16 |
| ATP synthase subunit beta, mitochondrial precursor | P06576 | 3.33 | 0.14 |
| 60S ribosomal protein L21 | P46778 | 3.33 | 0.13 |
| FK506-binding protein 3 | Q00688 | 3.23 | 0.15 |
| Apoptosis-inducing factor 1, mitochondrial precursor | O95831 | 3.23 | 0.00 |
| Heterogeneous nuclear ribonucleoprotein C-like 1 | O60812 | 3.23 | 0.21 |
| PERQ amino acid-rich with GYF domain-containing protein 2 | Q6Y7W6 | 3.23 | 0.14 |
| DNA damage-binding protein 1 | Q16531 | 3.13 | 0.07 |
| 60S ribosomal protein L23 | P62829 | 3.13 | 0.09 |
| Probable ATP-dependent RNA helicase DDX17 | Q92841 | 3.13 | 0.10 |
| Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial precursor | P36957 | 3.13 | 0.15 |
| Plasminogen activator inhibitor 1 RNA-binding protein | Q8NC51 | 3.13 | 0.19 |
| Hsp90 co-chaperone Cdc37 | Q16543 | 3.13 | 0.05 |
| 60S ribosomal protein L23a | P62750 | 3 | 0.10 |
| NMDA receptor-regulated protein 1 | Q9BXJ9 | 3 | 0.35 |
| Cytoskeleton-associated protein 4 | Q07065 | 3 | 0.15 |
| Acylamino-acid-releasing enzyme | P13798 | 2.94 | 0.07 |
| Proliferation-associated protein 2G4 | Q9UQ80 | 2.94 | 0.11 |
| ATP-dependent RNA helicase A | Q08211 | 2.94 | 0.06 |
| Vacuolar ATP synthase catalytic subunit A | P38606 | 2.94 | 0.07 |
| 60S ribosomal protein L31 | P62899 | 2.94 | 0.07 |
| 60S ribosomal protein L36 | Q9Y3U8 | 2.94 | 0.14 |
| 28 kDa heat- and acid-stable phosphoprotein | Q13442 | 2.94 | 0.09 |
| 60S ribosomal protein L10 | P27635 | 2.94 | 0.05 |
| Eukaryotic translation initiation factor 5 | P55010 | 2.86 | 0.24 |
| Translationally-controlled tumor protein | P13693 | 2.86 | 0.06 |
| Heterogeneous nuclear ribonucleoprotein Q | O60506 | 2.86 | 0.13 |
| Interleukin enhancer-binding factor 2 | Q12905 | 2.86 | 0.11 |
| 60S ribosomal protein L13 | P26373 | 2.86 | 0.04 |
| 60S ribosomal protein L19 | P84098 | 2.86 | 0.09 |
| 40S ribosomal protein SA | P08865 | 2.86 | 0.12 |
| 60S acidic ribosomal protein P2 | P05387 | 2.86 | 0.05 |
| 40S ribosomal protein S7 | P62081 | 2.86 | 0.08 |

**Table 3** *(continued)*

| Protein | Accession number | (Short tel/WT) | |
|---|---|---|---|
| | | Average | SDV |
| 60S ribosomal protein L12 | P30050 | 2.86 | 0.01 |
| Heterogeneous nuclear ribonucleoprotein A0 | Q13151 | 2.86 | 0.12 |
| Heterogeneous nuclear ribonucleoprotein K | P61978 | 2.86 | 0.12 |
| Transferrin receptor protein 1 | P02786 | 2.86 | 0.14 |
| Nuclease-sensitive element-binding protein 1 | P67809 | 2.86 | 0.10 |
| 60S ribosomal protein L26-like 1 | Q9UNX3 | 2.86 | 0.12 |
| 60S ribosomal protein L17 | P18621 | 2.86 | 0.13 |
| Catenin alpha-1 | P35221 | 2.86 | |
| *B. Proteins that were downregulated in cells with shortened telomeres compared to the intact wild type cells* | | | |
| Importin-8 | O15397 | 0.74 | 0.01 |
| WD repeat and HMG-box DNA-binding protein 1 | O75717 | 0.74 | 0.43 |
| Receptor expression-enhancing protein 5 | Q00765 | 0.74 | 1.22 |
| Glycyl-tRNA synthetase | P41250 | 0.73 | 0.54 |
| Protein mago nashi homolog 2 | Q96A72 | 0.72 | 0.33 |
| Protein phosphatase 1 regulatory subunit 7 | Q15435 | 0.71 | 0.60 |
| Cysteinyl-tRNA synthetase, cytoplasmic | P49589 | 0.69 | 0.46 |
| D-3-Phosphoglycerate dehydrogenase | O43175 | 0.63 | 0.41 |
| Aldehyde dehydrogenase, mitochondrial precursor | P05091 | 0.63 | 0.09 |
| Annexin A1 | P04083 | 0.61 | 0.57 |
| Serpin H1 precursor | P50454 | 0.59 | 0.70 |
| Phosphoserine phosphatase | P78330 | 0.59 | 0.02 |
| Phosphoserine aminotransferase | Q9Y617 | 0.47 | 0.36 |
| Succinyl-CoA:3-ketoacid-coenzyme A transferase 1, mitochondrial precursor | P55809 | 0.43 | 2.26 |
| Eukaryotic peptide chain release factor GTP-binding subunit ERF3A | P15170 | 0.39 | 1.51 |
| Asparagine synthetase [glutamine-hydrolyzing] | P08243 | 0.34 | 1.60 |
| Retinal dehydrogenase 1 | P00352 | 0.32 | 2.06 |
| Pseudouridylate synthase 7 homolog | Q96PZ0 | 0.27 | 4.62 |

which did not come up in our screen, as plausible mediators of the "short telomere" downstream phenotypes (Table S3, Supplemental material). While these proteins are predicted to be central, the network construction does not predict the direction of expression change of these proteins (their higher or lower expression) upon telomere shortening. To investigate this and validate the predictions of the networks, we chose to follow the expression of four proteins that were identified as most central and three proteins that were among the less central. Fig. 4 shows that the expression of the proteins identified by the network as central (but did not come up in the screen) did indeed change significantly upon telomere shortening. In contrast, the expression of the proteins deemed less central by the network was not significantly affected by telomere shortening. Additionally, inspection of the remaining high-scoring networks (highlighted in Supplemented Table S2B) revealed a notable consistency with regard to the high scoring proteins. In particular, the four proteins that we followed up on are included in several high scoring networks. On the other hand, proteins that received a low score in the apoptosis telomere network (*e.g.*, KU70, BCL2, PINx1) were very low scored or even missing in the other networks, implying that the network approach is robust.

### *3.5. Predicted nodal points are deregulated in cancer cells with short telomeres*

Several nodal proteins predicted by the network possessed a change in their expression in cells with shortened telomeres as shown in Fig. 4.

PRKDC (DNA-PKcs), a DNA damage repair protein, markedly increased the expression of its phosphorylated form (430%) in cells with shortened telomeres compared to the intact cells, a change that was not identified in the initial screen. Another nodal protein, c-Myc, a major transcription factor of hTERT expression was downregulated (about 30%) compared to the cells with intact telomere length. Two additional central proteins are members of the MAPK signaling pathway: JNK1 (MAPK8) acts as an integration point for proliferation, differentiation and regulation of transcription, meiosis, mitosis, and development. JNK1 expression was 50% higher in shortened telomere containing cells compared to the intact cells. RPS6KA1, a ribosomal protein S6 kinase, reduced its expression in the shortened telomere cells (to 60% of their length in the intact wild type cells). A possible explanation for these differences in protein expression belonging to the same signaling pathway is given later in the discussion.

### *3.6. The less central proteins and their expression in the cells*

The changes in the expression of three other proteins which were given a low centrality scoring by the network (connected only to one or two other proteins) were also mild according to the Western blot results. BCL2, an anti-apoptotic protein, was mildly downregulated (10% reduction) in the cells with shortened *versus* intact telomere length. Pinx-1 expression, a microtubule-binding protein essential for proper chromosome segregation, was reduced by ~20% compared to the intact cells. KU70 (XRCC6) which has a major role in DNA repair process and telomere maintenance did not affect its expression in the cells following telomere shortening (Fig. 4).

## 4. Discussion

In the present study we characterized the molecular changes occurring in cancer cells upon telomere shortening. We have concentrated on changes occurred in miRNA and protein expression and avoided the analysis of total gene expression due to the fact that miRNAs and proteins form the cellular end point (proteins) of phenotypic features and their regulatory elements (miRNA). Changes in the expression of genes are important but may not be necessarily expressed in the final amount of proteins in the cells, and likewise many proteins will have different functional concentrations in the cells that were not influenced by changes in their cognate RNA transcript but stem from other post-translationally regulatory factors.

The results of this study present for the first time an extensive miRNA and proteomic profiling reflecting the changes induced by telomere shortening in cancer cells. Since we wanted to avoid the effects of senescence (induced by the shortest telomeres) and to study the effects

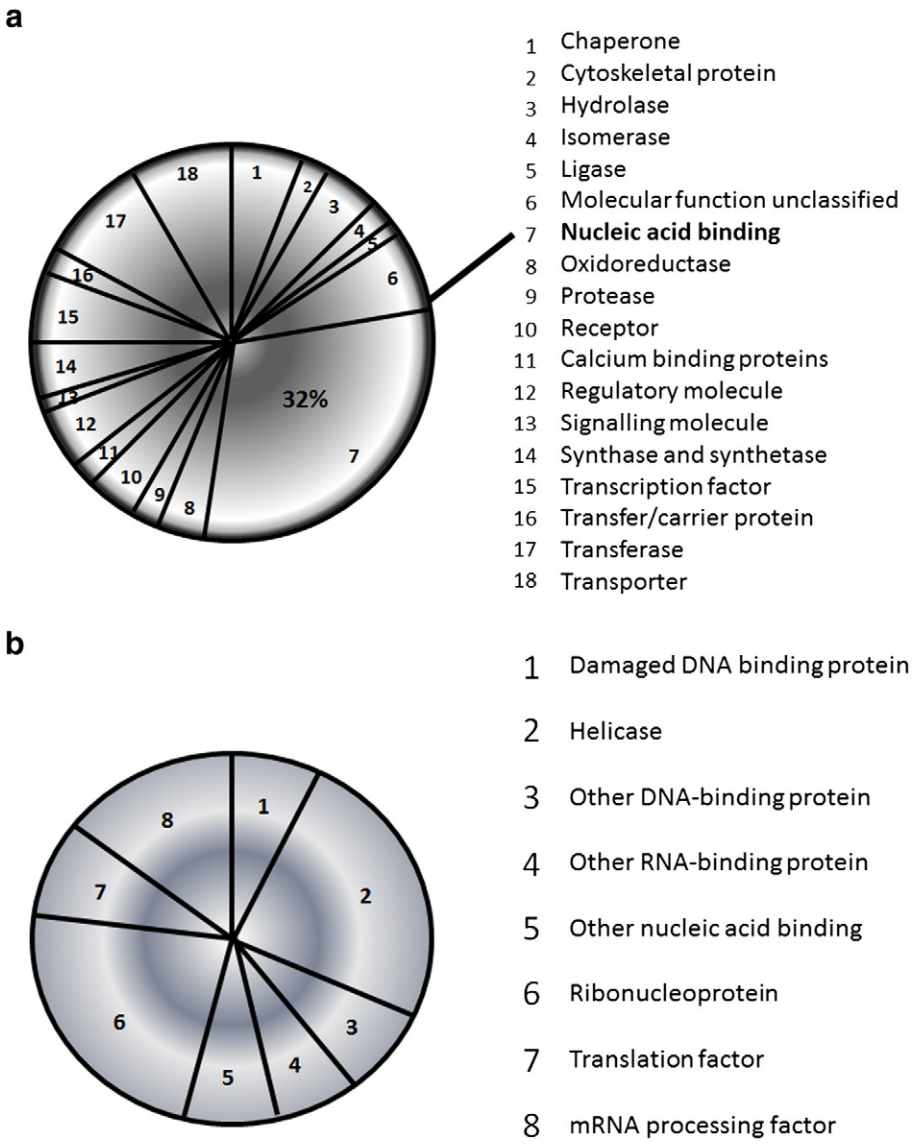


**Fig. 2.** Functional analysis of the proteins identified by the proteomic analysis. a. Analysis of the whole proteomic data. All 800 identified proteins were classified by the Panther software into functional groups. The largest group of proteins (32% of all proteins) is nucleic acid binding proteins indicated by the arrow. b. Sub-analysis of the nucleic acid proteins. Proteins belonging to the nucleic acid related protein group were classified by the Panther software.

of overall telomere shortening, we allowed for limited proliferation of the cells after reconstitution of telomerase activity, causing preferential elongation of the shortest telomeres [42]. The results of this study reflect the changes induced by the decreased telomere length *per se* and not by the telomerase inhibition or the dynamic process of telomere shortening. This is shown by the fact that re-elongated telomeres regained the original characteristics of the intact cells.

Our results uncovered changes in the regulation of many genes upon telomere shortening. These genes are distributed throughout the genome, and thus cannot be directly affected in cis by telomere length changes, as in TPE.

As shown in Table 1 the expression of 57 miRNAs was significantly changed by telomere shortening. Some of these miRNAs possess anti-growth effects and act as tumor suppressors, thus their expression in the cells with shortened telomeres may be directly relevant to the phenotype obtained from these cells (Table 2). Although there is no information in the literature regarding miRNA expression in cancer cells with shortened telomeres we found some similar data regarding miRNA expression in other cells, mostly somatic cells, in a senescent state. For example, three miRNAs (miR-92, miR-146b, miR-17) that were downregulated in stress induced senescent cells [43], were also downregulated in our cells with shortened telomeres. miR-146b is associated with inflammatory processes (Table 2); miRNAs 17 and 92 are a part of the 17–92 miRNA cluster implicated in proliferation and aggressiveness of malignancies. Another study compared several reports describing 12 miRNAs associated with senescence of somatic and cancer cells [45]. Among them, the Let7 family of miRNAs was upregulated in senescent mesenchymal stem cells, similar to the results in our cells with shortened telomeres. Additionally, a downregulation of the miRNA 17–92 cluster was shown in senescent cells and in tissues derived from young *versus* old individuals [44]. A recent review points to several miRNAs implicated in senescence, two of which (miR-125 and miR-21) were downregulated in our cells with shortened telomeres as well [45]. These results may imply that the cells with shortened telomeres in our experimental system may trigger processes leading to senescence.

The functional analysis of the differentially expressed proteins revealed that most of them (32%) are nucleic acid binding proteins (Fig. 2a). An analysis of this group shows that it consists mostly of ribonucleoproteins and helicases (about 25% each, Fig. 2b). In general,

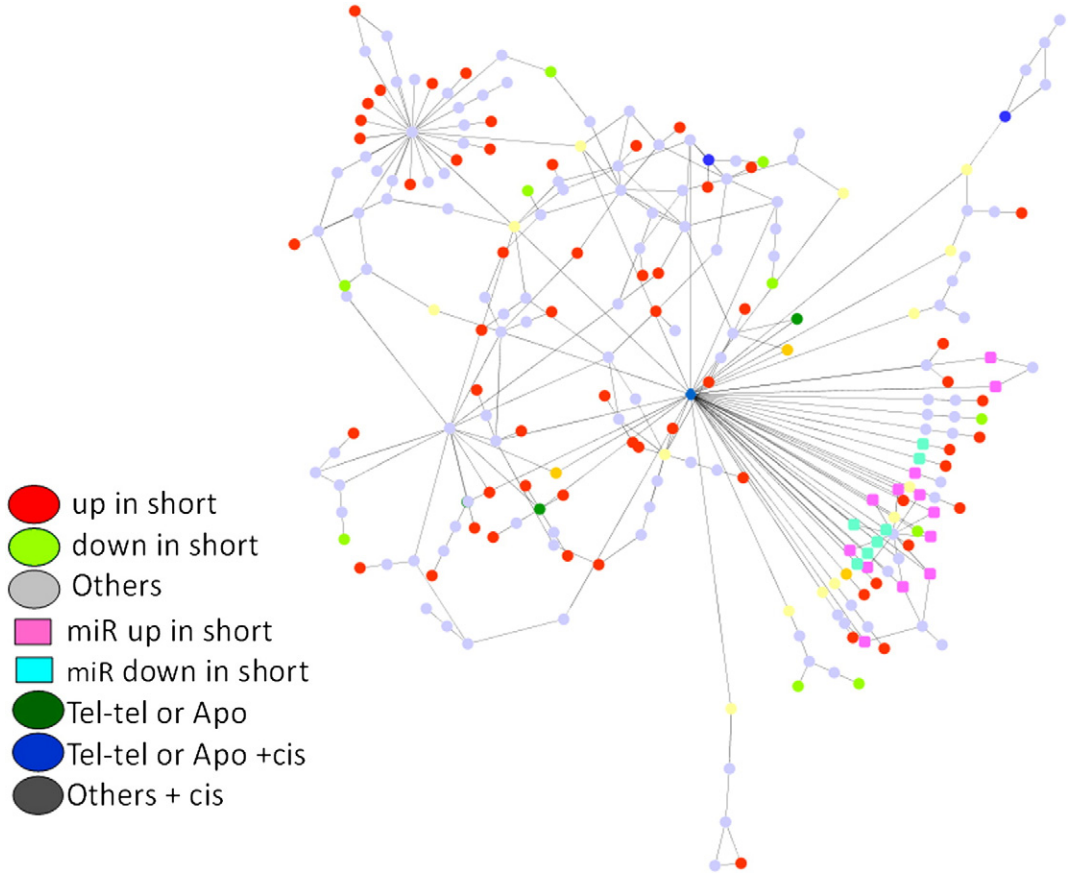


**Fig. 3.** The network connecting the proteomic and microRNA data combined with proteins related to apoptosis and the telomere–telomerase system. The colors of the nodes representing proteins and microRNAs divided into 8 categories are denoted at the side of the figure. Up or down in short — proteins that were either upregulated or downregulated in cells with shortened telomeres. Others — proteins that were selected by the algorithm used for the construction of the network from protein–protein interaction related databases. miRs up or down in short — microRNAs that were either upregulated or downregulated in cells with shortened telomeres. Tel-tel or Apo — proteins related to the telomeres–telomerase machinery or to apoptosis. Tel-tel or Apo + cis — proteins belonging to the telomere–telomerase system or to apoptosis and to the sensitivity to cisplatinum. Others + cis — proteins belonging to the sensitivity to cisplatinum. Please see the actual interactive network as well as a link for all the nodes and their interacting miRNAs or proteins in Supplemented materials.

ribonucleoproteins are nuclear proteins that contain RNA. This group of proteins includes the ribosome, telomerase, vault ribonucleoproteins, RNase P, hnRNP and small nuclear RNPs (snRNPs), which are implicated in pre-mRNA splicing. They may also participate in post-transcriptional regulation.

The meaning of these findings is not clear although ribonucleoproteins and helicases may play a role in the context of the higher DNA damage index identified in the cells with shortened telomeres.

The long-term aim of the study was to define the possible mechanisms and pathways contributing to the damage to the cancer cell due to shortening of telomeres. The initial step towards this goal was a characterization of the changes of miRNA and protein expression following telomere shortening. Next, we used network analysis in order to form a functional connection among our experimentally obtained data and known groups of proteins that may be biologically related to our experimental system. This includes proteins that are related to DNA-repair, migration, telomere and telomerase machinery, apoptosis and response to cisplatinum. We deliberately took this combinational approach in light of the relatively low number of proteins identified by the proteomic analyses (only 800 out of 100,000 cellular proteins). Adding the proteins from four functional groups that may be relevant to the phenotype of cancer cells with shortened telomeres may have broaden the spectrum of relevant proteins that may participate in the features of that phenotype. Indeed, this approach allowed us to single out a handful of differentially expressed proteins that were not identified by the proteomic results and may be important for telomere shortening mediated damage. We performed a preliminary validation of the network predictions by measuring the changes in the expression of the most central and least central scoring proteins. We found that the expression of the four "central" proteins significantly differed between the cells with shortened *versus* intact telomere length. These experimental findings strengthen the potential validity of the network results. It is of note that these four potentially important proteins showed up only in the network results and were not detected in the proteomic analysis. This finding also strengthens the potential usage of the networks in detection of important proteins. Inspection of the biological function of the proteins which were differentially expressed in our system or their connection according to the network prediction may underlie their relevance to the phenotype of cells with short telomeres. These include PRKDC (DNA-PKcs), a DNA damage repair protein and c-Myc, a major transcription factor of hTERT expression. PRKDC is connected to 16 differentially expressed proteins throughout the network. It is involved in DNA repair processes, in telomere maintenance and in the sensitivity to cisplatinum as well. These pathways are related to the proteins listed below, which were all upregulated according to the proteomic analyses of our cells. Protein examples include: NPM1, a protein that is related to proliferation and survival processes. EIF5, implicated in apoptosis; Cars, is a cysteinyl-tRNA synthetase related to the DNA repair process and DDB1, a DNA damage-binding protein which functions in nucleotide-excision repair imposed by cisplatinum. The overexpression of PRKDC in the cells with shortened telomeres (Fig. 4) probably reflects the constant DNA damage that these cells sense, which may have promoted the overexpression of their DNA repair mechanisms. c-Myc, a major transcription factor of hTERT expression plays a role in cell cycle progression, apoptosis and cellular transformation. Three out of the differentially expressed proteins that were connected to c-Myc may be

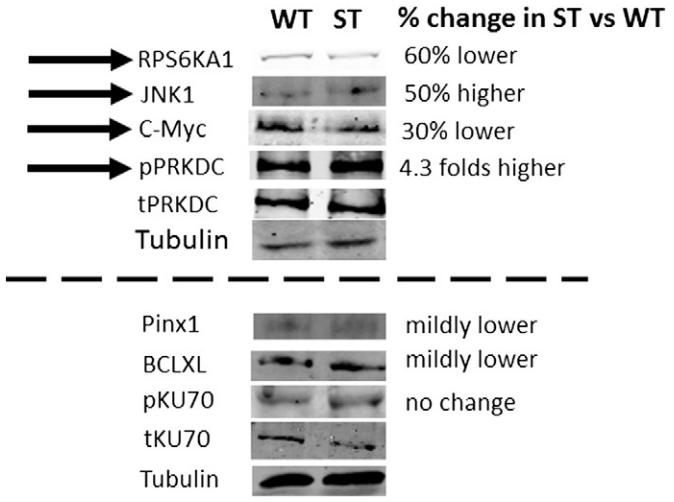


**Fig. 4.** Experimental validation of protein expression defined by the network. Western blot analyses were carried out in order to validate the predictions of the network. The expression of each protein was compared between the cells with shortened telomeres and the intact parental cells and normalized to the expression of a house-keeping gene product. The average changes in protein expression calculated from 3–4 independent experiments are denoted on the right in percentages. The proteins that significantly changed their expression after shortening of their telomeres are denoted by arrows and shown above the broken line.

relevant in conferring the "short telomere" phenotype. RPL10 can repress c-Jun-mediated transcriptional activation. RPL31 interacts with BRCA1 and may be implicated in DNA repair processes and RPSA, which is (as mentioned above) involved in cell adhesion, differentiation, migration, signaling, neurite outgrowth and metastasis. The relevance of c-Myc to the shortened telomere phenotype is not clear although it is involved in the transcriptional upregulation of telomerase. Interestingly, two other central nodes, JNK1 and RPS6KA1, belonging to the MAPK pathway, are regulated in an opposing manner in cells with shortened telomeres, implying that their putative function in the "telomere shortened" phenotype might not be related to the MAPK pathway. JNK1 is a central protein in the network and connected to 27 proteins, some of which exhibited changes in their expression in the shortened telomere cells according to the proteomic analyses. These proteins (RPL31, RPSA, RPL13, RPL26, PDCD8 and PGMRC1) are implicated in proliferation- and survival-related processes such as cell adhesion, differentiation, migration, signaling, neurite outgrowth, metastasis and anti-apoptosis. DDB1, also connected to JNK1, is a DNA damage-binding protein which functions in the nucleotide excision repair pathway, responsible for the repair of cisplatinum damage. The second MAPK related protein is RPS6KA1, connected to 16 proteins in the network. It phosphorylates various cellular substrates, including members of the MAPK signaling pathway. Importantly, it regulates mTOR, thus increasing life span and resistance to age-related pathologies. Its activity was implicated also in controlling cell growth and differentiation. RPS6KA1 is connected to several proteins which were differentially expressed in the cells with shortened telomeres. Among them are proteins that are involved in cancer cell survival or death (such as PGMRC1), proteins that are implicated in cell growth and tumorigenesis (DHX9, DDX17, SLC3A2), and those related to cell-adhesion and motility processes (CTNNA1, MARCKS). This may be relevant to the cells with shortened telomeres, which are moderately "poor growers" especially when grown in the presence of cisplatinum. Its network connections to proteins that were differentially expressed in the cells with shortened telomeres may highlight its importance in these cells.

The less central proteins according to the network scoring, BCL2 and Pinx-1, have no known biological role that may be implicated in the phenotype of cells with shortened telomeres. In contrast, KU70 (XRCC6) has a major role in DNA repair process and telomere maintenance. The fact that its expression did not change in our system is somewhat surprising since KU70 is one of the targeting factors of PRKDC (see above) which appeared to increase its expression in the cells with shortened telomeres. This may be explained by PRKDC being a limiting factor to their combined activity.

The experimental data combined with the information derived from the networks may clarify several pathways triggered by telomere shortening in cancer cells. However, the results of this study cannot be readily generalized to all cancer cells since it was performed on one cell line only. Therefore, the conclusions have to be confined only to that cell type and should be tested in other cancer cells.

In addition, we cannot differentiate at this stage whether these changes play a direct or indirect role in the short-telomere features obtained. A further study is needed to be able to pinpoint the mechanisms that serve as cause and not consequence to shortened telomeres of cancer cells.

Another point is the prolonged usage of GRN163 in our study which inhibited telomerase activity and therefore promoted the shortening of telomeres in the cancer cells. Theoretically there is a possibility that at least some of the molecular changes observed resulted from the prolonged exposure to GRN163 by itself and not necessarily to the shortening of telomeres. The best way to address this question is to study the long-term effect of this telomerase inhibitor on cells that lack telomerase activity such as U2OS and use the ALT (alternative lengthening of telomeres) to maintain their telomeres. We are not aware of such an experiment in the literature but in further studies it should be performed.

Further research in our laboratory is aimed at exploring the involvement of other proteins that appeared to be central in the network, and target them in order to directly obtain cancer cells with the shortened telomere phenotype. Validation of these results in additional cancer cells may assist in the identification of novel drug targets that may be utilized for future rational drug design against cancer.

## 5. Conclusions

In summary, we characterized the changes in miRNA and protein expression in order to define the possible mechanisms contributing to the damage to the cancer cell resulting from shortening of telomeres. The functional network analysis allowed us to pinpoint a number of differentially expressed proteins that may be important for telomere shortening mediated damage. The network findings were further validated experimentally. These experimental findings strengthen the potential validity of the network results.

Confirmation of these results in other cancer cells may lead to the identification of novel anti-cancer drug targets that might be clinically used to combat cancer.

## Competing interests

The authors declare that they have no competing interests.

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