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Protein interactions with *piALU* RNA indicates putative participation of retroRNA in the cell cycle, DNA repair and chromatin assembly

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Recent analyses suggest that transposable element-derived transcripts are processed to yield a variety of small RNA species that play critical functional roles in gene regulation and chromatin organization as well as genome stability and maintenance. Here we report a mass spectrometry analysis of an RNA-affinity complex isolation using a piRNA homologous sequence derived from Alu retrotransposal RNA. Our data point to potential roles for *piALU* RNAs in DNA repair, cell cycle and chromatin regulations.

Introduction

Genome-wide analyses have shown that a large proportion of the genome is transcribed to generate non-coding RNAs (ncRNAs), the biological role and significance of which remains largely unknown.[1] The genomes of humans and other complex eukaryotes have many transposon-derived sequences of various classes, commonly referred to as "repetitive elements" or simply "repeats." Until recently, the most prevalent view was that these repeats represent non-functional "junk DNA." However, this assumption is now being challenged.

A number of studies indicate that transcripts derived from transposable element (TE) sequences are processed through the cellular RNAi machinery.[2] The dis-regulation of TE-RNA processing is associated with diseases such as cancer,[3] macular degeneration,[4] autoimmunity[5,6] and human adult stem cell aging.[7] It has been proposed that RNA processing is necessary to silence TE transcription, and is mediated by several pathways including Argonaute-associated short interfering RNAs "siRNAs" in Arabidopsis[8] and *Saccharomyces pombe*,[9] Dicer-independent short RNA piRNA machinery in *Drosophila melanogaster*[10] and Dicer1 in mice.[4,11] All of these pathways converge at the level of chromatin to drive the heterochromatinization of TEs. However, modern analyses of retrotransposal RNA processing pathways have expanded our understanding of the degree to which TE elements are intimately linked to essential cellular signal-response and developmental functions. The RNAi-mediated processing of TE transcripts (in cis- or in trans-) is most likely involved in a broader spectrum of genomic regulations than simply TE silencing.

In light of new investigations, transcriptional activity from a number of retrotransposon families has been proposed to exert a beneficial function, suggesting that ncRNAs from retrotransposons, in a similar manner to the nuclear function of lncRNAs, may facilitate epigenetic regulation by orienting chromatin modifying factors/complexes to specific genomic locations in a cell type-, developmental stage- and signal-specific manner.[12-16] For example, mammalian LINE retrotransposons facilitate X chromosome inactivation[17] and LINE RNA products are essential structural and functional components for the formation of neocentromeric chromatin.[18] Also, SINEB2 and B1 elements define chromosomal boundaries[19-21] and regulate both mRNA production[22] and the heat shock response.[22,23] Interestingly, Alu elements provide a novel class of enhancer signals by mediating interchromosomal interactions[24-26] and also participate in chromatin remodeling[27] and DNA damage repair.[7,28,29] It is tempting to speculate that the assembly of protein complexes onto retrotransposal RNAs might be nucleotide-specific, implying that RNA-binding protein complexes may undergo conformational and/or functional changes dependent on the sequence composition, RNA length or frame of the processed intermediates, and could be sensitive to the presence of ssRNA, dsRNA or DNA-RNA hybrids.

Unlike the majority of long ncRNAs, which remain permanently in the nucleus, ncRNA from retrotransposons can be found in both nuclear and cytoplasmic compartments[29] where

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their regulation and processing, although scarcely understood, likely involves intricate feed-back loops between these two cellular compartments. This was elegantly demonstrated for the production of type I interferons in autoimmune disease.[6]

Recently, we have reported that the majority of the repairable DNA damage sites in self-renewing human adult stem cells is associated with the retrotransposal portion of the genome, in particular, Alu retrotransposons.[7] Moreover, it has been shown that the upregulation of transcriptional activity from Alu can be triggered by genotoxic stress-induced damage[30] as well as upon in-vivo and ex-vivo aging in human retinal pigment epithelium (RPE)[31] and human adult stem cells,[7] respectively. Intriguingly, evidence indicates that cellular cytotoxicity resulting from increased accumulation of *Alu* RNA is directly linked to Dicer-1 deficiency,[4] suggesting that such cytotoxicity may not necessarily be due to an increase in transcriptional activity, but rather the absence of concomitant transcript processing. Our recently published data indicate that the accumulation of unprocessed *Alu* transcripts triggers chromatin deterioration, loss of DNA repair in pericentromeric areas eliciting the persistent DNA damage response and, ultimately, cellular senescence.[7] Further, we have shown that human adult stem cells stably expressing an shRNA against *Alu* transcripts, sh-132Alu, override cellular senescence and reinstate their DNA repair capacity,[7] suggesting that RNAi machinery is involved in these events. This published data also implies two equally possible mechanisms through which an shRNA against *Alu* might mediate the observed function: (1) through the **p**ost **t**ranscriptional **g**ene **s**ilencing (PTGS) Dicer-dependent pathway via the cytoplasmic degradation of *Alu* RNA or (2) through facilitating transcriptional silencing by recruiting either the Dicer-dependent or Piwi-dependent arms of the RNAi pathway to act directly on the chromatin as shown in **Figure 1A**. Both of these pathways are plausible and either could depend on the assembly of single or multiple protein complexes capable of cross-talk with DNA-damage-sensing/repair and centromeric maintenance pathways. This hypothesis is plausible, considering that a recent study indicates that transcripts generated from telomere-repeat-encoded RNA (TERRA) interact with heterochromatin protein 1 (HP1), trimethlyated histone H3 lysine 9 (H3K9me3), core components of the Shelterin complex, as well as members of the DNA-damage-sensing pathway.[32]

Similarly, we hypothesize that this region of *Alu* RNA targeted by sh-132Alu, which is functionally relevant to overriding the senescent phenotype of human adult stem cells,[7] can mediate a broad array of downstream effects associated with retrotransposal transcription. Since the cytoplasmic role of *Alu* RNA in the assembly of the signal recognition particle (SRP) has been previously reported,[33] we have focused our efforts specifically on the discovery of nuclear complexes assembled on *Alu* RNA. Using an unbiased RNA affinity assay coupled with mass spectrometry, we provide evidence for the composition of molecular complexes assembled on processed *Alu* RNA transcripts. Our data implicate a number of molecular pathways through which processed intermediates of *Alu* RNA may participate in a multitude of nuclear processes within human adult stem cells.

Results

Alu RNA and its homology to human PIWI-interacting RNA (piRNA). Previously it was reported that *Alu* RNA participates in the cytoplasmic assembly of SRP in mammalian cells.[33] The mammalian SRP is composed of a single RNA, the *7SL* RNA and six proteins.[34] SRP9 and SRP14 bind to the 5' end of the RNA (*Alu*-domain), which functions in translational arrest.[35,36] However, the functional sh-132Alu reported by Wang et al.[7] has no complementarity to *7SL* RNA (**Fig. 1B**). In addition, the region of Alu that corresponds to the shRNA is more highly conserved between Alu repeats than is the rest of the element (**Fig. 1C**). This conservation is consistent with the reported ability of the shRNA to effectively knock down transcription from multiple Alu repeats,[7] including members of evolutionarily distinct young and old Alu subfamilies (**Fig. 1C**). All together, these data illuminate the functional relevance of the sh-132Alu sequence analyzed here, and indicate that this sequence may represent a naturally occurring processed *Alu* RNA species that functions in the nucleus.

Furthermore, we queried the sh-132Alu sequence analyzed here against PIWI-interacting RNA (piRNA) sequences previously reported by Girard et al.[37] The exact sequence of the shRNA was identified to be a PIWI-interacting RNA, pir-52207 and pir-57460[37] (NCBI accession #DQ585095), and is referred to herein as *piALU*. This suggests that *Alu* RNA is subjected to processing by the human PIWI complex, the molecular function of which is not fully understood. Nevertheless, the existence of this *piALU*, derived from processed *Alu* transcripts, indicates a possible nuclear function.

piALU RNA interaction with nuclear factors. We attempted to obtain a comprehensive list of nuclear proteins that interact, both directly and indirectly, with *piALU*. For this purpose, we utilized an unbiased RNA-affinity assay using synthetic biotinylated RNA oligos encompassing *Alu* nucleotides 140–185, shown in green in **Figure 1B**, with nuclear extracts from self-renewing human adult stem cells. This portion of Alu is conserved among different types of Alu retrotransposons: AluSx, AluJb, AluJo and AluY (**Fig. 1C**).

Our *piALU* RNA affinity assay was combined with an LC-MS/MS (liquid chromatography-electrospray tandem mass spectrometry) analysis of interacting proteins. To differentiate from non-sequence-specific interactions occurring with components of the nuclear RNA surveillance pathway protecting from transcriptional noise, we included a control hexanucleotide repeat control RNA "bait," as described in Materials and Methods. The experimental procedure was previously reported by Deng et al.[32] and is outlined in **Figure 2A**. A comparative analysis of RNA-protein binding by denaturing PAGE identified the presence of multiple *piALU* probe-specific bands (**Fig. 2B**) that were excised for LC-MS/MS identification. A list of the proteins identified is given in **Table S1**. A list of all identified peptides can be found in **Table S2**.

piALU RNA interacts with major chromatin modifiers, DNA repair complexes, centromeric chromatin/kinetochore assembly and transcriptional factors. Our mass spectrometry analysis of

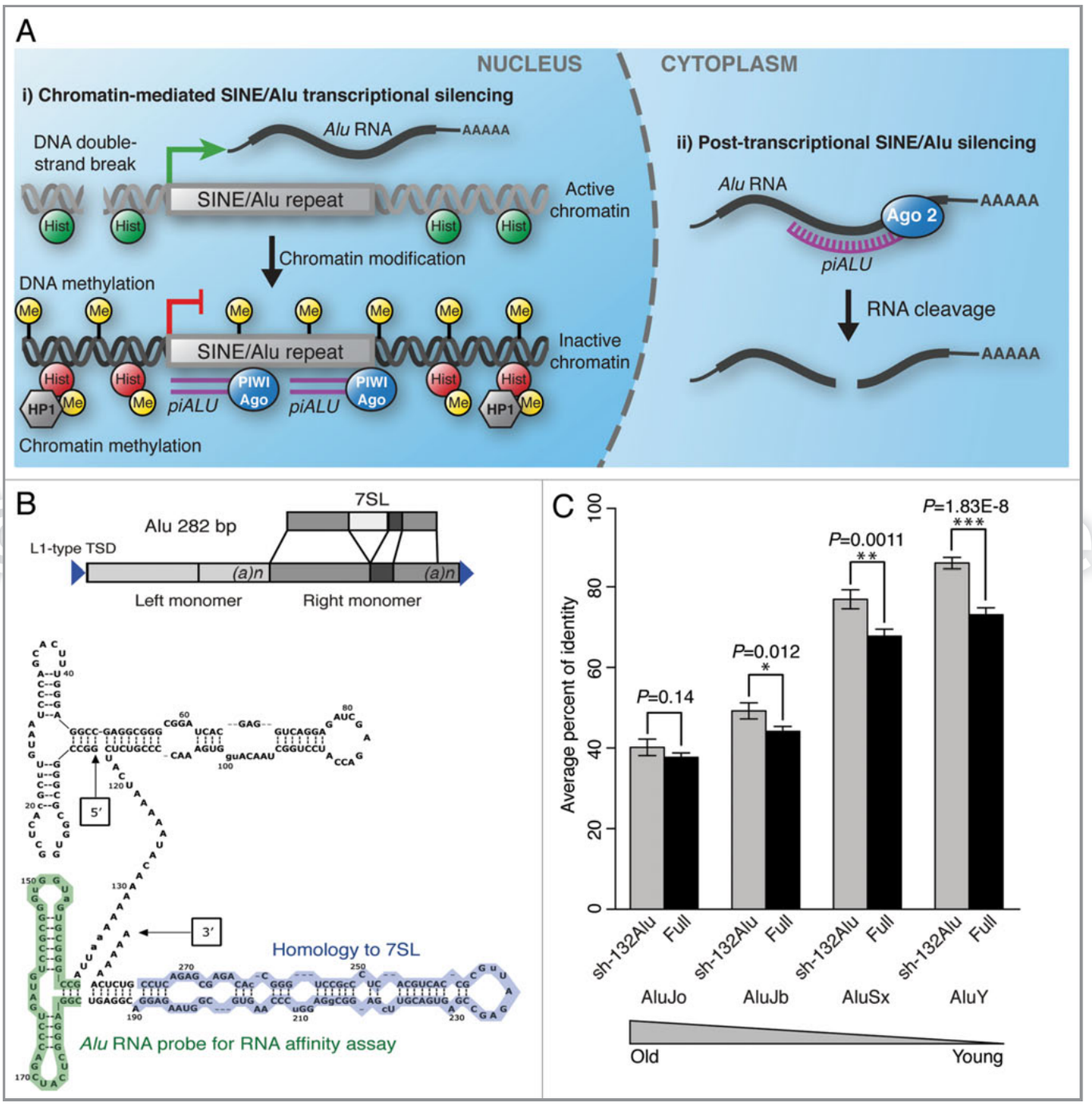


Figure 1. (A) Alternative models of sh-132Alu action7, (i) shRNA against Alu, which directs either nuclear PIWI or Dicer machinery to the genomic SINE/Alu repeat location, initiating transcriptional silencing via heterochromatinization involving both DNA methylation and histone modification. (ii) sh-132Alu acts through the PTGS Dicer-dependent Ago2 pathway, leading to the cytoplasmic degradation of unprocessed *Alu* RNA transcripts. (B) Top: Diagram representation of 7SL-conserved region within Alu full-length sequence. Bottom: Secondary structure of generic full-length *Alu* RNA. The segment highlighted in blue represents highly conserved 7SL-derived portion, while the segment highlighted in green represents region used for RNA affinity assay. (C) Comparison of sequence conservation for the region of Alu elements corresponding to the sh-132Alu sequence (grey bars: sh-132Alu) versus sequence conservation for the rest of the Alu sequence (black bars: Full). Average levels of sequence identity (y-axis) among dispersed Alu elements are shown for four Alu subfamilies of different relative ages (x-axis).

unbiased *piALU* RNA-affinity precipitates revealed the presence of major protein categories such as chromatin modifiers, DNA repair complexes, centromeric chromatin/kinetochore assembly and "others" (**Fig. 2C**). Although multiple peptides were identified for each protein with variable confidence scores (low, medium and high), shown in **Table S2**, only proteins with high confidence scores were included in **Figure 2C**. For example, the MS spectra for MLL protein can be viewed as **Figure S1** and others can be found in data online. All LC-MS/MS data has been uploaded to the Tranche database, and can be accessed as described in Materials and Methods.

The peptide representation of each component is depicted as a color gradient fading from true hues to black for singly represented peptides. It is important to note that the number of identified peptides does not necessarily indicate the abundance of the identified protein, as it is determined by the number of available tryptic cleavage sites. A larger protein with more tryptic sites has a greater chance of more peptide identifications than a shorter protein with fewer tryptic sites, even if their abundance is the same. Similar to protein interactions with telomeric RNA repeats (TERRA),[32] *piALU* binding proteins include the DNA damage response proteins DDB1, PARP and hnRNPM, suggesting that *piALU* RNA may be integral to DNA repair complexes.

To assess the relevance of *piALU* RNA to biological processes, we applied a Gene Ontology (GO)[38,39] analysis to the list of interacting proteins. All together, 19 cellular processes were significantly ($3.12 \times 10^{-13} \leq p \leq 1.5 \times 10^{-3}$) enriched among *piALU*-interacting proteins including: DNA repair, organelle organization, cell cycle, chromatin remodeling and transcription (**Fig. 3A**). These broader categories encompass nucleotide excision repair, **d**ouble-**s**tranded **b**reak (DSB) repair via homologous recombination, DNA recombination, V(D)J recombination, response to stress, chromosome segregation, cell cycle check-points, regulation of cell cycle checkpoint arrest, interphase of mitotic cycle and mitosis (**Fig. 3B**). Complexes related to DNA duplex unwinding, regulation of transcription and transcriptional elongation from RNA polymerase II promoters were also significantly enriched.

Several nuclear proteins were associated with multiple GO terms (n = 12) designated to cellular components including the centrosome, nucleoplasm, kinetochore, spindle, microtubule cytoskeleton, centromeric and telomeric regions of chromosomes, as well as the cohesin and DNA-directed RNA polymerase complexes (**Fig. 3B**).

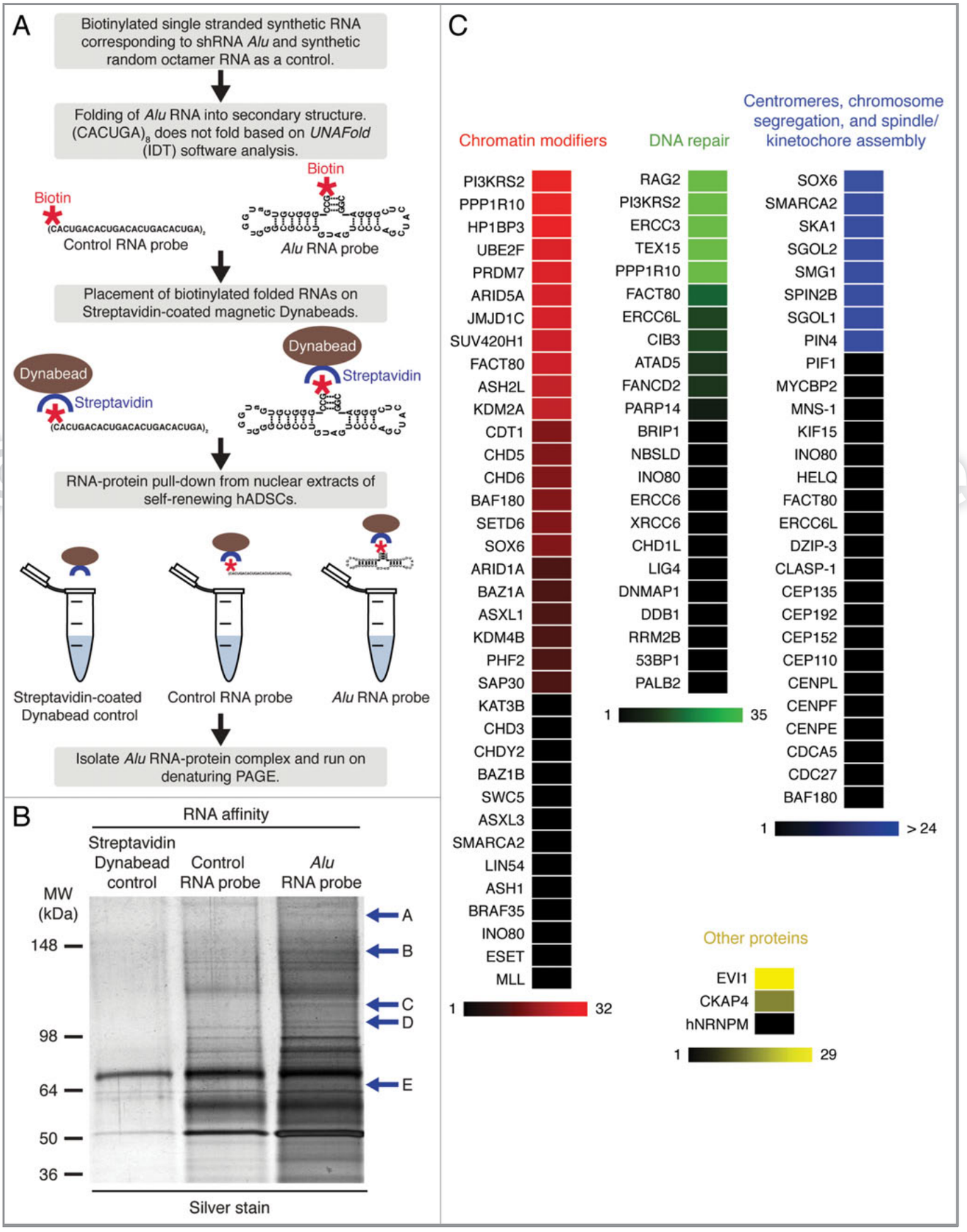
These data suggest a possible role for *piALU* RNA in the regulation of chromatin organization of centromeric/pericentromeric regions, DNA repair processes, and the transcriptional regulation of genes. All of these cellular processes have been proven to be indispensable to the regulation of cell cycle progression.

Protein-protein interaction network of the piALU RNA-affinity complex. An essential prerequisite for understanding the cellular function(s) of an isolated protein complex(es) is the ability to correctly uncover and annotate all functional interactions among LC-MS/MS identified proteins. To build a protein-protein interaction network among *piALU* RNA-affinity isolated proteins, we used the online database **S**earch **T**ool for the **R**etrieval of **In**teracting **G**enes (STRING) version 9.0. This tool provides uniquely comprehensive coverage and access to both experimental and predicted interaction information.[40] The STRING model of our LC-MS/MS data of nuclear *piALU* RNA-protein interactions is shown in **Figure 3C**. Only high confidence level (experimental) evidence of interactions/associations in curated databases in conjunction with the Markov clustering algorithm (MCL) were used to build the network and assign proteins into families (stronger associations are represented by thicker lines in **Figs. 3C and 4**).[41] Strikingly, nine out of the 64 *piALU* RNA interacting partners are involved in *chromosome segregation* and include SGOL1, SGOL2, CENPE, CENPF and CDCA5, with four more (PBRM1, CENPL, CDC27 and MAP3K8) directly linked to *mitosis*, while the addition of PIN4 and KIF15 depicts a complex protein network regulating *spindle assembly*. This observation suggests that *piALU* transcripts might form a molecular connection between centromere cohesion and microtubule dynamics at the kinetochore. We recently demonstrated that the accumulation of unprocessed Alu RNA in adult stem cells blocks the recruitment of the cohesin complex to centromeric areas, triggering cellular senescence.[7] Another group recently reported the loss of outer kinetochore proteins CENP-E and CENP-F upon knockdown of Sgo.[42] As CENP-E and CENP-F are members of the *piALU* interactome, it seems that Alu RNA, in its processed form, may be an RNA adaptor in spindle microtubule dynamics. This suggested involvement of *piALU* in chromosome segregation is intriguing in light of the high levels of cellular senescence and aneuploidy, due to a spindle checkpoint defect, seen in BubR1 deficient mice.[43] Further investigation into the regulation of chromosome segregation/spindle checkpoints by *piALU* may elucidate a novel role for TE-RNAs in cellular aging.

Data shown in **Figure 4** also indicate that 34 out of the 64 analyzed *piALU* RNA interacting partners are somehow involved in the regulation of the cell cycle. The presence of centrosomal proteins CEP192, CEP110, CEP152 and CEP135 points to the intriguing possibility that *Alu* RNA intermediates play a role in this cellular organelle and together with CUL4A, GMNN and CTD1 (**Fig. 4**), may participate in regulating both interphase and the G_2/M phase transition.

Excitingly, *piALU* RNA also pulled down components of the nucleotide excision repair (ERCC3, DDB1, MRE11A), non-homologous end joining (LIG4, XRCC6) and homologous

Figure 2 (See next page). (A) Step-by-step schematic representation of unbiased RNA affinity assay. (B) RNA affinity assay precipitants separated on a denaturing NuPAGE Novex 4–12% Bis-Tris gel, visualized by silver staining. Excised bands are labeled A–E. (C) Color-coded peptide representation of members in major functional categories. Proteins with the highest peptide representation within each group are depicted with the brightest color, fading to black for those with a representation of only 1. An extensive list of peptides and their spectra shown in **Table S2**, and all spectral data has been uploaded to the Tranche database.


A
Biotinylated single stranded synthetic RNA corresponding to shRNA Alu and synthetic random octamer RNA as a control.
Folding of Alu RNA into secondary structure. (CACUGA)8 does not fold based on UNAFold (IDT) software analysis.
Biotin
(CACUGACACUGACACUGACACUGA)2
Control RNA probe
Biotin
Alu RNA probe
Placement of biotinylated folded RNAs on Streptavidin-coated magnetic Dynabeads.
Dynabead
Streptavidin
(CACUGACACUGACACUGACACUGA)2
Dynabead
Streptavidin
RNA-protein pull-down from nuclear extracts of self-renewing hADSCs.
Streptavidin-coated Dynabead control
Control RNA probe
Alu RNA probe
Isolate Alu RNA-protein complex and run on denaturing PAGE.
B
RNA affinity
Streptavidin Dynabead control
Control RNA probe
Alu RNA probe
MW (kDa)
148
98
64
50
36
A
B
C
D
E
Silver stain
C
Chromatin modifiers
PI3KRS2
PPP1R10
HP1BP3
UBE2F
PRDM7
ARID5A
JMJD1C
SUV420H1
FACT80
ASH2L
KDM2A
CDT1
CHD5
CHD6
BAF180
SETD6
SOX6
ARID1A
BAZ1A
ASXL1
KDM4B
PHF2
SAP30
KAT3B
CHD3
CHDY2
BAZ1B
SWC5
ASXL3
SMARCA2
LIN54
ASH1
BRAF35
INO80
ESET
MLL
1
32
DNA repair
RAG2
PI3KRS2
ERCC3
TEX15
PPP1R10
FACT80
ERCC6L
CIB3
ATAD5
FANCD2
PARP14
BRIP1
NBSLD
INO80
ERCC6
XRCC6
CHD1L
LIG4
DNMAP1
DDB1
RRM2B
53BP1
PALB2
1
35
Centromeres, chromosome segregation, and spindle/ kinetochore assembly
SOX6
SMARCA2
SKA1
SGOL2
SMG1
SPIN2B
SGOL1
PIN4
PIF1
MYCBP2
MNS-1
KIF15
INO80
HELQ
FACT80
ERCC6L
DZIP-3
CLASP-1
CEP135
CEP192
CEP152
CEP110
CENPL
CENPF
CENPE
CDCA5
CDC27
BAF180
1
> 24
Other proteins
EVI1
CKAP4
hNRNPM
1
29

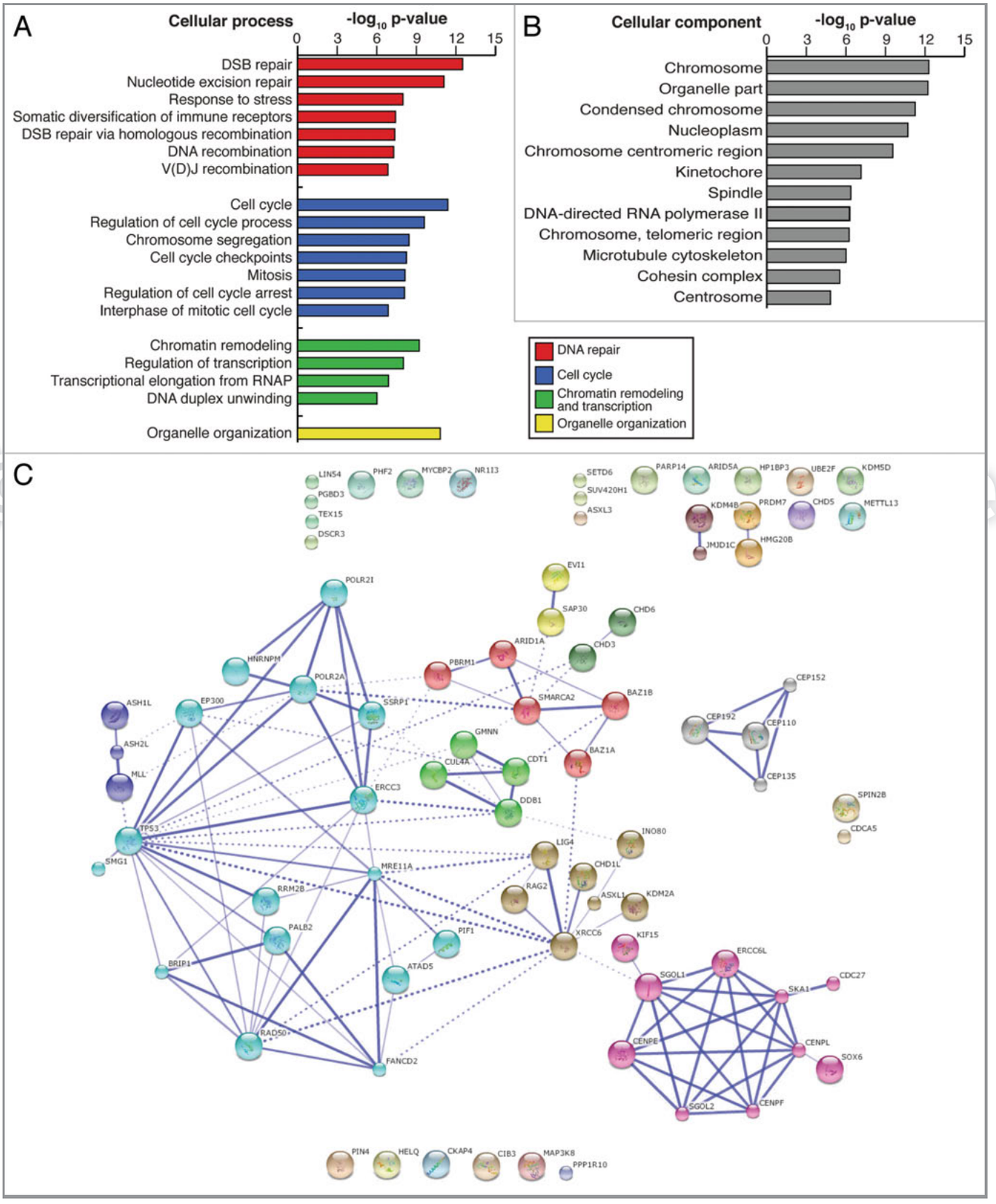


Figure 3. For figure legend, see page 32.

Figure 3 (See previous page). (A) Confidence levels suggesting the involvement of isolated complex proteins in various cellular processes, represented as a $\log_{10}$ (p value), p values calculated by STRING v9.0 software. (B) Confidence levels for the presence of isolated complex proteins in various cellular components, represented as a $\log_{10}$ (p value), as calculated using STRING v9.0 software. (C) Interaction web of isolated proteins produced in STRING 9.0 software. Thicker lines represent higher confidence (experimentally derived) interactions while dotted lines represent inferred (lower confidence) interactions.

recombination (PALB2, RAD50) DNA repair pathways as well as the BAZ1B protein, suggesting a putative role for *piALU* RNA in double-stranded break repair. The assembly of the DNA damage repair complexes described herein may be a critical event in adult stem cell aging.[4,7] It is tempting to speculate that a lack of proper assembly/function of these complexes together or separately with chromatin remodeling components (CHD6, SMARCA2, FACT80, BAZ1A, ARID1A, INO80, ASXL1 and KDM2A) may explain the gradual loss of DNA damage repair capacity with aging, triggering the persistent DNA damage response and cellular senescence.[44,45]

Discussion

A recent genome-wide ChIP-RNA sequencing project revealed that over a third of all known large conserved ncRNAs can be pulled down with just four different chromatin modifying proteins,[46] suggesting that most long ncRNAs associate with chromatin modifiers. The *piALU* RNA interactome described herein also includes a variety of chromatin modifiers, possibly adding another level of complexity to ncRNA/chromatin modifier protein complex formation with a preference for a shorter, processed form of *Alu* RNA. The full length sequence of *Alu* RNA can form four distinct stem-loop structures, each of which may act as individual nucleating centers for the formation of distinct RNA-protein complexes. Only one of the four stem-loops was used here as a synthetic RNA "bait," since it encompasses the previously reported human PIWI-interacting RNA[37] and is capable of nucleating protein complex(es) that are directly or indirectly responsible for reinstating the self renewal of senescent adult stem cells.[7]

Indeed, in accord with our previously published experimental data, the observed protein interactions support the relevance of these nuclear *piALU* RNA-protein complex(es) to cell cycle control, DNA repair, chromatin maintenance and the function of centromeres/pericentromeres in human adult stem cells. This data are currently lacking functional assessment, but nevertheless may hold the key to understanding how retrotransposal transcription/processing regulates human adult stem cell aging and senescence ex vivo.

Since no nuclear interactions with this portion of *Alu* RNA have been published, we cannot yet assess the technical or biological limitations of our reported data. It is possible that additional protein interactions were too unstable to withstand the stringent conditions used to purify *Alu* RNA-interacting components. In addition, some cellular proteins with unusual physical properties can be difficult to detect by LC-MS/MS analysis. Finally, some interactions could be cell type- or culture condition-dependent. Despite these drawbacks, our approach is suitable to probe nuclear *Alu* RNA-protein interactions in different cell types, culture conditions as well as upon various treatments that induce DNA-damage and/or cell cycle arrest.

The data reported in this manuscript point to a potentially interesting involvement of Alu retrotransposon-derived short RNA species in the regulation of cell cycle control, RNA-pol II transcription and the maintenance of chromatin architecture. It is also possible that DNA repair processes are intimately linked to *piALU* pathways. Further investigation will allow us to elucidate the functional significance of these intriguing observations.

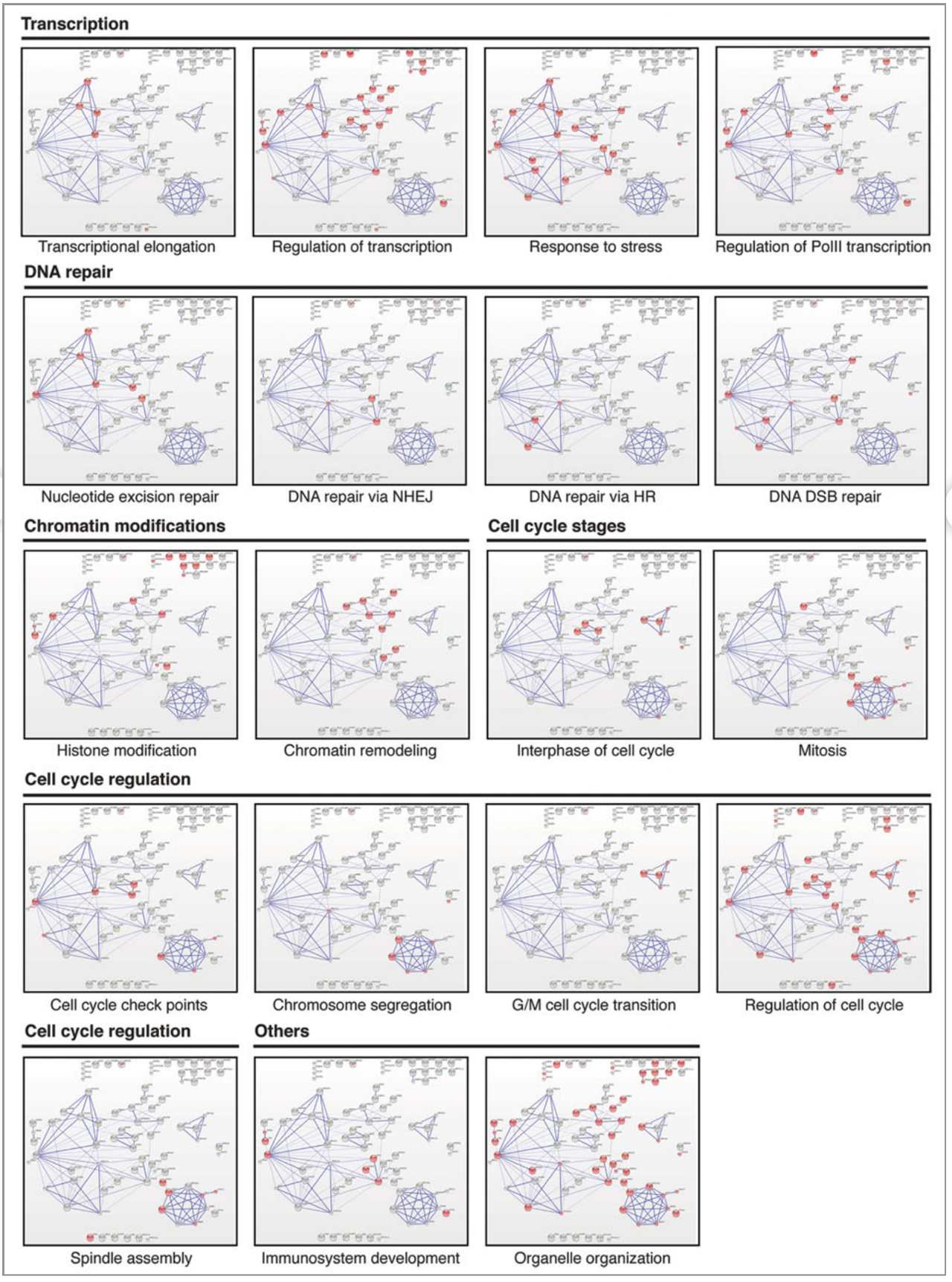
Materials and Methods

Human ADSC isolation and expansion. Human adipose-derived stem cells (hADSCs) were isolated from human subcutaneous white adipose tissue collected during liposuction procedures. The lipoaspirate was suspended in Hank's Buffered Salt Solution (HBSS), 3.5% Bovine Serum Albumin (BSA), 1% Collagenase, type II (Sigma) in 1:3 w/v ratio and shaken at 37°C for 50 min. The cells were filtered through a 70 μm mesh cell strainer (BD Falcon, 352350), treated with Red Blood Cell Lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.3) and expanded ex-vivo in DMEM/F12 complete medium (DMEM/F12, 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B; Invitrogen) in 10% CO_2 at 37°C and passaged at 80% confluency, changing medium every 72–96 h.

Nuclear chromatin extraction. Nuclear and cytoplasmic proteins were isolated from self-renewing hADSCs using the ProteoJET™ Cytoplasmic and Nuclear Protein Extraction Kit (Fermentas #K0311), according to the manufacturer's protocol.

RNA secondary structure folding. 5' biotinylated single stranded synthetic RNA probes corresponding to the 140–185 nucleotide region of full-length *Alu*, *piALU:* (5'-/Bio/-GCC GGGCGUGAUGGUGGGCGCCUGUAGUCCCAGCUACU CGGGAGGC-3') as well as a synthetic random octamer repeat 5'-/Bio/-$(CACUGA)_8$ as a linear control, were purchased from Integrated DNA Technologies (IDT). These oligos were purified via RNase-Free HPLC. Eight micrograms of each oligo were heated in a PCR tube with 15 μl of 2X Folding Buffer (40 mM HEPES pH 7.9, 0.4 mM EDTA, 2 M NaCl) and 9 μl DEPC water with 50 U/ml SUPERase-In RNase inhibitor (Ambion, #AM2694). The RNA oligonucleotides were heated to 78 °C to induce a linear conformation, then the reaction temperature was reduced to and held at 54 °C to accommodate and retain desired

Figure 4 (See opposite page). Representation of protein complex using Gene Ontology (GO) terms in String 9.0. For each process, involved proteins are highlighted in red. Each specific cellular process is grouped into one of the following five categories: transcription, DNA repair, chromatin modifications, cell cycle regulation and others.


Transcription
Transcriptional elongation
Regulation of transcription
Response to stress
Regulation of PolII transcription
DNA repair
Nucleotide excision repair
DNA repair via NHEJ
DNA repair via HR
DNA DSB repair
Chromatin modifications
Cell cycle stages
Histone modification
Chromatin remodeling
Interphase of cell cycle
Mitosis
Cell cycle regulation
Cell cycle check points
Chromosome segregation
G/M cell cycle transition
Regulation of cell cycle
Cell cycle regulation
Others
Spindle assembly
Immunosystem development
Organelle organization

secondary structure formation (mimicking the secondary stem-loop structure of the full length RNA), based on calculations by UNAFold software (IDT). The control repeat RNA 5'-/Bio/-$(CACUGA)_8$ is not capable of forming a secondary structure.

Protein pulldown (unbiased RNA affinity assay). One hundred microliters of streptavidin-coated Dynabeads (Dynabeads M-270 Streptavidin, #65306) per reaction were washed three times in 1× D150 buffer[32] (20 mM HEPES pH 7.9, 20% glycerol, 0.2 mM EDTA, 150 mM NaCl, 0.05% NP-40, 1 mM PMSF, 10 μM β-mercaptoethanol) before a final high-salt wash with 1× Folding Buffer (containing 1 M NaCl). Thirty μl of folded RNA solution was added to each reaction tube and incubated at RT for 15 min, then washed twice with 100 μl of low-salt Folding Buffer (containing 150 mM NaCl). Bead-bound RNA probes were incubated for 30min at RT with nuclear extracts from self-renewing hADSCs, along with 1 μl of 0.01M PMSF and 3 μl SUPERase-In. After incubation, the Dynabeads were washed five times in 1× D150 buffer, leaving only directly and indirectly interacting partners attached to the beads. As a negative control, nuclear extracts were also incubated with Dynabeads alone to control for any interaction between nuclear molecules and the beads themselves.

Denaturing PAGE analysis of affinity purified protein complexes. XT Sample buffer 12 (BioRad, 161-0791) and XT Reducing Agent (BioRad, 161-0792) were added to 10 μg of protein. After denaturation for 5 min at 100°C, protein samples were loaded into a precast 4–12% NuPAGE Novex 4–12% Bis-Tris gel (Invitrogen, NP0321BOX) and run out at 150 V for two hours. Gels were stained with ProteoSilver Plus Silver Stain Kit (Sigma, Prot-SIL1). A SeeBlue Plus2 Pre-Stained Standard (Invitrogen, LC5925) was used for visualization and approximation of molecular weights of protein samples. Bands unique to the *piALU* probe lane were excised and the analyzed by LC-MS/MS for protein content.

Sample preparation for MS analysis. The gel samples were first rinsed with acetonitrile, and then reduced using DTT followed by alkylation using iodoacetamide. We rinsed gel samples with three alternating washes of 50 mM ammonium bicarbonate and acetonitrile, then cooled and resuspended each gel slice in trypsin (5.5 μg/mL in 50mM ammonium bicarbonate/10% acetonitrile) and incubated at 37°C for 24 h for digestion of proteins. We extracted peptides with one rinse of 50 mM ammonium bicarbonate/10% acetonitrile followed by one rinse of 50% acetonitrile/0.1% formic acid, and prepared samples for mass spectrometry by lyophilization and rehydration in 20 μL 5% acetonitrile/0.2% formic acid.

Identification of protein partners by high resolution LC-MS/MS. High resolution LC-MS/MS analysis was performed on an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific) as previously described.[47] Briefly, we loaded excised and digested samples into 96-well plates for mass spectrometry analysis on an LTQ-Orbitrap XL (Thermo Fisher Scientific) instrument as previously described.[47] We injected 10 μL of each re-constituted sample using a Thermo Scientific EASY-nLC Autosampler. Reverse phase chromatographic separations were performed using Hypersil GOLD™ C18™ 3 μm media packed into a fused silica 75 μm inner diameter, 20 cm long column running at 250 nL/min. A gradient was produced using 5–40% acetonitrile, 0.2% formic acid over 150 min. The LTQ-Orbitrap was run in a top 8 configuration at 60 K resolution for a full scan, with monoisotopic precursor selection enabled and +1 and unassigned charge state rejected. The analysis on the LTQ-Orbitrap instrument was performed with CID fragmentation.

LC-MS/MS data analysis and protein identification. LC-MS/MS data analysis, protein identification and peaklist generation were performed using Proteome Discoverer (Thermo Fisher Scientific) algorithm incorporating the SEQUEST® search engine and Percolator™[48] as previously described.[47] MS/MS data were searched using 10 ppm mass accuracy on precursor m/z and a 0.5 Da window on fragment ions. Fully enzymatic tryptic searches with up to three missed cleavage sites were allowed. Oxidized methionines were searched as a variable modification and alkylated cysteines were searched as a fixed modification. Human databases were downloaded from NCBI and supplemented with common contaminants. We filtered peptides for each charge state to a false discovery rate (FDR) of 1%, and then grouped peptides into proteins using Occam's razor logic.

Protein interaction network analysis. All highly represented members of the RNA-protein complex that were isolated were analyzed using STRING 9.0 software.[40] The obtained interaction network was subsequently analyzed with BINGO2 plug-in to determine statistical enrichments for 14 Gene Ontology (GO) categories.[38,39]

LC-MS/MS data deposition in Tranche database. The data associated with this manuscript may be downloaded from ProteomeCommons.org Tranche using the following hash:

tgT9dlthcEjHrjgXb7syYw37XxF8qlIbhlffHXYyxbs2IrcUAT2axC3qlPM+yyyAEIGMthap579qtgdh9JSvXjkMqqQAAAAAAAAB+A==

More information about this upload:
Network: ProteomeCommons.org Tranche
Title: piALU_Interacting Proteins Complete
Description: Supplementary spectral database for publication.
Uploaded: Dec 7 2011, 07:42:08.457 p.m.[49]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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