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SAL-RNAs: Senescence-associated long non-coding RNAs

Kotb Abdelmohsen^{*}, Amaresh Panda, Min-Ju Kang, Jason Xu, Roza Selimyan, Je-Hyun Yoon, Jennifer L. Martindale, Supriyo De, William H. Wood 3rd, Kevin G. Becker, and Myriam Gorospe^{*}

Laboratory of Genetics, National Institute on Aging-Intramural Research Program, National Institutes of Health, Baltimore, MD 21224, USA

Abstract

Non-coding RNAs include small transcripts, such as microRNAs and piwi-interacting RNAs, and a wide range of long non-coding RNAs (lncRNAs). Although many lncRNAs have been identified, only a small number of lncRNAs have been characterized functionally. Here, we sought to identify lncRNAs differentially expressed during replicative senescence. We compared lncRNAs expressed in proliferating, early passage, ‘young’ human diploid WI-38 fibroblasts [population doubling (PDL) 20] with those expressed in senescent, late-passage, ‘old’ fibroblasts (PDL 52) by RNA sequencing (RNA-Seq). Numerous transcripts in all lncRNA groups (antisense lncRNAs, pseudogene-encoded lncRNAs, previously described lncRNAs and novel lncRNAs) were validated using reverse transcription (RT) and real-time, quantitative (q)PCR. Among the novel senescence-associated lncRNAs (SAL-RNAs) showing lower abundance in senescent cells, *SAL-RNA1* (*XLOC_023166*) was found to delay senescence, since reducing *SAL-RNA1* levels enhanced the appearance of phenotypic traits of senescence, including an enlarged morphology, positive β -galactosidase activity, and heightened p53 levels. Our results reveal that the expression of known and novel lncRNAs changes with senescence and suggest that SAL-RNAs play direct regulatory roles in this important cellular process.

Keywords

post-transcriptional gene regulation; transcriptome; non-coding; proliferation; senescence-associated gene expression patterns

INTRODUCTION

Untransformed cells divide in culture for a limited number of times, after which they cease proliferation and enter a state of long-term growth arrest named senescence (Hayflick, 1965). Cellular senescence can be triggered through two closely related processes: by telomere erosion (replicative senescence) and by exposure to damaging conditions (stress-induced or premature senescence) (Kuilman *et al.*, 2010). Although senescent cells do not divide, they can remain viable and metabolically active for a long time and often display a flat, enlarged morphology, and elevated activity of lysosomal β -galactosidase activity (Kuilman *et al.*, 2010). In addition, senescent cells exhibit nuclear senescence-associated heterochromatic foci (SAHF), cytoplasmic vacuoles, and enhanced autophagy (Campisi 2005; Gorospe & Abdelmohsen, 2011). Many senescent cells also display a characteristic

^{*}Correspondence: LMBI, NIA-IRP, NIH, 251 Bayview Blvd., Baltimore, MD 21224, USA, Tel: 410-558-8589; Fax: 410-558-8386, myriam-gorospe@nih.gov; abdelmohsenk@mail.nih.gov.

CONFLICT OF INTEREST

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senescence-associated secretory phenotype (SASP) (Rodier & Campisi, 2011; Tominaga et al., 2012); among the secreted factors are numerous cytokines and chemokines [e.g., granulocyte/macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-6, IL-8, and IL-1] and several matrix metalloproteases (e.g., MMP-1, and MMP-3). In addition, senescent cells often different types of oxidative and genotoxic damage; accordingly, they frequently express DNA damage-response proteins such as -H2AX, NBS1, MDC1, and 53BP1. Finally, cellular senescence is characterized by the presence of factors that govern tumor suppression networks, including the p53/p21 and the pRB/p16 tumor suppressor pathways (Kuilman *et al.*, 2010).

Although cellular senescence has been studied most extensively in cultured cells, there is broad appreciation that cellular senescence occurs *in vivo*. Since senescent cells accumulate in tissues as the organism ages, their metabolic behavior and their signature gene expression profile have been linked to a number of age-related physiologic and pathologic changes (Tyner *et al.*, 2002; Baker *et al.*, 2011). Among the age-related changes, the influence of senescence on carcinogenesis has been studied most extensively; it has revealed that senescence constitutes a strong tumor suppressive mechanism, although in some instances it can enhance different carcinogenic traits (e.g., tissue invasion) (Ohtani *et al.*, 2012). The influence of senescence on other diseases has also been recognized, particularly in pathologies associated with the chronic inflammatory phenotype seen in the elderly, like diabetes, cardiovascular disease, neurodegeneration, and declined immune function (Kuilman *et al.*, 2008; Freund *et al.*, 2010; Luo *et al.*, 2010; Gorospe & Abdelmohsen, 2011).

Given the impact of cellular senescence in age-associated processes, there is much interest in understanding how to modulate senescence for therapeutic purposes. A number of transcription factors have been implicated in driving senescence, including p53, and proteins in the (AP)-1, E2F, Id and Ets families. In addition, two prominent classes of post-transcriptional regulators of senescence have emerged in recent years. The first comprises RNA-binding proteins (RBP), such as human antigen R (HuR), AU-binding factor 1 (AUF1) and tristetraprolin (TTP), which associate with target mRNAs that encode senescence factors and influence cellular senescence (Abdelmohsen *et al.*, 2008; Sanduja *et al.*, 2009; Fabian *et al.*, 2010; Bartel *et al.*, 2009). The second comprises miRNAs, a large group of small (~22 nt), non-coding RNAs that are differentially expressed during senescence and control numerous senescence traits in cultured cells as well as in animal models (Lal *et al.*, 2008; Maes *et al.*, 2009; Marasa *et al.*, 2010; Bonifacio & Jarstfer, 2010; Wang *et al.*, 2010; reviewed in Gorospe & Abdelmohsen, 2011).

In addition to these two groups, long non-coding (lnc)RNA molecules (generally larger than 200 nucleotides), are increasingly recognized as a vast class of regulators of gene expression. LncRNAs comprise a highly heterogeneous group of transcripts with a wide range of sizes, structures, and subcellular locations. Accordingly, the emerging examples of lncRNA-controlled gene expression are presenting a complex spectrum of gene regulatory functions. LncRNAs can affect both transcriptional processes (including epigenetic activities) and post-transcriptional events (Lee *et al.*, 2012; Yoon *et al.*, 2012; reviewed by Schonrock *et al.*, 2012). Among the transcriptional regulatory functions, lncRNAs can target chromatin remodeling factors, coactivate or co-repress transcription factors, modulate the structure/function of bound proteins, form triple helices of DNA:RNA, and have scaffold or decoy functions affecting gene transcription (as seen for lncRNAs *NEATI*, *ANRIL*, *GAS5*, *HOTAIR*, *MALATI*) (Lee *et al.*, 2012). Among the post-transcriptional functions, lncRNAs that base-pair with mRNAs can modulate the translation and/or the stability of target mRNAs [e.g., *1/2-sbsRNAs*, *lincRNA-p21* (Gong and Maquat 2011; Yoon *et al.*, 2012)] while lncRNAs that do not base-pair can affect precursor mRNA splicing and translation by

acting as cofactors, competitors or decoys of RBPs or microRNAs [e.g., *lincMD1* (Cesana *et al.*, 2011)]. The lncRNA-modulated transcriptional and post-transcriptional mechanisms elicit differentiation, proliferation, and cell viability programs, highlighting the rising interest in the roles of lncRNAs in cell function, physiology and pathology. As reviewed comprehensively elsewhere (Wapinski & Chang, 2011; Harries *et al.*, 2012; Yan & Wang, 2012), the physiologic functions of lncRNAs include embryogenesis, cell proliferation, chromosomal imprinting (X chromosome inactivation), while pathologies implicating lncRNA function include cancer, neurodegeneration, immune dysfunction, and cardiovascular and metabolic diseases.

Despite rapidly rising interest in the expression and function of lncRNAs, their possible implication in senescence remain virtually unexplored. Therefore, we sought to identify senescence-associated lncRNAs (SAL-RNAs) using the fetal lung-derived WI-38 human diploid fibroblasts (HDFs) as model system (Wang *et al.*, 2001). Early-passage (proliferating, 'P') WI-38 cells can undergo a number of population doublings (PDLs) until they become terminally arrested (senescent, 'S'). Comparison of the patterns of expressed transcripts by deep sequencing of RNA expressed in proliferating and senescent fibroblasts (RNA-Seq analysis) revealed numerous SAL-RNAs previously annotated, including antisense transcripts, pseudogene-encoded transcripts, and other known lncRNAs, as well as many novel lncRNAs that were differentially expressed in the senescent population. We provide a comprehensive account of previously known SAL-RNAs and novel SAL-RNAs differentially expressed in senescent fibroblasts. Among the novel SAL-RNAs, we identified several transcripts that modulate the onset of senescence and protect senescent cell viability.

RESULTS

Proliferating and senescent WI-38 fibroblasts express different levels of antisense lncRNAs

Compared with proliferating (P), early-passage [population doubling (PDL) 22], senescent (S, PDL 52) WI-38 HDFs displayed a flattened and enlarged morphology and increased senescence-associated β -galactosidase (SA- β gal) activity, a widely used senescence marker (Debacq-Chainiaux *et al.*, 2009) (Fig. 1A). The flattened and enlarged morphology was also visualized by using rhodamine phalloidin, which stains filamentous (F-)actin (Fig. 1B). Western blot analysis revealed that S WI-38 cells expressed lower levels of Sirt1 and HuR, while they expressed higher levels of p21 and p53 (Fig. 1C), as previously reported (Marasa *et al.*, 2010). RNA-Seq analysis was performed using these cell populations and the subsequent characterization was focused on differentially expressed, senescence-associated lncRNAs (SAL-RNAs).

Among the differentially expressed lncRNAs were a number of naturally occurring antisense (NA) lncRNAs. Listed in Fig. 1D (and supplemental Table S1) are WI-38 NA-SAL-RNAs showing >5-fold higher in S compared with P (*top*) and those showing >4-fold higher in P relative to S (*bottom*). These differences were validated for a handful of NA-SAL-RNAs by reverse transcription (RT) using random hexamers and real-time, quantitative (q)PCR amplification using transcript-specific primer pairs. Validated NA-SAL-RNAs showing higher expression levels in senescent cells (Fig. 1E) included *RP11-346A9.1*, targeting the metalloproteinase ADAMTS19, and *OSTN-ASI-001*, complementary to the mRNA encoding the secreted protein Osteocrin. Validated NA-SAL-RNAs exhibiting lower abundance in senescent cells (Fig. 1F) included *VCAN-ASI-001*, complementary to Versican mRNA (encoding an extracellular matrix proteoglycan) and *LSAMP-ASI-001*, antisense to the mRNA encoding a limbic system-associated membrane protein (LSAMP). In several instances, the levels of the NA-SAL-RNA and the complementary mRNA showed positive correlation (not shown); whether this finding reflects a positive influence of one

transcript upon the other or simply joint transcriptional control awaits further investigation. In a different model of senescence, achieved by exposure of WI-38 fibroblasts to ionizing radiation (IR, 10 Gray) followed by culture for an additional 10 days (Fig. S1A), expression of NA-SAL-RNAs followed a similar trend, showing elevated levels of *RP11-346A9.1* and *OSTN-AS1-001* and reduced levels of *VCAN-AS1-001* and *LSAMP-AS1-001* in senescent (IR-treated) cells (Fig. S1B,C). These results indicate that the changes in lncRNA expression are not limited to the replicative exhaustion model (P and S fibroblasts).

Pseudogene-encoded and additional lncRNAs are differentially expressed with senescence

A number of lncRNAs encoded by pseudogenes were also found to be differentially expressed in senescent compared with proliferating WI-38 cells (Fig. 2A, supplemental Table S2). Validation of a subset of pseudogene-encoded (PE) transcripts using RT-qPCR analysis is shown in Fig. 2B (higher in senescent cells) and in Fig. 2C (higher in proliferating cells). Validated upregulated PE-SAL-RNAs include the carcinoembryonic antigen-related cell adhesion molecules *CEACAMP10* and *CEACAMP11*, which are highly expressed in solid tumors, down in senescent cells (Fig. 2B). Among the downregulated PE-SAL-RNAs are transcripts expressed from the ribosomal protein L21 pseudogene and from the heterogeneous nuclear ribonucleoprotein A1 (*HNRNPA1*) pseudogene (high in cancer cells, low in senescent cells). In IR-induced fibroblast senescence, the relative expression levels of PE-SAL-RNAs in IR-treated relative to untreated fibroblasts was the same as that seen in S relative to P fibroblasts (Fig. S1D,E); in a different cell model of replicative senescence (early-passage IMR-90 and late-passage IMR-90), PE-SAL-RNAs showed a similar trend (Fig. S2A,B).

Other well-annotated lncRNAs are listed in Fig. 3A (and supplemental Table S3). Validation of the differential expression in S cells for a subset of them is shown. *BX004987.5*, *CTD-2021J15.2*, and *RP11-314P12.2* were elevated with senescence (Fig. 3B) and *MALAT1*, *XIST*, *RP11-394O4.4*, *RP11-255A11.21*, and *MIAT* (also known as *GOMAFU*) were lower with senescence (Fig. 3C). Since the magnitude of changes in RNA levels differed between the RNA-Seq and RT-qPCR analyses, we assayed lncRNA changes by Northern blot analysis. As shown, *MALAT1* and *MIAT* were indeed reduced in S relative to P cells (Fig. 3D). As above, in WI-38 fibroblasts rendered senescent by DNA damage (IR) and in S and P IMR-90 fibroblasts, the relative expression levels of several previously reported SAL-RNAs largely recapitulated the trend seen in P and S fibroblasts (Fig. S1F,G and Fig. S2C,D).

The impact of these two lncRNAs, *MALAT1* and *MIAT*, was tested by lowering their abundance in early-passage (~PDL 22) HDFs; 5 days after transfection, cells were split and re-transfected to ensure the downregulation of the non-coding RNA. As shown in Fig. 3E, by 10 days of silencing *MALAT1* or *MIAT* using small interfering (si)RNAs directed to the respective RNAs, which reduced their levels by 70% or 50%, respectively (Fig. 3E), cultures displayed an increase in the numbers of senescent, SA- β gal positive cells (Fig. 3F, *left*) and changes in protein markers of senescence [p53 and p21 were upregulated, HuR and Sirt1 were downregulated (Fig. 3F, *right*)]. These results suggest that the reduction in some of these SAL-RNAs could directly contribute to implementing the senescent phenotype.

Identification of novel SAL-RNAs, both higher and lower in senescent cells

A number of novel SAL-RNAs were also identified in the RNA-Seq dataset as being upregulated in proliferating fibroblasts (Fig. 4A; supplemental Table S4); among them, an example of the RNA-Seq coverage plot (*XLOC_023166*, 'SAL-RNA1') and several validated transcripts of novel senescence-reduced lncRNAs are shown (Fig. 4B,C). Analysis

of the subcellular distribution of these senescence-downregulated SAL-RNAs by RT-qPCR analysis indicates that several of the transcripts were predominantly nuclear (e.g., *XXLOC_023166*, *XLOC_018371*, *XLOC_007522*, *XLOC_045655*, and *XLOC_064216*), while others (*XLOC_068343* and *XLOC_041539*) showed extensive presence in both the nucleus and the cytoplasm, with modest change in their subcellular distribution in senescence (Fig. 4D); the predominantly cytoplasmic lncRNA *7SL* and nuclear lncRNA *7SK* were included as controls in the fractionation experiment.

A comparably long list of SAL-RNAs was found to be upregulated in senescent fibroblasts (Fig. 5A, supplemental Table S5). A coverage plot for one such SAL-RNA showing higher levels in senescent cells (*XLOC_025931*; 'SAL-RNA2') and a number of validated SAL-RNAs are shown (Fig. 5B,C). Many validated lncRNAs in this group showed a primarily nuclear presence (e.g., *XLOC_025932*, *XLOC_024912*, *XLOC_025922*, *XLOC_025931*), but most of them are found in both the nucleus and the cytoplasm, with modest changes in the subcellular distribution during senescence. The nuclear lncRNA *XIST* was included as a control. Analysis in other models of senescence, the aforementioned IR-treated (senescent) versus untreated (proliferating) WI-38 fibroblasts and IMR-90 fibroblasts at early (proliferating, PDL 25) and late (senescent, PDL 49), indicated that the trends of expression of novel upregulated and downregulated SAL-RNAs (Fig. 4,5) were also conserved among senescence models (Fig. S3). Limited SAL-RNA analysis by Northern blotting is shown in Fig. S4. In sum, our results suggest that the RNA-Seq approach effectively identifies lncRNAs differentially expressed during senescence (Fig. 5D).

Modulation of cell fate by SAL-RNAs

In order to begin to study if SAL-RNAs affect any aspect of cellular senescence (e.g., proliferation, survival, gene expression, SA- gal activity), we designed siRNAs directed at *SAL-RNA1*, *SAL-RNA2*, or *SAL-RNA3* (*XLOC_025918*, all three sequences in supplemental Fig. S5), which achieved reductions to ~50% of the original transcript concentration by 5 days after transfection (Fig. 6A). Other lncRNAs tested (*XLOC_025932* and *XLOC_018371*) did not achieve sufficient silencing (not shown). As shown in Fig. 6B, by 5 days after transfecting senescent fibroblasts with the siRNAs, silencing *SAL-RNA2* or *SAL-RNA3* caused changes in cell morphology (Fig. 6B) and lowered cell numbers significantly (Fig. 6C). These changes were consistent with increased apoptosis, which was confirmed by the enhanced levels of cleaved PARP [Poly (ADP-ribose) polymerase], a marker of apoptosis (Fig. 6D) and by the increased expression of p53 (Fig. 6E). By contrast, 5 days after silencing *SAL-RNA1*, cells still displayed a senescent phenotype and cell numbers did not decline compared with the control group (Fig. 6B,C); no PARP cleavage was seen in these cells, underscoring the absence of apoptosis in this transfection group (Fig. 6D). However, the levels of p53 protein and mRNA also rose in early-passage fibroblasts 10 days after transfection with *SAL-RNA1*-directed siRNA (Fig. 6F); interestingly, in these fibroblasts, silencing *SAL-RNA1* elicited a senescent phenotype, as revealed by the enhanced expression of senescence protein markers p21 and p16 and the accumulation of SA- gal-positive cells (Fig. 6G). Together, these results indicate that *SAL-RNA2* and *SAL-RNA3* (*XLOC_025931* and *XLOC_025918*) likely protect the survival of senescent fibroblasts, while *SAL-RNA1* (*XLOC_023166*) helps to prevent the untimely onset of senescence.

DISCUSSION

We have used RNA-Seq to identify lncRNAs differentially expressed in senescent relative to proliferating human diploid fibroblasts. The collections of lncRNAs showing altered levels included several antisense transcripts (NA-SAL-RNAs), several pseudogene-encoded (PE-SAL-RNAs), and previously described lncRNAs (Figs. 1–3; Figs. S1–S3). Numerous

novel lncRNAs were also found, both increased and decreased with replicative senescence (Figs. 4,5). Among this group, three randomly chosen SAL-RNAs were found to affect the fate of the cell. While lowering each of the three triggered an increase in p53 levels, only two of them (*SAL-RNA2* and *SAL-RNA3*) were implicated in cell survival, while the third (*SAL-RNA1*) delayed senescence.

At present, it is unclear how many SAL-RNAs influence cellular senescence and what their relative impact is. Some SAL-RNAs likely do not affect directly the senescent phenotype. However, many other SAL-RNAs likely affect one or another senescence trait, including terminal growth arrest, cell survival, autophagy, DNA damage and repair, and senescence-associated protein expression patterns. Additional experiments are needed in order to establish which SAL-RNAs have stronger impact upon the process of cellular senescence and through which senescence trait. In this regard, SAL-RNAs are expected to function in coordination with other proteins (for example, RBPs and transcriptional regulators) and with other ncRNAs in order to control the aforementioned aspects of the senescence program. However, the most likely influence of SAL-RNAs is on senescence-associated gene expression programs. The specific gene expression pathways affected by the SAL-RNAs are unknown, but likely include those reported for other lncRNAs (Wapinski and Chang, 2011; Harries *et al.*, 2012; Yoon *et al.*, 2012), such as changes in chromatin structure, transcription factor activity, post-transcriptional gene regulation, and perhaps also post-translational gene regulatory processes. In a recent example of the latter function, the lncRNA *MALAT1* was reportedly reduced in senescent fibroblasts, in keeping with the observation that lowering *MALAT1* triggered cellular senescence in fibroblasts (Tripathi *et al.*, 2013). This effect was attributed in part to the decline in the oncogenic transcription factor b-Myb/Mybl2 caused by aberrant B-MYB pre-mRNA splicing when *MALAT1* levels were low. In turn, the reduced levels of b-Myb reduced the progression of cells through the G2/M cell cycle compartments and promoted cellular senescence (Tripathi *et al.*, 2013). It was interesting to note that longer lncRNAs in all groups (including NE-SAL-RNAs, PE-SAL-RNAs and novel and previously reported SAL-RNAs) showed a tendency to be downregulated in S cells, while shorter lncRNAs showed a tendency to be upregulated in S cells. Additional work is needed to fully elucidate the mechanisms that explain this pattern, but it is tempting to speculate that lncRNAs follow the same general rules of stability as eukaryotic mRNAs (e.g., human) and prokaryotic mRNAs (e.g., *E. coli*), where a negative correlation was noted between long mRNAs and stability (Feng and Niu, 2007). Perhaps RNA endonucleases and/or mechanical damage could also lower the abundance of longer lncRNAs, as proposed by Feng and Niu for mRNAs (2007); in light of our results, this differential stability appears to be more pronounced in senescent cells (Fig. 1D, 2A, 3A, S4, S5).

The results of experiments testing three chosen examples of SAL-RNAs (*SAL-RNA1*, *SAL-RNA2*, and *SAL-RNA3*) indicate that these lncRNAs could have a direct impact on the senescent phenotype by promoting cell survival (*SAL-RNA2*, *SAL-RNA3*) and by preventing premature senescence (*SAL-RNA1*). However, the specific mediators through which they modulate these cellular processes are unknown. The identification of the proteins or RNAs that interact with these transcripts after they are labeled with MS2 RNA or with another RNA tag represents a systematic way forward in this analysis. Such an approach will reveal critical factors that help elicit SAL-RNA function, whether it be one of scaffold for assembly of macromolecular complexes (e.g., chromatin remodeling machinery), competitor of another gene expression factor (e.g., a microRNA), or other regulatory functions. High-throughput analyses to find the specific changes in gene expression programs (e.g., mRNA profiling or protein profiling) taking place after silencing or overexpression of these SAL-RNAs are also important. Down the road, searches for disease-associated mutations in these transcripts, as well as the generation of knockout mice in which these transcripts are deleted may also be potentially informative. Additionally, it will

also be important to investigate the mechanisms that control the expression of *SAL-RNA1-3*, including the transcription factors and post-transcriptional regulators that affect their levels and their subcellular distribution.

Finally, it remains to be studied if the SAL-RNAs identified here in cultured models of cellular senescence are also differentially expressed and influence senescence *in vivo* in tissues and organs. With increasing appreciation that senescence underlies many of the changes that characterize the aging process (Kuilman *et al.*, 2010; Baker *et al.*, 2011) further studies of the possible implication of SAL-RNAs in age-associated disease are warranted.

EXPERIMENTAL PROCEDURES

Cell culture, transfection, small interfering RNAs and SA- β -galactosidase activity assay

WI-38 human diploid fibroblasts were obtained from Coriell Cell Repositories and in DMEM (Invitrogen) supplemented with 10% (v/v) FBS, antibiotics, and 0.1 mM non-essential amino acids (Invitrogen). All siRNAs were transfected at 20 nM final concentration using Lipofectamine-2000 (Invitrogen); UUCUCCGAACGUGUCACGUGdTT (Ctrl siRNA) was from Ambion. Duplexes (dsiRNA, each sense and antisense, respectively) obtained from Integrated DNA Technologies were used to silence *MALAT1* [GGAGCAGAGAGGUAUGGGAAGCAGA and CCUCGUCUCUCCAUACCCUUCGUU], *MIAT* [CUGUUUAAACAUUUCCACUUGCCAG and GACAAAUUUGUAAAGGUGAACGGUC], *XLOC_023166/SAL-RNA1* [CAUGCAUAUCAGCUCAGGUCUUAA and GUACGUAUAGUCAGUCCAGAAUU], *XLOC_025931/SAL-RNA2* [UUCUUUCCAGAUUUGUGUCACCUG and AAGAAAGGUCUAAACACAGUGGAG], and *XLOC_025918/SAL-RNA3* [UUUUGCUAAGUUCCACGAUCAGC and AAAACGAUUAAGGGUGUCUAGUCG]. In each case, 5 days after transfection, cells were split and re-transfected to ensure the downregulation of SAL-RNAs. Senescence-associated β -galactosidase activity in WI-38 cells was assessed using a kit from Cell Signaling, following the manufacturer's protocol.

Western blot analysis

Whole-cell lysates, prepared in RIPA buffer, were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto PVDF membranes (Invitrogen iBlot Stack). Incubations with primary antibodies recognizing p21 (Millipore), HuR, Sirt1, p53, p16, cleaved PARP, GAPDH (Santa Cruz Biotech) or β -actin (Abcam), were followed by incubations with the appropriate secondary antibodies conjugated with horseradish peroxidase (HRP; GE Healthcare) and by detection using enhanced luminescence (GE Healthcare).

RNA isolation, RT-qPCR and Sequencing

Trizol (Invitrogen) was used to extract total RNA from young proliferating and senescent cells according to the manufacturer's protocol. Total RNA was used for gene expression analysis by reverse transcription (RT) followed by quantitative (q)PCR analysis or by RNA deep sequencing (RNA-Seq). RT was performed by using random hexamers and reverse transcriptase (Maxima Reverse Transcriptase, Fermentas) and qPCR was carried out using gene-specific primers and SYBR green master mix (Kapa Biosystems) in an Applied Biosystems 7300 instrument.

For RNA sequencing, total RNA quality and quantity was assessed using the Agilent 2100-Bioanalyzer; 100 ng of RNA was used for first-strand and second-strand cDNA synthesis followed by single-primer isothermal amplification (SPIA) using NuGEN Ovation RNA-Seq

System V2 kits according to the manufacturer's protocol. The kit amplified both polyA-tailed and non-polyA-tailed RNA and removed ribosomal RNA. The amplified cDNA was sheared using Bioruptor (Diagenode) to an average size of 250–450 bases. The sequencing library was prepared using Illumina ChIP-Seq kits according to the manufacturer's protocol (Illumina, San Diego, CA). In short, the ends of the fragments were repaired using T4 DNA polymerase, E. coli DNA Pol I large fragment (Klenow polymerase), and T4 polynucleotide kinase (PNK) and adenines were added to the 3' end. Adapters were ligated to the DNA fragments, which were size-selected (250–300 bases) after electrophoresis through a 4% agarose gel. Eighteen cycles of PCR amplification was performed, followed by cluster generation and sequencing with Illumina Genome Analyzer (GA-II). Sequencing was performed for 42 cycles and the images generated were analyzed with the Firecrest program followed by base calls using the Bustard program; Firecrest and Bustard are part of the Illumina Analysis Pipeline package.

For RNA-Seq analysis, the quality of the bases was checked using FASTQC program and called bases were aligned to the human HG19 genome using the Tophat program, the Bowtie1 algorithm, and Ensembl hg19 (v62) as gene model annotations followed by genomic mapping (Trapnell *et al.*, 2010). The aligned reads were assembled into transcripts (both known and novel) using Cufflinks program with Ensembl hg19 (v62) transcripts as a guide (Roberts *et al.*, 2011a). FPKM (fragments per kilobase of exon model per million mapped reads) values were calculated after fragment bias correction and normalization to total hits. Significant changes in transcript expression levels were calculated using Cuffdiff program with a cut-off of $fdr < 0.1$ and minimum number of 5 alignments. Data were visualized in the UCSC genome browser (Roberts *et al.*, 2011b).

Northern blot analysis

Northern blot analysis was performed as previously described (Abdelmohsen *et al.*, 2007). Briefly, whole-cell RNA was isolated by using Trizol (Invitrogen), denatured, size-fractionated using 1.2% agarose-formaldehyde gels, and transferred. Oligonucleotides TTGCCGACCTCACGGATT for *MALAT1*, CACCAACTCTCCCACTAGGCTATAA for *MIAT*, and CCAATGGATCCTCGTTAAAGGATTT for 18S rRNA were end-labeled with [³²P]dATP and terminal transferase and used to detect these RNAs.

lncRNA PCR sequencing

PCR sequencing primers TGCATGTGTGTGTGTGTGTG and CTCTGGGAATCTGGAACCAA (forward and reverse, respectively) were used to amplify *SAL-RNA1/XLOC-166*. The PCR product was resolved by electrophoresis through a 2% agarose gel and the DNA was extracted from the gel using QIAquick Gel Extraction Kit (Qiagen). The purified PCR product (500 ng) was combined with 6 μ l of BigDye Terminator v1.1/3.1 Sequencing Buffer, and 3.2 pmol of forward or reverse primer as used for qPCR. The PCR sample was purified and analyzed using an ABI sequencer; sequences were analyzed by an ABI sequence scanner.

Primer pairs for lncRNA RT-qPCR

Primer pairs (all forward and reverse, respectively) were as follows:

CTD-2021J15.2: TGCTCATGACTCCTCTGTGG and
GACCAGAAGTCGTGCCTAGC

BX004987.5: CCATCGGCTGTAAACTTGGT and CAGAATATGGGCCAGCCTTA

RP11-314P12.2: CACAGGAGGATGTGTTGTG and
GAGGCTGCTGCAAGTTCAAGGTC

RP11-39404.2: TGCTTCCAGCACTGATGTTC and CCCCATCCTACCCTTCATTT

RP11-255A11.21: TATGTTGCCACCATCTTGGA and
CATAGGCCCTGCAGAAACAT

XLOC_023166/SAL-RNA1: TGCATGTGTGTGTGTGTGTG and
CTCTGGGAATCTGGAACCAA

OSTN-AS1-001: AGACGGGGTTTCACCATATT and
GGTGCAGTGGTTCACAGTTG

LSAMP-AS1-001: ATGATGAGGCAGCAAGAAGG and
ACAGTTCTGGGGTTGTTGGA

VCAN-AS1-001: TGCATTGATCGGTAACCTTATG and
AGGCCTGAATGGAGGACTTT

RP11-346A9.1: CTCTGGACAGGCAGAGGACT and
CCTAAGGAGCGTGAGGAGTG

AC079949.1: GCCTCGGATACCTCGGATAG and ATGGTTTAGCGCCAGGTTC

RP11-262D11.2: ATGCAAAGAGGGGAAAACCT and
TTGGAAGGTGGGTCCTAGTG

RP11-30B1.1: GGCCACATACCTGCAAATCT and GTGCCCTGACCTTGTGTTTGT

HNRNPA1P29: TGGTGGCATCAAAGAAAACA and
GAATCACGGCCATCAAAAAGT

CEACAMP10: TGGGAAAACCTTGTACCTG and
AGGCATGAGCAAGGACAGTT

CEACAMP11: CCCAGGCTAAAGATGTCCAA and
GGGTCCTGTGGCTGGTACT

XLOC_068343: CACGTTACCAAGTCGTCTG and
GGAGAGGGGCAGGAATTAAG

XLOC_018371: AGGAGCAAACCATCAGGAA and
GGCAGTGATTGTCTCCGAAT

XLOC_007522: GCCAAGGCCTGTGTAAATGT and
CTGGCCTGCTGATTTATGGT

XLOC_045655: CAAGGAAGGGCAGGAGATTA and
CTTTTTGGGCAGCTAGCACT

XLOC_041539: GTCTCCAGCATGACGTCTCA and
CCAGGCCTTAGGAGACAGG

XLOC_064216: GCTGGAAGCAAGGTTGAGAA and
CATGAGGCTCAGTTCTGTCTG

XLOC_014487: CTTCCCCTCAAATCCTGACA and
GGACAGAAGAACGGGTGAGA

XLOC_025927: TGATTTCTGGCCATTTTACA and
ACACCAGTCCACCTCCTCAC

XLOC_025919: GCACAAATGGAGCAGTCAAG and
GACAGGTAGAGGTGCACACG

XLOC_024912: ACAGAGAGCCTCGGTGGAT and AGGGTGCCTACATCCTTGGT

XLOC_025922: GTTGGAGTACAGTGGCATGA and
TGGTGGTACACACTTATGGTCCT

TP53: AGGCCTTGGAAGCAAGGAT and TGAGTCAGGCCCTTCTGTCT

7SK: GACGACCATCCCCGATAGAG and GGGAGCGCAGCTACTCGTAT

7SL: CAAAACCTCCCGTGCTGATCA and GGCTGGAGTGCAGTGGCTAT

Malat1: GCAGGCGTTGTGCGTAGAG and TTGCCGACCTCACGGATT

Xist: GTGTGTGAGTGTACCTACCGCTTT and CGACTAGCCCTAAGCCGAGTT

GAPDH: ATGGAAATCCCATCACCATCTT and CGCCCCACTTGATTTTTGG

MIAT: TCCTGAAACTCCTCTTTGTTTAACTG and
CACCAACTCTCCCACTAGGCTATAA

XLOC_025918/SAL-RNA3: ACTGCTGGGATAACGGTGAC and
TCTGTGCTCAGCTCTGCAAT

XLOC_025932: TGAAGAAGCAAGAGCCATGA and
TGGCACCACTGTGACTTGAT

XLOC_025931/SAL-RNA2: GGATGCTGTGAGCTTTGTGA and
GAAACCCCCAGAGCTGAGAC

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CR	coding region
RBP	RNA-binding protein
RISC	RNA-induced silencing complex
RNP	ribonucleoprotein complex
UTR	untranslated region
miRNA	microRNA

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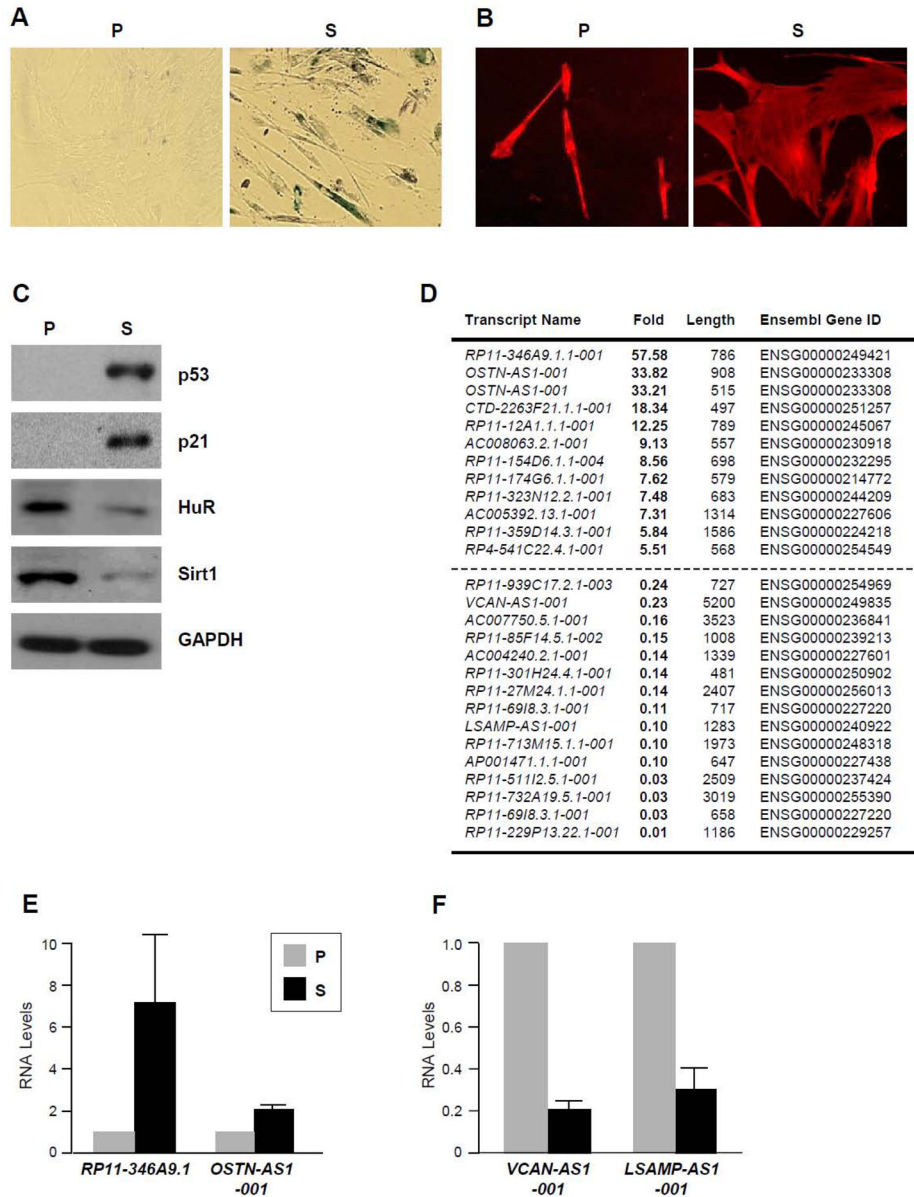


Figure 1. Characterization of proliferating and senescent WI-38 human diploid fibroblasts
(A) Micrographs to visualize senescence-associated -galactosidase activity in early-passage, proliferating (P) [population doubling (PDL) 20], and late-passage, senescent (S) (PDL 52) WI-38 cells. **(B)** Detection of F-actin to visualize the cytoskeleton using rhodamine phalloidin in fixed and permeabilized WI-38 cells. **(C)** Western blot analysis of the levels of p21, p53, HuR, Sirt1, and loading control -Actin in P and S cells. The even loading and transfer of the samples was confirmed by Coomassie blue and Ponceau red staining of the gel and membrane, respectively (not shown). **(D)** Naturally occurring antisense transcripts (NA) lncRNAs differentially expressed in P relative to S WI-38 fibroblasts, as identified by using RNA-Seq analysis; NA-SAL-RNAs elevated in S cells (*top*) and in P cells (*bottom*) are listed. **(E,F)** Individual validation of NA-SAL-RNAs displaying higher abundance in S cells (E) or in P cells (F).

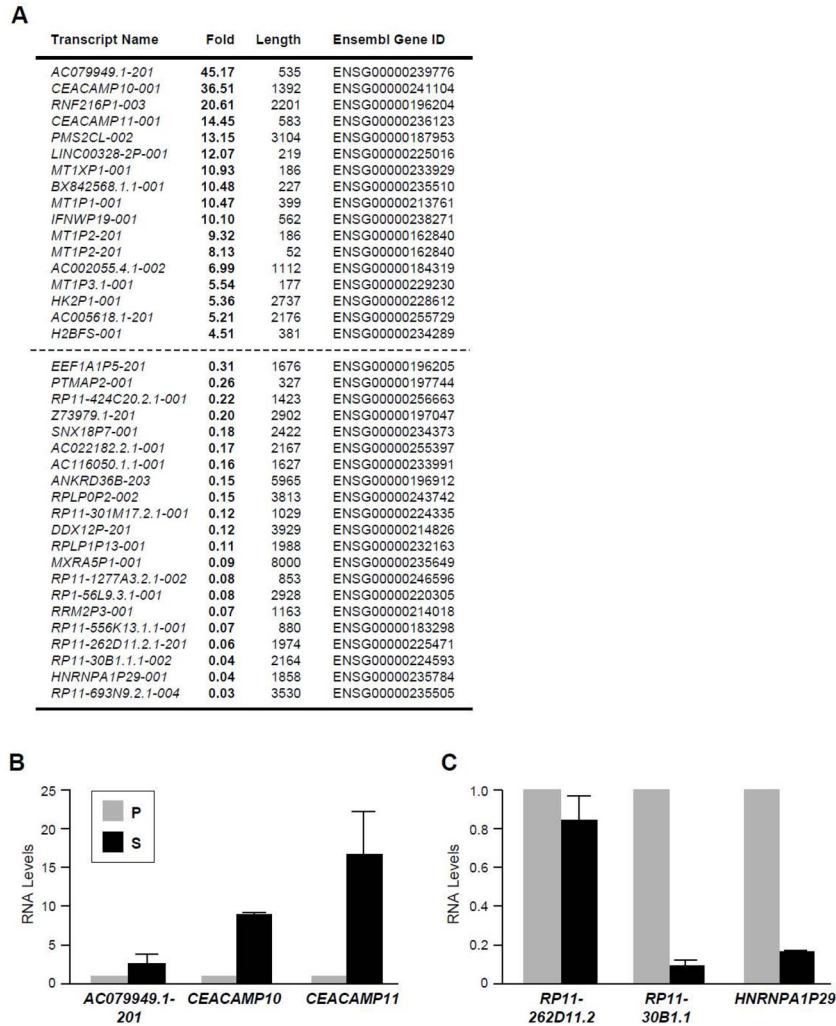


Figure 2. Pseudogene-encoded transcripts differentially expressed in proliferating and senescent WI-38 fibroblasts

(A) Pseudogene-encoded (PE)-SAL-RNAs transcript lncRNAs differentially expressed in P relative to S WI-38 fibroblasts, as identified by using RNA-Seq analysis; PE-SAL-RNAs elevated in S cells (*top*) and in P cells (*bottom*) are listed. (B,C) Individual validation of PE-SAL-RNAs displaying higher abundance in S cells (B) or in P cells (C).

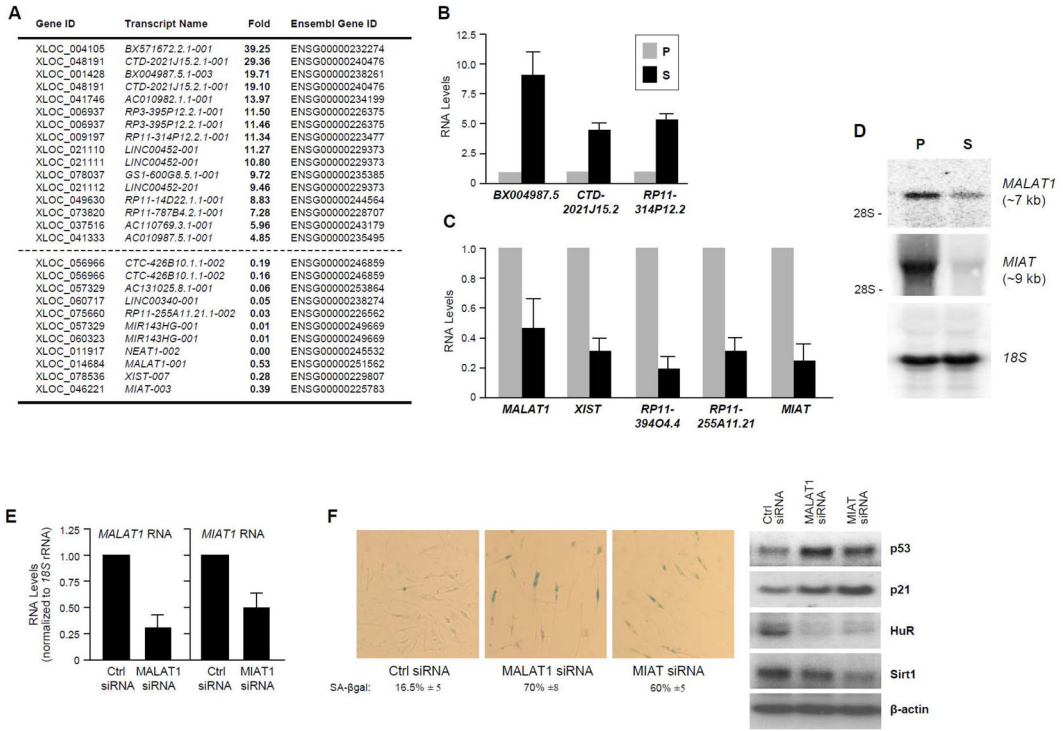


Figure 3. Known lncRNAs differentially expressed in proliferating and senescent WI-38 fibroblasts
(A) LncRNAs differentially expressed in P relative to S WI-38 fibroblasts, identified by using RNA-Seq analysis; lncRNAs elevated in S cells (*top*) and in P cells (*bottom*) are listed. **(B,C)** Individual validation of lncRNAs displaying higher abundance in S cells (B) or in P cells (C). **(D)** Northern blot analysis of the levels of lncRNAs *MALAT1*, *MIAT*, and loading control *18S* rRNA; the position of *28S* rRNA is indicated for size reference. **(E,F)** Ten days after transfection with siRNAs to silence *MALAT1* or *MIAT*, the levels of remaining lncRNA were monitored by RT-qPCR analysis (E), and the senescent phenotype was assessed by assessing SA- gal-positive cells (quantified below) and protein senescence markers p21 and p53 (upregulated), as well as HuR and Sirt1 (downregulated) (F).

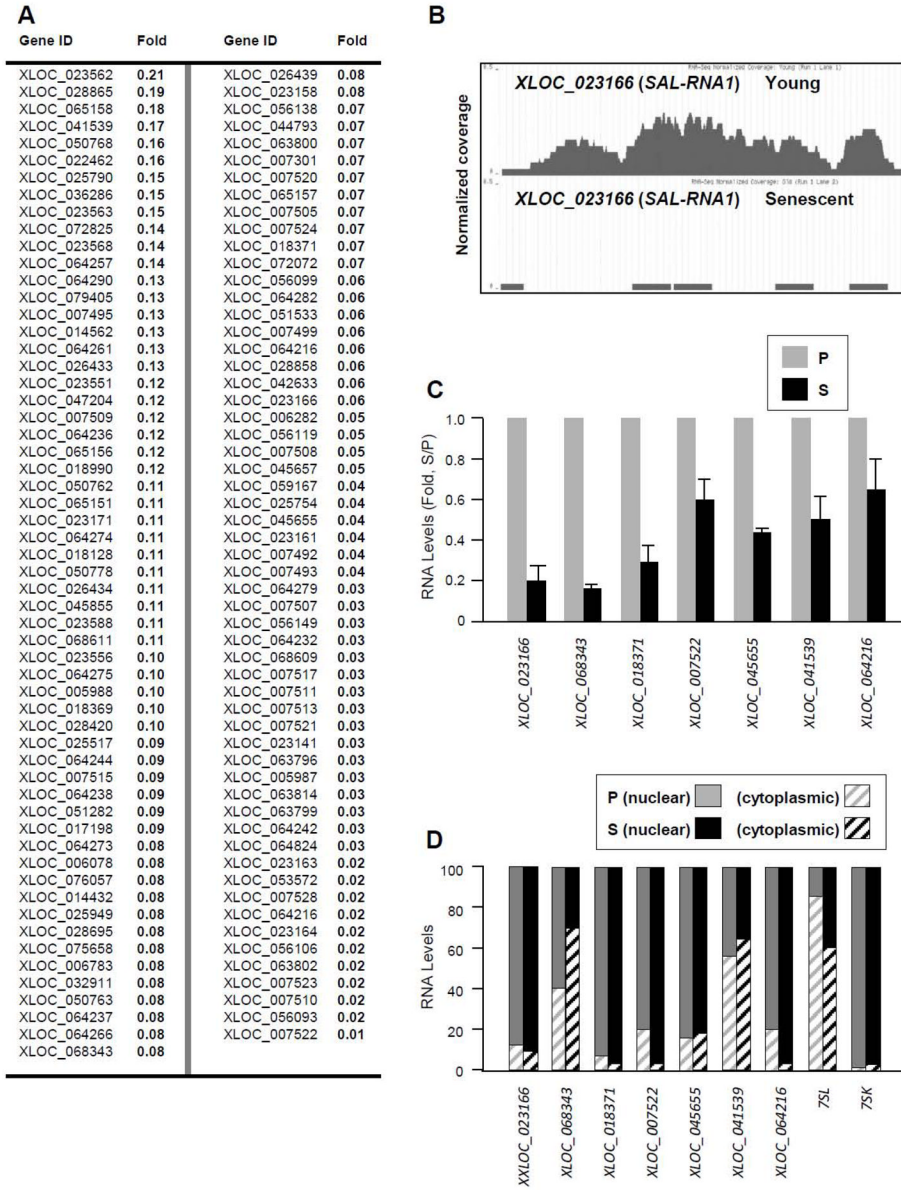


Figure 4. Novel SAL-RNAs preferentially expressed in proliferating WI-38 fibroblasts
(A) SAL-RNAs more abundant in P than in S WI-38 fibroblasts, identified by using RNA-Seq analysis. **(B)** Example of a novel SAL-RNA discovered by RNA-Seq analysis of WI-38 fibroblasts; coverage plot illustrating the increased presence of *XLOC_023166* (*SAL-RNA1*) in P compared with S cells. **(C)** Validation of seven novel SAL-RNAs preferentially expressed in P cells. **(D)** Relative abundance in nucleus and cytoplasm of the SAL-RNAs measured in (C). The mainly cytoplasmic lncRNA *7SL* and the mainly nuclear lncRNA *7SK* were included as controls for sample fractionation.

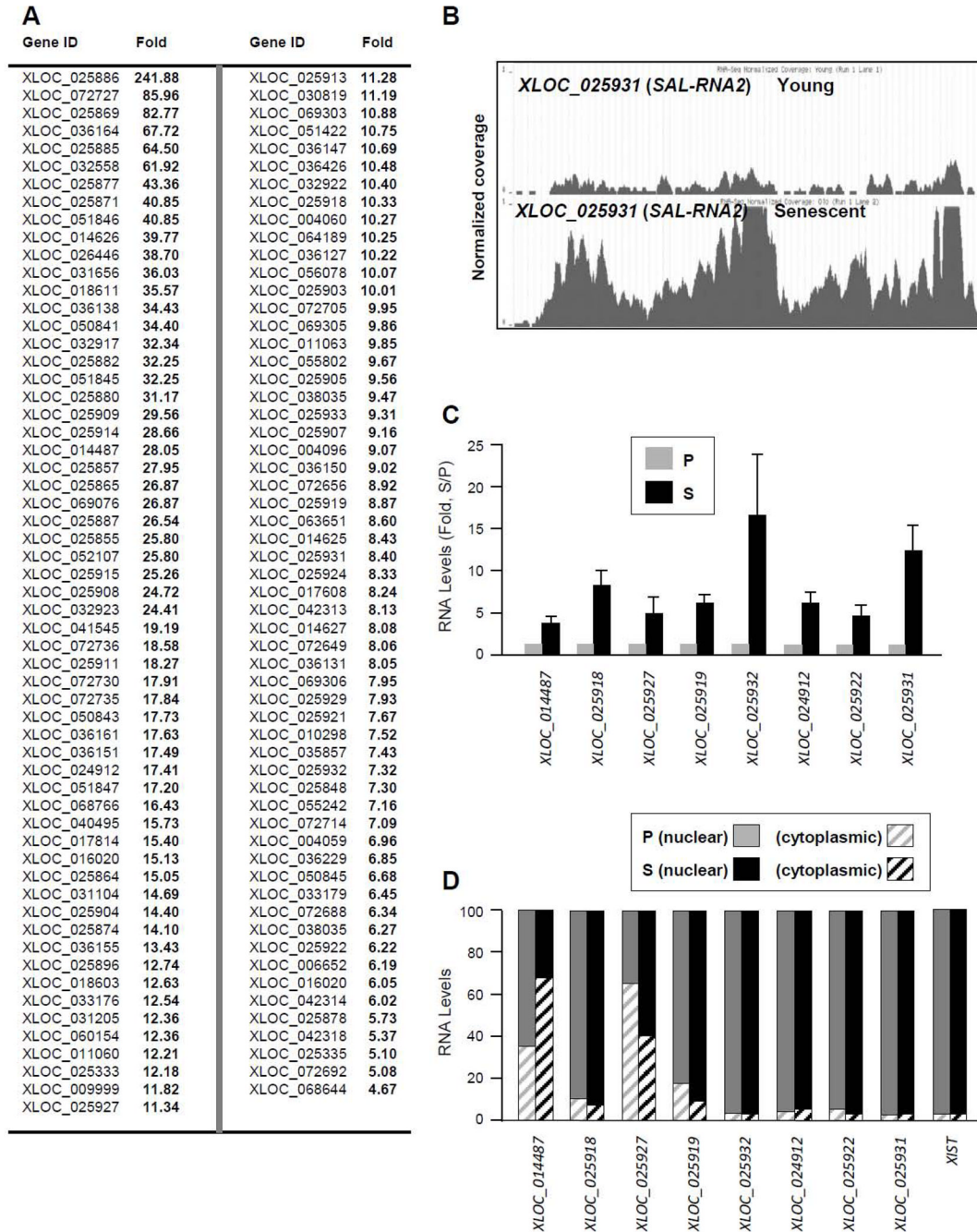


Figure 5. Novel SAL-RNAs preferentially expressed in senescent WI-38 fibroblasts

(A) SAL-RNAs more abundant in S than in P WI-38 fibroblasts, identified by using RNA-Seq analysis. (B) Example of a novel lncRNA discovered by RNA-Seq analysis of WI-38 fibroblasts; coverage plot illustrating the increased presence of *XLOC_025931* (*SAL-RNA2*) in S compared with P cells. (C) Validation of seven novel SAL-RNAs preferentially expressed in S cells. (D) Relative abundance in nucleus and cytoplasm of the SAL-RNAs measured in (C). The mainly nuclear lncRNA *XIIST* was included as a control for sample fractionation.

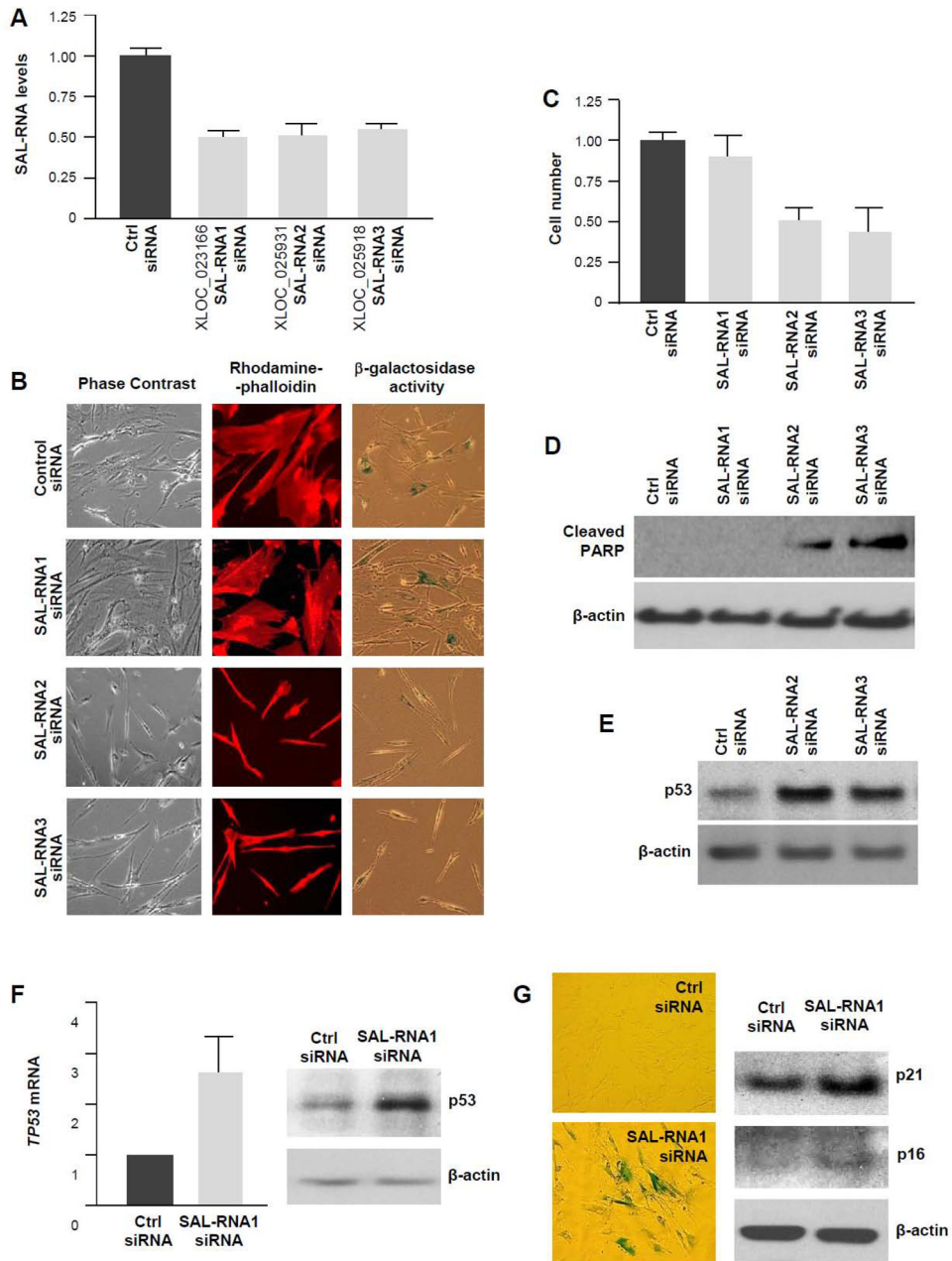


Figure 6. Novel SAL-RNAs preferentially expressed in senescent WI-38 fibroblasts

(A) Degree of silencing achieved 5 days after transfecting WI-38 cells with siRNAs directed at *SAL-RNA1*, *SAL-RNA2*, and *SAL-RNA3*, as assessed by RT-qPCR analysis. (B–D) In cells treated as explained in (A), F-actin was detected using rhodamine phalloidin in order to visualize the cytoskeleton (B), SA- gal activity was assessed in order to monitor cellular senescence (B), proliferation was measured by monitoring changes in cell number (C) and apoptosis was examined by measuring the level of cleaved PARP using Western blot analysis (D). (E) Western blot analysis to detect the levels of p53 in WI-38 10 days after silencing SAL-RNAs preferentially expressed in senescent cells. (F,G) By 10 days after transfecting WI-38 fibroblasts (P), with *SAL-RNA1*-directed siRNA, the levels of *TP53* mRNA and p53 were measured by RT-qPCR and Western blot analyses, respectively (F), and senescent cells were visualized by assessing SA- gal-positive cells (G, left) and protein

senescence markers p21 and p53 (upregulated), as well as HuR and Sirt1 (downregulated) (G, *right*).