A novel approach to assess the dynamics of extra-chromosomal circular ribosomal DNA in human cells

Joerg Leheste, Emily Forbes, Kristin DiGregorio, Victoria Katz, Alyssa Miceli, Christopher Husko, Raddy L Ramos, German Torres

Several nutrient-signaling pathways that extend life span have been described in model organisms. Thus, parallel and redundant signaling pathways that are similar across species might be subject to experimental manipulation. Here, we develop a PCR-based technique for testing the hypothesis that mitotic accumulation of extra-chromosomal ribosomal DNA circles might also determine life span in human cells. Using resveratrol, a phytochemical that counters age-related signs, we find treatment-dependent subcellular accumulations of extra-chromosomal 5S ribosomal DNA in human cell lines. These data suggest an association between DNA circles and intrinsic aging and demonstrate the utility of a PCR-based technique for studying the accumulation of dysfunctional molecules that promote senescence.

PeerJ PrePrints | http://dx.doi.org/10.7287/peerj.preprints.638v1 | CC-BY 4.0 Open Access | rec: 26 Nov 2014, publ: 26 Nov 2014

Authors

Joerg R. Leheste[1+], Emily Forbes[1], Kristin DiGregorio[1], Victoria Katz[1], Alyssa Miceli[1], Christopher Husko[1], Raddy L. Ramos[1], German Torres[1]

[1] Department of Biomedical Sciences
New York Institute of Technology College of Osteopathic Medicine
Northern Boulevard
P.O. Box 8000
Old Westbury, NY 11568-8000
USA

[+] Corresponding author Joerg R. Leheste
Phone: (001) 516-686-3764
Fax: (001) 516-686-1454
E-mail: jleheste@nyit.edu

1. Introduction

Aging appears to be plastic and can be manipulated by genetic and nutritional intervention. For example, activation of the silent information regulator T1 (SIRT1) pathway increases the life span of model organisms such as yeast and mice. SIRT1 is a NAD^+-dependent deacetylase that directly links transcriptional regulation to intracellular metabolism (Howitz et al., 2003). Among the signaling cues that activate SIRT1 pathways is the polyphenol molecule, resveratrol. SIRT1 activation by resveratrol triggers a broad range of transcription factors and co-regulators that mediate key mechanisms in the cell cycle, cell growth and apoptotic and autophagic programs of cell death (Baur, 2010; Torres et al., 2008; Torres et al., 2011). Other intracellular mechanisms activated by SIRT1-dependent pathways are those related to DNA stability and DNA replication. For example, using the budding yeast (*Saccharomyces cerevisiae*) as a model for elucidating signaling pathways that control life span, Sinclair and Guarente (1997) showed that increased activity of SIR2 (the yeast ortholog of mammalian SIRT1) suppresses the gradual accumulation of extra-chromosomal circular DNA (eccDNA). As its name implies, eccDNA are circular molecules propagated extra-chromosomally from repetitive and non-repetitive genomic regions of various species including, humans (Cohen and Segal, 2009; Cohen et al, 2010; Flores et al., 1988; Meyerink et al. 1979).

As in yeast, human ribosomal DNA (rDNA) is also organized in tandem rDNA repeats with five 43 kb rDNA (*rna45s5*) clusters encoding a large 45S rRNA precursor that is post-transcriptionally processed into 28S, 18S and 5.8S rRNAs (Henderson et al., 1972; Gonzalez and Sylvester, 1995). 5S rRNA is encoded by a separate 5S rDNA (*rn5s1*) cluster with 100-150 copies of a 2.2kb rDNA tandem repeat (Little and Braaten, 1989; Sorensen and Frederiksen, 1991). Investigations into the genomic architecture of human rDNA clusters reveal significant meiotic re-arrangement of about 11% per generation per gene cluster (Strults et al., 2008), with 5SrDNA molecules undergoing significant replicative steps (Cohen et al., 2010). In humans, SIRT1 expression is also associated with rDNA re-arrangements suggesting a conserved composition and function of anti-aging pathways. However, there is little experimental evidence for these association pathways, in particular whether activation of SIRT1 or reduction of eccrDNA could be considered for the prevention of specific diseases. In addition, there is no sensitive platform for functional screening of human eccrDNA which allows for the precise and accurate analysis underlying the formation of eccrDNA molecules. To address this technical limitation, we developed and validated a PCR-based protocol in combination with a nuclear transport assay for the quantitative analysis of eccrDNA molecules. To further support its practical use, we tested whether this technique could identify the occurrence of additional eccDNA species in human cell lines.

2. Materials & Methods

2.1 Cell Culture, Drug Treatment and Collection of Human Cells

The following adherent human epithelial cell lines were grown under standard culture conditions at 37°C with 5% CO_2: HEK-293 (embryonic kidney, CRL-1673); SH-SY5Y (neuroblastoma, CRL-2266) and; MCF7 (mammary adenocarcinoma, HTB-22), (ATCC, VA, USA). HEK-293 and MCF7 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) and SH-SY5Y in a 1:1 mixture of Eagle's Minimum Essential Medium (EMEM), and F12 Medium. Additionally, all media were supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% glutamine (Glutamax), 1% anti-mycotic (Fungizone) and 1% non-essential amino acids. Cells were propagated 1:10 using 0.25% (w/v) Trypsin/0.53 mM EDTA upon reaching a cellular density of approximately 70% - 80% (tissue culture reagents and supplements: Invitrogen, Carlsbad, CA, USA). Experiments were conducted with cells on standard 6-well multi-well plates. Resveratrol was dissolved in DMSO at a concentration of 100 mM, aliquotted and stored in light-proof containers at - 20 °C. Resveratrol treatment (50 µM, final concentration), was initiated at a cellular confluence level of about 70% for 6h or 48h. For nuclear transport studies, the lectin wheat germ agglutinin (WGA) was used to block nuclear transport (0.1 mg/ml, final concentration; 12h) and the lectin concavalin A (ConA) as control (0.1 mg/ml, final concentration; 12h). At the end of a given treatment period, cells were washed with 1X PBS, scraped off their dishes and collected by centrifugation for further analysis or stored at -80 °C.

2.2 Resveratrol-dependent Changes in Gene Expression using Quantitative PCR (QPCR)

Following 48h of resveratrol-treatment of human cells (HEK-293, MCF7, SH-SY5Y) on multi-well dishes, cells were collected and total RNA was prepared using the RNeasy RNA-isolation system/Qia shredder according to the manufacturer's specifications (Qiagen, Valencia, CA, USA) and as previously described by our group (Torres et al., 2008). RNA concentrations and integrity were determined *via* standard spectrophotometry and agarose gel-electrophoresis. Reverse transcription of RNA into complementary cDNA was carried out with the Superscript III First Strand Synthesis System for RT PCR (Invitrogen, Carlsbad, CA, USA). QPCR was conducted on a Mastercycler ep gradient S (Eppendorf AG, Hamburg, Germany) using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) with a total volume for each sample of 20 µl. Gene-specific DNA primers were either used as previously reported (Murayama et al., 2008) or designed using the integrated DNA Technologies Primer Quest tool (IDT, Coralville, IA, USA). Expression was accessed for *sirt1*, *rn5s1* (5SrRNA) and *rna45s5* (45S pre-ribosomal RNA) using the following primers: *β-actin* forward: 5'-CAG CCA TGT ACG TTG CTA TCC AGG-3'; *β-actin* reverse: 5'-AGG TCC AGA CGC AGG ATG GCA TG-3'; *rn5s1* forward: 5'-GAT CTC GTC TGA TCT CGG AAG CTA AG-3'; *rn5s1* reverse: 5'-AAA GCC TAC AGC ACC CGG TAT T-3'; *rna45s5* forward: 5'-GAA CGG TGG TGT GTC GTT C-3' *rna45s5* reverse: 5'-GCG TCT CGT CTC GTC

TCA CT-3'; *sirt1* forward: 5'-CTG TAG ACT TCC CAG ATC TTC CAG-3'; *sirt1* reverse: 5'-GTG ACA GAG AGA TGG CTG GAA TTG-3'.

2.3 Extraction of total eccDNA and Relative Quantification of eccDNA Circles using QPCR

Resveratrol-treated HEK-293, MCF7 and SH-SY5Ycells and DMSO (solvent) controls were separated into their nuclear and cytoplasmic fractions according to the manufacturer's instructions (CelLytic Nuclear Extraction Kit, Sigma, St. Louis, MO, USA). Both fractions were exposed to standard Na-acetate/ethanol precipitation and carefully re-suspended in 100 µl TE buffer. To eliminate DNA chromosomes and linear fragments, individual samples were purified with the MinElute Reaction Cleanup Kit (Qiagen, Valencia, CA, USA). Then, samples were sequentially treated with exonuclease III (20U/µl, 1h, 37°C), RNase A (50 µg/ml, 30 min, 37°C) and proteinase K (100µg/ml, 1h, 37°C) followed by another MinElute Reaction Cleanup purification and a final elution in 20 µl of a 10 mMTris buffer, pH 8.5. QPCR was carried out as described above with the same DNA primer pairs for *rn5s1* and *rna45s5* only this time measured against *sstI* satellite and *alu* repeats (Cohen et al., 2010): *sstI* forward: 5'-GTG GTG GTG CAT GGC CCC C-3'; *sstI* reverse: 5'-GAG CTC CAG GAT CAC CAC AGC-3'; *alu* forward: 5'-GGC GGG CGG ATC ACG AGG TCA G-3'; *alu* reverse: 5'-CCC GGG TTC ATG CCA TTC TCC TG-3'.

2.4 Isolation and PCR-amplification of Nuclear and Cytoplasmic eccDNA

For the isolation of eccDNA, HEK-293 cells were grown to confluence on 6-well multi-well plates and then separated into nuclear and cytoplasmic fractions with the CelLytic Nuclear Extraction Kit (Sigma, St. Louis, MO, USA) followed by Na-acetate/ethanol precipitation as described above. All samples were sequentially purified and enzymatically treated with exonuclease III, RNase A and proteinase K as previously described. To linearize 5SrDNA circles (there is a single BamHI restriction site) one half of each sample (10 µl) was treated with BamHI for 4h, 37°C while the other half was sonicated 3 times with 10s pulses on wet ice (30% maximum power, 2mm tip; Cell Disruptor, Heat-Systems Ultrasonics, Plainview, NY, USA). Samples were then purified using the MinElute Reaction Cleanup Kit (see above) and eluted in a final volume of 10 µl. To generate blunt ends, samples were treated with T4-DNA polymerase and dNTPs (Roche Applied Science, Indianapolis, IN, USA) for 20 min at 16°C and immediately purified with the MinElute Reaction Cleanup Kit. EcoRI adaptor oligonucleotides were ligated to the linearized and blunt-ended DNA pieces for 18h at 16°C and excess adaptors subsequently removed by gel filtration using Sephacryl S-400 spin columns according to the manufacturer's instructions (both: Universal Riboclone cDNA Synthesis System, Promega, Madison, WI, USA). Derivatives of eccDNA were then amplified using a high fidelity PCR system (Expand 20 kb plus PCR System, Roche Applied Science, Mannheim, Germany) and DNA primer matching the EcoRI adaptor sequence (forward and reverse primer: AAT TCC GTT GCT GTC G; Promega, Madison, WI, USA).

Reactions destined for DNA cloning and sequencing were exclusively recruited from the sonicated samples and subject to PCR elongation reactions at 4 min. Reactions used for Southernblot analysis were recruited from sonicated as well as BamHI restricted samples and subject to 15 min PCR elongation reactions.

2.5 Southern blot Analysis of eccDNA

Linearized and amplified eccDNA samples were slowly spread on ethidium bromide-stained 0.8% agarose gels (20-30mA). Then, gels were bathed in 0.25M HCl for 15min followed by transfer solution (0.5 M NaOH, 1.5 M NaCl) for 30 min before being assembled for alkaline upward DNA transfer onto a BrightStar-Plus nylon membrane (Ambion/Life Technologies, Carlsbad, CA, USA) with transfer solution for 12-18h. After a 5 min-bath in neutralizing solution (0.5 M Tris•CL, pH8.0), the membranes were dried and baked at 80 °C between blotting paper for 3h. Then, blots were pre-hybridized with UltraHyb solution (Ambion/Life Technologies, Carlsbad, CA, USA) at 42°C for 30min and then hybridized for 12-16h with a PCR-generated and gel-extracted DNA probe specific for rDNA (Strults et al., 2008) labeled using the BrightStar Psolaren-Biotin System (Ambion/Life Technologies, Carlsbad, CA, USA). For signal evaluation, autoradiographs were scanned and images imported into Adobe Photoshop CS5.1 (Adobe Systems Incorporated, Mountain View, CA).

2.6 Identification and Screening of eccDNA in HEK-293 Cells

Linearized and amplified eccDNA samples were briefly separated on ethidium bromide-stained 1.5% agarose gels from which a fragment cluster ranging from approximately 100 bp – 4kb was removed using the QIAquick Gel Extraction Kit (Qiagen,Valencia, CA, USA). Purified DNA was ligated into the pGEM-T Easy vector system (Promega, Madison, WI, USA) and transformed into chemically competent *E. coli* DH5α (Invitrogen/Life Technologies, Carlsbad, CA, USA). Plasmid DNA from ampicillin-resistant and beta-galactosidase negative trans-formants (blue-white screening) was treated with EcoRI restriction endonuclease for 1h at 37°C and analyzed via standard agarose gel electrophoresis (1.5%) to confirm presence of an insert. Only confirmed clones were selected for DNA sequencing using standard T7 sequencing primer (T7 RNA polymerase promoter). DNA sequences were screened for identity and genomic origin with the "Basic Local Alignment Search Tool" (BLAST; http://www.ncbi.nlm.nih.gov/blast/). To determine a potential driving-force for eccDNA mobilization, all confirmed sequences were analyzed with the repetitive sequence screening tool CENSOR (Kohany et al. 2006; http://www.girinst.org/censor/index.php).

3. Results and Discussion

While yeast SIR2 has a stabilizing effect on the organism's rDNA locus, the exact role of SIRT1 on human eccrDNA dynamics is unknown. In line with our previous findings demonstrating a close association of SIRT1 with specific human DNA targets

(Torres et al., 2008; Torres et al., 2011), SIRT1 has been linked to the transcriptional regulation of rna45s5 by the energy-sensing eNoSC protein complex (Murayama et al., 2008; Song et al., 2013). In order to understand the epigenetic regulation of rn5s1, we determined the transcriptional activity of rn5s1 and rna45s5, as well as sirt1, in HEK-293 cells following resveratrol treatment for 48h (Fig. 1A). Consistent with our previous work, we found that the transcriptional activity of sirt1 increased by 1.9-fold ($P = 5x10^{-6}$), while expression of rn5s1 increased by 3.5 fold ($P = 0.02$) and rna45s5 by 1.8 fold ($P = 0.03$). Parallel experiments in MCF7 and SH-SY5Y cells showed similar outcome trends, but failed to reach statistical significance at the $P < 0.05$ level (data not shown). Whereas expression of rn5s1 has not been studied in this context, the expressional increase at rna45s5 loci is not consistent with findings related to the eNoSC protein complex, which is associated with heterochromatin formation and transcriptional repression (Murayama et al., 2008). This discrepancy may be related to the fact that Murayama's group manipulated the nucleomethylin component of the eNoSC protein complex, whereas we manipulated SIRT1 through resveratrol treatment. It should be noted, however, that it is not clear what the cellular dynamics are between SIRT1 and rna45s5 or rn5s1 in human cells. In gain-of-function mouse models of disease, over-expression of SIRT1 increases homologous recombination of the entire rodent genome (Palacios et al., 2010). This suggests that DNA stability/mobility depends, in part, on the activation of SIRT1-dependent protein complexes and signaling pathways, particularly in those cells involved in nutrient metabolism and cellular growth.

Due to the low abundance of human eccrDNAs, we sought to develop a quantitative PCR-based approach for maximizing eccrDNA content. Investigations into appropriate internal standards for linearized eccDNA molecules revealed human SstI satellite repeats as well as Alu repeats as potential candidates. The SstI family consists of 2.5 kb repeating units with approximately 400 copies within genomic clusters (Epstein et al., 1987). Alu repeats are approximately 280bp long and are the most common repetitive element with about 1 million copies per haploid genome. Their average genomic separation of only about 3 kb increases their potential for genomic re-arrangement and eccDNA formation in mammalian cells. Such non-tandem repeats have previously been detected in eccDNA pools of human cells (Jones & Potter 1985; Riabowol et al., 1985). As noted earlier, however, comprehensive characterization of eccDNAs has been severely hampered due to their relatively low abundance in human cells and tissues (Cohen et al., 2010), and the lack of convenient and high-throughput readout. When comparing Alu-eccDNA or SstI-eccDNA to β-actin expression in HEK-293 cells using our novel eccDNA isolation/cloning strategy in conjunction with quantitative PCR (QPCR), we reliably detected robust levels of Alu-eccDNA across cellular compartments, whereas nuclear SstI-eccDNA was detectable in most cases but rarely in the cytoplasm (data not shown). To ensure that the internal standard was unaffected by our independent variable, resveratrol-treated cells (50 μM in culture medium) and controls were separated into their cytoplasmic and nuclear fractions and processed to

isolate eccDNA and cDNA (via reverse transcription of RNA). Comparing Alu-eccDNA concentration against β-actin expression of the same cells resulted in a relative Alu-eccDNA index which was independently derived for resveratrol-treated and -untreated cells. We found that resveratrol treatment did not significantly affect the concentration of Alu-eccDNA in either the nuclear or cytoplasmic compartment (Fig. 1B). We therefore continued using Alu-eccDNA as an internal standard in subsequent experiments.

Next, we tested if resveratrol-dependent SIRT1 activation, which had caused significant transcriptional increases at both types of rDNA loci, could also trigger concomitant changes in the concentration of cellular eccrDNA from the aforementioned loci (Fig. 2A-C). For this experiment, we used either total nuclear or cytoplasmic eccDNA for QPCR with primers specific for rn5s1, rna45s5, and the Alu consensus sequence. In HEK-293, MCF7 and SH-SY5Y cells, the abundance of rn5s1-eccrDNA in the cytoplasmic fraction consistently increased by 2.2-fold ($P = 0.01$), 3.4-fold ($P = 0.002$) and 1.5-fold ($P = 0.048$), respectively. At the same time, we found that rn5s1-eccDNA in the nuclear fraction of HEK-293 cells was significantly decreased by about 60% ($P = 0.01$), but unaffected in the nuclear compartment of MCF7 and SH-SY5Y cells. Changes in rna45s5-eccrDNA were not statistically significant under any experimental condition (data not shown).

To independently confirm the presence of rn5s1-eccrDNA in HEK-293 cells, we analyzed subcellular eccDNA extracts amplified with long-range PCR through Southern-blot analysis (Fig. 2D) using a previously published and labeled rn5s1-eccrDNA probe for detection (Strults et al., 2008; Cohen et al., 2010). Initially in this process, the eccDNA samples had either been sonicated or treated with BamHI restriction endonuclease (rn5s1-eccrDNA has a unique BamHI restriction site) to open up circular DNA. All samples showed a hybridization signal in the 2.2 kb range which is consistent with the published length of one rn5s1-eccrDNA monomer (Little & Braaten, 1989). Cytoplasmic but not nuclear samples displayed an additional signal in the higher DNA fragment size range at approximately 12 kb. Due to an initial filtration step, such large DNA piece could have been only extracted if they were supercoiled or otherwise condensed. However, the specificity of the signal indicates multicopy rn5s1eccDNA which could result from recombination-dependent concatemeric rn5s1eccrDNA replication as previously described in Drosophila (Cohen et al., 2005). Both, the resistance to full BamHI restriction and cytoplasm-specific location of higher-order rn5s1-eccrDNA need to be addressed in future studies.

To further investigate the observed resveratrol-induced cytoplasmic rn5s1-eccrDNA increase in HEK-293 cells, we sought to distinguish between nuclear import and cytoplasmic replication. We thus implemented a 12h treatment regimen involving combinations of resveratrol (50 μM) and WGA (0.1mg/ml) to block nuclear transport, and control lectin ConA (0.1mg/ml) (Fig. 3 A, B). In cytoplasmic fractions, we confirmed an

252 average increase of 180% for rn5s1-eccDNA (P = 0.01) and 100% (P = 0.01) above
253 control levels with resveratrol alone and in combination with ConA, respectively. WGA
254 alone significantly reduced cytoplasmic rn5s1-eccrDNA to about 50% (P = 0.03) of
255 control levels, whereas WGA in combination with resveratrol restored levels to 80%
256 baseline. Resveratrol treatment without ConA depleted nuclear rn5s1-eccrDNA
257 significantly to about 30% (P = 0.01) of the control baseline. Treatment with WGA or
258 WGA in combination with resveratrol resulted in rescue frequencies of between 70%-
259 80% which were, however, not statistically significant. These results indicate transport of
260 rn5s1-eccrDNA across the nuclear envelope but do not rule out compound tributaries,
261 such as cytoplasmic rn5s1-eccDNA amplification which needs yet to be investigated.

262 While testing our novel isolation protocol in combination with standard
263 recombinant DNA technology, we were able to capture and analyze 48 eccDNA
264 molecules ranging from 420 bp to 870 bp (average: 636 bp). We traced eccDNAs to
265 chromosomes 1, 2, 4, 5, 6, 8, 9, 11, 12, 13, 14, 17, 20, X (HEK-293 cells are female)
266 without showing any particular prevalence and only 1 duplication. Sequence analysis
267 revealed that the majority of eccDNA came from unique, non-repetitive genomic loci
268 (Fig. 4). Three of the identified sequences originated from tandemly arranged gene
269 clusters: 5S rDNA (rn5S1) with an average copy number of 98 repeats (Stults et al.,
270 2007) and, once again, adding validity to our QPCR findings; protocadherin-α (pcdhα1)
271 with 15 repeats (Wu and Maniatis, 1999) and sperm protein 4 associated with the
272 nucleus on the X chromosome (spanx4) with 5 repeats (Kouprina et al., 2004). A total of
273 3 sequences were mitochondrial. The majority of the 45 non-mitochondrial sequences
274 (30) were intergenic, 12 mapped to intron/regulatory region boundaries and 3 to coding
275 exon/intron boundaries. The identity and characteristics of all 16 eccDNAs mapping to
276 specific genes, including rn5s1 (~300 bp upstream of transcriptional start site), are
277 summarized (Tab. 1). Through further analysis with the repetitive DNA sequence mining
278 tool CENSOR (Kohany et al., 2006), we found that the majority of the 33 genomic
279 sequences (31 unique; 2 tandem repeats) included 1-4 short repetitive DNA elements
280 (1.7 on average) of which 91% are defined as transposable elements and 9%
281 interspersed DNA repeats (aluS, aluJ). Of the transposable elements, 94% were class-I
282 retrotransposons, including long interspersed nuclear elements (LINEs; includes l1),
283 short interspersed nuclear elements (SINEs; most abundant class in mammals; includes
284 aluJb, aluJo, aluSc) and endogenous retroviruses (erv1, erv2). Only 6% were class-II
285 non-autonomous DNA transposons (hAT superfamily). We believe that the abundance of
286 these elements in the genome could contribute to the formation and mobilization of
287 eccDNA via homologous recombination (Smit, 1999). The 3 eccDNA clones that
288 overlapped with actual coding exons did not include any such repetitive DNA elements
289 and therefore belong to the recently discovered class of microDNAs (Shibata et al.,
290 2012). There is a possibility, that the identified eccDNAs are byproducts of discontinuous
291 lagging strand synthesis during replication. Shibata and colleagues (Shibata et al.,
292 2012), however, were able to link the formation of certain microDNAs with corresponding
293 micro-deletions making a case for excision from the genome. The finding of rn5S1 and

other tandemly arranged genes in our eccDNA isolates is in line with our newly-developed QPCR-based approach and in line with our Southern blot findings. The abundance of interspersed repeats and transposons is suggestive of an involvement in the mobilization of eccDNAs. This and their potential involvement in genetic mosaics and partial aneuploidy need to be investigated in subsequent studies. The confirmation of microDNAs in human eccDNA pools opens the possibilities of studying these molecules using our experimental protocols.

4. Conclusions

There are several competing hypotheses about human aging, the inevitability of death and underlying cellular and molecular mechanisms. Here, we are introducing a novel PCR-based approach with cultured human cells and reveal the subcellular dynamics of rn5s1-eccrDNA in response to resveratrol with important analogies to an aging paradigm involving eccDNA accumulation in yeast. These findings point to rn5s1-eccrDNA as a potential candidate in the search for a stimulus factor that regulates age-related maintenance of telomeres and other genomic mechanisms responsible for organismic senescence and death and also points to the possibility of using rn5s1-eccrDNA as therapeutic target for the treatment of age-related pathologies. The unexpected finding of other eccDNA molecules, such as the recently discovered class of microDNAs, emphasizes the dynamics of the human genome which may bring benefits to some DNA loci but detrimental damage others.

5. Acknowledgements

We thank all of our students and colleagues for their critical discussion of the project. This work was supported by intramural funding to Joerg R. Leheste.

6. References

- Baur JA, Chen D, Chini EN, Chua K, Cohen HY, de Cabo R, Deng C, Dimmeler S, Gius D, Guarente LP, Helfand SL, Imai S, Itoh H, Kadowaki T, Koya D, Leeuwenburgh C, McBurney M, Nabeshima Y, Neri C, Oberdoerffer P, et al. 2010. Dietary restriction: standing up for sirtuins. *Science* 2010 329: 1012-3.

- Buck SW, Sandmeier JJ, Smith JS .2002. RNA polymerase I propagates unidirectional spreading of rDNA silent chromatin. *Cell* 111: 1003-14.

- Cockell MM, Perrod S, Gasser SM. 2000. Analysis of sir2p domains required for rDNA and telomeric silencing in saccharomyces cerevisiae. *Genetics* 155: 2021.

- Cohen S, Agmon N, Yacobi K, Mislovati M, Segal D. 2005. Evidence for rolling circle replication of tandem genes in Drosophila. *Nucleic Acids Res* 33: 4519-26.

- Cohen S, Agmon N, Sobol O, Segal D. 2010. Extrachromosomal circles of satellite repeats and 5S ribosomal DNA in human cells. *Mob DNA* 1:11.

- Cohen S, Segal D. 2009. Extrachromosomal circular DNA in eukaryotes: possible involvement in the plasticity of tandem repeats. *Cytogenet Genome Res* 124: 327-38.

- Colman RJ, Anderson RM, Johnson SC, Kastman EK, Kosmatka KJ, Beasley TM, Allison DB, Cruzen C, Simmons HA, Kemnitz JW, Weindruch R. 2009. Caloric restriction delays disease onset and mortality in rhesus monkeys. *Science*. 325: 201-4.

- Collins K, Mitchell JR. 2002. Telomerase in the human organism. *Oncogene* 21: 564-79.

- Epstein ND, Karlsson S, O'Brien S, Modi W, Moulton A, Nienhuis AW. 1987. A new moderately repetitive DNA sequence family of novel organization. *Nucleic Acids Res* 15: 2327-41.

- Flores SC, Sunnerhagen P, Moore TK, Gaubatz JW. 1988. Characterization of repetitive sequence families in mouse heart small polydisperse circular DNAs: age-related studies. *Nucleic Acids Res* 16:3889-906.

- Flores SC, Sunnerhagen P, Moore TK, Gaubatz JW. 1988. Characterization of repetitive sequence families in mouse heart small polydisperse circular DNAs: age-related studies. *Nucleic Acids Res* 16: 3889-906.

- Fritze CE, Verschueren K, Strich R, Easton Esposito R. 1997. Direct evidence for SIR2 modulation of chromatin structure in yeast rDNA. *EMBO J* 16: 6495-509

- Froy O, Miskin R (2010) Effect of feeding regimens on circadian rhythms: implications for aging and longevity. *Aging* 2:7-27.

- Gaubatz JW. 1990. Extrachromosomal circular DNAs and genomic sequence plasticity in eukaryotic cells. *Mutat Res* 237: 271-92.

- Gonzalez IL, Sylvester JE. 1995. Complete sequence of the 43-kb human ribosomal DNA repeat: analysis of the intergenic spacer. *Genomics* 27: 320-8.

- Gotta M, Strahl-Bolsinger S, Renauld H, Laroche T, Kennedy BK, Grunstein M, Gasser SM. 1997. Localization of Sir2p: the nucleolus as a compartment for silent information regulators. *EMBO J* 16: 3243-55.

- Gottlieb S, Esposito RE. 1989. A new role for a yeast transcriptional silencer gene, SIR2, in regulation of recombination in ribosomal DNA. *Cell* 1989 56:771-6.

- Henderson AS, Warburton D, Atwood KC. 1972. Location of ribosomal DNA in the human chromosome complement. *Proc Natl Acad Sci* 69:3394-8.

- Hochstrasser T, Marksteiner J, Humpel C. 2012. Telomere length is age-dependent and reduced in monocytes of Alzheimer patients. *Exp Gerontol* 47:160-3.

- Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, Zipkin RE, Chung P, Kisielewski A, Zhang LL, Scherer B, Sinclair DA. 2003. Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. *Nature* 425:191-6.

- Hubbard BP, Gomes AP, Dai H, Li J, Case AW, Considine T, Riera TV, Lee JE, E SY, Lamming DW, Pentelute BL, Schuman ER, Stevens LA, Ling AJ, Armour SM, Michan S, Zhao H, Jiang Y, Sweitzer SM, Blum CA, Disch JS, et al. 2013. Evidence for a common mechanism of SIRT1 regulation by allosteric activators. *Science* 339: 1216-9.

- Jiang WQ, Zhong ZH, Henson JD, Neumann AA, Chang AC, Reddel RR. 2005. Suppression of alternative lengthening of telomeres by Sp100-mediated sequestration of the MRE11/RAD50/NBS1 complex. *Mol Cell Biol* 25: 2708-21.

- Jones RS, Potter SS. 1985. L1 sequences in HeLa extrachromosomal circular DNA: evidence for circularization by homologous recombination. *Proc Natl Acad Sci* 82: 1989-93.

- Kohany O, Gentles AJ, Hankus L, Jurka J. 2006. Annotation, submission and screening of repetitive elements in Repbase: Repbase Submitter and Censor *Bioinformatics* 7: 474.

- Kouprina N, Mullokandov M, Rogozin IB, Collins NK, Solomon G, Otstot J, Risinger JI, Koonin EV, Barrett JC, Larionov V. 2004. The SPANX gene family of cancer/testis-specific antigens: rapid evolution and amplification in African great apes and hominids. *Proc Natl Acad Sci* 101: 3077-82.

- Lam YY, Peterson CM, Ravussin E. 2013. Resveratrol vs. calorie restriction: Data from rodents to humans. *Exp Gerontol* 48: 1018-24.

- Little RD, Braaten DC. 1989. Genomic organization of human 5 S rDNA and sequence of one tandem repeat. *Genomics* 4: 376-83.

- Mair W, Dillin A. 2008. Aging and survival: the genetics of life span extension by dietary restriction. *Annu Rev Biochem* 77: 727-54.

- Mangialasche F, Kivipelto M, Solomon A, Fratiglioni L. 2012. Dementia prevention: current epidemiological evidence and future perspective. *Alzheimers Res Ther* 4: 6.

- McEachern MJ, Krauskopf A, Blackburn EH. 2000. Telomeres and their control. *Annu Rev Genet* 34: 331-358.

- Meyerink JH, Klootwijk J, Planta RJ, van der Ende A, van Bruggen EF. 1979. Extrachromosomal circular ribosomal DNA in the yeast Saccharomyces carlsbergensis. *Nucleic Acids Res* 7:69-76.

- Minamino T, Miyauchi H, Yoshida T, Komuro I. 2003. Endothelial cell senescence in human atherosclerosis: role of telomeres in endothelial dysfunction. *J Cardiol* 41:39-40.

- Murayama A, Ohmori K, Fujimura A, Minami H, Yasuzawa-Tanaka K, Kuroda T, Oie S, Daitoku H, Okuwaki M, Nagata K, Fukamizu A, Kimura K, Shimizu T, Yanagisawa J. 2008. Epigenetic control of rDNA loci in response to intracellular energy status. *Cell* 2008 133: 627-39.

- Palacios JA, Herranz D, De Bonis ML, Velasco S, Serrano M, Blasco MA. 2010. SIRT1 contributes to telomere maintenance and augments global homologous recombination. *J Cell Biol* 191: 1299-313.

- Riabowol K, Shmookler Reis RJ, Goldstein S. 1985. Interspersed repetitive and tandemly repetitive sequences are differentially represented in extrachromosomal covalently closed circular DNA of human diploid fibroblasts. *Nucleic Acids Res* 13: 5563-84.

- Savale L, Chaouat A, Bastuji-Garin S, Marcos E, Boyer L, Maitre B, Sarni M, Housset B, Weitzenblum E, Matrat M, Le Corvoisier P, Rideau D, Boczkowski J, Dubois-Randé JL, Chouaid C, Adnot S. 2009. Shortened telomeres in circulating leukocytes of patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 179: 566-71.

- Shibata Y, Kumar P, Layer R, Willcox S, Gagan JR, Griffith JD, Dutta A. 2012. Extrachromosomal microDNAs and chromosomal microdeletions in normal tissues. *Science* 336: 82-6.

- Sinclair DA, Guarente L. 1997. Extrachromosomal rDNA circles--a cause of aging in yeast. *Cell* 91:1033-42.

- Smit AF. 1999. Interspersed repeats and other mementos of transposable elements in mammalian genomes. *Curr Opin Genet Dev* 9: 657-63.

- Song T, Yang L, Kabra N, Chen L, Koomen J, Haura EB, Chen J. 2013. The NAD+ synthesis enzyme nicotinamide mononucleotide adenylyltransferase (NMNAT1) regulates ribosomal RNA transcription. *J Biol Chem* 288: 20908-17.

- Sorensen PD, Frederiksen S. 1991. Characterization of human 5S rRNA genes. *Nucleic Acids Res* 19: 4147-5.

- Stults DM, Killen MW, Pierce HH, Pierce AJ. 2008. Genomic architecture and inheritance of human ribosomal RNA gene clusters. *Genome Res* 18: 13-8.

- Suh JH, Sieglaff DH, Zhang A, Xia X, Cvoro A, Winnier GE, Webb P. 2013. SIRT1 is a Direct Coactivator of Thyroid Hormone Receptor β1 with Gene-Specific Actions. *PLoS One* 8: e70097.

- Torres G, Frisella PD, Yousuf SJ, Sarwar S, Baldinger L, Zakhary SM, Leheste JR. 2008. A ChIP-cloning approach linking SIRT1 to transcriptional modification of DNA targets. *Biotechniques* 44: Pxii-Pxi.

- Torres G, Dileo JN, Hallas BH, Horowitz JM, Leheste JR. 2011. Silent information regulator 1 mediates hippocampal plasticity through presenilin1. *Neuroscience* 179:32-40.

- Viret JF, Bravo A, Alonso JC. 1991. Recombination-dependent concatemeric plasmid replication. *Microbiol Rev* 55: 675-83.

- Wachtler F, Schöfer C, Schedle A, Schwarzacher HG, Hartung M, Stahl A, Gonzales I, Sylvester J. 1991. Transcribed and nontranscribed parts of the human ribosomal gene repeat show a similar pattern of distribution in nucleoli. *Cytogenet Cell Genet* 57: 175-8.

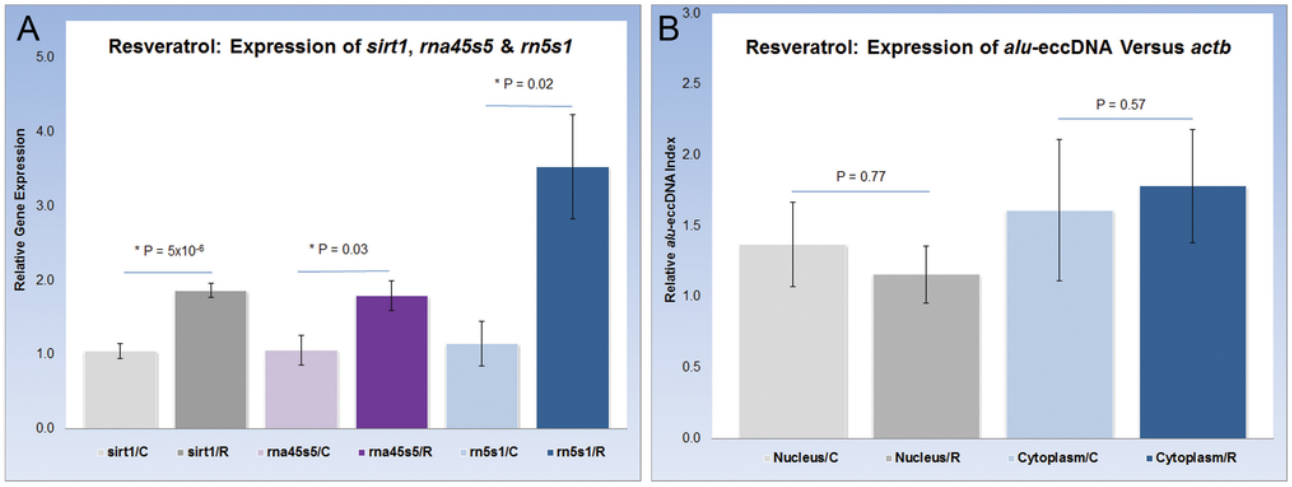
- Wu Q, Maniatis T. 1999. A striking organization of a large family of human neural cadherin-like cell adhesion genes. *Cell* 97: 779-90.

- Yang L, Song T, Chen L, Kabra N, Zheng H, Koomen J, Seto E, Chen J. 2013. Regulation of SirT1-NML binding by rRNA coordinates ribosome biogenesis with nutrient availability. *Mol Cell Biol* 33: 3835-48.

- Zellinger B, Akimcheva S, Puizina J, Schirato M, Riha K. 2007. Ku suppresses formation of telomeric circles and alternative telomere lengthening in Arabidopsis. *Mol Cell* 27: 163-9.

Figure 1

Figure 1 Resveratrol treatment induces the expression of sirt1 and rDNA genes and does not alter Alu-eccDNA in HEK-293 cells. **A)** QPCR-analysis reveals increases in relative gene expression for sirt1 (1.9-fold), rna45s5 (1.8-fold) and rn5s1 (3.5-fold) with resveratrol (R) treatment (50 µM in DMSO, 48h) compared to control (C) conditions (DMSO). **B)** The same treatment has no significant effect on Alu-eccDNA in either nuclear or cytoplasmic compartments of HEK-293 cells. The relative Alu-eccDNA index compares Alu-eccDNA with β-actin expression of the same treatment group via QPCR. Values are means ± SEM. * indicates statistical significance of annotated P; n = 20 for all groups.



2

Figure 2

Figure 2 Resveratrol treatment significantly increases cytoplasmic rn5s1-eccDNA in human cells. QPCR analysis (relative to Alu-eccDNA) comparing resveratrol (R) treatment (50 µM in DMSO, 48h) with control (C) conditions (DMSO) reveals significantly increased rn5s1-eccDNA in the cytoplasm of **A.** HEK-293 cells (2.2-fold), **B.** MCF7 cells (3.4-fold) and **C.** SH-SY5Y cells (1.5-fold). Concomitantly, rn5s1-eccDNA decreases significantly (-60%) in HEK-293 cells nuclei but not in other cells. **D.** Southern-blot using a gene-specific, labeled DNA probe confirms rn5s1eccDNA in HEK-293 cells (à). Cytoplasmic and nuclear eccDNA extracts are linearized (sonication or BamHI), electrophoresed and blotted. Lower bands are consistent with 2.2kb rn5s1eccDNA monomers. Only cytoplasmic samples display an additional signal around 12kb consistent with rn5s1eccDNA multimers. QPCR-values are means ± SEM. * indicates statistical significance of annotated P; n = 20 per group (**A-C**).

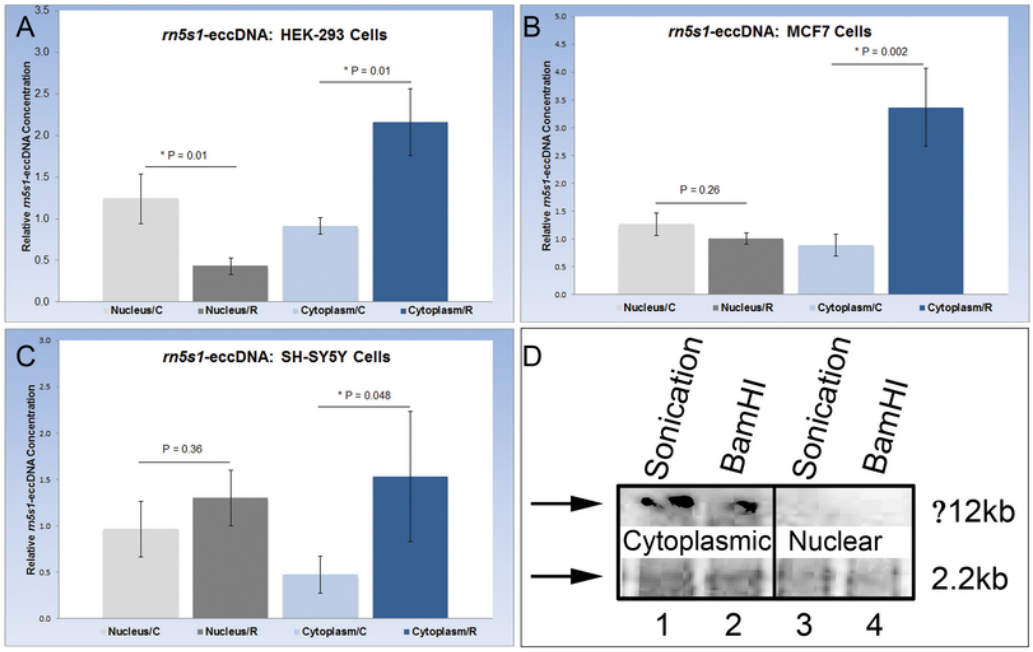


Figure 3

Figure 3 Increases in cytoplasmic rn5s1-eccDNA in HEK-293 cells involve nuclear transport. QPCR analysis (relative to Alu-eccDNA) of rn5s1-eccDNA under various conditions relative to untreated controls (baseline). Chemical reagents used: Resveratrol (R); concavalin A (ConA; control lectin); wheat germ agglutinin (WGA; nuclear transport inhibitor). **A.** Nuclear extracts show a significant resveratrol-dependent rn5s1-eccDNA reduction (-70%; R) which is WGA-sensitive (-20%; R&WGA). **B.** With resveratrol, cytoplasmic rn5s1-eccDNA surges (+180% R; +100% R&ConA) and returns to baseline with WGA (R&WGA). WGA by itself reduces baseline levels of rn5s1-eccDNA significantly (-50%). Values are means ± SEM. * indicates statistical significance of annotated P; n = 12 per group.

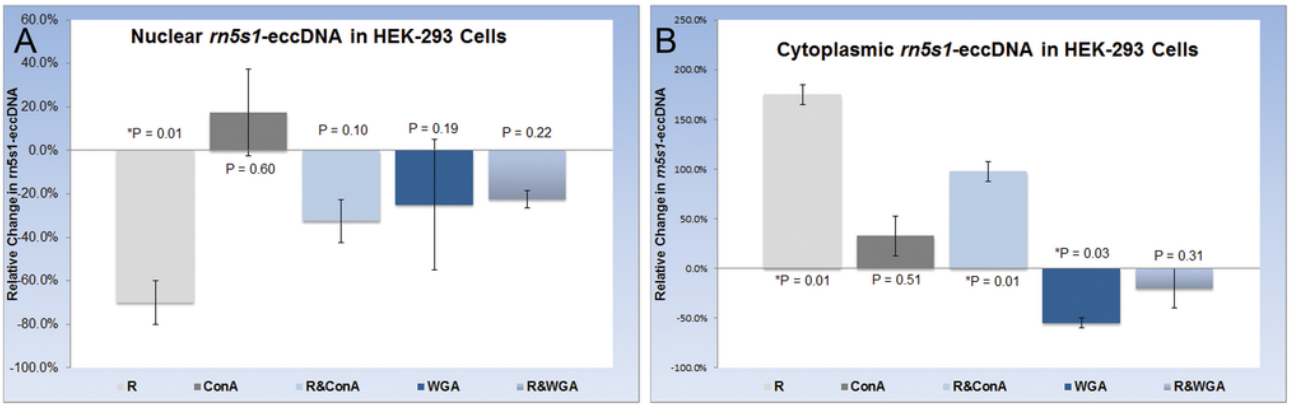


Figure 4

Figure 4. HEK-293 cells display genome-wide formation of eccDNA. This flowchart identifies the different types of eccDNA found in HEK-293 cells and classifies short repetitive DNA elements included. Our novel isolation and cloning strategy identified 48 eccDNA clones of which 13 map to coding genes (see Table 1). The majority of the non-mitochondrial clones (31 with tandem repeats and 3 with unique sequences) contain 1-4 short repetitive DNA elements (with an average of 1.7) possibly responsible for their mobilization. The majority (91%) encode transposable elements with class-I retrotransposons (94%; LINE, SINE, ERV and Alu elements) being dominant over class-II DNA transposons (6%; hAT). The minority (9%) includes interspersed repeats such as *aluS* and *aluJ*.

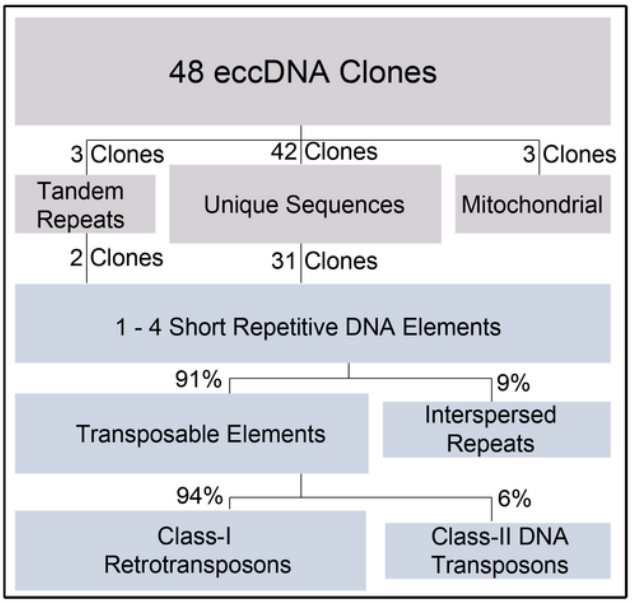


Table 1(on next page)

Table 1

Table 1 Identification and characterization of eccDNA linked to specific genes. Overlapping exons (ex) and introns (int) are sequentially numbered and the upstream (5') position of the untranslated region (utr) in rn5S1 is indicated. Genes and repetitive elements are abbreviated according to standard convention. Other abbreviations: SINE: short interspersed nuclear elements; hAT: a class-II DNA transposon superfamily; LINE: long interspersed nuclear elements; ERV: endogenous retrovirus; IR: Interspersed repeat.

Gene	Chromosome	Contains	Process Involvement	Size [bp]	Repetitive Element
acp6	1	int2	mitochondrial lipid biosynthesis	598	*aluSc* (SINE); *mer5A1* (hAT)
cdh9	5	int5	promotes cell-to-cell adhesion	650	*l1hS* (LINE); *lipmA2* (LINE)
snx24	5	int3	vesicular membrane transport	761	*herv*l (ERV)
aldh3a2	17	ex3-int3	fatty alcohol oxidation	481	-
bicD1	12	int2	cytoskeleton-based RNA sorting	781	*aluS* (IR); *l1me1* (LINE)
cpE	4	int1	neurotransmitter & peptide hormone production	700	*l1mb8* (LINE)
lacc1	13	int2	leprocy & Crohn's disease	560	*aluJb* (SINE)
nfyb	12	int6	transcription factor (repressor)	460	*aluJo* (SINE)
parva	11	int1	kidney development; F-actin binding	672	-
pcdhα1	5	int3	neuronal cell-cell interaction	870	-
plekhm3	2	int1	intracellular signal transduction	437	*aluJo* (SINE)
rn5S1	1	5'utr	ribosomal & mitochondrial function	661	*hervip10fh* (ERV)
sash1	6	int19-ex20	tumor suppression	629	-
specc1	17	int3	structural function	737	*lipA8* (LINE)
spy1	7	int3	cell-cycle regulation	708	*aluJ*(IR); *aluJo* (SINE)
tdrd7	9	ex20-ex21	lens development	633	-