Genomic stability, anti-inflammatory phenotype, and up-regulation of the RNAseH2 in cells from centenarians

Genomic stability, anti-inflammatory phenotype and up-regulation of the RNAseH2-dependent RNA:DNA hybrid degrading enzyme in cells from centenarians.

Gianluca Storci*[1,2,3], Sabrina De Carolis[1,3], Alessio Papi[4], Maria Giulia Bacalini[5], Noémie Gensous[1], Elena Marasco[1], Anna Tesei[6], Chiara Arienti[6], Michele Zannoni[6], Anna Sarnelli[7], Spartaco Santi[8,9], Fabiola Olivieri[10,11], Emanuela Mensà[10], Silvia Latini[10], Manuela Ferracin[1], Stefano Salvioli[1], Paolo Garagnani[1], Claudio Franceschi[1,5], Massimiliano Bonafè*[1,3,6]

[1]Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Italy;

[2]Interdepartmental Centre "L. Galvani" (CIG), University of Bologna, Italy;

[3]Center of Applied Biomedical Research (CRBA), Policlinico Universitario S.Orsola-Malpighi, Bologna, Italy;

[4]Department of Biological, Geological and Environmental Science, University of Bologna, Italy;

[5]IRCCS Istituto delle Scienze Neurologiche di Bologna, Bologna, Italy;

[6]Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST), IRCCS, Biosciences Laboratory, Meldola (Forlì) Italy.

[7]Medical Physics Unit, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola, Italy.

[8]Institute of molecular genetics, National Research Council (CNR), Bologna, Italy.

[9]Laboratory of Musculoskeletal Cell Biology, Rizzoli Orthopedic Institute, Bologna, Italy.

[10]Department of Clinical and Molecular Sciences, DISCLIMO, Università Politecnica delle Marche, Ancona, Italy.

[11]Center of Clinical Pathology and Innovative Therapy, Italian National Research Center on Aging, INRCA-IRCCS, Ancona, Italy;

***Correspondence to:** Gianluca Storci and Massimiliano Bonafé, Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Italy

Summary

Longevity is tightly linked across evolution to the maintenance of genomic stability. Centenarians are long-living subjects who reach the extreme limits of human lifespan, while escaping the inflammatory age-related diseases. Current literature underpins that genomic damage elicits inflammation by engaging cytoplasmic sensors *via* the misplacement of nucleic acids (including RNA:DNA hybrids) outside the nucleus. Here, we report that high genomic stability (*i.e.* preserved telomere length) and blunted DNA-damage-induced inflammation characterize centenarian's fibroblasts. This molecular make-up is associated with reduced amounts of cytoplasmic RNA:DNA hybrids. These cells convey high levels of the RNA:DNA degrading enzyme subunit RNAseH2C, whose knock-down up-regulates inflammatory mediators. The RNAseH2C locus is hypo-methylated in centenarian's cells, hyper-methylated in senescent cells, and in atherosclerotic plaques and cancer tissues. Noteworthy, RNAseH2C expression and anti-inflammatory phenotype can be elicited in different cell types exposed to Extracellular Vesicles isolated from centenarian's cell cultures. These data provide evidence on the molecular mechanisms that allow centenarians to escape the deleterious effects of inflamm-aging and to avoid inflammatory age-related diseases.

Running title: RNAseH2-driven genomic stability in centenarians

Keywords

Inflamm-aging, RNAseH2, RNA:DNA hybrids, Interferon beta-1, Interleukin-6, Telomeres

Introduction

Human longevity represents the capability of exceptional individuals to reach the extreme limits of human lifespan, while avoiding age-related diseases, such as atherosclerosis and cancer [1]. A common feature of age-related diseases is the activation of inflammatory pathways that recalls the systemic pro-inflammatory status that develops with age, named inflamm-aging [2]. At cellular level, inflammatory activation has been linked to DNA damage, a phenomenon which is epitomized in cell senescence, a state of permanent growth arrest in which a pro-inflammatory secretome is sustained by DNA-damage response (DDR) [3]. Speculatively, this phenomenon is likely to contribute to inflamm-aging [4]. Recently, DDR-induced inflammation has been linked to the misplacement of DNA into the cytoplasm as a consequence of genomic damage. [5]. In the cytoplasm, nucleic acids are recognized by evolutionary-conserved innate immunity sensors that ignite inflammation and type-I interferon (IFN) response [6]. A crucial role of such cytoplasmic

machinery has been recognized in senescent cells [7]. A particular kind of DNA damage-induced nucleic acids that can be misplaced into the cytoplasm are RNA:DNA hybrids [5,8]. These nucleic acids are highly capable to elicit inflammation and IFN response [9], and accumulate in cells carrying mutations in genes involved in the DNA repair mechanisms or lacking the RNA:DNA hybrids degrading machinery *i.e.* RNAseH2 [10, 11]. Genetic defects (*i.e.* RNAseH2C mutations) that lead the accumulation of RNA:DNA hybrids causes early-onset inflammatory diseases (fatal auto-inflammatory diseases), and are likely linked to the pathogenesis of age-related auto-immune diseases (*e.g.* systemic lupus erythematosus and psoriasis) [12, 13]. Current literature is concordant in considering that degradation of misplaced RNA:DNA hybrids is a crucial physiologic phenomenon to preserve normal cell functioning [12]. Noteworthy, cells defective for such machinery are not only sources of inflammatory mediators because of nucleic acid accumulation in the cytoplasm, but also accumulate RNA:DNA hybrids in the nucleus, a phenomenon that cause genomic instability and telomere derangement, thus creating the premises to further ignite inflammation and promoting cell senescence [11, 14]. Centenarians are a very informative model of longevity to understand factors that protect from age-related pathologies. Indeed, centenarians escape the majority of age-related diseases and avoid the detrimental effects of inflamm-aging [1]. A possible explanation for this phenomenon stands in their molecular setup that may restrain the activation of the innate immunity/inflammation in response to the accumulation of stress response byproducts [15]. In our previous investigation we showed that centenarians are endowed with highly efficient DNA repair capacity mainly due to the activation of 53BP1-dependent Non Homologous End Joining (NHEJ) [16]. Moreover, we previously reported that centenarians are endowed of well-preserved telomeres, an established marker of genomic integrity [17]. Here, we hypothesize that centenarians may be endowed with a molecular setting that allows them to maintain genomic stability while avoiding the activation of IFN/inflammatory response that accompanies DNA damage and occurs in senescent and stressed cells [3, 18]. To this purpose we measured the level of interleukin-6 (IL-6), as the most representative cytokine involved in aging and inflamm-aging ("the cytokine for gerontologist") [19], whose level associates with mortality and morbidity in aging and in the age-related pathologies, such as atherosclerosis and cancer [20]. We also measured IFN, which is a major mediator of *in vitro* senescence and elicits systemic progeric features in mice [21]. We then assessed the level of cytoplasmic RNA:DNA hybrids in fibroblasts of people of different ages and the expression of the cognate degrading enzyme RNAseH2 [12]. Finally, owing to the capability of extracellular vesicles (EV) to modulate IFN [22] and to systemically affect the inflammatory response [23] [24], we assessed the contribution of EV

in the capability of centenarian's fibroblasts to propagate their longevity-promoting molecular setup to recipient cells.

Materials and Methods

Cell cultures

Primary dermal fibroblasts (DF, **Supplementary Table 1**) were obtained from healthy young (18-40 years), old (58-98 years), and centenarian (>99 years) subjects. DF were cultured in 10% FCS DMEM (Euroclone, Milan, Italy). All the experiments were performed between the 3rd and 6th *in vitro* passage. DF were collected upon signed informed consent. The study was approved by the local Ethics Committee (S.Orsola-Malpighi Hospital Prot. n. 2006061707, amendment 08/11/2011; Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico, Prot. n. 2035, amendment 30/11/2011; University of Calabria 9/9/2004 amendment on 24/11/2011). Early passages and senescent human embryonic diploid lung fibroblasts were cultured as previously described [25]. THP1 cells were maintained in 20% FCS RPMI 1640 medium. Cells were seeded in 24-well plates at a density of 500.000 cells/ml. For M1-M2 monocyte polarization assay, THP1 were exposed to $1x10^3$ CD9+ EV/cell for 72h. MCF-7 cells were cultured in 10% RPMI 1640 medium. MCF-7 MS were generated by seeding single cell suspension (1-5x 10^3 cells per well) in low attachment plates filled with Mammocult^{tm} (Stem cell technologies, Vancouver, CA) and assessed at day 7 [26]. HUVECs were cultured as previously described (Prattichizzo *et al*., 2016) [27].

Exposure of DF to DNA damaging agents

DF were exposed to a single radiation dose of 7.5Gy using the linear acceleration Elekta Synergy Platform system (Elekta Oncology Systems, Stockholm, Sweden) as previously described [28]. DNA damage was evaluated 72h after treatment. Doxorubicin (Sigma, St Louis, MO) was used at a concentration of 5μM for 24h.

Comet Assay

The assay was performed according to the manufacturer's protocol (Comet assay, Trevigen, Gaithersburg, MD). Briefly, at the end of the treatments, $5x10^5$ cells were suspended in LMA garose (at 37°C) at a ratio of 1:10 (v/v), and 75 μl were immediately transferred onto the comet slide. The slides were immersed for 1 hr at 4°C in a lysis solution, washed in the dark for 1 hr at room temperature in alkaline solution, then electrophoresed for 30 min at 20V. Slides were immersed twice in dH2O for 5 minutes each, then dipped in 70% ethanol and stained with the Silver Staining Kit (Trevigen). The extent of DNA damage was evaluated quantitatively by Olympus IX51

microscope 10x, Olympus Corporation, Tokyo, Japan by scoring at least 100 nucleoids of different categories, using the free software Cell Profiler (version 2.1.1). Percentage of DNA in tail for different categories of comets was expressed.

Confocal microscopy analysis

Cells were fixed and permeabilized with ice cold methanol for 10' and acetone for 1' on ice, blocked with 2% BSA, and incubated with primary anti-S9.6 antibody (1:100 dilution, Kerafast, Boston, MA, USA) and secondary goat anti-mouse Alexa Fluor 488 (1:250; Life Technologies). Alternatively, cells were fixed with paraformaldehyde 4% for 20 min at room temperature and incubated overnight at 4°C with anti Rad51 diluted 1:100 (Millipore, Milan, Italy). The confocal imaging was performed with a Nikon A1 confocal laser scanning microscope, equipped with a 60x, 1.4NA objective and with 405, 488 and 561 nm laser lines. Rad51 foci were enumerated using Fiji (ImageJ).

Transient gene Knock-down and over-expression

RNAseH2C (HSS149830) and Snai2/Slug (HSS109993/109995/185949) specific siRNA, the GC matched content control (siSCR) were transiently transfected with lipofectamine 2000 (Life technologies, Carlsbad, CA, USA) following manufacturer instructions. All transfections were performed onto 70% confluent cells. The cells were harversted after 72h of siRNA transfection and stored in TRIzol® (Life technologies, Carlsbad, CA, USA).

Real time PCR analysis

Total RNA was extracted from cells using TRIzol® (Life technologies, Carlsbad, CA, USA). Taqman probes for Rad51, Snai2/Slug, IL-6, RNAseH2A/B/C, Interferon beta-1, TREX1, IL-1, PPRalpha, PPRgamma, RXRalpha, Alox-15, Jagged-1, IL-10, CD163, CD68 and Glucuronidase, were purchased from Life technologies (Carlsbad, CA, USA). Quantitative PCR analysis was performed on Light-Cycler using iQ software (BIORAD, Hercules, CA, USA), mRNA levels were calculated with $2^{\wedge -ddct}$ method.

Telomere Length assessment

Telomere length was measured as telomere-to-single copy gene ratio (T/S), using quantitative real-time PCR as previously described [29] with some modifications. For cellular samples 5 µl aliquots containing 20 ng DNA and 10 µl of master mix per sample were processed. For EV samples 5 µl aliquots containing 0.8 ng DNA and 10 µl of master mix per sample were processed. For each

standard curve, one reference DNA sample was diluted serially in water by 1.68-fold dilution to produce 5 DNA concentrations ranging from 30 to 2 ng in 5 µl. To reduce inter-assay variability, the telomere and the single-copy gene (36B4) were analysed on the same plate. Primer sequences and concentrations for telomere and 36B4 were as previously described [29]. The thermal cycling profile was: (1) one cycle of 10 s at 95 °C; (2) 30 cycles of 5 s at 95 °C, 15 s at 57 °C and 20 s at 72 °C. Measurements were performed in duplicate and reported as T⁄S ratio relative to a calibrator sample (Roche, Milano, Italy) to allow comparison across runs. EV telomeric sequencies were quantified based on the RT-PCR CT, without the comparison with single gene copy. To date no single gene copy was described in EV. The real-time Chromo4 MJ Research system (Bio-Rad Laboratories, Hercules, CA, USA) was used for all PCRs. The coefficients of variation within duplicates of the telomere and single-gene assay were 2% and 1.8%, respectively. Approximately 30% of samples were repeated on different plates to assess T/S measurement reproducibility. The inter-assay coefficient of variation was < 10%. All analyses were blind. The correlation coefficient between T⁄S and the telomere restriction fragment was R2 = 0.88

ELISA test

IL-6 ELISA was performed with Quantikine ELISA kit for human IL-6 (R&D System, Minneapolis, MN, USA) according to manufacturer instructions. Concentrations of Maresin 1 and Resolvin D1 (RvD1) in cell culture supernatants were measured using commercially available ELISA kits (Cayman Chemical Co., Ann Arbor, MI, USA) according to manufacturer instructions.

Western Blot analysis

Proteins were extracted with radio-immunoprecipitation assay buffer, with the exception of γ-H2AX extraction which was performed by adding HCl to the lysis buffer [10 mmol/L HEPES (pH 7.9); 1.5 mmol/L $MgCl_2$; 10 mmol/L KCl] to a final concentration of 0.2 mol/L. The following antibodies were used: anti-actin (C4- Santa Cruz, CA); anti-phospho-(gamma)-Histone H2AX (Ser139, clone JBW301, Millipore, Billerica, MA, USA); anti-Rad51, clone 3C10 (Millipore); anti-Snai2/Slug (L40C6, Cell Signalling, Beverly, MA, USA); anti-RNASeH2C (Protein-tech, IL, USA) and anti-actin (clone C4, Santa Cruz, CA, USA) as loading control.

DNA Methylation analysis

DNA methylation of RNASEH2C locus (genomic region: chr11:65,486,750-65,487,179, GRCh37/hg19) was evaluated using the EpiTYPER assay (Agena Bioscience). Briefly, the EZ-96 DNA Methylation Kit (Zymo Research Corporation) was used to perform bisulfite-conversion of

500 ng of genomic DNA. This treatment specifically converts unmethylated cytosines to uracils, while methylated cytosines remain unaffected. Ten ng of converted DNA were amplified using the following primers for bisulfite-converted DNA : RNASEH2C_F_aggaagagagATGGGGTTGAGGATAGTTTAAAAAG;RNASEH2C_R_cagtaatacg actcactatagggagaaggctCCCAATAAAAAAACTCTTCACAACA. PCR products were then processed and analysed according to EpiTYPER protocol. The following Gene Expression Omnibus datasets were analysed for RNASEH2C methylation according to Illumina HumanMethylation450 BeadChip array measurements: GSE40279, which includes whole-blood DNA methylation data from 656 healthy subjects with age ranging from 19 to 101 years; GSE46394, which includes DNA methylation data from 15 atherosclerotic lesion tissues and matched normal aortic tissue, plus 19 carotid atherosclerotic samples; GSE66695, which includes DNA methylation data from 40 normal and 80 breast cancer samples.

Small RNA seq analysis

Small RNA libraries were prepared from 6 dermal fibroblast samples using TruSeq Small RNA Library PrepKit v2 (Illumina, RS-200-0012/24/36/48), according to manufacturer's indications. Briefly, 35 ng of purified RNA were linked to RNA 3' and 5' adapters, converted to cDNA and amplified using Illumina primers containing unique indexes for each sample. Each library was quantified using Agilent Bioanalyzer and High Sensitivity DNA kit (Agilent, 5067-4626) and equal amount of libraries were pooled together. A size-selection was performed to keep fragments between 130-160 bp. After ethanol precipitation, the library pool was quantified with Agilent High Sensitivity DNA kit, diluted to 1.8 pM and sequenced using NextSeq® 500/550 High Output Kit v2 (75 cycles) (Illumina, FC-404-2005) on Illumina NextSeq500 platform. Raw base-call data generated from the Illumina NextSeq 500 system were demultiplexed by Illumina BaseSpace Sequence Hub (https://basespace.illumina.com/home/index) and converted to FASTQ format. After a quality check, which was performed with FastQC tool (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), the adapter sequences were trimmed using Cutadapt (http://cutadapt.readthedocs.io/en/stable/index.html). In this step, sequences shorter than 10 nucleotides were also removed. Read mapping was performed using STAR algorithm (https://www.ncbi.nlm.nih.gov/pubmed/23104886). The reference genome was constituted by human microRNAs sequences from the miRbase 21 database (http://www.mirbase.org/). Counts of raw mapped reads were obtained using the htseq-count script from the HTSeq tools (http://www-huber.embl.de/HTSeq/doc/overview.html); raw count were further normalized using DESeq2 bioconductor package (http://bioconductor.org/packages/release/bioc/html/DESeq2.html). Data

were analyzed using Genespring GX software v. 14.8 (Agilent Technologies). Differentially expressed miRNAs were identified using moderated t-test (FDR 10% with Benjamini-Hochberg correction), Principal Component Analysis and Cluster Analysis, with Manhattan correlation, were performed using GeneSpring GX software.

CD9+ Exosome Isolation and phenotypization from DF supernatants

CD9+ exosomes from young, old and centenarians DF supernatants were isolated by CD9 immunobeads isolation kit (HansaBioMed Life Sciences Ltd; cod: HBM-BOLC-CC/20-1) according to manufacturer's protocol. EVs were diluted to approximately 1 ml of PBS, loaded into the sample chamber of an LM10 unit (Nanosight, Malvern, UK) and three videos of either 30 or 60 seconds were recorded of each sample. Exosomal-surface antigens were investigated with the MACSPlex Exosome kit (Miltenyi Biotec GmbH, Gladbach, Germany). Briefly, after isolation, Exosomes were diluted in MACSPlex buffer and stained according to manufacturer instructions. The samples were analyzed with a FACSCanto flow cytometer (Beckton Dickinson). At least 10,000 events per sample were recorded. Data were analyzed with FACSDiva software. The median fluorescence values plotted in the graph were background corrected and normalized on CD63/81/9 median signal intensity. Negative values were excluded from the plot.

Statistical analysis

The exact p-value calculated by un-paired t-test (two groups comparisons) and one-way ANOVA (>2 groups comparisons). Post-hoc t-test values were corrected for multiple comparison according to *Bonferroni* correction. The statistical analysis was performed with Graph-pad Prism 6 software.

Results

Reduced level of Interleukin-6 and high genetic stability characterize cells from centenarians.

The first observation of our investigation regards the reduced IL-6 protein level in the supernatant of centenarian's primary dermal fibroblasts (DF) compared to supernatants from aged people ones (**Figure 1a**). The finding above was confirmed by real time PCR analysis, which also conveyed reduced IL-6 mRNA level in DF and peripheral blood mononuclear cells (PBMC) from centenarians, as compared to aged people (**Figure 1b**). These data suggested that cells from centenarians are characterized by a restrained pro-inflammatory phenotype. To further confirm this hypothesis, we performed small RNA-seq analysis, which showed the expression of an anti-inflammatory micro-RNA profile in centenarian's DF (**Supplementary Figure 1a**) [30-34]. We

then tested if such a peculiar molecular setup persisted even after induction of a DNA damage: upon exposure to a single radiation dose of 7.5Gy, we substantiate that centenarian's DF show a dramatically hampered capability to express IL-6, with respect to cells from old subjects (2 folds vs 80 folds, **Figure 1c**). Similar results were observed when cells were exposed to Doxorubicine (**Figure 1d**). These data indicate that centenarian's cells are endowed with hampered pro-inflammatory response to damaging agents. This finding is in agreement with our previous observation that centenarian's DF have a high capability to promptly repair DNA damage [16]. In our set of centenarian's DF, and in agreement with our previous observations [16] we found low level of the DNA double strand breaks (DSB) sensor Ser139-phosphorylated γ-H2AX (**Figure 2a**), accompanied by low level of overall DNA damage measured by comet assay (**Figure 2b**). Importantly, low levels of DNA damage and Ser139-phosphorylated γ-H2AX were persistent in centenarian's DF even after 7.5Gy of gamma radiation (**Figure 2c**) and doxorubicine exposure (**Figure 2d**). Interestingly, we previously reported that centenarian's DF display an up-regulation of the DNA damage repair protein 53BP1 [16]. 53BP1, while promoting the error-prone NHEJ machinery, switches-off the error-free Homologous (HR) DNA repair machinery [35]. Accordingly, we observed a substantial decrease of the major player of HR protein, *i.e.* Rad51 in centenarian's DF compared to old people ones (**Figure 2e**). This observation was confirmed by confocal microscopy analysis, showing a decrease of Rad51 positive foci in centenarian's DF even upon 7.5Gy of gamma radiation exposure (**Figure 2f**). Finally, since telomeres are major source of DNA damage signalling and HR is a major mechanism of telomere maintenance, we assessed telomere length in DF by PCR analysis. In keeping with previous observations [17], we found that centenarian's DF display higher than expected telomere length, being more similar to young people than old people ones (**Figure 2g**). These data show that blunted inflammatory activation in centenarian's DFs is accompanied by a remarkable genomic stability.

High level of RNAseH2C and low level of RNA:DNA hybrids are present in centenarian's cells

DNA damage is a powerful trigger of inflammation and IFN response as a consequence of the misplacing of nucleic acids into the cytoplasm [5, 8]. When DF from young, old and centenarians were assessed for the expression of IFN (and IL-6 for comparison), DF from centenarians showed a reduced expression of IFN, strikingly paralleling that of IL-6 (**Figure 3a**). Notably, IL-6 and IFN levels in young subjects were remarkably similar to those of centenarians (**Figure 3a**). A similar age-related trend (an up-regulation in aged people compared to young and centenarian's cells) was observed for the IFN-regulated gene TREX1 (**Figure 3b)**. RNA:DNA hybrids are processed by

RNAseH2 enzyme [12]. Remarkably, all three components of the RNAseH2 enzymatic complex were up-regulated in centenarian's DF compared to old people (**Figure 3c**). In particular, the up-regulation of RNAseH2C subunit in centenarian's cells, was confirmed by western blot analysis **(Figure 3c)**. Accordingly, the amount of RNA:DNA hybrids, assessed by confocal microscopy analysis, resulted reduced in the cytoplasm of centenarian's DF, with respect to old people's ones (**Figure 3d**). Moreover, after 7.5Gy of gamma radiation exposure, a dramatic increase of the amount of RNA:DNA hybrids in the cytoplasm of young and old people's DF, but not in centenarian's DF, was observed (**Figure 3d**). Importantly, we observed an opposite relationship between RNAseH2C and IL-6 expression in cells exposed to 7.5Gy of gamma radiation (**Figure 3e-f**). In centenarian's cells the transient knock-down of RNAseH2C leads to a marked dose-dependent increase of IL-6 and INF expression **(Figure 3g)**. These data indicate that RNAseH2C expression drives the anti-inflammatory phenotype of centenarian's cells.

Opposite correlation between RNAseH2C locus Methylation and disease-free longevity

Prompted by the above described results, we measured RNAseH2C expression in senescent cells, the best characterized model of *in vitro* aging [3]. A down-regulation of RNAseH2 subunits expression was observed in senescent primary human umbilical endothelial cells (HUVEC), in which a significant up-regulation of IFN was also found (**Figure 4a**). Accordingly, we found reduced RNAseH2C expression in senescent foetal lung fibroblasts [25] compared to early passage cells (**Figure 4b**). Epigenetic age-related changes are deeply involved in the aging process [36]. Inspired by the opposite RNASeH2C expression found in centenarians DF and senescent cells, DNA methylation at RNAseH2C locus was assessed by EpiTYPER assay (**Figure 4c**). A marked hypomethylation of the first 3 CpG sites of the investigated amplicon in centenarian's DF was observed (**Figure 4d**). Accordingly, we also observed an age-related decrease of RNAseH2C methylation level in PBMC (assessed by the Illumina Infinium 450K platform (GSE40279)), at least at cg11637721 (corresponding to the CpG3 site explored by the EpiTYPER assay) (**Figure 4e**). As expected, senescent lung fibroblasts displayed a higher degree of methylation in the same RNAseH2C sites that were hypomethylated in centenarian's DF (**Figure 4f**). We thus reasoned that RNAseH2C gene methylation may be increased in pathologic tissues. To verify this assumption, we searched Gene Expression Omnibus (GEO) database for DNA methylation datasets and analyzed data from GSE46394 and GSE66695 (see Methods). The analysis conveyed that the RNAseH2C CpG site cg11637721 is hypermethylated in tissues obtained from atherosclerotic plaques (**Figure 4g**) and breast cancer tissues, compared to cognate healthy ones (**Figure 4h**). These data indicate that disease-free longevity is associated with an unmethylated state of RNAseH2C locus.

Anti-inflammatory activity of extracellular vesicles from centenarians

EV have been recently reported to modulate INF/inflammatory response [37, 22]. To test the role of EV in our experimental setting, we isolated EV from young, old and centenarian's DF supernatants by anti-CD9 coated immunomagnetic beads. EV were counted by Nanosight and phenotypically characterized by FACS analysis (**Supplementary Figure 2a)**. EV were then administered to primary DF of people of different ages, THP1 myelomonocytic and MCF-7 breast cancer cells (**Figure 5a)**. We found that EV from centenarians were able to reduce IL-6 expression and to induce RNAseH2C in young people's DF, as well in THP1 and MCF-7 cells (**Figure 5b-c, Supplementary Figure 2b**). In THP1 cells exposed to centenarian's EV, we observed an over-expression of M2-polarization markers (CD68, CD163, IL-10 and PPAR-gamma, (**Figure 5d**), an up-regulation of anti-inflammatory enzyme (Alox-15) and RXRalpha transcription factor (**Figure 5e**), as well as a down-regulation of pro-inflammatory cytokines (IFN, IL-1) and PPR-alpha transcription factor (**Figure 5f**). Such an anti-inflammatory capacity of centenarian's EV was confirmed by high levels of pro-resolving mediators (maresin-1 and resolvin D1) in THP1 cells administered with centenarian's EV and exposed to 7.5Gy of gamma radiation (**Figure 5g**). Finally, we observed that EV from centenarians contained more than expected amounts of TTAGGG telomeric repeats (**Figure 5j**), that have long been characterized for their peculiar anti-inflammatory activity [38-40]. These data show the anti-inflammatory activity of centenarians EV.

Discussion

This paper reports the reduced inflammatory setup (low IL-6, INF and inflamma-miR expression) of DF from centenarians. Such exceptional individuals are the living proof that it is possible to reach the extreme limits of human life span by escaping the detrimental effects of major age-related dysfunctions and diseases [1]. These latter (*e.g.* atherosclerosis, diabetes and cancer), albeit extremely heterogeneous, share an inflammatory pathogenesis [4]. Hence, the data here reported suggest that the molecular setup of centenarians may allow these individuals to avoid or postpone inflamm-aging, *i.e.* the seemingly unavoidable activation of the inflammatory response that occurs during life [2]. Along the myriads of agents that may ignite inflammation, DNA damage response has emerged a crucial mechanism to explain the pro-inflammatory secretome of senescent cells [3]. More in general, DNA damage is associated with inflammation in a variety of physiologic and pathologic conditions [5, 10, 12, 18, 41]. Here we show remarkable genomic stability in cells from centenarians, *i.e.* almost absent comet tails and barely detectable levels of γ-H2AX

phosphorylation, even after exposure to DNA damaging agents. These peculiar features associate with a well-preserved telomere length. All these features extend and confirm previous reports [16, 17], showing that centenarians are likely to be endowed with peculiar capability to repair DNA damage, an activity that is associated with longevity across evolution [42]. Based on the tight relationship between inflammation and DNA damage, the maintenance of the genome is expected to shelter the individuals from a variety of inflammatory diseases, and more in general to prevent or post-pone inflamm-aging. Recent literature shows that DNA damage-induced inflammatory and IFN responses depend upon the displacement of nucleic acids into the cytoplasm, where they engage cognate sensors [5, 8, 12, 18]. The phenomenon has been recently linked to the induction of the pro-inflammatory secretome of senescent cells [7]. A particular kind of DNA damage-induced misplaced nucleic acids are DNA:RNA hybrids [5, 8]. Here we show that cells from centenarians are almost devoid of cytoplasmic RNA:DNA hybrids even upon the exposure to gamma radiations. Accordingly, the enzyme involved in the clearance of RNA:DNA hybrids, namely RNAseH2, is strongly up-regulated in cells from centenarians. Then we report that the knock-down of RNAseH2C elicits IL-6 and IFN expression, thus demonstrating that the up-regulation of this enzyme plays a substantial part in the anti-inflammatory setup of centenarian's cells. These data are in agreement with current literature showing that RNaseH2C mutations that impair cytoplasmic RNA:DNA hybrids degradation are major trigger for the inflammatory/IFN response [12]. Notably, RNA:DNA hybrids, albeit essential intermediates of the HR-dependent repair of telomere ends, must be proficiently removed to ensure genomic and telomeric stability [43]. Noteworthy, cells of centenarians display almost undetectable levels of Rad51 protein, the major player of HR, thus suggesting that they may act as HR deficient cells. This apparently paradoxical finding, perfectly fits with our previous observation that centenarian's cells show the up-regulation of 53BP1, the major mediator of NHEJ, that switches off Rad51 expression and function [35]. In such a peculiar genetic background, the up-regulation of the RNAseH2 enzyme, by promoting RNA:DNA hybrid degradation, is likely leading to genomic stability, thus assuring telomere maintenance [44]. In this regard, the unbalancing of DNA-repair mechanisms towards NHEJ, associated with higher expression of RNAseH2C is expected to be advantageous for human longevity as it may ensure adequate DNA repair, while avoiding the deleterious effects of Rad51-generated RNA:DNA hybrids [45]. As far as the Rad51 down-regulation in centenarian's cells, based on our previous observations [26], we noticed an up-regulation of Rad51 repressor SLUG, whose down-regulation up-regulated Rad51 mRNA level (**Supplementary Figure 3a-b**). Noteworthy, SLUG expression was also capable to up-regulate RNAseH2C expression (**Supplementary Figure 3c**). Thought these data may be considered preliminary, it may be speculated that SLUG-dependent mechanisms may

control DNA stability and the cognate inflammatory response in aging by modulating RNAseH2 expression. In this regard, we provide evidence that the expression of *RNAseH2C* is epigenetically regulated. In particular, *RNAseH2C* is hypo-methylated in centenarians, but hyper-methylated in *in vitro* senescent cells, atherosclerotic plaques and breast cancer tissues. Notably, all these cell types represent pathologic conditions associated with aging and with the up-regulation of inflammatory response [4, 46]. Notably, our data on the positive relationship between RNAseH2C and longevity are reinforced by literature data showing that RNAseH-dependent RNA:DNA hybrids clearance extends lifespan in the yeast [47]. Notably, RNAseH2C locus (as well as IL-6 receptor) were identified in a recent genome-wide analysis on human longevity as potential polymorphisms that may explain part of the inter-individual variability in attaining healthy aging and longevity [48]. A major conundrum in aging studies is the relationship between cellular and organismal mechanisms of aging. In this paper we show that centenarian's cells can cast anti-inflammatory signals to other distal cell types, such as fibroblasts, monocytes and cancer cells, via EV. Such subcellular nano-scaled structures have been extensively characterized [23] and contain a wealth of cellular components, including nucleic acids [33]. In all cellular models here tested, EV isolated from centenarian's DF are capable to induce RNAseH2C and reduce IL-6 expression. Seemingly, this phenomenon allows to conclude that the longevity-assuring molecular setup of centenarians can be propagated from cell to cell, and may therefore act as a systemic spreading that may counteract the pro-inflammatory secretome of senescent cells [4]. EV from centenarians also skew monocyte polarization towards the M2 phenotype which exhibits anti-inflammatory, pro-resolution and reparative capability [49]. These data are reinforced by our observation regarding the capability of EV from centenarians to up-regulate two omega 3–derived lipidic mediators, Resolvin-D1 and Maresin, which are tightly linked to the M2 macrophage phenotype [49, 50]. These pro-resolution anti-inflammatory mediators are currently regarded as molecules with powerful capability to taper inflammation and to promote tissue integrity and repair [50]. Noteworthy, EV from old people have been recently shown to induce M1-like features in monocytes [24]. Interestingly, in MCF-7 MS grown *in vitro* as 3D culture, a model for IL-6-dependent breast cancer stem cells growth [46], MS growth was blunted upon the administration of EV from centenarians (**Supplementary Figure 4a**) and showed also a reduced level of the IL-6 dependent MS growth factor JAG-1 (**Supplementary Figure 4b) [46]**. These data suggest that long-living individuals may escape cancer by setting-up an anti-inflammatory niche that halts or thwarts cancer cell growth and/or aggressiveness. Finally, we aimed at investigating whether the content of centenarian's EV may account for their anti-inflammatory activity. Unfortunately, the amount of RNA did not allow us to assess whether the microRNA content of such EV may recapitulate the one of the cells of origin, and thus propagating

the anti-inflammatory phenotype to target cells. However, as a support of the centenarian's EV anti-inflammatory activity, we report the presence of higher than expected telomeric sequences TTAGGG in centenarian's EV. Indeed, extracellular telomere sequence is anti-inflammatory since it engages cytoplasmic DNA sensors, but exerts inhibitory effects on inflammatory/IFN response [51, 52]. These data strongly suggest that EV (and telomeric EV) may spread the anti-inflammatory setup of centenarian's cells at systemic level, thus providing cues on the molecular mechanisms that taper inflamm-aging and promote longevity in humans.

Acknowledgements

The authors acknowledge Prof. John Sedivy (department of Molecular Biology, Brown University, Providence, RI, USA) for providing DNA of foetal lung fibroblasts, Antonino Romeo and Anna Sarnelli (Radiotherapy unit, IRST-IRCCS Meldola, Forlì) for their technical assistance for gamma radiation exposure experiments; Sara Pignatta, Michela Cortesi and Francesco Fabbri (Bioscience laboratory, IRST-IRCCS Meldola, Forlì) for their technical assistance in comet assay and cytofluorimetric analysis experiments. A particular acknowledge to Michele Guescini (University of Urbino) for his technical assistance on EV isolation and characterization;

This work has been supported by: 1) the EU-FP7 Projects under the grant agreements No. 613979 "MyNewGut"; No. 602757 "HUMAN" to CF; 2) by the EU-H2020 Project under the grant agreement No. 634821 "Propag-ageing" to CF; 3) by Roberto and Cornelia Pallotti Legacy for Cancer Research (University of Bologna) to MB and SS.

Conflict of Interest

All authors have no conflict of interest to declare

References

1. Franceschi C, Bonafe M. Centenarians as a model for healthy aging. *Biochem Soc Trans* 2003, **31**:457-61.
2. Franceschi C, Bonafè M, Valensin S, Olivieri F, De Luca M, Ottaviani E *et al*. Inflammaging. An evolutionary perspective on immunosenescence. *Ann N Y Acad Sci* 2000; **908**:244-54.
3. Rodier F, Coppe JP, Patil CK, Hoeijmakers W, Munoz DP, Raza SR *et al*. Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion. *Nat Cell Biol* 2009; **11**: 973-79.
4. Franceschi C, Campisi J. Chronic inflammation (inflammaging) and its potential contribution to age-associated diseases. *J Gerontol A Biol Sc Med Sci* 2014; **69**: Suppl 1, S4-9.
5. Shen YJ, Le Bert N, Chitre AA, Ko CX, Nga XH, Ho SS *et al*. Genome-derived cytosolic DNA mediates type I interferon-dependent rejection of B cell lymphoma cells. *Cell Rep* 2015; **11**: 460-73.
6. Schlee M, Hartmann G. Discriminating self from non-self in nucleic acid sensing. *Nat Rev Immunol* 2016; **16**: 566-80.
7. Gluck S, Guey B, Gulen MF, Wolter K, Kang TW, Schmacke NA *et al*. Innate immune sensing of cytosolic chromatin fragments through cGAS promotes senescence. *Nat Cell Biol* 2017; **19**: 1061-70.
8. Koo CX, Kobiyama K, Shen YJ, LeBert N, Ahmad S, Khatoo M *et al*. RNA polymerase III regulates cytosolic RNA:DNA hybrids and intracellular microRNA expression. *J Biol Chem* 2015; **290**: 7463-73.
9. Mankan AK, Schmidt T, Chauhan D, Goldeck M, Höning K, Gaidt M *et al*. Cytosolic RNA:DNA hybrids activate the cGAS-STING axis. *EMBO J* 2014; **33**: 2937-46.
10. Brégnard C, Guerra J, Déjardin S, Passalacqua F, Benkirane M, Laguette N. Upregulated LINE-1 Activity in the Fanconi Anemia Cancer Susceptibility Syndrome Leads to Spontaneous Pro-inflammatory Cytokine Production. *EBioMedicine* 2016; **8***,* 184-194.
11. Günther C, Kind B, Reijns MA, Berndt N, Martinez-Bueno M, Wolf C *et al*. Defective removal of ribonucleotides from DNA promotes systemic autoimmunity. *J Clin Invest* 2015; **125**: 413-24.
12. Crow YJ, Manel N. Aicardi-Goutieres syndrome and the type I interferonopathies. *Nat Rev Immunol* 2015; **15**: 429-40.

13. Molès JP, Griez A, Guilhou JJ, Girard C, Nagot N, Van de Perre P *et al*. Cytosolic RNA:DNA Duplexes Generated by Endogenous Reverse Transcriptase Activity as Autonomous Inducers of Skin Inflammation in Psoriasis. *PLoS One* 2017; **12**: e0169879.
14. Keskin H, Storici F. Defects in RNase H2 Stimulate DNA Break Repair by RNA Reverse Transcribed into cDNA. *Microrna* 2015; **4**: 109-16.
15. Franceschi C, Garagnani P, Vitale G, Capri M, Salvioli S. Inflammaging and 'Garb-aging'. *Trends Endocrinol Metab* 2017; **28**: 199-12.
16. Lattanzi G, Ortolani M, Columbaro M, Prencipe S, Mattioli E, Lanzarini C *et al*. Lamins are rapamycin targets that impact human longevity: a study in centenarians. *J Cell Sci* 2014; **127**: 147-57.
17. Mondello C, Petropoulou C, Monti D, Gonos ES, Franceschi C, Nuzzo F. Telomere length in fibroblasts and blood cells from healthy centenarians. *Exp Cell Res* 1999; **248**: 234-42.
18. Erdal E, Haider S, Rehwinkel J, Harris AL, McHugh PJ. A prosurvival DNA damage-induced cytoplasmic interferon response is mediated by end resection factors and is limited by Trex1. *Genes Dev* 2017; **31**: 353-369.
19. Ershler WB. Interleukin-6: a cytokine for gerontologists. *J Am Geriatr Soc* 1993; **41**: 176-81.
20. Knupfer H, Preiss R. Significance of interleukin-6 (IL-6) in breast cancer (review). *Breast Cancer Res Treat* 2007; **102**: 129-35.
21. Yu Q, Katlinskaya YV, Carbone CJ, Zhao B, Katlinski KV, Zheng H *et al*. DNA-damage-induced type I interferon promotes senescence and inhibits stem cell function. *Cell Rep* 2015; **11**:785-97.
22. Kitai Y, Kawasaki T, Sueyoshi T, Kobiyama K, Ishii KJ, Zou J *et al*. DNA-Containing Exosomes Derived from Cancer Cells Treated with Topotecan Activate a STING-Dependent Pathway and Reinforce Antitumor Immunity. *J Immunol* 2017; **198**: 1649-59.
23. Tkach M, Théry C. Communication by Extracellular Vesicles: Where We Are and Where We Need to Go. *Cell* 2016; **164**: 1226-32.
24. Eitan E, Green J, Bodogai M, Mode NA, Baek R, Jorgensen MM *et al*. Age-Related Changes in Plasma Extracellular Vesicle Characteristics and Internalization by Leukocytes. *Sci Rep* 2017; **7**: 1342.
25. Bacalini MG, Deelen J, Pirazzini C, De Cecco M, Giuliani C, Lanzarini C *et al*. Systemic Age-Associated DNA Hypermethylation of ELOVL2 Gene: In Vivo and In

Vitro Evidences of a Cell Replication Process. *J Gerontol A Biol Sci Med Sci* 2017; **72**: 1015-23.

26. Storci G, Bertoni S, De Carolis S, Papi A, Nati M, Ceccarelli C *et al*. Slug/beta-catenin-dependent proinflammatory phenotype in hypoxic breast cancer stem cells. *Am J Pathol* 2013; **183**: 1688-97.
27. Prattichizzo F, Giuliani A, Recchioni R, Bonafe M, Marcheselli F, De Carolis S *et al*. Anti-TNF-alpha treatment modulates SASP and SASP-related microRNAs in endothelial cells and in circulating angiogenic cells. *Oncotarget* 2016; **7**: 11945-58.
28. Tesei A, Sarnelli A, Arienti C, Menghi E, Medri L, Gabucci E *et al*. In vitro irradiation system for radiobiological experiments. *Radiat Oncol* 2013; **8**, 257.
29. Cawthon RM. Telomere measurement by quantitative PCR. *Nucleic Acids Res* 2002; **30**: e47.
30. Huang T, Kang W, Zhang B, Wu F, Dong Y, Tong JH *et al*. miR-508-3p concordantly silences NFKB1 and RELA to inactivate canonical NF-kappaB signaling in gastric carcinogenesis. *Mol. Cancer*. 2016; **15**: 9.
31. Kim SW, Ramasamy K, Bouamar H, Lin AP, Jiang D, Aguiar RC. MicroRNAs miR-125a and miR-125b constitutively activate the NF-kappaB pathway by targeting the tumor necrosis factor alpha-induced protein 3 (TNFAIP3, A20). *Proc Natl Acad Sci U S A* 2012; **109**: 7865-7870.
32. Luo Y, Tong L, Meng H, Zhu W, Guo L, Wei T *et al*. MiR-335 regulates the chemo-radioresistance of small cell lung cancer cells by targeting PARP-1. *Gene* 2017; **600**: 9-15.
33. Song X, Wang Z, Jin Y, Wang Y, Duan W. Loss of miR-532-5p in vitro promotes cell proliferation and metastasis by influencing CXCL2 expression in HCC. *Am J Transl Res* 2015; **7**: 2254-61.
34. Olivieri F, Albertini MC, Orciani M, Ceka A, Cricca M, Procopio AD *et al*. DNA damage response (DDR) and senescence: shuttled inflamma-miRNAs on the stage of inflamm-aging. *Oncotarget* 2015; **6**: 35509-21.
35. Ciccia A, Elledge SJ. The DNA damage response: making it safe to play with knives. *Mol Cell* 2010; **40**: 179-204.
36. Horvath S, Pirazzini C, Bacalini MG, Gentilini D, Di Blasio AM, Delledonne M *et al*. Decreased epigenetic age of PBMCs from Italian semi-supercentenarians and their offspring. *Aging (Albany NY)* 2015; **7**: 1159-70.

37. Takahashi A, Okada R, Nagao K, Kawamata Y, Hanyu A, Yoshimoto S *et al*. Exosomes maintain cellular homeostasis by excreting harmful DNA from cells. *Nat Commun* 2017; **8**: 15287.
38. Gursel I, Gursel M, Yamada H, Ishii KJ, Takeshita F, Klinman DM. Repetitive elements in mammalian telomeres suppress bacterial DNA-induced immune activation. *J Immunol* 2003; **171**: 1393-00.
39. Shirota H, Gursel I, Gursel M, Klinman DM. Suppressive oligodeoxynucleotides protect mice from lethal endotoxic shock. *J Immunol* 2005; **174**: 4579-83.
40. Goldfarb IT, Adeli S, Berk T, Phillippe M. Fetal and Placental DNA Stimulation of TLR9: A Mechanism Possibly Contributing to the Pro-inflammatory Events During Parturition. *Reprod Sci* 2017; **25**: 788-796
41. Bonafè M, Storci G, Franceschi C. Inflamm-aging of the stem cell niche: breast cancer as a paradigmatic example: breakdown of the multi-shell cytokine network fuels cancer in aged people. *Bioessays* 2012; **34**: 40-9.
42. MacRae SL, Zhang Q, Lemetre C, Seim I, Calder RB, Hoeijmakers J *et al*. Comparative analysis of genome maintenance genes in naked mole rat, mouse, and human. *Aging Cell* 2015; **14**: 288-91.
43. Ohle C, Tesorero R, Schermann G, Dobrev N, Sinning I, Fischer T. Transient RNA-DNA Hybrids Are Required for Efficient Double-Strand Break Repair. *Cell* 2016; **167**: 1001-13 e1007.
44. Balk B, Maicher A, Dees M, Klermund J, Luke-Glaser S, Bender K *et al*. Telomeric RNA-DNA hybrids affect telomere-length dynamics and senescence. *Nat Struct Mol Biol* 2013; **20**: 1199-205.
45. Wahba L, Gore SK, Koshland D. The homologous recombination machinery modulates the formation of RNA-DNA hybrids and associated chromosome instability. *Elife* 2013; **2**: e00505.
46. Sansone P, Storci G, Tavolari S, Guarnieri T, Giovannini C, Taffurelli M *et al*. IL-6 triggers malignant features in mammospheres from human ductal breast carcinoma and normal mammary gland. *J Clin Invest* 2007; **117**: 3988-02.
47. Salvi JS, Chan JN, Szafranski K, Liu TT, Wu JD, Olsen JB *et al*. Roles for Pbp1 and caloric restriction in genome and lifespan maintenance via suppression of RNA-DNA hybrids. *Dev Cell* 2014; **30**: 177-91.

48. Pilling LC, Kuo CL, Sicinski K, Tamosauskaite J, Kuchel GA, Harries LW *et al*. Human longevity: 25 genetic loci associated in 389,166 UK biobank participants. *Aging (Albany NY)* 2017; **9**: 2504-20.
49. Mantovani A, Biswas SK, Galdiero MR, Sica A, Locati M. Macrophage plasticity and polarization in tissue repair and remodelling. *J Pathol* 2013; **229**: 176-85.
50. Serhan CN. Pro-resolving lipid mediators are leads for resolution physiology. *Nature* 2014; **510**: 92-101.
51. Marwaha V, Chen YH, Helms E, Arad S, Inoue H, Bord E *et al*. T-oligo treatment decreases constitutive and UVB-induced COX-2 levels through p53- and NFkappaB-dependent repression of the COX-2 promoter. *J Biol Chem* 2005; **280**: 32379-88.
52. Steinhagen F, Zillinger T, Peukert K, Fox M, Thudium M, Barchet W *et al*. Suppressive oligodeoxynucleotides containing TTAGGG motifs inhibit cGAS activation in human monocytes. *Eur J Immunol* 2018; **48**: 605-611.

Figure legend

Figure 1. Reduced level of Interleukin-6 and high genetic stability in cells from centenarians.

A. IL-6 Elisa test in aged people (old) and centenarians (cen) DF supernatants. **B.** Real-time PCR analysis of IL-6 mRNA level in old and cen DF (left panel) and Peripheral Blood Mononuclear cells, PBMC (right panel). **C.** IL-6 mRNA level in old and cen DF exposed to gamma rays (7.5Gy); **D.** IL-6 mRNA level in old and cen DF exposed to Doxorubicine (5 μM for 24h); data are presented as mean ± s.d.;

Figure 2. Reduced level of DNA damage in cells from centenarians

A. phosho-Serine139-γ-H2AX (γ-H2AX) and actin protein level in DF from young (yng), aged (old) and centenarian (cen). **B-C.** Comet assay in yng, old and cen DF under basal or 7.5Gy of gamma radiation exposure. **D.** γ-H2AX and actin protein level in yng, old and cen DF treated or untreated with 5 μM Doxorubicine for 24h. **E.** Rad51 protein level in yng, old and cen DF and Rad51 mRNA level in old and cen DF. Actin protein level is reported in Fig 2A. **F.** Confocal microscopy analysis of Rad51 in yng, old, cent DF at basal condition and upon exposure of 7.5Gy of gamma radiation **G.** Telomere/single copy gene ratio (T/S) in DNA from yng (n=8), old (n=4), cent (n=5) DF; data are presented as mean ± s.d;

Figure 3. High level of RNAseH2C and low level of RNA:DNA hybrids in cells from centenarians

A. IFN and IL-6 mRNA level in DF from young (yng), aged (old) and centenarian (cen) in a different set of samples respect to Figure 1B. **B.** TREX1 mRNA level in yng, old and cen DF. **C.** RNAseH2A-H2B-H2C mRNA level in yng, old and cen DF; RNAseH2C and actin protein level in yng, old and cen DF; **D.** Confocal microscopy analysis of RNA:DNA hybrids (S9.6 antibody) in yng, old and cen DF under basal or upon 7.5Gy gamma radiation exposure. **E.** RNASeH2C mRNA level in yng, old and cen DF under basal or upon 7.5Gy gamma radiation exposure. **F.** IL-6 mRNA level in yng, old and cen DF under basal or upon 7.5Gy gamma radiation exposure **G.** IL-6, IFN and RNAseH2C mRNA level in cen DF upon RNAseH2C knock-down (20 and 60 ng siRNA); data are presented as mean ± s.d;

Figure 4. Opposite correlation of RNAseH2C locus methylation with longevity and age-related diseases

A. RNAseH2C and IFN mRNA level in early passage and senescent HUVEC cells; **B.** RNAseH2C mRNA level in early and senescent (sen) lung fibroblasts; **C.** UCSC view (Human GRCh37/hg19) of the RNaseH2C CpG locus explored with EpiTYPER assay and Illumina Infinium 450K corresponding probes. **D.** Percent of methylation for each RNAseH2C CpG unit, measured in young (yng), aged (old) and centenarian (cen) DF. **E.** Percent of methylation of the Illumina Infinium 450K CpG3 unit probe in whole-blood of people of different ages. **F**. Percent of methylation for each RNAseH2C CpG unit, in early and sen human embryonic diploid lung fibroblasts. **G.** Percent of methylation of the Illumina Infinium 450K CpG3 unit probe in normal aortic, aortic and carotid atherosclerotic samples (dataset GSE46394). **H.** Box Plot analysis of percent methylation of CpG3 unit probe in normal and ER-positive or ER-negative breast cancer samples (dataset GSE66695); data are presented as mean ± s.d;

Figure 5. Anti-inflammatory activity of extracellular vesicles from centenarians

A. Schematic of the experimental design showing CD9+ EV isolated from young (yng), aged (old) and centenarian (cen) DF and administered to DF, THP1 myelomonocitic cells and MCF-7 breast cancer cell lines (See Supplementary Figure 2); **B.** IL-6 and RNAseH2C mRNA level in DF exposed to yng-EV, old-EV, cen-EV. **C.** IL-6 and RNAseH2C mRNA level in THP1 cells exposed to yng-EV, old-EV, cen-EV. **D.** IL-10, CD163, CD68 and PPARgamma mRNA level in THP1 cells administered with yng-EV, old-EV, cen-EV. **E.** Alox-15 and RXR-alpha mRNA level in THP1 cells exposed to yng-EV, old-EV, cen-EV. **F.** IFN, IL-1beta and PPARalpha mRNA level in THP1 cells administered with yng-EV, old-EV, cen-EV. **G.** Elisa test of Maresin1 and Resolvin D1 on supernatant of THP1 cells administered with yng-EV, old-EV, cen-EV under basal or upon 7.5Gy gamma radiation exposure. **J.** Real Time PCR analysis of Telomeric repeat (T/S) in EV isolated from of yng, old, cent DF; data are presented as mean ± s.d;

Supplementary Figure 1

A, B. Small RNA-seq heat-map and Principal Component Analysis (PCA) in old and cen DF (n=2 in duplicates); **C,D** Relative expression level of specific microRNAs up-regulated (**C**) or down-regulated (**D**) in old and cen DF;

Supplementary Figure 2

A. Cytofluorimetric analysis of EV isolated from supernatant of primary fibroblasts from people of different ages; **B,** MCF-7 cells exposed to yng-EV, Old-EV and cen-EV: RNAseH2C and IL6 mRNA level; data are presented as mean ± s.d;

Supplementary Figure 3

A. Snai2/Slug protein level in yng, old and cen DF and Snai2/Slug mRNA level in old and cent DF. Actin protein level is reported in Fig 2A. **B.** Snai2/Slug and Rad51 mRNA level in cen DF upon Slug gene knock-down; **C.** RNAseH2C mRNA level in cen DF upon Snai2/Slug knock-down and RNAseH2C mRNA level in old DF transiently transfected with Snai2/Slug encoding plasmid; data are presented as mean ± s.d;

Supplementary Figure 4

A. MS assay in MCF-7 cells administered with yng-EV, old-EV, cen-EV; **B.** Jagged-1 mRNA expression in DF administered with yng-EV, old-EV, cen-EV; data are presented as mean ± s.d;