TITLE: Molecular profiling of glioblastoma-derived extracellular vesicles identifies small nucleolar RNAs as candidate liquid biomarkers for radiation- induced senescence

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ABSTRACT

Radiation-induced senescence (RIS) in glioblastoma (GBM) is an undesirable cell fate that inhibits tumor cell death and supports resistance and outgrowth. While senescence-targeting drugs are promising adjuvants, their clinical application will require proper patient selection based on post-treatment RIS burden. Current methods to evaluate senescence, however, are tissue-based, and given GBM's difficult anatomical location, post-treatment biopsies are impractical. Therefore, novel and less invasive biomarkers for TIS are urgently needed. To this end, we aimed to identify candidate extracellular vesicle (EV) liquid biomarkers for TIS by profiling senescence-associated cargo changes within GBM EVs. Using a panel of GBM patient-derived cell lines, we show that RIS is the primary functional state following radiation exposure and is associated with significant alterations in the cargo of senescent-derived EVs (senEVs). In particular, senEV transcriptomes have an increased abundance of senescence-associated RNA species and enrichment of senescence-associated gene sets. Most striking, however, was that senEVs are most differentiated by the significant enrichment of a panel of snoRNAs. This signature was conserved in 4/5 GBM models of RIS and was validated by qRT-PCR. Further analysis by mass spectrometry revealed that snoRNAs are likely co-packaged with their associating proteins, as senEVs had concurrent increases in these binding partners. Finally, in a preliminary patient cohort comparing plasma EVs obtained prior to surgery to those obtained after completion of their radiation therapy, we identified increased senescence-associated RNA such as CDKN2B and GLB1 and the snoRNA SNORA49 in post-radiation EVs. Altogether, this data suggests that senEV RNA species, and particularly snoRNAs, are a promising analyte for RIS-biomarker development. With further study, this work may open avenues for a companion diagnostic for senotherapeutics.

Keywords: glioblastoma, senescence, extracellular vesicles, snoRNA

1. INTRODUCTION

Glioblastoma treatment options have failed to advance within the last decade, and barring enrollment to a clinical trial, patients are generally constrained to surgical resection, radiation (IR), and temozolomide (TMZ) chemotherapy[1,2]. There is a dire need for new therapeutics, but the drastic heterogeneity, stemness, and physical location of GBM behind the blood-brain-barrier all pose hindrances to the drug development process[3,4]. While novel first-line therapies are in development and hold promise[2], an alternative is to instead exploit vulnerabilities induced by standard-of-care (SOC). This includes leveraging druggable persister states[5], such as therapy-induced senescence (TIS).

TIS—or when promoted by radiation, RIS—is a cell stress response characterized by a prolonged growth arrest, cytoskeletal changes, enhanced lysosomal biogenesis, metabolic dysregulation, epigenetic rewiring (senescence-associated-heterochromatic-foci formation, SAHF), and an enhanced and altered secretome (senescence associated secretory phenotype, SASP)[6]. There are multiple cell-intrinsic and extrinsic roles for TIS. Intrinsically, TIS tumor cells may serve as a reservoir for recurrent disease due to the potential for proliferative recovery[7]. While this is in contrast to the historical definition of senescence as an irreversible growth arrest, proliferative recovery from TIS has been documented in lung, breast, AML, prostate, and GBM cells[7–12]. Extrinsically, the SASP from both senescent tumor and senescent host cells can modulate the tumor microenvironment and contribute to immune evasion, chemotherapy-associated side effects, and tumor recurrence[13–17]. The ablation or modulation of senescent cells by senotherapeutics has been shown to enhance *in vitro* and *in vivo* drug response and longevity in various tumor types[11,18–21]. This includes promising preclinical results demonstrating the potency and tumor-control capacity of senolytic therapies in GBM[14,22–25].

Senotherapeutic potency, by definition, is dependent upon having an appreciable senescent burden. For example, senolytics have minimal toxicity to proliferating cells and sensitivity following exposure to senescence-stimuli is time sensitive. For example, Rahman *et al.* found that potency to the senolytic ABT-263 increases with time as GBM cells enter senescence following radiation exposure[23], while Saleh *et al.* demonstrated that potency decreases with time as lung cancer cells recover from etoposide-induced senescence[20]. TIS is likely also heterogeneous between patients, as evaluations of breast and lung cancer have shown that neoadjuvant chemotherapy induces TIS in ~40% of patients[8,26,27]. However, the prevalence of senescence and the incidence promoted by SOC in GBM patients are not well understood. While early studies in resected tissue

support a minimal pre-existing senescent fraction in treatment-naive tumors (<10% in any given section)[17,28], studies of TIS have been hampered by the inaccessibility of post-treatment tissue prior to recurrence. Further, biopsies are apt to miss TIS in regions that are unresectable, such as adjacent normal brain tissue. A tissue-independent assay for TIS in GBM patients might therefore i) enable a better understanding of the heterogeneous presentation and clinical consequences of TIS and ii) serve as a companion diagnostic for senotherapeutics by identifying patients with an increased senescent burden post-SOC.

Extracellular vesicles (EVs) have gained increased interest as a liquid biopsy for diagnosis and disease monitoring, including in GBM[29–31]. EVs are membrane-bound particles secreted by both tumor and host cells that contain RNA, DNA, and protein[32]. EVs from senescent cells (senEVs) *in vitro* have been shown to contain unique cargo[33–36], and EV protein cargo has been explored as a potential biomarker for senescent fibroblasts and Warner's syndrome[37]. However, to our knowledge, it has yet to be established if and how EVs may report on TIS in GBM, where markers may differ from noncancerous cell types.

Here, we investigate the elaboration of senEVs and their cargo in radiation-induced senescent GBM cells to qualify the potential of an EV-based TIS/RIS bioassay. Our results show that senescence-associated RNA species and snoRNAs are significantly enriched in senEVs. Importantly, snoRNA enrichment was conserved across 4/5 GBM models, which is promising for a heterogenous phenotype, in a heterogenous cancer. Using paired patient samples obtained at surgical resection and following completion of SOC, we further show the feasible detection of these species in patient plasma EVs. We conclude that radiation-induced senescence in GBM results in the elaboration of EVs that are differentiated by a signature comprised of senescence-associated RNA species and small nucleolar RNA. These EV-transcriptome changes warrant additional investigation and development as a more targeted biomarker/bioassay for RIS.

2. MATERIALS AND METHODS

2.1. Cell culture

GBM patient-derived cultures (GBM6, GBM43, GBM102, and GBM120) were acquired from Mayo Clinic[38], A172 cells were acquired from ATCC, and normal human astrocytes (NHAs) were acquired from Lonza (CC-2565). Media components are listed in Table S1. Patient-derived cultures were grown and generally tested as spheroids, but plates or slides were pre-coated with

laminin (Sigma Aldrich, L2020) for microscopy endpoints to aid visualization. All tumor lines were authenticated by STR and tested negative for mycoplasma.

2.2. Radiation treatments

Cells were irradiated with the X-Rad320 system (Precision X-ray, Inc), housed at University of Arizona, Phoenix Biomedical Campus. Time of treatment is considered day/hour 0. Controls are harvested on day 3, when they are in logarithmic and viable growth, and treated cells on day 7.

2.3. Drug dose response

Cells were plated in triplicate wells of a 96-well plate, allowed to adhere overnight, and then irradiated. Shields were used to ensure appropriate dosing. Relative viability was evaluated with CellTiter-Glo (Promega, G7570).

2.4. Live cell imaging

Live cell imaging was performed in 96-well plates on the CellCyte X™ (Cytena) at 10X. Analysis was performed by the CellCyte software, using the cell confluency program. Conditions for each independent experiment were analyzed in duplicate, at minimum.

2.5. SA-β-galactosidase staining

Histochemical staining by XGal (Invitrogen, 15520018) was performed as described previously[39]. Cells were imaged on a Keyence BZ-X800 at 20X and white balancing/brightening was performed in the Keyence analysis software. Quantification was performed in ImageJ after converting images to 8-bit and then thresholding and measuring percent of image area for thresholds that captured either majority cellular area vs blue staining.

2.6. Western Blotting

Cells or EVs were lysed in 1X RIPA (Millipore, 20-188) with cOmplete protease inhibitor cocktail (Roche, 4693116001) and phosphatase inhibitor cocktails I and II. Protein quantification was performed with the Pierce (Micro) BCA Protein Assay Kit (Thermo Scientific, 23225 and 23235). Equivalent protein quantities were separated by SDS-PAGE with NuPAGE 4-12% Bis-Tris gels (Invitrogen, NP0335) and then transferred to 0.45 micron polyvinylidene membranes (Invitrogen, LC2005). Membranes were blocked with 5% BSA and probed overnight at 4℃ for primary antibodies and probed for 2 hours at room temperature for secondary antibodies. Antibody information can be found in Table S1. Signal was detected with the Super Signal West Dura

Extended Duration Substrate (Thermo Scientific, 34075) with a LI-COR Odyssey Fc imaging system (LI-COR).

2.7. Immunofluorescence

8-well chamber slides (Ibidi, 80826) were fixed with 4% PFA, permeabilized with 0.5% Triton X-100, and then blocked with 5% BSA. Slides were incubated in primary at 4℃ overnight, and secondary at room temperature for at least 2 hours. Antibody information can be found in Table S1. Cells were counterstained with Hoechst and imaged at 60X on the Keyence BZ-X800. Images were sharpened in Inkscape v.1.4.2 evenly across all presented images.

2.8. EV and EV analyte isolation from cell culture supernatants

Cell culture supernatants were harvested on day 3 from proliferative control cells and day 7 from irradiated cells. Irradiated cells received a full media change on day 3. 10 mL supernatant was centrifuged and then concentrated to ~500 µL by ultrafiltration with a 100K molecular weight cut off (Thermo Scientific, 88533). The resulting filtrate was separated with the Izon qEV original 70nm columns (Izon, ICO-70) and fractions 7-11 were collected (500 µL fractions). For downstream protein analysis, the isolated fractions were further concentrated by ultrafiltration (Thermo Scientific, 88524) for western blot or by speedvac for proteomics. For downstream RNA analysis, isolated fractions were processed through exoRNeasy (Qiagen, 77144), per manufacturer's instructions.

2.9. Patient plasma collection and EV RNA isolation

Patients were consented and samples were acquired as approved by the Baylor Scott & White Health institutional review board (020-325). Whole blood was collected in K2 EDTA tubes with subsequent (single-spin) plasma isolation. Plasma was stored at -80℃ until processing. EVs and RNA were directly extracted from 1mL patient plasma by exoRNeasy, per manufacturer's instructions.

2.10. qRT-PCR

Whole cell RNA was isolated by RNeasy with manufacturer's instructions (Qiagen, 74106) and EV RNA was isolated by exoRNeasy with manufacturer's instructions (Qiagen, 77144). qRT-PCR was performed with the iTaq™ Universal SYBR Green One-Step Kit (Bio-Rad, 1725151). Primer information can be found in Table S2. For SASP, analysis was performed with the $2^{-\Delta\Delta Ct}$ method (β-actin as housekeeping). For EVs, there is no well-accepted housekeeping RNA to perform

normalization[40]. Therefore, relative expression was evaluated by calculating $2^{-\Delta Ct}$ (senEV-proEV) and weighting this expression by the ratio of input RNA (ng_proEV/ng_senEV), which was acquired by Qubit HS-RNA (Invitrogen, A32852) of the prepared RNA dilutions used during qRT-PCR. A synthetic cel-miR-39 cDNA (Norgen, 59000) was spiked in at a 1:10 dilution to identify any pipetting errors or presence of PCR inhibitors.

2.11. Total RNA library preparation and sequencing

Whole cell and EV RNA were cleaned with on-column DNase digestion (Zymo, R1013). RNA quality and quantity were determined with high-sensitivity RNA screentape (Agilent, 5067-5579). Total RNA libraries were prepared with SMART-Seq Total RNA Pico Input with ZapR Mammalian rRNA depletion and unique dual index kits (Takara, 634357 and 634752). Whole cell samples were prepared with 5 ng RNA, a 4 minute fragmentation, 5 adapter ligation cycles, and 14 PCR cycles. EV samples were prepared with 250-500 pg RNA, no fragmentation due to RNA size distributions, 5 adapter ligation cycles, and 18 PCR cycles. Library size was determined with high sensitivity D5000 screentape (Agilent, 5067) and quantity was determined by qPCR (Roche, KK4873). Equimolar pools with 5% PhiX were sequenced on the NovaSeq X Series platform. Paired-end sequencing was performed with 11 index cycles and 151 read cycles with NovaSeq Control Software 1.2.2.48004. Illumina BCL Convert v4.2.7 was used to convert base call (BCL) files into FASTQ files.

2.12. RNAseq analysis

High duplication rates are a known issue with low-input library prep[41]. However, deduplication without UMIs can cause increased noise and false negatives with little gain in accuracy[42–44]; this analytical risk is likely exacerbated in our non-fragmented libraries, as without random fragmentation, all identical biological transcripts with n >1 will appear indistinguishable from technical duplicates. Given these considerations, we proceeded without deduplication. Combined Read 1/Read 2 FASTQs were trimmed with cutadapt v4.4, and rRNA removed with sortmerna v4.3.4. Non-rRNA reads were quasimapped with Salmon v1.10.1 against a non-redundant genome annotation combined from GENCODE v40 and LNCipedia5.2, described originally by Rodosthenous *et al.*[45]. Transcript abundances were imported and converted to gene-level count matrices with tximport v1.18.0 in Rstudio 4.3.0. DESeq2 v1.42.0 provided normalized counts, and genes with less than 10 total reads across samples were removed. Differential expression analysis was performed with the Wald test with Benjamini-Hochberg correction. Designs included covariates of cell line/patient. Shrunken $\log_2$fold-change ($\log_2$FC), values were obtained with

apeglm v1.12.0[46]. BiomaRt v2.62.1 was used to pull biotypes of the significant genes. Gene ontology over-representation was performed with clusterProfiler v4.14.6 enrichGO(). Gene set enrichment analysis (GSEA) was performed with normalized counts extracted from DESeq2 that, where applicable, were averaged across biological replicates of the same cell line and condition. GSEA was performed with the Broad GSEA 4.3.3 software using the Signal2Noise ranking and gene set permutation with H and C2 MSigDB collections.

2.13. Mass Spectrometry and Analysis

24µg (GBM43), 16µg (GBM102) and 1.6µg (A172) of protein was digested for cell line lysates using a 2% deoxycholate-based lysis and in-solution trypsin digestion. Data acquisition was performed on RSLCnano U3000 LC system coupled to Thermo Orbitrap Eclipse mass spectrometer with FAIMS Pro interface. 500 ng peptides from each sample were directly loaded on a 50 cm C18 column (EasySpray ES903) and separated by a 2 hr. LC-MS/MS method. Full scans were acquired in the Orbitrap at a resolution of 120,000 over a mass range of 375-1500 m/z. Using Data-Dependent Acquisition, most abundant precursors from the full scan were fragmented by HCD (Collision Energy of 32%) and detected in the ion-trap. FAIMS Compensation Voltage (CV) of -40/-60/-80 was enabled with 1 sec cycle time per CV. Raw spectra were queried in Proteome Discoverer (ThermoFisher Scientific, v2.4) using Mascot search engine (MatrixScience, v2.7) against a human protein database (UP000005640, downloaded Oct 2020) with the following parameters: tryptic peptide cleavage with up to two missed cleavages, oxidation of methionine as a variable modifications and carbamidomethyl on cysteine as a fixed modification, match between runs disabled. Peptide and peptide spectral matches (PSMs) were filtered using a false discovery rate of less than 1%, calculated using Percolator. The resulting protein abundances from the raw spectra search were normalized using variance stabilizing normalization in R (v.4.1.2). Significance was determined with Welch's t-test followed by Benjamini-Hochberg (q-val < 0.05, $|\log_2 FC| > 2$). GSEA was performed with Broad's GSEA 4.2.3 software and then visualized using Cytoscape v3.10.2.

2.14. Graphing and Statistics

RNAseq and mass spectrometry analyses were performed as formerly indicated. All other graphing and statistical analyses were performed in GraphPad Prism v10.1.1, and results are shown as mean +/- the standard deviation of 3-5 independent experiments unless noted otherwise. Specific tests performed are indicated in the appropriate figure legends. Final figures were created with Inkscape v.1.4.2 .

3. RESULTS

3.1. Radiation promotes senescence across GBM patient-derived models

To confirm that senescence is a relevant drug-induced functional state in GBM, we examined the response to IR in 5 GBM cell lines (Table S3). We evaluated sensitivity to 2-10GY IR by relative viability at 24 hours, 72 hours, and 7 days (Figure 1A). Notably, effects were minimal until 7 days, suggesting a more delayed response like senescence than immediate cell death. This was supported by dose- and time-dependent effects on cell growth and senescence-associated-β-galactosidase (SA-β-gal) staining in the A172 cell line (Supplemental Figure 1A and 1B). Based on these data, we chose to further characterize senescence induction in all 5 GBM lines on day 7 following 6GY. As shown by live-cell imaging, 6GY did not result in large drops in cell viability but rather a sustained growth arrest through at least day 7, with most lines undergoing eventual proliferative recovery (Figure 1B); this is consistent with growth patterns documented in other models of tumor cell TIS[7]. Day 7 IR-treated cells were also larger and more granulated (Figure 1C), had increased SA-β-gal activity (Figure 1C), and showed increased H3K9Me3 expression, suggestive of SAHF (Figure 1D). Further, we observed downregulated PCNA (Figure 1E, top panel), upregulated p21 in a p53-dependent manner (Figure 1E, bottom panel), and upregulated IL6, IL1B, and MMP3, although this upregulation reached variable statistical significance (Figure 1G).

Finally, we profiled IR-induced transcriptional changes in A172, GBM43, and GBM102 by gene ontology and GSEA. Overrepresented gene ontologies in IR conditions (based on species with q-val <0.05 and log2FC>0), belonged to biological processes associated with viral response, interferon response, and cell size and organization (Figure 2A, left panel). These gene ontologies in response to radiation have been previously observed[47–49]. As expected for senescent cells, we found that underrepresented ontologies were associated with proliferation arrest, including DNA replication, chromosomal segregation, and cell cycle transitions (Figure 2A, right panel). GSEA further identified that post-IR cells were enriched in cell growth and cell stress hallmarks (Figure 2B), as well as multiple senescence-specific gene sets (Figures 2C). Altogether, our data supports robust RIS at both the transcriptomic and phenotypic levels, confirming the relevance of this phenotype in GBM and enabling biomarker discovery.

3.2. GBM RIS is accompanied by elaboration of EVs that reflect the senescent phenotype

To identify potential signatures of RIS in GBM-derived EVs, we harvested EVs from proliferative cells (proEVs) and senescent cells (senEVs) in our five GBM lines. EVs were isolated by size-exclusion chromatography prior to RNA extraction/sequencing to ensure a high degree of purity and enrichment of small EVs (Supplemental Figure 2A). EV cargo includes a variety of RNA types[50], and while long intact mRNAs have been identified, GBM EV mRNAs have previously been shown to be mostly fragmented[51]. This was also observed in our sample traces (Supplemental Figure 2B). As we were interested in both small noncoding RNA (<200nt) and mRNA markers of senEVs, we chose a total RNA approach with no fragmentation to minimize fragmentation-driven loss of small-mid length RNAs. While this approach does bias against intact long species, we posited that this would give us a broad view of putative senEV markers.

As expected, EVs clustered based on cell line of origin, with some separation between proEVs and senEVs for each line (Figure 3A). Still, when controlling for the effect of cell identity, we identified 580 differentially expressed species (q-val <0.1), though many of these had low magnitude changes (Figure 3B). Of these, 477 (82.2%) belong to the Ensembl biotype “protein coding”. To identify whether the senEV transcriptome recapitulates that of senescent cells, we identified overrepresented gene ontologies in our significant species and enriched gene sets across the entire transcriptome. Overrepresented biological processes in the senEV group were related to protein assembly and processing, MHC processing, and cartilage/collagen (Figure 3C, left panel); there was minimal overlap between these and the top biological processes we identified in our whole cell transcriptomes (Figure 2A, left panel). However, downregulated DEGs belonged to biological processes associated with DNA/chromosomal regulation and segregation, echoing what we found in the whole cell transcriptomes and what may be expected during senescence (Figure 3C, right panel). GSEA further revealed significantly enriched G2M checkpoint, E2F targets, and mitotic spindle gene sets in the senEV transcriptomes (Figure 3D). Of most interest, senEVs were also significantly enriched in three senescence gene sets (Figure 3E). We identified 23 species belonging to these senescent gene sets, as well as the broader Cell Age senescence database (build 2)[52], that met both a q-value threshold of <0.1 and had $\log_2$FC ≥ 1 in either direction (Table 1). Of these, 17/23 changed in the expected direction based on the gene set(s) to which they belong. Overall, these combined data suggest that senEV transcriptomes are reflective of– though not identical to– the senescent cell population from which they were derived.

Table 1. Senescence associated DEGs in senEVs. DEGs were screened against the gene sets found to be enriched in senEVs, as well as the larger CellAge Expression Database. Species with opposing directionality of expected are shaded gray.

Gene	Log2FC	shrunk Log2FC	q-val	Gene Set
IFI27	2.941	2.240	0.014	CellAge_UP
SULF1	2.847	2.875	0.050	CellAge_UP
CD163L1	2.653	1.435	0.060	CellAge_UP
PPL	2.326	1.742	0.014	p53_correlated
BGN	2.000	1.803	0.036	CellAge_DN
APLP1	1.709	1.307	0.046	CellAge_UP
COL5A3	1.680	1.376	0.016	CellAge_DN
IGFBP5	1.609	1.441	0.000	CellAge_UP, p53_correlated
NPDC1	1.496	1.362	0.000	CellAge_UP
PRIM1	-1.406	-1.195	0.011	CellAge_DN
CPPED1	1.362	1.046	0.060	CellAge_UP
YEATS4	-1.238	-1.013	0.025	CellAge_DN
H2AC4	-1.142	-0.875	0.044	DNA_Damage_Sen
MKI67	-1.139	-0.972	0.012	CellAge_DN
CDKN1A	1.112	1.012	0.000	CellAge_UP, DNA_Damage_Sen, SAHF
IGFBP3	1.074	0.749	0.078	CellAge_UP
H2BC8	-1.062	-0.682	0.091	DNA_Damage_Sen
LOXL2	1.061	0.692	0.084	CellAge_UP
BAZ1A	-1.052	-0.869	0.015	CellAge_DN
H2AC16	-1.049	-0.884	0.011	p53_correlated
CENPE	-1.019	-0.808	0.025	CellAge_DN, p53_correlated
H1-5	-1.013	-0.894	0.003	DNA_Damage_Sen, SAHF
SMURF2	-1.003	-0.881	0.004	CellAge_UP

As an orthogonal approach to senEV biomarker discovery, A172, GBM43, and GBM102 EVs were subjected to mass spectrometry to evaluate protein cargo (Supplemental Figure 3). Interestingly, senEVs from A172 and GBM43 had increased numbers of unique proteins compared to proEVs (Supplemental Figure 3A). This increased protein abundance has been previously observed in EVs from senescent oral squamous carcinoma cells[53]. Previous work has also suggested that ATP6V0D1 and RTN4 may serve as biomarkers from senescent fibroblasts[37]; however, this was not found to be the case in our GBM senEVs, as we observed no appreciable upregulation of ATP6V0D1 and upregulation of RTN4 in only GBM43 (Supplemental Figure 3B). We identified no shared upregulated and only three shared downregulated proteins (q-val <0.05) across lines: CEP55, KIF23, and RACGAP1 (Supplemental Figure 3C). Additionally, we found only modest signals of senescence at the protein level in our senEVs, both when considering gene set enrichment (Supplemental Figure 3D) and individual significant species (p-val <0.05, none of which met q-val <0.05) (Supplemental Figure 3E). Given this, we concluded that the senEV transcriptome was a stronger candidate for senEV biomarker development.

3.3. GBM senEVs have increased abundance of snoRNAs

Encouraged by the signatures of senescence-associated RNA cargo in senEVs, we wanted to identify additional markers that strongly differentiate senEVs from proEVs. We ranked significant species by their shrunken $|\log_2 FC|$ values and examined the top 20 (Figure 4A). Four species were protein coding, and two of these four were senescence-associated (*SULF1, IFI27*). Strikingly, of the 16 remaining non-coding species, 12 belonged to the snoRNA class. Of particular interest, *SNORA13* has recently been implicated in senescence[54].

To test the rigor of the RNAseq results, we performed qRT-PCR validation in an independent collection of GBM43, GBM102, and GBM120 EVs. Limited RNA yield from GBM6 and A172 EVs precluded evaluation, and primers for SNORD116-2 and -6 could not be built with confidence, so these were omitted in follow-up. As shown in Figure 4A, snoRNAs in the RNA sequencing cohort were upregulated in GBM43 and GBM120 senEVs, but not GBM102 (with the exception of one replicate). This was confirmed by qRT-PCR, as increased snoRNA expression was found in only GBM43 and GBM120 senEVs (Figure 4B). We also mined our proteomic data for snoRNA associating proteins (SRPs)[55], which would suggest co-packaging and provide additional indirect evidence of snoRNA incorporation in senEVs. Indeed, we found SRPs enriched in, and unique to, GBM43 and A172 senEVs (Table 2), but not in GBM102 senEVs. Overall, these validated results argue for snoRNA enrichment as a putative biomarker for senEVs. While not shared by

the GBM102 model, we consider the enrichment of snoRNA across 4⁄5 models as likely appropriate for capturing a molecularly diverse functional state in a very heterogenous tumor type[56–58].

Table 2. snoRNPs in GBM EVs identified by mass spectrometry. Log2FC values represent change of senEVs compared to proEVs. Shaded Log2FC boxes indicate that the protein was not identified in either condition, whereas shaded p-val and q-val boxes indicate that the protein was not identified in at least 2/3 of either condition, limiting significance testing.

	A172			GBM43			GBM102		
Gene	**Log2FC**	**p-val**	**q-val**	**Log2FC**	**p-val**	**q-val**	**Log2FC**	**p-val**	**q-val**
FBL	1.138	0.216	0.325	UNIQUE 6GY			UNIQUE 0GY		
NHP2	-0.391	0.053	0.123	2.836					
NOP56	UNIQUE 6GY			3.135	0.002	0.007			
NOP58	UNIQUE 6GY			3.204	0.007	0.018			
GAR1	UNIQUE 6GY			2.936			UNIQUE 0GY		
SNU13	UNIQUE 6GY			4.929			UNIQUE 0GY		
DKC1	UNIQUE 6GY			UNIQUE 6GY					
NOP10				UNIQUE 6GY					

3.4. GBM patient plasma EVs post SOC support further development of a senEV bioassay

Translation of a biomarker for clinical RIS detection in GBM will be difficult without commensurate IHC approaches to assess senescence in post-treatment biopsied tissue. However, signals of RIS-associated gene sets and/or snoRNAs in patient plasma EVs after completion of radiotherapy encourage future studies and motivate development of senEVs as a biomarker for RIS in patients. Therefore, we performed total RNA sequencing on EVs from 4 paired GBM patient plasma samples collected at time of surgical resection (PreOP) and following completion of 46GY IR with concurrent temozolomide (PostSOC). Patient demographics are included in Table S4. To

streamline patient sample preparation and ensure capture of rarer species in the plasma, we omitted double-selection by SEC followed by exoRNeasy; patient samples were instead prepared by directly processing plasma with exoRNeasy.

Interestingly, plasma EV transcriptome profiles showed greater dispersion between PreOP samples and less dispersion between PostSOC samples (Figure 5A). While this is potentially aligned with prior work showing that patient PBMCs exposed to radiation *ex vivo* cluster by dosage and not donor[59], we further examined the effects of final library size and sample storage duration as potential confounders. However, we did not find strong evidence supporting these factors as covariates (Supplemental Figure 4A and 4B). This clustering suggests that patient exposure to radiation and systemic temozolomide is associated with drastic cargo changes in plasma EVs.

We found that upregulated species in PostSOC samples with q-val <0.1 were enriched in biological processes associated with the immune system, particularly T-cell related, and cell cycle regulation (Supplemental Figure 4D). In fact, *CDKN1B*, which encodes for the cyclin dependent kinase inhibitor p27, was amongst the top most differentially expressed species (Supplemental Figure 4D). To determine whether elevated signals of senescence were present in the PostSOC EVs as a group, we again turned to GSEA. However, senescence gene sets were not significantly enriched (data not shown). We screened significant DEGs (q-val <0.1 and $|\log_2 FC| \geq 1$) against the CellAge database, theorizing that individual senescence-associated species may still be detectable, despite lack of whole gene set enrichment. 109 DEGs overlapped with the Cell Age Database, but a large percentage were discordant in their directionality (Figure 5B). We identified 41 species that aligned with CellAge (Figure 5B and Table S5). This includes well-known *CDKN2B* (the cell cycle inhibitor p15), for which we observed increased abundance in 4/4 paired samples; *GLB1* (β-galactosidase), for which we observed increased abundance in 3/4 paired samples; and *DDB2* (damage specific dna binding protein 2), for which we observed increased abundance in 4/4 paired samples (Figure 5C). We acknowledge the absence of counts for these genes in some PreOP samples limits confidence in the extent of upregulation, which is reflected in their low shrunken log2FC values (Table S5); however, given that the genes are not lowly expressed in the PostSOC samples (all samples have normalized counts > 10), we believe that the trend towards upregulation is likely real. Finally, we queried DEGs for snoRNAs and identified three species: *SNORA49, SNORA22, SNORA77A*, of which the two former were upregulated. Notably, *SNORA49* was identified in our *in vitro* senEVs, as well. 3/4 patients showed increased *SNORA49* normalized counts in their PostSOC sample compared to their PreOP (Figure 5C). Of

interest, the patient that did not show upregulated *SNORA49* is the only patient with definitive radiographic evidence of progressive disease (Table S4). While these data are not evidence of a snoRNA or senescence enrichment, per se, we find that the increase in detectable species of interest in PostSOC EVs from an unenriched plasma source with an untargeted sequencing effort is encouraging.

4. Discussion

Detection of therapy/radiation-induced senescence in cancer patients is difficult. TIS is temporally and spatially dynamic, meaning rigorous evaluation requires capture across time points and regions. However, current methodologies for evaluating senescence in patients are histochemical or transcriptomic, thereby requiring tissue[17,27,60,61]; for certain tumor types like GBM, this requirement precludes evaluation after SOC. The development of a minimally invasive liquid bioassay for TIS would open avenues for both an improved understanding of TIS clinically and the potential for a precision medicine approach for senotherapeutics. EVs have shown promise as biomarkers for binary findings like the presence or absence of GBM[29,62], but we questioned whether EVs may also be able to capture more complex phenotype information. Further, while previous studies have examined cargo in EVs released by senescent tumor cells, the focus has primarily been on functional biological outcomes of EV release and uptake, rather than identifying to what extent EV cargo reflects or can report on the senescent cell phenotype[33,63].

We found here that EVs elaborated by senescent GBM contain transcriptomic features that may enable their use as an analyte for RIS detection. Specifically, senEVs contain increased abundance of senescence-associated RNA species and decreased abundance of species associated with DNA replication and chromosome separation. This very closely mimics what was observed in the originating cell populations. Unexpectedly, we also found that senEVs contain an enrichment of snoRNAs, which are likely packaged with their protein binding partners. The noncanonical roles of snoRNAs are understudied, but at least one snoRNA identified in our senEVs, *SNORA13,* has been implicated in senescence[54].

It is important to emphasize that at least two separate classes of RNAs may identify senEVs (coding RNAs of senescence genes and the noncoding snoRNAs). We posit that a combination of both RNA classes will offer the greatest sensitivity and specificity to detect senescence. Senescent cells, themselves, are highly heterogeneous at the transcriptional level and phenotype development can be variable between cell lines and between senescence-stimuli[56,64,65]. This is

underscored by our own data here, where all five GBM models undergo senescence but with variable presentations of each evaluated phenotype. As a biomarker platform, senEVs, which encapsulate multiple different signals associated with senescence, may therefore be at an advantage. Future assay development will likely incorporate a targeted sequencing panel against multiple of our identified RNAs, combined with an algorithmic/machine learning–based approach to establish robust thresholds for defining a 'positive' signal of senescence.

Excitingly, from four matched longitudinal patient plasma samples we were also able to detect increased abundance of both senescence-associated and snoRNA species in postSOC EVs compared to pre-operation EVs. While broad conclusions are limited due to minimal cohort size and lack of UMIs to enable more confident transcript quantitation, these preliminary results demonstrate the feasibility of detecting putative markers of cell stress and functional states from plasma EVs. Further, our approach here was performed without enrichment for brain or GBM-derived EVs. Given the systemic administration of temozolomide, it is likely that a portion of the detected signals reflect stress imposed on host blood and non-brain tissues. We believe these signals are still of importance to patient health, as senescence outside of the tumor tissue of origin has previously been implicated in therapy-associated side effects, such as chemotherapy-induced neuropathy and cardiotoxicity[66,67]. Rather than moving forward with enrichment of GBM/brain-derived EVs in future work, we posit that retaining signals of TIS in both GBM/brain and other host tissues will be beneficial. To this end, recent advancements in single EV sequencing[68,69] may allow for the simultaneous detection of TIS associated signals alongside tissue-specific markers to help distinguish host cell and tumor cell senescence.

Perhaps the most difficult aspect of translating a senEV bioassay for RIS– or any clinical evaluation of RIS– will be the capacity to assess specificity and sensitivity of the markers. Future studies should certainly include larger patient cohorts with more robust time point acquisition, alongside matched radiographic response data and tissue sampling. Tumor types that allow for repeated tissue biopsy, such as breast cancer, may therefore be an ideal additional patient cohort. This would enable concurrent standard detection of tissue senescence to allow for true measure of senEV capabilities. While preliminary, our results in GBM patients presented here support such an undertaking.

Finally, it is important to acknowledge that radiation promotes multiple functional states in GBM, including but not limited to autophagy, persister states that depend on lipid peroxidase pathways,

and stem cell-like transitions[70–72]. Notably, these functional states are not mutually exclusive, and indeed, autophagy and senescence are closely intertwined[73]. While our current work has focused on RIS, the senEV signature likely represents only one opportunity to identify appropriate adjuvant therapies for GBM patients. Our data here provides proof-of-concept that EVs recapitulate and relay transcriptional information about complex processes that the originating cell population is experiencing. We posit that similar signatures can be developed for additional functional states, which would broaden the applications for a post-SOC EV assay for GBM and other cancer patients. While disentangling the markers for concurrent phenotypes will present a challenge, the data here ultimately supports extracellular vesicles as promising analytes for the development of liquid biopsies detecting drug-induced functional states, particularly radiation induced senescence.

5. Acknowledgments

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6. Data availability

All data is currently available upon request from the corresponding author. GEO accession numbers will be provided upon publication.

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8. Main Figure Legends

Figure 1. Evaluation of phenotypic markers of RIS following 6GY IR in GBM cells. A) Dose responses for five GBM cell lines following 0-10GY radiation at 24 hours, 72 hours, and 168 hours (D7) post-exposure, demonstrating that response to IR is delayed. The dotted lines mark 50%

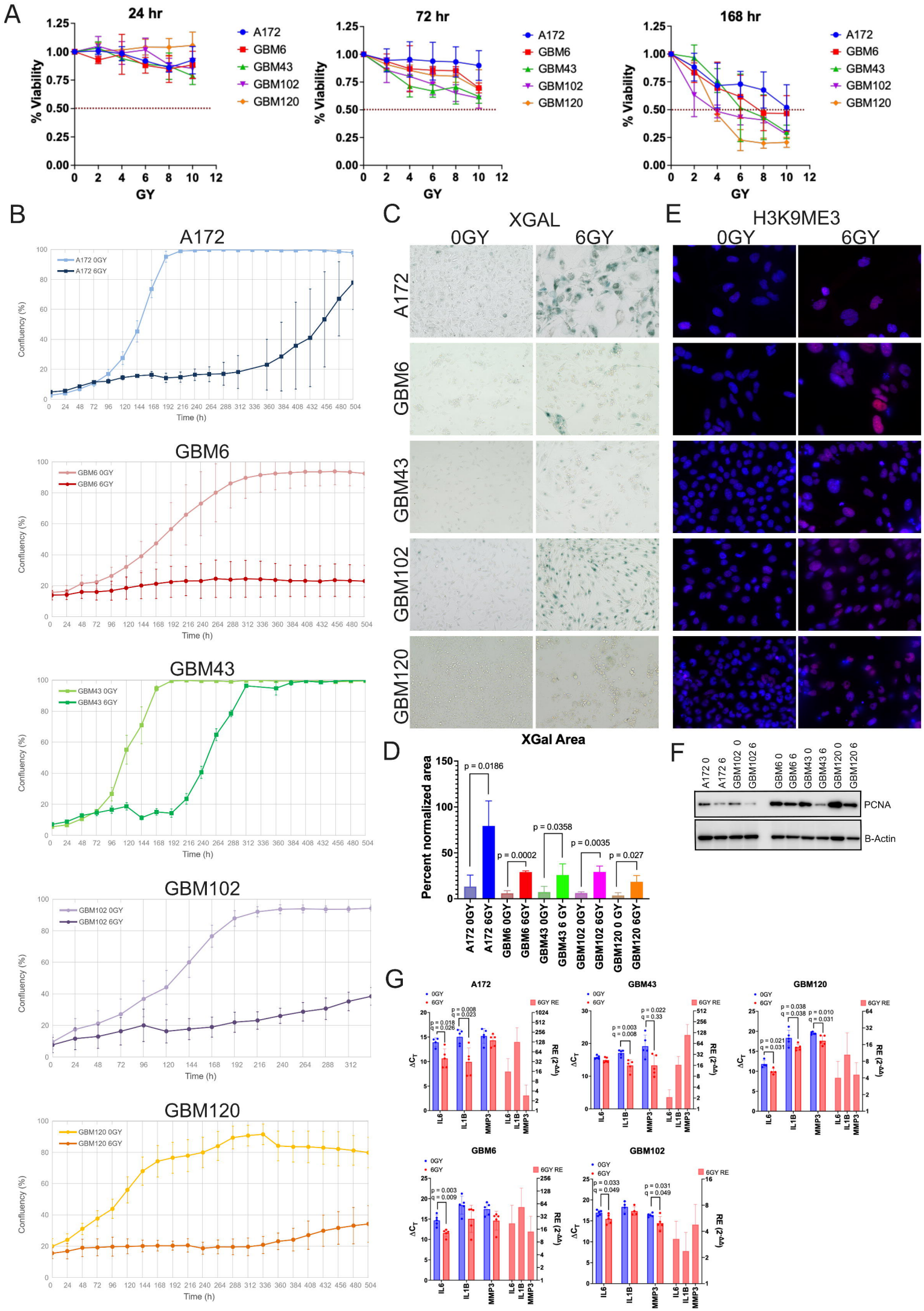
relative viability. **B)** Live-cell imaging demonstrating growth arrest in 6GY treated GBM. Graphs are shown as the mean confluency +/- SD of individual wells from a representative experiment (the chosen representative experiment for A172 is shown here and in supplemental figure 1). **C)** Brightfield microscopy shows phenotypic alterations in D7 treated GBM and increased SA-β-gal activity. **D)** Image J quantification of image area positive for XGal signal, normalized against cell area. Graph is mean +/- SD of at least 3 independent experiments with unpaired student's t-test p-values. **E)** H3K9ME3 immunofluorescence reveals increased SAHF on D7 **F)** 6GY leads to reduced PCNA and cell-line dependent increases in p21. **G)** SASP factors are increased in all 5 GBM lines post-radiation. ΔCt bar graphs (left axis) are shown as mean +/- SD with independent experiments represented by data points. Significant findings (q-val < 0.05) are shown by their p-value and q-value (unpaired t-test with Benjamini-Hochberg FDR). Relative expression (right axis) is shown as mean +/- SD across the independent experiments.

Figure 2. Profiling the senescent transcriptome in GBM. A) Significant differentially expressed genes (q-val <0.05) were queried for overrepresented (based on a positive log2FC) and underrepresented (based on a negative log2FC) gene ontologies. **B and C)** GSEA identifies expected gene sets for senescent cells. Significant hallmark gene sets are shown in **B,** and significant senescence-specific gene sets are shown in **C.**

Figure 3. EVs secreted by senescent GBM cells have distinct transcriptomes from those secreted by proliferative GBM cells. A) Principal component analysis following variance-stabilization transformation reveals that variation by EVs is driven primarily by cell line of origin, though there is some distinction between senEVs and proEVs within cell line. **B)** MA plot, with significance indicated by blue color, showing that the majority of identified RNA species have either low magnitude log2FC or do not reach significance. Red lines are drawn at Log2FC= -1,1 and orange are drawn at Log2FC= -0.585, 0.585. **C)** Queries of significant DEGs, as performed in Figure 2A, showing that upregulated DEGs in senEVs belong to biological processes distinct from the whole cell transcriptomes. However, there is overlap in the underrepresented processes, as there is a loss (downregulated DEGs) in biological processes related to DNA replication and chromosome segregation. **D and E)** GSEA identified hallmark gene sets that echo those of the whole cell transcriptomes during senescence, as well as significantly enriched senescence gene sets.

Figure 4. senEVs are generally enriched in snoRNAs. A) snoRNAs are the predominant class of RNAs that have large magnitude changes between sen and proEVs. Heatmap of log2(normalized_count+1), scaled by row, is shown for the top 20 species after ranking DEGs by shrunken log2FC values. **B)** snoRNA expression patterns observed in RNAseq were validated by qRT-PCR in GBM43, GBM102, and GBM120. cel-miR-39 cDNA was used as a qPCR spike in control. Ct values (left axis) and modified relative expression (right axis, see methods for details) are shown as mean +/- SD with independent experiments represented by data points. Significance was tested by paired t-test to account for the differences between independent experiments caused by variable RNA yield and assay input. Findings with p-value <0.05 are reported. Benjamini-Hochberg FDR q-values are also reported.

Figure 5. Species of interest are upregulated in GBM patient plasma EVs post-radiation. A) Principal component analysis following variance-stabilization transformation indicates clustering driven by treatment, particularly in postSOC EVs. **B)** Significant DEGs (PostSOC vs. PreOP) were screened against the Cell Age senescence expression database. 39 genes were observed to be upregulated as expected and 2 genes were observed to be downregulated as expected. **C)** Normalized counts for *CDKN2B, GLB1, DDB2,* and *snoRNA49.*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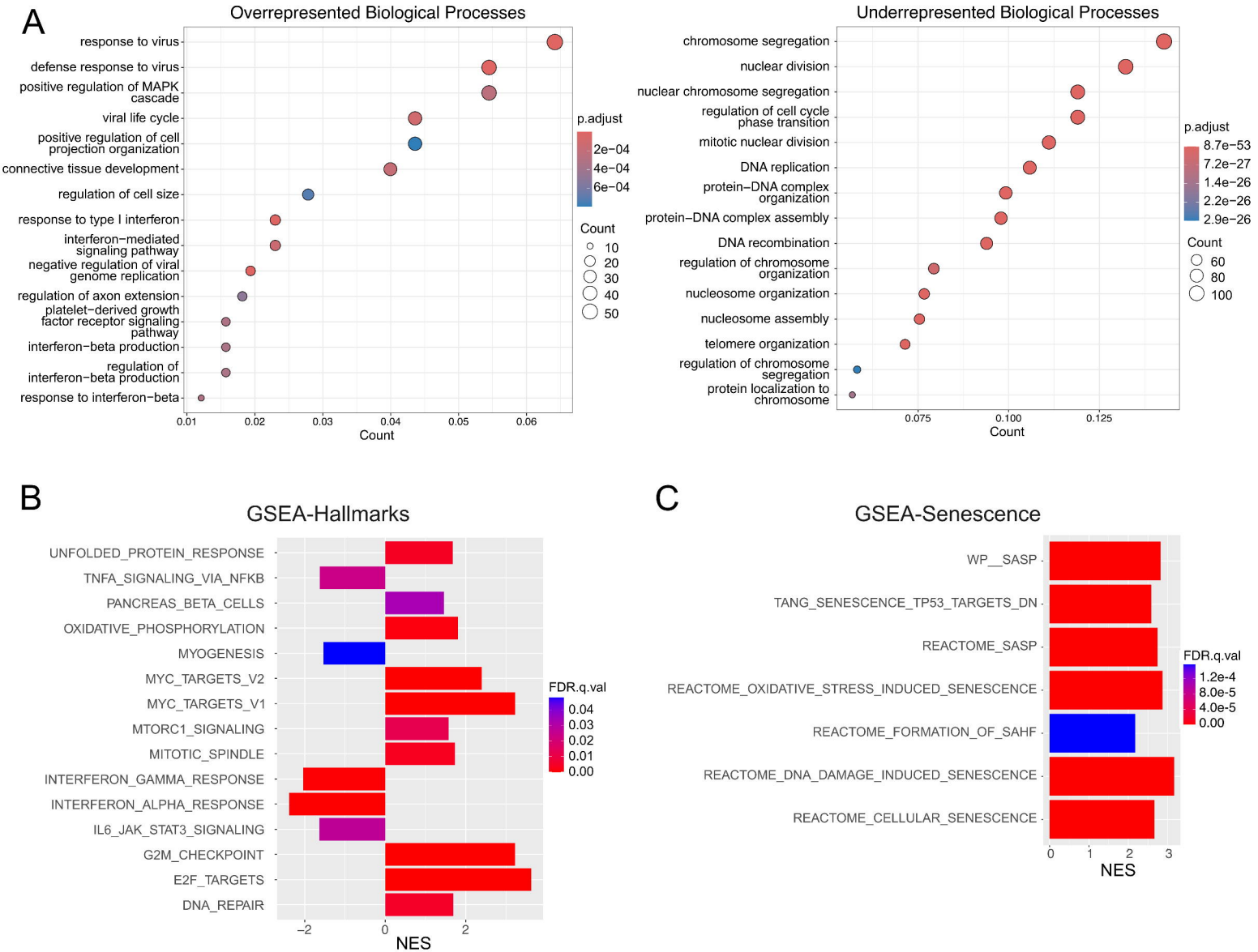
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Overrepresented Biological Processes
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positive regulation of MAPK cascade
viral life cycle
positive regulation of cell projection organization
connective tissue development
regulation of cell size
response to type I interferon
interferon−mediated signaling pathway
negative regulation of viral genome replication
regulation of axon extension
platelet−derived growth factor receptor signaling pathway
interferon−beta production
regulation of interferon−beta production
response to interferon−beta
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Underrepresented Biological Processes
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nuclear division
nuclear chromosome segregation
regulation of cell cycle phase transition
mitotic nuclear division
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protein−DNA complex assembly
DNA recombination
regulation of chromosome organization
nucleosome organization
nucleosome assembly
telomere organization
regulation of chromosome segregation
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UNFOLDED_PROTEIN_RESPONSE
TNFA_SIGNALING_VIA_NFKB
PANCREAS_BETA_CELLS
OXIDATIVE_PHOSPHORYLATION
MYOGENESIS
MYC_TARGETS_V2
MYC_TARGETS_V1
MTORC1_SIGNALING
MITOTIC_SPINDLE
INTERFERON_GAMMA_RESPONSE
INTERFERON_ALPHA_RESPONSE
IL6_JAK_STAT3_SIGNALING
G2M_CHECKPOINT
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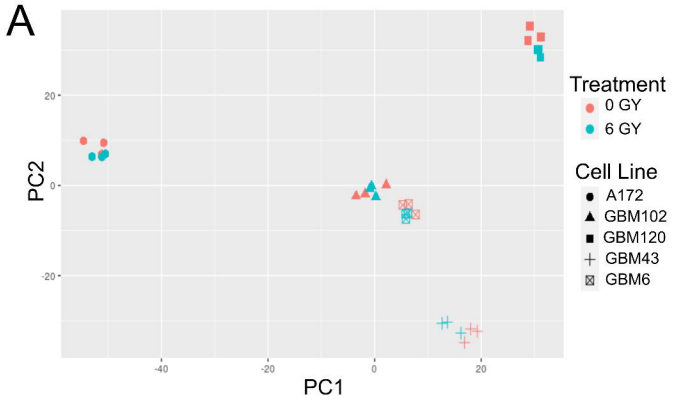
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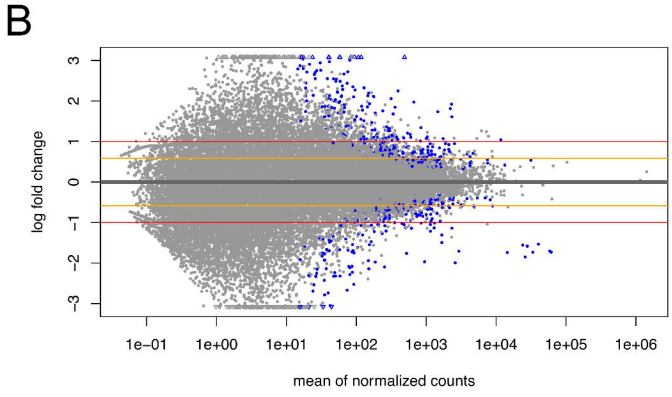
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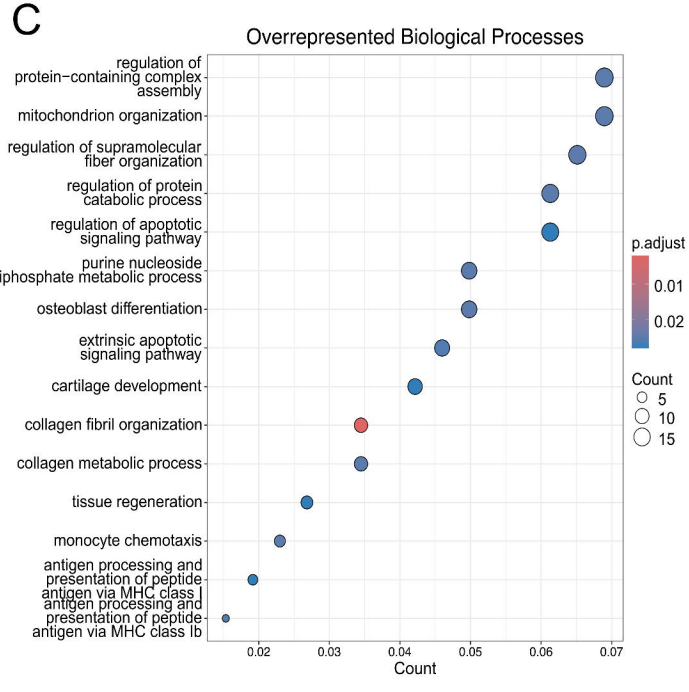
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regulation of protein−containing complex assembly
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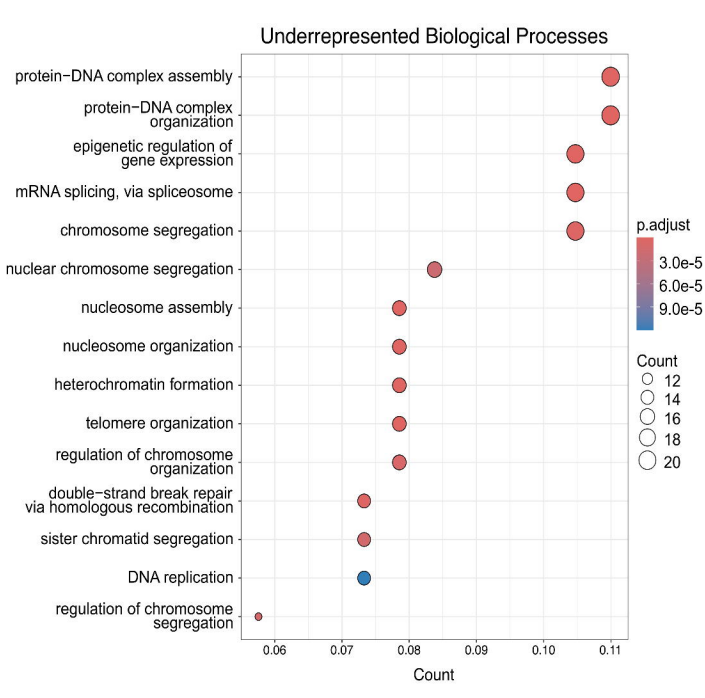
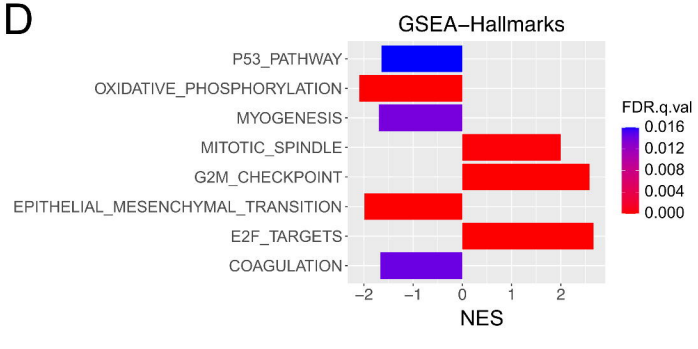
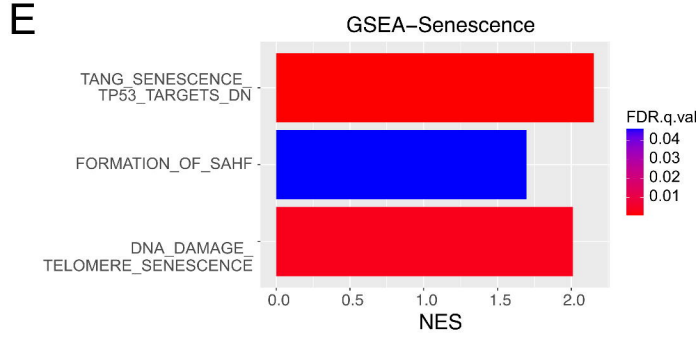
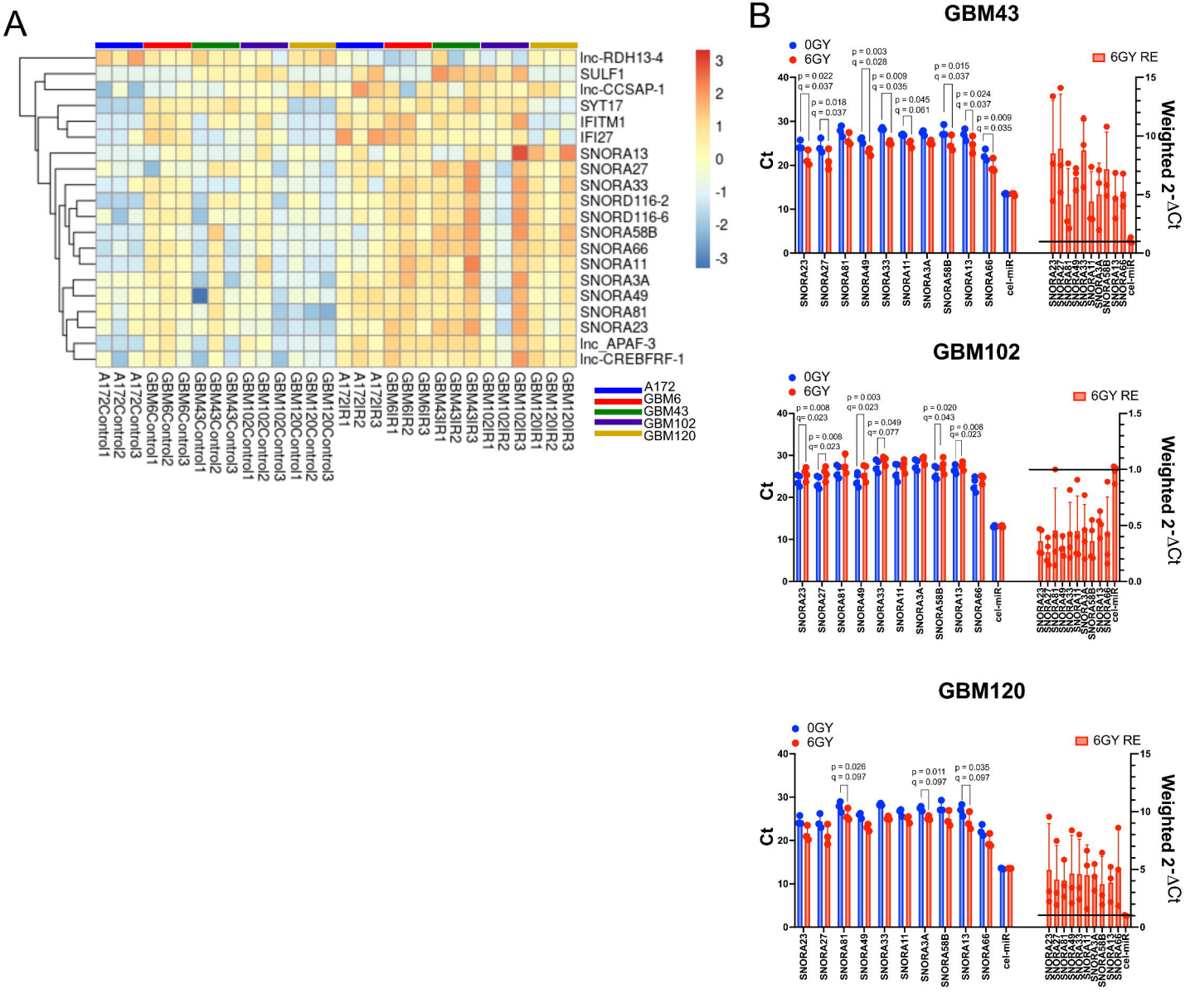
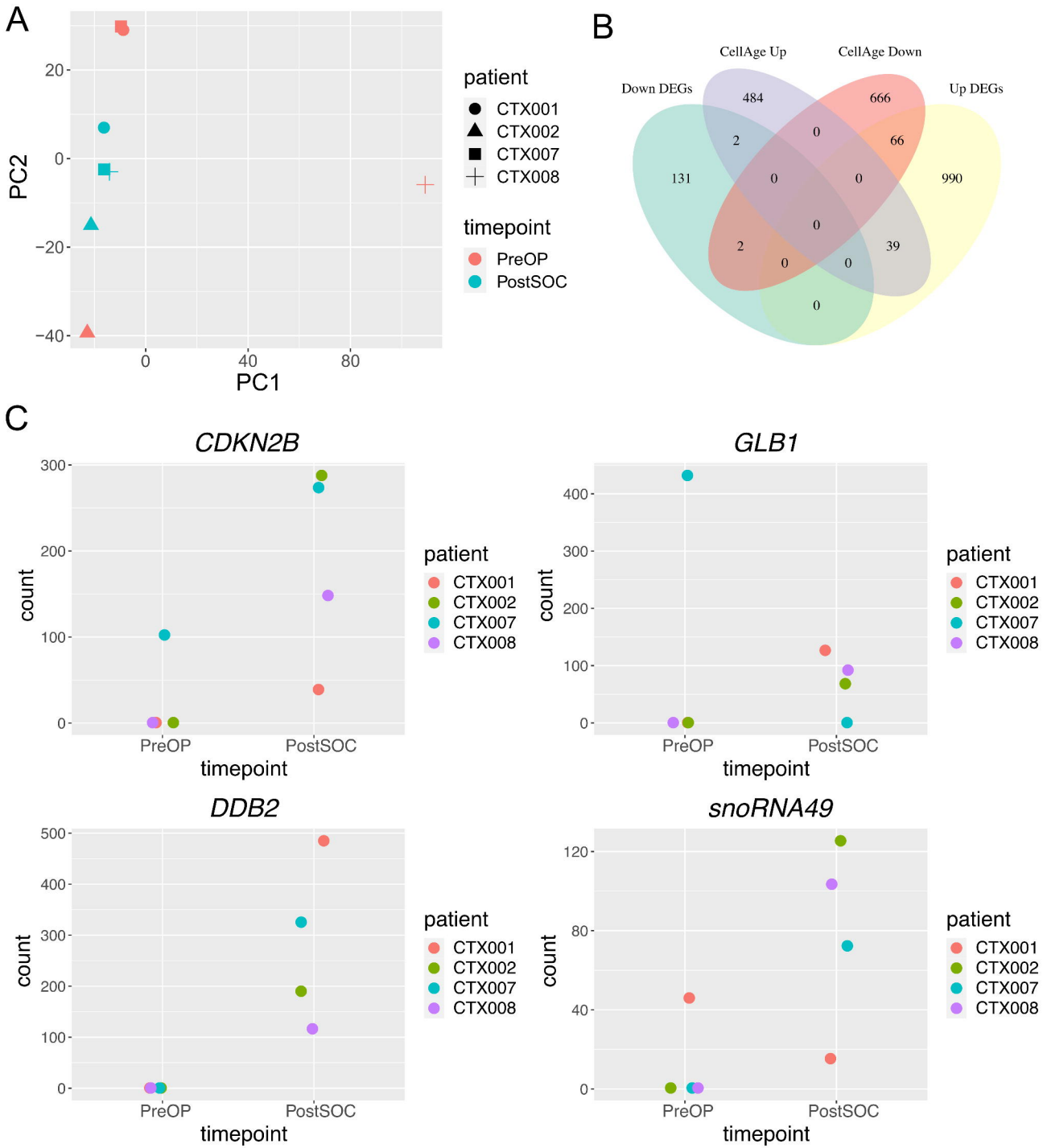
regulation of supramolecular fiber organization
regulation of protein catabolic process
regulation of apoptotic signaling pathway
purine nucleoside triphosphate metabolic process
osteoblast differentiation
extrinsic apoptotic signaling pathway
cartilage development
collagen fibril organization
collagen metabolic process
tissue regeneration
monocyte chemotaxis
antigen processing and presentation of peptide antigen via MHC class I
antigen processing and presentation of peptide antigen via MHC class Ib
0.02
0.03
0.04
0.05
0.06
0.07
Count
p.adjust
0.01
0.02
Count
5
10
15


Underrepresented Biological Processes
protein−DNA complex assembly
protein−DNA complex organization
epigenetic regulation of gene expression
mRNA splicing, via spliceosome
chromosome segregation
nuclear chromosome segregation
nucleosome assembly
nucleosome organization
heterochromatin formation
telomere organization
regulation of chromosome organization
double−strand break repair via homologous recombination
sister chromatid segregation
DNA replication
regulation of chromosome segregation
0.06
0.07
0.08
0.09
0.10
0.11
Count
p.adjust
3.0e-5
6.0e-5
9.0e-5
Count
12
14
16
18
20


D
GSEA−Hallmarks
P53_PATHWAY
OXIDATIVE_PHOSPHORYLATION
MYOGENESIS
MITOTIC_SPINDLE
G2M_CHECKPOINT
EPITHELIAL_MESENCHYMAL_TRANSITION
E2F_TARGETS
COAGULATION
−2
−1
0
1
2
NES
FDR.q.val
0.016
0.012
0.008
0.004
0.000


E
GSEA−Senescence
TANG_SENESCENCE_TP53_TARGETS_DN
FORMATION_OF_SAHF
DNA_DAMAGE_TELOMERE_SENESCENCE
0.0
0.5
1.0
1.5
2.0
NES
FDR.q.val
0.04
0.03
0.02
0.01


A
lnc-RDH13-4
SULF1
lnc-CCSAP-1
SYT17
IFITM1
IFI27
SNORA13
SNORA27
SNORA33
SNORD116-2
SNORD116-6
SNORA58B
SNORA66
SNORA11
SNORA3A
SNORA49
SNORA81
SNORA23
lnc_APAF-3
lnc-CREBFRF-1
3
2
1
0
-1
-2
-3
A172Control1
A172Control2
A172Control3
GBM6Control1
GBM6Control2
GBM6Control3
GBM43Control1
GBM43Control2
GBM43Control3
GBM102Control1
GBM102Control2
GBM102Control3
GBM120Control1
GBM120Control2
GBM120Control3
A172IR1
A172IR2
A172IR3
GBM6IR1
GBM6IR2
GBM6IR3
GBM43IR1
GBM43IR2
GBM43IR3
GBM102IR1
GBM102IR2
GBM102IR3
GBM120IR1
GBM120IR2
GBM120IR3
A172
GBM6
GBM43
GBM102
GBM120
B
GBM43
0GY
6GY
6GY RE
Ct
Weighted 2-ΔCt
p = 0.022
q = 0.037
p = 0.018
q = 0.037
p = 0.003
q = 0.028
p = 0.009
q = 0.035
p = 0.045
q = 0.061
p = 0.015
q = 0.037
p = 0.024
q = 0.037
p = 0.009
q = 0.035
SNORA23
SNORA27
SNORA81
SNORA49
SNORA33
SNORA11
SNORA3A
SNORA58B
SNORA13
SNORA66
cel-miR
GBM102
0GY
6GY
6GY RE
Ct
Weighted 2-ΔCt
p = 0.008
q= 0.023
p = 0.008
q= 0.023
p = 0.003
q= 0.023
p = 0.049
q= 0.077
p = 0.020
q= 0.043
p = 0.008
q= 0.023
SNORA23
SNORA27
SNORA81
SNORA49
SNORA33
SNORA11
SNORA3A
SNORA58B
SNORA13
SNORA66
cel-miR
GBM120
0GY
6GY
6GY RE
Ct
Weighted 2-ΔCt
p = 0.026
q = 0.097
p = 0.011
q = 0.097
p = 0.035
q = 0.097
SNORA23
SNORA27
SNORA81
SNORA49
SNORA33
SNORA11
SNORA3A
SNORA58B
SNORA13
SNORA66
cel-miR


A
PC2
PC1
20
0
−20
−40
0
40
80
patient
CTX001
CTX002
CTX007
CTX008
timepoint
PreOP
PostSOC
B
CellAge Up
CellAge Down
Down DEGs
Up DEGs
484
666
2
0
66
131
0
0
990
0
2
39
0
0
0
C
CDKN2B
GLB1
DDB2
snoRNA49
count
timepoint
PreOP
PostSOC
patient
CTX001
CTX002
CTX007
CTX008