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Elucidating Proteoform Dynamics Underlying the Senescence Associated Secretory Phenotype

Peter F. Doubleday,

Department of Molecular Biosciences, Proteomics Center of Excellence, Northwestern University, Evanston, Illinois 60208, United States

Luca Fornelli,

Department of Biology, University of Oklahoma, Norman, Oklahoma 73019, United States

Neil L. Kelleher

Department of Molecular Biosciences, Proteomics Center of Excellence, Northwestern University, Evanston, Illinois 60208, United States

Abstract

Primary diploid cells exit the cell cycle in response to exogenous stress or oncogene activation through a process known as cellular senescence. This cell-autonomous tumor-suppressive mechanism is also a major mechanism operative in organismal aging. To date, temporal aspects of senescence remain understudied. Therefore, we use quantitative proteomics to investigate changes following forced HRAS^{G12V} expression and induction of senescence across 1 week in normal diploid fibroblasts. We demonstrate that global intracellular proteomic changes correlate with the emergence of the senescence-associated secretory phenotype and the switch to robust cell cycle exit. The senescence secretome reinforces cell cycle exit, yet is largely detrimental to tissue homeostasis. Previous studies of secretomes rely on ELISA, bottom-up proteomics or RNA-seq. To date, no study to date has examined the proteoform complexity of secretomes to elucidate isoform-specific, post-translational modifications or regulated cleavage of signal peptides. Therefore, we use a quantitative top-down proteomics approach to define the molecular complexity of secreted proteins <30 kDa. We identify multiple forms of immune regulators with known activities and affinities such as distinct forms of interleukin-8, as well as GRO α and HMGA1, and temporally resolve secreted proteoform dynamics. Together, our work demonstrates the complexity of the secretome past individual protein accessions and provides motivation for further proteoform-resolved measurements of the secretome.

Corresponding Author: Neil L. Kelleher – Department of Molecular Biosciences, Proteomics Center of Excellence, Northwestern University, Evanston, Illinois 60208, United States; n-kelleher@northwestern.edu.

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jproteome.9b00739>.

B55-confirms +32 Da mass shift in di-S-sulhydrated or di-S-sulfinic acid modified IL8 and whole gel images of blots from Figure 1D (PDF)

Quantitative bottom-up proteomics of HRAS^{G12V} induced senescence (XLSX)

GO Enrichment (XLSX)

Proteoforms of the SASP (XLSX)

Complete contact information is available at: <https://pubs.acs.org/doi/10.1021/acs.jproteome.9b00739>

The authors declare no competing financial interest.

Graphical Abstract

Keywords

top-down proteomics; senescence; quantitative proteomics; secretome; proteoform

INTRODUCTION

Cellular senescence refers to a state of durable cell-cycle arrest in response to numerous intrinsic and extrinsic stressors in otherwise proliferation-competent cells. Senescent phenotypes can be induced by genotoxic stimuli, chemotherapeutic agents, loss of tumor-suppressive mechanisms, and oncogene activation or overexpression.¹ Several tumor-suppressive hallmarks including the upregulation of P16^{INK4}, p53, Rb, and chromatin remodeling proteins like HMGB1 accompany oncogene-induced senescence (OIS).¹ Further, a dynamic NF- κ B and interleukin-dependent secretory phenotype known as the senescence-associated secretory phenotype (SASP) reinforces senescence both in vitro and in vivo.² The SASP has pleiotropic functions and spurs proliferation in cancer cells, is largely detrimental to tissue homeostasis in aged animals, and yet aids in wound healing.³ In vivo, the SASP disrupts normal tissue function by inducing fibrosis and by inhibiting stem cell function.⁴ Given the desire to avoid the detrimental effects of senescent cells in vivo, a more robust definition of their net protein output, that is, the cadre of secreted factors and signaling programs underlying their complexity would clarify context-dependent effects and further define disease mechanisms underlying aging.

Large-scale quantitative investigations of senescent cells and of the SASP have been carried out using bottom-up proteomics and RNA-seq and by affinity-enrichment coupled with proteomics.^{2,5} These studies have defined the SASP of OIS as a set of ~200 gene products that change dynamically in time.⁶ These studies have also made the major conclusion that proteomic and mRNA signatures of the secretome are very poorly correlated using SILAC and RNA-seq approaches.² To date, no study has profiled temporal aspects of the secretome in detail nor has any top-down proteomics study described the proteoform complexity of SASP factors.^{7,8} As secreted factors exhibit distinct receptor affinities manifested through

post-translational modification, pro-peptide cleavage, and further regulated processing, understanding the complexity of individual SASP factors in space and time has the potential to better define mechanisms underlying age-related pathologies.^{9–11} Many secreted factors and immunoregulatory proteins are of the ideal molecular weight for high-throughput top-down proteomic studies run with chromatography in denaturing mode.¹²

Here, given fundamental unanswered questions about the complexity of the temporal dynamics of senescence induction and of the SASP, we use tandem mass tag (TMT)-based bottom-up proteomics and leverage label-free quantitative top-down proteomics to characterize and quantify proteoform-level changes across senescence induction spurred by expression of the oncogene, HRAS^{G12V}. From TMT-based proteomics, we find that SASP factors emerge after 3 days following initial stress. Through sufficiency studies, we further determine that cells require culture with forced HRAS^{G12V} expression and conditioned media for at least 4 days to become fully senescent. This time-point reflects major intracellular proteomic changes and the emergence of SASP factors such as interleukin-8 (IL8). Using top-down proteomics, we identify secreted proteoforms of IL8 and HMGA1 proteins as well as GRO α and CXCL5, proteins known to reinforce the senescent phenotype. While the study of the secretome and secreted factors is met with experimental hurdles like interfering serum proteins and large changes in expression levels, our results provide a temporal atlas of senescence induction and highlight some proteoform biology associated with SASP.

MATERIALS AND METHODS

Cell Culture

Human IMR90 lung fibroblast cells (ATCC CCL-186) expressing 4-OHT-inducible ER:HRAS^{G12V} were grown in Dulbecco's modified Eagle media (DMEM) supplemented with 10% heat-inactivated, sterile-filtered fetal bovine serum (Life Technologies). HRAS^{G12V} expression was induced under an estrogen receptor-driven system by treatment with 10 nM 4-hydroxytamoxifen (4OHT) (Sigma) as previously described.¹³ IMR90 ER:HRAS^{G12V} cells were a kind gift of Masashi Narita, CRUK, Cambridge.

Senescence-Associated β -Galactosidase Staining and Visualization

Endogenous β -galactosidase activity for senescent cell detection was monitored by a previously described cytochemical method.¹⁴ Briefly, subconfluent IMR90 cells were washed twice with warm Dulbecco's phosphate buffered saline (dPBS), and fixed in freshly prepared 0.2% glutaraldehyde/2% formaldehyde solution for 5 min. Adherent cells were then washed with dPBS and incubated with freshly prepared staining solution (20 mM citric acid, 50 mM Na₂HPO₄, 5 mM K₄[Fe(CN)₆]·3H₂O, 5 mM K₃[Fe(CN)₆], 150 mM NaCl, 2 mM MgCl₂, 1 mg/mL X-Gal, pH 6.0) for 12 h at 37 °C provided atmospheric O₂ and CO₂. To quench enzyme activity, cells were washed three times with dPBS and dried by washing once with 100% MeOH. Cells were visualized on a Evos microscope (Thermo). For sufficiency studies, cells were also stained with DAPI ProLong Gold (Thermo Fisher), and 15 random fields were visualized under UV and bright field settings across biological triplicate samples and counted using ImageJ software.

TMT Labeling and Off-Line Peptide Fractionation

Cells from each time point were separately lysed and labeled with 10-plex TMT reagents, per manufacturer instructions with several modifications. First, cells were washed with ice-cold dPBS, lysed in 8 M urea lysis buffer (8 M urea, 25 mM Tris pH 8.0, PierceNet protease/phosphatase inhibitors), and flash frozen. Thawed samples were disrupted through sonication using a sonicating probe tip (Kontes) with four, 5 s pulses with 5 s delay between pulses. Lysates were clarified by centrifugation at 21,000g for 10 min at 4 °C. Protein concentration was normalized by bicinchoninic acid (BCA) assay. Samples were then reduced with the addition of 200 mM Tris(2-carboxyethyl)phosphine) at 55 °C for 1 h and alkylated with iodoacetamide for 30 min at room temperature in the dark. Proteins were then acetone precipitated overnight and allowed to air dry. Proteins were resuspended in triethylammonium bicarbonate buffer pH 8.0 and digested to peptides with overnight digest with trypsin at a ratio of 1:40. Tryptic peptides were desalted on C18 resin as previously described and concentrated to near-dryness by vacuum centrifugation.¹⁵ TMT reagents were dissolved in anhydrous acetonitrile (MeCN) and added to peptide at a final concentration of 30% MeCN in 70% TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA). Reactions proceeded for 1 h at room temperature and were quenched with hydroxylamine to 0.3% (v/v) final concentration. TMT samples were combined at a 1:1:1:1:1:1:1:1:1:1 ratio (10-plex TMT experiment). The multiplexed sample was dried by vacuum centrifugation and desalted on C18 as previously described.^{15,16} Labeled peptides were resuspended in 7 mM KH₂PO₄/33% MeCN pH 2.65 and applied to preconditioned PolySULFOETHYL A columns (PolyLC Inc., Columbia, MD). Eluted fractions were collected across increasing steps of KCl molarity at 0, 10, 25, 50, 150, and 250 mM KCl in 7 mM KH₂PO₄/33% MeCN at pH 2.65. Fractions were dried by vacuum centrifugation, resuspended in 0.1% TFA, and desalted on C18 resin.

Secretome Sample Preparation for LC–MS/MS

Conditioned media was collected at select times following initial 4OHT administration. At 18 h prior to each sample collection time point, each sample of 5×10^6 cells was washed five times with sterile PBS, and media was replaced with 12 mL of DMEM. At the time of collection, conditioned media was carefully collected and precipitated with the addition of four volumes of –20 °C acetone and incubated overnight at –20 °C. Protein was pelleted by centrifugation at 14,000g for 10 min at 4 °C, and precipitated protein was resuspended in 1% sodium dodecyl sulfate and subjected to quantification by the BCA method (Pierce). An equal volume of each sample, corresponding to less than 350 µg of protein, was subjected to molecular weight based fractionation on an 8% GELFREE cartridge. Protein was precipitated from the first GELFREE fraction, corresponding to 0–30 kDa, by the MeOH/CHCl₃/H₂O method prior to liquid chromatography with tandem mass spectrometry (LC–MS/MS) as previously described.^{17,18}

Liquid Chromatography and Mass Spectrometry for Top-Down Proteomics

For quantitative top-down proteomics, injections for biological replicates were randomly ordered consistent with good practices for study design and execution. Protein samples were resolved by reversed-phase liquid chromatography using an Ultimate 3000 LC system

(Thermo Scientific, San Jose, CA). Samples were first loaded onto a trap column (30 mm, 150 μm inner diameter, i.d.) packed with PLRP-S resin for protein measurements (Agilent, Santa Clara, CA). To separate proteoforms, an in-house packed capillary PLRP-S column (350 mm, 75 μm i.d.) was used and proteoforms were resolved on a 75 μm $\times$ 30 cm analytical column packed with the same trap column material. Trap and analytical columns were heated at 35 $^{\circ}\text{C}$ for protein measurements. Proteoforms were resolved over a gradient consisting of a ramp of solvent B from 15 to 42% in 85 min, with a total run time of 120 min, including column wash (at 85% solvent B) and re-equilibration. Solvent A consisted of 5% acetonitrile and 0.2% formic acid in water, while solvent B was composed of 5% H_2O and 0.2% formic acid in acetonitrile. The outlet of the column was coupled to a PicoTip spray emitter, packed with 2 mm of PLRP resin (New Objective, Woburn, MA). The spray emitter was coupled online to a nanoelectrospray ionization source, to which a ~ 1.7 kV potential on an LTQ Fusion Lumos Orbitrap mass spectrometer (Thermo Fisher Scientific) operated in data-dependent mode. For top-down experiments, full MS1 data were collected from 500 to 2000 m/z with four μscans for each full scan at a resolution of 120,000 (at 200 m/z) with automatic gain control (AGC) set at 2×10^5 and a maximum injection time of 800 ms. The top-two most abundant precursor ion populations were selected for MS/MS by fragmentation with HCD in a 3 m/z centered window at a normalized collisional energy of 22. Data-dependent scans were acquired from 400 to 2000 m/z with 4 μscans , at a resolution of 60,000 (at 200 m/z) and a maximum injection time of 800 ms. Dynamic exclusion was implemented with a repeat count of 1 and a duration of 60 s.

Liquid Chromatography and Mass Spectrometry for Bottom-Up Proteomics

For TMT-based proteomic analyses, desalted peptides were loaded onto a 3 cm by 150 μm id trap column and separated over a linear gradient of on a 30 cm by 150 μm id analytical column packed with 1.8 μm 300 $\text{\AA}$ C18 resin (Sepax). For all peptide measurements, column and precolumns were heated to 65 $^{\circ}\text{C}$. A PicoTip emitter (New Objective) packed with 2 mm PLRP resin was coupled to a nanoelectrospray ionization source that provided ~ 1.4 kV potential on an Orbitrap Elite hybrid mass spectrometer (Thermo Fisher Scientific). TMT data were collected in a “top-10” data-dependent fashion. Full MS1 scans were collected from 300 to 1800 m/z with 1 μscan at a resolution of 60,000 (at 400 m/z) with AGC set to 1×10^6 and a maximum injection time of 200 ms. Mass spectra of tandem mass tag and sequence-specific ions were generated at the MS2 level following precursor isolation in a 2 m/z window and stepped collisional energy fragmentation (HCD: 35, 37, 39). Tandem spectra were acquired from 100 to 2000 m/z with 1 μscan , a target AGC of 1×10^5 , and a maximum injection time of 200 ms at a resolution of 30,000 (at 400 m/z). Three technical replicate injections were acquired for each crude SCX fraction, for a total of 18 injections for TMT-based experiments.

Data and Statistical Analyses

Peptide-level data were processed with MS Amanda on Proteome Discoverer 2.2 against the Swiss-Prot human database (release “2014_03”, March 19, 2014). Searches were performed using a 10 ppm precursor ion tolerance and a 4 m/z product ion tolerance. Additional peptide mass from TMT tags on N-termini (+229.1629) and carbamidomethylation of cysteine residues (+57.0215 Da) were set as static modifications while methionine oxidation

(+15.9949 Da) was set as a variable modification. Peptide spectral matches were filtered below a 1.0% FDR cutoff using a previously described target-decoy strategy.¹⁹

Top-down proteomic files were processed through TDPportal to identify intact proteins and to characterize proteoforms by database and scoring at a 1% proteoform level false discovery rate as previously described.²⁰ Briefly, TDPportal generated linked sets of precursor and fragmentation data from RAW files, which were searched against a highly annotated version of the Human UniProt Knowledge Base using a three-tiered search tree (files accessible at MASSIVE: MSV000084530). A three-tiered search strategy was employed for this study, consisting of a narrow precursor mass search (2.2 Da) and two “biomarker”-type searches. To characterize proteoforms that were not identified by our high throughput search, MS2 spectra were deconvoluted using the Xtract deconvolution algorithm (Thermo Fisher) and fragmentation was mapped using ProSight Lite software as previously described.²¹

RESULTS AND DISCUSSION

Temporal Proteomic Dynamics of HRAS^{G12V}-Induced Senescence

Recent work has identified large-scale transcriptional and proteomic changes in HRAS^{G12V}- and BRAF^{V600E}-driven senescence.^{22,23} Yet discrete, time-resolved mapping of the senescence transcriptome and proteome have remained elusive. Therefore, to better understand the temporal regulation and global proteomic changes in HRAS^{G12V}-induced senescence, we used a 10-plex TMT strategy to examine the whole proteome of IMR90 cells in response to tamoxifen-driven HRAS^{G12V} expression across 1 week. Cell lysate from each time-point was normalized at the protein level and subjected to chemical labeling with TMT reagents, multiplexing, and off-line strong-cation exchange prior to liquid chromatography tandem mass spectrometry on a linear ion trap-orbitrap hybrid mass spectrometer (Figure 1A). We conducted MS Amanda database searches using a target-decoy search to a 1.0% protein-level false discovery rate.^{19,24} In our large-scale dataset, we identified 4039 protein groups. Proteome-wide alterations were compared to growing IMR90 ER:RAS cells, treated with ethanol as a mock-treatment, and mark the $t = 0$ time point (Figure 1E, Table S1). Control cells were not cell-cycle arrested and therefore represent a heterogeneous population of growing IMR90 cells. Quantitative protein profiles were subjected to hierarchical clustering to identify biological processes and protein groups that show similar trends over time. Many proteins display little to no change across senescence, while several clusters of proteins reliably respond to HRAS^{G12V} expression. The biggest shifts were seen at 3–4 days following forced HRAS^{G12V} expression. Induction of HRAS^{G12V} expression was independently confirmed by Western blot relative to a tubulin control (Figure 1D). Further senescence phenotypes were confirmed in ER:HRAS^{G12V} IMR90 cells treated with 10 nM 4OHT for 1 week by probing for senescence-associated, pH-dependent β -galactosidase activity (SA- β G) (Figure 1B).¹⁴ To confirm cell cycle arrest in response to oncogene expression, the cell number was independently tracked over 8 days of cell culture. By approximately day 4, proliferation in IMR90 cells expressing HRAS^{G12V} is reduced (Figure 1C). Taken together, the results shown in Figure 1 suggest that the proteomic state of senescence is largely established by day 3 or 4 following forced expression of the HRAS^{G12V} oncogene.

SASP Factors and Sufficiency for Onset of Senescence

Given temporal proteomic changes and the established roles of secreted factors as senescence regulators, we first examined TMT data at the protein group-level for changes in secreted proteins (Figure 2). As a positive control, we identified HRAS as significantly upregulated at all time-points relative to untreated cells and relative to β -actin which remains unchanged across senescence (Figure 2A). Interestingly, we identified significant upregulation of innate immune kinases EIF2AK2 and TBK1, which are upregulated in response to cytosolic DNA sensed by cGAS-STING and aid in robust NF- κ B activation (Figure 2B).^{25,26} We found that SASP regulators IL8, IL1B, and SERPINB2 are only upregulated at 4 days following 4OHT administration and remain upregulated at all subsequent time-points, while MMP1 is upregulated even after 1 day of HRAS^{G12V} overexpression (Figure 2C).³ In support of previous findings, we also identified significant upregulation of HMGA1 and HMGA2. Interestingly, HMGA1 and HMGA2 display distinct kinetics from each other and many of the other proteins. These differences may arise from transcriptional programs later in senescence, or due to increased post-translational modification of these factors affecting their stability and export from the cell. Mechanistically, these proteins cooperate with p16^{INK4a} to establish heterochromatic foci and repress E2F target genes required for proliferation.²⁷ Further, we identified dynamic expression of ESDN (DCBLD2), which is upregulated following 7 days of forced HRAS^{G12V} expression (Figure 2C). A transmembrane protein ESDN is upregulated in many cancers and promotes cell motility in lung cancer, yet its cleaved secreted form binds growth factors and is thought to modulate paracrine signaling, although it has no acknowledged role in senescence.²⁸ In support of senescence as an autonomous mechanism of apoptotic resistance,^{29–31} we identified downregulation of pro-apoptotic executioner caspases, caspase-3 and caspase-7, while we saw upregulation of inflammatory caspase-4 and the integrated stress response proteins EIF2AK2 and PRKRA (Figure 2D).

We did not identify upregulation of alarmin HMGB proteins, another group of nonhistone chromatin associated proteins that undergo intracellular relocalization and secretion in response to oncogenic stress (Table S1).³² For many known SASP factors, we observed limited fold-change across senescence. Indeed, the intracellular amount of IGFBP5, THSB1, THSB2, and IL6ST were not identified to change significantly, suggesting distinct pseudo-steady-state levels of transcription, translation, and export for different classes of SASP factors (Table S1). This observation may additionally be attributed to several mass spectrometry-driven factors. For example, co-isolation and subsequent fragmentation of more than one precursor ion population in TMT experiments compresses reporter ion abundances and creates an underestimation of peptide abundances between conditions. As the majority of the proteome remains unchanged, reporter ion compression acts to suppress significant changes between labeled states.^{33,34} This experimental caveat is in part overcome by synchronous precursor selection and MS3-based quantification of reporter ions; however, this technology was not available on the Orbitrap Elite used in this study.³⁵ Nonetheless, elevated expression of canonical senescence regulators IL8, MMP1, HMGA2, and IL1B was observed and correlates with the largest global proteomic changes identified in Figure 1. Interestingly, while we identified IL8, we did not identify the other robustly expressed SASP factor, IL6.³⁶

As the SASP has an acknowledged role in reinforcing senescence phenotypes and further driving robust cell-cycle exit, we wondered if removal of conditioned media and 4OHT would allow previously stressed cells to continue mitotic cell division. Indeed, secreted factors and their receptors such as CXCR2 act in autocrine and paracrine manners in positive feedback loops to self-amplify the secretory network and strengthen signal transduction pathways that lead to cell cycle arrest.^{37,38} It is possible that only a small amount of stress or time under stress and transient loss of homeostasis is required to drive senescence. Alternatively, reinforcing signals from SASP must be established across at least one cell division. Therefore, in biological triplicate, we provided early passage ER:HRAS^{G12V} IMR90 cells with 10 nM 4OHT and then replaced the media following stringent washes in warm, sterile PBS at 10 discrete time points and continued culturing cells for one additional week (Figure 3A). This strategy removes 4OHT and all secreted proteins and extracellular regulators of senescence, so that the state at which cells possess an adequate transcriptional or intracellular signaling program leading to senescence can be determined. Intriguingly, this sufficiency study demonstrated that cells are locked into the senescence program following 3–4 days of HRAS^{G12V} over-expression in conditioned media, as we see a robust switch-like change to senescent phenotypes after 3 days (Figure 3B). To determine the temporally corresponding biological processes and proteomic state at the time of sufficiency, proteins upregulated by over twofold at day 3, relative to day 2 of forced HRAS^{G12V} expression, were subjected to gene ontology enrichment. Under this approach, we identified negative regulation of growth, cellular response to zinc and cadmium, regulation of anion transport, chromatin silencing, and several other biological processes as significantly enriched (Figure 3C, Table S2). Some of these biological processes such as negative regulation of cell growth and chromatin silencing are known processes in cellular senescence.³⁹ However, several processes such as cellular response to zinc and positive regulation of nitric oxide biosynthetic process are poorly understood in the context of senescence and represent new areas of research toward understanding the regulation and induction of the biological process.

Characterization and Quantification of Secreted IL8 Proteoforms

While secreted proteins reinforce senescence and adversely affect tissue homeostasis, the total number of reported secreted gene products is relatively small and therefore likely exhibits additional complexity, regulation, and tropism through cleavage and additional post-translational modifications. Therefore, we turned to a label-free top-down proteomic approach to characterize secreted proteins in their intact forms first identified by bottom-up proteomics. Only proteins under 30 kDa were examined in this study given that currently the 0–30 kDa mass range can robustly be quantified. This technical limitation imposes mild limitations on the proteomic coverage of SASP proteoforms. Indeed, a molecular weight-based analysis of a generalized compendium of secretome factors compiled by Eichelbaum et al. suggests that the median molecular weight distribution of these factors is below 30 kDa.⁴⁰ Further, this strategy is well suited to identify secreted proteins with near-complete molecular specificity, as concurrent regulated cleavages are retained in top-down proteomic experiments and classical immunology has identified different affinities and tropisms of distinct cleaved forms of interleukins.^{9,41} To gain a greater understanding of the number and complexity of SASP factors, 2 million early passage fibroblasts were first grown on 10 cm²

plates in full media. At $t = 0$, expression of HRAS^{G12V} was induced with 4OHT in IMR90 cells. Secreted proteoforms of the SASP were identified using a three-tiered search strategy to (1) identify precursor masses with a 2.2 Da precursor window and 10 ppm fragment ion tolerance or (2) with a 200 Da precursor window and 10 ppm fragment ion tolerance or (3) 10 ppm precursor tolerance and 10 ppm fragment ion tolerance, as previously described.⁴² From this top-down proteomic study, we identified 55 proteins and 258 proteoforms at a 1% proteoform-level FDR (Table S3).²⁰

Interestingly we identified and characterized several IL8 proteoforms by top-down proteomics at latter time points across senescence induction, including high sequence coverage of noncanonical IL8 proteoforms by HCD-based fragmentation (Figure 4A). IL8 is a known regulator of the SASP and plays a role in RAS-dependent tumor-associated inflammation and is further differentially processed to generate distinct secreted versions of the protein with different receptor affinities (Figure 4A).^{41,43} However, to date, IL8 expression has only been examined by ELISA and by bottom-up approaches. N-terminally truncated forms of IL8 cannot routinely be detected by bottom-up as only tryptic peptides, and missed cleavages are identified through database searching. Moreover, traditional ELISA assays report IL8 and other secreted markers as single gene products. Therefore, distinct IL8 proteoforms represent a level of complexity past what could be detected with a bottom-up proteomics approach. Full scan data for a single technical replicate injection at 6 days post 4OHT administration demonstrates the existence of five distinct IL8 proteoforms that can be robustly characterized (Figure 4B,C). Interestingly, of the abundant high stoichiometry proteoforms that were used for quantitative comparisons, we identified IL8 proteoforms (23–99) with one or two cysteine S-sulphydration or S-sulfinic acid events (+SSH or SO₂H⁺ 32 Da) (Figure 4). These post-translational modifications have not been previously identified on IL8, and S-sulphydration nor sulfinic acid modification of cysteine are not widely studied PTMs. However, S-sulphydration is fairly common and 10–25% of liver proteins are S-sulphydrated under physiological conditions.⁴⁴ Moreover, the PTM has acknowledged and emerging roles in inflammation and ischemia/reperfusion biology.⁴⁵ Indeed, S-sulphydration of NF- κ B helps mediate its antiapoptotic activity through increased DNA binding and gene activation, and recently, H₂S has been identified as a regulator of paracrine senescence.^{46,47} As reactive oxygen species (ROS) are increased in senescence and have recently been identified to induce paracrine senescence and telomere dysfunction, it is likely that nonenzymatic modification such as S-sulphydration or sulfinic of secreted proteins can alter the senescent phenotype.⁴⁸ Sulfinic acid modification has been identified as a post-translational modification, but it remains a largely understudied ROS-induced modification.⁴⁹ This finding represents an additional level the proteoform-resolved complexity of the SASP and suggests further study of redox modifications on secreted protein, in addition to intracellular regulators in senescence. Indeed, soluble gas regulators such as H₂S and H₂O₂ may help elicit a SASP response that does not fully activate an immune response in vivo.

Quantitative Analysis of SASP Factors

The total number of SASP factors appears to be relatively small given this study and previous investigations, and outside of IL8, we identified several known senescence

regulators.^{36,50} We also identified several additional secreted proteins that have a role in the SASP or aging biology, including β -2 microglobulin, BASP1, BCAP31, TMSB4, TMSB10, CXCL5, S100A6, S100A13, HMGA1, HMGA2, CXCL1 (GRO α), and fragments of MMP1 and MMP14 (Table S3).^{2,51–53} Interestingly, however, we did not identify IL6, IL13, IL1A, nor IL1B by top-down proteomics even though they are canonical SASP factors. It is likely that the abundance of these and other secreted factors is outside of the dynamic range and limit of detection for our proteomic approach or may be on the limit of the 8% GELFREE molecular weight-based enrichment which has a cutoff close to the molecular weight of these proteins. Indeed, the study of secretomes by proteomics is met with several technical hurdles. Serum proteins are several orders of magnitude more abundant than biological regulators and can obfuscate the detection and quantification of biologically relevant components. Serum-starvation represents an attractive approach to mitigate the contribution of exogenous proteins; however, translation of secreted factors is linked to mTORC1 and therefore sensitive to changes in serum.⁵⁴ Nonetheless, we identified several new proteoforms of known SASP factors. Beyond IL8, we quantified the proteoforms of CXCL5, GRO α (CXCL2), HMGA1, and S100A13 across 8 days following initial 4OHT administration. Of interest, all SASP factors were identified with N-terminal cleavages and additional post-translational modifications. Indeed, like IL8, major GRO α and CXCL5 proteoforms were identified without signal peptide sequences. In line with our intracellular proteomic findings, no validated SASP factors were identified prior to day 3.

Distinct from our TMT-based assessment of the senescent proteome, we identified and quantified eight distinct versions of HMGA1 by top-down proteomics (Figure 5A,B). HMGA proteins stabilize senescence by accumulating on chromatin and forming senescence-associated heterochromatic foci,²⁷ and interestingly, previous studies employing bottom-up proteomics have identified and confirmed mono- and dimethylation of arginine-25, as well as phosphorylation of serine-98, serine-101, and serine-102 on HMGA1.⁵⁵ Phosphorylation of HMGA1 by casein kinase (CK2) and protein kinase C (PKC) significantly reduces the protein binding affinity to AT-rich DNA.^{55,56} Moreover, monomethylation of HMGA1 at arginine 25 (Arg25) is associated with the execution of apoptosis in cancer cells,⁵⁵ and further, dimethylated Arg25 has been examined in breast cancer cell lines, and its methylation status strongly correlates with metastatic potential.⁵⁷ Although HMGA1 has been identified at the peptide-level in the context of senescence, the specific phosphorylation and methylation state of secreted HMGA1 has not been examined.²⁷ A significant increase in HMGA1 protein was identified as early as day 3; however, robust secretion as detected by top-down proteomics occurs following day 4 (Figure 5B). Interestingly, relative to day 4 and day 6 time points when high stoichiometry HMGA1 proteoforms are triply phosphorylated, at day 8, the proportion of nonphosphorylated and doubly phosphorylated proteoforms is significantly increased. The significant change in phosphorylation stoichiometry of secreted HMGA1 likely results from different chromatin binding affinity and may also modulate SASP signaling. Indeed, hyperphosphorylated HMGA1 has been observed in leukemic cell lines actively undergoing apoptosis and noted to change dynamically in time in response to agents that also induce senescence.⁵⁸

Past the quantification of HMGA1, dynamic quantitative changes in GRO- α and CXCL5 were observed between 6 and 8 days following forced HRAS^{G12V} expression. Taken

together, while senescence is established in these cells (as was identified by sufficiency assay, Figure 3), the dynamic change in SASP proteoforms is highly suggestive. As senescent cells can be kept alive in culture long after reaching their replicative arrest, it is likely that senescent cells cultured for 40 or more passages at the Hayflick limit display additional, unique proteoforms that influence cell fate. While outside of the scope of this study, it is possible that these factors influence the durability of the senescent phenotype.⁵⁹

CONCLUSIONS

We report untargeted quantitative bottom-up proteomics across HRAS^{G12V}-induced senescence as a resource for further investigations of OIS dynamics. Moreover, we employ top-down proteomics to understand regulated modification of proteins, given that concurrent modifications and their stoichiometries cannot be addressed by bottom-up proteomics. We demonstrate that presenescent cells require oncogenic stimuli and conditioned media components for at least 72 h to become robustly senescent, corresponding to a proteomic state of upregulated secretome factors such as IL8 and IL1B. Further, we report quantitative top-down proteomics of the secretome to identify distinct forms of individual secreted factors that may be important for senescence establishment and positive-feedback mechanisms, including multiple IL8 proteoforms some of which carrying a previously non-annotated cysteine PTM (S-sulphydration or S-sulfinic acid). Our data highlight an additional level of complexity in the secretome and provide the first evidence for proteoform-level alterations in the context-dependent and pleiotropic secretome of senescent human cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Quantitative temporal proteomic alterations in HRAS^{G12V}-driven senescence (A) HRAS^{G12V} was induced in transgenic IMR90 cells and samples were collected at 10 time points across 7 days. Proteins from each time point were labeled with TMT-reporter tags and subjected to multiplexed proteomic analysis. (B) To confirm senescence induction, concurrently grown cells were stained for senescence-associated β -galactosidase activity. (C) Cell number was tracked across senescence induction. (D) HRAS^{G12V} induction was confirmed by western blot, relative to tubulin. (E) A total of 4039 protein groups were identified across 7 days of senescence induction and were further clustered based on intensity profiles, normalized relative to $t = 0$ days.

Figure 2.

Upregulation of SASP factors correlates with sufficient cellular state of senescence TMT quantification of (A) HRAS and actin, (B) innate immune proteins EIF2AK2 and TBK1, (C) SASP proteins, IL8, IL1B, MMP1, SERPINB2, HMGA1, HMGA2, HMGB1, ESDN and (D) caspase proteins, across senescence induction relative to non-induced control cells.

Figure 3.

Sufficient cellular state for senescence phenotype (A) in biological triplicate, ER:HRAS^{G12V} cells were separately provided 4OHT for 0, 1, 2, 3, 4, 5, 6, or 7 days, stringently washed with buffer and provided with fresh media. Cells were then cultured for 1 week, and subjected to SA- β G staining. (B) Percent of cells positive for SA- β G staining across all time points was calculated for random fields. (C) Proteins upregulated by >2-fold at 3 days post-HRAS^{G12V} expression were subjected to Gene Ontology enrichment to identify significantly upregulated biological processes. Protein number for each process is provided above each bar.

Figure 4.

Complexity and quantification of IL8 proteoforms in senescence (A) IL8 is processed through regulated cleavage C-terminal to signal peptide (see arrows) into several proteoforms with different activities. Annotated HCD fragmentation spectrum of intact IL8 (6–77 aa) is shown. (B) Fragmentation maps for IL8 proteoforms identified in this study. (C) MS1 full scan spectra of co-eluting IL8 proteoforms from 6 days post-4OHT administration are shown. IL8 proteoforms co-elute (inset). Precursors selected for fragmentation colored to reflect fragmentation maps. (D) Workflow for quantitative proteomic study. (E) IL8 proteoforms were quantified across the induction of senescence.

Figure 5.

Quantitative top-down proteomics of SASP factors (A) observed (top) and theoretical (bottom) 15⁺ charge state distribution for secreted proteoforms corresponding to methylated and phosphorylated forms of HMGA1. Quantification of (B) HMGA1, (C) GRO- α , (D) S100A13, and (E) CXCL5 proteoforms across senescence induction.