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RESEARCH ARTICLE

Bioinformatic framework for analysis of transcription factor changes as the molecular link between replicative cellular senescence signaling pathways and carcinogenesis

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Abstract Cellular senescence is a natural condition of irreversible cell cycle arrest and apoptotic resistance that occurs in cells exposed to various stress factors, such as replicative stress or overexpression of oncogenes. Unraveling the complex regulation of senescence in cells is essential to strengthen senescence-related therapeutic approaches in cancer, as cellular senescence plays a dual role in tumorigenesis, having both anti- and pro-tumorigenic effects. In our study we created a model of replicative cellular senescence, based on transcriptomic data, including an extra intermediate time-point prior to cells entering senescence, to elucidate the interplay of networks governing cellular senescence with networks involved in tumorigenesis. We reveal specific changes that occur in transcription factor activity at different timepoints before and after cells entering senescence and model the signaling networks that govern these changes.

Keywords Replicative senescence · Transcriptomic analysis · Pathway analysis · Cell signaling

Introduction

Senescent cells are cells that are fully viable but have entered an irreversible cell cycle arrest and display apoptotic resistance. Cellular senescence occurs in response to various forms of cellular stress, which activate different distinct senescence programs (Hernandez-Segura et al. 2018). The best currently available model to study human aging in vitro is the replicative senescence (RS) model, which is based on the fact that young primary fibroblasts can perform a limited number of duplications in vitro before they become senescent (Cristofalo et al. 2004). The main cause of RS has been suggested to be telomere erosion that is the progressive shortening of the telomeres with every cell division (Deng and Chang 2007). Various extrinsic stress factors, such as DNA damaging agents and oxidative stress may also promote senescence, a

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process called stress-induced premature senescence (SIPS) (Kuilman et al. 2010). Moreover, expression of oncogenes or loss of tumor suppressor genes can trigger oncogene-induced cellular senescence (OIS) and the exposure of cancer cells to different types of therapy, such as chemotherapeutic agents and radiotherapy can lead to therapy-induced senescence (TIS) (Lee and Schmitt 2019). Classical OIS is mediated by the well-known $p16^{INK4a}$ (*CDKN2A*) and $p19^{ARF}$ (alternative splicing product of the *CDKN2A* gene) pathways (Collado and Serrano 2006).

Cellular programs, activated during RS and OIS, share a significant overlap (Zeng et al. 2018). Both in RS and OIS, the DNA damage response is activated and leads to the induction of cyclin-dependent kinase (CDK) inhibitors ($p16^{INK4A}$, $p19^{ARF}$ and $p27^{KIP1}$ (*CDKN1B*)) via the mitogen-activated protein signaling cascade and the activation of *TP53* pathways (Bartkova et al. 2006). Additionally, transcription of the E2F family genes is repressed through pathways regulated by *RB1* (Narita et al. 2003).

While induction of cellular senescence appears to be a promising target in cancer therapy, cellular senescence should be treated with caution as it can play two distinct roles in cancer progression; although it is a potent mechanism for cells to avoid malignant transformation, the senescence-associated secretory phenotype (SASP) can also lead to alterations in the cellular microenvironment that in turn promote cancer development by maintaining chronic inflammation (Rodier and Campisi 2011). The relationship of cellular senescence to cancer development appears to be affected by temporal factors. For example, it has been shown that the synthesis of the SASP-related secretome of senescent cells can change overtime (Hoare et al. 2016). It has been suggested that early stage senescence has a protective role against cell transformation while prolonged cellular senescence promotes cancer development (Zeng et al. 2018). Overtime, senescent cells may exhibit cell cycle re-entry or stemness reprogramming or lead to SASP related chronic inflammation (Lee and Schmitt 2019). Recent studies have shown that pre-senescence or early stage senescence can be reversed and cells with low $p16^{INK4a}$ levels can re-enter the cell cycle following *TP53* inactivation (Beauséjour et al. 2003). Senescent cells have been found to promote the incidence of stem cell markers-positive cells in their proximity and the senescence-associated programs in the cells undergoing senescence themselves have been linked to cancer stemness (Ritschka et al. 2017; Milanovic et al. 2018).

These findings highlight the need of removal of latent senescent cells after cancer therapy and has led to research into compounds that can selectively eliminate senescent cells in vivo, called senolytics (Lee and Schmitt 2019).

The complexity of the different temporal states of senescence regulation during cancer development and the utilization of cellular senescence research in cancer treatment has proved challenging. Hence, the need for a model of the progression of changes in the signaling pathways of cells during senescence to discover the distinct pathways active in pre-senescent cells and cells that have entered senescence, becomes apparent.

Cellular signaling networks play a fundamental role governing the alterations associated with the senescent phenotype. While we can probe the dynamic state of those signaling pathways through phosphoproteomic experiments, these only interrogate a limited number of phosphoproteins and come at a very high cost in comparison to global gene expression profiling. Nowadays, there are bioinformatic tools available to aid the creation of descriptive models of the activity of the signaling pathways in the cells based on the transcriptome activity. Here, we have designed a study to create a model of in vitro aging through transcriptomic measurements, adding an extra intermediate data point between the extreme conditions of young and senescent cells to observe which signaling pathways are affected before and after cells entering senescence.

Methods

Experimental process

HFL-1 human embryonic fibroblasts were obtained from the European Collection of Cell Cultures and were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 2 mM glutamine and 1% non-essential amino acids (complete medium). HFL-1 cells were maintained at 37 °C, 5% CO2 and 95% humidity and they were subcultured when they reached confluence at a

split ratio of 1:2. Cell number for each assay was determined in duplicates using a Coulter Z2 counter (Beckman, Brea, CA, USA). We maintained human primary fetal lung fibroblasts (HFL-1) in culture from a young stage until they reached a senescent stage after 50 replication cycles. The cells were collected at three different time points while undergoing RS. In the first young stage the cells were replicating every 24 h (1 population doubling/24 h), in the second middle-aged stage the cells were replicating every 7 days (1 population doubling/week) and in the third stage the cells had not replicated for 14 days (0 population doubling/2 weeks) and thus they were considered senescent. RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) following replenishment with complete medium 24 h before RNA isolation. We profiled gene expression of the cells of each stage in biological duplicates using the Illumina expression microarray HumanHT12_V4.

Differential expression analysis

We performed normexp background correction and quantile normalization using the neqc function. We continued with a non-specific intensity filtering procedure, in order to remove probesets which are consistently non-expressed in most samples. The *limma* R-package was used to determine the significantly differentially expressed genes. Linear models to compare gene expression between Middle-aged vs Young cells (hence on Middle-aged gene list), and Senescent vs Young cells (hence on Senescent gene list) were fitted & moderated t-statistics with adjusted p-values were computed for multiple testing correction (Benjamini and Hochberg 1995; Ritchie et al. 2015). The same double cutoff was used in both comparisons: FDR value < 0.05 & an absolute value of $\log_2$-fold change > 0.5.

BioInfoMiner noise reduction

We filtered the gene lists derived from the differential gene expression analysis through the BioInfoMiner platform interpretation algorithm, which prioritizes regulatory hub genes with broader systemic impact in cellular physiology, standing in the middle of many perturbed gene subsets, as described by ontological terms, that are found consistently significant through enrichment statistical tests (Koutsandreas et al. 2016). Through BioInfoMiner we detected and ranked the significantly altered biological processes using four different biological ontologies (Gene Ontology (GO) (Ashburner et al. 2000), Reactome (Fabregat et al. 2018), MGI Mammalian Phenotype (Smith and Eppig 2012), and Human Phenotype Ontology (HPO) (Köhler et al. 2019) and then extracted the genes that support each of the term's enrichment, and used the union of these genes, as a compact, noise-free subset of the initial, input list. For the enrichment scores we used a cutoff of BioInfoMiner bootstrap corrected p-value < 0.05.

Transcription factor activity enrichment analysis

To estimate transcription factor (TF) activity, from the levels of expression and the enrichment of their direct target genes we used TF-regulons derived from DoRothEA version 2 developed by Garcia-Alonso et al. (2019). A regulon is a collection of signed interactions of transcription factors with their transcriptional target genes. The developers of DoRothEA have assigned different levels of confidence to the TF-regulons, based on the supporting evidence for each interaction ranging from level A (highest confidence) to level E (lowest confidence). In the present analysis we have used the DoRothEA level A and level B TF-regulons as the input to the statistical method VIPER from the viper R package (Alvarez et al. 2016) to produce continuous network enrichment scores for each transcription factor based on the changes in the expression of their direct target genes (Garcia-Alonso et al. 2019). We then selected the statistically significantly enriched transcription factors for downstream analysis based on a cutoff of FDR < 1.

Pathway activity

To calculate predicted pathway activity, we applied the PROGENy tool (Schubert et al. 2018) to the total moderated t-values derived from the differential gene expression analysis. For our analysis we used the extended model matrix for 14 pathways (pathways presented in Table 2) as already described (Holland et al. 2019). The PROGENy tool returns relative pathway scores based on the distance of the provided gene expression matrix from the model matrix, scaled to have a mean of zero and standard deviation of one (Schubert et al. 2018).

Network inference

The normalized enrichment scores (NES) for significantly enriched transcription factors from DoRothEA and the pathway scores from PROGENy were used as an input for the CARNIVAL pipeline (Liu et al. 2019). We opted to use the inverse CARNIVAL pipeline due to the lack of known input perturbations to the cells. As a prior knowledge network (PKN) we used the signed and directed human signaling network from Omnipath (Türei et al. 2016). CARNIVAL is a tool designed to work with the outputs from DoRothEA and PROGENy along with a PKN as inputs. It predicts the signaling network topology and node activities by generating linear constraints from the provided inputs and applying an ILP solver to return the highest scoring networks that best explain the transcription factor activities inferred through DoRothEA. The presented figure of CARNIVAL results combines the results from multiple solutions.

Results

Transcription factor enrichment analysis

To calculate transcription factor activity enrichment, from the gene expression of their direct target genes we used the DoRothEA version 2 regulons combined with the viper R package to produces normalised enrichment scores for each transcription factor. In comparison to young cells we found the activity of the *E2F4*, *E2F1*, and *FOXM1* transcription factors to be consistently downregulated in middle-aged and senescent cells, observing an increase in downregulation through the consecutive time points. *TFAP2A* was also consistently downregulated both in middle-aged and senescent cells. In contrast, the transcription factors *HNF4A*, *RUNX1*, *IRF1*, and *STAT1* were consistently upregulated in middle-aged and senescent cells. In comparison, *PPARG*, *MITF*, and *TAL1* only begun to appear upregulated after the cells have entered senescence. The same behavior was observed in the downregulation of *E2F2*, *E2F3*, *MYC* and *TFDP1* (Table 1). Five different shapes of NES variations were observed; a consistent downregulation as we move from young to middle-aged and then to senescent cells (), a sharp downregulation () or upregulation () in middle-aged cells that is then maintained in senescent cells, a sharp downregulation () or upregulation () directly in the senescent cells.

Pathway activity

To infer pathway activity from our gene expression data we used PROGENy, a tool that calculates pathway scores based on genes that are perturbed after the activation of these pathways, derived from perturbation-based experiments. We present the pathway scores inferred from PROGENy in Table 2 for the genes derived from the two DE comparisons. The pathways with the highest increase in middle-aged and senescent cells were the JAK/STAT, Hypoxia, TNFα, and p53 pathways, followed by the WNT and NFκB pathways. On the other hand, we observed the highest decrease in the activity of the PI3K, TGFβ, Estrogen, Androgen and MAPK pathways, and a smaller decrease in the EGFR, Trail and VEGF pathways. Comparing the two time-points, the increase in NFκB pathway activity was higher in the middle-aged cells than in the senescent cells, while the decrease in Trail and VEGF pathway activities was higher in the senescent cells.

Signaling networks

Based on the predicted transcription factor and pathway activities we applied an ILP solver using the Inverse CARNIVAL pipeline to identify the upstream signaling network topology. In the first stage of middle-aged cells that replicate every week we observed an increase in the activity of *STAT1,* which is effected through the Janus kinases *JAK2* and *JAK3*, which in turn are upregulated due to the downregulation of suppressors of cytokine signaling *SOCS3* and *SOCS4*. The increased activity of *STAT1* led to increase in the activity of *IRF1* and inhibition of the activity of *MYC,* which in turn led to downstream downregulation of *E2F4* and *FOXM1* through the downregulation of *CDK4* and *RBL1*. The upregulation of the activity of *RUNX1* is mediated through the upregulation of *KAT6A* activity. Downregulation of WNT7A led to downstream downregulation of *PRKACA*, *TFAP2A* and *SRC*. The decrease in *SRC* activity further led to increased activation of *HNF4A* and downregulation of *PI3KCA*.

Table 1 Transcription factor enrichment scores and number of target genes affected per transcription factor in middle-aged and senescent cells VS young cells

Transcription factor	Middle-aged cells		Senescent cells		Sparkline
	NES	# of target genes affected	NES	# of target genes affected	
E2F4	− 7.131250252	126	− 9.584556428	138	
E2F1	− 3.522634472	93	− 5.537028539	104	
HNF4A	3.508604467	39	3.204686775	61	
FOXM1	− 2.984562247	14	− 4.480605609	14	
RUNX1	3.155641635	18	3.564097195	27	
STAT1	3.076945801	59	2.740430311	65	
IRF1	2.873399677	6	3.028900505	7	
TFAP2A	− 2.511192823	5	− 2.238451879	6	
TFDP1	–	–	− 3.402635334	5	
E2F2	–	–	− 3.224658734	9	
PPARG	–	–	2.612624793	7	
E2F3	–	–	− 2.441148109	6	
MITF	–	–	2.376295717	51	
MYC	–	–	− 2.370638506	62	
TAL1	–	–	2.28257674	43	

The number of target genes found affected and the significant Normalized Enrichment Scores (NES) produced using DoRothEA for the two gene sets derived from the DEGs for the comparisons of middle-aged vs young cells and senescent vs young cells. Sparklines present the general shape of the variation of the NES overtime. The full lists of affected target genes for each trancription factor, along with their log fold changes and adjusted p-values, are presented in the tables in Online Resource 1 and Online Resource 2

As can be seen from the overlay of the two networks illustrated in Fig. 1, all effects observed in middle-aged cells (a state that could be roughly nominated as the first stage of senescence) remained present in the predicted signaling network for the cells of the next stage that have entered senescence and have not duplicated for 2 weeks; moreover the observed changes were expanded to include downregulation of MAP kinases *MAP2K1* and *MAPK3*, mediated through *SRC* and *BRAF*, that led to the increase of *PPARG*, *TAL1* and *MITF* transcription factors activity. The increase of *TAL1* activity was further enhanced through the upregulation of *LMO1* and *LMO2*. Additionally, upregulation of *TP53* and *RB1* activity led to downstream downregulation of the E2F family members *E2F1*, *E2F2* and *E2F3*.

Discussion

In this work we attempt a new approach in the analysis of networks that regulate cellular senescence based on transcriptomic data, considering early changes in pre-senescent middle-aged cells and later changes in cells that have reached the cellular senescence state. Our findings highlight the complexity of the relationship of the RS-related networks to pathways involved in tumorigenesis and OIS. We observe a gradual deregulation of the signaling networks that are active in

Table 2 Pathway activity scores in middle-aged and senescent cells

Pathway	Middle-aged cells	Senescent cells
EGFR	-0.4184	− 0.3944
Hypoxia	0.9994	1
JAK/STAT	1	1
MAPK	− 0.8616	− 0.7112
NFκB	0.7828	0.4928
PI3K	− 0.9998	− 0.9994
TGFβ	− 0.9498	− 0.4678
TNFα	0.9966	0.9882
Trail	− 0.182	− 0.2974
VEGF	− 0.0008	− 0.277
p53	1	1
Androgen	− 0.8556	− 0.769
Estrogen	− 0.9466	− 0.9434
WNT	0.8246	0.799

The scaled PROGENy relative pathway scores calculated based on the differential gene expression lists for middle-aged vs young cells and senescent vs young cells

different consecutive temporal stages. This higher temporal resolution of changes that occur in the senescence regulatory networks at different stages before and after cells enter senescence is important for the utilization of cellular senescence mechanisms as a therapeutic strategy in cancer management.

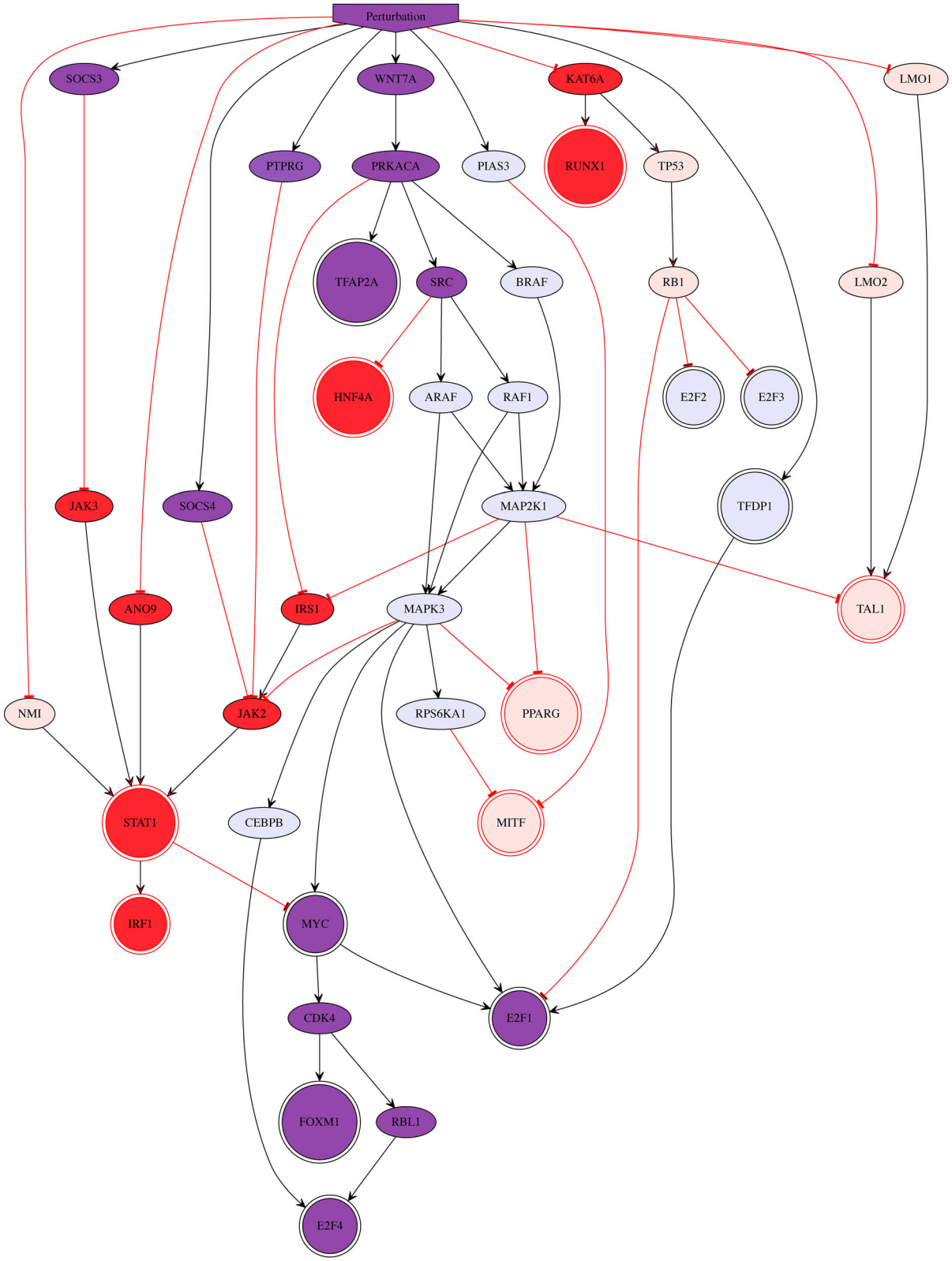
As soon as cells reach the middle-aged stage, we predict increased activity of the JAK/STAT pathway. This pathway plays a central role in cell proliferation and survival and its persistent activation has been implicated in various types of cancers (Bousoik and Montazeri Aliabadi 2018). On the other hand, it has also been found to be more active in senescent than non-senescent cells and its inhibition alleviates age-related tissue dysfunction (Xu et al. 2016). Its dual role does not come as a surprise, as it is involved in extensive cross-talk with numerous other pathways and its role in cellular senescence and carcinogenesis remains to be elucidated. In our network, *STAT1* is activated from the middle-aged stage. *STAT1* is known to promote the expression of genes that enhance cell cycle arrest and apoptosis (Bousoik and Montazeri Aliabadi 2018). It also enhances the expression of *IRF1* and together they create a positive feedback loop that leads to the constant expression of a number of

Fig. 1 Overlay of the CARNIVAL results for the middle-aged ▶ cells in comparison to young cells and for the senescent cells in comparison to young cells. Nodes predicted to be up-regulated in middle-aged and senescent cells are indicated in red and pink while predicted down-regulated nodes are colored in blue and purple. Activatory reactions are indicated with black arrows while inhibitory edges are colored in red. Double circled nodes correspond to trancription factors. Red and purple nodes are present in both the middle-aged and senescent cells networks, while light blue and pink nodes are present only in the network of senescent cells. The two original networks are presented in the figure in Online Resource 3

cytokines involved in the SASP. In support, inhibition of the JAK/STAT pathway has been found to suppress the SASP in endothelial cells (Kandhaya-Pillai et al. 2017).

While the WNT pathway, a well-known pathway involved in cellular proliferation and tumorigenesis, appears to have increased pathway activity in our results, the *WNT7A* activity, a protein member of the WNT family, is predicted to be decreased. The WNT pathway plays an important role in stemness reprogramming of the cells. It is interesting to mention that *WNT7A*, contrary to other WNT protein family members, has been suggested to have a protective role against tumorigenesis (Winn et al. 2006). *WNT7A* has been found to induce β-catenin-independent tumor suppressive cellular senescence in lung cancer, through an alternate senescence pathway not involved in OIS, and therapies using *WNT7A* have been suggested as a novel therapeutic pro-senescence strategy (Bikkavilli et al. 2015).

Downstream of *WNT7A* we observe the increase in the activity of *HNF4A* in the middle-aged cells and additionally, of *PPARG* and *MITF* in the cells that have entered senescence. *HNF4A* has been found to induce OIS and TIS in prostate cancer cells and has been suggested as a potential therapeutic strategy for cellular senescence in cancer cells (Wang et al. 2019). *PPARG* has been found to induce and accelerate cellular senescence by inducing *CDKN2A* expression (Gan et al. 2008). Furthermore, its activity is altered by cellular senescence. *PPARG* activity is involved in a number of metabolic pathways of the cell and has been proposed as a therapeutic target in multiple cancer studies (Furth 2018; Goldstein et al. 2017). *MITF* also plays a complicated dual role in proliferation and senescence. On one hand, it induces cellular senescence, in the presence of *TP53*, through the upregulation of *CDKN1A* expression and on the other hand it


Perturbation
SOCS3
WNT7A
KAT6A
LMO1
PTPRG
PRKACA
PIAS3
RUNX1
TP53
TFAP2A
SRC
BRAF
RB1
LMO2
HNF4A
ARAF
RAF1
E2F2
E2F3
JAK3
SOCS4
MAP2K1
TFDP1
ANO9
IRS1
MAPK3
TAL1
NMI
JAK2
RPS6KA1
PPARG
STAT1
CEBPB
MITF
IRF1
MYC
CDK4
E2F1
FOXM1
RBL1
E2F4

has an oncogenic role in melanomas and other types of cancers. Nevertheless, overexpression of *MITF* in melanoma cells can lead to OIS (Wang et al. 2016).

KAT6A activity appears upregulated in both middle-aged and senescent cells. However, it appears to activate only the *RUNX1* transcription factor in middle-aged cells and to lead to the additional activation of *TP53* and *RB1* in senescent cells. *RUNX1* is a member of the RUNX family of transcription factors and has been found to be a potent inducer of senescence in primary human fibroblasts. Premature senescence induced by the RUNX family differs significantly from OIS, suggesting it as a potent cancer fail-safe mechanism target (Wolyniec et al. 2009). A recent study demonstrated that *KAT6A* interacts with *PML* and *TP53*, and this interaction promotes *TP53* acetylation and subsequent changes lead to *TP53*-dependent cellular senescence. Phosphorylation of *KAT6A* by *AKT* can inhibit *PML* binding, which is a potential mechanism through which *TP53* activation (through *KAT6A*) is avoided, in the middle-aged cells (Rokudai et al. 2013). However, in another study focusing on OIS, the opposite results have been suggested; *KAT6A* suppresses cellular senescence through the regulation of suppressors of the *CDKN2A* locus characterized by gene expression alterations that are typical of loss of function that accompany *CDKN2A*-dependent senescence (Baell et al. 2018). The role of *KAT6A* activity in different stages of RS and OIS and its potential as a cancer therapy target remains to be further evaluated.

FOXM1 activity is found reduced both in middle-aged and senescent cells. *FOXM1* is a downstream target of *MYC* and regulates proliferation and senescence both in normal and cancer cells. Its expression is found reduced in RS and its depletion leads to senescence, while its overexpression suppresses senescence and leads to tumorigenesis (Smirnov et al. 2016; Li et al. 2008).

RB1 signaling, activated only in the senescent cells, is one of the main hallmarks of senescence needed for the irreversible establishment of senescence (Beauséjour et al. 2003). It is known to promote cellular senescence by repressing the transcription of E2F regulated proliferation promoting genes (Hernandez-Segura et al. 2018). In our network we observe the E2F transcription factor family activity to decrease during RS. While *E2F1* and *E2F4* activity appear to be downregulated from the middle-aged stage through the downregulation of *MYC*, *E2F2* and *E2F3* are downregulated in the senescent stage through *RB1*. *E2F1-3* act in a pro-proliferative manner, while *E2F4* has a repressive function. Loss of the three activating E2Fs *E2F1-3* results in acute cell cycle arrest (Wu et al. 2001). However, *E2F1* has also been found to be involved in apoptosis induction and $E2F1^{-/-}$ knock out mice display a predisposition to cancer development (DeGregori 2002). Although *E2F4* has been characterized as a repressor of cell cycle progression, it appears to act as an oncogene in rapidly proliferating cancer cells. *E2F4* possibly drives cell cycle progression in these cells by switching its role from a repressor to an activator of the expression of cell cycle genes (Hsu and Sage 2016).

Overall, the predicted networks propose specific signaling alterations orchestrating the senescent phenotype at different timepoints in primary human fibroblasts, with some of these pathways being common with known OIS processes, while others, which are not active in OIS could serve as potential pro-senescence targets. Increasing the understanding of the complex progression of senescence is valuable for senescence-related therapeutic approaches in cancer, as cellular senescence is a double-edged sword; it may act as tumor suppressor but it also leads to chronic inflammation which promotes cancer depending on the time frame (Rodier and Campisi 2011). Detailed knowledge of the exact senescence-related pathways that are activated or inhibited in a time-dependent manner will enable researchers to take advantage of the phenomenon of senescence as part of cancer therapy strategies without however increasing the health risks that may arise due to the pro-tumorigenic potential of senescent cells.

Author contributions IB, NC and AC contributed to the study conception and design. Material preparation and data collection were performed by ML. Data analysis was performed by IB. The first draft of the manuscript was written by IB, review and editing were performed by IB, NC and AC. All authors read and approved the final manuscript.

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Data availability The dataset supporting the conclusions of this article is available in the GEO repository, under the accession number GSE144703.

Code availability The BioInfoMiner platform is available online at the website https://bioinfominer.com. DoRothEA, PROGENy and CARNIVAL are published tools, available online on the Saez lab Github page https://github.com/saezlab.

Compliance with ethical standards

Conflict of interest Aristotelis Chatziioannou is the founder and CEO of e-NIOS PC. Other authors declare that they have no conflict of interest.

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