Identification of Autophagy-Related Genes as Targets for Senescence Induction Using a Customizable CRISPR-Based Suicide Switch Screen

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ABSTRACT

Pro-senescence therapies are increasingly being considered for the treatment of cancer. Identifying additional targets to induce senescence in cancer cells could further enable such therapies. However, screening for targets whose suppression induces senescence on a genome-wide scale is challenging, as senescent cells become growth arrested, and senescence-associated features can take 1 to 2 weeks to develop. For a screen with a whole-genome CRISPR library, this would result in billions of undesirable proliferating cells by the time the senescent features emerge in the growth arrested cells. Here, we present a suicide switch system that allows genome-wide CRISPR screening in growth-arrested subpopulations by eliminating the proliferating cells during the screen through

activation of a suicide switch in proliferating cells. Using this system, we identify in a genome-scale CRISPR screen several autophagy-related proteins as targets for senescence induction. We show that inhibiting macroautophagy with a small molecule ULK1 inhibitor can induce senescence in cancer cell lines of different origin. Finally, we show that combining ULK1 inhibition with the senolytic drug ABT-263 leads to apoptosis in a panel of cancer cell lines.

Implications: Our suicide switch approach allows for genome-scale identification of pro-senescence targets, and can be adapted to simplify other screens depending on the nature of the promoter used to drive the switch.

Introduction

Cellular senescence is a state of stable cell-cycle arrest, which can be induced by several cellular stresses, like telomere shortening, oncogene activation, DNA damage, and oxidative stress (1). Besides upregulating cell-cycle inhibitors, senescent cells usually develop several senescence-associated features, for example, the formation of senescence-associated heterochromatin foci, elevated lysosomal activity, positive staining of senescence-associated β -galactosidase (SA- β -gal), and the adoption of a senescence-associated secretory phenotype (SASP). The SASP is characterized by elevated secretion of chemokines, cytokines, and growth factors and can vary by cell type and stressor (2).

Senescence induction provides an important barrier against tumor development as it limits the proliferative potential of damaged cells. However, long-term presence of senescent cells in tissues may promote tumor invasion and metastasis (3). The deleterious long-term effects of senescence-inducing cancer therapy can be countered by combining it with a follow-up therapy such as a senolytic agent (4, 5). We have recently demonstrated the utility of cancer treatments consisting of a

combination of a pro-senescence therapy and a treatment that selectively eliminates the senescent cancer cells (6, 7). Moreover, pro-senescence therapies may be synergistic with checkpoint immunotherapies as the SASP can chemo-attract several immune cells (8), and senescence induction has been shown to cause vascular remodeling leading to enhanced drug delivery and T-cell infiltration (9).

Identifying additional targets to induce senescence in cancer cells could further enable such therapies. However, screening for such targets is challenging, as senescent cells become growth arrested, and senescence-associated features can take 1 to 2 weeks to develop. For a screen with a whole-genome CRISPR library, this would result in billions of cells by the time the senescent features emerge. Here, we present a suicide switch system that allows genome-wide CRISPR screening in growth-arrested subpopulations and identify autophagy-related proteins as targets for senescence induction.

Materials and Methods

Cell lines

The A549 lung cancer cell line, PANC1 pancreatic cancer cell line, RKO colon cancer cell line, and 293T human embryonic kidney cell line were obtained from ATCC. The Hep3B liver cancer cell line was obtained from S. Huang (NKI). A549 and RKO were cultured in RPMI-based medium supplemented with 10% FBS and 1% penicillin/streptomycin. Hep3B and PANC1 were cultured in DMEM-based medium supplemented with 10% FBS and 1% penicillin/streptomycin. All cell lines were validated by STR profiling (Eurofins) and regularly tested for Mycoplasma spp using a PCR-based assay.

Plasmids

The suicide switch plasmid was constructed using the following plasmids: backbone: pLV-EF1a-IRES-Puro (Addgene #85132), inducible caspase 9: pMSCV-F-del Casp9.IRES.GFP (Addgene #15567), RFP: pMSCV-pBabeMCS-IRES-RFP (Addgene #35395). The Ki-67

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promoter was amplified from genomic DNA using KI67_promoter_fwd GGGAGCCAAGCTCCAAGGGTTGCTGG and KI67_promoter_rev ATCCGGCCCCGAAGGCCACTTGT. The miR-146a-EGFP plasmid was a gift from Stephen Elledge.

Lentiviral transduction

Viral particles were produced in 293T cells by cotransfection of the suicide switch plasmid with second-generation packaging plasmids as described in <https://www.addgene.org/protocols/lentivirus-production>. Destination cells were infected with lentiviral supernatants at a multiplicity of infection of 0.3 using and with 8 µg/mL polybrene. After 24 hours of incubation, the supernatant was replaced by medium containing 10 µg/mL BSD or 2 µg/mL puromycin.

FACS-assisted genetic screen

A549-CID-mClover3 cells were infected in three independent biological replicates. Twenty-four hours after infection, cells were selected with puromycin for 48 hours. At day 6 after infection, the suicide switch was activated by addition of 10 nmol/L CID. At day 8 after infection, remaining cells were reseeded and cultured for 6 more days in the presence of 10 nmol/L CID. At day 14 after infection, cells were sorted into a mClover3-positive and a mClover3-negative fraction. Genomic DNA was isolated using the DNasy Blood and Tissue Kit (#69506, Qiagen) and sgRNA inserts were recovered from DNA by PCR amplification. Each PCR reaction consisted of 500 ng DNA, 10 µL GC buffer (5×), 1 µL forward primer (10 µmol/L), 1 µL reverse primer (10 µmol/L), 1 µL dNTPs (10 mM), 1.5 µL DMSO, and 0.5 µL polymerase in a total volume of 50 µL. Barcoded forward primers (10) were used to be able to deconvolute multiplexed samples after next-generation sequencing. PCR program consisted of initial denaturation at 98°C for 2 minutes, 16 cycles of 30 seconds denaturation at 98°C, 30 seconds of annealing at 60°C, and 30 seconds elongation at 72°C, with a final extension at 72°C for 5 minutes. PCR product purification was performed using the High Pure PCR Product Purification Kit according to manufacturers' instructions (#11732676001, Roche). PCR products of each sample were pooled, and 2.5 µL was used for a second PCR reaction in technical duplicates. Primers in this reaction contained barcodes and an adaptor sequence for next-generation sequencing. Sample concentrations were measured on a BioAnalyzer and were pooled equimolarly. sgRNA sequences were identified by Illumina HiSeq 2500 genome analyzer at the Genomics Core Facility (NKI). For sequence depth normalization, a relative total size factor was calculated for each sample by dividing the total counts of each sample by the geometric_mean of all totals. After normalization, a differential test between the t14pos and t14neg for each sgRNA was performed using DESeq2 (11). The output from the DESeq2 analysis contains the DESeq2 test statistic. Positive DESeq2 test statistic indicate positive log2FoldChange value, negative DESeq2 test statistic indicate negative log2FoldChange value. We sorted the output of DESeq2 on the test statistic in decreasing order, putting the most significant enriched sgRNA at the top. We then used the MAGeCK Robust Rank Algorithm (12) to determine for each gene if its sgRNAs are enriched towards the top of the result list. The resulting enrichment *P* values were corrected for multiple testing using the Benjamini-Hochberg correction, resulting in a FDR value. As hits we took the genes that had a FDR ≤ 0.1 and took along the borderline hit RAB14, which had a FDR of 0.102.

Incucyte cell proliferation assay and apoptosis assay

Cell lines were seeded into 384-well plates at a density of 500 to 2,000 cells per well, depending on growth rate and the design of the

experiment. Drugs were added at the indicated concentrations using the HP D300 Digital Dispenser (HP). Cells were imaged every 4 hours using the Incucyte ZOOM (Essen Bioscience). Phase-contrast images were analyzed to detect cell proliferation based on cell confluence. For the cell apoptosis assay, caspase-3/7 green apoptosis assay reagent (Essen Bioscience, #4440) was added to the culture medium and cell apoptosis was analyzed based on green fluorescent staining of apoptotic cells. The percentage of apoptotic cells was quantified based on images generated from biological triplicates.

Protein lysate preparation and western blots

Cells were washed with PBS and lysed in RIPA buffer supplemented with Complete Protease Inhibitor (Roche) and Phosphatase Inhibitor Cocktails II and III (Sigma). Protein quantification was performed with the BCA Protein Assay Kit (Pierce). All lysates were freshly prepared and processed with Novex NuPAGE Gel Electrophoresis Systems (Thermo Fisher Scientific) followed by Western blotting.

qRT-PCR

Total RNA was extracted from cells using the Quick-RNA MiniPrep Kit (Zymo Research). cDNA synthesis was performed using Maxima Universal First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. qPCR reactions were performed with FastStart Universal SYBR Green Master Rox (Roche). Sequences of the primers used for qRT-PCR analyses: KI67_fwd ATCAGCCGAAGTCAACATGA; KI67_rev GAGTTTGCCTGG-CCTGTACT; ATG101_fwd TTCATCGACTTCACTTATGTGCG; ATG101_rev GATGCACTCGTCTGAGAATGG; ATG9A_fwd CTGCCCTTCCGTATTGCAC; ATG9A_rev CTCACGTTTGTG-GATGCAGAT; RAB14_fwd TATGGCTGATTGTCCTCACACA; RAB14_rev CTGTCCTGCCGTATCCCAAAT; RB1CC1_fwd ATC-GAAGAGTGTGTACCTACAGT; RB1CC1_rev GCAGGTGGACG-ATCACATAAGAT.

Staining for senescence associated β-galactosidase activity

Senescence associated β-galactosidase activity in cells was detected using the Senescence Cells Histochemical Staining Kit (Sigma-Aldrich, CS0030), according to the manufacturer's instructions. The percentage of SA-β-gal staining positive cells was quantified by manual counting of positive and negative cells.

RNA sequencing

RNA (one sample per cell line/condition) was isolated using TRizol, and cDNA libraries were sequenced on an Illumina HiSeq2500 to obtain 65-bp single-end sequence reads. Reads were aligned to the GRCh38 human reference genome. Gene set enrichment analysis (GSEA) was performed using GSEA software as described previously (13). The FRIDMAN_SENESCENCE_UP gene set was used to assess the enrichment of senescence-associated genes (14). Enrichment scores were corrected for gene-set size (normalized enrichment score). The *P* value estimates the statistical significance of the enrichment score for a single gene set as described previously (13). The exact *P* value is shown in the figures unless the *P* value < 0.001.

Compounds and antibodies

Alisertib, ABT263, and SBI0206965 were purchased from MedKoo. The chemical inducer of dimerization CID/AP20187 was purchased from Takara Bio. Antibodies against HSP90 (sc-13119), p53 (sc-126), and p21 (sc-271610) were purchased from Santa Cruz Biotechnology. Antibodies against LC3B (#2775) and BCL2 (#2872) were purchased from Cell Signalling Technology.

CRISPR-cas9 knockout and TIDE analysis

A549 cells were infected with virus containing pLentiCRISPR with a guide RNA for the specified targets. One day after infection cells were selected with puromycin for 2 days. Nine days after infection, cells were lysed for Western blot and TIDE analyses (<https://tide.nki.nl/#about>; ref. 15). Sequences used for knockout and TIDE analyses: ATG101_guide CACCTACTCCATTGGCACCG; ATG101_fwd ATTCTGACCTGGGCTCCTT; ATG101_rev GGGAAAGGAAAAGTGGAAGC; ATG9A_guide CCGTTTCCAGAACTACATGG; ATG9A_fwd GCTCACAACCCCTGAATCTC; ATG9A_rev ATGGTGAGGGCAATAAGCAC; RAB14_guide AGCGATTAGGGCTGTACA; RAB14_fwd GGATTTTCCCCCTCTAGGC; RAB14_rev CAAACACCTTCAGATGCAAGC; RB1CC1_guide GACCAGATGATTGCTAGCTG; RB1CC1_fwd TTCCCATGCTTTTGCTTTT; RB1CC1_rev TAGCCATTCCATCACCTTCC. All mouse study protocols were approved by the NKI Animal Welfare Body.

Results

To enable genome-wide CRISPR screening for targets whose inhibition induces senescence, we aimed to remove proliferating cells during the screen. The most common way to eliminate proliferating cells is by cytotoxic drugs that inhibit cell division. Alternatively, there are inducible methods, for example, based on BrdU incorporation and visible light (16), or on expression of the Herpes Simplex Virus Thymidine Kinase gene in combination with treatment with ganciclovir (17). However, the elimination of cells using these methods relies on cell-cycle arrest caused by DNA damage, which by itself may induce senescence and lead to noise in a senescence focused genetic screen. To circumvent this problem, we utilized the inducible caspase 9 system (iCasp9) that was originally developed as a safety switch for T-cell therapy (18). This construct contains the intracellular portion of the human caspase 9 protein, fused to a drug-binding domain derived from the human FK506-binding protein (FKBP; **Fig. 1A**). Addition of the chemical induction of dimerization (CID) drug AP20187 leads to dimerization of the caspase 9 homodimers, resulting in cellular apoptosis. To specifically induce apoptosis in proliferating cells, we needed a suitable gene promoter to drive the expression of the safety switch. Previous work from our laboratory has identified alisertib, an aurora kinase A inhibitor, as an inducer of senescence in non-small cell lung cancer A549 cells (7). We tested whether the widely-used marker of proliferation, Ki-67 (19), was reduced in expression in these cells upon induction of senescence with alisertib. **Figure 1A** shows that expression of mRNA for Ki-67 is strongly reduced in senescent A549 cells. We therefore generated a suicide construct that contains iCasp9 driven by the Ki-67 promoter. The resulting proliferation-driven suicide switch was combined with an IRES-RFP element to enable sorting of infected cells (**Fig. 1A**).

To validate the suicide switch vector, proliferating A549 cells were infected with the suicide switch construct and RFP positive cells were sorted by FACS to generate single cell clones, which we named “A549-CID” cells. Clones were selected in which addition of the CID drug rapidly induced apoptosis as judged by changes in morphology and an increase in the signal of the Caspase-3/7-activated green fluorescent dye (**Fig. 1B**). Upon treatment with alisertib, the cells became growth-arrested with a flattened morphology characteristic of senescence. Addition of the CID drug to alisertib-treated senescent cells did not induce apoptosis, indicating that the iCasp9 homodimers of the suicide switch, under the control of Ki-67 promoter, are not expressed to sufficient levels to effectively induce apoptosis in these cells (**Fig. 1B**).

In addition to eliminating proliferating cells, we aimed to distinguish senescent cells from other growth arrested populations. We have shown previously that a lentiviral vector with a miR-146a promoter driving expression of the green fluorescent mClover3 can be used to mark senescent cells (7). We inserted the miR-146a-mClover3 vector into the A549-CID cells, yielding A549-CID-mClover3 cells. To determine the optimal timing to develop sufficient green fluorescent reporter signal, parental cells and A549-CID-mClover3 cells were treated with alisertib. Upon treatment, the cells increased in size as can be seen in the forward- and side scatter plots (FSC-A, SSC-A; **Fig. 1C** and **F**). Although the enlargement of the senescent cells resulted in increased auto-fluorescence (**Fig. 1D** and **G**), after 10 days of treatment with alisertib, the mClover3 signal could readily be detected over background (**Fig. 1E** and **H**).

To perform a genome-scale CRISPR screen for pro-senescence targets, we followed a strategy as outlined in **Fig. 1I**. In short, we infected A549-CID-mClover3 cells with the Brunello CRISPR library with low multiplicity of infection as described (20). After infection, the cells were selected with puromycin for 48 hours. At day six post infection, the CID drug was added to eliminate the proliferating cells. At day 14, the remaining cells were sorted by FACS into a mClover3 positive and a negative fraction. DNA was isolated from cells after puromycin selection (t0) and from the flow-sorted fractions (t14pos & t14neg). Subsequently, sgRNAs were recovered by PCR and quantified by sequencing.

There was a small group of sgRNAs with very high abundance, which were mostly enriched in the mClover3 negative fraction (Supplementary Fig. S1). These sgRNAs targeted the suicide switch, either by knocking out FKBP or caspase-9, which are part of the switch, or caspase-3 and -7, which are the downstream effectors of the switch. The enrichment of these sgRNAs provided an additional control for proper functioning of the suicide switch. We focused on sgRNAs that were enriched in the mClover3 positive fraction, as these could target genes whose suppression induces senescence. Differentially expressed sgRNAs were analyzed by DESeq2 as described (11, 21), and the MAGeCK Robust Rank Algorithm (12) was used to determine hit genes, shown in the volcano plot in **Fig. 2A**. The four hit genes: ATG9A, RB1CC1, ATG101, and RAB14 are all associated with early autophagy and were selected for further validation. Validation of these hit genes was performed in A549 cells without the suicide switch to rule out potential artifacts of this system. shRNA knockdown of the four targets all induced a flattened morphology and an increase in the percentage of SA- β -gal positive cells (**Fig. 2B–D**). In addition, we knocked out ATG9A, RB1CC1, ATG101, and RAB14 using CRISPR-Cas9 and analyzed senescence markers. We demonstrate a reduction of expression of LaminB1 and phospho-RB, and an increase in P21 expression in the knockout cells (Supplementary Fig. S2). To further certify the senescent nature of the cells, RNA-seq was performed on mRNA isolated from cells with shRNA knockdown of the top hit, ATG9A, and parental controls. GSEA showed an enrichment of the Fridman (14) senescence gene signature in the ATG9A knockdown cells, confirming their senescent state (**Fig. 2E**).

Autophagy is a tightly regulated intracellular degradation process that has an important role in cellular homeostasis (22). The main autophagy pathway, commonly referred to as macroautophagy, removes damaged, toxic or excess cellular components and also serves as a recycling pathway to maintain protein synthesis under nutrient starvation conditions. The process is initiated by the formation of isolation membranes, that eventually form double membrane-bound autophagosomes and fuse with lysosomes to degrade their contents (23). ATG9A, RB1CC1, and ATG101 are directly involved in

Figure 1.

A proliferation driven suicide switch facilitates positive selection of growth-arrested populations. **A**, Schematic representation of the proliferation driven suicide switch plasmid and the iCasp9 homodimers. Expression of Ki-67 mRNA in A549 cells was analyzed upon treatment with 0.5 $\mu\text{mol/L}$ of the senescence inducer alisertib. Error bars represent SEM of three biological replicates. **B**, A549-CID cells before and after 24 hours CID treatment. Alisertib treated cells were treated for 4 days with alisertib before addition of CID. Scale bars represent 100 μm . **C-E**, Flow cytometry analysis of A549-CID-mClover3 cells representing the cell size (**C**), autofluorescence (**D**), and signal of the miR-146a reporter (**E**). **F-H**, As in **C-E**, but for cells treated with alisertib for 10 days. **I**, Schematic overview of the CRISPR screening setup.

Figure 2.

Identification and validation of autophagy-related genes as targets for senescence induction. **A**, Volcano plot showing hit genes of the screen (FDR). **B** and **C**, shRNA knockdown of top hit genes in A549 cells (**B**) induces a flattened morphology and increases the percentage of SA- β -gal positive cells (**C**). Scale bars in images represent 100 μ m. **D**, Quantification of the percentage of SA- β -gal positive cells. Error bars represent SEM of three biological replicates. Asterisks represent significance as analyzed by *t* test (**, $P < 0.01$; ***, $P < 0.001$). **E**, GSEA of RNA-seq data obtained from A549 cells infected with a vector with shRNA against ATG9A vs. control shows enrichment of senescence associated genes. Samples were sequenced 8 days after infection.

autophagosome formation (24). RB1CC1 and ATG101 are members of the ULK1 complex, fundamental for the early steps of autophagosome biogenesis, whereas ATG9A transiently interacts with the forming autophagosome (Fig. 3A). In addition, RAB14 may be indirectly involved in early autophagy due to its role in intracellular membrane trafficking which is necessary for autophagosome growth, positioning and fusion (25).

There are currently no small molecule inhibitors available to inhibit the function of any of these proteins. However, the main effector of the ULK1 complex, the kinase ULK1, can be inhibited by SBI0206965. A549 cells treated with SBI0206965 show a dose

dependent reduction in proliferation (Fig. 3B) and adopt a flattened morphology with strong SA- β -gal activity, indicative of senescent cells (Fig. 3C). In addition, the comparison of mRNA expression of treated with untreated cells shows an enrichment of the Fridman senescence gene signature (Fig. 3D). We observed similar effects on growth arrest, flattened morphology and the percentage of SA- β -gal positive cells in cancer cell lines derived from liver (Hep3B), pancreas (PANC1), and colon (RKO; Fig. 3E–J). In addition, we performed ULK-1 inhibition in MCF7, PC9, T47D, and nontransformed BJ fibroblasts, and demonstrate that SBI0206965 can inhibit tumor growth and induce SA- β -gal activity in all three cancer cell

Figure 3.

SBI0206965 treatment induces senescence. **A**, Schematic overview of essential players in autophagophore nucleation and elongation. **B**, IncuCyte proliferation assay of A549 cells treated with different doses of SBI0206965. **C**, SA-β-gal staining of treated cells and untreated controls after 4 days of treatment. The number inset represents the percentage of SA-β-gal positive cells. Scale bars represent 100 μm. **D**, GSEA of RNA-seq data obtained from A549 cells treated with SBI0206965 vs. control shows enrichment of the Fridman senescence signature. **E–J**, As in **B** and **C**, but for Hep3B (**E** and **H**), PANC1 (**F** and **I**), and RKO (**G** and **J**). Scale bars represent 100 μm.

lines, but does not induce senescence in BJ cells (Supplementary Fig. S3). Unfortunately, pharmacokinetic analysis in two different mouse strains showed rapid depletion of SBI0206965 from the blood plasma (Supplementary Fig. S4). Due to this unfavorable pharmacokinetic profile, we did not pursue further *in vivo* experimentation with this compound.

To gain insight into how SBI0206965 induces senescence, we looked at expression changes in A549 cells over time of proteins involved in autophagy and cellular stress (Fig. 4A). LC3B plays a central role in the early stages of autophagosome formation and phagophore membrane

elongation, and is eventually degraded in the autolysosomal lumen. Turnover of LC3B is often used as a marker to monitor autophagy and autophagy-related processes (26). The accumulation of LC3B after several days of treatment with SBI0206965 indicates that autophagy is compromised and stalled in the early stage of autophagosome formation. In addition, after 2 days of treatment, we observed an increase in the levels of p53 and p21, which are pivotal for the establishment of senescence (27). Furthermore, we observed a significant increase in the expression of the apoptosis inhibitory protein BCL2, commonly elevated in senescent cells (28). BCL2 family members can be targeted

Figure 4.

Treatment with SBI0206965 sensitizes cells to senolytic treatment. **A**, Western blot analysis of A549 treated with 5 $\mu\text{mol/L}$ SBI0206965 for different durations up to 6 days. **B**, Crystal violet staining of parental A549 cells and cells treated with SBI0206965 for 4 days, with and without addition of ABT-263. The quantification of the elimination of cells, represented by the decrease in staining intensity, is shown in red. **C**, Representative images for A549, Hep3B, PANC1, and RKO cell lines showing control cells and SBI0206965 pretreated cells, before and 24 hours after addition of ABT263. Apoptotic cells were determined by caspase-3/7 apoptosis assay. Scale bars represent 100 μm .

with the BH3 mimetic ABT-263 (29). Parental A549 were not affected by ABT-263, whereas cells pretreated with SBI0206965 were eliminated (**Fig. 4B**). The same effect was observed in Hep3B, PANC1, and RKO cells. Combination of SBI0206965 with ABT-263 induced a significant increase in the percentage of apoptotic cells, assessed by fluorescence of the Caspase-3/7 Green Dye (**Fig. 4C**). To validate that this effect was not due to potential off target effects of SBI0206965, we confirmed our findings using the ULK1 inhibitor MRT68921 (Supplementary Fig. S5). These data highlight the utility of a combination therapy consisting of a pro-senescence and a senolytic agent.

Discussion

The suicide switch we describe here can be used to deplete proliferating cell populations, which is useful in performing screens for

proliferation arrest by enriching the growth-arrested population. We used this concept to perform genome-wide screens with positive selection of senescent cells. This allowed discovery of targets for pro-senescence therapy on a genome-wide scale. Using the proliferation-driven suicide switch we could culture our cells of interest for two weeks without overgrowth of proliferating cells. This timing and the retention of growth-arrested cells was crucial to identify sufficient senescent cells to call hits using a genome-wide library. A whole-genome approach in pro-senescence CRISPR screens is desirable as senescence is involved in a variety of physiological and pathological processes and is regulated by manifold pathways. In addition, we did not want to limit ourselves to the usual established drug target classes, as recent developments like proteolysis targeting chimeras and molecular glues are gradually increasing the playing field for targets with therapeutic potential (30).

Our genome-wide approach led to the identification of several targets related to autophagy that when inhibited can induce senescence. Autophagy plays an important role in quality control of macromolecules and energy homeostasis and the connection between autophagy and senescence has been described extensively. However, the jury is still out on whether autophagy has a positive or negative impact on senescence, or both (31). On the one hand, autophagy has been suggested to mediate the acquisition of the senescence phenotype (32). The associated protein turnover and recycling of cellular material would be essential to facilitate the mass synthesis of secretory proteins. On the other hand, impairment of autophagy with siRNA or shRNA has been described to induce senescence (33). The senescence induction is potentially caused by accumulation of damaged macromolecules and an increase in cytotoxic stresses, as ROS scavenging or inhibition of p53 activation delayed its onset upon autophagy impairment. Small molecule inhibition of autophagy with SBI0206965 mimicked this effect, with senescence induction in combination with increased levels of p21 and p53.

Combination of SBI0206965 with the BH3 mimetic ABT-263 induced apoptosis in a panel of cancer cell lines *in vitro*. Further experiments will have to be done to determine whether autophagy inhibition can induce senescence in cancer cells *in vivo* and whether such therapy is effective when combined with a senolytic treatment. Nevertheless, the identification and validation of new targets and a small molecule to induce senescence in cancer cells underscores the potential of using a suicide switch in screens. This switch system can help to perform large-scale gene knockout screens in a rapid and

cost-effective manner. Furthermore, the concept of the suicide switch could also be extrapolated from growth arrest to other traits depending on the nature of the promoter used to drive the inducible suicide switch.

Authors' Disclosures

No disclosures were reported.

Authors' Contributions

A. Schepers: Conceptualization, formal analysis, validation, investigation, visualization, methodology, writing—original draft, writing—review and editing. **F. Jochems:** Investigation. **C. Liefink:** Data curation, formal analysis. **L. Wang:** Investigation. **Z. Pogacar:** Investigation. **R. Leite de Oliveira:** Investigation. **G. De Conti:** Investigation. **R.L. Beijersbergen:** Supervision. **R. Bernards:** Supervision, writing—review and editing.

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References

- Hernandez-Segura A, Nehme J, Demaria M. Hallmarks of cellular senescence. *Trends Cell Biol* 2018;28:436–53.
- Wang B, Kohli J, Demaria M. Senescent cells in cancer therapy: friends or foes? *Trends Cancer* 2020;6:838–57.
- Coppé JP, Desprez PY, Krtolica A, Campisi J. The senescence-associated secretory phenotype: the dark side of tumor suppression. *Annu Rev Pathol Mech Dis* 2010;5:99–118.
- Leite de Oliveira R, Bernards R. Anti-cancer therapy: senescence is the new black. *EMBO J* 2018;37:e99386.
- Sieben CJ, Sturmlechner I, van de Sluis B, van Deursen JM. Two-step senescence-focused cancer therapies. *Trends Cell Biol* 2018;28:723–37.
- Wang C, Vegna S, Jin H, Benedict B, Liefink C, Ramirez C, et al. Inducing and exploiting vulnerabilities for the treatment of liver cancer. *Nature* 2019;574:268–72.
- Wang L, Leite de Oliveira R, Wang C, Fernandes Neto JM, Mainardi S, Evers B, et al. High-throughput functional genetic and compound screens identify targets for senescence induction in cancer. *Cell Rep* 2017;21:773–83.
- Kale A, Sharma A, Stolzing A, Stolzing A, Desprez PY, Desprez PY, et al. Role of immune cells in the removal of deleterious senescent cells. *Immun Ageing* 2020;17:16.
- Ruscetti M, Morris JP, Mezzadra R, Russell J, Leibold J, Romesser PB, et al. Senescence-induced vascular remodeling creates therapeutic vulnerabilities in pancreas cancer. *Cell* 2020;181:424–41.
- Evers B, Jastrzebski K, Heijmans JPM, Grønrum W, Beijersbergen RL, Bernards R. CRISPR knockout screening outperforms shRNA and CRISPRi in identifying essential genes. *Nat Biotechnol* 2016;34:631–3.
- Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014;15:550.
- Li W, Xu H, Xiao T, Cong L, Love MI, Zhang F, et al. MAGeCK enables robust identification of essential genes from genome-scale CRISPR/Cas9 knockout screens. *Genome Biol* 2014;15:554.
- Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* 2005;102:15545–50.
- Fridman AL, Tainsky MA. Critical pathways in cellular senescence and immortalization revealed by gene expression profiling. *Oncogene* 2008;27:5975–87.
- Brinkman EK, Chen T, Amendola M, Van Steensel B. Easy quantitative assessment of genome editing by sequence trace decomposition. *Nucleic Acids Res* 2014;42:e168.
- Burner GC, Norwood TH. Selective elimination of proliferating cells in human diploid cell cultures by treatment with BrdU, 33258 Hoechst and visible light. *Mech Ageing Dev* 1980;12:151–9.
- Tieng V, Cherpil O, Gutzwiller E, Zamboni AC, Delgado C, Salmon P, et al. Elimination of proliferating cells from CNS grafts using a Ki67 promoter-driven thymidine kinase. *Mol Ther Methods Clin Dev* 2016;6:16069.
- Straathof KC, Pulè MA, Yotnda P, Dotti G, Vanin EF, Brenner MK, et al. An inducible caspase 9 safety switch for T-cell therapy. *Blood* 2005;105:4247–54.
- Menon SS, Guruvayoorappan C, Sakthivel KM, Rasmi RR. Ki-67 protein as a tumour proliferation marker. *Clin Chim Acta* 2019;491:39–45.
- Doench JG, Fusi N, Sullender M, Hegde M, Vaimberg EW, Donovan KF, et al. Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. *Nat Biotechnol* 2016;34:184–91.
- Anders S, Huber W. Differential expression analysis for sequence count data. *Genome Biol* 2010;11:R106.
- Mizushima N. Autophagy: process and function. *Genes Dev* 2007;21:2861–73.
- Hamasaki M, Furuta N, Matsuda A, Nezu A, Yamamoto A, Fujita N, et al. Autophagosomes form at ER-mitochondria contact sites. *Nature* 2013;495:389–93.
- He C, Klionsky DJ. Regulation mechanisms and signaling pathways of autophagy. *Annu Rev Genet* 2009;43:67–93.
- Junutula JR, De Mazière AM, Peden AA, Ervin KE, Advani RJ, Van Dijk SM, et al. Rab14 is involved in membrane trafficking between the Golgi complex and endosomes. *Mol Biol Cell* 2004;15:2218–29.
- Tanida I, Ueno T, Kominami E. LC3 and autophagy. *Methods Mol Biol* 2008;445:77–88.

27. Kasthuber ER, Lowe SW. Putting p53 in context. *Cell* 2017;170:1062–78.
28. Yosef R, Pilpel N, Tokarsky-Amiel R, Biran A, Ovadya Y, Cohen S, et al. Directed elimination of senescent cells by inhibition of BCL-W and BCL-XL. *Nat Commun* 2016;7:11190.
29. Zhu Y, Tchkonja T, Fuhrmann-Stroissnigg H, Dai HM, Ling YY, Stout MB, et al. Identification of a novel senolytic agent, navitoclax, targeting the Bcl-2 family of anti-apoptotic factors. *Aging Cell* 2016;15:428–35.
30. Churcher I. Proteasome-induced protein degradation in drug discovery: breaking the rules or just making new ones? *J Med Chem* 2018;61:444–52.
31. Kang C, Elledge SJ. How autophagy both activates and inhibits cellular senescence. *Autophagy* 2016;12:898–9.
32. Young ARJ, Narita M, Ferreira M, Kirschner K, Sadaie M, Darot JFJ, et al. Autophagy mediates the mitotic senescence transition. *Genes Dev* 2009;23:798–803.
33. Kang HT, Lee KB, Kim SY, Choi HR, Park SC. Autophagy impairment induces premature senescence in primary human fibroblasts. *PLoS One* 2011;6:e23367.