An RNA interference screen for identifying downstream effectors of the p53 and pRB tumour suppressor pathways involved in senescence

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Abstract

Background: Cellular senescence is an irreversible cell cycle arrest that normal cells undergo in response to progressive shortening of telomeres, changes in telomeric structure, oncogene activation or oxidative stress and acts as an important tumour suppressor mechanism.

Results: To identify the downstream effectors of the p53-p21 and p16-pRB tumour suppressor pathways crucial for mediating entry into senescence, we have carried out a loss-of-function RNA interference screen in conditionally immortalised human fibroblasts that can be induced to rapidly undergo senescence, whereas in primary cultures senescence is stochastic and occurs asynchronously. These cells are immortal but undergo a rapid irreversible arrest upon activation of the p53-p21 and p16-pRB pathways that can be readily bypassed upon their inactivation. The primary screen identified 112 known genes including p53 and another 29 shRNAmirs targeting as yet unidentified loci. Comparison of these known targets with genes known to be up-regulated upon senescence in these cells, by micro-array expression profiling, identified 4 common genes TMEM9B, ATXN10, LAYN and LTBP2/3. Direct silencing of these common genes, using lentiviral shRNAmirs, bypassed senescence in the conditionally immortalised cells.

Conclusion: The senescence bypass screen identified TMEM9B, ATXN10, LAYN and LTBP2/3 as novel downstream effectors of the p53-p21 and p16-pRB tumour suppressor pathways. Although none of them has previously been linked to cellular senescence, TMEM9B has been suggested to be an upstream activator of NF-κB signalling which has been found to have a causal role in promoting senescence. Future studies will focus on determining on how many of the other primary hits also have a causal role in senescence and what is the mechanism of action.

Keywords: Cellular senescence, RNA interference screen, senescence bypass, conditionally immortal cells

Background

Normal somatic cells undergo a finite number of divisions before entering a state of irreversible growth arrest termed cellular senescence [1]. This is triggered in response to a variety of intrinsic and extrinsic stimuli including progressive telomere shortening or changes in telomeric structure at the ends of chromosomes or other forms of genotoxic stress such as oncogene activation, or DNA damage or oxidative stress, resulting in a DNA damage response and growth arrest via activation of the p53 tumour suppressor pathway [2,3]. Non-genotoxic stress induces senescence by a telomere independent mechanism involving activation of the p16-pRB pathway by up-regulation of p16INK4a [3,4].

Cellular senescence acts as an important tumour suppressor mechanism. Overcoming senescence and acquiring a limitless replicative potential has been proposed to be one of the key events required for malignant transformation [5]. Senescence is thought to have evolved as an example of antagonistic pleiotropy, whereby its beneficial traits in a reproductively active individual have deleterious effects later in life [6,7]. The underlying
mechanisms that control cellular senescence, the signal transduction pathways involved and how the diverse signals that result in senescence are all integrated, remain poorly defined. Moreover, the downstream effectors of the p53-p21 and p16-pRB pathways that result in the irreversible growth arrest and entry into senescence are unknown.

The discovery of RNA interference as a mechanism to silence gene expression has revolutionized studies on mammalian gene expression and has permitted loss-of-function genome-wide genetic screens, to identify genes involved in a variety of cellular processes, to be performed [8-12]. A number of shRNA libraries have been developed for carrying out such genome-wide screens, one of which is the pSM2 Retroviral shRNAmir library [13] (Thermo Scientific Open Biosystems). This library has several unique features that make it very versatile and efficient for large-scale screens particularly the human microRNA-30 (miR30) adapted design which increases knockdown specificity and efficiency [14].

Here we present a RNA interference screen using the human pSM2 retroviral shRNAmir library, carried out in the conditionally immortal HMF3A human fibroblasts, to identify genes whose silencing bypasses senescence arrest induced by activation of the p53-p21 and p16-pRB pathways. The primary screen identified 112 known genes and another 29 shRNAmir targets targeting as yet unidentified loci. Comparison of the known targets with genes known to be up-regulated upon senescence by micro-array expression profiling, identified 4 common genes whose expression was reversed when senescence was bypassed upon inactivation of the p53-p21 and p16-pRB pathways.

Results
To directly identify the downstream effectors of the p53-p21 and p16-pRB pathways, we have utilized the conditionally immortal HMF3A human fibroblasts, to identify genes whose silencing bypasses senescence arrest induced by activation of the p53-p21 and p16-pRB pathways. The primary screen identified 112 known genes and another 29 shRNAmir constructs targeting as yet unidentified loci. Comparison of the known targets with genes known to be up-regulated upon senescence by micro-array expression profiling, identified 4 common genes whose expression was reversed when senescence was bypassed upon inactivation of the p53-p21 and p16-pRB pathways.

Figure 1 Characteristics of CL3\textsubscript{EcoR} cells a. CL3\textsubscript{EcoR} cells are immortal at 34°C but undergo a senescence arrest upon shift up 38°C. b. Senescence is bypassed upon silencing of p21\textsuperscript{CIP1} using pRSp21F or sequestration of RB family of proteins by HPV16 E7.
densely growing colonies (Figure 2c) indicating that CL3EcoR cells and the procedure were sufficiently sensitive to generate colonies in which senescence had been overcome.

**ShRNA interference screen**

The formula: ln [1-0.95]/ln [1-1/(Library Size)] recommended for genetic screens by the Nolan lab (http://www.stanford.edu/group/nolan/screens/screens.html), suggested that approximately 1000 independent infectious events would be sufficient for a 99% confidence that all shRNAs within a pool had been sampled. To ensure that the screen would be saturating, virus sufficient to yield 10,000 infectious events was utilised for each pool (shown in Additional File 1). The screen was performed in successive waves of 10 pools. To minimise variation and background reversion, CL3EcoR cells were used at the same passage for every pool. Virus prepared from pRSp21F and pRSLamin A/C was used as positive and negative controls respectively to evaluate the level of background and ensure that the complementation assay worked for each round of the screen. Stably transduced cells were trypsinised and reseeded. Three weeks after reseeding, flasks were examined to identify growing colonies; a representative colony is shown in Figure 3. Each colony was examined microscopically to ensure it comprised growing cells and the number of
colonies obtained for each pool determined. The number of stably transduced cells, the number of flasks reseeded and the number of colonies obtained for each flask at 38°C are shown in Additional File 2. The flasks which contained more densely growing/bigger colonies (indicated in red in Additional File 2) were trypsinised, replated and used for extracting genomic DNA when confluent. 34 out of 100 pools yielded healthy growing colonies; pools 13, 78 and 82 particularly gave a higher number of colonies which were also larger. Pools 16, 18, 19, 21 and 80 also yielded colonies but they were smaller. For each pool that contained growing colonies, 1 to 4 flasks containing the highest number of growing colonies, were reseeded for extracting genomic DNA and resulted in a total of 81 sub-pools.

Identification of shRNAmir

The shRNAmir proviral inserts were by amplified by two rounds of nested PCR using pSM2 specific primers, the amplified products TOPO cloned and plasmid DNA extracted from at least six colonies sequenced to identify all shRNAs present within each pool; for some pools, DNA from many more colonies was sequenced. The hair pin sequence was determined by searching for the miR-30 context and the miR-30 loop that are common to all inserts and frame the hair pin. The sequence of the hair pin was used to identify the target gene by searching the pSM2 data base or by BLASTN analysis of the NCBI human genome data base (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences that could not be linked back to the list of hair pin sequences within the Genbank were not pursued and reseeded for growing colonies when confluent.

Overlap of the primary candidates of the shRNA screen with microarray data for genes up-regulated upon senescence in CL3 Eco/iB

To prioritise the candidates identified from the primary screen for functional validation, they were compared to genes up-regulated upon senescence and whose expression was reversed when senescence was abrogated upon inactivation of the p53 and pRB pathways ([16]; microarray expression profiling data is available from Gene Expression Omnibus database accession number GSE24810]. This identified 4 common genes, ATXN10, LAYN, LTBP2/3 and TMEM9B. The microarray expression profiling data presented in Table 2 showed that they were all up-regulated upon senescence growth arrest: ATXN10 by 1.3 fold (p-value 2 × 10^-3), LAYN by 2 fold (p-value 2 × 10^-4), LTBP2/3 by 2.5 (p-value 9 × 10^-9), 1.7 (p-value 5 × 10^-9), 1.5 (p-value 8 × 10^-4) and 1.4 fold (p-value 5 × 10^-5) and TMEM9B by 1.4 (p-value 1 × 10^-7) and 1.3 fold (p-value 2 × 10^-4) respectively. The data in Table 2 further show that up-regulation of these candidates was reversed when senescence was bypassed upon inactivation of the p53-p21 and p16-pRB pathways by silencing p53 (pRS_p53) or p21^{CIP1} (pRS_p21) or by sequestration of the RB family of proteins by HPV16 E7 or by expression of the dominant negative E2F-DB protein.

The identification of TMEM9B was particularly remarkable because the microarray analysis has suggested that senescence growth arrest in CL3 Eco/iB cells is associated with activation of the NF-κB signalling pathway and TMEM9B has previously been shown to be able to activate NF-κB dependent reporter constructs [18,19]. To determine if silencing of TMEM9B would bypass senescence, 4 GIPZ lentiviral silencing constructs
| Pool | Insert | Gene name | Gene symbol | Freq | Cons per Gene | Library location |
|------|--------|-----------|-------------|------|---------------|------------------|
| 3    | v2HS_63142 | keratin associated protein 5-9 | KRTAP5-9 | 3/9  | 1             | match            |
|      | v2HS_119967 | LOC155004 | LOC155004 | 4/9  | 2             | pool S2          |
|      | v2HS_56766 | acyl-CoA synthetase medium-chain family member 3 | ACSM3 | 1/9  | 2             | match            |
|      | v2HS_59294 | glycoprotein hormone alpha 2 | GPHA2 | 1/9  | 1             | match            |
|      | v2HS_53974 | PRO0255 protein | PRO0255 | 5/8  | 1             | match            |
|      | v2HS_63482 | paired box gene 1 | PAX1 | 2/8  | 1             | match            |
| 4    | v2HS_119967 | LOC155004 | LOC155004 | 2/10 | 2             | pool S2          |
|      | v2HS_66751 | LOC344082 | LOC344082 | 4/10 | 1             | match            |
|      | v2HS_70011 | serum amyloid A-like 1 | SAAL1 | 2/10 | 2             | match            |
|      | v2HS_98079 | human solute carrier family 22 | SLC22A3 | 1/10 | 2             | pool 79          |
| 5    | v2HS_108647 | LOC349868 | LOC349868 | 3/8  | 1             | pool 79          |
|      | v2HS_70473 | polymerase (DNA directed), mu | POLM | 2/8  | 1             | match            |
|      | v2HS_71958 | human olfactory receptor, family S, subfamily P, member 3 | OR5P3 | 3/8  | 1             | match            |
|      | v2HS_119967 | LOC155004 | LOC155004 | 7/12 | 2             | pool S2          |
|      | v2HS_66652 | protein phosphatase 3, catalytic subunit, B isoform | PPP3CB | 1/12 | 1             | match            |
|      | v2HS_70473 | polymerase (DNA directed), mu | POLM | 4/12 | 1             | match            |
| 7    | v2HS_112910 | human cyclin-dependent kinase 8 | CDK8 | 1/10 | 3             | match            |
|      | v2HS_98079 | solute carrier family 22, member 3 | SLC22A3 | 1/10 | 2             | pool 79          |
|      | v2HS_97017 | sterile alpha motif containing 4A | SAMD4A | 1/10 | 1             | match            |
|      | v2HS_69776 | cytochrome P450, family 4, subfamily Z, polypeptide 2 pseudogene | CYP4Z2P | 1/10 | 2             | match            |
| 9    | v2HS_108647 | LOC349868 | LOC349868 | 1/8  | 1             | pool 79          |
|      | v2HS_119967 | LOC155004 | LOC155004 | 1/8  | 2             | pool S2          |
|      | v2HS_62506 | family with sequence similarity 181, member B | FAM181B | 1/8  | 1             | match            |
|      | v2HS_55950 | prostate stem cell antigen | PSCA | 2/13 | 1             | match            |
|      | v2HS_119967 | LOC155004 | LOC155004 | 3/13 | 2             | pool S2          |
|      | v2HS_70312 | TRK-fused gene | TFG | 4/13 | 3             | match            |
|      | v2HS_71740 | ataxin 10 | ATXN10 | 1/13 | 1             | match            |
|      | v2HS_98079 | solute carrier family 22, member 3 | SLC22A3 | 1/13 | 2             | pool 79          |
|      | v2HS_65121 | sorting nexin 12 | SNX12 | 1/13 | 1             | match            |
|      | v2HS_64384 | amyloid beta precursor protein binding family A member 2 | APBA2 | 1/6  | 1             | match            |
|      | v2HS_97017 | sterile alpha motif containing 4A | SAMD4A | 1/6  | 1             | match            |
| 12   | v2HS_119967 | LOC155004 | LOC155004 | 5/11 | 2             | pool S2          |
|      | v2HS_58950 | signal-regulatory protein beta 2 | SRPB2 | 3/11 | 2             | match            |
|      | v2HS_119967 | LOC155004 | LOC155004 | 5/10 | 2             | pool S2          |
| 13   | v2HS_55731 | phenylalanine-tRNA synthetase-like, beta subunit | FARSIB | 1/12 | 2             | match            |
|      | v2HS_59258 | dynein, light chain, roadblock-type 1 | DYNLRB1 | 3/12 | 1             | match            |
|      | v2HS_59891 | TAO kinase 1 | TAOK1 | 5/12 | 3             | match            |
|      | v2HS_162164 | iodotyrosine deiodinase | IYD | 1/12 | 1             | match            |
|      | v2HS_68714 | cell cycle exit and neuronal differentiation 1 | CEND1 | 1/12 | 1             | match            |
|      | v2HS_55731 | phenylalanine-tRNA synthetase-like, beta subunit | FARSIB | 1/15 | 2             | match            |
|      | v2HS_64320 | Smith-Magenis syndrome chromosome region, candidate 7 | SMCR7 | 1/15 | 1             | match            |
|      | v2HS_71174 | peroxisome proliferator-activated receptor, gamma | PPARGC1A | 3/15 | 1             | match            |
|      | v2HS_71453 | similar to IAP-associated factor VIAF1, phosphocandin-like | 2/15 | 1             | match            |
|   | v2HS_68478 | v2HS_63107 | v2HS_61750 | v2HS_62831 |
|---|-----------|-----------|-----------|-----------|
| 16 | CD28 antigen | dopamine beta hydroxylase | LOC100129230 | membrane bound O-acyltransferase domain containing 1 |
|    | v2HS_68478 | CD28 | 8/12 | 2 pool 13 |
|    | v2HS_63107 | DBH | 2/12 | 1 match |
|    | v2HS_61750 | LOC100129230 | LOC9142 | LOC345672 |
|    | v2HS_62831 | MBOAT1 | 3/13 | 2 match |
| 18 | v2HS_63989 | STAR-related lipid transfer (START) domain containing 6 | LOC730256 |
|    | v2HS_63989 | STARD6 | 1/14 | 2 match |
|    | v2HS_58958 | TMEM9 domain family, member B/C11orf15 | TMEM9B |
|    | v2HS_57051 | RNA binding motif, single stranded interacting protein 1 | RBMS1 |
|    | v2HS_106158 | LOC345672 | LOC345672 | LOC345672 |
|    | v2HS_59560 | chromosome 13 open reading frame 1S | C13orf1S |
| 19 | v2HS_55312 | glucosamine-phosphate N-acetyltransferase 1 | LOC100129563 |
|    | v2HS_55312 | GNPNAT1 | 6/9 | 2 match |
|    | v2HS_68437 | LOC155004 | LOC155004 | LOC155004 |
|    | v2HS_55312 | LOC155004 | 2/9 | 3 match |
| 21 | v2HS_119967 | V2HS_58958 | LOC155004 | LOC155004 |
|    | v2HS_119967 | LOC155004 | 6/14 | 2 pool 52 |
|    | v2HS_34338 | protein phosphatase 4, regulatory subunit 2 | PPF4R2 |
|    | v2HS_119967 | LOC155004 | 1/13 | 2 match |
|    | v2HS_34338 | LOC155004 | 7/14 | 3 match |
| 30 | v2HS_42104 | citrate lyase beta like (CLYBL), transcript variant 1 | CLYBL |
|    | v2HS_42104 | LOC155004 | LOC100129563 | LOC155004 |
|    | v2HS_42104 | LOC155004 | 6/7 | 1 match |
|    | v2HS_42104 | LOC155004 | 5/7 | 1 match |
| 32 | v2HS_48278 | choline kinase-like | CHKL |
|    | v2HS_48278 | 6/6 | 1 match |
| 54 | v2HS_112629 | basic transcription factor 3, like 1 | BTF3L1 |
|    | v2HS_121153 | LOC221399 | LOC221399 | LOC221399 |
|    | v2HS_125538 | LOC350103 | 2/6 | 1 match |
|    | v2HS_129527 | LOC351851 | LOC351851 | LOC351851 |
|    | v2HS_119967 | LOC155004 | 6/6 | 1 match |
|    | v2HS_112629 | LOC155004 | 6/6 | 1 match |
| 55 | v2HS_117465 | SH3 domain binding glutamic acid-rich protein like 2 | SH3BGRL2 |
|    | v2HS_119051 | LOC90841 | LOC90841 | LOC90841 |
|    | v2HS_116174 | YTH domain containing 2 | YTHDC2 | YTHDC2 |
|    | v2HS_119120 | hypothetical protein FLJ0032 | FLJ0032 | FLJ0032 |
|    | v2HS_117465 | LOC350103 | LOC350103 | LOC350103 |
| 56 | v2HS_120429 | six-twelve leukemia (STL), non-coding RNA | STL |
|    | v2HS_115231 | rab23, member RAS oncogene family | RAB23 |
|    | v2HS_117914 | transketolase-like 2 | TKTL2 |
|    | v2HS_117229 | chromosome 9 open reading frame 58 | C9orf58 |
|    | v2HS_120429 | six-twelve leukemia (STL), non-coding RNA | STL |
|    | v2HS_119206 | ion peptidase 2, peroxisomal | LONP2 |
|    | v2HS_120429 | six-twelve leukemia (STL), non-coding RNA | 2/6 | 1 match |
|    | v2HS_119206 | six-twelve leukemia (STL), non-coding RNA | 6/6 | 1 match |
| 58 | v2HS_116174 | YTH domain containing 2 | YTHDC2 |
|    | v2HS_118722 | laylin | LAYN |
|    | v2HS_112838 | ectonucleoside triphosphate diphosphohydrolase 3 | ENTPD3 |
|    | v2HS_112982 | chromodomain helicase DNA binding protein 3 | CHD3 |
|    | v2HS_116174 | YTH domain containing 2 | YTHDC2 |
|    | v2HS_112838 | ectonucleoside triphosphate diphosphohydrolase 3 | ENTPD3 |
|    | v2HS_118254 | WD repeat and FYVE domain containing 2 | WDFY2 |
|    | v2HS_122548 | similar to ribosomal protein S3A | RPS3A |
|    | v2HS_120982 | coiled coil domain containing 129 | CCDC129 |
|    | v2HS_111554 | interleukin 2 | IL2 |
|    | v2HS_115544 | DEAD (Asp-Glu-Ala-Asp) box polypeptide 47 | DDX47 |
|    | v2HS_116377 | transmembrane protein 135 | TMEM135 |
|    | v2HS_117064 | CD1E molecule | CD1E |
|    | v2HS_119967 | LOC155004 | LOC155004 | LOC155004 |
|    | v2HS_119967 | LOC155004 | 4/9 | 2 pool 52 |
| v2HS_120757  | olfactory receptor, family 8, subfamily K, member 1 | OR8K1  | 1/9  | 2         | match |
| v2HS_108647  | LOC349868                                          |        |      |           |       |
| v2HS_115544  | DEAD (Asp-Glu-Ala-Asp) box polypeptide 47          | DDX47  | 3/8  | 4         | match |
| v2HS_116833  | intermediate filament protein syncoilin            | SYNC   | 1/8  | 2         | match |
| v2HS_115185  | dual specificity phosphatase 3                     | DUSP3  | 10/11 | 1         | match |
| v2HS_117903  | glutamine rich 2                                   | QRICH2 | 4/11 | 1         | match |
| v2HS_119967  | LOC155004                                          |        |      |           |       |
| v2HS_116833  | DEAD (Asp-Glu-Ala-Asp) box polypeptide 47          | DDX47  | 3/8  | 4         | match |
| v2HS_115544  | DEAD (Asp-Glu-Ala-Asp) box polypeptide 47          | DDX47  | 3/8  | 4         | match |
| v2HS_98079   | solute carrier family 22, member 3                 | SLC22A3| 1/12 | 1         | match |
| v2HS_117675  | latent transforming growth factor \(\beta\) binding protein 2/3 | LTBP2/3 | 15/15 |           |       |
| v2HS_118530  | RNA exonuclease 1 homolog like 1                   | REXO1L1| 5/13 | 3         | match |
| v2HS_119967  | latent transforming growth factor \(\beta\) binding protein 2/3 | LTBP2/3 | 5/13 | 3         | match |
| v2HS_118530  | RNA exonuclease 1 homolog-like 2                   | REXO1L1| 5/13 | 3         | match |
| v2HS_103818  | LOC284804                                          |        |      |           |       |
| v2HS_119967  | LOC155004                                          |        |      |           |       |
| v2HS_97981   | LOC51152                                          |        |      |           |       |
| v2HS_102441  | adenylate cyclase 1                                 | ADCY1  | 5/11 | 1         | match |
| v2HS_130882  | glutamate receptor, metabotropic 3                  | GRM3   | 1/11 | 3         | match |
| v2HS_97368   | yippee-like 5                                     | YPEL5  | 2/11 | 1         | match |

Table 1 Primary screen (Continued)
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| Gene ID | Gene Name | Reference | Isolation Frequency | Pool | Match |
|---------|-----------|-----------|---------------------|------|-------|
| v2HS_106158 | LOC349975 | 1/11 | 2 matches | pool 84 |
| v2HS_98525 | PRICKLE2 | 3/11 | 1 match |
| v2HS_100096 | EIF4A1 | 1/11 | 1 pool |
| v2HS_99423 | CCDC70 | 1/11 | 1 match |
| v2HS_109596 | ENSMUSG00000020331 | 1/14 | |
| v2HS_108399 | LOC349811 | 3/14 | 2 match |
| v2HS_184999 | EIF4A1 | 1/14 | |
| v2HS_93536 | PLF1 | 5/14 | 1 match |
| v2HS_109096 | SAMD4A | 1/14 | |
| v2HS_97017 | LOC349975 | 1/14 | |
| v2HS_93615 | PLP1 | 5/14 | 1 match |
| v2HS_95112 | RASA4 | 3/14 | 1 match |
| v2HS_99525 | BCL2L12 | 3/14 | 2 pool |
| v2HS_94640 | ARNTL | 3/14 | 2 match |
| v2HS_95019 | ZNF16 | 3/14 | 1 match |
| v2HS_97152 | UBE2J1 | 3/14 | 1 match |
| v2HS_106409 | LOC345700 | 1/14 | |
| v2HS_141367 | FLJ37626 | 1/11 | 1 match |
| v2HS_133299 | IGFBP6 | 1/11 | 1 match |
| v2HS_135564 | CXORF57 | 1/11 | 1 match |
| v2HS_141495 | ZNF454 | 7/12 | 2 pool |
| v2HS_130457 | RSPH10B | 6/7 | 3 match |
| v2HS_131154 | KCNJ2 | 1/7 | 3 match |
| v2HS_184999 | LOC345700 | 1/11 | 1 match |
| v2HS_95019 | ZNF16 | 9/11 | 1 match |
| v2HS_97152 | UBE2J1 | 1/11 | 1 match |
| v2HS_95019 | ZNF16 | 10/11 | 1 match |
| v2HS_119967 | LOC155004 | 2/11 | 2 pool |
| v2HS_133299 | IGFBP6 | 3/11 | 2 match |
| v2HS_135564 | CXORF57 | 3/11 | 2 match |

For each candidate the DNA pool from which it was isolated, the insert reference, target gene, frequency of isolation, as well as the number of shRNA targets for that gene within the SM2 library are indicated. The last column shows if the recovered insert was a match to a hair pin in that particular pool ("match") or if it was from another pool ("pool X").

Table 2 Microarray expression profiling data for common genes

| Probe | Symbol | GA p-value | HS p-value | pRS_p53 p-value | pRS_p21 p-value | E7 p-value | E2F_DB p-value |
|-------|--------|------------|------------|-----------------|-----------------|-------------|--------------|
| 208832_at | ATXN10 | 0.36 | 1E-03 | 0.25 | 3E-02 | 0.01 | -0.18 | 2E-01 | -0.35 | 2E-03 | 0.02 | 9E-01 | -0.23 | 4E-02 |
| 228080_at | LAYN | 1.04 | 2E-04 | 0.30 | 3E-01 | -0.95 | 1E-03 | -0.70 | 2E-02 | -0.56 | 6E-02 | -0.11 | 8E-01 |
| 219922_s_at | LTBP2/3 | 1.32 | 9E-08 | 0.66 | 5E-03 | -1.04 | 2E-05 | -1.32 | 3E-07 | -1.19 | 1E-06 | -1.41 | 4E-08 |
| 223690_at | LTBP2/3 | 0.75 | 5E-09 | 0.14 | 3E-01 | -0.52 | 1E-05 | -0.91 | 2E-10 | -1.27 | 1E-14 | -1.22 | 3E-14 |
| 204682_at | LTBP2/3 | 0.57 | 8E-04 | 0.48 | 8E-03 | -0.39 | 4E-02 | -0.94 | 3E-06 | -1.62 | 3E-12 | -2.02 | 4E-15 |
| 227308_s_at | LTBP2/3 | 0.46 | 5E-05 | 0.03 | 9E-01 | -0.43 | 3E-04 | -0.57 | 4E-06 | -0.28 | 2E-02 | -0.05 | 4E-06 |
| 218065_s_at | TMEM98 | 0.44 | 1E-07 | -0.11 | 2E-01 | -0.33 | 6E-05 | -0.38 | 9E-06 | -0.20 | 1E-02 | -0.18 | 2E-02 |
| 222507_s_at | TMEM98 | 0.39 | 2E-04 | -0.19 | 1E-01 | -0.28 | 1E-02 | -0.34 | 2E-03 | -0.12 | 3E-01 | -0.07 | 6E-01 |

Log2 fold changes in gene expression in CL3EcoR cells upon senescence arrest are indicated as GA and upon shift up of control temperature insensitive HMF3S cells, from 34°C to 38°C, as HS respectively. Also indicated are the data obtained for these genes from CL3EcoR cells in which senescence had been bypassed by silencing of p53 (pRS_p53) or p21CIP1 (pRS_p21) or by inactivation of pRB using HPV16 E7 (E7) or by expression of the dominant negative E2F-DB protein.
(v2LHS_247318, 58957, 58958 and 58959; Thermo Scientific Open Biosystems) targeting TMEM9B were obtained, pooled and introduced into CL3EcoR cells after packaging as lentiviruses. The GIPZ lentiviral library contains the same hair pins as the retroviral library but is more stable and the constructs are packaged as lentiviruses rather than retroviruses. Lentiviral human GIPZ Lamin A/C shRNAmir (v2LHS_62719) was used as a negative control. Silencing of TMEM9B was clearly able to bypass senescence (Figure 4a). Moreover each of the constructs was individually able to overcome senescence arrest with v2LHS_58957 being the most efficient [16].

To determine if ATXN10, LAYN and LTBP2/3 were also able to directly bypass senescence, lentivirus constructs from the GIPZ lentiviral shRNAmir library were used. The complementation assay for LTBP2/3, in Figure 4b, showed that silencing LTBP2/3 with the one available silencing construct (v2LHS_34089) clearly yielded healthy growing colonies. Silencing of ATXN10 was tested using 4 different silencing constructs v2LHS_71735, 71736, 71737 and 71740. All four constructs were able to overcome senescence and yielded more growing colonies than the Lamin A/C negative control (Figure 4c). Silencing of LAYN was
tested using 2 different silencing constructs v2LHS_265009 and 118722; both constructs were able to bypass growth arrest and produced growing colonies (Figure 4d).

Taken together our results showed that silencing of TMEM9B, ATXN10, LAYN and LTBP2/3 was able to bypass senescence in the conditionally immortal human fibroblasts.

Discussion

To directly identify the downstream effectors of the p53-p21 and p16-pRB pathways crucial for mediating entry into senescence, we carried out a loss-of-function RNA interference screen in the conditionally immortal HMF3A human fibroblasts. This identified 112 known genes including p53 and another 29 shRNAmers targeting unidentified loci. Comparison of these known targets with genes up-regulated upon senescence in these cells identified 4 common genes TMEM9B, ATXN10, LAYN and LTBP2/3. Direct silencing of these common genes using lentiviral shRNAmers bypasses senescence in the HMF3A cells. Although none of these genes has previously been linked to cellular senescence, TMEM9B has been suggested to be an upstream activator of NF-κB signalling which we have found to have a causal role in promoting senescence.

The 112 known targets identified by the shRNA screen comprise a wide variety of genes but most importantly one of them was the only p53 shRNAmir (v2HS_936i15 from pool 82) present within this library, thereby internally validating the screen. Moreover all of the primary targets were identified from single shRNAmers even though we have subsequently shown that other shRNAmers corresponding to these targets present within the library are able to bypass senescence. It is not clear why other shRNAmers were not isolated in the screen; however this is exactly what has been observed previously by others such as Westbrook and colleagues [20]. Nevertheless it remains to be demonstrated which of the targets identified by the primary screen are able to bypass senescence when assayed individually.

TMEM9B was one of the 4 genes in common between the shRNA screen and genes known to be up-regulated upon senescence in HMF3A cells [16]. Moreover expression of TMEM9B was down-regulated when senescence was bypassed upon abrogation of the p53-p21 or p16-pRB pathways. TMEM9B is a glycosylated protein that localises to lysosome membranes and partially to early endosomes. It has been shown to be a component of TNF signalling and a module shared between the interleukin-1 and Toll-like receptor pathways. It is also essential for TNF activation of both NF-κB and MAPK pathways by acting downstream of RIP1 and upstream of the MAPK and IkB kinases at the level of the TAK1 complex [19]. TMEM9B was also identified in a large scale study to identify genes activating NF-κB and MAPK signalling pathways [18]. These results are all consistent with our finding that in the conditionally immortal HMF3A cells, senescence arrest which was reversed upon silencing of p53 and p21CIP1 or ectopic expression of the dominant negative E2F-DB protein. Spincocerebellar ataxia type 10 (SCA10) is a dominantly inherited disorder characterized by ataxia, seizures and anticipation caused by an intronic ATTCT pentanucleotide repeat expansion. The product of SCA10 encodes the novel protein, ATXN10, previously known as E46L, which is widely expressed in the brain and belongs to the family of armadillo repeat proteins. Although clinical features of the disease are well characterized, very little is known about ATXN10. ATXN10 knock down by RNAi has recently been shown to cause increased apoptosis in primary cerebellar cultures, implicating in SCA10 pathogenesis [27,28]. This is in contrast to our finding that silencing of ATXN10 in HMF3A cells by four different shRNAmers did not cause apoptosis but promoted growth and permitted a bypass of senescence.

Layilin (LAYN) identified from pool 58 was 2 fold up-regulated upon senescence arrest, which was reversed upon abrogation of the growth arrest by inactivation of the latent TGFβ-binding protein 2/3 (LTBP2/3) hairpin sequence was identified from pool 66. Up-regulation of LTBP2/3 expression upon growth arrest was reversed when senescence was overcome. LTBP2 are secreted proteins initially identified through their binding to TGFβ and may be involved in their assembly, secretion and targeting [21]. LTBP2/3 in particular has been found to play an essential role in the secretion and targeting of TGFβ1 [22]. Since silencing of LTBP2/3 can bypass senescence in HMF3A cells, it suggests that LTBP2/3 may be linked with the control of cell growth and be playing a role in suppressing tumour progression perhaps through regulation of TGFβ. This is in accordance with the identification of TGFβ as a senescence-inducing factor in the human lung A549 adenocarcinoma cells [23]. It is also in accordance with several other reports suggesting that TGFβ1 is capable of inducing cellular senescence. For instance, stimulation of human diploid fibroblasts with TGFβ1 triggers the appearance of senescence associated-β-galactosidase activity and increases steady state mRNA levels of senescence associated genes including APO J, fibronectin, and M22 [24-26]; both APO J (clusterin) and fibronectin are up-regulated in CL3 cells upon senescence arrest and this is reversed when senescence is bypassed [16].
either the p53-p21 or the p16-pRB pathways. Moreover two different LAYN shRNA-mirs were found to directly bypass senescence in HMF3A cells. Layilin is a widely expressed integral membrane hyaluronan receptor, originally identified as a binding partner of talin located in membrane ruffles. Talin is responsible, along with its adaptor proteins, for maintaining the cytoskeleton-membrane linkage by binding to integral membrane proteins and to the cytoskeleton. Recently layilin has been suggested to play a crucial role in lymphatic metastasis of lung carcinoma A549 cells [29].

In addition to the genes described above, a number of other interesting genes particularly TAOK1, RAS4A and ARMCX2 were identified. TAOK1 is a micro-tubule affinity-regulating kinase required for both chromosome congression and checkpoint-induced anaphase delay [30]. It is known to activate the p38MAPK pathway through the specific phosphorylation of MKK3. This is a complex pathway responsive to stress stimuli and involved in cell differentiation and apoptosis and has been shown to have an important causative role in senescence [31]. RAS4A encodes a member of the GAP1 family of GTPase-activating proteins that have been identified to suppress the Ras/mitogen-activated protein kinase pathway in response to an elevation of Ca^{2+} ions. Stimuli that increase intracellular Ca^{2+} levels result in the translocation of this protein to the plasma membrane, where it activates Ras GTPase activity resulting in Ras being converted from the active GTP-bound state to the inactive GDP-bound state and suppression of downstream signalling [32]. ARMCX2 encodes a potential N-terminal transmembrane domain and a single Armadillo repeat; Armadillo repeat containing proteins are involved in development, maintenance of tissue integrity and suppressing carcinomas [33].

Conclusions
The RNA interference screen has identified 112 known candidate proteins including p53 and another 29 shRNA-mirs targeting as yet unidentified loci. Although none of them except p53 had previously been linked to senescence or known to be downstream effectors of the p53-p21 and p16-pRB tumour suppressor pathways, directly silencing four of these candidates, TMEM9B, ATXN10, LAYN and LTBP2/3 bypassed senescence in CL3^{EcoR} cells. It remains to be determined whether direct silencing of any of the other primary candidates will also bypass senescence. Any genes that can bypass senescence upon silencing are novel starting points for identifying the signalling networks that act downstream of p53 and pRB to induce cellular senescence. The genes/proteins identified in the screen are also potential tumour suppressors, and a mechanistic dissection of their mode of action and role in cancer will undoubtedly provide new avenues for further research.

Methods

Cell Culture

CL3^{EcoR} cells were maintained at 34°C ± 0.5°C [15]. Temperature shift experiments were performed at 38°C ± 0.5°C. Phoenix ecotropic and HEK293T cells were obtained from the ATCC and maintained at 37°C. Cells were grown in Dulbecco’s modified Eagles medium (DMEM) supplemented with 10% (v/v) heat-inactivated foetal bovine serum, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. All media and components were obtained from Invitrogen.

Viral packaging and infection

Lentiviruses were prepared according to Besnier et al. [34]. Ecotropic viruses were prepared by transfecting 10 μg of retroviral plasmid DNA into phoenix ecotropic cells by FuGENE 6 Transfection reagent (Roche), according to the manufacturer’s instructions. 24 hrs post-transfection, media was changed and fresh medium added. 48 hrs post-transfection, retroviral supernatant was harvested, filtered through a 0.45 μm filter and either used immediately or frozen at -80°C. A second harvest was prepared by adding fresh media to the plates and harvesting the virus supernatant the next day.

Cells were infected with virus supernatants for 24 hrs at 34°C. Four days post-infection, antibiotic selection was added (2 μg/ml puromycin for pRS and pSM2 retroviruses or 6 μg/ml puromycin for pGIPZ shRNA-mir lentiviruses; Invitrogen). Selection of cells infected with human GIPZ lentiviral shRNA-mir constructs in puromycin at 6 μg/ml, enriches for cells with higher levels of shRNA-mir expression. For the senescence bypass assay, the stably transduced cells were plated at 5 × 10^4 cells in T-75 flasks or at 1.6 × 10^4 cells in T-25 flasks and incubated overnight at 34°C. Next day the medium was changed and the cells shifted to 38°C for 3 weeks. Flasks which contained more densely growing or bigger colonies were trypsinised, replated and used for extracting genomic DNA when confluent.

Additional material

Additional file 1: Volume of virus supernatants used for the screen
The table shows the volume of virus supernatants used for each of the pools.

Additional file 2: Primary screen This table shows the pool number, the number of cells obtained after puromycin selection, the number of T-75 and T-25 cm2 flasks reseeded and the number of growing colonies observed after 3 weeks at 38°C. Numbers indicated in red correspond to flasks that were reseeded for extracting genomic DNA.
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Authors’ contributions

ER and LM carried out the screen. ER and PSJ rescued the inserts. C JL and AA amplified and provided the shRNAmir library. PSJ wrote the manuscript. All authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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