Simultaneous expression of MMB-FOXM1 complex components enables efficient bypass of senescence

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Cellular senescence is a stable cell cycle arrest that normal cells undergo after a finite number of divisions, known as the Hayflick limit. Senescence is triggered in response to a variety of intrinsic and extrinsic stimuli including progressive shortening of telomeres and other changes in telomeric structure, as well as various forms of stress that lead to a persistent DNA damage response such as oncogene activation and oxidative stress. Senescence is largely established and maintained by engaging either one or both p53/p21 and pRB/p16INK4A tumour suppressor pathways. Both pathways involve many upstream regulators and downstream effectors along with various interlinked side branches.

Previously, we identified several genes including some components of the DREAM complex and its associated factors that are differentially expressed in cellular senescence. DREAM is a multi-subunit complex, formed by the assembly of p130 or p107 (RB family of pocket proteins) together with Dimerization partner 1–2 (DP), E2F4-5, and a Multivulval class B (MuvB) core. The MuvB core comprises LIN9, LIN37, LIN52, LIN54, and RBBP4. During G0/G1, the MuvB core binds to p130/p107 and E2F4/DP to form the DREAM complex, which inhibits cell cycle-dependent gene expression thereby arresting cell division. When cells exit G0/G1, phosphorylation of p130 leads to disruption of the DREAM complex and the MuvB core, allowing activator E2Fs to upregulate genes required for progression into S phase. Upon entry into the cell cycle, the MuvB core binds to B-MYB to form the MMB complex, thereby indicating a central role for assembly of the DREAM complex in senescence.

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activator complex and regulate late S phase genes. In G2 phase, MMB recruits FOXM1; this is followed by proteasomal degradation of B-MYB, whereas active FOXM1 remains bound to MuvB and regulates expression of genes required for the G2/M transition9,11,12.

Although a role for the DREAM complex in cellular senescence has not been widely studied, it has been shown to contribute to Ras-induced senescence13. Here we have examined the role of the DREAM complex and its associated components in senescence using human breast fibroblasts (CL3EcoR) conditionally immortalised using a thermolabile SV40 large T (LT) antigen (U19tsA58) along with the catalytic subunit of human telomerase (hTERT)14. These cells are immortal if grown at 33.5 ± 0.5 °C, but undergo a stable growth arrest within 7 days upon inactivation of the thermolabile LT antigen at the non-permissive temperature 39 ± 0.5 °C. At the restrictive temperature, these inducibly senescent cells acquire classical features of senescence including changes in morphology, appearance of senescence-associated β-galactosidase, and expression of genes shared by other senescent cells7,14,15. Senescence can be bypassed in these cells by inactivation of the p53/p21 WAF1/CIP1 and pRB/p16INK4A pathways7. Recently it was shown that overexpression of B-MYB or expression of a non-phosphorylated form of LIN52-S28 disrupts DREAM assembly, promotes MMB formation, and upregulates FOXM113,16. Here we show that simultaneous expression of MMB-FOXM1 complex components, allowed cells to bypass senescence efficiently and that the critical components are: non-phosphorylated LIN52, B-MYB and an active FOXM1.

Results

Reconstitution of the DREAM associated MMB-FOXM1 complex bypasses senescence. Previously, we used expression profiling to identify genes differentially expressed upon cellular senescence in the human CL3EcoR fibroblasts17. We found that senescence-induced growth arrest was associated with increased expression of p53 target genes, whereas expression of target genes of the DREAM, RB-E2F, and MMB-FOXM1 complexes were decreased (Fig. 1a), supporting an important role for the p53/p21 WAF1/CIP1, DREAM/RB-E2F and p16INK4A pathways in establishing the senescence phenotype. We identified 549 up-regulated and 685 down-regulated genes whose expression was reversed when senescence was bypassed by inactivation of the p53/p21 WAF1/CIP1 and pRB/p16INK4A pathways7. When these genes were overlapped with published lists of p53, DREAM, RB-E2F, and MMB-FOXM1 target genes7, we found that DREAM (49%), RB-E2F (16%), and MMB-FOXM1 (22%) targets indeed accounted for a large proportion (87%) of the downregulated genes (Fig. 1b and Supplementary Table 1). Direct p53 target genes, however, accounted for only a small fraction (12%) of the highly upregulated genes, indicating an involvement of other transcription factors or indirect pathways. These findings further suggested that re-expression of cell cycle genes regulated by the DREAM, RB-E2F, and MMB-FOXM1 complexes may be key for bypassing senescence.

LIN9 and LIN52, two components of the MuvB core, along with B-MYB and FOXM1, were significantly downregulated upon senescence whereas LIN37 was upregulated (Fig. 2a); these changes were reversed when senescence was bypassed by inactivation of the p53/p21 WAF1/CIP1 and pRB/p16INK4A pathways. LIN proteins associate to form the MuvB core which binds to B-MYB upon destabilization of DREAM to assemble the MMB complex and subsequently to FOXM1, to form the MMB-FOXM1 complex9,20. Interestingly p107 (RBL1) and Dual specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A), a kinase that phosphorylates LIN52 at serine 28, were also significantly downregulated (Fig. 2a). We have previously used a Human Signal Transduction Open Array7 to confirm the expression profiling data by simultaneously analysing 600 genes by real—time qPCR7. After filtering to eliminate growth arrest genes whose expression was very low (Ct > 22), nearly 80% of the
genes showed concordant changes in expression by real time qPCR or expression profiling although the actual Log2Fold Changes obtained by the two methods were different. To determine if changes in gene expression were also reflected by alterations in the protein level, cell lysates prepared from CL3EcoR cells actively dividing at 34 °C and after shift up to 39 °C for 4 days were examined by western blotting. To rule out changes in expression due to the temperature shift, lysates were simultaneously prepared and analysed from HMF3S cells. These cells were derived by immortalisation of the same batch of human breast fibroblasts with a wild type SV40 U19 LT antigen along with hTERT and do not undergo senescence arrest upon shift up 39 °C. Since senescence is associated with changes in cell size, cell lysates equivalent to equal numbers of cells were compared. The results in Fig. 2b show that expression of FOXM1 and LIN9 were clearly reduced upon senescence arrest in accordance with downregulation at the RNA level. Expression of B-MYB was also reduced, while a reduction was also seen in HMF3S cells upon shift up even though the reduction was much greater in the CL3EcoR cells upon senescence. In accordance with the RNA data, LIN37 was clearly upregulated upon senescence whereas LIN54 expression was slightly increased due to the temperature shift. LIN54 RNA was not significantly altered upon senescence. In contrast to the above, the reduction in RNA levels upon senescence arrest for LIN52 was also observed.

Figure 2. Senescence growth arrest in CL3EcoR cells. (a) Senescence specific changes in expression at the RNA level as determined by expression profiling are shown. GA indicates Log2 fold changes in RNA expression upon senescence growth arrest. Positive numbers for GA indicate up-regulation and are shaded in red, (log2 fold change less than 0.5 are shaded in light red). Negative numbers indicating down-regulation are shaded in green (log2 fold change less than – 0.5 are shaded in light green). Fold changes in expression of LIN9 and RBL1 are shown for 2 and 3 independent features whereas all the others are for a single feature only. Also shown is the effect on expression when senescence was bypassed by inactivation of the pRB/p16INK4A and p53/p21WAF1/CIP1 pathways. The pRB/p16INK4A pathway was inactivated using SV40 wild type LT (wt_LT), Adenovirus 5 E1A 12S (E1A), Human Papilloma Virus (HPV) 16 E7 (E7) or a dominant negative E2F-DB protein. The p53/p21WAF1/CIP1 pathway was inactivated using SV40 wild type LT, a p53GSE element that inactivates p53 (GSE-p53) or short-hair pin RNAs targetting p53 (pRS_p53) or p21WAF1/CIP1 (pRS_p21). The changes in expression were reversed upon senescence bypass. (b) Representative immunoblots assessing changes in protein levels of FOXM1, B-MYB, LIN9, LIN37, LIN52, LIN54, DYRK1A, RB1, pRBS780, p107, and p130 in CL3EcoR and HMF3S cells. Images of uncropped gels and blots are shown in the Supplementary Figures.
and DYRK1A did not translate into a change in protein levels, with western blots revealing similar if slightly higher levels of protein in lysate from cells displaying senescence compared to proliferating cells. In conclusion, many of the changes in RNA for components of the DREAM complex translate into changes at the protein level. 

In accordance with the activation of the pRB/p16INK4A pathway playing a central role in the induction of senescence in CL3EcoR cells and this was associated with loss of RB1 phosphorylation, indicated by overt loss of reactivity with anti RB1 serine 780 antibody (Fig. 2b). Although expression of the RB1-related p107 was clearly reduced at the RNA level upon senescence (Fig. 2a), the protein level was only slightly decreased and the protein appeared to migrate faster, consistent with loss of phosphorylation leading to its activation as a growth suppressor. The level of the RB1-related p130 increased upon senescence in the CL3EcoR cells, but this was also observed in the HMF35 cells indicating that the change in expression was due to the temperature shift. Together these results indicate that the functional engagement of RB1 family of proteins upon cell senescence is not seen in proliferating cells including temperature shifted HMF35 cells.

We have previously found that inactivation of the p53/p21WAF1/CIP1 and pRB/p16INK4A pathways in CL3EcoR cells bypasses senescence. Inactivation of the p53/p21WAF1/CIP1 pathway with p53GSE that inactivates p53, or shRNAs that target p53 or p21WAF1/CIP1 bypassed senescence very efficiently22,23, as indicated by densely growing colonies observed after plating <3000 stably transduced cells. In contrast, bypass of senescence was much less efficient upon inactivation of the pRB/p16INK4A pathway22,24,25 where growing cells were only obtained after plating 35,000–50,000 stably transduced cells. Since disruption of the DREAM complex by ectopic expression of a LIN52 mutant that cannot be phosphorylated at serine 28 (LIN52-S28A) can suppress Ras-induced senescence13, our aim was to examine whether disruption of the DREAM complex would contribute to the bypass of senescence in CL3EcoR cells in a long-term senescence bypass assay22,24,25, thereby supporting the idea that re-expression of cell cycle genes regulated by the DREAM, RB-E2F, and MMB-FOXM1 complexes may be key for bypassing senescence.

We first tested the potential of the individual components of the MMB-FOXM1 complex to bypass senescence using full-length, wild-type ORFs for B-MYB, LIN9, LIN37, and LIN54 or constitutively active forms of FOXM1 (ΔNΔKEN) and LIN52 (S28A). FOXM1ΔNΔKEN is constitutively active as it lacks the amino terminal auto-inhibitory domain and the KEN box, required for the proteolytic degradation of FOXM126. Substitution of serine 28 with alanine in LIN52-S28A leads to an inability to bind p130 and assemble the DREAM complex thereby interfering with Ras-induced senescence in hTERT immortalized human BJ fibroblasts8. Recently, it has been shown that overexpression of B-MYB disrupts the DREAM complex, dependent upon its ability to interact with MuvB16. It is not known if overexpression of constitutively active FOXM1 can disrupt the DREAM complex.

We found that lentiviral (pLEX-MCS) mediated stable transduction of CL3EcoR cells with high titres of either LIN52-S28A and B-MYB produced growing colonies upon plating 35,000 stably transduced cells (Fig. 3a), in marked contrast to a short-hairpin RNA targeting p21WAF1/CIP1 which resulted in confluent flasks and a large number of colonies that were too numerous to count as observed previously7. Expression of wild type LIN9, LIN37, LIN54 or the constitutively active FOXM1ΔNΔKEN yielded either no colonies or a few colonies similar to the empty vector control. The lack of bypass observed with the FOXM1ΔNΔKEN was rather surprising since we have previously observed that its expression from the retroviral vector, pLPCX, exhibited some bypass potential. However the senescence bypass assays presented here were carried out under more stringent conditions than previously to minimize background due to leakiness of the U19tsA58 LT antigen.

Phosphorylation of LIN52 at serine 28 by DYRK1A is crucial for regulating the switch between the repressive DREAM complex and the activating MMB/MMB-FOX1 complexes13. Structural analysis has shown that this phosphorylation supports the binding of LIN52 to p130 and p107 allowing assembly of the DREAM complex27. To confirm that loss of phosphorylation of Ser-28 in LIN52 was required for bypass of senescence in these CL3EcoR cells, synthetic constructs for LIN52 wild type, LIN52-S28A and LIN52-S28E were prepared, inserted into pLEX-MCS and assayed. The results (Fig. 3b) showed that in contrast to LIN52-S28A, wild type LIN52 was unable to bypass senescence and did not yield any growing colonies, confirming that phosphorylation of serine 28 enabling the assembly of the DREAM complex was required to promote senescence. To further confirm the role of assembly of the DREAM complex in promoting senescence, we replaced Serine 28 in LIN52 with glutamic acid since LIN52-S28E binds p130 very inefficiently28. No significant reduction in the number of growing colonies was observed compared to LIN52-S28A (Fig. 3b). Together these results confirmed the importance of the disruption of the repressive DREAM complex for bypassing senescence in these inducibly senescent human cells.

Since the number of growing colonies obtained was quite low in comparison to inactivation of the p53/p21WAF1/CIP1 pathway, we wanted to determine if transduction of CL3EcoR cells with a mixture of lentiviruses prepared from a pool of components of the MMB-FOX1 complex would bypass senescence more efficiently compared to single components alone. Lentiviruses were prepared from pools of DNA comprising the different gene constructs at one sixth the amount of DNA used above for the single constructs; used to infect CL3EcoR cells, synthetic constructs for LIN52 wild type, LIN52-S28A and LIN52-S28E were prepared, inserted into pLEX-MCS and assayed. The results (Fig. 3b) showed that in contrast to LIN52-S28A, wild type LIN52 was unable to bypass senescence and did not yield any growing colonies, confirming that phosphorylation of serine 28 enabling the assembly of the DREAM complex was required to promote senescence. To further confirm the role of assembly of the DREAM complex in promoting senescence, we replaced Serine 28 in LIN52 with glutamic acid since LIN52-S28E binds p130 very inefficiently28. No significant reduction in the number of growing colonies was observed compared to LIN52-S28A (Fig. 3b). Together these results confirmed the importance of the disruption of the repressive DREAM complex for bypassing senescence in these inducibly senescent human cells.

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than the canonical LIN9 (Fig. 4b). However, expression of the constitutively active FOXM1 was more efficient than wild type FOXM1 for overcoming senescence when combined with MMB components (Fig. 4c).

LIN52, FOXM1, and B-MYB contribute to bypassing senescence. To identify the components of the MMB-FOXM1 complex crucial for the highly efficient bypass, reconstruction experiments were undertaken where one component was left out in turn. The rationale was that if a critical component was omitted, its absence would reduce colony formation. Using this drop-out approach, we observed that absence of LIN52-S28A, FOXM1ΔNΔKEN, or B-MYB led to a significantly reduced bypass of senescence (Fig. 5a). The strongest effect was observed when LIN52-S28A was omitted followed by FOXM1ΔNΔKEN and B-MYB, thereby identifying a key role for these three factors in bypassing senescence. Drop-out of LIN9, LIN37, or LIN54 did not signifi-
Figure 4. Senescence bypass assays after reconstitution of the MMB-FOXM1 complex. (a) Reconstitution with all the components of MMB-FOXM1 complex, indicated as RC, exhibited an efficient senescence bypass. The bar charts depict an overall average (+/- SD) of three independent repeat experiments. Single representative flasks from the internal repeats are shown. Empty pLEX-MCS vector, CL3EcoR cells and RAD51 were used as negative controls. Statistical analysis was conducted using One-way ANOVA, Tukey's Multiple Comparison Test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). (b) No significant difference in the senescence bypass potential for full length (FL-LIN9) and the canonical LIN9 (that lacked sixteen amino acids present in FL-LIN9 N-terminus due to a different start codon) was observed when examined individually or as part of the MMB-FOXM1 complex. The bar charts depict an overall average (+/- SD) of two independent repeat experiments. Empty pLEX-MCS vector was used as a negative control. Statistical analysis was conducted using One-way ANOVA, Tukey's Multiple Comparison Test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). (c) Reconstitution of the MMB-FOXM1 complex with constitutively active FOXM1 (FOXM1ΔNΔKEN) exhibited a significantly greater number of colonies than the complex reconstituted with wild type FOXM1 whereas when studied individually, wild type and constitutively active FOXM1 exhibited no significant difference, as very few colonies were obtained. Empty pLEX-MCS vector was used as the negative control. Statistical analysis was conducted using One-way ANOVA, Tukey's Multiple Comparison Test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

Discussion

We have employed a stable senescence bypass assay using conditionally immortalised human breast fibroblasts (CL3EcoR) to examine the role of the DREAM complex and its associated components in senescence. DREAM is a multi-subunit complex that inhibits cell cycle-dependent gene expression thereby arresting cell division leading to quiescence. Upon exit from quiescence, the DREAM complex dissociates enabling the MuvB core to bind B-MYB and form the MMB complex for regulating late S phase genes. Later in the cell cycle MMB recruits FOXM1 to regulate expression of genes required for G2 and the G2/M transition. Here we report that simultaneous expression of MMB-FOXM1 complex components efficiently bypasses senescence and that LIN52, B-MYB and FOXM1 are the crucial components. Moreover, bypass of senescence requires that serine 28 of LIN52 is not phosphorylated, indicating a central role for the DREAM complex in inducing senescence.

FOXM1 and B-MYB are both significantly down-regulated upon senescence in CL3EcoR cells. FOXM1 is also down-regulated upon replicative senescence in primary human fibroblasts. Both B-MYB and FOXM1 play important roles in cell proliferation and cancer. FOXM1 is a Forkhead transcription factor that regulates G2-specific gene expression, promotes cell proliferation and contributes to tumour progression. B-MYB, a member of the Myb family, is ubiquitously expressed in proliferating cells and a key physiological regulator of cell survival and cell cycle progression. Silencing of B-MYB in primary human foreskin fibroblasts induces senescence, whereas overexpression rescues Ras-induced premature senescence in primary rodent cells and disrupts the DREAM complex in human cells. B-MYB, upon association with MuvB, binds and regulates transcription of genes containing cell cycle genes homology region (CHR) elements in their promoters. As the MMB complex does not contain any E2F proteins, it binds exclusively to the CHR element via the LIN52 motif of LIN52 binds directly with LxCxE in the pocket domain of p107 and p130 during assembly of the DREAM complex. Phosphorylation of LIN52 at Ser-28 by DYRK1A promotes DREAM assembly, whereas phosphorylation of p107/p130 by CDKs facilitates disassembly to promote cell cycle progression. LIN52 therefore plays a central role in the assembly of repressive DREAM complex. It is also required for MuvB to bind B-MYB to form the MuvB activator complex but this is independent of Ser-28 phosphorylation. LIN52-S28A disrupts assembly of the DREAM complex thereby interfering with Ras-induced senescence. None of the MuvB components, apart from non—Ser-28—phosphorylated LIN52 exhibited a key role towards bypass of senescence. Phosphorylation of Ser-28 in LIN52 is important since the LxxSxExL motif of LIN52 binds directly with LxCxE in the pocket domain of p107 and p130 during assembly of the DREAM complex. Phosphorylation of LIN52 at Ser-28 by DYRK1A promotes DREAM assembly, whereas phosphorylation of p107/p130 by CDKs facilitates disassembly to promote cell cycle progression. LIN52 therefore plays a central role in the assembly of repressive DREAM complex. It is also required for MuvB to bind B-MYB to form the MuvB activator complex but this is independent of Ser-28 phosphorylation. LIN52-S28A disrupts assembly of the DREAM complex thereby interfering with Ras-induced senescence. In accordance with our finding that LIN52-S28A promotes bypass of senescence probably by disrupting DREAM and promoting assembly of the MMB complex. It was recently shown that overexpression of B-MYB disrupts the DREAM complex by increasing LIN52 protein levels, dependent upon the MuvB binding domain of B-MYB and the Ser-28 phosphorylation site of LIN52. However it remains to be investigated if overexpression of constitutively active FOXM1 can also disrupt the DREAM complex in a similar manner. This study has shown that simultaneous expression of MMB-FOXM1 complex components, particularly dephosphorylated LIN52, B-MYB and active FOXM1, enables an efficient bypass of senescence, by promoting both DREAM disassembly and activation of mitotic gene expression thereby highlighting a central role for the DREAM complex and the importance of the p53/p21-DREAM/RB-E2F and p16/RB pathways in establishing the senescence phenotype.
**Materials and methods**

**Cell culture.** CL3EcoR and HMF3S cells and derivatives thereof were maintained at 33.5 ± 0.5 °C and temperature shift experiments were performed at 39 ± 0.5 °C. They were developed by our group and have been published previously. CL3EcoR cells are a single cell clone of HMF3A cells that express the full length murine ecotropic retroviral receptor and closely mirror the parental cells in their temperature dependent growth characteristics. HMF3A cells were developed by conditional immortalisation of freshly isolated mammary fibroblasts using a thermolabile SV40 LT antigen along with hTERT. HMF3S cells were derived by immortalisation of the same
batch of human breast fibroblasts as HMF3A cells with a wild type SV40 U19 LT antigen along with hTERT15. Breast fibroblasts were prepared from normal breast tissue obtained with written informed consent from patients undergoing cosmetic surgery14 in accordance with UCL Institutional guidelines and regulations. All experimental procedures were undertaken in microbiological containment level 2 and level 3 facilities with strict adherence to safety protocols and guidelines. The study was approved by ‘The Joint UCL/UCLH Committees on the Ethics of Human Research.’

HEK293 and HEK293T cells were obtained from the American Type Culture Collection and maintained as recommended.

**Preparation of lentiviral constructs.** Lentiviruses expression constructs were used to stably transduce CL3EcoR cells. The ORF for each gene of interest was inserted into pLX301 or pLEX-MCS lentiviral vectors by gateway recombinatorial cloning or by DNA manipulation. Vectors pLX301 (catalogue number 25895) and pLEX-MCS (catalogue number OHS4735) were from Addgene and Thermo Scientific Open Biosystems respectively. Hygromycin resistant expression constructs for LIN9, LIN37, LIN52-S28A and LIN54 were provided by LL and JAD. Since CL3EcoR cells were hygromycin resistant, each of the inserts was sub-cloned into the puromycin resistant pLEX-MCS vector. Full length MYBL2 clone (HsCD00045539) and LIN9 (FL-LIN9) ORF, corresponding to a predicted LIN9 ORF 16 amino acids longer at the amino terminus than the original LIN9 were obtained from DNASU, a plasmid repository (https://dnasu.org/DNASU/). Gateway recombination cloning was used to insert these ORFs into the pLX301 destination vector. Both the constitutively active and the wild type form of FOXM1 were provided by Prof. Rene Medema (Netherlands Cancer Institute, Amsterdam, Netherlands) and sub-cloned into pLEX-MCS lentiviral vector. WT-LIN52-S28 and LIN52-S28E mutants were designed and ordered from GeneArt Gene Synthesis and Services [ThermoFisher Scientific (https://www.thermofisher.com/)]. The ORFs were cloned into pLEX-MCS vector.

**Lentiviral packaging and infection.** Lentiviruses were prepared by transfecting 1.5 μg of recombinant lentiviral vector constructs in pLEX-MCS, pLX301 or pGIPZshRNAmir, 1 μg of Gag/Pol expression vector and 1 μg of VSV-G envelope expression vector (pMDG2) into HEK293T cells by FuGENE 6 Transfection reagent (Promega), according to the manufacturer’s instructions.

10^5 CL3EcoR cells seeded in T-25 flasks were doubly infected with lentiviral supernatants for 24 h at 33.5 ± 0.5 °C to maximise the transduction efficiency. Stably transduced cells were selected using puromycin (2 μg/ml). For the senescence bypass assay, 3.5–5 × 10^4 stably transduced cells plated in T-75 flasks were cultured at the non-permissive temperature (39 ± 0.5 °C) for 3 weeks. To measure the extent of senescence bypass, the numbers of dense growing colonies were identified by methylene blue staining.

**Gene set analysis.** Microarray data on differential gene expression following senescence-induced growth arrest and its bypass were published previously7. They are available from Gene Expression Omnibus database accession number GSE24810. The 343 direct p53 target genes were taken from Supplementary Table S3 published in18. The DREAM, MMB-FOXM1, and RB-E2F target gene sets were taken from Supplementary Tables S7, S8, and S9, respectively, published in19. Venn diagrams were generated using BioVenn17. 549 up and 685 downregulated genes, whose expression was reversed upon senescence bypass, correspond to the 816 up and 961 downregulated microarray probes previously published in7.

**Immunoblot analysis.** CL3EcoR and HMF3S cells were plated at 34 °C and cultured. The following day, the cultures were shifted to 39 °C, 96 h after the temperature shift, each 10 cm dish of cells was lysed in 400 μl ice-cold ELB-SDS buffer (250 mM NaCl, 50 mM HEPES, 5 mM EDTA, 5 mM DTT, 0.1% Triton X-100, 0.1% SDS) supplemented with complete protease inhibitor cocktail (Roche) and phosphatase inhibitors (10 mM NaF, 10 mM β-glycerophosphate and 1 mM Na3VO4). One parallel dish of cells for each condition was trypsinized and counted to determine the cell number. Lysates corresponding to equal numbers of cells (4.375 × 10^5 cells per condition) were fractionated on SDS-PAGE gels and transferred to a 0.2 µm nitrocellulose membrane. Membranes were incubated with primary antibodies overnight at 4 °C, washed with TBST and probed with HRP secondary antibodies. The imaging of protein bands was undertaken using ChemiDoc MP imaging system and X-ray films.

**Antibodies.**

| Target | Resource | Identifier |
|--------|----------|------------|
| FOXM1  | Cell Signaling Technology | Cat# 5436, RRID: AB_10692483 |
| B-MYB  | Millipore | Cat# MABE886 |
| LIN9   | Bethyl   | Cat# A300-BL2981 |
| LIN37  | Santa Cruz Biotechnology | Cat# sc-515686 |
| LIN52  | Bethyl   | Cat# A300-BL1372 |
| LIN54  | Bethyl   | Cat# A303-799A, RRID: AB_11218173 |
| RB1    | Cell Signaling Technology | Cat# 9309, RRID: AB_823629 |
| pRB79a | Abcam    | Cat# ab32513, RRID: AB_777635 |
| p107   | Santa Cruz Biotechnology | Cat# sc-317, RRID: AB_632093 |
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Author contributions
PJ. and R.K. conceived the experiments. R.K. conducted the experiments. H.H. and M.F. undertook the Bio-informatics work presented in Figs. 1 and 2a respectively. X.S. and S.M. carried out the western blot analysis presented in Fig. 2b. L.L. and J.D. provided some of the constructs used in the experiments. M.F., L.L. and J.D. helped with revision of manuscript. All authors reviewed the manuscript.

Competing interests
The authors declare no competing interests.

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