The long non-coding RNA MIR31HG regulates the senescence associated secretory phenotype

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Oncogene-induced senescence provides a barrier against malignant transformation. However, it can also promote cancer through the secretion of a plethora of factors released by senescent cells, called the senescence associated secretory phenotype (SASP). We have previously shown that in proliferating cells, nuclear IncRNA MIR31HG inhibits p16/CDKN2A expression through interaction with polycomb repressor complexes and that during BRAF-induced senescence, MIR31HG is overexpressed and translocates to the cytoplasm. Here, we show that MIR31HG regulates the expression and secretion of a subset of SASP components during BRAF-induced senescence. The SASP secreted from senescent cells depleted for MIR31HG fails to induce paracrine invasion without affecting the growth inhibitory effect. Mechanistically, MIR31HG interacts with YBX1 facilitating its phosphorylation at serine 102 (p-YBX1S102) by the kinase RSK. p-YBX1S102 induces IL1A translation which activates the transcription of the other SASP mRNAs. Our results suggest a dual role for MIR31HG in senescence depending on its localization and points to the IncRNA as a potential therapeutic target in the treatment of senescence-related pathologies.
Cellular senescence is an irreversible state of growth arrest that can be driven by several stimuli including telomere shortening due to extensive replication, DNA damage, oxidative stress or oncogene overexpression. In addition, it has been recently shown that senescence can play a role in differentiation and tissue regeneration. Oncogene-induced senescence (OIS) was firstly reported by Serrano et al. when they observed that expressing an oncogenic form of Ras in primary fibroblast-induced senescence. Later work demonstrated that other oncogenes such as mutated BRAF promoted OIS both in vivo and in vitro. OIS has been considered as a barrier to prevent tumour progression and additional mutations in tumour suppressor genes are required in order to bypass senescence to promote malignancy.

Senescence has been reported to upregulate the expression of several inflammatory cytokines and angiogenic factors. This process has been named senescence-associated secretory phenotype (SASP). The SASP can promote malignancy, angiogenesis, and also SASP cells transplanted with control siRNA or siRNA targeting MIR31HG. As expected, many genes were differentially expressed comparing control versus senescent cells (Fig. 1c and Supplementary Data 1). Moreover, the transcriptional profile of MIR31HG knock-down senescent cells resembled the senescent profile with several clusters of differentially expressed genes (Fig. 1c and Supplementary Data 1). Among the GO categories significantly represented on the genes downregulated in the MIR31HG knock-down conditions we found cytokine and chemokine-related pathways (Supplementary Fig. 1a). Furthermore, we observed that senescent MIR31HG-depleted cells failed to upregulate part of the main SASP components previously defined.

To investigate a broader effect of MIR31HG in another senescence model, we assessed doxorubicin-induced senescence in B1 cells. As expected, cells showed senescence markers after 48 h treatment with 500 nM doxorubicin as seen by a reduced cell growth (Supplementary Fig. 1c) and the presence of β-galactosidase-positive cells (Supplementary Fig. 1d). Doxorubicin induced the expression of MIR31HG and also SASP components such as IL6, IL8 or CXCL1 (Supplementary Fig. 1e). p21 mRNA was also induced consistent with decreased cell proliferation (Supplementary Fig. 1e). Interestingly, depletion of MIR31HG with two different siRNAs resulted in decreased expression of SASP components but not p21 (Supplementary Fig. 1e).

In order to analyse the composition of the SASP at the protein level, we knocked down MIR31HG in BJ ER-BRAF cells and induced senescence by 72 h 4-OHT treatment in serum-free medium followed by mass spectrometry analysis of secreted proteins. Untreated cells transfected with control siRNA were used as control. Control senescent cells, when compared to control proliferating cells, showed differential secretion of factors (Supplementary Fig. 1f and Supplementary Data 2). The secretome of senescent MIR31HG knock-down cells resembled that of control senescent cells with discrete differences (Fig. 1f and Supplementary Fig. 1f-h). The reduced secretion of these factors and other components of the SASP (IL8 and MMP3) were validated by enzyme-linked immunosorbet assay (ELISA) and western blotting using two different siRNAs for MIR31HG (Fig. 1g and Supplementary Fig. 1i). Altogether our results demonstrate that MIR31HG...
knock-down reduces the production of several SASP components during OIS already at the transcriptional level and these changes are reflected in the secretome.

Paracrine effect of the SASP of MIR31HG-depleted cells. To determine the paracrine effect of the SASP we induced senescence in cells transfected with control siRNA or siRNAs targeting MIR31HG. After 72 h 4-OHT treatment CM was harvested and used for subsequent analysis. The CM from proliferating cells without 4-OHT induction was also harvested at the same time point as control. To assess the potential of the SASP in inducing paracrine senescence, we incubated BJ and IMR90 WT cells with the different CM for 72 h. Protein analysis of whole-cell extracts by western blot revealed that BJ and IMR90 cells growing in CM...
**Fig. 1** MIR31HG knock-down decreases the induction of SASP components during BRAF-induced senescence. **a** Relative MIR31HG expression normalized to housekeeping genes (HPRT1 and RPLPO) in BJ ER:BRAF cells were treated with ethanol (Control) or 1 μM 4-OHT for 48 h (n = 4). **b** Box plot showing MIR31HG expression in log2 (1pkm + 1) units in thyroid carcinoma (THCA) and colorectal carcinoma (CRC) comparing BRAF mutant and BRAF wild-type tumours. The box plot represents the median (middle line), the box indicates the first and third quartiles and the whiskers indicate ±1.5 × interquartile range. Two-tailed Wilcoxon test was performed to compare the expression differences in the BRAF mutant and BRAF wild-type tumours (CRC BRAF_wt = 309; CRC BRAF_mutant = 49; THCA BRAF_mutant = 290; THCA BRAF_wt = 199). **c** Heat map showing relative expression of differentially expressed genes in BJ ER:BRAF cells (control or siMIR31HG), treated with ethanol (Control 1-3) or 1 μM 4-OHT for 48 h (Sen 1-3 and Sen siMIR31HG 1-3). **d** Heat map showing relative expression in RPMKs of a subset of SASP genes previously defined 13 from the data provide in a, where differentially expressed genes (‘DE’, FDR < 0.01) are indicated in red, unchanged (non-significant, ‘ns’) in white. **e** qRT-PCR analysis of selected components of the SASP normalized to housekeeping genes (HPRT1 and RPLPO) in BJ ER:BRAF cells transfected with the indicated siRNAs (Control or siMIR31HG1-2), treated with ethanol (Control) or 1 μM 4-OHT for 48 h. The graphs show results compared to control ethanol-treated set to 1 (n = 4). **f** Mass spectrometry-based secretome analysis of senescent BJ ER:BRAF (1 μM 4-OHT) treated with siMIR31HG or control siRNA. The volcano plot shows differentially secreted proteins in MIR31HG knock-down senescent cells (Sen siMIR31HG) compared to control senescence cells (Sen) (−log10 p value along y-axis and log fold change along x-axis). Names are displayed for significantly changed between proteomes (pval < 0.05) and SASP proteins are marked in red. **g** Secreted IL6, CXCL1, IL8 and MMP3 (pg/ml) measured by ELISA in the CM of BJ ER:BRAF (Control or siMIR31HG1-2) treated for 72 h with ethanol (Control) or 1 μM 4-OHT (n = 3-5). All statistical significances were calculated using two-tailed Student’s t-tests, except **b** where two-tailed Wilcoxon test was used. *p < 0.05; **p < 0.01; ***p < 0.001; ns non-significant. All error bars represent means ± s.d. Source data are provided as a Source Data file.

**Fig. 2** The SASP of senescent MIR31HG knock-down cells induces senescence but not invasion in a non-autonomous manner. **a** Western blot analysis for p53 and GAPDH. In WT BJ cells incubated for 72 h with the CM collected from BJ ER:BRAF cells (Control or siMIR31HG1-2) treated with ethanol (Control) or 1 μM 4-OHT for 72 h. Molecular weight marker is shown in kDa. **b** Quantification of the western blot band intensities for p53 relative to GAPDH (n = 3). **c** Representative images of β-galactosidase staining from the same condition as indicated in a (n = 3). Scale bar: 50 μm. **d** Quantification of the% of β-galactosidase-positive cells from c (n = 3). **e** Relative growth of the% of invading cells from control senescent cells and MIR31HG knock-down senescent cells measured by crystal violet staining dissolved in acetic acid and measured at 590 nm in WT BJ cells incubated for 72 h with the CM collected from BJ ER:BRAF cells (Control or siMIR31HG1-2) treated with ethanol (Control) or 1 μM 4-OHT for 72 h. The graph shows the absorbance relative to the control cells set as 1 (n = 3). **f** qRT-PCR analysis of a subset of components of the SASP normalized to housekeeping genes (HPRT1 and RPLPO) in total RNA extracted from cells described in a. The graph shows the RNA expression relative to control ethanol-treated cells set to 1 (n = 4). **g** MDA-MB-231 invading cells through a matrigel membrane in contact with the CM collected from BJ ER:BRAF cells (Control or siMIR31HG1-2) treated for 72 h with ethanol (Control) or 1 μM 4-OHT. Representative images are shown in the figure (n = 4). Scale bar: 50 μm. **h** Quantification of the invading cells relative to control ethanol-treated cells (n = 3). All statistical significances were calculated using two-tailed Student t-tests, *p < 0.05; **p < 0.01, ns, non-significant. All error bars represent means ± s.d. Source data are provided as a Source Data file.

From control senescent cells and MIR31HG knock-down senescent cells were able to mildly upregulate the senescence marker p53 (Fig. 2a, b and Supplementary Fig. 2a) as well as β-galactosidase (Fig. 2c, d) compared to proliferating cells. Consistently, a subset of p53 target genes 31 was upregulated in BJ and IMR90 cultured in CM from senescent cells in the presence and absence of MIR31HG (Supplementary Fig. 2b, c). Moreover, cells growing in CM from senescent cells showed decreased cell number after 72 h, independently on MIR31HG expression (Fig. 2e and Supplementary Fig. 2d). This was consistent with...
decreased expression of cell cycle-related genes (Supplementary Fig. 2e, f). However, the upregulation of inflammatory cytokines that occurs upon 4-OHT induction is reduced after MIR31HG depletion (Fig. 2f). Acosta et al.\textsuperscript{13} reported that the transforming growth factor-β (TGF-β) signalling pathway is responsible for the paracrine senescence effect. As expected, RNA levels of TGFβ target genes were upregulated in B) ER:BRAF upon 4-OHT treatment compared to control proliferating cells (Supplementary Fig. 2g), while MIR31HG knock-down did not affect this increase. Moreover, the protein level of pSMA2, downstream effector of TGFβ signalling, was upregulated during OIS in control as well as in MIR31HG knock-down senescent cells (Supplementary Fig. 2h). Similarly, WT B) cells discretely activated TGFβ signalling when incubated with CM from control senescent cells and also with CM from MIR31HG knock-down senescent cells (Supplementary Fig. 2i, j). These results demonstrate that the TGFβ signalling pathway remains active in MIR31HG knock-down senescent cells suggesting that its activation could be responsible for the paracrine senescence induction. To determine the role of the SASP in promoting cancer cell invasion, we performed transwell invasion assays using the different CM described above as chemoattractant in the lower chamber of the transwell for MDA-MB-231 breast cancer cells. After 48 h incubation a higher number of MDA-MB-231 cells exposed to CM from senescent cells invaded through the matrigel membrane compared to proliferating CM (Fig. 2g, h). In contrast, the CM of MIR31HG knock-down senescent cells did not promote invasion (Fig. 2g, h). These results demonstrate that the paracrine senescence effect is retained in the SASP of MIR31HG knock-down senescent cells but not the induction of paracrine invasion. These findings suggest that the effect of MIR31HG in the SASP production does not occur at a general level but that only a subset of components is inhibited.

MIR31HG depletion reduces CEBPB and NF-κB activation in OIS. As NF-κB and CEBPB regulate the expression of many SASP genes by stimulating their transcription\textsuperscript{11,32,33}, we investigated the possibility that MIR31HG knock-down affected the abundance or the activation of these transcription factors. As expected, the protein levels of CEBPB and activated phosphorylated RELA (p-RELA) (component of the NF-κB complex) increased during BRAF activation (Fig. 3a, b). Interestingly, MIR31HG knock-down in senescent cells reduced CEBPB protein expression and activated p-RELA (Fig. 3a, b) without affecting their mRNA levels (Supplementary Fig. 3a). In accordance, binding of CEBPB to the IL6 promoter, a canonical CEBPB target, decreased significantly upon MIR31HG knock-down compared to control senescent conditions (Fig. 3c). Consistently, NF-κB translocation to the nucleus was inhibited upon MIR31HG depletion (Fig. 3d and Supplementary Fig. 3b). Moreover, the signalling pathway upstream NF-κB activation was affected in MIR31HG knock-down senescent (Supplementary Fig. 3c). These results suggest that MIR31HG acts upstream of both CEBPB and NF-κB transcriptional activation.

MIR31HG depletion decreases IL1A translation in OIS. IL1A has been reported to function as an upstream regulator of the SASP\textsuperscript{34}. In line with this, addition of human recombinant IL1A (hr-IL1A) in our model system induced expression of SASP components at the RNA level (Supplementary Fig. 3d). Blocking IL1A signalling using siRNA against IL1A reduced the levels of CEBPB expression upon senescence as well as the RNA levels of different components of the SASP (Supplementary Fig. 3e, f). To validate the paracrine role of IL1A in the SASP induction, we knocked down IL1A in senescent cells and use this CM to assess the expression of SASP components by qRT-PCR in recipient B) and IMR90 cells. The CM from IL1A knock-down senescent cells was not able to induce paracrine expression of SASP components in both cells lines B) and IMR90 (Supplementary Fig. 3g). Interestingly, while IL1A mRNA was not affected by the levels of MIR31HG (Fig. 1e), IL1A protein levels were strongly reduced in MIR31HG knock-down senescent cells compared to control senescent cells as measured by western blot of the total lysate (Fig. 3e, f) and by immunofluorescence staining (Fig. 3g). ICAM1, which was not affected by MIR31HG depletion at the RNA level (Fig. 1e), did not show any protein decreased, suggesting some degree of specificity (Supplementary Fig. 3h, i).

Addition of human recombinant IL1A (hr-IL1A) rescued the incapacity of MIR31HG knock-down senescent cells to induce SASP RNA transcription (Fig. 3h), IL6 secretion (Fig. 3i), and NF-κB nuclear translocation (Supplementary Fig. 3j). To test whether MIR31HG would be regulating IL1A at a translational level, we performed polysome profiling in control senescent cells and senescence cells upon depletion of MIR31HG. The profiles obtained upon sucrose gradient separation in both conditions showed no major changes in polysome distribution, indicating no changes in global protein synthesis (Fig. 3j). Analysing the distribution of IL1A mRNA through the gradient we observed the majority of the transcript present in the heavy polysome fractions, indicative of high level of translation (Fig. 3k). Remarkably, a significant decrease of IL1A mRNA in these fractions was observed in MIR31HG knock-down senescent cells compared to control senescent cells (Fig. 3l and Supplementary Fig. 3k). Distribution of ACTB mRNA through the gradient did not show any difference (Fig. 3l and Supplementary Fig. 3k). Despite the decrease in mRNA levels of other cytoxines such as IL6, the similar distribution of their mRNAs through the gradient indicates an equal translation efficiency suggesting that the effect of MIR31HG on translation is specific for IL1A (Fig. 3l and Supplementary Fig. 3k). To validate that reduced IL1A protein levels was due to an effect of MIR31HG on translation and not other related processes such as protein stability, we performed AHA pulse labelling and coupling of newly synthesized proteins to biotin followed by streptavidin pull-down and immunoblotting. We confirmed that MIR31HG-depleted senescent cells contained less newly synthesized IL1A compared to control senescent cells, whereas the amount of GAPDH or Vinculin remained unaltered (Fig. 3m). MTOR signalling has been recently involved in SASP regulation through IL1A signalling\textsuperscript{35}. However, MIR31HG knock-down did not affect mTOR signalling activation (Supplementary Fig. 3l), indicating an alternative mechanism for MIR31HG regulation of IL1A. These findings suggest that MIR31HG is implicated in the modulation of the SASP by regulating IL1A translation independent of the mTOR signalling pathway.

MIR31HG interacts with YBX1. In order to elucidate the mechanism by which MIR31HG exerts its function in regulating the SASP, we purified endogenous MIR31HG with its associated proteins from UV-crosslinked senescent B) ER:BRAF cells using antisense oligonucleotides containing locked nucleic acid (ASOs) complementary to the MIR31HG sequence coupled to magnetic beads (Fig. 4a). Oligonucleotides against the RNA sequence of luciferase, not expressed in BJ ER:BRAF cells, were used as control. Mass spectrometry analysis of MIR31HG-associated proteins revealed a low number of significantly enriched proteins among which several RNA-binding proteins (Fig. 4b and Supplementary Data 2). MIR31HG has been previously shown to interact with IκBα during osteogenic differentiation regulating NF-κB activation\textsuperscript{26}. However, we did not detect IκBα in our pulldown experiments. The majority were heterogeneous nuclear
Ribonucleoproteins or other predominantly nuclear proteins involved in RNA processing. Recent reports have demonstrated the role of PTBP1 in regulating the SASP composition through the regulation of the alternative splicing of genes implicated in intra-cellular trafficking. However, since MIR31HG localizes mainly in the cytoplasm during OIS (Supplementary Fig 4a) we focused on YBX1 as a protein with de
defined cytoplasmic functions (Fig. 4b).

YBX1 knock-down phenocopies MIR31HG depletion. YBX1 has been implicated in several cytoplasmic processes, such as translation or mRNA stability among others. According to previous findings, YBX1 prevents senescence in epidermal progenitors and knock-down of YBX1 induces senescence features in keratinocytes. Depletion of YBX1 in untreated proliferating BJ ER:BRAF resulted in decreased proliferation and p21 increase,
Fig. 3 MIR31HG knock-down decreases IL1A translation. a Western blot for p-RELA, RELA, CEBPB and GAPDH of BJ ER:BRaf cells (Control or siMIR31HG-1-2) treated with ethanol (Control) or 1 μM 4-OHT for 72 h. Molecular weight marker is shown in kDa. b Quantification of the p-RELA and CEBPB western blot band intensities relative to GAPDH (n = 3). c Chromatin immunoprecipitation followed by qPCR for CEBPB binding to the IL6 promoter and to an unrelated region in BJ ER:BRaf cells (Control or siMIR31HG-1) treated with ethanol (Control) or 1 μM 4-OHT for 48 h. The graph shows the percentage of the input that binds CEBPB (n = 3). d Immunofluorescence analysis for RELA and DAPI of BJ ER:BRaf cells (Control or siMIR31HG-1-2) treated with ethanol (Control) or 1 μM 4-OHT for 72 h. Representative images are shown in the figure (n = 3). Scale bar: 20 μm. e Western blot for IL1A in BJ ER:BRaf cells (Control or siMIR31HG-1-2) were treated with ethanol (Control) or 1 μM 4-OHT for 72 h. Molecular weight marker is shown in kDa (n = 3). f Quantification of IL1A western blot band intensities relative to Vinculin (n = 3). g Immunofluorescence of IL1A and DAPI in BJ ER:BRaf cells (Control or siMIR31HG-1-2) treated with ethanol (Control) or 1 μM 4-OHT for 72 h. Representative merged images are shown in the figure (n = 3). Scale bar: 50 μm. h Relative RNA expression for the indicated SASP factors in BJ ER:BRaf cells transfected with the indicated siRNAs (Control or siMIR31HG-1, -2), treated with ethanol (Control) or 1 μM 4-OHT for 48 h, in the absence or presence of 10 ng/ml of h-rIL1A for 2 h before RNA extraction (n = 3). i Western blot for IL6 from precipitated proteins from the media in the conditions indicated in h in the absence or presence of 10 ng/ml of h-rIL1A for 24 h before harvesting the CM (n = 3). j e. short exposure, i. long exposure. j Polysome profile performed by sucrose gradient separation in senescent BJ ER:BRaf control cells (Sen Control, black) or siMIR31HG cells (Sen siMIR31HG, red) treated with 1 μM 4-OHT for 72 h. Y-axis shows the absorbance at 260 nM and X-axis shows the number of the fractions collected. Red area shows the heavy polysome fractions and grey area shows the light polysome fractions. One representative experiment is shown in the figure (n = 3). k Distribution of the amount of IL1A mRNA in the heavy polysome (red) light polysome (grey) or other fractions (white) from the experiment in j. l Relative amount of IL1A, IL6 and ACTB mRNA present in the heavy polysome fractions in the experiment described in j. m Western blot analysis of newly synthesized IL1A, Vinculin and GAPDH levels in control senescent cells and MIR31HG- depleted senescent cells purified by AHA pulse-labeling and coupling to biotin followed by streptavidin pull-down. As control, proliferating cells without AHA labelling is shown. HRP-streptavidin shows the uniform labelling of newly synthesized proteins coupled to biotin. A representative experiment is shown (n = 3). n Vinculin, GAPDH and Western blot intensities of IL1A from pulldown samples in senescence (Sen, white) and senescence MIR31HG KD cells (Sen-MIR31HG KD, grey) (n = 3). All statistical significances were calculated using two-tailed Student’s t-tests, *p < 0.05; **p < 0.01; ns non-significant. All error bars represent means ± s.d. Source data are provided as a Source Data file.

although p53 levels were strongly downregulated (Supplementary Fig. 4b, c). Interestingly, knock-down of YBX1 in BRAF-induced senescent cells mimicked MIR31HG knock-down phenotype. SASP components were decreased at RNA level in YBX1 knock-down senescent cells compared to control senescent cells (Fig. 4c, d and Supplementary Data 3). Likewise, reduction in the secretion of IL6 and CXCL1 upon YBX1 knock-down were comparable to the levels following MIR31HG knock-down (Fig. 1e and 4e). Furthermore, IL1A protein levels were strongly reduced (Fig. 4f, g) whereas the mRNA level remained unaltered (Fig. 4d).

Formaldehyde crosslinked RNA immunoprecipitation (CLIP) of GFP-tagged YBX1 validated its interaction with MIR31HG (Fig. 4h). As a positive control, we found YBX1 binding its own RNA as previously reported. Interestingly, YBX1 binds IL1A mRNA whereas other cytokine mRNAs are bound to a lesser extent (Fig. 4h). Furthermore, no binding to abundant nuclear RNAs (MALAT1) or the mitochondrial RNAs previously used as negative controls was identified. To identify the YBX1-binding sites in MIR31HG we performed in silico prediction based on previous iCLIP studies, and identified five putative binding sites (Fig. 4i). We generated MIR31HG mutants harbouring deletions including the predicted binding sites (Fig. 4i) to perform electrophoretic mobility shift assays (EMSA), incubating the corresponding in vitro-transcribed RNA with recombinant YBX1. ND4, a mitochondrial transcript previously used as a negative control (Fig. 4h), did not produce a shift indicating the absence of binding, whereas the full-length MIR31HG (WT) resulted in a band shift when incubated with increasing amounts of YBX1 (Fig. 4j). Mutant 1 and 5 (Mut1 and Mut5) were able to bind YBX1 to the same extent as the WT, whereas mutants 2, 3 and 4 (Mut2, Mut3 and Mut4) were not (Fig. 4j). To validate these results, we performed competition assays incubating 32P-labelled MIR31HG WT transcript with increasing amounts of unlabelled Mut5 or Mut3. We observed that only mut5 competed for the binding of YBX1 (Fig. 4k). These results indicate that the predicted binding site BS3 in MIR31HG is required for YBX1 binding.

To study the biological function of the MIR31HG and YBX1 interaction, we analysed the stability and localization of MIR31HG upon YBX1 knock-down, since stabilization of RNAs is a well-described function of cytoplasmic YBX1 (refs. 43,44). We did not observe changes in MIR31HG expression nor localization upon YBX1 knock-down (Supplementary Fig. 4d, e). Moreover, protein levels of YBX1 did not change upon depletion of MIR31HG (Supplementary Fig 4f).

YBX1 is phosphorylated at serine 102 by RSK in OIS. Several modifications which can affect YBX1 function have been reported. Phosphorylation of serine in position 102 has been shown to be involved in the regulation of translation. We therefore studied the phosphorylation status of YBX1 and the putative implication of MIR31HG in YBX1 regulation. In order to analyse p-YBX1 in BRAF-induced senescence, we used an antibody that recognizes p-YBX1 at position S102 (p-YBX1S102). Interestingly, we detected increased levels of this modification at different time points after BRAF induction (Fig. 5a and Supplementary Fig. 5a). Different kinases have been reported to be responsible for S102 YBX1 phosphorylation such as AKT and RSK. Moreover, BI-D780, a more specific RSK inhibitor, was able to completely abolish phosphorylation of YBX1 in senescent cells (Fig. 5b and Supplementary Fig. 5c). Furthermore, overexpression of RSK resulted in an increased level of p-YBX1S102 (Supplementary Fig. 5d). Our results conclude that YBX1 is phosphorylated by RSK kinase in a BRAF-dependent manner.

pYBX1S102 induces translation of IL1A. It has been previously shown that YBX1 can promote translation of specific transcripts. We next wondered whether p-YBX1S102 could impact IL1A translation. As expected, BRAF-induced senescence
resulted in an upregulation of IL1A protein levels compared to control untreated cells (Fig. 5c and Supplementary Fig. 5e). Interestingly, treatment with the RSK inhibitor BI-D1780 failed to upregulate IL1A during senescence (Fig. 5c and Supplementary Fig. 5e). YBX1 has been recently implicated in cytokine translation by binding the 3'UTR of their mRNAs\(^{38}\). Using reporter plasmids harbouring the 3'UTR of their mRNAs we demonstrated that a WT version of YBX1 is able to increase luciferase translation in response to 4-OHT induction (Fig. 5d). A mutant that is not able to be phosphorylated at S102 (S102A) showed a similar binding to their targets suggesting that phosphorylation at S102 is not affecting YBX1 binding capacity (Fig. 5e and Supplementary Fig. 5g). We next characterized the binding capacity of the different phosphorylated versions of YBX1 to IL1A mRNA and to MIR31HG by RIP. All the proteins showed a similar binding to their targets suggesting that phosphorylation at S102 is not affecting YBX1 binding capacity (Fig. 5e and Supplementary Fig. 5g). We next characterized the role of the p-YBX1\(^{S102}\) in invasion using transwell assays. The CM from ethanol treated (control) or 1 μM 4-OHT BJ ER:BRAF cells transfected with control siRNAs or siRNAs against RSK, YBX1 or MIR31HG were used as chemoattractant in the lower chamber of the transwell for MDA-MB-231 breast cancer cell invasion. After 24 h a higher number of MDA-MB-231 cells exposed to CM from senescent cells invaded through the matrigel
membrane compared to control CM (Supplementary Fig. 5h, i). In contrast, the CM of RSK and YBX1 knock-down senescent cells promoted limited invasion as well as the CM of MIR31HG-depleted cells (Supplementary Fig. 5h, i). These results show that RSK and YBX1 are required for invasion. In order to further analyse the role of YBX1 phosphorylation, we performed transwell assays using BJ ER:BRAF cells expressing the different YBX1 constructs described above. Overexpression of all forms of YBX1 increased invasion independently of its phosphorylation status (Fig. 5f, g). However, under senescence conditions, overexpression of WT YBX1 promoted invasion to a similar extent as that of the S102D phosphor-mimic mutant, whereas the invasion was reduced when overexpressing the S102A mutant (Fig. 5f, g). Although endogenous YBX1 was depleted using siRNAs targeting the 3’UTR, residual YBX1 might explain the fact that all the constructs show increased invasion in 4-OHT-treated conditions compared to control. Altogether these results suggest a role of p-YBX1 in promoting invasion.

MIR31HG knock-down inhibits YBX1–RSK interaction. We next examined whether MIR31HG might have an impact on YBX1 phosphorylation. We performed cellular fractionation to analyse the localization of phosphorylated YBX1 in senescent cells and in senescent cells where MIR31HG was depleted. Interestingly, we observed that upon MIR31HG knock-down p-YBX1 is reduced in the cytoplasm (Fig. 6a, b), suggesting a role for MIR31HG in the phosphorylation process of YBX1. Several cytoplasmic lncRNAs have been shown to act as scaffolds for bringing molecules into close proximity. In order to analyse whether MIR31HG was binding both YBX1 and its kinase RSK, we performed native RIP using GFP-tagged version of RSK. We could not detect RSK binding to MIR31HG (Supplementary Fig. 6a, b). To further analyse the role of MIR31HG in the interaction of YBX1 with its kinase RSK, we performed proximity ligation assays (PLAs) using antibodies against total YBX1 and total RSK. Importantly, the interaction was higher in senescence compared to control (Fig. 6c, d). Interestingly, MIR31HG knock-down reduced the level of interaction confirming our hypothesis that MIR31HG mediates YBX1 interaction with its kinase RSK during OIS (Fig. 6c, d). In order to corroborate this result, we have performed in vitro kinase assays using recombinant RSK and recombinant YBX1, and observed that YBX1 is phosphorylated in a RSK-dependent manner (Supplementary Fig. 6c). We could detect RSK auto-phosphorylation as previously shown (Fig. 6e and Supplementary Fig. 6c). Addition of in vitro-transcribed MIR31HG resulted in a moderate but significant increase in p-YBX1 phosphorylation compared to ND4 and Mut3, lacking YBX1-binding site (Fig. 6e). To demonstrate that the interaction between YBX1 and MIR31HG is responsible for YBX phosphorylation, we created BJ ER:BRAF cell lines expressing doxycycline-inducible MIR31HG constructs WT, Mut5 and Mut3 (Supplementary Fig. 6d). Interestingly, overexpression of WT and Mut5 transcripts showed increased levels of p-YBX1 compared to an empty cell line and Mut3 overexpression, which was unable to bind MIR31HG (Figs. 4k and 6f, g). These results confirm that the interaction between MIR31HG and YBX1 facilitates its phosphorylation.

Discussion

The SASP is a hallmark of senescent cells and responsible for mediating the patho-physiological effects in the surrounding tissues. For a long time, efforts in the field have been focused on trying to induce senescence in cancer cells in order to prevent cancer progression. In contrast hereto, more recent reports have shown that targeting senescent cells strongly improve age-related diseases. Although recent work has extended our knowledge of the signalling network upstream the transcriptional induction of the SASP components and the complexity and diversity of the SASP suggest that additional regulators might be involved. It is crucial to acquire further knowledge of the detailed mechanism to be able to design therapies for cancer, inflammation and aging treatment. Here, we contribute to understanding the SASP regulation by describing the molecular mechanism by which the lncRNA MIR31HG regulates a subset of the SASP components during Braf-induced senescence by modulating I1A translation. It is known that most of the SASP components are regulated at the transcriptional level, although a small fraction may additionally be regulated by post-transcriptional mechanisms. In this study, we focus on the senescence process initiated following the expression of a mutated Braf. This is of importance as somatic mutations in Braf occur in approximately 7% of human cancer. We observed that MIR31HG expression is higher in thyroid and colorectal cancer tumour samples harbouring Braf mutations, as compared to Braf wild type, suggesting the relevance of this lncRNA in cancer.

We find that the secretome from BJ ER:BRAF senescent cells and BJ ER:BRAF MIR31HG knock-down senescent cells show differences in the levels of key SASP components. Whereas most
of the genes deregulated at the transcription level are also altered at the protein level, a small proportion is regulated only at RNA or protein level. This, together with the comparable polysome profiles of these cell lines, suggests no changes in global translation upon MIR31HG depletion. Interestingly, IL1A, an upstream regulator of the SASP, is reduced at the protein level following MIR31HG knock-down whereas the mRNA level is not significantly altered. No IL1A was detected in the conditioned media by mass spectrometry or by ELISA, likely due to the fact that IL1A remains attached to the membrane during OIS as previously described. In our cellular system addition of hr-IL1A induces the transcription of several cytokines and instigates the SASP. Intriguingly, it also rescues the decreased transcription of SASP components caused by MIR31HG depletion during BRAF-
induced senescence. These results strongly support the idea of MIR31HG regulating IL1A as an upstream regulator of the SASP transcriptional program. Work from the Campisi lab has demonstrated that the translation of IL1A during RAS-induced senescence is regulated by mTOR35. Although the mTOR pathway is increased in our OIS model, it is not altered in MIR31HG knock-down conditions. Instead, we found the lncRNA MIR31HG to affect IL1A translation through YBX1.

**Fig. 6 MIR31HG promotes YBX1 phosphorylation by facilitating YBX1 interaction with its kinase RSK.**  
A Western blot analysis for p-YBX1, total YBX1, GAPDH and H3 in total and cytoplasmic fractions of BJ ER:BRAF cells (Control or siMIR31HG1-2) treated with ethanol (Control) or 1 μM 4-OHT for 72 h (n = 3). b Quantification of the pYBX1 band intensities relative to total YBX1 and Vinculin from three independent experiments. c PLA representative images showing the interaction (red dots) between YBX1 and RSK in BJ ER:BRAF cells (Control or siMIR31HG1) treated with ethanol (Control) or 1 μM 4-OHT for 72 h. DAPI staining (blue) shows the nucleus. d Quantification of the number of interactions (dots) per cell in the conditions indicated in a representative experiment from four independent replicates (n = 4). Scale bar: 20 μm. e Left, in vitro kinase experiment incubating recombinant YBX1 in the presence of 32p-ATP and recombinant RSK1 in the presence of the indicated in vitro-transcribed RNA, run in 4–12% NuPAGE Bis–Tris gel and exposed to an Amersham Hyperfilm ECL film. Right, quantification of the band intensities represented as p-YBX1 related to p-RSK (n = 4). f Western blot for p-YBX1, total YBX1 and Vinculin in BJ ER:BRAF cells expressing a doxycycline-induced empty, MIR31HG WT, Mut3 or Mut5 treated for 36 h with ethanol (Control) or 1 μM 4-OHT. g Quantification of the p-YBX1 band intensities relative to total YBX1 and Vinculin (n = 3). h Scheme of the working model: (1) During OIS MIR31HG is induced and locates in the cytoplasm. (2) At the same time, transcription of IL1A mRNA is induced. (3) IL1A mRNA is exported to the cytoplasm where it interacts with YBX1. MIR31HG facilitates YBX1 interaction with the kinase RSK promoting its phosphorylation which in turns promotes IL1A translation. (4) IL1A signalling induces the transcription of other SASP mRNAs through CEBPB and NF-κB. (5) These RNAs will be translated in the cytoplasm and the SASP components will be secreted (6). Statistical significance was calculated using two-tailed Student’s t-tests, *p < 0.05; **p < 0.01; ***p < 0.001. Error bars represent means ± s.d. Source data are provided as a Source Data file.
partner. Importantly, YBX1 depletion mimics the MIR31HG knock-down phenotype in BRAF-induced senescence. This includes the reduced translation of IL1A and therefore the downregulation of downstream SASP components at RNA level, such as CXCL1, IL8 or IL6 among others. Recent work has shown that YBX1 prevents cytokine translation in cycling keratinocytes by binding their 3′UTR. In these studies, YBX1 knock-down results in increased translation of several cytokines and therefore induction of senescence38. Knock-down of YBX1 in cells subjected to BRAF-induced senescence resulted in reduced RNA levels of most of the cytokines previously annotated as SASP components13. Interestingly, in BRAF-induced senescence we observe that YBX1 binds a high fraction of the IL1A mRNA while binding other cytokines to a lesser extent. YBX1 binds AU-rich motifs present in the 3′UTR of cytokine mRNAs38,62, which is consistent with our IL1A 3′ UTR luciferase reporter assays. However, the stronger binding of YBX1 to IL1A mRNA compared to other cytokines show some degree of specificity towards IL1A mRNA. The mechanism underlying this specificity remains unknown. In a recent study, Dominguez et al.53 determined the importance of contextual features in RNA recognition by RBPs. Perhaps RNA secondary structures, the flanking nucleotide composition or the proximity to other RNA-binding proteins might be influencing the binding of YBX1 to the IL1A mRNA.

We observe an increase in p-YBX1S102 in BRAF-induced senescence and, importantly, p-YBX1S102 is reduced in the cytoplasm upon MIR31HG knock-down. Our results indicate that this post-transcriptional modification of YBX1 is involved in promoting IL1A translation during BRAF-induced senescence through its 3′UTR, which could explain the previously reported opposite roles of YBX1 in cytokine translation38. The role of p-YBX1 in translation remains to be fully understood. It is known that YBX1 binds the mRNA cap structure by displacing the eukaryotic translation factor 4E (eIF4E) and eIF4G and hence promotes translational repression44,64. p-YBX1S102, on the other hand, binds the mRNA cap structure less tightly allowing translation of repressed mRNAs46. However, not much is known about its role in promoting translation. Our luciferase reporter assay indicates that during senescence, p-YBX1S102 may have a role in promoting IL1A translation through the 3′UTR since the phosphomutant YBX1S102A fails to induce luciferase induction. Importantly, invasion is reduced when overexpressing YBX1S102A as compared to p-YBX1S102 and YBX1S102D. Although we have observed increased invasion upon YBX1 overexpression independently on its phosphorylation status, confirming previous findings of YBX1 involved in metastasis and invasion49,65,66, this result suggests that the YBX1 reduced phosphorylation is, at least, partly responsible for the SASP-induced invasion observed in MIR31HG-depleted senescent cells. Mutation of S102 did not alter YBX1 binding capability to IL1A or other mRNAs, consistently with previous reports demonstrating that phosphorylation at S102 does not affect the general RNA binding capacity of YBX1 (ref. 44). Phosphorylation of translation factors are determining events occurring during translation regulation67 and perhaps p-YBX1 is involved in differentially recruiting other translation factors with a role in IL1A mRNA translational regulation. Further phospho-proteomic studies would help to address this question. We cannot exclude that phosphorylation at other residues occur during senescence. In fact, S165 and S176 have been shown to activate NF-κB signalling68,69. It would be interesting to investigate whether these modifications have any impact in senescence or in translation regulation.

LncRNAs can modulate post-transcriptional modifications of proteins70–73. Our results suggest that during BRAF-induced senescence RSK appears to be the main kinase responsible for YBX1 phosphorylation. PLA experiments show an increase in RSK–YBX1 interaction during OIS correlating with an increased level of p-YBX1. Despite at a lower level, the presence of interaction in proliferating conditions suggests that the interaction may occur without BRAF induction. The signalling cascade activated during OIS might be necessary to phosphorylate YBX1 at least at S102. We cannot exclude that RSK phosphorylates other residues in proliferating cells. Nevertheless, our results indicate that the interaction between MIR31HG and YBX1 is important for the phosphorylation of YBX1 by RSK and its role in senescence, when the lncRNA is expressed and predominantly located in the cytoplasm74.

YBX1 is a very abundant protein whereas MIR31HG is expressed at low levels. The stoichiometry does not correspond for YBX1. Moreover, we do not detect MIR31HG directly binding RSK, likely excluding a role for MIR31HG as scaffold bridging together YBX1 and its kinase. Our results suggest that MIR31HG–YBX1 is a dynamic interaction that might infer an optimal conformation of YBX1 to facilitate its phosphorylation. Although the in vitro kinase experiments support this model, structural studies or more exhaustive phospho-proteomic analysis would be required to confirm this hypothesis.

The SASP has been reported to either limit or promote tumour progression. Dissociation of the good and bad sides of the SASPs is difficult since many of its components can have both roles depending on the cellular context14. Besides, many of the senescence effectors are activated by the same pathways that activate the SASP. Therefore, the identification of factors that can affect the SASP without altering the tumour-suppressive effects associated with senescence is a promising strategy for senescence-related therapies. Several reports have described factors that can uncouple the senescence growth arrest from the SASP35,36,57,59. Here, we identify an lncRNA that, when located in the cytoplasm during OIS, is responsible for the production of a distinct subset of SASP components. Its depletion during OIS leads to a decrease of interleukins, chemokines and other factors preventing invasion in vitro without reverting growth arrest. Interestingly, we have previously described a mechanism for the nuclear MIR31HG in proliferating cells in repressing p16INK4A expression by recruiting polycomb group proteins58. Altogether, our results suggest that the lncRNA MIR31HG has a dual role in inducing and buffering senescence depending on the cellular localization, which highlights the complexity of the regulation of senescence and the SASP. Interestingly, MIR31HG has been reported to be upregulated in different types of cancer74–76. The fact that depletion of MIR31HG induces p16 expression without activation of a SASP response38 and decreases pro-invasive factors during SASP induction makes it an interesting target for therapeutic purposes. Inhibition of MIR31HG as well as other anti-SASP therapies could be potentially used to ameliorate the detrimental effects of senescence cells.

Methods

Cell cultures, treatments, siRNA transfections. The human diploid cell lines TIG3 and BJ were immortalized using pbabe-hTERT and pMSCV-ER:B-RAF retroviral constructs. Hek-293T were transfected using calcium phosphate transfection with 10 μg of each plasmid. Forty-eight hours later, virus were harvested and polybrene. Twenty-four hours post-transduction the cells were selected with 50 μg/ml neomycin (h-TERT) and 5 μg/ml blastidicine (B-RAF).

Bi) ER:BRAF, TIG3:BRAF, BJ wild type (WT), IMR90 and MDA-MB-231 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% foetal bovine serum (FBS) (Hyclone) and penicillin/streptomycin (Invitrogen).

Bl and TIG3 were authenticated using IDEXX services (IMPACT II PCR Profile; CellCheck 9—human (9 Marker STR Profile and Inter-species Contamination Test). MDA-MB-231 were obtained from Janine Erler (BRIC,
University of Copenhagen) and IMR90 from Maite Huarte (CIMA, Pamplona, Spain). Senescence was induced by treatment with 1 µM 4-hydroxymethoxifen (4-OHT; Sigma) 24 h after transfection for 48 h or RNA analysis or 72 h for protein analysis. Rsk inhibitors, Bi-D1780 (Axon 1528) and FMK (Gift from Morten Frodin), were used at 10 µM for the time indicated in each experiment.

**Dosycycline-inducible cell lines.** YBX1-GBP and RSK-GFP overexpressing cell lines: To generate the lentiviral constructs, we cloned the coding sequence of YBX1 amplified from cdNA, into the inducible vector pLVX-TetOne Puro Vector (Clontech) that already contained GFP-fusion protein. We use In-Fusion HD Cloning designing primers according to the manufacturer’s indications (see primer sequences in Supplementary Table 1).

For the lentiviral production, HEK293T cells were transfected using lipofectamine 2000 (LifeTechnologies) with 7 µg of pLVX-YBX1-GBP or pLVX- empty-GFP, together with 6 µg of VacVG and 5 µg Pax8 viral plasmids. After 48 h supernatants containing the viral particles were filtered through a 0.45 µm filter. Confluent BJ ERF:RAB cells were then transduced with 1/3 of the viral supernatant that induced the YBX1 expression, cells were maintained in tetracycline-free media (serum). For the expression of YBX1, 100 ng/ml doxycycline was used for 72 h unless otherwise indicated. To transduce the phosphomutant (S102A) and phosphomimic (S102D) versions of the protein, directed mutagenesis was performed using QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene) directly from the pLVX-YBX1-GFP construct. Primers with the respective mutations were designed according to the manufacturer’s indications (see sequences in Supplementary Table 1). The pLVX-RSK-GFP cell line was generated as described for YBX1, amplifying the coding sequence from cdNA with specific primers (sequences in Supplementary Table 1).

**MIR31HG expression analysis in tumour samples.** Gene expression (RNAseq) and somatic mutation data for tumours in The Cancer Genome Atlas (TCGA Pan-Cancer) were downloaded from UCSC Xena platform77. Thyroid carcinoma (TTFCA) and colorectal adenocarcinoma (COAD & READ) were used for analysis because these tumours have high frequency of BRAF mutations (>10%) and also expressed MIR31HG (CRC BRAF_wt n = 309; CRC BRAF_mutant n = 49; THCA BRAF_wt = 290; THCA BRAF_mutant = 199). Expression values for all the samples were converted to log2(250 + 1) unit. Wilcoxon test was performed to compare the expression differences in the BRAF mutant and BRAF wild-type tumours, using R.

**RNA extraction and qRT-PCR analysis.** Total RNA was isolated using Trizol reagent (Invitrogen), treated with TURBO DNase (Ambion, Lifetecnologies) and reverse transcribed using TaqMan Reverse Transcription kit (Applied Biosystems) with random hexamer primers. Quantitative real-time PCR was performed using Sybr Green PCR Master Mix 2x and Deep Blue One Plus real-time PCR and software v2.3 (Applied Biosystems). The housekeeping genes HPRT1 and RPLPO were used for normalization of qRT-PCR data, unless otherwise stated.

**Cell fractionation.** Cells were grown in 15 cm dishes (NUNC). Nuclear/cytoplasmic fractionation was performed using Nuclei EZ Lysis Buffer (Sigma) followed by the addition of 10% acetic acid. The amount of crystal violet staining was quantified by measurement of the absorbance at 570 nm.

**Senescence-associated β-galactosidase staining.** Cells were seeded and transferred in 6-well or 12-well plates (NUNC). 24, 48 and 72 h after transfection or treatment cells were washed twice in PBS and fixed with 10% formalin for 10 min and stained with 0.1% X-gal solution for 30 min. Excess crystal violet stain was removed by several washes with water. The plates were allowed to dry and crystal violet was extracted by the addition of 10% acetic acid. The amount of crystal violet staining was quantified by imputation of missing data performed using MimProb setting based on minimal intensity values observed for each sample.

**Western blot analysis.** Cells were seeded and reverse transfected in 6-well or 12-well plates (NUNC). At 72 h post-transfection they were fixed and stained using the β-galactosidase staining kit (Cell Signaling) according to the manufacturer’s protocol.

**Invasion assay.** In all, 50 µl of 0.5 µg/ml Matrigel LDEV-Freet(Corning) was added to the transwell (Croning). Four hundred microliters of the desired CM was placed at the bottom of the transwell with 8 µm pore size. In total, 50,000 MDA-MB-231 cells were resuspended in 200 µl DMEM without PBS and placed on top of the transwell, and 200 µl Matrigel then the invaded cells were washed in PBS and fixed in cold 70% ethanol for 15 min and stained using crystal violet staining as indicated above.

**Cell fractionation.** Cells were grown in 15 cm dishes (NUNC). Nuclear/cytoplasmic fractionation was performed using Nuclei EZ Lysis Buffer (Sigma) following the manufacturer’s protocol.

**Immunofluorescence.** Cells were seeded on multichamber slides (Nunc) and transfected or treated as described. Seventy-two hour cells were washed with PBS 1x and fixed in 4% paraformaldehyde (PFA) (Sigma) for 15 min. The cells were permeabilized using 0.1% Triton X-100 in PBS (Sigma) following antibody incubation. Images were taken using a Zeiss fluorescence microscope and ZenPro software 2011. Antibodies and dilutions are listed in Supplementary Table 2.
A agaroase (Thermo Fisher Scientific) for 2 h. One per cent of the sample was used as the input control, and the remaining precleared chromatin was incubated overnight at 4 °C with 5 μg/ml of antibody (see Supplementary Table S3) by incubation with salmon sperm DNA/protein A agaroase (50% slurry) and centrifugation. The bead pellets were washed in low- or high-salt conditions (0.1% SDS, 1% Triton X-100, 2 mM EDTA pH 8.0, 20 mM Tris-HCl pH 8.0, and 150 mM low/500 mM high NaCl) followed by two washes with Tris-EDTA buffer. Elution buffer (0.1% SDS, 0.1% NaHCO3) was added to the bottom of antibody (see Supplementary Table S3) by incubation at 68 °C overnight. Samples were incubated 1 h at 37 °C with RNase A (Sigma) and 45 min at 50 °C with proteinase K (Ambion). The DNA was purified using Minulete PCR Purification Kit (Qiagen) and then amplified by qPCR using primers listed in Supplementary Table I.

RNA sequencing and bioinformatic analysis. RNA integrity was confirmed on an Agilent 2100 Bioanalyzer using Agilent RNA 6000 Nano kit (Agilent Technolo-
gies). Concentrations of the libraries were measured using the Qubit fluorometer (Invitrogen) and fragment size was assessed on an Agilent 2100 Bioana-
lyzer using Agilent High Sensitivity DNA kit (Agilent Technologies). The libraries were trimmed using Cutadapt80 to remove adapters and minimum read length after trimming was set to 18. Trimmed reads were mapped to hg38 using STAR aligner81 (version 2.5.1a). Uniquely mapped reads were counted towards genes using featureCounts82 (version 2.5.1a) and prior to the test correction. Only genes differentially expressed between Senescence and Senescence MIR31HG KD and [log(g-fold-change)] > 0.75 were used for GO-
term analysis.

MIR31HG oligonucleotide-based pull down. Cells were grown in 150 and 500 cm2 dishes in 25 or 90 ml media, respectively, up to ~90% confluency. Prior to collection cell were irradiated with UV light at 254 nm, then scraped in ice-cold PBS, spun down at 800 × g for 5 min and at 4 °C and stored at —80 °C. Amino-C12-
LNA-containing oligonucleotides against MIR31HG and luciferase (five per target, Exonuclease assay was performed using a 50 μg/ml RNA with CATS-Seq-seq
Kit (with poly-a selection) v2 x24 (Diagenode) according to the manufacturer's protocol. Concentrations of the libraries were measured using the Qubit fluor-
ometer (Invitrogen) and fragment size was assessed on an Agilent 2100 Bioana-
lyzer using Agilent High Sensitivity DNA kit (Agilent Technologies). The libraries were trimmed using Cutadapt80 to remove adapters and minimum read length after trimming was set to 18. Trimmed reads were mapped to hg38 using STAR aligner81 (version 2.5.1a). Uniquely mapped reads were counted towards genes using featureCounts82 (version 2.5.1a) and prior to the test correction. Only genes differentially expressed between Senescence and Senescence MIR31HG KD and [log(g-fold-change)] > 0.75 were used for GO-
term analysis.

MIR31HG constructs for in vitro transcription. pGEM-MIR31HG plasmid was established by PCR amplification of MIR31HG sequence (NR_027054.2) from cDNA using primers listed in Supplementary Table I and cloned using TA cloning strategy with pGEM-T easy vector system kit (Promega), according to the manu-
ufacturer’s protocols. MIR31HG truncations were established by PCR amplification from pGEM-MIR31HG plasmid with primers indicated in Supplementary Table I and cloned in In-fusion HD cloning kit (Clontech).

In vitro transcription and labelling. pGEM-MIR31HG WT and truncation con-
structs were linearized with NotI restriction enzyme. For ND4 negative control the sequence was from cDNA using T7 promoter included in the forward primer sequence (Supplementary Table I). The in vitro transcription reaction was performed by using 1 μg of the linearized plasmid or PCR product together with 1× T7 RNA polymerase buffer, 1 mM of NTPs), 1 U/μl of RNase inhibitors murine, and 2 U/μl of T7 RNA polymerase in a total volume of 20 μl for 20 at 37 °C. After 30 min DNAse treatment with TURBO DNA free at 37 °C the reactions were stopped by adding 0.5 mM EDTA. Samples were mixed with formamide loading dye and boiled 1 min at 95 °C before running on 1% agarose gel in 1× TBE. The RNA products were purified using NucleoSpin Gel and PC Clean-up kit (Macherey-Nagel) with T7C buffer for RNA extraction. RNAs were dephosphorylated with 1 U of calf Intestinal Alkaline Phosphatase (Invitrogen) and T4 Polynucleotide Kinase (NEB) and 32P-32P labelling was carried out following the protocol. Labelled RNAs were purified using NucleoSpin Gel and PC Clean-up kit. Radiolabelled RNAs were checked on agarose gel in 1× TBE buffer and quantified.

Electrophoretic mobility shift assay. Final concentration 2 nM of radiolabelled RNA was adjusted to 5 ml with Milli-Q water, incubated 1 min at 95 °C and cooled on ice for 2 min. RNA was allowed to fold for 30 min at 37 °C in binding buffer (in mM Tris-HCl pH 7.5, 50 mM KCl, 5 mM MgCl2, 0.1 mM CaCl2, 1 mM DTT, 0.1 μg/ml BSA, 0.4 mg/ml fragmented yeast RNA (Sigma), 5% glycerol, 0.025% SDS, 0.1% NCS) following the binding reaction was carried out at 30 °C for 30 min. Samples were loaded on a non-denaturing 0.7% agarose gel in cold 1× TBE buffer. After 2 h at 120 V of gel electrophoresis, the gels were vacuum dried for 90 min at 80 °C and exposed to an Amersham Hyperfilm ECL film.
measured using the Dual-Glo Luciferase Assay System (Promega) and the Glo-Max multi-detection system with Instinct software v3.1.1 (Promega).

For the transfection of NF-κB to the nucleus, 500 ng of a luciferase reporter plasmid containing four NF-kB-binding sites (Addgene Plasmid #111216) was transfected using Lipofectamine 3000 (Life Technologies) in B/ER/BRAF cells 24 h after siRNA transfection (control or siMIR31HG1). One hundred and fifty nanograms of pRL-TK Renilla luciferase reporter vector was co-transfected with control and luciferase assay normalization.

Twenty-four hours after transfection fresh media containing 1 µM 4-OHT was added to the cells. Forty-eight hours after treatment Firefly and Renilla luciferase units were measured using the Dual-Glo Luciferase Assay System and the Glo-Max multi-detection system with Instinct software v3.1.1 (Promega).

Proximity ligation assay. Cells were reverse transfected and seeded in eight-well multichamber slides (Nunc Lab-Tek II Chamber Slide®). Twenty-four hours later media was changed and 1 µM 4-OHT was added to the corresponding wells. After 72 h cells were fixed in 4% formaldehyde for 15 min and processed for PLA using Duolink™ in Situ PLA® Probe Anti-Rabbit PLUS (Sigma) according to the manufacturer’s instructions. The antibodies are listed in Supplementary Table 2. Images were obtained using a fluorescence microscope (Zeiss) and ZenPro software 2011 and quantified using Cell Profiler software v3.1.5. Five to 10 images were quantified from four independent experiments.

In vitro kinase assay. One microgram of recombinant YBX1 (Abcam) was incubated with 20 ng of recombinant RSK1 protein (R&D) and the corresponding in vitro-transcribed RNA in kinase buffer (25 mM TRIS/HCl pH 7.5, 5 mM glycerophosphate, 2 mM DTT, 10 mM MgCl2, 50 µM ATP, 10 µCi µc-32P (Perkin Elmer), protease inhibitor mixture Complete (Roche), phosphoSTOP (Roche)) for 15 min at 30 °C. Laemmli buffer was added to stop the reaction and boiled at 95 °C for 5 min. Proteins were separated by electrophoresis in 4–12% NuPAGE Bis–Tris gels (Invitrogen). Subsequently, the gel was dried at 80 °C for 1 h and then exposed to an Amersham Hyperfilm ECL film. The band intensities were measured using ImageJ/Fiji software v2.0.0.

Formaldehyde crosslinked RNA immunoprecipitation. Cells were seeded in 15 cm plates and treated with 1 µM 4-OHT the day after for 48 h. The cells were washed twice with PBS and crosslinked with 1% formaldehyde in PBS shaking for 15 min. Crosslinking was stopped incubating with glycine 0.25 M for 5 min. Eighty to 100 mg of cell pellet was resuspended in lysis buffer (50 mM TRIS/HCl pH 7.4, 100 mM NaCl, 0.5% Triton X-100, 5 mM EDTA, 0.25% Na-deoxycholate, Protease Inhibitor cocktail, RNase Inhibitor (NEB)) and sonicated three cycles of 10 s using a BRANSON sonicator at setting 15%. After 15 min centrifugation at 16,000 × g the supernatant was collected in a new tube. Ten per cent of the extract was saved as RNA input. The rest of the cell extract was incubated with 10 µl of GFP-Trap™ Magnetic Agarose beads (Chromotek) rotating at 4 °C for 2 h. The beads were then collected using a magnetic rack and washed once with lysis buffer and five times with high-salt buffer (50 mM TRIS/HCl pH 7.4, 1 M NaCl, 0.5% Triton X-100, 1 M Urea, 5 mM EDTA, 1 mM DTT). After the last wash, the beads were resuspended in 100 µl of RIP buffer (50 mM HEPES pH 7.5, 0.1 M NaCl, 5 mM EDTA, 10 mM DTT, 0.5% Triton X-100, 1% SDS) and incubated 45 min at 70 °C to revert crosslinking. Proteins were digested incubating at 37 °C for 15 min at 37 °C to 100 mg of cell pellet was resuspended in lysis buffer (50 mM TRIS/HCl pH 7.4, 1 M NaCl, 0.5% Triton X-100, 1 M Urea, 5 mM EDTA, 1 mM DTT). After the last wash, the beads were resuspended in 100 µl of RIP buffer (50 mM HEPES pH 7.5, 0.1 M NaCl, 5 mM EDTA, 10 mM DTT, 0.5% Triton X-100, 1% SDS) and incubated 45 min at 70 °C to revert crosslinking. Proteins were digested incubating at 37 °C for 15 min at 37 °C. Laemmli buffer was added to stop the reaction and boiled at 95 °C. 15 min at 30 °C. Laemmli buffer was added to stop the reaction and boiled at 95 °C. 15 min at 30 °C. 12% NuPAGE Bis–Tris gels were run and proteins were visualized using the GloMax Multi detection system with Instinct software v3.1.1 (Promega).

Native RNA immunoprecipitation. Cells were seeded in 15 cm plates and treated with 1 µM 4-OHT the day after for 48 h. The cells were washed twice with PBS and centrifuged at 500 × g for 5 min. The pellet was resuspended in lysis buffer (50 mM TRIS/HCL pH 7.4, 100 mM NaCl, 0.5% Triton X-100, 5 mM EDTA, 0.25% Na-deoxycholate, Protease Inhibitor (Roche), RNase Inhibitor (NEB)). Ten per cent of the extract was saved as RNA input. The rest of the cell extract was incubated with 10 µl of GFP-Trap™ Magnetic Agarose beads (Chromotek) rotating at 4 °C for 2 h. The beads were then collected using a magnetic rack and washed three times with lysis buffer. RNA was extracted adding 600 µl of TRIzol reagent (Invitrogen, Thermo Fisher) using the manufacturer’s protocol.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The MS data associated with this study has been deposited to PRIDE ProteomeXchange: PXD017475. RNA-seq data have been deposited in GEO with the accession number: GSE144752. Publicly available data was downloaded from UCSC Xena platform: https://xenabrowser.net/datasets?dataset=tagset_RSEM_genome&fpknnhost=http%3A%2F%2Ftool.xena.hub.net&removeFVhub=https%3A%2F%2Fpancanatlas.xenahubs.net&removeFVhub=https%3A%2F%2Fpancanatlas.xenahubs.net&removeFVhub=https%3A%2F%2Fpancanatlas.xenahubs.net&removeFVhub=https%3A%2F%2Fpancanatlas.xenahubs.net&removeFVhub=https%3A%2F%2Fpancanatlas.xenahubs.net&removeFVhub=https%3A%2F%2Fpancanatlas.xenahubs.net&removeFVhub=https%3A%2F%2Fpancanatlas.xenahubs.net&removeFVhub=https%3A%2F%2Fpancanatlas.xenahubs.net&removeFVhub=https%3A%2F%2Fpancanatlas.xenahubs.net&removeFVhub=https%3A%2F%2Fpancanatlas.xenahubs.net&removeFVhub=https%3A%2F%2Fpancanatlas.xenahubs.net&removeFVhub=https%3A%2F%2Fpancanatlas.xenahubs.net&removeFVhub=https%3A%2F%2Fpancanatlas.xenahubs.net&removeFVhub=https%3A%2F%2Fpancanatlas.xenahubs.net&removeFVhub=https%3A%2F%2Fpancanatlas.xenahubs.net&removeFVhub=https%3A%2F%2Fpancanatlas.xenahubs.net&removeFVhub=https%3A%2F%2Fpancanatlas.xenahubs.net&removeFVhub=https%3A%2F%2Fpancanatlas.xenahubs.net&removeFVhub=https%3A%2F%2Fpancanatlas.xenahubs.net&removeFVhub=https%3A%2F%2Fpancanatlas.xenahubs.net&removeFVhub=https%3A%2F%2Fpancanatlas.xenahubs.net&removeFVhub=https%3A%2F%2Fpancanatlas.xenahubs.net&removeFVhub=https%3A%2F%2Fpancanatlas.xenahubs.net&removeFVhub=https%3A%2F%2Fpancanatlas.xenahubs.net&removeFVhub=https%3A%2F%2Fpancanatlas.xenahubs.net&removeFVhub=https%3A%2F%2Fpancanatlas.xenahubs.net&removeFVhub=https%3A%2F%2Fpancanatlas.xenahubs.net&removeFVhub=https%3A%2F%2Fpancanatlas.xenahubs.net&removeFVhub=https%3A%2F%2Fpanc...
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**Author contributions**
M.M. and A.H.L. designed the experiments and analysed the data. M.M. wrote the manuscript and all the coauthors gave comments and input. M.M., M.L., F.S.A., B.M., and S.T. conducted experiments. F.S.A. analysed the RNA-seq data. M.L. performed the mass spectrometry experiments and analysis with help from L.M.H. and J.S.A. N.R. and A.J.S. analysed MIR31HG expression in THCA and CRC from TCGA public available data.

**Competing interests**
The authors declare no competing interests.

**Additional information**
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