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The lncRNA *MIR31HG* regulates p16^{INK4A} expression to modulate senescence

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Oncogene-induced senescence (OIS) can occur in response to oncogenic insults and is considered an important tumour suppressor mechanism. Here we identify the lncRNA *MIR31HG* as upregulated in OIS and find that knockdown of *MIR31HG* promotes a strong p16^{INK4A}-dependent senescence phenotype. Under normal conditions, *MIR31HG* is found in both nucleus and cytoplasm, but following B-RAF expression *MIR31HG* is located mainly in the cytoplasm. We show that *MIR31HG* interacts with both *INK4A* and *MIR31HG* genomic regions and with Polycomb group (PcG) proteins, and that *MIR31HG* is required for PcG-mediated repression of the *INK4A* locus. We further identify a functional enhancer, located between *MIR31HG* and *INK4A*, which becomes activated during OIS and interacts with the *MIR31HG* promoter. Data from melanoma patients show a negative correlation between *MIR31HG* and p16^{INK4A} expression levels, suggesting a role for this transcript in cancer. Hence, our data provide a new lncRNA-mediated regulatory mechanism for the tumour suppressor p16^{INK4A}.

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The mammalian genome encodes thousands of long non-coding RNAs (lncRNAs), which are transcribed and processed in a manner similar to messenger RNAs, but lack the protein-coding potential. Some of these transcripts are essential factors for the regulation of important cellular processes such as proliferation, differentiation or development, as well as a broad range of diseases including cancer^{1–3}. lncRNAs participate in a broad spectrum of gene-regulatory functions such as epigenetic activities, translational regulation or scaffolding/decoy functions^{4–6}. As transcriptional regulators, a number of lncRNAs regulate the transcription of neighbouring genes by *cis*-acting mechanisms^{7–9}, but may also act in *trans* without affecting neighbouring genes^{10–13}.

Cellular senescence is a state of irreversible growth arrest¹⁴, which can be triggered by different stimuli, such as telomere shortening, DNA damage, oxidative stress or oncogene activation¹⁵. In addition, senescence has recently been demonstrated to have a role in tissue patterning and remodelling during normal development^{16,17}. Oncogene-induced senescence (OIS) was first reported by Serrano *et al.*¹⁸ when they observed primary human and mouse fibroblasts undergoing senescence following expression of an oncogenic form of RAS. Later reports showed that many other oncogenes might cause OIS both *in vitro* and *in vivo*^{19,20}. OIS is considered a barrier against tumorigenesis. In support of this, senescent cells are present in pre-malignant tissues and not in tumour cells, indicating that senescence must be bypassed by additional tumorigenic alterations to progress towards malignancy^{19,20}. Senescent cells have been found to secrete interleukins, metalloproteases and other cytokines as part of the senescence-associated secretory phenotype (SASP) and these factors are known to stimulate cancer progression in a non-cell autonomous manner^{15,21,22}. Therefore, senescence may either fuel or halt tumour progression and this may depend on the age of the organism^{23,24}. Cellular senescence is primarily dependent on two tumour suppressor pathways: p14^{ARF}/p53 and p16^{INK4A}/pRB, both of which mediate cell cycle arrest. The upstream proteins of both pathways, p16^{INK4A} and p14^{ARF}, together with an additional tumour suppressor protein p15^{INK4b}, are encoded by the same locus *INK4B-ARF-INK4A* at human chromosome 9p21. This locus is tightly regulated by the Polycomb group proteins (PcGs)^{25–27}, which are responsible for its transcriptional repression in normal cells. During senescence, the Polycomb repressive complexes 1 and 2 (PRC1 and PRC2) are dissociated from the chromatin, resulting in transcriptional activation²⁶. Notably, the lncRNA *ANRIL* (antisense non-coding RNA in the *INK4* locus) is encoded in antisense orientation by the same locus²⁸ and affects the epigenetic silencing of p15^{INK4b} (ref. 29). *ANRIL* has been demonstrated to mediate the recruitment of chromatin silencing machinery to the *INK4* locus and to suppress the expression of p15^{INK4b} (refs 29,30); however, its role during senescence remains unclear. Recently, the lncRNA *UCA1* has been reported to interact with hnRNPA1 and to increase p16^{INK4A} mRNA stability by preventing its degradation³¹. In another recent report, *PANDA* lncRNA has been shown to regulate senescence entry and exit, and to interact with the scaffold-attachment factor A³². Other lncRNAs have been identified to be involved in cellular senescence, although their mechanisms of action remain uncharacterized³³. The importance of the *INK4/ARF* locus and the interplay between OIS and cancer prompted us to further investigate lncRNAs deregulated in OIS.

We identify a number of deregulated lncRNAs during B-RAF-induced OIS. Among these, *MIR31HG/LOC554202* is upregulated in OIS, whereas the knockdown of *MIR31HG* promotes a p16^{INK4A}-dependent senescence phenotype. Under normal

conditions *MIR31HG* is located in both the nucleus and the cytoplasm, whereas upon B-RAF expression *MIR31HG* is almost exclusively localized in the cytoplasm. We demonstrate that the *MIR31HG* transcript interacts with the genomic regions *INK4A* and *MIR31HG*, and with PcG proteins, and that *MIR31HG* is required for PcG binding and repression of the *INK4A* locus. We further identify an enhancer, located between *MIR31HG* and the *INK4A* loci, which is activated during OIS and directly interacts with the *MIR31HG* locus. Finally, we found an anti-correlation between *MIR31HG* and p16^{INK4A} RNA expression in melanoma, suggesting a role for this transcript in cancer. Together, our results provide a new lncRNA-mediated regulatory mechanism for the tumour suppressor p16^{INK4A} during OIS. Our findings highlight the role of lncRNAs acting as transcriptional regulators in critical processes such as cellular senescence and cancer.

Results

***MIR31HG* is induced during OIS.** To identify lncRNAs with a role in OIS, we used immortalized human diploid fibroblasts, TIG3-hTERT, expressing a constitutively activated form of the mouse B-RAF (V600E) fused to the oestrogen receptor (ER:B-RAF). Addition of 4-hydroxitamoxifen (4-OHT) activates B-RAF and consequently induces OIS. This senescent phenotype includes activation of the enzyme β -galactosidase, induction of p16^{INK4A} expression, growth arrest and formation of senescence-associated heterochromatin foci (SAHFs) characterized by high levels of H3K9me3 (Supplementary Fig. 1a–d). RNA sequencing in OIS and control cells revealed that 655 protein-coding and 131 non-coding RNAs were affected by B-RAF overexpression (log₂-fold change > $\pm$ 1.5, false discovery rate < 0.05; Fig. 1a and Supplementary Data 1). Quantitative reverse transcriptase-PCR (qRT-PCR) validation of a gene set known to be relevant for senescence confirmed the robustness of the model (Fig. 1b). The majority of the lncRNAs deregulated in OIS corresponded to long-intergenic RNAs (lincRNAs) and antisense transcripts (Fig. 1c). One of the top hits identified was the lncRNA *MIR31HG* (previously known as *LOC554242* (ref. 34; Fig. 1d). Interestingly, this lncRNA is located on chromosome 9 (9p21.3), ~400 kb away from the *INK4A* locus, which prompted us to investigate it further. We confirmed by qRT-PCR that this transcript was more than eightfold upregulated in OIS (Fig. 1e). Using absolute quantification with *in-vitro*-transcribed *MIR31HG* as a standard curve for qRT-PCR analyses, we observed that there are ~40 copies of *MIR31HG* in control cells and 250 in 4-OHT-treated cells (Fig. 1f). Further characterization of *MIR31HG* revealed that it is poly-adenylated (Supplementary Fig. 1e) and located both in the cytoplasm and the nucleus (Fig. 1g). Using BJ foreskin fibroblast cells from early, intermediate and late passages, we observed an upregulation of *MIR31HG* during replicative senescence, suggesting that the induction of this lncRNA may be a common feature of different types of senescence (Supplementary Fig. 1f).

***MIR31HG* knockdown induces a senescence phenotype.** To analyse the physiological relevance of this lncRNA in senescence, we knocked down *MIR31HG* in TIG3-hTERT-ER:B-RAF without 4-OHT treatment, using two independent small interfering RNAs (siRNAs) and assessed a senescence phenotype 72 h post transfection, where we obtained a knockdown efficiency of ~70%–80% (Fig. 2a). We observed that knockdown of *MIR31HG* reduces cell growth in a manner similar to the induction of B-RAF (Fig. 2b). This decrease in cell number was not due to apoptosis, as no difference in the percentage of sub-G1 phase cells was detected (Supplementary Fig. 2a). Notably, a decrease in the S phase was evident as measured by the diminished incorporation

Figure 1 | The lncRNA *MIR31HG* is induced during OIS. (a) Pie chart representation of the number of protein-coding and non-coding RNAs deregulated during OIS ($\log_2$ -fold change $> \pm 1.5$, false discovery rate (FDR) < 0.05). (b) qRT-PCR analysis of *IL-6*, *p16^{INK4A}*, *IL-8*, *CDK4* and *CDK6* in cells treated with 4-OHT relative to cells treated with ethanol as vehicle (control). Data were normalized to housekeeping genes and the levels in control cells set to 1. (c) Diagrammatic representation of the different classes of lncRNAs deregulated during senescence. (d) Representation of all of the lncRNAs altered during OIS with FDR < 0.05 . *MIR31HG* (red) was identified as one of the top hits. The dotted lines indicate $\log_2$ FC = 1. (e) *MIR31HG* RNA levels relative to GAPDH in ethanol- (control) or 4-OHT-treated cells measured by qRT-PCR. (f) The number of molecules of *MIR31HG* transcript in control cells and in 4-OHT-treated cells measured by qRT-PCR using dilutions of *in-vitro*-transcribed *MIR31HG* as standard curve. (g) Percentage of nuclear and cytoplasmic RNA levels of *MIR31HG*, *GAPDH* and *MALAT1* measured by qRT-PCR after cell fractionation. The graph shows the average of three independent experiments. (b,e-g) The results are from three independent biological experiments and are represented as mean $\pm$ s.e.m. Student's *t*-test, $**P < 0.01$.

of EdU (Fig. 2c). In addition, *MIR31HG* knockdown cells presented several senescence features including increased β -galactosidase activity (Fig. 2d) and significantly increased levels of *p16^{INK4A}* expression as detected by immunofluorescence, western blotting and mRNA quantification (Fig. 2e–g and Supplementary Fig. 2b). Changes in *p14^{ARF}* protein levels were not observed following *MIR31HG* knockdown (Supplementary Fig. 2c). Furthermore, the formation of SAHF^s confirmed a strong senescence phenotype in *MIR31HG*-depleted cells (Fig. 2h). Similarly, *MIR31HG* knockdown in BJ cells led to reduced cell growth and an increase in *p16^{INK4A}* protein levels, corroborating our results observed in TIG3 cells (Supplementary Fig. 2d,e).

The *MIR31HG* locus also encodes the microRNA miR-31 in the first intron. The transcription of both the lncRNA and the miRNA is known to be driven by the same promoter³⁴. Furthermore miR-31 has previously been reported to be

upregulated in OIS³⁵. We confirmed that miR-31 expression is increased during OIS but miR-31 expression levels were unaffected by the siRNAs designed against exonic regions of *MIR31HG* transcript (Supplementary Fig. 2f). These results demonstrate that the senescence phenotype induced by the siRNAs is due to *MIR31HG* knockdown and not due to miR-31 depletion.

To address the impact of *MIR31HG* overexpression in senescence, we created a stable TIG3-B:RAF cell line that overexpressed *MIR31HG* in a doxycycline-inducible manner. We observed that 72 h doxycycline treatment increases the levels of both nuclear and cytoplasmic *MIR31HG* in a similar manner (Supplementary Fig. 3a). In control proliferating cells, overexpression of *MIR31HG* did not have an impact on cell proliferation or *p16^{INK4A}* protein levels (Supplementary Fig. 3b, lanes 1–4). However, we observed a diminished *p16^{INK4A}* upregulation following 4-OHT treatment (Supplementary

Figure 2 | *MIR31HG* knockdown induces a senescence phenotype. (a) Relative *MIR31HG* RNA levels in cells transfected with the indicated siRNAs analysed by qRT-PCR in control or 4-OHT-treated cells. Control values were set as 1. Results are represented as mean $\pm$ s.e.m. from >3 independent experiments. (b) Absorbance levels of crystal violet solution measured at 560 nm from cells transfected with the indicated siRNAs at the designated time points post transfection. (c) Percentage of EdU-positive staining in siRNA-transfected cells with or without 4-OHT treatment. (d) Representative images of the β -galactosidase staining of cells under the indicated conditions 72 h post transfection. Scale bar, 100 μ m. (e) Percentage of p16^{INK4A}-positive cells analysed by immunofluorescence 72 h post transfection. (f,g) Western blotting and qRT-PCR analysis of p16^{INK4A} protein and mRNA expression, respectively, 72 h post transfection in ethanol or 4-OHT-treated cells. (h) Left, representative images of DAPI (4,6-diamidino-2-phenylindole) and H3K9me3 staining by immunofluorescence in siRNA-transfected cells after 72 h. Right: quantification of the percentage of SAHF-positive cells. At least 150 cells from three independent experiments were counted. Scale bar, 10 μ m. All experiments show the mean $\pm$ s.e.m. from >3 independent experiments. Student's *t*-test, **P* < 0.05, ***P* < 0.01.

Fig. 3b, lanes 5–8, and Supplementary Fig. 3c) without affecting cell growth (Supplementary Fig. 3d). To investigate whether exogenous *MIR31HG* could rescue *MIR31HG* knockdown-induced senescence, we silenced *MIR31HG* in these cells. The levels of *MIR31HG* increased approximately fourfold after 72 h doxycycline treatment in cells transfected with control siRNAs (Supplementary Fig. 3e). These control cells did not show any evident phenotype measured by p16^{INK4A} levels and cell proliferation (Supplementary Fig. 3f–h). As expected, RNA levels decreased after 72 h knockdown in non-treated cells and were recovered in doxycycline-treated cells for the same period of time (Supplementary Fig. 3e). Under these conditions, knockdown of *MIR31HG* increased p16^{INK4A} expression at RNA (Supplementary Fig. 3f) and protein levels (Supplementary

Fig. 3g) as previously observed, but not when *MIR31HG* was simultaneously expressed (Supplementary Fig. 3f,g). Moreover, the growth defects observed in knockdown cells after 72 and 96 h transfection were reverted by *MIR31HG* overexpression (Supplementary Fig. 3h). These data demonstrate the specificity of the siRNAs employed against *MIR31HG* and suggest that cytoplasmatic *MIR31HG* is not essential for the senescence phenotype.

***MIR31HG* effect in senescence is p16^{INK4A} dependent.** To identify the pathways affected by *MIR31HG* depletion, we performed RNA sequencing from cells transfected with control or *MIR31HG* siRNAs. From the 83 genes significantly altered by

Figure 3 | The effect of *MIR31HG* in senescence is p16^{INK4A} dependent. (a) Venn diagram representing the number of genes altered following 4-OHT treatment and si*MIR31HG* transfection after 72 h ($\log_2$ -fold change $> \pm 1.5$, false discovery rate (FDR) < 0.05). (b) Pie chart showing the number of genes commonly upregulated (red), commonly downregulated (green) or oppositely regulated (grey) by 4-OHT and siRNA knockdown. (c) qRT-PCR analysis of components of the SASP 72 h after 4-OHT treatment or transfection with the siRNAs indicated. (d) Absorbance measurements of crystal violet solution at 560 nm, 72 h post transfection with the indicated siRNAs. (e) Quantification of the number of SAHF-positive cells in cells transfected with the siRNAs or treatment indicated. At least 150 cells from three independent experiments were counted. All the graphs represent the mean $\pm$ s.e.m. from at least three independent experiments. Student's *t*-test, **P* < 0.05 , ***P* < 0.01 .

MIR31HG knockdown ($\log_2$ -fold change $> \pm 1.5$, false discovery rate < 0.05), 21 were also affected on B-Raf induction (Fig. 3a and Supplementary Table 1), with only 2 commonly upregulated and 1 commonly downregulated (Fig. 3b). We did not find evidence of the induction of broad senescence-related programmes in *MIR31HG* knockdown cells. In contrast, of the 18 genes oppositely regulated between the two conditions, many encoded cytokines, interleukins and other proteins known to be part of the SASP (Supplementary Table 2)³⁶. These were upregulated during OIS but downregulated or unaltered in *MIR31HG* knockdown cells (Supplementary Table 2). To confirm the absence of SASP in the *MIR31HG* knockdown-mediated senescence phenotype, we measured the RNA expression levels of several genes belonging to the SASP by qRT-PCR. As expected, the expression of all these genes were upregulated following B-Raf activation, whereas their levels were unaffected or decreased in *MIR31HG* knockdown cells (Fig. 3c). It has been reported previously that p16^{INK4A} is able to induce cellular senescence without expressing the associated inflammatory phenotype³⁷. We therefore speculated whether the cellular senescence observed after *MIR31HG* depletion was mediated by increased p16^{INK4A} expression. Indeed, the cell growth phenotype and the formation of SAHFs induced by *MIR31HG* knockdown could be rescued by p16^{INK4A} depletion (Fig. 3d,e and Supplementary Fig. 4a,b). p16^{INK4A} activation

during senescence results in repression of the transcriptional activity of the E2F transcription factors and, in consequence, of its pro-proliferative target genes. By analysing gene expression by qRT-PCR, we observed that a set of E2F target genes were reduced after *MIR31HG* depletion to similar levels as in senescence cells (Supplementary Fig. 4c), indicating that the cell growth defect observed on *MIR31HG* knockdown are due to an increased level of p16^{INK4A}. Moreover, p53 and phosphorylated γ H2A.X were not activated by *MIR31HG* knockdown, indicating the absence of DNA damage (Supplementary Fig. 4d,e), which may explain the lack of SASP³⁶ and supporting a p16^{INK4A}-dependent senescence phenotype.

***MIR31HG* is exported to the cytoplasm during senescence.** To investigate the mechanisms whereby *MIR31HG* expression is increased in senescence and how its silencing results in senescence, we performed cellular fractionation in TIG3 cells with and without B-Raf activation. In control cells, *MIR31HG* was located both in the nucleus and the cytoplasm, with slight enrichment in the nuclear fraction. In contrast, following B-Raf activation, *MIR31HG* was almost exclusively cytoplasmic (Fig. 4a). To bolster these data in an independent cell line, we turned to immortalized primary BJ fibroblasts. As OIS has been shown

Figure 4 | *MIR31HG* is exported to the cytoplasm on senescence induction by a general RNA export pathway. (a) Percentage of nuclear and cytoplasmic RNA levels of *MIR31HG*, *GAPDH* and *MALAT1* measured by qRT-PCR after subcellular fractionation in cells treated with ethanol (control) or 4-OHT for 72 h. (b) Nuclear (left) and cytoplasmic (right) RNA fraction from total RNA in control cells (white) and 4-OHT-treated cells (black) for the indicated time points. Values at time 0 for both conditions were set as 1. (c) Percentage of nuclear and cytoplasmic RNA levels of *MIR31HG* in control or 4-OHT treated cells 72 h after transfection with the indicated siRNAs. (d) Percentage of nuclear and cytoplasmic RNA levels of *GAPDH* and *MALAT1* in the experiment indicated in c. All the graphs show the mean $\pm$ s.e.m. from at least three independent experiments. Student's *t*-test, **P* < 0.05, ***P* < 0.01.

to be p16^{INK4A} independent in BJ cells³⁸, one hypothesis could be that *MIR31HG* remains in the nucleus during senescence, thus preventing p16^{INK4A} expression. To test this hypothesis, we used BJ-hTERT-ER:B-RAF cells, which responded to 4-OHT with an increase of *MIR31HG*, together with induction of other senescence markers such as *IL8* or *MMP1* to a similar extent as observed in TIG3 cells (Supplementary Fig. 5a). By cellular fractionation, we assessed the distribution of *MIR31HG* in BJ cells undergoing OIS. Similar to what we observe in TIG3 cells, *MIR31HG* in BJ cells is located about equally in the nucleus and in the cytoplasm under normal conditions but almost exclusively in the cytoplasm after 72 h 4-OHT-treatment (Supplementary Fig. 5b). However, in our hands, these cells also showed an increase of p16^{INK4A} RNA and protein levels during senescence (Supplementary Fig. 5c), in agreement with a relocation of *MIR31HG* away from the *INK4A* locus. To further investigate this translocation we carried out cell fractionation assays in a time-course experiment during 4-OHT treatment. We observed that the amount of *MIR31HG* in control cells remains largely unaltered throughout the treatment period in both compartments (Fig. 4b). However, after 36 h of 4-OHT treatment, the levels of nuclear *MIR31HG* started to decrease (Fig. 4b, left panel). Inversely, at this time point after the treatment an increase in cytoplasmic *MIR31HG* was detected (Fig. 4b, right panel). In the same conditions we measured *GAPDH* and *MALAT1* as cytoplasmic and nuclear controls, respectively, and observed that both transcripts behaved as expected throughout the experiment, with no changes detected in response to 4-OHT treatment (Supplementary Fig. 5d,e).

It has been recently reported that a subset of lncRNAs are exported to the cytoplasm via common mRNA pathways³⁹. To corroborate the export of *MIR31HG* during senescence, we

depleted a set of export factors that have been previously involved in RNA export^{40,41} (Supplementary Fig. 5f). Among the factors tested, the knockdown of Aly using two independent siRNAs significantly increased the retention of *MIR31HG* in the nucleus under senescence induction, indicating that this export factor is involved in *MIR31HG* export to the cytoplasm (Fig. 4c,d and Supplementary Fig. 5f-h). These experiments show that *MIR31HG* is exported to the cytoplasm in senescence by an Aly-dependent RNA export pathway.

***MIR31HG* is required for PcG binding to the *INK4A* locus.** To investigate the role of *MIR31HG* in regulating p16^{INK4A} expression, we measured p16^{INK4A} expression levels from total RNA in the time-course experiment mentioned above. We observed *MIR31HG* total expression increased up to threefold after 24 h 4-OHT treatment (Fig. 5a, left panel). However, upregulation of p16^{INK4A} occurs later (Fig. 5a, right panel), concomitantly with the decrease in nuclear *MIR31HG* levels (Fig. 5b).

Therefore, we investigated a nuclear function for *MIR31HG* in regulating p16^{INK4A} expression in normal cells. As p16^{INK4A} is tightly regulated at the transcriptional level by PcG complexes^{25–27}, we hypothesized that *MIR31HG* participates in PcG-mediated repression. Towards this, we first investigated whether PcG recruitment to the *INK4A* locus was affected by *MIR31HG* knockdown. Using chromatin immunoprecipitation (ChIP) experiments, we assessed the binding of RING1B and SUZ12, components of PRC1 and PRC2, respectively, to several regions of the *INK4A* locus. As expected, RING1B, SUZ12 and the repressive mark established by PRC2, H3K27me3 were present throughout the locus in control cells and with reduced presence in senescent cells (Fig. 5c and Supplementary Fig. 6a).

Figure 5 | *MIR31HG* is required for PcG binding to the *INK4A* locus and interacts with PcG proteins. (a) *MIR31HG* (left) and *p16^{INK4A}* (right) RNA levels from total RNA extracted from cells treated with 4-OHT for the time indicated measured by qRT-PCR. The data are represented as the expression in 4-OHT versus control, setting untreated as 1. (b) Nuclear *MIR31HG* levels from experiment in Fig. 4b and total *p16^{INK4A}* transcript levels in cells treated with 4-OHT for the indicated time points. The graph represents the data from the treatment versus the control. Levels in cells without treatment have been set to 1 (dotted red line). (c) ChIP-qPCR experiments for RING1B, SUZ12 and H3K27me3 throughout the *INK4/ARF* locus or at an unrelated region (unrel) in ethanol (control), 4-OHT-treated and siMIR31HG-transfected cells. The position of primers used in the analysis are shown in the schematic diagram (red) and the sequences are present in Supplementary Table 4. (d) RNA immunoprecipitation analysis monitoring EZH2 and SUZ12 (left), and RING1B (right) binding to *MIR31HG* RNA and *GAPDH* RNA in control and 4-OHT-treated cells. All the graphs (a–d) show the mean $\pm$ s.e.m. from three independent experiments. Student's *t*-test, **P* < 0.05, ***P* < 0.01. (e) Electrophoretic mobility assay showing the *in vitro* binding of 2 nM *MIR31HG* and *Luciferase* (*Luc*) transcript to the indicated amounts of recombinant PRC2 complex. BSA at the highest concentration (160 nM) was used as a negative control.

Intriguingly, *MIR31HG* knockdown cells showed a significant reduction in the presence of PRCs and H3K27me3, comparable to that seen in senescence conditions (Fig. 5c), which correlates with the increase in *p16^{INK4A}* transcription. As controls, we did not observe any RING1B, SUZ12 and H3K27me3 enrichment at different positions of the *MIR31HG* promoter (Supplementary Fig. 6b), nor at an unrelated region located several megabases away (Fig. 5c). The amounts of PcG proteins bound to the *INK4B* locus were also very low and no significant differences were observed on *MIR31HG* depletion (Fig. 5c).

***MIR31HG* interacts with PcG complexes.** It is well known that lncRNAs can contribute to the recruitment of PcG complexes to repress gene transcription of target genes, and in some cases this regulation may be dependent on direct lncRNA interaction with PcG proteins such as EZH2, SUZ12 and CBX7 (refs 8,42,43). To investigate binding of *MIR31HG* to PcG complexes, we carried out RNA immunoprecipitation using antibodies towards SUZ12, EZH2 (PRC2) and RING1B (PRC1). *MIR31HG* bound all three PcG proteins in control cells, suggesting that this transcript is part of the repressive complexes (Fig. 5d and Supplementary Fig. 6c).

Importantly, this interaction was decreased in senescence (Fig. 5d and Supplementary Fig. 6c), consistent with the activation of *p16^{INK4A}* transcription and with the reduced levels of *MIR31HG* in the nucleus after senescence. Although the PRC2 protein levels are decreased in OIS compared with control cells, we did not observe differences in the amount of protein immunoprecipitated in both conditions (Supplementary Fig. 6d and data not shown). Next, we investigated whether *MIR31HG* was able to directly interact with PcGs. Using *in-vitro*-transcribed full-length *MIR31HG* and purified recombinant reconstituted PRC2 complex (Supplementary Fig. 6e), we performed RNA electrophoretic mobility shift assays. Adding increasing amounts of PRC2 to 2nM ³²P-labelled *MIR31HG* transcript we observed a shift, indicating direct binding of *MIR31HG* to PRC2 (Fig. 5e). Purified BSA was used as a negative protein control. *In-vitro* interactions with PRC2 have been shown to occur in a promiscuous manner⁴⁴. Indeed, when we used an *in-vitro*-transcribed *Luciferase* transcript, we still observed PRC2 binding; however, the strength of the binding is higher for *MIR31HG* than for *Luciferase* (Fig. 5e and Supplementary Fig. 6f). These results indicate that *MIR31HG* is able to interact directly with PRC2.

***MIR31HG* interacts with the *INK4A* and *MIR31HG* loci.** To gain more insight into the regulatory mechanism, we investigated RNA–chromatin interactions using chromatin isolation by RNA purification (ChIRP)^{45,46}. This technique uses biotinylated antisense oligonucleotides to purify endogenous lncRNAs and their associated chromatin complexes. Using two non-overlapping oligonucleotide pools targeting *MIR31HG*, we were able to enrich *MIR31HG* but not the negative control mRNA *GAPDH* (Fig. 6a). Almost undetectable levels of transcripts were recovered using oligonucleotides targeting *Luciferase* mRNA, demonstrating the specificity of the approach (Fig. 6a). RNA purification followed by qPCR revealed interactions between *MIR31HG* RNA and both *p16^{INK4A}* and *MIR31HG* promoters in control cells. Importantly, these interactions were diminished in senescence (Fig. 6b,c) and reduced to background levels at an unrelated region situated several megabases away (Fig. 6d).

To investigate whether *MIR31HG* acts together with PRC2 in regulating *p16^{INK4A}* expression, we performed ChIRP experiments in RING1b or EZH2 knockdown cells (Supplementary Fig. 7a). In these conditions, we could retrieve ~15%–25% of the endogenous transcript (Fig. 6e) and observed that *MIR31HG* interaction with *p16^{INK4A}* promoter is diminished in the absence of PcG (Fig. 6f) correlating with *p16^{INK4A}* expression (Supplementary Fig. 7b). These experiments suggest that *MIR31HG* and PcG proteins cooperate in repressing *INK4A* expression.

An OIS-activated enhancer interacts with *MIR31HG* promoter.

As the above results suggested a possible role for *MIR31HG* in mediating chromatin looping between *MIR31HG* and *INK4A* chromatin regions, situated 400 kb apart, we performed circular chromosome conformation capture followed by deep sequencing (4C-seq)⁴⁷, using the *MIR31HG* and *INK4A* promoters as viewpoints. No obvious interaction between these two regions was detected. However, we identified a strong interaction between the *MIR31HG* promoter and a region located ~100 kb upstream *MIR31HG* (Fig. 7a and Supplementary Fig. 8a). To validate these data, we repeated the 4C-seq experiments using the identified region as a viewpoint and confirmed the existence of a physical interaction between *MIR31HG* promoter and that chromosomal location (Fig. 7b and Supplementary Fig. 8a). Examining ENCODE data, we observed that the identified interacting

region contains features characterizing enhancers, such as high levels of the histone marks H3K27ac and H3K4me1, and low presence of H3K4me3. It is also characterized by DNA hypersensitivity and by high transcription factor occupancy (Encode project consortium⁴⁸ (Supplementary Fig. 8b)). To validate the enhancer features in senescent TIG3 cells, we carried out ChIP experiments for H3K4me1 and H3K4me3 in control and senescence cells, respectively. Using seven pairs of primers spanning the 20-kb region, we observed low signals for H3K4me3 and high signals for H3K4me1, as expected for an enhancer, in both situations (Supplementary Fig. 8c). However, the levels of H3K4me1 were significantly higher under senescence conditions, suggesting an activation of the enhancer during senescence (Fig. 7c). In contrast, in three different regions of the *INK4A* promoter, H3K4me1 and H3K4me3 levels were similar in control cells and only the levels of H3K4me3 increased in senescence, correlating with the transcriptional activation of *p16^{INK4A}* after B-Raf activation (Fig. 7c). Recent studies have reported that many enhancers can bidirectionally transcribe non-coding RNAs with low expression levels (enhancer RNAs (eRNAs))⁴⁹ and our RNA-seq data indicated that bidirectional transcription was occurring from the enhancer. By qRT-PCR, we were able to detect these transcripts almost exclusively during senescence (Fig. 7d). To corroborate the functionality of these enhancer, we created luciferase reporter constructs carrying different fragments of the 20-kb region, corresponding to peaks in enhancer marks in fibroblast data from ENCODE (Supplementary Fig. 8b). As enhancers are shown to act in both directions, we cloned both the forward and the reverse sequences and measured the activation of a luciferase gene driven by the SV40 promoter. A pGL3-basic vector containing both the SV40 promoter and enhancer was used as a positive control. Following transient transfections into TIG3 cells, we observed enhancer activity from the reporters containing fragment 2 and fragment 3 in both directions, but not fragment 1 (Fig. 7e). Taken together, these results characterize a new functional enhancer with direct interaction with *MIR31HG* during senescence.

MIR31HG and *p16^{INK4A}* inversely correlate in melanoma.

MIR31HG has been previously reported to be deregulated in human cancers^{34,50,51}. In our model system, *MIR31HG* is driven by B-Raf oncogene activation. We therefore hypothesized a role for *MIR31HG*-mediated *p16^{INK4A}* regulation in melanoma, where 40%–60% of the patients carry B-Raf mutations. We analysed RNA expression data sets in melanoma tumour samples from The Cancer Genome Atlas (TCGA Research Network: <http://cancergenome.nih.gov/>). In this data set, *INK4A* is mutated or homozygously deleted in ~40% of cases. The deletions are strongly centred at *INK4A*, often extending up or downstream of *INK4A*; however, *MIR31HG* is rarely affected. Importantly, focusing exclusively on tumours holding normal diploid *INK4A* loci with no mutations or copy number changes, we observed a negative correlation between *MIR31HG* and *p16^{INK4A}* RNA expression (Pearson's $r = -0.33$, $P = 0.03$, two-tailed test) (Supplementary Fig. 8d). This finding suggests that *MIR31HG* might be repressing *p16^{INK4A}* expression in human melanoma.

Discussion

Over the last few years, a number of lncRNAs have been identified and characterized as important factors in fundamental physiological and pathological processes. Here we contribute to the understanding of the function of lncRNAs by describing a role for *MIR31HG*, which is located 400 kb upstream from the *INK4A* locus on chromosome 9, in OIS. Consistent with a recent report⁵⁰, *MIR31HG* downregulation reduces cell growth and,

Figure 6 | *MIR31HG* interacts with the *INK4A* and *MIR31HG* loci in control cells but not in senescence or in the absence of PcG proteins. (a) ChIP experiments using two independent probe pools *MIR31HG*-asDNA (odd and even) retrieved ~15%–20% of *MIR31HG* endogenous RNA and undetectable levels of *GAPDH*. *Luc*-asDNA probes retrieved undetectable levels of both RNAs. ChIP DNA signal for *MIR31HG* and *Luc* binding on the *INK4A* promoter (b), *MIR31HG* promoter (c) and on an unrelated region (d) in ethanol (control) and 4-OHT-treated cells. Positions of the primers used for qPCR are shown in red and the sequences provided in Supplementary Table 4. All the graphs show the mean $\pm$ s.e.m. from three independent experiments. (e) *MIR31HG* retrieval in a ChIP experiments with the same probes as in b from ethanol (control), 4-OHT-treated cells or cells transfected with siRNAs against *EZH2* or *RING1B*. (f) ChIP DNA signal for *MIR31HG* binding at the *INK4A* promoter in the conditions described in e. The position of the primers are shown in b. The graphs (e,f) show the mean $\pm$ s.e.m. from two independent experiments.

intriguingly, promotes a strong senescence phenotype. Although this may seem paradoxical given the global upregulation of *MIR31HG* during senescence, our data demonstrates a relative nuclear depletion of *MIR31HG* during senescence. Here we describe a nuclear function for *MIR31HG* in repressing the cell cycle inhibitor $p16^{\text{INK4A}}$ at the transcriptional level by facilitating the binding of PcG proteins to the locus. We furthermore show that the general RNA export factor Aly plays a role in the exporting *MIR31HG* to the cytoplasm during senescence. We do not see changes in Aly RNA or protein levels in senescence. As it has been shown that Aly is methylated to reduce its RNA-binding affinity favouring the downstream export of RNAs⁵², we speculate that Aly is posttranslationally modified during senescence to facilitate *MIR31HG* export. However, the detailed

mechanism by which Aly performs this function requires further investigation.

The overexpression of *MIR31HG* in control cells, where $p16^{\text{INK4A}}$ is already repressed, does not show any evident senescence phenotype. The lack of phenotype, even when the cytoplasmic *MIR31HG* fraction is increased up to fourfold, suggests that cytoplasmic *MIR31HG* might not contribute to senescence.

We have not been able to revert OIS by overexpressing *MIR31HG*, although $p16^{\text{INK4A}}$ levels appear to be decreased. This indicates that the mere $p16^{\text{INK4A}}$ reduction in this system is not enough to revert B:RAF-induced senescence. However, *MIR31HG* knockdown-dependent senescence is reverted by the overexpression, where $p16^{\text{INK4A}}$ is restored to control levels. These results

suggest that that p16^{INK4A} is the main factor in *MIR31HG*-dependent senescence, while the molecular mechanisms involved in B-RAF-induced senescence might be more complex⁵³.

As mentioned above, the role of *MIR31HG* in senescence is dependent on p16^{INK4A}, as we are able to revert these effects by depleting p16^{INK4A}. Indeed, the senescence phenotype induced by loss of *MIR31HG* resembles the p16^{INK4A}-dependent

senescence previously described by the Campisi lab, marked by the absence of both DNA damage and SASP³⁷. The *MIR31HG* regulatory mechanism appears specific for p16^{INK4A} and not for the other tumour suppressor proteins p15^{INK4B} and p14^{ARF} encoded by the *INK4-ARF* locus. However, we cannot exclude that *MIR31HG* might be acting *in trans* affecting other loci. It is currently unclear whether the interaction of *MIR31HG* with the

INK4A locus is direct or mediated by additional factors, such as PcG proteins. Although the *MIR31HG* transcript contains repetitive sequence elements that might be important for RNA:DNA interactions, as has been shown for Alu elements^{54,55}, we do not observe any obvious complementarity between *MIR31HG* and the *INK4A* locus.

Although the *MIR31HG* transcript binds the genomic regions *MIR31HG* and *INK4A*, we could not detect a direct interaction between the two loci using 4C-seq, which in turn rules out a chromatin looping model, as has recently been shown for other lncRNAs^{56,57}. In contrast, our 4C-seq, as well as previously published HiC-seq data⁵⁸, indicates that the two genes belong to separate topological domains and therefore are likely to not have physiological relevant interactions. Moreover, we do not observe any major changes in chromosome conformation after *MIR31HG* knockdown, indicating that *MIR31HG* is not likely to affect the overall chromatin structure of the locus. Hence, the *MIR31HG* interaction with its own genomic locus might merely reflect nascent transcription. Importantly, our 4C analyses identified chromatin interactions between *MIR31HG* and different neighbouring regions. The most intriguing interaction corresponds to an enhancer located ~100 kb from *MIR31HG*, which appears to be functional under normal conditions and further activated during senescence. The interaction appears to already exist in normal cells, allowing a basal *MIR31HG* expression level. The activation of the enhancer drives *MIR31HG* upregulation and the increased interaction frequency might play a more humble additive role. The region encompassing the identified enhancer was recently characterized as a putative super enhancer⁵⁹. Enhancers and super enhancers, large clusters of enhancers, have been demonstrated to bind master transcription factors and to interact with genes that define cell identity⁵⁹. Several enhancers have been shown to respond to diverse stimuli such as neuronal depolarization⁴⁹ or ER α signalling⁶⁰. In a recent report Rosenfeld and colleagues⁶¹ demonstrated that ER α -responsive enhancers are characterized by the recruitment of a complex of transcription factors. The expression of key transcription factors in response to B-Raf activation may be involved in the enhancer activation. In the functionality assay, we have not observed differences in the activity of the enhancer fragments in proliferating and in senescent cells. This could be due to the massive overexpression of the reporters, a very artificial system. To support our hypothesis that the identified enhancer is active during senescence, we detect transcription from the enhancer primarily during senescence. A large portion of enhancers can be transcribed into eRNAs⁴⁹, which have been proposed to participate in gene activation^{9,60,62,63}. Future experiments will address whether these eRNAs play a functional role in the activation of the enhancer and *MIR31HG*. Enhancers and super enhancers have been described to be responsible for cell identity⁵⁹. An interesting possibility may be that the enhancers

activated in specific tissues or specific conditions might be regulating the expression of other lncRNAs that could exert their actions at a genome level, adding a layer of regulation on transcriptional regulation.

By analysing melanoma cancer data, we observed that patients with higher levels of *MIR31HG* often have reduced *p16^{INK4A}* expression. In concordance with our results, this suggests that *MIR31HG* repression of *p16^{INK4A}* in these patients favours cancer development. These data and previous reports from other groups link *MIR31HG* to cancer. In lung and breast cancers *MIR31HG* was shown to be upregulated^{50,51}, whereas in triple-negative breast cancer it appears to be downregulated due to promoter hypermethylation³⁴. The mechanism for *MIR31HG* upregulation in breast and lung cancer remains unknown. Analysis of the enhancer status and the correlation between *MIR31HG* and *p16^{INK4A}* in other types of cancer might reveal a general role for *MIR31HG* in cancer. The implication of *MIR31HG* in senescence raises the interesting possibility of its involvement in development, although this may be unlikely, as no evidence of *p16^{INK4A}* expression has been detected in developmental senescence in mouse embryos¹⁷.

Altogether, our results provide a new mechanism for *p16^{INK4A}* transcriptional regulation by the lncRNA *MIR31HG*. The importance of *p16^{INK4A}* as a tumour suppressor emphasizes the relevance of adding new knowledge to the complex regulatory mechanism of the *INK4A* locus regulation. The identification of new lncRNAs influencing cell cycle regulators or other tumour suppressors will aid to advance our understanding of the basic biology of cancer.

Methods

Cell cultures, treatments and siRNA transfections. The human diploid cell lines TIG3 and BJ were immortalized with telomerase (hTERT) and transduced with a retrovirus generated from pMSCV-ER-B-Raf previously described³⁵. Cells were maintained in DMEM medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone) and penicillin/streptomycin (Invitrogen). Senescence was induced by treatment with 1 μ M 4-OHT (Sigma) 24 h after seeded for at least 48 h (unless otherwise indicated). When additional incubation time was required, media with fresh 4-OHT was added. Control cells were treated with ethanol for the same period of time. siRNA oligonucleotides were transfected at a final concentration of 50 nM by reverse transfection using RNAiMAX (Invitrogen), according to the manufacturer's instructions. The siRNAs sequences are listed in Supplementary Table 4.

***MIR31HG*-overexpressing cell lines.** To generate lentiviral constructs we cloned the full-length *MIR31HG* sequence into the inducible vector pLVX-TetOne Puro Vector (Clontech). We use In-Fusion HD Cloning designing primers according to the manufacturer's indications (see primer sequences in Supplementary Table 4).

For the lentiviral production, HEK293T cells were transfected using Lipofectamine 2000 (Life Technologies) with 7 μ g of pLVX-*MIR31HG* or pLVX-empty, together with 6 μ g of VsVg and 5 μ g Pax8 viral plasmids. After 48 h, supernatants containing the viral particles were filtered through a 0.45- μ m filter. Confluent TIG3 cells were then infected with one-third of the viral supernatant and 8 μ g μ l⁻¹ polybrene. Twenty-four hours post infection, the cells were selected with 1 μ g μ l⁻¹ puromycin. Cells were maintained in tetracycline-free

Figure 7 | A 20-kb enhancer region activated during OIS interacts with the *MIR31HG* promoter. (a,b) 4C-seq representation of the chromosome interactions detected using *MIR31HG* (a) or the enhancer region (b) as viewpoints. At the top, the ideogram represents the human chromosome 9. The location of the 4C-seq profiles is shown by the vertical red mark. Position of *MIR31HG* promoter is shown in red and the enhancer region in green. RefSeq *hg19* genes are indicated by rectangles (exons) and arrows (introns) that point the direction of transcription. Interactions occurring in ethanol-treated cells (control) are shown in red and in 4-OHT-treated cells in blue. (c) ChIP-qPCR experiments monitoring H3K4me3 (left) and H3K4me1 (right) binding throughout the enhancer region in ethanol-treated cells (control) (white) and in 4-OHT-treated cells (black). Primer positions are indicated in the diagram and in Supplementary Fig. 8b, and the sequences are provided in Supplementary Table 4. (d) qRT-PCR analysis of an eRNA transcribed from the enhancer identified, in control cells or in 4-OHT-treated cells. The data show the enrichment in senescence compared with control cells. No product was detected when the reverse transcriptase enzyme was omitted. (e) Activation of *Luciferase* reporter constructs containing three different fragments of the enhancer cloned in forward (Fwd) and reverse (Rev) orientation. A control plasmid containing SV40 enhancer was used as positive control. Luciferase values normalized to *Renilla luciferase* are shown relative to the pGL3-promoter empty vector set as 1 (dotted black line). All the graphs (c–e) show the mean $\pm$ s.e.m. from at least three independent experiments.

tested serum (Clontech). For the expression of the lncRNA 100 ng ml^{-1} doxycycline was used for 72 h, unless otherwise indicated.

Luciferase reporter assays. For the enhancer reporter assays, three different fragments from the enhancer region were amplified by PCR from genomic DNA (see primer list). The PCR constructs were cloned into the pGL3-promoter *Firefly* luciferase reporter vector (Promega), using XhoI restriction enzyme (primer sequences are shown in 5). TIG3 cells (3×10^5) were seeded in a 24-well plate. After 24 h, the cells were transfected using Lipofectamine 3000 (Life Technologies) with 500 ng of the PGL3-promoter, PGL-3 control or the enhancer-luc constructs, together with 150 ng of pRL-Tk *Renilla* luciferase reporter vector for transfection control and luciferase assay normalization. Six hours after transfection, fresh media containing $1 \mu\text{M}$ 4-OHT or EtOH as vehicle was added to the cells. Forty-eight hours after treatment *Firefly* and *Renilla* luciferase units were measured, using Dual-Glo Luciferase Assay System and Glo-Max Multi Detection System (Promega).

Crystal violet staining. Cells were seeded and reverse transfected in 12-well plates (NUNC). Twenty-four, 48, 72 and 96 h after transfection, cells were washed twice in PBS and fixed with 10% formalin for 10 min, and stained with 0.1% crystal violet solution. 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 measurement of the absorbance at 570 nm.

Senescence associated β -galactosidase staining. Cells were seeded and transfected in 12-well plates (NUNC). After 72 h post transfection, they were fixed and stained using the β -galactosidase staining kit (Cell Signaling), according to the manufacturer's protocol.

EdU incorporation assays and flow cytometry analysis. Cells grown in six-well plates (NUNC) were pulsed with EdU ($33 \mu\text{M}$) for 30 min before fixation. The EdU was detected using Click-IT EdU Alexa Fluor-647 Flow Cytometry Assay Kit (Molecular Probes, Life Technologies), according to the manufacturer's protocol. Data were generated by flow cytometric analysis of 10,000 cells per treatment. For propidium iodide staining, harvested cells were fixed in 70% ethanol overnight before incubation in propidium iodide and RNase-A solution for 30 min. Data analysis was performed using the FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Immunofluorescence. Cells were seeded on rounded coverslips placed in 24-well plates (NUNC). Seventy-two hours post transfection, the cells were washed with PBS $\times 1$, fixed in 4% paraformaldehyde (Sigma) for 10 min and then 0.1% Triton X-100 in PBS (Sigma) was added for 10 min. The coverslips were washed twice with PBS $\times 1$, blocked for 30 min with 5% normal goat serum in PBS before incubation with the primary antibodies in 2% normal goat serum for at least 1 h at room temperature. After intensive washing with PBS $\times 1$, the secondary antibody was incubated in 2% goat serum for 1 h at room temperature in the dark. Next, the coverslips were washed, drained and mounted on glass slides using Prolong Gold Antifade Reagent with DAPI (Life Technologies). Antibodies and dilutions are listed in Supplementary Table 5.

Western blot analysis. Cells were seeded, reverse transfected in six-well plates (NUNC) and treated the following day with $1 \mu\text{M}$ 4-OHT or equivalent volumes of ethanol. After 48/72 h, cells were harvested, washed once with PBS and the pellets lysed in RIPA buffer (150 mM NaCl, 0.1% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 8), 1 mM EDTA) containing protease inhibitors (Complete Mini Protease Inhibitor Cocktail; Roche Applied Science). Proteins were separated by electrophoresis in 4%–12% NuPAGE Bis-Tris gels (Invitrogen) and transferred to nitrocellulose membranes. The antibodies used for western blotting are listed in Supplementary Table 5.

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.

RNA extraction and qRT-PCR analysis. Total RNA was isolated using Trizol reagent (Invitrogen), treated with TURBO DNase (Ambion, Life Technologies) and reverse transcribed using TaqMan Reverse Transcription kit (Applied Biosystems) with random hexamer primers. Quantitative real-time PCRs were performed using Syber Green PCR Fast PCR Master Mix 2x (Applied Biosystems). The house-keeping genes *GAPDH* and *RPLP0* were used for normalization of qRT-PCR data, unless otherwise stated. For miR-31 qPCR, total RNA was prepared using Trizol reagent and qPCR analysis was performed using TaqMan miRNA assays (Applied Biosystems), using primers for hsa-miR-31 and *RNU44*.

To calculate the number of molecules per cell, *MIR31HG* was *in vitro* transcribed (see *in vitro* RNA transcription). The concentration of the transcript

was measured by A260 values and converted to the number of copies using the molecular weight of the RNA. Dilutions of this transcript were retro-transcribed as described above and the complementary DNA was used as standard curve for qPCR. The primers used for qPCR are listed in Supplementary Table 4.

RNA sequencing. TIG3-hTERT-ER:B-RAF were treated with $1 \mu\text{M}$ 4-OHT or equal volumes of EtOH for 48 h, or they were transfected with Allstar negative control (Qiagen) or *MIR31HG* siRNAs for 72 h. RNA was extracted from three biological replicates, the quality assessed on the 2100 expert Bioanalyzer (Agilent) and sent for library preparation and sequencing on the Illumina HiSeq2000 by BGI Genomics (Hong Kong).

ChIP experiments. Cells were fixed with 1% formaldehyde for 10 min. Cross-linking was arrested by adding glycine (0.125 M) for 5 min at room temperature. The cells were subsequently harvested in SDS lysis buffer (0.5% SDS, 100 mM NaCl, 50 mM Tris-Cl pH 8.1, 5 mM EDTA pH 8.0, protease inhibitor mixture Complete (Roche) and 1 mM phenylmethylsulfonyl fluoride (PMSF)). Nuclei were pelleted and resuspended in IP buffer (2 volumes SDS lysis buffer: 1 volume Triton-X buffer (100 mM Tris-Cl, pH 8.6, 100 mM NaCl, 5 mM EDTA pH 8.0, 5% Triton X-100)). The lysates were sonicated using BIORUPTOR sonicator for 12 cycles of 30 s and centrifuged at maximum speed. The sheared chromatin was diluted to 1 ml with IP buffer and precleared with salmon sperm DNA/recombinant protein A-agarose (Thermo Scientific) for 2 h. One per cent of the sample was used as the input control and the remaining precleared chromatin was incubated overnight with $10 \mu\text{g}$ by incubation with salmon sperm DNA/protein-A agarose (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). The beads were then washed once with LiCl buffer (0.25 M LiCl, 1% NP-40, 1% Na-deoxycholate, 1 mM EDTA and 10 mM Tris-HCl pH 8.0) followed by two washes with Tris-EDTA buffer. Elution buffer (0.1% SDS, 0.1 M NaHCO_3) was added to the samples and the cross-linking was reverted 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, Life Technologies). The DNA was purified using Minelute PCR Purification Kit (Qiagen) and then amplified by qPCR using primers listed in Supplementary Table 4.

Native RNA immunoprecipitation. Cells were collected in cold PBS by scraping and lysed in lysis buffer (20 mM Tris-HCl pH 7, 150 mM NaCl, 100 mM KCl, 5 mM MgCl_2 , 0.5% Nonidet P-40, SupersesIn (Ambion, Life Technologies) 100 U ml^{-1} , dithiothreitol (DTT) 1 mM and $1 \times$ protease inhibitor mixture Complete (Roche)). After centrifugation, NT2 buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM MgCl_2 , 0.05% Nonidet P-40, 200 U ml^{-1} SupersesIn (Ambion, Life Technologies), 1 mM DTT, 15 mM EDTA pH 8) was added to the supernatant to 1 ml total volume and the samples were precleared with yeast RNA/recombinant protein A or G-agarose (Thermo Scientific) for 2 h. One per cent of the sample was used as the input control and the remaining extracts were incubated with the corresponding antibodies (Supplementary Table 5) overnight at 4°C . RNA-antibody complexes were collected by incubation with protein-A or protein-G agarose. After extensive washing, $1/5$ of the sample was used for western blotting control and the remaining for RNA extraction and qRT-PCR analysis.

In-vitro RNA transcription and labelling. Templates containing the T7 promoter were generated by PCR with high-fidelity DNA polymerase (Phusion HF, Thermo Scientific) and the primers listed in Supplementary Table 4. PCR products were purified from agarose gels and $1 \mu\text{g}$ of template was *in vitro* transcribed using T7 RNA polymerase and the MAXIscript kit (Ambion, Life Technologies) for 2 h 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 buffer and boiled 3 min at 37°C before running on 1% agarose gel in $1 \times$ TBE. The RNA products were purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) with NTC buffer for RNA extraction. RNAs were dephosphorylated with 1 U of Calf Intestine Alkaline Phosphatase (Invitrogen) and 5'-end labelled with T4 Polynucleotide Kinase (NEB) and $\gamma\text{-}^{32}\text{P}$ (Perkin Elmer). Labelled RNAs were precipitated following phenol:chloroform extraction. Radiolabelled RNAs were checked on agarose gel in $1 \times$ TBE buffer and quantified.

In-vitro binding and electrophoretic mobility assays. Final concentration (2 nM) of radiolabelled RNA was adjusted to $5 \mu\text{l}$ 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 (50 mM Tris-HCl pH 7.5, 100 mM KCl, 5 mM MgCl_2 , 0.1 mM CaCl_2 , 1 mM DTT, 0.1 mg ml^{-1} BSA, 0.2 mg ml^{-1} fragmented yeast RNA (Sigma), 5% glycerol, 0.025% bromophenol blue and 0.025% xylene cyanol). The protein was added at the concentrations indicated and 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 $1 \times$ TBE buffer. After 90 min at 90 V of gel electrophoresis, the gels were vacuum dried for 90 min at 80°C and exposed to an Amersham Hyperfilm ECL film.

A five-member PRC2 complex was obtained through co-expression of human Ezh2, His + Flag-tagged Eed, Suz12, Aebp2 and Rbbp4 in High Five cells using a baculovirus expression system (pVL1392-derived transfer vector and Sapphire baculovirus DNA (Allele Biotechnology)). The complex was purified on anti-Flag M2 beads (Sigma) (Supplementary Fig 5e). Purified BSA (NEB) was used as negative control.

Chromatin isolation by RNA purification. CHIRP assays were performed as previously described⁴⁶.

Probe design. The antisense oligonucleotide probes were designed using the online probe designer at single molecule fish (www.singlemoleculefish.com) using the following parameters: (1) number of probes: 1/100 bp of RNA length; (2) target GC% = 45; (3) oligonucleotide length = 20; (4) spacing length = 60–80. The probes, modified at 3'-end with BiotinTEG were obtained from Stanford University (http://pan.stanford.edu). To assess the specificity of the oligonucleotides, the probes were divided into two pools, odd and even. All the experiments were carried out using both pools independently. Antisense oligonucleotides targeting luciferase RNA were used as negative control.

RNA immunoprecipitation. Cells (15×10^7) were grown in 15 cm dishes (NUNC), harvested by trypsinization and cross-linked using 1% glutaraldehyde for 15 min. The cross-linking was quenched by the addition of 1.25 M glycine at room temperature for 5 min. The cells were lysed with 1 ml per 100 mg cell pellet of lysis buffer (50 mM Tris-HCl pH 7, 10 mM EDTA, 1% SDS, 1 mM PMSF, 200 U ml⁻¹ Superscript II (Ambion, Life Technologies) and 1 × protease inhibitor mixture Complete (Roche)) and sonicated using BIORUPTOR sonicator for 75 cycles of 30 s. Sonicated samples were centrifuged at 16100 r.c.f. for 10 min and 1% of the lysate was removed as RNA input and DNA input. Two millilitres of hybridization buffer (750 mM NaCl, 1% SDS, 5 mM Tris-HCl pH 7, 1 mM EDTA, 15% formamide, 1 mM PMSF, 200 U ml⁻¹ Superscript II (Ambion, Life Technologies) and 1 × protease inhibitor mixture Complete (Roche)) together with 100 pmol of each pool of probes were added to 1 ml of chromatin, mixed and incubated at 37 °C for 4 h, while shaking. RNA was pulled down using the DynaMag-2 magnet strip after incubating with C1 magnetic beads (Invitrogen, Life Technologies) for 30 min at 37 °C, while rotating. Pre-warmed washing buffer (2 × NaCl and sodium citrate, 0.5% SDS and 1 mM PMSF) was used to wash the beads. After the last wash, 100 µl were used for RNA isolation and 900 µl for DNA fraction.

RNA isolation. RNA samples were treated with Proteinase K (Ambion, Life Technologies) in PK Buffer (100 mM NaCl, 10 mM Tris-HCl pH 7.0, 1 mM EDTA and 0.5% SDS) at 50 °C for 45 min while shaking and then boiled for 10 min at 95 °C, and RNA was extracted using TRIzol and MIRNeasy mini columns (Qiagen), and then treated with DNase (Qiagen). One microlitre of RNA was used for qRT-PCR analysis, to confirm RNA retrieval. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is used as a negative control.

DNA isolation. DNA was eluted with Elution Buffer supplemented with RNase A (10 mg ml⁻¹) and 10 µl RNase H (10 U µl⁻¹) per ml of DNA Elution Buffer (50 mM NaHCO₃ and 10% SDS). After PK treatment at 50 °C for 45 min, DNA was isolated using PhOH:Chloroform:Isoamyl (Sigma) and used for subsequent qPCR analysis with primers indicated in Supplementary Table 4.

4C and high-throughput sequencing. The 4C-seq protocol was followed essentially as previously described⁶⁴. Briefly, 10^7 cells were cross-linked with 4% formaldehyde in PBS/10% FBS for 10 min at room temperature. After quenching the reaction with 0.125 M glycine, cells were lysed in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Igepal, 1% Triton X-100, 1 mM PMSF and protease inhibitor mixture Complete (Roche)) using a douncer. The cross-linked DNA was digested with a 4-bp cutter restriction enzyme site (*DpnII* and *NlaIII*) followed by proximity ligation and cross-linking removal. A secondary restriction enzyme digestion was performed with a 4-bp restriction enzyme (*NlaIII* and *Csp6I*, respectively) followed again by proximity ligation. Primers from the 4C-seq primer database⁴⁷ with additional 5' overhangs of the adapter sequences for Illumina single-read sequencing were used as viewpoint. The sequences are listed in Supplementary Table 4. Sixteen PCR reactions were carried out using 200 ng of the resulting 4C template per reaction. The products were pooled together and purified using QIAquick PCR purification kit (Qiagen). The quality was assessed using a 2100 expert Bioanalyzer (Agilent) and the samples were sent for deep sequencing using Illumina HiSeq2000 by BGI Genomics (Hong Kong).

Bioinformatic analysis. For the RNA-seq analysis, we ran TopHat (ref1) (v. 2.0.10) in single-end mode with the option library-type = fr-secondstrand. No GTF file was given to Tophat. Bowtie⁶⁵ (v. 2.1.0.0) and SAMtools⁶⁶ (v. 0.1.19.0) were called by TopHat.

For the differential expression analysis, we used the pipeline⁶⁷. Briefly, bam file output from TopHat were sorted and converted to SAM files using SAMtools and reads per gene were counted using HTSeq with a GTF file from GENCODE⁶⁸ (v. 15), with all pseudogenes removed. EdgeR⁶⁹ was used for differential expression analysis. Genes with less than one read per million in more than three samples were removed before analysis. Pairwise contrasts were calculated between all experimental groups (see RNA sequencing).

For analysis of *MIR31HG* and *p16^{INK4A}* expression levels in human melanoma tumours, we used molecular profiles of 226 melanoma tumour samples from The Cancer Genome Atlas. We used DNA copy number calls and somatic mutations from the cBioportal (PMID 22588877) to exclude samples with altered *INK4A* copy number or with mutations targeting *INK4A*. These filters selected 43 *INK4A* wild-type tumour samples for further mRNA expression analysis (Supplementary Table 3). We used normalized RNA-seq read counts estimated by the RSEM algorithm (21816040; http://gdac.broadinstitute.org/runs/analyses__2013_09_23/data/SKCM/20130923/). We then estimated expression levels of individual genes by computing using log2 read counts per million from the RSEM-normalized read counts in each sample.

The 4C-seq mapping and normalization was carried out as described in van de Werken *et al.*⁴⁷ In brief, the 4C-Seq reading primer sequences were demultiplexed from the 4C-seq data and trimmed, from the 5'-end to the first restriction enzyme recognition site. The sequences were mapped, while ignoring quality score, to a database of digested genome fragment ends using the human reference genome build hg19. The interactions near the viewpoint were used to calculate the mean of the normalized coverage for a running window of size 5 kb (indicated by a black line) and a sliding window of 2–50 kb (colour coded in multiscale domainogram⁷⁰). All mean values represent enrichment compared with the maximum 5-kb mean value. The 20th and 80th percentiles were computed and depicted in grey areas around the 5-kb running window (Supplementary Fig. 7a). The 4C-seq contact profile comparison plots (Fig. 7a,b) were generated by combining the mapping⁶⁴ and normalization⁴⁷ as described previously. A running trimmed mean of 10% was calculated to smoothen the 4C-seq data. The R statistical package version 3.1.0 was used for the statistical calculations and for generating the 4C-seq plots. (R-Core-Team: R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2013). The R Gviz package version 1.8.4. was used for plotting the annotation data.

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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 coauthors commented. M.M. and G.M. conducted the experiments. M.M.N. and J.S.P. performed RNA-seq bioinformatics analysis. A.J. analysed publically available data sets. S.A. and K. Hansen helped designing the 4C-seq experiment and H.J.G.v.d.W. designed primers and analysed the 4C-seq data. J.H. and K. Helin provided reagents.

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