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# Small Interfering RNAs Targeting a Chromatin-Associated RNA Induce Its Transcriptional Silencing in Human Cells

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1 **siRNAs targeting a chromatin-associated RNA induce its transcriptional silencing in**  
2 **human cells**

3

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5

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11

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13

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15

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24

25 **Abstract**

26

27 **Transcriptional gene silencing by small interfering RNAs (siRNAs) has been widely**  
28 **described in various species, such as plants or fission yeasts. In mammals, its extent**  
29 **remained somewhat debated. Previous studies showed that siRNAs targeting gene**  
30 **promoters can induce the silencing of the targeted promoter, although the involvement**  
31 **of off-target mechanisms was also suggested. Here, by nascent RNA capture and RNA**  
32 **Pol II ChIP, we show that siRNAs targeting a chromatin-associated non-coding RNA**  
33 **induce its transcriptional silencing. Deletion of the sequence targeted by one of these**  
34 **siRNAs on the two alleles by genome editing further show that this silencing is due to**  
35 **base pairing of the siRNA to the target. Moreover, by using cells with heterozygous**  
36 **deletion of the target sequence, we show that only the wild type allele, but not the deleted**  
37 **allele, is silenced by the siRNA, indicating that transcriptional silencing occurs only in**  
38 **cis. Finally, we demonstrate that both Ago1 and Ago2 are involved in this**  
39 **transcriptional silencing. Altogether, our data demonstrate that siRNAs targeting a**  
40 **chromatin-associated RNA at distance from its promoter induce its transcriptional**  
41 **silencing. Our results thus extend the possible repertoire of endogenous or exogenous**  
42 **interfering RNAs.**

43

44

45 **Introduction**

46

47 RNA interference (RNAi), first discovered in plants during the 1990s and observed later in  
48 other systems, like worms, yeast and mammals is a rapid and efficient tool to induce the  
49 knockdown of specific transcripts. This main mechanism of post-transcriptional gene  
50 silencing is largely conserved throughout evolution. In mammals and non-mammalian  
51 organisms, exogenous or endogenously synthesized small double-stranded RNA molecules of  
52 19 to 24 nucleotides, called small-interfering RNA (siRNA), are loaded on the RISC (RNA-  
53 induced silencing complex) complex containing argonaute proteins (Ago) (1). The two  
54 strands of siRNAs are then dissociated and the passenger strand is ejected. The complex is  
55 then tethered to target RNAs containing a sequence complementary to the guide strand. When  
56 sequence complementarity is high enough, the endoribonuclease activity present in Ago2  
57 cleaves the target RNA between bases 10 and 11 of the siRNA complementary region,  
58 leading to the degradation of the target RNA (2, 3).

59

60

61 Given that the above-described processes usually take place in the cytoplasm, RNA  
62 interference is in this respect a cytoplasmic process functioning at a post-transcriptional level,  
63 and leading to RNA degradation. Nevertheless, in some organisms such as yeast or plants,  
64 RNAi has been described to work also in the nucleus to silence repeated elements and to  
65 nucleate heterochromatin (4). However, the presence of the nuclear RNAi in mammals is  
66 more controversial. Indeed, initial data concluded that intronic sequences cannot be targeted  
67 with RNAi (5, 6). This observation together with the findings that some RISC proteins were  
68 mostly cytoplasmic led to the conclusion that RNA interference is restricted to the cytoplasm  
69 in human cells (5). However, in the last two decades, various studies have suggested the

70 presence and the action of the RNAi machinery in the nucleus of mammalian cells. Indeed,  
71 whereas siRNA loading factors into RISC are exclusively cytoplasmic, other RNAi factors,  
72 such as Dicer, Ago1 and Ago2, have been found in the nucleus and can lead to the siRNA-  
73 mediated cleavage of chromatin-associated RNAs (7-9). Moreover, the preferential nuclear  
74 localization of Ago2 has been evidenced in specific human tissues (10). It was later found that  
75 Ago-miRNA complexes can target and silence post-transcriptionally thousands of pre-  
76 mRNAs in the nucleus in mouse ES cells that contain high levels of nuclear Ago proteins (11,  
77 12), although it is still unclear whether all these genes are directly targeted by Ago proteins.  
78 Co-transcriptional processes such as alternative splicing can also be affected by siRNA  
79 targeting a specific splice site of pre-mRNA [39] Moreover, siRNAs have been successfully  
80 used to decrease the expression of nuclear ncRNAs (7, 13), including by ourselves (14-16).

81 In addition, transcriptional gene silencing by siRNAs was also reported in mammalian cells.  
82 siRNAs directed against the promoter of the elongation factor 1 $\alpha$  gene repress the expression  
83 of a reporter gene driven by this promoter (17). Further studies confirmed for other genes that  
84 siRNAs can repress transcription when targeting promoters (18-25) or first exons (13). This  
85 was also extended to miRNAs or siRNAs targeting downstream regions of protein-coding  
86 genes (26, 27). Transcriptional gene silencing requires an RNA template (28, 29), such as  
87 promoter-associated antisense transcripts (30) or ncRNAs overlapping the gene promoter or  
88 downstream regions (25, 31) and involves either Ago1 or Ago2 (18, 31, 32). The targeting of  
89 the siRNA to promoters leads to the deposition of repressive chromatin marks at these  
90 promoters, such as H3K9me3 and H3K27me3 (9, 20, 24, 32-34) or CpG methylation (19, 22),  
91 resulting in transcriptional gene repression. Note that it was proposed that the previously  
92 reported transcriptional repression by siRNAs of the VEGF gene promoter and HIV LTR  
93 could be due to off-target effects, since a similar effect was observed on reporter systems with  
94 a deleted or mutated target site (35). For the HIV LTR, a later study showed that siRNAs

95 targeting HIV1 LTR are specific to the HIV1 LTR and do not affect the highly related HIV2  
96 LTR neither a panel of other genes regulated in a similar way, arguing against purely off  
97 target effects (36).

98 Despite these studies, to our knowledge, siRNAs repressing promoters while targeting RNAs  
99 far downstream the promoter region has not been reported so far. Here, we show that siRNAs  
100 directed against a chromatin-associated RNA from the vlincRNA (very long intergenic non-  
101 coding RNA) family can silence its promoter even when targeting sequences are located  
102 thousands of bases downstream from the promoter. Moreover, through the use of genome  
103 editing, we found that this effect occurs in cis and is not due to off target mechanisms,  
104 therefore demonstrating RNA interference mechanisms acting at distance in mammals.

105

106

107

## 108 **Results**

109

### 110 **siRNAs can efficiently decrease the expression of VINK nuclear RNA**

111

112 In previous works, we successfully used siRNAs to deplete the expression of various RNAs  
113 associated with chromatin (14-16). This includes depletion of VAD, a senescence-specific  
114 ncRNA which belongs to the vlincRNA family (16). More recently, we investigated another  
115 vlincRNA also strongly associated with chromatin with more than 85 % present in the  
116 chromatin fraction and very weakly spliced, which we named VINK (Ouvrard et al., in  
117 preparation). During the course of this investigation, we designed and transfected siRNAs  
118 directed against this vlincRNA in RAF1 oncogene-induced senescent WI38 human cells. By

119 RT-qPCR, we observed that an siRNA against VINK (VINK-1 siRNA) efficiently inhibits  
120 VINK expression when measured at various places along the VINK RNA (Figure 1A and  
121 1B). A similar result was obtained with a second independent couple of control and VINK  
122 siRNAs (VINK-2 siRNA), although VINK inhibition was slightly less efficient with this  
123 siRNA (Figure 1A and 1C). A dose response showed that the effect was maximal from 10 nM  
124 of siRNA (data not shown). The efficient depletion of VINK using each of these two siRNAs  
125 was also observed on RNA-Seq experiments (Figure 1D). Thus, siRNAs against the VINK  
126 vlnrcRNA efficiently repress VINK expression.

127 From these RNA-seq datasets, analysis of reads containing at least one spliced junction  
128 showed that VINK is indeed poorly spliced with less than 0.4 % of spliced reads relative to all  
129 reads aligned in VINK region. For comparison, we previously showed that the median  
130 percentage of spliced reads in annotated genes is around 20 % (15). The main splicing  
131 junctions are indicated in Fig1A and listed in Table S3 along with the locations of primers and  
132 siRNA sequences. The most abundant splicing event occurs between a region close to the  
133 TSS and a region around 100 kb downstream (see the sharp peaks in RNA -seq datasets in  
134 Fig. 1D and Table S3).

135 Data showing that siRNAs can efficiently decrease VINK expression , as well as the  
136 expression of other nuclear RNAs, which we have published previously (14-16), were  
137 obtained in senescent WI38 cells expressing the hTERT, the catalytic subunit of the human  
138 telomerase and an ER-RAF1 fusion protein. To test whether the depletion of VINK RNA by  
139 siRNAs can be observed in other cell types, we made use of the human osteosarcoma U2OS  
140 cell line that also expresses VINK. In U2OS cells, VINK is truncated from its 5' end, the  
141 transcript starting around 75 nt upstream the region targeted by the siRNA-1 (data non  
142 shown). Transfection of each of the two siRNAs against VINK efficiently repressed VINK  
143 expression (Figure 1E).

144 Thus altogether, these data indicate that siRNAs can efficiently induce the knock-down of the  
145 chromatin-associated VINK RNA.

146

#### 147 **siRNAs affect the transcription of their targeted nuclear RNAs**

148

149 Given that the use of siRNAs to target nuclear RNAs is debated, we intended to investigate  
150 the mechanism of this effect. To that goal, we first analyzed whether siRNAs directed against  
151 VINK induced its degradation. We transfected senescent WI38 cells with the VINK-1 siRNA  
152 (which is the most efficient, see Figure 1B) or control siRNA and treated transfected cells  
153 with actinomycin D to block the transcription. The decrease of VINK expression along time  
154 reflects its stability. Using this experimental setting, we found that the half-life of VINK was  
155 between 100 and 200 minutes, depending on the region of VINK analyzed (Figure 2A). This  
156 half-life was longer when we analyzed the 5' extremity of VINK (VINK-A and VINK-B),  
157 certainly because these regions are found in spliced forms of VINK (Fig 1A Suppl Tables ).  
158 However, wherever investigated, VINK half-life was not significantly changed in cells  
159 transfected with the VINK-1 siRNA. Moreover, no difference in its half-life was observed  
160 upon VINK-1 siRNA transfection following either short or long periods of actinomycin  
161 treatment (Figure 2A and data not shown). Thus, these experiments suggest that the stability  
162 of VINK RNA is not affected by the presence of VINK siRNAs.

163

164 We next assessed whether VINK siRNAs affected VINK transcription. To that goal, we  
165 analysed ongoing transcription by nascent RNA capture, which relies on metabolic labelling  
166 of RNAs being transcribed with a nucleotide analogue, allowing their biotinylation and  
167 purification on streptavidin beads. Analysis of recovered nascent RNAs thus provides an  
168 accurate measure of ongoing transcription. We transfected senescent WI38 cells with each of



169 the two siRNAs against VINK and measured nascent transcripts at different locations along  
170 VINK (Figure 2B). We found that the presence of VINK nascent RNA was strongly  
171 decreased upon transfection of the two VINK siRNAs in all regions analyzed (Figure 2B).  
172 Thus, siRNAs targeting VINK RNA decrease its transcription. This effect was also observed  
173 in U2OS cells (Figure 2C), indicating that it is not a specific feature of the WI38 cell line.

174

175 To test whether this effect is restricted to VINK or to RNAs from the vlincRNA family, we  
176 transfected senescent WI38 cells with a siRNA directed against the ARHGAP18 read-through  
177 RNA belonging to the family of START RNAs that we characterized in a previous study (14).  
178 Read-through RNAs are produced by transcription beyond the transcription termination site  
179 and are known to be nuclear/chromatin associated (37). We also observed that the presence of  
180 ARHGAP18 START nascent RNA was strongly decreased upon transfection of the siRNA  
181 targeting it (Figure 2D). ARHGAP18 nascent transcript from which the START RNA  
182 originates was also decreased, as measured close to its TSS in its first exon and intron located  
183 about 170 kb upstream the siRNA targeted site (Figure 2D). These results indicate that the  
184 effect of the siRNA directed against ARHGAP18 START RNA is also transcriptional.

185 Thus, we conclude from these experiments that siRNAs can repress the transcription of the  
186 chromatin-associated RNA they target, even when the targeted site is far from the promoter.

187

188

### 189 **siRNAs targeting VINK RNA influence RNA polymerase II presence at VINK TSS**

190

191 Inhibition of nascent RNA upon siVINK transfection was observed throughout the VINK  
192 transcribed region, including when analyzing regions upstream of the siRNA-targeted  
193 sequence (Figures 1 and 2). siRNA-mediated inhibition of nascent transcription by premature

194 transcription termination was recently observed in drosophila (38). To analyse whether VINK  
195 siRNAs induce premature transcription termination or involve the repression of VINK  
196 promoter, we analyzed the recruitment of RNA pol II to TSS which would be unaffected by  
197 the induction of premature termination. We thus transfected the VINK-1 or VINK-2 siRNAs  
198 and performed chromatin immunoprecipitation (ChIP) using an antibody that recognizes total  
199 RNA pol II. We found that both siRNAs against VINK inhibited the presence of RNA pol II  
200 at VINK TSS compared to control GAPDH TSS (Figure 3B and data not shown for VINK-2  
201 siRNA), indicating that the VINK siRNAs affect RNA pol II recruitment at VINK promoter.  
202 We next analyzed whether this was also associated with the regulation of chromatin marks.  
203 We found that the VINK-1 siRNA decreased the presence of the transcription-associated  
204 H3K27ac, H3K4me3 and H3K36me3 marks at VINK TSS, whereas the repressive mark  
205 H3K9me3 was unaffected (Figure 3C). H3K27me3 was undetectable at VINK TSS, even in  
206 the presence of VINK-1 siRNA (data not shown). Thus, these data indicate that siRNAs  
207 targeting VINK decrease the presence of transcription-associated chromatin marks and of  
208 RNA Pol II at VINK TSS, therefore decreasing VINK transcription. To our knowledge, such  
209 an effect on a promoter chromatin landscape induced by siRNAs targeting RNAs thousands  
210 of bases downstream has never been reported so far.

211

212

213 **Repression of transcription by VINK siRNA is dependent on base-pairing to its target**  
214 **site**

215

216 We next tested whether the effect of VINK siRNA on VINK transcription is due to base-  
217 pairing and not to off-target effects. Indeed, in some instances, transcriptional silencing using  
218 siRNAs was attributed to off-target effects (35).

219

220 To analyze whether pairing of the siRNA to its target site is involved, we raised, by genome  
221 editing in WI38 cells, a stable cell line that we called WI38- $\Delta/\Delta$  #1, in which 210 bp  
222 encompassing the target site for the VINK-1 siRNA are deleted on the two alleles (data not  
223 shown). We next transfected WI38- $\Delta/\Delta$  #1 cells with the siRNAs directed against *VINK*.  
224 Whereas the VINK-2 siRNA works as in parental cells (compare Figures 4A and 1C), the  
225 VINK-1 siRNA, for which the sequence is not present in WI38- $\Delta/\Delta$  #1 cells, did not repress  
226 VINK expression (Figure 4A). Importantly, a similar result was also observed in another  
227 independent clone deleted for the VINK-1-targeted sequence on the two alleles (WI38-  
228  $\Delta/\Delta$  #2) (Figure 4B). The absence of effects of the VINK-1 siRNA on VINK expression in  
229 WI38- $\Delta/\Delta$  #2 cells was also obvious from RNA-seq data when comparing the effect of the  
230 VINK-1 siRNA in parental WI38 (+/+) and deleted WI38- $\Delta/\Delta$  #2 cells (Figure 4C).

231 Thus, removal of its target sequence abolished the effect of the VINK-1 siRNA on VINK  
232 expression, indicating that it represses the expression of its target RNA through base pairing  
233 to its target sequence. Of note, in the conditions we used to achieve VINK knock-down,  
234 VINK-1 siRNA induces prominent off-target effects with significant changes in the  
235 expression of thousands of genes upon siRNA transfection in the cell line in which the siRNA  
236 target site was deleted (Figure 4D).

237

238

### 239 **VINK siRNA requires cis-targeting to repress transcription of VINK**

240

241 All together the above-described data unambiguously demonstrate that the VINK-1 siRNA  
242 represses VINK transcription by a process involving base-pairing. This suggests that VINK-1  
243 siRNA targets VINK RNA and induces chromatin modifications at VINK promoter in cis that

244 would lead to the repression of transcriptional initiation. However, we cannot formally rule  
245 out the possibility that the VINK-1 siRNA targets and induces the degradation by  
246 conventional RNAi mechanisms of a sub-population of VINK, an effect that would not be  
247 observed in our previous experiments because it only represents a minor proportion of VINK.  
248 If this population was able to indirectly regulate VINK expression, its depletion could then be  
249 signaled to the VINK promoter to repress VINK transcription. Of note, the VINK-1 siRNA  
250 targets a region located 300 bases upstream from a donor splice site (see Table S1 and S3),  
251 and may thus target a spliced product of VINK.

252 To formally demonstrate that VINK siRNAs act in cis, we raised by genome editing a cell  
253 line in which only one of the two alleles encoding VINK can be targeted by the VINK-1  
254 siRNA, the other allele being deleted for the target sequence (that we called WI38- $\Delta$ /+). If the  
255 effects of siRNAs were due to base pairing at chromatin inducing local chromatin  
256 modifications, only the siRNA-targeted allele would be repressed. On the contrary, if the  
257 repression of VINK transcription was indirect, then the two alleles should be similarly  
258 regulated. We thus transfected WI38- $\Delta$ /+ cells with VINK-1 and VINK-2 siRNAs and  
259 performed RT-qPCR monitoring allele-specific VINK expression (see Figure 5A for the  
260 design of PCR primers). Each of the two siRNAs inhibited the expression of the wild type  
261 allele with a higher effect of the VINK-1 siRNA compared to the VINK-2 siRNA (Figure  
262 5B), as in previous experiments (see Figure 1B-D). By contrast, on the deleted allele, while  
263 we still observed repression by the VINK-2 siRNA, repression by the VINK-1 siRNA was  
264 abolished (Figure 5C). The effect of the VINK-1 siRNA on total VINK expression in WI38-  
265  $\Delta$ /+ was weaker than on the wild type allele (compare Figure 5D with Figure 5B) or than in  
266 parental cells (compare Figure 5D with Figure 1B), as expected since only one allele was  
267 affected.

268 Altogether, these data thus demonstrate that the effect of VINK-1 siRNA on VINK  
269 transcription is mediated in *cis* by base-pairing of the siRNA to its target site on the  
270 chromatin-associated VINK RNA.

271

### 272 **Ago1 and Ago2 are involved in siRNA-mediated VINK transcriptional repression**

273

274 It was shown that transcriptional silencing by siRNA targeting promoters can involve either  
275 Ago1 or Ago2 (18, 31, 32). We thus investigated whether these proteins also participate in the  
276 transcription repression by siRNAs targeting VINK. We first tested whether Ago1 or Ago2  
277 can be associated with chromatin in WI38 cells, since the nuclear localization of Ago proteins  
278 is somewhat debated. To that goal, we performed cell fractionation experiments to obtain  
279 cytoplasmic, nuclear soluble and chromatin protein fractions (Figure 6A). Cell fractionation  
280 was effective, since  $\alpha$ -tubulin and GAPDH were mostly found in the cytoplasmic fraction,  
281 PARP and HDAC1/2 in the nuclear soluble fraction and histone H3 in the chromatin fraction.  
282 Interestingly, both Ago1 and Ago2 were detected in the three fractions, with an important  
283 amount present at chromatin. Comparison with the GAPDH or  $\alpha$ -tubulin profiles indicates  
284 that this amount is unlikely due to contamination of the chromatin fraction with cytoplasmic  
285 proteins, and thus indicates that Ago1 and Ago2 are present at the chromatin in significant  
286 amounts. To test whether Ago1 or Ago2 expression was required for transcriptional  
287 repression by VINK1 siRNA, we made use of siRNAs targeting either Ago1 or Ago2. These  
288 two siRNAs were efficient and specific as observed by western blot (Figure 6B). We thus  
289 tested the effect of Ago1 and/or Ago2 knockdown on VINK repression by the VINK-1  
290 siRNA. We found that siRNAs against Ago1 and Ago2 have a similar efficiency on their  
291 respective target whether or not they were transfected together with control or VINK-1  
292 siRNAs, indicating that transfection of many siRNAs together did not affect their efficiency

293 (Figure 6C and D). In contrast, the effect of the VINK-1 siRNA was weaker in the presence  
294 of either Ago1 or Ago2 siRNA (Figure 6C) and was nearly abolished when knocking-down  
295 both Ago1 and Ago2 (Figure 6D). Importantly, this last result was also observed when  
296 analyzing nascent RNA expression, confirming that it occurs at the transcriptional level  
297 (Figure 6E). Thus, these results indicate that both Ago1 and Ago2 are involved in siRNA-  
298 mediated transcriptional repression of VINK.

299

300

### 301 **Discussion**

302

303 In this study, we show that siRNAs can induce efficient and specific transcriptional silencing  
304 of an RNA associated with chromatin. We observed the silencing of VINK promoter using  
305 siRNAs targeting the RNA in regions located thousands of bases downstream of the promoter,  
306 a phenomenon that, to our knowledge, has never been described so far in mammals. We  
307 further demonstrate the involvement of Ago1 and Ago2 in this process. This silencing was  
308 lost when the sequences targeted by the siRNA were deleted, ruling out off-target effects and  
309 demonstrating that base-pairing to its target sequence is required. Note that we cannot  
310 formally conclude whether this base pairing occurs on VINK RNA or on its transcribed  
311 template DNA.

312

313 The first question raised by our finding is whether the interference we have shown here with  
314 exogenously delivered siRNAs can also take place in natural conditions with endogenous  
315 siRNAs. Indeed, in various species, RNAi mechanisms have been shown to be involved in  
316 transcriptional silencing occurring at specific chromatin domains such as pericentric  
317 heterochromatin in fission yeast (4). Moreover, the involvement of endogenous antisense

318 RNAs in the establishment or maintenance of heterochromatin silencing is well established  
319 (34, 39). Interestingly, it was recently proposed that tRNA-derived small RNAs (tsRNA)  
320 could be endogenous regulators of many genes, albeit by a different interference mechanism  
321 requiring cleavage by Ago2 (12), and other examples of endogenous siRNAs produced from  
322 protein-coding gene bodies have been described (40). Thus, we can speculate that endogenous  
323 RNAs complementary to chromatin-associated RNAs could be important regulators of the  
324 expression of these RNAs in mammals by triggering RNAi and transcriptional gene silencing.

325

326 To our knowledge, our study is the first to show that interfering RNAs targeting a RNA in  
327 regions far downstream of a promoter can induce its transcriptional silencing in  
328 mammals. Such long-distance effects have been widely described in other species, in  
329 particular in plants. In these cases, it relies on RNA-dependent RNA polymerase (RdRP)  
330 which mediates amplification and propagation of double strand RNAs towards the 5' end of  
331 RNAs (4). However, in mammals, RdRP is not conserved. What could thus be the mechanism  
332 involved in the process we describe here? It is known that double strand RNAs targeting the  
333 vicinity of a promoter (upstream promoter or the first exon) can lead to transcriptional  
334 silencing of the promoter through the induction of repressive epigenetic marks in a manner  
335 dependent on some RISC components (19, 22, 24). Here, we can speculate that the silencing  
336 of the promoter at distance of the siRNA target site could be achieved by chromatin folding  
337 that would bring the VINK/siRNA hybrid and the promoter in close proximity. As a  
338 consequence, this would change the chromatin landscape at the promoters, as proposed for  
339 miRNAs targeting the 3' end of genes (26, 27). Of note, the siVINK-1 siRNA used in our  
340 studies targets a sequence close to an internal promoter, which is active in U2OS cells (data  
341 not shown) and probably also in WI38 (Ouvrard et al., in preparation). This second VINK  
342 TSS could favor its spatial proximity with the VINK promoter located more than 30 kb

343 upstream. However, the fact that we observe transcriptional silencing with many different  
344 siRNAs argues against the importance of specific preexisting chromatin folding that would  
345 drive this silencing.

346

347 Contrary to the studies showing transcriptional gene silencing by miRNA targeting  
348 downstream regions of genes (26, 27), we observed a decrease in the presence of marks  
349 associated with transcription rather than an increase in repressive marks. Our data suggest the  
350 involvement of Ago proteins in this silencing. First, mere steric blocking by the VINK/siRNA  
351 hybrid is probably not sufficient for this silencing, since an antisense oligonucleotide  
352 targeting the same sequence as the VINK-1 siRNA does not induce transcriptional silencing,  
353 despite readily decreasing VINK expression (data not shown). Moreover, we found that Ago1  
354 and Ago2 proteins depletion partially reversed VINK repression and that significant levels of  
355 Ago1 and Ago2 proteins are present at the chromatin in WI38 cells. However, by ChIP, we  
356 were not able to detect binding of Ago1 or Ago2 to the site targeted by the siRNA. We thus  
357 cannot formally rule out the possibility that the requirement of Ago proteins is indirect. Along  
358 this line, it would be interesting to analyse the relative importance of the siRNA seed domain  
359 versus other parts of the siRNA and whether 100% complementarity is required for silencing.

360 Despite the fact that intronic sequences are also found associated with chromatin, siRNAs  
361 directed against intronic sequences of pre-mRNAs are usually not effective to decrease the  
362 expression of pre-mRNAs (5, 6, 41). This finding first indicates that transcription of the target  
363 sequence is not sufficient to induce transcriptional silencing by siRNA, suggesting that the  
364 target of the siRNA is chromatin-associated RNA but not the DNA being transcribed. Splicing  
365 occurs co-transcriptionally leading to the degradation of introns, which thus greatly decrease  
366 the window of opportunity for the siRNA to bind efficiently its target RNA or to induce



367 chromatin modifications when targeted. Moreover, siRNAs directed against a lncRNA did not  
368 induce transcriptional silencing, except when targeting the first exon (13). Thus, whatever  
369 the mechanism involved, it does not operate on any RNA associated with chromatin and we  
370 can speculate that the difference lies in the time of residence of the targeted RNA at  
371 chromatin.

372

373 The use of siRNAs to silence nuclear RNAs has been debated for long, because of the low  
374 levels of some RNAi proteins in the nucleus. As a consequence, siRNA-mediated degradation  
375 of RNA is mostly efficient in the cytoplasm. Nonetheless, as discussed above, siRNAs  
376 targeting promoters can in some instances repress transcription from the promoter they target.  
377 Here, we show that the promoter of chromatin-associated RNAs can be silenced using  
378 siRNAs targeting these RNAs in regions located far downstream of the promoter. Thus,  
379 siRNAs can represent interesting tools to investigate the function of chromatin-associated  
380 RNA by allowing their efficient and specific knock-down, even if the promoter is not well  
381 characterized or if the RNA is produced through multiple promoters. In addition, and coupled  
382 to a method allowing the degradation of RNA without interfering with any transcription step,  
383 it may help to distinguish between the role of the RNA itself and the role of its transcription,  
384 an ever-raising question in the field. Most importantly, since we found that siRNAs inhibit the  
385 transcription of the allele they target but not of the allele they do not target, they could allow  
386 studying *cis* effects of nuclear non coding RNAs or of their transcription, provided that an  
387 existing polymorphism or heterozygous genome editing, as we have done here, allows to  
388 target a single allele.

389

390 Note however that in the conditions we used to achieve the knock-down of VINK, VINK-1  
391 siRNA induces prominent off-target effects with significant changes in the expression of

392 thousands of genes upon siRNA transfection in the cell line in which the siRNA target site  
393 was deleted. Moreover, the use of two independent siRNAs was not sufficient to cope with  
394 off-target effects. Indeed, most of the genes showing expression changes with two  
395 independent siRNAs directed against VINK in wild type cells have a tendency to be changed  
396 in a similar way in the cell line deleted for the siRNA-1 target site (data not shown). These  
397 off-target effects are probably due to the fact that an siRNA can bind to and degrade target  
398 RNAs without perfect sequence complementarity, the seed region of only 6-7 nucleotides in  
399 the siRNA being involved in targeting numerous RNAs (42). It is possible that they are  
400 exacerbated when siRNAs do not have any target in the cytoplasm, such as for siRNAs  
401 directed against chromatin-associated RNAs or control siRNAs. Indeed, we also found  
402 thousands of DE genes between two different control siRNAs (data non shown). Importantly,  
403 we have not been able to dissociate the base pairing-dependent repression of VINK  
404 expression to off-target effects by changing the amount of siRNAs or the time after  
405 transfection at which we harvested cells (data not shown). It will thus be critically important  
406 for functional studies to design an experimental strategy ruling out off-target effects, such as  
407 overexpression of a siRNA-resistant form of the targeted RNA, or, as we performed here,  
408 removal of the siRNA target site by genome editing. When possible, this latter strategy is  
409 probably better since it maintains the normal production of the RNA at the normal place in the  
410 genome and in the nucleus, which is sometimes critical for the function of non-coding RNAs.

411

412 Finally, our findings suggest that siRNAs targeting chromatin-associated RNAs may have  
413 therapeutic potential. Indeed, the therapeutic value of siRNAs is currently widely investigated  
414 (43). Moreover, the relation of chromatin-associated RNAs with cancer progression has been  
415 documented in many instances, such as PVT1, H19, MALAT1 and HOTAIR (for a recent  
416 review see (44)). RNAs from the vlincRNA family are also overexpressed in cancer cells

417 (45), and this overexpression may participate in allowing these cells to survive genotoxic  
418 stress (46). Targeting these RNAs with siRNAs may thus have therapeutic potential.  
419 Moreover, by modifying the chromatin landscape at their promoters and thus potentially  
420 reversing epigenetic information, it may have long-term benefits.  
421

422

## 423 **Materials and Methods**

424

### 425 **Cell culture**

426

427 WI38-hTERT/ER-RAF1 immortalized fibroblastic cell line was kindly provided by C. Mann  
428 (47). They were grown in MEM supplemented with L-glutamine, non-essential amino acids,  
429 sodium pyruvate, penicillin-streptomycin and 10% of fetal bovine serum under 5% CO<sub>2</sub> and  
430 5% O<sub>2</sub>. Senescence was induced by treating cells with 20 nM of 4-hydroxy-tamoxifen for  
431 three days. U2OS cells (ATCC) were grown in DMEM containing Glutamax supplemented  
432 with sodium pyruvate, penicillin-streptomycin and 10% of foetal bovine serum under 5%  
433 CO<sub>2</sub>. WI38 cells and derivatives were transfected using the siRNA transfection reagent  
434 Dharmafect 4 (Dharmacon) following the manufacturer's instructions with 100 nM of siRNA  
435 unless indicated in Figure legend and harvested 72 hours later. U2OS cells were transfected  
436 with the Interferin transfection reagent (Polyplus) or with Dharmafect 4 according to the  
437 manufacturers' instructions with 50 nM of siRNA and harvested 48 hours later. For RNA  
438 stability analysis, actinomycin D (Sigma) was added at 10 µg/mL. siRNAs used are listed in  
439 Table S1:

440

### 441 **Cell fractionation, western blots and antibodies**

442

443 Phosphatases and proteases inhibitors (thermofisher, # 78441) were added in all buffers. Fresh  
444 trypsinized cell were lysed in 5 cell volumes of lysis buffer (10 mM Tris pH8; 10 mM NaCl;  
445 2 mM MgCl<sub>2</sub>). After 5 min on ice, 0.5% NP-40 was added. After another 10 min on ice, one  
446 third of the volume was kept as whole cell extract, the remaining lysate was centrifuged 10

447 min at 5000 rpm. The supernatant corresponded to the cytosolic fraction. The pellet (nuclei)  
448 was resuspended in 1 volume of buffer 3 (20 mM Hepes pH 7.9 ; 420 mM NaCl ; 1.5 mM  
449 MgCl<sub>2</sub> ; glycerol 10%), then incubated 30 min on ice, shaking every 5 min. After 10 min of  
450 centrifugation at max speed, the supernatant was kept as nuclear soluble fraction. The pellet  
451 was the chromatin fraction. All the fractions (cytosol, nuclear soluble and chromatin) were  
452 equilibrated to the same final volume and same buffer compositions, adding 1% SDS in each.  
453 The whole cell extract was also adjusted to the same composition. The whole cell extract and  
454 chromatin were sonicated until viscosity disappeared. Laemmli sample buffer was added to  
455 the samples and the same volume of each fraction was loaded on an SDS-PAGE, except for  
456 the whole cell extract for which twice the volume of fractions was loaded.

457

458 Western blots were performed using standard protocols. Ago1 and Ago2 were detected using  
459 the monoclonal anti-AGO1 (6H1L4) antibody from Invitrogen (used at 1/1000) and the  
460 monoclonal anti-AGO2 (R.386.2) antibody from Invitrogen (1/1000), GAPDH was detected  
461 using the monoclonal anti-GAPDH (MAB374) antibody from Millipore (1/10000), PARP  
462 with anti-PARP from Cell signaling at 1/1000 (9542), HDAC1, HDAC2 and HDAC3 with  
463 anti-HDAC-3 from BD transduction lab at 1/500 (611125) , H3 with anti-H3 from Abcam at  
464 1/1000 (Ab1791) and  $\alpha$ -tubulin with anti-tubulin from Sigma at 1/1000 (T6199).

465

466 For ChIP experiment, anti-RNA pol II was from Bethyl laboratories (A304-405A), anti-  
467 H3K27ac from Abcam (Ab4729), anti-H3K4me3 from Diagenode (pAb-003), anti-H3K9me3  
468 from Abcam (Ab8898) , anti-H3 from Abcam (Ab1791)and anti-H3K36me3 from Abcam  
469 (Ab9050). Purified rabbit IgG control was from Millipore (PP64B).

470

471 **Genome Editing**

472

473 Genome editing of WI38 was performed with CRISPR-Cas9 tools in ribonucleic particle  
474 (RNP) format. Briefly, the Alt-R S.p. HiFi Cas9 Nuclease V3 (IDT) was assembled with  
475 equimolar amount of hybridized sgRNA (tracrRNA and crRNA from IDT) to generate RNPs.  
476 According to IDT recommendations, RNPs were transfected at 5 nM each with Lipofectamine  
477 CRISPRMAX (Thermofisher). Cells were harvested one week later to analyse the efficiency  
478 of genome editing. Genomic DNA was extracted with Master Pure purification kit (Epicentre)  
479 according to the manufacturer's instructions, except that 250 µg of Proteinase K were used for  
480 cell lysis. Genome editing efficiency was checked by PCR on genomic DNA from the  
481 transfected population with the GoTaq G2 polymerase (Promega) using primers surrounding  
482 the 210 bp deleted region. PCR products were visualized on an agarose gel. Clones were then  
483 isolated and screened by PCR as described above. Two homozygous clones and one  
484 heterozygous clone were selected. The sequences of sgRNAs are the following ones:

485 sgRNA-1 : TAGGAGGCCAGTGCTCCAGG

486 sgRNA-2 : TTCCTGACTCTTGAAAACCA

487

488

489

490

491

#### 492 **RNA extraction, reverse transcription and qPCR**

493

494 Except when indicated, total RNA was prepared using the MasterPure RNA Purification Kit  
495 (Epicentre) according to the manufacturer's instructions, except that 250 µg of Proteinase K  
496 was used for cell lysis. In Figure 2A, TRIzol (Sigma) was used. Briefly, adherent cells are  
497 directly lysed with 0.1 mL/cm<sup>2</sup> of TRIzol during 10 min, the lysate is harvested before adding

498 0.1 mL of chloroforme per mL of TRIzol. After centrifugation, the upper phase is collected  
499 and the Epicentre kit is used for the following DNase digestion and subsequent precipitation.  
500 After total nucleic acids recovery, DNA was removed with a cocktail of DNaseI and Baseline  
501 Zero DNase supplemented with Riboguard RNase Inhibitor for 45 min at 37°C.  
502 500 ng of total RNA were reverse transcribed with Superscript III reverse transcriptase  
503 (Invitrogen) according to manufacturer's instructions. A condition without Superscript III was  
504 performed for each sample and analysed by qPCR using GAPDH exon 9 primers to check for  
505 DNA contamination. qPCR was performed in triplicate with TB green Premix Ex Taq from  
506 Takara and the Biorad CFX thermocycler. GADPH mRNA expression was used to normalize  
507 the results of RT-qPCR. Primers are listed in Table S2.

508

#### 509 **Nascent RNA capture**

510

511 Cells were incubated with 0.2 mM of ethynyl-uridine, a uridine analogue, for 1h. Cells were  
512 then harvested, total RNA was prepared and nascent RNA was purified from 2 µg of total  
513 RNA with the Click IT Nascent RNA capture kit from Life Technologies according to the  
514 manufacturer's recommendations.

515

516

#### 517 **Chromatin immunoprecipitation (ChIP)**

518

519 ChIP was performed on  $10 \cdot 10^6$  transfected senescent WI38 cells as previously described (16),  
520 except that the nuclear lysis buffer was diluted twice before use, and sonicated nuclei were  
521 diluted 5 times in the dilution buffer. 10 million cells transfected with siRNA were  
522 crosslinked for 15 min using 1% formaldehyde directly in the culture medium. 0.125 M of

523 glycine were then added for 5 min. After two washes with PBS, cells were scraped and frozen  
524 at -80°C. Cells were lysed with 3 ml of lysis buffer (5 mM Pipes pH 8, 85 mM KCl, 0.5%  
525 NP40) and homogenized 40 times with a dounce (20 times, pause 2 minutes, 20 times). After  
526 centrifugation, nuclear pellets were resuspended in 1.5 ml of nuclear lysis buffer (25 mM Tris  
527 pH 8.1, 5 mM EDTA, 0.5% SDS), and sonicated 10 times for 5s (power setting 0.5 ON 0.5  
528 OFF and 50% amplitude, Branson Sonifier 250), to obtain DNA fragments of about 500 bp.  
529 DNA concentration was determined using a Nanodrop and samples were adjusted to the same  
530 concentration of chromatin. Samples were diluted five times in dilution buffer (0.01% SDS,  
531 1.1% Triton X-100, 1.2 mM EDTA pH 8, 17 mM Tris pH 8.1, 167 mM NaCl) and precleared  
532 for 2 h with 250 µL of previously blocked 50% protein-A and protein-G beads (Sigma P-7786  
533 and P-3296 respectively). Blocking was achieved by incubating the beads with 0.5 mg/ml of  
534 Ultrapure BSA and 0.2 mg/mL of salmon sperm DNA for 4 h at 4°C. 100 µL of chromatin  
535 were kept for inputs. 100 µg of pre-cleared samples per CHIP were incubated overnight for  
536 RNA pol II CHIP or 40 µg for histones CHIP with 4µg of antibody at 4°C. A mock sample  
537 without antibody was processed similarly. Then, 40 µL of blocked 50% A/G beads were  
538 added for 2h at 4°C to recover immune complexes. Beads were washed once in dialysis buffer  
539 (2 mM EDTA, 50 mM Tris pH 8, 0.2% Sarkosyl), five times in wash buffer (100 mM Tris pH  
540 8.8, 500 mM LiCl, 1% NP40, 1% NaDoc) and twice in TE buffer (10 mM Tris pH 8, 1 mM  
541 EDTA). The bead/chromatin complexes were resuspended in 200 µL of TE buffer and  
542 incubated 30 min at 37 °C with 10 µg of RNase A (Abcam), as well as input DNA.  
543 Formaldehyde crosslink was reversed in the presence of 0.2% SDS at 70°C overnight with  
544 shaking. After 2 h of proteinase K (0.2 mg/ml) treatment at 45°C, immunoprecipitated and  
545 input DNA were purified on columns using Illustra GFX kit (GE Healthcare). All buffers for  
546 CHIP experiment were supplemented with EDTA-free protease inhibitor cocktail (Roche) and  
547 filtered 0.2 µM. Results were analysed by qPCR. Primers are listed in Table S2.



548

549

550 **Statistics**

551

552 In all figures with statistical analyses, data were normalized to 1 relative to a control which  
553 was included in each experiment (usually a control siRNA). For significance analysis, the  
554 Log<sub>2</sub> of fold change compared to this control was calculated in order to obtain data with a  
555 normal distribution. All experiments were then pooled together to calculate the variance of  
556 these experiments with a sufficient number of points. We then calculated using the student t  
557 test with this fixed variance the probability that the mean of each data set is equal to 0 (the  
558 value of the control), which represents the p value of the difference to the control. In some  
559 cases, we calculated using the paired student t test the probability that the mean of two  
560 populations is equal, which represents the p value of the difference between the two  
561 populations. A star is included in the Figure when the p value is below 0.05. The p value is  
562 indicated in the figure when it is between 0.05 and 0.1. When above 0.1, the difference is  
563 considered as non-significant and “ns” is indicated.

564

565 **RNA-seq**

566

567 We performed strand-specific RNA-Seq method, relying on UTP incorporation in the second  
568 cDNA strand. For each sample, 5-10 µg of total RNA was submitted to EMBL-GeneCore,  
569 Heidelberg, Germany. Paired-end sequencing was performed by Illumina’s NextSeq 500  
570 technology. Two replicates of each sample were sequenced.

571 The quality of each raw sequencing file (fastq) was verified with FastQC (48). Files were  
572 aligned to the reference human genome (hg38) in paired-end mode with STAR Version 2.5.2b

573 and processed (sorting and indexing) with samtools (49). rDNA contamination was removed  
574 in the alignment process. Uniquely aligned reads were counted, per gene\_id, using HT-seq  
575 Version 0.6.1 in a strand specific mode with the union count method parameter (50). The  
576 features' annotation used for counts was constructed from NCBI refseq annotation gtf file  
577 from UCSC, taking the entire locus of reference for each gene. After removing lowly  
578 expressed counts (> 4 reads over the 4 samples), differential analysis was performed with  
579 DESeq2 Bioconductor R package, Version 1.22.1 (51). Counts were normalized using  
580 DESeq2 normalization method. Both treatment (siRNA VINK/siRNA Ctrl) and batch  
581 (replicates) effects were taken into account to estimate parameters and fit the model, in order  
582 to apply Wald test. Bigwig files were generated using rtracklayer Bioconductor-R package,  
583 Version 3.10, with 1 base pair binning and normalization based on the total number of read  
584 aligned. Spliced read of VINK were obtained by using GenomicAlignments (version 1.22.1)  
585 library in R script and human reference genome (BSgenome.Hsapiens.UCSC.hg38, version  
586 1.4.1). Mapped paired-end reads were read from BAM with "readGAlignmentPairs" function.  
587 By using "njunc" and "junction" functions, the number of spliced reads and genomic  
588 coordinates of junctions were obtained (52). Spliced junctions were analysed as described in  
589 (15).

590

#### 591 **Data availability**

592

593 RNA-Seq data are available in the Geo database under the accession number # GSE197308

594

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603

604

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- 758

759 **Figure legends**

760

761 **Figure 1 - Small interfering RNAs can efficiently decrease VINK expression**

762

763 **A:** Schematic representation of VINK genomic locus, with the localization of siRNA binding  
764 sites, qPCR primers, and main splicing events detected in Fig. 1D RNA-Seq experiments  
765 indicated. The exact locations of siRNAs, primers and main splicing events relative to the  
766 TSS of VINK estimated from RNA-Seq read coverage (Ouvrard et al., manuscript in  
767 preparation) are indicated in Table S1, S2 and S3, respectively. **B:** Senescent WI38-RAF1  
768 cells were transfected with the VINK-1 or control (Ctle-1) siRNAs. 72 hours later, total RNA  
769 was prepared and analysed by RT-qPCR for the expression of GAPDH mRNA or VINK  
770 (using the indicated primers). The amount of VINK RNA was standardized to GAPDH  
771 mRNA and calculated relative to 1 for cells transfected with the Ctle-1 siRNA. The means  
772 and standard deviations from 5 independent experiments are shown. \*: p value < 0.05  
773 compared to control. **C:** Same as in B, except that siVINK-2 and another control siRNA  
774 (Ctle-2) were used. The means and standard deviations from 7 independent experiments are  
775 shown. \*: p value < 0.05 compared to control. **D:** WI38-RAF1 cells were treated as in B and  
776 C, and total RNA was analysed by RNA-Seq. Two duplicates of RNA-seq experiments were  
777 visualized in the IGB browser showing the normalized read coverage for the VINK locus.  
778 Splicing events consistently detected in RNA-Seq experiments are indicated above the  
779 profiles. **E:** U2OS cells were transfected with the indicated siRNAs. 48 hours later, total RNA  
780 was prepared and analysed by RT-qPCR for the expression of GAPDH mRNA or VINK  
781 (using the indicated primers). The amount of VINK RNA was standardised to GAPDH  
782 mRNA and calculated relative to 1 for cells transfected with the Ctle-1 siRNA. The means

783 and standard deviations from 3 independent experiments are shown. \*: p value < 0.05  
784 compared to Ctle-1 siRNA ; ns: non-significant, p value > 0.1.

785

786 **Figure 2 - Small interfering RNAs affect VINK transcription**

787

788 **A:** Senescent WI38-RAF1 cells were transfected with the VINK-1 or control (Ctle-1)  
789 siRNAs. 48 hours later, actinomycin D (10 µg/mL) was added or not. Cells were harvested  
790 after the indicated time. Total RNA was prepared with Trizol and analysed by RT-qPCR for  
791 the expression of GAPDH mRNA or VINK (using the indicated primers). The amount of  
792 VINK RNA was standardised to GAPDH mRNA (which does not vary during this time  
793 course of actinomycin D) and calculated relative to 100 for the untreated sample. The means  
794 and standard deviations from 3 independent experiments are shown. Student t test was  
795 performed to compare VINK1 or Ctle-1 siRNAs samples at each time point of actinomycin D  
796 treatment. ns: p value > 0.1. **B:** Senescent WI38-RAF1 cells were transfected with the  
797 indicated siRNAs. Nascent RNAs (Biotin-pull down) were prepared and analysed by RT-  
798 qPCR for the expression of GAPDH mRNA or VINK (using the indicated primers). The  
799 amount of VINK RNA was standardised to GAPDH mRNA and calculated relative to 1 for  
800 the Ctle-1 siRNA. The means and standard deviations from 3 independent experiments are  
801 shown. \*: p value < 0.05 compared to Ctle-2 siRNA. **C:** Same as in B, except that U2OS cells  
802 were used. \*: p value < 0.05 compared to Ctle-1 siRNA ; ns: non-significant, p value > 0.1. **D:**  
803 Senescent WI38-RAF1 cells were transfected with Ctle-1 siRNA or an siRNA directed  
804 against ARHGAP18 START RNA (14). Nascent RNAs (Biotin-pull down) were prepared and  
805 analysed by RT-qPCR for the expression of GAPDH mRNA or the indicated primers on  
806 ARHGAP18. The amount of ARHGAP18 or START RNA was standardised to GAPDH  
807 mRNA and calculated relative to 1 for the Ctle-1 siRNA. The means and standard deviations



808 from 3 independent experiments are shown. \*: p value < 0.05 compared to Ctle-1 siRNA ; ns:  
809 non-significant, p value > 0.1; number: p value compared to Ctle-1 siRNA.

810

811 **Figure 3 – VINK siRNAs affect the presence of RNA polymerase II at VINK TSS**

812

813 **A:** Schematic representation showing the localization of the TSS (transcriptional start site) of  
814 VINK and primers used to analyse ChIP experiments. **B:** Senescent WI38-RAF1 cells were  
815 transfected with the indicated siRNAs (four experiments were performed with Ctle-2 and two  
816 with Ctle-1). 72 hours later, fixed chromatin was prepared and subjected to a ChIP experiment  
817 using an antibody directed against total RNA pol II or control IgG or no antibody as a control,  
818 as indicated. The amount of VINK TSS sequence was measured by qPCR with the indicated  
819 primers, standardized to the amount of GAPDH TSS sequence and calculated relative to 1 for  
820 the RNA pol II ChIP from cells transfected by the Ctle siRNA. The means and standard  
821 deviations from six independent experiments are shown (four controlled with no antibody  
822 (NoAb) and two with a control IgG. **C:** Same as in B, except that anti-H3K27ac, anti-  
823 H3K4me3, anti-H3K36me3, anti-H3K9me3 antibodies were used. The Ctle siRNAs were the  
824 siCtle-1 siRNA except for H3K27ac experiments for which three experiments were  
825 performed with Ctle-1 and three with Ctle-2. The amount of VINK TSS sequence was  
826 standardised to the amount of GAPDH TSS sequence and to nucleosome occupancy measured  
827 by ChIP using an antibody recognising total histone H3. The result was calculated relative to  
828 1 for relevant ChIPs from cells transfected by the Ctle siRNA. The means and standard  
829 deviations from six (H3K27ac) or three (all other antibodies) independent experiments are  
830 shown. \*: p value < 0.05 compared to Ctle siRNA.

831

832 **Figure 4 – The siRNA target site is required for VINK repression**

833

834 **A:** Senescent WI38- $\Delta/\Delta$  #1 cells were transfected with the indicated siRNAs. 72 hours later,  
835 total RNA was prepared and analysed by RT-qPCR for the expression of GAPDH mRNA or  
836 VINK (using the indicated primers). The amount of VINK RNA was standardized to GAPDH  
837 mRNA and calculated relative to 1 for cells transfected with the Ctle-1 siRNA. The means  
838 and standard deviations from 3 independent experiments are shown. \*: p value < 0.05  
839 compared to Ctle-2 siRNA ; ns: non-significant, p value > 0.1. **B:** Same as in A, except that  
840 senescent WI38- $\Delta/\Delta$  #2 cells were used. The means and standard deviations from five  
841 independent experiments are shown. \*: p value < 0.05 compared to Ctle-2 siRNA ; ns: non-  
842 significant, p value > 0.1; number: p value compared to Ctle-2 siRNA. Note that siCtle1 leads  
843 to a slightly higher VINK expression than the siCtle2 in the cell line, probably due to off  
844 target effects exemplified in D. **C:** Senescent parental WI38-RAF1 (WI38-RAF1 WT) or  
845 WI38- $\Delta/\Delta$  #2 cells were transfected with the indicated siRNA, and total RNA was analysed  
846 by RNA-Seq. Two duplicates of RNA-seq data for the VINK locus are shown. Note that data  
847 from WI38-RAF1 WT are the same as the four upper lines in Figure 1D. **D:** Senescent wild  
848 type WI38 cells were transfected with siVINK-1, siVINK-2, siCtle-1 and siCtle-2, whereas  
849 homozygously deleted ( $\Delta/\Delta$  #2) cells were transfected with siVINK-1 and siCtle-1. Total  
850 RNAs were prepared from two independent experiments and subjected to RNA-Seq.  
851 Differential gene expression (DGE) analysis was performed with the DESeq2 package. 1616  
852 genes were found significantly deregulated by the two VINK siRNAs compared to the two  
853 controls in wild-type cells, and 2216 genes were significantly deregulated by the siVINK-1  
854 siRNA in the  $\Delta/\Delta$  #2 cell line. These 2216 genes represent off-target genes since the siVINK-  
855 1 siRNAs does not decrease VINK expression in this cell line (see above). The Venn diagram  
856 shows the intersection between these two gene sets. Note that 720 out of the 1616 genes

857 significantly deregulated by the VINK1 and VINK2 siRNAs in wild type cells are also  
858 significantly changed upon VINK-1 siRNA transfection in the deleted cell line.

859

860

861

862

863 **Figure 5 - VINK siRNA requires a cis-targeting to chromatin to repress VINK**  
864 **transcription**

865

866 **A:** Schematic representation of the two alleles of VINK present in WI38- $\Delta$ /+ with primers  
867 allowing their specific detection. The deleted allele is recognized by a primer located on the  
868 deletion junction. **B:** Senescent WI38- $\Delta$ /+ cells were transfected with the indicated siRNAs. 72  
869 hours later, total RNA was prepared and analysed by RT-qPCR for the expression of GAPDH  
870 mRNA or VINK wild type allele. The amount of VINK allele was standardised to GAPDH  
871 mRNA and calculated relative to 1 for cells transfected with the Ctle-2 siRNA. The means  
872 and standard deviations from 3 independent experiments are shown. \*: p value < 0.05  
873 compared to Ctle-2 siRNA ; ns: non-significant, p value > 0.1. **C:** Same as in B, except that  
874 the deleted VINK allele was analysed. The means and standard deviations from 3  
875 independent experiments are shown. \*: p value < 0.05 compared to Ctle-2 siRNA ; ns: non-  
876 significant, p value > 0.1; number: p value compared to Ctle2 siRNA. **D:** Same as in B and C,  
877 except that total VINK expression was analysed with the indicated primers. The means and  
878 standard deviations from 3 independent experiments are shown. \*: p value < 0.05 compared  
879 to Ctle-2 siRNA ; ns: non-significant, p value > 0.1; number: p value compared to Ctle2  
880 siRNA.

881

882 **Figure 6 – Ago1 and Ago2 are involved in VINK transcriptional repression by VINK**  
883 **siRNA**

884

885 **A:** Senescent WI38 cells were recovered and subjected to a fractionation experiments. Whole  
886 cell extracts (WCE) and cytoplasmic (cyto), nuclear soluble (NS) and chromatin (CHR)  
887 fractions from the same amount of cells were analysed by western blot using the indicated  
888 antibodies. **B:** Senescent WI38 cells were transfected with 30 nM of the indicated siRNAs. 72  
889 hours later, cells were harvested and total cell extracts were subjected to a western blot  
890 analysis with antibodies directed against Ago1, Ago2 or GAPDH as a loading control. The  
891 stars (\*) indicate non-specific bands. Identical results were obtained in three independent  
892 experiments **C:** Senescent WI38 cells were transfected with 30 nM of the indicated siRNAs.  
893 The amount of siRNAs was kept constant at 60 nM using Ctle-1 siRNA. 72 hours later, total  
894 RNA was prepared and analysed by RT-qPCR for the expression of GAPDH, Ago1 or Ago2  
895 mRNA or VINK. The amount of Ago1, Ago2 or VINK were standardised to GAPDH mRNA  
896 and calculated relative to 1 for cells transfected with the Ctle-1 siRNA. The means and  
897 standard deviations from 3 independent experiments are shown. \*: p value < 0.05 when  
898 comparing the effect of siVINK-1 in cells depleted for Ago1 or Ago2 to its effect alone  
899 (paired student t test). **D:** Senescent WI38 cells were transfected with 25 nM of the indicated  
900 siRNAs. The amount of siRNAs was kept constant at 75 nM using Ctle-1 siRNA. 72 hours  
901 later, total RNA was prepared and analysed by RT-qPCR for the expression of GAPDH,  
902 Ago1 or Ago2 mRNA or VINK. The amount of Ago1, Ago2 mRNA or VINK were  
903 standardised to GAPDH mRNA and calculated relative to 1 for cells transfected with the Ctle-  
904 1 siRNA. The means and standard deviations from 6 independent experiments are shown. \*: p  
905 value < 0.05 when comparing the effect of siVINK-1 in cells depleted for Ago1 and Ago2 to  
906 its effect alone (paired student t test). **E** Same as in D, except that nascent RNAs were

907 recovered. The amount of VINK was standardised to GAPDH and calculated relative to 1 for  
908 cells transfected with the Ctle-1 siRNA. The means and standard deviations from three  
909 independent experiments are shown. \*: p value < 0.05 when comparing the effect of siVINK-  
910 1 in cells depleted for Ago1 and Ago2 to its effect alone (paired student t test).

911

912













