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

Julien Ouvrard[1,2], Lisa Muniz[1,2], Estelle Nicolas[1,2]*, Didier Trouche[1,2]§*

[1]MCD, Centre de Biologie Integrative (CBI), University of Toulouse, CNRS, UPS, Toulouse, France

[2]Equipe labellisée Ligue Contre le Cancer, Team « Chromatin and Cell Proliferation », Toulouse, France

*** These authors contributed equally to this work**

Running head : Silencing of a nuclear RNA by siRNAs

Keywords: siRNA, RNA interference, nuclear RNA, transcriptional silencing

§ Correspondence to :

Didier Trouche

MCD UMR 5077, Centre de Biologie Intégrative

Université Paul Sabatier

118 Route de Narbonne

31062 Toulouse Cedex, France

E-mail : didier.trouche@univ-tlse3.fr

Abstract

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

Introduction

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

Given that the above-described processes usually take place in the cytoplasm, RNA interference is in this respect a cytoplasmic process functioning at a post-transcriptional level, and leading to RNA degradation. Nevertheless, in some organisms such as yeast or plants, RNAi has been described to work also in the nucleus to silence repeated elements and to nucleate heterochromatin (4). However, the presence of the nuclear RNAi in mammals is more controversial. Indeed, initial data concluded that intronic sequences cannot be targeted with RNAi (5, 6). This observation together with the findings that some RISC proteins were mostly cytoplasmic led to the conclusion that RNA interference is restricted to the cytoplasm 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 1α 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 vlincRNA 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-Δ/Δ #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-Δ/Δ #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-Δ/Δ #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 Δ/Δ #2) (Figure 4B). The absence of effects of the VINK-1 siRNA on VINK expression in
229 WI38-Δ/Δ #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-Δ/Δ #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 signalized 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-Δ/+). 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-Δ/+ 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 Δ/+ 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 a-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 α-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 phenomenom 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 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% CO2 and
430 5% O2. 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 CO2. 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 MgCl2). 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 MgCl2 ; 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 α-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^2 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 10.10^6 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 Log2 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 bining 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
595 **Acknowledgements**
596 This work was supported by a grant fom the Ligue Nationale Contre le Cancer to DT as an
597 "équipe labellisée". JO is supported by studentships from the French Ministry of Science and

598 from the Fondation pour la Recherche Médicale (FRM). LM was supported by a fellowship
599 from the Fondation de France. EN is a researcher at the INSERM.
600 The authors wished to thank Antonin Morillon, Hervé Seitz and all members of D Trouche's
601 lab for helpful discussions. We thank the genomic facility (BigA) from the CBI and Virginie
602 Jouffret for RNA-Seq data analysis.

603

604

605 **References**

606

607 1. **Martinez J, Patkaniowska A, Urlaub H, Lührmann R, Tuschl T.** 2002. Single-stranded
608 antisense siRNAs guide target RNA cleavage in RNAi. Cell **110:**563-574.
609 2. **Liu J, Carmell MA, Rivas FV, Marsden CG, Thomson JM, Song JJ, Hammond SM,**
610 **Joshua-Tor L, Hannon GJ.** 2004. Argonaute2 is the catalytic engine of mammalian
611 RNAi. Science **305:**1437-1441.
612 3. **Meister G, Landthaler M, Patkaniowska A, Dorsett Y, Teng G, Tuschl T.** 2004. Human
613 Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. Mol Cell **15:**185-
614 197.
615 4. **Castel SE, Martienssen RA.** 2013. RNA interference in the nucleus: roles for small
616 RNAs in transcription, epigenetics and beyond. Nat Rev Genet **14:**100-112.
617 5. **Zeng Y, Cullen BR.** 2002. RNA interference in human cells is restricted to the
618 cytoplasm. RNA **8:**855-860.
619 6. **Vickers TA, Koo S, Bennett CF, Crooke ST, Dean NM, Baker BF.** 2003. Efficient
620 reduction of target RNAs by small interfering RNA and RNase H-dependent antisense
621 agents. A comparative analysis. J Biol Chem **278:**7108-7118.
622 7. **Gagnon KT, Li L, Chu Y, Janowski BA, Corey DR.** 2014. RNAi factors are present and
623 active in human cell nuclei. Cell Rep **6:**211-221.
624 8. **Kalantari R, Hicks JA, Li L, Gagnon KT, Sridhara V, Lemoff A, Mirzaei H, Corey DR.**
625 2016. Stable association of RNAi machinery is conserved between the cytoplasm and
626 nucleus of human cells. RNA **22:**1085-1098.
627 9. **White E, Schlackow M, Kamieniarz-Gdula K, Proudfoot NJ, Gullerova M.** 2014.
628 Human nuclear Dicer restricts the deleterious accumulation of endogenous double-
629 stranded RNA. Nat Struct Mol Biol **21:**552-559.
630 10. **Sharma NR, Wang X, Majerciak V, Ajiro M, Kruhlak M, Meyers C, Zheng ZM.** 2016.
631 Cell Type- and Tissue Context-dependent Nuclear Distribution of Human Ago2. J Biol
632 Chem **291:**2302-2309.
633 11. **Sarshad AA, Juan AH, Muler AIC, Anastasakis DG, Wang X, Genzor P, Feng X, Tsai PF,**
634 **Sun HW, Haase AD, Sartorelli V, Hafner M.** 2018. Argonaute-miRNA Complexes
635 Silence Target mRNAs in the Nucleus of Mammalian Stem Cells. Mol Cell **71:**1040-
636 1050.e1048.

637 12. **Di Fazio A, Schlackow M, Pong SK, Alagia A, Gullerova M.** 2022. Dicer dependent
638 tRNA derived small RNAs promote nascent RNA silencing. Nucleic Acids Res **50:**1734-
639 1752.
640 13. **Stojic L, Niemczyk M, Orjalo A, Ito Y, Ruijter AE, Uribe-Lewis S, Joseph N, Weston S,**
641 **Menon S, Odom DT, Rinn J, Gergely F, Murrell A.** 2016. Transcriptional silencing of
642 long noncoding RNA GNG12-AS1 uncouples its transcriptional and product-related
643 functions. Nat Commun **7:**10406.
644 14. **Muniz L, Deb MK, Aguirrebengoa M, Lazorthes S, Trouche D, Nicolas E.** 2017.
645 Control of Gene Expression in Senescence through Transcriptional Read-Through of
646 Convergent Protein-Coding Genes. Cell Rep **21:**2433-2446.
647 15. **Muniz L, Lazorthes S, Delmas M, Ouvrard J, Aguirrebengoa M, Trouche D, Nicolas E.**
648 2021. Circular ANRIL isoforms switch from repressor to activator of p15/CDKN2B
649 expression during Raf1 oncogene-induced senescence. RNA Biol **18:**404-420.
650 16. **Lazorthes S, Vallot C, Briois S, Aguirrebengoa M, Thuret JY, St Laurent G, Rougeulle**
651 **C, Kapranov P, Mann C, Trouche D, Nicolas E.** 2015. A vlincRNA participates in
652 senescence maintenance by relieving H2AZ-mediated repression at the INK4 locus.
653 Nat Commun **6:**5971.
654 17. **Morris KV, Chan SW, Jacobsen SE, Looney DJ.** 2004. Small interfering RNA-induced
655 transcriptional gene silencing in human cells. Science **305:**1289-1292.
656 18. **Janowski BA, Huffman KE, Schwartz JC, Ram R, Nordsell R, Shames DS, Minna JD,**
657 **Corey DR.** 2006. Involvement of AGO1 and AGO2 in mammalian transcriptional
658 silencing. Nat Struct Mol Biol **13:**787-792.
659 19. **Castanotto D, Tommasi S, Li M, Li H, Yanow S, Pfeifer GP, Rossi JJ.** 2005. Short
660 hairpin RNA-directed cytosine (CpG) methylation of the RASSF1A gene promoter in
661 HeLa cells. Mol Ther **12:**179-183.
662 20. **Ting AH, Schuebel KE, Herman JG, Baylin SB.** 2005. Short double-stranded RNA
663 induces transcriptional gene silencing in human cancer cells in the absence of DNA
664 methylation. Nat Genet **37:**906-910.
665 21. **Janowski BA, Huffman KE, Schwartz JC, Ram R, Hardy D, Shames DS, Minna JD,**
666 **Corey DR.** 2005. Inhibiting gene expression at transcription start sites in
667 chromosomal DNA with antigene RNAs. Nat Chem Biol **1:**216-222.
668 22. **Suzuki K, Shijuuku T, Fukamachi T, Zaunders J, Guillemin G, Cooper D, Kelleher A.**
669 2005. Prolonged transcriptional silencing and CpG methylation induced by siRNAs
670 targeted to the HIV-1 promoter region. J RNAi Gene Silencing **1:**66-78.
671 23. **Zhang MX, Ou H, Shen YH, Wang J, Coselli J, Wang XL.** 2005. Regulation of
672 endothelial nitric oxide synthase by small RNA. Proc Natl Acad Sci U S A **102:**16967-
673 16972.
674 24. **Weinberg MS, Villeneuve LM, Ehsani A, Amarzguioui M, Aagaard L, Chen ZX, Riggs**
675 **AD, Rossi JJ, Morris KV.** 2006. The antisense strand of small interfering RNAs directs
676 histone methylation and transcriptional gene silencing in human cells. RNA **12:**256-
677 262.
678 25. **Napoli S, Pastori C, Magistri M, Carbone GM, Catapano CV.** 2009. Promoter-specific
679 transcriptional interference and c-myc gene silencing by siRNAs in human cells.
680 EMBO J **28:**1708-1719.
681 26. **Younger ST, Corey DR.** 2011. Transcriptional regulation by miRNA mimics that target
682 sequences downstream of gene termini. Mol Biosyst **7:**2383-2388.

683 27. **Yue X, Schwartz JC, Chu Y, Younger ST, Gagnon KT, Elbashir S, Janowski BA, Corey**
684 **DR.** 2010. Transcriptional regulation by small RNAs at sequences downstream from 3'
685 gene termini. Nat Chem Biol **6:**621-629.
686 28. **Han J, Kim D, Morris KV.** 2007. Promoter-associated RNA is required for RNA-
687 directed transcriptional gene silencing in human cells. Proc Natl Acad Sci U S A
688 **104:**12422-12427.
689 29. **Hawkins PG, Santoso S, Adams C, Anest V, Morris KV.** 2009. Promoter targeted
690 small RNAs induce long-term transcriptional gene silencing in human cells. Nucleic
691 Acids Res **37:**2984-2995.
692 30. **Schwartz JC, Younger ST, Nguyen NB, Hardy DB, Monia BP, Corey DR, Janowski BA.**
693 2008. Antisense transcripts are targets for activating small RNAs. Nat Struct Mol Biol
694 **15:**842-848.
695 31. **Chu Y, Yue X, Younger ST, Janowski BA, Corey DR.** 2010. Involvement of argonaute
696 proteins in gene silencing and activation by RNAs complementary to a non-coding
697 transcript at the progesterone receptor promoter. Nucleic Acids Res **38:**7736-7748.
698 32. **Kim DH, Villeneuve LM, Morris KV, Rossi JJ.** 2006. Argonaute-1 directs siRNA-
699 mediated transcriptional gene silencing in human cells. Nat Struct Mol Biol **13:**793-
700 797.
701 33. **Benhamed M, Herbig U, Ye T, Dejean A, Bischof O.** 2012. Senescence is an
702 endogenous trigger for microRNA-directed transcriptional gene silencing in human
703 cells. Nat Cell Biol **14:**266-275.
704 34. **Giles KE, Ghirlando R, Felsenfeld G.** 2010. Maintenance of a constitutive
705 heterochromatin domain in vertebrates by a Dicer-dependent mechanism. Nat Cell
706 Biol **12:**94-99; sup pp 91-96.
707 35. **Moses J, Goodchild A, Rivory LP.** 2010. Intended transcriptional silencing with siRNA
708 results in gene repression through sequence-specific off-targeting. RNA **16:**430-441.
709 36. **Suzuki K, Ishida T, Yamagishi M, Ahlenstiel C, Swaminathan S, Marks K, Murray D,**
710 **McCartney EM, Beard MR, Alexander M, Purcell DF, Cooper DA, Watanabe T,**
711 **Kelleher AD.** 2011. Transcriptional gene silencing of HIV-1 through promoter
712 targeted RNA is highly specific. RNA Biol **8:**1035-1046.
713 37. **Vilborg A, Passarelli MC, Yario TA, Tycowski KT, Steitz JA.** 2015. Widespread
714 Inducible Transcription Downstream of Human Genes. Mol Cell **59:**449-461.
715 38. **You J, Song Z, Lin J, Jia R, Xia F, Li Z, Huang C.** 2021. RNAi-directed knockdown
716 induces nascent transcript degradation and premature transcription termination in
717 the nucleus. Cell Discov **7:**79.
718 39. **Lee JT, Lu N.** 1999. Targeted mutagenesis of Tsix leads to nonrandom X inactivation.
719 Cell **99:**47-57.
720 40. **Zhang MX, Zhang C, Shen YH, Wang J, Li XN, Chen L, Zhang Y, Coselli JS, Wang XL.**
721 2008. Effect of 27nt small RNA on endothelial nitric-oxide synthase expression. Mol
722 Biol Cell **19:**3997-4005.
723 41. **Qiu L, Hu X, Jing Q, Zeng X, Chan KM, Han J.** 2018. Mechanism of cancer:
724 Oncohistones in action. J Genet Genomics **45:**227-236.
725 42. **Birmingham A, Anderson EM, Reynolds A, Ilsley-Tyree D, Leake D, Fedorov Y,**
726 **Baskerville S, Maksimova E, Robinson K, Karpilow J, Marshall WS, Khvorova A.**
727 2006. 3' UTR seed matches, but not overall identity, are associated with RNAi off-
728 targets. Nat Methods **3:**199-204.

43. **Traber GM, Yu AM.** 2022. RNAi based therapeutics and novel RNA bioengineering technologies. J Pharmacol Exp Ther.
44. **Chen Y, Li Z, Chen X, Zhang S.** 2021. Long non-coding RNAs: From disease code to drug role. Acta Pharm Sin B **11:**340-354.
45. **St Laurent G, Shtokalo D, Dong B, Tackett MR, Fan X, Lazorthes S, Nicolas E, Sang N, Triche TJ, McCaffrey TA, Xiao W, Kapranov P.** 2013. VlincRNAs controlled by retroviral elements are a hallmark of pluripotency and cancer. Genome Biol **14:**R73.
46. **Cao H, Xu D, Cai Y, Han X, Tang L, Gao F, Qi Y, Cai D, Wang H, Ri M, Antonets D, Vyatkin Y, Chen Y, You X, Wang F, Nicolas E, Kapranov P.** 2021. Very long intergenic non-coding (vlinc) RNAs directly regulate multiple genes in cis and trans. BMC Biol **19:**108.
47. **Jeanblanc M, Ragu S, Gey C, Contrepois K, Courbeyrette R, Thuret JY, Mann C.** 2012. Parallel pathways in RAF-induced senescence and conditions for its reversion. Oncogene **31:**3072-3085.
48. **Andrews S.** 2010. FastQC: a quality control tool for high throughput sequence data. *Available online at :* http://wwwbioinformaticsbabrahamacuk/projects/fastqc/.
49. **Li H, Handsaker B, Wysoker A, Fennel T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R.** 2009. The sequence alignment /Map (SAM) format and SAMtools. BioinformaticsR and 1000 Genome Project Data Processing Subgroup **25:**2078-2079.
50. **Anders S, Pyl P, Huber W.** 2014. HTSeq - A Python framework to work with high throughput sequencing data. Bioinformatics ***doi:10.1093/bioinformatics/btu638***.
51. **Love MI, Huber W, Anders S.** 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol **15:**550.
52. **Lawrence M, Huber W, Pagès H, Aboyoun P, Carlson M, Gentleman R, Morgan MT, Carey VJ.** 2013. Software for computing and annotating genomic ranges. PLoS Comput Biol **9:**e1003118.

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**

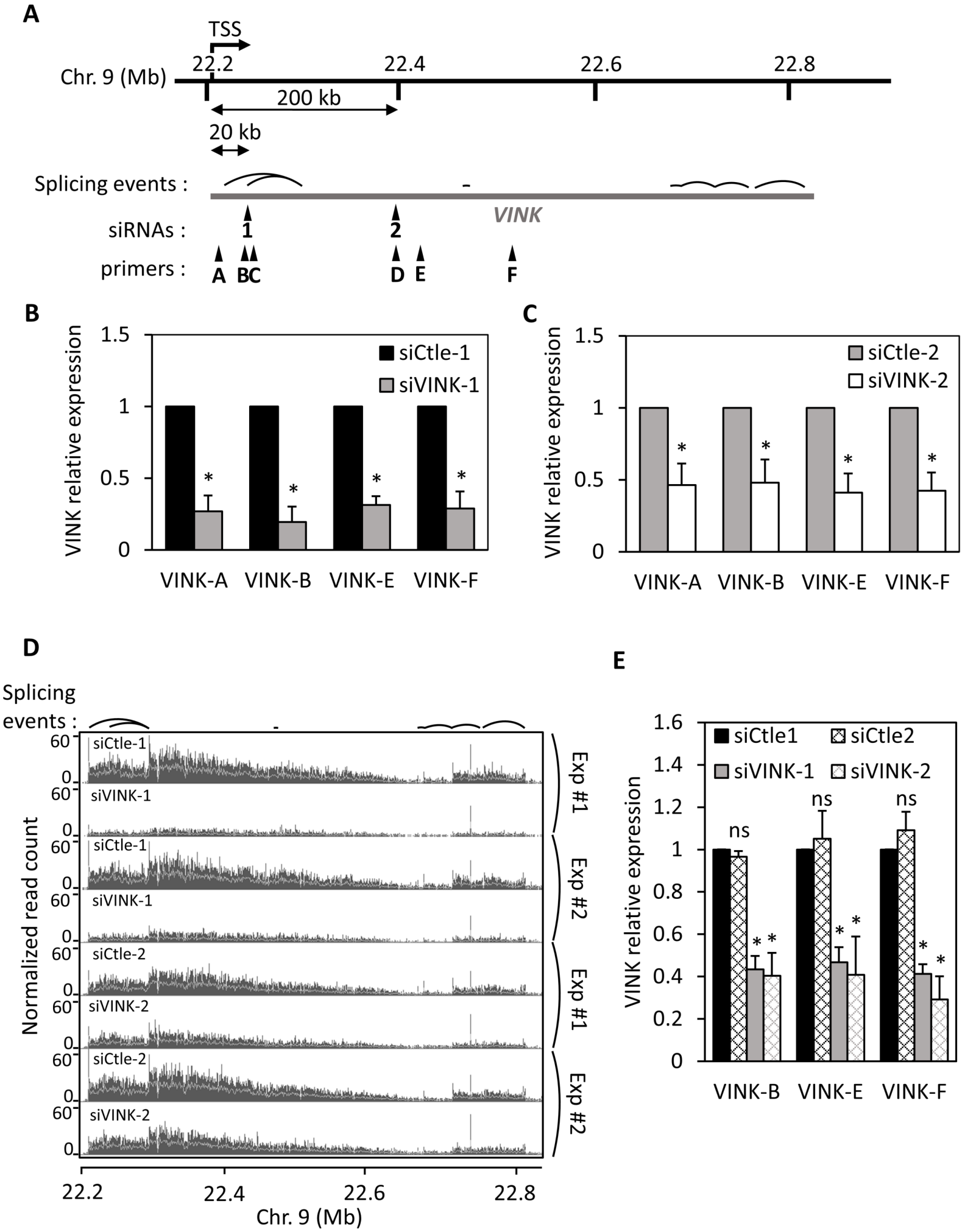
833

834 **A**: Senescent WI38-Δ/Δ #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-Δ/Δ #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-Δ/Δ #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 (Δ/Δ #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 Δ/Δ #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

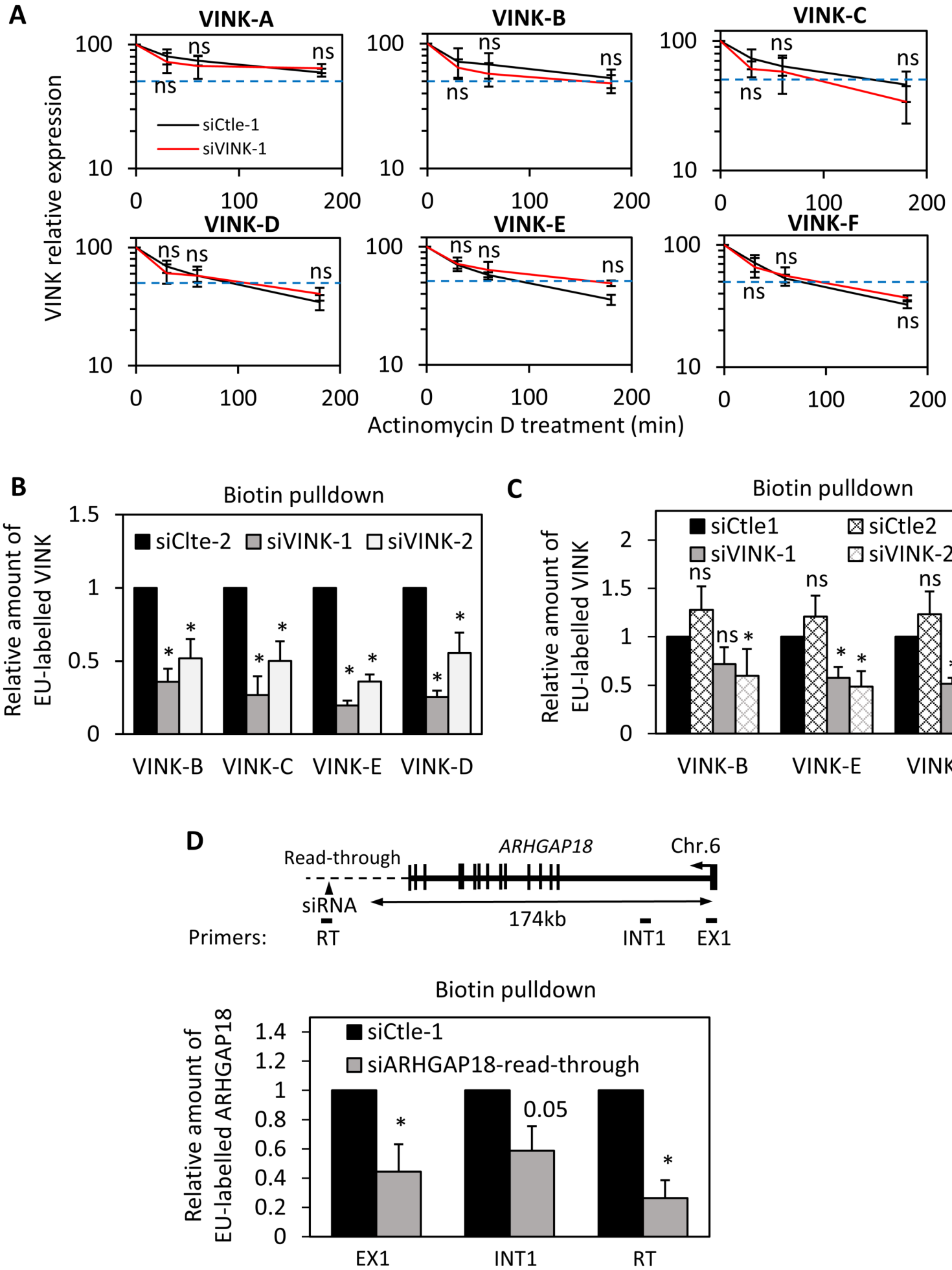
857 significantly deregulated by the VINK1 and VINK2 siRNAs in wild type cells are also
858 significantly changed upon VINK-1 siRNA transfection in the deleted cell line.
859
860
861
862
863 **Figure 5 - VINK siRNA requires a cis-targeting to chromatin to repress VINK**
864 **transcription**
865
866 **A**: Schematic representation of the two alleles of VINK present in WI38-Δ/+ with primers
867 allowing their specific detection. The deleted allele is recognized by a primer located on the
868 deletion junction. **B**: Senescent WI38-Δ/+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

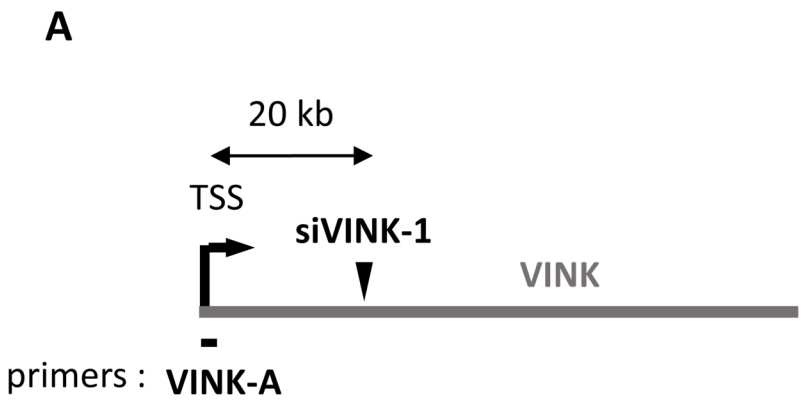
907 recovered. The amount of VINK was standardised to GAPDH and calculated relative to 1 for
908 cells transfected with the Ctle-1 siRNA. The means and standard deviations from three
909 independent experiments are shown. *: p value < 0.05 when comparing the effect of siVINK-
910 1 in cells depleted for Ago1 and Ago2 to its effect alone (paired student t test).
911
912

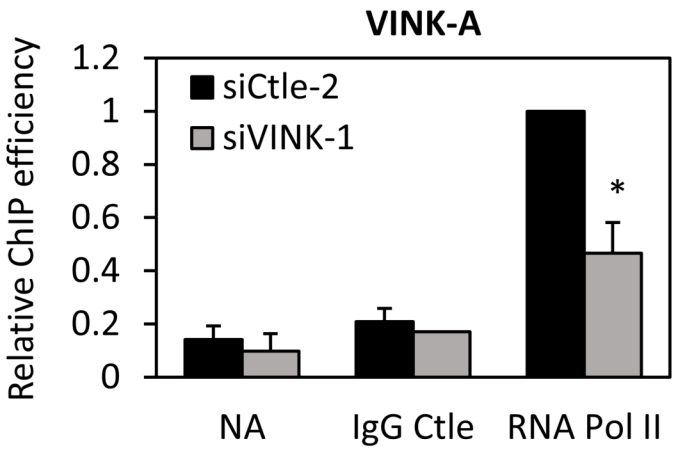


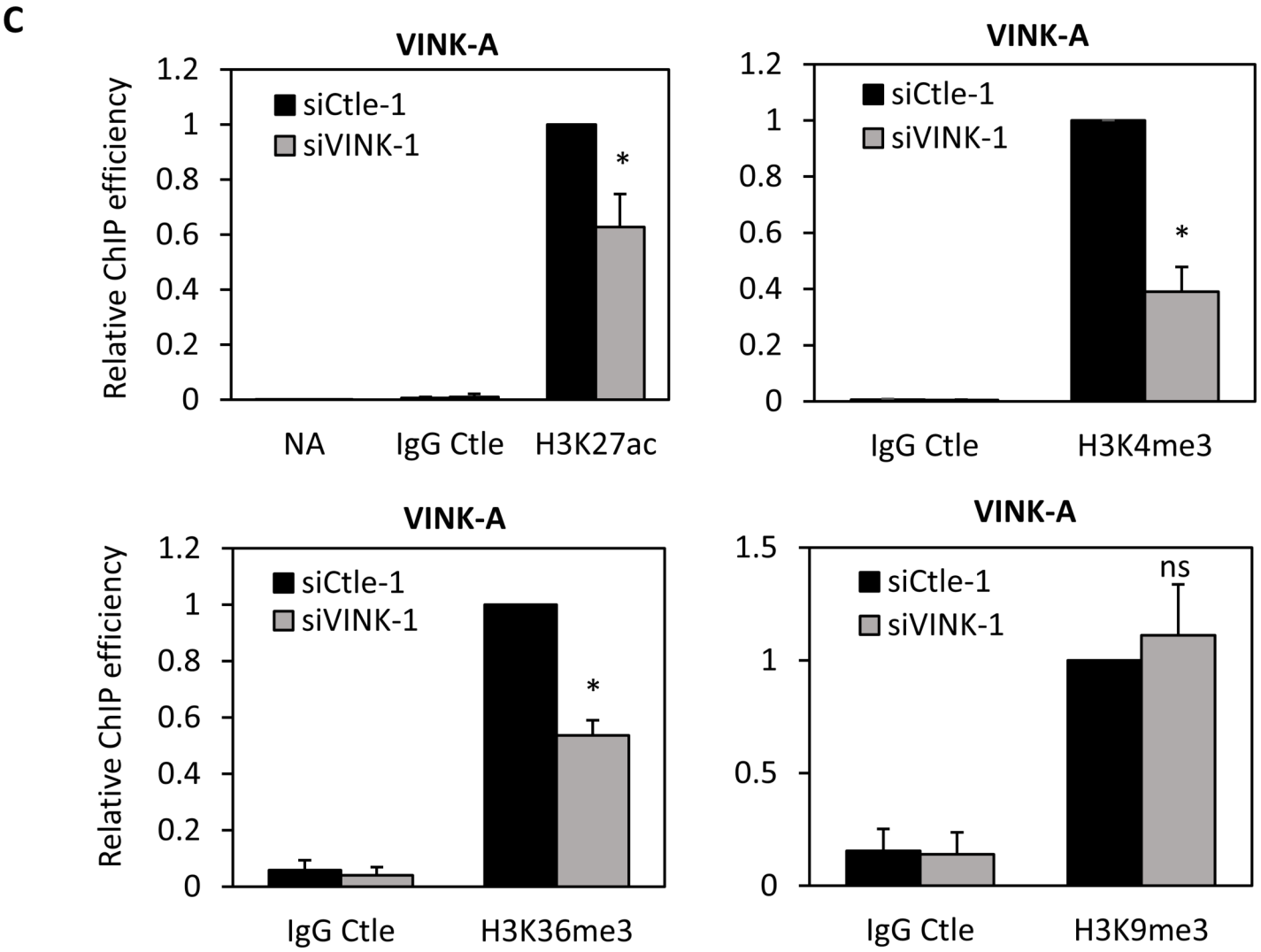
Ouvrard et al. Figure 1



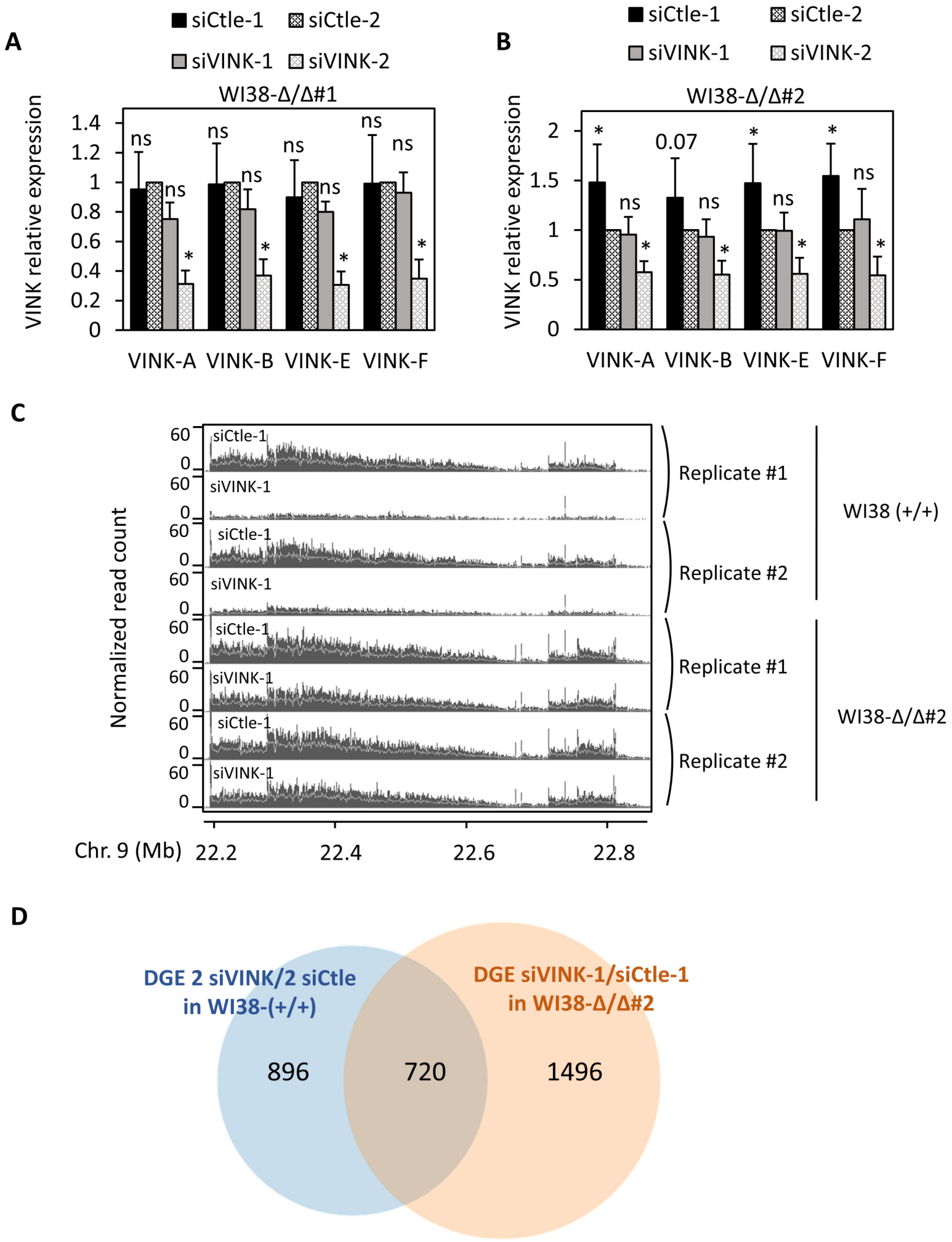
Ouvrard et al. Figure 2



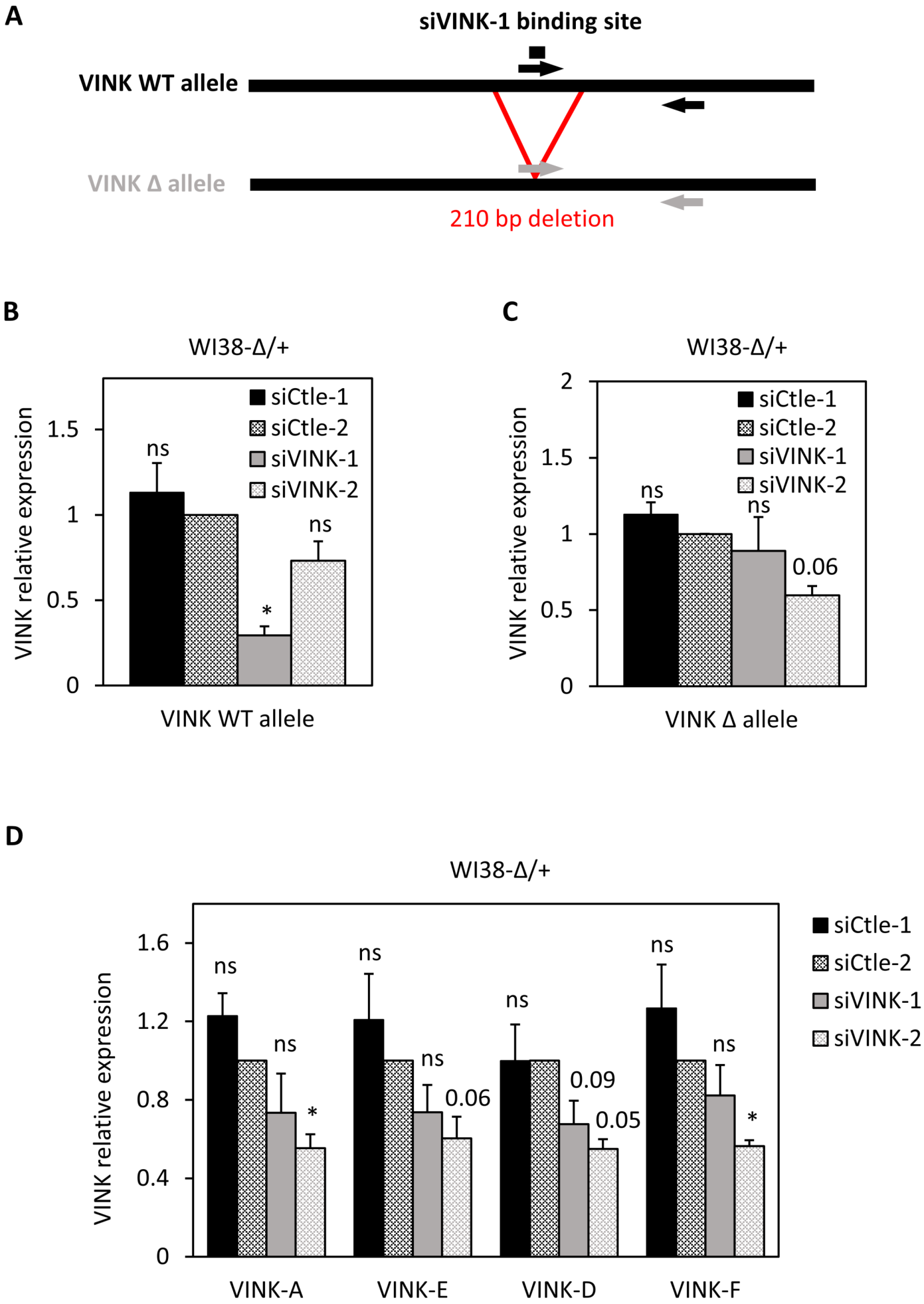




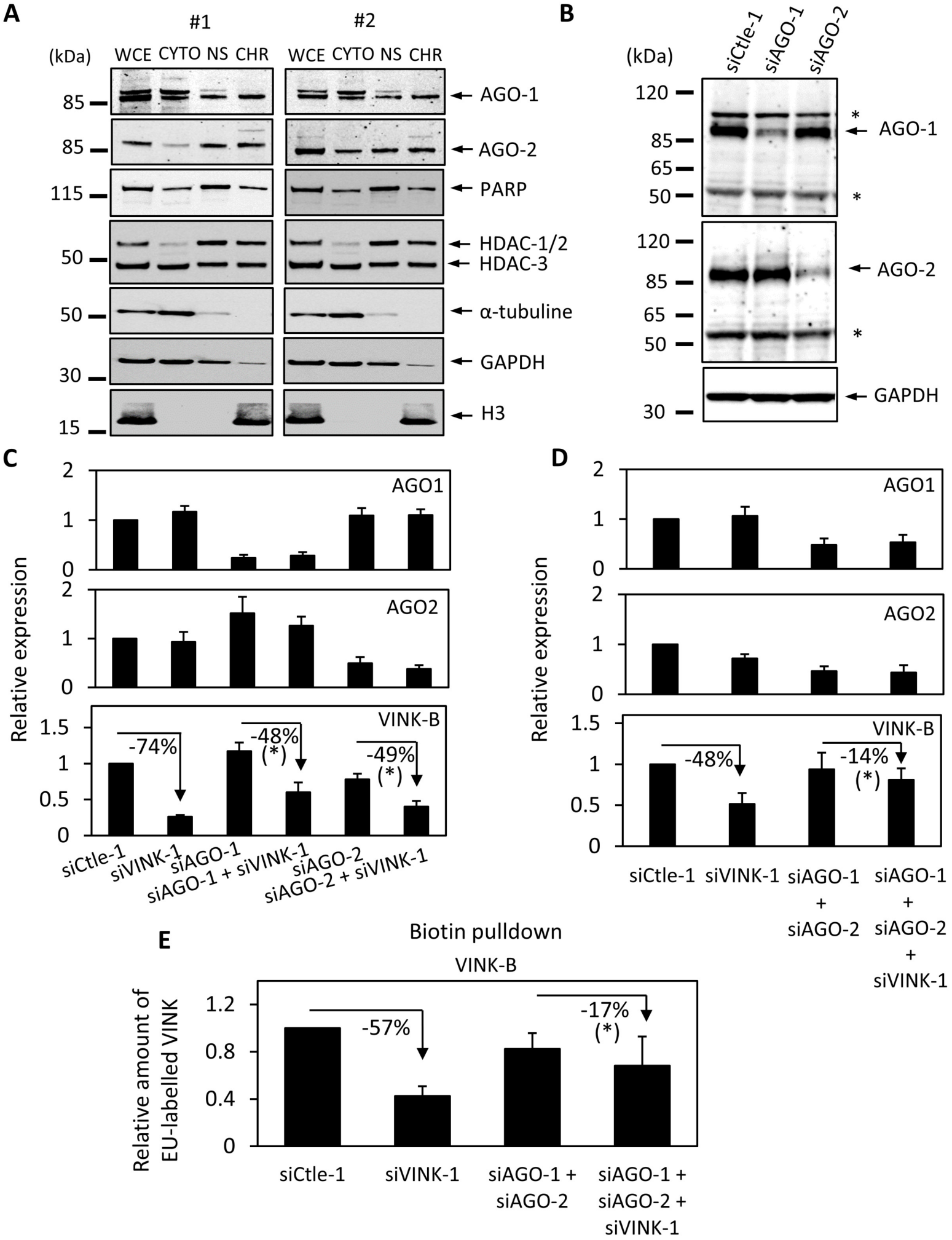
Ouvrard et al. Figure 3



Ouvrard et al. Figure 4



Ouvrard et al. Figure 5



Ouvrard et al. Figure 6