Biochimica et Biophysica Acta 1790 (2009) 1170–1178

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

# Reduction of gene expression by a hairpin-loop structured oligodeoxynucleotide: Alternative to siRNA and antisense

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ARTICLE INFO

*Article history:*
Received 30 October 2008
Received in revised form 29 April 2009
Accepted 22 May 2009
Available online 6 June 2009

*Keywords:*
Gene silencing
Reverse transcriptase-RNase H
Polypurine tract
siRNA
Antisense

ABSTRACT

*Background:* We previously described the inhibition of HIV-1 replication by a 54-mer hairpin-loop structured oligodeoxynucleotide (ODN) A, which binds the polypurine tract (PPT) on HIV-1 RNA. ODN A was shown to lead to reduced viral RNA in virions or early during infection.
*Methods and results:* Here we demonstrated that ODN A was able to cause hydrolysis of viral RNA not only by retroviral RT-associated RNase H but also cellular RNase H1 and RNase H2 *in vitro*. Furthermore, ODN A reduced gene expression in a dose-dependent manner in a cell-based reporter assay where a PPT sequence was inserted in the 5′ untranslated region of the reporter gene. The efficacy of ODN A was higher than that of its siRNA and antisense counterparts. By knocking down cellular RNases H, we showed that RNase H1 contributed to the gene silencing by ODN A but the possibility of a partial contribution of RNase H-independent mechanisms could not be ruled out.
*General significance:* Our findings highlight the potential application of hairpin-loop structured ODNs for reduction of gene expression in mammalian cells and underscore the possibility of using ODN A to trigger the hydrolysis of HIV RNA in infected cells by cellular RNases H.



## 1. Introduction

Ribonucleases H (RNases H) are ubiquitous endonucleases found in retroviruses, bacteria and mammalian cells, which specifically hydrolyze the RNA strand in RNA–DNA hybrids [1]. The HIV-1 reverse transcriptase (RT) comprises both polymerase and RNase H activities and is essential for retroviral replication [2,3]. We have previously applied the small hairpin-loop structured ODN A, to cause hydrolysis of HIV-1 RNA by the RNase H activity of HIV-1 RT [4–8]. ODN A consists of an antisense strand and a second strand linked by a stretch of four thymidines (Fig. 1A). The antisense strand of ODN A specifically binds and forms a RNA–DNA hybrid with the 3′ polypurine tract (PPT) of HIV-1 RNA by Watson–Crick base pairing [9]. Binding of ODN A to PPT generates a substrate for the RNase H moiety of HIV-1 RT, leading to premature destruction of the RNA template and hence inhibition of viral replication [4–8]. The inhibitory effect of ODN A is highly sequence-specific and more potent compared to that exhibited by asA, which is the antisense strand of ODN A [4–8].

The RNase H domain of HIV-1 RT shares striking structural homology with the RNase H domain of human RNase H1 [3,10,11], an enzyme, which plays a key role in post-transcriptional gene silencing [3,10,11]. Another known human RNase H, RNase H2, seems to play a role in replication [12] and mismatch control [12]. Mutations in the RNase H2 are associated with the severe neurological disease Aicardi–Goutieres syndrome [13]. The structure of human RNase H2 has not been determined and its role in antisense-mediated post-transcriptional gene silencing remains unclear [11]. The RNase H moieties of HIV-1 RT and human RNase H1 share structural similarities but differ in some of their functional properties. For example, the RNase H domain of HIV RT requires the polymerase domain for RNA degradation [14], whereas the RNase H domain of human RNase H1 alone shows significant activity [15]; molecular modeling based on crystallographic data predicts that the RNA/DNA hybrid would fit onto HIV RT and human RNase H1 with different trajectories [15]; HIV RT but not human RNase H1 is able to cleave double-stranded RNA in the presence of $Mn^{2+}$[15,16].

*Abbreviations:* as, antisense; ODN, hairpin-loop structured oligodeoxynucleotide; siRNA, small interfering RNA; PPT, polypurine tract; RNase H, ribonuclease H; RT, reverse transcriptase; nt, nucleotide; EGFP, enhanced green fluorescent protein; RT-PCR, reverse transcriptase polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; FACS, fluorescence activated cell sorting; PBS, phosphate buffered saline; NP-40, Nonidet P-40; HA, hemaglutinin

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doi:10.1016/j.bbagen.2009.05.017

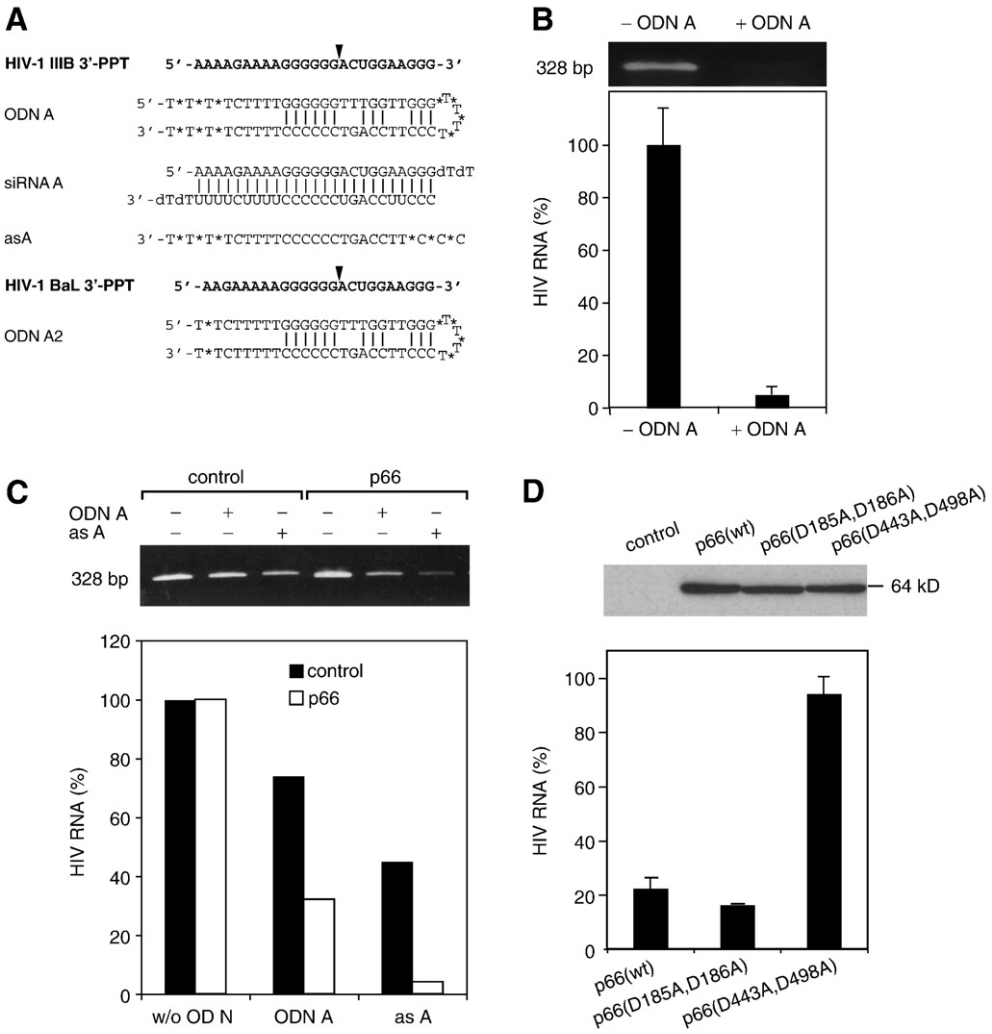


**Fig. 1.** RT (p66) enhanced hydrolysis of HIV-1 RNA mediated by ODNs in cellular lysates. (A) Sequences of the ODNs and controls used. Phosphorothioation of the phosphodiester backbone is indicated by asterisks. The HIV-1 IIIB 3′-PPT RNA targeted by ODN A, asA and siRNA A and the HIV-1 BaL 3′-PPT RNA targeted by ODN A2 are shown. Vertical bars indicate Watson–Crick bonds. Arrowheads indicate the primary cleavage site of RNase H of HIV-1 RT within the PPT. (B) ODN A-mediated degradation of HIV-1 RNA in lysate of HIV-1-infected C81-66/45 T-cells. Lysates of C81-66/45 infected with HIV-1 (m.o.i. = 0.01) were incubated with and without ODN A (250 nM) in a lysate supplementation assay (Materials and methods). The amount of unhydrolysed PPT RNA was quantified by RT-PCR using primers PU3.1 and PU3.4, which hybridize to regions flanking the cleavage site (Materials and methods). The resultant 328-bp RT-PCR products are shown in the top panel and their relative amounts are shown in the graph. (C) Lysate of HEK293 cells with or without expressing p66 was incubated with HIV-1 genomic RNA and 250 nM ODN A, asA or TE buffer in a lysate supplementation assay. One representative experiment of three independent experiments is shown. (D) The RNase H activity of p66 enhanced ODN-mediated degradation of HIV-1 RNA. Lysates (10 μg) of RNase H1-knockdown HEK293 transfected with empty vector (control) or vector expressing HA-tagged p66 wild-type (p66wt) or p66 mutants were incubated with 250 nM ODN A in a lysate supplementation assay. The amounts of HA-tagged p66 wild-type and mutants expressed were analyzed by immunoblotting using anti-HA antibody (upper panel). The graph shows the relative amounts of PPT RNA normalized against the empty vector control. Error bars represent standard deviations of duplicates.

These differences prompted us to ask whether the RNA/ODN A hybrid is substrate not only for the HIV RT-associated RNase H but also for cellular RNases H.

Here we extended our previous studies by investigating whether the RNA/ODN A hybrid may be recognized by human RNase H, which would then hydrolyze the target RNA, in order to test, whether ODN A or similar ODNs could be applied to reduce gene expression in human cells. Our findings demonstrate that the HIV-1 RT-associated RNase H, human RNase H1 and human RNase H2 *in vitro* could all recognize the RNA/ODN A hybrid as well as the RNA/asA hybrid leading to cleavage of the RNA. ODN A reduced gene expression in a live-cell reporter assay where a PPT sequence was inserted in the 5′ untranslated region of the reporter gene. ODN A, compared with its antisense and siRNA counterparts, mediated significantly more potent reduction of reporter gene expression. Finally, our findings indicate that RNase H1 contributed to the gene silencing by ODN A. This study sheds new light on the ability of ODN A to cause cellular RNases H to degrade target RNAs and provides evidence also that ODNs may be promising tools for reduction of cellular and/or viral RNAs in mammalian cells.

## 2. Results

### *2.1. HIV RT enhanced ODN A-mediated RNA hydrolysis in mammalian cell lysate*

The PPT is a specialized region of the HIV RNA specifically recognized by the HIV RT during retroviral replication [9]. In previous studies, we targeted the PPT of HIV with ODN A for the degradation of the RNA genome in HIV viral particles [6] and inhibition of viral replication [4,5,7]. Here we used the PPT, which is known to be accessible to hybridization with ODN A, as the model target site for studying the role of cellular RNases H for gene silencing by ODNs. The ODNs tested and their target sequences are indicated in Fig. 1A.

Recombinant HIV RT was shown to cleave synthetic PPT RNA in the presence of ODN A by its RNase H activity [6,8]. Thus, we first

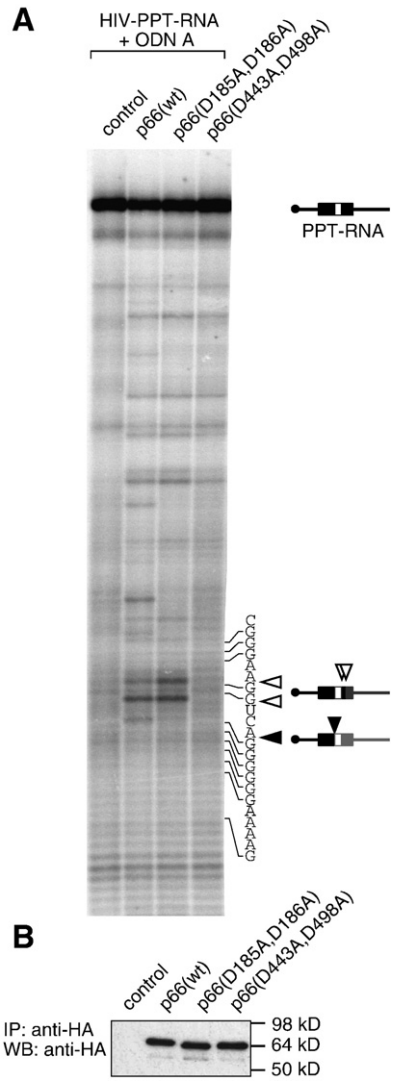


**Fig. 2.** The RNase H activity of RT was required for ODN A-mediated cleavage of target RNA. (A) 5′ labelled synthetic PPT RNA of HIV-1 was incubated with immunoprecipitated HA-tagged p66 wild-type, HA-tagged p66(D443A,D498A) or HA-tagged p66 (D185A,D186A) in the presence of ODN A (50 nM) for 30 min at 37 °C. Cleavage products were analyzed in a 6% urea-polyacrylamide gel. The scheme depicts the major RNA species. Closed and open rectangles represent the purine-rich and ACU sequences of PPT, respectively. Closed arrowheads indicate the cleavage at the ACU sequence. Open arrowheads indicate the additional cleavage sites. The non-labeled cleavage product is shown in grey. (B) The relative amounts of immunoprecipitated HA-tagged p66 wild-type, HA-tagged p66(D443A,D498A) or HA-tagged p66(D185A,D186A) in the cleavage assay were analyzed by immunoblotting.

examined whether the PPT-specific ODN A could cause hydrolysis of HIV-1 RNA by endogenous HIV RT in the cell lysate of HIV-infected cells. Results of lysate supplementation assays show that ODN A led to a 98% degradation of PPT RNA in the HIV-infected cell lysate (Fig. 1B). No degradation of the target RNA was observed in the mock control in the absence of ODN A. These results provide the first line of evidence that ODN A caused target RNA degradation in mammalian cell lysate in the presence of endogenous RT. To further confirm the ability of RT-associated RNase H to catalyze ODN A-mediated RNA cleavage in mammalian cell lysate, we expressed in human kidney embryonic (HEK293) cells the catalytically active p66 subunit of HIV RT which consists of the polymerase and RNase H domains [3]. Compared to the mock control, expression of p66 enhanced the ODN A-mediated RNA

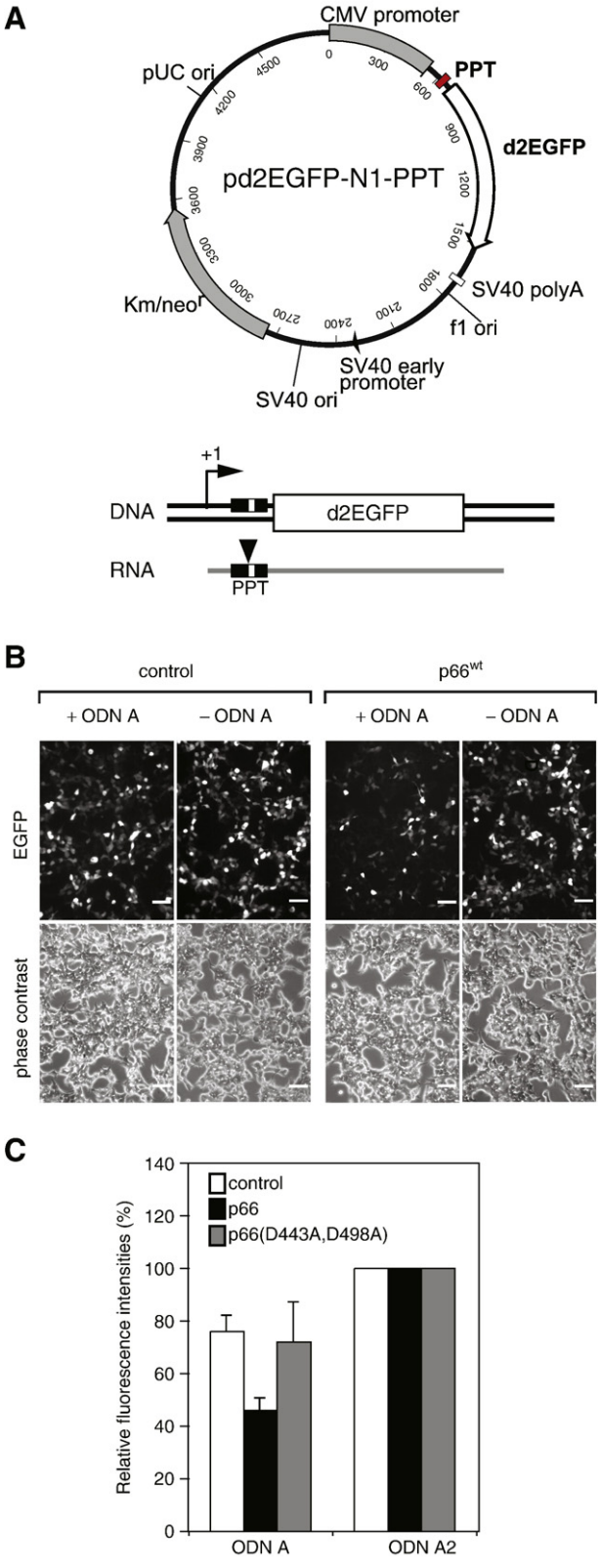


**Fig. 3.** The RNase H activity of RT was required for enhanced transgene silencing by ODN A. (A) The schematics represent the plasmid map of pd2EGFP-N1-PPT (circular map) and the enlarged 5′ untranslated region of the *d2egfp* gene (open rectangle) containing an inserted PPT region (closed rectangle). Expression of EGFP is driven by a *cytomegalovirus* (CMV) promoter (arrow; "+1" indicates the transcription start site). (B) Fluorescence and phase contrast micrographs of HEK293 and HEK293 expressing p66 wild-type at 22 h post-transfection with pd2EGFP-N1-PPT in the absence or presence of 50 nM ODN A. (C) The EGFP expression levels in ODN A-treated cells were quantified by FACS and normalized against those in the negative control ODN A2-treated cells (graph). Error bars represent standard deviations of triplicates.

degradation from 26% to 68% (Fig. 1C). In comparison with ODN A, the antisense strand of ODN A, asA, led to even higher levels of target RNA degradation both in the absence and presence of p66. Expression of the native p66/p51 heterodimer instead of a p66 homodimer gave rise to similar results (data not shown). The expression of mutant p66 (D443A,D498A) defective in its RNase H activity [17] failed to enhance RNA hydrolysis mediated by ODN A (Fig. 1D), whereas the D185A, D186A double mutation destroying the polymerase activity [18] was similar to wild-type p66. Taken together, these results demonstrate that the RNase H activity of HIV RT is essential for the ODN A- and asA-mediated degradation of target RNA in mammalian cell lysates. The role of the RNase H activity of RT in the ODN A-mediated cleavage of RNA was further confirmed by the observation that immunoprecipitates of wild-type p66 (p66wt) or the polymerase mutant p66(D185A, D186A), but not the RNase H mutant p66(D443A,D498A), cleaved PPT RNA in the presence of ODN A (Fig. 2A and B). These findings confirm that ODN A causes hydrolysis of the RNA strand in the RNA/DNA hybrid by the RNase H activity of HIV RT.

### 2.2. HIV RT potentiated the effect of ODN A on gene silencing in cell culture

We then used the reporter plasmid pd2EGFP-N1-PPT encoding a d2EGFP mRNA that contains a PPT sequence in its 5′ untranslated region to allow targeting of the transcript as a cell-based model to examine the effects of ODN A on gene expression in mammalian tissue culture cells (Fig. 3A). d2EGFP is a short-lived variant of the enhanced green fluorescent protein (EGFP) useful for kinetic gene expression studies [19]. HEK293 cells were co-transfected with ODN A and pd2EGFP-N1-PPT. Addition of ODN A led to a reduction in fluorescence intensity (Fig. 3B) in comparison with the control, which was not treated with ODN (Fig. 3B). Expression of p66 enhanced this effect. Furthermore a reduction in fluorescence intensity was observed for ODN A, but not for the control ODN A2 (Fig. 3C) indicating that the ODN A-mediated downregulation was sequence-specific. The ODN A-mediated reduction in fluorescence intensity was enhanced by the expression of p66 wild-type but not by the RNase H-defective mutant p66(D443A,D498A). Taken together, these results suggest that ODN A causes gene silencing in intact mammalian cells and the ability of ODN A to reduce gene expression can be enhanced by the RNase H activity of p66.

### 2.3. Human RNase H1 and RNase H2 contribute to ODN A-mediated hydrolysis of target RNA

ODN A was able to cause hydrolysis of HIV RNA even in the absence of p66 in HEK293 cytoplasmic lysate (Fig. 1C) as well as in intact cells, suggesting that cellular RNases H or RNase H-like enzymes (Fig. 4A) are likely to contribute to the ODN A effect. RNase H1 and RNase H2 are found ubiquitously in mammalian cells [13,20] and are involved in the removal of RNA primers from Okazaki fragments, the processing of R loops to modulate replication initiation, mitochondrial DNA replication and cell growth [12,21,22]. RNase H1 is present in cytoplasm, mitochondria and nucleus [11,22,23]. RNase H2, which is located predominantly in the nucleus [24–26], exists as a heterotrimer consisting of three subunits, RNase H2A, RNase H2B and RNase H2C

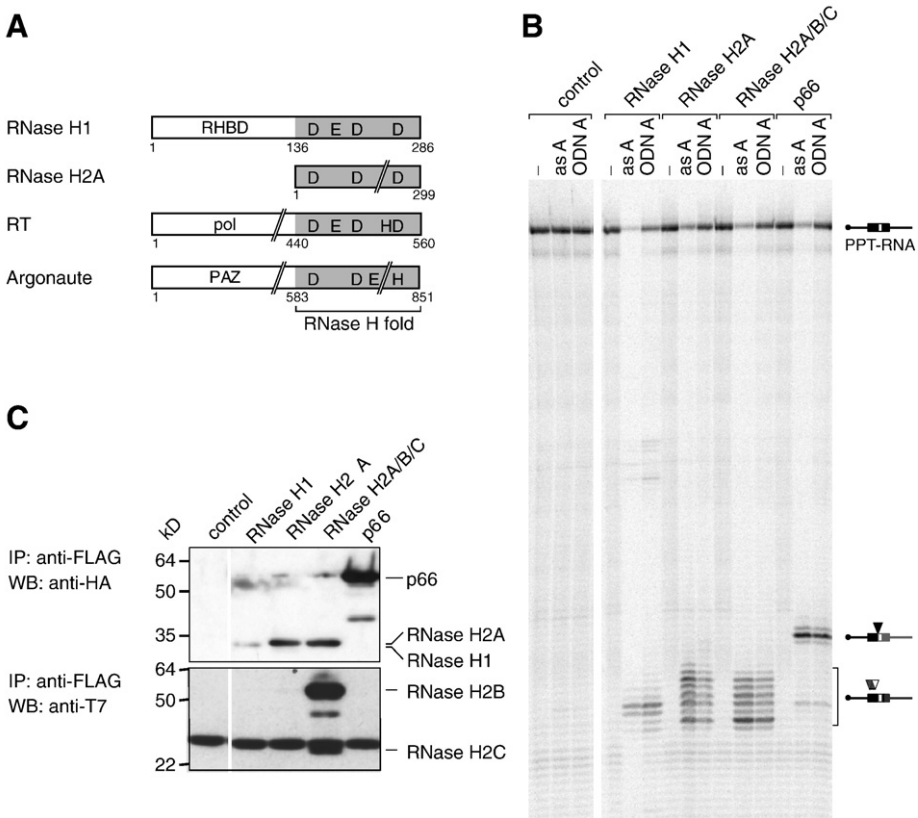


**Fig. 4.** Roles of cellular RNases H in ODN A-mediated hydrolysis of target RNA. (A) Scheme of the RNase H or RNase H-like domains (grey rectangles) of eukaryotic RNase H1, RNase H2A, HIV-1 RT or Argonaute 2 (Ago2) [47]. The bars are not in scale. Amino acid residue numbers are shown below each bar. The catalytic amino acid residues are shown in capital letters. Ago2 is a highly conserved protein which plays a key role in RNA interference and is a critical component of the RNA-induced silencing complex (RISC) [27]. The RNA–DNA hybrid binding domain (RHBD) of RNase H1, the polymerase (pol) domain of RT, and the PAZ domain of Ago2 are indicated. (B) For *in vitro* cleavage assays HA/FLAG-tagged RNase H1, RNase H2A, p66, and T7-tagged RNase H2B and T7-tagged RNase H2C were expressed in HEK293 cells as indicated and RNase H1, RNase H2A and p66 were immunoprecipitated. 5′-labelled HIV-1 PPT RNA was incubated with immunoprecipitates of either MOCK-transfected-(control), RNase H1-, RNase H2A-, RNase H2A/B/C complex-, or p66-transfected cells in the presence of ODN A (50 nM) for 30 min at 37 °C. Cleavage products were analyzed in 6% urea-polyacrylamide. Schematics are as described in Fig. 2A legend. (C) The amount of proteins in the immunoprecipitates was determined by Western Blotting using anti-HA or anti-T7 antibody.

[13]. In order to test, whether cellular RNases H would cleave PPT-containing RNA in the presence of ODN A or asA *in vitro*, we transfected HEK293 cells with plasmids expressing HA-tagged RNase H constructs for RNase H1, RNase H2A and p66. In the case of RNase H2, we also co-transfected the cells with T7-tagged RNase H2B and T7-tagged RNase H2C. All RNase H-containing immunoprecipitates were able to cleave the target RNA in the presence of ODN A or asA (Fig 4B). Surprisingly, in this experiment RNase H2A did not require T7-tagged RNase H2B and T7-tagged RNase H2C for cleavage, most likely because it co-precipitated with the endogenous RNase H2B and RNase H2C subunits in a trimeric complex (Fig. 4B, lanes 7–9), implying a low expression of the exogenous proteins. RNase H1, RNase H2 and RNase H of p66 showed slightly different cleavage patterns, regardless of whether the RNA/ODN hybrid substrates consisted of ODN A or asA (Fig. 4B). Differences in specificity between RNase H1 and RNase H2 in RNA hydrolysis triggered by antisense ODNs have been described previously [20]. p66 cleaved at a specific site as indicated in Fig. 1A.

### *2.4. ODN A-mediated gene silencing occurred via an RNase H-dependent mechanism*

To test for a contribution of RNase H1 to ODN A-dependent RNA hydrolysis in a lysate supplementation assay, we knocked down

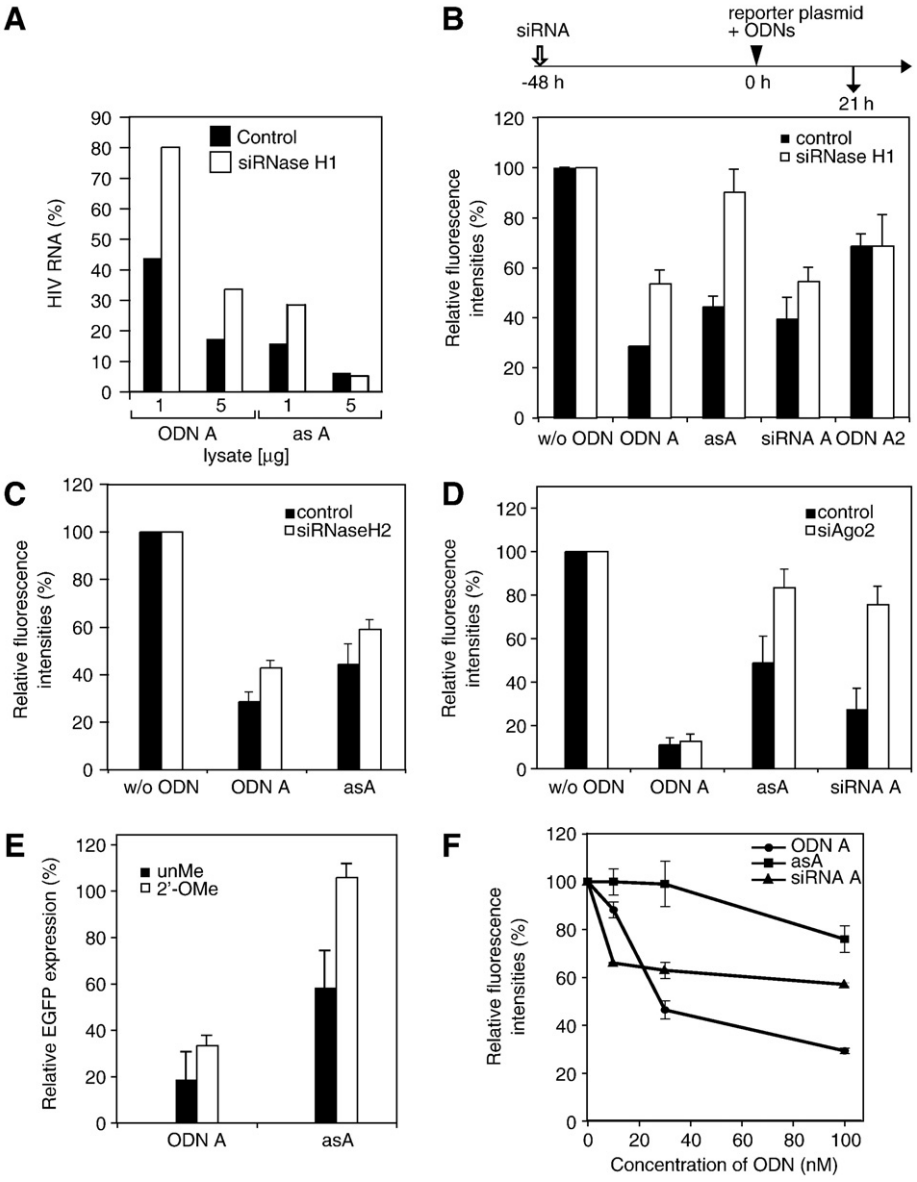


**Fig. 5.** Roles of cellular RNases H in ODN A-mediated transgene silencing. (A) Cytoplasmic lysate (1 or 5 µg) of HEK293 and RNase H1-knockdown HEK293 was incubated with HIV-1 RNA in the presence of 250 nM ODN A, asA or TE buffer as control for 2 h at 37 °C in a lysate supplementation assay (Materials and methods). Graph shows the relative amounts of PPT RNA relative to the control. One representative experiment of two is shown. (B–D) HEK293 cells pre-transfected with negative control siRNA (control) and (B) RNase H1-specific siRNA (siRNase H1), (C) RNase H2A-specific siRNA (siRNase H2A) or (D) Ago2-specific siRNA (siAgo2) for 48 h were transfected with pd2EGFP-N1-PPT and 100 nM oligonucleotides for 21 h prior to FACS. The time-line of the transgene expression assay is shown. EGFP expression levels were normalized against those of null-ODN controls (w/o ODN). The average level of knockdowns was 58% for RNase H1 and 95% for RNase H2a. (E) HEK293 cells were transfected with pd2EGFP-N1-PPT and 100 nM unmethylated (unMe) or fully 2′-*O*-methylated (2′-*O*-Me) ODN A or asA for 21 h. The relative EGFP mRNA levels determined by qRT-PCR are shown. (F) Comparison of the dose-dependent reduction of d2EGFP levels by ODN A, asA and siRNA A. HEK293 cells were transfected with pd2EGFP-N1-PPT and ODN A, asA or siRNA A (0, 10, 30, and 100 nM) for 21 h prior to FACS. Representative results from three independent experiments are shown. Error bars represent standard deviations of duplicates. The *P*-values of the various differences in EGFP mRNA levels were determined by Student's *t*-test. The corresponding *P*-values for ODN A versus asA are: 0.1 (10 nM), 0.002 (30 nM) and 0.002 (100 nM); the corresponding *P*-values for ODN A versus siRNA are: 0.0005 (10 nM), 0.01 (30 nM) and 0.04 (100 nM).

endogenous RNase H1 by siRNA in HEK293 cells. Indeed knockdown of RNase H1 reduced the level of RNA hydrolysis observed with either ODN A or asA (Fig. 5A). The extents of RNA degradation were slightly higher in the presence of asA compared to ODN A, indicating that asA was slightly more effective under the conditions of this assay.

To investigate the molecular mechanism by which ODN A mediated gene silencing in living cells, we analyzed the effects of ODN A and control oligonucleotides on the levels of d2EGFP in the HEK293 cells whose RNase H1 (Fig. 5B), RNase H2 (Fig. 5C) or Argonaute 2 (Ago2) (Fig. 5D) had been knocked down. Ago2, which contains a PIWI domain exhibiting an RNase H fold (Fig. 4A), is part of the RNA-induced silencing complex (RISC) and plays a key role in RNA interference [27]. Knockdown of the various RNA processing enzymes was achieved by pre-transfection of the cells with the corresponding specific siRNAs. In the time frame tested, knockdown of the enzymes was not toxic as verified by trypan blue exclusion and proliferation assays (data not shown). For control cells, a negative control siRNA was used. Treatment of the control cells and RNase H1-knockdown cells with ODN A resulted in $74 \pm 0.1\%$ and $47 \pm 5\%$ reduction, respectively, in the level of d2EGFP (Fig. 5B). In contrast, knockdown of RNase H1 led to an almost complete abrogation of the gene silencing effect of asA (Fig. 5B). RNase H2 knockdown had a minor effect on gene silencing by both ODN A and as A (Fig. 5C). Ago2-knockdown did not affect gene silencing by ODN A, but significantly reduced gene silencing by siRNA and asA (Fig. 5D). The RNase H1-dependent gene silencing effect of ODN A is sequence-specific, since RNase H1-knockdown had no effect on the level of d2EGFP in cells treated with the negative control ODN A2 (Fig. 5B). The effects of the ODNs on d2EGFP expression at the protein level mirrored those evaluated at the RNA level using quantitative RT-PCR (data not shown). Taken together, the results of the lysate supplementation assay (Fig. 5A) and reporter assays (Fig. 5B–F) suggest that the gene silencing effect of ODN A in intact cells is due to ODN A-induced cleavage of RNA by RNase H1. The results of gene expression assays in RNase H1 knockdown cells suggest that the gene silencing effect of ODN A was only partly dependent on RNase H1 (Fig. 5B) whereas that of asA was predominantly RNase H1-dependent (Fig. 5B). Therefore we cannot rule out the possibility that an RNase H-independent mechanism might also contribute to the gene silencing effect of ODN A. Our data indicate a minor role of RNase H2 and no role of Ago2 for ODN A-dependent gene silencing (Fig. 5B, C and D), and surprisingly, Ago 2 appeared to have contributed to gene silencing by asA to a small extent (Fig. 5B and D).

To further examine whether RNase H-independent pathways could contribute to the gene silencing effects observed, we compared the effects of ODN A and asA with those of their 2′ O-methylated variants, the latter of which do not allow cleavage of the RNA by RNase H due to the 2′ O-methylation. Consistent with the results shown in Fig. 5B and C, a fully 2′ O-methylated ODN A, compared to control ODN A, led to only a slight reduction in gene silencing whereas the 2′ O-methylation modification completely abolished the ability of asA to reduce the level of d2EGFP (Fig. 5E). These observations support our hypothesis that RNase H1 plays an essential role in the asA-mediated gene silencing whereas the gene silencing effect of ODN A in intact cells is less RNase H1-dependent and might involve a partial contribution of RNase H-independent mechanisms.

To further compare the potency of these oligonucleotides in gene silencing, we examined in parallel the effects of ODN A, asA and siRNA A on the level of d2EGFP in the reporter assay in a dose-dependent manner. The results show that ODN A, siRNA A and asA, at a concentration of 100 nM, downregulated the expression of d2EGFP by $71 \pm 2\%$, $41 \pm 2\%$ and $22 \pm 11\%$, respectively (Fig. 5F). Although ODN A, asA and siRNA A all reduced the expression of d2EGFP in a dose-dependent manner, ODN A showed significantly stronger gene silencing effects compared with those of siRNA and asA at 30–100 nM ($p<0.05$) (Fig. 5F).

## 3. Discussion

RNases H play a key role in antisense technology. Previous studies aiming to characterize the interaction of RNases H with RNA/as hybrids have stimulated the development of antisense for applications such as the reduction of cellular gene expression by post-transcriptional gene silencing [28–31] and inhibition of viral replication [32–37]. Antisense analogs with modifications such as phosphorothioation [38], ODN circularization [39] and introduction of a hairpin-loop structure [5,32,35,40] have been found to show enhanced efficacy compared to unmodified antisense ODNs. In particular, Kuwasaki et al. showed that hairpin antisense ODNs, compared to single-stranded antisense ODNs, were more resistant to nuclease degradation and resulted in a stronger anti-HIV activity [32]. Consistent with these results, previous work in our laboratory showed that the hairpin-loop structured ODN A, which specifically forms a RNA–DNA hybrid with the 3′ polypurine tract (PPT) of HIV-1 RNA [9] and triggers hydrolysis of the HIV-1 RNA by the RNase H moiety of RT, showed increased efficacy compared with its antisense strand alone [4–8]. Here we extended the previous findings by showing that the hairpin-loop structured ODN A can reduce gene expression in the human kidney epithelial cell line HEK293.

While the general utility of hairpin ODNs for gene silencing remains to be further delineated, this proof-of-concept study shows that ODN A was more potent than its antisense counterparts in HEK293 cells. Since ODN A was less effective than asA in lysate supplementation assays, the increased potency of ODN A compared to asA in cells may be partly explained by a higher intracellular stability of ODN A due to a higher nuclease resistance [32,40]. Also the siRNA A used here was less efficient than ODN A. However this siRNA, which was designed analogously to ODN A, might not be representative for RNA interference. Although its size of 27 nucleotides is in agreement with effective siRNAs which require dicer for their processing and incorporation into RISC, its nucleotide sequence has not been optimized. This study has provided preliminary insights into the possible mechanisms by which ODN A exhibits its gene silencing effects. Knockdown of RNases H1 reduced the ODN A-mediated gene silencing effect, demonstrating that cellular RNase H1 plays a role. The ability of ODN A-like hairpin-loop ODNs to co-opt cellular RNase H for gene silencing is supported by our other recent observations: (i) an ODN designed to target the polymerase RNA of *influenza* virus, which does not possess any viral RNase H activity, was able to inhibit influenza replication in cell culture and in mice [37]; (ii) similarly, ODNs targeting essential genes of herpes simplex virus inhibited viral replication in mammalian cells [41] and (iii) an ODN targeting human or murine telomerase RNA induced rapid inhibition of proliferation and inhibited tumor formation in a surrogate metastasis model by reducing telomerase RNA levels [42] However, in contrast to the gene silencing effects of asA and siRNA A which were predominantly RNase H1-and Ago2-dependent, respectively, the gene silencing effects of ODN A exhibited only a partial dependence on RNase H1 or RNase H2. This suggests that some forms of RNase H-independent mechanisms may contribute to the gene silencing effects of ODN A, e.g. through translational inhibition, a mechanism which is responsible for the gene silencing effects of some ODNs [43]. We hypothesize that ODN A may be able to inhibit translation by one of the following mechanisms. The presence of a second strand in ODN A might enable ODN A to cause a more effective physical blockade of the translational machinery compared to asA. Since the second strand of ODN A is G-rich, G-quadruplex formation may further contribute to maintain this blockade. The second strand of ODN A alone showed a G-quadruplex-specific UV absorption spectrum *in vitro* [44]. In the presence of the antisense strand, G-quadruplex formation by ODN A was however not detectable, probably due to the formation of Watson–Crick base pairs which may stabilize the partially double-stranded structure in preference to the G-quadruplex structure (data not shown). Consistent with this, electrophoretic mobility analyses of

the ODN A in native gels did not indicate G-quadruplex formation. The binding of the antisense strand of ODN A to the PPT may however enable the second strand of ODN A to adopt higher-ordered structures. Another possibility is that ODN A and the PPT RNA may together form G-quadruplexes which could then lead to translational inhibition. Alternatively, ODN A may form a triplex structure with the PPT RNA on the basis of Hoogsteen rules [5]. Although we have not been able to demonstrate triplex formation of ODN A-PPT RNA *in vitro*, it is possible that an ODN A-PPT RNA triplex might exist transiently *in vivo* and hence contribute transiently to translational inhibition. The inhibition of gene expression observed with the 2′ O-methylated ODN A, which was RNase H-independent, may also be due to translational inhibition mediated by G-quadruplex formation because 2′ O-methylated ODNs compared to unmethylated ODNs have a greater tendency to form G-quadruplexes. However, given that structural differences exist between 2′ O-methylated ODN A and unmethylated ODN A, it is plausible that 2′ O-methylated ODN A may elicit gene silencing via mechanisms distinct from those of unmethylated ODN A.

The findings in this study have implications on the roles of the various cellular RNases H in oligonucleotide-mediated reduction of gene expression. Our data support the previous observation that RNase H1 rather than RNase H2 plays a major role in antisense gene silencing [11]. The hydrolysis of mRNA by RNase H occurs predominantly in the cytoplasm [23] and our confocal microscopy analyses showed that the transfected ODN A and asA were found mostly in the cytoplasm (data not shown). Recently it has been described that HEK293 cells express higher levels of RNase H1 than RNase H2 and RNase H1 is found throughout the cell, whereas RNase H2 localization is restricted to the nucleus [24]. This might explain the minor role of RNase H2 for gene silencing observed here. A previous systematic study has shown that RNase H-dependent antisense ODNs but not siRNAs were active towards exon targets in the pre-mRNA in the cell nucleus [45]. We do not exclude the possibility that RNase H2 may play a larger role during processes such as cell division when the ODNs can more readily enter the nucleus. Whether ODNs could also be effective towards pre-mRNA in the nucleus is an interesting question to be addressed in the future.

This proof-of-concept study underscores the potential utility of hairpin-loop structured ODNs for reduction of gene expression, particularly of ODN A to recruit cellular RNase H for degradation of HIV-1 mRNA in chronically infected cells. Future investigations should aim to further characterize the modes of action and the *in vivo* efficacy of this type of ODNs. A systematic study using ODN A-like ODNs targeting different target sites on mRNA is underway to explore the general utility of this class of ODNs.

## 4. Materials and methods

### 4.1. Cell cultures and viruses

The human embryonic kidney (HEK293) cell line, the HTLV-1-transformed T-cell lymphocytic cell line C81-66/45 and HIV-1 strain IIIB were cultivated as described previously [5,46].

### 4.2. Oligonucleotides

The ODNs except the 2′-*O*-methyl (2′-*O*-Me)-modified ODNs were synthesized as described [7] by Operon Biotechnologies. The 2′-*O*-methyl (2′-*O*-Me)-modified ODNs, which were phosphorothioated at the last two bases on both 5′ and 3′ ends and at the four-thymidine linker, were synthesized by Integrated DNA Technologies. siRNA A was synthesized by Dharmacon Inc.

### 4.3. Plasmids

The oligonucleotides NR1 5′GATCTTTTAAAAGAAAAGGGGGGACTGGAAGGGCTAG3′ and NR2 5′TCGACTAGCCCTTCCAGTCCCCCCTTTTCTTTTAAAA3′ were annealed, digested with Bgl II and Sal I and cloned into the Bgl II and Sal I sites in pd2EGFP-N1 (Clontech) to generate the reporter plasmid pd2EGFP-N1-PPT. The p66 gene of the codon-optimized 96ZM651.8 clone (AIDS Reagents Cat# 6173) was cloned into pIRESneoHA/FLAG and mutagenized using D498A: 5′GGTGAACATCGTGACCGCCAGCCAGTACGC3′; D443A: 5′CCTTCTACGTGGCCGGCGCCGCCAAC3′; D185A/D186A: 5′CTACCAGTACATGGCCGCCCTGTACGTGGGC3′.

The RNaseH1 insert was amplified from pET15b-RnH1 (from R. Crouch) by PCR using primers RnH1upper 5′GATTACGCTAGCGGCCGCATGTTCTATGCCGTGAGGAGGGGC3′ and RnH1lower 5′CCACTGAATTCTCAGTCTTCCGATTGTTTAGCTCCTTCTC3′. The NheI/EcoRI fragment was cloned into pIRESneoHA/FLAG-Ago2(NheI/EcoRI) replacing Ago2. RNase H2A was cloned from the plasmid pcDNA3.1-mychis-Dest/RNase H2A (from A. Jackson) using RnH2upper 5′GATTACGCTAGCGGCCGCATGGATCTCAGCGAGCTG3′ and RnH2lower 5′CCACTGAATTCCTAGAGGCTGGTTGCTGACTCCA3′.

### 4.4. Cell lysis

C81-66/45 was infected by HIV-1 IIIB as described [4]. Cells were pelleted by centrifugation and lysed in buffer C (10 mM HEPES pH7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF, Complete™ protease inhibitor cocktail (Roche), 0.2% NP-40). The supernatant obtained by centrifugation at 1000 *g* for 5 min was used in lysate supplementation assays. To prepare the cytoplasmic lysate of HEK293 or RNase H1-knockdown HEK293, cells were harvested in ice-cold PBS, centrifuged at 1000 *g* for 5 min at 4 °C and lysed in buffer C without NP-40. The cell suspension was homogenized and ultracentrifuged as described [46].

### 4.5. Lysate supplementation assays

Cell lysate was incubated with 80 ng HIV-1 genomic RNA and 250 nM oligonucleotides or TE in cleavage buffer (50 mM HEPES, pH 7.9, 5 mM ATP, 1 mM GTP, 50 U/ml RNasin, 500 mM KCl, 7.5 mM $MgCl_2$, 2.5 mM DTT and 50 μM $ZnCl_2$) for 2 h at 37 °C (in 20 μl). HIV-1 RNA was then extracted using QIAamp Viral RNA Mini Kit (QIAGEN). PPT RNA was amplified by RT-PCR using primers PU3.1 and PU3.4 which flank the cleavage site located 5′ to the ACU sequence within the PPT sequence [7]. The resultant RT-PCR product (328 bp) was electrophoresed in 2% agarose and quantified by densitometry.

### 4.6. Immunoprecipitation and RNase H assays

HEK293T cells transfected with plasmids expressing the various RNases H for 36 h were lysed in 500 μl lysis buffer (20 mM Tris HCl pH 7.2, 150 mM KCl, 2 mM $MgCl_2$, 0.5 mM DTT, 0.5% NP-40 supplemented with the Complete™ protease inhibitor cocktail (Roche)) for 10 min and centrifuged for 10 min at 13,000 rpm at 4 °C. The HA/FLAG-tagged RNases H constructs were immunoprecipitated using anti-FLAG agarose (Roche) which were then washed sequentially with Lysis buffer, buffer R (50 mM Tris HCl pH 7.2, 500 mM NaCl, 5 mM $MgCl_2$, 0.5 mM DTT, 0.5% NP-40) and buffer S (50 mM Tris HCl pH 7.2, 50 mM NaCl, 5 mM $MgCl_2$, 0.5 mM DTT). The beads were used for cleavage reactions in RNase H buffer containing 0.2 U/μl RNasin (Roche) and 10 nM 5′-labeled HIV-PPT RNA pre-annealed with 50 nM ODN as described [6,36]. Beads were analyzed by Western blotting using 10% SDS-PAGE and anti-HA-horseradish peroxidase (Roche) or anti-T7 (Novagen) antibodies.

### 4.7. Knockdown of RNase H1 and RNase H2A in HEK293 cells

RNase H1- and RNase H2A-deficient HEK293 cells were generated by transfection of HEK293 in 10-cm plate with 2–5 nM siRNAs (QIAGEN, except where stated) using HiPerfect (QIAGEN) according to

manufacturer's instructions. The siRNAs for knocking down RNase H1 expression: siRnH1_1 s 5′GUUUGCCACAGAGGAUGAGdTdT 3′; siRnH1_1as 5′CUCAUCCUCUGUGGCAAACdTdG3′ (synthesized by Dharmacon Inc); siRnH1_2s 5′CGAUAAAUGGUAUAACUAAdTdT3′ siRnH1_2as 5′UUAGUUAUACCAUUUAUCGdTdA3′; siRnH1_3s 5′GGUUAAGUAUAUAAUAAAUdTdT3′ siRnH1_3as 5′AUUUAUUAUAUACUUAACCdAdT3′. The siRNA for knocking down RNase H2A expression: siRnH2A_1s 5′GGACUUGGAUACUGAUUAUdTdT3′; siRnH2A_1as 5′AUAAUCAGUAUCCAAGUCCdTdG3′; siRnH2A_2s 5′GGAUUGAGGUGACGGUCAAdTdT3′; siRnH2A_2as 5′UUGACCGUCACCUCAAUCCdCdG3′ siRnH2A_3s 5′GGGUCAAAUACAACCUGAAdTdT3′; siRnH2A_3as 5′UUCAGGUUGUAUUUGACCCdGdC3′. The siRNAs siAgo2-1 (Dharmacon) was used for Ago2 knockdown [27]. The AllStars negative control siRNA was purchased from QIAGEN.

### 4.8. Quantification of RNA by real-time RT-PCR (qRT-PCR)

Cellular RNA was isolated using QIAamp RNA-Blood Mini Kit (QIAGEN). cDNA synthesis and qRT-PCR were performed as described [36]. Primers and Taqman probes for amplifying *gapdh* cDNA: forward 5′GAAGGTGAAGGTCGGAGTC3′; reverse 5′ GAAGATGGTGATGGGATTTC3′; probe: 5′FAM-CAAGCTTCCCGTTCTCAGCC-TAMRA3′. For amplifying human *rnase H1* cDNA: forward 5′GGTTTCCTGCTGCCAGATTTAA3′; reverse 5′GGCTTGCAGATTTCCTGACAA3′; probe 5′FAM-TTTGCCACAGAGGATGAGGCCTGG-TAMRA3′. For amplifying human *rnase H2A* cDNA: forward 5′GTGGCAGACTCAAAGACCCTAT3′; reverse 5′CACGAATACCTGGGTGACGTT3′; probe: 5′FAM-GGGCGGGTCAAATACAA-TAMRA3′. For amplifying *d2egfp* cDNA: forward 5′CTGCTGCCCGACAACCAC3′; reverse 5′TCACGAACTCCAGCAGGAC3′; probe 5′VIC-CCAGTCCGCCCTGAGCAAAGACC-TAMRA3′. Cycling conditions used: 50 °C, 2 min (1 cycle); 95 °C, 10 min (1 cycle); and 95 °C for 15 s followed by 60 °C for 1 min (40 cycles). Primers and probes were synthesized by Microsynth and Applied Biosystems, respectively.

### 4.9. Transgene expression assays

HEK293 or HEK293 transfected with siRNAs for 48 h were seeded in 12-well plates ($5\times10^5$ cells per well). After overnight incubation, the cells were transfected with pd2EGFP-N1-PPT (360 ng) and oligonucleotides (100 nM unless otherwise stated) using 2.5 μl lipofectamine 2000 (Invitrogen) per μg DNA. At 21 h post-transfection, EGFP expression was analyzed by FACS or qRT-PCR. Amount of EGFP mRNA was normalized against the amount of GAPDH mRNA and expressed as percentages of EGFP expression compared to that of control cells transfected with vector alone (null-ODN control).

### 4.10. Fluorescence activated cell sorting (FACS)

Single cells, after trypsinisation, were fixed in PBS containing 2% paraformaldehyde. Mean fluorescence intensities (based on 10000 counts) were determined using a FACSCalibur® flow cytometer and the software CellQuest® (BD Biosciences).

## Acknowledgements

We are grateful to Robert J. Crouch and Andrew P. Jackson for providing the human RNase H1 and human RNase H2 expression constructs, respectively. We thank Firouzoh Farahmand and Kolja Schaale for their technical support.

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