Supporting Information

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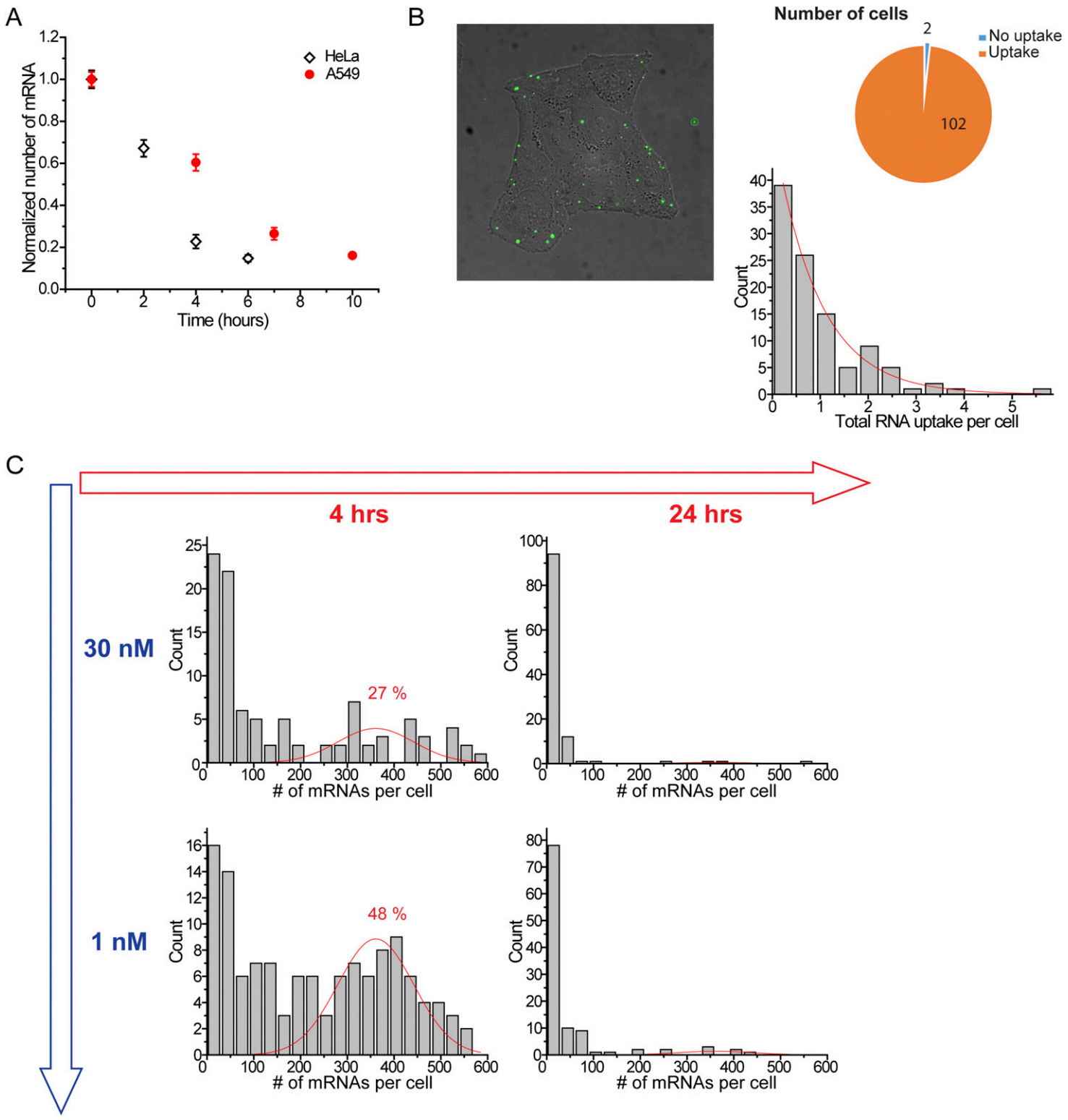


Fig. S1. (*A*) mRNA reduction in A549 cells vs. HeLa cells treated by 3 nM of U27 RNA continuously without washing shows the different silencing kinetics according to cell lines. (*B*) Cell images of labeled iRNA uptake show nearly 100% of transfection efficiency. (*C*) iRNA concentration-dependent gene silencing suggests that the unsilenced population seen at the early time point comes from the more slowly silencing population. We fit the unsilenced population to the Gaussian curve using the same mean and peak width of cytoplasmic mRNA as in a population of cells that were not treated with any iRNAs.

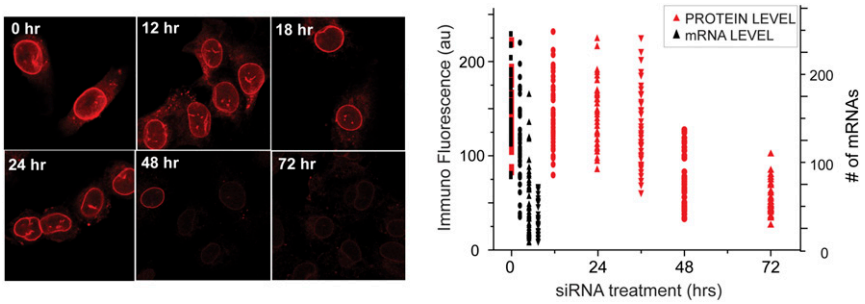


Fig. S2. Immunofluorescence of lamin A protein and smFISH of lamin A mRNA shows the extensively delayed silencing kinetics of the protein compared with that of the mRNA.

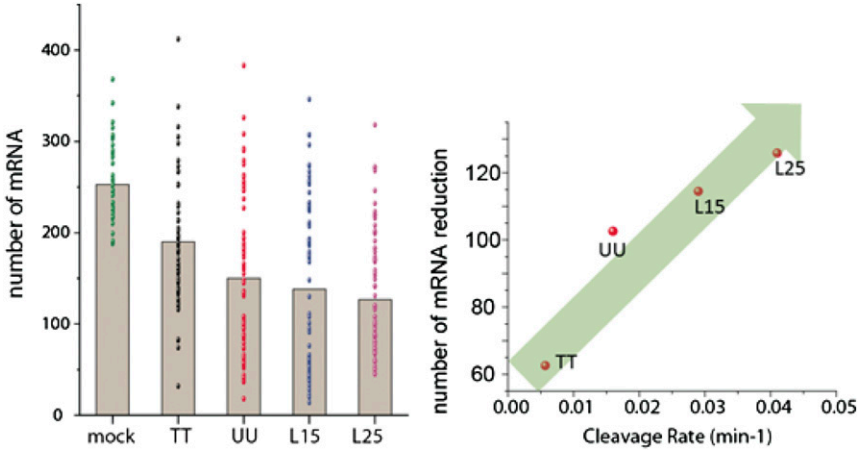


Fig. S3. The faster gene silencing with a longer loop and the positive correlation between dicing rate and the silencing efficiency are observed not only for the loop of polyuracil but also for random sequences.

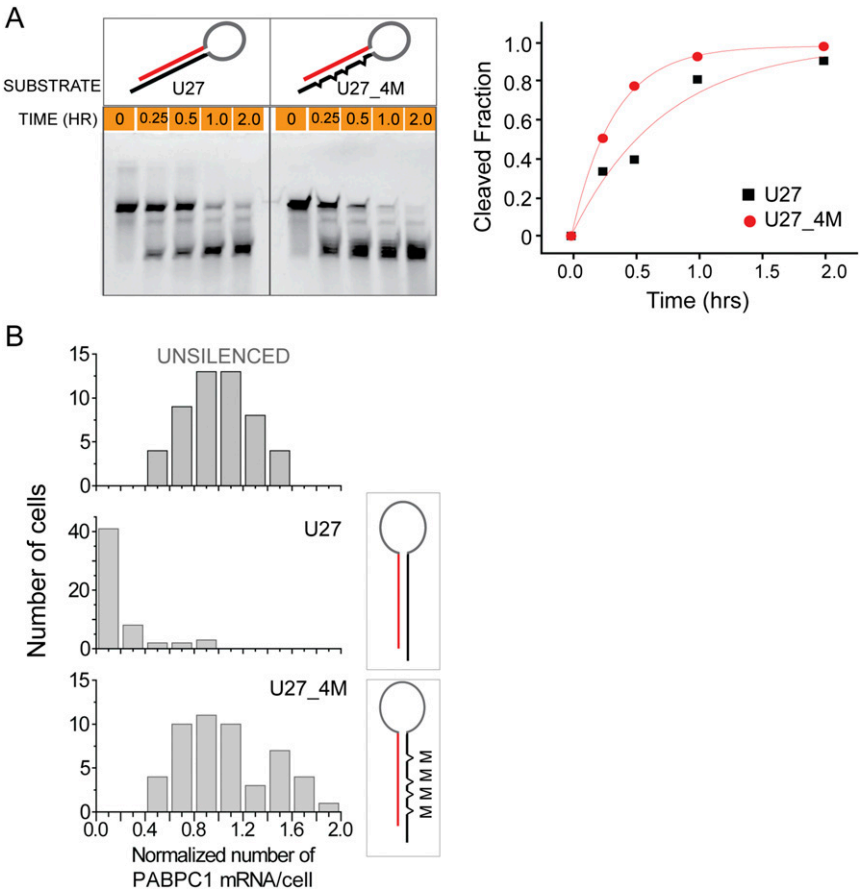


Fig. S4. The decoupling between dicing and silencing according to stem mismatch is shown in the *PABPC* gene as well, suggesting that the decoupling is not gene specific. The stem mismatch of iRNA against the *PABPC* gene shows comparable or faster dicing kinetics in comparison with the complementary iRNA (*A*) but displays much more inefficient gene silencing (*B*).

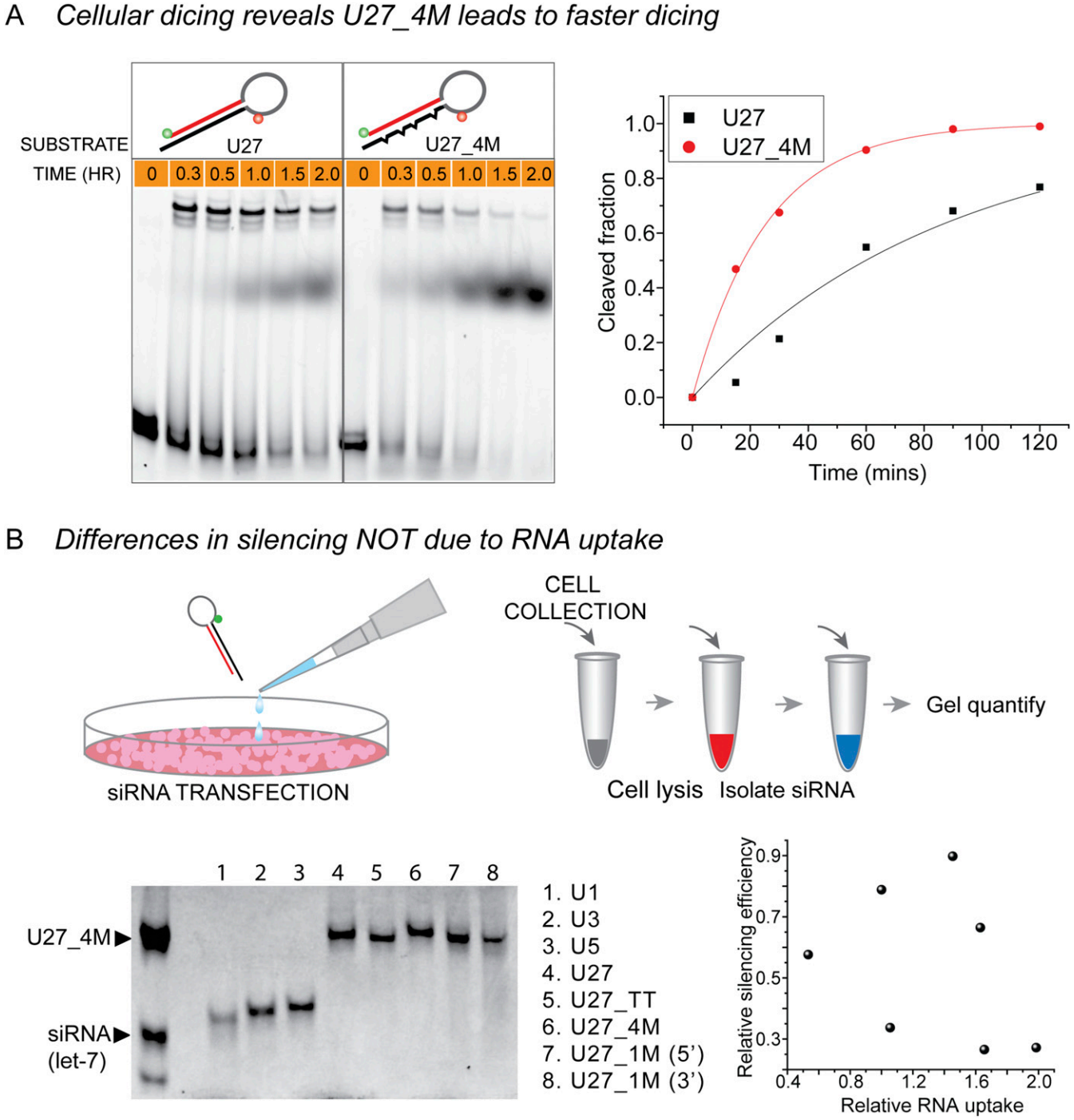
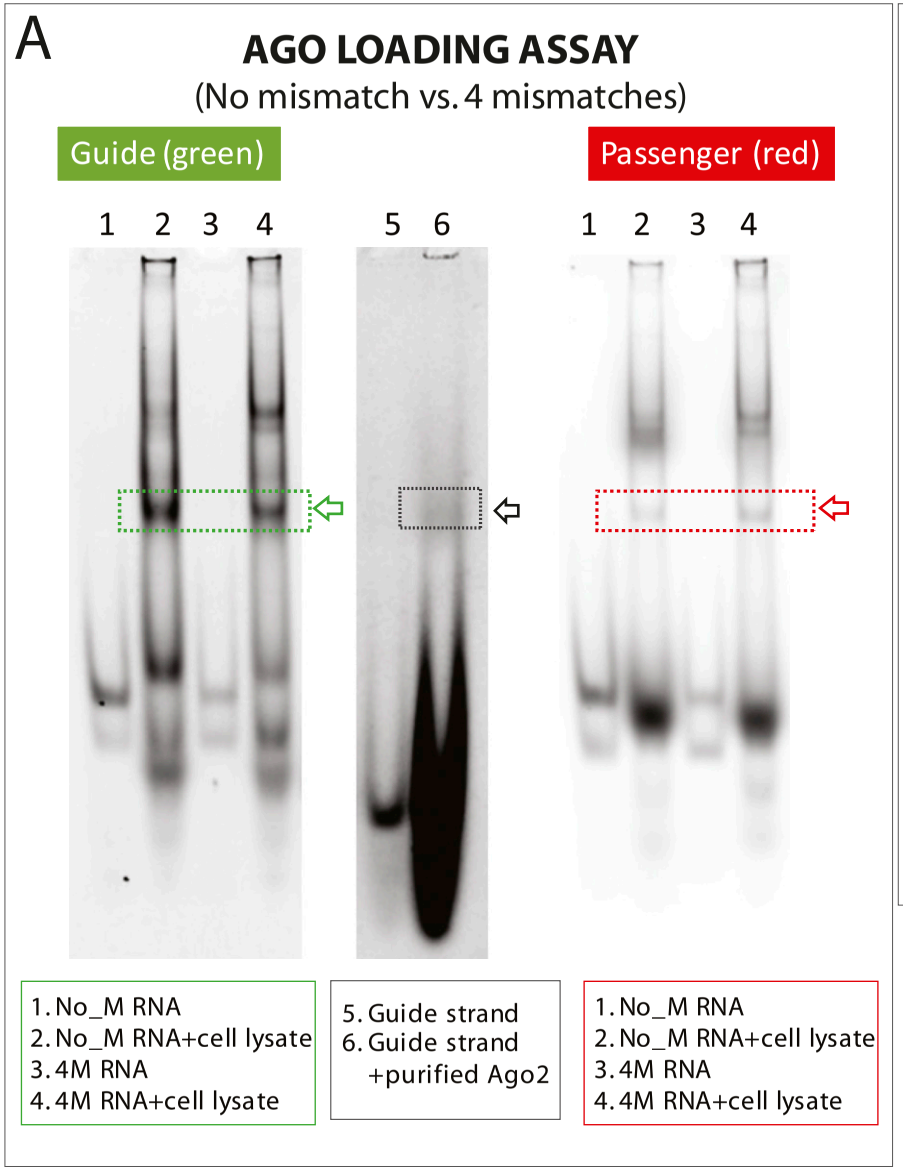


Fig. S5. Assays to measure the cellular dicing rate and the relative RNA uptake for iRNAs. (*A*) Cellular dicing assay using HeLa cell lysate confirms that the stem mismatches do not reduce the dicing rate. (*B*) The relative RNA uptake was measured for iRNAs by quantifying iRNAs extracted from cells that are transfected with labeled iRNAs. We found no correlation between the silencing efficiency and RNA uptake.



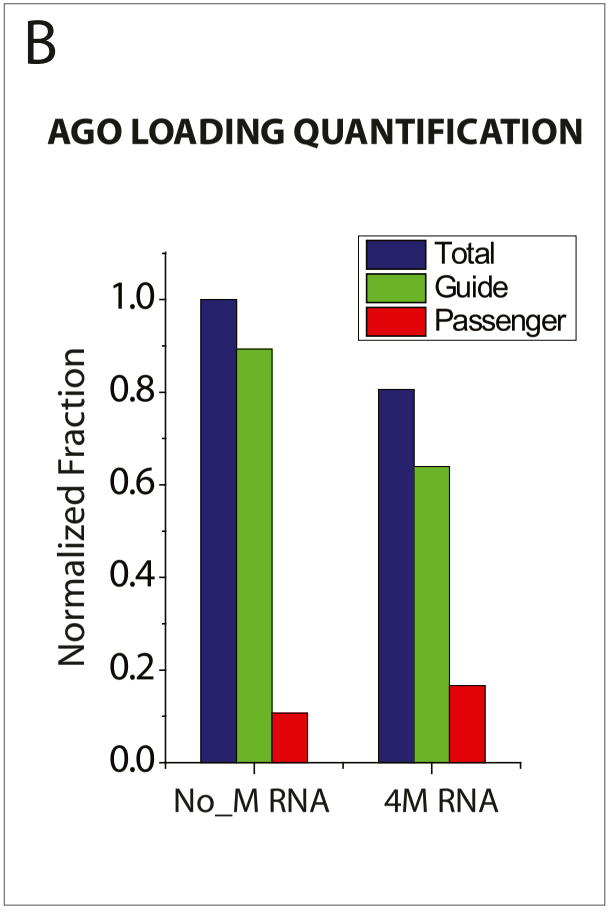


Fig. S6. Ago loading assay using Dicer substrates with no mismatch vs. Dicer substrates with four mismatches. (*A*) An EMSA was performed using HeLa cell lysates and a dual-labeled Dicer substrate that has Cy3 dye at its guide strand and Cy5 at its passenger strand. (*B*) The gel bands marked by green and red arrows in *A* were quantified. The stronger guide-Ago gel band and weaker passenger-Ago band suggest that the mismatches in the stem could reduce the proper Ago loading of the guide strand.

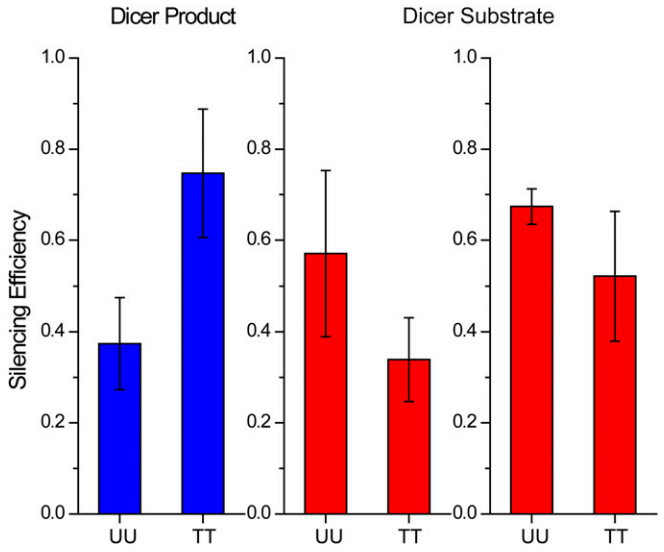


Fig. S7. DNA substitution at the 3′ overhang of the Dicer product enhances the silencing efficiency, which is the opposite of the effect on the Dicer substrate. Two different Dicer substrates were tested, one with a loop (*Center*) and the other without a loop (*Right*).

Table S1. Sequences of FISH probes for lamin A gene

Name	Sequence	Name	Sequence
lmna_1	tattgcagccctcagagttc	lmna_intron_1	aagagggacagacactgtag
lmna_2	ctgagcagctatcaggtcac	lmna_intron_2	ctgatgccagatatggaagc
lmna_3	cttctcactgagagcagtgc	lmna_intron_3	ccatcactggcaaatctgat
lmna_4	ctagggctgcctcaagcttg	lmna_intron_4	ggaaatcctctagtgctcac
lmna_5	atggtctgcagcctgttctc	lmna_intron_5	ctagcagggaccaaagtaga
lmna_6	ttctggaagtccagttcctc	lmna_intron_6	ccaagagatgactaggggta
lmna_7	cagctcctcactgtagatgt	lmna_intron_7	ggtctccaaggaaagtaagg
lmna_8	caccagtcgggtctcatgac	lmna_intron_8	ctgtaccactttggcacaag
lmna_9	gctgcttcccattgtcaatc	lmna_intron_9	gctgatgagcacagaagttt
lmna_10	cacctggtcctcatgctggg	lmna_intron_10	catagatgcatgatccctgt
lmna_11	ggcagaataagtcttctcca	lmna_intron_11	tttcagaaggtgtctcaagg
lmna_12	gactgcctggcattgtccag	lmna_intron_12	ctaagccacaagttccagag
lmna_13	caggttgctgttcctctcag	lmna_intron_13	ctctctaccctgaaggctaa
lmna_14	gactgctgcagctcctcgtg	lmna_intron_14	gcaacttagattccgagctc
lmna_15	gctgccagctgcttctggag	lmna_intron_15	gctggtaaagtgaagcaact
lmna_16	aggtctcgaagcttcgcctc	lmna_intron_16	gaaccggagaatgtattccc
lmna_17	ctcacgggccagtgagtcct	lmna_intron_17	atagagtgacactcagaggg
lmna_18	gtttgcgctttttggtgacg	lmna_intron_18	agcaattcactgtgaagagg
lmna_19	ctgcggctctcagtggactc	lmna_intron_19	gctgccatctgtaactcatc
lmna_20	ggtcctcattggacttgttg	lmna_intron_20	ctgggcccctgattattttt
lmna_21	gatctgccaattgcccatgg	lmna_intron_21	gctataaccagtcgaacagc
lmna_22	gatcatctccattctggcgc	lmna_intron_22	cctcaaagtgggtaaagctt
lmna_23	gggaaccggtaagtcagcaa	lmna_intron_23	atctggttccagatgtggat
lmna_24	gtgttctgtgccttccacac	lmna_intron_24	ctgatctgcactgaacagag
lmna_25	ttgatgagagccgtacgcag	lmna_intron_25	ggatgaccaggcacaatatt
lmna_26	ttgcgcatggccacttcttc	lmna_intron_26	aagtctggttagagctcaga
lmna_27	aaccacagtcactgagcgca	lmna_intron_27	tttacacatgtgggactgtg
lmna_28	cgagcgcaggttgtactcag	lmna_intron_28	aaaggtactggtcacctctc
lmna_29	cgctggcagatgccttgtcg	lmna_intron_29	tagcacttctcgatctgagc
lmna_30	cgagtgaccgtgacactgga	lmna_intron_30	ttacttagccttctccctgg
lmna_31	tgtatgatgctgcagttctg	lmna_intron_31	ttaggttggtttgcatctgc
lmna_32	ggaaggcagctcaaactcac	lmna_intron_32	gctatgcctgcaattaccag

Table S2. Sequences of FISH probes for the PABPC gene

Name	Sequence	Name	Sequence
pabpc1_1	ggaagttcacatacgcgtag	pabpc1-intron_1	cgagaaaatggtcgcaaagg
pabpc1_2	aattcatggtgtccaaagca	pabpc1-intron_2	ccctaaagtttgagagcgtc
pabpc1_3	ctggcttgccctttataaca	pabpc1-intron_3	atcgccacaggaactttatc
pabpc1_4	tactccacttttgcgaagtg	pabpc1-intron_4	cactgatgagttctgggaag
pabpc1_5	tagcccttggaaccattttc	pabpc1-intron_5	gaaaccccaaatgcatgtct
pabpc1_6	ctgcgtctcaaagtgtacaa	pabpc1-intron_6	aaaactggccttcaaagtgg
pabpc1_7	caatagctctttcagctgct	pabpc1-intron_7	aaaatggtctaagttgcctg
pabpc1_8	tttgcgatcatttaggagca	pabpc1-intron_8	ctcgaaaaccaccagtactg
pabpc1_9	ctagctccaagttcagcttc	pabpc1-intron_9	acagataagactggtccagt
pabpc1_10	ccaaagagatccttaaggcg	pabpc1-intron_10	gcaactcaacagaccacaga
pabpc1_11	actttcacacttaaggcagg	pabpc1-intron_11	taagctgacttctcaaagcc
pabpc1_12	agctttctgtgcatcttcat	pabpc1-intron_12	gacatgtattcaagtggcca
pabpc1_13	ctttttctgagctcgaccaa	pabpc1-intron_13	cacagtgaagagctgatcag
pabpc1_14	cctggtatctggtgatccta	pabpc1-intron_14	ttacaacttctggctctgtg
pabpc1_15	ggagaaaactctttccggag	pabpc1-intron_15	actgcttacagagcaaaagg
pabpc1_16	ctttagtggcttcttctggg	pabpc1-intron_16	tgcctaaggttgacaagaac
pabpc1_17	cgtacacttgccattctctg	pabpc1-intron_17	cccatctgacatgcattgat
pabpc1_18	tgccatgaagtaacctgaag	pabpc1-intron_18	agctgagaaagagtaacact
pabpc1_19	gatagtatgcagcacggttc	pabpc1-intron_19	tggtctactggtacagactg
pabpc1_20	ggacttggtcttagttgagc	pabpc1-intron_20	aactggtcactttcagaagc
pabpc1_21	gatagcaccgggcatatttt	pabpc1-intron_21	tgagtcacctcttcctatga
pabpc1_22	gacccattgtctgtgttgat	pabpc1-intron_22	gtccctttaagccctgaaa
pabpc1_23	tttatactgtggaacggtgc	pabpc1-intron_23	aaaagactagtccccgaaga
pabpc1_24	gctgtgcattaagatgttgc	pabpc1-intron_24	acctgtaccattaaaggctc
pabpc1_25	aggctgttgcattgtaactt	pabpc1-intron_25	ttcagtgattcatggcacaa
pabpc1_26	tttgcttttgctcttgagga	pabpc1-intron_26	attcttgacgcaatctctcg
pabpc1_27	ataagaggaaacagccgttc	pabpc1-intron_27	aagacaaataccagaagccc
pabpc1_28	acatgccagtgattttacca	pabpc1-intron_28	agtatcacacactagtgtgg
pabpc1_29	tggagactcgagcatatgaa	pabpc1-intron_29	aagcttcaagatggctagac
pabpc1_30	caaccttagaacggagtgac	pabpc1-intron_30	acatggtctcacagttgaac
pabpc1_31	gcttgtagtacagctacagc	pabpc1-intron_31	acttcaaaatttttgctggc
pabpc1_32	actgttaactgctttctggg	pabpc1-intron_32	gcaggaaggacattttcaga

Table S3. Sequences of various iRNAs

Category	Name	Sequence
Loop size variants	U1	AUAAGUCUUCUCCAGCUCCUUA/T/UAAGGAGCUGGAGAAGACUUAUUU
	U3	AUAAGUCUUCUCCAGCUCCUUAUU/T/UAAGGAGCUGGAGAAGACUUAUUU
	U5	AUAAGUCUUCUCCAGCUCCUUAUUUU/T/UAAGGAGCUGGAGAAGACUUAUUU
	U15	AUAAGUCUUCUCCAGCUCCUUAUUUUUUUUUUUUUU/T/UAAGGAGCUGGAGAAGACUUAUUU
	U27	AUAAGUCUUCUCCAGCUCCUUAUUUUUUUUUUUUUUUUUUUUUUUUU/T/UAAGGAGCUGGAGAAGACUUAUUU
Stem variants	U27_3′sm	AUAAGUCUUCUCCAGCUCCUUAUUUUUUUUUUUUUUUUUUUUUUUUU/T/UAAGGAGCUGGAGAAGACUUCUUU
	U27_5′sm	AUAAGUCUUCUCCAGCUCCUUAUUUUUUUUUUUUUUUUUUUUUUUUU/T/UAAGUAGCUGGAGAAGACUUAUUU
	U27_4m	AUAAGUCUUCUCCAGCUCCUUAUUUUUUUUUUUUUUUUUUUUUUUUU/T/UAAGGAGCUGAAGAAGGUUUGUUU
3′ overhang variants	U27_TT	AUAAGUCUUCUCCAGCUCCUUAUUUUUUUUUUUUUUUUUUUUUUUUU/T/UAAGGAGCUGGAGAAGACUUAUTT
siR_Stem variants	siR	(AS) 5′–AUAAGUCUUCUCCAGCUCCUU–3′ (S) 3′–UUUAUUCAGAAGAGGUCGAGG–5′
	siR_3′sm	(AS) 5′–AUAAGUCUUCUCCAGCUCCUU–3′ (S) 3′–UUUCUUCAGAAGAGGUCGAGG–5′
	siR_5′sm	(AS) 5′–AUAAGUCUUCUCCAGCUCCUU–3′ (S) 3′–UUUAUUCAGAAGAGGUCGAUG–5′
	siR_csm	(AS) 5′–AUAAGUCUUCUCCAGCUCCUU–3′ (S) 3′–UUUAUUCAGAAGAGGUCGAGG–5′
	siR_4m	(AS) 5′–AUAAGUCUUCUCCAGCUCCUU–3′ (S) 3′–UUUGUUUGGAAGAAGUCGAGG–5′
siR_3′ overhang variants	siR_TT	(AS) 5′–AUAAGUCUUCUCCAGCUCCUU–3′ (S) 3′–TTUAUUCAGAAGAGGUCGAGG–5′
Control measurement	Cmyc_siR	AACAGAAAUGUCCUGAGCAAUUUUAGGGUCACACCCACCACUGGGAGAUAAUUGCUCAGGACAUUUCUGUUCC
	PABPC_U27	AUUAUCAAUGGAUUUGUCCUUAUUUUUUUUUUUUUUUUUUUUUUUUU/T/UAAGGACAAAUCCAUUGAUAAUUU
	PABPC_U27_4m	AUUAUCAAUGGAUUUGUCCUUAUUUUUUUUUUUUUUUUUUUUUUUUU/T/UAAGGACAAACCCAUUUGUAGUUU
Loop size variants with different loop sequence	L5	AUAAGUCUUCUCCAGCUCCUUUUAGGA/T/AAGGAGCUGGAGAAGACUUAUUU
	L15	AUAAGUCUUCUCCAGCUCCUUUUAGGGUCAUGGGAGA/T/AAGGAGCUGGAGAAGACUUAUUU
	L25	AUAAGUCUUCUCCAGCUCCUUUUAGGGUCACACCCACCACUGGGAGA/T/AAGGAGCUGGAGAAGACUUAUUU

Green letters indicate the guide strand; red letters indicate the mismatch position or DNA overhang; shaded letters indicate the loop; /T/, amino modifier with C6 linker for a dye labeling. A, antisense; S, sense.