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Temporal and Spatial Regulation of MicroRNA Activity with Photoactivatable Cantimirs

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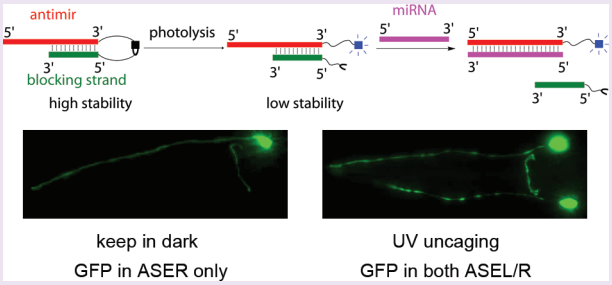
Supporting Information

ABSTRACT: MicroRNAs (miRNAs) are small non-coding RNAs that play numerous important roles in physiology and human diseases. During animal development, many miRNAs are expressed continuously from early embryos throughout adults, yet it is unclear whether these miRNAs are actually required at all the stages of development. Current techniques of manipulating microRNA function lack the required spatial and temporal resolution to adequately address the functionality of a given microRNA at a specific time or at single-cell resolution. To examine stage- or cell-specific function of miRNA during development and to achieve precise control of miRNA activity, we have developed photoactivatable antisense oligonucleotides against miRNAs. These caged oligonucleotides can be activated with 365 nm light with extraordinarily high efficiency to release potent antisense reagents to inhibit miRNAs. Initial application of these caged antimirs in a model organism (*C. elegans*) revealed that the activity of a miRNA (*lsy-6*) is required specifically around the comma stage during embryonic development to control a left/right asymmetric differentiation program in the *C. elegans* nervous system. This suggests that a transient input of *lsy-6* during development is sufficient to specify the neuronal cell fate.



MicroRNAs (miRNAs) are small non-coding RNAs, ~22 nucleotides long, that play diverse roles in development, physiology, and human diseases.[1] A specific miRNA can target many messenger RNAs (mRNAs) by binding to their 3′-untranslated region (3′ UTR) to inhibit mRNA translation and/or stability.[2] Because of their complex involvement in numerous pathways in different cell types, it is important to test the functionality of a given microRNA at single-cell resolution. In addition, during animal development, many microRNAs are found to be expressed from early embryos throughout adults. While studies based on phenotypic readouts (forward genetics) can provide functional and mechanistic characterization of miRNAs whose loss of function mutations are available, it remains challenging to address whether these miRNAs are continuously required during development or if there are specific developmental windows in which these tiny genes play essential roles. Answers to this question not only provide mechanistic insights of how miRNAs act *in vivo* but also are crucial for us to understand how genetic circuitries involving miRNAs are assembled in space and over time in order to provide highly specific biological outputs. To tackle this question it is necessary to manipulate miRNA activity with the desired spatial and temporal resolution.

The current paradigm of inhibiting miRNA involves both chemical and genetic approaches. In the former case, metabolically stable nucleotide analogues such as 2′-*O*-methyl oligoribonucleotides, peptide nucleic acids, morpholinos, and locked nucleic acids (LNA) have been developed as antisense reagents against miRNAs. These miRNA antagonists (antagomirs or antimirs) are quite effective in knocking down miRNAs both *in vitro* and in living organisms including primates.[3−9] Applying antimirs to inhibit miRNAs is limited in its spatial and temporal resolution, so it remains challenging to inactivate a specific microRNA in a selected cell or time. The only current genetic strategies for inactivating a microRNA in a spatially and temporally controlled manner may utilize either Cre/Lox-mediated gene knockout in mice or transgenic expression of so-called miRNA sponges, which reduce miRNA function through competitive interaction with artificial target sites.[10] Both strategies are labor- and time-intensive and require not only appropriate model systems but also the availability of promoters with desired spatial and temporal resolution.

To gain precise spatial and temporal control of manipulating miRNA activity *in vivo*, we resort to the technique of photoactivation by exploiting the superb maneuverability and precision of a light beam. Different photonic approaches have been

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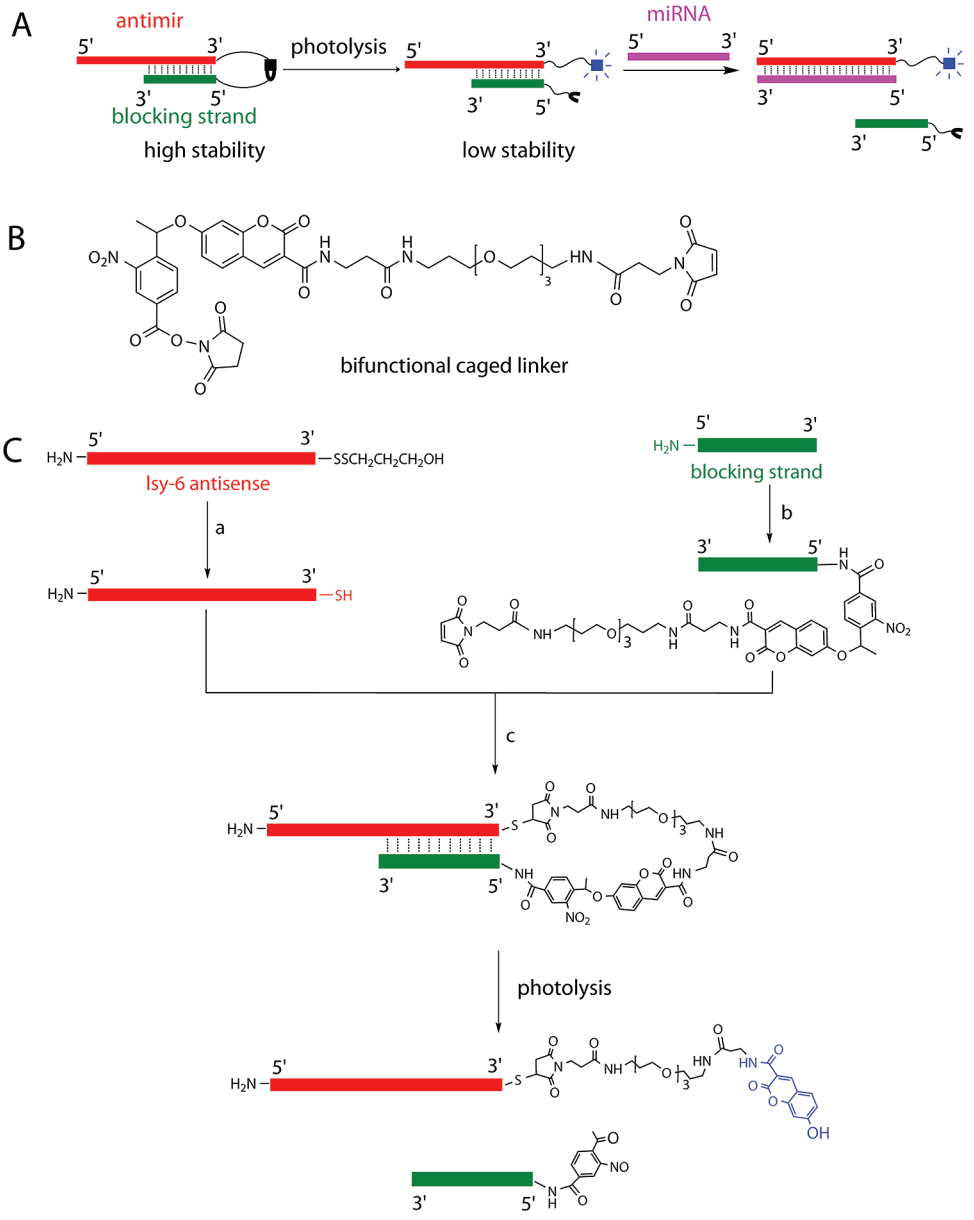

Figure 1. Design and synthesis of caged antimir (cantimir). (A) Design of cantimir for the light controlled inhibition of microRNA. The black and blue square represents caged coumarin (non-fluorescent) and coumarin, respectively. (B) Structure of a bifunctional caged linker. (C) Synthesis and photolysis of cantimirs against *lsy-6* miRNA. Reagents and conditions: (a) 5 mM tris(2-carboxyethyl)phosphine, phosphate buffer(50 mM, pH 7.0), argon, 1 h, 95%; (b) 0.1 M caged linker in DMSO, 3 mM oligoribonucleotide in phosphate buffer (100 mM, pH 7.24), 2:1 (v/v); 35 °C, 35 min, 85%; (c) water, RT, overnight, 31%.

developed for controlling gene expression or the level of mRNAs in living model organisms,[11−17] yet no attempt has been reported for regulating miRNA activity *in vivo* with light. To fill this technology gap, we considered photoactivatable antisense reagents as an efficient and versatile tool for a number of reasons. First, miRNAs are particularly susceptible to inhibition by antisense oligonucleotides.[3] Second, a number of nucleotide analogues such as 2′-*O*-methyl oligoribonucleotides, LNA, or their phosphorothioate derivatives are known to be stable *in vivo* to induce highly specific down-regulation of a miRNA through base complementation.[3−9] In addition, the chemistry for preparing these nucleotide analogues is well developed so they are easily accessible. Finally, antisense oligonucleotides are highly charged and hydrophilic molecules so they can not diffuse across hydrophobic cell membranes on their own. Once generated from their photocaged precursors, they ought to stay in the cells (and their progenies) where photoactivation is executed. This retains the spatial resolution of photoactivation at the cellular level and is advantageous over other methods based on caging hydrophobic ligands or using heat to induce gene expression.[15,16]

To develop photoactivatable antisense reagents to inhibit miRNA activity *in vivo*, we considered *C. elegans* as a particularly suitable model system for testing these caged antimirs (cantimirs) because of its optical transparency, invariant developmental program, ease of reagent delivery, and several well-characterized miRNA pathways for the reagent validation.[18] In particular, we show that cantimirs can be used to effectively inhibit the function of the miRNA *lsy-6*, which is expressed in a single neuron

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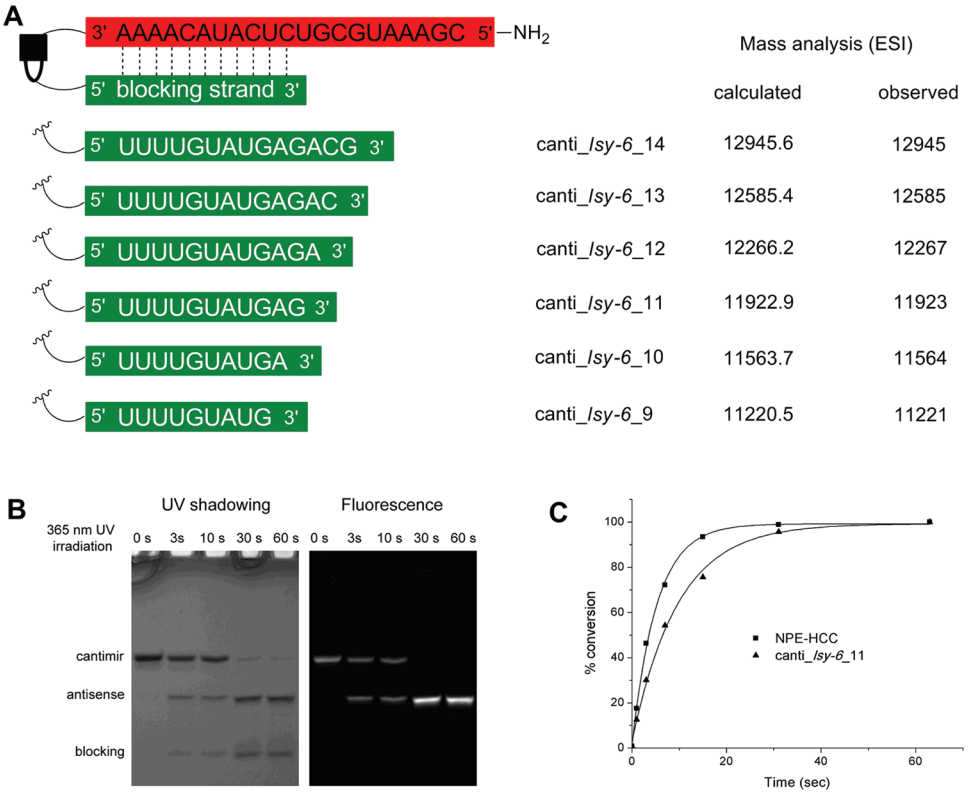

Figure 2. *In vitro* characterization of *lsy-6* cantimirs. (A) Structures, sequences, and mass analyses of *lsy-6* cantimirs containing blocking strands of different lengths. (B) UV-dose-dependent photolysis of canti_*lsy-6*_11 analyzed by the gel shift assay. The sample that has not been UV uncaged (0 s) prior to the gel shift assay showed fluorescence on the gel due to UV exposure from the gel documentation system. (C) Time courses of photoconversion of canti_*lsy-6*_11 and a reference caged coumarin, NPE-HCC (ref 20).

in *C. elegans* where it is required for proper neuronal fate specification.

Results and Discussion. Since miRNAs of *C. elegans* can be effectively inhibited by 2′-*O*-methyl oligoribonucleotides,[5] we designed and constructed these cantimirs using two strands of 2′-*O*-methyl oligoribonucleotides (Figure 1A): one strand is an antisense oligoribonucleotide with sequence complementary to a specific miRNA, and the other is a blocking strand of shorter length complementary to the 3′-terminus of the antisense strand, so the sequence of the blocking strand overlaps with the "seed" sequence of a miRNA, a region of miRNA that has been thought to be important for the target recognition and inhibition.[2] When the antisense and the blocking strands are covalently connected by a caged linker, the relatively short blocking strand is expected to bind tightly to the complementary antisense strand, thus preventing the antisense oligoribonucleotide from hybridizing with its target.[19] Photolysis splits the caged linker, reduces the interaction between the blocking strand and the antisense strand, and makes the antisense oligonucleotide accessible to hybridization with its target miRNA of equal length (Figure 1A).

To prepare these cantimirs, we first synthesized a bifunctional photocleavable coumarin linker that contains an amine reactive NHS ester and a thiol reactive maleimide (Figure 1B and Supplementary Figure 1). The 1-(2-nitrophenyl)ethyl (NPE) caged coumarin exhibits very high uncaging efficiency by either UV light or two photon excitation.[20,21] This facilitates photoactivation, thus minimizing phototoxicity in living cells. The superb uncaging efficiency of these caged coumarins results from the process of "substrate-assisted photolysis", in which the coumarin fluorophore transfers the absorbed energy to the nearby coupled NPE cage for the photolytic reaction.[20] Another advantage of employing the caged coumarin as a photocleavable linker is that, after photolysis, we can instantly judge the extent and the location of photouncaging from the resulting coumarin fluorescence. To enhance the flexibility and the water solubility of the linker, a triple repeat of oxyethylene was incorporated into the caged linker. This caged linker was first reacted with a blocking strand of 2′-*O*-methyl oligoribonucleotide containing a 5′-amino group. The reaction was carried out at neutral pH only for ∼30 min to minimize the degradation of maleimide. HPLC and PAGE analysis of the reaction mixture suggested high conversion of the starting material (Supplementary Figure 2). Since the conjugated product contained a caged coumarin, it displayed intense blue fluorescence on the polyacrylamide gel (PAGE) upon UV illumination. Subsequently we reacted this intermediate with an antisense 2′-*O*-methyl oligoribonucleotide (against the miRNA *lsy-6*) containing a terminal thiol group (Figure 1C). To examine how the length of the blocking strand affects the activity of these cantimirs, we prepared a total of six *lsy-6* cantimirs using blocking oligonucleotides varying from a 9-mer (canti_*lsy-6*_9) to a 14-mer (canti_*lsy-6*_14). These products were purified by PAGE and HPLC (Supplementary Figure 2) and confirmed by electrospray ionization (ESI) mass spectrometry (Figure 2A).

In vitro photolysis by UV light (365 nm) confirmed that these cantimirs were efficiently uncaged in a UV-dose-dependent manner to generate two photocleaved oligonucleotides: the fluorescent antisense strand (containing coumarin) and the nonfluorescent blocking strand (Figure 2B). Overall the fluorescence

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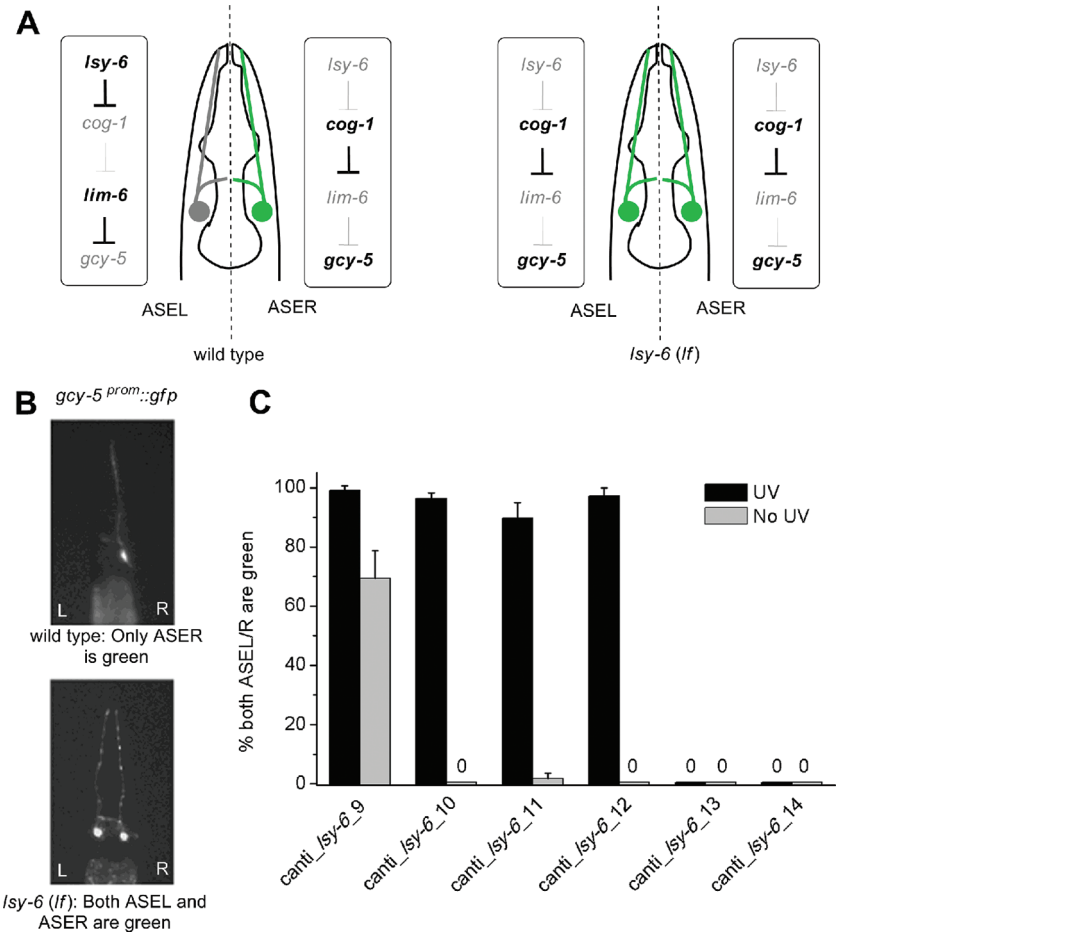

Figure 3. *In vivo* assay of the background and light-activated antisense activity of *lsy-6* cantimirs. (A) *lsy-6* mediates inhibition of *gcy-5* gene expression in the ASE class of chemosensory neurons (ref 23). A simplified version of a more complex gene regulatory network[24] is shown. (B) Example fluorescence images of *gcy-5*^{prom}*::gfp* expression in ASE neurons. (C) Embryos labeled with *lsy-6* cantimirs with different blocking strands were either kept in the dark (no UV) or illuminated with 365 nm UV light (UV) prior to the comma stage and scored for the *lsy-6(lf)* phenotype when they reached adults. All experiments were repeated at least twice, and in each time at least 50 worms were scored. The error bars are standard deviations.

intensity of the cantimir increased over 80 times upon exhaustive photolysis (Supplementary Figure 3). By following the time course of coumarin fluorescence enhancement after photolyzing the cantimir or a reference caged coumarin (NPE-HCC, ref 20) with known uncaging efficiency (Figure 2C), we quantified the uncaging quantum yield (Q_u) of the cantimir to be 37%, and its uncaging cross-section (product of Q_u and the extinction coefficient at 365 nm) to be 7,350 M^{-1} cm^{-1}. Thus, these cantimirs, like their parent caged coumarins, manifest very high photolytic efficiency that is ideally suitable for the small animal uncaging applications, as we have previously shown that the amount of UV light required to photolyze these caged coumarins was at least an order of magnitude below the level of UV dose that might even start to cause cell damage. [22]

We had anticipated that the length of the blocking strand had to be optimized in order to make an ideal cantimir: on one hand, a longer blocking strand binds more tightly to an antisense strand, thus completely switching "OFF" the antisense activity of a cantimir; on the other, to switch "ON" the antisense activity after photolysis, a shorter blocking strand is preferred because it would dissociate from the antisense oligonucleotide more rapidly. To evaluate the *in vivo* performance of this series of canti_*lsy-6*, we injected them into the gonad of a reporter worm strain expressing GFP in the ASER neuron (*gcy-5*^{prom}*::gfp*, *i.e.*, the expression of GFP is driven by the promoter of *gcy-5* gene, which is only expressed in ASER).[23] The *lsy-6* miRNA regulates left-right asymmetry of ASE neurons, a pair of chemosensory neurons that share many bilaterally symmetrical features yet differ in their ability to discriminate different ions by expressing distinct sets of chemoreceptors of the *gcy* gene family.[24] In wild type animals, *lsy-6* miRNA is only present in ASEL (left ASE) and restricts *gcy-5* expression to ASER through repression of the transcription factor *cog-1*, a direct target of *lsy-6*.[25] By contrast, deletion[25] or knockdown[5] of *lsy-6* induces ectopic *gcy-5*^{prom}*::gfp* expression in ASEL, resulting in GFP labeling of both ASEL and ASER ("Lsy phenotype"; Figure 3A,B).

After injecting cantimirs into the gonad of adult worms, we collected labeled early stage embryos and illuminated them with UV light (365 nm). We then assessed adult animals for a Lsy phenotype. Among these cantimirs, only canti_*lsy-6*_9, the cantimir that cantains a 9-mer blocking strand, showed high background antisense activity even without UV photolysis (Figure 3C), probably because the 9-mer blocking strand is too short to effectively shield the antisense oligoribonucleotide from hybridizing with *lsy-6*. Increasing the length of the blocking strand drastically reduced the background activity, yet UV photolysis

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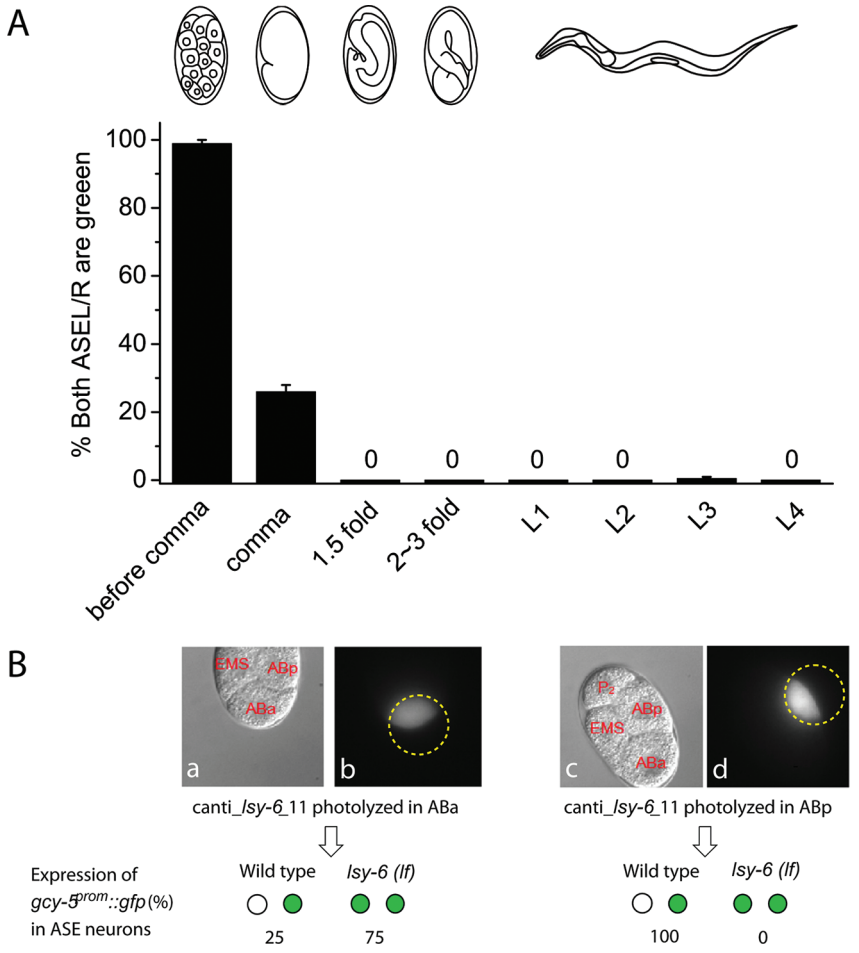

Figure 4. Stage- and cell-specific inhibition of *lsy-6* in developing *C. elegans*. (A) Worms (*gcy-5::gfp)* labeled with canti_*lsy-6*_11 were photolyzed with UV light (365 nm) at five developmental stages. The *lsy-6(lf)* phenotype was scored similarly as in Figure 3. (B) Cell-specific inhibition of *lsy-6* in developing *C. elegans*. Local photolysis of canti_*lsy-6*_11 in ABa (a, b), but not in ABp (c, d), of 4-cell stage embryos resulted in *lsy-6(lf)* in the adult worms. Fluorescence images (b, d) of 4-cell embryos during local photolysis where a restricted beam of UV light (indicated by the yellow circle) was shone only on the selected cell. $N = 12$ for each condition.

of canti_*lsy-6*_10, _11, and _12 turned on their antisense inhibitory activity to knock down *lsy-6* effectively. Interestingly, uncaging canti_*lsy-6*_13 or canti_*lsy-6*_14 failed to inhibit *lsy-6*, likely because 13-mer and 14-mer blocking strands remained tightly bound with the antisense oligonucleotide even when they were no longer covalently linked. This was supported by the native gel-shift analysis, which showed that, upon photolysis, the 13-mer and the 14-mer blocking strands, but not 12-mer or shorter ones, remained bound with the antisense oligonucleotide on the native gel (Supplementary Figure 4A). Further analyses of the thermal stability of the duplexes formed from the antisense strand and the blocking strand oligonucleotides suggested that duplexes with melting points above 60 °C are likely to remain hybridized *in vivo* and hence are ineffective in inhibiting miRNAs (Supplementary Figure 4B).

Studies using a *lsy-6*^{prom}*::gfp* reporter have suggested that *lsy-6* expression started at embryonic stage and persisted though larval and adult stages.[26] It was unclear, however, whether *lsy-6* expression is continuously required throughout development in order to specify the expression of *gcy* genes in the ASE neurons. To address this question, we photolyzed canti_*lsy-6*_11 in developing worms at different developmental stages: before the comma stage, during the comma stage (~380 min of development), 1.5-fold (~420 min), 2- to 3-fold (~450 to 520 min), and larval stage 1 to 4 (L1 to L4). Uncaging the cantimir before the comma stage was highly effective in blocking *lsy-6* to induce the ectopic expression of *gcy-5::gfp* in ASEL. In striking contrast, inhibition of *lsy-6* after the comma stage essentially failed to block the conversion of ASEL into ASER (Figure 4A). This result argues that *lsy-6* activity prior to the comma stage is essential for regulating the asymmetric expression of *gcy* genes in adult worms and suggests that a transient input from *lsy-6* prior to the comma stage is sufficient to produce a stable ASEL fate in adult worms.

A few additional lines of evidence further support that *lsy-6* acts during embryonic development and is no longer required later on to specify the asymmetric expression of the *gcy* chemoreceptors in the ASE neurons. First, a fosmid-based fluorescent reporter for the direct target of *lsy-6*, the *cog-1* homeobox gene,[27] shows that *cog-1* expression is repressed in ASER from the time the reporter initiates expression at about the 2-fold stage (Supplementary Figure 5), suggesting that *lsy-6* function is indeed exerted around that time. Second, experiments using a strong temperature-sensitive allele of *cog-1* revealed that *cog-1* function is necessary only around the comma stage, but at no later stage, coincident with the birth of the ASE neurons or shortly after.[28] These data are consistent with the results obtained with the *lsy-6*

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cantimir, arguing that the expression pattern and timing of activity of *cog-1* are consistent with a critical role for *lsy-6* around the comma stage.

In addition to the temporal feature of miRNA action, the cellular or subcellular distribution of miRNAs represents another level of complexity in their regulation and function. To test the spatial selectivity of applying these cantimirs to block miRNAs in cells of interest, we locally photolyzed canti_*lsy-6*_11 in a blastomere, ABa or ABp, of a 4-cell stage embryo. ABa and ABp are the precursors of ASEL and ASER neurons, respectively.[28] Since uncaging canti_*lsy-6*_11 in ABa or ABp was expected to inhibit *lsy-6* only in their corresponding daughter cells, and because *lsy-6* functions by inhibiting *gcy-5* expression in ASEL, blocking *lsy-6* in ABa, but not in ABp, would affect the expression of *gcy-5*. Indeed, local uncaging with a narrow beam of light in ABa caused ectopic expression of *gcy-5::gfp* in ASEL, while local uncaging in ABp had no effect (Figure 4B). Thus, these cantimirs are capable of blocking miRNAs in both stage- and cell-specific manners.

In summary, we have developed a new class of caged antimirs with high uncaging efficiency for regulating miRNAs *in vivo*. Initial applications of a cantimir against the *lsy-6* miRNA revealed that a transient *lsy-6* activity around the comma stage is sufficient for specifying the fate of ASE neurons later on. The modular design and facile assembly (Figure 1C) of these cantimirs also offers an ideal template for constructing photoactivatable antisense oligonucleotides against numerous miRNAs or perhaps even other types of non-coding RNAs. Combined with the uncaging techniques of high three-dimensional selectivity (two photon excitation for example) and appropriate cellular delivery methods, these cantimirs should offer us new approaches to perform functional analysis of miRNAs with unprecedented spatial and temporal resolution in living cells and in different biological systems.

METHODS

Photoactivation of Cantimir and Light-Controlled Silencing of *lsy-6* in *C. elegans*. Purified cantimirs (20 μM) were injected into both gonads of young adult transgenic hermaphrodites expressing GFP in the ASER neuron (*gcy-5*^{prom}*::gfp*, strain OH 3192). Rhodamine dextran (40 KD, 8 mg mL^{-1} final concentration) was included in the injection solution as a marker. To minimize photolysis of cantimirs during injection, a long-pass filter was placed in the excitation light path of the inverted microscope to prevent the sample from exposing to light shorter than 410 nm. For each experiment, we routinely injected a cantimir into ~20 worms. About 16 h postinjection, we collected rhodamine-labeled embryos laid from injected worms under a fluorescence dissection scope (SteREO Discovery V12, CarlZeiss). These embryos were then separated into different groups based on the stages of development. Grouped embryos or larvae were photolyzed with UV light (365 nm) from a mercury lamp (B-100AP, UVP) for a total of 60 s (12 s × 5, spaced 10 s apart). The lamp typically puts out UV light (365 nm) at an intensity of ~0.4×10^{-8} einsteins cm^2 s^{-1}. The *lsy-6(lf)* phenotype was scored when these worms reached adults. UV light illumination itself had no effect on *gcy-5*^{prom}*::gfp* expression, nor did it have any observable effect on animal development or behavior.

To selectively activate a cantimir in a blastomere, after worm injection, we cut injected worms to collect 2-cell stage labeled embryos. The embryos were quickly mounted on a 2% agar pad. We then performed local uncaging in ABa or ABp cell when the embryo developed to the 4-cell stage, using a field diaphragm to limit the size of the uncaging beam and following the same protocol as previously described.[22] The UV-illuminated (360 ± 20 nm, 5 s) embryos were then recovered from the agar pad, transferred to an agar dish, and allowed to develop in the dark until *lsy-6(lf)* phenotype was scored at the adult stage.

ASSOCIATED CONTENT

Supporting Information. Supplementary figures and synthesis and *in vitro* characterization of cantimirs and expression of *cog-1*. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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