TECHNOLOGY REPORT

PhotoMorphs™: A Novel Light-Activated Reagent for Controlling Gene Expression in Zebrafish

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Summary: Manipulating gene expression in zebrafish is critical for exploiting the full potential of this vertebrate model organism. Morpholino oligos are the most commonly used antisense technology for knocking down gene expression. However, morpholinos suffer from a lack of control over the timing and location of knockdown. In this report, we describe a novel light-activatable knockdown reagent called PhotoMorph™. PhotoMorphs can be generated from existing morpholinos by hybridization with a complementary caging strand containing a photocleavable linkage. The caging strand neutralizes the morpholino activity until irradiation of the PhotoMorph with UV light releases the morpholino. We generated PhotoMorphs to target genes encoding enhanced green fluorescent protein, No tail, and E-cadherin to illustrate the utility of this approach. Temporal control of gene expression with PhotoMorphs permitted us to circumvent the early lethal phenotype of E-cadherin knockdown. A splice-blocking PhotoMorph directed to the *rheb* gene showed light-dependent gene knockdown up to 72 hpf. PhotoMorphs thus offer a new class of laboratory reagents suitable for the spatiotemporal control of gene expression in the zebrafish. genesis 47:736–743, 2009. © 2009 Wiley-Liss, Inc.

Key words: morpholino; gene expression; zebrafish; gene knockdown; light activatable; caged morpholino

Morpholino oligos are the most commonly used antisense technology for gene knockdown in zebrafish and *Xenopus* (Nasevicius and Ekker, 2000). The success of this technology lies in the lack of cellular toxicity and the simplicity of its application. Once the sequence of a gene is known, a morpholino can be designed and injected, and gene function can be determined by examining the "morphant" phenotype. However, morpholino-mediated knockdown is limited by a lack of temporal and spatial control. This can be problematic when gene knockdown produces an early lethal phenotype, precluding analysis of gene function later during development. Previous efforts to generate conditionally active morpholinos have focused mainly on using photolabile modifications to cage the knockdown reagent.

Shestopalov *et al.* (2007) recently described the synthesis of a modified morpholino consisting of a hairpin loop joined at the end by a photolabile group. Upon photolysis, the hairpin dissociates, allowing for the morpholino to target its complementary RNA and to block gene expression. A similar approach was described by Tang *et al.* (2007) in which a negatively charged peptide nucleic acid was used as the sequence-specific gene-knockdown reagent. This was caged using *O*-methyl RNA linked via a photocleavable group in a hairpin configuration. These efforts have provided proof of principle for this general approach. Both methods offer efficient caging and knockdown, but each knockdown reagent must be synthesized de novo.

Currently there is no technology reported that can convert existing morpholinos into conditional gene-knockdown reagents. To address this need, we developed PhotoMorphs, which are easily generated through simple Watson-Crick base pairing of a conventional morpholino with a complementary caging oligonucleotide. The caging strand contains a centrally placed photocleavable linker that bisects the oligonucleotide when irradiated with 365 nm light, releasing the morpholino (see Fig. 1). Here, we describe the development and application of this technology.

RESULTS

Caging Strand Composition

Because the caging strand is bound to the morpholino by Watson-Crick base pairing, we were at liberty to

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investigate a wide variety of caging backbones while using the same morpholino. We generated caging strands consisting of many variations of the phosphoribose backbone, including all DNA, all RNA, DNA deletions, RNA deletions, alternating RNA and DNA, varying percentages of RNA and DNA in different orientations, substituting ribothymidine for ribouridine in the RNA backbone, and substituting deoxyuridine for deoxythymidine in the DNA backbone. We also tried variations of the normal linkages, including no 3′ OH group, the addition of a 2′ *O*-methyl group, and a phosphorothioate linkage in the DNA backbone. Each oligo was injected at 100 μM and embryos screened for toxicity at 24 hours post fertilization (hpf) (see Fig. 2). In general, we found greater toxicity with increasing DNA content. A 10-mer of DNA without RNA in the backbone killed 25% of the embryos. However, if RNA makes up the rest of the backbone, 10 DNA bases are not as toxic, killing less than 10% of the embryos. Most importantly, we found that backbones comprised entirely of RNA were the least toxic. Addition of DNA to the backbone increased toxicity, and this result was more severe when RNA was removed from the backbone.

GFP PhotoMorph

To assess the effectiveness of caging, we used the GutGFP transgenic line, in which green fluorescent protein is expressed in all endoderm-derived structures (Ng *et al.*, 2005). The morpholino was directed to the ATG start site of the GFP mRNA. First, we determined the lowest concentration of morpholino that could produce effective knockdown based on green fluorescence in the live embryo at 48 hpf, testing a range of concentrations from 0.2 to 200 μM. We achieved acceptable knockdown with no evident toxicity with a concentration of 10 μM, about twice the ED_{50}. As we found subsequently, in this dynamic part of the dose-response curve, the addition of the caging strand results in >90% inhibition of morpholino activity. Then, upon uncaging, the morpholino regains nearly all of its activity. Thus, "sub-saturation" concentrations of morpholino provided robust caging and uncaging as shown in the experiments to follow.

To test for caging of the morpholino, we hybridized morpholino to varying concentrations of caging strand ranging from 75 to 200 μM. Lower concentrations resulted in inadequate caging, with GFP still knocked down. A caging strand concentration of 100 μM produced effective caging, with GFP expression comparable to uninjected embryos. Thus, optimal caging was achieved at a 1:10 morpholino to caging strand molar ratio.

After demonstrating that RNA could be used to effectively cage the morpholino, we then determined if placing a photocleavable linker in the caging strand could enable light-inducible knockdown. One variable we examined was the optimal substitution of the photocleavable linker into the backbone. We inserted a nitrophenyl group in the middle of the backbone, with 0, 1, or 2 standard bases removed. Using the GutGFP line, we found that both caging and uncaging were successful with each of the strand variations (Fig. 3a). Uncaging PhotoMorphs with 0 and 2 bases removed resulted in similar reductions in GFP expression (74% and 72%, respectively) when compared with GFP in the caged condition. We saw the greatest reduction in fluorescence between caged and uncaged conditions using the PhotoMorph with 1 base removed (90% reduction). We showed that GFP is knocked down by the morpholino, and the caging strand (1 base removed) effectively cages the morpholino, as shown by the high GFP expression in the gut, similar to uninjected control fish. After irradiation with UV light, the morpholino is released and GFP is knocked down (Fig. 3b). On the basis of these results, we concluded that removing 1 base from the caging strand backbone was ideal for incorporating the photocleavable linker.

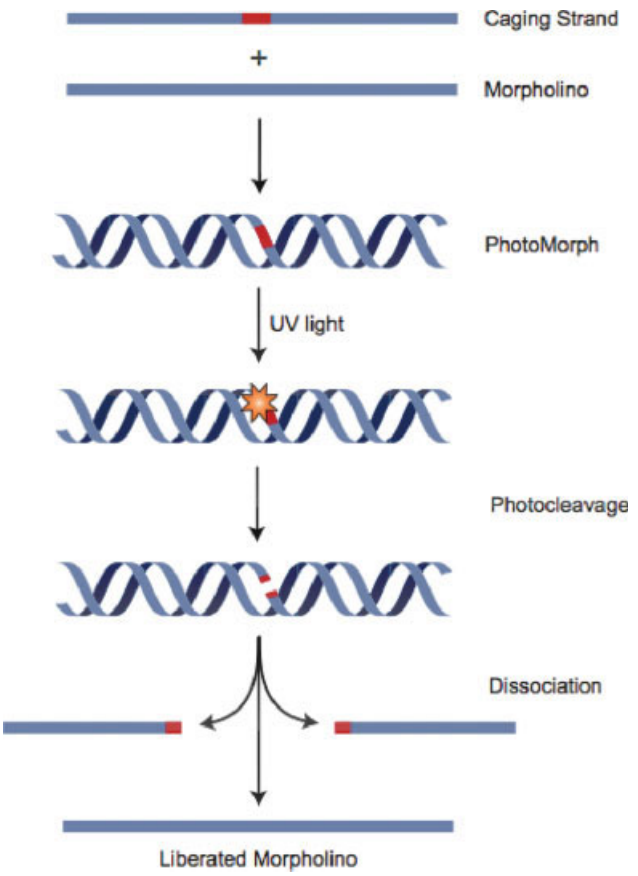

FIG. 1. Schematic diagram of PhotoMorph function. The morpholino and caging strand are hybridized to form a heteroduplex called a PhotoMorph. UV irradiation results in cleavage of the caging strand at the photocleavable linker, dissociation of the caging strand, and liberation of the morpholino. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

ntl PhotoMorph

Having established proof of principle with GFP, we then tested other genes using a similar algorithm: first identifying the lowest concentration of morpholino that

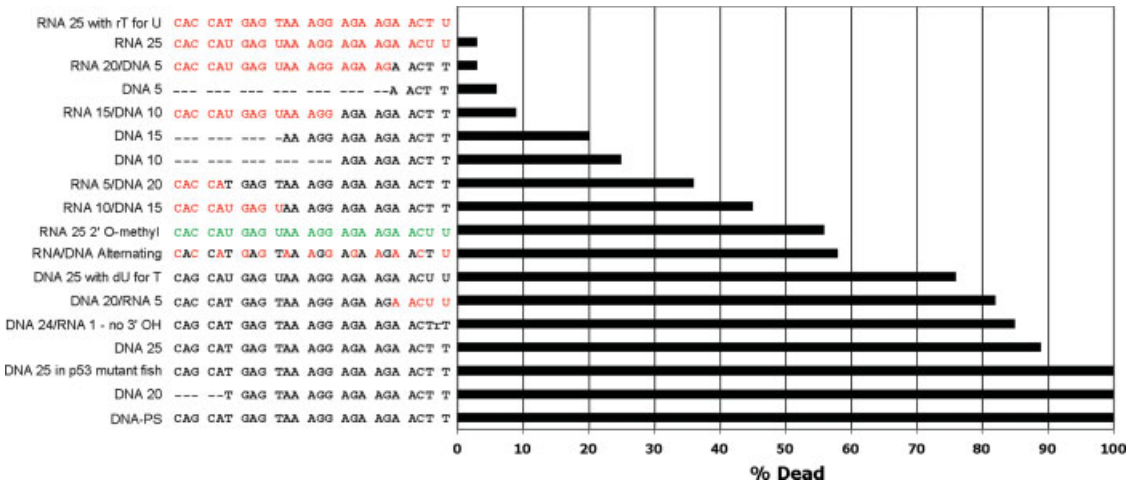

FIG. 2. Optimization of the caging strand backbone. Zebrafish embryos were injected with caging strand and scored for survival at 24 hpf. Toxicity correlated with DNA content, while RNA was nontoxic. Caging strands were made to GFP morpholino sequence, where RNA bases are shown in red, and DNA in black. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

gives acceptable knockdown, then generating the PhotoMorph by hybridizing a molar excess of caging strand. We chose the *no tail* gene because of the clear phenotype produced by its knockdown (Halpern *et al.*, 1993). We injected *ntl* morpholino ranging from 12.5 μM to 100 μM and found that 12.5 μM was sufficient to produce adequate knockdown and a strong phenotype. We hybridized the morpholino (12.5 μM) with the caging strand (100 μM) to generate the *ntl* PhotoMorph. We uncaged the PhotoMorphs at various timepoints and scored the embryos at 79 hpf. We divided the embryos in each condition into groups according to phenotypic severity (see Fig. 4). Embryos injected with the PhotoMorph were indistinguishable from the uninjected group, demonstrating the effectiveness of the caging. After uncaging, many embryos became severely affected, while others develop relatively normally. The phenotype varied as a function of the stage of uncaging, with milder effects the later the uncaging was performed, consistent with the role of *ntl* early in development. Scoring the knockdown phenotype at 27 hpf produced a similar result.

E-cadherin PhotoMorph

We then tested a PhotoMorph directed to the *cdh1* gene, which encodes E-cadherin. This knockdown phenotype features gastrulation defects and mimics the *half-baked* mutant (Kane *et al.*, 2005). We tested morpholino concentrations ranging from 1 μM to 100 μM. At concentrations of 10 μM and above, most embryos died by 24 hpf. Below 10 μM, the majority of embryos were unaffected. We used 10 μM morpholino and 100 μM caging strand to generate the PhotoMorph. We scored clutches by sorting embryos into groups according to phenotypic severity, including normal, mildly deformed (presence of head and tail), severely deformed (absence of head and tail), and dead (Fig. 5a). Although we observed variable morpholino efficacy between clutches, only 6% of the PhotoMorph-injected embryos were dead at 24 hpf (Fig. 5b). For both clutches, the percent of affected embryos was similar between the unmodified and uncaged morpholino (84 and 90% uncaging efficiency for clutch 1 and 2, respectively).

In three separate experiments, we uncaged at pre- and postgastrulation timepoints (4 and 8 hpf, respectively). On average, only 4% of Photomorph-injected embryos were dead at 24 hpf. Uncaging at 4 hpf resulted in death of an average of 36% of the embryos, while uncaging at 8 hpf resulted in death of 10% of the embryos, illustrating stage-specific knockdown. This result demonstrates the ability to circumvent early lethal phenotypes by controlling the timing of PhotoMorph activation.

To quantify the effectiveness of the E-cadherin PhotoMorph, we compared knockdown using morpholino, caged PhotoMorph and uncaged Photomorph by Western blot (10 μM morpholino and 100 μM caging strand) (see Fig. 6). At two timepoints (8 hpf raised at 28°C and 24 hpf raised at room temperature, indicated by asterisk), the unmodified and the uncaged morpholinos gave comparable knockdown, and the caged morpholino was comparable to uninjected control. However, we saw better knockdown for both morpholino and uncaged PhotoMorph at 24 hpf* (Fig. 6a), likely due to the time needed for decay of preexisting protein. For this PhotoMorph, using higher concentrations of morpholino (20 μM) and PhotoMorph (20 μM morpholino and 200 μM caging strand) resulted in better conventional morpholino knockdown, but less effective caging and uncaging.

Splice-Blocking PhotoMorph

We used a PhotoMorph directed to the *rheb* gene to monitor the time course for uncaging by RT-PCR. *Rheb* encodes a G-protein that mediates signaling through the TOR pathway. At 100 μM, we achieved acceptable

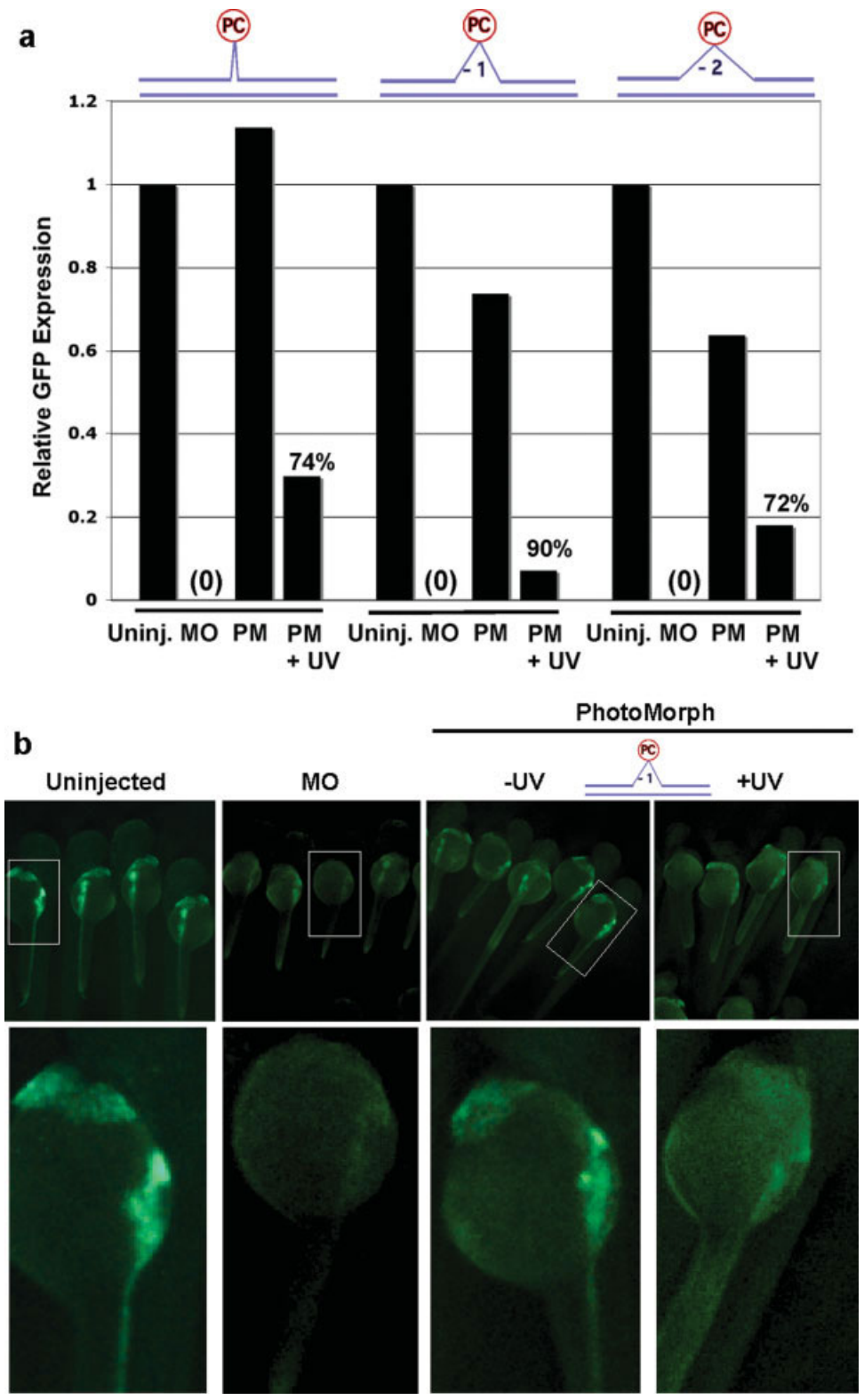

FIG. 3. GFP PhotoMorph efficacy. We tested the efficacy of PhotoMorphs with 0, 1, or 2 bases removed from the backbone. **a:** Results indicated that each PhotoMorph (PM) is effective at both caging and uncaging the morpholino (MO). The 1-base substitution PhotoMorph provided the greatest difference between caged and uncaged GFP morpholino. **b:** Representative Gut-GFP larvae are shown uninjected, injected with GFP morpholino, and with 1-base substitution PhotoMorph, with and without irradiation at 4 hpf. White boxes show the area enlarged in the corresponding panels below.

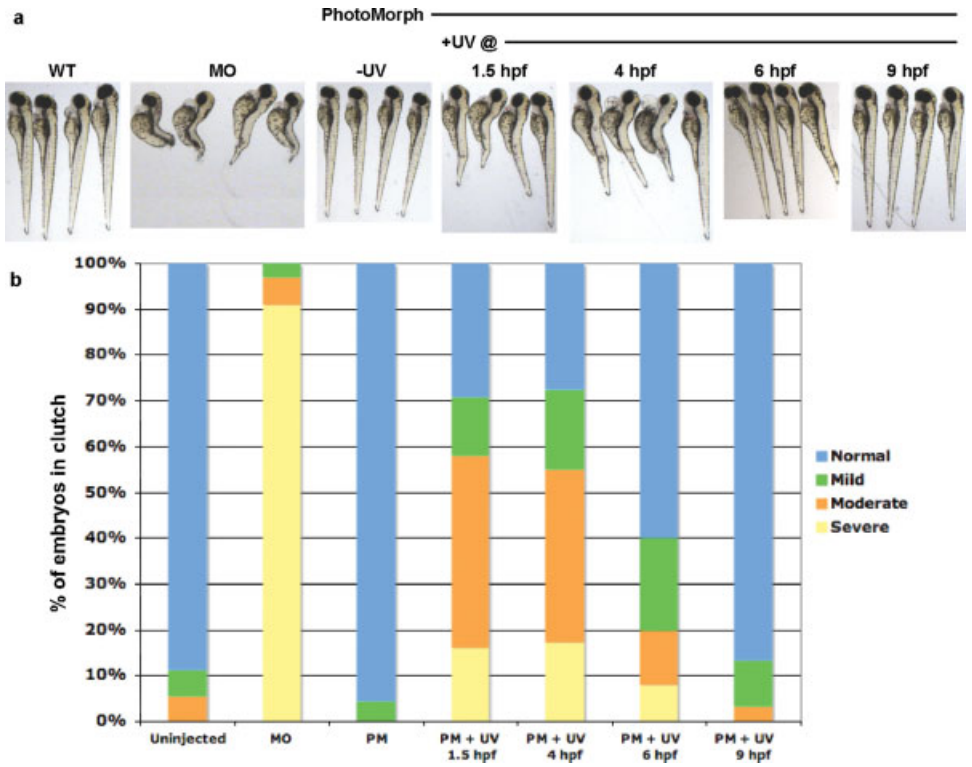

FIG. 4. *ntl* PhotoMorph efficacy. **a:** Embryos were irradiated at 1.5 hpf, 4 hpf, 6 hpf, or 9 hpf, and all embryos were imaged at 79 hpf. **b:** The graph shows the percentage of embryos of varying phenotypic severity for each condition, ranging from normal to severely affected. Embryos were injected with *ntl* morpholino or PhotoMorph and irradiated at the indicated timepoint. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

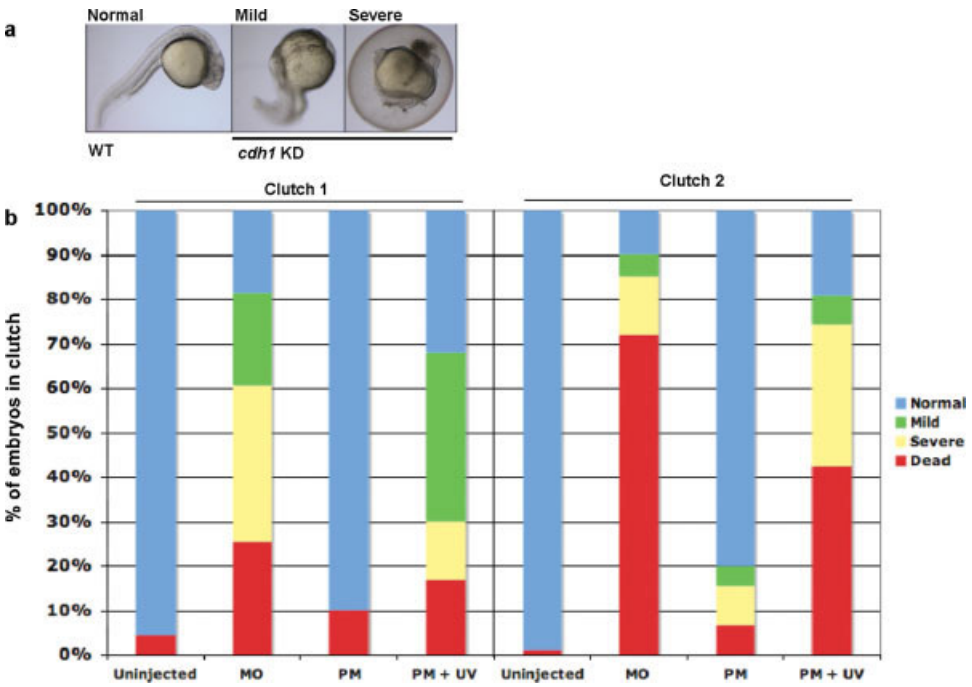

FIG. 5. E-cadherin PhotoMorph efficacy. **a:** Representative embryos exhibiting normal, mild, and severe E-cadherin knockdown phenotypes. Embryos were imaged at 24 hpf. **b:** Embryos were scored at 24 hpf based on phenotype. Results indicated that the PhotoMorph was effective at caging and uncaging the morpholino, regardless of baseline morpholino efficacy. Embryos were injected with E-cadherin morpholino or PhotoMorph and irradiated at 4 hpf as indicated. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

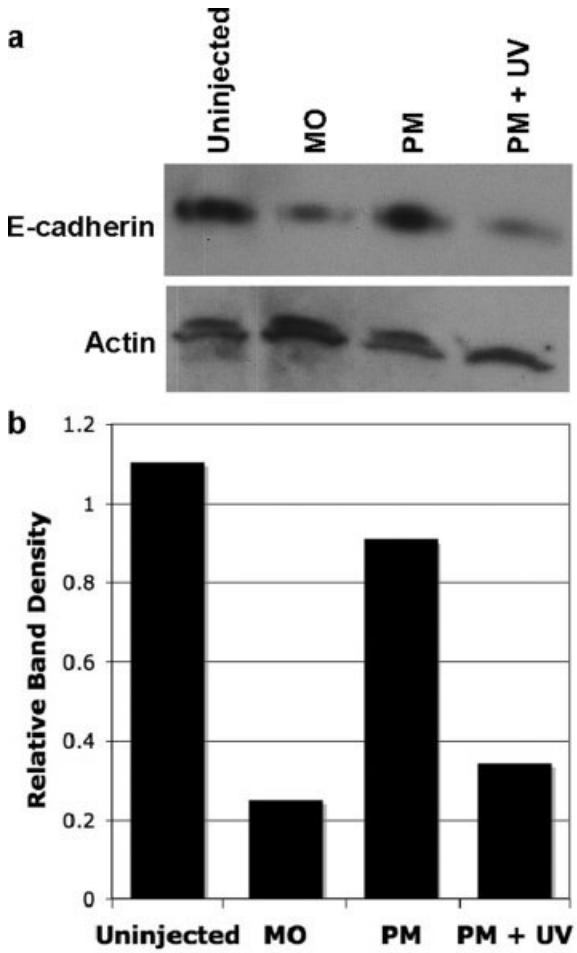

FIG. 6. E-cadherin Western blot and densitometry. **a:** Protein samples were collected at 24 hpf*. The asterisk indicates a developmental delay of ~8 h (embryos developed at 22°C to extend viability until a later timepoint). Results showed E-cadherin levels, relative to actin, are knocked down by the morpholino and by uncaging the PhotoMorph. **b:** Relative band density is shown as the ratio of E-cadherin to actin. Embryos were injected with E-cadherin morpholino or PhotoMorph and irradiated at 4 hpf as indicated.

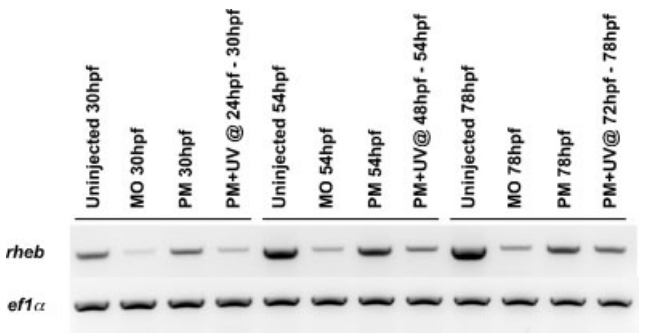

FIG. 7. Deferred activation of a splice-blocking PhotoMorph. PhotoMorph (100 μM morpholino/500 μM caging strand) directed to a splice site in the *rheb* gene was injected and RNA samples collected 6 h after irradiation at 30, 54, and 78 hpf as indicated. Results showed *rheb* levels, relative to *ef1-α*, are knocked down by the morpholino and by uncaging the PhotoMorph. Results showed effective caging at the earlier timepoints, but caging ability of the PhotoMorph is reduced by 78 hpf. The *ef1-α* gene served as loading control.

knockdown with minimal toxicity at 24 hpf. We generated the PhotoMorph by hybridizing 100 μM morpholino to 500 μM caging strand and found effective caging at 30 and 54 hpf, even at a morpholino:caging strand molar ratio of 1:5. At later time points (i.e. 72 hpf), caging is detectable, but less robust (see Fig. 7). We then tested uncaging of *rheb* PhotoMorph by UV irradiation at 24, 48, and 72 hpf and collected RNA 6 h post-irradiation. We detected excellent UV-dependent knockdown of *rheb* at 24 and 48 hpf. At 72 hpf, UV-induced gene knockdown was detectable but less pronounced due to less efficient caging. One explanation for this observation is a dilution-driven dissociation of the PhotoMorph heteroduplex at later stages of development.

These data nonetheless demonstrate the feasibility of using PhotoMorphs to defer the temporal window of knockdown relative to conventional morpholinos, a long sought-after capability. One strategy to combat the postulated late-stage dissociation of the PhotoMorph heteroduplex is to directly and covalently cage the exocyclic Watson–Crick H-bond donors and acceptors with photolabile protecting groups as recently described by the Deiters group (Young *et al.*, 2008). Efforts toward the development and testing of covalently caged knockdown agents are underway in our laboratories.

CONCLUSIONS

In this study, we demonstrate that conventional morpholinos can be converted into conditional knockdown reagents by hybridization to a complementary oligonucleotide strand containing a photocleavable linker. We identified critical variables for optimal caging and uncaging, including morpholino concentration, the chemical composition of the caging strand, and the molar ratio of morpholino to caging strand. The main advantage of PhotoMorph technology over previous conditional knockdown strategies is its accessibility to laboratories that already use morpholinos. Caging strands can be synthesized for virtually any morpholino sequence, circumventing the need for a specialized hairpin that is not commercially available as of this writing. PhotoMorphs will thus enable laboratories to use existing resources to perform novel experiments.

The main application of PhotoMorph technology is spatial and temporal gene regulation. Researchers can use PhotoMorphs to control timing of gene knockdown, delaying morpholino activation until a specified time in development. For example, uncaging after gastrulation can produce a morphant phenotype that would have otherwise been inaccessible due to early lethality caused by pregastrulation knockdown. Spatially restricted conditional gene knockdown is also a possible application for PhotoMorphs. Coinjecting a photoconvertible lineage tracer, such as Kaede protein or caged fluorescein

dextran, with a PhotoMorph may offer a novel approach to creating genetic mosaics. Selectively irradiating and tracking a spatial target would then allow testing of gene function in a subset of cells. Together, these technologies can be used to control gene expression in single cells, individual organs, or any location in the specimen accessible to a beam of light.

METHODS

PhotoMorph Synthesis

Caging strands were generated with standard solid phase oligonucleotide synthesis (SuperNova Life Science, www.supernovalifescience.com). The photocleavable nitrophenyl group was introduced as a phosphoramidite during synthesis. Caging strands were injected into the yolk of the one-cell stage embryo at 100 μM (4.6 nL per injection), using a Drummond Nanoject II microinjector. Embryos were scored for death at 24 hpf.

To generate PhotoMorphs, caging strands were hybridized to morpholinos by incubation at 70°C for 30 min in Danieau's solution (Westerfield, 1995) and phenol red (0.1%), followed by slow cooling and storage at 4°C overnight. Molar ratios were typically 1:5–1:10 as indicated. Mixes were injected into the yolk of a one-cell stage embryo (4.6 nL per injection). PhotoMorphs are light sensitive; therefore, all work was done under reduced lighting conditions, and injections were performed using a yellow filter to prevent photocleavage during injection. Embryos were irradiated with 365-nm light using a UVL-56 UV lamp at a distance of 5 cm, which delivers 1350 $\mu W/cm^2$ per the manufacturer's specifications. Irradiation was performed at various timepoints for 30 min, with periodic swirling of the plate to ensure uniform irradiation. This delivers 2.43 J/cm^2 of UVA radiation, which is below the measured LD_{50} of 850 J/cm^2 UVA as described previously (Dong *et al.*, 2007). UV treatment alone had no detectable adverse effects on development, consistent with previous reports. Embryos were imaged for data analysis at various timepoints.

Scoring Morphant Phenotypes

To quantify the efficacy of the GFP PhotoMorph, we used Adobe Photoshop to pinpoint the brightest point of fluorescence in the embryo (GutGFP transgenic line, Zebrafish International Resource Center, www.zebrafish.org). We calculated the average luminosity within a clutch of treated embryos and then normalized the values relative to the control (1) and the morpholino knockdown (0) to graph relative GFP expression. To determine the efficacy of the *ntl* PhotoMorph at 79 hpf, we grouped the embryos within each condition according to severity of the *ntl* phenotype. We counted the number of embryos in each phenotype group to express *ntl* PhotoMorph efficacy as a percentage. To score the phenotype at 27 hpf, we used Canvas X to measure the length of the embryo, from head to tail. To determine the efficacy of the E-cadherin PhotoMorph at 24 hpf, we grouped the embryos within each condition according to severity of the *cdh1* knockdown phenotype. We counted the number of embryos in each phenotype group to express E-cadherin PhotoMorph efficacy as a percentage. Experiments were performed using embryos from the same clutch for all conditions within each experiment.

Protein Collection and Western Blotting

We homogenized 20 embryos in 200 μL of 2× SDS sample buffer containing a cocktail of protease inhibitors (Roche, catalog no. 11836170001, www.roche-applied-science.com) and then sonicated the samples two times for 30 s. Samples were boiled, spun down, and frozen for Western blot analysis.

Samples were loaded on a 7.5% gel for SDS-PAGE, and then transferred to PVDF. We used E-cadherin mouse monoclonal antibody (BD Transduction Laboratories, catalog no. 610181, www.bdbiosciences.com) at 1:2,500 in 5% milk. We stripped the blot using Restore Western Blot Stripping Buffer (Pierce, www.piercenet.com), then re-probed using mouse monoclonal β-actin antibody [AC-15] (Abcam, catalog no. ab6276, www.abcam.com). HRP-conjugated secondary antibodies were used at 1:15,000, and blots were developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce, www.piercenet.com) according to the manufacturer's protocol.

We performed densitometry using Scion Image 1.63. We measured band density in a fixed area, subtracted background signal, and normalized to actin.

RNA Collection and RT-PCR

We collected RNA from 10 embryos per condition using TRIzol, according to the manufacturer's protocol (Invitrogen, www.invitrogen.com). Reverse transcription reactions were performed using oligo$(dT)_{20}$ primers and SuperScript™ II, according to the manufacturer's protocol (Invitrogen). Primer sequences were: *rheb*-f: GGGG ACA AGT TTG TAC AAA AAA GCA GGC TCC ATG CCG CAG CCG AAA TCG C, *rheb*-rev: GGGG AC CAC TTT GTA CAA GAA AGC TGG GTC CAT CAT GGA GCA GGG CGT C, *ef1α*-f: CTTCTCAGGCTGACTGTGC, *ef1α*-rev: CCGCTAGCATTACCCTCC. We used the following temperature cycle: 94°C for 3 min, 94°C for 30 s, 58°C for 30 s, 72°C for 1 min, 34 times to step 2, 72°C for 10 min, and hold at 4°C.

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