Supporting Information

Genetic Code Expansion in Zebrafish Embryos and its Application to Optical Control of Cell Signaling

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Experimental Methods

Materials. The zebrafish experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh. The AB* strain was maintained under standard conditions at the University of Pittsburgh School of Medicine in accordance with Institutional and Federal guidelines. Unnatural amino acid **1** was purchased from Chem-Impex International. Unnatural amino acids **2** to **4** were synthesized as described previously.[1]

Statistical methods. Error bars represent s.e.m. for the N numbers shown in figures. Sample sizes were chosen because preliminary experiments suggested that it would be sufficient to indicate incorporation of unnatural amino acid. No statistical methods were used to predetermine sample size. Unpaired t-tests were used to calculate statistical significance.

Plasmid construction. The *Renilla* luciferase (Rluc) gene was amplified from pGL4.7 (Promega) using primers P1 and P2 (for sequence information see Table S1), and was cloned into BamHI and XbaI sites of the pCS2+ plasmid through an in-fusion cloning kit (Clontech). The L95TAG mutation was introduced into pCS2-Rluc by QuikChange site-directed mutagenesis (Agilent) using P3 and P4. PylRS (WTRS, HCKRS, and OABKRS) was amplified using P5 and P6, and was cloned into the pCS2+ plasmid as described above. The firefly luciferase (Fluc) gene was amplified from pGL3 (Promega) using P7 and P8, and was cloned into the pCS2+ plasmid. The K206TAG mutation was introduced into pCS2-Fluc using P9 and P10. pCS2-Fluc-K206TAG-Rluc was assembled from two overlapping fragments, pCS2-Fluc-K206TAG (amplified with P11 and P12) and Rluc (amplified with P13 and P14), using the Gibson assembly method. Maltose binding protein (MBP) tagged MEK1 gene (MBP-MEK1) was

amplified from Addgene plasmid # 68300,[2] using primers P15 and P16, and was cloned into NcoI and AseI sites of the pBAD-pylT plasmid. The K97TAG mutation was introduced using P17 and P18. Constitutively active caMEK1 (containing S218D and S222D mutations) and MEK1-K97TAG were amplified using P19 and P20, and were cloned into the pCS2+ plasmid as described above. All plasmids were confirmed by Sanger sequencing. For map of newly constructed plasmid, see Figure S12.

Protein expression. pBAD-MBP-MEK1-K97TAG-pylT was co-transformed with pBK-HCKRS into *E. coli* Top10 cells. A single colony was grown in LB media overnight, and the entire culture (250 µl) was added to LB media (25 ml) supplemented with **3** (1 mM), Tet (25 µg ml^{-1}), and Kan (50 µg ml^{-1}). Cells were grown at 37 ºC, 250 rpm, and protein expression was induced with arabinose (0.1 %) when OD_{600} reached 0.4. After overnight expression at 27 ºC, cells were pelleted and resuspended in phosphate lysis buffer (pH 8.0, 50 mM, 6 ml). Triton X-100 (60 µl, 10%) was added to the mixture. The lysate was incubated on ice for 1 h, sonicated, and then centrifuged (13,000 g) at 4 ºC for 10 min. The supernatant was transferred to a 15 ml conical tube and Ni-NTA resin (Qiagen, 100 µl) was added. The mixture was incubated at 4 ºC for 2 h with mild shaking. The resin was then collected by centrifugation (1,000 g, 10 min), and washed with lysis buffer (800 µl) and wash buffer (800 µl) containing imidazole (20 mM). The protein was eluted with elution buffer (200 µl) containing imidazole (250 mM). Expression of MBP-MEK1-K97TAG-**3** was confirmed by SDS-PAGE (8%) (Figure S7). For protein decaging, purified proteins (20 µl) were irradiated in a PCR tube for 5 min with a 365 nm UV transilluminator (8 mW/cm^2).

Protein MS/MS: The non-irradiated and irradiated protein samples were analyzed by SDS-PAGE (8%), and stained with Coomassie Brilliant Blue. Regions corresponding to the expected molecular weight of MBP-MEK1 were excised and sent for LC-MS/MS analysis (MS Bioworks).

The in-gel typsin digests were analyzed by nano LC-MS/MS with a Waters NanoAcquity HPLC system interfaced to a ThermoFisher Q Exactive. Peptides were loaded on a trapping column and eluted over a 75 μm analytical column at 350 nL/min. The mass spectrometer was operated in data-dependent mode, with MS and MS/MS performed in the Orbitrap at 70,000 FWHM resolution and 17,500 FWHM resolution, respectively. The fifteen most abundant ions were selected for MS/MS. The peptide mass tolerance was set as 10 ppm, and the fragment mass tolerance was set as 0.02 Da. Data were filtered using a minimum protein value of 90%, a minimum peptide value of 50% and requiring at least two unique peptides per protein.

mRNA synthesis. The corresponding pCS2+ plasmid was linearized through NotI digestion and was purified by phenol:choloform:isoamyl alcohol (PCIA) extraction. Linear DNA (1 μg) was used to generate mRNA in a 20 μl reaction with the mMESSAGE mMACHINE SP6 Transcription Kit (Ambion). The mRNA was purified through PCIA extraction, G-50 sephadex spin column (Roche, #11274015001), and ethanol precipitation. The mRNA pellet was dissolved in 15 μl of water and the quality was verified by 1% agarose gel.

PylT synthesis. The PylT sequence contained a U25C mutation in order to increase incorporation efficiency.[3] The PylT DNA with a truncated T7 promoter was amplified from a PylT oligonucleotide (IDT), using P21 and P22. The PylT DNA was purified by phenol extraction, and 1.5 μg of DNA was used as the template to generate PylT RNA using the MEGAscript T7 Transcription Kit (Ambion). PylT RNA was purified through PCIA extraction, followed by ethanol precipitation. The RNA pellet was dissolved in 10 μl of water, and the quality was verified by 1.5% agarose gel. The PylT RNA without CCA was generated in a similar manner. Chemically synthesized PylT RNA (HPLC purified) was purchased from Integrated DNA Technologies (IDT). It was used directly without further purification. The PylT sequence is shown in Table S2.

Microinjection of embryos. For Rluc and Fluc experiment, the injection mixture (3 µl) was prepared as 50 ng/µl of reporter mRNA, 100 ng/µl of PylRS mRNA, and 1,000 ng/µl of PylT. For the unnatural amino acid incorporation, 0.15 µl of unnatural amino acid (100 mM stock) was added to 1.5 µl of injection mixture (assuming that the volume of 1 to 2 cell stage embryos is around 20 nL, the expected final concentration of the unnatural amino acid is 1 mM). Embryos from natural mating were obtained and microinjected at the 1 to 2 cell stage with 2 nL of the injection mixture using a World Precision Instruments Pneumatic PicoPump injector. For Fluc activation experiments, at 48 hpf the embryos were irradiated for 2 minutes with a 365 nm UV transilluminator (8 mW/cm^2). For constitutively active MEK1 experiments, 2 nL of 10 ng/µl caMEK1 mRNA was injected per embryo. For caged MEK1 experiments, the injection mixture was prepared as 50 ng/µl of MEK1-K97TAG mRNA, 100 ng/µl of HCKRS mRNA, 1,000 ng/µl of PylT, and 2 nL of mixture were injected per embryo. The embryos were irradiated for 30 seconds at 2 h, 5 h or 8 h post injection. Embryos were dechorinated and imaged at 10 hpf using a stereomicroscope (Leica MZ 16 FA). Embryos irradiated after 5 h post injection were used for whole-mount RNA in situ hybridization. For all injections, zebrafish embryos with early mortality (< 6 hpf) were excluded from further analysis. Zebrafish embryos were randomly assigned to experimental groups during irradiation. Blinding was not used for scoring dorsalized embryos.

Embryo toxicity assay. For toxicity tests of unnatural amino acids, embryos were injected with 2 nL of injection solution (0.15 µl of 100 mM unnatural amino acid in DMSO or just DMSO (vehicle control), 0.75 µl of phenol red (0.5% in Dulbecco's phosphate-buffered saline), and 0.6 µl of water). Live (phenotypically normal) and dead embryos were counted at 24 hpf.

Luciferase assay. Luciferase assays for zebrafish embryos were performed as described previously.[4] Briefly, 4 embryos were collected at 48 hpf, washed twice with 1 ml of phosphate-

buffered saline (PBS), and were incubated with 50 µl of 1x passive lysis buffer (Promega) for 30 minutes. After incubation, the embryos were manually homogenized with pipette tips, and the extract was centrifuged for 3 minutes at 13,000 rpm to remove cellular debris. For Rluc assays, 10 µl of lysate was mixed with 10 µl of Renilla Luciferase Assay Reagent (Promega), and luminescence was read immediately on a microplate reader (Tecan M1000 PRO), with an integration time of 1,000 ms. For Fluc activation assays, a dual luciferase reporter assay system (Promega) was used. Fluc activity was measured by mixing 10 µl of lysate with 50 µl of LAR II reagent. Rluc activity was measured by adding 50 µL of Stop & Glo reagent. Luminescence was read on a Tecan M1000 PRO microplate reader.

Western blotting. For detection of caged MEK1 expression, zebrafish embryos were collected at 6 h post injection and washed twice with Ringer's solution. For time-course analysis of MEK1 expression and ERK phosphorylation, zebrafish embryos were collected 30 min after UV irradiation at specified time points. The samples were incubated with 80 µl of 2% SDS buffer for 20 minutes, homogenized manually with pipette tips, heated at 95 ºC for 5 minutes, and centrifuged for 1 minute at 13,000 rpm. The protein extract was analyzed by Western blot. Briefly, after gel electrophoresis and transfer to a nitrocellulose membrane (GE Healthcare), the membrane was blocked in Tris-buffered saline with 0.1% Tween 20 and 5% milk powder for one hour. The blots were probed with an anti-phospho-ERK antibody (1:1,000 dilution, Cell Signaling, #9101S), an anti-ERK antibody (1:1,000 dilution, Sigma, #M-7431), an anti-HA antibody (1:1,000 dilution, Cell Signaling, #3724), or an anti-β-actin antibody (1:1,000 dilution, Santa Cruz, #81178) overnight at 4 ºC, followed by incubation with secondary antibodies (goat anti-rabbit IgG-HRP for phospho-ERK blot and HA blot, goat anti-mouse for ERK blot and β-actin blot, 1:20,000 dilution) conjugated to horseradish peroxidase (HRP) for 1 hour at room temperature. The blots were further incubated with the SuperSignal West Pico working solution (mixture of the Stable Peroxide Solution and the Luminol/Enhancer Solution, 500 µl each,

Thermo Scientific) for 5 min at room temperature. The luminescence signal was detected on a ChemiDoc (setting: Chemi Hi Sensitivity, manual exposure time: 10 sec).

Whole-mount RNA in situ hybridization. Embryos collected at shield stage (6 hpf) or bud stage (10 hpf) were fixed overnight in 4% paraformaldehyde/PBS at 4 ºC. Whole-mount *in situ* hybridization was performed with standard procedures, using described probes for *ta* and *chd*.[5] The embryos were incubated with anti-digoxigenin-AP (1:2,000 dilution, Roche #11093274910) for 4 hours at room temperature, and were stained in 500 µl of BM Purple alkaline phosphatase substrate (Roche #11442074001) for 1 hour at room temperature. The embryos were transferred into 1x PBS and imaged using a stereomicroscope (Leica MZ 16 FA). All stained zebrafish embryos were scored without blinding.

Supporting Figures & Tables

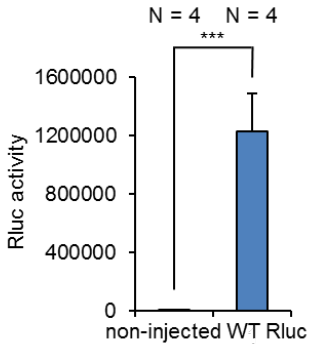

Figure S1. Activity of wild-type Rluc in zebrafish embryos. Embryos were injected with wild-type Rluc mRNA (100 pg). Embryos were collected at 48 hpf, and luciferase assays were performed. N indicates the number of pooled samples (4 embryos each). Statistical significance is indicated by *** $p < 0.001$ (unpaired t-test).

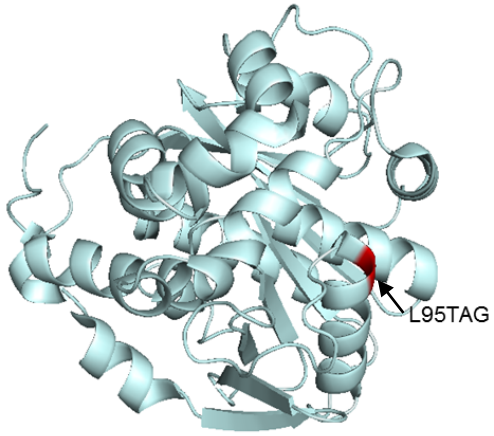

Figure S2. Renilla luciferase as a reporter for unnatural amino acid incorporation. The unnatural amino acids were incorporated at a permissive leucine residue (L95) located at the protein surface (shown in red). PDB: 2PSD.

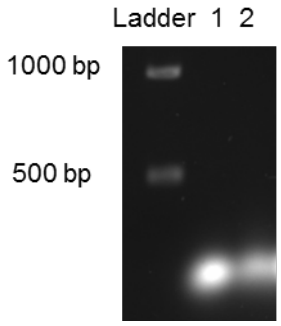

Figure S3. Gel analysis of PylT-CCA used in the embryo injections. Lane 1: chemically synthesized PylT-CCA. Lane 2: *in vitro* transcribed PylT-CCA.

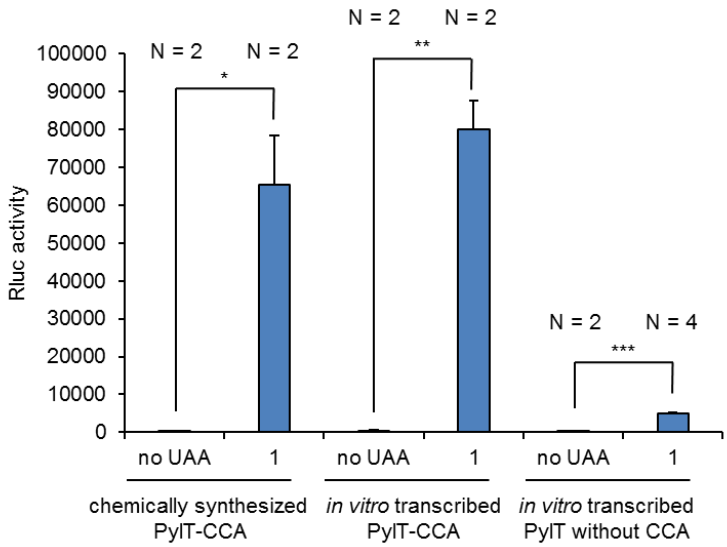

Figure S4. Comparison of incorporation efficiency with different PylTs. The unnatural amino acid **1** was incorporated into Rluc-L95TAG. N indicates the number of pooled samples (4 embryos each). Statistical significance is indicated by * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ (unpaired t-tests).

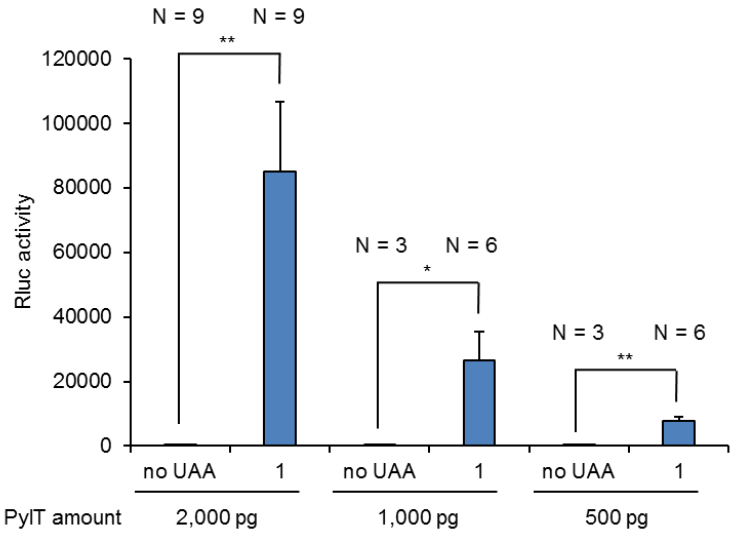

Figure S5. Comparison of incorporation efficiency with different amounts of PylT-CCA (*in vitro* transcribed). The unnatural amino acid **1** was incorporated into Rluc-L95TAG. N indicates the number of pooled samples (4 embryos each). Statistical significance is indicated by * $p < 0.05$ and ** $p < 0.01$ (unpaired t-tests).

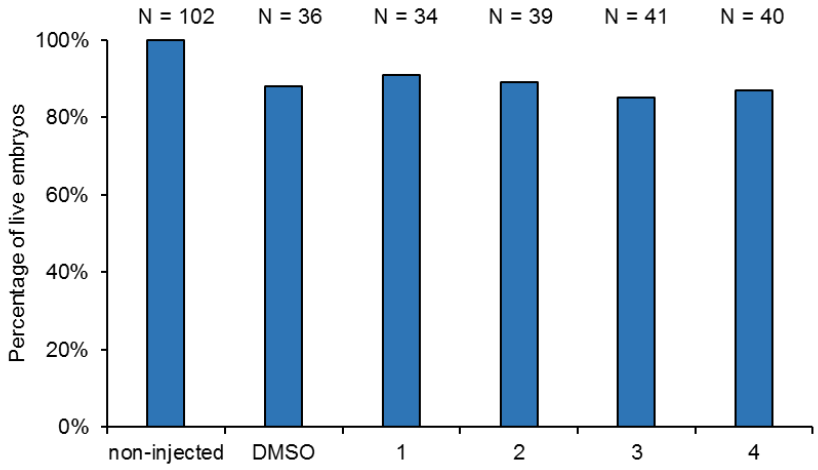

Figure S6. Toxicity test of the unnatural amino acids **1**-**4**. Embryos were injected with 1% DMSO or unnatural amino acid, and the numbers of live and dead embryos were counted at 24 hpf. No developmental defects were observed in live embryos. N represents the number of embryos observed.

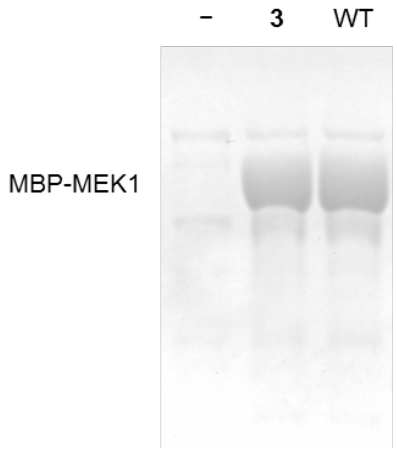

Figure S7. Coomassie stained gel of Ni-purified MBP-MEK1-K97TAG-**3** expressed in *E. coli*. No protein expression was detected in the absence of **3**. MBP-MEK1-WT was used as a positive control.

a

MKIEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKLEEKFPQVAATGDGPDIIFWAHDRFGGYAQSGLLAEITPD
KAFQDKLYPFTWDAVRYNGKLIAYPIAVEALSLIYNKDLLPNPPKTWEEIPALDKELKAKGKSALMFNLQEPYFTWPLIAADG
GYAFKYENGKYDIKDVGVDNAGAKAGLTFLVDLIKNKHMNADTDYSIAEAAFNKGETAMTINGPWAWSNIDTSKVNYGVTVL
PTFKGQPSKPFVGVLSAGINAASPNKELAKEFLENYLLTDEGLEAVNKDKPLGAVALKSYEEELAKDPRIAATMENAQKGEI
MPNIPQMSAFWYAVRTAVINAASGRQTVDEALKDAQTNSSSNNNNNNNNNNLGIEGRENLYFQGHMPKKKPTPIQLNPAP
DGSAVNGTSSAETNLEALQKKLEELELDEQQRKRLEAFLTQKQKVGELKDDDFEKISELGAGNGGVVFKVSHKPSGLVMA
RKLIHLEIKPAIRNQIIRELQVLHECNSPYIVGFYGAFYSDGEISICMEHMDGGSLDQVLKKAGRIPEQILGKVSIAVIKGLTYLR
EKHKIMHRDVKPSNILVNSRGEIKLCDFGVSGQLIDSMANSFVGTRSYMSPERLQGTHYSVQSDIWSMGLSLVEMAVGRYP
IPPPDAKELELMFGCQVEGDAAETPPRPRTPGRPLSSYGMDSRPPMAIFELLDYIVNEPPPKLPSGVFSLEFQDFVNKCLIK
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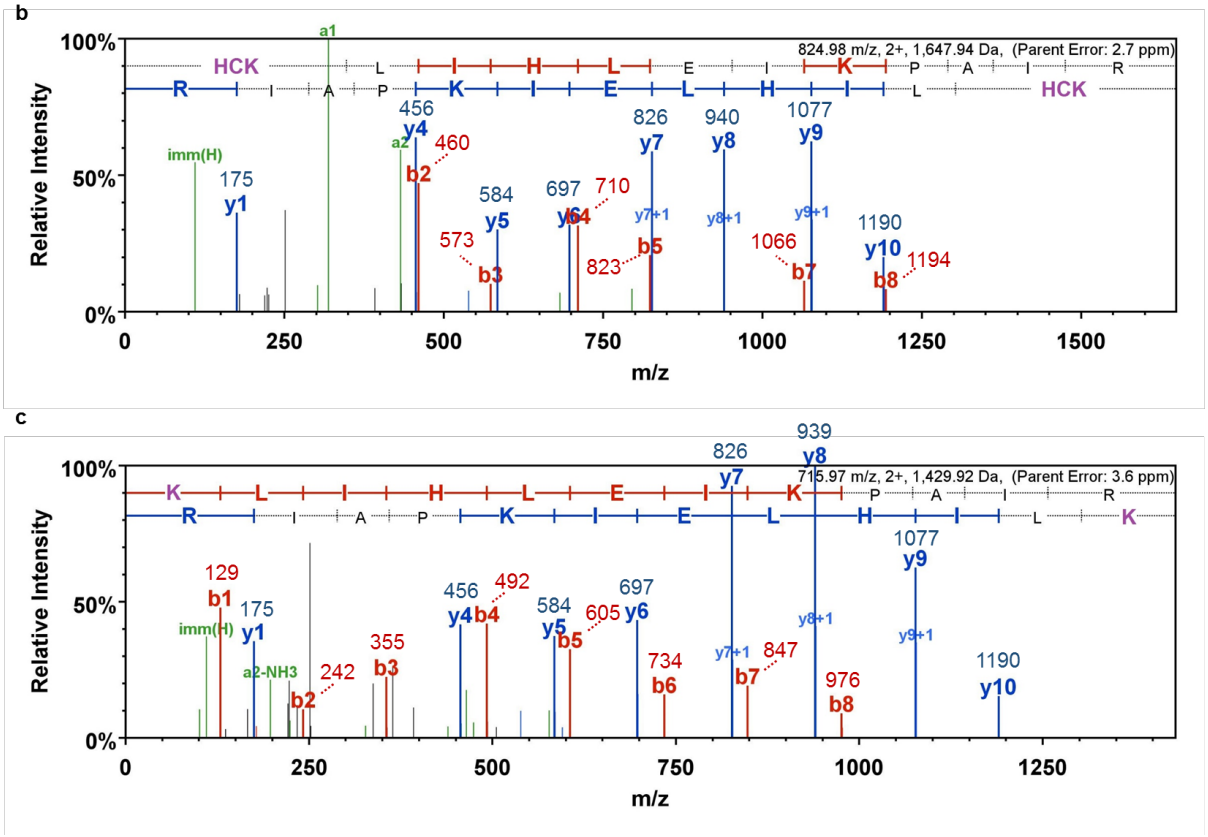

Figure S8. MS/MS analysis confirms incorporation of **3** into MEK1 and subsequent decaging with UV light. (a) Full-length protein sequence of MBP-MEK1. Target fragment with **3** is highlighted in yellow, and the K97 position is labeled in red. (b) The spectrum before UV irradiation indicates **3** at the 97 position. (c) The spectrum after UV irradiation indicates lysine at the 97 position. The MBP-MEK1-K97TAG-**3** protein was recombinantly expressed in *E. coli*. MS/MS analyses were conducted by MS Bioworks (Ann Arbor, MI).

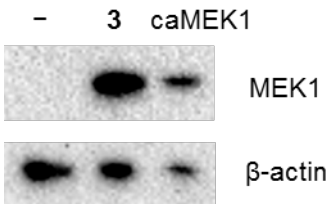

Figure S9. Western blot analysis of **3**-dependent expression of MEK1 in zebrafish embryos, with TAG amber codon at position lysine 97.

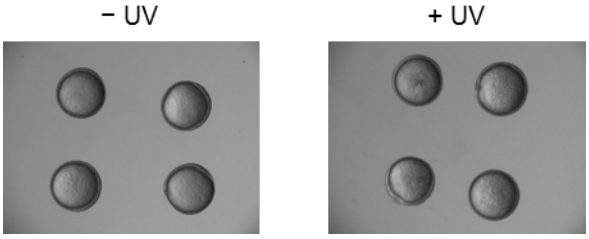

Figure S10. Embryos developed normally when injected with caMEK1-K97TAG mRNA, HCKRS mRNA and PylT, but not **3**. Embryos were either kept in the dark or irradiated at 5 h post injection. Images were taken at 10 hpf.

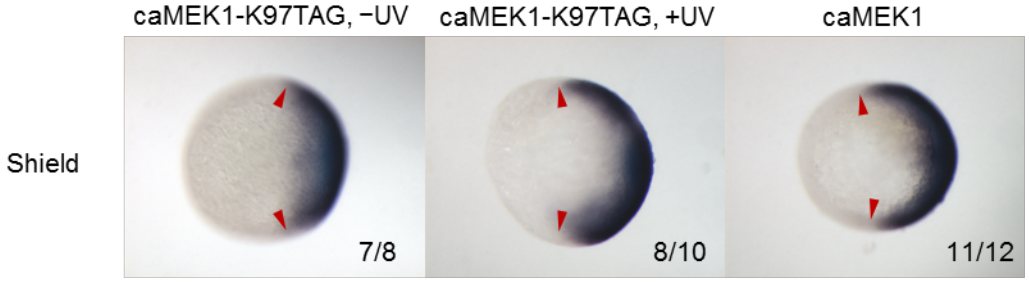

Figure S11. Upregulation of *chordin* (*chd*) expression in response to light-activation of MEK1, imaged at shield stage. Embryos that express caged MEK1 show expanded expression after exposure to UV light, compared to embryos kept in the dark (see red arrows). Animal views with the dorsal side to the right. Number of embryos that displayed the shown expression pattern and total number of embryos are shown.

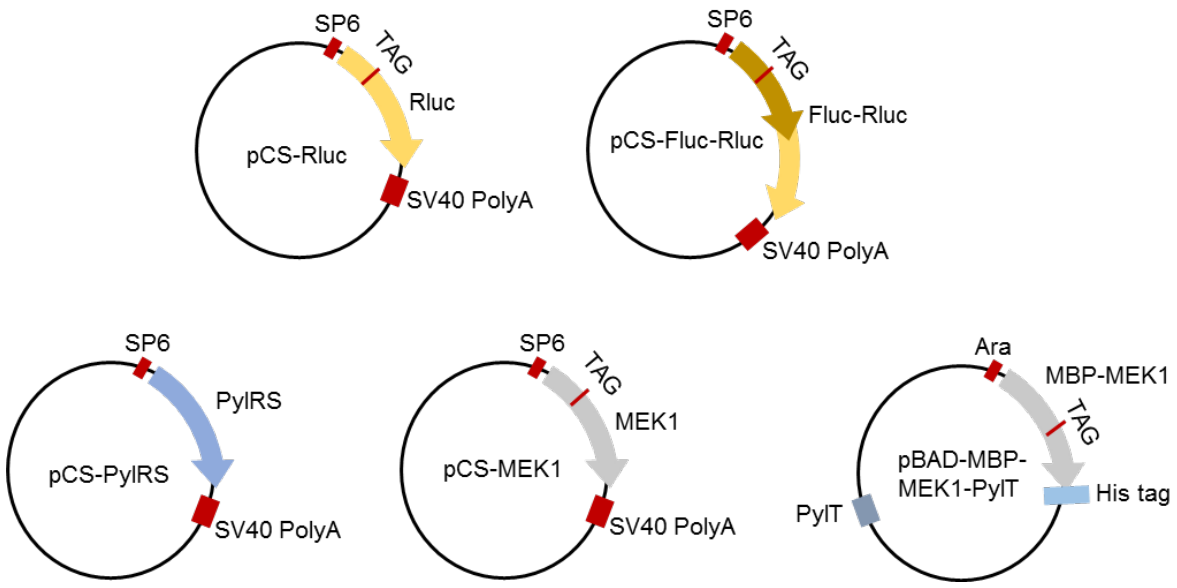

Figure S12. Generated constructs for *in vitro* synthesis of injected mRNAs and for bacterial expression of MBP-MEK1. In addition to the TAG codon containing plasmids, the corresponding non-TAG constructs for expression of wild-type proteins were constructed as well.

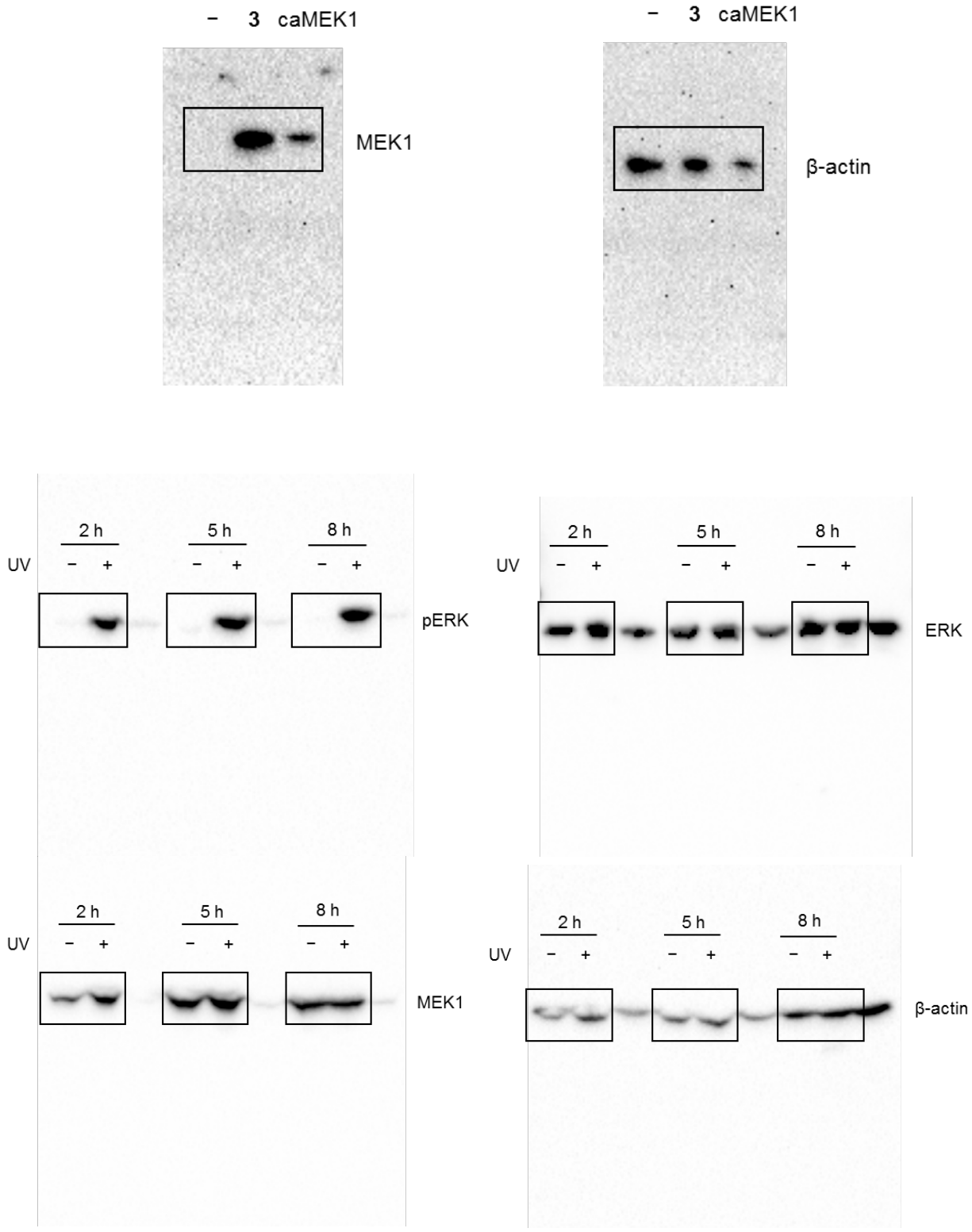

Figure S13. Full western blots of the cropped regions shown in Figure 2c and Figure S9.

Supporting Tables

P1	stctttttgcaggatccatggcttccaaggtgtacgacc
P2	tcactatagttctagatcattactgctcgttcttcagcacg
P3	ggaatggctcatatcgcctctaggatcactacaagtacctcacc
P4	ggtgaggtacttgtagtgatcctagaggcgatatgagccattcc
P5	tctttttgcaggatccatggactacaaggacgacg
P6	tcactatagttctagatcacaggttggtgctgatg
P7	tctttttgcaggatccatggaagacgccaaaaacataaag
P8	tcactatagttctagatcattacacggcgatctttccg
P9	ggatctactggtctgccttagggtgtcgctctgcctcatag
P10	ctatgaggcagagcgacaccctaaggcagaccagtagatcc
P11	gcgtgctgaagaacgagcagtacccatacgatgttccagattacgcttaatgatctagaactatagtg
P12	ggtcgtacaccttggaagcgccggagcccacggcgatctttccgccc
P13	gggcggaaagatcgccgtgggctccggcgcttccaaggtgtacgacc
P14	cactatagttctagatcattaagcgtaatctggaacatcgtatgggtactgctcgttcttcagcacgc
P15	agctccatgggcaaaatcgaagaaggtaaactgg
P16	agctattaatttagtgatggtggtgatgatgcgc
P17	gtccggtctggttatggctcgttagctgatccacctggaaatcaaacc
P18	ggtttgatttccaggtggatcagctaacgagccataaccagaccggac
P19	tctttttgcaggatccatgcccaagaagaagccgacgccc
P20	tcactatagttctagattatcattaagcgtaatctggaacatcgtatgggtacatgacgccagcagcatgggttggtgtg
P21	taatacgactcactatagga
P22	cggaaaccccgggaatctaa

Table S1. List of primers (shown 5’ to 3’).

PylT DNA (without CCA)	TAATACGACTCACTATAGGAAACCTGATCATGTAGATC GAACGGACTCTAAATCCGTTCAGCCGGGTTAGATTCC CGGGGTTTCCG
PylT DNA (with CCA)	TAATACGACTCACTATAGGAAACCTGATCATGTAGATC GAACGGACTCTAAATCCGTTCAGCCGGGTTAGATTCC CGGGGTTTCCGCCA
Synthetic PylT RNA	GGAAACCUGAUCAUGUAGAUCGAACGGACUCUAAAU CCGUUCAGCCGGGUUAGAUUCCCGGGGUUUCCGCCA

Table S2. Sequence of PylT DNA and chemically synthesized PylT RNA (shown 5' to 3'). Underlined letters in the PylT DNA indicate a truncated T7 promoter.

References

(1) (a) Nguyen, D. P.; Lusic, H.; Neumann, H.; Kapadnis, P. B.; Deiters, A.; Chin, J. W. *J. Am. Chem. Soc.* **2009,** *131*, 8720-1. (b) Luo, J.; Uprety, R.; Naro, Y.; Chou, C.; Nguyen, D. P.; Chin, J. W.; Deiters, A. *J. Am. Chem. Soc.* **2014,** *136*, 15551-8. (c) Luo, J.; Liu, Q.; Morihiro, K.; Deiters, A. *Nat. Chem.* **2016,** *8*, 1027-1034.

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(3) Chatterjee, A.; Sun, S. B.; Furman, J. L.; Xiao, H.; Schultz, P. G. *Biochemistry.* **2013,** *52*, 1828-37.

(4) Alcaraz-Perez, F.; Mulero, V.; Cayuela, M. L. *BMC Biotechnol.* **2008,** *8*, 81.

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