SUPPORTING INFORMATION

Nitroreductase-activatable morpholino oligonucleotides for \textit{in vivo} gene silencing

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Supplementary Figure 1. NfsB-dependent linearization of 4-NB cyclic cMOs. Mass spectra of in vitro reaction mixtures initially composed of 2 µM 4-NB ntlα cMO, 100 µM NADH, and various concentrations of NfsB-mCherry. The reactions were incubated at 28.5 °C for the indicated times and then analyzed by LC-MS. Mass peaks associated with the starting cyclic cMO and its linearized 4-hydroxylaminophenyl (4-HAP)-derived product are shown with black circles and bars, respectively.
Supplementary Figure 2. Estimation of NfsB-mCherry levels in zebrafish embryos injected with nfsB-mCherry mRNA. (a) Western blot analysis of purified mCherry and NfsB-mCherry isolated from 5-hpf embryos previously injected with nfsB-mCherry mRNA. Signal intensities were measured with a Bio-Rad ChemiDoc XRS system, and the estimated NfsB-mCherry levels are shown. (b) The mCherry standard curve used to estimate NfsB-mCherry levels.
**General synthetic procedures.** All reactions were carried out in flame-dried glassware under a nitrogen atmosphere and stirred magnetically. Reactions were monitored by thin layer chromatography (TLC), using glass-backed silica gel 60F254 (Merck 250-µm thickness). Reagents and solvents obtained from commercial sources were stored under nitrogen and used directly without further purification. Yields refer to chromatographically and spectroscopically pure compounds unless otherwise stated. Flash chromatography was performed with silica gel (EM Science silica gel 60 Å, 70-230 mesh) as a stationary phase. The $^1$H NMR and $^{13}$C NMR spectra were recorded on a 300 MHz, 400 MHz, or 500 MHz Varian NMR spectrometers. Chemical shifts are in δ units (ppm) for $^1$H NMR spectra and $^{13}$C NMR spectra. Electrospray (ESI) mass spectra were obtained using a Micromass ZQ single quadrupole liquid chromatography-mass spectrometer (LC-MS) and a Micromass Q-TOF hybrid quadrupole LC-MS.

**4-NB linker synthesis**

1-(4-nitophenyl)but-3-en-1-ol (2). To a stirred solution of 4-nitrobenzaldehyde (1.00 g, 6.62 mmol) in dry CH$_2$Cl$_2$ (20 mL) was added TiCl$_4$ (16 mL of a 1 M solution in CH$_2$Cl$_2$) and allyltrimethylsilane (2.40 mL, 15.7 mmol) at -78 °C. After the reaction mixture was stirred for 30 minutes, it was washed twice with aq. 2 N HCl and once with water. Solvent was removed *in vacuo*, and the residue was purified by silica gel chromatography (acetone/CH$_2$Cl$_2$) to obtain 2 as a yellow gum (1.14 g, 89%). $^1$H NMR (400 MHz, CDCl$_3$) δ 8.17 (d, $J = 9.2$ Hz, 2H), 7.52 (d, $J = 9.2$ Hz, 2H), 5.75 (m, 1H), 5.15 (m, 2H), 4.84 (m, 1H), 2.52 (m, 1H), 2.45 (m, 1H), 2.22 (brs, 1H). HRMS-ESI: m/z calculated for C$_{10}$H$_{12}$NO$_3$ [M + H]$^+$: 194.0812; observed: 194.0819.
1-(4-nitrophenyl)propane-1,3-diol (3). A solution of 2 (0.960 g, 4.97 mmol) in MeOH (40 mL) was cooled to -78 °C and then saturated with ozone gas for 5 minutes, producing a blue-colored reaction mixture. Sodium borohydride (1.03 g, 27.8 mmol) was added to the solution at -78 °C, and the solution allowed to warm to room temperature over a period of 30 minutes. The reaction was then quenched with saturated aq. NH₄Cl (20 mL). MeOH was removed in vacuo, and the aqueous layer was extracted with EtOAc. The combined organic layers were removed in vacuo, and the residue was purified by silica gel chromatography (MeOH/CHCl₃ = 1:19) to yield 3 as a brown foam (792 mg, 82%). ¹H NMR data was identical to that previously reported¹: (400 MHz, CDCl₃) δ 8.22 (d, J = 8.8 Hz, 2H), 7.56 (d, J = 8.8 Hz, 2H), 5.15 (m, 1H), 3.94 (m, 2H), 3.39 (brs, 1H), 2.00 (brs, 1H), 1.98 (m, 2H). HRMS-ESI: m/z calculated for C₉H₁₁NNaO₄ [M + Na]⁺: 220.0580; observed: 220.0588.

3-(Methylamino)-1-(4-nitrophenyl)propan-1-ol (4). To a stirred solution of 3 (500 mg, 2.54 mmol) in dry pyridine (4 mL) was added p-toluenesulfonyl chloride (662 mg, 3.46 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 3 hours and pyridine was removed in vacuo. The resulting residue was dissolved in EtOAc (50 mL), washed with cold aq. 1 N HCl and concentrated aq. NaCl, and dried over anhydrous Na₂SO₄. Solvent was removed in vacuo, and the monotosylated product was purified by silica gel chromatography (CHCl₃/acetone, stepwise gradient from 1:0 to 20:1) to yield 3-hydroxy-3-(4-nitrophenyl)propyl 4-methylbenzenesulfonate as colorless flakes with slight impurities (713 mg, 80%). HRMS-ESI: m/z calculated for C₁₆H₁₇NNaO₆S [M + Na]⁺: 374.0669; observed: 374.0662. A solution of 3-hydroxy-3-(4-nitrophenyl)propyl 4-methylbenzenesulfonate (220 mg, 0.626 mmol) and methylamine (12.5 mL of a 2.0 M solution in THF, 20.0 mmol) was stirred at 80 °C overnight.
The solvent was removed *in vacuo*, co-evaporated twice with MeOH, trititated with dry diethyl ether, and dried *in vacuo* to yield the tosylate salt of 4 as a colorless solid (191 mg, 80%). $^1$H NMR (400 MHz, CDCl$_3$) δ 8.10 (d, J = 7.0 Hz, 2H), 7.73 (d, J = 6.8 Hz, 2H), 7.56 (m, 1H), 7.51 (d, J = 7.0 Hz, 2H), 7.21 (d, J = 6.8 Hz, 2H), 5.12 (dd, J = 8.0, 2.4 Hz, 1H), 3.23 (m, 1H), 5.12 (dd, J = 8.0, 2.4 Hz, 1H), 3.23 (m, 1H), 2.72 (s, 3H), 2.55 (brs, 1H), 2.39 (s, 3H), 2.22-2.17 (m, 2H), 2.12-2.05 (m, 2H). HRMS-ESI: m/z calculated for C$_{10}$H$_{15}$N$_2$O$_3$ [M + H]$^+$: 211.1077; observed: 211.1080.

**Methyl 6-((3-hydroxy-3-(4-nitrophenyl)propyl)(methyl)amino)-6-oxohexanoate (5).** Compound 4 (100 mg, 0.262 mmol) and N,N-diisopropylethylamine (91.0 µL, 0.524 mmol) were dissolved in CH$_2$Cl$_2$ (1.00 mL) and the solution was cooled to 0 °C. Methyladipoyl chloride (40.6 µL, 0.262 mmol) was added over 10 minutes, and the reaction mixture was stirred for 6 hours at room temperature. After the reaction solvent was removed *in vacuo*, the resulting residue was dissolved in EtOAc, washed twice with saturated aq. NaHCO$_3$ and then dried over anhydrous Na$_2$SO$_4$. Solvent was removed *in vacuo*, and the residue was purified by silica gel chromatography (CHCl$_3$/EtOAc, stepwise gradient from 1:0 to 5:1) to yield 5 as a colorless oil (47.9 mg, 52%). $^1$H NMR (500 MHz, CDCl$_3$) δ 8.15 (d, 2H, J = 8.8 Hz), 7.52 (d, 2H, J = 8.8 Hz), 5.18 (brs, 1H), 4.56 (d, 1H, J = 7.0 Hz), 4.18 (m, 1H), 3.64 (s, 3H), 3.03 (s, 3H), 3.00 (m, 1H), 2.39-2.28 (m, 4H), 1.95 (m, 1H), 1.72-1.62 (m, 5H). HRMS-ESI: m/z calculated for C$_{17}$H$_{25}$N$_2$O$_6$ [M + H]$^+$: 353.1707; observed: 353.1707.

**Methyl 1-chloro-12-methyl-9-(4-nitrophenyl)-2,7,13-trioxo-8-oxa-3,6,12-triazaoctadecan-18-oate (6).** Compound 5 (43.0 mg, 0.122 mmol) was dissolved in CH$_2$Cl$_2$ (370 mL) and added to 1,1’-carbonyldiimidazole (51.5 mg, 0.318 mmol) in CH$_2$Cl$_2$ (555 µL). The reaction mixture
was stirred for 3 hours at room temperature under nitrogen, diluted with CHCl₃, washed twice with water, and dried over anhydrous MgSO₄. Solvent was removed in vacuo to yield crude imidazole carbamate as a yellow gum (55.4 mg, 0.124 mmol). The imidazole carbamate was dissolved in CH₂Cl₂ (720 μL) and the solution was cooled to 0 °C. Ethylenediamine (26.6 μL, 0.398 mmol) was added, and the reaction mixture was stirred for 4 hours at room temperature under nitrogen. Solvent was removed in vacuo to yield the crude amine as a colorless oil (53.0 mg, 0.121 mmol). HRMS-ESI: m/z calculated for C₂₀H₃₁N₄O₇ [M + H]⁺: 439.2187; observed: 439.2184. Without further purification, the obtained amine (53.0 mg, 0.121 mmol) was dissolved in CH₂Cl₂ (900 μL) and triethylamine (92.3 μL, 0.664 mmol) and cooled to 0 °C. 2-Chloroacetyl chloride (21.0 μL, 0.265 mmol) dissolved in CH₂Cl₂ (300 μL) was added slowly to this solution. The mixture was stirred at room temperature for 20 minutes, at which time, 5% saturated aq. NaHCO₃ was added. The organic layer was dried over anhydrous MgSO₄, solvent was removed in vacuo, and the residue was purified by silica gel chromatography (CHCl₃/acetone, stepwise gradient from 1:0 to 1:1) to yield 6 as a colorless oil (40.1 mg, 64%).

1H NMR (400 MHz, CDCl₃) δ 8.11 (d, J = 8.4 Hz, 2H), 7.61 (brs, 1H), 7.43 (d, J = 8.4 Hz, 2H), 5.64 (brs, 1H), 5.55 (m, 1H), 4.21 (m, 1H), 3.94 (brs, 2H), 3.71 (m, 1H), 3.59 (s, 3H), 3.41-3.24 (m, 4H), 3.19 (m, 2H), 2.92 (s, 3H), 2.28-2.22 (m, 4H), 1.64-1.34 (m, 4H). HRMS-ESI: m/z calculated for C₂₂H₃₂ClN₄O₈ [M + H]⁺: 515.1906; observed: 515.1908.

2,5-dioxopyrrolidin-1-yl 1-chloro-12-methyl-9-(4-nitrophenyl)-2,7,13-trioxo-8-oxa-3,6,12-triazaoctadecan-18-oate (7). Compound 6 (40.1 mg, 0.0780 mmol) was dissolved in THF (270 μL), and cooled to 0 °C. To this solution, an aqueous solution of LiOH (4.00 mg, 0.0952 mmol in 360 μL water) was added slowly, and allowed to stir at room temperature for 2 hours.
At this time point, the reaction mixture was diluted with EtOAc, washed with 2 M HCl (360 µL), and organic layer was dried over MgSO₄. Solvent was removed in vacuo to afford crude 1-chloro-12-methyl-9-(4-nitrophenyl)-2,7,13-trioxo-8-oxa-3,6,12-triazaoctadecan-18-oic acid as a colorless oil (34.6 mg, 89%). ¹H NMR (400 MHz, CDCl₃) δ 8.59 (brs, 1H), 8.20 (d, J = 8.5 Hz, 2H), 7.63 (brs, 1H), 7.50 (d, J = 8.5 Hz, 2H), 5.71 (brs, 1H), 5.68 (m, 1H), 4.18 (m, 1H), 4.03 (brs, 2H), 3.75 (m, 1H), 3.51-3.22 (m, 6H), 3.01 (s, 3H), 2.43-2.30 (m, 4H), 1.73-1.64 (m, 4H).

Without further purification, the carboxylic acid (21.2 mg, 0.0424 mmol) was dissolved in CH₃CN (525 µL) and reacted with N,N'-disuccinimidyl carbonate (45.0 mg, 0.176 mmol) and pyridine (35.0 µL, 0.439 mmol) at room temperature overnight. The remaining residue was dissolved in EtOAc, washed with 0.1 N aq. HCl, washed with saturated aq. NaHCO₃ and dried over anhydrous MgSO₄. Solvent was removed in vacuo, and the residue was purified by silica gel column chromatography (CHCl₃/acetone, stepwise gradient from 1:0 to 1:1) to yield 7 as a colorless oil (19.2 mg, 76%). ¹H NMR (400 MHz, CDCl₃) δ 8.22 (d, J = 8.7 Hz, 2H), 7.62 (brs, 1H), 7.52 (d, J = 8.7 Hz, 2H), 5.64 (m, 1H), 5.36 (m, 1H), 4.24 (m, 1H), 4.04 (brs, 2H), 3.86 (m, 1H), 3.60 (m, 1H), 3.51-3.32 (m, 4H), 3.28 (m, 1H), 3.01 (s, 3H), 2.86 (brs, 4H), 2.42-2.32 (m, 4H), 1.88-1.75 (m, 4H). HRMS-ESI: m/z calculated for C₂₅H₃₃ClN₅O₁₀ [M + H]⁺: 598.1910; observed: 598.1911.

**Cyclic cMO syntheses**

Synthetic procedures identical to those previously reported for a DMNB cyclic ntlα cMO² were applied to 4-NB cyclic cMOs targeting ntlα, pdxl, or mnx1, using bifunctional linker 7 and the 5’ amine- and 3’-disulfide-functionalized MOs indicated below. DMNB cMOs targeting pdxl or mnx1 were similarly prepared with the corresponding DMNB-containing linker.² In brief, the
25-base targeting MO was conjugated to the 4-NB or DMNB linker in 0.1 M Na₂B₄O₇, pH 8.5 buffer overnight, and the resulting linker-MO intermediate was purified on a NAP-5 gel filtration column and lyophilized. The resulting white solid was dissolved in water, acidified with acetic acid, washed sequentially with CHCl₃ and EtOAc, neutralized with 10% aq. NH₄OH, and lyophilized again. MO cyclization was next induced by dissolving the linker-MO intermediate in 0.1 M Tris-HCl, pH 8.4 buffer and treating it overnight with resin-immobilized TCEP. The reaction supernatant was then purified on a NAP-5 column and lyophilized to afford the final cyclized product.

**4-NB ntlα cMO (10a).** *ntlα* MO: 5’-GACTTGAGGCAGACATATTTCGGAT-3’. The 4-NB linker-ntlα MO intermediate 9a was obtained in 75% yield. MS-ESI: m/z calculated for C₃₃₈H₅₂₃N₁₁₇O₁₁₂P₂₅S₂Cl [M+H]⁺, 9453; observed 9453. The cyclized cMO 10a was obtained in 86% yield. MS-ESI: m/z calculated for C₃₃₄H₅₁₅N₁₁₅O₁₁₁P₂₅S [M + H]⁺: 9299; observed: 9299.

**4-NB pdx1 cMO (10b).** *pdx1* MO: 5’-GATAGTAATGCTCTTCCCGATT-3’. The 4-NB linker-pdx1 MO intermediate 9b was obtained in 64% yield. MS-ESI: m/z calculated for C₃₄₅H₅₃₂N₁₄₉O₁₁₅P₂₅S₂Cl [M+H]⁺, 9482; observed 9482. The cyclized cMO 10b was obtained in 73% yield. MS-ESI: m/z calculated for C₃₃₃H₅₁₆N₁₄₈O₁₁₄P₂₅S [M + H]⁺: 9224; observed: 9225.

**4-NB mnx1 cMO (10c).** *mnx1* MO: 5’-TTTTTAGATTTCCTCCAGATCTGGCAC-3’. The 4-NB linker-mnx1 MO intermediate 9c was obtained in 84% yield. MS-ESI: m/z calculated for C₃₄₄H₅₃₄N₁₄₁O₁₁₉P₂₅S₂Cl [M+H]⁺, 9424; observed 9424. The cyclized cMO 10c was obtained in 89% yield. MS-ESI: m/z calculated for C₃₃₂H₅₁₈N₁₄₀O₁₁₈P₂₅S [M + H]⁺: 9166; observed: 9167.
DMNB *pdx1* cMO. *pdx1* MO: 5’-GATAGTAATGCTCTTCCCGATTcAT-3’. The DMNB linker-*pdx1* MO intermediate was obtained in 57% yield. MS-ESI: m/z calculated for C$_{347}$H$_{537}$N$_{149}$O$_{117}$P$_{25}$S$_2$Cl [M+H]$^+$, 9542; observed 9545. The cyclized cMO was obtained in 97% yield. MS-ESI: m/z calculated for C$_{335}$H$_{521}$N$_{148}$O$_{116}$P$_{25}$S [M + H]$^+$: 9284; observed: 9285.

DMNB *mnx1* cMO. *mnx1* MO: 5’-TTTTTAGATTTCTCCTCCATCTGGCCCA-3’. The DMNB linker-*mnx1* MO intermediate was obtained in 64% yield. MS-ESI: m/z calculated for C$_{346}$H$_{539}$N$_{141}$O$_{121}$P$_{25}$S$_2$Cl [M+H]$^+$, 9484; observed 9485. The cyclized cMO was obtained in 87% yield. MS-ESI: m/z calculated for C$_{334}$H$_{523}$N$_{140}$O$_{120}$P$_{25}$S [M + H]$^+$: 9226; observed: 9225.
REFERENCES

