Supplementary Information Guide

1. **Supplementary Methods**. Additional Methods including a Primer Table providing all oligo sequences and a Transgenic Line Table providing names and references for all transgenic lines used or created.

2. **Supplementary Data File**. Nucleotide sequences of all NTR variants tested, vectors created, and transgenes created.

3. **Supplementary Table 1**. Statistical analysis of graphed components of Fig. 3.

4. **Supplementary Table 2**. Statistical analysis of graphed components of Fig. 4.

5. **Supplementary Table 3**. Statistical analysis of graphed components of Fig. 5.

6. **Supplementary Table 4**. Statistical analysis of graphed components of Fig. 6.

7. **Supplementary Table 5**. Statistical analysis of graphed components of Extended Data Fig. 4.

Supplementary Methods

NTR variants – sequence information
The protein accession numbers for the native NTRs used in this study are: NfsB_Ec = WP_000351487.1; NfsB_Vv = WP_011081872.1; NfsB_St = WP_000355874.1; NfsB_Ck = WP_047458272.1; NfsB_Kp = WP_004178896.1; NfsB_Pp = WP_010953384.1; NfsB_Cs = WP_004384956.1; FraseI_Vf = P46072.3; NfsB_Vh = WP_005435698.1; YfkO_Bs = ADE73858.1; YdgI_Bs = WP_003225379.1.

DNA sequences and corresponding protein sequences of all engineered NTRs used in this study have been deposited with GenBank. Nucleotide sequences of all NTR variants tested here, as well as vectors created, and transgenes used to create new lines are provided in Supplementary Data File 1.

NTR variants – sequence comparisons
Amino acid sequence identity cladograms of the eleven NfsB orthologs, grouped according to percent shared amino acid identity with NfsB_Ec, were generated using the ClustalW2 tool with default settings (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Protein alignments were performed using Clustal Omega sequence alignment tool (default settings; http://www.ebi.ac.uk/Tools/msa/clustalo).

NTR variants – cloning
Native NTR candidates were PCR amplified and cloned into the *Nde*I and *Sal*I sites of two plasmids: pUCX (Addgene #60681), for bacterial overexpression assays, and pET28a(+) (Addgene #69864-3), for His_6-tag protein purification. Engineered gene variants were synthesized as fragments by Twist Bioscience (San Francisco, CA, USA).

For expression in human cells, NTR genes were Phusion™ PCR amplified using primers that introduced a mammalian Kozak consensus sequence, a TAG stop codon and Gateway™

(Invitrogen) BP recombination sites. PCR fragments were then recombined into the pDONR221™ vector (Invitrogen) using BP Clonase™ II enzyme mix (Thermo Fisher Scientific), and subsequently into the F279-V5 mammalian expression destination vector (a Gateway™ compatible plasmid which expresses inserted genes from a constitutive CMV promoter and has suppressor read through capabilities to incorporate a V5 epitope at the C-terminus)[59] using LR Clonase™¬ II enzyme mix (Thermo Fisher Scientific).

For expression in zebrafish, NTR genes were PCR amplified from the pET28a(+) vector using primers NfsB_Vv pTOL2_Fw and NfsB_Vv_Rv, digested with *Xma*I and *Sal*I and cloned into the *Xma*I and *Xho*I (an isoschizomer of *Sal*I) sites of a pTol2-5xUAS-GAP-tagYFP-P2A-*nfsB*_Ec vector, to swap out the parental *nfsB*_Ec for the new NTR variants. Note, the P2A element allows equimolar co-expression of the YFP reporter and NTR variants from a single transgene[60], avoiding possible loss of NTR activity caused by fusion protein oligomerization; the GAP element is a 20 bp dual palmitoylation sequence from the zebrafish *gap43* gene used to target reporters to the plasma membrane.

The pTol2 5xUAS:GAP-ECFP,he:GAP-ECFP plasmid was created by cutting ECFP from a 14xUAS:GAP-ECFP plasmid with *Xma*I and *Bsr*GI and cloning it into the same sites to replace the 5' EYFP reporter in a pTol2-5xUAS:GAP-EYFP,he:GAP-EYFP vector[61]. Similarly, the hatching enzyme promoter (he) driven ECFP "marker" reporter was cut from pTol2-1xUAS-GAP-tagBFP2gi,he:GAP-tagBFP2 with *Cla*I and *Eco*RV and cloned into the same sites to replace the 3' EYFP of the same parent vector[61]. Note, hatching enzyme (*he*) promoter driven "marker" reporters provide a means of tracking germline transmission of UAS and QUAS reporter/effector transgenes in the absence of a Gal4 or QF driver, respectively. Marker reporters are strongly expressed in the hatching gland from 1-3 dpf, then fade since the hatching gland is a temporary structure[61].

The rho:GAP-tagYFP-P2A-nfsB_Vv F70A/F108Y plasmid was generated by PCR amplifying the 3.7 kb rhodopsin promoter from the rho:YFP-NTR plasmid using primers rho_promoter_F2 and rho_promoter_R, and cloning it into the FseI and SnaBI sites of pTOL2-5xUAS-GAP-tagYFP-P2A-NfsB_Vv F70A-F108Y.

The pTol2 5xUAS:GAP-tagYFP-P2A-nfsB_Vv F70A/F108Y,he:GAP-tagBFP2 plasmid was generated by cutting the he:GAP-tagBFP2 element from pTol2-1xUAS-GAP-tagBFP2gi,he:GAP-tagBFP2 with *Hpa*I and *Eco*RV, and cloning it into the same sites downstream of the NTR 2.0 polyA in the vector pTol2 5xUAS:GAP-tagYFP-P2A-NfsB_Vv F70A/F108Y. The mCherry version, pTol2 5xUAS:GAP-mCherry-P2A-NfsB_Vv F70A/F108Y,he:GAP-tagBFP2, was generated by cloning the *Bsm*BI flanked portion of pTol2-5xUAS-GAP-mCherry-P2A-NTRmut,he:TagRFP into the same sites in the pTol2 5xUAS:GAP-tagYFP-P2A-NfsB_Vv F70A/F108Y,he:GAP-tagBFP2 vector. The EGFP version, pTol2 5xUAS:GAP-EGFP-P2A-NfsB_Vv F70A/F108Y,he:GAP-tagBFP2 was generated by using overlap extension PCR to fuse EGFP amplified with primers *Sbf*I-GFP_FOR and GFP_REV (from -6ascl1a:EGFP plasmid, kind gift of Dr. Dan Goldman) and 2A-NTR2.0 amplified by FW-P2A and KflI-Bglobin_REV (from the pTol2 5xUAS:GAP-tagYFP-P2A-NfsB_Vv F70A/F108Y,he:GAP-tagBFP2 vector), cutting the product with *Sbf*I and *Kfl*I and cloning it into the same sites within the pTol2 5xUAS:GAP-tagYFP-P2A-NfsB_Vv F70A/F108Y,he:GAP-tagBFP2 vector.

The pTol2 5xQUAS:GAP-tagYFP-P2A-NfsB_Vv F70A/F108Y,he:ECFP vector was created by amplifying the tagYFP to *he* promoter section of the UAS vector with the *Mlu*I-YFP_FOR and *AvrII*-He1Pro_REV primers, digesting with *Mlu*I and *AvrI*I and cloning into the same restriction sites within the pDestT2 QUAS-nls-mApple,he:ECFP plasmid (kindly provided by Marnie Halpern). All relevant primers are listed in the Primer Table below.

Zebrafish husbandry and transgenic resources

Zebrafish were maintained under standard growth conditions at 28.5 °C in a 10 h dark / 14 h light cycle. All zebrafish were treated in accordance with approved protocols reviewed by the Johns Hopkins Animal Care and Use Committee. Unless otherwise noted, all zebrafish were in the *roy orbison* (*roy; mpv17*$^{a9/a9}$) mutant background and treated with 200 nM phenylthiourea (PTU) at 16-24 hours post-fertilization (hpf) to facilitate live imaging and quantification of fluorescent reporters. New zebrafish transgenic lines were created by co-injecting miniTol2-based expression plasmids and 25 pg of Tol2 mRNA into one-cell stage zebrafish embryos and screening for germline transmission. UAS reporter lines, *Tg(5xUAS:GAP-tagYFP-P2A-nfsB_Vv F70A/F108Y)jh513* and *Tg(5xUAS:GAP-ECFP,he:GAP-ECFP)gmc1913,* were created by injecting into eggs from the *Et(2xNRSE-fos:KALTA4)gmc617* driver line to allow visualization of reporter expression. The *Tg(rho:GAP-tagYFP-P2A-nfsB_Vv F70A/F108Y)jh405* line was created by injecting into *roy*$^{a9/a9}$ eggs. A full list of all zebrafish transgenic lines used or created here, as well as abbreviations used herein, delineation of NTR variant, and references are provided in Transgenic Line Table below.

Primer Table | Primers used in this study

Primer Name	***Sequence (5' → 3')***
Primers for insertion of nitroreductase genes into vectors pUCX and pET28a+	
NfsB_Vv Fw	GGGGCATATGACTATTGTTCAAGCT
NfsB_Vv Rv	GGGGTCGACTTAGATTTCGGTAAAAACAG
NfsB_Ec Fw	GGGCATATGGATATCATTTCTG
NfsB_Ec Rv	GGGGAATTCTTACACTTCGGTTAAG
NfsB_Kp_Fw	GGGGCATATGGATATCGTATCGGTC
NfsB_Kp_Rv	GGGGCTCGAGTTAAATCTCGGTAATGGT
NfsB_Pp_Fw	GGGGCATATGGATACCGTATCGCTG
NfsB_Pp_Rv	GGGGCTCGAGTCAGAGGAAGGTGAACACTT
NfsB_St_Fw	GGGGCATATGGATATCGTTTCTGTC
NfsB_St_Rv	GGGGGTCGACTTAGACTTCCGTCAGTG
NfsB_Ck_Fw	CCCCCATATGGATATCGTTTCTGTCGC
NfsB_Ck_Rv	CCCGTCGACTCAGACTTCCGTCAGGGTG
FraseI_Vf Fw	GGGGCATATGACGCATCCAATTATTCA
FraseI_Vf Rv	GGGGGTCGACCTAAAGAATGGTAATTAC
NfsB_Vh_Fw	CCCCCATATGTCTCATCAAATCATTACAGAC
NfsB_Vh_Rv	CCCCTCGAGTTAAAGAGTAGTAATTACGTC
YfkO_Bs_Fw	GGGGCATATGGCAGATCTAAAGACACA
YfkO_Bs_Rv	CTAGGTCGACTTAAACCCACTTCACAACAT
YdgI_Bs_Fw	GGGGCATATGATCAAAACAAACGATTTTATGG
YdgI_Bs_Rv	GGGGGTCGACTTATTTCCATTCTGCAATTG
NfsB_Cs_Fw	CCCCCATATGAACCTTAATGAGATCATTCGC
NfsB_Cs_Rv	CCCGTCGACTCAGAGCTGCGTGATCAC

Primers for insertion of nitroreductase genes into vector F279-V5	
NfsB_Vv GW_Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATGG GCACTATTGTTCAAGCT
NfsB_Vv GW_Rv	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAGATTTCGGTAAA
NfsB_Ec GW_Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATGG GCGATATCATTTCTGTCGCCTT
NfsB_Ec GW_Rv	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTACACTTCGGTTAAGGTGAT GT
Primers for insertion of nitroreductase variants into vector pTOL2-5xUAS-GAP-tagYFP-P2A-nfsB_Ec	
NfsB_Vv pTOL2_Fw	CCCCCCGGGCCTATGACTATTGTTCAA
NfsB_Vv_Rv	GGGGTCGACTTAGATTTCGGTAAAAACAG
Primers for insertion of rhodopsin promoter into vector pTOL2-5xUAS-GAP-tagYFP-P2A-nfsB_Ec	
rho_promoter_F2	AATTTAGGGTAATGGCCGGCCCGACCTGCAGGTCAACGG
rho_promoter_R	AGTCTACCCTGCTACGTAGGCTGCGGTTGGATGTGG
Primers for swapping eGFP gene into 5xUAS:YFP-2A-NTR2.0 vector	
*Sbf*I-GFP_FOR	GCAGCCTGCAGGAAGCACAGCTACAATGATGGTGAGCAAGGGCGAG
GFP-oe_REV	CTCCGCTTCCCTTGTACAAGTACTTGTACAGCTCGTCCATGC
FW-P2A	TACTTGTACAAGGGAAGCGGAGCTA
*Kfl*I-Bglobin_REV	CAAGGGTCCCCAAACTCACC
Primers for insertion of YFP-2A-NTR 2.0 gene into 5xQUAS vector	
*Mlu*I-YFP_FOR	TTAACGCGTAGCAGGGTAGACTGTAGAGG
*Avr*II-He1Pro_REV	GGCCCTAGGGTTGCCTCA

Underlined sequences are restriction, recombination, or overlap extensions.

Transgenic Line Table | Transgenic lines used and established in this study

Individual Transgenic Lines			
Full Transgene Name	**NTR**	**Abbreviation** (here)	**Ref**.
Tg(5xUAS:GAP-tagYFP-P2A-nfsB_Vv F70A/F108Y)jh513	2.0	*UAS:YFP-NTR2.0*	here
Et(2xNRSE-fos:KALTA4)gmc617	na	*NRSE:KalTA4*	61
Tg(5xUAS:GAP-ECFP,he:GAP-ECFP)gmc1913	na	*UAS:CFP*	here
Tg(14xUAS:nfsB_Ec-mCherry)c264	1.0	*UAS:NTR1.0-Cherry*	62
Tg(mpeg1.1:EYFP-nfsB_Ec T41Q/N71S/F124T)w202	1.1	*mpeg:NTR1.1-YFP*	63
Tg(rho:EYFP-nfsB_Ec)gmc500	1.0	*rho:YFP-NTR1.0*	64
Tg(rho:GAP-tagYFP-P2A-nfsB_Vv F70A/F108Y)jh405	2.0	*rho:YFP-NTR2.0*	here
Tg(mpeg1.1:Gal4-VP16)gl24	na	*mpeg:Gal4*	65
Double Transgenic Lines			
Full Transgene Name	**NTR**	**Abbreviation** (here)	Ref.
Et(2xNRSE-fos:KALTA4)gmc617; *Tg(5xUAS:GAP-tagYFP-P2A-nfsB_Vv F70A/F108Y)jh513*	2.0	*NRSE:KalTA4;* *UAS:YFP-NTR2.0*	61 here
Et(2xNRSE-fos:KALTA4)gmc617; *Tg(5xUAS:GAP-ECFP,he:GAP-ECFP)gmc1913*	na	*NRSE:KalTA4;* *UAS:CFP*	61 here
Tg(mpeg1.1:Gal4-VP16)gl24; *Tg(5xUAS:GAP-tagYFP-P2A-nfsB_Vv F70A/F108Y)jh513*	2.0	*mpeg:Gal4;* *UAS:YFP-NTR2.0*	65 here
Triple Transgenic Lines			
Full Transgene Name	**NTR**	**Abbreviation** (here)	Ref.
Et(2xNRSE-fos:KALTA4)gmc617; *Tg(5xUAS:GAP-tagYFP-P2A-nfsB_Vv F70A/F108Y)jh513;* *Tg(5xUAS:GAP-ECFP,he:GAP-ECFP)gmc1913*	2.0	*NRSE:KalTA4;* *UAS:YFP-NTR2.0* *UAS:CFP*	61 here here
Et(2xNRSE-fos:KALTA4)gmc617; *Tg(5xUAS:GAP-tagYFP-P2A-nfsB_Vv F70A/F108Y)jh513;* *Tg(14xUAS:nfsB_Ec-mCherry)c264*	2.0 1.0	*NRSE:KalTA4;* *UAS:YFP-NTR2.0* *UAS:NTR1.0-Cherry*	61 here 62

New Reporter/Effector Transgenic Lines				
Full Transgene Name	**NTR**	**Expression system**	**Reporter**	**Ref.**
Tg(5xUAS:GAP-EGFP-P2A-NfsB_Vv F70A/F108Y,he:tagBFP2)jh551	2.0	Gal4/UAS	EGFP	here
Tg(5xUAS:GAP-tagYFP-P2A-NfsB_Vv F70A/F108Y,he:tagBFP2)jh552	2.0	Gal4/UAS	tagYFP	here
Tg(5xUAS:GAP-mCherry-P2A-NfsB_Vv F70A/F108Y,he:tagBFP2)jh553	2.0	Gal4/UAS	mCherry	here
Tg(5xQUAS:GAP-tagYFP-P2A-NfsB_Vv F70A/F108Y,he:ECFP)jh562	2.0	QF/QUAS	tagYFP	here

References

59. Prosser, G. A. *et al.* Creation and screening of a multi-family bacterial oxidoreductase library to discover novel nitroreductases that efficiently activate the bioreductive prodrugs CB1954 and PR-104A. *Biochem. Pharmacol.* **85**, 1091–103 (2013).
60. Provost, E., Rhee, J. & Leach, S. D. Viral 2A peptides allow expression of multiple proteins from a single ORF in transgenic zebrafish embryos. *Genesis* **45**, 625–629 (2007).
61. Xie, X., Mathias, J. R., Smith, M. A., Walker, S. L., Teng, Y., Distel, M., Köster, R. W., Sirotkin, H. I., Saxena, M. T. & Mumm, J. S. Silencer-delimited transgenesis: NRSE/RE1 sequences promote neural-specific transgene expression in a NRSF/REST-dependent manner. *BMC Biol.* **10,** 93 (2012).
62. Davison, J. M., Akitake, C. M., Goll, M. G., Rhee, J. M., Gosse, N., Baier, H., Halpern, M. E., Leach, S. D. & Parsons, M. J. Transactivation from Gal4-VP16 transgenic insertions for tissue-specific cell labeling and ablation in zebrafish. *Dev. Biol.* **304,** 811–24 (2007).
63. Petrie, T. A., Strand, N. S., Yang, C.-T., Tsung-Yang, C., Rabinowitz, J. S. & Moon, R. T. Macrophages modulate adult zebrafish tail fin regeneration. *Development* **141,** 2581–91 (2014).
64. Walker, S. L., Ariga, J., Mathias, J. R., Coothankandaswamy, V., Xie, X., Distel, M., Köster, R. W., Parsons, M. J., Bhalla, K. N., Saxena, M. T. & Mumm, J. S. Automated reporter quantification in vivo: High-throughput screening method for reporter-based assays in zebrafish. *PLoS One* **7,** e29916 (2012).
65. Ellett, F., Pase, L., Hayman, J. W., Andrianopoulos, A. & Lieschke, G. J. Mpeg1 Promoter Transgenes Direct Macrophage-Lineage Expression in Zebrafish. *Blood* **117,** e49-56 (2011).