

Supplemental Data

Supplemental Experimental Procedures

Construction of a mock vector. The pTL26 plasmid was constructed by replacing the murine, erythroid ALAS active site loop-encoding sequence with a non-ALAS-encoding sequence. pGF42, a ferrochelatase expression plasmid (1), was digested with *Xba* I and *Bam* HI and the generated fragment was ligated into the ALAS expression plasmid, pGF23 (2), previously digested with the same two enzymes.

Construction of the ALAS synthetic shuffled library. The construction of the synthetic shuffled library depended on the design of five overlapping partially overlapping oligonucleotides, which contain DNA degeneracies to allow multiple mutations to be introduced in 10 of the 18 positions of the ALAS active site loop (Table S1). The primers used in this study were the following (positions with base changes are indicated in *bold*):

Fwd Primer MALAS216: 5'-CTG CTC TCC AAG CAC AGC ATC TAT **VTK** CAG **VSS** ATC
AAC-3'

Rev Primer RALAS217: 5'-GGG GGC CAA GCG **MAR** **BHK** CWS **VBB** WYY **NHB** **VBB**
CAC AGT TGG **RHD** GTT GAT **SSB** CTG-3'

Fwd Primer MALAS218: 5'-CGC TTG GCC CCC TCC CCC CAC CAC AGC CCT CAG ATG
ATG GAA AAC TTT-3'

Fwd Primer MALAS219: 5'-ATC TGT GAT CTT CTG CTC TCC AAG CAC AGC AT-3' **Rev**

Primer RALAS220: 5'-CTT CTC CAC AAA GTT TTC CAT CAT CTG A-3'

where R = 50%A + 50%G; Y = 50%C + 50%T; M = 50%A + 50%C; K = 50%G + 50%T; S = 50%C + 50%G; W = 50%A + 50%T; H = A + 25%C,T; B = 33%C + 33%G + 33%T; V = 33%A + 33%C + 33%G; D = 33%A + 33%G + 33%T; N = 25%A + 25% C + 25% G + 25%T.

An annealing reaction (20 μ L) containing 100 pmol of each of the five oligonucleotides was incubated at 65 °C for 2 min and then slowly cooled to room temperature. The annealed oligonucleotides were extended with 2 units of thermostable DNA polymerase and dNTPs, at final concentration of 55 μ M each, in a 20min reaction at room temperature.

The generated double-stranded DNA was used as template in a PCR reaction using the outermost forward and reverse primers (MALAS219 and RALAS220) and following the cycling parameters: a total of 30 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute was followed by an extension of 5 minutes at 72°C. The shuffled DNA product was further amplified by PCR and using 2 primers (MALAS166 and RpBREvo2 – *sequence below*), which annealed to the 5' and 3' ends of the shuffled DNA product. The annealing sites for MALAS166 and RpBREvo2 were upstream and downstream of the sequences for the restriction endonucleases, *Xba* I and *Bam* HI, respectively. PCRs were performed using a MJ Research Minicycler and following the cycling parameters: a total of 30 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute was followed by an extension of 5 minutes at 72°C. The amplified, shuffled DNA product was digested with *Xba* I and *Bam* HI, purified and ligated into pTL26, the mock ALAS expression vector, previously digested with the same two enzymes, as described in (3).

Fwd Primer MALAS166: 5'-TGC AGG CCA TAG AGG AGA CC-3'

Rev Primer RpBREvo2: 5'-TAC GAG TTG CAT GAT AA-3'

Table S1. Designed mutations for incorporation at indicated positions within the ALAS active site loop¹

| <i>Position</i> | <i>WT</i> | <i>Mut1</i> | <i>Mut2</i> | <i>Mut3</i> | <i>Mut4</i> | <i>Codon</i> |
|-----------------|-----------|-------------|-------------|-------------|-------------|--------------|
| 423 | V | I | L | | | VTK |
| 425 | A | S | P | | | VSS |
| 428 | Y | F | H | S | | HDY |
| 432 | P | A | S | D | R | VVB |
| 433 | R | I | V | K | S | VDN |
| 434 | G | K | | | | RRW |
| 435 | E | Q | T | S | D | VVB |
| 436 | E | L | | | | SWG |
| 437 | L | R | K | M | | MDV |
| 438 | L | | F | | | YTK |

¹WT denotes amino acid found in mALAS-2 active site loop. Amino acids indicated in the columns labeled *Mut1-Mut4* reflect all *naturally* occurring amino acids found in the ALAS active site loop. Codon indicates the nucleotide codon used to obtain the indicated mixture of amino acid residues. DNA degeneracies are represented in the IUB code: Y, C/T; M, A/C; K, G/T; R, G/A; S, C/G; W, A/T; V, A/C/G; H, A/C/T; D, A/G/T; B, C/G/T; N, A/C/G/T.

Table S2. Thermodynamic activation parameters of wild-type ALAS and the SS2 variant

| Enzyme | Slope/(K) | E_a (kcal/mol) | ΔH^\ddagger (30°C) (kcal/mol) | ΔG^\ddagger (30°C) (kcal/mol) | ΔS^\ddagger (30°C) (cal/mol·K) |
|--------|-----------|---------------------|---|---|--|
| SS2 | -9200 | 18 | 18 | 19 | -4.4 |
| WTALAS | -24000 | 48 | 48 | 19 | -95 |

Figure S1

Active Site Loop Y422.....R439

| | | | | | | | | | |
|----|-------------|-----|------------------|-----------------|-----------------|------------------|-------------|-----------------|-----|
| 1 | ALAS_DELLEU | 486 | LLLSKHGIYVQAINY | PTVPRG-EELLRLAP | SPHH-SPQMMEFVE | KLLAAWTEVGLPLQD | -VSIAACNF | FELMSEWERSYFGNM | 573 |
| 2 | ALAS_DLLLEU | 486 | LLLSKHGIYVQAINY | PTVPRG-EELLRLAP | SPHH-SPQMMEFVE | KLLAAWTEVGLPLQD | -VSIAACNF | FELMSEWERSYFGNM | 573 |
| 3 | ALAS_HOMSAP | 491 | LLLSKHGIYVQAINY | PTVPRG-EELLRLAP | SPHH-SPQMMEFVE | KLLLAWTAVGLPLQD | -VSVAACNF | FELMSEWERSYFGNM | 578 |
| 4 | ALAS_RATNOR | 491 | LLLAKHSIYVQAINY | PTVPRG-EELLRLAP | SPHH-SPQMMEFVE | KLLLAWTEVGLPLQD | -VSVAACNF | FELMSEWERSYFGNM | 578 |
| 5 | ALAS_RATRAT | 491 | LLLAKHSIYVQAINY | PTVPRG-EELLRLAP | SPHH-SPQMMEFVE | KLLLAWTEVGLPLQD | -VSVAACNF | FELMSEWERSYFGNM | 578 |
| 6 | ALAS_MUSMUS | 491 | LLLSKHSIYVQAINY | PTVPRG-EELLRLAP | SPHH-SPQMMEFVE | KLLLAWTEVGLPLQD | -VSVAACNF | FELMSEWERSYFGNM | 578 |
| 7 | ALAS_DANRER | 486 | ILLEKHNIYVQAINY | PTVPRG-EELLRLAP | SPFH-NPIMMNYFAE | KLLDVWQEVGLPLNG | -PAQASCTF | FDLMSEWEKSYFGNM | 573 |
| 8 | ALAS_DANROS | 486 | ILLEKHNIYVQAINY | PTVPRG-EELLRLAP | SPFH-NPIMMNYFAE | KLLDVWQEVGLPLNG | -PAQASCTF | FDLMSEWEKSYFGNM | 573 |
| 9 | ALAS_OPSTAU | 486 | SLLEKHNIYVQAINY | PTVPRG-QELLRLAP | SPHH-HPAMMEYFVD | KLVEVWQEVGLPLNG | -PATVSCCTF | FDLMSEWEKSYFGNM | 573 |
| 10 | ALAS_HOMSPN | 545 | ELMSRHNIYVQAINY | PTVPRG-EELLRIAP | TPHH-TPQMNNYFLE | NLLVTWKQVGLLEKLP | -HSSAECNF | FEVMSEREKSYFSGM | 632 |
| 11 | ALAS_DELDEL | 545 | ELMSRHNIYVQAINY | PTVPRG-EELLRIAP | TPHH-TPQMNNYFVE | NLLATWKRVGLELKP | -HSSAECNF | FEVMSEREKSYFSGM | 632 |
| 12 | ALAS_MOUDEM | 546 | ELMTRHNIYVQAINY | PTVPRG-EELLRIAP | TPHH-TPQMNNYFVE | KLLVTWKRVGLELKP | -HSSAECNF | FEVMSEREKSYFSGM | 633 |
| 13 | ALAS_GALVAR | 540 | KLMSQHSIYVQAINY | PTVPRG-EELLRIAP | TPHH-TPQMMSYFLE | KLLATWKDVGLELKP | -HSSAECNF | FEVMSERERSYFSGM | 627 |
| 14 | ALAS_XENLAE | 308 | KLMDYSIYVQAINY | PTVPRG-EELLRIAP | TPHH-NPQ----- | ----- | ----- | ----- | 344 |
| 15 | ALAS_OPSBET | 532 | LMMSHHNIYVQAINY | PTVARG-DELLRIAP | TPHH-TPEMMKYFVD | RLVQTWKEVGLLEKLP | -HSSAECTFC | FEVMNEREKSYFSGM | 619 |
| 16 | ALAS_OPSPAR | 532 | LMMSHHNIYVQAINY | PTVARG-DELLRIAP | TPHH-TPEMMKYFVD | RLVQTWKEVGLLEKLP | -HSSAECTFC | FEVMNEREKSYFSGM | 619 |
| 17 | ALAS_DANDAN | 518 | IMMSRYNIYVQAINY | PTVARG-EELLRIAP | TPHH-TPQMNNYFVE | KLTQTWTEVGLPLKP | -HSSAECNF | FEIMSEREKSYFSGM | 605 |
| 18 | ALAS_MYXGLU | 565 | ELMSRHNIYVQAINY | PTVPRG-EEMLRVVV | TPHH-TPQMNNYFVE | HLTNSWKDIGLNLRP | -HASAECNY | FELMSEHDQVYFDGM | 652 |
| 19 | ALAS_STRDRO | 512 | SLLEEHNIVQAINY | PTVPSG-EEKLRVAA | SPXH-TPDMMDFRVA | SLSEVWAKSGLRFNT | PICPRECEFC | FEELSSRERSFAEES | 578 |
| 20 | ALAS_DROMEL | 463 | VLIEQFGHVLQSIY | PTVARG-QEKLRLAP | TPFH-TFEMMNALVT | DLKKVWEMVDLSTNV | PISPNACMFC | WHQDTSPLD | 529 |
| 21 | ALAS_LIMPOL | 494 | ELISMHGHIYVQAINY | PTVPRG-EEKLRVAA | TPFH-TRPMMEAFVR | DLVSVWRGLKPLLRD | GICEKKECF | FEHLESRVLP | 560 |
| 22 | ALAS_SEPOFF | 540 | DLLIKHNIYVQAINY | PTVARG-EEKLRVAA | TPHH-TKEMMDHFVD | CVVVKWLEHGLTLNP | -DSTRPAEF | FSI----- | 603 |
| 23 | ALAS_GLYDIB | 516 | ELMEEHGIYVQAINY | PTVPRG-QELLRVAP | TPHH-TKEMMDSFVN | ATLSVFLNNNIELKS | -TCGINCLY | CEAFTNRER | 586 |
| 24 | ALAS_GALGAL | 420 | ALLEEHGLYVQAINH | PTVPRG-QELLRIAP | TPHH-SPMLENLAD | KLSECWGAUGLPLRD | -PPGPS | LSLLSPLER | 508 |
| 25 | ALAS_SINMEL | 346 | LLLDNFGIYVQAINY | PTVPRG-TERLRITP | TPMH-SDADIDLVS | ALHSLWSRCALARAV | A----- | ----- | 405 |
| 26 | ALAS_SMRMEL | 275 | LLLDNFGIYVQAINY | PTVPRG-TERLRITP | TPMH-SDADIDLVS | ALHSLWSRCALARAV | A----- | ----- | 334 |
| 27 | ALAS_AGRTUM | 364 | ILLDNHGIYVQAINY | PTVPRG-TERLRITP | TPMH-SDADIDLVS | ALHSLWSRCALARAV | A----- | ----- | 423 |
| 28 | ALAS_AGRRAD | 346 | ILLDSHGVIYVQAINY | PTVPRG-TERLRITP | TPMH-SDADIEHLVQ | ALHSLWSRCALARAV | A----- | ----- | 405 |
| 29 | ALAS_RHIRAD | 276 | ILLDSHGVIYVQAINY | PTVPRG-TERLRITP | TPMH-SDADIEHLVQ | ALHSLWSRCALARAV | A----- | ----- | 335 |
| 30 | ALAS_RHOPSE | 344 | ELINRYGIYVQAINY | PTVPRG-TERLRITP | SPQH-TDADIEHLVQ | ALSEIWARVGLAKAA | ----- | ----- | 403 |
| 31 | ALAS_RHOPSE | 346 | ALLARHAIYVQAINY | PTVPRG-QERFRITP | TPFH-TTSHMEALVE | ALLAVGRDLGWAMSR | RAA----- | ----- | 407 |
| 32 | ALAS_EUGGRA | 343 | LLLKRFGIYVQAINY | PTVDVG-TERLRITP | SPVH-TNEHMATLIT | ALLQVWEEELGLPLRP | PVFDTEGY | WLPNSAMWR | 431 |
| 33 | ALAS_BRAJAP | 348 | LLLEEHLGIYVQAINY | PTVAKG-SERLRITP | SPYH-DDGLIDQLAE | ALLQVWDRGLGLPLKQ | KSLAAE----- | ----- | 409 |
| 34 | ALAS_BRAJAP | 275 | LLLEEHLGIYVQAINY | PTVAKG-SERLRITP | SPYH-DDGLIDQLAE | ALLQVWDRGLGLPLKQ | KSLAAE----- | ----- | 339 |
| 35 | ALAS_BRUMEL | 345 | RLLEEVHGIYVQAINY | PTVPRG-TERLRITP | SPLH-DPKLIDGLKD | ALLEVWNEELGIPFAE | PSAPQAANS | MVSKAGG----- | 425 |
| 36 | ALAS_ZYMMOB | 287 | ILLNEYGAIYVQAINF | PTVPRG-TERLRITP | GPTH-NEAMLRELTD | SLVAIWHRLDMRFAA | ----- | ----- | 345 |
| 37 | ALAS_RHOCAP | 348 | MLLSDYGVYVQAINF | PTVPRG-TERLRITP | SPVH-DLKQIDGLVH | AMDLLWARCA----- | ----- | ----- | 401 |
| 38 | ALAS_RHOCAP | 278 | MLLSDYGVYVQAINF | PTVPRG-TERLRITP | SPVH-DLKQIDGLVH | AMDLLWARCA----- | ----- | ----- | 331 |
| 39 | ALAS_PARDEN | 292 | MLLADFSIYVQAINF | PTVPRG-TERLRITP | SPVH-DPKQIDHLVK | AMDLSWSQCKLNRST | SAA----- | ----- | 339 |
| 40 | ALAS_PARZEA | 360 | MLLADFSIYVQAINF | PTVPRG-TERLRITP | SPVH-DPKQIDHLVK | AMDLSWSQCKLNRST | SAA----- | ----- | 409 |
| 41 | ALAS_RHOPSE | 364 | MLLIHFGIYVQAINF | PTVPRG-TERLRITP | SPVH-DSGMIDHLVK | AMDVLWQHCHALNRAE | VVA----- | ----- | 407 |
| 42 | ALAS_EMENID | 510 | KLLEEHLGIYVQAINY | PTVPRG-EERLRITP | TPGH-TQELRDHLVE | AVNTVWVDLGIKRAS | DWKAMGGF | AELENQPI | 598 |
| 43 | ALAS_EMENID | 440 | KLLEEHLGIYVQAINY | PTVPRG-EERLRITP | TPGH-TQELRDHLVE | AVNTVWVDLGIKRAS | DWKAMGGF | AELENQPI | 528 |
| 44 | ALAS_ASPNID | 440 | KLLEEHLGIYVQAINY | PTVPRG-EERLRITP | TPGH-TQELRDHLVE | AVNTVWVDLGIKRAS | DWKAMGGF | AELENQPI | 528 |
| 45 | ALAS_ASPORI | 508 | KLLEEHLGIYVQAINY | PTVPRG-EERLRITP | TPGH-TQELRDHLVQ | AVQTVWNEELGIKRST | DWEAQGGF | AAEENQPI | 596 |
| 46 | ALAS_NEUCRA | 334 | KLLNDHQIYVQAINY | PTVPRG-QERLRITP | TPGH-TKQFRDHLVA | ALDSIWTELGIKRST | DWAAE | EPVAPLW | 421 |

| | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|-----|-------------|-----|-------|-------|--------|--------|-----|-----|-------|-------|-------|-------|---------|-------|-------|--------|-------|-------|------|------|------|--------|-------|-------|---------|--------|-----|
| 47 | ALAS_GIBFUJ | 393 | MLLN | YGI | YVQAIN | Y | PTV | FVG | QERLR | VTP | TPGH | IKEYR | DQLVE | AIDEI | WTRLD | DIKRTS | DWAAE | GGF | IGV | GEQD | --NV | QEPL | WTDK | QLN | 479 | | |
| 48 | ALAS_YARLIP | 404 | LLLTK | HQI | YVQAIN | F | PTV | FIG | QERLR | VTP | TPGH | HEGLC | DDELVA | ALEDV | VQEL | DLKRV | DWTAE | GGC | CGV | GEV | --E | VEPL | WSEE | QLS | 489 | | |
| 49 | ALAS_CANALB | 451 | LLL | NKHD | YVQAIN | F | PTV | FIG | EERLR | VTP | TPGH | GP | ELSKQ | LVE | AVDSV | FTEL | NLNR | DWKKL | GGLV | GVG | VEG | -AAK | VEHI | WTEE | QLA | 538 | |
| 50 | ALAS_CANALB | 381 | LLL | NKHD | YVQAIN | F | PTV | FIG | EERLR | VTP | TPGH | GP | ELSKQ | LVE | AVDSV | FTEL | NLNR | DWKKL | GGLV | GVG | VEG | -AAK | VEHI | WTEE | QLA | 468 | |
| 51 | ALAS_DEBHAN | 392 | LLLD | KYNI | YVQAIN | F | PTV | FIG | QERLR | VTP | TPGH | GP | ELSNQ | LIG | ALDSV | FNEL | SLSR | DWEGK | GGLC | GVG | EPD | -IEPI | EHI | WTEE | QLA | 479 | |
| 52 | ALAS_SACCR | 435 | ILINK | HQI | YVQAIN | F | PTV | ARG | TERLR | VTP | TPGH | TNDL | SDILIN | AVDDV | FNEL | QLPR | VR | DWESQ | GGLL | GVG | ESG | -FVE | ESNL | WTSS | QLS | 522 | |
| 53 | ALAS_SACCAS | 365 | ILINK | HQI | YVQAIN | F | PTV | ARG | TERLR | VTP | TPGH | TNDL | SDILIN | AVDDV | FNEL | QLPR | VR | DWESQ | GGLL | GVG | ESG | -FVE | ESNL | WTSS | QLS | 452 | |
| 54 | ALAS_CANGLA | 347 | ILMEK | HRI | YVQAIN | F | PTV | SRG | TERLR | VTP | TPGH | TNDL | SDILIA | AVDDV | FNEL | QLPR | IR | DWEMQ | GGLL | GVG | DKN | -FVPE | PNL | WTEE | QLS | 434 | |
| 55 | ALAS_KLULAC | 387 | ILMDK | HRI | YVQAIN | F | PTV | ARG | TERLR | VTP | TPGH | TNDL | SDILMD | ALEDV | VSTL | QLPR | VR | DWEAQ | GGLL | GVG | DPN | -HVP | QPNL | WTKD | QLT | 474 | |
| 56 | ALAS_EREGOS | 373 | ILMEK | HRI | YVQAIN | F | PTV | PRG | TERLR | VTP | TPGH | TNDL | SDVLLD | AMDDV | VKTL | QLPR | V | DWAAH | GGLL | GVG | EPD | -YVPE | ANL | WTEE | EQMS | 460 | |
| 57 | ALAS_AGABIS | 460 | KLLSE | HDI | YVQAIN | Y | PTV | ARG | EERLR | ITV | TPRH | TMEQ | MEGLIR | SLNQV | F | EELN | NRLS | DWKL | AGGR | AGV | GIPG | AADDV | QPI | WTDE | QIG | 548 | |
| 58 | ALAS_AGADIV | 390 | KLLSE | HDI | YVQAIN | Y | PTV | ARG | EERLR | ITV | TPRH | TMEQ | MEGLIR | SLNQV | F | EELN | NRLS | DWKL | AGGR | AGV | GIPG | AADDV | QPI | WTDE | QIG | 478 | |
| 59 | ALAS_SCHPOM | 475 | SLLHD | HNI | YVQAIN | F | PTV | SVG | TERLR | VTP | TPAHN | TEHY | VQSLTN | AMNDV | VSKF | NINR | ID | GWEK | R | IDV | GRL | CKF | PVLP | PFTT | TH----- | 558 | |
| 60 | ALAS_SCHMIK | 405 | SLLHD | HNI | YVQAIN | F | PTV | SVG | TERLR | VTP | TPAHN | TEHY | VQSLTN | AMNDV | VSKF | NINR | ID | GWEK | R | IDV | GRL | CKF | PVLP | PFTT | TH----- | 488 | |
| 61 | ALAS_RICPRO | 342 | MLLNE | YGI | YVQHIN | F | PTV | PRG | TERLR | VTP | TPAH | TDK | MINDLST | ALVHI | F | DEL | DI | ELSS | AKEL | N | KEV | RL | H | L | I | A----- | 414 |
| 62 | ALAS_RICTYP | 342 | MLLNE | YGI | YVQHIN | F | PTV | PRG | TERLR | VTP | TPAH | TDK | MINDLST | ALVHI | F | DEL | DI | ELSS | TKEL | N | KEV | RL | H | L | I | A----- | 414 |
| 63 | ALAS_RICPRO | 272 | MLLNE | YGI | YVQHIN | F | PTV | PRG | TERLR | VTP | TPAH | TDK | MINDLST | ALVHI | F | DEL | DI | ELSS | AKEL | N | KEV | RL | H | L | I | A----- | 344 |
| 64 | ALAS_RICCON | 354 | MLLNE | YGI | YVQHIN | F | PTV | PRG | TERLR | VTP | TPAH | TDK | MINDLSV | ALVQI | F | DEL | DI | ELSS | AKEL | N | EEV | RL | N | L | I | A----- | 426 |
| 65 | ALAS_CHRVIO | 354 | RLLEE | F | DI | YVQPIN | Y | PSV | PRG | GERFR | LT | V | GPRR | SHEE | I | QRF | V | AL | KH | C | L | A----- | ----- | ----- | 403 | | |
| A8 | | 491 | LLL | SKHSI | YVQAIN | Y | PTV | PRG | EELL | R | L | L | L | L | L | L | L | L | L | L | L | L | L | L | L | L | 578 |
| D8 | | 491 | LLL | SKHSI | YVQAIN | Y | PTV | PRG | EE | Q | L | R | L | L | L | L | L | L | L | L | L | L | L | L | L | L | 578 |
| G7 | | 491 | LLL | SKHSI | YVQAIN | Y | PTV | PRG | EELL | R | L | L | L | L | L | L | L | L | L | L | L | L | L | L | L | L | 578 |
| F1 | | 491 | LLL | SKHSI | YVQAIN | Y | PTV | P | PK | Q | E | Q | L | R | L | L | L | L | L | L | L | L | L | L | L | L | 578 |
| F10 | | 491 | LLL | SKHSI | YVQAIN | Y | PTV | P | Q | N | T | E | N | L | R | L | L | L | L | L | L | L | L | L | L | L | 578 |
| A4 | | 491 | LLL | SKHSI | YVQAIN | Y | PTV | R | K | G | N | E | L | L | R | L | L | L | L | L | L | L | L | L | L | L | 578 |
| F3 | | 491 | LLL | SKHSI | YVQAIN | Y | PTV | P | H | N | K | E | K | L | R | L | L | L | L | L | L | L | L | L | L | L | 578 |
| H1 | | 491 | LLL | SKHSI | YVQAIN | Y | PTV | N | I | E | K | E | K | L | R | L | L | L | L | L | L | L | L | L | L | L | 578 |
| SS2 | | 491 | LLL | SKHSI | YVQAIN | Y | PTV | E | I | N | Q | E | K | L | R | L | L | L | L | L | L | L | L | L | L | L | 578 |

Fig S1. Alignment of sixty-five different species specific ALAS active site loop sequences with the hyperactive variants isolated in this study. The naturally occurring amino acid sequences were obtained from public databases (NCBI) using a BLAST search and aligned using CLUSTAL W (4). The 10 positions within the 18-amino acid sequence targeted for mutagenesis are high-lighted in cyan, and changes observed in hyperactive variants are high-lighted in magenta. The amino acid numbering in red refers to that of murine erythroid ALAS (mALAS-2). Represented proteins are: ALAS_DELLEU: *Delphinapterus leucas* ALAS (20138447); ALAS_DLLLEU: *Delphinapterus leucas* cook inlet subspecies 2 (5281116); ALAS_HOMSAP, *Homo sapiens* ALAS erythroid (4557299); ALAS_RATNOR, *Rattus norvegicus* erythroid ALAS (51980582); ALAS_RATRAT, *Rattus rattus* erythroid ALAS (6978485); ALAS2_MUSMUS, *Mus musculus* erythroid ALAS (33859502); ALAS_DANRER, *Danio rerio* ALAS (18858263); ALAS_DANROS, *Danio roseus* ALAS (20138448); ALAS_OPSTAU, *Opsanus tau* ALAS (1170202); ALAS_ORYLAT, *Oryzias latipes* ALAS (49022596); ALAS_HOMSPN, *Homo sapiens* erythroid ALAS (4502025); ALAS_DELDEL, *Delphinus delphis* erythroid ALAS (20138445); ALAS_MOUDOM, *Mus musculus domesticus* erythroid ALAS (23956102); ALAS_GALVAR, *Gallus varius* erythroid ALAS (122821); ALAS_XENLAE, *Xenopus laevis* erythroid ALAS (44968228); ALAS_OPSEBET, *Opsanus beta* ALAS (1170206); ALAS_OPSPAR, *Opsanus pardus* ALAS (532630); ALAS_DANDAN, *Danio danglia* ALAS (32451642); ALAS2_MYXGLU, *Myxine glutinosa* erythroid ALAS (4433550); ALAS1_BRALAN, *Branchiostoma lanceolatum* ALAS 1 (28630217); ALAS1_STRDRO, *Strongylocentrotus droebachiensis* ALAS 1 (4433548); ALAS1_DROMEL, *Drosophila melanogaster* ALAS 1 (2330591); LAS1_LIMPOL, *Limulus polyphemus* ALAS 1 (4433540); ALAS1_SEPOFF, *Sepia officinalis* ALAS 1 (4433546); ALAS2_GLYDIB, *Glycera dibranchiate* ALAS 2 (4433544); ALAS2_GALGAL, *Gallus gallus gallus* ALAS 2 (1170201); ALAS_SINMEL, *Sinorhizobium meliloti* 1021 ALAS (15966742); ALAS_SMRMEL, *Sinorhizobium meliloti* ALAS (18266808); ALAS_AGRUM, *Agrobacterium tumefaciens* ALAS (889869); ALAS_AGRRAD, *Agrobacterium radiobacter* ALAS (95069); ALAS_AGRUM, *Agrobacterium tumefaciens* ALAS (122818); ALAS_RHOPAL,

Rhodopseudomonas palustris ALAS (4001678); ALAS_RHOSPO, *Rhodobacter sporagenes* ALAS (541302); ALAS_EUGGRA, *Euglena gracilis* ALAS (12620813); ALAS_BRAELK, *Bradyrhizobium elkanii* ALAS (66534); ALAS_BRAJAP, *Bradyrhizobium japonicum* ALAS (30179569); ALAS_BRUMEL, *Brucella melitensis* ALAS (25286547); ALAS_ZYMMOB, *Zymomonas mobilis* ALAS (4511998); ALAS_RHOGLU, *Rhodobacter gluconicum* ALAS (97435); ALAS_RHOCAP, *Rhodobacter capsulatus* ALAS (122828); ALAS_PARDEN, *Paracoccus denitrificans* ALAS (1170207); ALAS_PARZEA, *Paracoccus zeaxanthinifaciens* ALAS (537435); ALAS_RHOSPH, *Rhodobacter sphaeroides* ALAS (541301); ALAS_EMENID, *Emericella nidulans* ALAS (418756); ALAS_EMCNID, *Emericella nidulans* ALAS (585244); ALAS_ASPNID, *Aspergillus nidulans* ALAS (40745239); ALAS_ASPORY, *Aspergillus oryzae* ALAS (5051989); ALAS_NEUCRA, *Neurospora crassa* ALAS (52782908); ALAS_GIBFUJ, *Gibberella fujikuroi* ALAS (15721883); ALAS_YARLIP, *Yarrowia lipolytica* ALAS (52782857); ALAS_CANBER, *Candida berate* ALAS (7493758); ALAS_CANALB, *Candida albicans* ALAS (10720014); ALAS_DEBHAN, *Debaryomyces hansenii* ALAS (52782855); ALAS_SACCER, *Saccharomyces cerevisiae* ALAS (6320438); ALAS_SACCAS, *Saccharomyces castellii* ALAS (122831); ALAS_CANGLA, *Candida glabrata* ALAS (52782865); ALAS_KLULAC, *Kluyveromyces lactis* ALAS (52788271); ALAS_EREGOS, *Eremothecium gossypii* ALAS (52782894); ALAS_ASPBIS, *Agaricus bisporus* ALAS (1679599); ALAS_AGADIV, *Agaricus divoniensus* ALAS (2492846); ALAS_SCHPOM, *Schizosaccharomyces pombe* ALAS (7492336); ALAS_SCHMIK, *Schizosaccharomyces mikatae* ALAS (52782853); ALAS_RICCON, *Rickettsia conorii* ALAS (7433712); ALAS_RICTYP, *Rickettsia typhi* ALAS (51474008); ALAS_RICPRO, *Rickettsia prowazekii* ALAS (6225494); ALAS_RICRIC, *Rickettsia rickettsia* ALAS (2528635); ALAS_CHRVIO, *Chromobacterium violaceum* ALAS (34102112).

Figure S2

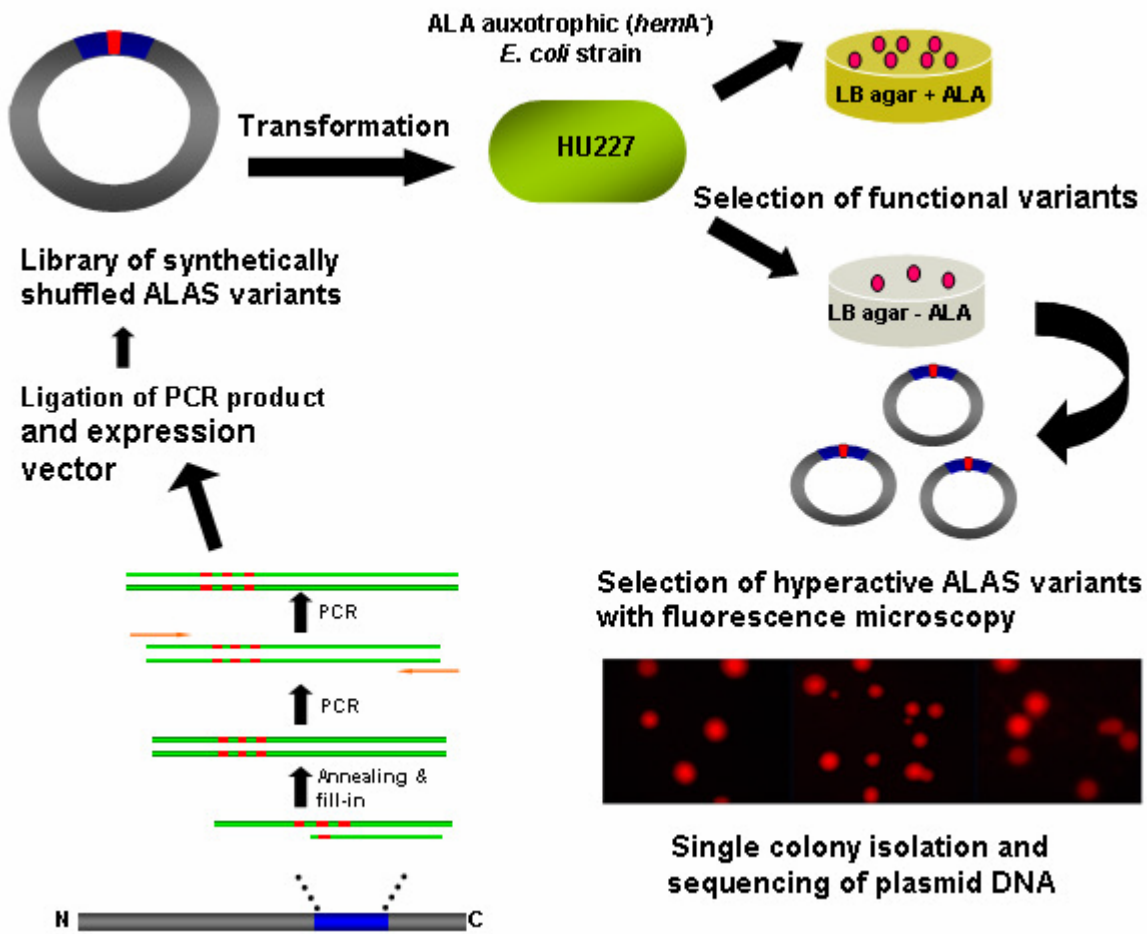
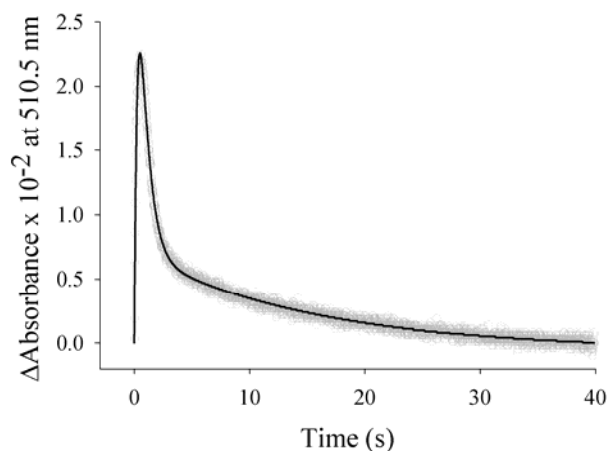


FIG S2. The generation and screening of the library.

A library of over 100,000,000 possible ALAS variants was constructed with PCR using a series of degenerate mixed base oligonucleotides. The PCR product was ligated into an expression vector. The resulting plasmids were transformed into *Escherichia coli* strain HU227 and plated on LB + ampicillin agar with and without ALA. The colonies that grew in the absence of ALA were identified as functional variants. Functional variants were screened for porphyrin overproduction by fluorescence microscopy.

Figure S3



Time course for the reaction of wild-type ALAS-glycine complex with succinyl-CoA under single turnover conditions. The reaction was monitored by following the absorbance changes at 510 nm, which was previously assigned to the quinonoid intermediate.

REFERENCES

1. Ferreira, G. C. (1994) *J. Biol. Chem.* **269**, 4396-4400
2. Ferreira, G. C., and Dailey, H. A. (1993) *J. Biol. Chem.* **268**(1), 584-590
3. Gong, J., and Ferreira, G. C. (1995) *Biochemistry* **34**(5), 1678-1685
4. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res.* **22**, 4673-4680