Supporting Information

Cammett et al. 10.1073/pnas.0915057107

SI Text

Supplemental Methods ***Vector and mutant construction.*** All construction details are available from the authors upon request. To facilitate cloning of retroviral libraries expressing E5-like proteins, we first constructed a modified full-length BPV E5 gene from synthetic oligonucleotides and inserted it into the hygromycin-resistant retroviral vector, pRV-Hyg^R (Riese and DiMaio, *Oncogene* 10:1431–1439 [1995]), to generate pRVY-E5P2A. This modified gene contained a canonical Kozak sequence for optimal translation, resulting in a proline to alanine substitution at the second position of the E5 protein, as well as silent mutations to introduce or remove restriction endonuclease cleavage sites for cloning. Codons 14–44 of E5P2A were then replaced with an in-frame 1.3 kb IRES-GFP "stuffer" fragment from pCMMP-IRES-GFP (a gift from Bill Sugden, University of Wisconsin). This new retroviral vector was named pT2H-F13 and expresses a bicistronic transcript encoding the first 13 residues of the E5P2A protein as well as GFP.

Retroviral vectors pBABE-puro containing the wild-type hEPOR gene and pMX containing the mEPOR gene were gifts from Bernard Forget (Yale University). The mEPOR was subcloned into pMSCVneo (Clontech) by using unique EcoRI and HpaI sites. The HA-tagged HA-hEPOR gene was excised from pMX-HA-hEPOR (obtained from S. Constantinescu) and cloned into pBABE-puro by using unique EcoRI and SalI sites. A mutant segment of the naturally occurring New Britain (NB) hEPOR variant that confers familial polycythemia and is 59 amino acids shorter than the full-length protein (obtained from B. Forget) (Arcasoy et al., *Blood* 89: 4628–35 [1997]) was inserted into HA-hEPOR gene. The double cysteine-to-serine mutation was constructed in TC2-3 cloned in pT2H-F13 by using double-stranded oligonucleotides, to generate TC2-3CCSS. Oligonucleotides used for mutagenesis and cloning shown in Table S1. To construct HA-hEPOR (5A) and HA-hEPOR(mPR), oligonucleotide pairs E and F in Table S1, respectively, were annealed, extended, digested with AgeI and BglII, and ligated into HA-hEPOR engineered with silent mutations to contain AgeI and BglII sites flanking the transmembrane domain.

Cells and viruses. Human 293T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, 20 mM HEPES (pH 7.3), and 1X penicillin/streptomycin (DMEM-10). Recombinant Vesicular stomatitis virus (VSV)-G pseudotyped retroviruses were prepared by co-transfecting 293T cells with a retroviral plasmid (or the TJC-5 library) and pantropic pVSVg (Clontech) and pCL-Eco (Imgenex) packaging plasmids (Naviaux et al., *J. Virol* 70:5701–5705 [1996]). After culture in OptiMEM (Gibco) for 48 h at 37 °C, the viral supernatant was recovered, filtered through a 0.45 μm filter (Millipore), concentrated approximately 20X, and used immediately or stored at −80 °C. Murine BaF3 cells were maintained in RPMI-1640 supplemented with 10% heat inactivated FBS, 6% WEHI-3B cell conditioned medium, 2 mM L-glutamine, 0.06 mM β-mercaptoethanol, and 1X penicillin/streptomycin (RPMI-IL-3). In some experiments, IL-3 was reduced or removed or EPO was added.

Selection of growth factor-independent cells. Freshly thawed BaF3/hEPOR cells were maintained in RPMI-EPO for one week and then washed and transferred to RPMI-IL-3 medium. Twenty-four hours later, 500,000 cells in 700 μL of medium were added to each well of a 12-well tissue culture plate. Cells were infected with 300 μL of freshly prepared concentrated stocks of the TJC-5 library at a multiplicity of infection of 0.2 (estimated by counting GFP$^+$ cells generated by infection in parallel with a control vector). Polybrene was added to a final concentration of 4 μg/mL. Cells were incubated at 37 °C for 4 h and then transferred to individual 25 cm^2 flasks (Falcon) containing 9 ml of RPMI-IL-3 with polybrene. Two days later, 2 million cells of each sample were harvested, washed once in PBS, and resuspended in 10 mL selection medium [RPMI with reduced IL-3 (0.15% WEHI conditioned medium) and 1 mg/mL hygromycin B]. On days six and eight post-infection, cells were passaged 1:3 into fresh selection medium. On day 14, when mock-infected cultures were dead, 2.5 mL of cells were harvested from the three TJC-5-infected pools that contained the highest number of viable cells and cultured for 3 d in full-strength RPMI-IL-3 to rescue surviving cells. Genomic DNA was isolated (Qiagen, DNEasy) from these pools, and integrated TJC-5 insert sequences were recovered from genomic DNA by PCR (Roche, Expand Long Template PCR kit) using primers specific for the fixed portions of the E5 gene and the vector (primers listed in Table S1). The PCR products were purified and cloned into pT2H-F13 between XhoI and BamHI sites, and packaged to generate secondary libraries.

Each concentrated secondary library was used to infect four wells of BaF3/hEPOR cells as described above, with 1:2 cell splits in selection medium on days two and seven after infection. On day 12, the cells from all four infections for each library were combined and viable cells were isolated by preparative sorting on a BD FACSAria. Genomic DNA was purified from these cells, and inserts were recovered as above, cloned into pT2H-F13, and packaged into retrovirus to generate tertiary libraries.

Two hundred and fifty thousand BaF3/hEPOR cells were infected separately with each concentrated tertiary library. Infected cells were incubated in selection medium for 21 d with passaging every 3 to 4 d. Two hundred thousand cells were then washed and transferred to 10 mL RPMI medium lacking IL-3 and EPO and cultured for 8 d. Wells infected with libraries from two pools grew in the absence of IL-3, and viable cells were isolated by sorting as above. Inserts were recovered from genomic DNA purified from the viable cells, cloned into pT2H-F13, and sequenced.

Testing recovered clones for growth-factor independence. Recovered clones in pT2H-F13 were individually packaged into retrovirus and used to infect BaF3/hEPOR cells. For each infection, approximately 250,000 cells/well in RPMI-IL-3 medium were infected with concentrated retrovirus as described above. After selection in hygromycin, 200,000 viable cells of each infection were washed twice and resuspended in 10 ml of RPMI lacking IL-3 and EPO and cultured. Viable cells were counted daily by using a Beckman Coulter Counter. As shown in supplemental Fig S1, cells infected with T2H-F13 died after growth factor removal, whereas TC2-3 and related proteins conferred varying degrees of growth-factor independence. To test the requirement of the two cysteines in TC2-3, T2H-F13 expressing TC2-3 or TC2-3CCSS were individually packaged into retrovirus and tested for their ability to confer growth-factor independence, as described above.

To test individual recovered clones for activity when expressed from a high-expression vector, 500 μL RPMI-IL-3 containing 500,000 cells of various cell lines were plated in 12-well plates. 500 μL of concentrated CMMP-based retrovirus or RPMI-IL-3 (mock-infected) were added to each well containing 4 μg/mL polybrene. After 4 h, individual infections were transferred to 9 mL RPMI-IL-3 containing polybrene. Typically, >90% of the

cells were GFP-positive after infection. Two days later, 200,000 cells from each infection were washed and transferred to 10 mL RPMI medium lacking IL-3 and EPO. Viable cells were counted daily by using a Beckman Coulter Counter.

TOXCAT assay. The sequence encoding amino acids 8 –35 of TC2-3 was cloned into the TOXCAT chimeric construct between sequences encoding the N-terminal DNA-binding domain of ToxR and the maltose binding protein (Russ and Engleman, *PNAS* 96:863–868 [1999]) . This fusion protein (and controls containing the transmembrane domain of wild-type glycophorin A [GpA], which forms a strong dimer in the membrane, and the dimerization-defective GpA G83I mutant) were expressed in *E. coli*. The level of oligomerization was determined via enzymatic CAT activity quantification, employing ^{3}H-chloramphenicol, as described (Russ and Engelman, 1999). CAT activity was normalized to the expression level of each chimera as determined by Western blotting with an antibody that recognizes maltose binding protein (ZYMED Laboratories) (Fig S3).

Immunoprecipitation and immunoblotting. The following antibodies were used: anti-HA affinity matrix (Roche), HA-tag; C-20 (Santa Cruz), full-length hEPOR (but not the NB variant); rabbit polyclonal antibody α-NB (gift of M. Arcasoy), the NB variant of the hEPOR (but not the full-length receptor); rabbit polyclonal antibody recognizing 16 C-terminal amino acids of the E5 protein, E5 and TC2-3; monoclonal antibody 4G10 (Millipore), phosphotyrosine.

BaF3/HA-hEPOR or parental Ba/F3 cells expressing TC2-3 or the E5 protein were grown in RPMI-IL-3. For phosphotyrosine blotting, cells were treated with medium containing 50 μM pervanadate for 30 min at 37 °C prior to harvest and, in some cases, acutely stimulated with 5 units/mL EPO for 5 min at 37 °C. Cells were collected by centrifugation and washed twice in PBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF). Cell lysates were prepared in RIPA-MOPS buffer (20 mM morpholinepropanesulfonic acid [pH 7.0], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1% deoxycholate, and 1% SDS) buffer containing protease and phosphatase inhibitors (Roche Complete Protease Inhibitor Cocktail and Sigma Phosphatase Inhibitor Cocktail 2) on ice. Lysates were centrifuged for 30 min at 14,000 rpm in a tabletop centrifuge at 4 °C, and concentration of total protein in the supernatant was determined with the bicinchoninic acid (BCA) protein assay kit (Pierce).

To immunoprecipitate HA-tagged hEPOR, 50 μL of an anti-HA affinity matrix (Roche) was added to 1 mg of protein lysate, and rotated overnight at 4 °C. To immunoprecipitate EPO receptor, 20 μL of a rabbit antibody against the C-terminal domain of the hEPOR (C-20, Santa Cruz) was added to 1 mg of protein lysate and rotated overnight at 4 °C. Immune complexes were recovered by adding 50 μL of protein A sepharose bead slurry for at least one hour at 4 °C. To immunoprecipitate TC2-3 or E5, 10 μL of a rabbit antibody against the fixed 16 C-terminal residues of the E5 protein was used and samples were processed as for C-20 immunoprecipitation. Immunoprecipitated samples were washed three times with NETN buffer (100 mM NaCl, 0.1 mM EDTA, 20 mM Tris-HCl [pH8.0], and 0.1% Nonidet P-40) supplemented with 1 mM PMSF, pelleted, and resuspended in 2x Laemmli sample buffer with or without 200 mM DTT and 5% β-mercaptoethanol. Proteins were resolved by SDS-PAGE on a 12% polyacrylamide gel for EPO receptor and phosphotyrosine blotting, or a 20% polyacrylamide gel for E5 blots. The resolving gel was transferred to a 0.45-μm polyvinylidene fluoride (PVDF) membrane by using standard procedures (the E5 gels were transferred without SDS).

Membranes were blocked with gentle agitation for 1 h at room temperature in 5% nonfat dry milk or 5% bovine serum albumin (BSA) (for 4G10 blotting only) in 1X tris buffered saline plus 0.1% Tween-20 containing sodium azide (TBST). A 1 : 250 dilution of a rabbit anti-E5 polyclonal antibody in 5% milk/TBST was used to detect E5 protein and TC2-3. Anti-phosphotyrosine 4G10 antibody (Millipore) was used at a 1 : 1000 dilution in 5% BSA/TBST to detect tyrosine phosphorylated HA-hEPOR. A 1 : 500 dilution of rabbit C-20 antibody in 5% milk/TBST was used to detect HA-hEPOR. A 1 : 1000 dilution of rabbit α-NB antibody in 5% milk/TBST was used to detect NB HA-hEPOR. Membranes were incubated in primary antibody overnight at 4 °C with gentle agitation, washed three times in TBST, and then incubated with gentle agitation for 1 h at room temperature with horse radish peroxidase (HRP)-coupled secondary antibodies (1 : 10, 000 donkey anti-mouse [for 4G10] or donkey anti-rabbit [for anti-E5] antibody [both from Jackson Immunoresearch]), or with 1 : 8, 000 protein A HRP (for anti-C-20) (Amersham), diluted in blocking buffer lacking azide. All membranes were incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce) to detect protein bands.

Analysis of human CD34^{+} cells. Adult CD34^{+} cells were obtained by G-CSF-mobilized peripheral blood apheresis and cryopreserved. Thawed cells were cultured for 4 d in expansion medium [20 ng/ml recombinant human (rh)-IL-6, 100 ng/ml rh-stem cell factor (SCF), 100 ng/mL rh-FLT-3 ligand, and 20 ng/mL rh-IL-3 in StemSpan serum-free medium (Stem Cell Technologies)]. Five hundred thousand cells in 500 μL expansion medium per well of a 12-well plate were infected with 500 μL of concentrated CMMP-IRES-GFP or CMMP-IRES-GFP/TC2-3 by spinoculation in the presence of 8 μg/mL polybrene. The infected cells were incubated overnight at 37 °C and then transferred to a 6-well dish with fresh expansion medium. Forty-eight hours post-infection, GFP-expressing cells were isolated by sterile cell sorting on a BD FACS Vantage SE at 488 nm.

Infected GFP-positive cells were seeded at 3×10^5 cells/mL in differentiation medium [20 ng/mL rh-SCF, 5 ng/mL rh-IL-3, 0.2 μM β-Estradiol, and 2 μM dexamethasone in StemSpan serum-free medium] in the absence or presence of 1 unit/mL EPO. Viable cells were counted at various days. The cell cultures were diluted over time with fresh medium, as necessary, to maintain the cell concentration at approximately 3×10^5 cells/mL, and cell counts were corrected for dilution. After various times, 10^5 cells were washed in 0.5% BSA/PBS and incubated with a mouse monoclonal anti-human GpA antibody (eBioscience, clone HIR2) on ice for 20 min in the dark. The cells were then washed with 0.5% BSA/PBS, incubated with allophycocyanin-conjugated donkey anti-mouse polyclonal antibody (eBioscience) on ice for 20 min in the dark, and cells positive for GpA were determined by flow cytometry on a BD FACS Calibur at 633 nm. 10^4/mL GFP-positive infected cells in Iscoves-Modified Dulbecco's Medium (L-glutamine, 25 mM HEPES, 3.024 mg/L Na_2CO_3) (Gibco) plus 2% FBS were diluted 1 : 10 in methylcellulose medium (Methocult H4531; Stem Cell Technologies) containing 50 ng/mL rh-SCF, 20 ng/mL rh-IL-3, and 20 ng/mL rh-IL-6 in the presence (3 units/mL) or absence of EPO. One thousand cells were plated per 35 mm dish, and colony formation and benzidine staining were scored at day 14.

For analysis of β-globin transcripts, QiaShredder, RNeasy Mini and RNase-free Dnase kits (Qiagen) were used to isolate total RNA from 5×10^5 GFP-expressing HPCs grown for 6 d in differentiation medium. One μg RNA was used as a template for cDNA synthesis using an iScript cDNA synthesis kit (Bio-Rad). Using the Bio-Rad MyiQ Single-color, qRT-PCR was performed with iQ SYBR Green Supermix (Bio-Rad) and 40 ng cDNA per 20 μL reaction. Samples were heated 3 min at 95 °C and then subjected to 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 60 °C for 1 min. Gene-specific primers designed by using the Universal ProbeLibrary ProbeFinder software (Roche) are listed in the supplementary material. Results were normalized to expression of glyceraldehyde 3-phosphate dehydrogenase mRNA. Primers are listed in Table S1.

Receptor	none	IL-3	EPO	TC2-3	BPV E5
none	-	+	-	-	-
PDGFβR	-	+	-	-	+
mEPOR	-	+	+	-	-
hEPOR	-	+	+	+	-
HA-hEPOR	-	+	+	+	-
HA-hEPOR(5A)	-	+	+	-	nt
HA-hEPOR(mPR)	-	+	+	-	nt

Fig. S1. Summary of response of cells to various growth factors and transmembrane proteins. BaF3 cells expressing the indicated exogenous receptors are listed on the *Left*. The medium was supplemented with no exogenous growth factors or with IL-3 or EPO, as indicated, or TC2-3 or the BPV E5 protein were stably expressed in the cells. *Plus Sign* indicates that the cells proliferated robustly, *Minus Sign* indicates that the cells died within a few days. *NT*, not tested.

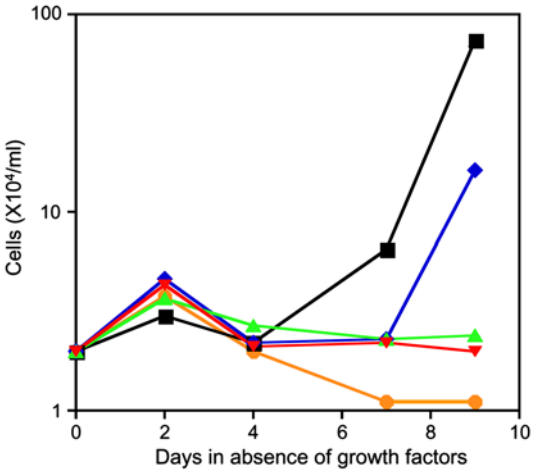

Fig. S2. Ability of small transmembrane proteins to confer growth-factor independence in cells expressing the hEPOR. BaF3/hEPOR cells infected with empty pT2H-F13 or the same vector expressing small transmembrane proteins were selected in medium containing hygromycin and IL-3, and then cultured in the absence of EPO and IL-3. Viable cells were counted after the indicated number of days in the absence of growth factors. *Orange*, vector; *Blue*, TC1-9; *Black*, TC2-3; *Red*, TC1-10; and *Green*, TC2-1. The higher activity of TC2-3 was observed in multiple independent experiments.

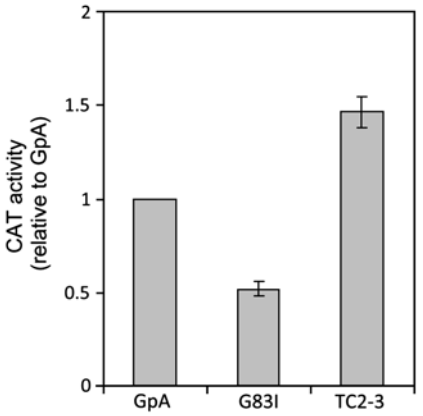

Fig. S3. TOXCAT analysis of TC2-3. Transmembrane amino acids 8–35 from TC2-3 were inserted into the maltose binding protein/toxR chimera and expressed in *E. coli* containing a toxR-dependent chloramphenicol acetyl transferase (CAT) gene. CAT activity was measured on extracted protein after normalizing for the amount of fusion protein in the extract. Wild-type glycophorin A (GpA) and the dimerization-defective G83I glycophorin A mutant were used as standards.

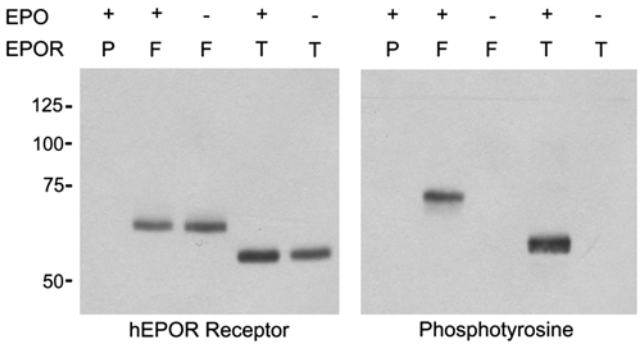

Fig. S4. Identification of the activated form of the hEPOR. Parental BaF3 cells (*P*) and cells expressing HA-tagged full-length hEPOR (*F*) or HA-tagged truncated NB hEPOR (*T*) were acutely treated with EPO (*Plus Sign*) or left untreated (*Minus Sign*), as indicated. RIPA/pervanadate extracts were immunoprecipitated with anti-HA, electrophoresed, and immunoblotted with 4G10 anti-phosphotyrosine monoclonal antibody to visualize activated receptor (*Right*). Antibody was stripped from the membrane, which was then reprobed with a mixture of anti-EPOR (C-20) (to visualize full-length hEPOR) and α-NB (to visualize truncated hEPOR) (*Left*). EPO treatment of HA-hEPOR cells induces tyrosine phosphorylation of a band that migrates more slowly than the receptor band. The truncation causes a marked increase in the electrophoretic mobility of both the receptor band and the tyrosine phosphorylated band in comparison to the wild-type hEPOR. Molecular weight markers (in *kDa)* are shown on the *Left*.

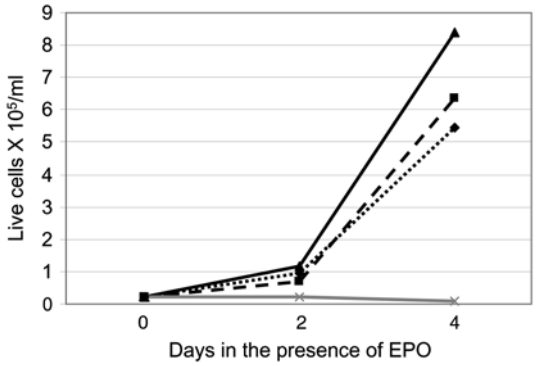

Fig. S5. hEPOR transmembrane mutants support EPO-dependent growth. Parental BaF3 cells and BaF3 cells expressing the wild-type HA-hEPOR, HA-hEPOR (5A), and HA-hEPOR(mPR) were infected with MSCV expressing TC2-3. After hygromycin selection, pooled cell populations were cultured in medium lacking IL-3 and supplemented with 0.5 U/mL EPO. Viable cells were counted after 2–4 d in medium with EPO. The graph shows cells expressing TC2-3 and no hEPOR (*Gray Line*), wild-type HA-hEPOR (*Dotted Black Line*), HA-hEPOR(5A) (*Dashed Black Line*), or HA-hEPOR(mPR) (*Solid Black Line*). Similar results were obtained in an additional independent experiment.

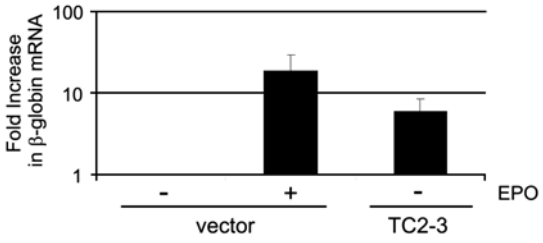

Fig. S6. TC2-3 induces β-globin mRNA expression in HPCs. Total RNA was isolated from HPCs incubated in differentiation medium for 6 d. Vector, infected with CMMP-IRES-GFP; TC2-3, infected with same vector expressing TC2-3. EPO was added where indicated. Levels of human β-globin mRNA were determined by qRT-PCR and expressed relative to vector-infected cells in the absence of EPO. Results shown are the averages of two independent experiments.

Table S1. Oligonucleotides used in library, clone and mutant construction; recovery of inserts from selected cells; and measurement of mRNA levels.

	Oligonucleotide sequence
A.	Fwd: 5′-AGTGTCCTAGGALXZLXZLXZLXZLXZLXZLXZLXZLXZLXZLXZLXZLXZLXZLXZLXZLXZTACTGGVASCATTTCGAATGCTCCTG-3′ Rev: 5′-GCAGCTCGAGGATCCTTAAAAGGGCAGACCTGTACAGGAGCATTCGAAATG-3′ L = A:C:G:T ratio, 1:1:1:0.5; X = A:C:G:T ratio, 0.1:0.25:0.1:1; Z = A:C:G:T ratio, 0:1:0.1:0; V = A:C:G ratio, 1:1:1; S = C:G ratio, 1:1
B.	Fwd: 5′-CGCAAGTGTCCTAGGA-3′ Rev: 5′-GCAGCTCGAGGATCC-3′
C.	Fwd: 5′-CGTTAACTCGAGCAATTGACCATGG-3′ Rev: 5′-GCCGGATCCTTAAAAGGGCAGACC-3′
D.	Fwd: 5′-CGAATCCTCGTCGACAGGTCTGCCCTTTTAAG-3′ Rev: 5′-GATCCTTAAAAGGGCAGACCTGTCGACGAGGATT-3′
E.	Fwd: 5′-GCGGAACCGGTGTCGCTGCTGACGCCTAGCGACCTGGACCCCCTCATCCTGACGCTCTCCCTCATCCTCGTGGTCGCTGCGGC-3′ Rev: 5′-CCAGGCCAGATCTTCTGCTTCAGAGCCCGGCGGTGGGAGAGCAGCGCGAGCACGGTAGCCGCTGCCGCAGCGACCACGAGGATG-3′
F.	Fwd: 5′-GCGGAACCGGTGTCGCTGCTGACGCCTAGCGACCTGGACCCCGTGGTGATCTCAGCCATCCTGGCCTTAGTGGTCCTTACCGTC-3′ Rev: 5′-CCAGGCCAGATCTTCTGCTTCAGAGCCCGGCGGTGGGACATGATGAGGATGATGAGAGAGATGACGGTAAGGACCACTAAGGC-3′
G.	Fwd: 5′-GGTGGTGAGGCCCTGGGCAGG-3′ Rev: 5′-GGCCATCACTAAAGGCACCG-3′
H.	Fwd: 5′-CTGCACCACCAACTGCTTAG-3′ Rev: 5′-GTCTTCTGGGTGGCAGTGAT-3′

A. Degenerate oligonucleotides used to construct TJC5 library.
B. Short (amplification) primers used to insert randomized segment into TJC5 library.
C. Oligonucleotides used to rescue inserts from genomic DNA.
D. Oligonucleotides used to insert cysteine-to-serine mutations into TC2-3.
E. Oligonucleotides used to construct HA-hEPOR (5A)
F. Oligonucleotides used to construct HA-hEPOR (mPR)
G. Oligonucleotides used to measure β-globin mRNA by qRT-PCR.
H. Oligonucleotides that amplify GAPDH mRNA for qRT-PCR normalization.