Co-transfection of murine NXPE2 and murine glycophorin A confers reactivity with Ter-119

Glycophorin A (GPA) is known to play a central role in erythrocyte biology. In addition to maintaining the negatively charged glycocalyx, GPA is a major component of the “macrocomplex” that facilitates RBC adaptation to different oxygen environments (e.g., membrane deformability, metabolic regulation, water balance, and release of mediators that regulate vascular tone). TER-119 was one of the earliest antibodies that could delineate murine erythroid lineages and has been reported to recognize murine glycophorin A (mGPA). However, some lines of evidence have suggested that TER-119 binds instead to a distinct mGPA-associated protein of unknown identity rather than mGPA itself. In this report, we demonstrate that the epitope recognized by TER-119 is reconstituted by co-expression of mGPA and murine neurexophilin and PC-esterase domain family member 2 (NXPE2), but not by either mGPA or NXPE2 alone. In addition to solving a longstanding debate over what is required for a cell to be recognized by TER-119, and thus adding depth to numerous published studies, these findings are the first report of NXPE2 being expressed in erythrocytes, raising the possibility that NXPE2 plays a functional role in erythropoiesis and/or red blood cell biology.

TER-119 is highly specific to the erythroid lineage in mice and has been instrumental in the study of erythropoiesis and other red blood cell (RBC) functions in hundreds of primary research papers. Auffray *et al.* have reported that TER-119 recognizes mGPA[1] based on the observation that TER-119 both fails to react with RBC from band 3 knockout mice (which lack RBC surface mGPA)[2] and TER-119 recognizes 2 proteins by Western blot (52 kD and 32 kD). The 32 kD band has a similar size as mGPA and continues to be detected by Western blot at increasing SDS concentrations, a known property of mGPA.[1] In contrast, Kina *et al*. reported that both anti-sera to mGPA and TER-119 immunoprecipitate the same 4 bands (100 kD, 60 kD, 52 kD, and 32 kD), but TER-119 only recognizes the 52 kD band by Western blot. Because the 52 KD band was not consistent with known properties of mGPA, Kina *et al*. concluded that TER-119 recognizes an mGPA-associated protein but not mGPA itself.[3] Consistent with this interpretation, TER-119 was not reactive with any erythroid cell line tested, including those with high levels of mGPA expression.[3,4]

Thus, there is lack of agreement on the precise target of TER-119. However, the data support the hypothesis that mGPA is necessary but not alone sufficient to generate the epitope that is bound by TER-119.

While screening a population of genetically diverse outbred (DO) mice for traits associated with RBC, we serendipitously observed that peripheral blood from approximately 5% of mice was only weakly reactive with TER-119. Using TER-119 reactivity as a trait, we mapped a quantitative trait locus (QTL) with a peak logarithm of the odds (LOD) score of 27.8 (genome-wide adjusted *P* value < 2.22×10^{-16}) on chromosome 9 at 48.1 Mbp (47.8-49.2 Mbp support interval) (Figure 1A). The TER-119^{weak} phenotype correlated with genetic variations inherited from the WSB founder strain (Figure 1B). Notably, the TER-119^{weak} phenotype followed a recessive pattern of inheritance – only DO mice that had 2 copies of the WSB allele (i.e. *homozygous “WSB carrier”*) were TER-119^{weak} (Figure 1C). Subsequent flow cytometry confirmed that RBC from WSB mice are also TER-119^{weak}; all other parental strains that were used to found the DO population were strongly TER-119 reactive (Figure 1D). All studies were carried out under protocols approved by the University of Virginia Animal Care and Use Committee.

The identified locus was a gene rich region with no deletions between strains (MGI; https://www.informatics.jax.org), containing 12 protein expressing genes plus 2 predicted genes (Table 1, Figure 2A). Of these genes, 5 are transmembrane proteins, 3 of which flanked the peak QTL (i.e., *neurexophilin and pc-esterase domain family member 2* (*Nxpe2*), *5-hydroxytryptamine receptor 3A* (*Htr3a*), and *5-hydroxytryptamine receptor 3B* (*Htr3b*). Although not predicted to be transmembrane, *Nxpe4* surrounds the *Nxpe2* gene in B6 mice. Thus, we included NXPE4 in phenotypic assessment. NXPE2, NXPE4, HTR3A and HTR3B each have amino acid polymorphisms between B6 and WSB mice. Expression vectors for B6 and WSB variants of each of the 4 candidate genes were transfected into HEK cells followed by staining with TER-119. Because HTR3A and HTR3B can form heterodimers, they were also co-transfected. Similarly, NXPE2 and NXPE4 were each co-transfected with an expression vector of mGPA. mGPA amino acid sequence does not vary in any of the strains used to generate DO mice. In all transfections, an expression vector for GFP was included to confirm transfection efficiency and to allow gating on the transfected population. TER-119 reactivity on viable GFP^{+} cells was assessed by flow cytometry (Figure 2B). Co-transfection of NXPE2 and mGPA conferred TER-119 reactivity. We interpret the very broad histogram shift to be due to use of transient transfection methods that generate highly heterogeneous populations. No significant TER-119 reactivity was seen under any other transfection condition, although positive controls to confirm protein expression were not performed.

Both the WSB and B6 variants of NXPE2 conferred TER-119 binding to HEK cells when co-transfected with mGPA, rejecting the hypothesis that the TER-119^{weak} phenotype

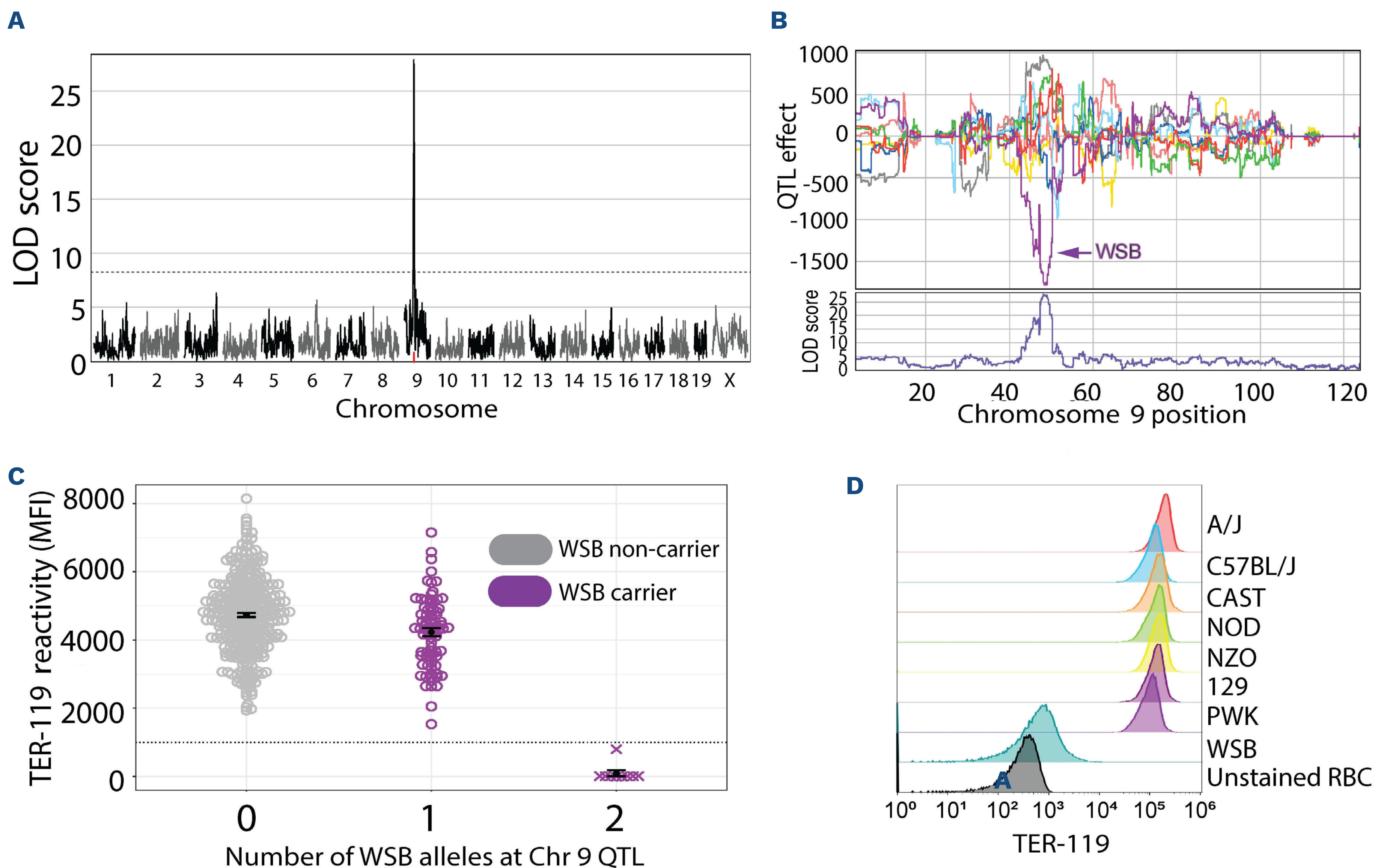


Figure 1. Mapping of TER-119 reactivity quantitative trait locus on chromosome 9. (A) Quantitative trait locus (QTL) for TER-119 reactivity, measured as mean fluorescence intensity (MFI) through flow cytometry, using 427 mice from the Diversity Outbred (DO) population.[13] Peripheral blood was stained with TER-119 conjugated to BV421 (BioLegend Cat# 116234) at a dilution of 1:100 and analyzed by flow cytometry using an attune cytometer. Data were analyzed using FloJov10. DO mice (Jackson Strain #009376) were from the 45th and 46th generation of the DO population. The total sample of 427 mice had 273 females and 154 males. All DO mice were genotyped on the GigaMUGA array (approx. 143,000 markers).[14] QTL analysis was performed using the qtl2 R package. A QTL was detected with a peak LOD score of 27.8, corresponding to a genome-wide adjusted *P* value $<2.22\times10^{-16}$, on chromosome (Chr) 9 at 48.1 Mbp (47.8-49.2 Mbp support interval). (B) The TER-119^{weak} phenotype associates with WSB allele of the QTL. (C) The TER-119^{weak} phenotype follows an autosomal recessive inheritance. (D) Red blood cells (RBC) from WSB mice (but none of the other DO founder strains) have the TER-119^{weak} phenotype. The DO population was generated through interbreeding 8 inbred strains of mice (abbreviated names in parentheses): A/J (AJ), C57BL/6J (B6), 129S1/SvlmJ (129), NOD/ShiLtJ (NOD), NZO/HILtJ (NZO), CAST/EiJ (CAST), PWK/PhJ (PWK), and WSB/EiJ (WSB). Staining of each strain was carried out twice with equivalent results. LOD: logarithm of the odds.

was due to an alteration of amino acid(s) in WSB mice that disrupted the epitope recognized by TER-119 (*Online Supplementary Figure S1*). This led us to hypothesize that WSB mice have decreased expression of NXPE2. The only available antisera to NXPE2 did not detect a specific band in membrane preps from either B6 or WSB RBC by Western blot (*data not shown*). However, DO gene expression data and eQTL show very low NXPE2 expression in liver (10.1038/nature18270; https://churchilllab.jax.org/qtlviewer/svenson/DOHFD) and bone (https://churchilllab.jax.org/qtlviewer/DO/bone) only in DO mice that are homozygous for the WSB allele of NXPE2 (*Online Supplementary Figure S2A, B*), matching the TER-119 reactivity observed in RBC. Also consistent with the RBC specific expression of TER-119, analysis of curated RNAseq data from hematopoiesis demonstrates that *Nxpe2* has an approximate 3-fold increase during differentiation of HSC into the erythroid lineage (*Online Supplementary Figure S3A, B*).[5]

Our current understanding is limited to the observation that co-transfection of NXPE2 and mGPA confer TER-119 reactivity. The simplest explanation is that TER-119 binds to an epitope generated from an NXPE2/mGPA complex (either an epitope to which both contribute or an epitope on one molecule allosterically induced by complexing with the other molecule). Given that NXPE2 is a known transmembrane protein, we favor this explanation. However, there are alternate hypotheses, all of which are equally consistent with the current data, such as NXPE2 expression

Table 1. Protein coding genes contained in quantitative trait locus for TER-119 expression in diversity outbred population.

Chr.	Start (bp)	End (bp)	cM	Strand GRCm39	MGI ID	Feature type	Symbol	Name	Protein type
9	48073321	48311325	26.39	+	MGI:1924792	Protein coding	*Nxpe4*	*Neurexophilin and PC-esterase domain family, member 4*	Secreted
9	48229303	48264749	26.38	-	MGI:1925502	Protein coding	*Nxpe2*	*Neurexophilin and PC-esterase domain family, member 2*	Transmembrane
9	48379812	48391911	26.42	-	MGI:1888981	Protein coding	*Rexo2*	*RNA exonuclease 2*	Nucleus/Nucleolus
9	48400001	48406599	26.43	-	MGI:1914260	Protein coding	*Rbm7*	*RNA binding motif protein 7*	Nucleus/ Nucleoplasm
9	48406742	48407272	26.43	+	MGI:3643566	Protein coding	*Gm5617*	*Predicted gene, 5617*	-
9	48503177	48516453	26.45	-	MGI:1099443	Protein coding	*Nnmt*	*Nicotinamide N-methyltransferase*	Cytoplasm
9	48565597	48747522	26.47	-	MGI:103222	Protein coding	*Zbtb16*	*Zinc finger and BTB domain containing 16*	-
9	48581383	48585989	-	+	MGI:5591439	Protein coding	*Gm32280*	*Predicted gene, 32280*	-
9	48810513	48822399	26.53	-	MGI:96282	Protein coding	*Htr3a*	*5-Hydroxytryptamine (serotonin) receptor 3A*	Transmembrane
9	48846308	48876290	26.54	-	MGI:1861899	Protein coding	*Htr3b*	*5-Hydroxytryptamine (serotonin) receptor 3B*	Transmembrane
9	48896675	48953817	26.56	+	MGI:2442293	Protein coding	*Usp28*	*Ubiquitin specific peptidase 28*	Nucleus/ Nucleoplasm
9	48958902	48959594	26.58	-	MGI:3642767	Protein coding	*Cldn25*	*Claudin 25*	Transmembrane
9	48966913	48990075	26.58	+	MGI:1349478	Protein coding	*Zw10*	*Zw10 kinetochore protein*	Cytoplasm
9	49013994	49028891	26.59	+	MGI:1933407	Protein coding	*Tmprss5*	*Transmembrane protease, serine 5 (spinesin)*	Transmembrane

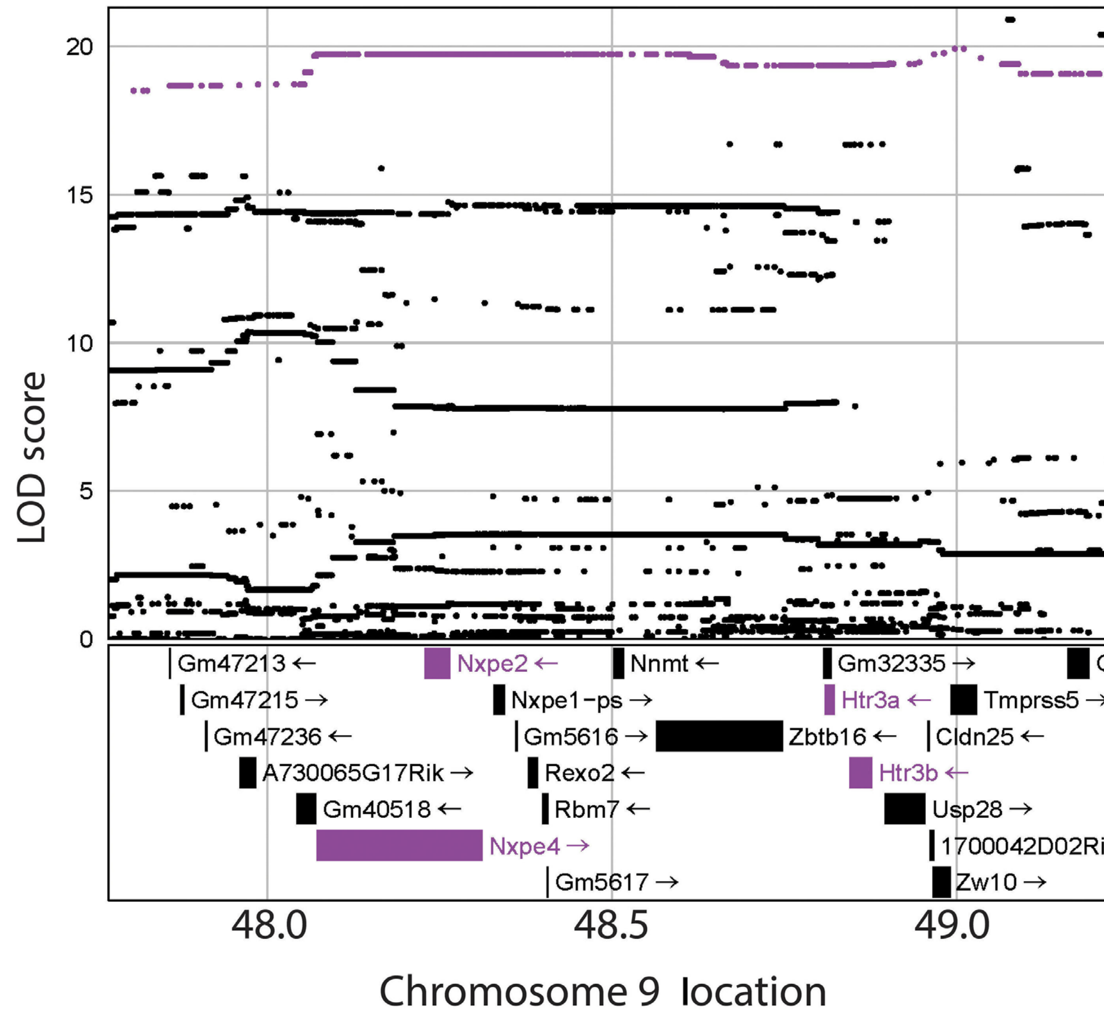
Chr: chromosome; bp: base pair; cM: centimorgan; GRCm39: Genome Reference Consortium Mouse Build 39; MGI: Mouse Genome Informatics.

resulting in modification of mGPA generating the TER-119 epitope (e.g., folding, processing, or post-translational modification – either directly or through induction/activation of other gene products). Ongoing studies will be required to distinguish between these hypotheses.

The current findings do not indicate what, if any role NXPE2 plays in erythropoiesis or mature RBC function (alone or in complex with mGPA). WSB mice have a normal phenotype with regards to hematocrit, hemoglobin, reticulocyte count, and mean corpuscular volume.[6] However, WSB mice have decreased (but not absent) expression of NXPE2 and binding of TER-119. Thus, NXPE2 may play a functional role for which lower expression is sufficient. *Nxpe2* knockout mice are viable and have been reported as part of a gene trap library;[7] with no phenotypes noted other than decreased proliferation of skin fibroblasts.[8] However, no characterizations of hematologic parameters were reported.[8] Finally, it is worth noting that *Nxpe2* has not been identified in genome-wide association studies (GWAS) using changes in erythropoiesis as a trait; however, for a gene to be identified in GWAS it must vary within the population being studied in a way that alters function. As such, while a pertinent negative, the absence of GWAS hits does not rule out a role of NXPE2 in erythropoiesis. Future studies will be required to assess erythropoiesis and RBC function in the absence of NXPE2.

GPA is known to be an essential component of the RBC macrocomplex; however, the current model of the murine macrocomplex does not contain NXPE2.[9-11] We speculate that NXPE2 / mGPA form dimers that are distinct from the macrocomplex, as TER-119 immunoprecipitates far fewer proteins than anti-mGPA.[3] However, we cannot rule out the possibility that NXPE2 participates in the macrocomplex in a fashion not hitherto detected.

NXPE2 has not been reported in the human RBC proteome.[11] This may indicate that NXPE2 is not expressed in human hematopoiesis, but its failure to be detected for technical reasons cannot be ruled out. NXPE2 has not been detected in the mouse RBC proteome either, suggesting its particular biochemistry may make it difficult to detect at the biochemical level. Even well-known abundant proteins (e.g., beta actins) can be difficult to detect depending upon



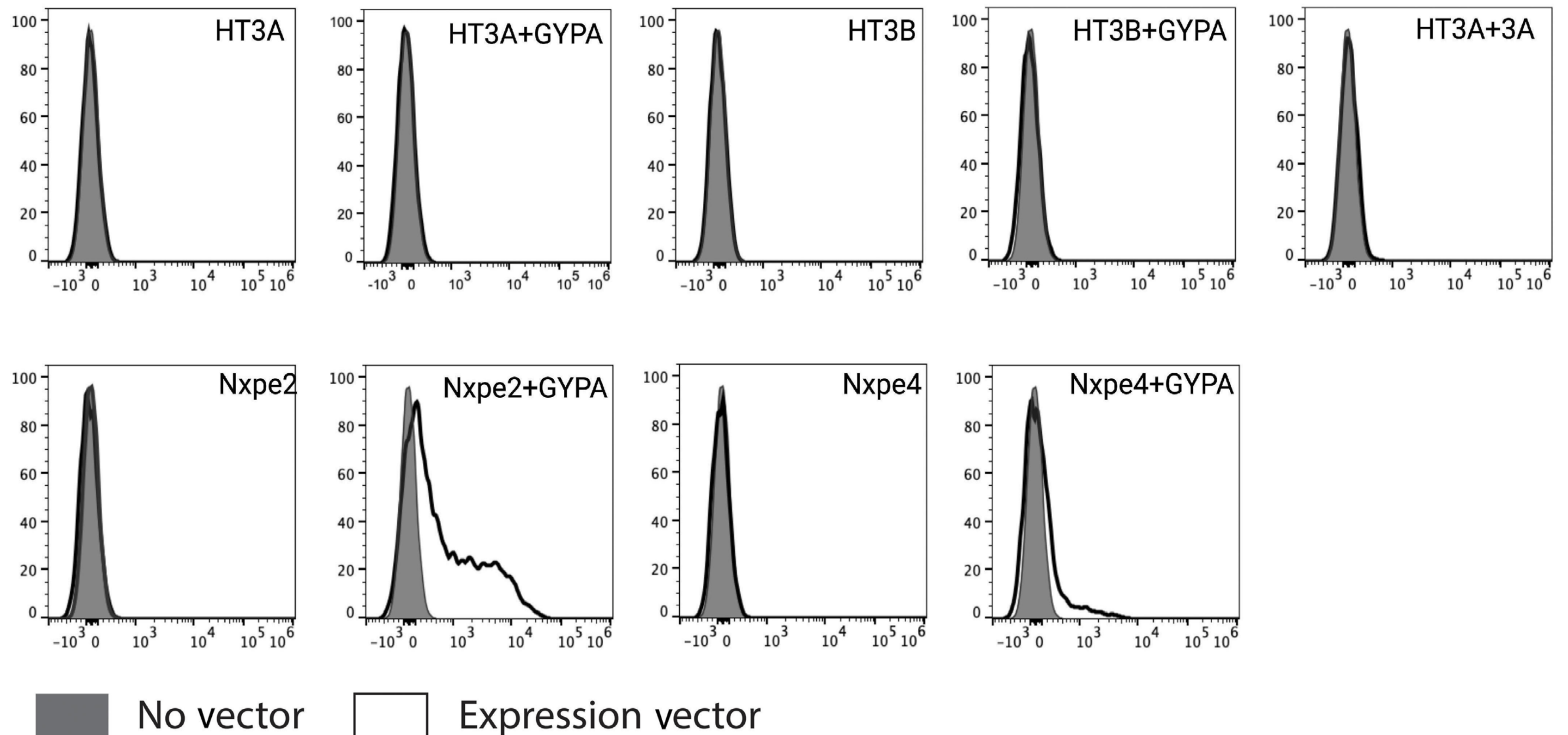


Figure 2. Co-transfection of NXPE2 and murine glycophorin A results in TER-119 immunoreactivity. (A) The genetic composition of the identified quantitative trait locus (QTL) (see Figure 1A) included 3 transmembrane proteins in close proximity to the peak QTL (indicated in purple). NXPE4 is not predicted to be a transmembrane protein but was included in subsequent hypothesis testing due to its relation to NXPE2. (B) HEL cells were transfected with each of the indicated expression vectors, alone or in combination, stained with TER-119, and analyzed by flow cytometry. An expression vector for GFP was included in all transfections and the histograms shown are gated on viable GFP$^+$ HEK cells. WSB and B6 sequences for each of the tested gene products were acquired from the Mouse Genome Informatics (MGI) database (https://www.informatics.jax.org). Open reading frames for each gene were synthesized by Genewiz, with Kozak consensus elements upstream of the start codon and ligated into a eukaryotic expression vector driven by the CMV promoter. HEK cells were transfected using FuGENE 6 transfection reagent (Promega Cat# E2691) according to the manufacturer's instructions and stained with TER-119 as above. The transfection experiment was carried out twice with equivalent results. LOD: logarithm of the odds.

methodologies used.[11] The only published report regarding human NXPE2 is its identification in an RNAseq study assessing markers in gastric cancer in humans.[12] Future studies will be required to investigate if NXPE2 is expressed on human erythrocytes and what function, if any, it plays in either murine or human RBC biology.

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Disclosures

James Zimring is a co-founder of Svalinn Therapeutics that develops products unrelated to the current work and had no involvement with the current studies. AD is a founder of Omix Technologies Inc. and an advisory board member for Hemanext and Macropharma.

Contributions

JCZ conceived the project. JCZ, AD, SEE, GRK and GAC designed experiments. AMH, NH, and MD carried out physical experiments. GRK, GAC, CO and MV performed all data analysis for genetic mapping. Although major categories of contribution are listed above, this was a group project with ongoing dialog and all authors participated in experimental design, data interpretation, and writing the manuscript.

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Data-sharing statement

All data and code for producing results have been made publicly available at figshare (https://doi.org/10.6084/m9.figshare.24456619.v1). Processed data and results can also be downloaded from the QTLViewer (https://churchilllab.jax.org/qtlviewer/Zimring/RBC).

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