Science Advances
AAAS

Supplementary Materials for

In vivo multidimensional CRISPR screens identify *Lgals2* as an immunotherapy target in triple-negative breast cancer

Peng Ji *et al.*

Corresponding author: Zhi-Ming Shao, zhi_ming_shao@163.com; Xin Hu, xinhu@fudan.edu.cn; Gen-Hong Di, genhong_di@163.com

Sci. Adv. **8**, eabl8247 (2022)
DOI: 10.1126/sciadv.abl8247

The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

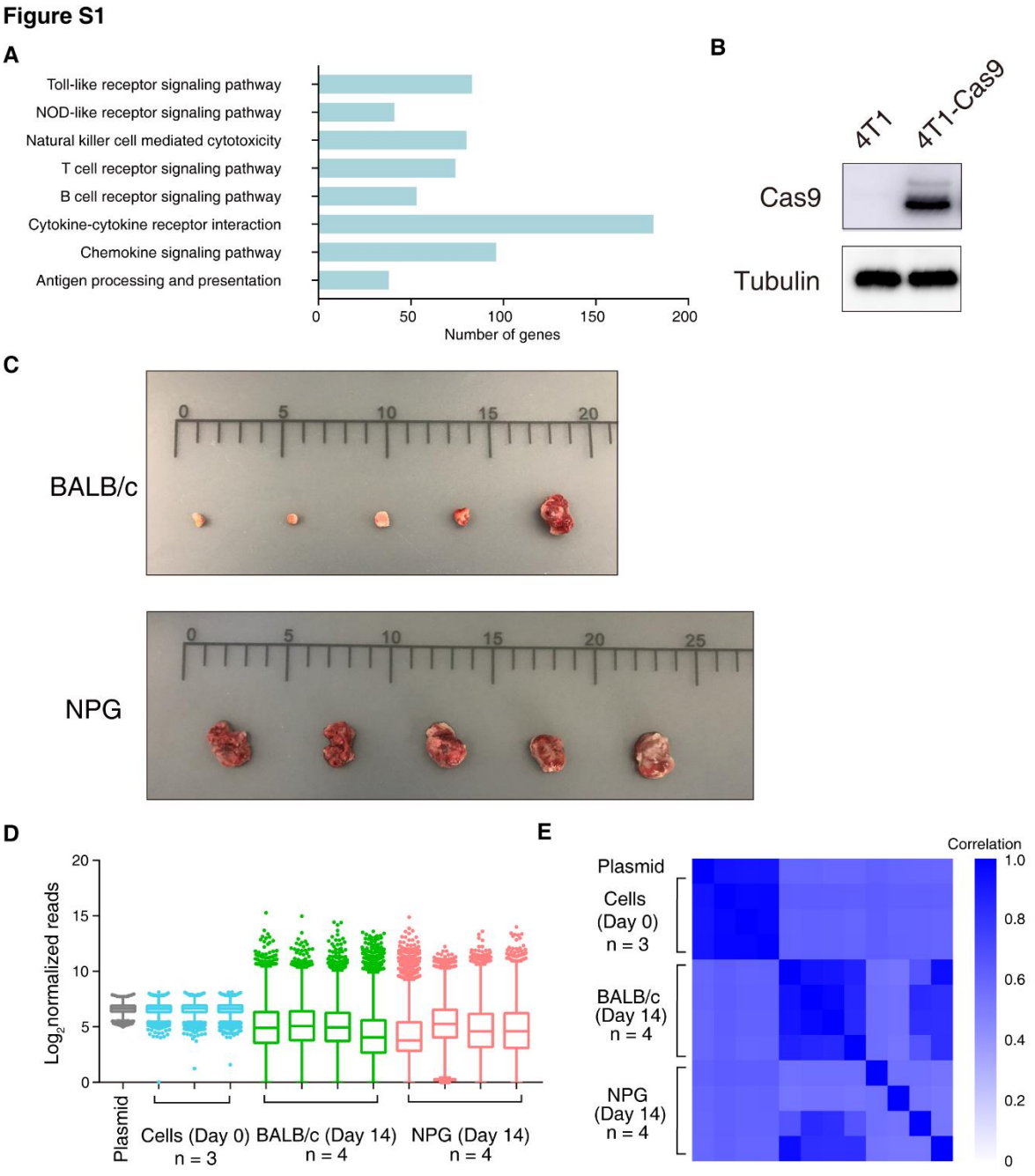

Fig. S1. Analysis of DrIM screen performance.

(A) Histogram showing the number of human disease-related immune genes targeted in the screen in each of the KEGG pathways indicated.

(B) Western blot analysis of 4T1 cell lysate of uninfected cells or after transduction with a lentiviral vector encoding Cas9 for Cas9 and tubulin expression.

(C) *Ex vivo* images of resected tumors from BALB/c mice and NPG mice.

(D) Boxplot of the sgRNA normalized read counts for the DrIM plasmid library, transduced cells before transplantation, tumors in BALB/c mice and tumors in NPG mice. Outliers are shown as colored dots for each respective sample.

(E) Spearman correlation coefficient of the normalized sgRNA read counts from the DrIM plasmid library, transduced cells before transplantation, tumors in BALB/c mice and tumors in NPG mice.

Figure S2

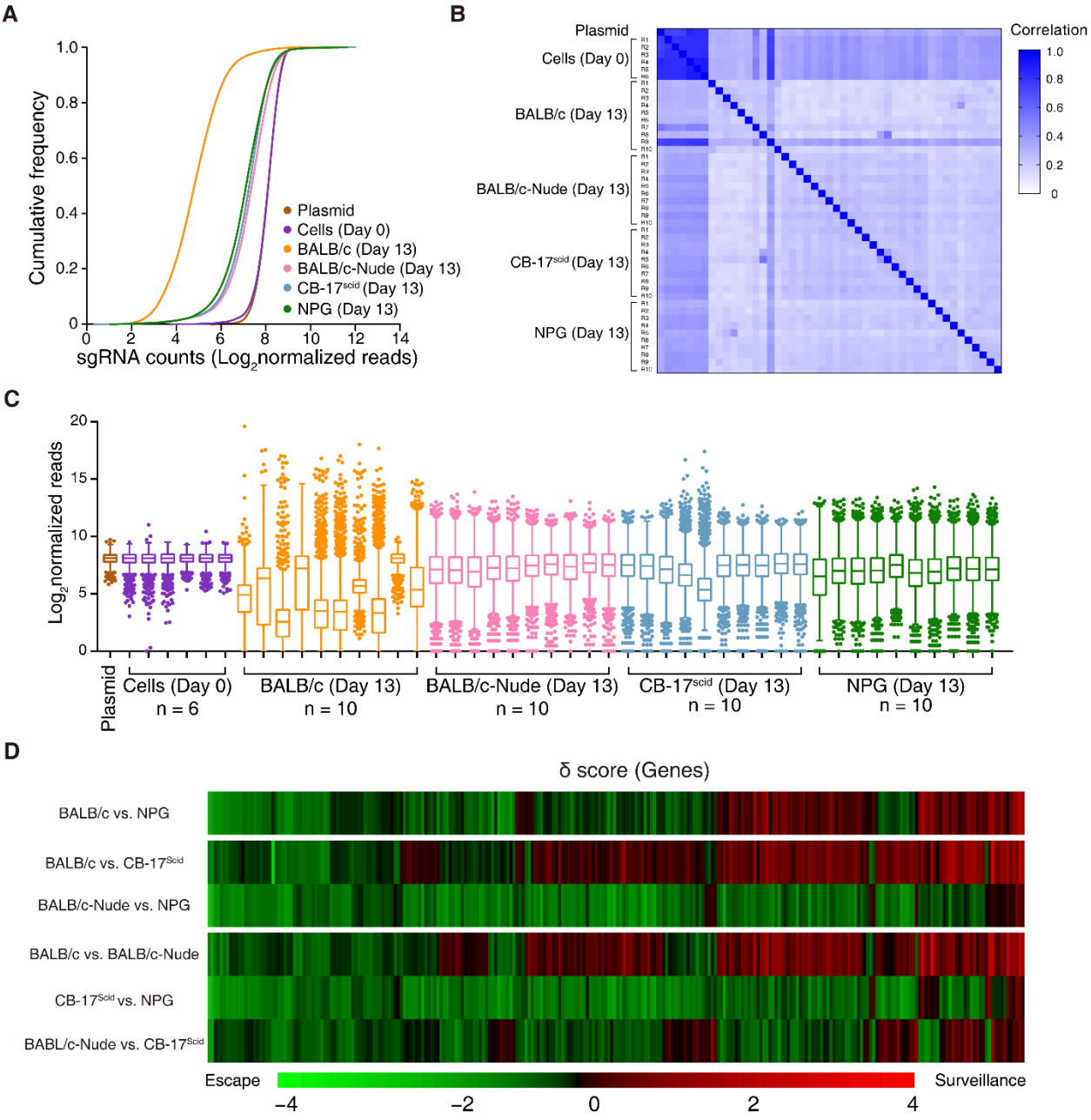

Fig. S2. Analysis of mini-DrIM screen performance.

(A) Cumulative distribution function (CDF) plots of mini-DrIM library sgRNAs in the plasmid, cells before transplantation, tumors in BALB/c mice, tumors in BALB/c-Nude mice, tumors in CB-17^{scid} mice and tumors in NPG mice. Distributions for each sample type are averaged across individual mice and infection replications.

(B) Spearman correlation coefficient of the normalized sgRNA read counts from the mini-DrIM plasmid library, transduced cells before transplantation, tumors in BALB/c mice, tumors in BALB/c-Nude mice, tumors in CB-17^{scid} mice and tumors in NPG mice.

(C) Boxplot of the sgRNA normalized read counts for the mini-DrIM plasmid library, transduced cells before transplantation, tumors in BALB/c mice, tumors in BALB/c-Nude mice, tumors in CB-17^{scid} mice and tumors in NPG mice. Outliers are shown as colored dots for each respective sample.

(D) Heatmap of δ score under different immune-selection pressures.

Figure S3

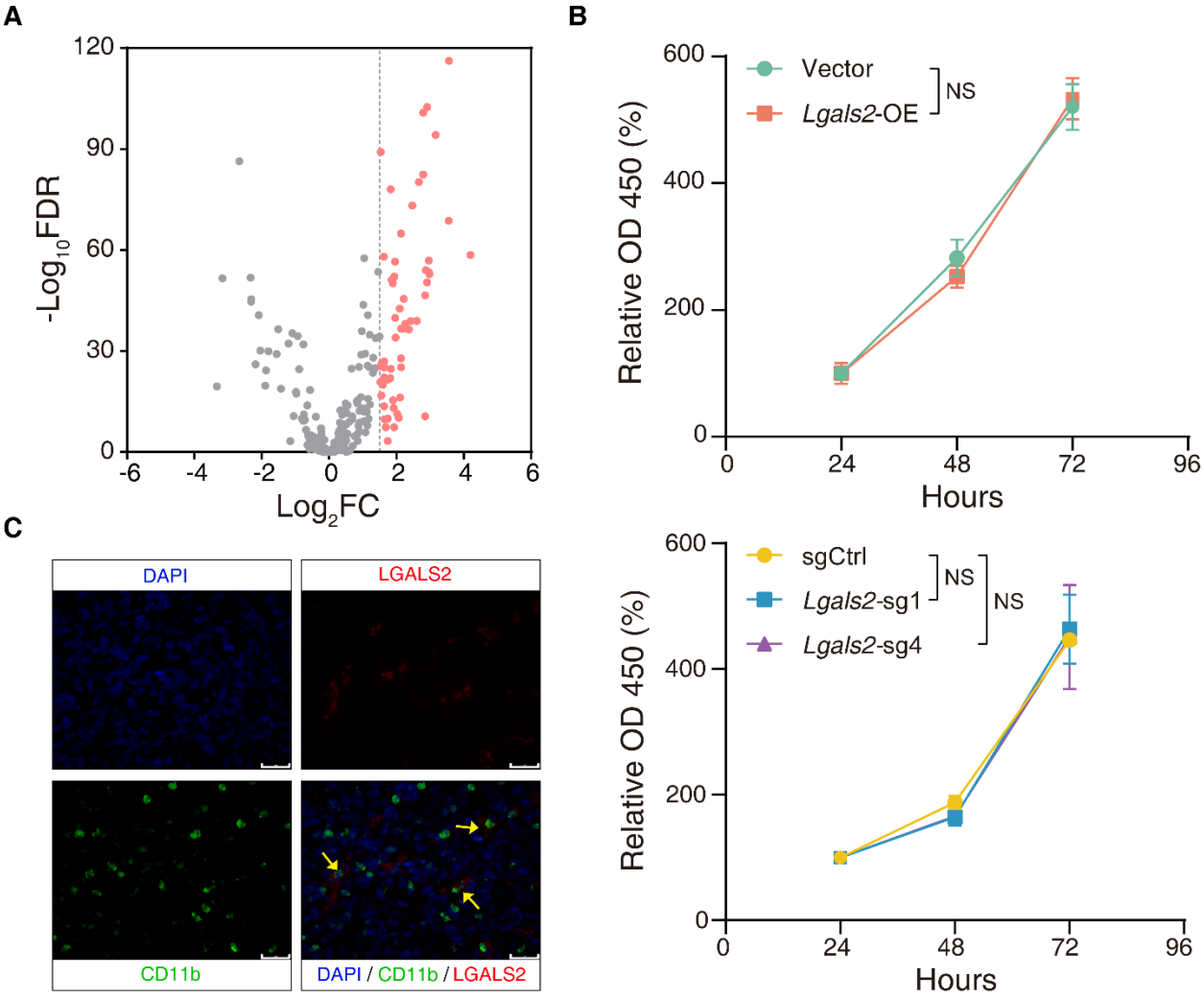

Fig. S3. Identification of putative essential genes mediating immunosuppression in TNBC.

(A) Volcano plot showing comparison of mRNA expression of mini-DrIM library genes between TNBC tumor tissue samples (n = 360) and paired normal samples (n = 88) in the FUSCC cohort.

(B) *In vitro* cell growth curve of 4T1 cells over-expressing *Lgals2* (top panel) or lacking *Lgals2* (bottom panel). NS $P \geq 0.05$. Data are presented as mean ± s.e.m.

(C) Representative immunofluorescence staining of LGALS2 (red) in combination with CD11b (green) in resected tumors from 4T1 cells transplanted BALB/c mice. Arrows indicate LGALS2 did not express on $CD11b^+$ cells. Scale bars, 25 μm.

Figure S4

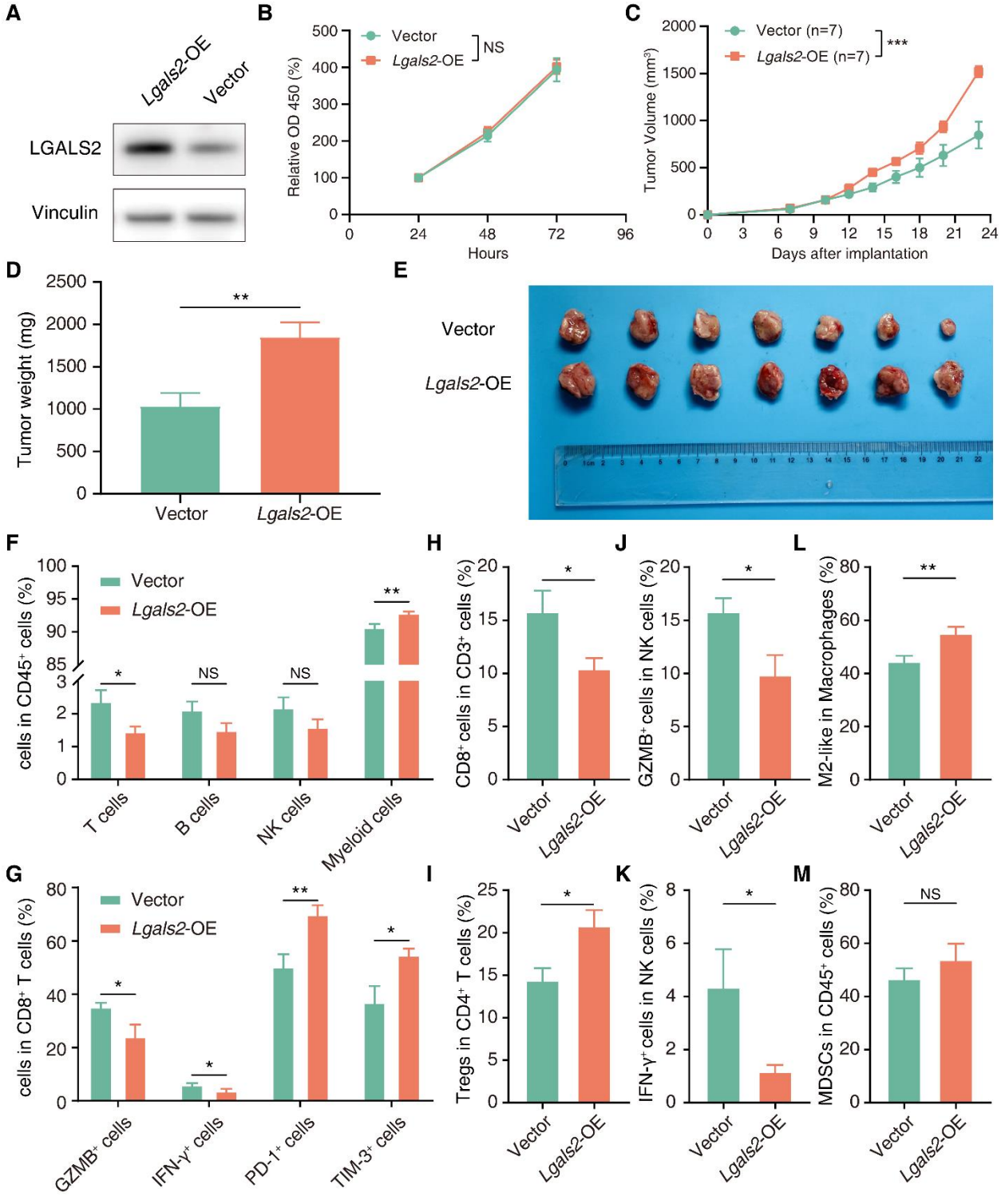
A
Lgals2-OE
Vector
LGALS2
Vinculin
B
Relative OD 450 (%)
Vector
Lgals2-OE
NS
Hours
C
Tumor Volume (mm³)
Vector (n=7)
Lgals2-OE (n=7)
***
Days after implantation
D
Tumor weight (mg)
**
Vector
Lgals2-OE
E
Vector
Lgals2-OE
F
cells in CD45⁺ cells (%)
Vector
Lgals2-OE
T cells
B cells
NK cells
Myeloid cells
H
CD8⁺ cells in CD3⁺ cells (%)
J
GZMB⁺ cells in NK cells (%)
L
M2-like in Macrophages (%)
G
cells in CD8⁺ T cells (%)
GZMB⁺ cells
IFN-γ⁺ cells
PD-1⁺ cells
TIM-3⁺ cells
I
Tregs in CD4⁺ T cells (%)
K
IFN-γ⁺ cells in NK cells (%)
M
MDSCs in CD45⁺ cells (%)
NS

Fig. S4. Identification of *Lgals2* function on EMT6 breast cancer mouse models.

(A) Western blot of *Lgals2* protein level in EMT6 cells transduced with either vector or *Lgals2*-overexpressing plasmid.

(B) *In vitro* cell growth curve of EMT6 cells over-expressing *Lgals2*. NS $P \geq 0.05$. Data are presented as mean ± s.e.m.

(C-E) *In vivo* analysis of transplanted EMT6 cells with *Lgals2* overexpressing in BALB/c mice including (E) tumor growth curve, (F) tumor weight and (G) *ex vivo* images of resected tumors. *** $P < 0.001$, ** $P < 0.01$. Data are presented as the mean ± s.e.m.

(F-M) The primary tumors of vector control and *Lgals2*-overexpressing EMT6 tumors of BALB/c mice were harvested for flow cytometry to determine the percentages of (H) T cells, B cells, NK cells and myeloid cells among $CD45^+$ cells, (I) Granzyme B^+ ($GZMB^+$) cells, IFN-γ^+ cells, PD-1^+ cells and TIM-3^+ cells among $CD8^+$ T cells, (J) $CD8^+$ T cells among $CD3^+$ T cells, (K) $FOXP3^+CD25^+$ Tregs among $CD4^+$ T cells, (L) $GZMB^+$ cells among NK cells, (M) IFN-γ^+ cells among NK cells, (N) M2-like macrophages among total macrophages and (O) MDSCs among $CD45^+$ cells. ** $P < 0.01$; * $P < 0.05$; NS $P \geqslant 0.05$. Data are presented as the mean ± s.e.m.

Figure S5

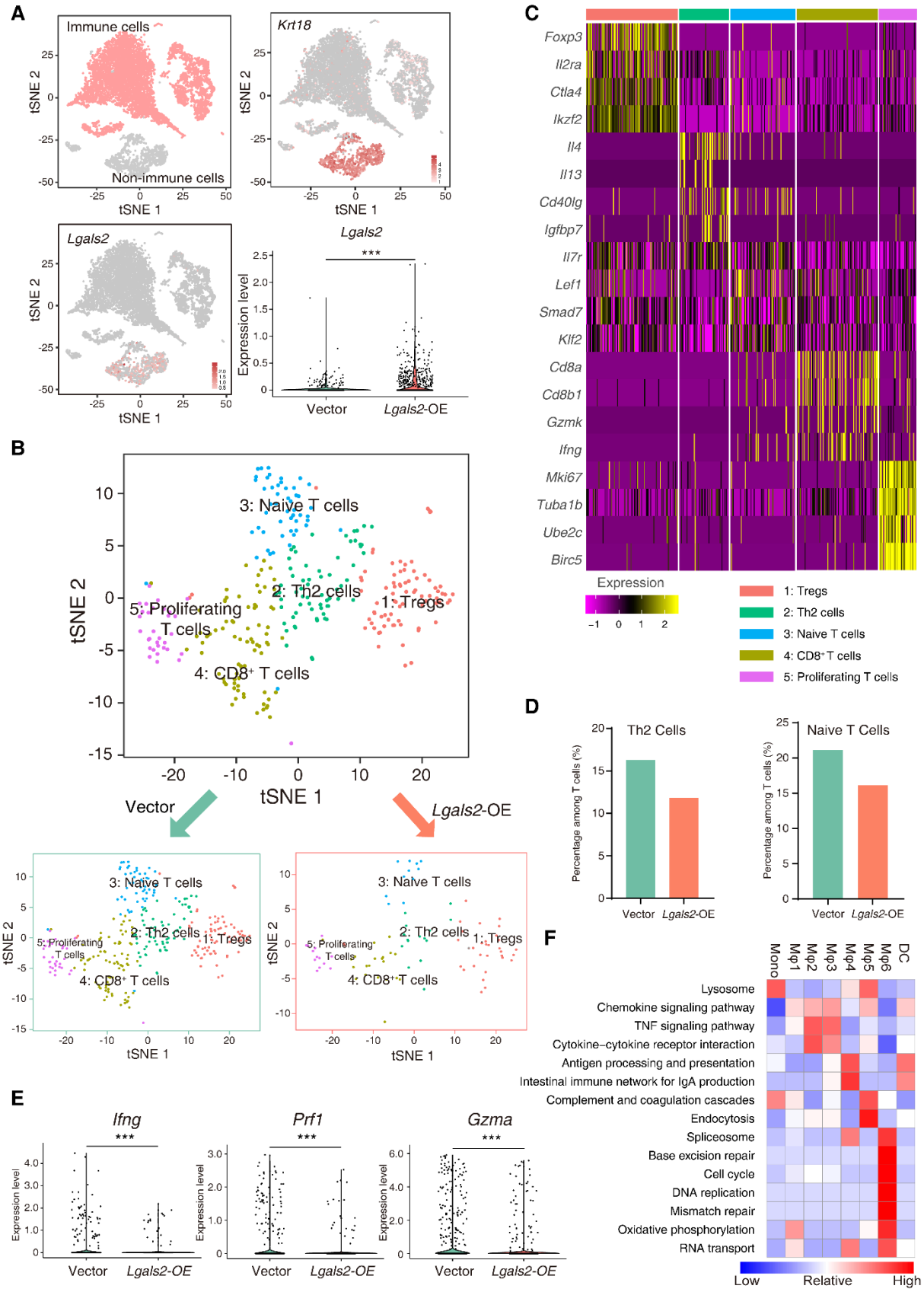
A
Immune cells
Non-immune cells
Krt18
Lgals2
tSNE 1
tSNE 2
Expression level
***
Vector
Lgals2-OE
B
3: Naive T cells
2: Th2 cells
1: Tregs
5: Proliferating T cells
4: CD8⁺ T cells
Vector
Lgals2-OE
C
Foxp3
Il2ra
Ctla4
Ikzf2
Il4
Il13
Cd40lg
Igfbp7
Il7r
Lef1
Smad7
Klf2
Cd8a
Cd8b1
Gzmk
Ifng
Mki67
Tuba1b
Ube2c
Birc5
Expression
1: Tregs
2: Th2 cells
3: Naive T cells
4: CD8⁺ T cells
5: Proliferating T cells
D
Th2 Cells
Naive T Cells
Percentage among T cells (%)
Vector
Lgals2-OE
E
Ifng
Prf1
Gzma
F
Mono
Mφ1
Mφ2
Mφ3
Mφ4
Mφ5
Mφ6
DC
Lysosome
Chemokine signaling pathway
TNF signaling pathway
Cytokine–cytokine receptor interaction
Antigen processing and presentation
Intestinal immune network for IgA production
Complement and coagulation cascades
Endocytosis
Spliceosome
Base excision repair
Cell cycle
DNA replication
Mismatch repair
Oxidative phosphorylation
RNA transport
Low
Relative
High

Fig. S5. Single-cell RNA-seq analysis of 4T1 tumors with vector or *Lgals2* overexpression.

(A) t-SNE plot showing identification of immune cells and non-immune cells with marker genes and violin plot of *Lgals2* mRNA levels.

(B) t-SNE plot of re-classification of intratumoral T cells.

(C) Heatmap of key significant genes in clusters of T cells.

(D) The distribution of Th2 and Naïve T cells between Vector and *Lgals2*-OE tumor cells.

(E) Violin plot of *Ifng*, *Prf1* and *Gzma* mRNA levels.

(F) Heatmap of GSEA identifying pathway enrichment by monocytes/dendritic cells/macrophages cluster.

Figure S6

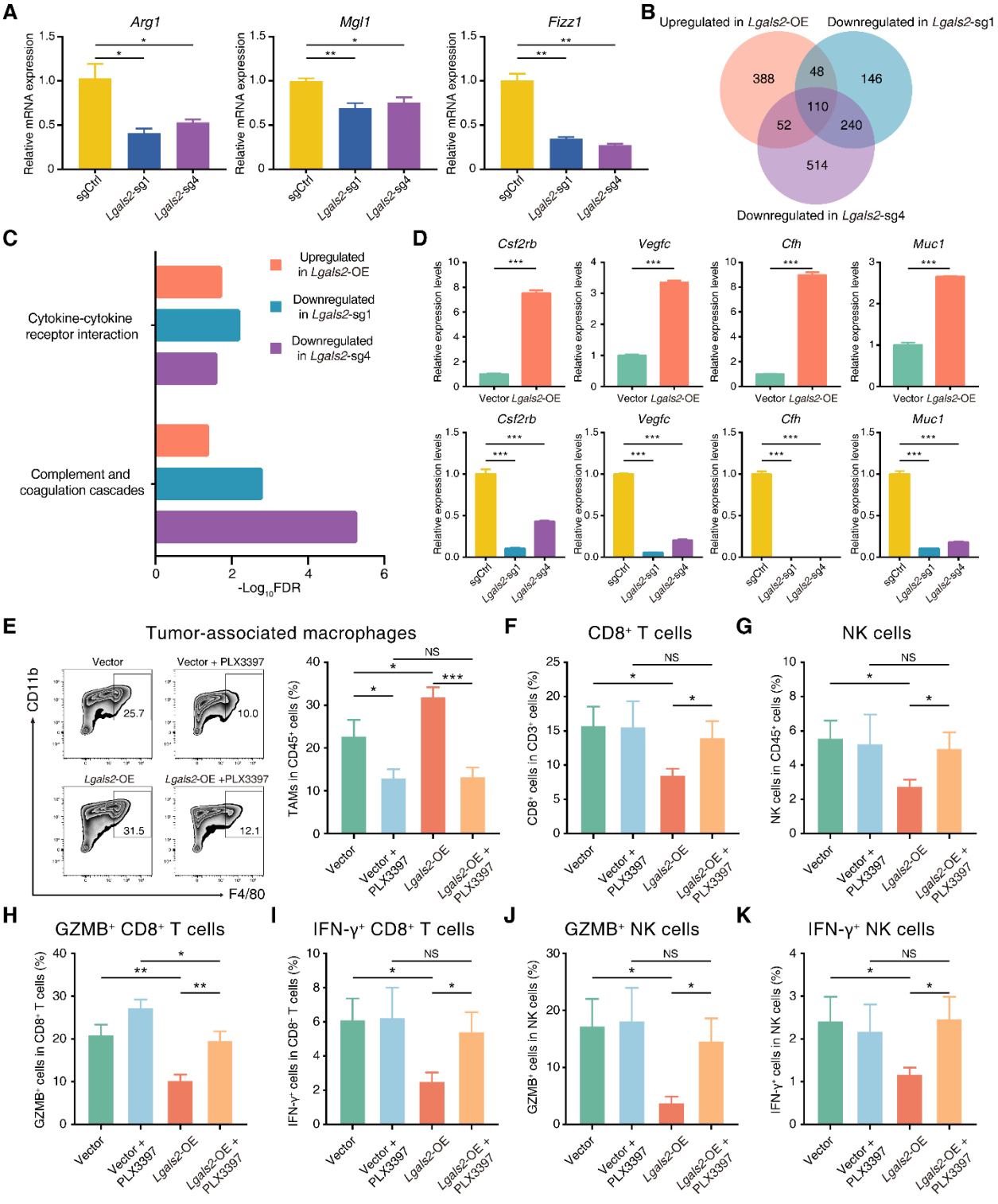
A
Arg1
Mgl1
Fizz1
Relative mRNA expression
sgCtrl
Lgals2-sg1
Lgals2-sg4
B
Upregulated in Lgals2-OE
Downregulated in Lgals2-sg1
Downregulated in Lgals2-sg4
388
48
146
110
52
240
514
C
Upregulated in Lgals2-OE
Downregulated in Lgals2-sg1
Downregulated in Lgals2-sg4
Cytokine-cytokine receptor interaction
Complement and coagulation cascades
-Log10FDR
D
Csf2rb
Vegfc
Cfh
Muc1
Relative expression levels
Vector
Lgals2-OE
E
Tumor-associated macrophages
Vector
Vector + PLX3397
Lgals2-OE
Lgals2-OE +PLX3397
CD11b
F4/80
25.7
10.0
31.5
12.1
TAMs in CD45+ cells (%)
F
CD8+ T cells
CD8+ cells in CD3+ cells (%)
G
NK cells
NK cells in CD45+ cells (%)
H
GZMB+ CD8+ T cells
GZMB+ cells in CD8+ T cells (%)
I
IFN-γ+ CD8+ T cells
IFN-γ+ cells in CD8+ T cells (%)
J
GZMB+ NK cells
GZMB+ cells in NK cells (%)
K
IFN-γ+ NK cells
IFN-γ+ cells in NK cells (%)
Vector
Vector + PLX3397
Lgals2-OE
Lgals2-OE + PLX3397
NS

Fig. S6. *Lgals2* facilitates M2-like polarization and proliferation of macrophages through CSF1.

(A) *Arg1*, *Mgl1* and *Fizz1* mRNA in mouse peritoneal macrophages co-cultured with *Lgals2*-knockout and vector control 4T1 cells for 72 hours were analyzed by qPCR. ** $P < 0.01$, * $P < 0.05$. Data are presented as the mean ± s.e.m.

(B) Venn diagram of significantly upregulated genes in *Lgals2* over-expressing 4T1 tumor cells and significantly downregulated genes in *Lgals2* knockout 4T1 cells *in vitro* from bulk RNA-seq.

(C) GO analysis of significantly upregulated genes in *Lgals2*-overexpressing 4T1 tumor cells and significantly downregulated genes in *Lgals2* knockout 4T1 cells *in vitro* from bulk RNA-seq.

(D) qPCR single-gene validation of representative significantly upregulated or downregulated genes, including *Csf2rb*, *Vegfc*, *Cfh* and *Muc1* in 4T1 cells. *** $P < 0.001$. Data are presented as mean ± s.e.m.

(E) After study endpoint, the $F4/80^+CD11b^+$ tumor-associated macrophages in 4T1 tumors of BALB/c mice from each group were measured by flow cytometry. Representative plots of individual tumors are shown on the left, and bar graphs of the summary data for all tumors are shown on the right. *** $P < 0.001$; * $P < 0.05$. Data are presented as the mean ± s.e.m.

(F-K) The primary tumors of 4T1 models from each group were harvested for flow cytometry to determine the percentages of (F) $CD8^+$ T cells among $CD3^+$ T cells, (G) NK cells among $CD45^+$ cells, (H) Granzyme B^+ ($GZMB^+$) cells among $CD8^+$ T cells, (I) IFN-γ^+ cells among $CD8^+$ T cells, (J) $GZMB^+$ cells among NK cells and (K) IFN-γ^+ cells among NK cells. ** $P < 0.01$; * $P < 0.05$; NS $P \geqslant 0.05$. Data are presented as the mean ± s.e.m.

Figure S7

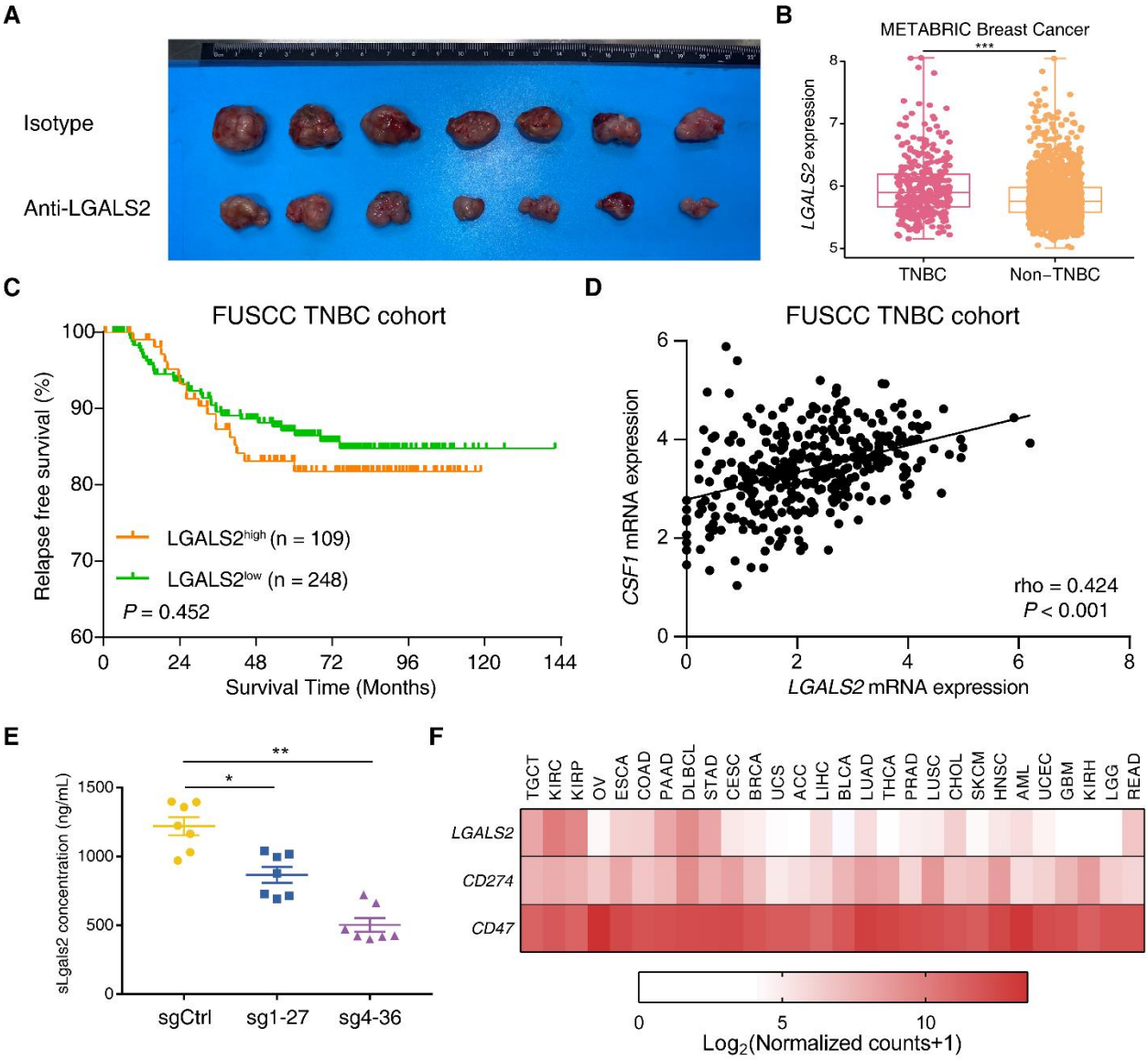

Fig. S7. Blockade of LGALS2 enhances antitumor immune response and shows immunotherapeutic potential of LGALS2 as a target in TNBC cohorts.

(A) Ex vivo images of resected 4T1 tumors receiving isotype or anti-LGALS2 antibody treatment from BALB/c mice.

(B) Box plot showing *LGALS2* mRNA expression level across TNBC and non-TNBC samples in the METABRIC cohort. Whiskers indicate the minimum and maximum values. *** $P < 0.001$.

(C) Kaplan-Meier curves of relapse-free survival between high and low IHC score of LGALS2 for TNBC in the FUSCC cohort.

(D) The association between *LGALS2* and *CSF1* mRNA expression levels in TNBC patient samples from FUSCC (n = 360).

(E) Comparison of serum LGALS2 concentration in the 4T1 transplanted BALB/c mice with either vector control or Lgals2-targeting sgRNAs. * $P < 0.05$, ** $P < 0.01$.

(F) Heat map of expression ($\log_2$[normalized counts + 1]) of *LGALS2* from bulk TCGA and TARGET studies, as compared to known immune checkpoint molecules *CD274* and *CD47*.

Table S1 (separate file). DrIM sgRNA library annotation.

Table S2 (separate file). δ scores of genes in DrIM sgRNA library.

Table S3 (separate file). Mini-DrIM sgRNA library annotation.

Table S4 (separate file). δ scores of different immune-selection pressures in mini-DrIM sgRNA library.

Table S5 (separate file). Eleven candidate genes related to tumor immune escape in TNBC.

Table S6 (separate file). Correlation between clinicopathologic variables and expression of LGALS2 in 357 cases of TNBC.

Table S7 (separate file). Abbreviations, full names, and number of tumor and healthy samples for TCGA, TARGET, and GTEX studies analyzed in this study.

Table S8 (separate file). Primers and sequences for qPCR.