Supporting Information

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SI Materials and Methods

Cloning and Mutagenesis of SMAD1, SMAD3, and SMAD5 3′ UTRs. 3′ UTR fragments cloned into pMIR vector (Ambion) are shown below; oligonucleotides used for PCR amplification and mutagenesis (QuikChange Site-Directed Mutagenesis Kit; Stratagene) are underlined; nucleotides within the seed sequence that were mutated are colored in red.

SMAD1. GCAGGACTTTGTGTACAGTTAAAGGAGAGATGG
CCAAGCCAGGGACAAATTGTCTATTAGAAAACGGTCC
TAAGAGATTCTTTGGTGTTTGGCACTTTAAGGTCATCG
TTGGGCAGAAGTTTAGCATTAATAGTTGTTCTGAAACG
TGTTTTATCAGGTTTAGAGCCCATGTTGAGTCTTCTTTT
CATGGGTTTTCATAATATTTTAAAACTATTTGTTTAGCGA
TGGTTTTGTTCGTTTAAGTAAAGGTTAATCTTGATGATA-
TACATAATAATCTTTCTAAAATTGTATGCTGACCATACTT
GCTG

SMAD3. CAGCATCCAGAAACACCAAACCAGGCTGGCTAAA-
CAAGTGGCCGCGTGTAAAAACAGACAGCTCTGAGTCAA-
ATCTGGGCCCTTCCACAAGGGTCCTCTGAACCAAGCCCC-
ACTCCCTTGCTAGGGGTGAAAGCATTACAGAGAGATGG-
AGCCATCTATCCAAGAAGCCTTCACTCACCTTCACTGCT-
GCTGTTGCAACTCGGCTGTTCTGGACTCTGATG

SMAD5. GTTCTTCAGTGTAATGTGACTTCATGCTATATATCT-
TTTGTAAGACATTTCCTTTTTTAAAAAAATTTTTGCAAAT-
AACTGATCTCAAGTATATGTCATTTACTCAAAATCTGT
CATAAGCATTACTTTATAGCTAGTGACAGTGCATGCA-
CAGCCTTGTTCAACTATGTTTGCTGCTTTTGGACAATGTT-
GCAAGAACTCTATTTTTGACATGCATTAATCTTTTATTT-
TGCACTTTTATGGGTGACAGTTTTTAGCATAACCTTTGA
TAAAATACACTCAAGTGACTTGGACTTAGATGCTTATCC
TTACGTCC

Electroporation of Anti-miR-155 and Anti-miR-Negative Control RNA Oligonucleotides (Ambion) in OCI-Ly3 and OCI-Ly10 DLBCL Cell Lines. Twenty-four hours before experiment started the culture media (20% FBS Iscove's modified Eagle's medium) was changed and cells maintained at 1×10^6 cells/mL. Immediately before electroporation the cells were washed twice in ice-cold PBS and 5×10^6 of cells resuspended in 500 μL of serum-free medium with 100 nM of relevant oligos in 4-mm cuvettes. Subsequently, electroporation was performed using a Bio-Rad Gene Pulser (Bio-Rad Laboratories) at 250 V, 975 μF, and ∞ resistance and cells kept on ice for 10 min, followed by culturing under usual conditions. At 24 h after electroporation the cells were harvested and RNA (quantification of miR-155) and protein (SMAD5 immunoblot) isolated. Experiments were performed in duplicate and repeated three times. Parallel assays using a similarly designed and chemically modified oligonucleotide, Cy3-labeled Anti-miR (Ambion), were performed for monitoring uptake of these miRNA inhibitors. FACS analysis consistently showed transfection efficiencies close to 70%, which was indirectly confirmed by the marked knockdown of miR-155 expression.

SMAD5 Immunoprecipation. Cell lysates from TGF-β1–treated (1 ng/mL for 1 h) and untreated DLBCL cell lines were incubated with anti-SMAD5 antibody (R&D Systems) or control goat Ig overnight, followed by incubation with protein A/G-Sepharose beads (1 h) at 4 °C. Immunoblotting of whole-cell lysate (input) and immunopreciptates was performed with anti-phospho SMAD1/5, anti-total SMAD1, or total-SMAD5 antisera.

Expression of TGF-β and BMP Receptors in DLBCL and Other Cell Lines—Semiquantitative RT-PCR. RNA was isolated from the Ly1, Ly18, Ly19, HaCaT, and A2058 (melanoma cell line) cell lines using TRIzol (Invitrogen), and cDNA generated with M-MLV Reverse Transcriptase and Random Primers (Promega). RT-PCR was performed as previously described (1), and products were visualized on an AlphaImager apparatus (Alpha Innotech).

Primers for the reactions were as follows:

ALK1F: 5′-CTGGCTCTGAGGCTAGCTGT-3′
ALK1R: 5′-TTGGGCACCACATCATAGAA-3′

ALK2F: 5′-GACGTGGAGTATGGCACTATC-3′
ALK2R: 5′-GAACCATGACTTCTCATCACG-3′

ALK3F: 5′-GGGGTCCGGACTTATGAAAA-3′
ALK3R: 5′-TCGGAGTCTGATTTCATCCC-3′

ALK4F: 5′-CTGACACCATTGACATTGCC-3′
ALK4R: 5′-GCACCCTAAGCACAGCTACC-3′

ALK5F: 5′-ACCCTTCATTAGATCGCCCT-3′
ALK5R: 5′-TCAAAAAGGGATCCATGCTC-3′

ALK6F: 5′-CTTGCGTTGTAAATGCCACC-3′
ALK6R: 5′-CCGACACTGAAAATCTGAGC-3′

ALK7F: 5′-CTGCTATTGCTCATCGAGAC-3′
ALK7R: 5′-CAACAGAATAGATGTCAGCTCG-3′

TBR2F: 5′-CCACCTGTGACAACCAGAAATC-3′
TBR2R: 5′-CTTTGGAGAAGCAGCATCTTCC-3′

BMPR2F: 5′-GTTGACAGGAGACCGTAAAC-3′
BMPR2R: 5′-CTTTTACAGCAACTGGACGC-3′

TBPF: 5′-TATAATCCCAAGCGGTTTGCTGCG-3′
TBPR: 5′-AATTGTTGGTGGGTGAGCACAAGG-3′.

TGF-β and BMP Signaling in Murine and Human Normal Mature B Cells. Isolation of murine mature B cells was performed as previously described (2). In brief, spleens were harvested from WT C57BL/6 mice. Subsequently, B cells were purified from the total mononuclear cell population using the Mouse B-Cell Enrichment Kit in the RoboSep Automated Cell Separator (Stem Cell Technologies). Purity and degree of enrichment was determined by FACS-based measurement of CD19-positive cells, in pre- and postseparation aliquots. These primary cells were cultured in B cell media (RPMI-1640 supplemented with 20% FBS, 100 μM β-mercaptoethanol, 10 mM Hepes, 2μM L-glutamine, 0.1% penicillin/streptomycin, 20 μg/mL LPS, and 2.5 ng/mL murine IL-4) and exposed to 5 ng/mL TGF-β, 200 ng/mL BMP2/4 (both for 1 h) or left untreated. Protein was harvested and immunoblots performed for phospho-SMAD1/5, total SMAD5, phospho-SMAD2, and total SMAD2. For isolation of human mature B cells, tonsils were obtained from patients undergoing elective tonsillectomy and processed as previously described (3). In brief, tonsillar mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation and depleted of non-B cells in a magnetic field after incubation with murine anti-CD3 (Zymed) and anti-CD11b (Coulter) and magnetic beads coated with sheep anti-mouse IgG. The purity of the isolated tonsillar B cells was greater than 95%, as

determined by subsequent immunostaining with anti-CD19 (Coulter). Subsequently, frozen aliquots of these cells were grown in B cell media (RPMI-1640 supplemented with 20% FBS, 100 μM β-mercaptoethanol, 10 mM Hepes, 2 μM L-glutamine, 0.1% penicillin/streptomycin, 20 μg/mL LPS, and 2.5 ng/mL human IL-4) and after overnight culture exposed to 5 ng/mL TGF-β, 200 ng/mL BMP2/4 (both for 2 h) or left untreated. Protein was harvested and immunoblots performed for phospho-SMAD1/5, total SMAD5, phospho-SMAD2, and total SMAD2.

RNAi-Mediated Knockdown of ALK2, ALK3, and ALK5. SiRNAs directed to these genes were obtained from Dharmacon (SMARTpool). For each gene, four independent sequences were included in pool. Next, 5×10^6 of the relevant DLBCL cell lines were electroporated with 200 nM of relevant oligos using a Bio-Rad Gene Pulser (Bio-Rad Laboratories) at 250 V, 975 μF, and ∞ resistance. At 72 h after transfection, an aliquot of cells was harvested for RNA isolation and subsequent real-time RT-PCR–based quantification of these genes' knockdown, in comparison with DLBCLs transfected with a control siRNA oligo (Dharmacon). The remaining cells were left untreated or exposed to TGF-β (1 ng/mL for 30 min), protein was isolated, and Western blotting performed with phospho and total SMAD5 antibodies.

siRNA oligos sequences:

TGFBR1 (ALK5):
Target sequence: GGAGUUGGAUUGCUGAAUU
Target sequence: GCUGAAAGUCUCUAUGUUA
Target sequence: CAGCUAGGCUUACAGCAUU
Target sequence: CUGAAAGUCUCUAUGUUAA

ACVR1 (ALK2):
Target sequence: GAACUGGGCAUUUCACGAA
Target sequence: GAAACGGAAUUGUACAACA
Target sequence: ACAGUUAGCUGCCUUCGAA
Target sequence: CCACGUGACACCACCGAAU

BMPR1A (ALK3):
Target sequence: GGACUCAGCUCUAUUUGAU
Target sequence: CAGCUACGCCGGACAAUAG
Target sequence: CGAGUGAUCCGUCAUACGA
Target sequence: CCGCAAUUGCUCAUCGAGA

Antibody-Mediated Blockade of TGF-β Receptors. Relevant DLBCL cell lines were culture in the presence of 50 μg/mL of the anti-human TGF-βRII antibody (R&D Systems; AF-241-NA) for 1 h. Subsequently the cells were exposed to TGF-β (0.25 ng/mL for 30 min), protein isolated, and Western blotting performed with anti-phospho and total SMAD5 antibodies.

Expression Analysis of *CDKN1A* (p21) and *MYC* After Exposure to TGF-β1. Relevant DLBCL cell lines were grown in 2% serum overnight and treated with TGF-β1 2.5ng/mL for 45 min (Ly1) and 90 min (Ly18). RNA was isolated and cDNA synthesized as we described (1). Real-time RT-PCR amplifications were performed in triplicate using the SYBR green method in the Bio-Rad iCYCLER real-time PCR detection system. The expression of target genes was normalized to that of house-keeping controls (Cyclophilin A and TATA-Binding protein, TBP), relative quantification was achieved using the ΔΔCT method and expression defined as $2^{-\Delta\Delta CT}$, where the untreated samples represented the baseline.

Oligonucleotides used were:

CDKN1AF: 5′-TGGAGACTCTCAGGGTCGAAA-3′
CDKN1AR: 5′-GGCGTTTGGAGTGGTAGAAATC-3′

c-MYCF: 5′-CAGCTGCTTAGACGCTGGATT-3′
c-MYCR: 5′-GTAGAAATACGGCTGCACCGA-3′

CyclophilinAF: 5′-CTCCTTTGAGCTGTTGCAG-3′
CyclophilinAR: 5′-CACCACATGCTTGCCATCC-3′

TBPF: 5′-TATAATCCCAAGCGGTTTGCTGCG-3′
TBPR: 5′-AATTGTTGGTGGGTGAGCACAAGG-3′.

Cell Cycle and Apoptosis Analyses After Exposure to TGF-β1. Cell cycle profile was determined by propidium iodide staining, followed by FACS analyses. Cells were harvested at 72 h (Ly1 and Ly19) or 96 h (Ly18), after exposure to TGF-β1: 2.0 ng/mL Ly1, 2.5 ng/mL Ly18, and 0.5 ng/mL Ly19. For apoptosis studies, the DLBCL cell line Ly18 stably expressing small hairpin (sh) vector control or two independent SMAD5 shRNA sequences (Ly18 sh-control, Ly18 sh-SMAD5#1, and Ly18 shSMAD5#3) were cultured in 2% RPMI with or without TGF-β (5 ng/mL) for 48 h. Subsequently, the cells were harvested and Annexin V staining and FACS-based measurements performed as previously described (4).

SMAD5-Specific shRNA Sequences. Complementary single-stranded oligonucleotides were combined, heated to 95 °C in Dharmacon's 5× siRNA Buffer [300 mM KCl, 30 mM Hepes (pH 7.5), and 1.0 mM MgCl2] and annealed at room temperature for 16 h. The pLTR-H1-eGFP vector was digested with BglII and SalI and double-stranded oligos cloned with standard techniques and sequencing confirmed.

Oligos sequences are as follows:

SMAD5 shRNA #1 target sequence: AGTCTTACCTCCAGTATTA

Top strand:
5′GATCTCCAGTCTTACCTCCAGTATTATTCAAGAGATAATACTGGAGGTAAGACTTTTTTGGAAG 3′

Bottom strand:
5′TCGACTTCCAAAAAAGTCTTACCTCCAGTATTATCTCTTGAATAATACTGGAGGTAAGACTGGA 3′
SMAD5 shRNA #3 target sequence: GATTCACAGATCCTTCAAA

Top strand:
5′GATCTCCGATTCACAGATCCTTCAAATTCAAGAGATTTGAAGGATCTGTGAATCTTTTTGGAAG 3′

Bottom strand:
5′TCGACTTCCAAAAAGATTCACAGATCCTTCAAATCTCTTGAATTTGAAGGATCTGTGAATCGGA 3′

Expression of SMAD5 Targets Genes in Primary DLBCLs and DLBCL Cell Lines—Real-Time RT-PCR and Microarray-Based Gene Expression Analysis. ***Real-time RT-PCR.*** RNA was isolated, cDNA synthesized, and real-time RT-PCR performed as described above. Putative SMAD5 (or SMAD1/5) direct targets (*ID1*, *ID2*, *ID4*, *BMPR2*, *BMPR1B*, *BMP4*, *BMP6*, *SMAD6*, and *SMAD7*) were identified from three independent studies (5–7) that used gene expression profiling to gain insight into the downstream events associated with SMAD1/5 activation, including (*i*) a zebrafish model of SMAD1- or SMAD5-specific knockdown used to investigate embryonic hematopoiesis; (*ii*) a HUVEC model of ectopic expression of constitutively active ALK1 or ALK5, to segregate TGF-β effects on SMAD2/3 vs. SMAD1/5; and (*iii*) a murine

model of SMAD1/5/8 knockout used to define the contribution of SMADs to granulosa cell tumors.

The genes selected for further analyses were present in more than one of the reports and/or were members of a family of genes with known association with SMAD5 signals (e.g., *ID*s, *BMP*s, *BMPR*s). Before measuring their expression in our primary tumors, for which limited amount of material was available, all RT-PCR conditions were optimized in a set of DLBCL cell lines. Of the nine genes listed above, *ID4*, *BMP4*, and *BMPR1B* were not expressed at substantial levels in the DLBCL cell lines analyzed and therefore were not used in primary tumors. Thus, we defined the expression of *ID1*, *ID2*, *BMPR2*, *BMP6*, *SMAD6*, and *SMAD7* in 15 primary DLBCLs and in the Ly18 DLBCL cell line ectopically expressing miR-155 or SMAD5-shRNA constructs.

Oligonucleotide sequences:

ID1F: 5′-CTGAACTCGGAATCCGAAG-3′
ID1R: 5′-AGGAACGCATGCCGCCTCG-3′

ID2F: 5′- CTGTCCTTGCAGGCTTCTGAATTC-3′
ID2R: 5′-CATGAACACCGCTTATTCAGCCAC-3′

ID4F: 5′-GCTCACTGCGCTCAACAC-3′
ID4R: 5′-CTCTTCCCCCTCCCTCTCTA-3′

BMPR2F: 5′-GTTGACAGGAGACCGTAAAC-3′
BMPR2R: 5′-CTTTTACAGCAACTGGACGC-3′

BMPR1BF: 5′-CTTGCGTTGTAAATGCCACC-3′
BMPR1BR: 5′-CCGACACTGAAAATCTGAGC-3′

BMP4F: 5′-CACTGGTCTTGAGTATCCTG-3′
BMP4R: 5′-GAGGAGATCACCTCGTTCTC-3′

BMP6F: 5′-TCTCCAGTGCTTCAGATTAC-3′

BMP6R: 5′-GAACCAAGGTCTGCACAATC-3′

SMAD6F: 5′-GTACAAGCCACTGGATCTGTC-3′
SMAD6R: 5′-CATGCTGGCGTCTGAGAATTC-3′

SMAD7F: 5′-CATCACCTTAGCCGACTCTG-3′
SMAD7R: 5′-GAAGTTGGGAATCTGAAAGCC-3′

Expression-based microarray analysis. To further characterize the effects of miR-155 on the expression of SMAD5 targets in DLBCLs, we used a well-annotated and publicly available independent dataset of primary DLBCLs, generated using Affymetrix U133A and U133B platforms (8). These data were normalized to achieve a mean of zero and SD of 1 using dChip software, as previously described (9). We next used a curated list of 18 putative SMAD5 target genes and the BIC gene (primary miR-155), totaling 27 unique probe sets, to interrogate the subset of DLBCLs with the highest and lowest primary miR-155 expression ($n = 88$), as previously described (1). Sample distribution was determined by hierarchical clustering, and the strength of the clusters was measured by the t-statistics function of dChip software. In brief, the t-statistic was calculated as (mean1 − mean2)/sqrt[SE(mean1)^2 + SE(mean2)^2], its P value computed according to the t-distribution, and the degree of freedom set according to Welch modified two-sample t test (10). Significance was set at $P < 0.05$. To confirm the statistical validity of hierarchical clustering, we used GSEA (version 2.0 with 1,000 permutations) (11). Therein, an enrichment score is calculated as a weighted Kolmogorov-Smirnov test. The significance of the enrichment score is estimated by a permutation test procedure adjusted for multiple hypotheses testing (normalized enrichment score, NES) and displayed as an FDR corresponding to each NES. Parameters were set for 1,000 permutations, and significance was defined as FDR < 0.10. The resulting heat map (clustering analysis) and enrichment plot (GSEA analysis) are shown in Fig. S8.

1. Rai D, Karanti S, Jung I, Dahia PL, Aguiar RC (2008) Coordinated expression of microRNA-155 and predicted target genes in diffuse large B-cell lymphoma. *Cancer Genet Cytogenet* 181:8–15.
2. Kim SW, Rai D, McKeller MR, Aguiar RC (2009) Rational combined targeting of phosphodiesterase 4B and SYK in DLBCL. *Blood* 113:6153–6160.
3. Aguiar RC, et al. (1999) PTPROt: An alternatively spliced and developmentally regulated B-lymphoid phosphatase that promotes G0/G1 arrest. *Blood* 94: 2403–2413.
4. Chen L, Juszczynski P, Takeyama K, Aguiar RC, Shipp MA (2006) Protein tyrosine phosphatase receptor-type O truncated (PTPROt) regulates SYK phosphorylation, proximal B-cell receptor signaling and cellular proliferation. *Blood* 108: 3428–3433.
5. Ota T, et al. (2002) Targets of transcriptional regulation by two distinct type I receptors for transforming growth factor-beta in human umbilical vein endothelial cells. *J Cell Physiol* 193:299–318.
6. Pangas SA, et al. (2008) Conditional deletion of Smad1 and Smad5 in somatic cells of male and female gonads leads to metastatic tumor development in mice. *Mol Cell Biol* 28:248–257.
7. McReynolds LJ, Gupta S, Figueroa ME, Mullins MC, Evans T (2007) Smad1 and Smad5 differentially regulate embryonic hematopoiesis. *Blood* 110:3881–3890.
8. Monti S, et al. (2005) Molecular profiling of diffuse large B-cell lymphoma identifies robust subtypes including one characterized by host inflammatory response. *Blood* 105:1851–1861.
9. Li C, et al. (2009) Copy number abnormalities, MYC activity, and the genetic fingerprint of normal B cells mechanistically define the microRNA profile of diffuse large B-cell lymphoma. *Blood* 113:6681–6690.
10. Li C, Wong WH (2001) Model-based analysis of oligonucleotide arrays: Expression index computation and outlier detection. *Proc Natl Acad Sci USA* 98:31–36.
11. Subramanian A, Kuehn H, Gould J, Tamayo P, Mesirov JP (2007) GSEA-P: A desktop application for Gene Set Enrichment Analysis. *Bioinformatics* 23:3251–3253.

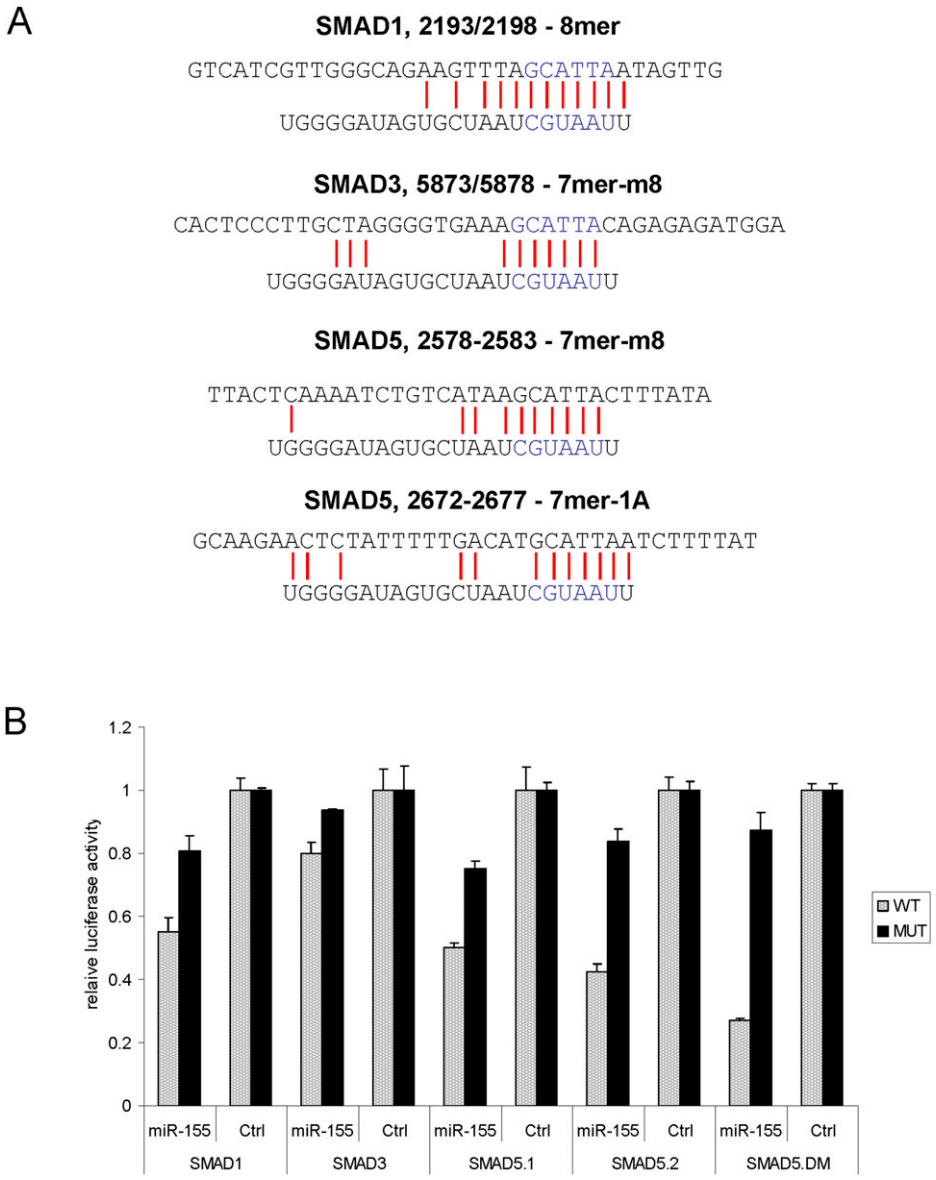

Fig. S1. Interaction between miR-155 and SMAD5. (*A*) Alignment of miR-155 sequences with the 3′ UTRs of the indicated *SMAD* genes. Seed sequence is highlighted in blue, nucleotide position in the 3′ UTR and seed region type, as defined by Lewis et al. [Lewis BP, Burge CB, Bartel DP (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 120:15–20], are also shown. (*B*) Luciferase reporter constructs containing the 3′ UTR of the indicated *SMAD* genes [WT or with point mutations on the putative miR-155 binding sites (MUT)] were cotransfected in HEK-293 cells with pre-miR-155 specific or control pre-miRNA oligos. MiR-155 did not significantly modify the activity of the *SMAD3* construct but inhibited the *SMAD5* constructs. The luciferase activity of both WT and MUT *SMAD1* constructs was decreased by miR-155, albeit more in the former. The lack of in vivo interaction between miR-155 and *SMAD1* was confirmed in DLBCL genetically modified to overexpress this miRNA (Fig. S2). Data shown are mean ± SD of the relative luciferase activity - ratio of intensity between premiR-155 and control oligos. SMAD5.1 and SMAD5.2 represents constructs with mutation in only one of the miR-155 binding sites; SMAD5.DM is double mutant.

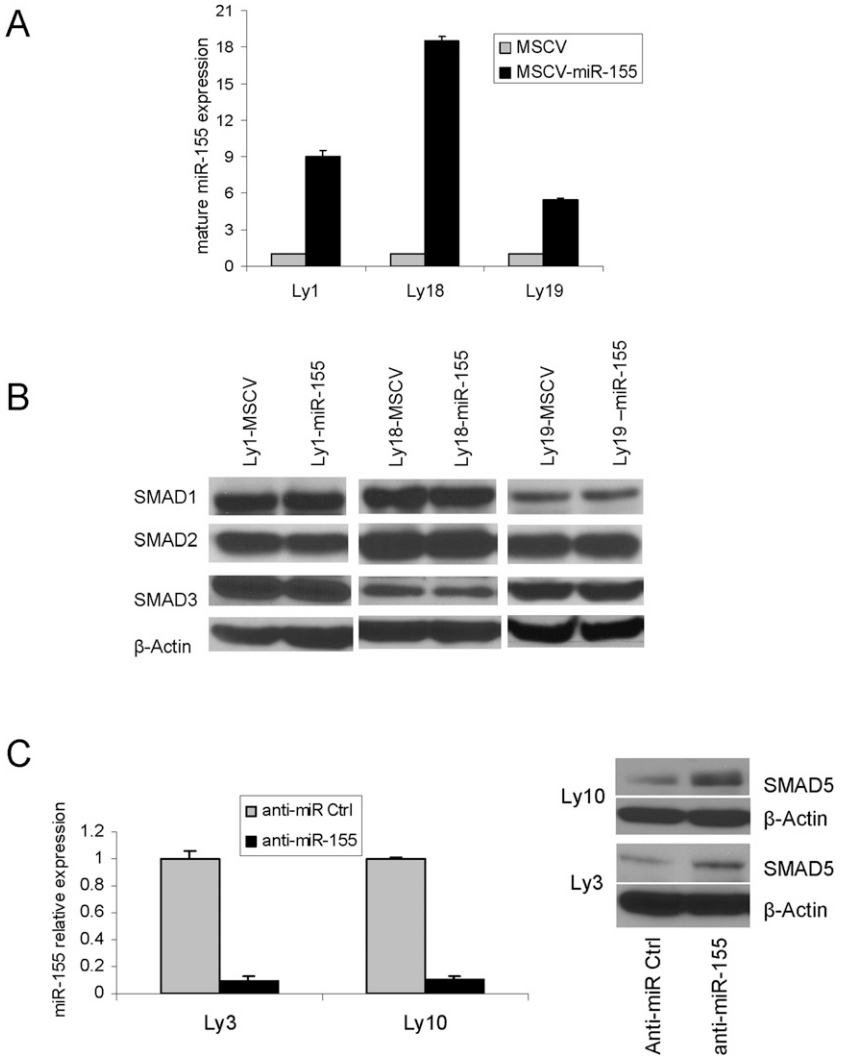

Fig. S2. Genetic modulation of miR-155 in DLBCL influences the expression of SMAD5 but not that of SMAD1/2/3. (*A*) Three independent DLBCL cell lines stably expressing primary miR-155 (MSCV-miR-155) or empty vector (MSCV) were generated, and overexpression of mature miR-155 confirmed with stem-loop real-time RT-PCR (TaqMan MicroRNA Assay; Applied Biosystems). Down-regulation of SMAD5 is shown in Fig. 1*B* in the main text. (*B*) Western blot analyses show that the expression levels of SMADS 1, 2, and 3 are not modified in the three DLBCL cell lines ectopically expressing miR-155. (*C*) Transient transfection of chemically modified anti-miR-155 or anti-miR-control oligonucleotides in two independent DLBCL cell lines expressing high endogenous levels of miR-155 resulted in decrease in miR-155 expression (stem-loop real-time RT-PCR; *Left*) and consequent elevation of SMAD5 expression (Western blot). In *A* and *C*, the expression of mature miR-155 was normalized to that U6 small nucleolar RNA, the quantification defined with the ΔΔCT method, and relative expression reported with the $2^{-\Delta\Delta CT}$ formula. Data shown are mean ± SD.

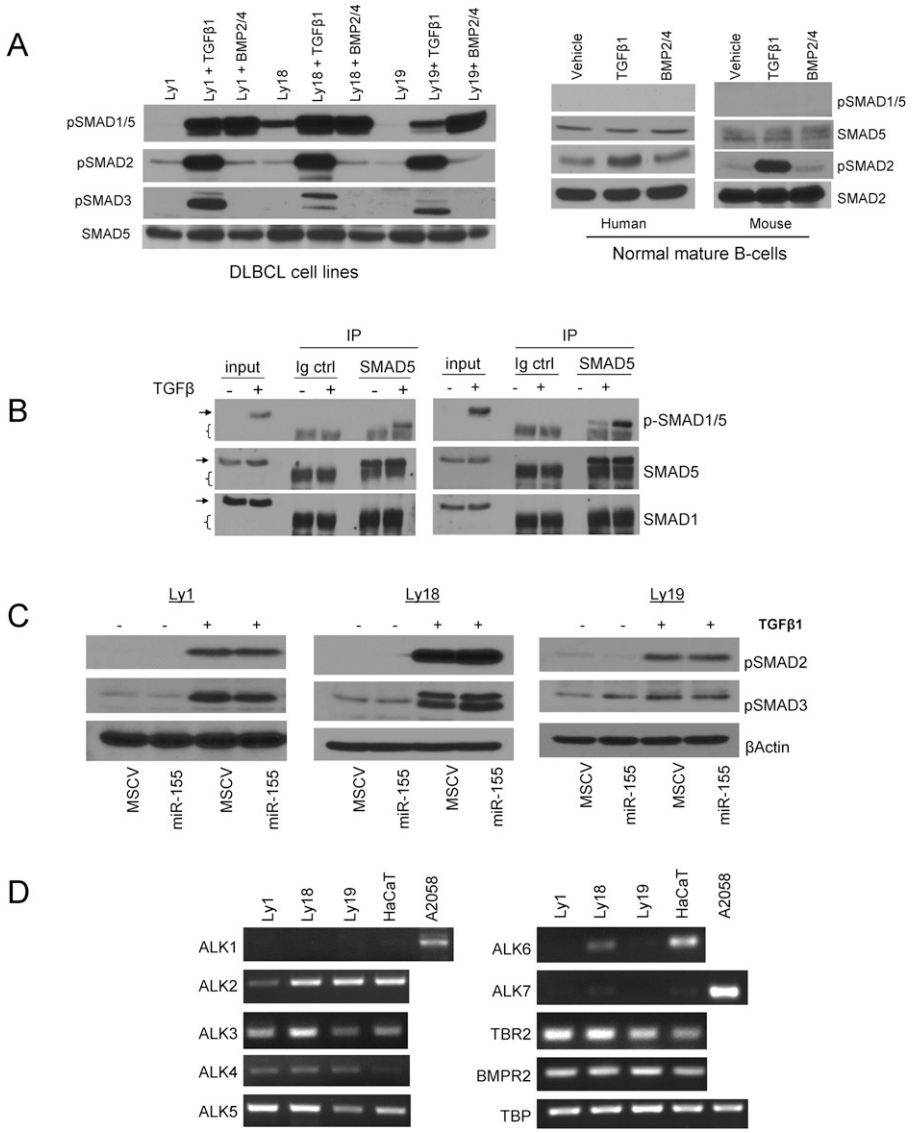

Fig. S3. Canonical and noncanonical TGF-β signaling in normal B-cells and DLBCLs (*A*) Western blot analyses of multiple DLBCL cell lines (*Left*) treated with TGF-β1 (1 ng/ml) or BMP2/4 (50 ng/ml). TGF-β1 induced SMAD2 and SMAD3 phosphorylation but, surprisingly, also SMAD1/5, whereas BMP signals activated only SMAD1/5. Total SMAD5 confirms that loading does not account for the observed results. Human and mouse normal mature B-cells (*Right*) were exposed to TGFβ (5 ng/ml) or BMP2/4 (200 ng/ml), and induction of SMAD1/5 or SMAD2 phosphorylation defined by western blotting. Total SMAD5 and SMAD2 expression confirm equal loading and demonstrate the significantly higher expression of SMAD2 in these cell populations (*B*) Immunoprecipation (IP) of total SMAD5 in whole cell lysate of the cell lines Ly1 (left) and Ly18 (right) followed by immunoblotting with p-SMAD1/5 antibody confirmed that TGF-β1 (1 ng/ml for 1 hour) signals through SMAD5 in these cells. Specificity of the IP was confirmed by the use of an Ig negative control (middle lanes) and by reprobing with SMAD5 or SMAD1 antibodies. The arrow indicates the phospho or total SMAD5 bands, and the bracket the Ig heavy chain. (*C*) Western blot analyses show that the phosphorylation of SMAD2/3 following TGF-β1 treatment (1 ng/ml for 1 hour) is not modified in three DLBCL cell lines ectopically expressing miR-155. (*D*) Semi-quantitative RT-PCR for type I and type II TGF-β and BMP receptors was performed in the cell lines Ly1, Ly18, Ly19, and, as controls, in the immortalized human keratinocyte HaCaT and A2058 (melanoma). The type II receptors, *TBR2* and *BMPR2*, were readily expressed in all DLBCL cell lines examined. Amongst the type I receptors, *ALK5* (TGFβ) and *ALK2* and *ALK3* (BMP) were the most prominently expressed. The *TBP* (Tata-binding protein) control confirms that all cDNAs are intact.

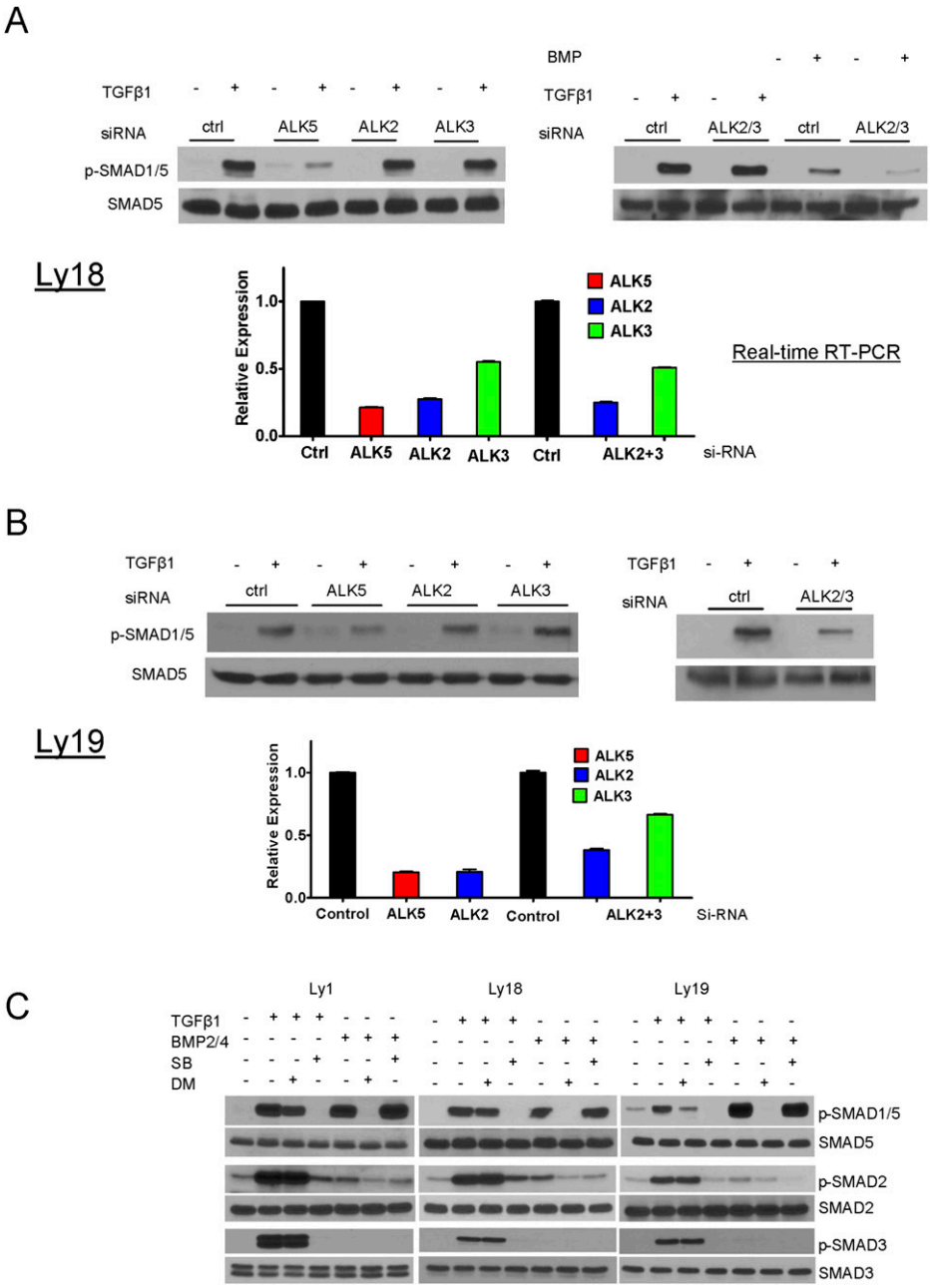

Fig. S4. Canonical and noncanonical TGF-β signaling in DLBCL: Role of TGF-β and BMP receptors. (*A* and *B*) Western blot analyses of phosho-SMAD1/5 levels after TGF-β1 activation in the Ly18 (*A*) and Ly19 (*B*) cell lines after transient transfection of a control siRNA, or ALK5, ALK2, ALK3, and combined ALK2/ALK3 RNAi sequences. ALK5 knockdown significantly blocked TGF-β1–mediated SMAD1/5 phosphorylation in both cell lines, whereas double ALK2/3 knockdown was effective in Ly19. Real-time RT-PCR measurement of the ALK genes confirms efficacy of siRNA strategy, and blockade of BMP signal demonstrates the functional relevance of ALK2/3 inhibition in Ly18 (*A*, *Right*). (*C*) Western blot analyses of phospho and total SMAD1/5, 2, and 3 levels in DLBCL cell lines exposed to TGF-β1 or BMP2/4, with or without pretreatment with the type I TGF-β or BMP receptor kinase inhibitors, SB-431542 (SB) and dorsomorphin (DM). The canonical TGF-β1 and BMP2/4 signals induce phosphorylation of SMAD2/3 and 1/5, which are readily inhibited by SB and DM, respectively. The noncanonical activation of SMAD1/5 by TGF-β1 was abolished by SB in all cell lines examined (lane 4 in each of the upper panels), whereas DM (lane 3) effects were more prominent in Ly19.

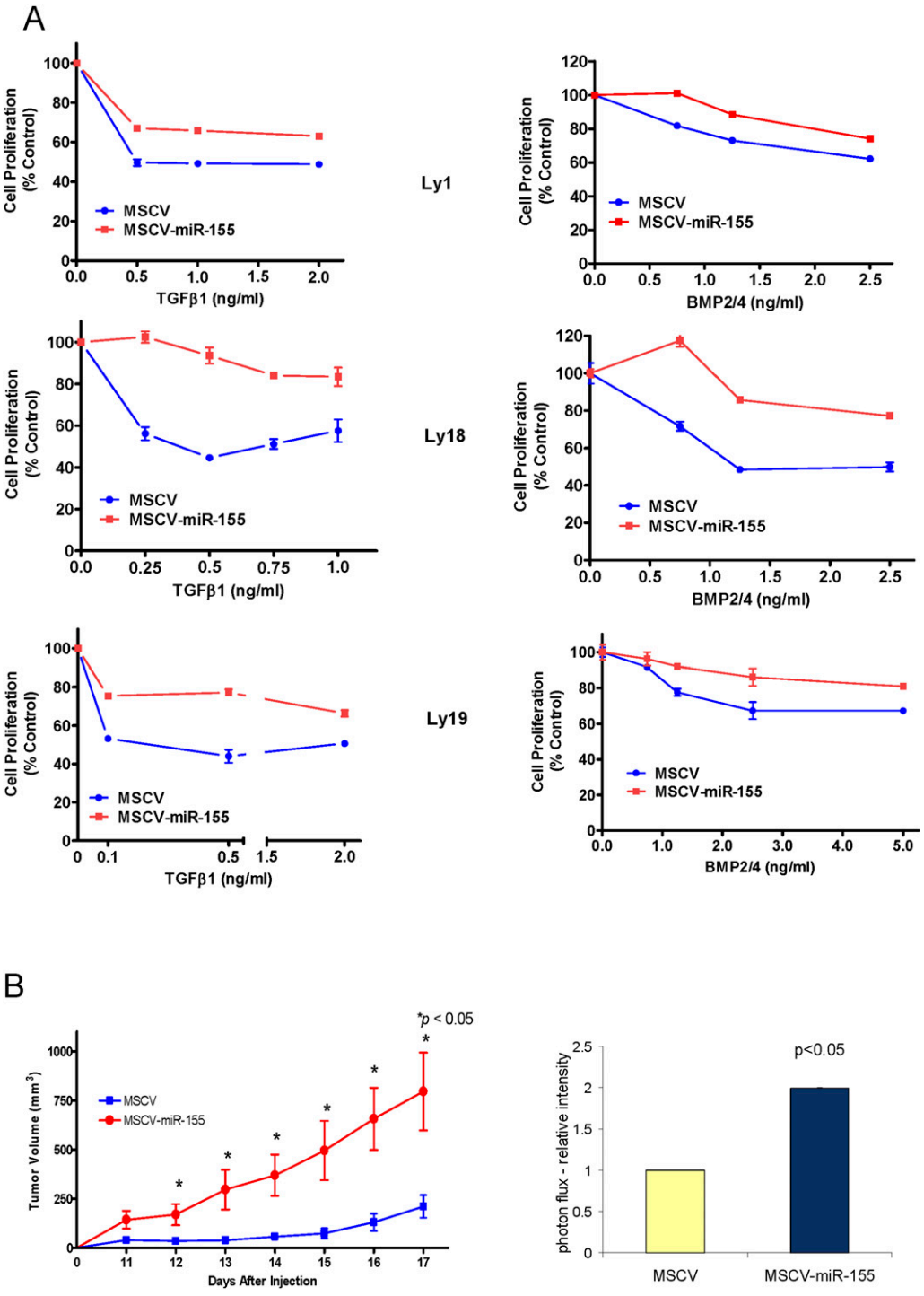

Fig. S5. MiR-155 expression limits the growth-inhibitory effects of TGF-β1 and BMP2/4 in vitro and promotes DLBCL growth and spread in vivo. (*A*) In vitro. The inhibitory effects of TGF-β1 (*Left*) or BMP2/4 (*Right*) on the proliferation of DLBCL cell lines genetically modified to overexpress miR-155 and their isogenic controls are shown. The multiple doses of TGF-β1 and BMP2/4 used are displayed on the *x* axis; TGF-β1 data for Ly1 were collected at 72 h, and for Ly18 and Ly19 at 96 h; BMP data for Ly1 and Ly18 were collected at 96 h, and at 72 h for Ly19. Expression of miR-155 significantly limited the growth-inhibitory activities of these cytokines in all cell line models analyzed, and at all tested doses ($P < 0.05$, Student's *t* test). Data shown are mean ± SEM of the percentage inhibition of cells exposed to TGF-β1 or BMP2/4 normalized by vehicle-treated cells. (*B*) In vivo. *Left:* Eight mice were inoculated s.c. with the isogenic Ly18 cells expressing miR-155 or an empty vector (MSCV). Tumors became visible 10 days after implantation and were measured daily thereafter with an electronic caliper. MiR-155–expressing cells consistently gave rise to larger tumors ($P < 0.05$, Student's *t* test). Data are represented as mean ± SEM. *Right:* Quantification of bioluminescence emitted (photon flux) by the tumors displayed in Fig. 4*A* in main text confirms that the miR-155–expressing DLBCLs grow larger and more widespread than their isogenic controls ($P < 0.05$, Student's *t* test). Data shown are mean ± SD of ratio of photon flux intensity detected in miR-155–expressing DLBCLs (MSCV-miR-155), normalized by signal obtained from MSCV-only tumors.

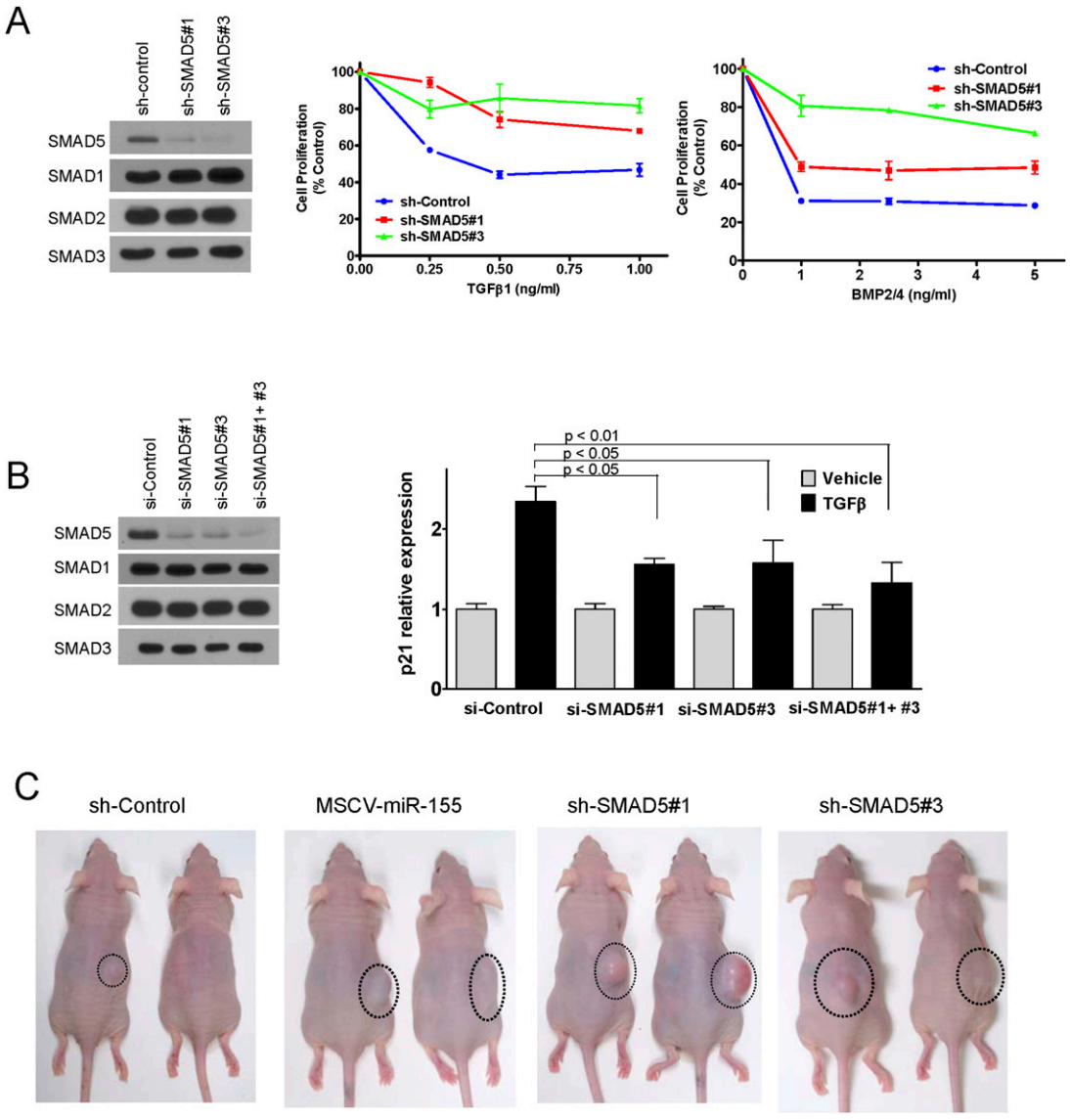

Fig. S6. SMAD5 knockdown recapitulates miR-155 overexpression in DLBCL. (*A*) Stable shRNA-mediated down-regulation of SMAD5 in the Ly18 cell line significantly blocked the growth-inhibitory effects of TGF-β1 and BMP2/4 at various doses ($P < 0.05$, Student's *t* test). Western blot (*Left*) confirms the down-regulation of SMAD5 but not of other R-SMADs. Data shown are mean ± SEM of the percentage inhibition of cells exposed to TGF-β1 or BMP2/4 normalized by vehicle-treated cells. Data were collected at 72 h. (*B*) SiRNA-based knockdown of SMAD5 significantly blocked the TGF-β1–medited (2.5 ng/mL for 90 min) induction of *p21* expression. Western blot (*Left*) confirms the down-regulation of SMAD5 but not of the other R-SMADs. Data shown are mean ± SEM of cells exposed to TGF-β1 normalized by vehicle-treated cells. (*C*) Ectopic expression of miR-155 or stable knockdown of SMAD5 expression enhances DLBCL growth in vivo. Sublethally irradiated mice were inoculated s.c. with Ly18 cells stably expressing two independent SMAD5 shRNA constructs, a shRNA-vector control, or miR-155. The mice shown are on day 22 after injection, and the tumors location is indicated.

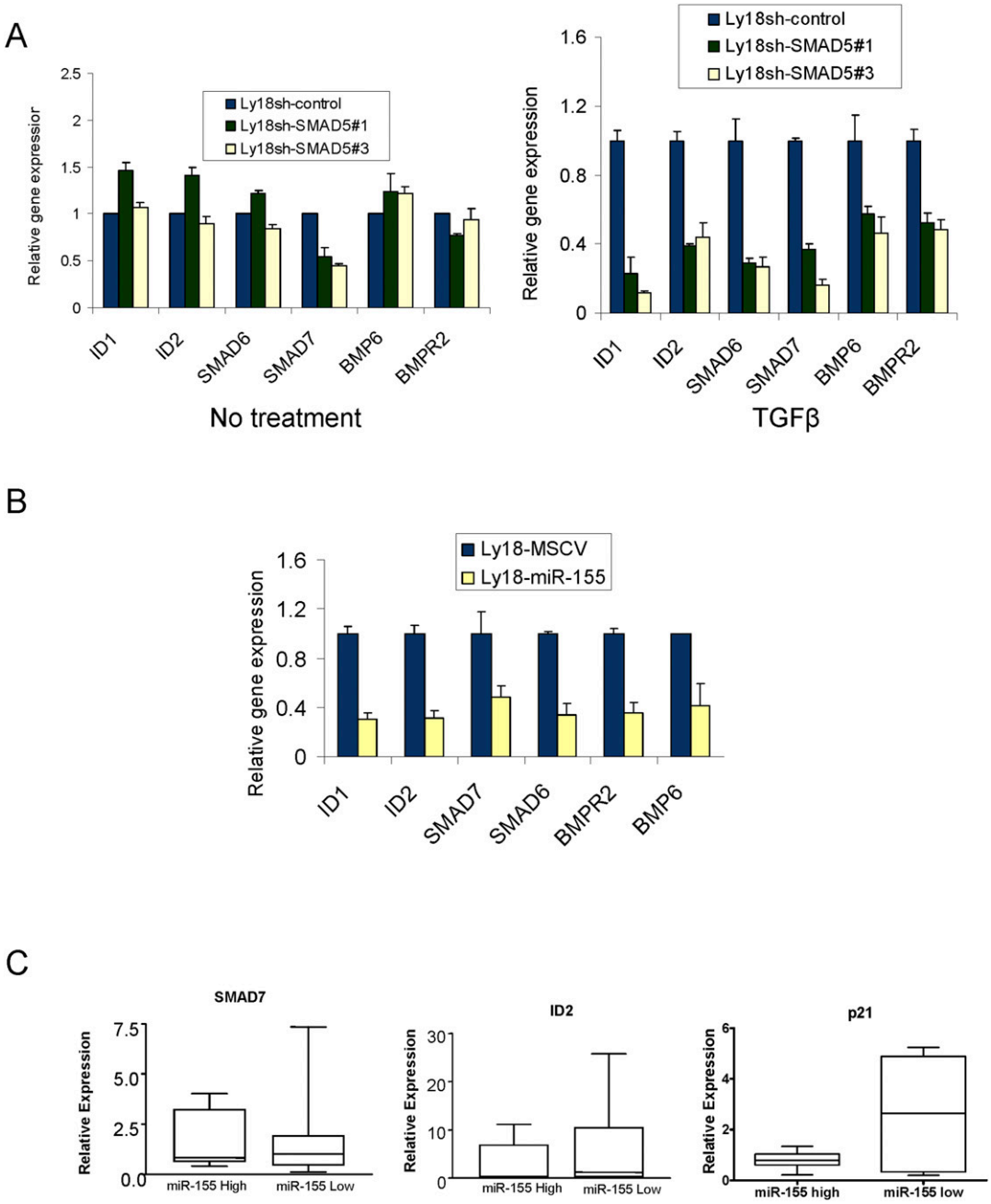

Fig. S7. SMAD5 knockdown or miR-155 expression blocks the TGF-β1–mediated induction of SMAD5 transcriptional targets. Quantitative real-time RT-PCR measurement of putative SMAD5 target genes (*ID1*, *ID2, BMP6, BMPR2, SMAD6*, and *SMAD7*) in the Ly18 cell line stably expressing SMAD5 shRNA constructs (*A*) or miR-155 (*B*). The TGF-β1–induced (2.5 ng/mL for 2 h) gene expression was significantly lower in SMAD5 knockdown and miR-155 cells when compared with their isogenic control counterparts ($P < 0.05$, Student's *t* test). Data shown are mean ± SD gene expression in cells exposed to TGF-β1 relative to vehicle-treated cells and normalized to sh-controls (*A*) or MSCV (*B*) cells. *A*, *Left*: Expression of these targets genes at baseline (i.e., without TGF-β treatment). (*C*) The mean expression of three putative SMAD5 transcriptional targets, *SMAD7* and *ID2*, was higher in primary DLBCLs with low levels of miR-155 than in tumors with high expression of this miRNA. However, these differences did not reach statistical significance. *Far Right:* Significantly different expression of *p21* ($P < 0.05$, two-sided Student's *t* test) in primary DLBCL with distinct miR-155 levels.

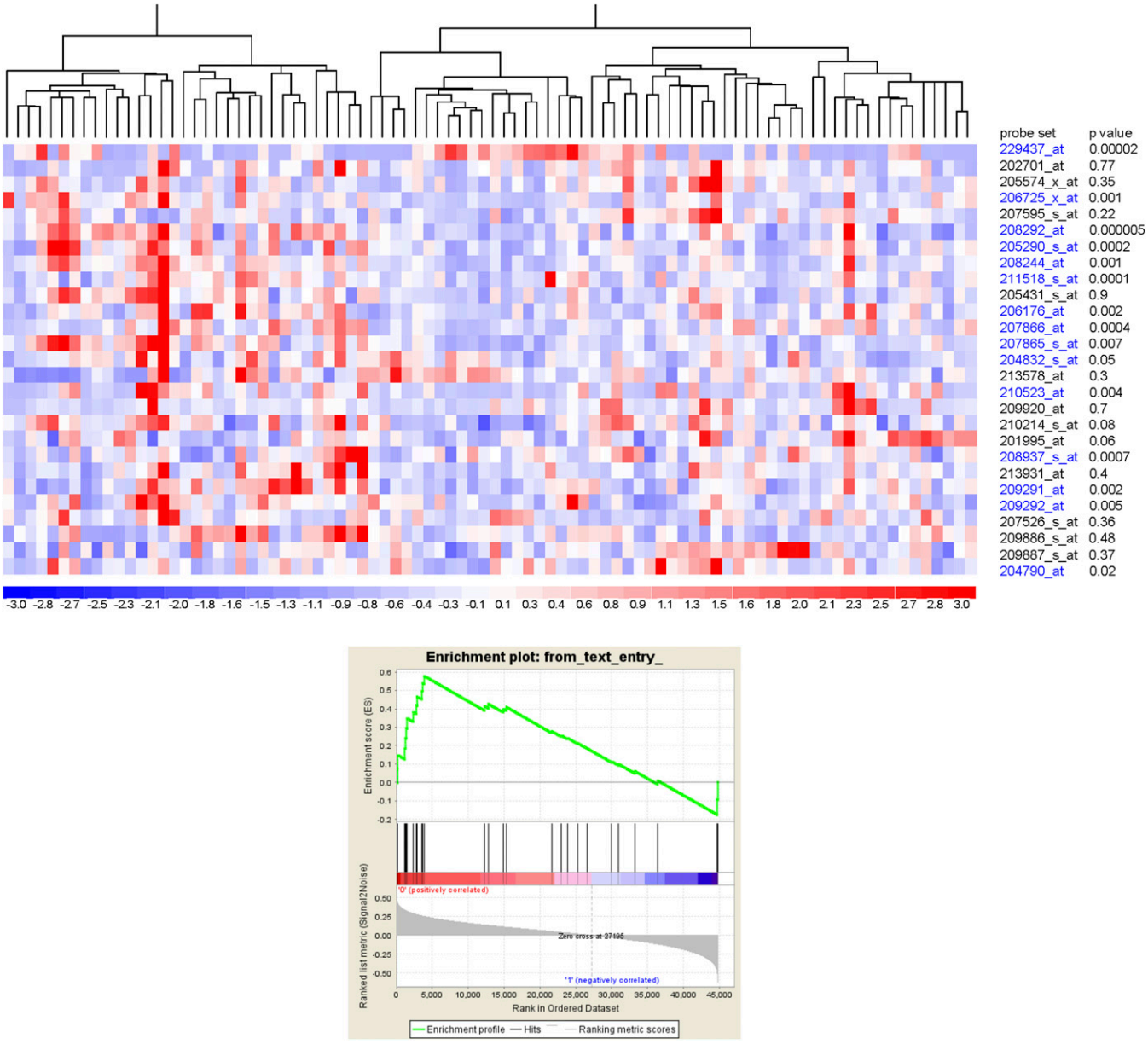

Fig. S8. Hierarchical clustering and Gene Set Enrichment Analysis (GSEA) GSEA of the correlation between miR-155 and SMAD5 target gene expressions in DLBCL. Expression of miR-155 drove the identification of three clusters predominantly characterized by (left to right): (*i*) low miR-155 expression and high expression of SMAD5 target genes; (*ii*) high miR-155 expression and low expression of SMAD5 targets, or (*iii*) low miR-155 expression and high expression of subsets of SMAD5 targets. In this analysis, the expression of approximately two thirds(13 of 19, 68%) of the putative SMAD5 targets studied (probesets labeled blue, and including *ID1*, *SMAD7, ID4, BMP1*, *BMP2*, *BMPRIA*, *BMPRIB, BMP3*, *BMP4*, *BMP6*, *BMP8a*, *BMP8b*, and *BMP10*) was significantly higher in DLBCLs with low miR-155 expression (clusters 1 and 3) than in those with abundant miR-155 expression ($P < 0.05$, *t* statistic; see *SI Materials and Methods* for details). The statistical significance of this association was confirmed with GSEA (*Bottom*). In this investigation, a statistically significant enrichment (nominal *P* value = 0.02, FDR q value = 0.02; see *SI Materials and Methods* for details) of putative SMDA5 target genes was found in DLBCLs expressing low levels of miR-155, as depicted in the enrichment plot (*Bottom*).

Table S1. Primary DLBCL characteristics

Tumor ID	Site	Age	Sex	Molecular classification*	Mature miR-155 relative expression
642	Stomach	61	Female	GCB	11.76
1326	Lymph node	48	Female	Non-GCB	4.29
2485	Gastroesophageal	78	Female	GCB	0.15
3271	Lymph node	70	Female	GCB	2.25
3279	Soft tissue, back	59	Male	Non-GCB	11.93
3409	Thyroid	76	Female	GCB	1.15
3522	Small bowel	49	Female	GCB	0.41
4221	Lymph node	74	Male	Non-GCB	0.11
4236	Axillary mass	65	Male	GCB	0.28
4350	Lymph node	65	Female	Non-GCB	1.91
4450	Lymph node	62	Male	GCB	0.13
5028	Stomach	72	Female	GCB	6.26
5081	Stomach	51	Male	GCB	0.15
5186	Colon	39	Female	GCB	17.95
5204	Stomach	51	Male	GCB	9.76
6614	Lymph node	66	Female	Non-GCB	0.14
6902	Lymph node	75	Male	Non-GCB	4.07
7089	Lymph node	61	Male	GCB	0.09
7572	Lymph node	60	Female	GCB	0.13
7924	Lymph node	78	Male	GCB	0.13

Based on the algorithm defined by Hans et al. [Hans CP, et al. (2004) Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. Blood 103:275–282]. GCB, germinal center B-cell-like OK.