Supplementary Methods

Drugs and reagents. Doxorubicin (Sigma), 5-aza-2'-deoxycytidine (decitabine, Sigma), azacitidine (Celgene), rituximab (Roche), cisplatin (Sigma), etoposide (Sigma), dexamethasone (Sigma), vincristine (Sigma), mechlorethamine (Sigma) were added from a concentrated stock solution to the 10% serum-containing culture medium. Drugs were dissolved in DMSO (Sigma) or distilled pure water. Decitabine and azacitidine were dissolved in distilled water and used within an hour of preparation. Recombinant human TGFβ1 (carrier-free) (BioLegend, San Diego, CA) and recombinant human BMP2 (BioLegend) were prepared in PBS with 1% BSA.

Gene expression profiling and data processing. Microarray were performed as previously published. Briefly, total RNA was extracted from 5 x 10^6 cells using the RNeasy Plus Mini kit (Qiagen) and reverse transcribed using the Superscript III (Invitrogen). Samples were labeled and hybridized to NimbleGen human arrays (Roche) according to the manufacturer's protocol. Pair files were processed with NimbleScan version 12.2.0. The trimmed mean target intensity of each array was set to 500.

Real-time qPCR. Total RNA was extracted from 5 x 10^6 cells with the use of the RNeasy Mini Plus kit (Qiagen) and eluted in RNAse-free water. cDNA was synthesized using high capacity RNA-to-cDNA kit (Applied Biosystems). Primer sequences are shown in Table S3. RT qPCR conditions and data analysis following ∆∆Ct method were published elsewhere. Data is presented as fold to control (vehicle treated cells).

Mice studies. The Research Animal Resource Center of the Weill Cornell Medical College of Medicine approved all mouse procedures. Adult (6 to 8 week-old, male, weighing average of 21 to 24 grams) SCID mice (CBBySmn.CB17-Prkdcscid/J) were purchased from the US National Cancer
Institute and subcutaneously injected in the left flank with $10^7$ low-passage human DLBCL cells (OCI-Ly7 and OCI-Ly1). Tumor size was recorded every other day using electronic digital calipers. We calculated tumor volume with the formula: tumor volume = (smallest diameter$^2$ x largest diameter) / 2.

Treatments were performed as described in the Results section. Drugs were dissolved in PBS and administered intraperitoneally. Mice were weighed every other day. C57BL/6 mice (NCI) were used for toxicity studies. At the end of the experiment, mice were killed by CO$_2$ inhalation or by cervical dislocation under anesthesia overdose.

**Clinical trial and patient samples.** A phase I study of epigenetic priming with azacitidine prior to R-CHOP in patients with newly diagnosed DLBCL was approved by the Institutional Review Board of Weill Cornell Medical College (ClinicalTrials.gov identification number NCT01004991). Patients were eligible if they had not received previous therapy, had preserved organ function, and did not have active viral hepatitis. Patients were treated with subcutaneous azacitidine daily for 5 days at escalating doses, followed by standard R-CHOP (rituximab 375 mg/m$^2$, cyclophosphamide 750 mg/m$^2$, doxorubicin 50 mg/m$^2$, vincristine 1.4 mg/m$^2$ capped at 2 mg, and prednisone 100 mg daily for 5 days), administered on day 8. Cycles were repeated every 21 days for 6 cycles. Hematopoietic growth factor was administered according to ASCO guidelines. Azacitidine dose escalation was undertaken according to a modification of the Continuous Reassessment Model called the TiTE-CRM. The TiTE-CRM method assumes a simple model for the probability of a DLT as a function of the combination, and uses the occurrence of toxicities in the patients enrolled in the trial to sequentially determine which combination to allocate to a new patient. New subjects are continuously recruited without pauses for complete follow-up of currently-enrolled subjects. Azacitidine doses were escalated using the following dose levels: dose level 1: 25 mg/m$^2$ daily; dose level 2: 50 mg/m$^2$ daily; dose level 3: 75 mg/m$^2$ daily. Dose limiting toxicity (DLT) was defined as grade 3 or greater non-hematologic toxicity or grade 4 hematologic toxicity lasting more than 7 days in the setting of myeloid growth factor use. Neutropenic fever was considered DLT only if grade 4. Responses were defined according to the Revised Response Criteria for Malignant Lymphoma. Tumor and blood samples for correlative studies were obtained prior to initiation of treatment and
after cycle one of azacitidine, prior to initiation of R-CHOP.

**Ex vivo treatment.** We obtained single cells suspensions from lymph node biopsies by physical disruption of tissues followed by cell density gradient separation (Fico/Lite LymphoH, Atlanta Biologicals). CD19 cells were positively selected from the mononuclear cell suspension using CD19 magnetic beads and an auto-MACS cell separator (Myltenyi Biotech). Cell number and viability were determined by a dye-exclusion method (Easycount, Immunicon). Primary DLBCL cells were cultured in 96-well chambers featuring two compartments separated by a 0.4 μm-porous polyester membrane (Corning Inc.). The lower chamber contained HK dendritic cells to support the growth of DLBCL cells contained in the upper chamber. Cells were grown in advanced RPMI medium with 10% human serum supplemented with antibiotics, L-glutamine and HEPES for 48 h. For long-term culture (96 h) IL-4 1ng/ml was added to the culture medium. For the 48 h experiment, primary cells were seed in HK conditioned medium for 2 h followed by to 2 h of drug (or vehicle) exposure and transferred to the co-culture system for the remaining of the experiment. Cells were treated with single drugs (doxorubicin, rituximab) or combination of drugs (CHOP) or vehicle in 4 replicates. For the 96 h sequential experiment, primary cells were seed in HK conditioned medium for 2 h followed by to 2 h of DAC 100 nM (or vehicle) exposure and transferred to a co-culture 6-well system for 48 h. After 48 h DAC and vehicle treated cells were counted and exposed to doxorubicin or vehicle for 2 h and transferred to a co-culture 96-well plate system for the remaining of the experiment. At the end of the experiment, viability was determined by using an ATP-based luminescent method (CellTiter-Glo, Promega) and dye-exclusion based method (Easycount, Immunicon).

**Immunohistochemistry (IHC).** IHC was performed as previously described with modifications for the phospho-SMAD1 antibody (rabbit polyclonal anti-human SMAD1 phosphorylated at Ser463/465, Cell Signaling). Briefly, antigen retrieval in individual samples and tissue microarray (TMA) samples were performed in alkaline buffer pH: 9 (Target Retrieval Solution, Dako) at 95°C (in an steamer) for 15 minutes. Cases were blinded scored by two researchers. Individual de-identified cases and cases
from the clinical trial were from the Weill Cornell Medical College - New York Presbyterian Hospital and TMAs were from the British Columbia Cancer Agency.

Statistical analysis. Analysis was performed using the R open source Statistical software version 2.11.1 and Prims version 5.0d. To determine the genes differentially expressed and methylated between sensitive and resistant cell lines to doxorubicin we computed the fold-change (difference in logs of means sensitive – resistant) of normalized arrays (after filtering for probes with a standard deviation greater than 0.5 for gene expression arrays). Publicly available datasets were used to determine SMAD1 gene expression (GSE10846 and Ref.5) and methylation (GSE2396) in DLBCL patients. The DLBCL GSE10846 gene expression database contains information of 233 patients, 201 of them classified as ABC (n = 93) vs. GCB (n = 108) and 152 contained IPI information (69 ABC vs. 83 GCB). The DLBCL GSE10846 gene expression database contains information of 233 patients, 201 of them classified as ABC (n = 93) vs. GCB (n = 108); while the Ref5 gene expression database contains information of 119 patients (34 ABC vs. 85 GCB). The methylation database GSE10846 contains information of 69 patients, 57 of them classified as ABC (n = 17) vs. GCB (n = 40). The HELP assay was also performed in a dataset of 231 DLBCL patients from the British Columbia Cancer Agency. The percent of SMAD1 methylation was determined from the probeset MSPI0406S00245619 as previously published6.

References


