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Supplemental Information

Wilms' Tumor Blastemal Stem Cells Dedifferentiate to Propagate the Tumor Bulk

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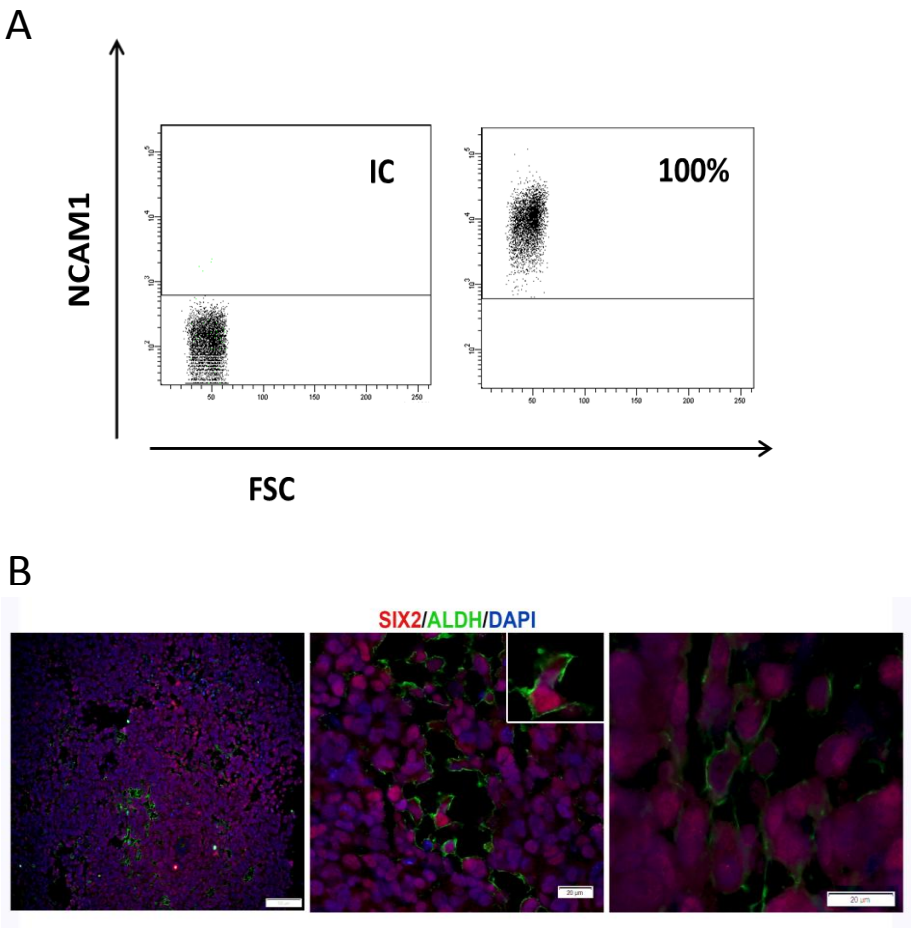
Supplemental figures and figure legends

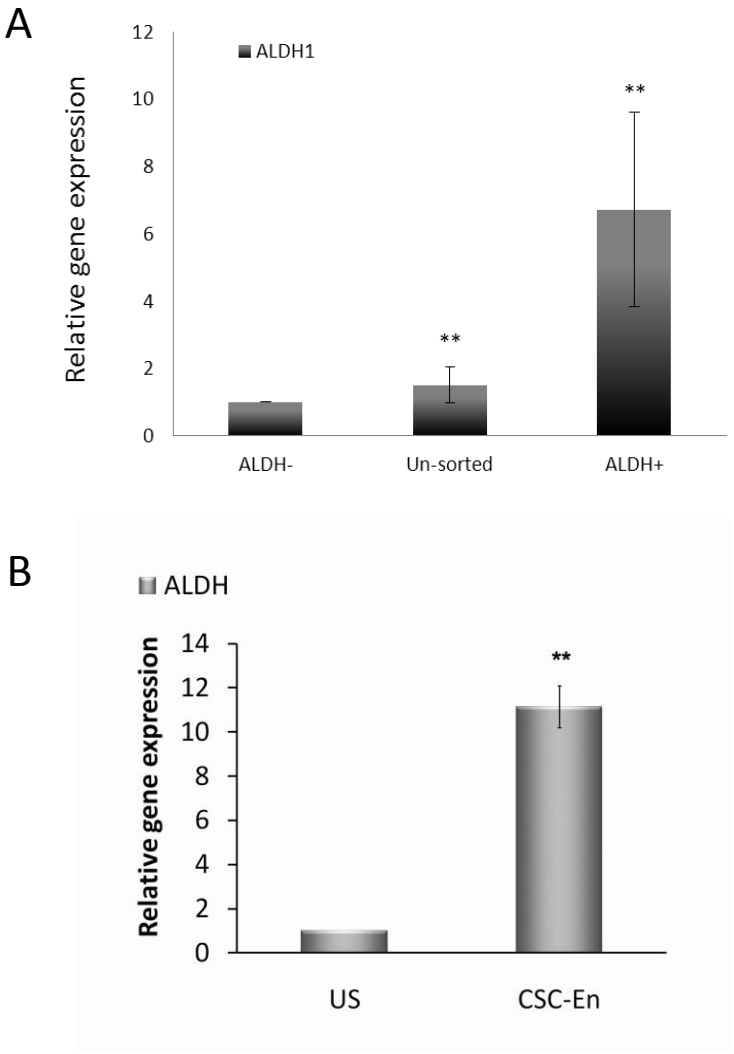
Figure S1: NCAM1, SIX2 and ALDH1 expression in WT Xn



(A) All late WT Xn cells express high levels of NCAM1. FACS analysis of late WT Xn cells, demonstrating high expression of NCAM1 in all tumor cells, emphasizing that late Xn are composed solely of blastemal cells. IC, Isotype control.

(B) SIX2 and ALDH1are both expressed by the WT CSC. Immuno-fluorescent staining of pure-blastema WT Xn for SIX2 (red), ALDH1 (green) and DAPI nuclear staining (blue). All the tumor cells are SIX2+ while ALDH1+ cells are scattered within these blastemal sheets. Scale bar; 100µm (left panel), 20µm (middle and right panels).

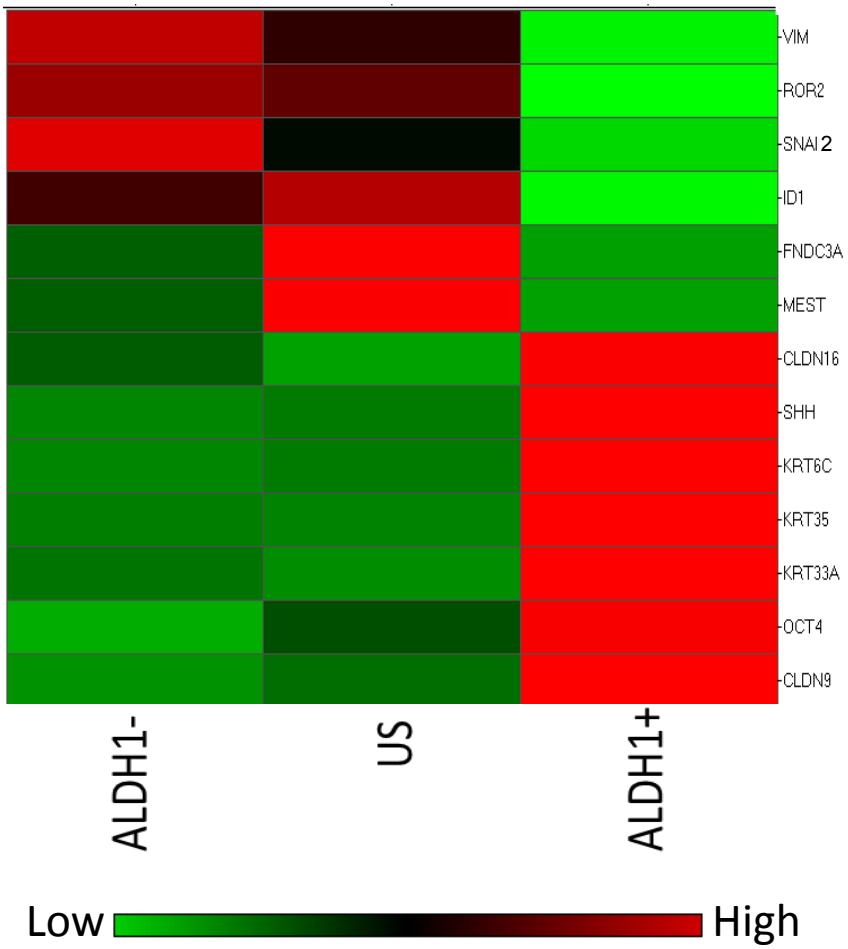
Figure S2: Validation of ALDH1 gene expression levels



(A) Validation of ALDH1 FACS-sorting quality. Quantitative real-time PCR analysis of *ALDH1* expression levels following FACS-sorting according to ALDH1 enzymatic activity, confirming high efficiency enrichment of ALDH1+ cells. The values for ALDH1- cells were used to normalize (therefore=1) and all other values were calculated with respect to them. Results are presented as the mean ± S.E.M of three separate experiments; **, $p<0.01$

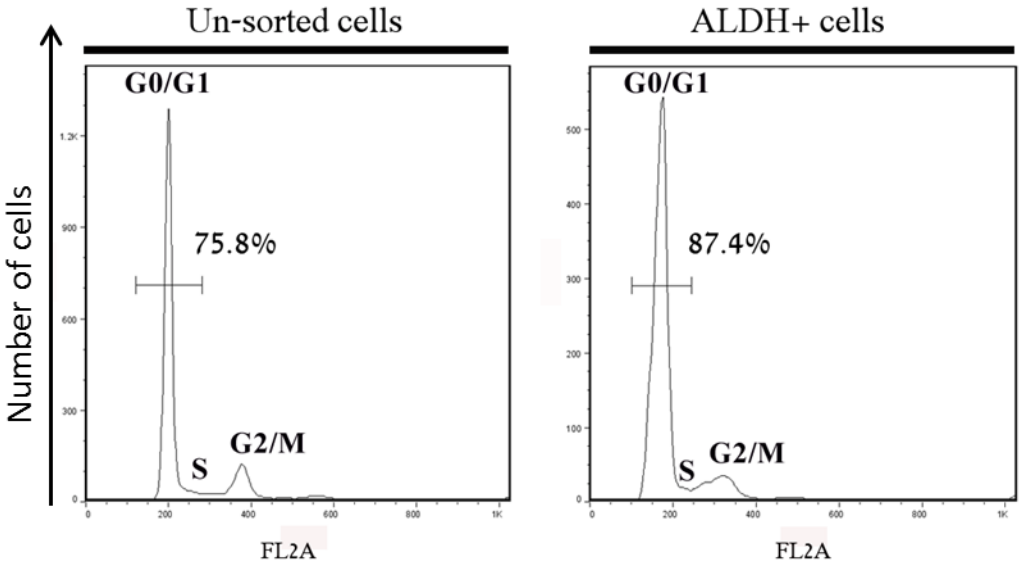
(B) Validation of ALDH1 expression in the different Xn models. Validation via qRT-PCR of ALDH1 expression in un-sorted derived (US) and ALDH1+ derived (CSC-En) Xn, showing up-regulation in the latter. Results are presented as the mean ±SEM of experiments obtained from three different patients; ** $p<0.01$

Figure S3: microarray gene expression analysis



Microarray gene expression analysis of WT CSCs. Representative Heat map of differentially expressed genes between: 1. sorted ALDH+ cells, 2. sorted ALDH- cells and 3. Un-sorted Xn cells (US). This comparison revealed a unique profile of the ALDH+ CSCs including high levels of stemness genes (*OCT4 and SHH*), along with high expression of epithelial differentiation markers (*E-Cadherin, Keratin 6c,33a,35 and Claudine 7,9,13*) and lower levels of mesenchymal markers (*Vimentin, SNAI2, MEST*).

Figure S4: ALDH+ WT CSC present a quiescent nature



Reprehensive flow cytometric cell cycle analysis revealed that the ALDH1+ fraction demonstrated a higher percentage of cells in the G0/G1 phase compared to unsorted cells (87.4% and 75.8%, respectively), further supporting their quiescent nature.

Supplemental tables

Table S1| Proteomic analysis of ALDH1+ CSC enriched WT Xn:

Proteins up-regulated in ALDH1$^+$ CSC enriched WT xenografts		
Function/Pathway WT cells	**Gene symbol**	**Protein name**
MET (Epithelial)	*CDH1, EGFR*	E-Cadherin; EGFR_pY1068, EGFR
Stemness (self renewal, hESC)	*NOTCH1*	Notch1
NFκB/Akt pathway	*AKT1, AKT2 AKT3; NFKB1*	Akt_pS473; NF-kB-p65_pS536;
Wnt/beta catenin pathway	*GSK3A, GSK3B*	GSK3-alpha-beta
PI3K/PKC pathway	*PIK3CA; PIK3R1; PRKCA*	PI3K-p85; PI3K-p110-alpha; PKC-alpha
mTOR signaling pathway	*TSC1*	TSC1
Onco-proteins	*RAF1; SRC; EGFR; CCNB1*	C-Raf; Src_pY527, Src; EGFR; EGFR_pY1068; Cyclin_B1
Tumor suppressor proteins	*RB1; SMAD3;*	Rb_pS807_S811 ; TAZ
Proteins down-regulated in ALDH1+ CSC enriched WT xenografts		
Function/pathway	**Gene symbol**	**Protein name**
MET (Mesenchymal)	*COL6A1; SNAI2*	Collagen VI; Snai2
Endothelial	*PECAM1*	CD31
DNA repair genes	*RAD50*	Rad50
Proliferation	*PCNA*	PCNA
Onco-proteins	*YAP1, ; WWTR1 RAB25; MET*	YAP; WWTR1; Rab25; c-Met_pY1235
Tumor suppressor proteins	*CAV1*	Caveolin-1

Shown are up-regulated (upper panel) and down-regulated (lower panel) proteins, according to their ascribed function.

Table S2| 10 most up- and down-regulated genes in CSC-En Xn in comparison to un-sorted derived Xn and human fetal kidney (hFK):

10 most up-regulated genes in CSC-En Xn in comparison to un-sorted derived Xn and human fetal kidney (hFK):

	Gene Symbol	Gene Title	Un-derived Xn	CSC-En Xn	hFK
1	*GPR89B/C*	G protein-coupled receptor 89B/C	7.9	98.5	15.4
2	*GFRAL*	GDNF family receptor alpha like	7.7	57.9	4.1
3	*C6orf115*	chromosome 6 open reading frame 115	5.3	37.7	42.0
4	*COL6A5*	collagen, type VI, alpha 5	10.2	44.4	4.1
5	*IGHV7-81*	immunoglobulin heavy variable 7-81	5.2	18.0	4.6
6	*THBS4*	thrombospondin 4	62.8	211.5	10.4
7	*FOXF1*	forkhead box F1	4.6	14.7	4.7
8	*SNORA33*	small nucleolar RNA, H/ACA box 33	4.8	14.9	9.5
9	*SHH*	sonic hedgehog	5.7	16.1	5.3
10	*XKR9*	XK, Kell blood group complex subunit, member 9	157.5	429.8	4.8

10 most down-regulated genes in CSC-En Xn in comparison to un-sorted derived Xn and human fetal kidney (hFK):

1	*PCBP4*	poly(rC) binding protein 4	99.0	22.1	28.3
2	*NPY*	neuropeptide Y	361.0	109.0	5.3
3	*LGALS1*	lectin, galactoside-binding, soluble, 1	30.3	9.2	333.2
4	*GTF2B*	general transcription factor IIB	109.3	37.3	129.4
5	*ALDOA*	aldolase A, fructose-bisphosphate	22.6	7.8	152.5
6	*MANF*	mesencephalic astrocyte-derived neurotrophic factor	27.9	10.4	176.1
7	*UGP2*	UDP-glucose pyrophosphorylase 2	30.6	11.4	221.0
8	*RNF20*	ring finger protein 20	12.5	4.9	98.3
9	*ENO1*	enolase 1, (alpha)	416.7	164.1	227.7
10	*ETV5*	ets variant 5	215.5	85.7	13.4

Table S3| 10 most up- and down-regulated genes in ALDH+ in comparison to ALDH- and un-sorted Xn cells:

10 most up-regulated genes in ALDH+ in comparison to ALDH- and un-sorted Xn cells:

	Gene Symbol	Gene Title	ALDH+	ALDH-	US
1	*BNIP3L*	BCL2/adenovirus E1B 19kDa interacting protein 3-like	106.0	7.8	11.2
2	*HSD17B8*	hydroxysteroid (17-beta) dehydrogenase 8	51.9	5.2	4.2
3	*NUDT16*	nudix-type motif 16	52.7	5.6	53.9
4	*ACPL2*	acid phosphatase-like 2	41.3	4.7	4.9
5	*PABPC1L2A*	poly(A) binding protein, cytoplasmic 1-like 2A	65.0	7.7	5.1
6	*TRIM33*	tripartite motif-containing 33	151.6	18.2	91.4
7	*LOC100128185*	PNAS-19	58.8	7.4	4.5
8	*C6orf25*	chromosome 6 open reading frame 25	38.3	5.7	16.7
9	*GP1BA*	glycoprotein Ib (platelet), alpha polypeptide	30.0	4.8	5.5

10 most down-regulated genes in ALDH+ in comparison to ALDH- and un-sorted Xn cells:

1	*DAOA-AS*	DAOA antisense RNA (non-protein coding)	7.0	191.2	144.0
2	*PTTG1/3P*	pituitary tumor-transforming 1/3	23.2	569.4	826.7
3	*CTRB2*	chymotrypsinogen B2	5.9	143.9	188.5
4	*IGSF21*	immunoglobin superfamily, member 21	8.8	170.0	153.2
5	*DNMT3L*	DNA (cytosine-5-)-methyltransferase 3-like	16.8	320.2	292.2
6	*NME1*	non-metastatic cells 1, protein (NM23A)	6.6	122.4	176.6
7	*CHST1*	carbohydrate (keratan sulfate Gal-6) sulfotransferase 1	9.1	160.7	268.4
8	*ANKRD36*	ankyrin repeat domain 36	5.5	92.1	81.3
9	*ECHDC3*	enoyl CoA hydratase domain containing 3	18.6	309.6	453.3
10	*LETMD1*	LETM1 domain containing 1	11.5	184.3	182.1

Supplemental experimental procedures

Ethics statement

This study was conducted according to the principles expressed in the Declaration of Helsinki and was approved by the Institutional Review Boards of Sheba, Hadassah-Ein Kerem and Asaf Harofeh Medical Centers.

In vivo xenograft formation

Initial WT xenografting to 5-8 weeks old, female, nonobese diabetic immunodeficient mice v mice was performed as previously described (15). Briefly, primary WT tissue was cut into 2-5mm pieces and implanted subcutaneously in the back of the mouse. Tumors were harvested approximately 3-6 month post implantation or when they reached a size of 1.5cm diameter. Single cells suspensions were obtained by mincing the samples in Iscove's modification of Dulbecco's medium (IMDM) containing antibiotics (penicillin and streptomycin), followed by treatment with collagenase IV for 2h at 37◦C. Enzymatically treated tissue was triturated using IMDM at twice the volume of the collagenase solution and the suspension filtered (100μm cell strainer) and washed twice with IMDM containing antibiotics. Erythrocytes were removed by ACK RBS lysis buffer.

Late passages Xn were formed by serial injection of approximately 106 dissociated cells from freshly retrieved WT Xn. Cells were injected in 100μl 1:1 serum free medium/Matrigel (BD Biosciences, San Jose, CA).

Fluorescence-activated cell sorting (FACS) analysis

FACS analysis of fresh WT cells was performed as previously described (15). Small tumor pieces were dissociated into single cells, washed in RBCs lysis solution (comprised of: 8.3g NH_4Cl, 1.0g $KHCO_3$, 1.8ml of 5% EDTA in double distilled H_2O) at 1ml/5ml cell suspension ratio was applied for 2min in 4C◦. Cells were then filtered through a 30μm nylon mesh before final centrifugation.

Assessment of the percentage of NCAM1 surface marker:

All cells were re-suspended in FACS buffer consisting of 0.5% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis) and 0.02% sodium azide in PBS. NCAM1 Surface antigen was labeled by incubation with fluorochrome conjugated antibody (eBioscience, San Diego, CA) at a concentration of 1µg antibody per 106 cells for 30min, in the dark, at 4°C to prevent internalization of antibodies. In addition, we used 7-amino-actinomycin-D (7AAD; eBioscience, San Diego, CA) for viable cell gating. All washing steps were performed in FACS buffer. All Quantitative measurements were made in comparison to IgG isotype antibody (eBioscience, San Diego, CA).

Assessment of the percentage of cells with high ALDH1 enzymatic activity

Detection of cells with high ALDH1 enzymatic activity was performed using the ALDEFLUOR kit (StemCell Technologies, Durham, NC, USA) as previously described (28, 29). Cells were suspended in Aldefluor assay buffer containing BODIPY-aminoacetaldehyde (BAAA), an uncharged ALDH1 substrate followed by incubation for 30-45 min at 37°C, in the dark. BAAA is taken up only by living cells through passive diffusion and then converted intracellular by ALDH1 into BODIPY-aminoacetate, a negatively charged reaction product, which is retained inside cells expressing high levels of ALDH1, resulting in these cells becoming brightly fluorescent. The fluorescent of these ALDH1 expressing cells (ALDH1+) can be detected by the green fluorescence channel (520-540 nm) of the FACSAria (BD Biosciences, San Jose, CA). As a negative control, for each sample of cells an aliquot treated in the same conditions was additionally incubated with diethylaminobenzaldehyde (DEAB), a specific ALDH1 inhibitor. Incubation of cells with the BAAA without the addition of DEAB resulted in a shift in BAAA fluorescence defining the ALDH1+ population. Since only cells with an intact cellular membrane could retain the Aldefluor reaction product, only viable ALDH1+ cells were identified.

FACS sorting

Cells were harvested as described above, filtered through a 30µm nylon mesh before final centrifugation, and then re-suspended in either in a FACS buffer or in an

ALDEFLUOR buffer. When WT Xn cells were sorted, possible contaminating mouse cells were eliminated from cells by discarding H2K+ (with an anti-H2Kd antibody - Miltenyi Biotech, Germany,). FACSAria was used in order to enrich for cells expressing surface markers and ALDH1 high activity. A 100-µm nozzle (BD Biosciences, San Jose, CA), sheath pressure of 20–25 pounds per square inch (PSI), and an acquisition rate of 1,000–3,000 events per second were used as conditions optimized for WT cell sorting. Single viable cells were gated on the basis of 7AAD, and then physically sorted into collection tubes for all subsequent experiments. Data was additionally analyzed and presented using FlowJo software.

Chip Array

The chip array data is deposited in publicly library (GEO); accession numbers GSE57269. All experiments were performed using Affymetrix HU GENE1.0st oligonucleotide arrays (Rivera and Haber, 2005). Total RNA from each sample was used to prepare biotinylated target DNA, according to the manufacturer's recommendations. The target cDNA generated from each sample was processed as per manufacturer's recommendation using an Affymetrix Gene Chip Instrument System. Details of quality control measures can be found online. Significantly changed genes were filtered as changed by at least twofold (p-value: 0.05). See also Supplemental Experimental Procedures.

Quantitative Real Time reverse transcription PCR analysis – Gene expression analysis

Quantitative reverse transcription PCR (qRT-PCR) was carried out to determine fold changes in expression of a selection of genes. Total RNA from cells was isolated using an RNeasy Micro Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. cDNA was synthesized using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, California USA) on total RNA. Real-time PCR was performed using an ABI7900HT sequence detection system (Perkin-Elmer/Applied Biosystems, California, USA) in the presence of TaqMan Gene Expression Master Mix (Applied Biosystems, California, USA). PCR amplification was performed using gene specific TaqMan Gene Expression Assay-Pre-Made kits (Applied Biosystems, California, USA). Each analysis reaction was performed in

triplicate. HPRT1 or GAPDH were used as an endogenous control throughout the experimental analyses. PCR results were analyzed using SDS RQ Manager 1.2 software. Statistical analysis was performed using a non-paired 2-tails T-test. Statistical significance was considered at $P<0.05$.

Reverse phase protein array (RPPA)

Phosphorylated and unphosphorylated components of cell signaling pathways were quantified by RPPA as previously described (Tibes R, et al. Reverse phase protein array: validation of a novel proteomic technology and utility for analysis of primary leukemia specimens and hematopoietic stem cells. Mol Cancer Ther. 2006;5(10):2512–2521.). Briefly, protein extracts were prepared from un-sorted and ALDH1+ derived WT Xn. Following quantification, extracts were denatured and diluted in 5 serial 2-fold serial dilutions, which were then arrayed on multiple slides along with positive and negative controls prepared from mixed cell lysates or dilution buffer. Each slide was probed with a validated primary antibody and with a biotin-conjugated secondary antibody. After staining, slides were scanned, and spot intensities were analyzed, quantified, and normalized.

H&E staining

H&E staining of paraffin-embedded tissues: 5µm sections of paraffin-embedded tissues were mounted on super frost/plus glass and incubated at 60ºC for 40 minutes. After deparaffinization, slides were incubated in Mayer's Hematoxylin solution (Sigma-Aldrich) and incubated with 1% HCl in 70% ethanol for 1 minute. Slides were then incubated for 10 seconds in Eosin (Sigma-Aldrich). Images were produced using Olympus BX51TF.

Immunohistochemical staining of primary WT and WT Xn.

Sections, 4-µm thick, were cut from primary WT and WT Xn for immunohistochemistry. Immunostainings were performed as previously described (9). Brifly, the sections were processed within 1 week to avoid oxidation of antigens. Before immunostaining, sections were treated with 10mM citrate buffer, PH 6.0 for 10 min at 97ºC for antigen retrieval, followed by 3% H2O2 for 10 min. The slides were subsequently stained using the labeled strepavidin-biotin (LAB-SA) method using a Histostain plus kit (Zymed, San Francisco, CA, USA). Anti human NCAM

antibody (LifeSpan Biosciences, Inc. Seattle, WA, USA) was used at a dilution of 1:250. The immunoreaction was visualized by an HRP-based chromogen/substrate system (liquid DAB substrate kit – Zymed, San Francisco, CA, USA).

Western Blot

PWT, hFK and WT Xn cells were lysed in a buffer containing 50mM Tris-HCl, 150mM NaCl, 0.1mM EDTA, 0.5mM DTT, 1.5mM MgCl, 0.5% Triton-X. Total protein concentration was determined using the BCA protein assay kit (Thermo Scientific, Kalamazoo, MI). Proteins (100μg) were denatured in laemmli sample buffer (Bio-rad, Hercules, CA) (5 min, 950 C) and then separated on SDS-10% polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose membranes, which were then blocked overnight at 4°C, with 5% nonfat dry milk .Membranes were then incubated with anti-SIX2 (Affinity Bioreagents, Golden, CO, 1:200) / anti-E-Cadherin (Cell Signaling technology, #3195, 1:500) / anti VIMENTIN (abcam, #ab8978, 1:100) primary antibodies for 60 min and later with, goat anti-rabbit IgG secondary antibodies for 60 min. The membrane was reacted with ECL substrate (Thermo scientific) and then exposed to medical x-ray film (Fuji) for detection of antibody-bound protein bands.

IF staining of cells

Cells were fixed with 2% PFA with 3% sucrose in PBS (for SIX2 primary antibody) or with 4% PFA (for MNF and E-cadherin) for 10 min, and washed with PBS. Cells were blocked with 5% human serum, 5% donkey serum and 1% BSA in PBS–Tween (0.05%) followed by incubation a primary antibodies for SIX2 (Novus, cat# aa264-277), MNF (Dako, cat# M0821) or E-cadherin (Cell Signaling, cat#24E10) for 1 h in room temperature. Cells were washed and then incubated with a secondary antibody for 1 h in room temperature. Mounting containing DAPI (Dapi Fluoromount-G; SouthernBiotech, #0100-20) was applied. Images were obtained by Olympus BX51 fluorescence microscope using Olympus DP72 camera and cellSens standard software.