## Impact of *Sox11* over-expression in Ba/F3 cells

*Martin Lord,[1] Gustav Arvidsson,[2] Agata M. Wasik,[1] Birger Christensson,[1] Anthony P. Wright,[2] Alf Grandien[3] and Birgitta Sander[1]*

*[1]Department of Laboratory Medicine, Division of Pathology, Karolinska Institutet and Karolinska University Hospital; [2]Department of Laboratory Medicine, Clinical Research Center, Karolinska Institutet and [3]Department of Medicine, Center for Hematology and Regenerative Medicine (HERM), Karolinska Institutet, Stockholm, Sweden*

*Correspondence: birgitta.sander@ki.se*
*doi:10.3324/haematol.2018.197467*

# Supplementary Information

## Impact of Sox11 overexpression in Ba/F3 cells

Martin Lord[1)], Gustav Arvidsson[2)], Agata M. Wasik[1)], Birger Christensson[1)], Anthony P. Wright[2)], Alf Grandien[3)] and Birgitta Sander[1)]

1) Department of Laboratory Medicine, Division of Pathology, Karolinska Institutet and Karolinska University Hospital, Stockholm, Sweden

2) Department of Laboratory Medicine, Clinical Research Center, Karolinska Institutet, Stockholm, Sweden

3) Department of Medicine, Center for Hematology and Regenerative Medicine (HERM), Karolinska Institutet, Stockholm, Sweden

## Complete Materials and Methods

### PCR amplification and cloning

Mouse full CDS fragments encompassing specific cloning sites with a Kozak sequence were generated from purified plasmids containing the mouse *Sox11* gene (Q7M6Y2, plasmid kindly provided by Dr. Veronique Lefebvre, Dept. of Cell Biology, Cleveland clinic, Cleveland, OH, USA). PCR fragments were prepared by AccuPrime PCR kit (Invitrogen) following manufacturers recommendations. Amplified fragments were purified using GeneJET PCR Purification Kit (Thermo Scientific) and double restricted at 37°C for 5 min by Fast Digest restriction enzymes (MluI/MluI, Thermo Scientific) to generate complementary ends before introduced into the retroviral pSIREN-cPPT-TRE-PGK1-rtTA2-IRES-YFPZeoR vector.[1] Restricted fragments and vectors were purified on gel using GeneJET Gel Extraction Kit (Thermo Scientific) and DNA estimated on gel before ligation. Insert and vector were ligated at room temperature by T4 ligase (Thermo Scientific) according to manufacturer's protocol. Before electroporation into TOPO10 electrocompetent bacteria, ligated samples were again purified using GeneJET PCR Purification Kit (Thermo Scientific). After electroporation bacteria were inoculated on a shaker in SOC medium for 1 h at 37°C. Bacteria solution was subsequently plated on agar plates supplemented with 100 µg/ml carbenicillin overnight (O/N) at 37°C. Isolated colonies were picked and inoculated O/N at 37°C in LB-medium with 100 µg/ml carbenicillin. Plasmids were purified by GeneJET Plasmid Miniprep Kit and subjected to restriction analysis. Clones were verified using BigDye 3.1 terminators (Applied Biosystems) and Sanger sequencing. Plasmids intended for transfection were maxi prepped using EndoFree Plasmid Maxi Kit (Qiagen).

### Virus preparation and transduction of Ba/F3 cells

For virus production, phoenix cells (293T) were maintained in complete DMEM (10% FCS, 2mM L-glutamine, 1 mM Na-pyruvate, 50 U/ml penicillin, 50 µg/ml streptomycin, 500 µl 2-mercaptoethanol (Gibco) and $2.5x10^6$ cells were seeded in a 6 cm dish the day prior transfection. Transfections were carried out in OptiMEM (Gibco) using 10 µg DNA and 20 µl Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol and incubated O/N, 37°C 5% $CO_2$. After 24 h incubation, medium was replaced with DMEM complete medium O/N. Virus was concentrated 30X by ultracentrifugation

O/N at 6000 x g. Ba/F3 cells,[2] were maintained in RPMI supplemented with 10% FBS and 5% IL3 (supernatant from transfected X63 cells as described by Karasuyama et al.[3]) Cells ($1x10^6$) were transduced in two rounds using 50 µl concentrated virus (45 min, 2000 rpm) together with polybrene (Sigma) followed by a second round of transduction 24 h later. Zeocin (Invitrogen) was used for negative selection. The transfection was validated by YFP expression analyses by flow cytometry using an LSRFortessa (BD).

**Protein estimation and Western Blot**

Ba/F3 cells were harvested, washed and lysed in RIPA buffer supplemented with a protease inhibitor cocktail (Sigma Aldrich) and protein concentrations were determined by BCA assay (Pierce Protein Research kit). A total of 20 µg protein was loaded and resolved by electrophoresis under reducing conditions on NuPage 10% Bis-Tris gels (Invitrogen) in MES running buffer (Invitrogen). Proteins were transferred onto PVDF membranes (Millipore) using semidry technique, followed by 1 h blocking at room temperature in 10% milk in TBST (SOX11) or 5% BSA in TBST (GAPDH). SOX11 protein was detected by rabbit polyclonal anti-SOX11 antibody (HPA000536 Sigma-Aldrich, 1:2000) diluted in 5% milk in TBST, at 4°C O/N, followed by incubation of HPR-conjugated anti-rabbit secondary antibody (NA934, GE Healthcare, 1:5000 in 5% milk, 2 h at RT). Membranes were then treated with SG substrate (Vector Laboratories) and incubated with GAPDH antibody (mAbcam9484, 1:2000 in 5% BSA, O/N at 4°C) as loading control. GAPDH was visualized by HPR-conjugated anti-mouse secondary antibody (NA931, GE Healthcare, 1:5000 in 5% milk, 2 h at RT). Membranes were incubated in ECL solution (Western Lightning Plus-ECL, PerkinElmer) for 5 min and imaged with an ODYSSEY® Fc LI-COR Imaging System's CCD camera.

**Proliferation**

In all experiments, $2.5x10^4$ Ba/F3 cells/ml were plated and cultured in medium containing IL-3, ±80 µg/ml doxycycline ("Doxyferm", Nordic Drugs AB) in 6-well plates. Trypan blue exclusion assay was performed at 24 h, 48 h and 72 h on cells in culture. XTT analysis, (Roche) was performed in accordance with the manufacturer's instruction at 72 h. The absorbance (450 nm and 650 nm) was measured 4 h later with the FLx800™ Multi-Detection Microplate Reader (Fisher Scientific). $^3$H-Thymidine

incorporation was performed using 200 µl cell suspension 68 h post seeding and cells were harvested 4 h later (total 72 h post seeding) by a Tomtec harvester and the radioactivity measured by a 1450 MicroBeta Trilux liquid scintillation and luminescence counter.

**Live Cell Imaging (LCI)**

*Sox11* transduced Ba/F3 cells (doxycycline$^{+/-}$) were grown in 48-well plates at 2.5x10$^4$ cells/ml at 37°C in 5% $CO_2$ and tracked by live cell imaging (LCI) for 72 h with images captured every 30 min at 10x magnification using phase contrast microscopy (Nikon Eclipse Ti-E). Pictures of all time points were compiled and processed in ImageJ (v.1.50i).

**Microarray sample preparation and analysis**

RNA was extracted from Ba/F3 cells using RNeasy Plus mini kit (Qiagen), measured and quantified on Nanodrop ND-100 (RIN > 9.3 for all samples). Four different groups, each with four repeats, were analyzed using Affymetrix's MTA-1 mouse array (genome version mm10, NetAffx Build 34): NT-OFF (non-transduced Ba/F3 in 5% IL-3), NT-ON (non-transduced Ba/F3 cells in 5% IL-3 and doxycycline), Sox11-OFF (*Sox11* transduced cells in 5% IL-3) and Sox11-ON (*Sox11* transduced cells in 5% IL-3 and doxycycline). CEL files were imported in to the transcriptome analysis console program TAC (v.1.4.1) and normalized by RMA. Pair-wise comparisons between expression values for NT-ON vs. NT-OFF and Sox11-ON vs. Sox11-OFF were interrogated for differential gene expression by two-tailed unpaired Student's t-test and the results were adjusted for multiple testing by false discovery rate.[4] In non-transduced Ba/F3 cells (NT) treated with doxycycline, only *Higd1a* showed significantly altered transcript level (FDR q-value = 0.029, FC = -1.25). Raw data for the experiment in the form of CEL files has been uploaded to the Gene Expression Omnibus (GEO) data repository database[5] and can be found under accession number GSE108419.

**Gene set enrichment analysis (GSEA)**

Features with low microarray intensities were filtered out (log2(intensity) < 4) and mean intensity values per gene were subsequently imported into the GSEA software (v3.0, Broad Institute) after ortholog conversion of mouse gene symbols to official human gene symbols via the biomaRt bioconductor

package (biomaRt v2.30.0, Bioconductor v3.4, R v3.3.2) using the Ensembl annotation database for mouse and human ($n_{genes}$ = 15893). Gene expression value differences between Sox11-ON and Sox11-OFF were used to find overrepresentation within gene sets from GO Biological processes (c5.bp.v5.0.symbols.gmt) by 1000 permutations and shuffling the gene sets with default settings. Gene sets with less than 15 or more than 500 members were excluded from the analysis. Gene overlaps between the enriched gene sets were studied using the Cytoscape (v3.6.0) plugin Enrichment Map. Enriched gene sets were clustered using the Cytoscape plugin clusterMaker2 using MCL clustering and keywords for the 27 clusters ($n_{nodes} \geq 3$) were generated using the Cytoscape plugin AutoAnnotate.

**Overlap with stage specific B-cell differentiation associated genes**

Selected stage-specific genes assigned to the pro-B, pre-B, B-cell and plasma cell stage as defined by present (P) or absent (A) comparison by Affymetrix P/A analysis and dCHIP analysis[6] were used to investigate B-cell differentiation associated genes.

**Identification of adhesion related molecules in gene expression profile data**

Cell-adhesion genes (n = 794) were retrieved from OKCAM, an online database of evaluated human cell-adhesion molecules (CAMs).[7] For analysis, the MTA-1 array was filtered for highly differentially expressed genes (FDR q-value $\leq 0.05$, FC $\geq 1.5$ or FC $\leq -1.5$), mouse symbols were converted to official human gene symbols via the biomaRt Bioconductor package as previously described.

**Statistical methods**

Two tailed Student's t-test.

## Legends to Supplementary Tables and supplementary video

### Supplementary Table S1

**Differentially expressed genes.** The 872 most differentially expressed genes (534 genes up and 338 genes down, FDR q-value ≤ 0.05 and FC ≥ 1.5 or FC ≤ -1.5) between Sox11-ON (Sox11 expressing Ba/F3 cells) and Sox11-OFF (control Ba/F3 cells) were interrogated for differential gene expression on a MTA-1 mouse array. The columns represent: Symbol: Official gene symbol, Public ID: representative feature id, entrez: Mus Musculus entrez gene ID, Column 4–11 log2 transformed normalized microarray intensity values for Sox11-ON (column 4–7) and Sox11-OFF (column 8–11), avg_mSOX11ON: mean microarray intensity for Sox11-ON samples, avg_mSOX11OFF: mean microarray intensity for Sox11-OFF samples, FC: gene expression fold change for Sox11-ON relative to Sox11-OFF, *P*: significance from two-tailed unpaired Student's t-test, FDR q: false discovery rate adjusted *P*-value.

### Supplementary Table S2

**Results from Gene Set Enrichment Analysis (GSEA).** Transcript level differences between Sox11-ON and Sox11-OFF were used to find overrepresentation within gene sets from the gene ontology biological processes database (c5.bp.v5.0.symbols.gmt). Red indicates gene sets containing genes with increased transcript levels in Sox11-ON cells whereas blue indicates gene sets associated with genes with lower transcript levels. The columns represent: cluster: cluster identifier, GO term; enriched functional category, gs size; number of genes in functional category, ES; enrichment score from GSEA, NES; normalized enrichment score, *P*; gene set enrichment analysis *P*-value, FDR q; false discovery rate adjusted *P*-value, genes: all genes in functional category.

### Supplementary Table S3

**Summary of Gene Set Enrichment Analysis (GSEA) gene set genes per cluster.** Unique genes per cluster for the unified list of genes from all functional categories belonging to that particular cluster. The columns represent: cluster: cluster identifier, name_hg_GSEA: official Homo Sapiens gene symbol that was used for GSEA, name_mm: official Mus Musculus gene symbol: FC: mRNA fold change in Sox11-ON cells relative to Sox11-OFF, *P*: significance from two-tailed unpaired Student's t-test, FDR q: false discovery rate adjusted *P* value, leading edge: logical expression indicating whether the gene

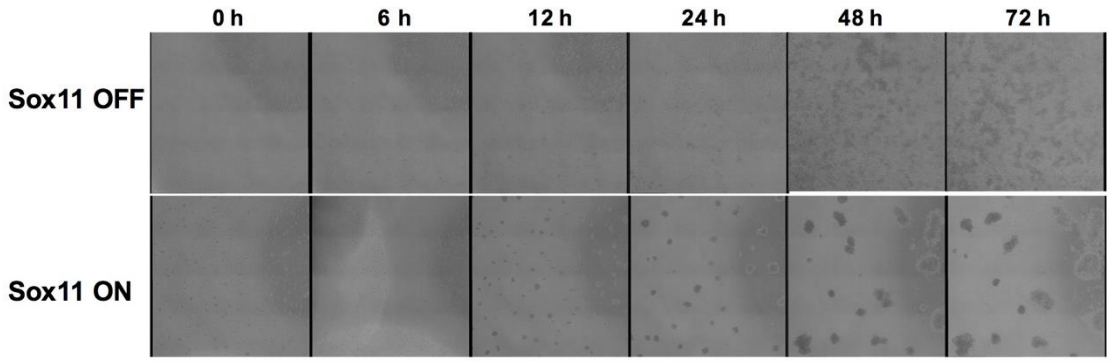
was part of the leading edge giving rise to the enrichment of at least gene set one gene sets in the cluster, column 8–15: normalized microarray intensity values for Sox11-ON cells (column 8–11) and Sox11-OFF cells (column 12–15).

**Supplementary video**

*Sox11* transduced Ba/F3 cells (doxycycline$^{+/-}$) were grown in 48-well plates at 2.5x10$^4$ cells/ml at 37°C in 5% $CO_2$ and tracked by live cell imaging (LCI) for 72 h with images captured every 30 min at 10x magnification using phase contrast microscopy (Nikon Eclipse Ti-E). Pictures of all time points were compiled and processed in ImageJ (v.1.50i).

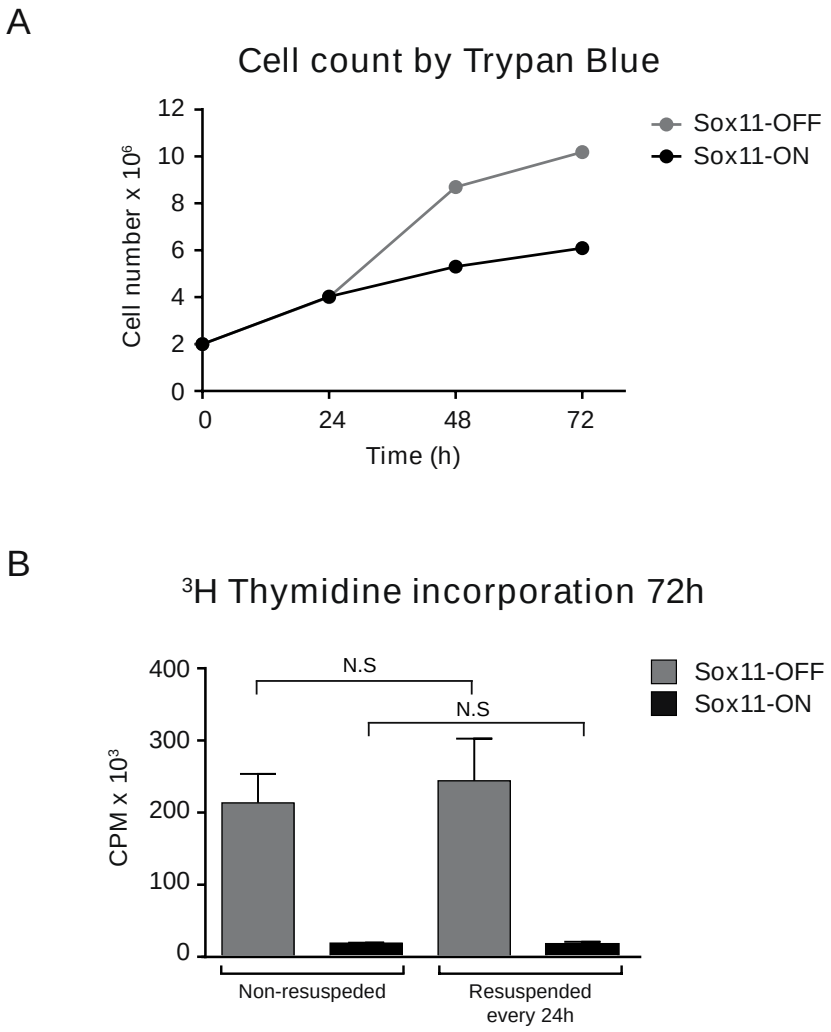
## Supplementary Figures

### Supplementary Figure S1



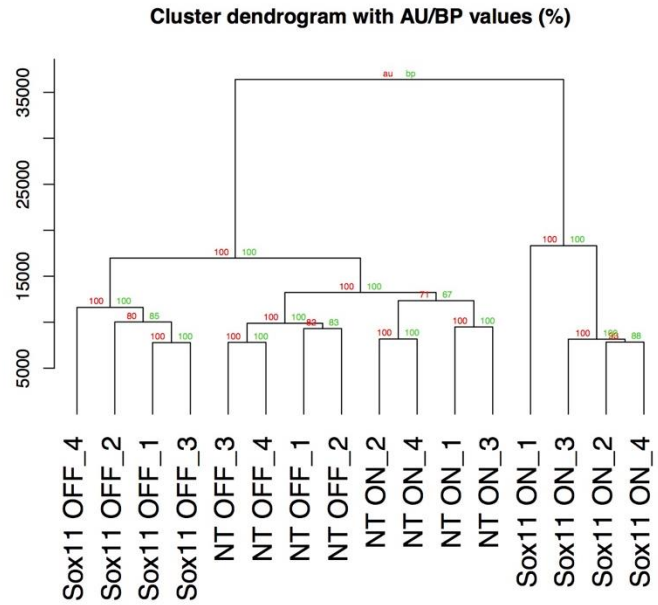

**Supplementary Figure S1:** Sox11-ON and Sox11-OFF Ba/F3 cells visualized by live cell imaging using a Nikon Ti-E microscope over the course of 72 h (4x). At 12 h small cell aggregates were observed for Sox11-ON cells and at 72 h large aggregates of cells had formed.

**Supplementary Figure S2**




**Supplementary Figure S2: A)** Sox11-ON cells proliferate less following 72 h of *Sox11* expression as compared to Sox11-OFF cells, measured by trypan blue exclusion (5 ml). Time points for readout: 24 h, 48 h and 72 h **B)** $^{3}$H-Thymidine incorporation at 72 h. No significant difference for cell aggregates resuspended every 24 h compared to non-resuspended (OFF vs OFF $P = 0.31$; ON vs ON $P = 0.41$).

**Supplementary Figure S3**




**Supplementary Figure S3:** Microarray analysis of non-transduced Ba/F3 cells and Sox11 transduced Ba/F3 cells. Four different groups of Ba/F3 cells were subject to gene expression analysis by Affymetrix MTA-1 array; Non-transduced Ba/F3 cells without doxycycline (NT-OFF), Non-transduced Ba/F3 cells with doxycycline (NT-ON), *Sox11*-transduced Ba/F3 cells without doxycycline (Sox11-OFF) and *Sox11*-transduced Ba/F3 cells with doxycycline (Sox11-ON), in quadruplicates. Cluster dendrogram based on microarray signal intensities showing approximately unbiased (AU) *P*-value and bootstrap probability (BP) value in red and green, respectively.

**Legend to Supplementary Video file (Supplementary_video.mp4, 42 s)**

Sox11-ON and Sox11-OFF Ba/F3 cells visualized by live cell imaging using a Nikon Ti-E microscope over the course of 72 h (4x) with time points highlighted every 6 h. At 12 h small cell aggregates were observed for Sox11-ON cells and at 72 h large aggregates of cells had formed.

## References to Supplementary Information


1. Nystrom S, Antoine DJ, Lundback P, et al. TLR activation regulates damage-associated molecular pattern isoforms released during pyroptosis. The EMBO journal. 2013;32(1):86-99.

2. Palacios R, Henson G, Steinmetz M, McKearn JP. Interleukin-3 supports growth of mouse pre-B-cell clones in vitro. Nature. 1984;309(5964):126-131.

3. Karasuyama H, Melchers F. Establishment of mouse cell lines which constitutively secrete large quantities of interleukin 2, 3, 4 or 5, using modified cDNA expression vectors. European journal of immunology. 1988;18(1):97-104.

4. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate - a Practical and Powerful Approach to Multiple Testing. J Roy Stat Soc B Met. 1995;57(1):289-300.

5. Barrett T, Wilhite SE, Ledoux P, et al. NCBI GEO: archive for functional genomics data sets--update. Nucleic acids research. 2013;41(Database issue):D991-995.

6. Tsapogas P, Breslin T, Bilke S, et al. RNA analysis of B cell lines arrested at defined stages of differentiation allows for an approximation of gene expression patterns during B cell development. Journal of leukocyte biology. 2003;74(1):102-110.

7. Li CY, Liu QR, Zhang PW, et al. OKCAM: an ontology-based, human-centered knowledgebase for cell adhesion molecules. Nucleic acids research. 2009;37(Database issue):D251-260.