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

In mantle cell lymphoma (MCL) there is controversy as to whether *SOX11* acts as an oncogene or a tumor suppressor. To investigate its function as a potential oncogene, *Sox11* was induced in the pro-B cell line Ba/F3, a cell-line previously used for the evaluation of the transformation capability of genes. In these cells Sox11 expression reduced proliferation and induced cell aggregation. 872 genes were differentially expressed, including cell adhesion genes. Among the upregulated genes was an enrichment of gene sets related to forwarding the cell cycle, notably, they also contained crucial checkpoint genes, consistent with the observed reduction in cell proliferation.

The *Sox11* gene belongs to the Sry-related high-mobility group (HMG) box (Sox) transcription factor family, and together with *Sox4* and *Sox12*, constitutes the SoxC group. The *SoxC* genes are of vital importance during organogenesis as they regulate developmentally important genes, such as protocadherin β, *PCDHB*.[1] Postnatally, *Sox11* expression is mainly restricted to neuronal tissue,[2] whereas *Sox4* is involved in early B-cell development and is necessary for the survival of pro-B cells.[3] SOX11 has no identified role in hematopoiesis or lymphopoiesis and is epigenetically silenced in most mature B cells, but is expressed in MCL and in rare reac-

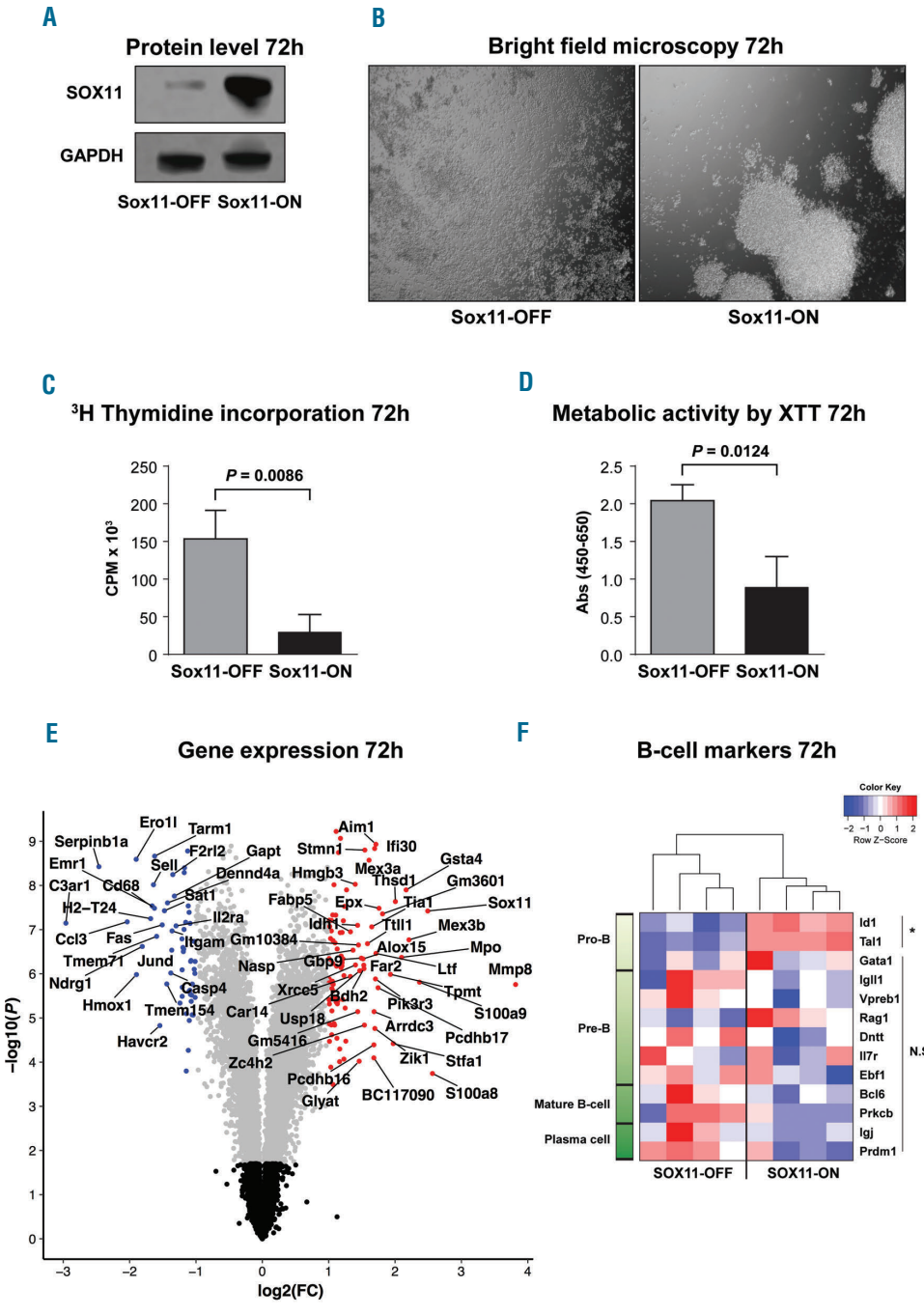


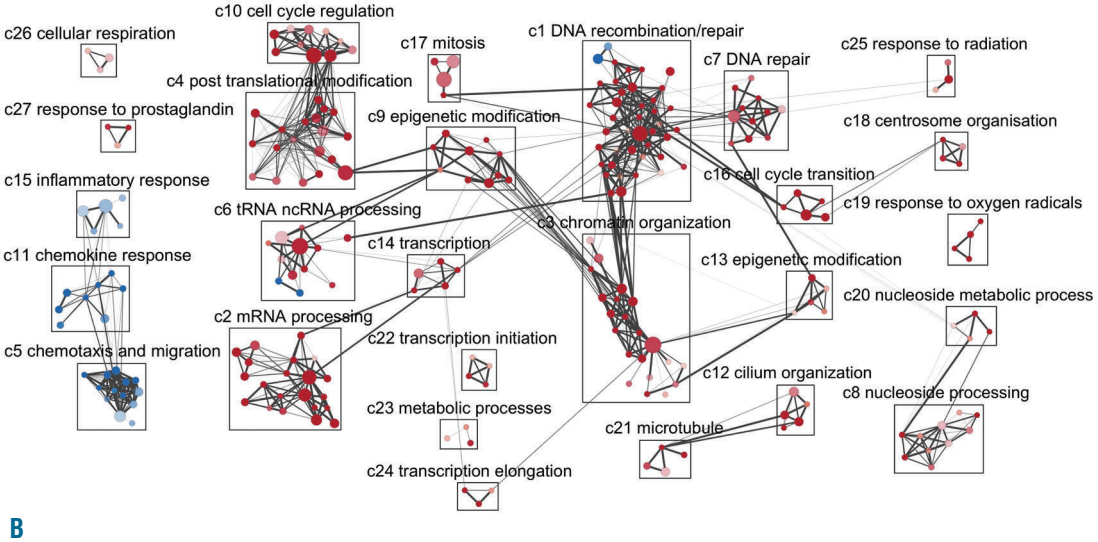
Figure 1. Phenotypical, proliferative and transcriptional changes in Ba/F3 cells upon 72 h of induced *Sox11* expression. (A) Western Blot of SOX11 protein expression in Sox11-ON (doxycycline supplemented medium) and Sox11-OFF (control medium) cells at 72 h, detected with the rabbit polyclonal anti-SOX11 antibody HPA000536, Sigma-Aldrich. B) Bright field microscopy images of cell aggregates following 72 h of continuous *Sox11* expression (10x), imaged by Nikon Ti-E microscope. C) Sox11-ON cells incorporates less ^{3}H-Thymidine at 72 h of *Sox11* induction following a 4 h pulse, as compared to Sox11-OFF cells, measured in counts per minute, error bars represent the standard deviation (*P*=0.0086, n=3). D) Mean relative absorption measured by XTT (n=3). Metabolic activity is significantly reduced (*P*=0.0124) in Sox11-ON cells as compared to Sox11-OFF cells. E) Volcano plot representation of transcript level differences by Affymetrix MTA-1 mouse arrays (Microarray data has been made available through the GEO database with accession number GSE108419). Names are shown for the genes with the largest transcript level fold change (log2FC ≥1.3 or ≤-1.3). Blue and red: genes with significantly altered transcript levels in Sox11-ON cells and a fold change below -2 (blue) (FDR q-value ≤0.05 and log2FC ≥-1) or above 2 (red) (FDR q-value ≤0.05 and log2FC ≥1); gray: genes significantly changed at the transcript level (FDR q-value ≤0.05); black: genes with non-significant transcript level changes. F) Expression levels after *Sox11* induction in genes specifically expressed at different stages of B-cell development. Only the pro-B restricted genes *Id1* and *Tal1* had significantly altered transcript levels in Sox11-ON cells (FDR q-value: 0.006 and 0.016, respectively). None of the other investigated pro-B and pre-B cell associated genes were altered at the transcript level. Genes associated with later B-cell developmental stages are shown for comparison. Transcript levels are presented as a gene-wise standardized expression (Z-score). FC: fold change.

tive lymphocytes.[4] SOX11 is also expressed in non-lymphoid malignancies, such as glioma, breast cancer and ovarian cancer. Both the oncogenic and tumor suppressor function of SOX11 has been reported in epithelial malignancies.[5,6] In MCL, it is proposed that *SOX11* acts as an oncogene, mainly by inducing cell proliferation, enforcing PAX5 expression and inhibiting terminal B-cell differentiation into plasma cells *via PRDM1* and *BCL6*.[7,8] SOX11 overexpression has also been associated with increased B-cell receptor (BCR) signaling and promotion of oncogenic transformation of B cells in a murine model.[9] However, other studies report reduced cell proliferation upon *SOX11* expression in MCL cells.[10,11] The non-malignant, IL-3 dependent pro-B cell line Ba/F3, which does not express immunoglobulins,[12] has previously been used for evaluating the transformation capability of potential oncogenes.[13] Herein we used the Ba/F3 cell line to investigate the functional and transcriptional changes resulting from induced *Sox11* expression.

Sox11 was expressed in the Ba/F3 cell line for 72 h (Sox11-ON) (Figure 1A). In contrast to the non-induced cells (Sox11-OFF), Sox11-ON cells began to form small clusters at 12 h (*Online Supplementary Figure S1*) with large and pronounced aggregates observed at 72 hours (Figure 1B and *Online Supplementary Video*). Conditioned medium from Sox11-ON cells did not induce cell aggregation in non-transduced Ba/F3 cells. We detected lower proliferation in Sox11-ON cells (*Online Supplementary Figure S2A*). Further experiments showed that Sox11-ON cells displayed significantly lower ^{3}H-Thymidine incorporation at 24, 48 and 72 hours (72 hours shown in Figure 1C) and lower metabolic activity than Sox11-OFF cells (Figure 1D). Re-suspending cell aggregates every 24 h did not affect ^{3}H-Thymidine incorporation at 72 h (*Online Supplementary Figure S2B*).

The global gene expression profile for Sox11-ON cells

A



B

cluster	name	keywords	nodes	genes	significant	leading edge
c1	DNA recombination/repair	repair dna excision nucleotide somatic strand recombination immune synthesis telomere	41	970	625	458
c2	mRNA processing	mrna splicing rna 3 end ribonucleoprotein spliceosomal spliceosome termination splice	21	721	486	333
c3	chromatin organization	histone chromatin disassembly methylation nucleosome remodeling complex organization beta catenin	21	805	529	357
c4	post translational modification	protein signaling small conjugation removal receptor pathway antigen regulation ligase	19	1043	616	404
c5	chemotaxis and migration	chemotaxis migration leukocyte regulation positive lymphocyte granulocyte neutrophil external stimulus	13	355	152	107
c6	tRNA ncRNA processing	ncrna endoplasmic reticulum trna snrna processing rrna iii localization transcription	12	549	367	268
c7	DNA repair	regulation dna double break homologous repair negative strand recombination replication	11	344	207	138
c8	nucleoside processing	biosynthetic nucleoside pyrimidine process compound nucleobase glycosyl purine monophosphate phosphate	10	199	115	80
c9	epigenetic modification	silencing gene epigenetic expression dsrna fragmentation heterotetramerization rdna posttranscriptional chromatin	10	253	189	141
c10	cell cycle regulation	cycle cell checkpoint phase mitotic transition integrity g1 regulation positive	10	474	294	190
c11	chemokine response	interferon gamma erk1 erk2 cascade interleukin 1 chemokine mediated monocyte	8	329	135	98
c12	cilium organization	cilium morphogenesis component axoneme assembly involved localization cellular organization protein	5	235	118	68
c13	epigenetic modification	negative chromosome modification acetylation histone regulation methylation organization chromatin protein	5	92	59	46
c14	transcription	capping transcription promoter elongation templated polymerase rna ii initiation dna	5	218	132	95
c15	inflammatory response	inflammatory acute wounding neuron projection regeneration maturation response regulation protein	5	378	155	113
c16	cell cycle transition	transition cycle phase cell g1 g2 involved mitotic initiation replication	5	241	167	113
c17	mitosis	sister chromatid cohesion reciprocal dna recombination mitotic nuclear division organelle	4	438	271	178
c18	centrosome organisation	centrosome organizing center duplication centriole microtubule organization assembly cycle	4	76	53	38
c19	response to oxygen radicals	hydrogen peroxide oxygen radical detoxification catabolic process metabolic response	4	77	34	20
c20	nucleoside metabolic process	deoxyribonucleotide deoxyribonucleoside triphosphate deoxyribose phosphate process metabolic catabolic pyrimidine	4	35	26	21
c21	microtubule	microtubule movement localization transport based establishment protein complex	4	190	104	54
c22	transcription initiation	initiation transcription ii promoter templated polymerase positive regulation rna dna	4	29	21	19
c23	metabolic processes	secondary quinone glycoside metabolic process	3	64	34	23
c24	transcription elongation	elongation templated transcription ii promoter polymerase regulation dna positive rna	3	37	26	21
c25	response to radiation	radiation ionizing ray response gamma	3	132	81	62
c26	cellular respiration	respiration tricarboxylic aerobic acid cellular metabolic process	3	140	91	68
c27	response to prostaglandin	prostaglandin fatty response cellular stimulus acid	3	53	16	13

Figure 2. Significantly enriched gene ontology terms from the gene set enrichment analysis (GSEA) clustered based on similarity in gene content. (A) 240 of the 288 significant functional categories were divided into 27 clusters (containing at least three functional categories). Functional categories representing up-regulated genes in Sox11-ON cells are presented in red, while gene sets representing down-regulated genes are presented in blue. Gene set sizes are represented by circle size, where gene sets containing few genes are represented by small circles and functional categories with more genes have larger circles. A darker shade is indicative of lower GSEA q-value. Darker edges represent a greater similarity between functional category nodes. Clusters are ranked according to size, where c1 contains the most functional categories (n=41) and c27 (n=3) the fewest. Functional categories have been positioned manually for increased legibility. B) Detailed information about clusters in A. The columns represent: 1. keywords, which are the most prevalent words among the gene ontology term names in each cluster, as generated by the Cytoscape plugin AutoAnnotate, 2. nodes, which indicate the number of functional categories in each cluster, 3. genes, which represent the number of unique genes for the unified list of genes from all functional categories in the cluster, 4. significant, which indicates the number of unique genes in the cluster that were differentially expressed (FDR q-value ≤0.05) and 5. leading edge, which indicates the number of unique genes per cluster that were present in the GSEA leading edge.

following 72 h of *Sox11* expression was distinct from both Sox11-OFF and non-transduced Ba/F3 cells (*Online Supplementary Figure S3*) with 872 significantly changed genes (534 genes up-regulated (fold change (FC) ≥1.5) and 338 genes down-regulated (FC ≤-1.5) in Sox11-ON cells false discovery rate (FDR) q-value ≤0.05) as shown in Figure 1E and *Online Supplementary Table S1*. The gene with the highest FC, *Mmp8*, is involved in proteolysis and is induced in peripheral blood mononuclear cells following incubation with S100A8 and S100A9 proteins, both of whose corresponding transcripts were among the most increased in the Sox11-ON cells. Additionally, the immunomodulatory cytokine, *Ccl3*, was down-regulated in Sox11-ON cells. In cancer, *S100A8/A9* overexpression has been associated with increased adhesion, reduced migration, impaired tumor growth and reduced transcript levels of *CCL3*.[14] Among the most differentially expressed genes were several adhesion-associated genes retrieved from the curated cell-adhesion database OKCAM. *Stfa1* and the SOX11 regulated protocadherin β genes[1] (*Pcdhb16* and *Pcdhb17*) were up-regulated, while *Sell* and *Itgam* were down-regulated (Figure 1E). Down-regulation of *Sell*, which is involved in leukocyte rolling, has also been reported in MCL.[15]

To investigate whether *Sox11* expression in Ba/F3 cells would influence the gene expression profile of B-cell developmental genes, the expression of genes characteristic for different stages of B-cell development was analyzed as described in the *Online Supplementary Materials and Methods*. Among those, *Sox11* induction increased transcript levels for two pro-B-cell restricted genes, *Id1* and *Tal* in Sox11-ON (FC: 1.2 and 1.3, respectively), but not any of the other genes typically associated with specific stages of B-cell development (Figure 1F). Even though expression of many genes was affected by *Sox11* expression, no significant changes in expression were observed for other investigated pro-B cell associated genes other than *Id1* and *Tal*, nor for other B-cell development associated genes that were included for comparison, such as *Prdm1* and *Bcl6*. Additionally, we observed no transcript level change for the previously reported *Sox11* target *Pax5*.[8]

To functionally classify genes with changed transcript levels a Gene Set Enrichment Analysis (GSEA) of Gene Ontology terms for biological processes resulted in 234 gene sets enriched for *Sox11*-induced genes and 54 gene sets enriched for *Sox11* down-regulated genes (FDR q-value ≤ 0.05, *Online Supplementary Table S2*). Two hundred and forty out of the 288 functional categories could be divided into 27 clusters (Figure 2, details in *Online Supplementary Table S2* and *Table S3*). The majority of clusters associated with genes that had increased transcript levels in Sox11-ON cells represented basal cellular functions. Several of those clusters are associated with aspects of the cell cycle: c1, c3, c7, c10, c16, c17 and c18. An analysis of the leading-edge genes in these clusters revealed a pattern largely associated with forwarding the cell cycle, but notably these clusters also contain important cell cycle checkpoint genes such as *Chek1* and *Chek2*, as well as DNA damage response genes such as *Trp53, Brca1* and *Brca2* (*Online Supplementary Table S3*). The functional outcome was reduced proliferation. Other clusters were indicative of epigenetic modifications (c9 and c13) and changes in transcription (c2, c14, c22 and c24). Only three clusters represent genes with lower transcript levels in Sox11-ON cells: c5, c11 and c15. These gene sets, associated with leukocyte function, contained genes encoding for cytokines and immunomodulatory pathways, including *Ccl3, Ccl4, Il6* and *Tnf*.

In conclusion, *SOX11* has been reported to have oncogenic properties in MCL,[8] however this has not been confirmed in other reports.[10,11,16] Oncogenic transformation associated with increased BCR signaling has been reported in murine B cells overexpressing *Sox11*.[9] While the pro-B cell line in the present study lack the BCR, *Sox11* was nevertheless able to significantly alter the global gene expression pattern, indicating that the implications of *Sox11* expression can be highly context dependent. In the context of a non-malignant pro-B cell line, *Sox11* expression markedly up-regulated transcript levels of genes involved in basal cell functions and down-regulated transcript levels of genes associated with leukocyte responses. The net results of induced *Sox11* expression in Ba/F3 cells was reduced proliferation and a marked cell aggregation. However, these results cannot be directly extrapolated to MCL, a lymphoma which is characterized by high genomic complexity. Consequently, the lack of oncogenic effects upon induced *Sox11* expression in the Ba/F3 cells does not exclude the possibility that *SOX11* exhibits oncogenic activity in other cell contexts where crucial cell cycle checkpoint genes are absent, or perhaps by cooperating with oncogenes, tumor suppressor genes or ongoing BCR-signaling mechanisms that are already deregulated in lymphoma.

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