Regulation of SOX11 expression through CCND1 and STAT3 in mantle cell lymphoma

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Key Points

- CCND1 binds to and reduces recruitment of HDAC1 and HDAC2 to the *SOX11* promoter, causing increased histone acetylation and *SOX11* transcription.

- STAT3 represses *SOX11* transcription by interacting directly with the *SOX11* gene promoter and enhancer.

Summary

The neural transcription factor SOX11 is usually highly expressed in typical mantle cell lymphoma (MCL), but it is absent in the more indolent form of MCL. Despite being an important diagnostic marker for this hard-to-treat malignancy, the mechanisms of aberrant SOX11 expression are largely unknown. Herein, we describe two modes of SOX11 regulation by the cell cycle regulator cyclin D1 (CCND1) and the signal transducer and activator of transcription 3 (STAT3). We found that ectopic expression of CCND1 in multiple human MCL cell lines resulted in increased SOX11 transcription, which correlated with increased acetylated histones H3K9 and H3K14 (H3K9/14Ac). Increased H3K9/14Ac and SOX11 expression were also observed after HDAC1 or HDAC2 was depleted by RNA interference or inhibited by the HDAC inhibitor vorinostat. Mechanistically, we showed that CCND1 interacted with and sequestered HDAC1 and HDAC2 from the *SOX11* locus, leading to SOX11 up-regulation. Interestingly, our data revealed a potential inverse relationship between phosphorylated Y705 (pY705) STAT3 and SOX11 expression in MCL cell lines, primary tumors and patient-derived xenografts. Functionally, inactivation of STAT3 by inhibiting the upstream Janus kinase (JAK) 1 or JAK2 or by STAT3 knockdown was found to increase SOX11 expression, whereas interleukin 21 (IL21)-induced STAT3 activation or overexpression of the

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constitutively active form of STAT3 decreased SOX11 expression. In addition, directly targeting SOX11 by RNA interference or indirectly by IL21 treatment induced toxicity in SOX11-positive MCL cells. Collectively, we demonstrate the involvement of CCND1 and STAT3 in the regulation of SOX11 expression, providing new insights and therapeutic implications in MCL.

Introduction

The high-mobility-group neural transcription factor SOX11 is predominantly expressed in the developing brain and has critical roles in neurogenesis and embryonic development.[1-4] Although SOX11 is not expressed in normal B cells and does not appear to play a role in lymphopoiesis, its aberrant expression has been found in several lymphoproliferative diseases including mantle cell lymphoma (MCL),[5-7] Burkitt lymphoma,[8] and B- and T-cell lymphoblastic leukemia.[7,8] SOX11 is also overexpressed in several types of solid tumors including ovarian carcinoma[9,10] basal-like breast carcinoma,[11,12] glioma,[13] medulloblastoma[14] and prostate cancer.[15] In MCL, SOX11 is highly expressed in most classical cases with nodal presentation, but is notably absent in indolent leukemic cases that display an IGVH-mutated phenotype.[16]

The role of SOX11 in MCL is incompletely understood. Previous studies have identified several direct targets of SOX11 in MCL including DBN1, SETMAR, HIG2 and WNT signaling.[17,18] Subsequent studies have revealed that SOX11 is essential for MCL xenograft growth *in vivo* and directly mediates transcription of the B-cell transcription factor PAX5 and, thus, is thought to promote lymphomagenesis through deregulated B-cell differentiation.[19] SOX11 also mediates the expression of platelet-derived growth factor alpha (PDGFA),[20] C-X-C motif chemokine receptor 4 (CXCR4), and focal

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adhesion kinase (FAK),[21] which promote angiogenesis, tumor cell migration and metastasis, respectively. Despite conflicting results regarding its prognostic value,[6,16,22] SOX11 is an established diagnostic marker for MCL.[7] In breast cancer, SOX11 is essential for proliferation and expression of a gene signature characteristic of aggressive basal-like breast cancer.[12]

Given the important biology of SOX11, several studies have investigated the mechanism of aberrant SOX11 expression. Gustavsson *et al.*[23] demonstrated that, while SOX11 is important in developing neurons, its expression is virtually absent in other tissues owing to promoter hypermethylation. Studies by Vegliante *et al.*[24] showed that SOX11 expression in embryonic stem cells and some B-cell lymphomas was associated with unmethylated DNA and active histones H3K9/14Ac and H3K4me3. SOX11 can be induced in MCL and breast cancer cell lines after treatment with the HDAC inhibitor vorinostat (also known as SAHA) or trichostatin A, suggesting that HDACs might participate in the regulation of SOX11 expression.[24,25] More recently, an elegant integrative analysis of the epigenome in primary MCL uncovered a distant regulatory element 675 kb downstream from the *SOX11* gene that appears to influence transcriptional activity at the *SOX11* promoter.[26] Using the circularized chromosome conformation capture sequencing (4C-seq) method to detect long-range chromatin interactions, Queiros *et al.*[26] demonstrated that this distant enhancer has three-dimensional contact with the *SOX11* gene promoter, but how it affects SOX11 expression remains to be determined.

In this study, we have investigated two potential mechanisms of SOX11 expression. By ectopically expressing CCND1 in human MCL cell lines, we demonstrate

that CCND1 mediates SOX11 expression through interaction with HDAC1 and HDAC2 at the *SOX11* locus. In addition, using genetic and pharmacological inhibition, we show that the signal transducer and activator of transcription 3 (STAT3) binds to the *SOX11* promoter and enhancer, and functions as a transcriptional repressor. These findings demonstrate two distinct modes of SOX11 regulation and may have implications for the treatment of MCL.

Materials and Methods

Cell lines and culture conditions

Human MCL lines Z-138, JEKO-1, UPN-1 and SP-53 were kindly provided by Dr. Louis Staudt. GRANTA-519, JVM-2, MINO and MAVER-1 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The MCL lines Z-138, JEKO-1, GRANTA-519 and UPN-1 were authenticated by short tandem repeat DNA profiling (ATCC, Manassas, VA) **(Supplementary Table S1)**. Other cell lines were not authenticated. Cells were cultured in RPMI-1640 medium, except GRANTA-519 in DMEM medium (Life Technologies, Grand Island, NY), supplemented with 10% fetal bovine serum, 100 IU/mL penicillin and 100 µg/mL streptomycin in a humidified incubator at 37°C with 5% CO_2.

Primary MCL samples and patient-derived xenografts

Primary cells were obtained from the tumor bank of the Pathology Department of City of Hope as de-identified samples after approval by the Institutional Review Board and prepared as previously described.[27] Briefly, frozen cells were thawed in 37°C water bath, washed in RPMI-1640 medium and cultured in RPMI-1640 medium supplemented

with 20% fetal bovine serum and 200 Kunits/ml of DNASe I (Sigma, St. Louis, MO) for 15 minutes in 37°C CO_2 incubator followed by washing. Cells were recovered overnight in CO_2 incubator before experiments. MCL patient-derived xenografts (PDX) were obtained from the public repository of xenografts (ProXe)[28] **(Supplementary Table S2)**. Samples were transplanted into sublethally irradiated NOD.Cg-*Prkdc^scid^Il2rg^tm1Wjl*/SzJ (NSG) mice (The Jackson Laboratory, Bar Harbor, ME) through tail vein injection. Lymphoma xenografts were frozen in aliquots and subsequently thawed and cultured in RPMI-1640 medium supplemented with 20% fetal bovine serum, 100 IU/mL penicillin and 100 µg/mL streptomycin in a humidified incubator at 37°C with 5% CO_2.

cDNA expression vectors

The retroviral expression vector pBMN-CCND1-HA-IRES-Hygro, encoding carboxy-terminus HA-tagged CCND1 wild type or mutants, was previously constructed.[27] HDACs-HA constructs were a kind gift from Dr. Yue Xiong (University of North Carolina, Chapel Hill), as previously described.[29] FLAG-tagged SOX11 expression vector was constructed by cloning the PCR-generated SOX11 products from Z-138-derived cDNA template into the pBMN-IRES-Hygro vector (a gift from Gary Nolan) at BamHI and XhoI restriction sites. FLAG-SOX11 PCR products were generated using the following primer pairs:

5'TAGTAGGGATCCGCCGCCACCATGGACTACAAAGACGATGACGACAAGGTGCAG
CAGGCGGAGAGCTTG   and
5'CTACTACTCGAGTCAATATGTGAACACCAGGTCGGAGAA. The final SOX11 construct was confirmed by DNA sequencing. The lentiviral STAT3 constitutive active construct EF.STAT3C.Ubc.GFP was a gift from Linzhao Cheng (Addgene plasmid #

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24983) and its retroviral subclone was a gift from Lixin Rui.

RNA interference reagents

CCND1 and HDAC1 shRNA constructs and sequences were obtained from a previously generated shRNA library.[30] STAT3 shRNA (#840) pKLO construct was kindly provided by Anna Scuto as previously reported.[31] SOX11 shRNA (#454) and HDAC2 shRNA (#1678) pKLO constructs were obtained from Sigma (Sigma, St. Louis, MO). RNAi sequences are listed in **Supplementary Table S3**. A DNA insert encoding a fusion puromycin N-acetyl-transferase-green fluorescence protein (GFP) was cloned into the SOX11 shRNA pKLO vector at the BamHI and KpnI restriction sites to produce a GFP-coexpressing vector.

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) reactions were performed using RT^2 SYBR® Green qPCR Master Mix (Qiagen, Valencia, CA) or Taqman Universal PCR Master Mix (Thermo Fisher Scientific, Waltham, MA) and analyzed by the StepOnePlus Real-time PCR system (Life Technologies, Grand Island, NY). Since SOX11 is encoded by an intronless gene, in addition to DNAse treatment before cDNA synthesis, a poly-A specific primer for SOX11[32] was used to minimize amplification of potential genomic DNA (gDNA) contamination. As a negative control, mock cDNA synthesis without addition of reverse transcriptase was also prepared to verify for the presence of contaminating gDNA. We demonstrated that the cycle threshold (Ct) values for the poly-A specific SOX11 primer in RT-positive cDNA samples were consistently >10 cycles less than those in mock cDNA samples (Ct 24 vs. 39) **(Supplementary Table S4)**. Additional primers that can amplify SOX11 or GAPDH from gDNA also yielded similarly

large differences in Ct values between RT-positive and mock cDNA samples (Ct 25 vs. 36 and 23 vs. 39, respectively) **(Supplementary Table S4)**. These results confirm the validity of our mRNA assessment using either SOX11 primers. Primer sequences for SOX11 and GAPDH are shown in **Supplementary Table S5**. Taqman probes for SOX11 (Hs00848583_s1), STAT3 (Hs00374280_m1), HDAC1 (Hs02621185_s1) and GAPDH (Hs02786624_g1) were purchased from ThermoFisher (ThermoFisher, Waltham, MA). Relative mRNA expression was normalized to GAPDH signals and calculated using the ddCt method.

Immunoblot and immunoprecipitation analyses

Cells were lysed in the presence of protease inhibitor cocktail (Sigma, St. Louis, MO) and Halt phosphatase inhibitor cocktail (Pierce Biotechnology, Rockford, IL) for 30 min. Lysates were cleared by centrifugation and protein concentrations were determined by BCA protein assay (Pierce Biotechnology, Rockford, IL). Twenty micrograms of lysates per lane were separated by 4-15% SDS-PAGE and immobilized on the nitrocellulose membranes (ThermoFisher, Waltham, MA) for immunoblotting. Immunoblot signals were developed by a chemiluminescent detection method (Pierce Biotechnology, Rockford, IL) and captured by standard autoradiographic films. For immunoprecipitation, see Supplementary Methods.

Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed using the ChIP Assay Kit (Millipore, Temecula, CA) according to manufacturer's instructions. Details are described in Supplementary Methods.

Statistical analyses:

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A two-tailed Student t-test or linear regression analysis was performed for comparison between two groups, using Prism Version 6.0b (GraphPad Software, La Jolla, CA). P values < 0.05 were considered statistically significant.

Additional detailed method descriptions are available in Supplementary Materials.

Results

CCND1 up-regulates SOX11 expression in human MCL cell lines.

To determine whether CCND1 induces SOX11, hemaglutinin (HA)-tagged CCND1 was ectopically expressed in the human MCL lines Z-138, JEKO-1 and GRANTA-519. The recurrent mutation CCND1 Y44D, which affects phosphorylation-dependent proteolysis and result in increased protein levels,[27] was also expressed in Z-138 and JEKO-1 cells. Compared to empty vector controls, both wild-type (WT) and mutant CCND1 increased protein expression levels of SOX11 in these cell lines by immunoblot analysis **(Figures 1A and Supplementary Figures S1A)**. To ensure the specificity of the SOX11 antibody used in the current study, depletion or overexpression of SOX11 was carried out in MCL cell lines, and specific loss or increase in SOX11 expression was confirmed by immunoblot analysis **(Supplementary Figures S2A, B)**. We next used reverse transcription (RT)-PCR assays to determine whether CCND1 mediated SOX11 transcription. Since SOX11 is encoded by an intronless gene, we used a mRNA-specific RT-PCR assay (see Materials and Methods) and demonstrated that overexpression of WT or mutant CCND1 increased SOX11 mRNA levels in these cell lines **(Figures 1B and Supplementary Figures S1B)**.

To determine whether CCND1 is required for SOX11 expression, we depleted CCND1 in MCL cells using an shRNA that has been validated in a previous study.[33]

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CCND1 depletion in both Z-138 and JEKO-1 cells resulted in reduced SOX11 protein levels **(Figures 1C)**, in addition to decrease in cell viability **(Figures 1D)** similar to previously observed in JEKO-1 cells.[33] Depletion of SOX11 also resulted in reduced cell viability in Z-138, JEKO-1 and an additional MCL line MINO **(Figure 1E)**. We next determined whether SOX11 up-regulation was due to altered protein stability by treating CCND1-HA-expressing Z-138 cells with cyclohexamide (CHX) and assessing SOX11 protein turnover by immunoblot analysis. In this experiment, WT and mutant CCND1 samples consistently expressed more SOX11 than empty vector controls before CHX treatment **(Figure 1F)**. However, the rate of SOX11 protein turnover in WT and mutant CCND1 samples was comparable to that of controls after 3 hours in CHX **(Figure 1F)**. This result excluded increased protein stability as a mechanism of increased SOX11 expression. Together, these data suggest a role for CCND1 in the regulation of SOX11 expression in MCL cell lines.

CCND1 affects histone modification at the *SOX11* locus.

Since SOX11 transcription is associated with histone acetylation,[24,25] we performed chromatin immunoprecipitation (ChIP) and quantitative PCR (qPCR) assays to examine whether CCND1 influences histone modification at the *SOX11* locus. To determine the chromatin regions on the *SOX11* gene that are likely reactive to the active histone mark H3K9/14Ac antibody, we searched for H3K9Ac ChIP signals in SOX11 expressing cells in the ENCODE database and identified two potential DNA regions for PCR amplification (amplicons) **(Figure 2A)**. ChIP-qPCR experiments were carried out in Z-138 and JEKO-1 cells that overexpress CCND1 using a previously-validated H3K9/14Ac antibody and PCR primers for the two amplicons.[24] Compared to

empty vector controls, overexpression of CCND1 significantly increased H3K9/14Ac signals in both cell lines **(Figures 2B-E)**. Enrichment of H3K9/14Ac at the *SOX11* locus was also observed in Z-138 cells treated with the HDAC inhibitor SAHA **(Figure 2F)**. In addition, SOX11 expression was positively correlated with H3K9/14Ac levels following treatment with SAHA in Z-138 cells **(Figures 2G)**. Thus, these data indicate that CCND1 mediates SOX11 expression through histone acetylation at the *SOX11* locus.

CCND1 interacts with HDAC1 and HDAC2.

The effects of CCND1 overexpression and HDAC inhibition on histone acetylation of the *SOX11* locus led us to examine whether CCND1 physically associates with members of the HDAC family to mediate SOX11 transcription. We co-expressed individual HDACs with CCND1 in HEK-293T cells and analyzed potential interactions using co-immunoprecipitation. **Figure 3A** shows that CCND1 strongly interacts with HDAC1, HDAC2 and, to a lesser extent, HDAC3, but not with other HDAC members. Validation of this interaction in Z-138 cells or in primary MCL samples by immunoprecipitation with CCND1 or HDAC1 antibody also showed CCND1 in the complex with HDAC1 and HDAC2 **(Figure 3B)**. In addition, shRNA-mediated depletion of HDAC1 from Z-138, JEKO-1 **(Figures 3C and Supplementary Figures S3A)** or GRANTA-519 **(Supplementary Figures S3B, C)** cells resulted in increased SOX11 mRNA and protein levels, further confirming the role of HDAC1 in modulating SOX11 expression. Increased SOX11 expression was also observed when HDAC2 was depleted in Z-138 and JEKO-1 cells **(Figures 3D).** Together, these results indicate that interaction of CCND1 with HDAC1 and HDAC2 plays a role in mediating SOX11 expression.

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Reduced chromatin recruitment of HDAC1 in CCND1 overexpressing cells.

We next asked whether CCND1 affected recruitment of HDAC1 at the *SOX11* locus. ChIP-qPCR assays were performed in CCND1-overexpressing Z-138 cells using anti-HDAC1 antibody and PCR primers located near the *SOX11* transcription start site. HDAC1 ChIP signals were significantly reduced in CCND1-expressing cells as compared to empty vector controls **(Figure 4A)**. As an alternative approach, cell fractionation was used to assess the distribution of HDAC1 and HDAC2 within cellular compartments. Protein extracts from soluble cytoplasmic and nuclear fractions, as well as from insoluble nuclear fractions, from equal numbers of control or CCND1 overexpressing Z-138 cells were evaluated by immunoblot analysis. The nuclear proteins histone H3 and LAMIN A and C were used as markers for the nuclear fractions. We found that both HDAC1 and HDAC2 resided predominantly in the insoluble nuclear fraction in the empty vector controls. However, in cells with CCND1 overexpression, there was increased accumulation of HDAC1 and HDAC2 in the soluble nuclear fraction **(Figure 4B)**. Similar increase of HDAC1 and HDAC2 protein levels in the soluble nuclear fraction was also observed in GRANTA-519 cells that overexpressed CCND1 **(Supplementary Figure S4)**. Taken together, these results indicate that CCND1 overexpression results in re-distribution of HDAC1 and HDAC2 from the chromatin environment, including the chromatin of the *SOX11* gene.

STAT3 negatively regulates SOX11 expression.

To further confirm the positive role of CCND1 in regulating SOX11 expression, we transduced CCND1-expressing lentivirus into the SOX11-negative MCL cell line JVM-2. Surprisingly, compared to an increase in SOX11 levels in JEKO-1 cells,

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ectopically expressed CCND1 did not induce SOX11 in JVM-2 cells (**Figure 5A**). Analysis of publicly-available gene expression data from SOX11-positive and -negative MCL cases[34] also revealed no correlation between SOX11 and CCND1 (**Figure 5B**). However, a positive correlation between CCND1 and SOX11 was observed in SOX11-positive cases albeit not statistically significant due to a small sample size (n=15) **(Supplementary Figure S5A)**. Analysis of relevant public data from another study,[16] in which SOX11 positivity was identified in 13 cases, also showed a positive correlation between CCND1 and SOX11 although, again, not statistically significant **(Supplementary Figure S5B)**.

These observations prompted us to investigate additional mechanisms of SOX11 regulation. Since SOX11-negative MCL cases typically have plasmacytic differentiation,[16,35] we hypothesized that SOX11 transcription might be negatively regulated during B-cell differentiation. To identify the molecules potentially involved in this process, we analyzed transcription factors that bind to the *SOX11* locus, including the recently identified *SOX11* enhancer,[26] using ChIP-Seq data from the ENCODE project.[36] Among *SOX11*-locus bound factors, STAT3 was chosen for further study because of its role in B-cell differentiation.[37] Interestingly, expression of SOX11 and the active pY705 STAT3 were inversely correlated in MCL cell lines (except JEKO-1) **(Figure 5C)**, in primary MCL samples (except samples #11 and #15) **(Figure 5D)**, and in MCL patient-derived xenographs (PDX) **(Figure 5D).** To determine whether SOX11 is negatively regulated by activated STAT3, we treated JEKO-1, GRANTA-519, MAVER-1 and JVM-2 cells, which express high pY705 STAT3 levels, with AZD1480, an inhibitor of the upstream kinases JAK1 and JAK2.[38] Immunoblot analysis showed that AZD1480

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effectively blocked STAT3 phosphorylation and resulted in increased SOX11 mRNA and protein levels in JEKO-1, GRANTA-519 and MAVER-1 cells **(Figure 5E, F)**, but not in JVM-2 cells **(Supplementary Figure S6A)**. Similar increases in SOX11 expression were also observed in GRANTA-519 cells after treatment with another STAT3 inhibitor, JAK inhibitor I (MilliporeSigma, Burlington, MA, USA) **(Supplementary Figure S6B)**. AZD1480-induced up-regulation of SOX11 was mediated by STAT3 inhibition, as depletion of STAT3 also led to increased SOX11 mRNA and protein expression in both JEKO-1 and GRANTA-519 cells **(Figures 5G, H)**.

In line with the repressive role of STAT3, interleukin (IL) 21-induced STAT3 activation[39] in MINO, SP-53, Z-138 **(Figures 6A, B)** and MCL PDX models **(Figures 6C)** or ectopic expression of a constitutively active form of STAT3[40] in Z-138 cells **(Supplementary Figure S7)** resulted in reduced SOX11 expression. Interestingly, IL21 also reduced viability of MCL lines (MINO, SP-53 and Z-138) or PDX models (#5 and #7) with low or negative STAT3 activity **(Figure 6D, E)** while it had little effect on MCL cells with high pY705 STAT3 expression (JEKO-1, MAVER-1 and GRANTA-519) **(Supplementary Figure S8)**. As depletion of SOX11 also reduced cell viability in MINO, Z-138 and JEKO-1 cells **(Figure 1E)**, we depleted SOX11 from the remaining MCL lines and determine their survival. We found that SOX11 depletion had little effect on the viability of GRANTA-519 cells and slightly increased cell growth in MAVER-1 cells **(Supplementary Figure S9A, B)**. Data from SP-53 cells were not available due to sensitivity of this cell line under our lentiviral transduction conditions. Thus, similar to IL21 treatment, SOX11 depletion is toxic in MINO and Z-138 cells while having little

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effect in GRANTA-519 and MAVER-1 cells. JEKO-1 cells appear to be an exception, as they are resistant to IL21 but sensitive to SOX11 depletion.

We next determined whether STAT3 was recruited directly to the *SOX11* gene by performing ChIP-qPCR experiments with an anti-pY705 STAT3 antibody in JEKO-1 cells. Since STAT3 phosphorylation is required for DNA binding[41] and this phosphorylation is efficiently inhibited by AZD1480, we used AZD1480-treated JEKO-1 cells as a negative control for the pY705 STAT3 ChIP-qPCR experiments. **Figure 7A** shows that pY705 STAT3 was specifically recruited to the *SOX11* gene and enhancer, and this recruitment was significantly impaired after AZD1480 treatment. Increased active histone H3K9/14Ac signals at the *SOX11* promoter and enhancer regions were also observed in AZD1480-treated MAVER-1 cells using H3K9/14Ac ChIP-qPCR **(Figure 7B)**. Taken together, these findings indicate that STAT3 represses SOX11 transcription through recruitment of pY705 STAT3 to the *SOX11* locus, and that manipulation of the STAT3-SOX11 axis directly through SOX11 or indirectly through STAT3 induces toxicity in SOX11^{+} MCL cells.

Discussion

The current study reveals two distinct regulatory mechanisms of SOX11 expression in MCL, specifically through CCND1 and STAT3. By genetically manipulating CCND1 levels using ectopic expression and gene knockdown, we have demonstrated that CCND1 is sufficient and necessary for SOX11 expression in the MCL cell lines Z-138, JEKO-1 and GRANTA-519. SOX11 expression is also negatively regulated by the post-germinal center B-cell differentiation factor STAT3, which may link SOX11 regulation to specific stages of B-cell differentiation.

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In our proposed model, CCND1 interacts with and sequesters HDAC1 and HDAC2 from regulatory elements in the *SOX11* locus, leading to increased histone acetylation and *SOX11* transcription **(Figure 7C)**. The ability of CCND1 to associate with transcriptional regulators and affect gene transcription is well recognized[42]. Fu *et al.*[43] demonstrated that CCND1 preferentially associates with HDAC1, HDAC2, HDAC3 and HDAC5, and recruits HDAC1 to the *PPAR-gamma* promoter to repress its transcription. We found that CCND1 consistently binds to HDAC1 and HDAC2 and, to a lesser extent, HDAC3, but not other HDAC members **(Figure 3A)**. In contrast to transcriptional repression as a consequence of HDAC1 recruitment to the gene promoter as reported by Fu *et al.,*[43] we have shown that elevated CCND1 levels in MCL cells result in reduced HDAC1 recruitment at the *SOX11* promoter and subsequent increased gene transcription. Mechanisms of gene expression through HDAC1 relocation from transcriptional regulators have been described. For example, Di *et al.*[44] reported that treatment with estrogen or the glycolysis inhibitor 2-deoxyglucose in the breast cancer cell line MCF-7 caused eviction of HDAC1 from a co-repressor complex, leading to increased histone acetylation at the *BRCA1* promoter and *BRCA1* transcription. In another study focusing on developing neurons, expression of Lmo4 led to displacement of Hdac1 from the transcriptional repressor complex NuRD, resulting in derepression of the Ctip2 locus.[45] Together, these observations support the removal of HDAC1 from regulatory elements as a common mechanism, and indicate that diverse signals can mediate this process depending on the cellular context. In line with this notion, we speculate that, in addition to CCND1, other abnormalities that interfere with HDAC1 function may also contribute to SOX11 expression. Characterization of new

mutations, particularly those affecting HDAC1-interacting proteins, may provide further insight into the mechanisms of deregulated SOX11 expression.

Although our data favor CCND1-mediated HDAC1 sequestration as the mechanism of SOX11 up-regulation, it is possible that CCND1 may influence SOX11 transcription by affecting enzymes that maintain the dynamic histone acetylation/deacetylation equilibrium, such as the histone acetyltranferases EP300, PCAF and GCN5. Indeed, CCND1 has been shown to physically associate with EP300[46] and PCAF[47] and regulate gene expression. Furthermore, through its cyclin-dependent kinase (CDK) partners CDK4 and CDK6, CCND1 elicits transcriptional changes by phosphorylating and activating GCN5.[48] In the current study, we found that treating MCL cell lines with the EP300 inhibitor C646 strongly down-regulated SOX11 expression **(Supplementary Figure S10)**, consistent with involvement of EP300 in regulating SOX11 expression. These observations warrant further studies into the molecular interactions between CCND1 or the CCND1/CDK4 complex and transcriptional regulators at the *SOX11* promoter.

Although ectopic expression of CCND1 can induce SOX11 expression in multiple MCL lines, SOX11 is not expressed in a subset of t(11;14)-positive MCL cases[16] or in any t(11;14)-positive multiple myeloma (MM) cases.[49] These observations suggest that SOX11 expression is regulated by additional factors and/or cellular contexts. To investigate additional mechanisms of SOX11 expression, we turned to a recent study profiling the methylome of MCL. In that study, Queirós *et al.*[26] found a potential SOX11 regulatory element 675 kb downstream of the *SOX11* gene that was hypomethylated and associated with the active enhancer mark H3K27ac in SOX11-positive, but not in

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SOX11-negative, MCL cells. We then examined the transcription factors that are associated with this putative enhancer, as previously reported by the ENCODE project.[36] Among factors that bind to this enhancer, we focused on STAT3 as a potential repressor of SOX11 because STAT3 is important for post-germinal center B-cell differentiation,[37] a commonly recognized phenotype of SOX11-negative MCL.[16,19] Indeed, our data revealed that STAT3 is recruited to both *SOX11* gene and enhancer loci, and functions as a transcriptional repressor in multiple MCL lines including JEKO-1, GRANTA-519 and MAVER-1. Our findings are consistent with previous reports that showed constitutively active STAT3 in the majority (70%) of indolent leukemic MCL cases,[50] which do not express SOX11.[16] As mentioned above, t(11;14)-positive MM is another example of the inverse correlation of STAT3 activation and SOX11 expression, as the majority of MM cases show constitutive activation of STAT3.[51] Our data, however, do not exclude additional mechanisms of SOX11 regulation as non-concordant cases do exist, including JEKO-1, MCL#15 and MCL#24 (pY705 STAT3^{+}, SOX11^{+}) and MCL#11 (pY705 STAT3^{-}, SOX11^{-}). Similarly, despite AZD1480 effectively reduced pY705 STAT3 levels in JVM-2 cells, little SOX11 induction was observed, implicating a repressive mechanism other than pY705 STAT3 **(Supplementary Figure S6A)**. The Epstein-Barr virus (EBV)-positive status of JVM-2 cells unlikely contributed to the lack of AZD1480-induced SOX11 expression, as EBV[+] GRANTA-519 cells readily up-regulated SOX11 expression after STAT3 inhibition **(Figure 5E, F and Supplementary Figure S6B)**. It is possible that mutations affecting SOX11 transcriptional machinery exist in MCL cells with little STAT3 activity. In support of this notion, many MCL tumors, including JVM-2 cells, were found to harbor frameshift

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mutations[52-54] that affect *mixed-lineage leukemia (MLL) 2* and/or *MLL4* genes, which encode enzymes that methylate histone 3 lysine 4 (H3K4) and positively regulate gene transcription.[55] Confirmation of these mutations and elucidation of the underlying mechanisms that regulate SOX11 expression are thus warranted. In addition, potential crosstalks between CCND1 and STAT3, which have been observed in other systems, may provide further clues to understand SOX11 regulation. For example, overexpression of a constitutively active form of STAT3 in HEK-293T cells[56] or of a dominant negative variant of STAT3 in mouse NIH-3T3 cells[57] directly activated or inhibited CCND1 promoter activity, respectively. Interestingly, CCND1 was also found to repress STAT3 activation in HepG2 cells.[58] Therefore, investigating how such crosstalks influence SOX11 expression in the context of MCL will be needed to further improve our understanding of the complex SOX11 regulation.

Our findings have implications for better understanding of the two clinically-distinct MCL subtypes, i.e., typical and indolent MCL. We believe that the previously-described post-germinal center phenotype of SOX11-negative, indolent MCL[16,19] could be related to upregulated STAT3 activity. In contrast, MCLs that initially express high levels of CCND1 and SOX11 are likely prevented from plasmacytic differentiation, possibly due to SOX11-mediated PAX5 upregulation,[19] and thus have low STAT3 activity. Our data also have implications for the development of new treatment strategies for MCL. While CCND1 and SOX11 are promising therapeutic targets, pharmacological inhibitors for these molecules are currently not available. In contrast, despite the availability of small-molecule inhibitors for HDAC1 and HDAC2 or STAT3 signaling, targeting these molecules would not be beneficial for MCL patients due to the

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undesired effect of increasing SOX11 levels. Indeed, clinical studies in mantle cell lymphoma using vorinostat as a single agent have shown very modest activity to date. For example, Kirschbaum *et al.* found that none of nine MCL patients responded to vorinostat.[59] Similarly, Ogura *et al.* showed that vorinostat had no effect on the overall survival of all four enrolled MCL patients.[60] While the efficacy of STAT3 inhibition remains unclear from one study with limited MCL patient enrollment,[61] the present study implicates that STAT3 may not be an ideal target in MCL owing to its negative role in SOX11 regulation. Instead, our data advocates for further studies that target the regulatory mechanisms of SOX11 to reduce SOX11 levels and potentially differentiate aggressive MCL tumors to an indolent phenotype. In support of this notion, we showed that IL21, a potent plasma cell-inducing cytokine,[62] effectively up-regulated STAT3 activity, leading to reduced SOX11 levels and viability in SOX11$^+$ MCL cells. Our data are thus consistent with previous studies that showed IL21-mediated toxicity in MCL through a STAT3-dependent mechanism.[63,64] However, IL21 susceptibility appears limited to MCL cell lines with low or negative STAT3 activity, as pY705 STAT3^{high} MCL cells are resistant to IL21 treatment. Thus, our results also reveals pY705 STAT3 as a potential biomarker for IL21-based therapy.

In summary, we have demonstrated that CCND1 and STAT3 play key roles in regulating SOX11 expression. CCND1 binds to and reduces recruitment of HDAC1 and HDAC2 to the *SOX11* promoter, leading to increased histone acetylation and *SOX11* transcription. In contrast, STAT3 directly interacts with the *SOX11* gene locus and its enhancer and functions as a transcriptional repressor. These findings have implications for our understanding of SOX11 deregulation in MCL and may have therapeutic

potential for MCL patients.

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Author's Contributions

AM and VNN designed the experiments. AM, NS, AP and VNN performed the experiments. AM, MM and VNN analyzed data. TVN provided essential research reagents. RWC, EB, MM, LP, LVP, LWK, DMW, DDW, STR, W-CC and MM provided and reviewed pathological data. DDW edited the manuscript. VNN directed the research and wrote the manuscript.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed by all authors.

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FIGURE LEGENDS

Figure 1. CCND1 upregulates SOX11 expression. A. Immunoblot analysis of Z-138 and JEKO-1 cells stably transduced with empty vector (EV), WT or Y44D mutant CCND1-HA constructs. Cell lysates (30 μg per lane) were separated by SDS-PAGE gel and immunoblotted with indicated antibodies. Arrow indicates a mobility shift of the CCND1-HA protein. Arrowhead indicates endogenous CCND1. **B.** Quantitative PCR (qPCR) analysis of SOX11 mRNA expression. Cell lines generated as described in (**A**) and mRNAs were harvested for SOX11 qPCR. Shown are the means of mRNA expression levels after normalization to GAPDH signals from four independent amplification experiments. Error bars, SD. *** $P<0.001$ by a 2-sided Student t-test. (See also **Supplementary Figures S1A, B**). **C.** CCND1 is required for SOX11 expression. Z-138 and JEKO-1 cells were stably transduced with control or CCND1 shRNA and protein expression was analyzed by immunoblotting with indicated antibodies 2 days after transduction. **D.** Effect of CCND1 knockdown on cell survival. Z-138 and JEKO-1 cells were stably transduced with control or CCND1 shRNA and propidium iodide (PI)-negative (viable) cells were assessed by flow cytometry over time. Shown are the means of PI negative fractions compared to day-2 samples from at least two independent experiments. **E.** Effect of SOX11 knockdown on MCL survival. Indicated MCL cell lines were transduced with control or SOX11 shRNA lentiviral vector that coexpresses GFP. Shown are the means of GFP+ fractions compared to Day 2 from two independent experiments. **F.** Z-138 cells expressing EV, WT or Y44D CCND1-HA were treated with 10 μM of cyclohexamide (CHX) for indicated times and cell lysates were prepared for immunoblot analysis with indicated antibodies. Numbers below

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immunoblots are relative densitometric values of corresponding bands after normalization to ACTIN or GAPDH and respective control signals.

Figure 2. CCND1 affects histone modifications at the *SOX11* locus. A. ENCODE H3K9Ac ChIP-Seq data for H1-hESC cells show *SOX11* gene regions that have positive ChIP peak signals. Arrows indicate regions where PCR primers were designed. **B-F.** H3K9/14Ac chromatin immunoprecipitation assays for the *SOX11* gene from indicated cells stably transduced with empty vector or CCND1 (**B-E**) or treated with 1μM of SAHA for 16 h (**F**). Bar graphs show means of qPCR signals of DNA regions 1 or 2 (amplicon 1 and 2) pulled down by the H3K9/14Ac antibody as fold enrichment relative to the background signals from the isotype control IgG antibody. Error bars, SD. *** P<0.001, **** P<0.0001 by a 2-sided Student t-test. **G.** Immunoblot analysis of Z-138 cells treated with 2 μM of SAHA for 3 h and immunoblotted with indicated antibodies.

Figure 3. CCND1 interacts with HDAC1 and HDAC2. A. HEK-293T cells were transiently co-transfected with untagged CCND1 and individual HA-tagged HDACs and immunoprecipitated with HA antibody followed by immunoblotting with indicated antibodies. Lysates before immunoprecipitation were used as input samples. Arrow indicates specific bands for HDAC4-HA **B.** Z-138 cells or primary MCL samples were immunoprecipitated with isotype control IgG, CCND1 or HDAC1 antibody and immunoblotted with indicated antibodies. Lysates before immunoprecipitation were used as input samples. Arrow, specific HDAC1 staining. * non-specific bands. **C, D.** Z-138

and JEKO-1 cells were stably transduced with control, HDAC1 (**C**) or HDAC2 (**D**) shRNA and protein expression was analyzed by immunoblot analysis with indicated antibodies 3 days after transduction (See also **Supplementary Figures S3A-C**). * non-specific bands.

Figure 4. Reduced chromatin localization of HDAC1 in CCND1 overexpressing cells. A. Chromatin immunoprecipitation assays for the *SOX11* gene from Z-138 cells stably transduced with empty vector (EV), WT or mutant Y44D CCND1. Bar graphs show means of quantitative PCR signals of region 1 (amplicon 1) pulled down by the HDAC1 antibody as fold enrichment relative to the background signals from the isotype control IgG antibody. Error bars, SD. **** P<0.0001 by a 2-sided Student t-test. **B.** Cytosolic, soluble and insoluble nuclear extracts were prepared as described in Materials and Methods from Z-138 cells that stably expressed empty vector or WT CCND1-HA. The extracts were immunoblotted with indicated antibodies. LAMIN A/C and histone H3 were used to confirm nuclear fractions. Cyto, cytoplasmic; Nu, soluble nuclear fraction; Insol, insoluble nuclear fraction (See also **Supplementary Figures S4**).

Figure 5. STAT3 negatively regulates SOX11 expression. A. JEKO-1 and JVM-2 cells were transduced with empty vector (EV) or CCND1-HA and cell lysates were immunoblotted with indicated antibodies. **B.** Box plots of relative SOX11 and CCND1 mRNA expression in primary MCL cases. Gene expression data for SOX11 and CCND1 were obtained from GSE16455[34] (see **Supplementary Table S6**) and plotted using

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GraphPad Prism v7.0a. **** P<0.0001 by a 2-sided Student t-test, n.s. non-significance (See also **Supplementary Figure S5A, B**). **C, D.** Immunoblot analysis of MCL cell lines (**C**) or MCL PDXs and primary MCL cases (**D**) with indicated antibodies. **E.** Indicated MCL cell lines were treated with indicated doses of the JAK1/2 inhibitor AZD1480 for 16 h and immunoblotted with indicated antibodies. (See also **Supplementary Figure S6A, B**). **F,** Indicated MCL cell lines were treated with AZD1480 as in (**E**) and SOX11 mRNA was analyzed by qPCR. Shown are the means of mRNA expression levels after normalization to GAPDH signals from four independent amplification experiments. **G.** JEKO-1 or GRANTA-519 cells were transduced with control or STAT3 shRNA and protein lysates were prepared for immunoblot analysis with indicated antibodies. **H.** Indicated MCL lines were treated with 500 nM of AZD1480 for 16 h and SOX11 mRNA was analyzed by qPCR. Bar graphs show the means of mRNA expression levels after normalization to GAPDH signals from four independent amplification experiments. Error bars, SD. ** P<0.01, *** P<0.001 by a 2-sided Student t-test.

Figure 6. Effects of IL21 on STAT3 activity, SOX11 expression and cell viability in MCL cells. A. Indicated MCL cell lines were treated with 50 ng/ml of IL21 for 96 h and SOX11 mRNA was analyzed by qPCR. Shown are the means of mRNA expression levels after normalization to GAPDH signals from four independent amplification experiments. **B.** Immunoblot analysis of indicated MCL cell lines treated as described in (**A**). **C.** Immunoblot analysis of MCL PDX models treated with 50 ng/ml of IL21 for 72 h. **D.** Indicated MCL cell lines were treated with 50 ng/ml of IL21 and viable cells (PI-negative) were assessed by flow cytometry at indicated times. Shown are the means of

PI-negative fractions compared to untreated samples from at least two independent experiments. **E.** MCL PDX cells were treated with IL21 and viable cells were analyzed as in (**D**) for the indicated times. Shown are the means of PI-negative fractions compared to untreated samples from at least two independent experiments. Error bars, SD. ** P<0.01, *** P<0.001, **** P<0.0001 by a 2-sided Student t-test.

Figure 7. pY705 STAT3 is directly recruited to the *SOX11* gene. A. Top panel, Diagram of the *SOX11* gene and enhancer (not drawn to scale). Arrows indicate STAT3 binding sites. Bottom panel, JEKO-1 cells were treated with 500 nM of AZD1480 or DMSO for 16 h and chromatin immunoprecipitation assays were performed using the pY705 STAT3 antibody. **B.** Chromatin immunoprecipitation assays using isotype IgG or H3K9/14Ac antibody for MAVER-1 cells treated with 500 nM of AZD1480 or DMSO for 16 h. Bar graphs (**A, B**) show means of qPCR signals from four independent amplification experiments using primers to regions 1-4 (Amplicons). Data are shown as the percentage of total input chromatin DNA. Error bars, SD. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001 by a 2-sided Student t-test. **C.** A proposed model of SOX11 expression through distinct mechanisms mediated by CCND1 and STAT3 in typical or indolent form of MCL.

Figure 1

Figure 2

Figure 3

A

HDAC1-HA HDAC2-HA HDAC3-HA HDAC4-HA
+   +   +   +
CCND1   CCND1   CCND1   CCND1   CCND1

Input IP: HA Input IP: HA Input IP: HA Input IP: HA Input IP: HA

150—
100—
75—
50—

IB: HA

37—

IB: CCND1

HDAC5-HA HDAC6-HA HDAC7-HA   HDAC8-HA   HDAC11-HA
+   +   +   +   +
CCND1   CCND1   CCND1   CCND1   CCND1

Input IP: HA Input IP: HA Input IP: HA   Input IP: HA   Input IP: HA

150—
100—

50—   50—
37—   37—

IB: HA

37—   37—   37—

IB: CCND1

B

Z-138   **Z-138**   MCL#24   MCL#15
      IP   IP   IP   IP

Input IgG CCND1 Input IgG HDAC1   Input IgG HDAC1 Input IgG HDAC1   IB

HDAC1   ← HDAC1
                                                                                * n.s.
HDAC2   HDAC2

CCND1   CCND1

C

Z-138 **JEKO-1**

shCon shHDAC1 shCon shHDAC1

HDAC1
  1   0.59   1   0.49

SOX11
  1   1.20   1   1.35

GAPDH
  1   1   1   1

D

Z-138 **JEKO-1**

shCon shHDAC2 shCon shHDAC2

HDAC2
n.s.* 1   0.17   1   0.50

SOX11
  1   1.61   1   3.36

GAPDH
  1   1   1   1

Figure 4

SOX11

Exon 1

1

A

Z-138

Fold enrichment

16
14
12
10
8
6
4
2
0

****
****

EV   WT   Y44D

■ IgG
■ HDAC1

B

Z-138

Empty Vector   CCND1-HA

Cyto   Nu   Insol   Cyto   Nu   Insol

HDAC2
0.78   6.47   1.87   3.51

HDAC1
0.51   6.89   1.06   4.46

CCND1

CCND1   Increased exposure

HA

Histone H3

LAMIN A/C
1   1   1   1

Figure 5

Figure 6

Figure 7

A

675 kb

1 2　　　3 4　Amplicons

SOX11 gene　　*SOX11* enhancer

pY705-STAT3 ChIP

****　****　****　*

■ DMSO
■ AZD 1480

Amplicons

B

■ DMSO
■ AZD1480

**　　　　　**　　　　　**　　　　　***

IgG　H3K9/14Ac　IgG　H3K9/14Ac　IgG　H3K9/14Ac　IgG　H3K9/14Ac

Amplicon 1　Amplicon 2　Amplicon 3　Amplicon 4

C

Typical MCL

CCND1

CCND1　HDAC1/2

TFs　SOX11　**ON**

Indolent MCL

CCND1

STAT3 STAT3 Ⓟ　SOX11　✕**OFF**　Ⓟ　STAT3 STAT3 Ⓟ

Ⓟ

SOX11 enhancer

Regulation of SOX11 expression through CCND1 and STAT3 in mantle cell lymphoma

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